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AN INTERPRETATION OF PARTIALLY COMPETITIVE INHIBITION OF THE ESTERASE ACTIVITY OF CARBOXYPEPTIDASE A

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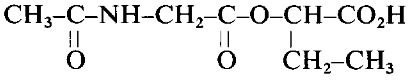
SUMMARY

The cyclohexylacetate ion is a partially competitive inhibitor for the hydrolysis of L-2-aceturoxybutanoic acid by bovine carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.12.2). The kinetics of hydrolysis in the presence of this inhibitor are similar for this ester, which does not display substrate inhibition, and for *O*-hippuryl-L-3-phenyllactic acid, for which pronounced substrate inhibition is apparent. This suggests that partially competitive inhibition kinetics are not related to the tendency of this latter substrate to form 2:1 substrate–enzyme complexes. It is shown that for this enzyme, partially competitive inhibition is not consistent with the formation of E–I–S complexes. Rather, all inhibition data can be rationalized in terms of a scheme involving the formation of an E–I_2 complex.

INTRODUCTION

We [1] recently reported a study of the reversible inhibition by carboxylate ions of the hydrolysis of *O*-hippuryl-L-3-phenyllactic acid by bovine carboxypeptidase A. For carboxylate ions having small aliphatic or aromatic hydrocarbon moieties, strictly competitive inhibition kinetics were observed. However, the anions of some larger aliphatic carboxylic acids gave inhibition kinetics that were consistent with partially competitive inhibition [2]. Thus, plots of E/v vs $1/S$ ($E =$ enzyme concentration; $v =$ initial velocity; $S =$ substrate concentration) at various concentrations of inhibitor anion (I) were linear, and all converged at the ordinate axis to $1/k_{cat}$ as is typical of competitive inhibitors [2]. However, calculation of an inhibition constant K_i at each inhibitor concentration, assuming strictly competitive inhibition kinetics, gave inhibition constants that were dependent on the inhibitor concentration; with K_i decreasing with increasing inhibitor concentration.

We have previously refrained from attempting a detailed interpretation of this phenomenon, since we could not eliminate the possibility that partially competitive inhibition kinetics with the substrate under study, *O*-hippuryl-L-3-phenyllactic acid, may have been related to the known tendency of this substrate to form higher order enzyme–substrate complexes with carboxypeptidase A. Such complexes become evident through the observation of substrate inhibition of the enzymic hydrolysis [3–6]. We have now eliminated this possibility by extending this work using a substrate that shows no tendency to form such higher-order complexes; L-2-aceturoxybutanoic acid (a). Reversible inhibition of the



$$\underset{\underset{O}{\|}}{CH_3-C}-NH-\underset{\underset{O}{\|}}{CH_2-C}-O-\underset{\underset{CH_2-CH_3}{|}}{CH}-CO_2H \qquad (a)$$

enzymic hydrolysis of this substrate corresponds closely to that for *O*-hippuryl-L-3-phenyllactic acid, and we now present a simple scheme for partially competitive inhibition of the esterase activity of this enzyme that is consistent with all presently available data.

METHODS

The experimental procedures closely followed the methods previously described [1]. Thus, all reactions were followed on the pH-stat at 25 °C, pH 7.5, ionic strength 0.2 (NaCl). DL-2-Aceturoxybutanoic acid was a gift from Mr J. Murphy. The synthesis of this ester and a detailed study of its properties as a substrate for carboxypeptidase A will be discussed separately (Bunting, J. W. and Murphy, J., unpublished). All substrate concentrations are given in terms of the L-enantiomer, since the D-isomer is neither a substrate nor an inhibitor for this enzyme.

Competitive inhibition constants (K_i^{app}) at constant inhibitor concentration were calculated from Eqn 1, where K_m^{app} is the apparent Michaelis constant in the presence of inhibitor of concentration I, and K_m the Michaelis constant at I = 0.

$$K_i^{app} = \frac{I}{(K_m^{app}/K_m - 1)} \qquad (1)$$

RESULTS

Reciprocal plots (E/*v* vs 1/S) of the dependence of initial velocity on substrate concentration for the hydrolysis of L-2-aceturoxybutanoic acid by carboxypeptidase A are shown in Fig. 1 in both the absence and presence of cyclohexylacetate anion. All plots are clearly linear and converge to the same intercept on the ordinate axis, and so suggest that the cyclohexyl acetate ion is a competitive inhibitor for this substrate. In the absence of inhibitor, the Michaelis–Menten parameters calculated for this substrate are: $K_m = 8.89 \cdot 10^{-3}$ M and $k_{cat} = 1.94 \cdot 10^4$ min^{-1} (pH 7.5, 25 °C, ionic strength 0.2). A more extensive study in our laboratory of the hydrolysis of this substrate by carboxypeptidase A has confirmed that this reciprocal plot is linear over the substrate concentration range 10^{-4}–10^{-1} M (Murphy, J., unpublished) and we conclude that the hydrolysis of this substrate is free of the substrate activation and inhibition effects that complicate the kinetics of the enzymic hydrolysis of many substrates of this enzyme.

Inhibition constants calculated for cyclohexylacetate ion at each inhibitor concentration in Fig. 1, assuming strictly competitive inhibition, are given in Table I along with similar data for the inhibition of *O*-hippuryl-L-3-phenyllactic acid by this same anion. For each substrate, it is apparent that the calculated K_i^{app} values decrease with

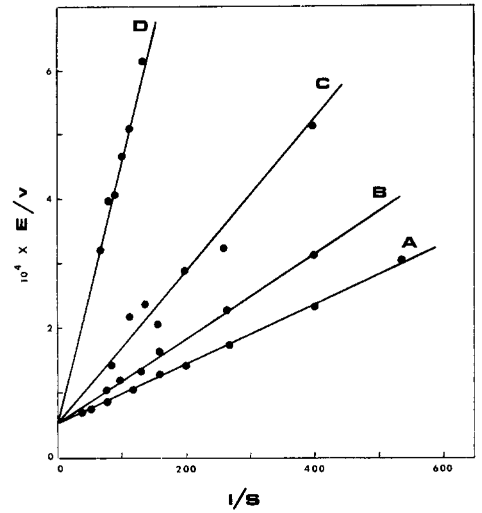


Fig. 1. Inhibition by cyclohexylacetate ion of the hydrolysis of L-2-aceturoxybutanoic acid by carboxypeptidase A. A, I = 0; B, I = 2.47 mM; C, I = 3.71 mM; D, I = 9.90 mM.

TABLE I

DEPENDENCE OF K_i^{app} ON I FOR INHIBITION OF CARBOXYPEPTIDASE A BY CYCLOHEXYLACETATE ION

Conditions: pH 7.5, 25 °C, ionic strength 0.2.

I (M)	K_m^{app} (M)	K_i^{app} (M)
(a) *O*-Hippuryl-L-3-phenyllactic acid*		
$3.23 \cdot 10^{-3}$	$1.69 \cdot 10^{-4}$	$2.46 \cdot 10^{-3}$
$5.54 \cdot 10^{-3}$	$2.63 \cdot 10^{-4}$	$2.07 \cdot 10^{-3}$
$6.86 \cdot 10^{-3}$	$3.57 \cdot 10^{-4}$	$1.73 \cdot 10^{-3}$
$8.31 \cdot 10^{-3}$	$6.03 \cdot 10^{-4}$	$1.12 \cdot 10^{-3}$
$1.20 \cdot 10^{-2}$	$1.04 \cdot 10^{-3}$	$8.82 \cdot 10^{-4}$
(b) L-2-Aceturoxybutanoic acid		
$2.47 \cdot 10^{-3}$	$1.19 \cdot 10^{-2}$	$7.14 \cdot 10^{-3}$
$3.71 \cdot 10^{-3}$	$2.27 \cdot 10^{-2}$	$2.38 \cdot 10^{-3}$
$9.90 \cdot 10^{-3}$	$8.32 \cdot 10^{-2}$	$1.18 \cdot 10^{-3}$

* Concentrations of cyclohexylacetate ion only in Table I and Fig. 3 of ref. 1 are actually 50% greater than indicated.

increasing inhibitor concentration, as is typical of partially competitive inhibitors. Furthermore, a plot of $1/K_i^{app}$ against inhibitor concentration is apparently linear within experimental error (Fig. 2), and the data for both substrates appear to lie on the same line. This is a clear indication that the phenomenon of partially competitive inhibition by this anion of the esterase activity of carboxypeptidase A is a property of the inhibiting ion and is not related to the particular substrate employed.

The dependence of the reciprocal of the initial velocity on cyclohexylacetate ion concentration at constant substrate concentration is shown in Fig. 3 for these two

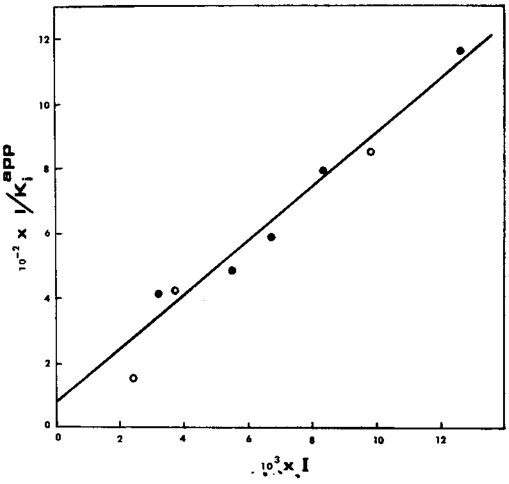


Fig. 2. Dependence of inhibition constant on inhibitor concentration for the inhibition by cyclohexylacetate ion of ester hydrolysis by carboxypeptidase A. (Data from Table I). ○, *O*-hippuryl-L-3-phenyllactic acid; ●, L-2-aceturoxybutanoic acid.

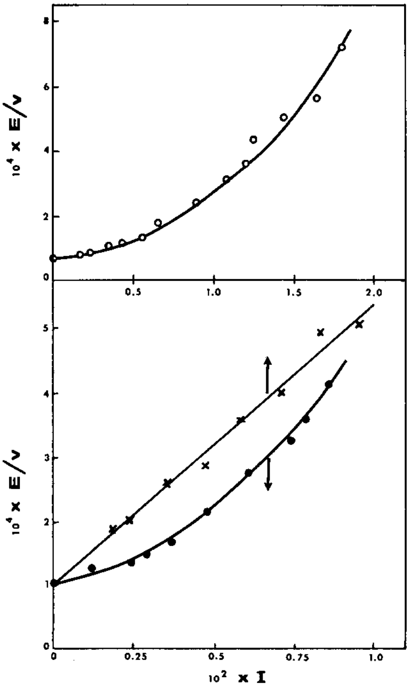


Fig. 3. Dependence of initial velocity on inhibitor concentration at constant substrate concentration for: ○, cyclohexylacetate ion and *O*-hippuryl-L-3-phenyllactic acid ($2.0 \cdot 10^{-4}$ M); ●, cyclohexylacetate ion and L-2-aceturoxybutanoic acid (0.01 M); ×, cyclopropanecarboxylate ion and L-2-aceturoxybutanoic acid (0.01 M). (Arrows indicate the relevant abscissa scales).

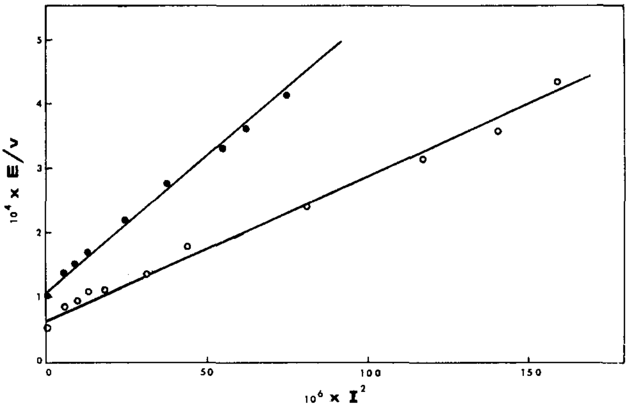


Fig. 4. Dependence of initial velocity on inhibitor concentration (I^2) for the inhibition by cyclohexyl acetate ion of ester hydrolysis by carboxypeptidase A; ○, *O*-hippuryl-L-3-phenyllactic acid ($2 \cdot 10^{-4}$ M); ●, L-2-aceturoxybutanoic acid (0.01 M).

substrates. For comparison, a similar study using the anion of cyclopropanecarboxylic acid as inhibitor for 2-aceturoxybutanoic acid is also included in Fig. 3. As is expected for a strictly competitive inhibitor, this Dixon plot is linear for the latter anion, and allows calculation of $K_I = 1.5 \cdot 10^{-3}$ M, which is in good agreement with the value obtained previously [1] ($K_i = 1.7 \cdot 10^{-3}$ M) using *O*-hippuryl-L-3-phenyllactic acid as substrate. The curved plots for the cyclohexylacetate ion suggest a greater than first-degree dependence on inhibitor concentration. In fact, replotting for both of these substrates as in Fig. 4 clearly shows that the reciprocal of the initial velocity in the presence of cyclohexylacetate ion is linear in I^2 within experimental error, up to greater than 80% inhibition in each case.

It is clear from the data presented, that the characteristics of partially competitive inhibiton by cyclohexylacetate ion of the enzymic hydrolysis of the two substrates under consideration are very similar. Since there is no evidence for the binding of more

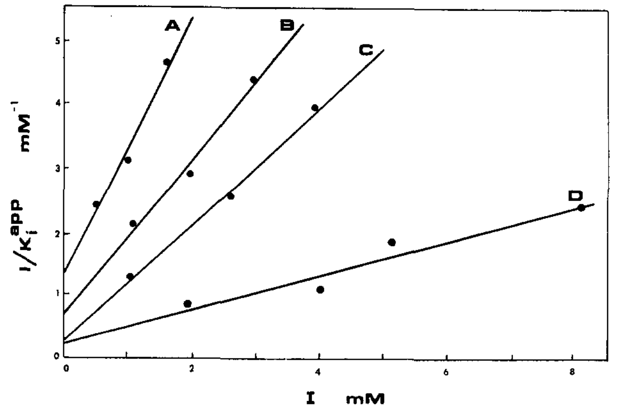


Fig. 5. Dependence of inhibition constant on inhibitor concentration for inhibition of hydrolysis of *O*-hippuryl-L-3-phenyllactic acid by carboxypeptidase A. A, 3-cyclohexylpropanoate ion; B, heptanoate ion; C, cyclohexane carboxylate ion; D, octanoate ion. (Data of ref. 1, Table I).

than one molecule of L-2-aceturoxybutanoic acid to carboxypeptidase A, we feel that our previous observations of partially competitive inhibition kinetics using *O*-hippuryl-L-3-phenyllactic acid as substrate cannot be dismissed as an artifact of the tendency of this latter substrate to bind to more than one site on the enzyme molecule. We have, therefore, re-analyzed our data on other partially competitive inhibitors of the hydrolysis of this substrate in the light of the relationships that are apparent in Figs 2 and 4. Although the data on these ions are not as detailed as for the cyclohexyl acetate ion, in each case the plots of $1/K_i^{app}$ against I (Fig. 5) and E/v against I^2 are apparently linear. Thus it seems clear that the phenomenon of partially competitive inhibition by each of these ions, and for the two substrates examined, should be explicable by a common kinetic scheme.

DISCUSSION

Partially competitive inhibition kinetics have usually been associated [2] with the reaction scheme that is outlined in Scheme 1*, in which the catalytic activities

Scheme 1

$E + S \rightleftharpoons E\text{–}S\cdot$	dissociation constant K_s
$E + I \rightleftharpoons E\text{–}I$	dissociation constant K_i
$E\text{–}I + S \rightleftharpoons E\text{–}I\text{–}S$	dissociation constant K_{is}
$E\text{–}S + I \rightleftharpoons E\text{–}I\text{–}S\cdot$	dissociation constant K_{si}
$E\text{–}S \xrightarrow{k} E + P$	
$E - I\text{–}S \xrightarrow{k} E - I + P$	

of the two complexes, E–S· and E–I–S, are the same. This scheme gives rise to the dependence of initial velocity on inhibitor and substrate concentrations in Eqn 2. This equation reduces to Eqn 3

$$\frac{v}{E} = \frac{k}{1 + \frac{K_s}{S}\cdot\frac{(1 + I/K_i)}{(1 + I\cdot K_s/K_i\cdot K_{is})}} \tag{2}$$

$$\frac{v}{E} = \frac{k}{1 + \frac{K_s}{S}(1 + I/K_i)} \tag{3}$$

for strictly competitive inhibition if $K_{is} = K_{si} = \infty$; i.e. E–I–S is not formed. In Eqn 2, the apparent substrate binding constant (K_s^{app}) in the presence of inhibitor is

$$K_s^{app} = \frac{K_s(1 + I/K_i)}{1 + I\cdot K_s/K_i\cdot K_{is}} \tag{4}$$

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[*] For convenience, in all schemes the experimentally measured Michaelis constant, K_m, is assumed to reflect the dissociation constant, K_s. If this is not true, the meaning of K_s and K_s^{app} will be different to the definitions given, but the overall forms of the rate equations and the conclusions reached will not be affected. For some substrates of this enzyme, there is direct evidence [7] that K_m is equivalent to K_s.

If, for a partially competitive inhibitor, one calculates K_i^{app} according to Eqn 1, then substitution of Eqn 4 into Eqn 1 gives

$$K_i^{app} = \frac{I}{\left(\frac{1 + I/K_i}{1 + I \cdot K_s/K_i \cdot K_{is}} - 1\right)} = \frac{K_i \cdot K_{is} + I \cdot K_s}{K_{is} - K_s} \tag{5}$$

Eqn 5 suggests that if Scheme 1 is operative K_i^{app} should be linearly dependent on I, whereas it is clear from Figs 2 and 5 that, in fact, $1/K_i^{app}$ is linearly dependent on I in the present cases.

Eqn 4 can be rearranged to Eqn 6,

$$\frac{K_s^{app} - K_s}{I} = \frac{K_s}{K_i} - \frac{K_s^{app} \cdot K_s}{K_i \cdot K_{is}} \tag{6}$$

which further suggests that a plot of $(K_s^{app} - K_s)/I$ against K_s^{app} will be linear with slope $-K_s/k_i \cdot K_{is}$ and ordinate intercept K_s/K_i if Scheme 1 is operative. Since K_s can be measured in the absence of inhibitor, all other binding constants in the above scheme can then be evaluated. A plot according to Eqn 6 of the data from Table I for the cyclohexylacetate ion and *O*-hippuryl-L-3-phenyllactic acid is shown in Fig. 6. Although the predicted linear relationship of Eqn 6 appears to be obeyed, the slope is positive rather than negative as is required by this equation. This leads to a negative value for K_{is}, which is, of course, physically meaningless. Furthermore, Eqn 2 predicts that at constant substrate concentration, a plot of E/v vs I should approach a limiting

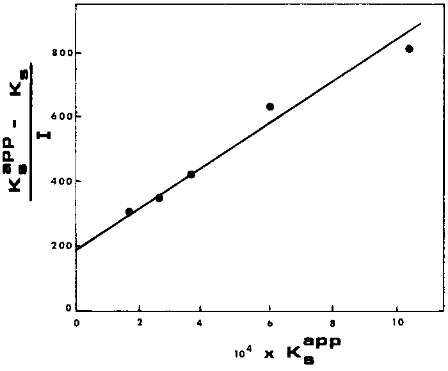

Fig. 6. Plot of data from Table Ia for cyclohexylacetate ion according to Eqn 6.

maximum value $(1/k_{cat} + K_s \cdot K_{si}/S \cdot k_{cat} \cdot K_i)$ at high inhibitor concentrations. However, the experimentally determined dependence of E/v on I for the present partially competitive inhibitors of carboxypeptidase A shows no approach to such a limiting maximum value (Figs 3 and 4).

The above three experimental tests clearly indicate that the most generally accepted scheme for partially competitive inhibition is not applicable to the inhibition of the esterase activity of carboxypeptidase A. The relationships that are apparent in Fig. 4 suggest that the binding of two inhibitor ions per enzyme molecule may be involved in the present case. We have found that the simplest scheme involving an E–I$_2$ complex that can be fitted to the present experimental observations is that outlined in Scheme 2. This scheme generates the

Scheme 2

$$\begin{array}{ll} \mathrm{E} + \mathrm{S} \rightleftarrows \mathrm{E{-}S} & \text{dissociation constant } K_s \\ \mathrm{E} + \mathrm{I} \rightleftarrows \mathrm{E{-}I} & \text{dissociation constant } K_i \\ \mathrm{E{-}I} + \mathrm{I} \rightleftarrows \mathrm{E{-}I_2} & \text{dissociation constant } K_{ii} \\ \mathrm{E{-}S} \xrightarrow{k} \mathrm{E\cdot} + \mathrm{P} & \end{array}$$

dependence of initial velocity on substrate and inhibitor concentrations in Eqn 7, and for this case, K_s^{app} is given by Eqn 8.

$$\frac{v}{\mathrm{E}} = \frac{k}{1 + \frac{K_s}{\mathrm{S}}\left(1 + \frac{\mathrm{I}}{K_i} + \frac{\mathrm{I}^2}{K_i \cdot K_{ii}}\right)} \qquad (7)$$

$$K_s^{app} = K_s\left(1 + \frac{\mathrm{I}}{K_i} + \frac{\mathrm{I}^2}{K_i \cdot K_{ii}}\right) \qquad (8)$$

Substitution of Eqn 8 into Eqn 1 gives

$$K_i^{app} = \frac{K_i \cdot K_{ii}}{K_{ii} + \mathrm{I}}$$

$$\text{i.e. } \frac{1}{K_i^{app}} = \frac{1}{K_i} + \frac{\mathrm{I}}{K_i \cdot K_{ii}} \qquad (9)$$

This predicted linear dependence of $1/K_i^{app}$ on I is as observed in Figs 2 and 5, and according to Eqn 9, the ordinate intercepts on the plots can be equated with $1/K_i$ and the slopes with $1/K_i \cdot K_{ii}$. Thus it is theoretically possible to evaluate both K_i and K_{ii} for the partially competitive inhibitors in Figs 2 and 5 in accordance with Scheme 2. Values for K_i and K_{ii} calculated in this way are given in Table II. Unfortunately, both

TABLE II

INHIBITION CONSTANT DATA FOR PARTIALLY COMPETITIVE INHIBITORS OF CARBOXYPEPTIDASE A

Carboxylate ion	K_i (M)	K_{ii} (M)	$K_i \cdot K_{ii}$*	$K_i \cdot K_{ii}$**	$K_i \cdot K_{ii}$***
Heptanoate	$1.5 \cdot 10^{-3}$	$5.3 \cdot 10^{-4}$	$8.1 \cdot 10^{-7}$	$6.5 \cdot 10^{-7}$	
Octanoate	$4.6 \cdot 10^{-3}$	$8.7 \cdot 10^{-4}$	$4.0 \cdot 10^{-6}$	$3.6 \cdot 10^{-6}$	
Cyclohexane carboxylate	$4.0 \cdot 10^{-3}$	$2.7 \cdot 10^{-4}$	$1.1 \cdot 10^{-6}$	$1.0 \cdot 10^{-6}$	
Cyclohexylacetate	$1.2 \cdot 10^{-2}$	$1.0 \cdot 10^{-3}$	$1.2 \cdot 10^{-5}$	$6.3 \cdot 10^{-6}$	$9.3 \cdot 10^{-6}$
3-Cyclohexylpropanoate	$8.0 \cdot 10^{-4}$	$6.2 \cdot 10^{-4}$	$5.0 \cdot 10^{-7}$	$3.6 \cdot 10^{-7}$	

* From $1/K_i^{app}$ vs I.

** From E/v vs I^2 for *O*-hippuryl-L-3-phenyllactic acid.

*** From E/v vs I^2 for L-2-aceturoxybutanoic acid.

of these values rely on the estimation of what, in some cases, is quite a small intercept, and small errors in the estimation of this intercept will be reflected as relatively large errors in its reciprocal, K_i, and also K_{ii}.

Eqn 7 may be re-arranged to Eqn 10.

$$\frac{E}{v} = \frac{1 + K_s/S}{k_{cat}} + \frac{K_s \cdot I}{S \cdot k_{cat} \cdot K_i} + \frac{K_s \cdot I^2}{S \cdot k_{cat} \cdot K_i \cdot K_{ii}} \tag{10}$$

The observed linear dependence of E/v on I^2 in Fig. 4 requires that, for Eqn 10 to be applicable, the term in I should be negligibly small relative to the term in I^2 over the inhibitor concentration range under study. If this is assumed to be the case, then the slopes of the lines in Fig. 4 can be equated to $K_s/S \cdot k_{cat} \cdot K_i \cdot K_{ii}$. Thus the product $K_i \cdot K_{ii}$ can also be independently evaluated from these plots. Values of $K_i \cdot K_{ii}$ calculated in each way for each inhibitor are included in Table II, and in general, reasonable agreement between the two values is apparent. In particular, the values of $K_i \cdot K_{ii}$ calculated in this way for cyclohexyl acetate ion from the data for the two different substrates, are in acceptable agreement considering the experimental errors involved.

We therefore feel that Scheme 2 is consistent with the available experimental data for these inhibitors. This scheme assumes an ordered binding of two inhibitor molecules to independent sites on the enzyme. Such ordered binding is, of course, kinetically indistinguishable from random binding of two inhibitor molecules as indicated in Scheme 3,

Scheme 3

$$E + S \xrightarrow{K_s} E\text{–}S \rightarrow E + P$$

$$\begin{tikzcd}
E + I \arrow[r, shift left, "K_i'"] \arrow[d, shift left] & E - I \arrow[l, shift left] \arrow[d, shift left] \\
I - E \arrow[u, shift left, "K_i''"] \arrow[r, shift left] \arrow[ur, phantom, "+I"] & E - I_2 \arrow[l, shift left, "K_{ii}''"] \arrow[u, shift left, "K_{ii}'"']
\end{tikzcd}$$

which generates rate equation 11, which has the

$$\frac{v}{E} = \frac{k}{1 + \frac{K_s}{S}\left(1 + I\left(\frac{1}{K_i'} + \frac{1}{K_i''}\right) + \frac{I^2}{K_i' \cdot K_{ii}'}\right)} \tag{11}$$

same form as Eqn 7, but with

$K_i = K_i' \cdot K_i''/(K_i' + K_i'')$, and $K_i \cdot K_{ii} = K_i' \cdot K_{ii}' = K_i'' \cdot K_{ii}''$.

We are unable to distinguish between Schemes 2 and 3 at the present time.

In view of the fact that partially competitive inhibition is most usually [2] associated with the formation of an E–I–S complex which reacts at the same rate as the corresponding E–S complex, modification of Schemes 2 and 3 should be considered in which an E–I–S species is also present. One possible case is given in Scheme 4, which

Scheme 4

$$\begin{array}{rl}
\mathrm{E + S \rightleftarrows E\text{–}S} & \text{dissociation constant } K_s \\
\mathrm{E + I \rightleftarrows E\text{–}I} & \text{dissociation constant } K_i \\
\mathrm{E\text{–}I + I \rightleftarrows E\text{–}I_2} & \text{dissociation constant } K_{ii} \\
\mathrm{E\text{–}S + I \rightleftarrows E\text{–}I\text{–}S} & \text{dissociation constant } K_{si} \\
\mathrm{E\text{–}S} \xrightarrow{k} \mathrm{E + P} & \\
\mathrm{E\text{–}I\text{–}S} \xrightarrow{k} \mathrm{E\text{–}I + P} &
\end{array}$$

leads to the rate equation 12. Re-arrangement of

$$\frac{v}{\mathrm{E}} = \frac{k}{1 + \dfrac{K_s}{\mathrm{S}} \dfrac{(1 + \mathrm{I}/K_i + \mathrm{I}^2/K_i \cdot K_{ii})}{(1 + \mathrm{I}/K_{si})}} \qquad (12)$$

this equation shows that for large I, E/v becomes linear in I, and that at no time does E/v approach linearity in I^2 as is experimentally observed (Fig. 4). A similar conclusion also holds for other such variations in Schemes 2 and 3, and so it seems that E–I–S species are not important in the reversible inhibition of carboxypeptidase A by these carboxylate anions.

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