Hydrophobic Interaction of Small Molecules with α-Chymotrypsin†

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ABSTRACT: Kinetic, binding, and optical rotatory dispersion data for a group of anionic and cationic inhibitors at pH 8 indicate that inhibition of α-chymotrypsin is a slow uncompetitive deactivation process which is controlled from a hydrophobic site separate from the active site. The control is manifested as an immediate partial reduction in enzyme activity followed by a slow continued first-order deactivation (ultimately complete), all as a result of the reversible hydrophobic binding of appreciably less than one inhibitor ion per molecule. Neutral inhibitors of comparable hydrophobicity lead primarily to precipitation. A mechanism for inactivation is proposed. Kinetic data for ten 3-(*n*-alkanoyl)-*O*-benzoate substrates indicate that with increasing hydrophobicity the derivatives *n*-octanoyl to *n*-dodecanoyl become predominantly more effective as inhibitors by the proposed mechanism. The *n*-propanoyl to *n*-heptanoyl derivatives act as Michaelis–Menten substrates, with more effective binding and greater reactivity at the active site with increasing hydrophobicity. Hydrophobic interaction at two sites may account, in general, for the parabolic structure–activity relationship that commonly occurs for homologous series substrates: 18 examples, with structure–activity correlations, are cited.

The initial purpose in undertaking the studies reported here was to give a better insight into the factors that contribute to the "parabolic relationship" that holds for a number of enzyme systems when, for a homologous series of substrates, enzyme activity is plotted against the number of carbon atoms in a carbon chain associated with the substrate. This matter is of particular importance to those who design biologically active compounds using linear regression analysis techniques based on structure–activity relationships (Hansch, 1969). α-Chymotrypsin was selected as the enzyme for study because its structure and properties have been so extensively characterized, it is so readily available in pure form, and because of the possibility that the results might be generally and directly applicable to other enzymes, at least to the other serine proteases. The 3-(*n*-alkanoyl)-*O*-benzoates[1] were selected as substrates because they are easily synthesized, their hydrolyses are easily followed analytically, and there existed published accounts (Hofstee, 1957, 1959) showing a parabolic relationship with α-chymotrypsin.

As experimental work progressed it became evident that the parabolic effect is in reality a reflection of the general way in which the activity of α-chymotrypsin is influenced by the hydrophobic interaction of the enzyme with small molecules. The bulk of this paper, therefore, is devoted to an examination of these interactions, with the conclusions being applied to an explanation of the parabolic effect.

Experimental Procedures

Materials. All purchased chemicals were of a research grade obtained from standard commercial sources. α-Chymotrypsin, a three times crystallized product prepared free of autolysis products and low molecular weight contaminants by the procedure of Yapel *et al.* (1966), was obtained from Worthington Biochemical Corp. α-Chymotrypsin stock solutions contained 2.0 mg/ml in 10^{-3} M HCl. Spectrophotometric assay at 282 nm (ε 2.03 $(mg/ml)^{-1}$ cm^{-1}) showed them to be 7.5×10^{-5} M. Titration of α-chymotrypsin with *trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961) showed the active-site content to be 89.6%. α-Chymotrypsin activity was shown to hold up in the absence of inhibitors for the periods and conditions needed for runs. The 3-(*n*-alkanoyl)-*O*-benzoic acid substrates were synthesized by procedures adapted from Kaufmann (1927). The melting points are summarized by Hofstee (1954); 3-(*n*-undecenoyl)-*O*-benzoic acid, not previously reported, had a melting point of 74.9–75.7°. The buffer solution of pH 8.0, referred to as 0.05 M Tris, was ~0.022 M Tris and 0.028 M Tris-HCl; each preparation was checked for exactness. Tetradecyltrimethylphosphonium bromide (mp 218–220°) was prepared by a method adapted from Jerchel and Kimmig (1950). Tetradecyldimethylsulfonium bromide (mp 88–94°) was prepared by first synthesizing its *p*-toluenesulfonate by a method adapted from Burness (1959) and then converting to the bromide by ion exchange using Dowex 1-X8 (Br^-).

Kinetic Measurements. The kinetic studies were made with a Gilford No. 2400S spectrophotometer, using automatic reference compensation and normally a full-scale absorbance of 0.1. The cell compartment was thermostated at 30.0°. Enzyme activity was recorded as rate of formation of 3-hydroxybenzoate as shown by change in absorbance at 300 nm (ε 1.0 $\times 10^3$ M^{-1} cm^{-1}). Reactions were initiated in the cell compartment by rapid mixing of a 0.10-ml aliquot of one component loaded onto a perforated Teflon platform (~0.95 cm^2) mounted at the bottom of a thin Teflon rod.

For noninhibition studies, 0.10 ml of α-chymotrypsin stock solution was added to 2.90 ml of buffered substrates. α-Chymo-

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[1] Abbreviations used are: the 3-(*n*-alkanoyl)-*O*-benzoates are referred to by the number of carbon atoms in the side chain, *e.g.*, no. 7 is the heptanoyl ester; *B*, molecules of inhibitor bound per molecule of enzyme; *P*, partition coefficient for a solute in the system 1-octanol–water; *F*, fraction of uninhibited initial rate of hydrolysis of no. 7 at 5.0×10^{-5} M that 2.50×10^{-6} M α-chymotrypsin is able to exhibit after having been in contact for *t* min with inhibitor at given concentration; [I], inhibitor concentration; V_0, initial rate of hydrolysis for a given set of conditions.

trypsin activity was frequently monitored by this procedure using 2.90 ml of 5.17×10^{-5} M no. 7 to give 5.00×10^{-5} M no. 7 and 2.50×10^{-6} M α-chymotrypsin in the reaction mixture. For inhibition studies, desired quantities of α-chymotrypsin and inhibitor solutions were rapidly mixed in 0.05 M Tris at $t = 0$ and maintained at 30.0°. Samples (2.90 ml) of mixture were used for analysis in the cell compartment, with 0.10-ml aliquots of 1.50×10^{-3} M no. 7 being added to each sample at appropriate time intervals (t) after mixing. In each instance initial rates were recorded for ~10 min. Although rates of uncatalyzed hydrolysis of the benzoate esters in 0.05 M Tris are very low (see Results), the ester solutions were never used for more than a few hours at a time.

Optical Rotatory Dispersion (ORD) Measurements. ORD measurements were made at room temperature (~23°) with a Cary Model 60 spectropolarimeter, using 1.0-cm cells. Each solution was made up with a freshly weighed sample of α-chymotrypsin dissolved in 0.05 M Tris and containing inhibitor at the desired concentration; the α-chymotrypsin concentration was 3.0×10^{-5} M. Because of a change in rotation that always occurs in the region of 285–300 nm just after the α-chymotrypsin is dissolved (especially in the first 2 or 3 hr), solutions were allowed to stand for 24 hr before taking the measurements presented in this paper. It was shown that the change in rotation resulting from dissolving alone was completely unrelated to the activity of the enzyme.

Equilibrium Binding Measurements. For the ^{14}C-labeled dodecanoate solutions equilibrium binding measurements were carried out with a modified Paulus (1969) apparatus fitted with Visking membranes; the α-chymotrypsin concentration was 2.0×10^{-5} M in 0.05 M Tris. For the ^{35}S-labeled dodecyl sulfate and the ^{14}C-labeled dodecyltrimethylammonium and tetradecyltrimethylammonium solutions, Bio-Rad Bio-Fiber 50 Minibeaker dialyzers with hollow cellulose fibers were adapted for use as ultrafilters; the α-chymotrypsin concentration was 3.0×10^{-5} M in 0.05 M Tris. Equilibria were run at room temperature (~23°). It was verified by activity assay that no enzyme passed through these membranes. Aliquots (0.10 ml) of both filtrate and original solution were added to 10.0 ml of Bray (1960) scintillation solution and counted on a Packard Tricarb liquid scintillation counter, Model 3314, using special low-K glass vials. For each run many aliquots of fresh filtrate were taken over extended periods of time to ensure that equilibrium had been reached and to improve the counting statistics. Of several types of membranes tried, none was found that would be satisfactory for measurements with [^{14}C]octanol or ^{14}C-labeled esters 11 and 12; very large and unpredictable quantities of these solutes were retained by the membranes.

Results

Figure 1 shows the parabolic relationship that exists for the 3-(*n*-alkanoyl)-*O*-benzoate substrates under a given set of conditions with α-chymotrypsin. There is an approximately 40-fold greater activity for a seven-carbon alkanoyl side chain than for no. 4 or 12. That the chemical reactivity of a given homologous series of substrates is little affected by the length of the carbon chain after the first two or three carbons is shown by the uncatalyzed hydrolysis of the 3-(*n*-alkanoyl)-*O*-benzoates in 0.05 M Tris at pH 8.0 at 30° which proceeds with the following rate constants: no. 2, 5.7×10^{-4} min^{-1}; no. 3, 4.9×10^{-4} min^{-1}; no. 4, 4.8×10^{-4} min^{-1}; no. 5 and higher, about $3.8 \pm 0.2 \times 10^{-4}$ min^{-1}. Conventional evaluation of $1/K_m$ for the 3-(*n*-alkanoyl)-*O*-benzoates shows about

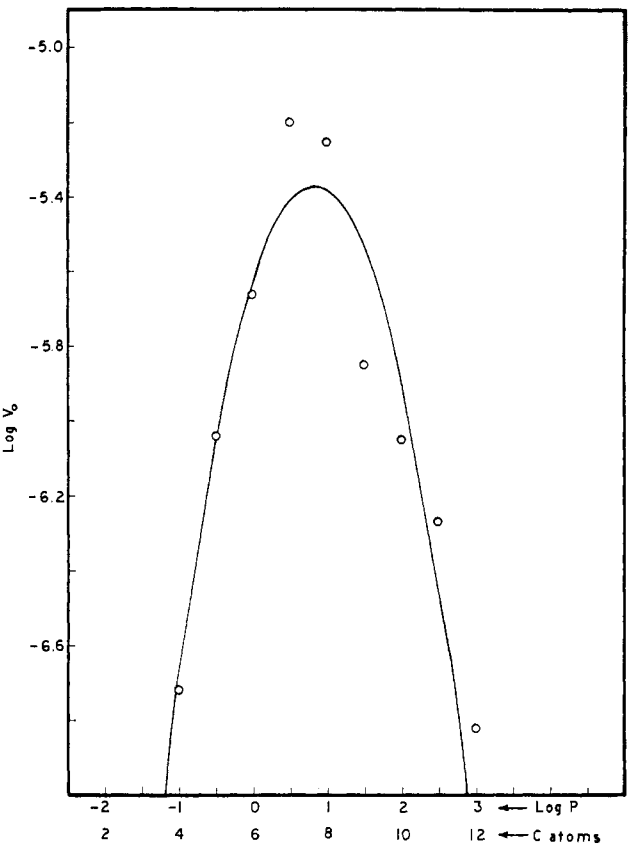

FIGURE 1: Log V_0 *vs.* log P and also the number of C atoms in the alkanoyl side chain of the 3-(*n*-alkanoyl)-*O*-benzoate substrates. V_0, initial rate, has units of M min^{-1} for the following conditions: 1×10^{-4} M substrate, 2.5×10^{-6} M α-chymotrypsin, 0.05 M Tris at pH 8.0, 30.0°. Points are experimental, curve is calculated from eq 8.

a fourfold increase in $1/K_m$ to an apparent value of about 3.7 $\times 10^{-3}$ M^{-1} for substrates with 7 or 8 carbons, followed by a similarly mild decrease from 9 to 12 carbons. Apparent k_{cat} values also show a parabolic relationship, with an approximately fivefold increase to a maximum of 8.8 min^{-1} for no. 7. When 3-(*n*-undecenoyl)-*O*-benzoate is used as a substrate (the double bond is between C-10 and -11), it is catalytically hydrolyzed as readily as no. 6, not extremely poorly like no. 11 which is comparable in size. These data imply that the large effects observed in Figure 1 result primarily from the hydrophobic characteristics of the substrates and not from their variation in size, chemical reactivity, or ability to form the Michaelis–Menten complex.

For this reason a study was made of the effect of nonacylating inhibitors with varying hydrophobicities, using no. 7 as a reference substrate. The fraction ($F = R/R_0$) of original α-chymotrypsin activity that exists in the presence of inhibitor after contact for t minutes was determined from the ratio of the initial rates of hydrolysis of no. 7 under standard conditions (2.50×10^{-6} M α-chymotrypsin and 5.00×10^{-5} M no. 7); R and R_0 represent the initial rate in the presence and absence of inhibitor, respectively. Figures 2–4 are typical of the relationship that exists between the first-order rate plots of log ($100F$) *vs.* post-mixing time (t) in minutes. Table I represents an effort to summarize the salient kinetic characteristics of six additional inhibitors at a variety of concentrations.

The general behavior pattern of the ionic inhibitors is as follows. Once a critical threshold concentration is exceeded an inhibitor produces a very fast (within a minute) partial reduc-

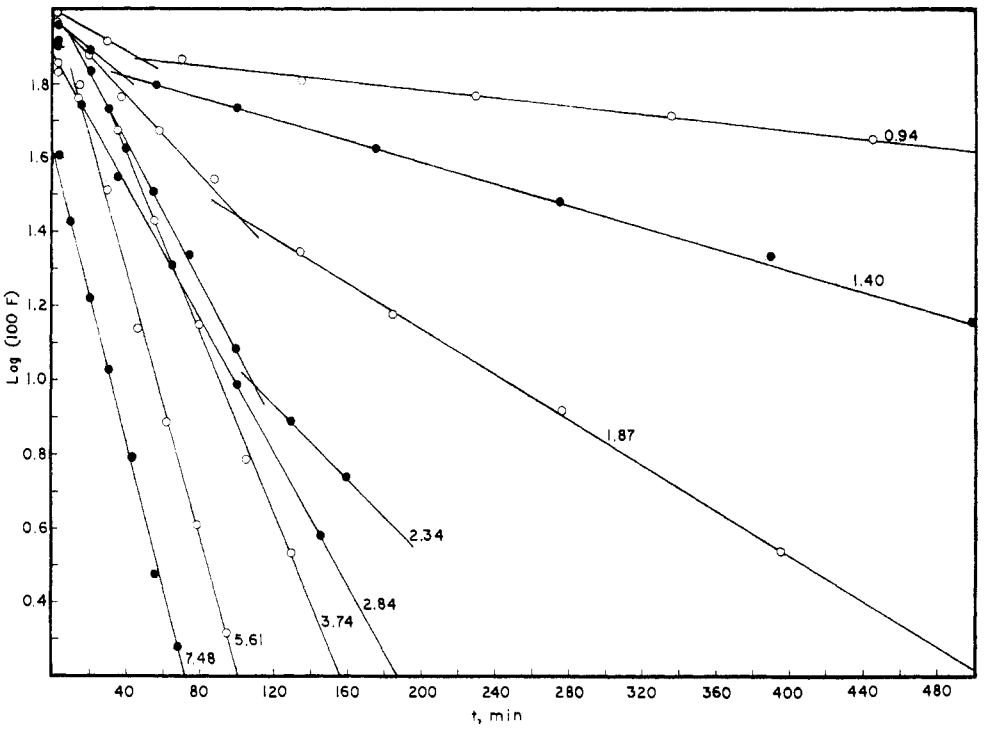

FIGURE 2.

FIGURES 2–4: Log (100F) *vs.* α-chymotrypsin–inhibitor contact time t. Each curve is labeled with the molar inhibitor concentration ($\times 10^4$) with which α-chymotrypsin is in contact; for all runs, 2.5×10^{-6} M α-chymotrypsin, 5.0×10^{-5} M no. 7 (reference substrate), 0.05 M Tris at pH 8.0, and 30.0°: (2) dodecyl sulfate; (3) dodecanoate; (4) tetradecyltrimethylammonium.

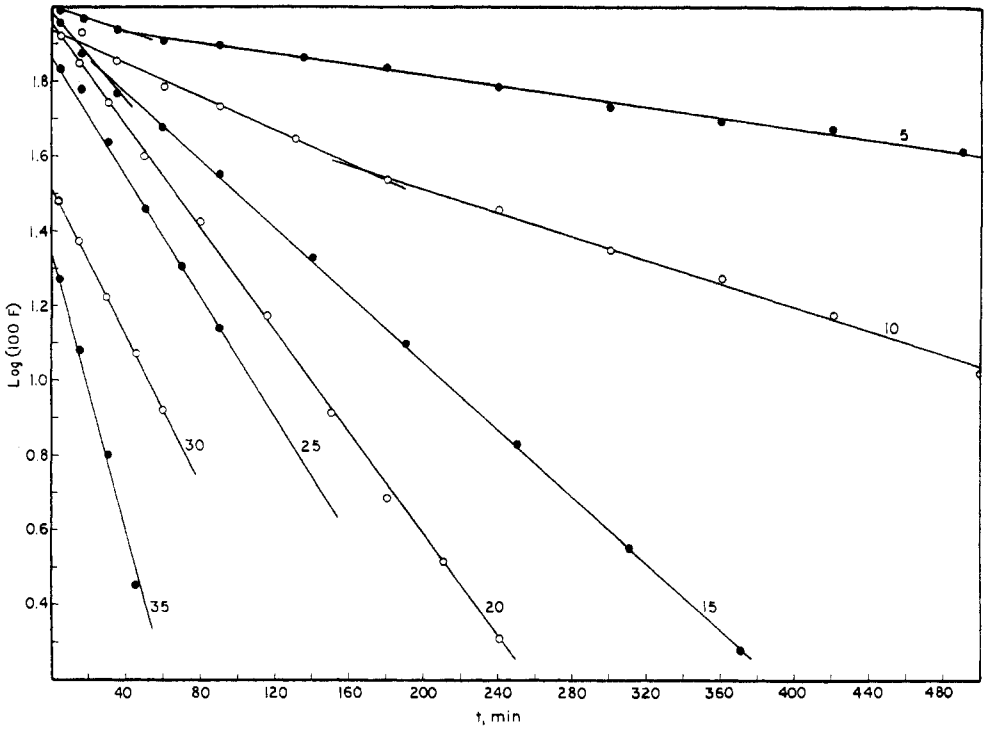

FIGURE 3 (see Figure 2).

tion in α-chymotrypsin activity followed by a relatively slow first-order reduction in activity; at lower concentrations there are frequently two first-order processes. The first-order processes are remarkably slow considering the easy access of the inhibitor to the enzyme. With increasing inhibitor concentration above the threshold concentration, the extent of immediate partial reduction in activity increases and the subsequent half-life for further inactivation decreases. The more hydrophobic the inhibitor in a given homologous series, the smaller the concentration required to produce a given amount of inhibition.

Experiments with neutral inhibitors having hydrophobicities comparable to those already discussed showed that these compounds (alcohols and amides) are not nearly as effective as the charged inhibitors, and that they basically lead to *precipitation* of the enzyme. Table I includes the effect

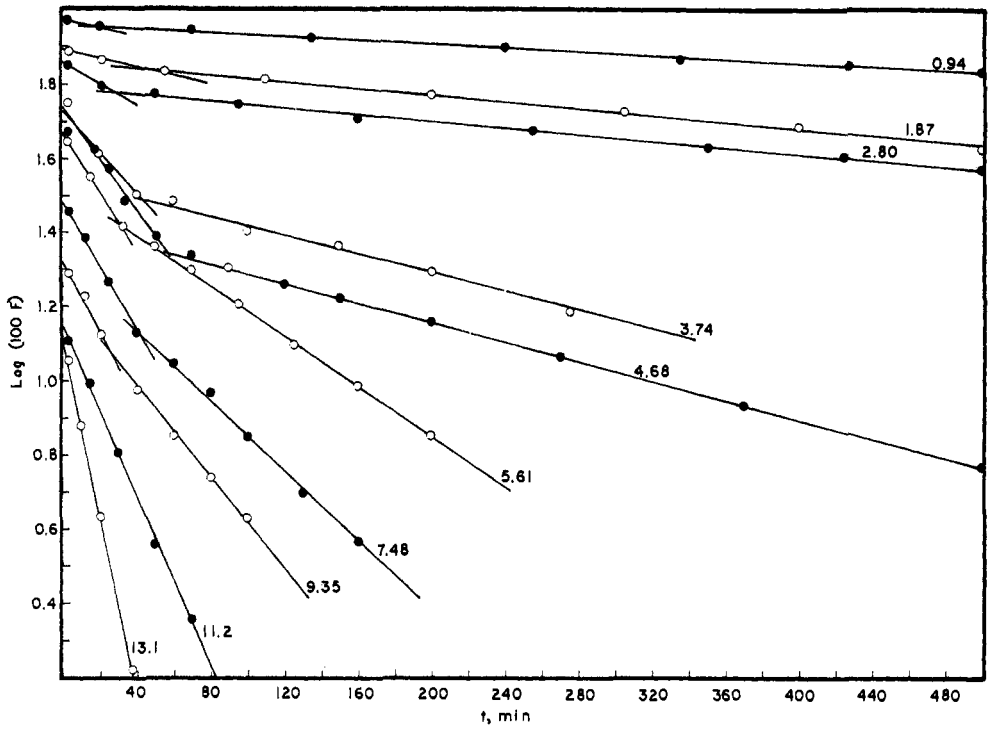

FIGURE 4 (see Figure 2).

of octanamide at near saturation; a high concentration (3.5×10^{-3} M) produces a small low rate of deactivation over a period of hours, and after a little more than 8 hr α-chymotrypsin begins to precipitate. At no concentration up to saturation (4.3×10^{-3} M) does 1-octanol bring about a reduction in enzyme activity within 1 hr as measured by the method used here, but precipitation will ultimately be observed at concentrations $>2.26 \times 10^{-3}$ M. Neither inhibition nor precipitation was observed within 1 hr with saturated solutions of 1-nonanol (1.4×10^{-3} M) or 1-decanol (4.5×10^{-4} M). For the lower 1-alkanols the following concentrations are the lowest ones observed to bring about precipitation of α-chymotrypsin within 1 hr; none of them caused an initial reduction in enzyme activity: 1-pentanol, 9.9×10^{-3} M; 1-hexanol, 7.8×10^{-3} M; 1-heptanol, 5.0×10^{-3} M. Concentrations greater than these caused some reduction in activity before precipitation occurred.

A useful measure of the hydrophobicity of a compound is log P, where P is the partition coefficient of that compound between 1-octanol and water (Leo *et al.*, 1971). Table I also lists values of log P for the inhibitors. Two inhibitors stand out in interesting ways. Although it is almost the least hydrophobic of all the inhibitors shown, dodecylsulfonate is about ten times more effective than any of the others, as indicated by the very low concentrations needed for inhibition. It also has a relatively small immediate partial deactivation but impressively short half-lives for first-order deactivation. Tetradecyldimethylsulfonium, almost the most hydrophobic of all the inhibitors shown, has impressive immediate partial deactivation effects but relatively poor first-order deactivation effects.

To check the competitive nature of the ionic inhibitors, the initial rates of hydrolysis of no. 7 with no inhibitor at six different concentrations over a concentration range of 0.1×10^{-4} to 2×10^{-4} M were determined and compared with the initial rates for the same concentrations of no. 7 for a period of 90 min in the presence of 7.5×10^{-4} M dodecyl sulfate. Two important conclusions can be drawn from these runs. The first is that over the 20-fold range in the concentration of no. 7, the immediate partial reduction in enzymic activity is remarkably constant and corresponds to 42% (with a random range from 40 to 46%) of the normal enzyme activity. The second conclusion is that the subsequent first-order deactivation of the enzyme is independent of the initial concentration of no. 7 (*i.e.*, $k = 0.0123$ min^{-1} with a random range of 0.0121–0.0127 min^{-1} corresponding to a half-life of 56.3 min). The first-order deactivation process is therefore determined solely by the inhibitor concentration as described in the earlier paragraphs. The implication of these observations is that the inhibitors are not acting competitively with the substrate at the active site.

Since α-chymotrypsin is known to dimerize under certain conditions (see Discussion) it might be concluded that the rate of deactivation is simply the rate of dimerization (assuming the dimer to be inactive), but if that were the case second-order rate constants should have been observed in all cases, not first order. Furthermore, when a fivefold increase in initial α-chymotrypsin concentration was used (along with 7.5×10^{-4} M dodecyl sulfate), the rate of deactivation was about one-third the normal rate instead of the approximately 25 times faster rate that might have been expected if dimerization occurs. The ready reversibility of the inhibitor–enzyme binding could be shown by making a fivefold dilution of the preceding reaction mixture at the point where the α-chymotrypsin retained only 2% of its original activity and again following the enzyme activity as a function of time. On dilution, the activity immediately rose to about 19%, corresponding to a little less than that which would have remained after the same period of contact had the solution been made originally at these concentrations (2.50×10^{-6} M α-chymotrypsin and 1.5×10^{-4} M dodecyl sulfate); it then continued to disappear by a first-order process at very nearly the rate expected for these concentrations.

Substrates 6, 9, 10, 11, and 12 were handled kinetically in the same manner as were the inhibitors in the work just described. As before, difference spectral changes were re-

TABLE I: Kinetic Characteristics of Several Inhibitors Taken from First-Order Plots at the Same Conditions as Those Shown in Figures 2–4.

Inhibitor, I (Hydrophobicity)	10^4[I] (M)	Intercept[a] (Log (100F))	Half-life[b] (min)	Intersection[c] (min)	Half-life[d] (min)
Tetradecanoate (log P = 1.80)	1.0	(53)[e]	387		
	2.0	(8)[e]	63		
	2.5	(2)[e]	43		
	3.0	1.57	14.1	15	55
	4.0	1.46	11.3	7	56
	5.0	1.35	9.8		
Tridecanoate (log P = 1.30)	1.0	(105)[e]	586		
	2.5	(30)[e]	555	306	365
	5.0	2.00	100	95	124
	7.5	1.99	53	205	55
	10.0	1.92	44		
	12.5	1.70	32.8		
	15.0	1.33	40.5	53	96
Dodecylsulfonate (log P = 0.96)	0.10	(32)[e]	348		
	0.20	(10)[e]	112	322	56
	0.40	1.90	34.7	45	27.4
	0.60	1.82	21.5		
	0.80	1.68	15.8		
	1.00	1.62	12.1		
Tetradecyltrimethylphosphonium (log P = 2.07)	2.0	1.96	868		
	4.0	1.81	240		
	6.0	1.62	91		
	7.0	1.48	62	50	95
	8.0	1.38	58		
	10.0	1.26	33.3		
	12.0	1.20	29.2		
Tetradecyldimethylsulfonium (log P = 2.70)	2.0	1.94	∞	180	5400
	4.0	1.78	∞	120	1020
	6.0	1.52	98	40	419
	8.0	1.24	45		
	10.0	1.02	31		
Octanamide (log P = 1.79)	9.4	1.95	406		
	34.6	1.92	337	270	990

[a] Intercept of the first first-order curve with the log (100F) axis. [b] Half-life of the first first-order process. [c] Intersection of the first first-order curve with the second one. [d] Half-life of the second first-order process. [e] No intercept (no "immediate partial reduction in activity"). Inactivation commences after a delay of the number of minutes shown in parentheses.

corded so as to give directly the initial rate of hydrolysis of no. 7 when it was added to substrate–enzyme mixtures that had stood in contact for measured lengths of time.

Qualitatively, Figure 5 shows that 10^{-3} M no. 6, originally present at 20 times the concentration of no. 7 as a reference, acts as would normally be predicted for a simple site-competitive Michaelis–Menten substrate. That is, as no. 6 is hydrolyzed over a period of time its concentration decreases and it competes less and less well with 7 for the active site; as a consequence, the initial rate of hydrolysis of 7 increases with time. Nevertheless, even when 6 is completely hydrolyzed, the initial rate of hydrolysis of 7 does not reach the value it would normally have in the absence of an inhibitor. The reason for this is that one of the products of 6 hydrolysis (the 3-hydroxybenzoate, not the hexanoate) has, in the period of the 5 hr required for the complete hydrolysis of 6, slowly and inexorably acted as an inhibitor (in the sense described in the earlier part of this paper) and reduced the enzyme activity from that which it was at the beginning of the experiment. In fact, the longer the enzyme stays in contact with the hydrolysis products after the initial 5-hr period the more it becomes additionally deactivated by the 10^{-3} M 3-hydroxybenzoate. This inactivation by the 3-hydroxybenzoate must not be confused with the usual concept of product inhibition associated with enzyme catalysis because the same concentration of 3-hydroxybenzoate when initially mixed with enzyme shows no inhibition of hydrolysis of 7, while on long contact it does.

In contrast with 6, substrates 9–12 (Figures 6–9) show dramatically different behavior with increasing hydrophobicity. All of these substrates lie to the right of the maximum in the parabolic activity curve, Figure 1. When compared with Figures 2–4 it is clear that they all act as inhibitors, with 11 and 12 having predominantly inhibitor characteristics. Ester 10 has a hydrophobicity (log P = 2.01) that is comparable to the ionic inhibitors dodecyl sulfate and tetradecylphosphonium, with the others ranging above and below this at 0.5 log P unit/CH_2 group. However, unlike the simple inhibitor studies in which the inhibitor concentration remained constant during the course of a run, these substrate studies are complicated by the fact that the substrate (*i.e.*, the inhibitor) concentration

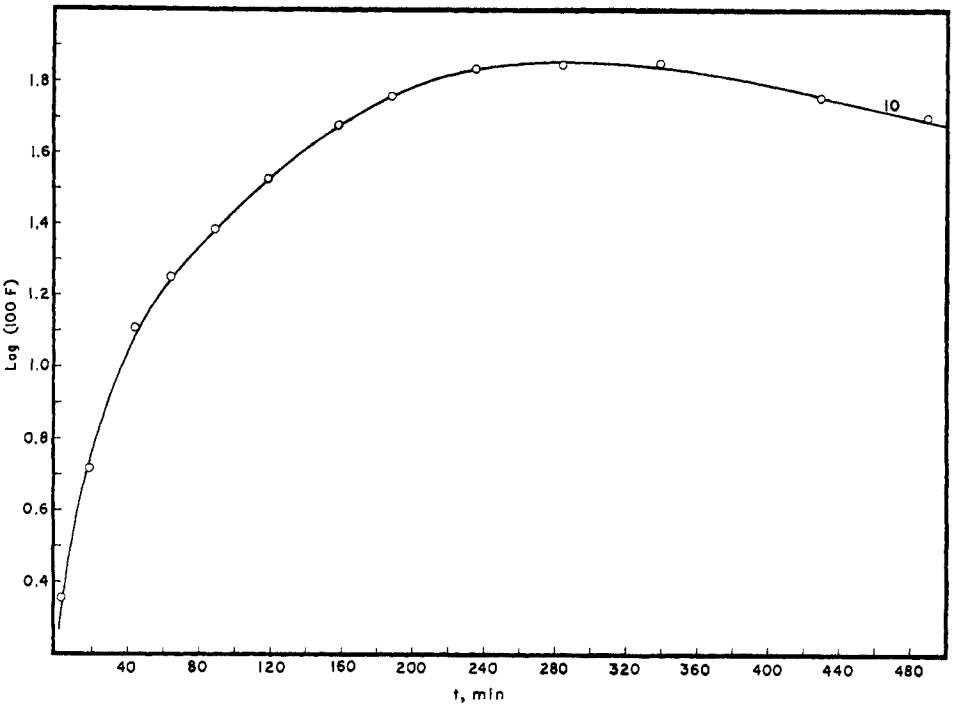

FIGURE 5.

FIGURES 5–9: Log (100F) *vs.* α-chymotrypsin-inhibiting substrate contact time t. Each curve is labeled with the molar concentration ($\times 10^4$) with which α-chymotrypsin is in contact; for all runs, 2.5×10^{-6} M α-chymotrypsin, 5.0×10^{-5} M no. 7 (reference substrate), 0.05 M Tris at pH 8.0, and 30.0°: (5) no. 6; (6) no. 9; (7) no. 10; (8) no. 11; and (9) no. 12.

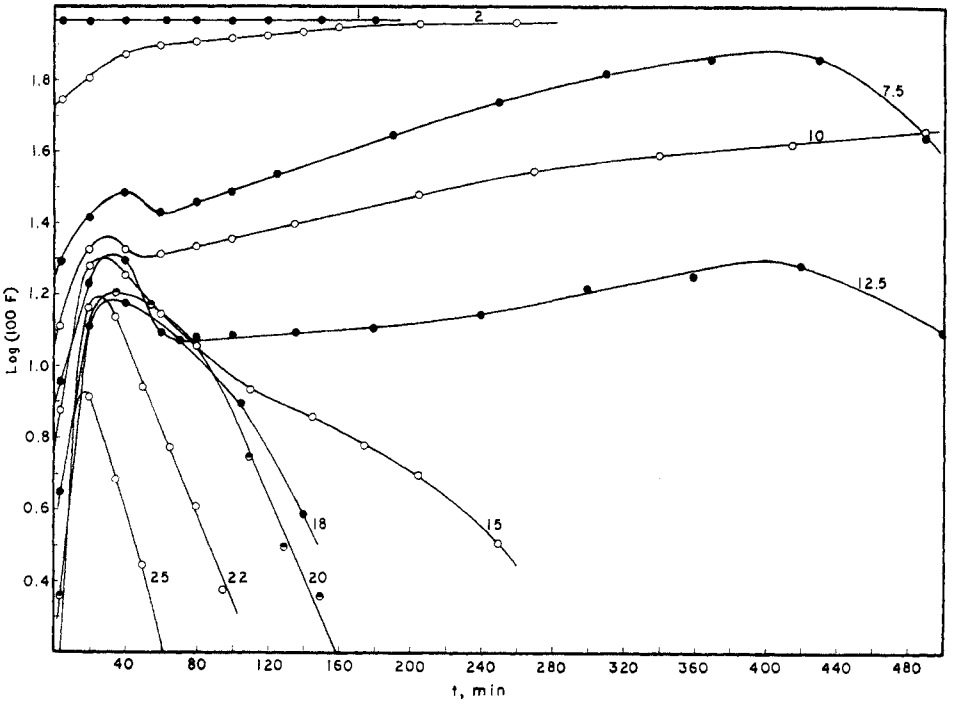

FIGURE 6 (see Figure 5).

decreases during the course of a run because of hydrolysis. These complications are particularly evident for 9 and 10 which initially hydrolyze appreciably fast relative to the rate of first-order deactivation. In the case of 9 it appears that at the higher concentrations 9 competes at the very outset much as a normal Michaelis–Menten competitor (just as 6 does in Figure 5), but also at these high concentrations the rate of first-order deactivation is so high that very shortly the deactivation process overshadows hydrolysis and the enzyme becomes rapidly deactivated. Of all these esters, 10 seems to possess the best balance between hydrolytic and inhibitor characteristics to most clearly demonstrate the effect of these competing forces as a function of concentration.

In spite of all the competitions and conformational changes that are indicated by Figures 6–9, all of these substrates normally have classical shapes for their enzymatic hydrolysis curves (concentration *vs.* time). However, careful selection of just the right concentrations can present a different picture;

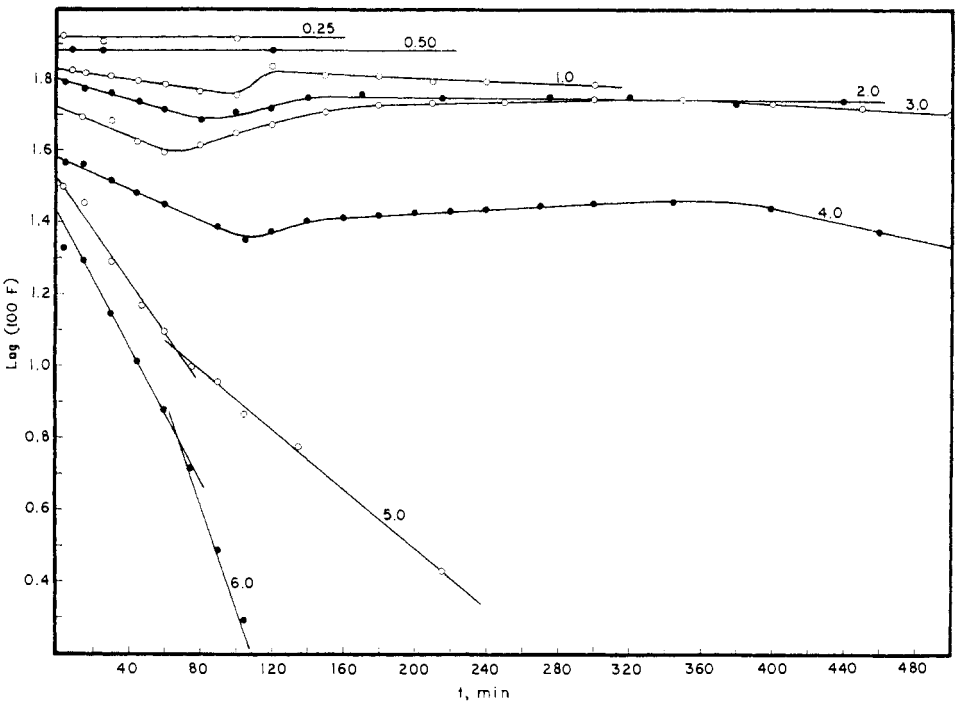

FIGURE 7 (see Figure 5).

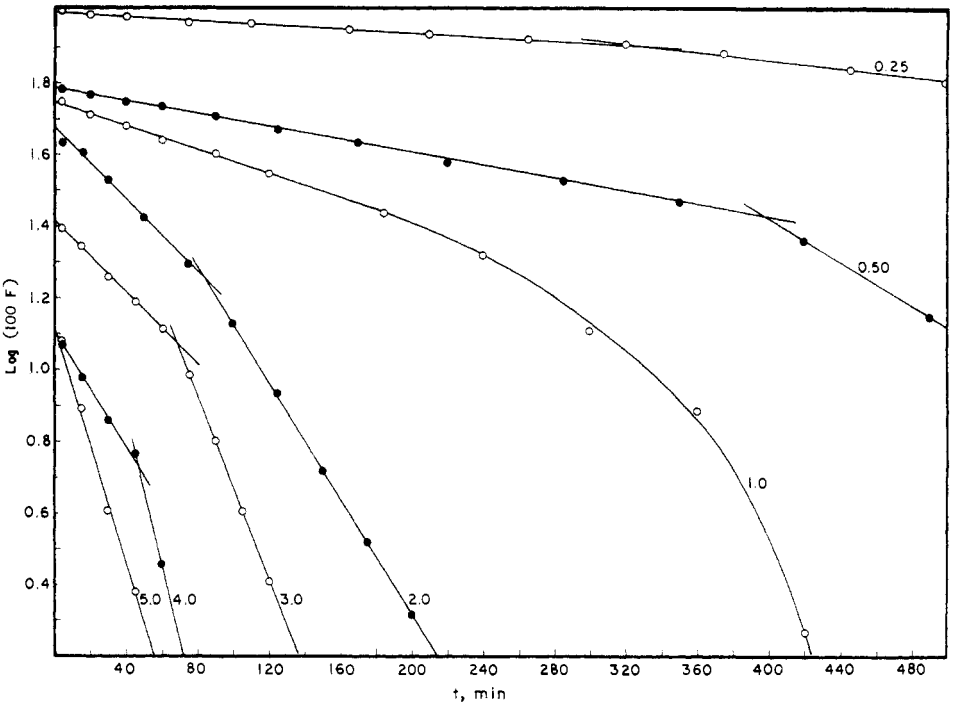

FIGURE 8 (see Figure 5).

for example, hydrolysis of 0.7×10^{-4} M 12 at an enzyme concentration of 2.50×10^{-5} M shows a sigmoid type curve. In addition, with this concentration of α-chymotrypsin the substrate is essentially completely hydrolyzed within 2 hr, while with the α-chymotrypsin concentration normally used (2.50×10^{-6} M) the enzyme is completely deactivated by the time half of the substrate is hydrolyzed. These and similar experiments also demonstrate the ready reversibility of the deactivating conformational changes as the inhibiting substrate concentration is reduced through hydrolysis. They further demonstrate the fact that the deactivation is not the result of enzyme dimerization. If the latter were the case, then the tenfold increase in α-chymotrypsin concentration should have caused something of the order of a 100-fold increase in the rate of enzyme *de*activation at the outset instead of the approximately sevenfold *in*crease in the initial rate of hydrolysis of 12 that was observed.

Critical micelle concentration (cmc) values were determined for some substrates in 0.05 M Tris in order to ascertain whether micelle formation plays an important role in the kinetic observations. This was accomplished by measuring the surface tension (bubble pressure method) of solutions of substrate and then plotting surface tension *vs.* the logarithm of concentration. The cmc values so obtained are approximately

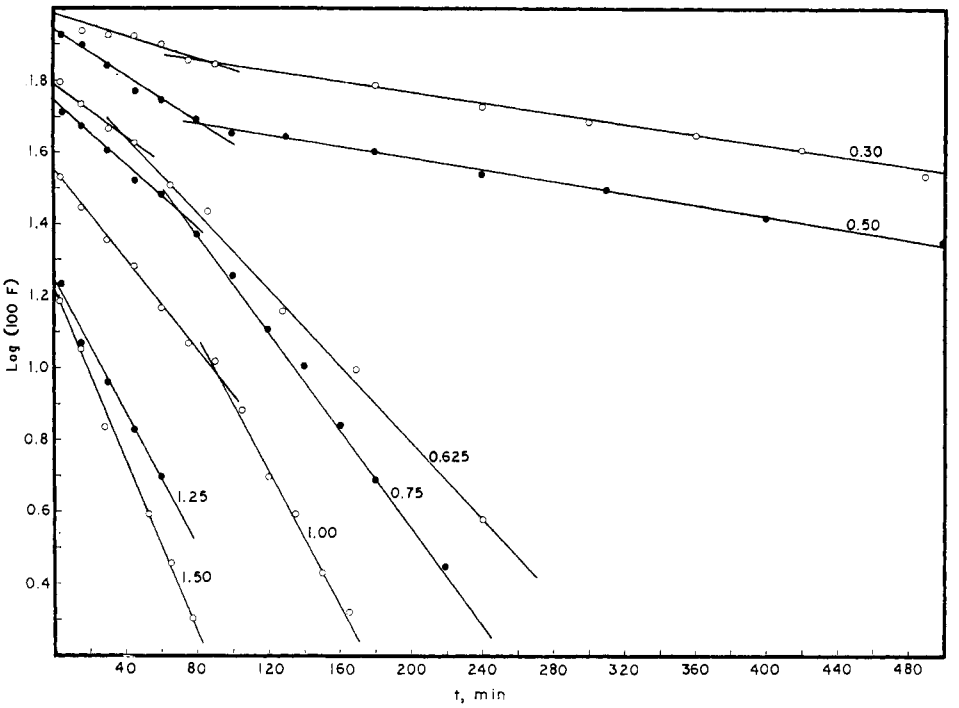

FIGURE 9 (see Figure 5).

as follows: 10, 2×10^{-3} M; 11, 6.3×10^{-4} M; 12, 2.5×10^{-4} M. Reliable values in the presence of α-chymotrypsin are difficult to obtain, especially at higher concentrations, because of the simultaneous occurrence of hydrolysis. However, it is fairly certain that the presence of α-chymotrypsin at 2.50×10^{-6} M has no effect on the surface tension or cmc, and that the presence of 2.50×10^{-5} M α-chymotrypsin probably lowers the surface tension very slightly. The conclusion is that all of the kinetic measurements were carried out under conditions which did not involve micelles.

The effect of low concentrations of inhibitors on the ORD spectrum was determined for several inhibitors. The principal results are as follows. The inhibitors have no significant effect on the ORD of α-chymotrypsin in the 230–235-nm region, but they do alter the Cotton effect in the 285–300-nm region. At low concentrations the inhibitors just slightly diminish this Cotton effect but above a characteristic threshold concentration, and within a relatively narrow range of concentrations, the 285–300-nm Cotton effect is virtually eliminated. In order to accentuate this effect, the difference in specific rotations observed at the two wavelengths, $\Delta[\alpha] = [\alpha]_{300} - [\alpha]_{285}$, brought about by a given concentration of inhibitor, [I], is plotted *vs.* log [I].

The results for three inhibitors are shown in Figure 10. The general conclusion is that the inhibitors induce some conformational changes in the secondary and tertiary structures of α-chymotrypsin that include the release of tryptophan chromophores from their asymmetric environment (Strickland *et al.*, 1969), but they do not appreciably affect the helical structure characteristic of the 230–235-nm region (Fasman *et al.*, 1966). At all 1-octanol concentrations for which it was possible to make ORD measurements without precipitation, there was no indication that [α] at 285–300 nm is affected.

Equilibrium binding measurements were undertaken in an effort to answer the question of how many inhibitor molecules per molecule of α-chymotrypsin does it take to bring about the kinetic and optical changes described above. The results are summarized in Figure 11, each point representing at least six independent runs. Noting the large binding at the higher inhibitor concentrations, an effort was made to get results at 2.50×10^{-6} M, the concentration used in the kinetic studies. Some results are shown in Table II.

Several conclusions can be drawn. One is that even though the inhibitors are well below their critical micelle concentrations (cmc values at 25° are: decanoate, 2.4×10^{-2} M; dodecyl sulfate, 8.2×10^{-3} M; dodecyltrimethylammonium, 1.4×10^{-2} M; tetradecyltrimethylammonium, 3.5×10^{-3} M), micelles do form in the presence of α-chymotrypsin,

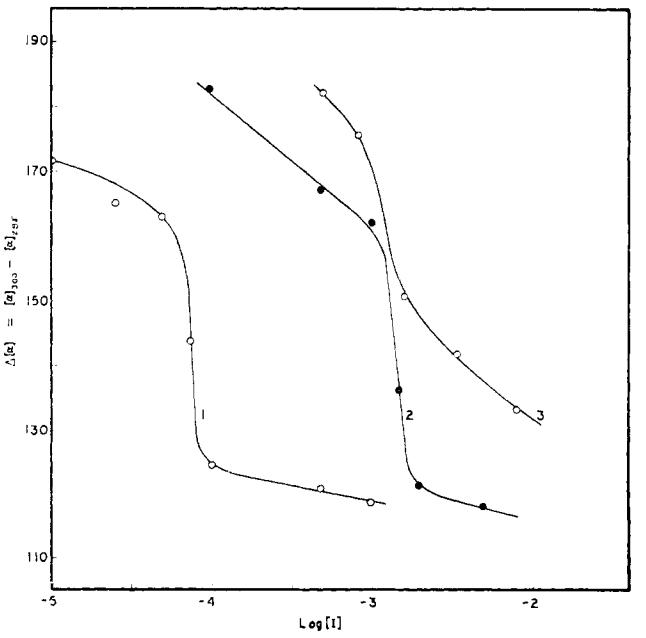

FIGURE 10: Difference in specific rotations, $\Delta[\alpha] = [\alpha]_{300} - [\alpha]_{285}$, for α-chymotrypsin as a function of log [I]. Conditions are 3.0×10^{-5} M α-chymotrypsin in 0.05 M Tris at pH 8.0. Curves are (1) dodecyl sulfate, (2) tetradecyltrimethylammonium, and (3) dodecanoate.

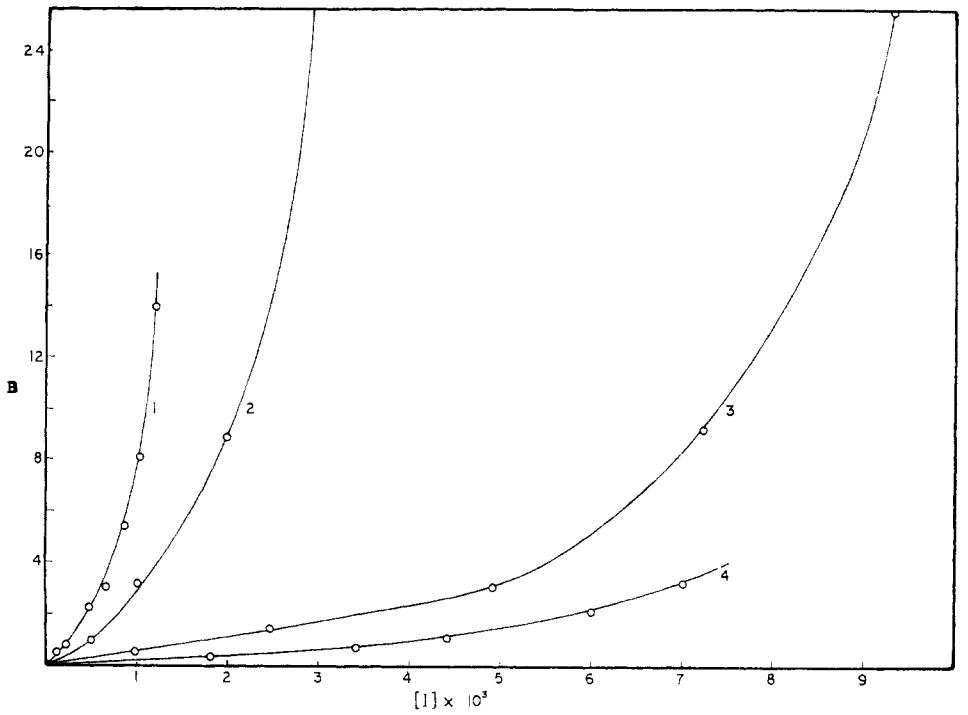

FIGURE 11: B, the number of molecules of inhibitor bound at equilibrium per molecule of α-chymotrypsin, plotted as a function of inhibitor concentration, [I]. Curves are (1) tetradecyltrimethylammonium, (2) dodecyl sulfate, (3) dodecyltrimethylammonium, and (4) dodecanoate. For curves 1–3, [α-chymotrypsin] = 3.0×10^{-5} M; for 4, [α-chymotrypsin] = 2.0×10^{-5} M; in each case, 0.05 M Tris at pH 8.0.

though it would be difficult to pick a concentration below which there is only simple binding and above which there is conventional micelle formation. For a given value of [I] one would normally expect B to decrease with decreasing α-chymotrypsin concentration, not increase. However, if it is assumed that on the average each α-chymotrypsin molecule acts as a nucleus for the formation of a micelle, then (for a given [I]) there are fewer inhibitor molecules for a micelle at the higher α-chymotrypsin concentration than at the lower, and the number of inhibitor molecules "bound" per molecule of α-chymotrypsin is less (as observed). An effort was made with dodecyl sulfate to find the concentration below which no micelle formation occurs in the presence of α-chymotrypsin. The counting statistics become very bad with low concentrations of enzyme *and* inhibitor, but the conclusion was drawn that no distinction could be made at 1×10^{-3} M dodecyl sulfate where $B \simeq 3$. It is concluded that the kinetic measurements were made at inhibitor concentrations below those which induce micelle formation.

Another conclusion to be drawn is that for those inhibitors for which kinetic, ORD, and equilibrium binding data have been obtained, *complete inactivation* of α-chymotrypsin as well as simple partial deactivation may be brought about by the statistical binding of appreciably *less* than one inhibitor ion per molecule of α-chymotrypsin. The figures shown in Table III show roughly the interrelationship of these data.

Discussion

The results described in the preceding section show that a variety of nonacylating ionic inhibitors reversibly inactivate (denature) α-chymotrypsin in a manner that may include an immediate partial reduction in activity followed by a slow first-order process (frequently two in tandem at the lowest concentrations). Neutral inhibitors of comparable hydrophobicity are poor inhibitors and lead to enzyme precipitation. In a given homologous series of inhibitors, the effectiveness increases with hydrophobicity. In terms of the number of inhibitor molecules bound per molecule of α-chymotrypsin, anionic inhibitors are much more effective than are the cationic. As a class, the cationic inhibitors appear to produce a more pronounced "immediate partial reduction in activity" than do the negative ions, but they more slowly deactivate the enzyme in the subsequent first-order process. There appears to be no neat relationship between charge density on groups attached to carbon chains of a given length ($N(CH_3)_3^+$, $P(CH_3)_3^+$, and $S(CH_3)_2^+$ on *n*-tetradecyl in one group, and OSO_3^-, SO_3^-, and CO_2^- on *n*-dodecyl in another, for example). Neither does there appear to be any quantitative relationship between hydrophobicity (log P) and immediate partial reduction in activity or the magnitude of the first-order rate constants for deactivation.

Since inhibition and deactivation do not result from competition with substrate at the Ser-195···His-57 active site, they must result from interaction with a control site. This control site must be hydrophobic in nature because, to be effective, an inhibitor must possess relatively high hydrophobicity; but high hydrophobicity itself is not enough because electrically neutral inhibitors lead primarily to precipitation. The simplest general conclusion is that when an ionic charge (+ or −) is hydrophobically bound to the control site there is an accompanying electrostatic interaction with the enzyme which results in an unfavorable conformational change and an active site that is less effective for enzymic action.

Although all of the kinetic measurements described under Results support the control site concept, and not deactivation by dimerization, it is worth mentioning supporting evidence from published work. First, McClure and Edelman (1967) showed, using the fluorescent hydrophobic anionic probe 2-

TABLE II: Equilibrium Binding of Inhibitors to α-Chymotrypsin.

Inhibitor ([I] (M))	*B*, mol of I Bound/mol of α-Chymotrypsin	
	At 3.00×10^{-5} M α-Chymotrypsin	At 2.50×10^{-6} M α-Chymotrypsin
Dodecyl sulfate (2.0×10^{-3})	8.2 ± 0.3	28 ± 8
Dodecyl sulfate (1.5×10^{-3})	5.0 ± 0.4	12 ± 10
Tetradecyltrimethylammonium (1.5×10^{-3})	13.2 ± 0.1	74.5 ± 2

TABLE III: Relationship between Kinetic, Binding, and ORD Data.

Inhibitor	*R* (M)	*B*	*C*[e]
Dodecanoate	$5.0–3.5 \times 10^{-4}$ [a]	0.06–0.67[c]	~0.25
	~12×10^{-4} [b]	~0.2[d]	
Dodecyl sulfate	$0.94–7.5 \times 10^{-4}$ [a]	0.12–1.75[c]	~0.25
	~2×10^{-4} [b]	~0.25[d]	
Tetradecyltrimethylammonium	$0.94–13.1 \times 10^{-4}$ [a]	0.25–9.0[c]	~9
	~5×10^{-4} [b]	~2.2[d]	

[a] The range of concentrations used in the kinetic studies. The lowest concentration produces only a small amount of inhibition; the highest produces total inactivation in about 1 hr. [b] The approximate concentration required to totally inactivate α-chymotrypsin in 8 hr. [c] The approximate number of inhibitor ions bound per molecule of α-chymotrypsin at the low and high concentrations shown under *R*. [d] The approximate number of inhibitor ions bound per molecule of α-chymotrypsin at the corresponding concentration shown under *R*. [e] The approximate number of inhibitor ions bound per molecule of α-chymotrypsin at the midpoint of the narrow range of inhibitor concentrations that produce the marked changed in Δ[α] in Figure 10.

p-toluidinylnaphthalene-6-sulfonate, that α-chymotrypsin possesses a pH-dependent hydrophobic binding site (which is not part of the active site) at which the sulfonate can noncompetitively inhibit the hydrolysis of acetyl-L-tyrosine ethyl ester. Second, the early work of Tinoco (1957) and Egan *et al.* (1957) indicates that α-chymotrypsin does not dimerize at the ionic strength and low enzyme concentrations used in the present experiments. Using the equilibrium dimerization constant of 2.1×10^{8} M^{-1} at pH 7.8 obtained by Nicol *et al.* (1972), one calculates that about 0.6% of the enzyme is in the form of a dimer. Further examination of their work indicates that there is less tendency for α-chymotrypsin to associate in the presence of 0.02 M β-phenylpropionate and 0.02 M phenylpropiolate (and presumably other hydrophobic anions also) than when they are absent. In fact, the present work with inhibitors appears to shed some light on the very early stages of the process in which proteins are ultimately converted to the random coil state. Figure 11 covers the concentration range just below that studied by Reynolds and Tanford (1970) and implies that the binding of dodecyl sulfate by proteins at a given ionic strength is not a simple two-stage affair but continuously incremental with increasing dodecyl sulfate concentration.

The observations described in the present work, namely that at a *given* pH the inhibitor ions deactivate α-chymotrypsin by interaction at some location other than the Ser-195···His-57 active site and that they cause the loss of the Cotton effect at 285–300 nm, parallels those observations for α-chymotrypsin with a *change* in pH. It is tempting to think that perhaps much the same conformational changes take place in each case even though the basic causes are quite different. For example, assume that the inhibitor ions bind to the hydrophobic environment of the Ile-16···Asp-194 ion pair and, through electrostatic interaction, displace the Asp-194 side chain to block and inactivate the Ser-195···His-57 active site as previously suggested by Sigler *et al.* (1968) for an increase in pH. This displacement of the Asp-194 side chain would also mechanically disrupt the His-40···Trp-141 interaction and thus eliminate the source of the 285–300-nm Cotton effect. [Oppenheimer *et al.* (1966) showed that the 285–300-nm Cotton effect disappears with increasing pH. Strickland *et al.* (1969) showed that tryptophan residues in an asymmetric environment are needed for the 285–300-nm Cotton effect. Shinitzky and Goldman (1967) showed that the characteristic fluorescence in polypeptides results from an imidazole–indole interaction that can be quenched by protonation of the imidazole. Hess *et al.* (1970) showed that in α-chymotrypsin it is His-40 which is related to tryptophan fluorescence. The H-bond assignments of Birktoft *et al.* (1970) implicate Trp-141 as the only possible Trp residue able to interact with His-40. The X-ray work of Freer *et al.* (1970) showed that an H bond is broken between Asp-194 and His-40 when the Asp-194··· Ile-16 ion pair is created.] The hydrophobic binding of anionic inhibitors would result in an effective electrostatic repulsion of the Asp-194 side chain, but the cationic inhibitors would tend to form an alternate ion pair with Asp-194 which would be a less effective mode for the displacement of the Asp-194 side chain. This view is in keeping with the observations that anionic inhibitors are more effective than cationic ones. The principal effect of neutral inhibitors seems to be to increase the total hydrophobicity (without serious interference with the Ile-16···Asp-194 ion pair) to the point where aggregation occurs, the inhibitors possibly acting as hydrophobic bridges between individual enzyme molecules.

Regardless of whether the inhibitor-induced deactivating conformational changes in α-chymotrypsin are those suggested in the previous paragraphs, or others that will be suggested in the future, there remains the interesting kinetic problem of how the complete deactivation of *all* the α-chymotrypsin molecules is brought about by appreciably less than one inhibitor ion physically and reversibly bound per α-chymotrypsin molecule. It is believed that the same basic principles are applicable here that underlie the slow transition model proposed by Ainslie *et al.* (1972) for relating transients and cooperative kinetics of enzymes. Of particular importance is the condition that both the binding and the isomerization steps of the mechanism be in a steady state but not at equilibrium; the net result is a reaction flux in one direction. It is suggested that the following circumstances exist when this condition is applied to the present studies.

A given inhibitor ion is incapable of bringing about the total deformation needed for complete deactivation during its residence time at the hydrophobic site. Being only reversibly bound, the inhibitor ion soon returns to solution, and the hydrophobic site is free to undergo relaxation and to lose the

TABLE IV: Adsorption Constants for Selected Inhibitors.[a]

Inhibitor	a	b
Dodecanoate	1.46×10^2	74.7
Dodecyl sulfate	1.36×10^3	591
Tetradecyltrimethylammonium	3.46×10^3	528
Dodecyltrimethylammonium	4.65×10^2	911

[a] Values of a and b are adsorption constants for eq 2. a is also the equilibrium binding constant for eq 6 for the limiting case of $[I] \rightarrow 0$ (see discussion of eq 7). For all equations, [I] is expressed in M.

deformation that had occurred during the residence time. The experimental conditions are such that at any given instant only a certain fraction of the enzyme molecules is bound to inhibitor ions, this fraction being a continually changing group of molecules. If the relaxation time of the enzyme is relatively long compared to the interval that elapses between the leaving of one inhibitor ion and the binding of another, then the second ion will bind at this site to a somewhat different conformational state than did the first. Thus, during its time of residence the second ion will bring about a little greater total change in conformation than the first was able to achieve before it left. The consequence of a whole succession of adsorptions and desorptions over a period of time is that *all* of the useful conformational states are eventually eliminated and the enzyme is completely deactivated. The higher the concentration of the inhibitor ions the more frequently will adsorptions and desorptions occur (*i.e.*, the total residence time per minute will increase, and B will be larger), the smaller will be the amount of relaxation between adsorptions, and the faster will be the rate of enzyme deactivation.

In the following effort to quantitatively formulate the foregoing suggestions, it will be helpful to think of the enzyme molecule as being able to exist in a very large number of conformational states capable of catalysis, each state differing slightly from the other in a continuous fashion, ranging from maximum activity (A_0) at the outset after the "immediate partial reduction in activity," to zero activity ($A_\infty = 0$) when the enzyme is completely inactive. The activity (A) that exists at post-mixing time (t) might then be construed to correspond to the remaining number of useful conformational states through which the enzyme will pass during the course of deactivation, the transition occurring in succession from the most active state to the least active. With this crude picture, the rate of loss of activity is related to B and A by equation 1.

$$-(dA/dt) = k'BA \tag{1}$$

The relationship of B to the concentration of inhibitor in solution, [I], is shown in Figure 11, and, over the range of concentrations used in the kinetic experiments, it is given accurately by the expression characteristic of cooperative adsorption at low coverage

$$B = a[I]/(1 - b[I]) \tag{2}$$

where a and b are constants characteristic of the given inhibitor (see Table IV). Substitution of eq 2 into eq 1 gives eq 3.

$$-(dA/dt) = ak'[I]A/(1 - b[I]) \tag{3}$$

During the course of any one run [I] remains constant so that in this circumstance the integrated rate equation assumes the simple first-order form

$$\log (A/A_0) = -kBt = -Kt \tag{4}$$

or, as related to Figures 2–4

$$\log (100F) = -Kt + 2 \tag{5}$$

For the three cases for which both kinetic data and adsorption constants are known, values of K from Figures 2–4 were plotted *vs.* values of B calculated from eq 2 in order to check the linear relationship, $K = kB$, that is suggested by eq 4. When two first-order processes were involved, K for the major contributor was selected. The results shown in Figure 12 are not impressive but they appear to support the views expressed above. The "true" rate constant (k in eq 4) may be taken from the slope of each line in Figure 12. For dodecanoate and dodecyl sulfate the value of k is about 1.83×10^{-2} min^{-1}, while for tetradecyltrimethylammonium k is about 1.08×10^{-3} min^{-1}. The two negative ions are about 17 times more effective than the positive ion, in keeping with the nature of the hydrophobic site previously discussed.

One consequence of a process that continually changes the nature of the active site is the impossibility of calculating binding constants for the inhibitors from kinetic data. If the inhibitor binding equilibrium is represented by

$$E + I \rightleftharpoons EI \tag{6}$$

the corresponding equilibrium binding constant (K_i) can be calculated, with the help of eq 2, from the expression

$$K_i = \frac{a}{1 - (a + b)[I]} \tag{7}$$

Since K_i depends on [I] when it should be independent of [I], it is clear that eq 7 does not properly represent the binding equilibrium of eq 6. Perhaps it would be more correct to say that eq 6 does not properly represent the true state of affairs; in fact, it cannot at concentrations that bring about the observed binding of more than one inhibitor ion per molecule of enzyme. However, for purposes of approximation, the values of K_i calculated from eq 7 for $[I] = 1 \times 10^{-4}$ are: decanoate, 1.41×10^2; dodecyl sulfate, 1.56×10^3; tetradecyltrimethylammonium, 5.26×10^3; dodecyltrimethylammonium, 4.97×10^2. Since $K_i \rightarrow a$ when $[I] \rightarrow 0$, it is probably as informative to simply use the values of a in Table IV.

When eq 3 is applied to the 3-(*n*-alkanoyl)-*O*-benzoates it can be seen that the term $k'a[I]/(1 - b[I])$ is no longer constant during a run because [I] = [S] continually changes. But even if [S] were determined as a function of time from separate direct measurements of catalyzed hydrolyses, it would be impossible to evaluate the constants a and b and k' without also determining the equilibrium binding curves for the substrates. Kinetic parameter evaluation would be still further complicated for these substrates because of the rapidly increasing hydrophobic inhibitory power of the *n*-alkanoate products with chain length going from 9 to 12 carbon atoms.

Figures 6–9 show directly how these substrates (9–12) affect the activity of the enzyme toward 7, but not necessarily how each substrate influences the activity of the enzyme toward itself. How much "immediate partial reduction in activity"

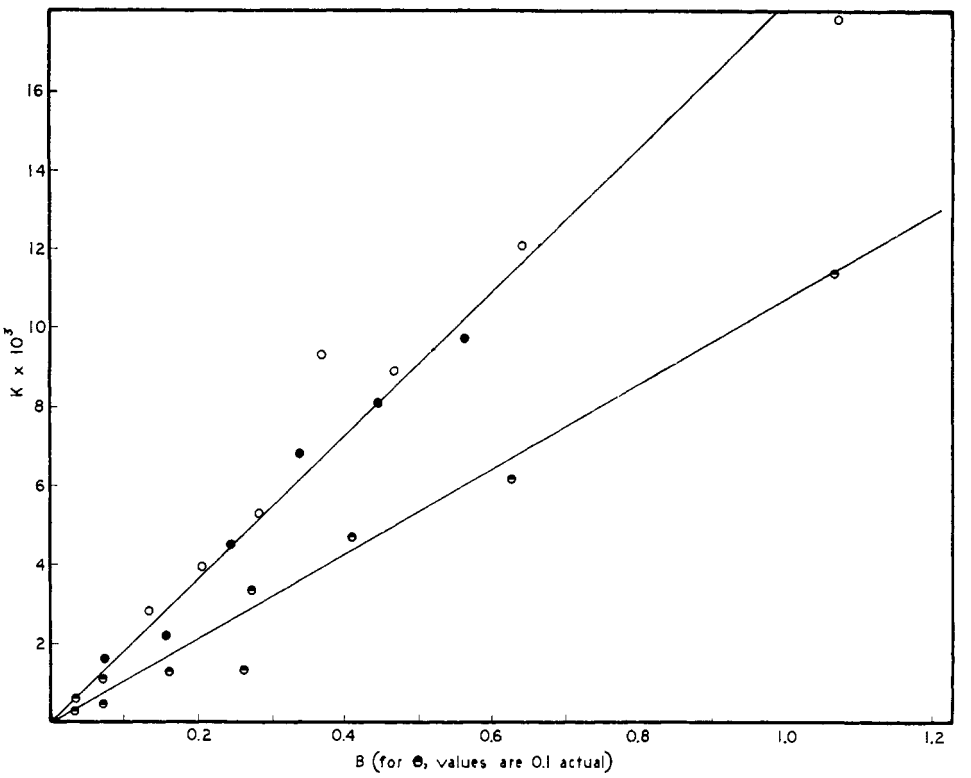

FIGURE 12: Plot of pseudo-first-order rate constant K in eq 4 *vs.* B. Values of K are taken from slopes of lines in Figures 2, 3, and 4. Values of B for a given [I] are taken from Figure 11 with the help of eq 2. The slopes of the lines for (○) dodecyl sulfate, (●) dodecanoate, and (⊖) tetradecyltrimethylammonium give values of k for eq 4.

is there at each concentration? Is it the same as for 7? For these substrates, the usual picture of a substrate molecule inducing a better fit at the active site is overshadowed by other conformational changes brought about by a *different* substrate molecule acting at another site. No doubt there are also some active-site conformational changes brought about by the substrate molecules that bind there, but the present experiments cannot differentiate between or assess the relative magnitude of the two effects. At any rate, it seems reasonable to state that the cause of the parabolic relationship as observed in Figure 1 is not so much a matter of variation in K_m and ability to acylate or deacylate as it is a deformation of the enzyme which results from the substrate's increased ability to hydrophobically bind at a separate site that controls the conformation and activity of the active site.

The present work relates to the recent observations of Gaudin and Viswanatha (1972) who performed two sets of experiments with sodium dodecyl sulfate. In the first set, 2-min assays at pH 8.0 following the 500-fold dilution of 10-min sodium dodecyl sulfate–α-chymotrypsin incubation mixtures at pH 4.0 showed a steady loss in α-chymotrypsin activity with increasing sodium dodecyl sulfate concentration. It is difficult to say just how much of what is observed in the assay can be attributed to the incubation period where the conditions and concentrations cause α-chymotrypsin dimerization (Aune and Timasheff, 1971), how much to the effect of great dilution and pH change, and how much to the conditions of the post-dilution period.

In the second set, Gaudin and Viswanatha (1972) used sodium dodecyl sulfate concentrations ranging from 10^{-5} to 10^{-2} M in the absence of KCl and buffer and performed 2-min assays at pH 8.0 immediately after adding α-chymotrypsin to give a concentration of 8×10^{-8} M. For the lower concentrations these experiments would be equivalent to looking in Figure 2 at how this family of curves intersects with the left-hand axis, corresponding to $t = 0$. The explanation of their results at low concentrations would appear to lie in the slow deactivation processes described in the present paper rather than to their suggestion that the substrate protects the enzyme against denaturation by sodium dodecyl sulfate. Their conclusion that the great enhancement of activity in the range of 10^{-3} to 5×10^{-2} M sodium dodecyl sulfate is related to the presence of micelles is probably correct, but it may also be related to the work described by Reynolds and Tanford (1970).

One way to characterize the reactivity of a system such as the enzymic hydrolysis of 3-*n*-alkanoyl *O*-benzoate substrates by α-chymotrypsin is by means of a Hansch-type structure–activity correlation equation, using techniques of linear regression analysis (Hansch, 1969). For example, in the case of the present homologous series of nine esters ($n = 9$), there can be generated the following equation (eq 8) that relates

$$\log V_0 = -5.66 + 0.63 \log P - 0.37 (\log P)^2 \quad (8)$$

the initial velocity (V_0) of the substrate at 10^{-4} M to its hydrophobicity (log P). Figure 1 shows the curve calculated from this equation; the points are experimental. If electronic and steric effects are important, then terms can also be included and weighted to the extent of their statistical importance. In eq 8, the correlation coefficient (r) is 0.960 and no improvement in correlation is derived from considering other parameters. The feature of eq 8 that is of special interest here is the $-(\log P)^2$ term, characteristic of all parabolic relationships. The implication is that there is a substrate hydrophobicity that is optimal for activity in the system at hand; in the present instance this corresponds to log $P = 0.866$.

There is a temptation to speculate about the general ap-

TABLE V: Some Enzyme–Substrate Systems Which Show a Parabolic Activity–Hydrophobicity Relationship of the Type: Log $X = a + b(\text{Log } P) - c(\text{Log } P)^2$.[a]

Enzyme	Substrate	n	X	a	b	c	r	Reference
Dehydrogenase								
Alcohol butyryl-CoA, bovine liver	Acyl-CoA derivatives	6	V_0	0.67 (±1.6)[b,c]	1.22 (±1.6)	0.32 (±0.34)	0.924	Green *et al.* (1964)
Hydrolases								
Acetylcholinesterase, bovine erythrocyte	*O*-Alkyl *O*,*O*-diethyl phosphate	9	$1/S_{50}$	1.49 (±1.5)	2.04 (±1.0)	0.22 (±1.6)	0.974	Bracha and O'Brien (1968)
	S-Alkyl *O*,*O*-diethyl phosphorothiolates	9	$1/S_{50}$	−1.57 (±2.7)	3.62 (±1.4)	0.39 (±0.18)	0.978	Bracha and O'Brien (1968)
Amidase, rabbit liver	Alkylamides	13	V_0	1.35 (±0.18)	0.64 (±0.18)	0.21 (±0.05)	0.942	Bray *et al.* (1950)
α-Chymotrypsin, bovine pancreatic	Alkyl *p*-nitrophenylethyl phosphonates	7	$1/S_{50}$	−3.12 (±1.9)[c]	5.32 (±1.3)	0.79 (±0.19)	0.986	Becker *et al.* (1963)
	3-(*n*-Alkanoyl)-*O*-benzoates	9	V_0	−5.66 (±0.22)	0.63 (±0.25)	0.37 (±0.11)	0.960	This work
	3-(*n*-Alkanoyl)-*O*-benzoates	6	V_0	−5.26 (±0.19)	0.51 (±0.18)	0.58 (±0.20)	0.986	Hofstee (1957)
	2-(*n*-Alkanoyl)-*O*-benzoates	5	V_0	−5.37 (±0.28)	0.58 (±0.58)	0.66 (±0.49)	0.973	Hofstee (1959)
Elastase, porcine pancreatic	*p*-Nitrophenyl alkanoates	5	k_{cat}	−5.79 (±6.4)	6.46 (±4.5)	1.13 (±0.74)	0.980	Marshall and Akgün (1971)
Esterase, goat intestine	*p*-Nitrophenyl alkanoates	9	$1/K_m$	1.22 (±1.3)	1.26 (±0.62)	0.09 (±0.07)	0.976	Malhotra and Philip (1966)
	p-Nitrophenyl alkanoates	10	V_{max}	1.38 (±0.93)	0.71 (±0.42)	0.12 (±0.04)	0.991	Malhotra and Philip (1966)
Fatty acid activating, bovine liver	*n*-Alkanoates	8	V_0	−0.88 (±0.14)	0.39 (±0.14)	0.12 (±0.03)	0.985	Mahler *et al.* (1953)
Trypsin, bovine pancreatic	3-(*n*-Alkanoyl)-*O*-benzoates	6	V_0	−4.96 (±0.21)	0.08 (±0.40)	0.30 (±0.24)	0.986	Hofstee (1957)
Lyase								
L-Cysteine sulfoxide lyase, onion	*S*-Alkylcysteine sulfoxides	4	$1/K_m$	1.02 (±0.16)[b]	1.77 (±0.29)	0.56 (±0.11)	1.000	Schwimmer *et al.* (1964)
Oxidases								
Monoamine oxidase, bovine liver	*n*-Alkylamines	8	V_0	1.33 (±0.27)	0.79 (±0.39)	0.21 (±0.15)	0.935	Alles and Heegard (1943)
Monoamine oxidase, rabbit liver	Alkyl- and phenylalkylamines	30	V_0	−1.64 (±0.98)	0.65 (±0.25)	0.13 (±0.06)	0.887[d]	Alles and Heegard (1943)
Synthetases								
Valyl-tRNA synthetase, *E. coli*	Aliphatic amines	10	$1/K_m$	2.73 (±0.36)	1.39 (±0.65)	2.10 (±0.70)	0.937	Owens and Bell (1970)
L-α-Glycerophosphate ester synthetase, guinea pig liver	Alkanoic acids	10	V_0	−0.47 (±0.74)	1.22 (±0.51)	0.16 (±0.08)	0.918	Kornberg and Pricer (1953)

[a] n = no. of substrates correlated; r = correlation coefficient. [b] Figures in parentheses represent the 95% confidence intervals on the regression constants a, b, and c. [c] In these examples the comparative hydrophobic constant π ($\pi = \log P_X - \log P_H$; Leo *et al.*, 1971) has been used in place of log P. [d] For this correlation an additional term, 0.28 (±0.10) pK_a is needed.

plicability to other enzyme systems of the hydrophobic control principles herein discussed for α-chymotrypsin. Considering the multitude of similarities between the serine proteases (Hartley, 1970), the most likely extension is in this direction. However, since it is not known how generally applicable these principles are, Table V summarizes the correlations for a wider variety of enzyme–substrate systems for which a parabolic relationship has been demonstrated. There are many other examples of parabolic relationships in the literature but most of them lack data suitable for structure–activity correlation. Table V includes regression coefficients for structure–activity correlation equations written in the form

$$\log X = a + b \log P - c(\log P)^2 \qquad (9)$$

where X is the measure of activity selected by the investigator. In each case the correlations have been shown to be significant by application of the F test at $\alpha = 0.1$ or better. The 95% confidence interval for each coefficient is given in parentheses following the value.

From these results it is clear that investigators who undertake structure–activity studies with α-chymotrypsin (and presumably many other enzymes) should select systems with low lipophilic character and, ideally, with no ionic charge.

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