Hydrolysis of Conformationally Homogeneous Substrates by α-Chymotrypsin. Consequences of the Enzymatic Specificity

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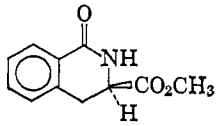
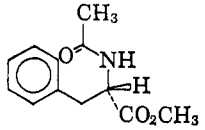
Abstract: The rates of acylation of α-chymotrypsin (ChT) (k_2' of Table II) by the nitrophenyl esters of *cis*- (axial) and *trans*- (equatorial) 4-*t*-butylcyclohexanecarboxylic, 4-*t*-butylbenzoic, cyclohexanecarboxylic, benzoic, and acetic acids stand in the ratio 1:68:36:121:13:16 in 20% methanol, pH 8 at 25°. Results of turnover studies are in good agreement with the acylation rates. The data suggest the hypothesis that D-3-carbomethoxy-3,4-dihydroisocarbostyril (D-CDIC) undergoes chymotryptic hydrolysis in the conformation with an equatorial carbomethoxy group. The necessary conditions for application of the models to D-CDIC are discussed. The equatorial D-CDIC hypothesis leads to proposals for the origin of the stereospecificity of ChT toward D- and L-CDIC and for the geometric disposition of methyl acetyl-L-phenylalaninate at the active site of ChT which can be tested experimentally.

Detailed studies on the interaction of low molecular weight substrates and inhibitors with α-chymotrypsin (ChT) represent one method for exploring the structural- and stereo-specificity of the enzyme.[3] One goal of such studies is definition of the geometrical disposition of a "specific" substrate at the active site of ChT.[4] The considerable number of degrees of freedom available to typical model compounds and the possibility of variation in the conformation of the enzyme with the nature of the adsorbed or covalently bound substrate (or inhibitor)[5] may severely limit the utility of this approach. The former difficulty has been cleverly circumvented, however, by investigations of the ChT-catalyzed hydrolysis of D- and L-3-carbomethoxydihydroisocarbostyril (CDIC) and related compounds.[4]



D-CDIC



L-APME

These investigations established that chymotryptic hydrolyses of D-CDIC and methyl acetyl-L-phenylalaninate (L-APME) are highly similar and that D-CDIC is ~4000 times more reactive than L-CDIC. The possibility then arises of employing the relatively rigid geometry of D-CDIC to define the geometric disposition of a specific substrate such as L-APME at the active site of ChT, provided the analogy between D-CDIC and L-APME is valid. Attempts in this direction have been described by Hein and Niemann[4b,c] and Awad, Neurath, and Hartley.[6,7]

Unfortunately, a single important ambiguity remains in the molecular conformation of D-CDIC. The carbomethoxy group can achieve either an axial or an equatorial position on the amide ring *via* the (half-) chair–chair interconversion of the ring.[8-10] Knowledge of which conformation is implicated in the ChT reaction is clearly crucial to understanding and using the CDIC results. Both Hein and Niemann and Awad, Neurath, and Hartley assume that it is axial D-CDIC which is the kinetically important conformation and which should serve as the L-APME model. We have attempted to test the suggestion that ChT, in contrast to hydroxide ion,[11] will preferentially catalyze the hydrolysis of an axial carboxylic ester by investigating the chymotryptic hydrolysis of two cyclohexyl derivatives with known conformation and of several related substances. The substrates employed were the nitrophenyl esters of *cis*- (axial) and *trans*- (equatorial) 4-*t*-butylcyclohexanecarboxylic,[11] 4-*t*-butylbenzoic, cyclohexanecarboxylic, benzoic, and acetic acids.[12] The validity of employing the 4-*t*-butylcyclohexane compounds as models for D-CDIC will be examined in the Discussion.

Results[13]

General. Rates of the enzyme-catalyzed (pH 8) and hydroxide ion catalyzed (pH 10–11) hydrolysis of the six nitrophenyl esters in a 20% methanol–3% acetonitrile aqueous solution at 25.1 ± 0.1° were determined spectrophotometrically at 400 mμ. Enzymatic rates were corrected for spontaneous ester hydrolysis when necessary. Both conventional turnover ($K_0 \simeq [S]_0 \gg [E]_0$, eq 1-4)[14] and attempted acylation[15] ($[E]_0 \geq [S]_0 \ll$

(1) Supported by Grant AM 08005-03 of the U. S. Public Health Service.

(2) Presented in part at the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965.

(3) For recent discussions of the specificity of ChT, see (a) J. R. Knowles, *J. Theoret. Biol.*, **9**, 213 (1965); (b) M. L. Bender and F. J. Kezdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).

(4) (a) G. Hein, R. B. McGriff, and C. Niemann, *J. Am. Chem. Soc.*, **82**, 1830 (1960); (b) G. Hein and C. Niemann, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1341 (1961); (c) G. E. Hein and C. Niemann, *J. Am. Chem. Soc.*, **84**, 4487, 4495 (1962).

(5) For example, D. E. Koshland, Jr., *Enzymes*, **1**, 332 (1959).

(6) E. S. Awad, H. Neurath, and B. S. Hartley, *J. Biol. Chem.*, **235**, PC35 (1960).

(7) The formulation of I. B. Wilson and B. F. Erlanger, *J. Am. Chem. Soc.*, **82**, 6422 (1960), has been criticized by Hein and Niemann.[4c] In our opinion, the major defect in the Wilson–Erlanger suggestion is postulation of a *cis*-amide bond for methyl benzoyl-L-phenylalaninate: see L. Pauling, "The Nature of the Chemical Bond," 3rd ed, Cornell University Press, Ithaca, N. Y., 1960, pp 281–282.

(8) The geometry of the amide ring of CDIC should be similar to that of 1,3-cyclohexadiene. For discussion of the latter, see ref 9.

(9) E. L. Eliel, N. L. Allinger, S. J. Angyal, and G. A. Morrison, "Conformational Analysis," Interscience Publishers, Inc., New York, N. Y., 1965, p 125.

(10) For brevity, the conformation of D-CDIC with the carbomethoxy group in the equatorial position will be termed equatorial D-CDIC, etc.

(11) See ref 9, Chapter 2.

(12) The esters will be abbreviated *cis*-4-*t*-Bu, *trans*-4-*t*-Bu, 4-*t*-Bu-Benz, Cyclohex, Benz, and NPA, respectively.

(13) Detailed discussion of some points raised in this section may be found in the Experimental Section.

(14) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3669 (1964).

(15) F. J. Kezdy and M. L. Bender, *Biochemistry*, **1**, 1097 (1962). Professor Bender has kindly pointed out that the additional important condition $[S]_0 >> K_0$ was inadvertently omitted from the apendix to this article and has given us some helpful suggestions on the acylation reactions.

Table I. α-Chymotrypsin-Catalyzed Hydrolysis of Some Nitrophenyl Esters in Turnover Reactions[a]

Substrate	Runs[b]	$10^6[S]_0$, M	Polynomial/visual 10^2k_0, sec^{-1}	Polynomial/visual 10^6K_0, M	First order 10^2k_0, sec^{-1}	First order 10^6K_0, M	k_0/K_0,[c] M^{-1} sec^{-1}
trans-4-*t*-Bu	32	2.98–9.53	2.93 ± 0.16[d]	8.43 ± 0.82	2.91 ± 0.11	6.75 ± 0.49	3476
4-*t*-BuBenz[e]	24	2.89–7.71	2.94 ± 0.53[f]	23.8 ± 5.1	1.40 ± 0.07	6.98 ± 0.65	2006
Cyclohex	28	3.98–35.8	10.1 ± 0.1[f]	16.1 ± 0.4			6273
Benz	28	3.71–33.4	0.65 ± 0.02[f]	9.82 ± 0.66			660
NPA[g]	20	3.82–99.4	11.7 ± 0.6[d]	163 ± 12	13.9 ± 0.3	193 ± 6	662

[a] In 20% methanol–3% acetonitrile, pH 8.01 ± 0.05, 25.1°, $[E]_0 = 5.19–5.25 \times 10^{-7}$ M. See Results and Experimental Section for details. [b] Number of points in Lineweaver–Burk plot. [c] Based on polynomial visual data except for 4-*t*-BuBenz. [d] Polynomial procedure. [e] Polynomial procedure gave $k_0 = 4.37 \pm 1.46 \times 10^{-2}$ sec^{-1}, $K_0 = 3.59 \pm 1.23 \times 10^{-5}$ M. [f] Visual procedure. [g] Visual procedure gave $k_0 = 1.35 \pm 0.03 \times 10^{-1}$ sec^{-1}, $K_0 = 2.04 \pm 0.06 \times 10^{-4}$ M.

K_m) experiments defined the enzymatic reactivity of the substrates. First- and second-order rate constants, where appropriate, were obtained by conventional

$$v_0 = k_0[E]_0[S]_0/(K_0 + [S_0]) \quad (1)$$

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (2)$$

$$K_m = (k_{-1} + k_2)/k_1 \simeq k_{-1}/k_1 \quad (3)$$

$$K_0 = (k_3/(k_2 + k_3))K_m \qquad k_0 = k_2k_3/(k_2 + k_3) \quad (4)$$

treatment of the data. All calculations were by the method of least squares and were carried out on the IBM 1620 and CDC 3600 computers at the University of Massachusetts Computing Center.

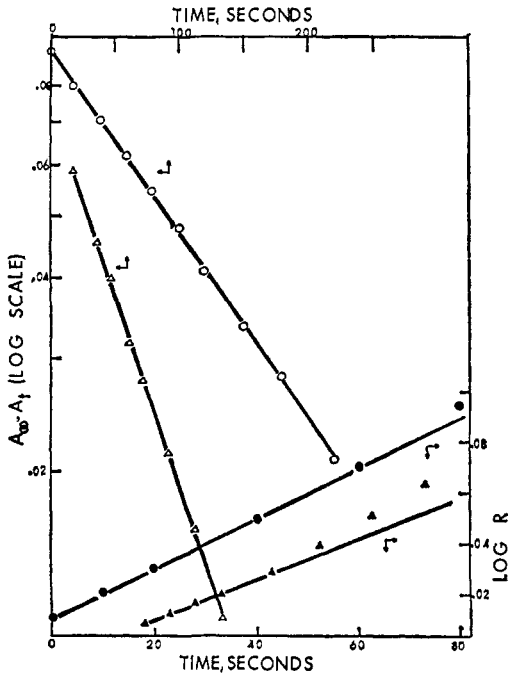


Figure 1. Plots of typical acylation runs: ○ and ●, first- and second-order plots for *p*-nitrophenyl acetate, respectively ($[E]_0 = 8.75 \times 10^{-6}$ M, $[S]_0 = 6.21 \times 10^{-6}$ M); △ and ▲, first- and second-order plots for *p*-nitrophenyl *trans*-4-*t*-butylcyclohexanecarboxylate, respectively ($[E]_0 = 4.83 \times 10^{-6}$ M, $[S]_0 = 4.33 \times 10^{-6}$ M). $R = (A_\infty - ([S]_0/[E]_0)A_t)/(A_\infty - A_t)$.

Turnover Experiments. A visual and/or polynomial[16] estimate of v_0, the initial velocity, was made for each optical density–time curve. Lineweaver–Burk[17] plots of $1/v_0$ *vs.* $1/[S]_0$ afforded k_0 and K_0 of eq 1. For some substrates, each turnover run gave a linear first-order kinetic plot of ln $(OD_\infty - OD_t)$ *vs.* time. In such instances, for each run the first-order rate constant k_1'' was calculated, and v_0 defined as $k_1''[S]_0$. The set of v_0's so obtained yielded an alternative Lineweaver–Burk determination of k_0 and K_0. Agreement between the various modes of treating the data was satisfactory except for 4-*t*-BuBenz, where the acylation results suggest that the first-order procedure is preferable. Turnover of *cis*-4-*t*-Bu was too slow to evaluate under the experimental conditions. Table I displays the results of the Lineweaver–Burk plots, which were analyzed by Wilkinson's method;[18] the small errors associated with most of the data indicate the general excellence of the plots.

Acylation Experiments. The acylation runs usually did not afford good second-order plots, apparently because the condition $[S]_0 \gg K_0$ could not be satisfied with the relatively insoluble substrates and some turnover intruded[15] (in the trivial case $[E]_0 \gg [S]_0$ good first- and second-order kinetics will of course obtain). Most runs in fact provided excellent first-order plots from the time recording of the optical density–time curve commenced (~15 sec after initiation of reaction, ~0–40% reaction already occurred) to 70–80% reaction. Treatment of the data therefore proceeded in two directions.

The initial portion, at minimum, of most runs obeyed second-order kinetics and provided an experimental k_2' (Figure 1). The part of each run so behaved was estimated visually from a second-order plot similar to those of Figure 1. No apparent second-order phase was seen with Cyclohex.

The first-order portion of each run was also estimated from a plot of the data (Figure 1), calculation afforded the experimental k_1', and a second-order rate constant was defined as $k_1'/[E]_0$. The primary justification for the first-order analysis is its success. Although in some instances there was a noticeable systematic dependency of $k_1'/[E]_0$ on $[E]_0$ and/or $[S]_0$, the standard deviations in $k_1'/[E]_0$ were small and $k_1'/[E]_0$ generally agreed with k_2' (Table II). The consistent tendency for $k_1'/[E]_0$ to be less than k_2' suggests that under our "acylation" conditions there is a "steady-state" [E], with $[E] < [E]_0$. Consequently, the second-order analysis of the data is probably more correct.

(16) K. A. Booman and C. Niemann, *J. Am. Chem. Soc.*, **78**, 3642 (1956).

(17) H. Lineweaver and D. Burk, *ibid.*, **56**, 658 (1934).

(18) G. N. Wilkinson, *Biochem. J.*, **80**, 324 (1961).

Table II. α-Chymotrypsin-Catalyzed Hydrolysis of Some Nitrophenyl Esters in Acylation Reactions[a,b]

Substrate	Runs	$10^6[S]_0$, M	$10^6[E]_0$, M	$k_1'/[E]_0$, M^{-1} sec^{-1}	k_2', M^{-1} sec^{-1}
cis-4-*t*-Bu	16	3.61–7.82	4.81–56.3	49 ± 3	52 ± 3
trans-4-*t*-Bu	11	3.67–7.94	4.83–8.84	2854 ± 189	3559 ± 470
4-*t*-BuBenz	12	3.56–8.89	4.81–53.1	1449 ± 83	1766 ± 90
Cyclohex	9	3.98–7.16	4.83–8.84	6839 ± 266	
Benz	16	3.71–6.68	4.78–52.8	557 ± 28	652 ± 28
NPA	21	3.82–6.21	4.78–56.3	774 ± 30	907 ± 76
	10[c]	3.82–6.21	4.78–8.75	1715 ± 191	2603 ± 152

[a] Footnote *a*, Table I except for $[E]_0$. [b] All uncertainties are standard deviations. [c] In 3% acetonitrile, pH 7.7.

Table III. Indole Inhibition of the α-Chymotrypsin-Catalyzed Hydrolysis of Some Nitrophenyl Esters in Turnover Reactions[a]

Substrate	Runs[b]	$10^6[S]_0$, M	$10^3[I]_0$, M	Method[c]	10^2k_0, sec^{-1}	10^6K_0, M	10^3K_i, M
trans-4-*t*-Bu	...[d]		0		2.93 ± 0.16	8.43 ± 0.82	
	15	3.18–7.43	6.54	Visual	1.29 ± 0.13	13.0 ± 1.9	2.6
				Poly	1.63 ± 0.52	17.8 ± 7.4	
				First	8.15 ± 0.57	5.29 ± 0.75	
4-*t*-BuBenz	...[d]		0		1.40 ± 0.07	6.98 ± 0.65	
	16	3.38–7.88	6.54	First	0.42 ± 0.07	6.46 ± 2.05	3.1
				Visual	0.53 ± 0.15	9.12 ± 4.69	
	10	3.38–7.88	6.54	First	0.38 ± 0.03	6.01 ± 1.04	3.0
				Visual	0.32 ± 0.02	4.16 ± 0.69	
Cyclohex	...[d]		0		10.1 ± 0.1	16.1 ± 0.4	
	14	3.70–33.3	4.22	Visual	11.0 ± 0.6	38.8 ± 3.3	3.5
				Poly	10.2 ± 0.8	33.4 ± 3.9	
	14	3.70–33.3	6.33	Visual	10.5 ± 0.5	46.0 ± 2.9	3.6
				Poly	10.4 ± 0.7	44.8 ± 4.3	
NPA	...[d]		0		11.7 ± 0.6	163 ± 12	
	13	4.69–98.4	4.15	Poly	14.5 ± 1.9	519 ± 75	3.0
				Visual	15.0 ± 1.8	539 ± 74	
				First	15.7 ± 0.8	508 ± 29	
	11	6.15–98.4	6.23	Poly	18.6 ± 2.9	905 ± 146	2.8
				Visual	35.6 ± 17.8	1730 ± 750	
				First	16.5 ± 3.3	703 ± 157	

[a,b] The same as footnotes *a,b* of Table I except $[E]_0 = 5.18–5.57 \times 10^{-7}$ *M*. [c] The initial velocity, v_0, was estimated either visually, by polynomial fit or from the integrated first-order rate expression, as described in the text. Where more than one method was used, the first set of results given is preferred and was used to calculate K_i. [d] From Table I.

Acylation of ChT with NPA in 3% acetonitrile (Table II) provided a check on our treatment of the acylation experiments. The pattern of results approximated those in the methanolic solvent and k_2' agreed with the value 2890 M^{-1} sec^{-1}, pH 7.8, estimated from the literature.[15,19] Even in this highly aqueous medium, where $K_m < [S]_0 \leq [E]_0 > K_0$ but not $\gg K_0$, first-order plots were superior to second-order ones. If deviations from second-order acylation kinetics arise from the incursion of turnover, then the effect should be even greater in the methanolic solvent than in the more aqueous one, since the ratio k_3/k_2 of eq 2 increases as the methanol content of the solvent rises.[19]

Kezdy and Bender[15] showed that on the basis of eq 1–4, $k_2' = k_2/K_m = k_0/K_0$. The appropriate columns of Tables I and II reveal the desired agreement between turnover and acylation results.

Indole Inhibition. The effect of indole on both turnover and acylation was determined and the data treated as in the uninhibited reactions. Application of eq 5 and 6 to the turnover and acylation experiments, respectively, provided the inhibitor constant K_i, which was essentially independent of the substrate or reaction employed. The small range of $[S]_0$ available for the *t*-butyl-containing substrates led to a degree of uncertainty in k_0 and K_0 as to preclude determination of the kind of inhibition encountered. Tables III and IV display the results.

$$K_i = [I]_0/(\{(k_0/K_0, [I] = 0)/(k_0/K_0, [I] = [I]_0)\} - 1) \quad (5)$$

$$K_I = [I]_0/(\{(k_2', [I] = 0)/(k_2', [I] = [I]_0)\} - 1) \quad (6)$$

Reactivity toward Hydroxide Ion. First-order rate constants for the hydrolysis of the esters in buffers of pH 10–11 were divided by the apparent $[OH^-]$ to give the results summarized in Table V (see Experimental Section for actual rate constants).

The rate of alkaline hydrolysis of Cyclohex should provide a means of determining the conformational free energy difference[11,20] for the carbo-*p*-nitrophenoxy group by the Winstein–Holness method.[11] A detailed study will be necessary before it can be decided if the present observations represent a breakdown[20] in this approach to the determination of conformational preferences. Molecular models suggest that the effective size of a carbo-*p*-nitrophenoxy and a carbomethoxy group on the cyclohexane ring should be similar; for the latter, $-\Delta G°_x = 1.1$ kcal/mole.[11] We conclude that Cyclohex has primarily an equatorial ester group and that all the cyclohexyl substrates should possess

(19) M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kezdy, *J. Am. Chem. Soc.*, **86**, 3697 (1964).

(20) But see H. Kwart and T. Takeshita, *ibid.*, **86**, 1161 (1964).

Table IV. Indole Inhibition of the α-Chymotrypsin-Catalyzed Hydrolysis of Some Nitrophenyl Esters in Acylation Reactions[a,b]

Substrate	Runs	$10^6[S]_0$, M	$10^6[E]_0$, M	$10^3[I]_0$, M	k_2', M^{-1} sec^{-1}	10^3K_i,[c] M
cis-4-*t*-Bu	8	3.92–5.88	9.38–56.3	4.15	24 ± 2	3.5
	8	3.92–5.88	9.38–56.3	6.23	19 ± 2	3.4
trans-4-*t*-Bu	7	3.11–7.11	4.83–53.1	4.22	1099 ± 183	1.9
	8	3.11–7.11	4.83–53.1	6.33	873 ± 140	2.1
	10	3.18–8.49	4.87–53.6	6.54	877 ± 133	2.1
4-*t*-BuBenz	10	3.06–9.78	4.83–53.1	4.22	809 ± 73	3.6
	8	3.06–9.78	4.83–53.1	6.33	656 ± 62	3.7
	9	3.38–9.00	4.87–53.6	6.54	622 ± 51	3.6
Cyclohex	8	3.70–6.67	4.76–8.72	4.22	3158 ± 85	3.6
	8	3.70–6.67	4.76–8.72	6.33	2498 ± 105	3.6
Benz	7	4.18–5.56	8.80–52.8	0.56	560 ± 13	3.4
	8	4.18–5.56	8.80–52.8	1.01	509 ± 22	3.6
	8	4.18–5.56	8.80–52.8	4.15	279 ± 15	3.1
	8	4.18–5.56	8.80–52.8	6.23	215 ± 11	3.1
NPA	11	3.78–6.15	9.38–56.3	4.15	329 ± 20	2.4
	12	3.78–6.15	9.38–56.3	6.23	268 ± 19	2.6

[a] Footnote *a*, Table I except for $[E]_0$. [b] All uncertainties are standard deviations. [c] Based on the second-order treatment of the acylation data.

Table V. Relative Reactivity of Six Nitrophenyl Esters toward α-Chymotrypsin and Hydroxide Ion

p-Nitrophenyl ester of	Conformation of ester group	Rel reactivity toward α-ChT[a]	Rel reactivity toward OH^-
cis-4-*t*-Bu-cyclohexaneCOOH	Axial	1	1
trans-4-*t*-Bu-cyclohexaneCOOH	Equatorial	68	10
4-*t*-Bu-benzoic acid	Coplanar to ring	36	8
CyclohexaneCOOH	Primarily equatorial	121	11
Benzoic acid	Coplanar to ring	13	18
Acetic acid		16	52

[a] Based on second-order acylation data except for the fourth entry, which is based on k_0/K_0 of the turnover experiments.

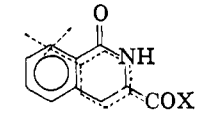
relatively strain-free chair forms, with twist or boat forms unimportant for the reactions here considered.

Discussion

Table V summarizes the results of the present study and demonstrates that the reactivity patterns of the six nitrophenyl esters toward α-chymotrypsin and hydroxide ion are markedly different. In the enzymatic reaction *cis*-4-*t*-Bu, with its carbo-*p*-nitrophenoxy group axially disposed to the cyclohexane ring, is less reactive than (a) the two other cyclohexane derivatives, in which the ester group must or may[21] assume an equatorial conformation, and (b) the two benzoic acid substrates, where the position of the ester group relative to the acyl ring is more reminiscent of an equatorial than an axial cyclohexyl ester. With the model compounds ChT discriminates *against* an axial ester group even more than does OH^-.

The subsequent discussion primarily considers under what conditions results of the model studies can be extrapolated to the conclusion that axial D-CDIC is not implicated in chymotryptic hydrolysis. The compounds here employed can be no more than crude models for the CDIC family, since they lack completely an α-acylamino chain and possess in only some distorted form a β-phenyl group, both of which play an important role[3,4] in orienting good substrates of ChT. The low values of K_0 and k_0 of Table I suggest that the absence of these orienting functions produces important unproductive binding of model substrates to enzyme.[4b,c] Such a possibility, coupled with the knowledge that k_0 and K_0 are complex quantities at best for nitrophenyl esters[14] (eq 4) and with the inability to measure k_0 and K_0 for *cis*-4-*t*-Bu, renders arguments in terms of k_0 and K_0 nearly meaningless. Most of our discussion will be based on the data in Table V; all of it is directed at evaluation of an assumed binding mode for the model substrates which places the ester group of the models near the position occupied by the ester group of either axial or equatorial D-CDIC and which is postulated to lead to hydrolysis.

Conditions for Applying the Models. The spatial orientation of the ester group of *cis*-4-*t*-Bu is closer to that of the ester group of axial D-CDIC than to that of the ester group of equatorial D-CDIC, and for *trans*-4-*t*-Bu the converse applies, so long as the approximate plane of the cyclohexane ring of the 4-*t*-butyl substrates is bound to ChT at about the same position as the dihydroisocarbostyril function of D-CDIC.[22] Molecular models confirm the validity of the foregoing statement for the extreme examples represented in **1** and **2**. In **1**,



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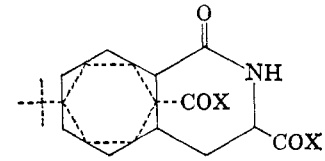


2

the cyclohexyl ring is more closely analogous to the amide ring, in **2**, to the aromatic ring of D-CDIC.

(21) To argue that Cyclohex undergoes enzymatic hydrolysis *via* an axial conformation seems unreasonable in view of the results with the other two cyclohexyl derivatives. Such an argument would be analogous to that put forth for D-CDIC in ref 4b,c and 6.

(22) (a) Carbon atoms 1, 3, 5 of the cyclohexane ring on one side of the imagined plane, atoms 2, 4, 6 on the opposite. (b) Likewise the benzoic acid derivatives more closely resemble equatorial D-CDIC.

For **1**, the resemblance between model compounds and D–CDIC is great, while for **2** the resemblance is not so good.[23] If the cyclohexane rings of *cis*-4-*t*-Bu and *trans*-4-*t*-Bu should be related to quite different parts of D–CDIC (*e.g.*, *cis* isomer to amide ring, *trans*, to aromatic ring) the utility of the models may vanish. Observation that ChT also preferentially hydrolyzes an equatorial ester group in the 3-*t*-butylcyclohexanecarboxylic acid family[24] strengthens our belief that the 4-*t*-butyl results can provide meaningful insight into the chymotrypsin mechanism.

The small effect of the 4-*t*-butyl groups (Table I) came as some surprise, and may ultimately prove to be a strong argument against the models. The high rates of chymotryptic hydrolysis of acetyl-L-hexahydrophenylalanine methyl ester[25] and of trypotophan derivatives[26] suggest that unfavorable steric interactions in the region of ChT to which **1** and **2** assign the *t*-butyl group should not be too severe, and that introduction of the *t*-butyl side chain might produce large favorable binding effects. A clearer understanding of the role of the *t*-butyl groups should be provided by investigations[24] on the chymotryptic hydrolysis of the nitrophenyl esters of *d*- and *l*-*trans*-3-*t*-butylcyclohexanecarboxylic acid (axial esters), where the *t*-butyl groups of the two enantiomers occupy very different positions in space when the ester functions are made to coincide.

Models *vs.* CDIC Family. Quantitative comparisons between the model and CDIC compounds, severely limited by the aforementioned ambiguities in the meaning of K_0 and k_0, lead to no firm decision on the validity of the models. We estimate that the k_0/K_0 reactivity of the methyl esters of *trans*-4-*t*-butylcyclohexanecarboxylic acids would lie between that of D–CDIC and L–CDIC.[27] This is a respectable magnitude of reactivity for such nonspecific substrates, especially since the ester group of *trans*-4-*t*-Bu (and of Cyclohex) can serve as a model for the equatorial ester group of either D– or L–CDIC (in terms of **1**) and still satisfy the previously defined conditions for applying the models. The transformation is effected by 180° rotation plus a slight displacement of the *t*-butyl group; perhaps herein lies the source of some of the ambiquity of the present experiments. Similar manipulations cannot cause *trans*-4-*t*-Bu to resemble axial D–CDIC.

Hopes that the transitions *trans*-4-*t*-Bu → 4-*t*-BuBenz and Cyclohex → Benz would mirror effects in the system D–CDIC → carbomethoxyisocarbostyril (CIC) were partially realized.[29] There is almost no difference in behavior for the first pair of model compounds but the decrease by a factor of 16 in k_0 for the second set begins to approach the D–CDIC/CIC results.

Investigations of indole inhibition represent a further examination of the relevance of the cyclohexyl models. Hein and Niemann[4c] used indole extensively in their D–CDIC study, and found competititive inhibition in the hydrolysis of D–CDIC (characteristic of "trifunctional" substrates) and noncompetitive inhibition in the hydrolysis of L–CDIC, with $K_i = 6\text{–}8 \times 10^{-4}$ *M*. We estimate[30] that in our solvent system K_i should be about $2.5 \times (6\text{–}8 \times 10^{-4}\ M) = 1.5\text{–}2 \times 10^{-3}\ M$, in fair agreement with the data of Tables III and IV. Although the type of inhibition manifested by the *t*-butyl compounds could not be reliably established (see Results), the nitrophenyl ester of cyclohexanecarboxylic acid, for which the turnover data are most reliable, exhibited clean competititve inhibition. The Cyclohex results substantiate our faith in the model compounds but suggest that distinctions among substrates, based on the kind of indole inhibition encountered, should be viewed with some skepticism. Although the inhibition data establish that hydrolysis of the nitrophenyl esters is definitely occurring at the active site of ChT, they neither prove nor disprove the validity of the models.

Conclusions. Statements 1–3 offer a simple but not unique rationalization of the data contained in this paper: (1) an axial ester group is the primary cause of the low enzymatic reactivity of *cis*-4-*t*-Bu; (2) axial D–CDIC is therefore eliminated as an important contributor to the rapid hydrolysis of D–CDIC by ChT; (3) by default, equatorial D–CDIC is the enzymatically reactive conformation (equatorial D–CDIC postulate).

Although these proposals must remain highly speculative in the absence of conclusive experiments, we can discern *no* experimental evidence to support the alternative axial D–CDIC postulate. Furthermore, exploration of the consequences of assuming the correctness of proposals 1–3 can lead to more definitive experimental tests of their validity.[31]

Let us first examine the origin of the stereospecificity of ChT toward CDIC.[29] The position of the carbonyl carbon of the ester group is taken to be the crucial variable, given the nature of the α-chymotrypsin mechanism.[3b] Direct measurements on Dreiding models, which are especially suited to studying the conformation problem, were made when the aromatic rings of D– and L–CDIC coincided precisely and the amide functions were superimposed upon each other. The carbonyl carbon–carbonyl carbon distance is ~2.5 A when axial D–CDIC and equatorial L–CDIC are compared (**3**, as in ref 4 and 6) and ~1.5 A for equatorial D–CDIC *vs.* equatorial L–CDIC (**4**), and one of these distances must be capable of causing D–CDIC

(23) The tacit assumption that the aromatic ring of D–CDIC binds at the hydrophobic site associated with the phenyl ring of L–APME and not at the polar binding site of the amide group is fundamental to using D–CDIC as a model for L–APME. The esters of the present study should certainly bind preferentially at the hydrophobic site; *cf.* ref 3b.

(24) Unpublished results with Dr. T. Sone. Detailed studies on a series of 3-*t*-butyl esters are underway.

(25) J. B. Jones and C. Niemann, *Biochemistry*, 2, 498 (1963).

(26) *E.g.*, F. J. Kezdy, G. E. Clement, and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3690 (1964).

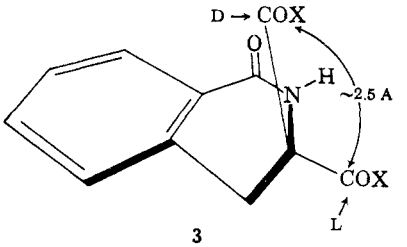
(27) *trans*-4-*t*-Bu and Cyclohex are 4–7.5 times more reactive than NPA under our reaction conditions. In aqueous solution at pH 7.8–7.9, NPA has[15] $k_0/K_0 = 5 \times 10^3\ M^{-1}\ \text{sec}^{-1}$ and D–CDIC has[4c] $k_0/K_0 = 4.3 \times 10^4\ M^{-1}\ \text{sec}^{-1}$. The values of k_0/K_0 for the model compounds and D–CDIC should therefore be approximately equal. The nitrophenyl esters of the models are perhaps 100 times more reactive than the corresponding methyl esters, since this factor is 100, 53, and 84 for derivatives of hippuric acid,[14] N-acetyl-L-tryptophan[26] and N-acetyl-L-phenylalanine,[26] respectively. The factor is 10^4 for esters of N-acetylglycine, however.[14]

(28) B. Zerner, R. P. M. Bond, and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3674 (1964).

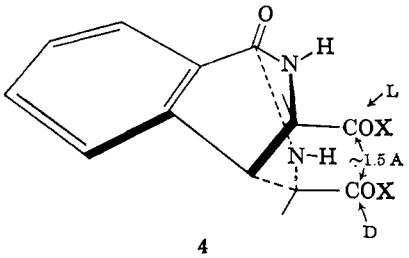
(29) K_0 is 22 and k_0 183 times more favorable for D–CDIC *vs.* L–CDIC; for D–CDIC *vs.* CIC, the figures are 3 and 169 times more favorable, respectively. The arguments probably relate primarily to the k_0 terms.

(30) The factor 2.5 is obtained from the data of S. Kaufman and H. Neurath, *J. Biol. Chem.*, **180**, 181 (1949), on the solvent dependence of K_0 (= K_m, probably) for the hydrolysis of N-acetyl-L-tyrosine amide by ChT.

(31) The equatorial D–CDIC postulate may eventually prove correct but the models, unjustified.



3



4

to be (at least)[32] 200–4000 times[29] more reactive than L-CDIC. The axial D-CDIC postulate maximizes the difference between the reactive conformation of D-CDIC and any accessible conformation of L-CDIC; the principle of maximum difference is alluring but to our knowledge without experimental support. A possible test of the proposition that the 1.5-A gap which results from the equatorial D-CDIC postulate is sufficient to explain the enzymatic stereospecificity is presented by the chymotryptic hydrolysis of the nitrophenyl esters of D- and L-*cis*-3-*t*-butylcyclohexanecarboxylic acid.[24] The equatorial ester groups of the enantiomers mimic in some measure the situation in equatorial D-CDIC *vis-à-vis* equatorial L-CDIC if the *t*-butyl groups are made to coincide.

A satisfactory explanation for the CDIC series of compounds must account for the behavior of CIC. Its (k_0/K_0) reactivity intermediate between that of D- and L-CDIC is nicely accommodated by **4**, but its low k_0 is more in accord with **3**. We are seeking further evidence for our belief that esters in these cyclic systems with the structure ⩾C—COX generally may be poor substrates for ChT (like Benz and unlike 4-*t*-BuBenz). Hein and Niemann's arguments on CIC are also unpersuasive, since their conclusion[4c] that L-CDIC undergoes reaction with an axial ester group and improperly oriented amide function leaves undisclosed how CIC ever undergoes chymotryptic hydrolysis.

Finally, a model for the geometric disposition of L-APME at the active site of ChT can be constructed on the basis of the equatorial D-CDIC postulate plus the following two assumptions: (a) corresponding ester and aromatic groups of L-APME and D-CDIC should nearly coincide, and (b) when the corresponding ester and aromatic groups of D-APME and D-CDIC are made to coincide, the acetamino chain of D-APME cannot be close to the position occupied by the acetamino group of L-APME in its reactive conformation. The CH→NHCO bonds of D-CDIC and of the L-APME model so constructed are found to point in nearly *opposite* directions. This leads to the conclusion that the amide function of D-CDIC can contribute little to the reactivity of D-CDIC.[33] Detailed discussion of this model for the reactive conformation of L-APME is deferred until studies on a series of 2-naphthoic acid derivatives are completed.

(32) L-CDIC does not necessarily undergo hydrolysis in the equatorial conformation of 3 and 4; see ref 4c for one alternative.

(33) Not too surprising, if true, since D-CDIC has a *cis* and L-APME, a *trans* amide bond.[7]

Experimental Section[34]

Chemicals. Buffer components were reagent grade, distilled water was redistilled through an all-glass apparatus, and methanol and acetonitrile were Eastman Kodak Spectro Grade. Cinnamoylimidazole had mp 132–134.5° (lit.[35] mp 133–133.5°), *p*-nitrophenol, mp 112–114° (lit.[36] mp 114°), and indole, mp 52–53° (lit.[37] mp 52°). The properties of the nitrophenyl esters are described in Table VI. Quantitative measurements of the nitrophenol liberated in the hydrolysis of the esters provided further proof of their purity. The esters were prepared from the corresponding acids (either commercial samples or synthesized by published procedures)[36] by a thionyl chloride–pyridine method. Particular attention was directed to the question of the stereochemical purity of *cis*-4-*t*-Bu and *trans*-4-*t*-Bu. A small amount of the acid chloride prepared as an intermediate in the synthesis of each nitrophenyl ester was added to methanol and the resultant methyl ester examined with vapor phase chromatography. Each methyl ester appeared to be >99% of the desired isomer.

Buffers. The following buffers were prepared:[39] (1) pH 5, 0.1 *M* (total) acetate; (2) pH 7.7, 0.067 *M* (total) phosphate; (3) pH 10 and 11 carbonate–borate; (4) 20% methanolic pH 8, 10, and 11 by diluting 20.0 ml of methanol to 100.0 ml with the appropriate aqueous buffer; all volumes were measured at 25°. The methanolic buffers were used for kinetics and the invariability of ϵ for *p*-nitrophenol in the pH 8 buffer provided proof of the reproducibility of the buffer preparation procedure.

Enzyme Solutions. Worthington 3 times crystallized ChT, Lots CDI 6058-59, 6079, and 6087-88, was used. Stock solution E was prepared by dissolving ~550 mg of ChT in 10 ml of pH 5 buffer and was stored in the refrigerator when not in use. The concentration of ChT in E was determined daily by titration.[35] Solutions E/6, E/11, and ET were prepared daily by mixing, respectively, the following milliliters of E and pH 7.7 buffer: 1.00 + 5.00, 0.500 + 5.00, 0.100 + 10.0.

pH. The apparent pH's of typical reaction mixtures (*e.g.*, for enzymatic runs, a mixture of 9.0 ml of buffer, 0.30 ml of the appropriate enzyme solution, and 0.30 ml of substrate solution) were measured with a Radiometer PHM 4c meter standardized against pH 9.18 borate buffer.[40] The following range of pH's was observed for different batches of buffer: E, 7.96–8.01; E/6, 8.02–8.04; E/11, 8.03–8.05; ET, 8.04–8.06; pH 10, 9.92–9.93; pH 11, 11.08–11.16.

Kinetic Procedure. Substrates were dissolved in acetonitrile. A typical enzyme-catalyzed run was initiated by adding 0.100 ml of substrate solution to an open cuvette containing 3.0 ml of buffer plus 0.100 ml of enzyme solution at 25.1 ± 0.1°. The cell was loosely stoppered and recording of the optical density–time curve commenced. In some instances, the enzyme solution was added to a solution of buffer plus substrate. For runs with indole either 0.050 ml of indole solution (in acetonitrile) plus 0.050 ml of substrate solution ("usual" method, assumed unless otherwise specified) or 0.100 ml of a solution containing indole plus substrate ("joint" method) was substituted for the substrate solution in the general procedure. The hydroxide ion catalyzed runs omitted the enzyme solution. A Cary Model 14 recording spectrophotometer equipped with thermostated cell compartment and cell holder and both 0–1, 1–2 and 0–0.1, 0.1–0.2 optical density slide wires was used.

The rest of the Experimental Section details the origin of the data displayed in Tables I–IV.

***cis*-4-*t*-Bu.** Turnover reactions were too slow to be meaningful since in 40–60 min, less than 1 mole of nitrophenol/mole of ChT was liberated ($[S]_0$ = 2.94–7.85 × 10^{-6} *M*, $[E]_0$ = 5.57 × 10^{-7} *M*).

(34) All melting points are uncorrected.

(35) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, 236, 2930 (1961).

(36) "Heilbron's Dictionary of Organic Compounds," A. H. Cook, H. M. Bunbury, and D. H. Hey, Ed., 4th ed, Oxford University Press, New York, N. Y., 1965, p 2477.

(37) See ref 30, p 1846.

(38) Professor E. Eliel kindly advised us on the preparation of *cis*- and *trans*-4-*t*-butylcyclohexanecarboxylic acids.

(39) W. M. Clark, "The Determination of Hydrogen Ions," The Williams and Wilkens Co., Baltimore, Md., 1928.

(40) R. G. Bates, *J. Res. Natl. Bur. Std.*, 66A, 179 (1962).

Table VI. Physical Properties of Nitrophenyl Esters

Compound	Mp, °C	Formula	Calcd, % C	Calcd, % H	Found, %[a] C	Found, %[a] H
cis-4-*t*-Bu[b]	94–95	$C_{17}H_{23}NO_4$	66.86	7.59	66.96	7.64
trans-4-*t*-Bu[c]	124–127	$C_{17}H_{23}NO_4$	66.86	7.59	67.32	7.62
4-*t*-BuBenz	123–125	$C_{17}H_{17}NO_4$	68.21	5.72	68.43	5.58
Cyclohex	49–51	$C_{13}H_{15}NO_4$	62.64	6.07	62.94	5.99
Benz	142–145.5[d]					
NPA	77–78[e]					

[a] Analyses by Micro-Tech Laboratories, Skokie, Ill. [b] The acid had mp 117–119.5°. H. H. Law and H. Hart, *J. Am. Chem. Soc.*, **81**, 4897 (1959), report mp 117–118°. [c] The acid had mp 173–174.5°; Law and Hart[b] give 174–174.5°. [d] Reference 36, p 2477, gives mp 142.5°. [e] Reference 36, p 2477, gives mp 81–82°.

In acylations, with $[E]_0 = 4.8\text{–}8.8 \times 10^{-6}$ *M*, up to 30%, but with $[E]_0 = 5.6 \times 10^{-5}$ *M* up to 85% total reaction was observed. Both first- and second-order plots were linear.

trans-**4-*t*-Bu.** Precipitation of substrate at $[S]_0 = 9.5 \times 10^{-6}$ *M* may have occurred in the uninhibited turnover reactions, but analysis of the data for the substrate range $3.0\text{–}7.9 \times 10^{-6}$ *M* gave values identical with those of Table I ($k_0 = 2.93 \pm 0.17 \times 10^{-2}$ sec^{-1}, $K_0 = 6.81 \pm 0.71 \times 10^{-6}$ *M*). First-order plots were linear to 50% or more reaction, and first-order and polynomial treatment of the data were in good agreement. Runs followed for a long time showed a diminution in the rate of liberation of nitrophenol at 50–60% reaction that was more rapid than was expected on the basis of the first part of the reaction. The effect was not investigated in detail, but seemed to occur at higher per cent reaction for lower $[S]_0$. It may have been caused by a small amount of a poisonous impurity but gross impurity is ruled out by the acylation runs which gave excellent plots to 85% reaction and liberated the theoretical amount of nitrophenol. Precipitation of substrate in the "usual" procedure caused considerable inconvenience in the indole-inhibited turnover studies, and resort had to be made to the "joint" procedure. The results in Table III refer to the latter technique and show that the first-order and less arbitrary visual treatments were not in too satisfactory agreement.

Acylations were straight forward except that at $[E]_0 = 8.84 \times 10^{-6}$ *M* some first-order plots showed initial curvature. For such runs, the first part of the reaction supplied k_2' and the tail, k_1' (plot usually linear from 40 to over 90% reaction). Similar observations hold for the inhibited acylations. In Table IV, the first two entries refer to the "usual," the third to the "joint" method of kinetics. Agreement between first- and second-order treatment of the acylations was consistently satisfactory.

4-*t*-BuBenz. Remarks made about turnover of *trans*-4-*t*-Bu apply to turnover here. Some reactions gave first-order plots which were linear to 50% reaction, with no sign of a tailing off. There is no obvious reason why the two methods of treating the turnover data were in such poor agreement for the uninhibited reactions but in reasonable agreement for the inhibited runs. The two series of runs in Table III were by the "joint" procedure and were carried out on different days; the Cary instrument was better behaved for the second set of runs. Acylations proceeded smoothly, with first-order plots linear from 15–90% reaction. The first two sets of data in Table IV refer to the "usual," the third, to the "joint" method.

Cyclohex. Turnover data were of excellent quality and the individual runs did not adhere to first-order kinetics. Acylations showed no trace of a linear second-order portion, and first-order plots were linear for 20–90% reaction.

Benz. Turnover was slow and, at most, 20% reaction was observed. No attempt was made to observe the inhibited turnover reaction. Acylations gave excellent first-order plots.

NPA. Turnover runs were first-order to at least 62% reaction ($[E]_0 = 5.57 \times 10^{-7}$ *M*, $[S]_0 = 9.84 \times 10^{-5}$ *M*) and the accuracy of the data was limited by the corrections necessary for the spontaneous hydrolysis. Acylations gave linear first-order plots to over 80% reaction. Reports[41] that indole promotes the deacylation of acetylchymotrypsin may explain the trend in k_0 of Table III.

Hydroxide Ion Catalyzed Reactions. Determination of the second-order rate constant for each ester was carried out in the pH range 9.9–11.2. For *cis*-4-*t*-Bu, *trans*-4-*t*-Bu, 4-*t*-BuBenz, Cyclohex, Benz, and NPA, k_{OH} in M^{-1} sec^{-1} was 1.45 ± 0.06 (6), 14.5 ± 0.8 (7), 11.2 ± 0.3 (4), 15.4 ± 0.6 (6), 26.0 ± 0.7 (6), and 75 ± 2 (7), respectively, where the uncertainties are standard deviations and the numbers in parentheses reflect the number of runs upon which k_{OH} is based.

Acknowledgment. Mr. Jon E. Rohde synthesized some of the substrates and carried out preliminary experiments on the hydrolysis of Cyclohex.

(41) (a) E. S. Awad, Ph.D. Thesis, University of Washington, 1959; (b) R. J. Foster, *J. Biol. Chem.*, 236, 2461 (1961).