The Kinetics of Carboxypeptidase B Activity

III. EFFECTS OF ALCOHOL ON THE PEPTIDASE AND ESTERASE ACTIVITIES; KINETIC MODELS

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Certain of the kinetic properties of carboxypeptidase B have been described in the previous papers of this series (1, 2). The experimental constants, \( K_a \) and \( k_0 \), were determined with a number of substrates, and the enzyme-inhibitor dissociation constants were evaluated with several competitive inhibitors. The present studies were undertaken in an effort to obtain knowledge of the affinity of carboxypeptidase B for its substrates through measurement of \( K_s \) values, the constants for the dissociation of enzyme-substrate complexes.

Slater has demonstrated that values for \( K_s \) may be obtained in systems where \( k_0 \), the rate constant for breakdown of enzyme-substrate complex to products, is varied independently of \( K_s \) (3). Main (4) interpreted the alcohol-promoted activation and reversible inhibition of hydrolysis of certain nitrophenyl carboxylic acid esters by human serum cholinesterase to be examples of this type of phenomenon. On the basis of this assumption, he assigned \( K_s \) values calculated from the common point of intersection obtained by extrapolations in Lineweaver-Burk plots (5) of activities at a number of alcohol concentrations.

With the hope of obtaining similar data from which kinetic constants could be derived for carboxypeptidase B, the peptidase and esterase activities of this enzyme were examined in the presence of low concentrations of butanol and other alcohols. The results of some of these studies were incompatible with changes in \( k_0 \) independent of \( K_s \) and prompted a re-investigation of the assumptions and derivations used in the determination of \( K_s \). It became evident that more than one model was compatible with the data for both peptidase and ester hydrolysis. Under these circumstances, the assignment of \( K_s \) values is justified only if additional experimental evidence for one model in preference to others is available.

EXPERIMENTAL PROCEDURE

The carboxypeptidase B was a sample of the enzyme used in previous studies (1). Rate measurements were conducted by means of a spectrophotometric procedure (1) with the peptide substrate, hippuryl-L-arginine, and the ester substrate, hippuryl-L-argininic acid, in 0.025 M Tris-acetate buffer, pH 8.0, at 23°C.

1-Butanol was prepared in 0.8 M stock solution and was incorporated into buffered substrate solution before addition of enzyme.

RESULTS

Peptide Substrate: Hippuryl-L-arginine—The effect of butanol on the hydrolysis of hippuryl-L-arginine catalyzed by carboxypeptidase B is shown graphically in Fig. 1. The activities at five different concentrations of peptide substrate were determined in 0, 0.025, 0.040, 0.10, and 0.33 M butanol. The data are plotted according to the Dixon modification (6) of the Lineweaver-Burk method (5). The lines drawn through the experimental points and extended to the \( 1/S \) axis, as required in the Dixon plot, intersect in a common point in the upper left quadrant. The activity of carboxypeptidase B toward the peptide substrate increases with increasing butanol concentrations; more than 100% increase in \( V_{max} \) is apparent at the 0.33 M butanol concentration. The apparent values for \( K_m \), on the other hand, decrease with increasing butanol concentrations; more than a 60% decrease in the \( K_m \) value is evident at the highest butanol concentration employed.

The values for \( V_{max} \) and apparent \( K_m \) obtained with other alcohols are recorded in Table I. Each alcohol activates the hydrolysis of hippuryl-L-arginine; the degree of activation at equivalent alcohol concentrations increases with increasing alcohol chain length. When the activities are plotted by the method used in Fig. 1, the lines approximate a common point of intersection in the upper left quadrant in close vicinity to that shown in Fig. 1.

Ester Substrate: Hippuryl-L-argininic Acid—The data, which are plotted by the Dixon method in Fig. 2, were obtained by estimation of the rates of hydrolysis at six different concentrations of ester substrate in the presence of 0, 0.05, 0.15, and 0.30 M butanol. The activity of carboxypeptidase B toward ester substrate decreases with increasing butanol concentration. The apparent \( K_m \) values also decrease as the butanol concentration is increased. The values for \( V_{max} \) and \( K_m \) in 0.3 M butanol are approximately 15 and 40%, respectively, of the uninhibited values.

Extrapolations of the lines drawn through the experimental points for each butanol concentration of Fig. 2, when extended into the lower left quadrant, appear to intersect at a common point in this quadrant.
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In the reactions catalyzed by the hydrolytic enzymes, the second substrate is presumably the water molecule, which is also the supporting medium and is always present in large excess. Attempts to vary \( k_0 \) by reducing the concentration of water have been generally unsuccessful, primarily because of the deleterious effects on proteins of the materials employed for this purpose.

Main (4) has stated that it is unlikely that the effect of low concentrations of alcohols in activating or inhibiting serum cholinesterase is in any way related to the concept of a second substrate. He has postulated, however, that the effect of alcohols in these cases is limited to modifications in rate of breakdown of enzyme-substrate complex to free enzyme and products.

Since \( K_m \) is equal to \((k_{-1} + k_0)/k_1\), a change in \( k_0 \) alone requires a change in \( K_m \) in the same direction. The results described for the hydrolysis of the ester substrate, hippuryl-L-arginine acid, as well as those reported for the hydrolysis of the nitrophenyl carboxylic acid esters (4), are consistent with this requirement.

However, the observation that \( K_m \) and \( k_0 \) change in opposite directions during the alcohol activation of hippurylarginine hydrolysis is not in agreement with this requirement.

As a result of a survey of possible schemes by which the effect of alcohols on peptide hydrolysis could be analyzed, a model of free enzyme modification was found applicable to all of the observations communicated here and also to those reported by Main (4). An alternate model of enzyme-substrate complex modification, on the other hand, was compatible only with the alcohol enhancement of peptide and nitrophenyl carboxylic acid ester hydrolysis. These models, along with that of Slater (3) and Main (4), are outlined briefly below.

**Model A: Free Enzyme Modification**

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_0} E + \text{products} + A
\]

An essential feature of this model is that enzyme, \( E \), reacts with a modifying compound, \( A \), to give rise to an altered enzyme species, \( EA \). The reaction with \( A \) is not necessarily stoichiometric. The altered enzyme binds substrate with an affinity, \( 1/K_m^* \), which is equal to \( k_3/k_4 \), and which may or may not differ from that of the unaltered enzyme. The enzyme-substrate complex, \( EAS \), formed with the altered enzyme, breaks down to altered enzyme and products. The rate constant for this dissociation, \( k_8^* \), may or may not differ from \( k_8 \), the rate constant for product-forming dissociation of \( ES \).

By assigning the following values,

\[
K_m = \frac{k_{-3} + k_0}{k_1}; \quad K_m^* = \frac{k_{-3} + k_4^*}{k_3}; \quad V_{max} = k_0 E_t
\]

where \( E_t \) is the total enzyme concentration, the reciprocal rate equation may be derived on the assumptions of the steady state and large amounts of substrate and modifying compound relative to

**TABLE I**

| Alcohol         | Concentration | \( V_{max} \) | \( K_m \)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.004 M</td>
<td>18.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.4 M</td>
<td>20.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.2 M</td>
<td>23.0</td>
<td>1.5</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>0.1 M</td>
<td>25.6</td>
<td>1.4</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>0.1 M</td>
<td>32.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Similar but less pronounced inhibition of the carboxypeptidase B-catalyzed hydrolysis of hippuryl-L-argininic acid is observed with 1-propanol.

**INTERPRETATIONS**

The method of Slater for determining \( K_s \) values requires that \( K_s \), i.e. \( k_{-1}/k_1 \), remain constant while \( k_0 \) is increased or decreased (3). These requirements have been fulfilled by Slater and Bonner (7) in experiments with succinic dehydrogenase in which the conversion of succinic acid to fumaric acid requires the simultaneous reduction of a hydrogen acceptor and where the effective concentration of this second substrate has been varied by the use of specific inhibitors.
to enzyme

\[ \frac{1}{v} = \frac{1 + \frac{k_2 K_m}{k_{-2} K_m} A + \frac{1}{S} \frac{k_2}{k_{-2}} A}{E_t \left( \frac{k_0}{k_{-2} K_m} - \frac{k_0}{k_{-2} K_m} A \right)} \]  

(1)

The intercept on the 1/V axis of the line obtained from a Lineweaver-Burk plot may change as the concentration of A changes. If \( k_o^* \) is greater than \( k_o \), the intercept decreases with increasing A, and if \( k_o^* \) is less than \( k_o \), the intercept increases. Since the reciprocal of this intercept is \( V_{\text{max}} \), one may say that, when \( k_o^* \) is greater than \( k_o \), A activates the reaction and, when \( k_o^* \) is less than \( k_o \), A inhibits the reaction.

The lines obtained at various concentrations of A in a Lineweaver-Burk plot meet in a common point, the coordinates of which are \((k_o - k_o^*)/(k_o^* K_m - k_o K_m^*)\) on the 1/S axis and \((K_m - K_m^*)/(E_t (k_o^* K_m - k_o K_m^*))\) on the 1/V axis. This may be verified by substituting the 1/S coordinate for 1/S in Equation 1 and obtaining the given 1/V coordinate for all values of A.

The quadrant containing the common point of line intersection is determined by the algebraic signs of these coordinates, which in turn depend on whether \( k_o^* \) is greater or less than \( k_o \), whether \( K_m \) is greater or less than \( K_m^* \), and whether \( k_o^* K_m \) is greater or less than \( k_o K_m^* \). The various possibilities are summarized in Table II.

Model B: Enzyme-Substrate Complex Modification

\[ E + S \xrightarrow{k_1} ES \xrightarrow{k_0} E + \text{products} \]

+ \[ A \]

\[ \xrightarrow{k_3 + k_{-3}} \]

\[ ESA \xrightarrow{k_0^*} E + A + \text{products} \]

In this scheme, the enzyme-substrate complex, ES, reacts with a modifying compound, A, to yield an altered enzyme-substrate complex, ESA. The reaction with A is again not necessarily stoichiometric. The altered enzyme-substrate complex is converted to E, A, and products. The rate constant for this dissociation, \( k_0^* \), may or may not differ from \( k_o \), the rate constant for product forming dissociation of ES.

The reciprocal rate equation may be derived on the assumptions of the steady state and large amounts of substrate and modifying compound relative to enzyme, 

\[ \frac{1}{v} = \frac{1 + \frac{A}{K_m^*} + \frac{1}{S} \frac{k_0^* A}{k_1 K_m^*}}{E_t \left( \frac{k_0}{k_1 K_m^*} \right)} \]  

(2)

where \( K_m^* = (k_{-1} + k_0^*)/k_2 \).

Although Equation 2 is not identical with Equation 1, it is of the same mathematical form and consequently has the same formal properties. As in Model A, when \( k_0^* \) is greater than \( k_0 \), increases in A increase \( V_{\text{max}} \) and, when \( k_0^* \) is less than \( k_0 \), increases in A decrease \( V_{\text{max}} \). All lines in a 1/S versus 1/V plot have a common point of intersection, which may fall in any one of three available quadrants. The coordinates of this point are \[(k_0(k_o - k_o^*)/(k_o^* k_{-1}))\] on the 1/S axis and \[k_{-1} + (k_0 - k_0^*)]/(E_t k_0 k_{-1})\] on the 1/V axis. A summary of the algebraic signs of these coordinates is given in Table III. It may be noted from this table that a value for \( k_0 \) greater than \( k_0^* \) implies that...
the point of intersection must be in the upper right quadrant. This is in contrast to Model A, in which a value for $k_0$ greater than $k_0^*$ is compatible with intersection in any one of the three quadrants.

Model C: Modification in Rate of Product-forming Dissociation of Enzyme-Substrate Complex (Slater, Main)—In this scheme, the modifying compound does not react with $E$ or $ES$ but, in some manner, changes the rate constant for the dissociation of $ES$ to $E$ and products. The change in rate is related to the concentration of modifying compound.

The reciprocal rate equation is the classical one,

$$
\frac{1}{v} = \frac{K_m^* + \frac{1}{S}}{k_o^* E_t}
$$

where $K_m^* = (k_+ + k_*)/k_0$ and $k_o^*$ is the modified rate constant for dissociation of $ES$ to $E$ and products.

Extrapolations of the lines obtained at various concentrations of modifying compound in Lineweaver-Burk plots meet in a common point, the coordinates of which are $-(k_0 - k_0^*)$ or $k_0^*$ on the $1/V$ axis and $-1/(-k_0 E_t)$ on the $1/S$ axis. For both the cases of activation and of inhibition, i.e. $k_0^*$ is greater than $k_0$ and $k_0^*$ is less than $k_0$, respectively, the common point of intersection of the lines must fall to the left of the $1/V$ axis and below the $1/S$ axis, i.e. in the lower left quadrant.

The values for the coordinates of the common point of line intersections in Lineweaver-Burk plots of the three models outlined above are summarized in Table IV. The quadrants into which these common points of intersection may fall in cases of activation ($V_{\text{max}}$ increased by the modifying compound) and inhibition ($V_{\text{max}}$ decreased by the modifying compound) are also shown diagrammatically in this table. Since under Model A, the common point of line intersection may fall in any of the three possible quadrants in either activation or inhibition, this model must be considered as a possible mechanism for any reactions of the type described in this communication. No estimate of $K_s$ or of individual rate constants may be obtained from data accumulated for systems which function in accord with this model, although estimates of $K_s$ are possible under Models B and C.

It should be emphasized that no attempt has been made to review or derive all models which might possibly apply to the data described. For a more comprehensive review of models, the reader is referred to Hearon et al. (9). The three models presented here are offered in evidence that there is no reason per se for accepting one model in preference to another when the common point of intersection of lines in a reciprocal plot falls in an area that is compatible with both schemes of catalysis.

### Table IV

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation for coordinates on axis</th>
<th>Possible quadrant of line intersection*</th>
<th>Increase in $V_{\text{max}}$ (activation)</th>
<th>Decrease in $V_{\text{max}}$ (inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (free enzyme modification)</td>
<td>$K_m - K_m^* / E_t(k_0^* k_m - k_e k_m^*)$</td>
<td>$k_0^* k_0 / k_e k_m - k_e k_m^*$</td>
<td>$X$</td>
<td>$X$</td>
</tr>
<tr>
<td>B (enzyme-substrate complex modification)</td>
<td>$k_1 + (k_2 - k_*) / E_t k_o k_2 k_3$</td>
<td>$k_2 (k_2 - k_*) / k_o k_2 k_3$</td>
<td>$X$</td>
<td>$X$</td>
</tr>
<tr>
<td>C (modification in rate of product-forming dissociation of enzyme-substrate complex)</td>
<td>$1 / E_t k_3$</td>
<td>$1 / K_4$</td>
<td>$X$</td>
<td>$X$</td>
</tr>
</tbody>
</table>

* In Model A, line intersection may occur on the $1/V$ or $1/S$ axis when $k_o^* = k_o$ or $K_m^* = K_m$, respectively. In Model A, lines may be parallel (intersect at infinity) when $k_o^* k_m - k_e k_m^* = 0$. In Model B, line intersection may occur on the $1/V$ or $1/S$ axis when $k_o^* = k_o$ or $k_+ + (k_0 - k_*) = 0$, respectively.

# Discussion

Values for $K_m$, the Michaelis constant, and $k_o$, the rate constant for dissociation of enzyme-substrate complex to free enzyme and products, may readily be calculated from velocity-substrate relationships. Estimates of $K_s$, the substrate constant, the reciprocal of which is a measure of affinity between enzyme and substrate, are not directly measurable but require additional information for their evaluation. This is apparent in the Briggs and Haldane interpretation of $K_m$ as $K_s + (k_o/k_0)$ (10).

The present experiments were conducted in an effort to obtain the additional information necessary for the determination of $K_s$ values. It is obvious that the system of free enzyme modification (Model A) must be considered as a possible explanation for the results reported here as well as for those reported by Main (4). Unfortunately, Model A does not permit evaluation of any of the individual rate constants, which could at least be compared with those derived by means of other models. It should be emphasized that rate constants calculated on the basis of Models B and C are of doubtful validity unless concrete experimental evidence as to the applicability of these models is forthcoming.

It is improbable that a single agent, such as alcohol, would affect two closely related reactions, catalyzed by the same enzyme, by two separate mechanisms. In the case of carboxypeptidase B, the inhibition of esterase activity by butanol is compatible with Model C but not with Model B, whereas the activation of peptidase activity fits the requirements of Model B but not Model C. Certainly the mechanism of free enzyme modification as described in Model A, which fits both the activation of peptidase and inhibition of esterase activity, would appear to be the most attractive of the three explanations given. Com-
parison of the physical characteristics of carboxypeptidase B in the presence and absence of butanol have revealed no clear-cut differences in sedimentation coefficients, ultraviolet absorbancies, or optical rotatory dispersion values. This is not surprising, however, in that one might expect subtle changes localized in the vicinity of the enzyme active site that might very well be undetectable by the present methods. It is significant in this respect that the activating and inhibiting effects of butanol are readily reversible.

There are several reports on the determination of rate constants for hydrolytic enzymes which are based upon changes in $K_m$, $k_0$, or both as a function of the alteration in solvent composition (11-13). In each case, the kinetic data were used as proof or support for a hypothesis that $K_m$ is equal to a ratio of unrelated rate constants, i.e. $k_0/k_1$. Certain of these analyses may warrant re-evaluation in light of the present findings.

**SUMMARY**

1. The carboxypeptidase B-catalyzed hydrolysis of the peptide, hippuryl-L-arginine, is activated by 1-butanol and several other alcohols, whereas the hydrolysis of the ester, hippuryl-L-argininic acid, catalyzed by this enzyme is inhibited by 1-butanol.

2. Reciprocal plots of velocity versus substrate concentration at several butanol concentrations with each substrate give straight lines, which upon extrapolation approximate a common point of intersection. The common point of intersection in the case of peptide substrate is in the upper left quadrant. That for ester substrate is in the lower left quadrant.

3. Examination of these results indicate (a) that the activation of peptidase activity is consistent with a model of enzyme-substrate complex modification or with a model of free enzyme modification and (b) that the inhibition of esterase activity is compatible with a model outlined by Slater and extended by Main wherein only $k_b$ (the rate constant for dissociation of enzyme-substrate complex to free enzyme and products) is modified or with a model of free enzyme modification.

4. It is not possible to establish the validity of constants for carboxypeptidase B calculated on the basis of any one model, since concrete experimental evidence for the applicability of any one of these models is not presently available.

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**REFERENCES**

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