[CONTRIBUTION FROM THE FERTILIZER INVESTIGATIONS UNIT, BUREAU OF CHEMISTRY AND SOILS, UNITED STATES DEPARTMENT OF AGRICULTURE]

The Determination of Enzyme Dissociation Constants

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Introduction

Kinetic studies of enzyme reactions have led to the theory of equilibrium intermediate compound formation between enzyme and substrate (general reference, Haldane,[1] p. 38). The simplest case of equilibrium may be represented by the dissociation constant

$$K_s = (E)\,(S)/(ES). \qquad (1)$$

On the basis of the assumed theory the rate of the observed reaction is directly proportional to the concentration of the enzyme-substrate compound, (ES), at all values of the concentration of the substrate, (S). It is proportional to (S) only at low values of (S). The numerical value of the dissociation constant is given by the substrate concentration at half-maximum velocity, where (E) = (ES).

The equilibrium in equation 1 may be heterogeneous or homogeneous. Hitchcock[1a] has pointed out the formal identity of the simplest Langmuir adsorption isotherm with an equation he derived expressing the application of the law of mass action to a reversible homogeneous reaction between two substances in solution, the total concentration of one, (E) + (ES), being kept constant and the other, (S), varied. In the latter case the observed initial reaction velocity v is given by the well-known equation (*cf.* Michaelis and Menten[2])

$$v = V_{max}(S)/(K_s + (S)) \qquad (2)$$

where V_{max} is a numerical constant representing the maximum velocity obtained when the enzyme E exists completely in the form ES ($V_{max} = k(E_{total})$). In the simplest Langmuir surface reaction, the velocity is proportional to the amount q of gas adsorbed at an equilibrium pressure p, where

$$q = ap/(b + p) \qquad (3)$$

a being the maximum amount of gas adsorbable when the adsorbing surface is saturated. **b** is a dissociation constant corresponding to K_s and represents the ratio of the velocity constants of evaporation and condensation of gas from and onto the surface, providing the observed reaction velocity is negligible compared to the velocity of evaporation. Agreement of experimental data with either equation, therefore, does not necessarily decide whether an enzyme-substrate equilibrium is heterogeneous or homogeneous since both represent rectangular hyperbolas. Data of an independent nature in addition to the kinetic studies are required.

Enzyme properties are determined chiefly by means of kinetic studies. In many cases investigated heretofore, the mechanism and equations of the simplest case just outlined have been assumed to hold without regard to other possibilities. The dissociation constant K_s, whether true or apparent, has been evaluated arbitrarily by plotting activity (initial velocity) against (S) or log (S) and taking the value of (S) at half-maximum activity. In certain cases detailed analytical methods have also been employed. Generally considered, however, there have been no convenient and direct methods available for ascertaining which of several mechanisms *may*, and which *may not*, be involved. Although (S) at half-maximum activity is in any case a characterizing property of an enzyme, it does not necessarily represent a thermodynamic dissociation constant. In many cases the kinetics involved do not correspond to equation 2 (Case I), but to Cases II to VII, briefly outlined as follows

Case I $E + S \rightleftharpoons ES$ (active)

Case II $E + nS \rightleftharpoons ES_n$ (active)

Case III $E + S \rightleftharpoons ES$ (active), $ES + (n-1)S \rightleftharpoons ES_n$ (inactive)

Case IV General inhibition: non-competitive; and competitive ($2E + S + I \rightleftharpoons ES$ (active) + EI (inactive))

Case V $E + (n + n')S \rightleftharpoons ES_n$ (active) + $ES_{n'}$ (active)

Case VI $E + S \longrightarrow ES$ (active), (steady-state concentration of ES)

Case VII $S \longrightarrow S'$, (steady-state (S')), $S' + E \rightleftharpoons ES$ (active)

In all these cases the velocity is assumed to be a direct function of the concentration of an active intermediate or active intermediates.

It is proposed in this paper to present graphical methods of testing velocity equations and evaluating constants involved in the various funda-

(1) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930.

(1a) D. I. Hitchcock, THIS JOURNAL, **48**, 2870 (1926).

(2) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 1333 (1913).

mental type mechanisms postulated above, by putting a given equation in a form that is linear and employing straight line extrapolations. The relative weighting of the experimental observations alters in a definite manner when the form of an equation is altered, and if not taken into account may alter slightly the parameter constants obtained, whether graphical or analytical methods are employed. This possible disadvantage will rarely outweigh the convenience of the graphical method, where proper weighting is less easily applied. In this connection it is often instructive to plot a given set of data in several ways. This is true whether straight lines are involved or not. Complementary analytical refinements leading to the determination of the actual probabilities of the constants and functions by means of Pearson's Chi test, involving proper weighting, are considered elsewhere[3a,3b] in connection with experimental studies of the writers on the nitrogen fixing enzyme nitrogenase in Azotobacter.

Each case will be illustrated by experimental data specifically consistent with, but not necessarily proving, that case only. Needless to say, caution is necessary in concluding that a reaction follows a certain mechanism because a set of data fits a certain equation. Lack of extensive data might also be responsible for a misleading agreement of data with an assigned mechanism. In any case, the direction of purposeful experimentation should be more evident. It is desired to encourage detailed analyses of kinetic data relating to enzymes in order to determine what mechanisms *may*, but not *necessarily do*, hold, and particularly to eliminate certain mechanisms which *definitely do not* hold. Non-conformity of a set of data with a given mechanism eliminates that mechanism unless closer analysis indicates that some consistent experimental error might be involved. The various experimental data discussed are given chiefly as illustrations of various types of kinetic data; their intrinsic interest is secondary here.

Various combinations of Cases I to VII will not be considered except by implication, nor cases involving reaction courses such as irreversible consecutive reactions, reaction product inhibition, irreversible inactivation. No consideration will be given to postulated enzyme mechanisms other than those involving formation of intermediate compounds or complexes.

(3) (a) H. Lineweaver, D. Burk and W. E. Deming, THIS JOURNAL, **56**, 225 (1934); (b) Dean Burk, "Azotase and Nitrogenase in Azotobacter," a review chapter in "Ergebnisse der Enzymforschung," by F. F. Nord and R. Weidenhagen, Vol. III, Leipzig, 1934.

Experimental

Case I. (Invertase, Raffinase, Amylase.)—Equation 2 (likewise equation 3) becomes linear in form upon taking the reciprocal of both sides

$$1/v = K_s/V_{max}(S) + 1/V_{max} \qquad (4)$$

When $1/v$ is plotted against $1/(S)$, the ordinate intercept is $1/V_{max}$ and the slope of the straight line is K_s/V_{max}, thus evaluating K_s. Multiplied through by (S) equation 4 becomes

$$(S)/v = (S)/V_{max} + K_s/V_{max} \qquad (5)$$

and when $(S)/v$ is plotted against (S), the ordinate intercept is K_s/V_{max} and the constant slope $1/V_{max}$. Hitchcock[4] suggested this latter method in testing the Langmuir equation cited. Hanes[5] determined K_s and V_{max} for the amylase system by the method of least squares based on equation 5, rather than graphically. Kuhn[6] also used an analytical method with invertase.

It has been our experience that usually the method of plotting based on equation 4 rather than 5 gives a better placement of the experimental values at low substrate concentrations. Figure 1 and Table I illustrate the use of this method.

TABLE I

K_s AND V_{max} VALUES IN CASE I

Enzyme	Figure 1 K_s	Figure 1 V_{max}	Original paper K_s	Original paper V_{max}	Observer
Invertase, Curve I	0.0166 *M*	3.94	0.0166 *M*		Michaelis and Menten[2]
Invertase, Curve II	.017 *M*	23.4	.017 *M*		Kuhn[6]
Raffinase, Curve III	.23 *M*	100	.24 *M*		Kuhn[6]
Amylase, Curve IV	.076%	0.478	.077%	0.478	Hanes[5]
Amylase, Curve V	.079%	.552	.079%	.554	Hanes[5]

Case II. (Citric Dehydrogenase.)—The velocity equation for Case II corresponding to equation 2 for Case I may easily be shown to be

$$v = V_{max}(S)^n/(K_s + (S)^n) \qquad (6)$$

A plot of $1/v$ against $1/(S)^n$ yields a straight line, the ordinate intercept and slope having the same significance as before, but K_s is now the nth power of the substrate concentration at half-limiting velocity. The data of Dann[7] on citric dehydrogenase appear to represent a case where n is 2, as

(4) D. I. Hitchcock, "Physical Chemistry," Thomas, Baltimore, 1931, p. 93.

(5) C. S. Hanes, *Biochem. J.*, **26**, 1406 (1932).

(6) R. Kuhn, *Z. physiol. Chem.*, **125**, 28 (1923).

(7) W. J. Dann, *Biochem. J.*, **25**, 178 (1931).

indicated in Curve I, Fig. 2. Any straight line extrapolation of Curve II (n = unity) would almost certainly intersect the abscissa at a finite

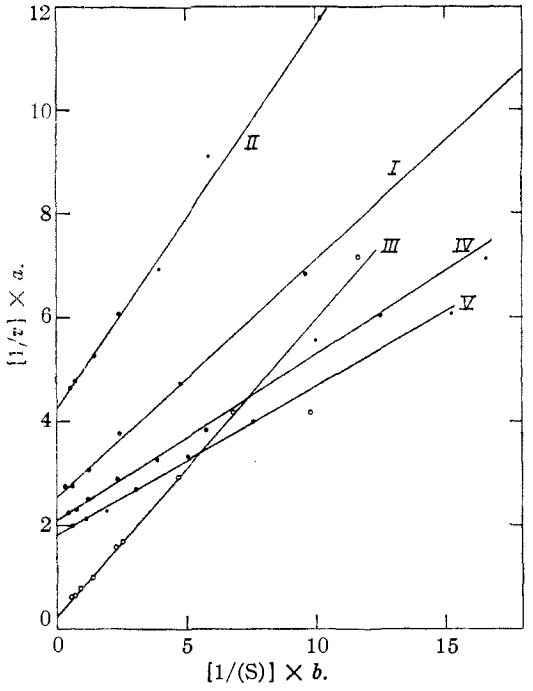

Fig. 1.—Evaluation of K_s and V_{max} in Case I. Curve I, invertase, data of Michaelis and Menten,[3] Table I, Fig. 1a, scale: $(1/v) \times 10$; $(1/(S)) \times 0.1$. Curve II, invertase, data of Kuhn,[6] Table 8, scale: $(1/v) \times 100$; $(1/(S)) \times 0.1$. Curve III, raffinase, data of Kuhn,[6] Table 14, scale: $(1/v) \times 25$; $(1/(S)) \times 0.1$. Curve IV, amylase, data of Hanes,[5] Table III, scale: $(1/v) \times 1$; $(1/(S)) \times 0.5$. Curve V, amylase, data of Hanes,[5] Table V, scale: $(1/v) \times 1$; $(1/(S)) \times 0.5$. v scale values in original investigators' relative units. (S) values: Curves I II, III, molal; Curves IV and V, %.

substrate concentration, implying attainable infinite velocity. The data in this case are probably not as trustworthy as those of the much studied hydrolytic enzymes. We do not wish to discourage possible reinterpretations, leading to either simpler or more complex mechanisms, by indicating that the velocity–substrate measurements, as reported by the original investigator, are consistent with a mechanism in which $n = 2$. In this connection, for instance, one might wish to investigate the effect of concomitant substances in the enzyme preparation which reduce methylene blue and which might compete with citrate for *citric* dehydrogenase and affect thereby the calculation of the desired true velocity, and thus possibly the value of n.

In cases where each component of the ratio of $(ES_n)/(E)$ can be evaluated experimentally, the equation

$$(ES_n)/(E) = (S)^n/K_s \qquad (7)$$

may be thrown into linear form by taking the log of both sides, and the slope obtained on plotting log $(ES_n)/(E)$ against log (S) evaluates n, equal to unity in the simplest case, and the intercept at S = 1 evaluates log $1/K_s$. Redfield and Ingalls[8] used this method in studying the oxygen dissociation of hemocyanin. For the enzymic case, if the maximum velocity observed experimentally ($V_{max\text{-}obs}$) corresponds accurately to kE_{total} (E saturated), then Case II may be solved in the same manner, using log $v/(V_{max\text{-}obs} - v)$ instead of log $(ES_n)/(E)$.

The case $2E + S \rightleftharpoons E_2S$, superficially the converse of Case II, would seem incapable of accounting for the observed rates of enzymic reactions. This mechanism kinetically requires the entrance into the reaction of two individual particles or colloidal carriers, each with one (or more) active E groups. If one active group, E, per single colloidal carrier of large molecular

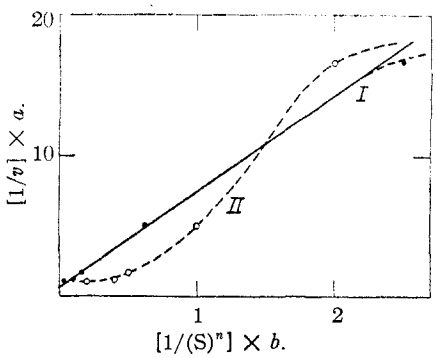

Fig. 2.—Evaluation of K_s and V_{max} in Case II. Citric dehydrogenase, data of Dann,[7] 25°, average of 5 experiments. Curve I, scale: $(1/v) \times 1$; $(1/(S)^2) \times 0.001$; $K_s = 0.007$ M; substrate concentration at half-maximum velocity = 0.084 M, V_{max} = 1.7. Curve II, scale: $(1/v) \times 1$; $(1/(S)^1) \times 0.04$. Dann's values: "K_s" = substrate concentration at half-maximum velocity = 0.172 M, V_{max} = 1.0. v scale values in relative velocity units of Dann. (S) values: molal.

weight is assumed, the molecular concentration, rate of diffusion, and probability of proper spatial orientation of the active group at the moment of collision would presumably be too low. The

(8) A. C. Redfield and E. N. Ingalls, *J. Cell. Comp. Physiol.*, 1, 253 (1932).

assumption of several E groups per single colloidal carrier acting independently or together is equivalent merely to one reactant from the standpoint of kinetic theory and represents cases of the types already considered.

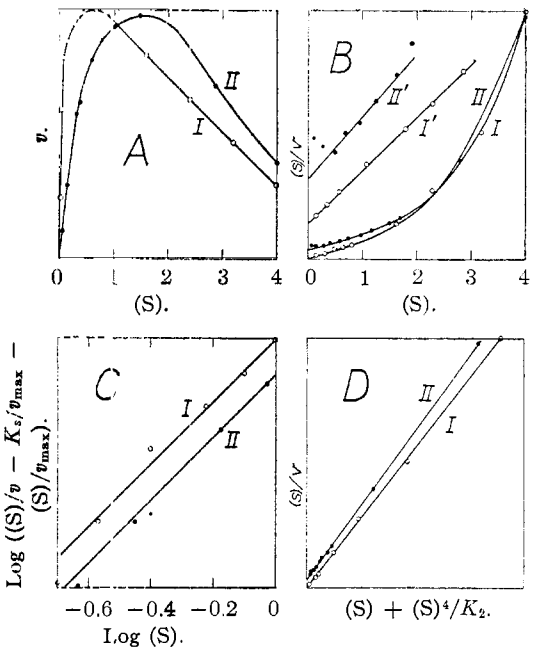

Fig. 3.—Evaluation of K_s, V_{max}, K_2, and n in Case III. Curve I, A, B, C, D, *Azotobacter chroococcum* respiration as a function of oxygen pressure, data of Burk.[9] Curve II, A, B, C, D, catalase activity as a function of H_2O_2 concentration, data of Stern.[10] v values: relative. (S) values: Curves IA, IB, I'B, $(O_2) \times 0.04$, 0.04, 0.1 %-atm., respectively; Curves IIA, IIB, II'B, $(H_2O_2) \times 8.6$, 8.6, 14.2 M, respectively. Log (S) values: Curve IC, (log % O_2) − 2; Curve IIC, [log (H_2O_2)] + 0.3.

Case III. (Oxygenase and Catalase.)—The velocity equation involved in substrate inhibition is (Ref. 1, p. 84)

$$v = V_{max}(S)/((S) + K_s + (S)^n/K_2) \qquad (8)$$

where K_2 is the dissociation constant $(ES)(S)^{(n-1)}/(ES_n)$ of the inactive enzyme-substrate compound. Equation 8 can be written in the form

$$(S)/v = K_s/V_{max} + (1/V_{max})((S) + (S)^n/K_2) \qquad (9)$$

A plot of $(S)/v$ against (S) yields a curve which becomes concave upward at high values of (S), where $(S)^n/K_2$ is no longer negligible. The linear limiting slope at low (S) values is $1/V_{max}$. (V_{max} is not the experimentally observed maximum velocity, see V_{opt} later.) The intercept is K_s/V_{max}, thus evaluating K_s. Figure 3B illustrates this method. Curves I and II are plots of data for oxygenase activity (Azotobacter respiration, Burk[9]) and catalase activity (Stern[10]) over the entire experimental substrate range. Curves I' and II', replots over the range of low (S), are used for the actual slope and intercept determinations. Figure 3A presents the original velocity data plotted directly against substrate concentration.

Equation 9 may be written in the logarithmic form

$$\log((S)/v - K_s/V_{max} - (S)/V_{max}) = n \log (S) - \log K_2 V_{max} \qquad (10)$$

A plot of the left-hand term against log (S) yields a straight line whose slope is n and whose intercept at S = 1 is $-\log K_2 V_{max}$, thus evaluating K_2. Figure 3C illustrates this method. Owing to the nature of the equation the greatest analytical accuracy will be obtained with high (S) values where the relative experimental error need be least.

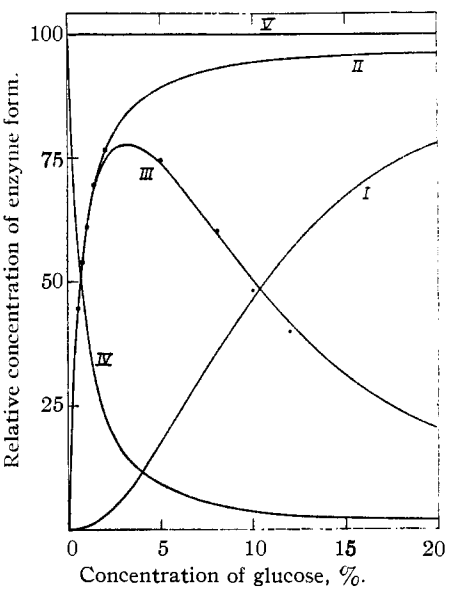

Fig. 4.—Distribution of E as a function of glucose concentration (Azotobacter respiration[9]). Curve I, calculated concentration of ES_3 (inactive). Curve II, calculated concentration of ES (active) without ES_3 formation. Curve III, calculated concentration of ES with ES_3 formation; (●), observed values. Curve IV, calculated concentration of E (Curve V − I − III). Curve V, total concentration of enzyme, free and combined, E + ES + ES_3 (Curve I + III + IV)

It follows from equation 9 that, having determined K_2 and n, the curves of Fig. 3A may be thrown into linear form by plotting $(S)/v$ against $((S) + (S)^n/K_2)$. Providing the mechanism in

(9) D. Burk, *J. Phys. Chem.*, **34**, 1207 (1930).
(10) K. G. Stern, *Z. physiol. Chem.*, **209**, 176 (1932).

Case III holds, a straight line will be obtained whose slope and intercept (as in Fig. 3B) are $1/V_{max}$ and K_s/V_{max}, respectively. Figure 3D illustrates this method of testing the applicability of this mechanism over the entire substrate concentration range.

Figure 4 shows the calculated relative distribution between E, ES (active) and ES_n (inactive) for Azotobacter respiration as a function of glucose concentration,[9] the curves being constructed on the basis of constants K_s, V_{max}, K_2 and n determined by the method illustrated in Fig. 3. Table II presents the various constants determined in connection with Figs. 3 and 4.

The optimum velocity (V_{opt}) is obtained at a finite value of (S) (see Table II) and hence is always less than V_{max}, which is theoretically obtained, even in the absence of inhibition, only at infinite (S). This is illustrated in Fig. 4, Curves III and V, and Table II, Columns 3 and 4.

Mechanisms are conceivable in which n need not be a whole number. *A priori*, also, n might be quite large, instead of 3 to 4 as found here, or 2 assumed by Stern. Haldane (Ref. 1, p. 85) assumed a value of 2 in the case of the data of Bamann[11] on sheep's liver lipase and Murray[12] confirmed this later for ethyl butyrate with more complete experimental data.

The case of Azotobacter respiration is especially interesting as a bimolecular reaction involving two substrates where high substrate concentration inhibition is given by both reactants, oxygen and glucose, and to fairly high powers of each. The oxygen function reported in Fig. 3A was obtained at a glucose concentration yielding approximately optimum rate (80% at 1% glucose instead of 100% at 3.2% as in Table II). The glucose function of Fig. 4 was obtained at an oxygen concentration yielding an approximately optimum rate (95 to 100% at 21% oxygen instead of 100% at 15%). As pointed out in a previous paper,[9] either chain reaction kinetics or contact catalysis might be involved in the conjugate maxima. Competitive inhibition between oxygen and glucose was held possible. The constants K_s and K_2 of Table II, however, were derived upon a basis of non-competitive inhibition between oxygen and glucose, and this postulated mechanism is apparently confirmed by the experimental data, Curve ID, Fig. 3, and Curve III, Fig. 4. Additional experiments of a direct nature substantiate this view. The percentage inhibition of respiration at different glucose concentrations was found to be practically independent of the oxygen pressure over a 22-fold range, 4.5 to 100×10^{-2} atm., and at different oxygen pressures independent of the glucose concentration over a 25-fold range, 0.2 and 5%, showing no competitive inhibition between substrates.

TABLE II

K_s, V_{max}, K_2 AND n VALUES IN CASE III

Column No.	1	2	3	4	5	6	7	8	9	10
Function Type figure	Substrate	K_s 3 B	V_{max} 3 B	V_{opt} 3 A	n 3 C	K_2 C 3	Low (S) at $1/2V_{opt}$ 3 A	High (S) at $1/2V_{opt}$ 3 A	$(S_{high})^{(n-1)}$ at $1/2V_{opt}$ 3 C	(S) at V_{opt}[a] 3 A
Azotobacter respiration[9]	O_2	0.75%	106	100	4.0	2×10^5%	0.70%	76%	4.4×10^5%	15%
Azotobacter respiration[9]	Glucose	.61%	100	78	3.0	10%	.45%	12.4%	154%	3.2%
Catalase (Fig. 3)	H_2O_2	.057 *M*	141	100	4.0	0.043 *M*	.033 *M*	0.4 *M*	0.064 *M*	0.17 *M*
Catalase (Stern[10])	H_2O_2	.033 *M*	...	100	2.0	.4 *M*	...	....	....	(.11 *M*)

[a] Calculated from $(S)V_{opt} = (K_sK_2/(n-1))^{1/n}$ (Ref. 1, p. 85).

The substrate concentrations at which $v = 1/2V_{opt}$ are not given by K_s (as in Case I) and $K_2^{1/(n-1)}$. Use of (S) at $1/2V_{opt}$ as an approximation to (S) at $1/2V_{max}$ will lead to low values of K_s and high values of K_2 for catalase activity, as shown by comparing Stern's values with those derived from Fig. 3 (Table II, Rows 6 and 7), and for all three cases in another manner comparing Columns 1 and 7, and Columns 6 and 9. Stern's value of $K_2 = (S)^{(n-1)} = 0.4$ *M* was based on a value of S at $1/2V_{opt}$ and an assumed value of $n = 2$ (as appears to be the case in certain lipases). Using $n = 4$, as found in Fig. 3, K_2 would be 0.064, still higher than the value $K_2 = 0.043$, from Fig. 3, because V_{opt} is less than V_{max}.

The experimental values of n in Table II are whole numbers to within the first decimal point and the values of K_2 reported are based upon intercepts at log (S) = 0 in Fig. 3C when the slopes are drawn with the exact whole number assumed.

(11) E. Bamann, *Ber.*, **62**, 1538 (1929).
(12) D. R. P. Murray, *Biochem. J.*, **24**, 1890 (1932).

Case III might be written, "E + S $\rightleftharpoons$ ES (active), E + nS $\rightleftharpoons$ ES$_n$ (inactive); $K_3 = (E)(S)^n/(ES_n)$." This introduces no change in the treatment except for employing the relation $K_3 = K_2K_s$ in Fig. 3C and 3D and Table II. There is also the more general case where the active form might be ES$_{n'}$ where n' is a number larger than 1 but less than n in the inactive form ES$_n$. This would involve some of the method of Case II, plotting $1/v$ against $1/(S)^{n'}$ or $(S)^{n'}/v$ against $(S)^{n'}$ to obtain K_s and V_{max}, etc.

Case IV. (Invertase.)—Competitive inhibition may be represented in the simplest case (*cf.* Case I) by the equation

$$1/v = (1/V_{max})(K_s + K_s(I)/K_I)(1/(S)) + 1/V_{max} \quad (11)$$

where (I) is the concentration of inhibitor and K_I the enzyme-inhibitor dissociation constant. The term $K_s(I)/K_I$ represents an increase in slope in the $1/v \times 1/(S)$ plot, when V_{max} is constant. Competitive inhibition occurs when the term "slope $\times$ V_{max}" is increased by inhibitor as in Curves 44 and 45, compared with the control 42, Fig. 5 and Table III (data of Kuhn and Münch[13]). Non-competitive inhibition is indicated by a lower V_{max} (higher ordinate intercept) as in Curve 43 (and possibly slightly in 44 and 45) compared to Curve 42, Fig. 5. The case of two or more competing substrates (Ref. 1, p. 85) is a special case of competitive inhibition where both enzyme-substrate complexes decompose irreversibly.

TABLE III

K_s, V_{max} AND K_I VALUES IN CASE IV

Curve	Inhibitor	$K_s + \frac{K_s(I)}{K_I}$	K_I	V_{max}	Apparent K_s (Kuhn and Münch[13])
42	.....	0.035	..	26.3	0.040
43	0.175 M α-glucose	.034	..	21.3	.040
44	.183 M β-glucose	.076	0.156	25.0	.070
45	.196 M fructose	.076	.167	25.0	.070

Case V. (Esterase.)—A given substrate forms more than one active enzyme complex. The velocity equation is

$$v = (V_{max-n}(S)^n/K_s + V_{max-n'}(S)^{n'}/K_{s-n'})/(1 + (S)^n/K_s + (S)^{n'}/K_{s-n'}) \quad (12)$$

V_{max-n} and $V_{max-n'}$ correspond to maximum velocities theoretically obtained when E exists completely in the active forms ES$_n$ and ES$_{n'}$, respectively, and K_s and $K_{s-n'}$ are corresponding dissociation constants. In this case a plot of v against (S) may pass through an optimum as in Case III, but approaches a constant, not zero, velocity, corresponding to $V_{max-n'}$. The values of the limiting slopes and intercepts of both $1/v \times 1/(S)$ and $(S)/v \times (S)$ plots may be used in evaluating the various constants occurring in equation 10. Schwab, Bamann and Laeverenz[14] worked out this case in connection with the action of human liver esterase on (−)-ethyl mandelic ester, the active forms being ES$_1$ and ES$_2$ ($n = 1$, $n' = 2$), K_s and $K_{s-n'}$ being 4.3×10^{-4} and 3.8×10^{-6} M. The ratio of V_{max-n} : V_{opt} : $V_{max-n'}$ is 1 : 0.81 : 0.584, V_{opt} occurring at about 3.9×10^{-3} M.

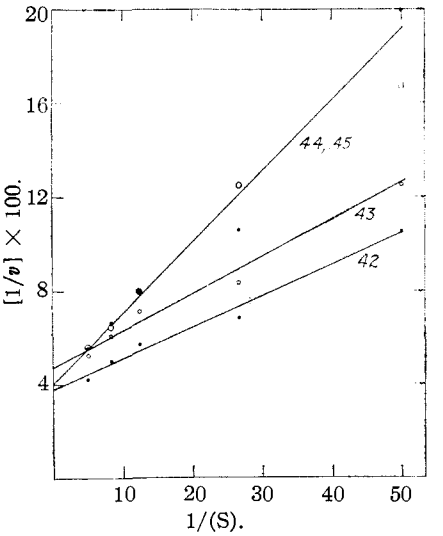

Fig. 5.—Competitive and non-competitive inhibition (Case IV). Curves 42, 43, 44, 45, invertase, data of Kuhn and Münch,[13] Tables 42, 43, 44, 45, respectively.

Case VI. (Urease.)—In velocity equations involving (ES) = steady state where $k_3 \doteq k_2$, K_s is replaced by an apparent dissociation constant $K_s' = (k_2 + k_3)/k_1 = K_s + k_3/k_1$ arrived at by Briggs and Haldane[15] for the mechanism

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \quad (13)$$

P is the final reaction product. A similar constant $K_s'' = k_3/k_1$ was obtained by Van Slyke and Cullen[16] assuming $k_2 = 0$. The velocity equation corresponding to equation 4 is (*cf.* Ref. 1, p. 40)

$$1/v = K_s'/V_{max}(S) + 1/V_{max} \quad (14)$$

A plot of $1/v$ against $1/(S)$ yields a straight line as in Case I but K_s' varies as a direct function of

(13) R. Kuhn and H. Münch, *Z. physiol. Chem.*, **168**, 1 (1927).

(14) G. M. Schwab, E. Bamann and P. Laeverenz, *Z. physiol. Chem.*, **215**, 121 (1933).

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

(16) D. D. Van Slyke and G. E. Cullen, *J. Biol. Chem.*, **19**, 141 (1914).

V_{max} (*i. e.*, of k_3), approaching a limiting value, K_s, at the ordinate intercept, which will be zero only if $k_2 = 0$. The substrate concentration at half-maximum velocity, $S_{V_{max}}/2$, varies with V_{max} also. Urease activity appears to involve either K_s' or K_s'' as a function of P_H (Ref. 1, p. 41), and competitive inhibition (Case IV) by hydrogen ion may also be involved. Instances of Case VI are probably uncommon. Obviously, K_s values obtained in Cases I-V are true dissociation constants *only* if they remain constant while the corresponding V_{max} is varied considerably.

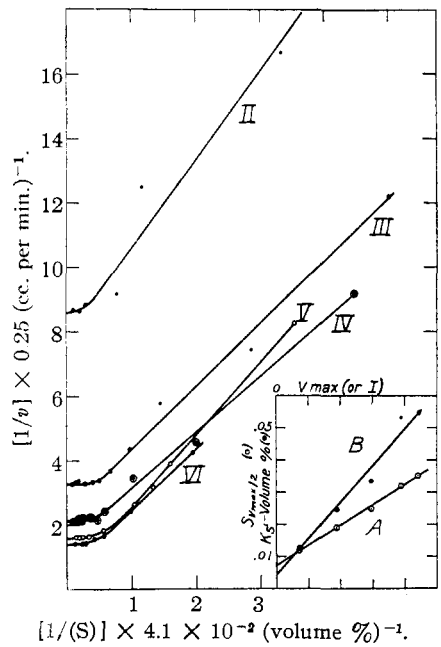

Fig. 6.—Evaluation of K_s and K_s', in Case VII (photosynthesis). Curves II, III, IV, V and VI correspond to relative light intensities I of 31.5, 80.4, 127, 175 and 191, respectively, data of Hoover, Johnston and Brackett,[17] 22°.

Case VII. (Diffusion.)—In this case the concentration of active intermediate is not directly proportional to (E)(S) but to (E)(S'), where S' is formed from S at a velocity comparable with ES ⟶ E + P. This case is very common and covers the activities of many intracellular enzymes, especially where rates of diffusion, as in tissues, often become limiting. Actually, the reaction S ⟶ S' may be either chemical or physical, but is more commonly the latter. The velocity equation can be shown to be

$$v = V_{max}k_1'(S)/(V_{max} + k_1K_s + k_1'(S) - v) \quad (15)$$

where k_1' is the velocity constant in S ⟶ S'. An explicit rather than the implicit solution of v in equation 15 involves a complex quadratic equation.

K_s, the true dissociation constant of ES (⇌ E + S), and k_1', may be evaluated by three methods (A, B, C). Placing $v = {}^1/_2V_{max}$ in equation 15 yields $S_{V_{max}}/2 = K_s + V_{max}/2k_1'$, so that plotting $S_{V_{max}}/2$ against V_{max} yields K_s as intercept and $1/2k_1'$ as slope (Method A, *cf.* Curve A, inset, Fig. 6). As in Case VI, $S_{V_{max}}/2$ varies directly with V_{max}, but in Case VII a plot of $1/v$ against $1/(S)$ no longer yields a straight line but a curve whose slope is $(1/k_1' + K_s/V_{max} - v/V_{max}k_1')/(1 - v^2/(S)V_{max}k_1')$. This is true since (ES) is no longer a rectangular hyperbolic function of (S). The limiting slope at low values of $1/(S)$ is K_s/V_{max} and the ordinate intercept is $1/V_{max}$ (Method B). As $1/(S)$ increases, the slope increases, approaching another limit, $K_s/V_{max} + 1/k_1' = K_{s'}/V_{max}$, as v becomes negligible. $1/k_1'$ may thus be evaluated by taking the difference between the two limiting slopes. A plot of $K_{s'}$ (= slope × V_{max} = $V_{max}/k_1' + K_s$) against V_{max} yields a line whose slope is $1/k_1'$ and whose intercept is K_s (Method C, *cf.* Curve B, inset, Fig. 6); it is also possible to plot the limiting slope $K_{s'}/V_{max}$ against $1/V_{max}$ (slope = K_s, intercept = $1/k_1'$).

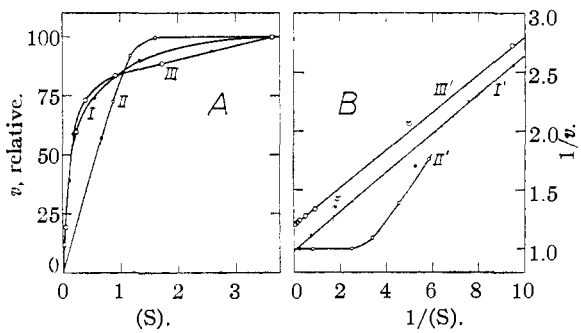

Fig. 7.—Elimination of diffusion steady state in Case VII (photosynthesis). Curves I and I', II and II', data of Van der Paauw,[19] *ca.* 20°, normal and narcotized Hormidium; Curve III and III', data of Warburg,[20] Table IV, Row 4, 25°. Curves I, II and III, (S) × 10%, 40% and 5 × 10^5 *M*. Curves I' and II', $(1/v) \times 100$, $(1/(S)) \times 0.1$; Curve III', $(1/v + 0.02) \times 50$, $(1/(S)) \times 5 \times 10^6$. $1/v$ values in investigators' units.

Figure 6 and Fig. 7B Curve II' illustrate the nature of the curves one might obtain in a diffusion-limited enzymic process (data of Hoover, Johnston and Brackett[17]). Van den Honert[18] showed by independent direct means that the rate

(17) W. H. Hoover, E. S. Johnston and F. S. Brackett, *Smithsonian Misc. Coll.*, **87**, No. 16 (1933).

(18) T. H. Van den Honert, *Rec. trav. bot. néerl.*, **27**, 149 (1930).

of diffusion of dissolved carbon dioxide was normally limiting at high light intensities and high temperatures in the case of the filamentous alga Hormidium. Van der Paauw[19] later confirmed this indirectly by lowering V_{max} sufficiently with narcotic (presumably without effect on the rate of carbon dioxide diffusion in the stomata-free Hormidium) to obtain a linear $1/v$ against $1/(S)$ plot (Curve I', compared to Curve II', Fig. 7B). Curve II, Fig. 7A, is a close approximation to that required by strict application of the well-known Blackman Principle of Limiting Factors (*cf.* Ref. 17, p. 17; Ref. 18, p. 149; Ref. 19, p. 500), whereas Curve I is the usual rectangular hyperbola. This general principle, which limits the extent of the transition range where photosynthesis is limited simultaneously by two factors such as carbon dioxide pressure and light intensity, would appear to receive analytical interpretation in the treatment of Case VII given here. The unicellular green alga chlorella studied by Warburg[20] and Emerson and Arnold[21] (continuous light) yielded a linear $1/v$ against $1/(S)$ plot, in agreement with Warburg's view that diffusion is not here limiting (*cf.* Curve III and III', Fig. 7A and 7B).

We do not propose that all of the known pertinent data on the influence of carbon dioxide pressure in the photosynthetic mechanism can be represented by equation 15, since, *a priori*, the nature of the light reaction, only partially understood, has not been included. The problem of photosynthesis, of widespread interest, involves much data and numerous postulated mechanisms which are generally of limited applicability and not easily correlated. For these reasons numerical values of the constant K_s for wheat and Hormidium, obtainable by Methods A, B, or C, are not for the time being given. Until more uniform postulates, based to some extent on data other than kinetic, can be found in the literature, this procedure seems desirable. The general methods presented, however, may be used in any detailed consideration of proposed or to-be-proposed photosynthetic mechanisms. The qualitative analysis given for wheat indicates that, as actually found for Hormidium, the rate of diffusion of the substrate carbon dioxide is, at low and intermediate pressures, normally limiting, even at low light intensities where the rate of photosynthesis at high carbon dioxide pressures is directly proportional to the light intensity. Further experimentation on the effect of temperature at high light intensities and low carbon dioxide pressures could assist in deciding this point further. Although Case VII is common, unfortunately no data on any case, particularly of a simpler nature than photosynthesis, have come to our attention which are susceptible of strict quantitative analysis. In this connection, interesting model experiments might be devised, using either enzymes or catalysts in general.

The treatment of Case VII does not resolve the data into a linear plot over the entire substrate concentration range. However, it possesses the advantage of offering three methods of obtaining K_s in the simple case, and is capable of evaluating additional constants as well in more complex cases. In applying Case VII, it is necessary to be able to vary V_{max}, as for example, by non-competitive inhibitors, P_H, possibly temperature, etc.

(19) F. Van der Paauw, *Rec. trav. bot. néerl.*, **29**, 497 (1932).
(20) O. Warburg, *Biochem. Z.*, **100**, 230 (1919).
(21) R. Emerson and W. Arnold, *J. Gen. Physiol.*, **15**, 409 (1932).

Discussion

If mathematical representation be given a set of kinetic data one should be in a superior position to test an assigned mechanism, first with respect to other known facts, and second with respect to experimentation suggested by such representations. The methods for correlating a given mechanism and set of data based on the linear graphical methods suggested in this paper place emphasis upon testing the qualitative nature of a mechanism before obtaining the numerical values of the related constants involved.

Mathematical representations of kinetic data are not necessarily limited to demonstrated cases of enzymic catalysis, but may cover complex physiological processes like respiration, photosynthesis, chemical autotropism, or nitrogen fixation, where the formal or arbitrary assignment of enzymic nomenclature, based on methods of physical rather than organic chemistry, may, or may not, be conventional. *In vitro* demonstrations need not be prerequisite to mathematical analysis of data obtained from *in vivo* systems.

The writers are indebted to Dr. A. K. Balls, Dr. P. H. Emmett and Dr. W. E. Deming of the Bureau of Chemistry and Soils for valuable criticism and suggestions.

Summary

1. Graphical methods involving constant slopes and straight line extrapolations have been

developed for testing and interpreting kinetic data, and for determining dissociation constants of enzyme-substrate and enzyme-inhibitor compounds and other related constants when the data are found to be consistent with an assigned mechanism.

2. Representative analyses are given for invertase, raffinase, amylase, citric dehydrogenase, catalase, oxygenase, esterase and lipase, involving substrate activation, substrate inhibition, general competitive and non-competitive inhibition, steady states and reactions of various orders.

3. A plot of the reciprocal of the observed velocity v against the reciprocal of the substrate concentration (S) yields in the simplest case (*e. g.*, invertase, amylase) a straight line whose slope and ordinate intercept yield K_s (Michaelis dissociation constant) and V_{max} (theoretical maximum velocity). In the presence of competitive inhibitors the slope is increased but the intercept is unchanged. With non-competitive inhibitors the intercept also is raised. When the active intermediate contains n molecules of S a straight line is obtained upon plotting the reciprocal of $(S)^n$, instead of the reciprocal of (S), which would yield a curve concave upward. A steady-state occurring before the formation of the active intermediate, to whose concentration v is proportional, will yield a curve with two limiting slopes. Curves of v plotted directly against (S), passing through an optimum and approaching a zero value, indicate the existence of an additional inactive intermediate (catalase), whereas approaching a constant value indicates two or more active intermediates (*l*-ethyl mandelic esterase). The constants involved in the three latter cases are determined from limiting slopes and intercepts of various plots.

4. The various methods described are applicable to general chemical catalysis, homogeneous or heterogeneous, and possess many advantages of usefulness and convenience over less extensively developed methods employed heretofore. They are capable of eliminating certain postulated mechanisms, and indicating what mechanism or mechanisms *may* be involved, though not necessarily proving what mechanism *is* involved, in a given case.

WASHINGTON, D. C. RECEIVED AUGUST 18, 1933

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

Orientation in the Furan Nucleus. VIII. β-Acylaminofurans

BY ROBERT R. BURTNER

This report is concerned with the synthesis of one of the more promising precursors of simple β-aminofurans, namely, the β-acylaminofurans. The increased availability of 3-furoic acid[1] opens an attractive avenue of approach to 3-acylaminofuran. The following sequence of reactions illustrates the synthesis of 3-benzoylaminofuran

$$3\text{-}C_4H_3OCOOH \longrightarrow 3\text{-}C_4H_3OCOOC_2H_5 \xrightarrow{NH_2NH_2}$$
$$3\text{-}C_4H_3OCONHNH_2 \xrightarrow{HNO_2} 3\text{-}C_4H_3OCON_3 \longrightarrow$$
$$3\text{-}C_4H_3ONCO \xrightarrow{C_6H_5MgBr} 3\text{-}C_4H_3ONHCOC_6H_5$$

In like manner, 2-methyl-3-benzoylaminofuran was prepared from 2-methyl-3-furoic acid, and the corresponding 2-methyl-3-acetaminofuran was obtained by the use of methylmagnesium iodide instead of phenylmagnesium bromide.

(1) Gilman and Burtner, THIS JOURNAL, 55, 2905 (1933).

Experimental Part

3-Furoyl Hydrazide.—This hydrazide was prepared from ethyl 3-furoate and hydrazine hydrate in a customary manner. The yield was 75% and the compound melted at 124–124.5° after crystallization from a benzene–methanol mixture.

Anal. Calcd. for $C_5H_6O_2N_2$: C, 47.61; H, 4.76. Found: C, 47.37; H, 5.01.

3-Furyl Isocyanate.—A solution of 12 g. (0.1 mole) of 3-furoyl hydrazide in 300 cc. of water was mixed with 75 cc. of 2 *N* sulfuric acid and the resulting solution chilled to 0°. To this was added dropwise and with shaking a solution of 7 g. (0.11 mole) of sodium nitrite in 30 cc. of water. The azide was removed by extraction with ether, and this extract was washed with water and then dried over sodium sulfate. Approximately three-fourths of the ether was removed by distillation; then 90 cc. of dry benzene was added; and, finally, the remaining ether was removed by distillation. The benzene solution of 3-furoyl azide was then carefully heated until the calculated volume of nitrogen was evolved for con-