Anal. Calcd. for $C_{20}H_{25}N_4O_{21}P_4Na_5 \cdot 5H_2O$ (986): P, 12.3. Found: P, 12.3.

The doubly labeled compound, *thymidylyl-2-C*^{14}*-(5′→3′)-H*^{3}*-thymidine 5′-triphosphate*, was similarly prepared but on a 10-μmole scale.

(f) 2′-Deoxycytidylyl-(5′→3′)-thymidine 5′-triphosphate[21] gave at pH 2 λ_{max} 273 mμ (ϵ (P) 4785).

Anal. Calcd. for $C_{19}H_{24}N_5O_{20}P_4Na_5 \cdot 7H_2O$ (1007): P, 12.3. Found: P, 12.3.

(g) Thymidylyl-(5′→3′)-thymidylyl-(5′→3′)-thymidine 5′-Triphosphate.[22] Paper chromatography of the sodium salt [λ_{max} 267 mμ (ϵ (P) 5560)] obtained from an apparently symmetrical peak on column chromatography gave a second spot, R_f 0.50, in solvent C. Gel filtration chromatography on Sephadex G-75[23] gave a single symmetrical peak which emerged somewhat in advance of tetrathymidylic acid (pTpTpTpT) used as a reference. Rechromatography on DEAE-cellulose, chloride form, with a linear gradient of lithium chloride buffered at pH 5.6 with 0.01 *M* lithium acetate, effected no noticeable change of the paper chromatography results. Elution and rechromatography of the triphosphate spot in solvent C gave the R_f 0.50 spot, in addition to the product spot, indicating, at least in part, an artifact of the chromatographic procedure. The hygroscopic lithium salt was isolated by evaporation of the column effluent and repeated extraction with methanol and acetone to remove lithium chloride and lithium acetate; at pH 2 λ_{max} 267 mμ (ϵ (P) 5660).

Anal. Calcd. for $C_{30}H_{37}N_6O_{28}P_5Li_6 \cdot 16H_2O$ (1414): P, 11.0. Found: P, 11.0.

(h) Thymidylyl-(5′→3′)-2′-deoxyadenosine 5′-triphosphate[24] gave at pH 8 λ_{max} 259 mμ (ϵ (P) 5500).

Acknowledgment. The authors express their thanks to A. Murray, III, V. N. Kerr, and D. L. Williams of this laboratory for preparation of some of the starting materials, and to Mrs. G. T. Fritz, Mrs. V. E. Mitchell, and Mrs. E. H. Lilly for skilled technical assistance.

(21) The starting material, d-pTpC, was prepared by D. L. Williams by condensation of N^4, O$^{3'}$-diacetyl-2′-deoxycytidine 5′-phosphate and 2-cyanoethyl 5′-thymidylate with DCC.[10]

(22) The starting material, pTpTpT, was obtained by polymerization of pT with DCC by the method of H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).

(23) F. N Hayes, E Hansbury, and V. E. Mitchell, *J Chromatog.*, **16**, 410 (1964).

(24) The starting material, d-pApT, was isolated following condensation of N^6-benzoyl-2′-deoxyadenosine 5′-phosphate and excess O$^{3'}$-acetylthymidine 5′-phosphate with DCC.[10]

On the Steady-State Method of Enzyme Kinetics

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A unified analytical treatment has been developed for the continuous description of the transient-state and steady-state phases of reaction according to the enzymic mechanism of Michaelis and Menten. This allows a separation of the two sources of errors attending the usual steady-state solution. The relative error δ_c arises from the omission of the complementary function from the unified solution, and the relative error δ_p accompanies an approximation to the particular integral. Equations and inequalities relating δ_c and δ_p to experimental observables have been derived. The consideration of errors indicates that the validity of the steady-state assumption does not depend upon the concentrations of reaction intermediates remaining stationary, but merely upon the elimination of the time as an explicit variable governing these concentrations.

Introduction

Since exact solutions to the differential equations arising from the formulation of enzyme kinetics are not obtainable, approximate solutions are required as the basis of experimental analysis. Among these, the steady-state solutions introduced by Briggs and Haldane[1] have come to constitute the principal means for defining the kinetic properties of enzymic systems. More recently, a complete generalization of the steady-state method has been achieved in the form of the schematic rule by King and Altman[2] describing the relative concentrations of enzymic species and the structural rules by Wong and Hanes[3] describing the structure of rate equations. The use of structural rules makes possible the direct interpretation of steady-state kinetics in terms of correlations between features of reaction mechanism and features of rate behavior. In contrast to these developments in the application of the steady-state solutions, there is only limited understanding of the underlying implications of the steady-state assumption itself. There is no continuous transition between the transient-state (or, presteady-state) and steady-state solutions now in general use, and, even during the steady-state phase, the validity of the solutions remains undefined by any rational analysis of errors. It is the purpose of the present study to consider the preliminary delineation of these problems.

Conventional Derivations of Approximate Solutions

The simplest representative of an enzymic mechanism is given by the mechanism of Michaelis and Menten[4] for irreversible one-substrate reactions

(1) G. E. Briggs and J. B. S. Haldane, *Biochem. J.*, **19**, 338 (1925).

(2) E. L. King and C. Altman, *J. Phys. Chem.*, **60**, 1375 (1956).

(3) J. T. Wong and C. S. Hanes, *Can. J. Biochem. Physiol.*, **40**, 763 (1962).

(4) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

where the substrate S combines reversibly with enzyme E to form a complex ES before its conversion to product P. The rates of change of the various concentration factors are described by eq. 1–4 where eq. 4 indicates the conservation of total enzyme. In what follows, the first time derivatives of the concentration factors are denoted by the superscript ′

$$[ES]' = k_1[E][S] - (k_2 + k_3)[ES] \quad (1)$$

$$[P]' = k_3[ES] \quad (2)$$

$$-[S]' = k_1[E][S] - k_2[ES] \quad (3)$$

$$[E] + [ES] = [E_0], \text{ the total enzyme} \quad (4)$$

Combining eq. 1 and 4 yield

$$\frac{[ES]'}{k_1[S] + k_2 + k_3} + [ES] = \frac{k_1[S][E_0]}{k_1[S] + k_2 + k_3} \quad (5)$$

There is no exact solution to eq. 5. Instead, two approximate solutions are usually employed, covering separately the earlier transient state and the later steady state.

The Steady-State Solution. The steady-state solution for [ES] is obtained by regarding the first left-hand term in eq. 5 to be negligible compared to [ES], *i.e.*

$$[ES] = \frac{k_1[S][E_0]}{k_1[S] + k_2 + k_3} \quad (6)$$

This in turn leads to, upon differentiating with respect to time,

$$[ES]' = \frac{k_1(k_2 + k_3)[S]'[E_0]}{(k_1[S] + k_2 + k_3)^2} \quad (7)$$

and, upon substituting into eq. 4

$$[E] = \frac{(k_2 + k_3)[E_0]}{k_1[S] + k_2 + k_3} \quad (8)$$

Upon substitutions with eq. 6 and 8, eq. 2 and 3 yield, respectively, eq. 9 and 10

$$[P]' = \frac{k_3k_1[S][E_0]}{k_1[S] + k_2 + k_3} \quad (9)$$

$$-[S]' = \frac{k_3k_1[S][E_0]}{k_1[S] + k_2 + k_3} \quad (10)$$

Equations 9 and 10 show [P]′ and −[S]′ as having identical expressions. However, it is already known from the study of Swoboda[5] that a small time lag actually holds between the largely parallel steady-state solutions for [P]′ and −[S]′. This becomes apparent in eq. 11

$$-[S]' - [P]' = [ES]' \quad (11)$$

Equation 11 is a result of the conservation of total substrate moiety, and the finite difference between [P]′ and −[S]′ is represented by [ES]′, already given in eq. 7.

The Transient-State Solution. It has been pointed out by Gutfreund and Roughton,[6] Laidler,[7] and Swoboda[5] that, during the earliest stages of the reaction, [S] does not deviate appreciably from its zero-time value of $[S_0]$, especially if $[S_0] >> [E_0]$. Setting [S] as $[S_0]$ in eq. 5 leads to the transient-state solution for [ES]

$$[ES] = \frac{k_1[S_0][E_0]}{k_1[S_0] + k_2 + k_3} - \frac{k_1[S_0][E_0]}{k_1[S_0] + k_2 + k_3} \cdot \exp\{-(k_1[S_0] + k_2 + k_3)t\} \quad (12)$$

(5) P. A. T. Swoboda, *Biochim. Biophys. Acta*, **23**, 70 (1957).

A Unified Treatment

Both the steady-state equation (6) and the transient-state equation (12) have found useful application in enzymic studies. However, there is no continuous transition between these equations, and the analytical description of the transition period between the transient-state and steady-state phases becomes problematic. This prompted a search for some unified treatment capable of giving a continuous description of these two phases. As will be seen such a treatment follows from the consideration of the hypothetical general solution to eq. 5.

The exact solution to eq. 5 is unknown. However, since eq. 5 is linear, its hypothetical general solution may be regarded as the sum of a complementary function $[ES]_c$ and a particular integral $[ES]_p$[8]

$$[ES] = [ES]_c + [ES]_p \quad (13)$$

Setting the right-hand side of eq. 5 to zero leaves the reduced equation which yields $[ES]_c$ upon rearrangement and integration

$$[ES]_c = (\text{constant}) \cdot \exp\left\{-k_1 \int_0^t [S]dt - k_2t - k_3t\right\} \quad (14)$$

As for $[ES]_p$, in order that eq. 6 will come to hold for the steady-state phase, the following approximation is made

$$[ES]_p \simeq \frac{k_1[S][E_0]}{k_1[S] + k_2 + k_3} \quad (15)$$

At the start of the reaction, [ES] is almost invariably zero, so eq. 13 requires the zero-time values of $[ES]_c$ and $[ES]_p$ to be equal but opposite in signs. This fixes the constant in eq. 14 as the negative of the zero-time value of $[ES]_p$, and eq. 13 can be rewritten as

$$[ES] = [ES]_p - (\text{zero-time value of } [ES]_p) \cdot \exp\left\{-k_1 \int_0^t [S]dt - k_2t - k_3t\right\} \quad (16)$$

Clearly any approximation to $[ES]_p$ also at once leads to a corresponding approximation to the general solution for [ES]. Substituting the approximation in eq. 15 into 16 yields

$$[ES] = \frac{k_1[S][E_0]}{k_1[S] + k_2 + k_3} - \frac{k_1[S_0][E_0]}{k_1[S_0] + k_2 + k_3} \cdot \exp\left\{-k_1 \int_0^t [S]dt - k_2t - k_3t\right\} \quad (17)$$

(6) H. Gutfreund and F. J. W. Roughton, cited in F. J. W. Roughton, *Discussions Faraday Soc.*, **17**, 116 (1954).
(7) K. J. Laidler, *Can. J. Chem.*, **33**, 1614 (1955).
(8) E. L. Ince, "Ordinary Differential Equations," Dover Publications, New York, N. Y., 1944, p. 115.

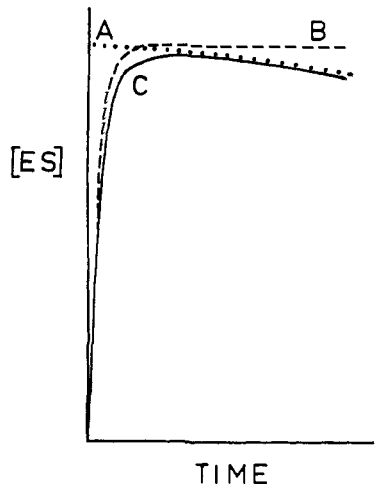

Figure 1. Sketches of different solutions to eq. 5. Curve A: steady-state solution (6); curve B: transient-state solution (12); curve C: unified solutions (17) and (18).

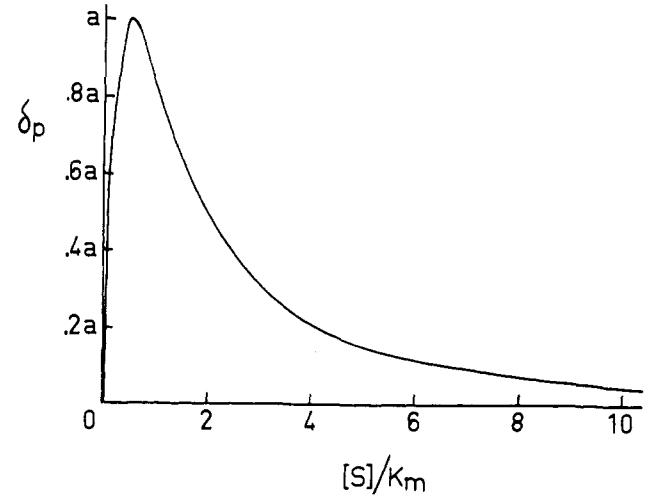

Figure 2. Variation of the relative error δ_p with $[S]/K_m$ as $[S]/[E_0]$ is maintained constant. The scale for δ_p is expressed in terms of a = the maximum value of δ_p at $[S] = 0.5K_m$.

The integral in eq. 17 simply represents the area under the progress curve for [S] from zero time to time t, and may be determined experimentally. Moreover, because $k_1\int_0^t [S]dt$, k_2t and k_3t are all positive, the exponential term representing $[ES]_c$ is significant only for small values of t; if it should vanish before [S] has deviated significantly from $[S_0]$, eq. 17 simplifies into 18

$$[ES] = \frac{k_1[S][E_0]}{k_1[S] + k_2 + k_3} \cdot \frac{k_1[S_0][E_0]}{k_1[S_0] + k_2 + k_3} \cdot \exp\{-(k_1[S_0] + k_2 + k_3)t\} \quad (18)$$

As can be seen from Figure 1, the unified solutions (17) and (18) describe both the rise of [ES] in the transient state and its decline in the steady state. In contrast, since the steady-state solution (6) does not include a term for $[ES]_c$, it describes only the decline but not the rise of [ES]. On the other hand, the transient-state solution (12) requires $[S] \backsimeq [S_0]$ in both $[ES]_c$ and $[ES]_p$, and therefore succeeds to describe only the rise but not the decline of [ES]. The reason is, whereas it may be justified to set $[S] \backsimeq [S_0]$ in $[ES]_c$ if $[ES]_c$ rapidly vanishes, the same cannot be said for setting $[S] \backsimeq [S_0]$ in $[ES]_p$, because $[ES]_p$ cannot vanish before [S] has significantly deviated from $[S_0]$.

Analysis of Errors

The unified solutions (17) and (18) incur the assumption that $[ES]_p$ as given in eq. 15 is an adequate particular solution to eq. 5. The steady-state solution (6) incurs the additional assumption of $[ES]_c$ being omissible, and therefore $[ES] \backsimeq [ES]_p$. The valid use of these solutions must rest on an understanding of the errors accompanying these assumptions.

The Relative Error δ_c. From eq. 18, the decay of $[ES]_c$ is seen to be characterized by the time constant τ

$$\tau = 1/(k_1[S_0] + k_2 + k_3) \quad (19)$$

and the omission of $[ES]_c$ at time t implies, considering that $[S] \backsimeq [S_0]$ during the transient state

$$1 \backsimeq 1 + \delta_c \quad (20)$$

$$\delta_c = \exp(-t/\tau) \quad (21)$$

where δ_c represents the relative error due to the omission of $[ES]_c$. δ_c does not depend on the enzyme concentration, but as the enzyme concentration is decreased and the duration of the steady state lengthens, the transient state wherein δ_c remains significant becomes *relatively* suppressed. In an experimental system, if the transient state is observable as an initial burst in substrate disappearance or lag in product formation, the decay of δ_c with time can be assessed, knowing that τ is of the order of the time required for [ES] to rise to 63% of its steady-state level, and δ_c will be reduced to below 0.01 (*i.e.*, a 1% error) at $t > 4.6\ \tau$. On the other hand, if the transient state is too brief to be observed (*e.g.*, often the time required for an adequate mixing of enzyme and substrate solutions is much longer than τ), δ_c will not be important and it becomes feasible to extrapolate the steady state back to zero time to give the "initial steady-state velocity" of reaction.

The Relative Error δ_p. To test the adequacy of $[ES]_p$ as given in eq. 15 as a particular solution of eq. 5, it is substituted into eq. 5 to yield

$$\frac{k_1[S][E_0]}{k_1[S] + k_2 + k_3} \backsimeq [ES]_p + \frac{[ES]_p'}{k_1[S] + k_2 + k_3} \quad (22)$$

Equation 22 can be rewritten as

$$1 \backsimeq 1 + \delta_p \quad (23)$$

where δ_p represents the relative error due to the particular-integral approximation of eq. 15 and has the following expression, obtained by inserting $[ES]_p$ and its first time derivative into eq. 22 and rearranging

$$\delta_p = \frac{(k_2 + k_3)[S]'}{(k_1[S] + k_2 + k_3)^2[S]} \quad (24)$$

The steady-state expression for [S]′ from eq. 10 may be employed, taking only its absolute value, to convert eq. 24 into 25

$$\delta_p = \frac{k_3}{(k_2 + k_3)} \cdot \frac{[S]/K_m}{(1 + [S]/K_m)^3} \cdot \frac{1}{[S]/[E_0]} \quad (25)$$

where $K_m = \frac{k_2 + k_3}{k_1}$, the Michaelis constant for substrate S. Evidently, for a given $[S]/K_m$, δ_p decreases as $[S]/[E_0]$ increases. On the other hand, for a given $[S]/[E_0]$, the variation of δ_p with $[S]/K_m$ is as shown in Figure 2, δ_p going through a maximum at $[S] = 0.5K_m$. Therefore δ_p can be made small by making $[S] >> [E_0]$, $[S] >> K_m$, or $[S] << K_m$.

In general, k_2 and k_3 are not known, and eq. 25 cannot be employed directly. Instead, the following inequality may be considered

$$\delta_p < \frac{[S]/K_m}{(1 + [S]/K_m)^3} \cdot \frac{1}{[S]/[E_0]} \quad (26)$$

At $[S] = 0.5K_m$, δ_p reaches its maximum limiting value

$$\text{limiting } \delta_p < \frac{1}{6.75[S]/[E_0]} \quad (27)$$

That is, δ_p cannot exceed 0.01 (*i.e.*, a 1% error) when the $[S]/[E_0]$ ratio exceeds 15.

Although the inequalities (26) and (27) are convenient in application, it may be noted that other expressions for δ_p also can be usefully derived from eq. 24. For example, when considering initial steady-state velocities, [S] will be close to $[S_0]$, and $(k_2 + k_3)$ must be smaller than $(k_1[S_0] + k_2 + k_3)$, and the following expression

$$\delta_p < \frac{\tau[S]'}{[S]} \quad (28)$$

results from eq. 24. Consequently, when the transient state is brief (*i.e.*, τ is small), and the steady state is prolonged (*i.e.*, [S] changes slowly with time), δ_p will be small and the steady-state method will be valid.

It is of interest to compare eq. 25 with the analysis by Miller and Alberty[9] of the reversible one-substrate mechanism under the special condition that the Michaelis constants for the substrate and the product are identical (exact solutions become obtainable under this special condition). In this case, the steady-state approximation has been shown to improve as $[S_0]/[E_0]$ becomes large or as $([S_0] + [E_0])$ becomes much smaller than K_m; it is not clear if the approximation also improves as $([S_0] + [E_0])$ becomes much larger than K_m. More recently, Hommes[10] and Walter and Morales[11] have suggested a direct interpretation of finite values of [ES]′ and [ES]′/[S]′ as representing the error of the steady-state solution. However, the justification for this interpretation is necessarily obscure, since eq. 7 in fact requires the ratio [ES]′/[S]′ to be finite in the steady-state phase.

Numerical Examples. In order to illustrate the quantitative behavior of δ_c and δ_p, the two following systems, the rate constants of which have been calculated by Dixon and Webb[12] from the measurements by Gutfreund,[13,14] may be considered: System I: Hydrolysis of benzoyl-L-arginine ethyl ester by trypsin ($k_1 = 4 \times 10^6\ M^{-1}$ sec.$^{-1}$; $k_2 = 25$ sec.$^{-1}$; $k_3 = 15$ sec.$^{-1}$); System II: Hydrolysis of acetyl-L-phenylalanine ethyl ester by chymotrypsin ($k_1 = 10^6\ M^{-1}$ sec.$^{-1}$; $k_2 = 90$ sec.$^{-1}$; $k_3 = 10$ sec.$^{-1}$).

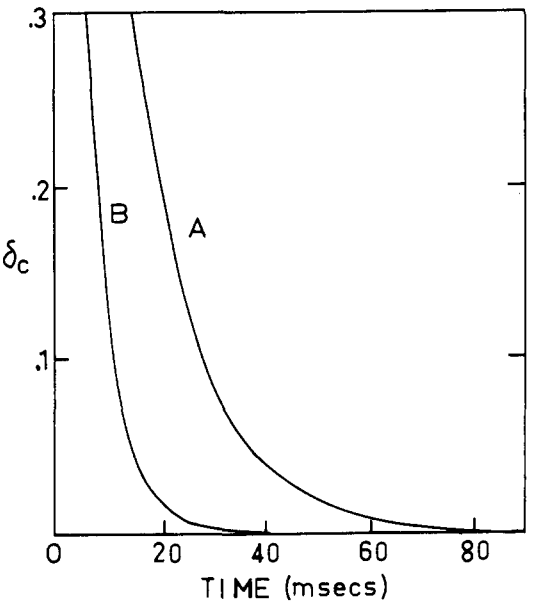

Figure 3. Variation of the relative error δ_c with time at $[S_0] = K_m$. Curve A: system I, hydrolysis of benzoyl-L-arginine ethyl ester by trypsin; curve B: system II, hydrolysis of acetyl-L-phenylalanine ethyl ester by chymotrypsin.

On the basis of these rate constants, $K_m = 10^{-5}\ M$ for System I and $10^{-4}\ M$ for System II. At $[S_0] = K_m$, $\tau = 12.5$ msec. for System I and 5 msec. for System II. These values of K_m and τ in turn enable the calculation of δ_c with eq. 21 and δ_p with eq. 25. The results are shown in Figures 3 and 4. In Figure 3, δ_c is seen to decrease with time; System I requires about 60 msec. for δ_c to be reduced to below 0.01, whereas System II requires about 25 msec. In Figure 4, δ_p is seen to decrease as $[S]/[E_0]$ increases; for the top curve at $[S] = 0.5K_m$, System I requires a substrate/enzyme ratio of about 6 for δ_p to be reduced to below 0.01, whereas System II requires a ratio of less than 2.

During the progress of a reaction, [S] changes with time, so δ_p also will change with time. Nevertheless, it is clear from Figure 4 that, for Systems I and II, δ_p will remain small for a significant portion of the reaction even when the initial substrate/enzyme ratio employed is as low as of the order of 10. Under such a condition of a low substrate/enzyme ratio, the relative duration of the transient state by no means will be negligible. It becomes necessary to restrict the steady-state solution to the later steady-state stages and abandon the measurement of "initial steady-state velocities," or to employ the unified solutions (17) and, to a lesser extent, (18).

Improvement of Approximation

When the substrate/enzyme ratio is too low and the particular-integral approximation in eq. 15 proves inadequate, the numerical solutions developed by

(9) W. G. Miller and R. A. Alberty, *J. Am. Chem. Soc.*, 80, 5146 (1958).
(10) F. A. Hommes, *Arch. Biochem. Biophys.*, **96**, 28 (1962).
(11) C. F. Walter and M. F. Morales, *J. Biol. Chem.*, **239**, 1277 (1964).
(12) M. Dixon and E. C. Webb, "Enzymes," Longmans, Green and Co., London, 1958, p. 110.
(13) H. Gutfreund, *Discussions Faraday Soc.*, **17**, 220 (1954).
(14) H. Gutfreund, *ibid.*, **20**, 167 (1955).

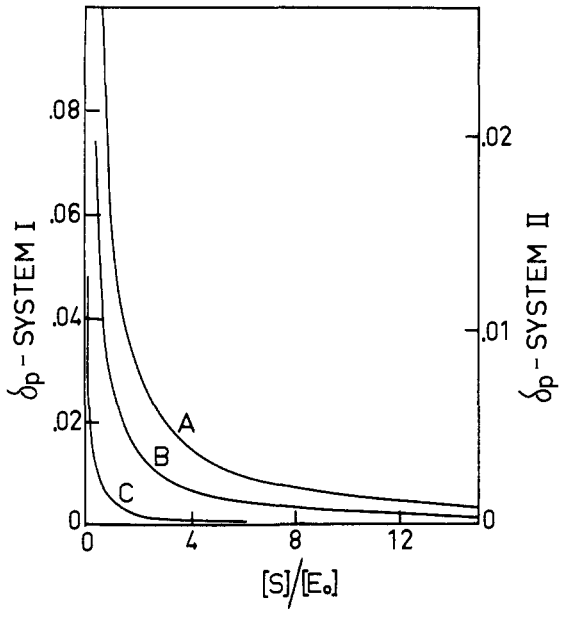

Figure 4. Variation of the relative error δ_p with $[S]/[E_0]$ for system I, hydrolysis of benzoyl-L-arginine ethyl ester by trypsin, and system II, hydrolysis of acetyl-L-phenylalanine ethyl ester by chymotrypsin. Curve A: $[S] = 0.5K_m$; curve B: $[S] = 2K_m$ or $0.098K_m$; curve C: $[S] = 8K_m$ or $0.0114K_m$.

Chance,[15] Yang,[16] and Swoboda[5] always may be resorted to. Starting with the expression for $[ES]_p$ in eq. 15, which may be written as $[ES]_{p(ss)}$ (where ss represents steady state), the procedure of successive substitutions suggested by Curtiss and Hirschfelder[17] and Hirschfelder[18] is also applicable to yield an improved approximation for $[ES]_p$, or $[ES]_{p(improved)}$, for use in eq. 16

$$[ES]_{p(improved)} = [ES]_{p(ss)} - \frac{[ES]'_{p(ss)}}{k_1[S] + k_2 + k_3} + \dots . \quad (29)$$

The two terms shown in the series in eq. 29 result from one round of substitution, and the series can be built up infinitely by further substitutions. Equation 29 is of special interest in showing that the steady-state solution may be regarded as the first term in an approximation series that can be employed for numerical integration. This property of the steady-state solution, pointed out by Hirschfelder[18] for chemical steady-state systems in general, serves to emphasize the close relationship between steady-state and numerical solutions, which often have distinct advantages and disadvantages. For enzymic systems, numerical solutions can describe reactions at low substrate/enzyme ratios and readily yield evaluations of individual rate constants. Steady-state solutions, on the other hand, are simple to apply and provide direct correlations between features of mechanism and of rate behavior for the characterization of reaction mechanisms.

(15) B. Chance, *J. Biol. Chem.*, **151**, 553 (1943).
(16) C. C. Yang, *Arch. Biochem. Biophys.*, **51**, 419 (1954).
(17) C. F. Curtiss and J. O. Hirschfelder, *Proc. Natl. Acad. Sci. U. S.*, 38, 235 (1952).
(18) J. O. Hirschfelder, *J. Chem. Phys.*, **26**, 271 (1957).

Discussion

In enquiring into the physical nature of the steady-state assumption, it is interesting to note that two different conditions can be invoked to obtain the steady-state solution (6) from the differential equation (5): condition I: $[ES]' = 0$; condition II: $([ES]'/(k_1[S] + k_2 + k_3)) << [ES]$.

In practice, condition I is most commonly employed, and gives rise to the widespread supposition that the steady-state method requires [ES] to remain stationary, its rate of formation being just balanced by its rate of disappearance.[19] This is an untenable supposition because eq. 7, which directly follows from the steady-state solution (6), requires $[ES]'$ to be finite as long as $[S]'$ is finite. Condition I is in fact a sufficient but unnecessary condition, in contrast to condition II which is both sufficient and necessary. It is noteworthy, however, that in eq. 7 $[ES]'$ is dependent on $[S]'$, and neither eq. 6 nor 7 explicitly contains time. The absence of time from steady-state expressions is generally the case with enzymic systems,[2,3] and also the case with other chemical systems (*e.g.*, systems discussed by Frank-Kamenetskii[20]). The suggestion therefore can be made simply to interpret the steady-state assumption, at least for enzymic systems, as *requiring* the elimination of time as an explicit variable governing the concentrations of steady-state intermediates. The error eq. 28 supports this interpretation.

From eq. 28, δ_p is expected to be small provided $\tau[S]'/[S]$ is small. At the start of the reaction the substrate concentration in the vicinity of the enzyme is made to undergo a step change from zero to $[S_0]$, and the speed of the response of [ES] toward this step change is characterized by the response time τ. If this initial response is rapid and τ small, and the subsequent relative change in substrate concentration is slow, δ_p will be small, and the steady-state solution will be valid. However these conditions are also the conditions expected to be prerequisite to regarding [ES] as explicitly dependent on [S] but not on time, because under these conditions [ES] will be able to adjust to changes in [S] constantly without significant lag. The elimination of time as an explicit variable for [ES] completes the analogy to an exact steady state in an open system. Interestingly, the situation is also not totally unlike that of tracing the reaction time course of the substrate with a recording instrument; the instrument must be capable of fast response and the substrate concentration must not change too rapidly in order for the recorded measurements to be valid, namely, to be explicitly dependent on the substrate concentration but not on time.

The interpretation of the steady-state assumption not as the stationary concentrations of enzymic intermediates but as the elimination of time as an explicit variable governing these concentrations is therefore in agreement with the general conclusion by Rice[21]

(19) For example, see review by F. M. Huennekens in "Techniques of Organic Chemistry," Vol. VIII, S. L. Friess, E. S. Lewis, and A. Weissberger, Ed., Part II, 2nd Ed., Interscience Publishers, Inc., New York, N. Y., 1963, p. 1245.
(20) D. A. Frank-Kamenetskii, "Diffusion and Heat Exchanges in Chemical Kinetics, translated by N. Thon, Princeton University Press, Princeton, N. J., 1955, p. 351.
(21) O. K. Rice, *J. Phys. Chem.*, **64**, 1851 (1960).

that the transient state must be brief for the steady-state method to be applicable. With enzymic reactions the relative duration of the transient state may be suppressed simply by increasing the substrate/enzyme ratio, and deviations from the steady state arising from the explosive accumulation of enzymic intermediates are also precluded.

For the mechanism of Michaelis and Menten for irreversible one-substrate reactions, the error equations derived in this study help define the errors of the steady-state method in terms of experimental observables and assess what may constitute a sufficiently high substrate/-enzyme ratio. For other enzymic mechanisms, it also would not be unexpected that the relative errors δ_c and δ_p will both increase with enzyme concentration. Since for all enzymic mechanisms the initial steady-state velocity is predicted to be strictly proportional to enzyme concentration,[2,3] the following operational criterion appears applicable. If velocity *vs.* enzyme concentration is observed to be linear, the errors of the steady-state theory may be *tentatively* accepted as within the errors of experimental observations. On the other hand, if experimental precision permits the detection of a nonlinear relationship of velocity *vs.* enzyme concentration unexplained by experimental factors, the adequacy of the steady-state theory over the nonlinear range of enzyme concentrations *must* be questioned.

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Communications to the Editor

Biosynthesis of the Tetracyclines. VII.[1] 4-Hydroxy-6-methylpretetramid, an Intermediate Accumulated by a Blocked Mutant of *Streptomyces aureofaciens*[2]

Sir:

In our continuing study of the biosynthetic pathways to the tetracycline antibiotics, we have found the phenomenon of cosynthesis[3] to be a useful tool both in characterizing new blocked mutants of the tetracyclines-producing *Streptomyces* and in demonstrating the presence of transferable intermediates in the biosynthetic process. This phenomenon has been used in the study of a new *Streptomyces aureofaciens*, strain V655, a dark-green-pigmented, spontaneous variant isolated directly from a 7-chlorotetracycline-producing parental strain. Significant cosynthetic activities of this new strain with two other blocked *S. aureofaciens* mutants are presented in Table I. The cosynthetic production of 7-chlorotetracycline in the mixed fermentation of strain V655 with the inherently nonchlorinating, blocked mutant T219 confirms that V655 has the chlorination potential indicated by its derivation from a chlorinating parental strain. This result, in conjunction with the further observation that V655 accumulated no chlorinated product of itself (as shown by fermentation in the presence of radioactive chloride ion, $^{36}Cl^-$), shows that the primary metabolic block in V655 is earlier than the chlorination step. The elaboration of 7-chlorotetracycline in mixed fermentation of V655 with the nonmethylating, blocked mutant ED1369 suggests the transfer of a 6-methyl-containing, unchlorinated intermediate from V655 to ED1369 and completion of the 7-chlorotetracycline molecule by the latter mutant. Addition of heat-killed, mature V655 mash to growing ED1369 again resulted in appearance of 7-chlorotetracycline, although in smaller amount, showing that a stable intermediate had accumulated in the V655 fermentation. The appearance of antibacterial activity in ED1369 fermentations upon adding V655-derived fractions was used as a biological assay method, by means of which we were able to isolate the active component of V655 fermentation mashes in pure form. In the accompanying paper[4] this substance is characterized as 1,3,-4,10,11,12-hexahydroxy-6-methylnaphthacene-2-carbox-

Table I. Cosynthesis of Tetracycline Antibiotics

	Strain		
	T219	ED1369	V655
Principal antibiotic product of parental strain	TC[a]	7-Chloro-6-demethyl-TC	7-Chloro-TC
Assay[b] when grown alone (μg./ml.)	<1.0	<1.0	2–5
Assay in mixed fermentation with strain V655 (μg./ml.)	460	370	—
Antibiotic produced in mixed fermentation with strain V655	7-Chloro-TC	7-Chloro-TC	—

[a] TC = tetracycline. [b] Antibacterial activity as determined by *Staphylococcus aureus* turbidimetric assay.

(1) Paper VI: J. R. D. McCormick, J. Reichenthal, S. Johnson, and N. O. Sjolander, *J. Am. Chem. Soc.*, 85, 1694 (1963).

(2) The results of this work were included in summary in a paper presented at the Congress for Antibiotics, Prague, Czechoslovakia, June 1964.

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