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The Inhibition of the α-Chymotrypsin-catalyzed Hydrolysis of Nicotinyl-L-tryptophanamide and of Methyl Hippurate by Indole[1]

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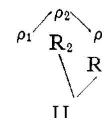
It has been observed that while indole competitively inhibits the α-chymotrypsin-catalyzed hydrolysis of the trifunctional specific substrate, nicotinyl-L-tryptophanamide, it causes an entirely different type of inhibition in the hydrolysis of the bifunctional specific substrate, methyl hippurate. A consideration of the steric implications of the three center hypothesis has led to the conclusion that only with a bifunctional specific substrate and a monofunctional inhibitor will there be formed, in addition to the binary complexes arising from the separate interaction of the inhibitor and the specific substrate with the enzyme, a ternary complex resulting from the concurrent interaction of both the inhibitor and the specific substrate with the enzyme. Detailed analyses of the experimental data indicate that the kinetic behavior of the system α-chymotrypsin-methyl hippurate-indole is consistent with the above prediction and, if it is assumed that the affinity of α-chymotrypsin for indole is identical in the two reactions studied, that the ternary complex ESI is hydrolyzed at a significantly lower rate than the binary complex ES. These results, therefore, provide support for the three center hypothesis as applied to α-chymotrypsin.

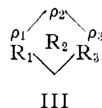
Our present knowledge[3-5] of the structural requirements of synthetic specific substrates of α-chymotrypsin is consistent with the hypothesis that a typical trifunctional specific substrate of the general formula $R_1CHR_2R_3$,[6] derived from an L-α-amino acid, combines with the catalytically active site of the enzyme at three centers, ρ_1, ρ_2 and ρ_3, which present a complementary aspect to the three characteristic structural features, R_1, R_2 and R_3, of the specific substrate.[7] Although the nature of the forces involved in each of the three interactions, $R_1-\rho_1$, $R_2-\rho_2$ and $R_3-\rho_3$, is by no means well understood, it may be inferred from purely steric considerations, that if a close fit does exist between the R groups and the ρ centers, a blocking of any one, or two of the three centers will result in an impairment of the ability of the active site as a whole to combine with a trifunctional specific substrate. Thus, it may be predicted that a compound which contains but two R groups separated from each other by the optimum distance, or one which may be considered to consist of only a single R group, should be capable of functioning as a competitive inhibitor in the α-chymotrypsin-catalyzed hydrolysis of a trifunctional specific substrate. A study of the effect of various compounds on the kinetics of hydrolysis of nicotinyl-L-tryptophanamide and of acetyl-L-tyrosinamide has provided examples of competitive inhibition of the α-chymotrypsin-catalyzed hydrolysis of a trifunctional specific substrate by all three possible types of bifunctional inhibitors, *i.e.*, $R_1CH_2R_2$,[8] $R_2CH_2R_3$[9,10] and $R_1CH_2R_3$,[11] and competitive inhibition of the α-chymotrypsin-catalyzed hydrolysis of a trifunctional specific substrate by at least one monofunctional inhibitor, *i.e.*, benzamide, has been observed.[10]

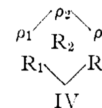
It is known that both types of bifunctional compounds in which a particularly susceptible R_3 group is present, *i.e.*, $R_1CH_2R_3$ as in methyl hippurate[11] and $R_2CH_2R_3$ as in methyl β-phenylpropionate,[12] can, when the second R group in the molecule possesses a relatively high affinity for the enzyme, serve as specific substrates for α-chymotrypsin. Thus the availability of bifunctional specific substrates and monofunctional inhibitors makes it possible to devise a more stringent test of the steric requirements of the three-center hypothesis than has been hitherto practicable.

In a system containing enzyme and a bifunctional specific substrate the intermediate complex ES,[13] which is subsequently transformed into free enzyme and reaction products, can be represented by formulas I or II. The three center hypothesis



requires that in each complex one of the ρ centers at the active site remains unoccupied. If the combining power of the vacant ρ center in I and II is retained, at least in part, the introduction of a monofunctional inhibitor, R_2 or R_1, into the system will lead to the formation of the ternary complex, III or IV. The kinetic behavior of such a system will be dependent upon the properties of the ternary





complex. It will, however, no longer assume the character of a classical competitive system as might be expected of one containing enzyme, a monofunctional inhibitor and a trifunctional specific substrate. It thus appears possible, from an analysis of kinetic data relative to the enzymatic hydrolysis of a suitable bifunctional specific substrate in the presence of an appropriate monofunctional inhibitor, to deduce the existence of a ternary complex of type III and IV, which would in turn provide an indirect

(1) Supported in part by a grant from Eli Lilly and Company.

(2) To whom inquiries regarding this article should be sent.

(3) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(4) H. J. Shine and C. Niemann, THIS JOURNAL, **74**, 97 (1952).

(5) H. T. Huang, R. J. Foster and C. Niemann, *ibid.*, **74**, 105 (1952).

(6) Where R_3 is a functional derivative of the carboxyl group; R_2, the α-amino acid side chain; and R_1, the remaining unspecified substituent of the α-carbon atom.

(7) H. T. Huang and C. Niemann, *ibid.*, **73**, 3223 (1951).

(8) H. T. Huang and C. Niemann, *ibid.*, **74**, 101 (1952).

(9) H. Neurath and J. Gladner, *J. Biol. Chem.*, **188**, 407 (1951).

(10) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 5963 (1952).

(11) H. T. Huang and C. Niemann, *ibid.*, **74**, 4634 (1952).

(12) J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949).

(13) For definition of the symbols used in this communication, *cf.* H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

demonstration of the plausibility of the three center hypothesis.

Preliminary Studies

Methyl hippurate, $R_1CH_2R_3$, where R_1 = benzamido and R_3 = carbomethoxy, was selected as the bifunctional specific substrate for the proposed study, because its kinetics of hydrolysis had been previously studied in aqueous media,[11] and it had had been shown to react with the same catalytically active site as that involved in the α-chymotrypsin-catalyzed hydrolysis of a number of trifunctional specific substrates of the acylated α-amino acid amide type.[11] In order to find a suitable monofunctional inhibitor of structure R_2 the inhibition of the α-chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tryptophanamide by several monofunctional compounds was examined, and the results of these experiments are summarized in Table I. It is apparent that of the compounds listed in Table I indole is the most useful inhibitor for the proposed study. It not only possesses a high affinity for the enzyme but is also sufficiently soluble in aqueous media to permit its use over a wide range of inhibitor concentrations. The observed order of affinity, indole >> phenol, was anticipated from previous studies.[14,15]

TABLE I

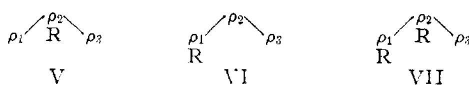
INHIBITION OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF NICOTINYL-L-TRYPTOPHANAMIDE BY SEVERAL MONOFUNCTIONAL COMPOUNDS[a-c]

Inhibitor	[I][d]	Inhibition, %	Approx. K_I[e]
Cyclohexanol	36	9	80
Pyridine	50	20	50
Nicotinamide	20	8	50
Phenol	20	38	7.0
Skatole	5	52	1.0
Indole	5	60	0.72

[a] In aqueous solutions at 25° and pH 7.9, 0.02 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer, with the rate determined by a formol titration.[13] [b] [S] = 10×10^{-3} M, $K_S = 2.7 \times 10^{-3}$ M.[13] [c] [E] equivalent to 0.208 mg. protein-nitrogen per ml. [d] [I] in units of 10^{-3} M. [e] Calculated on the assumption that the inhibition is competitive, K_I in units of 10^{-3} M.

Effect of Indole on the Hydrolysis of Nicotinyl-L-tryptophanamide

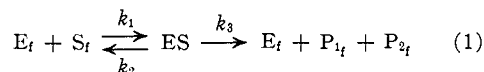
Although it is to be expected that indole will interact principally with the ρ_2 center, it cannot be assumed that it will do so exclusively. It should be realized that the exact mode of interaction of a monofunctional inhibitor of the type R_1, or R_2, with the active site of the enzyme as visualized in the three center hypothesis, is a matter of considerable uncertainty. For example, it has been noted previously[10] that a compound such as benzamide, because of its structual relationship to a number of common R_2 and R_1 groups, may be capable of interacting not only with ρ_2 but also with ρ_1, to form the binary complexes EI (V) and EI′ (VI).



(14) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1535 (1951).
(15) D. W. Thomas, R. V. MacAllister and C. Niemann, *ibid.*, **73**, 1548 (1951).

Either V or VI can react with a second molecule of the inhibitor to form the ternary complex VII. As the formation of all three of the above complexes may prevent the active site from combining with a trifunctional specific substrate it is, therefore, advisable that a detailed analysis be made of the effect that the formation of a ternary complex such as VII may have on the kinetics of hydrolysis of a trifunctional specific substrate in the presence of a monofunctional inhibitor such as indole. We have accordingly studied in detail the system α-chymotrypsin–nicotinyl-L-tryptophanamide–indole. The experimental conditions were identical with those employed in the preliminary investigation, with appropriate variations in the specific substrate and inhibitor concentrations.

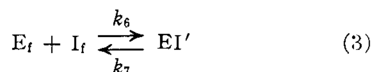
The reaction between the enzyme and the specific substrate may be formulated in terms of equation (1), where $K_S = (k_2 + k_3)/k_1$. Since in all experi-



$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1_f} + P_{2_f} \quad (1)$$

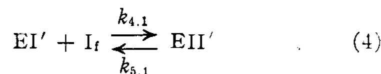
ments the extent of hydrolysis of the specific substrate was limited to within 30%, the reaction of the enzyme with one of the hydrolysis products[13] can be justifiably ignored. On the basis of the assumption that indole reacts with ρ_2 to give EI (V), and that it also reacts with ρ_1 to give EI′ (VI) we may introduce equations (2) and (3) where $K_I = k_5/k_4$ and

$$E_f + I_f \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI \quad (2)$$



$$E_f + I_f \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EI' \quad (3)$$

$K_\iota = k_7/k_6$. The ternary complex EII′ (VII) may be formed from either EI′ or EI according to equations (4) or (5).



$$EI' + I_f \underset{k_{5.1}}{\overset{k_{4.1}}{\rightleftharpoons}} EII' \quad (4)$$

$$EI + I_f \underset{k_{7.1}}{\overset{k_{6.1}}{\rightleftharpoons}} EII' \quad (5)$$

When all reactants possess unit activity coefficients it follows that

$$d[EI]/dt = k_4[E_f][I_f] + k_{7.1}[EII'] - k_5[EI] - k_{6.1}[EI][I_f] \quad (6)$$

$$d[EI']/dt = k_6[E_f][I_f] + k_{5.1}[EII'] - k_7[EI'] - k_{4.1}[EI'][I_f] \quad (7)$$

$$d[EII']/dt = k_{4.1}[EI'][I_f] + k_{6.1}[EI][I_f] - (k_{5.1} + k_{7.1})[EII'] \quad (8)$$

If it is assumed, as a first approximation, that $k_4 >> k_{4.1}$, $k_5 >> k_{5.1}$, $k_6 >> k_{6.1}$ and $k_7 >> k_{7.1}$ then for the steady state condition, $d[EI]/dt = d[EI']/dt = d[EII']/dt \rightarrow 0$, and with $K_S = [S_f][E_f]/[ES]$, $K_I = [I_f][E_f]/[EI]$ and $K_\iota = [I_f][E_f]/[EI']$, equations (6), (7) and (8) lead to the relations given in equations (9) and (10).

$$[EII'] = [I_f]^2 \times K_S/[S_f] \times [ES]/C_1 \quad (9)$$

$$1/C_1 = (k_{4.1}/K_\iota + k_{6.1}/K_I)(1/(k_{5.1} + k_{7.1})) \quad (10)$$

If by definition $k_{5.1}/k_{4.1} = k_{1.1}$ and $k_{7.1}/k_{6.1} = K_{\iota.1}$ equation (10) may be transformed into equation

(11). When $[S_f] = [S]$ and $[I_f] = [I]$, *i.e.*, when the system is in zone A with respect to both specific

$$1/C_I = 1/K_\iota K_I(K_I k_{5.1}/K_{I.1} + K_\iota k_{7.1}/K_{\iota.1})1/(k_{5.1} + k_{7.1}) \quad (11)$$

substrate and competitive inhibitor,[16,17] [EI], [EI′] and [EII′] may be expressed in terms of [ES] as in equation (12). If by definition $v = k_3[ES]$

$$[ES] = [E][S]/(K_S(1 + [I]/K_I + [I]/K_\iota + [I]^2/C_I) + [S]) \quad (12)$$

and $V = k_3[E]$ equation (12) can be transformed into equation (13).

$$1/v = K_S/V(1 + [I]/K_I + [I]/K_\iota + [I]^2/C_I)1/[S] + 1/V \quad (13)$$

Thus for a series of experiments at a constant inhibitor concentration a plot of $1/v_0$ *versus* $1/[S]_0$ would be expected to give a straight line with the same intercept as for the case when $[I] = 0$. Comparing equation (13) with the Lineweaver and Burk equation for competitive inhibition,[18] *i.e.*, equation (14) it is evident that the value of the

$$1/v = K_S/V(1 + [I]/K_I')1/[S] + 1/V \quad (14)$$

apparent enzyme–inhibitor dissociation constant K_I', determined from a $1/v_0$ *versus* $1/[S]_0$ plot assuming simple competitive inhibition, will be given by equation (15).

$$K_I' = K_I K_\iota/(K_I + K_\iota + K_I K_\iota[I]/C_I) \quad (15)$$

It is seen from equation (15) that the value of K_I' is not independent of [I] and will increase as [I] is increased. The actual contribution of the term containing [I] to the value of K_I' will be dependent upon the magnitude of the relative affinity ratio $K_I/K_\iota/C_I$ which may be considered as an index relating the affinity of the binary complexes and that of the free enzyme for the inhibitor. The relation between $K_I K_\iota$ and C_I may be appreciated from an examination of equation (11) and by comparing the affinity of the inhibitor for the free enzyme E_f, *i.e.*, the K_I and K_ι values, with that for the binary complexes EI and EI′, *i.e.*, the $K_{I.1}$ and $K_{\iota.1}$ values. Three possible conditions may exist, *viz.*, (a) $K_{I.1} < K_I$ and $K_{\iota.1} < K_\iota$ with $C_I < K_I K_\iota$; (b) $K_{I.1} = K_I$ and $K_{\iota.1} = K_\iota$ with $C = K_I K_\iota$; and (c), $K_{I.1} > K_I$ and $K_{\iota.1} > K_\iota$ with $C_I > K_I K_\iota$. For steric reasons it appears unlikely that an interaction between indole and ρ_1 or ρ_2 will facilitate the interaction of a second molecule of indole with one of the remaining ρ centers. Thus it may be assumed that the first condition considered above can be ignored and that the term $K_I K_\iota/C_I$ can be considered to be equal to or less than unity.

TABLE II

APPARENT ENZYME–INHIBITOR DISSOCIATION CONSTANTS OF α-CHYMOTRYPSIN AND INDOLE[a,b]

$[I]^c$	0.70	1.60	2.40	5.0
$K_I'^c$	0.71	0.68	0.68	0.72

[a] At 25° and pH 7.9 in aqueous solutions 0.02 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. [b] Specific substrate nicotinyl-L-tryptophanamide, $K_S = 2.7 \times 10^{-3}$ M. [c] In units of 10^{-3} M.

(16) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).

(17) A. Goldstein, *ibid.*, **27**, 52 (1944).

(18) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

The results of experiments at four different inhibitor concentrations are summarized in Fig. 1 and in Table II. These data indicate that the system is essentially one of competitive inhibition because within the range of the values of [I] studied, *i.e.*, 0.70 to 5.0 × 10^{-3} M, no apparent decrease in the K_I' value was observed.

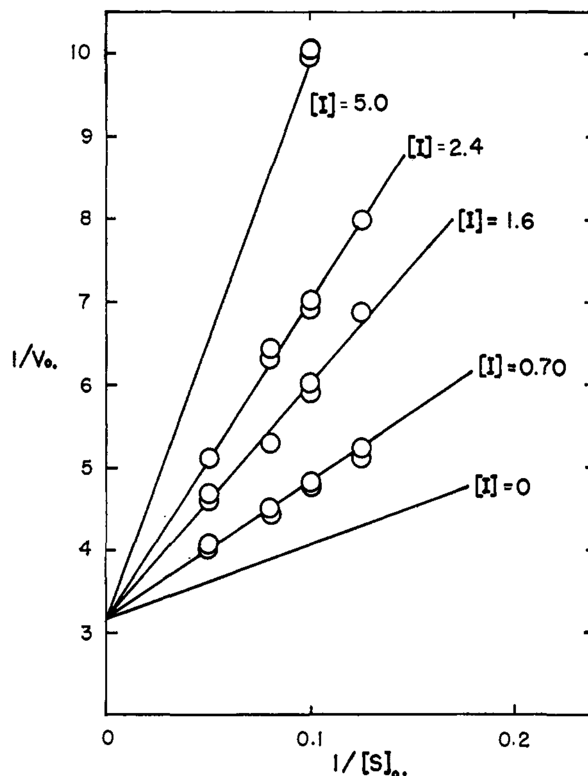


Fig. 1.—Competitive inhibition of the α-chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tryptophanamide by indole in aqueous solutions at 25° and pH 7.9; v_0 in units of 10^{-3} M/min.; [S] and [I] in units of 10^{-3} M; [E] equivalent to 0.208 mg. protein nitrogen/ml.; 0.02 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

Two sets of conditions will cause the term containing [I] in equation (15) to become negligible, *viz.*, (a) when the affinity of the inhibitor for one of the two reactive ρ centers is much greater than that for the other, *e.g.*, when $K_\iota >> K_I$ then $K_I' \rightarrow K_I$; and (b) when $C_I >> K_I K_\iota$ then $K_I \rightarrow K_I K_\iota/(K_I + K_\iota)$. It may be argued that if the condition $C_I >> K_I K_\iota$ is true, then $K_{I.1} >> K_I$ and $K_{\iota.1} >> K_\iota$, and the concentration of EII′ in the system will be experimentally unimportant. In other words, this condition implies that the combination of either ρ_1 or ρ_2 with indole will effectively hinder the vacant ρ centers from interacting with another indole molecule. While the structure and properties of the active site may be such that this condition can be accommodated, such a limitation makes it extremely improbable that any significant amount of a ternary complex of type III could be formed and the system α-chymotrypsin–methyl hippurate–indole would also be expected to behave as one involving only classical competitive inhibition. Since the results of studies on the latter system, which are presented later, do show marked deviations

from the kinetics of classical competitive inhibition, it appears reasonable to assume that the first condition postulated above is true, and that the interaction between indole and the active site principally involves the center ρ_2.

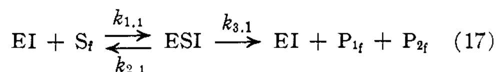
It may, of course, be contended that in the system under consideration both of the above conditions are only partially operative, *i.e.*, $K_\iota > K_I$ but not $>> K_I$ and $C_I >$ but not $>> K_I K_\iota$. While K_I, K_ι and C_I all have significant values relative to each other, the two factors are jointly effective in reducing the actual degree of dependence of the K_I' value on the value of [I]. It is of interest to define the limiting values for K_I/K_ι and $K_I K_\iota/C_I$. For an error of $\pm 5\%$ in the mean K_I' value it may be assumed that K_I' at $[I] = 0.70 \times 10^{-3}\ M$, is 0.70, and K_I' at $[I] = 5.0 \times 10^{-3}\ M$ is 0.655. C_I is then 50.6. Thus, if $K_I/K_\iota > 0.10$ then $K_I K_\iota/C_I$ must be <0.12. Considering that $K_I K_S/C_\sigma$ is substantially larger than 0.1, then $K_I K_\iota/C_I$ is also probably substantially larger than 0.1, and it appears that the assumption $K_\iota >> K_I$ is, for all practical purposes, a valid approximation for the system under consideration.

Effect of Indole on the Hydrolysis of Methyl Hippurate

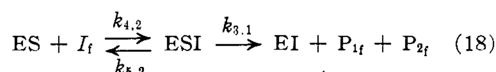
The system α-chymotrypsin–methyl hippurate–indole was studied under the same conditions and within the same limitations as those employed in the previous investigation of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate itself.[11] In the following discussion of various modes of interaction of the inhibitor, the specific substrate and the enzyme it is assumed that of the possible reactions between indole and α-chymotrypsin, as depicted by equations (2), (3) and (4), only (2) need be considered as significant, *i.e.*, $K_I' = K_I$. As has been pointed out previously,[11] methyl hippurate ($R_1CH_2R_3$, R_1 = benzamido and R_3 = carbomethoxy) may react with the active site of the enzyme in two ways to give: the susceptible complex ES (R_1 interacts with ρ_1 and R_3 with ρ_3), as in I, and the inert complex ES′ (R_1 interacts with ρ_2 and R_3 is free). Thus, in addition to equations (1) and (2) it is necessary to consider equation (16) where $K_\sigma = k_9/k_8$.

$$E_f + S_f \underset{k_9}{\overset{k_8}{\rightleftarrows}} ES' \qquad (16)$$

The ternary complex ESI (III) if it is formed in the system, may then be derived from both EI and ES according to equations (17) and (18) where $k_{3.1}$ is



$$EI + S_f \underset{k_{2.1}}{\overset{k_{1.1}}{\rightleftarrows}} ESI \xrightarrow{k_{3.1}} EI + P_{1f} + P_{2f} \qquad (17)$$



$$ES + I_f \underset{k_{5.2}}{\overset{k_{4.2}}{\rightleftarrows}} ESI \xrightarrow{k_{3.1}} EI + P_{1f} + P_{2f} \qquad (18)$$

assumed to be the specific rate constant for the transformation of ESI into EI and reaction products. With unit activity coefficients assumed for all reactants it is evident from equations (17) and (18) that d[ESI]/dt is given by equation (19).

$$d[ESI]/dt = k_{1.1}[EI][S_f] + k_{4.2}[ES][I_f] - (k_{2.1} + k_{5.2} + k_{3.1})[ESI] \qquad (19)$$

For the simplifying assumptions that $k_1 >> k_{1.1}$, $k_2 >> k_{2.1}$, $k_4 >> k_{4.2}$ and $k_5 >> k_{5.2}$, *i.e.*, $K_S = [E_f][S_f]/[ES]$ and $K_I = [E_f][I_f]/[EI]$, and that $[S_f] = [S]$ and $[I_f] = [I]$, *i.e.*, the system is in zone A with respect to both the specific substrate and the inhibitor, we have, when d[ESI]/dt = 0, equations (20), (21) and (22). If $K_{S.1}$ is defined as

$$[ESI] = [I](K_S[ES]/C_\sigma) \qquad (20)$$

$$1/C_\sigma = (k_{1.1}/K_1 + k_{4.2}/K_S)(1/(k_{2.1} + k_{3.1} + k_{5.2})) \qquad (21)$$

$$[ES] = [E][S]/(K_S(1 + [I]/K_1) + [S](1 + K_S/K_\sigma + K_S[I]/C_\sigma K_I)) \qquad (22)$$

$(k_{2.1} + k_{3.1})/k_{1.1}$ and $K_{I.2}$ as $k_{5.2}/k_{4.2}$ equation (20) may be rearranged to give equation(23). For the same reasons advanced to interpret the significance of

$$1/C_\sigma = 1/K_S K_I[(k_{2.1} + k_{3.1})K_S/K_{S.1} + k_{5.2}K_I/K_{I.2}][1/(k_{2.1} + k_{3.1} + k_{5.2})] \qquad (23)$$

C_I, it is probable that $K_S \leq K_{S.1}$ and $K_I \leq K_{I.2}$ so that the affinity ratio $K_S K_I/C_\sigma$ may be considered as being equal to or less than unity. If by definition $v = k_3[ES] + k_{3.1}[ESI]$, *i.e.*, $v = k_3([ES] + r[ESI])$, where $r = k_{3.1}/k_3$, we arrive at equation (24) where $V = k_3[E]$.

$$1/v = K_S/V(1 + [I]/K_I)(1/(1 + rK_S[I]/C_\sigma))1/[S] + 1/V(1/(1 + rK_S[I]/C_\sigma))(1 + K_S/K_\sigma + K_S[I]/C_\sigma) \qquad (24)$$

It will be recalled[11] that $1/V' = 1/V(1 + K_S/K_\sigma)$, $K_S/V = K_S'/V'$ and $V' = k_3'[E]$. Hence equation (24) can be rearranged to give the more useful equation (25). Thus, for the present system, a plot of $1/v_0$ *versus* $1/[S]_0$ will also give a straight

$$1/v = K_S'/V'(1 + [I]/K_I)(1/(1 + rK_S[I]/C_\sigma))1/[S] + (1/(1 + rK_S[I]/C_\sigma))(1/V' + (K_S'/V')([I]/C_\sigma)) \qquad (25)$$

line, but now both the slope and the intercept are functions of the inhibitor concentration. When equation (25) is compared with the equivalent equation for the case where [I] = 0, it will be appreciated that the value of the ratio r has a profound effect on the value of the slope and of the intercept. If $r = 0$, *i.e.*, $k_{3.1} = 0$, then the slope will be identical with that calculated on the basis of simple competitive inhibition, but the intercept will be raised by the amount $K_S'[I]/V'C_\sigma$. This situation is analogous to the case of so-called non-competitive inhibition so familiar in enzyme kinetics.[18–20] However, if r is finite, the value of $(1 + rK_S[I]/C_\sigma)$ is greater than unity, and the slope should be less than that calculated on the basis of simple competitive inhibition. However, the slope may be less than, equal to, or greater than the quantity K_S'/V', *i.e.*, the slope for the case where [I] = 0, depending upon whether the quantity C_σ/rK_S is greater than, equal to, or less than K_I. Similarly, the position of the intercept will be determined by the relative values of rK_S and K_S', *i.e.*, the intercept will be less than, equal to, or greater than $1/V'$ when rK_S is greater than, equal to or less than K_S'.

The experimental results obtained at two inhibitor concentrations are summarized in Fig. 2. It is evident that the observed plots indicate a direct proportionality between $1/v_0$ and $1/[S]_0$.

(19) P. W. Wilson in H. A. Lardy "Respiratory Enzymes," Burgess, Minneapolis, Minn., 1949.

(20) L. Marsart in J. B. Sumner and K. Myrbäck "The Enzymes," Academic Press, Inc., New York, N. Y., 1951.

However, the character of the intercepts clearly excludes any possibility of reconciling the data with the kinetics of a simple competitive system. In order to test the validity of the concepts which led to the formulation of equation (25), values for C_σ and rK_S/C_σ at each given [I] value have been calculated from the data given in Fig. 2 with the aid of equation (25) and the known values of K_S', *i.e.*, 8.5×10^{-3} *M*, and k_3', *i.e.*, 2.2×10^{-3} *M*/min./mg. protein-nitrogen/ml.[11] These calculations lead to the values given in Table III. The slope and intercept which should have been obtained if the system had been one of simple competitive inhibition are included to facilitate comparison with the observed values.

TABLE III

ANALYSIS OF THE KINETIC DATA FOR THE SYSTEM α-CHYMOTRYPSIN–METHYL HIPPURATE–INDOLE ACCORDING TO EQUATION (25)[a]

[I]	Observed value in Fig. 2 Slope	Intercept	Found for rK_S/C_σ	C_σ	Calcd. for comp. inhib.[b] Slope	Intercept
1.2	29.0	2.55	0.59	10.0	49.6	2.15
2.0	35.0	3.00	0.51	9.5	70.6	2.15

[a] [I], [S] in units of 10^{-3} *M*, $V' = 0.465 \times 10^{-3}$ *M*/min., $K_I = 0.70 \times 10^{-3}$ *M*, $K_S' = 8.5 \times 10^{-3}$ *M*, C_σ in units of $(10^{-3}\ M)^2$. [b] Slope $= K_S'(1 - [I]/K_I)/V'$, intercept $= 1/V'$.

The agreement in the two sets of values for rK_S/C_σ and C_σ obtained from the observed slopes and intercepts of the plots given in Fig. 2 may be regarded as satisfactory in view of the experimental limitations encountered in this investigation. The kinetic data are, therefore, consistent with the expectation that in addition to the binary complexes ES, ES′ and EI, a ternary complex ESI, resulting from the interaction of both inhibitor and specific substrate with the enzyme, is also formed in the system. The existence of such a ternary complex was predicted from a consideration of the steric requirements of the three center hypothesis and the structure of the compounds capable of interacting with the active site of the enzyme. The above results therefore provide indirect experimental support for the basic concepts of the three center hypothesis. It is, moreover, possible to extend the present analysis to include an estimate of the probable limiting values of r and K_S. The mean value for the quantity rK_S/C_σ is 0.55,[21] and that for C_σ is 9.75 from which it follows that $rK_S = 5.36$. Since, K_S must be greater than K_S', *i.e.*, $K_S > 8.5$, the affinity ratio $K_SK_IC_\sigma < 1$, and $K_I = 0.70$, it may be inferred that the following relationships will be valid: $K_SK_I/C_\sigma > 0.61$ but < 1.0, $K_S > 8.5$ but < 13.9, and $r < 0.64$ but > 0.39. The significance of these limiting values in terms of the three-center hypothesis will be briefly discussed.

The ratio K_SK_I/C_σ is a measure of the over-all affinity of the three ρ centers in ES and EI for the complementary R groups relative to that of the same three centers in the free enzyme. It will be recalled from equation (23) that $K_SK_I/C_\sigma = 1$ when $K_{S.1} = K_S$ and $K_{I.2} = K_I$. The calculated lower limit of about 0.6 for the quantity K_SK_I/C_σ may then be interpreted to mean that the over-all

(21) All values used in the discussion which follows are based upon units of 10^{-3} *M* for K_S and K_I.

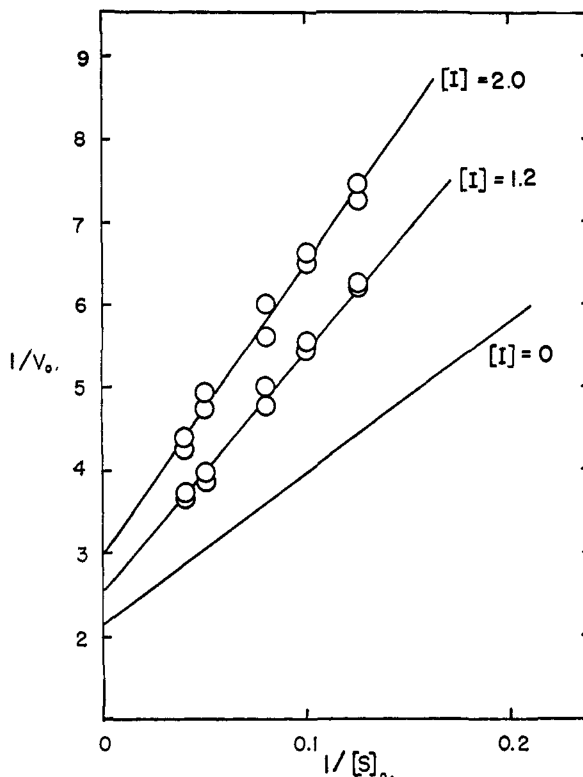


Fig. 2.—Effect of indole on the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate in aqueous solutions at 25° and *p*H 7.9; v_0 in units of 10^{-3} *M*/min.; [S] and [I] in units of 10^{-3} *M*; [E] equivalent to 0.208 mg. protein nitrogen/ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

combining ability of the binary complexes must compare favorably with that of the free enzyme. Two situations are conceivable wherein the formation of a binary complex of the type ES or EI may adversely affect the affinity of the vacant ρ center or centers for a second reacting molecule; (a) where the reaction with S or I materially distorts the unoccupied center or centers; and (b) where the presence of S or I at the active site hinders the approach of a second reacting molecule. In view of the probable proximity of the ρ centers to each other it is perhaps inevitable that the second situation must be operative to some extent. However, it does appear that the inherent combining power and the integral structure of a ρ center remains relatively unimpaired by the interaction of R groups with adjacent ρ centers. It should be emphasized that in the system under consideration both the inhibitor, *i.e.*, indole and the specific substrate, *i.e.*, methyl hippurate, possess structural features which can be considered to be entirely complementary to the ρ centers at the active site. Neither possess non-interacting structural features which may, by virtue of their mass effectively screen the neighboring ρ centers from the approach of other molecules containing one or more complementary R groups. Such an effect could, for example, be operative in the complex ES′ where the benzamido group interacts with ρ_2, and the carbomethoxy group is free, and hence is in a position to screen both ρ_3 and ρ_1. The extent to which the unoccupied ρ centers are

thus masked may be of another order of magnitude greater than that indicated above.

From the limiting values for K_S, *i.e.*, $K_S > 8.5$ but < 13.9, and from the relation, $K_S' = K_S K_\sigma / (K_S + K_\sigma)$[11] it is estimated that the value of K_σ, the enzyme–substrate dissociation constant for the non-susceptible complex ES′, must be greater than 22. These values indicate that of the reactions between α-chymotrypsin and methyl hippurate which lead to the formation of intermediate complexes, the formation of the susceptible complex ES is the more important reaction. For the system α-chymotrypsin–methyl hippurate $V = V'(1 + K_S/K_\sigma)$.[11] Substituting the appropriate limiting values for K_S and K_σ, it is evident that V must have a value between V' and 1.63 V'. Therefore k_3 lies between k_3' and 1.63 k_3', *i.e.*, between 2.2 and 3.6 × 10^{-3} M/min./mg. protein nitrogen/ml. The limits for r indicate the $k_{3.1}$ has a value of *ca.* 0.4 k_3 to 0.6 k_3. The rate of hydrolysis of ESI is thus approximately one-half that of ES. From a previous analysis of the factors which appear to be important in determining the susceptibility to hydrolysis of the α-chymotrypsin–specific substrate complexes[11] it appears that two effects may be responsible for the lesser susceptibility to hydrolysis of ESI relative to that of ES, *i.e.*, a diminution of the strain centered in the R_3–ρ_3 interaction, and a reduction in the accessibility of the R_3–ρ_3 locus to subsequent attack by other reactants.[7] Since it appears, at least for the case under consideration, that the interaction of a given R group with a particular ρ center has relatively little effect upon the structural integrity of the adjacent ρ centers there is little reason to postulate a lesser strain in the R_3–ρ_3 interaction of ESI than in the R_3–ρ_3 interaction of ES. Therefore, the principal reason for the decreased susceptibility to hydrolysis of ESI may be ascribed to the effectiveness of the indole–ρ_2 interaction in limiting the accessibility of the R_3–ρ_3 locus in ESI to subsequent attack. This interpretation suggests that a bimolecular mechanism may be the rate-determining step in the transformation of ES and ESI to free enzyme, reaction products and, in the latter instance, inhibitor or enzyme–inhibitor complex.

The treatment used in the preceding discussion, hereafter referred to as case I, is based upon the proposition that the ternary complex ESI can be formed from both ES and EI. In view of the fact that in most discussions of the mode of formation of ternary complexes in so-called non-competitive systems[18–20] it is tacitly assumed that the ternary complex can be formed from only one of the binary complexes, it is of interest to compare the initial rate equations for the special cases where ESI can only be formed from either EI or ES with that for case I.

In Case II the assumption is made that an R_1–ρ_1, R_3–ρ_3 interaction will effectively prevent an indole–ρ_2 interaction. ESI is then exclusively formed from EI and S. Of the reactions considered in case I the one represented by equation (18) can, therefore, be omitted. From the relation given in equation (26) it follows that the initial rate equation for case II is equation (27).

$$K_{S.1} = (k_{2.1} + k_{3.1})/k_{1.1} = [ES][S]/[ESI] \quad (26)$$

$$1/v = K_S'/V'(1 + [I]/K_I)(1/(1 + rK_S[I]/K_{S.1}K_I))1/[S] + (1/(1 + rK_S[I]/K_{S.1}K_I))(1/V' + (K_S'/V')([I]/K_{S.1}K_I)) \quad (27)$$

In case III the assumption is made that an indole–ρ_2 interaction will effectively prevent ρ_1 and ρ_3 from interacting with the bifunctional specific substrate, *i.e.*, ESI is exclusively formed from ES and I. By omitting equation (17) from case I and from the relation given in equation (28) the initial

$$K_{I.3} = (k_{5.2} + k_{3.1})/k_{4.2} = [ES][I]/[ESI] \quad (28)$$

rate expression for case III is that given by equation (29).

$$1/v = K_S'/V'(1 + [I]/K_I)(1/(1 + r[I]/K_{I.3}))1/[S] + (1/(1 + r[I]/K_{I.3}))(1/V' + (K_S'/V')([I]/K_S K_{I.3})) \quad (29)$$

The interpretation of the experimental data given in Fig. 2 in terms of equations (25), (27) and (29) is summarized in Table IV. Although all three equations lead to the same limiting values for r, *i.e.*, <0.64 and >0.39, and for K_S, *i.e.*, <13.9 and >8.5, it is seen that the values 9.75, mean of 10.0 and 9.5, and 0.55, mean of 0.59 and 0.51, have a different significance in each of the three equations. As it is clearly beyond the scope of the experimental data summarized in Fig. 2 to distinguish between cases I, II and III it should be realized that the selection of case I, *i.e.*, the general case, for the treatment of the system α-chymotrypsin–methyl hippurate–indole is based upon accessory arguments which deny the validity of the limitations inherent in cases II and III for the particular example being considered.

From considerations similar to those above it will be appreciated that in the so-called non-competitive systems the assumption that the enzyme–substrate–inhibitor complex is formed exclusively from the enzyme–substrate complex and the free inhibitor[18–20] is by no means sustained by the experimental data ordinarily available, even though the rate equation formulated on this basis is consistent with the experimental data expressed in terms of a $1/v_0$ *versus* $1/[S]_0$ plot. Unless there is good reason for imposing limitations of the kind illustrated in cases II and III the rate equations for the formation of ternary complexes should be based upon the general case.

TABLE IV

COMPARISON OF EQUATIONS (25), (27) AND (29)[a]

Case	Eq.	Values from data in Fig. 2 9.75[b]	0.55[c]	Ternary complex constant	Affinity ratio[d]
I	25	C_σ	rK_S/C_σ	$C_\sigma = 9.75$	$K_S K_I/C_\sigma$
II	27	$K_{S.1}K_I$	$rK_S/K_{S.1}K_I$	$K_{S.1} = 13.9$	$K_S/K_{S.1}$
III	29	$K_S K_{I.3}$	$r/K_{I.3}$	$K_{I.3} > 0.70 < 1.15$	$K_I/K_{I.3}$

[a] K_S, K_I, etc., in units of 10^{-3} M. [b] Mean of 10.0 and 9.5. [c] Mean of 0.59 and 0.51. [d] Affinity ratio in all cases <1> 0.6.

Experimental[22]

Specific Substrates and Inhibitors.—The preparation of nicotinyl-L-tryptophanamide[13] and methyl hippurate[11] has been described previously. Indole, m.p. 52–53°, skatole, m.p. 95–96°, and nicotinamide, m.p. 133–134°, were Eastman Kodak Co. reagent grade products and were recrystallized at least twice from appropriate solvents. Cyclohexanol, pyridine and phenol were reagent grade products and were redistilled prior to use.

(22) All melting points are corrected.

Enzyme Experiments.—All experiments were conducted at 25° and pH 7.9 ± 0.1 in aqueous solution 0.02 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. The methods employed for the experiments with nicotinyl-L-tryptophanamide, $K_S = 2.7 \times 10^{-3}$ M,[13] and with methyl hippurate, $K_S' = 8.5 \times 10^{-3}$ M,[11] were identical with those described previously for other experiments with these specific substrates. The enzyme preparation was an Armour product, lot no. 90402, and it will be noted that the specific enzyme concentrations E_S' and E_I' for all experiments were such as to satisfy zone A conditions.[13,16,17]

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING OF THE UNIVERSITY OF WASHINGTON]

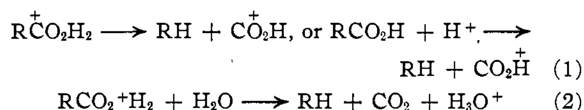
The Kinetics of the Decarboxylation of 2,4,6-Trihydroxybenzoic Acid in Perchloric Acid Solution

BY W. M. SCHUBERT AND J. D. GARDNER

RECEIVED AUGUST 18, 1952

Both the rate of decarboxylation of 2,4,6-trihydroxybenzoic acid and the ratio $[RCO_2^-]/[RCO_2H]$ were determined by an ultraviolet spectroscopic method in several concentrations of perchloric acid. The value of k_2' in the equation $k_2' = k_{obsd.}([RCO_2^-] + [RCO_2H])/[RCO_2H]$ was found constant in the region of 0.135 to 38.2% perchloric acid. This is consistent with the rate-controlling step being a first order decomposition of RCO_2H (or something proportional to RCO_2H), or the reaction of RCO_2^- with a proton released from the solution to give the products directly. The reaction is solvent dependent.

A study of the decarboxylation of hydroxy aromatic acids was undertaken with the view of determining (1) whether the reactions are acid-catalyzed, and (2) if they are acid-catalyzed, what is the rate controlling step; *i.e.*, is it represented by equation (1), (2) or (3). The acid-catalyzed

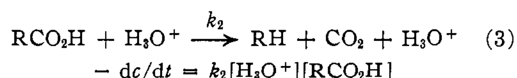


$$R\overset{+}{C}O_2H_2 \longrightarrow RH + \overset{+}{C}O_2H, \text{ or } RCO_2H + H^+ \longrightarrow RH + \overset{+}{C}O_2H \quad (1)$$

$$RCO_2{}^+H_2 + H_2O \longrightarrow RH + CO_2 + H_3O^+ \quad (2)$$

decarboxylation of hindered alkyl benzoic acids is complicated by the occurrence of a side ionization to the acyl ion, $R\overset{+}{C}O$, in at least the higher ranges of sulfuric acid solutions in which it has been studied.[1] It appeared that the decarboxylation of hydroxy aromatic acids could be examined over a range of acid concentration in which ionization to acyl ion does not occur.

In the meantime, Hammick, Brown and Elliot[2a] reported on the kinetics of the decarboxylation of 2,4,6-trihydroxybenzoic acid in 0 to 0.18 N HCl. The reaction rate was followed by a titration method. Earlier the kinetics of decarboxylation of this and other hydroxy aromatic acids had been studied in resorcinol as a solvent at higher temperature.[2b] It was concluded that two simultaneous mechanisms applied. The first of these, reaction (3), was pictured as proceeding *via* attack of a solvated proton on the α-carbon of the undissociated acid

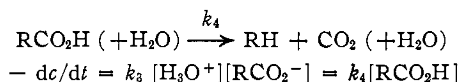


$$RCO_2H + H_3O^+ \xrightarrow{k_2} RH + CO_2 + H_3O^+ \quad (3)$$

$$-dc/dt = k_2[H_3O^+][RCO_2H]$$

The second reaction (4) was pictured as the attack of a solvated proton on the carboxylic acid anion, or the decomposition of the undissociated acid with or without the intervention of a molecule of water

$$RCO_2^- + H_3O^+ \xrightarrow{k_3} RH + CO_2 + H_2O \quad (4)$$



$$RCO_2H\ (+H_2O) \xrightarrow{k_4} RH + CO_2\ (+H_2O)$$

$$-dc/dt = k_3[H_3O^+][RCO_2^-] = k_4[RCO_2H]$$

Evaluation of k_2 and k_4 from the observed rate constants required a knowledge of the degree of ionization of RCO_2H to RCO_2^-, which was calculated from a value for the ionization constant, K, of 2×10^{-2}. On this basis k_2 was determined to be about five times as large as k_4 at 40°.

Since mechanisms (1) and (2) [or (3)] are kinetically indistinguishable in dilute mineral acids, the reaction was studied in these laboratories over a wide range of acid concentrations, from 0 to 58.5% perchloric acid. Perchloric acid was chosen because it is a strong acid for which values of H_0 are known, it is monobasic and is a non-sulfonating medium. It was found that an ultraviolet spectroscopic method could very readily be adapted to following the rate of disappearance of the carboxylic acid, which has limited solubility in aqueous mineral acids. Furthermore, the spectroscopic method could at the same time be used to determine directly the actual concentrations of RCOOH and $RCOO^-$ in the reaction media.

Experimental

Materials.—2,4,6-Trihydroxybenzoic acid prepared by carbonation of phloroglucinol[3] was recrystallized from acetonitrile, m.p. 205–206°. Perchloric acid solutions of desired strength were made by dilution of Baker C.P. 20% acid and Mallinckrodt C.P. 60% acid. The strength of each acid solution was determined by titration of a suitable aliquot against standard alkali.

Apparatus and Kinetic Method.—A stock solution was prepared by dissolving about 20 mg. of 2,4,6-trihydroxybenzoic acid in 100 ml. of water kept at constant temperature. For each kinetic run, 5 ml. of the stock solution was diluted quickly to 50 ml. with perchloric acid of appropriate strength and at constant temperature. A portion of this solution was transferred quickly to a quartz stoppered Beckman cell. This cell and the cell containing the blank solution, made by diluting 5 ml. of water to 50 ml. with the same strength perchloric acid, were placed in a small constant temperature water-bath that had been inserted between the

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(2) (a) B. R. Brown, W. W. Elliot and D. Ll. Hammick, *J. Chem. Soc.*, 1384 (1951); (b) B. R. Brown, D. Ll. Hammick and J. B. Scholefield, *ibid.*, 778 (1950).

(3) P. Holmes, D. E. White and I. H. Wilson, *ibid.*, 28 (1950).