The Combination of Chymotrypsin and Chymotrypsinogen with Fluorescent Substrates and Inhibitors for Chymotrypsin*

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ABSTRACT: The interaction of α-chymotrypsin, chymotrypsinogen, and diisopropyl-α-chymotrypsin (DIP-α-chymotrypsin) with amino acid conjugates of the fluorescent dye 1-dimethylaminonapthalene-5-sulfonyl chloride (DNS-chloride) has been investigated by means of depolarization of fluorescence. DNS-D-tryptophan ethyl ester, DNS-L-tryptophan ethyl ester, DNS-glycine ethyl ester, and DNS-amide were all reversibly bound by the proteins. The apparent binding strengths of DNS-D-tryptophan ethyl ester, an inhibitor of chymotrypsin, were in the general order of α-chymotrypsin > chymotrypsinogen > DIP-α-chymotrypsin and for a given protein DNS-D-tryptophan ethyl ester > DNS-amide ≃ DNS-glycine ethyl ester. DNS-glycine ethyl ester and DNS-amide were bound with about equal affinity to chymotrypsin and chymotrypsinogen but with lesser affinity to DIP-chymotrypsin. Identical changes in the emission spectra of the ligands were observed when the DNS group was either covalently bonded to the protein or when a ligand was bound to the protein in the equilibrium sense, suggesting that the site of attachment to the protein is the same in both cases, and no difference (other than that of quantum yield) was observed between chymotrypsin and chymotrypsinogen in the two cases. These, and other factors, were interpreted to mean that the substrate binding site is functional in the zymogen, and is similar to the binding site of the active enzyme.

While zymogens such as chymotrypsinogen and trypsinogen are evidently devoid of catalytic function, the ability of a zymogen to combine in a specific manner with substrates and competitive inhibitors has not been conclusively established. Vaslow and Doherty (1953) used equilibrium dialysis to determine the binding of the virtual substrate, acetyl-3,5-dibromotyrosine, to chymotrypsin and chymotrypsinogen, respectively, and found nearly equal free energies of binding of approximately −2.5 kcal/mole though the enthalpy of binding was twice as great for chymotrypsinogen as for chymotrypsin. Erlanger (1958) observed equal affinities of adsorption of chymotrypsinogen and DIP[1]-α-chymotrypsin onto *N*-carbobenzyloxy-L-leucyl-D-phenylalanine benzyl ester, an insoluble compound that meets the structural requirements for a competitive inhibitor of chymotrypsin, and a 40% increase in the adsorption of α-chymotrypsin relative to the two former proteins.

Massey *et al.* (1955) coupled chymotrypsin and chymotrypsinogen with DNS-chloride in varying amounts. They studied the conjugates by means of the fluorescence depolarization technique of Weber (1952-a,b) and concluded that a group in chymotrypsinogen capable of reacting with DNS[1] is identical with a part of the active center of chymotrypsin. More recently, after the present investigation had been completed, Weiner and Koshland (1965a) and Glazer (1965) reported independently on the combination of proflavine with chymotrypsin and chymotrypsinogen. Although two different experimental methods were employed, strong combination of the dye by chymotrypsin was found by both groups of investigators, in contrast to chymotrypsinogen which bound the dye only weakly. Proflavine had been previously reported by Wallace *et al.* (1963) to be among the most effective competitive inhibitors of chymotrypsin, and, according to Bernhard and Gutfreund (1965), is also an effective inhibitor of trypsin. The method of depolarization of fluorescence can also be used to measure the equilibrium binding of fluorescent ligands, and Laurence (1952) has measured the degree of binding from the changes in intensity and polarization of the fluorescence occurring when various ligands are bound to serum albumin. It should thus be possible, by means of synthetic substrates and inhibitors "tagged" with a fluorescent label, to study the binding of these compounds to chymotrypsin and chymotrypsinogen.

Experimental Section

Methods

Fluorescent intensities and polarizations were measured

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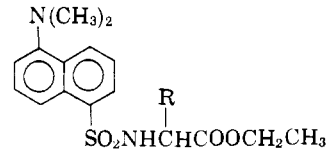
[1] The following abbreviations are used: ATEE, *N*-acetyl-L-tyrosine ethyl ester; DIP, diisopropylphosphoryl; DNS, 1-dimethylaminonapthalene-5-sulfonyl; glyEE, glycine ethyl ester; trpEE, tryptophan ethyl ester.

in a nullpoint apparatus similar to that described by Weber (1956) in which the linearly polarized component of fluorescence vibrating in the direction of propagation of the exciting light is used as a reference signal. The exciting light was in all cases 365 mμ from a GE H85A3 Hg arc, isolated by means of Corning 5860 and 7380 filters. Fluorescence was observed through Corning 3486 filters with EMI 6256B photomultiplier tubes. The details of the instrument design will be published elsewhere (Deranleau, 1966, submitted for publication). *Absorption spectra* were recorded on a Perkin-Elmer Model 350 spectrophotometer fitted with temperature-regulated cuvet holders. *Emission spectra* were measured as follows: light from the mercury arc was collimated with a quartz lens, and the 365 mμ Hg lines were isolated as described above. A second quartz lens, oriented at 90° to the collimated exciting light, focused the fluorescence from the cell on the entrance slit of a Bausch and Lomb 250-mm grating monochromator. The fluorescence was recorded as a function of wavelength with an EMI 6256B photomultiplier placed at the exit slit of the monochromator. The spectra so obtained were corrected for the spectral response of the photomultiplier (from the response curve supplied by the manufacturer) but not for the transmission of the monochromator. The latter correction is small for the relatively narrow emission bands studied here (Teale and Weber, 1957). The effective band width passed by the monochromator slits was 3 mμ.

Binding Measurements. Concentrated protein solutions were made up in cold buffer, the pH was adjusted to that of the buffer, and the solution was centrifuged at 5000*g* for 30 min at 4°. Quantitative dilutions of this stock solution were kept on ice until mixed with ligand. After a short time for temperature equilibration, the polarization and fluorescence intensity were determined as previously described. Protein concentrations were determined from extinction coefficients: chymotrypsinogen 50.2 (Wilcox *et al.*, 1957), α-chymotrypsin 48.2 and DIP-α-chymotrypsin 48.5 (calculated from chymotrypsinogen value), and lysozyme 37.0 (recalculated for mol wt 14,125 from the value given by Weber, 1961), in units of $cm^2\ mole^{-1} \times 10^6$ at 282 mμ. Fluorescent ligands were made up in 1,4-dioxane (Matheson Coleman and Bell spectroquality reagent) and the concentrations determined from the weight of ligand added. All measurements were done at 25°.

Activity Measurements. Enzymatic activities were measured with a Radiometer Titrator TTT1a and an Ole Dich recorder with syringe drive. The conditions of the assay for chymotryptic activity were as follows: 0.010 M ATEE[1] (Calbiochem) in 0.01 M Tris–HCl buffer containing 0.02 M $CaCl_2$, 0.0834 M KCl, and 2% ethanol, pH 7.80, ionic strength 0.15.

Fluorescent Ligands. Several amino acid ethyl esters were coupled to 1-dimethylaminonapthalene-5-sulfonyl chloride to form compounds of type I.



I

In a typical preparation, 1 g of sulfonyl chloride (representing a 10% molar excess) was dissolved in 100 ml of benzene, and the solution was added to a flask containing the relevant amino acid ethyl ester and 20–30 ml of dry pyridine. After refluxing the mixture for *ca.* 1 hr, the solvents were removed by vacuum distillation, and the resulting oil was dissolved in ether and washed successively with 5% sodium bicarbonate, 5% sodium acetate, and water.

The compounds were crystallized in dilute solution from approximately 30% ethanol–water mixtures with yields of 50–70%. The conjugates were recrystallized twice from ethanol–water and their properties are listed in Table I. The starting materials were purchased from Calbiochem, except glycine ethyl ester (Eastman Organics) and L-tryptophan ethyl ester hydrochloride (Mann Research Laboratories), and were of the highest purity obtainable. Ethyl esters that could not be obtained commercially were prepared by Fischer esterification. 1-Dimethylaminonapthalene-5-sulfonamide was prepared according to Weber (1952b). The absorption and emission spectra of the conjugates are shown in Figure 1, and Table II lists the various extinction coefficients.

TABLE I: Properties of Fluorescent Ligands.

Compound	Mol Wt	Melt. Range (°C)	Anal.[a] (%) C	H	N
DNS-glycine ethyl ester	336.4	74–75	57.40 (57.12)	6.07 (5.99)	9.14 (8.33)
DNS-L-tryptophan ethyl ester	465.6	118–119	64.60 (64.69)	6.15 (5.85)	9.28 (9.03)
DNS-D-tryptophan ethyl ester	465.6	123–123.5	64.01 (64.49)	6.12 (5.85)	9.21 (9.03)

[a] Microanalytical Laboratory, 164 Banbury Road, Oxford, England. Figures in parentheses are theoretical values. There was no significant weight loss on drying *in vacuo* over $CaSO_4$ for 7 days.

Proteins. Chymotrypsinogen A was prepared from a pooled eleven-times-crystallized sample which was recrystallized twice before use from 0.25 saturated $(NH_4)_2SO_4$ at pH 5 (Northrop *et al.*, 1948) in the presence of a 100 molar excess of DFP. The starting material contained about 4×10^{-4}% chymotrypsin as

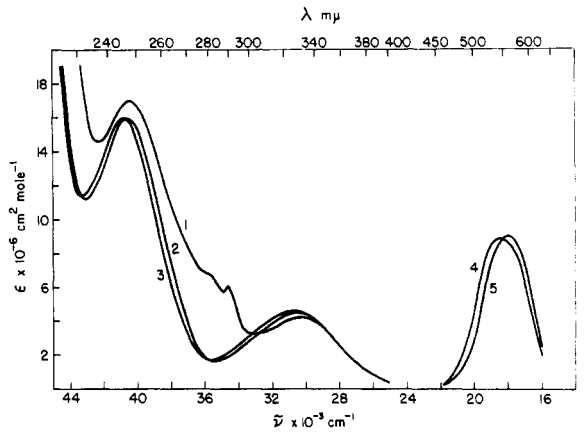


FIGURE 1: Absorption and emission spectra of fluorescent ligands. Extinction of DNS-D-trpEE and DNS-L-trpEE (1), DNS-glyEE (2), and $DNSNH_2$ (3). Emission spectra (in arbitrary units) of $DNSNH_2$ (4), and DNS-D-trpEE and DNS-glyEE (5).

TABLE II: Extinction Coefficients and Wavelength Maxima of Ligands.[a]

Compound		$\epsilon \times 10^{-6}$ (cm^2 $mole^{-1}$)	λ_{max}, mμ
DNS-glycine ethyl ester	I	15.9	246.5
	II	4.49	328
DNS-D-tryptophan ethyl ester	I	17.0	247.5
	II	(6.8)	(280)
	III	5.97	289
	IV	4.20	330
DNS-L-tryptophan ethyl ester	I	16.7	247.5
	II	(6.8)	(280)
	III	5.98	289
	IV	4.20	330
DNS-amide	I	16.0	245
	II	4.67	326

[a] In 20% dioxane–water (v/v). Values in parentheses refer to shoulders on absorption bands.

measured by its activity against ATEE. *$DNS_{0.26}$-chymotrypsinogen* (0.26 substituted chymotrypsinogen A) was made available through the courtesy of Miss R. A. Henriksen and Dr. Philip E. Wilcox. *α-Chymotrypsin* was a Worthington preparation, 3× crystallized, Lots No. CDI-6048 and CDI-6032. *$DNS_{0.26}$-chymotrypsin* (0.26 substituted) was prepared from α-chymotrypsin by reaction with a 9 molar excess of 1-dimethylaminonapthalene-5-sulfonyl chloride (on Celite, 10%, Calbiochem) in 0.1 M Na_2HPO_4 for 30 min at room temperature. A second preparation, *$DNS_{0.64}$-chymotrypsin* (0.64 substituted), was prepared under similar conditions, but without Celite adsorbent. Following centrifugation,

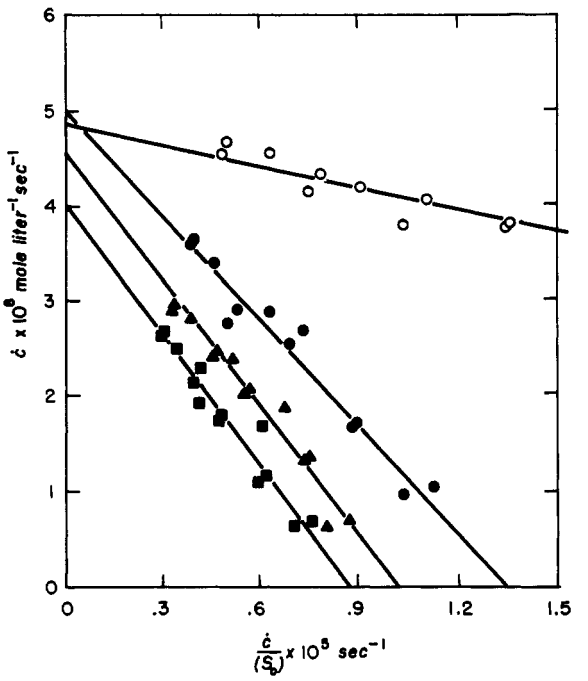


FIGURE 2: Eadie plots of the rates of hydrolysis ($\dot{c}$) of ATEE by chymotrypsin in the presence and absence (●) of DNS-D-trpEE (1.0×10^{-4} M (■) and 8.0×10^{-5} M (▲)) at 25° in 5% dioxane, 0.01 M Tris, 0.02 M $CaCl_2$, 0.083 M KCl, pH 7.8, ionic strength 0.15. The open circles correspond to an experiment in the absence of dioxane. Lines are drawn from a least-squares analysis, followed by a Newton–Raphson refinement of the coefficients by automatic digital computation.

the unreacted dye and the salt were removed on a Sephadex G-25 column, and the fluorescent product was lyophilized. The number of DNS groups coupled to the protein (degree of substitution) was determined from the absorption maximum of the 300–400 mμ band, using the extinction coefficient of 4.4×10^6 cm^2 $mole^{-1}$ for the coupled DNS group (average of values given in Table II and those of Weber, 1952b). Both $DNS_{0.26}$-chymotrypsinogen and $DNS_{0.26}$-chymotrypsin were stable during dialysis at pH 9.5 at room temperature for 20 hr, as indicated by the absence of any fluorescent material in the dialysate. *DIP-α-chymotrypsin*, purified by chromatography on CM-cellulose, was a gift from Dr. Virginia L. Richmond (Lot C) and contained 0.05% chymotrypsin (activity *vs* ATEE). *Lysozyme* was obtained from Worthington Biochemicals, 2× crystallized, Lot LY-596.

Results

Fluorescent Ligands as Substrates and Inhibitors. The hydrolysis of DNS-L-trpEE by chymotrypsin was demonstrated by incubating part of a solution of ligand with enzyme at pH 7.9, the remainder serving as a control. High-voltage flat-plate electrophoresis of the

TABLE III: Inhibition of Chymotryptic ATEE Hydrolysis by DNS-D-trpEE.[a]

Dioxane (%)	Concentration Inhibitor (M)	K_m, mole l.$^{-1}$	k_I, l. mole^{-1}	Average Fit Error[b]
0	0	$7.40 \pm 0.55 \times 10^{-4}$	...	0.024
5	0	$3.70 \pm 0.29 \times 10^{-3}$	...	0.056
5	1.0×10^{-4}	...	1900 ± 300	0.071
5	8.0×10^{-5}	...	2500 ± 100	0.069

[a] Values by least-squares fit, followed by Newton–Raphson refinement. [b] From the fitted line: (calcd value − obsd value)/calcd value.

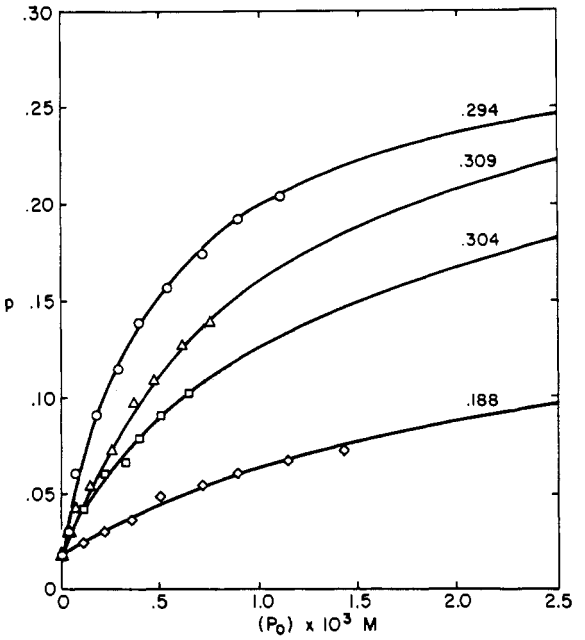


FIGURE 3: Effect of protein concentration on the polarization of fluorescence of DNS-D-trpEE, 2.00×10^{-5} M, in 5% dioxane, 0.01 M Tris, 0.02 M $CaCl_2$, 0.083 M KCl, pH 7.8, ionic strength 0.15, 25°. Numbers on the curves correspond to the predicted maximum value of the polarization as the protein concentration approaches infinity (see text). Chymotrypsin (○); chymotrypsinogen (△); DIP-chymotrypsin (□); and lysozyme (◇).

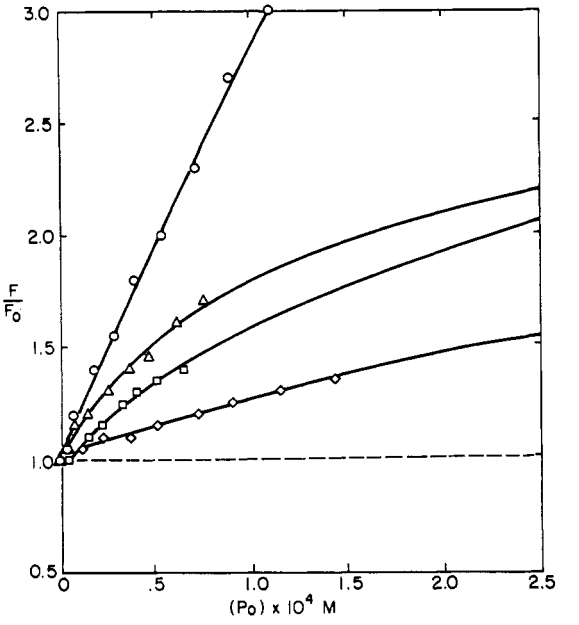


FIGURE 4: Effect of protein concentration on the observed fluorescent intensity F, relative to the fluorescent intensity F_0 of 2.00×10^{-5} M DNS-D-trpEE. Conditions and points same as for Figure 3.

samples in pyridine–acetic acid at pH 6.5 showed that the fluorescent spots corresponding to the controls remained at the origin ($^+R_{F(e)}$ 0.06), whereas those corresponding to the enzyme-treated portion moved toward the anode with an $^+R_{F(e)}$ of 0.34, indicating that a negatively charged group had been formed in the reaction with the enzyme. Chromatography of the solution in *t-amyl alcohol* saturated with 3% NH_3 (aqueous) showed a fluorescent spot corresponding to the enzyme-treated portion with R_F values of *ca.* 0.5; the spot corresponding to the control ran with the solvent front, again indicating hydrolysis of the ligand by the enzyme. Rate assays were not feasible due to the very low solubility of the ligand in water.

Kinetic measurements were carried out to test the ability of DNS-D-trpEE to function as an inhibitor of the hydrolysis of ATEE by chymotrypsin. Reaction rates were measured in the pH-stat in 5% 1,4-dioxane containing 0.01 M Tris–0.02 M $CaCl_2$–0.08 M KCl buffer, pH 7.80, ionic strength 0.15, at 25°, at varying substrate concentrations in the presence and absence of a constant amount of ligand. The results at two different ligand concentrations, plotted in Eadie coordinates, are shown in Figure 2, and demonstrate that DNS-D-trpEE is an inhibitor of chymotrypsin action under the conditions of measurement reported here. The values of the Michaelis constant, K_m, and the inhibitor association constant, k_I, are given in Table III.

Polarization and Fluorescent Intensity in Relation to

Binding. Typical results for the binding of DNS-D-trpEE to various proteins at pH 7.8 in 5% dioxane are given in Figures 3, 4, and 5. The experiments were carried out at constant ligand concentration (2.00×10^{-5} M) and varying protein concentration since the insolubility of the ligands prevented the inverse experiment. Furthermore, concentration quenching was observed for DNS-D-trpEE in solutions with concentrations greater than 5×10^{-5} M. The lines and experimental points shown in the figures have been automatically plotted by a digital computer plotting routine (University of Michigan Plot, adapted for the Fortran IV Compiler of an IBM 7094/7040 operating under Ibsys Monitor Control), and have a vertical plotting accuracy of ±2%, and a horizontal plotting accuracy of ±1%. In each case, the curves shown are the "best fit" curves obtained by a least-squares analysis of the experimental data, followed by a Newton–Raphson refinement of the coefficients of the equation for the curve. Figures 3 and 4 show the effect of increasing protein concentration on the polarization and total fluorescence, respectively, of DNS-D-trpEE, and the fitted curves are rectangular hyperbolas.

The polarization p (Figure 3) increases with increasing protein concentration as more ligand is bound from p_{min} at zero protein concentration, to p_{max}, corresponding to the point at which all the ligand is in the bound form. Due to insolubility of the proteins and the weakness of the binding, experimental data were unavailable in the region of p_{max}, and this value was obtained from the limit of the fitted hyperbola as the total protein concentration (P_0) approaches infinity. Since the saturation fraction of protein, *i.e.*, the number of moles of ligand bound per mole of protein, is a small and practically constant number in these experiments, it is more convenient to describe the binding in terms of the saturation fraction of ligand, $\bar{s}$, the number of moles of ligand bound per mole of ligand. If the polarization is used as a measure of the saturation fraction of ligand, then experimentally

$$\bar{s} = (p - p_{min})/(p_{max} - p_{min}) \qquad 0 \leqslant \bar{s} \leqslant 1 \quad (1)$$

In the case where there is only one ligand binding site per protein molecule, the binding constant k for the association reaction (A) + (P) $\rightleftharpoons$ (PA) can be obtained from the relation

$$\bar{s}/(P) = k(1 - \bar{s}) \quad (2)$$

where (P) is the concentration of unbound protein. Figure 5 shows the data of Figure 3 plotted according to eq 2, with $\bar{s}$ calculated by means of eq 1. The binding constant is obtained from the slope (or $\bar{s}/(P)$ intercept) of the line, and the observation that the line passes through 1.0 on the $\bar{s}$ axis indicates that the original assumption of a single binding site was justified.[2]

The results of all the experiments at pH 7.8 in 5% dioxane are summarized in Figure 6 in the form of a histogram in order to facilitate visual comparison of the

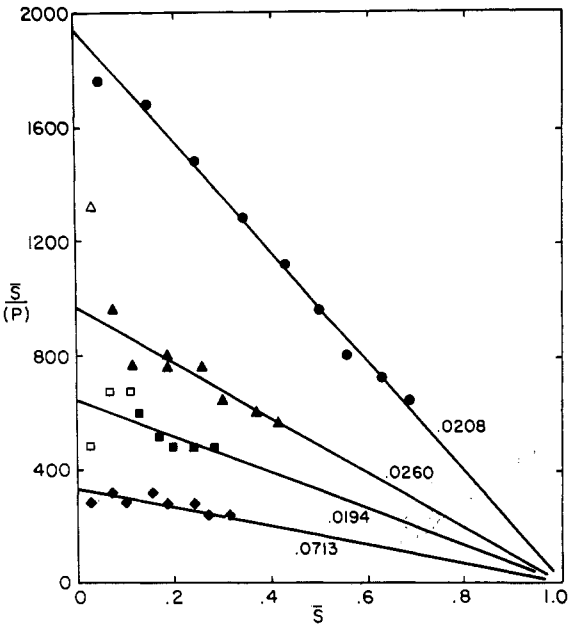


FIGURE 5: Modified Scatchard plot of the binding of DNS-D-trpEE to chymotrypsin (●), chymotrypsinogen (▲), DIP-chymotrypsin (■), and lysozyme (◆) calculated from the data of Figure 3. The numbers represent the average fitting error for each calculated line (average of the values of $(y_{calcd} - y_{obsd})/y_{obsd}$). See text for full details.

magnitudes of the binding constants for the various systems investigated. Of special interest are the decrease in the binding constant for DNS-D-trpEE from chymotrypsin to chymotrypsinogen, the similarity of the binding of DNS-glyEE and $DNSNH_2$ to the same two proteins, and the decrease in the binding constant of all three ligands between chymotrypsinogen and DIP-chymotrypsin. The values k_{av} given in Figure 6 are the average of binding constants obtained by three different computation techniques which fit the experimental data to eq 2. In general, the method of calculation does not introduce appreciable differences in the results. Complete details of the calculations discussed in this section are to be found in the Appendix.

The changes in both polarization and intensity caused by addition of protein to a given ligand were completely reversible. The same values for both parameters at a given protein concentration were obtained regardless whether this protein concentration was attained by dilution of a more concentrated ligand–

[2] In the figure shown, the lines have been forced through an intercept of 1.0 by adding fictitious points to the experimental data at this intercept value (hinge point method, see Appendix). If these fictitious points are omitted, the s intercepts are near or equal to 1.0 in virtually all the cases tested (21 out of 26 data sets obtained in 5% dioxane had uncorrected $\bar{s}$ intercepts between 0.85 and 1.15. Of all 26 sets, the range was from 0.845 to 1.37, with an average value of 1.02).

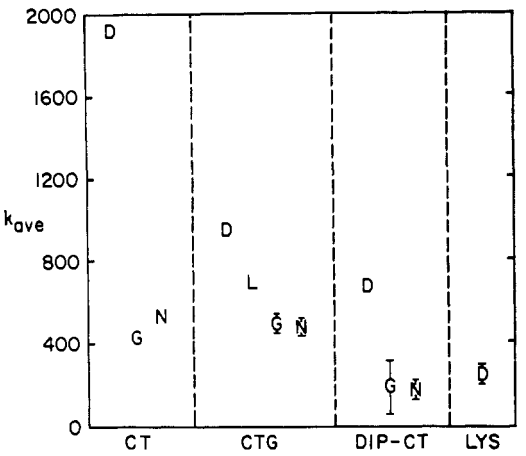


FIGURE 6: Histogram showing the distribution of the various average binding constants at pH 7.8 in 5% dioxane for DNS-D-trpEE (D), DNS-L-trpEE (L), DNS-glyEE (G), and $DNSNH_2$ (N) binding to chymotrypsin (CT), chymotrypsinogen (CTG), DIP-chymotrypsin (DIP-CT), and lysozyme (LYS). Vertical bars indicate average spread of the binding constant as determined by three different fitting techniques (see Appendix).

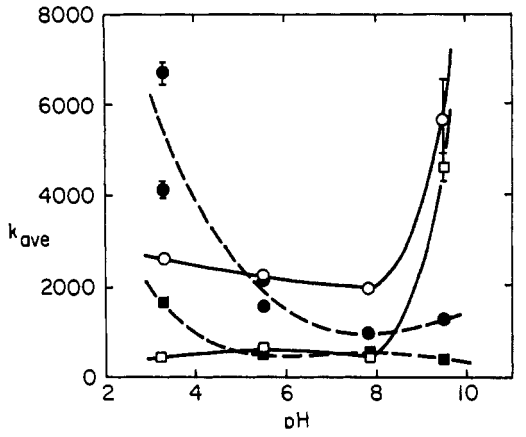


FIGURE 7: Approximate pH dependence of the average binding constants for DNS-D-trpEE on chymotrypsin (○) and chymotrypsinogen (●), and DNS-glyEE on chymotrypsin (□) and chymotrypsinogen (■). Data for chymotrypsinogen at pH 3.3 are uncertain (see text).

protein mixture or by addition of protein to a solution containing ligand only.

Effect of pH on Binding. It was of interest to determine whether titration of the various groups on the protein surface near or at the active site would influence the binding properties of the various dye conjugates. Binding studies were therefore carried out at four pH values, chosen as follows: pH 3.3, below the pK of COOH groups; pH 5.5, between the pK values of COOH and imidazolyl groups; pH 7.8, the pH of maximum activity of chymotrypsin toward synthetic substrates; and at the alkaline pH 9.5. These results are shown in Figure 7. At pH 3.3, chymotrypsinogen solutions display a high degree of light scattering in the presence of the dyes, indicating extensive aggregation, and hence the results at this pH are of doubtful validity. Furthermore, this phenomenon was time dependent, as the turbidity increased slowly over a 10–15-min period. To a smaller extent, this is also true at pH 5.5, and it is unknown whether the effects at these pH values are due to the dye or to the presence of 5% 1,4-dioxane. The intensity of the fluorescence of all the ligand conjugates is 55–60% lower at pH 3.3 than at pH 7.8, owing to the dimethylammonium group acquiring a proton below pH *ca.* 4 (Klotz and Fiess, 1960). It is of interest to note in this connection that these authors found a large shift in the pK of the dimethylammonium group (from 3.99 to 1.67) when DNS chloride was covalently coupled to serum albumin. At pH 9.5, the binding of DNS-D-trpEE and DNS-glyEE to chymotrypsin increased markedly, whereas the binding of the same two ligands to chymotrypsinogen was virtually unchanged relative to the binding at pH 7.8.

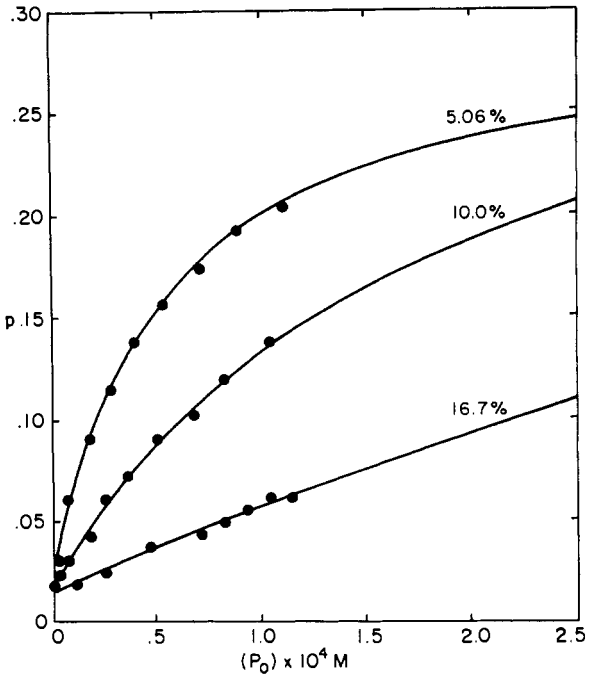


FIGURE 8: Effect of dioxane concentration on the binding of DNS-D-trpEE (2×10^{-5} M) to chymotrypsin at pH 7.8. Figures in per cent refer to the total dioxane concentration. Solutions adjusted to give final concentrations of 0.01 M Tris, 0.02 M $CaCl_2$, 0.083 M KCl, ionic strength 0.15.

Effect of Dioxane Concentration. The effect of dioxane on the apparent binding properties of chymotrypsin at pH 7.8 is shown in Figure 8. The binding constant is 14 times greater in 5% dioxane (k_{av} = 1920) than in 17% dioxane (k_{av} = 140), and 3 times greater than in 10%

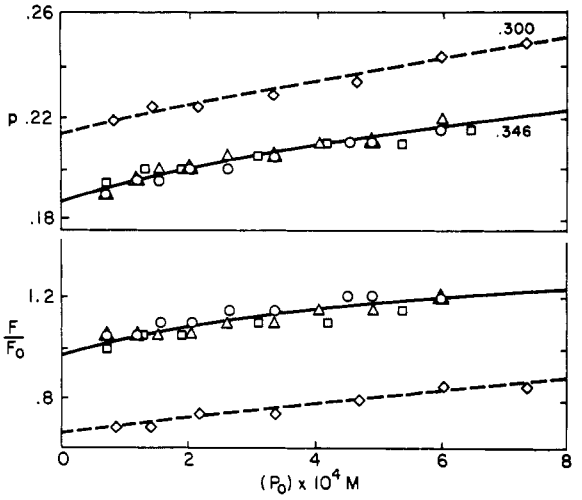


FIGURE 9: Concentration dependence of the polarization and relative fluorescence intensity of $DNS_{0.26}$-chymotrypsinogen (fixed DNS concentration, 2×10^{-5} M) plus unsubstituted chymotrypsinogen at various pH values: pH 3.3 (◇), pH 5.5 (△), pH 7.8 (○), pH 9.5 (□). F_0 was taken as the average fluorescent intensity of 2×10^{-5} M solutions of the free ligands at the pH values indicated. Numbers on curves refer to the predicted maximum values of the rectangular hyperbola fit.

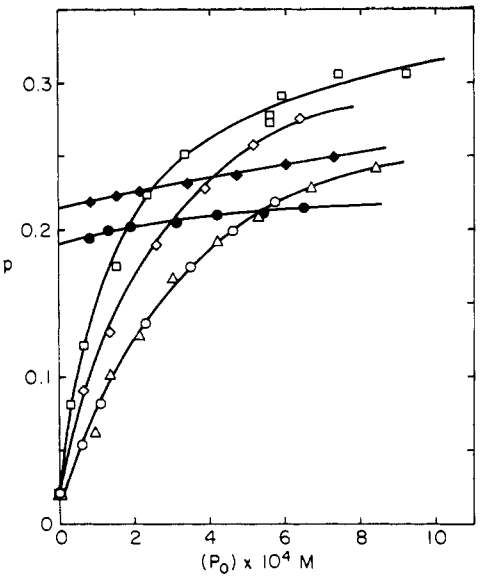


FIGURE 10: Relation of the maximum polarization to the binding of DNS-D-trpEE to chymotrypsinogen at pH 3.3 and 5.5. Chymotrypsinogen plus DNS-D-trpEE (2×10^{-5} M): pH 3.3 (□, ◇), pH 5.5 (△, ○). $DNS_{0.26}$ chymotrypsinogen (2×10^{-5} M) plus unsubstituted chymotrypsinogen: pH 3.3 (◆), pH 5.5 (●). Solid and open figures of a given kind (◆, ◇ and ●, ○) represent experiments performed with the same chymotrypsinogen solutions.

dioxane (k_{av} = 570). Studies of the polarization of DNS-D-trpEE in 10% dioxane at pH 7.8 in the presence of various proteins again revealed the series chymotrypsin > chymotrypsinogen > DIP-chymotrypsin with respect to their apparent binding capability. DIP-trypsin was also tested, and had a binding constant of about one-third of that of DIP-chymotrypsin.

Polarization of Covalently Coupled $DNS_{0.26}$-chymotrypsinogen. In the absence of rotation on the surface of the protein, or differences in the lifetime of the excited states, the polarization of a covalently coupled dye–protein complex should be the same as the maximum polarization observed when all the dye is bound to protein in the equilibrium situation. Laurence (1952) has presented evidence that this is indeed the case for serum albumin, where the covalently bound DNS group has a rigidity in the protein surface comparable to that of the adsorbed DNS group (free acid). To obtain an independent value for the maximum polarization that could be used in quantitating the results of the binding studies, experiments analogous to those already described were carried out in which $DNS_{0.26}$-chymotrypsinogen replaced the dye conjugates as the fluorescent species. The results of these measurements in 5% dioxane at various pH values are shown in Figure 9. At a constant $DNS_{0.26}$-chymotrypsinogen concentration, both the polarization and the relative fluorescent intensities increased slowly with increasing concentration of unlabeled chymotrypsinogen, the total changes amounting to about 13% over the range studied. The maximum value of the polarization predicted by fitting the pH 5.5, 7.8, and 9.5 data to a rectangular hyperbola was 0.35 ± 0.06, in reasonably good agreement, considering the extrapolations involved in these comparisons, with the average of the maximum polarizations found in the binding experiments at these pH values, 0.29 ± 0.07.

The increase in polarization with increasing unlabeled chymotrypsinogen concentration was not due to the contribution of the intrinsic fluorescence of the protein to the total fluorescence intensity, as was shown by a similar experiment in which the $DNS_{0.26}$-chymotrypsinogen concentration was varied over the entire range, thus providing a constant ratio of fluorescent signal to stray light (intrinsic fluorescence of the protein, solvent contributions, etc.). Simultaneous polarization and sedimentation measurements at pH 7.8 in 5% dioxane after equilibration by dialysis overnight at 4° against the solvent (5% dioxane, 0.01 M Tris chloride, 0.02 M $CaCl_2$, 0.083 M KCl, ionic strength 0.15) showed an increase in polarization with concentration similar to that found when the $DNS_{0.26}$-chymotrypsinogen concentration was held constant, although the entire curve was shifted to higher polarizations. The sedimentation coefficient showed a correlative decrease toward higher protein concentrations (9% over the range studied); this decrease, and the value of $s_{20,w}$ extrapolated to zero protein concentration (2.70 S), are in good agreement with the results obtained on unsubstituted chymotrypsinogen by Dreyer *et al.* (1955). These authors observed an extrapolated value of $s_{20,w}$ of 2.63 S (R. D.

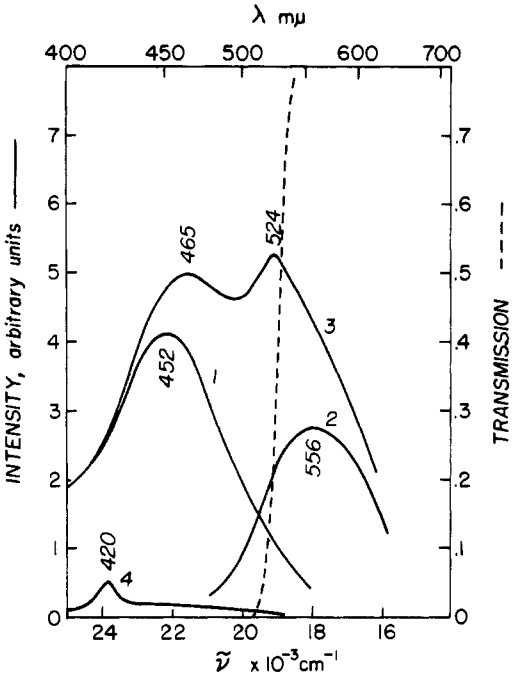


FIGURE 11: Emission spectra of chymotrypsinogen (20 mg/ml) (1), DNS-D-trpEE (2×10^{-5} M) (2), and a mixture of the two (3). The dashed curve represents the transmission of the fluorescence filter used in polarization and intensity measurements. The Raman spectrum of the solvent (5% dioxane, 0.01 M Tris, 0.02 M $CaCl_2$, 0.083 M KCl, pH 7.8, ionic strength 0.15) is shown for comparison (4). Excitation wavelength: 365 mμ.

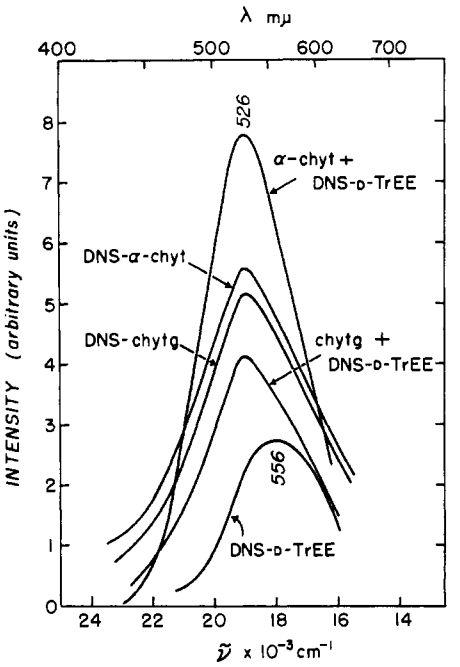


FIGURE 12: Emission spectra of equilibrium-bound and covalently coupled ligand–protein complexes corrected for protein contribution. The concentration of the DNS group (including DNS-D-trpEE) was approximately the same in all cases (2×10^{-5} M), and the total protein concentrations were: chymotrypsin and chymotrypsinogen, 20 mg/ml; $DNS_{0.26}$ chymotrypsin and $DNS_{0.26}$-chymotrypsinogen, 2 mg/ml.

Wade, private communication), and a similar concentration dependence of the sedimentation coefficient in phosphate buffer at pH 7.5 in the absence of dioxane.

Experiments at constant $DNS_{0.26}$-chymotrypsinogen concentration were also carried out at pH 3.3. Under these conditions, the solutions scattered light extensively, indicating a high degree of aggregation, and the observed polarizations were 20% greater than those observed at the other pH values, although the relative increase of polarization with concentration was approximately the same (Figure 10).

A curious feature of the results with chymotrypsinogen at pH 3.3 and 5.5 was that higher polarizations (and intensities) were observed with the equilibrium-bound dye than with the covalently coupled dye, as shown in Figure 10. The significance of these findings is considered in the Discussion.

Emission Spectra in Relation to Binding. The emission spectra of the various dye–protein complexes were measured to provide further information on the nature of the interactions discussed previously. In Figure 11 are shown the emission spectra of free DNS-D-trpEE, free chymotrypsinogen, and a mixture of the two, along with the transmission curve of the filter used in the polarization and intensity measurements. The two most important factors are the increase in fluorescence intensity on binding, as already noted, and the marked wavelength shift (30 mμ) of the fluorescence of the dye to shorter wavelengths. This introduces a problem in a quantitative treatment of binding by intensity data since, because of the shift in wavelength, the intensity observed in a binding experiment (above the cutoff point of the filter) will not be a direct measure of the amount of dye bound at any particular protein concentration.

When the observed emission spectra were corrected for the protein contribution, chymotrypsin and chymotrypsinogen were found to have nearly identical emission characteristics in the presence of DNS-D-trpEE, the only essential difference being in the intensity of the two (Figure 12). At approximately equal concentrations (20 mg/ml), the wavenumber maxima were at 19,000 cm^{-1} (526 mμ) for both proteins. The emission spectra of $DNS_{0.26}$-chymotrypsin and $DNS_{0.26}$-chymotrypsinogen at approximately equal concentrations (2 mg/ml, corresponding to *ca.* 2×10^{-5} M DNS-protein) were virtually identical, not only with each other, but also identical with the emission spectra of the unsubstituted proteins in the presence of DNS-D-trpEE. The peak wavenumber was 19,000 cm^{-1}, and both proteins had nearly equal fluorescent intensities. These results are shown in Figure 12, along with the spectrum of free

DNS-D-trpEE for comparison. The wavenumber maximum was 18,000 cm^{-1} (556 mμ) for the latter compound, and the frequency shift is thus 1000 cm^{-1} between the free and bound dye. It is important to note that the intensities here should be directly comparable, since the concentration of the DNS group was approximately the same in all cases. The measurements were made under the same conditions as were the binding and maximum polarization experiments, *i.e.*, 365 mμ excitation in 5% dioxane, 0.01 M Tris, 0.02 M $CaCl_2$, 0.083 M KCl, ionic strength 0.15, pH 7.8, at 25°.

Activity of DNS-chymotrypsin. The activities of $DNS_{0.26}$-chymotrypsin and the chymotrypsin from which it was prepared were measured in the pH-stat *vs.* 0.01 M ATEE at 20° in 0.01 M Tris buffer containing 0.02 M $CaCl_2$, 0.083 M KCl, and 2% ethanol. The zero-order rate constants $\dot{c}/(P_0)$, where $\dot{c}$ is the rate and (P_0) the total protein concentration, were: $DNS_{0.26}$-chymotrypsin 86 sec^{-1} and chymotrypsin 120 sec^{-1} (mean of six determinations each). Thus it was observed that $DNS_{0.26}$-chymotrypsin had 28% less "activity" as expressed by rates than the unsubstituted enzyme. The other preparation, $DNS_{0.64}$-chymotrypsin, had 63% less "activity" than the unsubstituted enzyme by the same criteria. In both cases, the loss of activity is in agreement with the degree of substitution as determined spectrophotometrically. The degree of substitution of $DNS_{0.64}$-chymotrypsin was checked independently by the "all or none" assay of Schonbaum *et al.* (1961). A value of 0.37 ± 0.01 mole of "active sites" per mole of protein was obtained, corresponding to a substitution in the active site of 63%.

Discussion

The conclusion of Vaslow and Doherty (1953) that chymotrypsinogen, as well as chymotrypsin, contains a specific site capable of binding a virtual substrate has been extended in this investigation to an actual substrate, DNS-L-trpEE, and a competitive inhibitor, DNS-D-trpEE. The present evidence for the existence of a binding site in chymotrypsinogen can be summarized as follows: (1) solutions of all the fluorescent ligands showed marked increases in both the polarization and the intensity of their fluorescence when mixed with chymotrypsinogen. (2) The observed changes were of the same general form, although differing in magnitude, as those occurring when the ligands were mixed with chymotrypsin. With the enzyme, the site of interaction is the active center, since DNS-D-trpEE is an inhibitor, and DNS-L-trpEE is a substrate. (3) A large shift in the emission spectrum of DNS-D-trpEE occurred toward shorter wavelengths when the ligand was mixed with either chymotrypsin or chymotrypsinogen. The close correspondence of the two spectra suggests that the interactions take place on similar, or nearly identical, binding sites.

The relative binding strengths of the various ligands toward chymotrypsin at pH 7.8 are of the order expected from the specificity requirements of the enzyme toward synthetic substrates (Neurath and Schwert, 1950), the tryptophanyl derivative being bound more strongly than the glycyl one (Figure 6). The fact that the latter, as well as DNS-amide, are bound at all is probably a manifestation of the contribution of the naphthalene function to binding. The relative order of binding, however, suggests that in DNS-D-trpEE the indole ring is bound in preference to the naphthalene group. In fact, Wallace *et al.* (1963) found that 1-amino-5-naphthalenesulfonate is a competitive inhibitor of chymotrypsin with a k_I of 320 l. $mole^{-1}$ which is the same order as the values reported herein for the binding of $DNSNH_2$ to chymotrypsin. It is interesting to note in this connection that DNS-glyEE can assume a configuration similar to that of tryptophan, in which the naphthalene ring can lie in nearly the same position with respect to the carbonyl group as can the indole nucleus.

With DNS-D-trpEE as ligand, the affinity decreases in the order chymotrypsin > chymotrypsinogen > DIP-chymotrypsin > lysozyme. While this order is in keeping with the fact that the protein with the highest affinity is the only one revealing catalytic function, the binding of this inhibitor by chymotrypsinogen as well as by DIP-chymotrypsin with affinities exceeding that of the control (lysozyme) suggest that the binding site is functional in these proteins, although with diminished avidity as compared to chymotrypsin. The DIP group undoubtedly overlaps the binding site to a certain extent, thus preventing sterically the close approach of the ligand to the binding site. The difference between chymotrypsinogen and chymotrypsin can be best explained by an alteration in the electronic structure of the binding site attendant activation, leading to increased binding strength, rather than to physical blocking of the binding site in chymotrypsinogen. The latter explanation would require that DNS-glyEE and $DNSNH_2$ have reduced binding to chymotrypsinogen relative to chymotrypsin, which is not observed (Figure 6). This hypothesis finds some support in the work of Erlanger (1958), who found that an insoluble compound (*N*-carbobenzyloxy-L-leucyl-D-phenylalanine benzyl ester, CLPB) satisfying the structural requirements of an inhibitor of chymotrypsin would adsorb the latter protein about 40% more strongly than it would adsorb chymotrypsinogen or DIP-chymotrypsin, which were bound with equal strength. In general, the data suggest that differences between the enzymatically active and inactive protein species become smaller as the ligands become less effective as inhibitors.

In chymotrypsin, and to a lesser extent in chymotrypsinogen, the binding of the virtual substrate acetyl-3,5-dibromo-L-tyrosine is controlled by protonation of a group with p*K* of about 6.7 (Vaslow, 1958). The binding is stronger at pH values below the p*K*, and since the virtual substrate has a negative charge, this probably corresponds to the acquisition of a positive charge in the active center resulting in increased binding through the increased electrostatic attraction. With the neutral compounds employed in this work, the binding should be relatively independent of such charge effects. This was approximately the case for the binding of DNS-D-

trpEE and DNS-glyEE to chymotrypsin at pH 5.5 and 7.8, where the apparent binding strengths did not differ from each other by more than 10%, in contrast to acetyl-3,5-dibromo-L-tyrosine, which binds more than four times more strongly at pH 5.5 than at pH 7.8 (Vaslow, 1958). However, at pH 9.5, in contrast to pH 7.8, there was little difference in the ability of chymotrypsinogen to bind DNS-D-trpEE and DNS-glyEE, respectively, whereas with chymotrypsin, the binding of either derivative was stronger at the higher pH than it was at the lower pH.[3] This is probably the result of conformational changes in chymotrypsin which do not occur in chymotrypsinogen at pH 9.5 (Neurath *et al.*, 1956).

Massey *et al.* (1955) have shown that the values of τ_0/ρ_h of covalently coupled dye–protein complexes are the same for DNS-chymotrypsin, DNS-chymotrypsinogen, and DNS-DIP-chymotrypsin at any given degree of labeling and, further, that τ_0/ρ_h (see Appendix) varies with the degree of labeling above 1 mole of dye per mole of protein. These results have been interpreted as indicating that the first molecule of dye combining with the protein is attached to a specific site, and that this site is the same for all three proteins. The present results provide further evidence that this may be the case for DNS-chymotrypsin and DNS-chymotrypsinogen since the fluorescence spectra of the two dye–protein complexes are identical. In view of the similarity of the fluorescence spectra of the covalently coupled and the equilibrium-bound DNS group, it is unlikely that this site is different from the substrate binding site. However, the actual group through which the dye is chemically bonded may be different. The DNS-chymotrypsinogen prepared by Hartley and Massey (1956) was fully activatable, whereas the DNS-chymotrypsin prepared by them and by us was inhibited corresponding to the degree of substitution by the dye. Hartley and Massey (1956) also found that β-phenylpropionate protected the activity of the enzyme during reaction with the fluorescent dye, but did not prevent substitution from taking place. It is thus possible that there are at least two separate groups in chymotrypsin available for chemical reaction with the dye, one of which is identical with the group available in chymotrypsinogen. The group which is common to both must be adjacent to the active center, for otherwise no difference in the fluorescence spectra should be observed between the dye covalently coupled to protein and that covalently coupled to a small amino acid. In addition, the observed spectral shift is in the proper direction (toward shorter wavelengths) for an interaction involving higher energy excited states, which might be expected from mutual polarization interactions of the hydrophobic binding site with the bound aromatic nucleus. Gold (1965) has presented evidence that covalent coupling of DNS-chloride to chymotrypsin results in sulfonylation of the active center serine.

The present conclusion of the existence of a substrate-binding site in chymotrypsinogen on first sight appears to be at variance with the findings of Weiner and Koshland (1965a) and of Glazer (1965) who found that proflavine, a competitive inhibitor of chymotrypsin, was only weakly bound by chymotrypsinogen. This contradistinction is more apparent than real. The binding constants observed herein for the interaction of chymotrypsinogen and DNS-D-trpEE, the most effective of the fluorescent ligands investigated in this study, correspond to a free energy of binding of such low magnitude as to be below the threshhold of the measurements of Glazer. The binding site of an enzyme, such as chymotrypsin, is large enough to permit binding of substrate and inhibitor to occur for a number of reasons, through a number of interactions with functional groups on the active site. If, in addition, the active site is assumed to be susceptible to conformational variations, in response to the nature of the interacting substrate or inhibitor (Weiner and Koshland, 1965b), a multitude of enzyme–inhibitor interactions, leading to partial or complete blocking of the active site, would give rise to the kinetic relationships characteristic of "inhibition." Moreover, while proflavine is among the most effective competitive inhibitors of chymotrypsin (Wallace *et al.*, 1963), it is by no means the most specific one since it also inhibits trypsin with great avidity (Bernhard and Gutfreund, 1965). Much like DFP, proflavine has no apparent structural similarity to specific substrates for chymotrypsin or trypsin, in contrast to the inhibitors used in the present investigation.

Acknowledgment

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Appendix

D. A. Deranleau

Fluorescence Polarization in Relation to Binding

A small fluorescent molecule in aqueous solution generally exhibits a small or even zero polarization, due to the fact that the lifetime of the excited state is large with respect to the relaxation time of rotational Brownian motion. If the small molecule (adsorbate) is bound to a protein or other macromolecule having a rotational relaxation time of the same order of magnitude as the lifetime of the excited state of the adsorbate, then the complex will show an appreciable degree of polarization in solution. The observed polarization in such a protein–ligand mixture will depend on the relative

[3] These results are in contradistinction to the results of Erlanger (1958), who found that the binding of chymotrypsin by CLPB decreased markedly above pH 8.5 and suggested that this was due to an alteration in the charge on an essential group in the active center. This seems unlikely since the binding of CLPB was constant over the pH range 1.5 to 8.5, and a charge alteration is known to take place in the active center in this pH region.

TABLE A: Binding Constants at pH 7.8 by Various Methods of Calculation.

System	Function Fitted: p or $\frac{F}{F_0} = \frac{a_0 + a_1(P_0)}{1 + b_1(P_0)}$			$\bar{\nu}/(P) = k(1 - \bar{\nu})$			
	Limiting Slope[a] as $(P_0) \rightarrow 0$		b_1				
	p vs. (P_0)	F/F_0 vs. (P_0)	p vs. (P_0)	k_{hyper}	k_{hinpt}	k_{int}	$k_{av} \pm$ av devn
DNS-D-trpEE *vs.*							
Chymotrypsin	2000	(2000)	2000	1920	1930	1910	1920 ± 10
Chymotrypsinogen	1000	(1000)	900	950	960	950	950 ± 3
DIP-chymotrypsin	700	(800)	600	690	640	740	690 ± 30
Lysozyme	200	(200)	300	220	330	230	260 ± 50
DNS-L-trpEE *vs.*							
Chymotrypsinogen	500	(600)	500	710	640	710	690 ± 30
DNS-glyEE *vs.*							
Chymotrypsin	700	(1000)	400	420	430	420	430 ± 5
Chymotrypsinogen	400	(300)	500	630	530	650	600 ± 50
DIP-chymotrypsin	200	(300)	400	110	420	80	200 ± 140
$DNSNH_2$ *vs.*							
Chymotrypsin	500	(800)	600	500	590	500	540 ± 40
Chymotrypsinogen	700	(600)	600	450	550	440	480 ± 50
DIP-chymotrypsin	200	(800)	100	220	240	200	220 ± 10

[a] Computed from relative values multiplied by the binding constant k_{av} for DNS-D-trpEE *vs.* chymotrypsin. Due to the nonlinearity of F/F_0 with concentration, the values in parenthesis are included for comparison only.

contribution which the free and bound adsorbate molecules make to the total observed fluorescent intensity $F = \Sigma_i F_i$ according to the relation

$$p = \Sigma_i p_i F_i / \Sigma_i F_i \qquad (1A)$$

(Weber 1952a), where $F_i = I_{\parallel i} + I_{\perp i}$ and $p_i = (I_{\parallel i} - I_{\perp i})/F_i$. In a binding experiment where ligand is "titrated" by the addition of protein, p, as defined by eq 1A, will vary between the polarization of unbound fluorescent ligand, p_{min}, and totally bound fluorescent ligand, p_{max}. Experimentally, the amount of binding can be described in terms of the saturation fraction of ligand $\bar{\nu}$ defined previously (eq 1).

A generalized theoretical description of $\bar{\nu}$ has been given by Deranleau (1964) for the case in which protein polymerization can occur during the binding process. Assuming that there is only one ligand binding site per protein species P_i, with association constant k_i, the following expression can be derived from the general definition

$$\bar{\nu} = \Sigma_i k_i[\Pi_i k_i'](P_1)^i / [1 + \Sigma_i k_i[\Pi_i k_i'](P_1)^i] \qquad (2A)$$

$$i = 1, 2, \ldots; k_i' = 1$$

Here (P_1) is the concentration of unbound protein monomer, and the k_i' are the protein–protein association constants. Combining the theoretical description given in eq 2A with the experimental definition of $\bar{\nu}$ (eq 1), we can write a phenomenological description of the binding process in terms of the dependent experimental probe p and the independent variable (P_1). This equation takes the form of a general rational function

$$p = \sum_{i=0}^{n} a_i(P_1)^i \Big/ \left[1 + \sum_{i=1}^{m} b_i(P_1)^i\right] \qquad (3A)$$

where, specifically, $a_0 = p_{min}$, $a_i = p_{max}k_i[\Pi_i k_i']$, and $b_i = k_i[\Pi_i k_i']$. In addition, the last equation has a maximum $a_n/b_m = p_{max}$, $n = m$, as the protein concentration becomes infinitely high. By fitting data to eq 3A, it should be possible to test for the effects of polymerization (if present); dimer formation should yield a second degree curve, trimer a third degree, etc., but the constants will not be separable, and an approximation must be made to (P_1) if this information is not available from separate experiments (see, for example, Deranleau, 1964). Equation 3A is also valid for other experimentally available measures of the saturation fraction that vary according to eq 1, such as changes in absorbance, fluorescent intensity, etc., and is in a convenient form for curve fitting on high-speed computing equipment.

Methods of Data Handling

In some cases, those in which the association constants are small or those in which solubility problems prevent using high concentrations of reactants, incomplete information may be obtained, and an experimental determination of values of p at or near p_{max} will be impossible, as in the present case. An approximate method of some use in the absence of such information makes use of the limiting slope of a plot of p *vs.* (P_0), the *total* protein concentration. Assuming simple one-to-one equilibria, and $(P_1) \simeq (P_0)$, the limiting slope of eq 3A with $i = 1$ is

$$\lim_{(P_0)\to 0} \partial p/\partial(P_0) = k(p_{max} - p_{min}) \qquad (4A)$$

If the $(p_{max} - p_{min})$ are approximately the same for a series of ligand–protein systems (proteins having similar rotational relaxation times), then a comparison of the *relative* binding constants can be made by referencing to any one of the series. This is the basis for the "limiting slope" approximations given in Table A.

Much more information can be obtained from an incomplete set of binding data if automatic curve-fitting routines can be used to examine the data. In the present case, we have made the approximation $(P_1) \simeq (P_0)$ and have fitted the data to eq 3A by means of a least-squares computer routine which scans all the rational functions up to degree 5/5 (numerator/denominator). The routine accepts a "fit" when the *average* error of the points, $(p_{calcd} - p_{obsd})/p_{obsd}$, does not exceed 0.05, allowing for two bad points in each data set. For 26 data sets in 5% dioxane, this preliminary scan yielded 15 sets which fit a 1/1 rational function (rectangular hyperbola) within the specified tolerances, and 4 additional sets which fit a rational function of degree 2/2. On the basis of this preliminary analysis, a computer program was designed around the fit of polarization data to the 1/1 rational function. Again using the approximation $(P_1) \simeq (P_0)$, the experimental data were fitted to eq 3A (with $i = 1$) by a least-squares subroutine, and the coefficients $a_0 = p_{min}$, $a_1 = p_{max}k$, and $b_1 = k$ were refined by a Newton–Raphson iteration technique. The maximum value of the function, $a_1/b_1 = p_{max}$, was computed from the refined coefficients. Although the values so obtained are approximations (except when the protein concentration is high), p_{max} and p_{min} from the fitted curve can be used to obtain a value of $\bar{\nu}$ according to eq 1, and the correctness of the initial guess of p_{max} can be checked by plotting the data according to a rearranged form of eq 2A. With $i = 1$, eq 2A can be written as

$$\bar{\nu}/(P_1) = k(1 - \bar{\nu}) \qquad (5A)$$

The latter is the usual equation for 1:1 binding (eq 2), where $(P_1) = (P_0) - \bar{\nu}(A_0)$ is the concentration of *unbound* protein, and (A_0) is the total ligand concentration. If the values of $\bar{\nu}$ calculated from the guessed p_{max} are correct, the last equation requires that the $\bar{\nu}/(P_1)$ *vs.* $\bar{\nu}$ plot be a straight line with an $\bar{\nu}$ intercept of 1.0. The values of $\bar{\nu}$ and (P_1) were accordingly computed, and the data with the p_{max} guessed from the maximum of the 1/1 rational function (hyperbola) were fitted to eq 5A. Data with more than 2.5 times the average error of a given set were rejected, the saved points refitted by least squares, and the coefficients refined as before. The slope of the resultant line was taken as the binding constant k_{hyper}. In several cases the data did not pass through the 1.0 intercept on the $\bar{\nu}$ axis required by eq 5A so a hinge point was provided by adding an equal number of points with coordinates $\bar{\nu}/(P_1) = 0$, $\bar{\nu} = 1.0$ to a given data set, and the data refitted to pass through the $\bar{\nu} = 1.0$ intercept. The slope of this line was designated as k_{hinpt}, the binding constant on the basis of the hinge point. Then a check on the maximum value found in fitting the data to a hyperbola was made by changing p_{max} until an $\bar{\nu}$ intercept of 1.0 was obtained for both the original data set and the set after rejection of the bad points. Least-squares fitting and refinement of these lines then yielded the slope k_{int}, the binding constant from the intercept calculations. Finally, an average value k_{av} for the binding constant was obtained by averaging the three constants from the straight line slopes. Table A contains a comparison of the results by the various methods of calculations. The computer used was an IBM 7094/7040, and the final program required 7 min of computation time to process 34 sets of data.

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The Reaction of Carboxypeptidase A with Chromophoric Substrates *

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ABSTRACT: The reaction of bovine pancreatic carboxypeptidase A with a series of chromophoric substrates has been studied. Newly synthesized substrates used include the amides *N-trans*-cinnamoyl-L-phenylalanine, *N-trans*-3-(2-furylacryloyl)-L-phenylalanine, and *N-trans*-3-(3-indoleacryloyl)-L-phenylalanine as well as two ester analogs, *O-trans*-cinnamoyl-DL-β-phenyllactate and *O-trans*-3-(2-furylacryloyl)-DL-β-phenyllactate. Using ultraviolet absorbance spectrophotometry, the rates of carboxypeptidase-catalyzed hydrolyses of these substrates have been examined as functions of pH, ionic strength, temperature, and enzyme and substrate concentrations. The variation of reaction rates with enzyme and substrate concentrations was found to obey Michaelis–Menten kinetics, even when enzyme and substrate concentrations were approximately equal, as was the case in studies involving amide substrates. The activation parameters calculated from the effect of temperature upon the reaction rates are within the range previously reported for the action of carboxypeptidase on other substrates. Gel filtration on Sephadex G-50 has provided evidence for the existence of intermediates in reactions involving cinnamoylphenylalanine. A new assay for carboxypeptidase with furylacryloylphenyllactate as substrate has been developed.

The reaction of carboxypeptidase A with amide, ester, and protein substrates has been the object of intensive studies by several investigators (reviewed by Neurath and Schwert, 1950; Smith, 1951; Neurath, 1960). The time course of these hydrolytic reactions has been mostly measured by the ninhydrin reaction or by potentiometric titration at constant pH. In order to exploit more sensitive methods of measurements as are required for a detailed investigation of the mechanism of action of this enzyme, a series of substrates for carboxypeptidase A has been developed which allow the reaction to be followed by absorbance spectrophotometry in a region of the spectrum where the protein does not measurably absorb light. The substrates used for this purpose are derivatives of either phenylalanine or of β-phenyllactate wherein the amino or hydroxyl group has been acylated by one of a series of β-aroylacrylic acids (Bernhard *et al.*, 1965). By use of these substrates it has proven possible to examine the mechanism of carboxypeptidase-catalyzed reactions with particular attention to the presence of intermediates.[1]

Experimental Section

Commercial reagents of the highest available quality were obtained from the following sources: *N*-carbobenzoxyglycyl-L-phenylalanine, Mann Research Laboratories; L-phenylalanine, Nutritional Biochemical Corp.; cinnamoyl chloride and cinnamic acid, Eastman

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[1] Abbreviations used in this paper are: CiPhe, cinnamoylphenylalanine; FAPhe, furylacryloylphenylalanine; IAPhe, indoleacryloylphenylalanine; CiPLA, cinnamoylphenyllactate; FAPLA, furylacryloylphenyllactate; Ammediol, 2-amino-2-methyl-1,3-propanediol; and carboxypeptidase, bovine pancreatic carboxypeptidase A.