Modification of Amino Groups in Inhibitors of Proteolytic Enzymes*

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ABSTRACT: Trinitrobenzenesulfonic acid (TNBS) was used to quantitate and modify free amino groups in several inhibitors of trypsin and chymotrypsin. The number of amino groups found by the TNBS method agreed well with that predicted by amino acid analysis. The trypsin-inhibitory activity of the following inhibitors was destroyed by modification with TNBS: lima bean inhibitor, bovine colostrum inhibitor, cassowary ovomucoid, duck ovomucoid, penguin ovomucoid, and turkey ovomucoid. The last three inhibitors, which also inhibit chymotrypsin, lost their trypsin-inhibitory activity but not their chymotrypsin-inhibitory activity. The activities of soybean trypsin inhibitor and chicken ovomucoid, both of which inhibit only trypsin, were relatively unaffected by modification with TNBS. Based on kinetic analysis of the data, it was concluded that either one or two "fast"-reacting amino groups are essential for the activity of four trypsin inhibitors, depending on the inhibitor. Correlated studies with deamination and carbamylation supported this conclusion.

The reagent 2,4,6-trinitrobenzenesulfonic acid (TNBS)[1] was developed by Okuyama and Satake (1960) and Satake *et al.* (1960) as a reagent for the determination of free amino groups in amino acids and peptides. They showed that TNBS reacts preferentially and quantitatively with free amino groups under mild conditions to give the corresponding trinitrophenyl derivatives. No reaction occurs with the imidazole nitrogen of histidine, the guanidinium group of arginine, or the hydroxyl group of tyrosine or threonine. TNBS, however, will react with free sulfhydryl groups, but at a much slower rate than with amino groups, to produce a very labile product under the required alkaline reaction conditions (Kotaki *et al.*, 1964). The trinitrophenyl derivative of amino groups can be quantitated spectrophotometrically with much greater accuracy than is possible with ninhydrin (Habeeb, 1966). TNBS also has been used as a reagent to modify the amino groups of xanthine oxidase (Greenlee and Handler, 1964), cytochrome *c* (Takemori *et al.*, 1962), and myosin adenosine triphosphate (Kubo *et al.*, 1960).[2]

Several inhibitors of proteolytic enzymes being studied in our laboratory were inactivated by modification of amino groups (Stevens and Feeney, 1963). We have used TNBS to study the essentiality of these groups for inhibition. Since the rates of modification and inactivation can be followed simultaneously when TNBS is used, it was possible to analyze the data kinetically in order to find how many amino groups are involved in the inhibition.

Materials and Methods

Materials. Salt-free crystalline preparations of bovine trypsin, bovine α-chymotrypsin, chicken lysozyme, and a crude preparation of soybean trypsin inhibitor were purchased from the Worthington Biochemical Corp. The ovomucoids used in this study were: chicken (*Gallus gallus*) and cassowary (*Casuarius aruensis*) ovomucoids, which inhibit only trypsin; tinamou (*Eudromia elegans*) and golden pheasant (*Chrysolophus pictus*) ovomucoids, which inhibit only chymotrypsin; and turkey (*Maleagres gallopavo*), penguin (*Pygoscelis adeliae*), and duck (*Anas platyrhynchos*) ovomucoids, which inhibit trypsin and chymotrypsin independently and simultaneously. All of these ovomucoids were prepared by trichloroacetic acid–acetone (one volume of 0.5 M trichloroacetic acid in water plus two volumes of acetone) precipitation (Lineweaver and Murray, 1947), CM-cellulose chromatography (Rhodes *et al.*, 1960), or a combination of both (Feeney *et al.*, 1963). The CM-cellulose and DEAE-cellulose were obtained from the Whatman Co. The lima bean inhibitor was prepared by the method of Jones *et al.* (1963). The bovine colostrum inhibitor was prepared by the method of Laskowski and Laskowski (1951), followed by gel filtration on Sephadex G-75 and chromatography on DEAE-cellulose.

The trinitrobenzenesulfonic acid was purchased from

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[1] Abbreviations used: TNBS, trinitrobenzenesulfonic acid; BTEE, benzoyl-L-tyrosine ethyl ester.

[2] Since completion of this manuscript, a paper has appeared (Goldfarb, 1966) describing a kinetic analysis of results obtained on TNBS modification of bovine serum albumin. In this case it was assumed that there were three classes of amino groups of varying reactivity.

Nutritional Biochemicals Corp. The trypsin substrate, TAME, was purchased from Mann Research Laboratories. BTEE, the substrate for chymotrypsin, was synthesized from benzoyl chloride and L-tyrosine ethyl ester according to a modification of the method described by Fox (1946) for the synthesis of benzoyldiiodotyrosine ethyl ester.

Inhibitor Assays. Assays for trypsin and chymotrypsin were made by the spectrophotometric method of Hummel (1959), modified for the assay of inhibitors of these enzymes.

Quantitation of Free Amino Groups. The quantitation of free amino groups was done essentially according to the method of Habeeb (1966). To 1 ml of protein solution (0.4–2.3 mg/ml) were added 1 ml of 4% $NaHCO_3$, pH 8.5, and 1 ml of 0.1% TNBS in water. The solution was incubated at 40° for 2 hr. Then 1 ml of 10% sodium dodecyl sulfate was added, followed by 0.5 ml of 1 N HCl. The absorbance of the solution, diluted with 0.01 N HCl if necessary, was read at 344 mμ against a blank treated as above but containing 1 ml of water instead of the protein solution. The wavelength selected was the wavelength at which the solution had maximum absorbance in a Bausch and Lomb Spectronic 600 spectrophotometer.

TNBS Modification. A protein concentration was chosen which would give a tenfold excess of reagent to free amino groups in the final mixture. Twelve 1-ml samples of the protein solution were pipetted into separate test tubes and the reaction mixtures made up as for quantitation. After incubation periods of 5, 10, 15, 20, 30, 40, 50, 65, 80, 100, and 120 min, respectively, the reaction in each of the 11 tubes was stopped as described above, and the number of modified amino groups determined. At each of the time intervals, 0.2 ml of solution was removed from the remaining tube, diluted with ice-cold deionized water to stop the reaction, and assayed for residual inhibitory activity. Under our experimental conditions, *i.e.*, TNBS excess at 40°, the modification of amino groups with TNBS and the loss of trypsin inhibitory activity were both first-order reactions.

Deamination. Deamination by nitrous acid was done in a manner similar to that described by Maurer and Heidelberger (1951). The extent of modification was determined on the intact protein with ninhydrin, and on acid hydrolysates by amino acid analyses. The loss of lysine as determined by amino acid analysis agreed closely with the loss of amino groups as determined by ninhydrin. The ninhydrin method was standardized against the Van Slyke method on chicken and turkey ovomucoids (Stevens and Feeney, 1963).

Carbamylation. Carbamylation was done according to the procedure used previously in this laboratory (Stevens and Feeney, 1963) for the modification of proteins. The inhibitors modified were penguin, turkey, cassowary, and duck ovomucoids. The loss of amino groups during the reaction was followed by treating the carbamylated proteins with TNBS as described above.

Kinetic Analysis. The curves for the time course of the experiments were analyzed kinetically by the method developed by Koshland *et al.* (1958) and Ray *et al.* (1960). By this method it is possible to differentiate two individual rate constants from a curve including both rates.[8]

Results

Quantitation. Absorbancy of the final solution, read at 344 mμ, was found to be linear with respect to the amount of protein in the reaction mixture to at least 2 mg. The molar extinction coefficient of one trinitrophenylamino group in a protein was found to be $1.09 \pm 0.01 \times 10^4$ in acidic solution, using lysozyme (mol wt 14,300, seven free amino groups) as a standard. This molar extinction coefficient correlates well with the value of 1.09 and 1.1×10^4 given by Okuyama and Satake (1960) for synthetic peptides. The number of free amino groups determined for several proteolytic enzyme inhibitors is given in Table I.

TABLE I: Comparison of Number of Amino Groups Found in Inhibitors by Amino Acid Analysis and by Reaction with Trinitrobenzenesulfonic Acid.

Inhibitor	Number of Amino Groups Found by[a]	
	Amino Acid Analysis[b]	TNBS[c]
Chicken ovomucoid	12 or 13	14
Turkey ovomucoid	12	12
Tinamou ovomucoid	16	16
Cassowary ovomucoid	16	16
Penguin ovomucoid	13	13
Duck ovomucoid	18	15
Lima bean inhibitor	5.0	3.1
Bovine colostrum inhibitor	—	3.1

[a] Figures rounded to two places. [b] Lysine plus N terminal. [c] Method is described in text.

TNBS Modification. Modification of free amino groups previously has been shown to cause a loss in the trypsin-inhibitory activity of three trypsin inhibitors, *i.e.*, turkey ovomucoid (Stevens and Feeney, 1963), pancreatic trypsin inhibitor (Kassel and Laskowski, 1965), and lima bean inhibitor (Fraenkel-Conrat *et al.*, 1952). In the present study, the amino groups of several other inhibitors of trypsin and chymotrypsin also were modified, and the loss of their inhibitory

[8] Although the amino groups in a protein probably all have different reactivities, it was assumed that the groups in any one class were similar enough to one another to permit analyses of the curve.

TABLE II: Effects of Modifications of Amino Groups with Trinitrobenzenesulfonic Acid on the Inhibitory Activities of Proteolytic Enzyme Inhibitors.

Types of Inhibitors	Loss of[a] Trypsin-Inhibitory Act.	Loss of[a] Chymotrypsin-Inhibitory Act.
Trypsin and chymotrypsin		
Turkey ovomucoid	+	−
Penguin ovomucoid	+	−
Duck ovomucoid	+	−
Trypsin		
Cassowary ovomucoid	+	*c*
Lima bean inhibitor	+	*c*
Colostrum inhibitor	+	*c*
Chicken ovomucoid	−[b]	*c*
Soybean inhibitor	−	*c*
Chymotrypsin		
Golden pheasant ovomucoid	*c*	−
Tinamou ovomucoid	*c*	−

[a] +, complete loss of inhibitory activity; −, no loss of inhibitory activity. [b] Essentially no loss of activity at degrees of modification where other inhibitors are almost completely inactivated; 50% loss of inhibitory activity at essentially 100% modification of amino groups. [c] These inhibitors do not inhibit the indicated enzymes.

activity followed (Table II). The time courses of modification and inactivation of turkey ovomucoid are shown in Figure 1. Similar curves for the loss of trypsin-inhibitory activity and modification were found for those trypsin inhibitors inactivated by modification of amino groups. Chicken ovomucoid and soybean inhibitor showed little loss of inhibitory activity even at high levels of modification of amino groups.

Deamination. The results of deamination of chicken and turkey ovomucoids with nitrous acid are shown in Table III. It is evident that there was a direct relationship between the losses of the trypsin inhibitory activity and amino groups of turkey ovomucoid. There was no significant loss of either the chymotrypsin inhibitory activity of turkey ovomucoid or the trypsin inhibitory activity of chicken ovomucoid upon deamination.

TABLE III: Effects of Deamination of Turkey and Chicken Ovomucoids with Nitrous Acid.[a]

Protein	Reaction Time (min)	Loss of (%) Amino Groups	Loss of (%) Trypsin-Inhibitory Act.
Turkey ovomucoid	30	18.2	10.8
	180	25.6	27.9
	480	47.0	48.3
Chicken ovomucoid	30	12.2	4.6
	180	13.8	7.2
	480	33.6	4.2

[a] Treatments with nitrous acid were done according to Maurer and Heidelberger (1951).

Carbamylation. In several experiments with car-

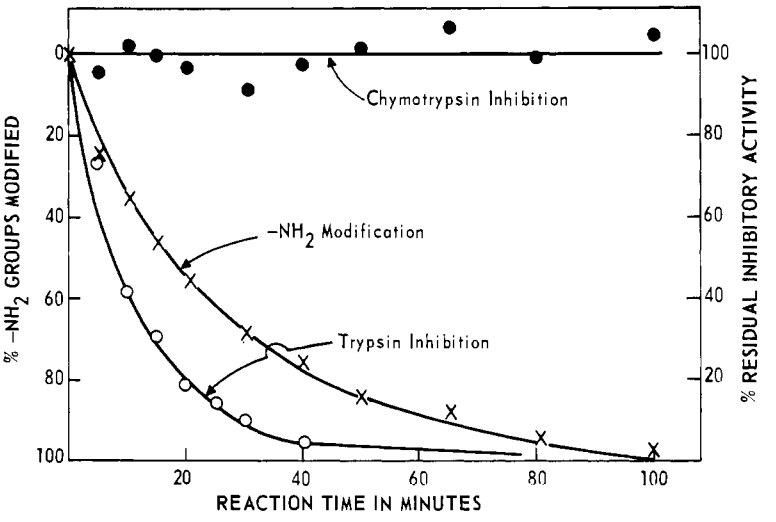

FIGURE 1: Time course of amino group modification with trinitrobenzenesulfonic acid and loss of inhibitory activity in turkey ovomucoid.

bamylation, it was found that the amino groups of four ovomucoids could be modified at about the same rate with cyanate. Penguin, turkey, and cassowary ovomucoids lost their trypsin inhibitory activity at a rate much faster than the loss of total amino groups. However, in duck ovomucoid the loss of trypsin inhibitory activity followed the loss of total amino groups very closely.

Kinetic Analysis of TNBS Reactions. The data obtained for turkey ovomucoid, plotted by the method used by Koshland *et al.* (1958), are given in Figure 2. Since the reaction was found to be pseudo first order with respect to amino groups, the nonlinearity of the plot for loss of total amino groups indicates that not all the amino groups are equally reactive. It was assumed for this work that there are two classes of amino groups, designated "fast" and "slow" reacting. The line for the loss of the "fast"-reacting amino groups was obtained by subtracting the extrapolated line for the "slow"-reacting class from the curve for loss of total amino groups. When the loss of trypsin-inhibitory activity was plotted on the same graph, a straight line was obtained. The data for bovine colostrum inhibitor, plotted by the Koshland method, are shown in Figure 3. The first-order rate constants for loss of the "fast" amino groups and for loss of trypsin-inhibitory activity calculated from such plots are given in Table IV. The first-order rate constants for the losses of "slow" amino groups were 15–25% the rates for "fast" amino groups.

TABLE IV: First-Order Rate Constants for Treatment of Inhibitors with Trinitrobenzenesulfonic Acid.

Inhibitor	First-Order Rate Constant (k min^{-1}) Modification of "Fast" Amino Groups	Loss of Trypsin-Inhibitory Act.
Turkey ovomucoid	0.087	0.081
Penguin ovomucoid	0.094	0.092
Cassowary ovomucoid	0.043	0.059
Colostrum inhibitor	0.058	0.122
Duck ovomucoid	0.089	0.051
Lima bean inhibitor	*a*	0.092
Chicken ovomucoid	0.041	<0.006
Tinamou ovomucoid	0.042	*b*

[a] Difficulties encountered in distinguishing between rates of three differently reacting amino groups. [b] Tinamou ovomucoid inhibits chymotrypsin, not trypsin. No loss in activity against chymotrypsin was observed.

Discussion

TNBS has been shown by Habeeb (1966) to be a valuable reagent for determining the number of free amino groups in proteins. Our work reaffirms his findings. The numbers of amino groups found by the TNBS method agree quite well with the numbers obtained by amino acid analysis (Table I). However, some discrepancies are evident. The difference of one or two amino groups in chicken ovomucoid cannot be explained at the present time. The proteins for which the values are lower by the TNBS method, *i.e.*, duck ovomucoid and lima bean inhibitor, could have amino groups which are "buried" and thus inaccessible to the reagent, as has been shown for native bovine serum albumin with TNBS (Harris and Becker, 1965). Also, they might have amino groups which react very slowly and which, even after 2 hr, are not all modified by TNBS.

Although modification of the amino groups with TNBS caused a loss in inhibitory activity, the possibility still exists that this loss might have been due to modification of groups other than amino groups or to steric, electrostatic, or conformational effects. However, stable derivatives with groups other than amino or sulfhydryl groups under these conditions have not been previously reported (Okuyama and Satake, 1960; Satake *et al.*, 1960). No sulfhydryl groups have been found in these inhibitors. In addition, it has been shown previously (Simlot and Feeney, 1966) that amidination which results in no perceptible change in charge upon modification also inactivates trypsin inhibitors susceptible to modification of amino groups. The results obtained by deamination with nitrous acid confirm that a positively charged free amino group must be present in turkey ovomucoid in order for inhibition to occur. The retention of complete chymotrypsin-inhibitory activity of turkey ovomucoid after modification of all the amino groups indicates that major conformational changes, while not being impossible, are unlikely.[4]

The very rapid loss of trypsin-inhibitory activity by several inhibitors modified with TNBS indicated that one or more amino groups might be important for this activity. An attempt was made, therefore, to estimate the number of amino groups involved in the inhibition. Three models were considered: (a) one amino group at the active site, modification of which causes inactivation; (b) two amino groups at the active site, modification of either causing inactivation; and (c) two amino groups at the active site, modification of both required for inactivation. Although other models are possible, those selected were thought to be most

[4] In the case of turkey ovomucoid, modification with reagents which make the protein more acidic, such as acetylation or iodination, actually change the inhibitor into a better inhibitor for chymotrypsin by increasing the rate of reaction with chymotrypsin (Simlot and Feeney, 1966). On the other hand, requirement of lysines (or arginines) for trypsin inhibitors is further evident by the fact that modification of amino groups of chymotrypsin inhibitors has not been found to cause inactivation (Stevens and Feeney, 1963; Simlot and Feeney, 1966). Chymotrypsin does not ordinarily hydrolyze peptide bonds involving carboxyl groups of lysine and arginine.

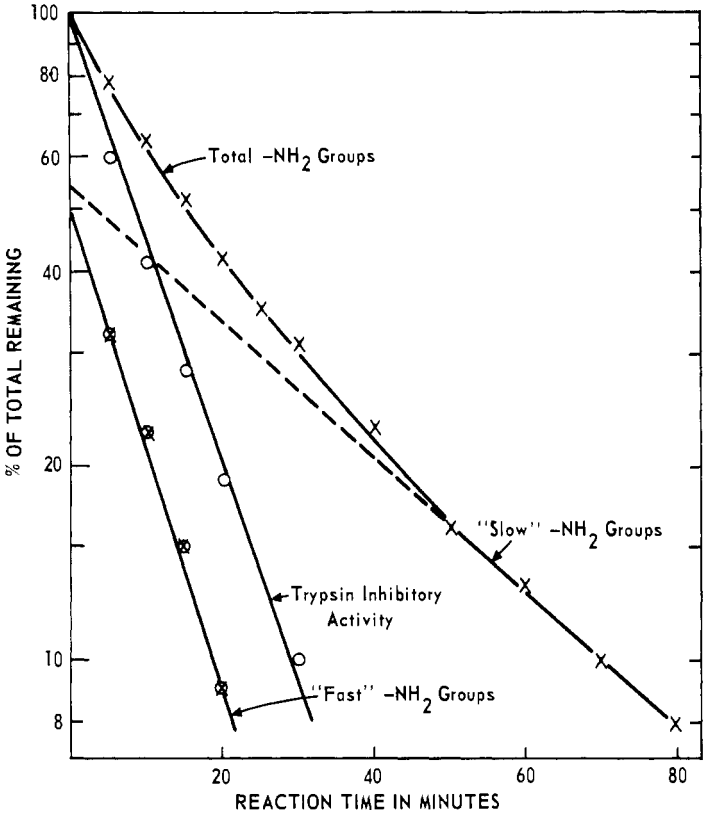

FIGURE 2: Semilogarithmic plot for loss of amino groups and loss of trypsin-inhibitory activity in turkey ovomucoid by modification with trinitrobenzenesulfonic acid.

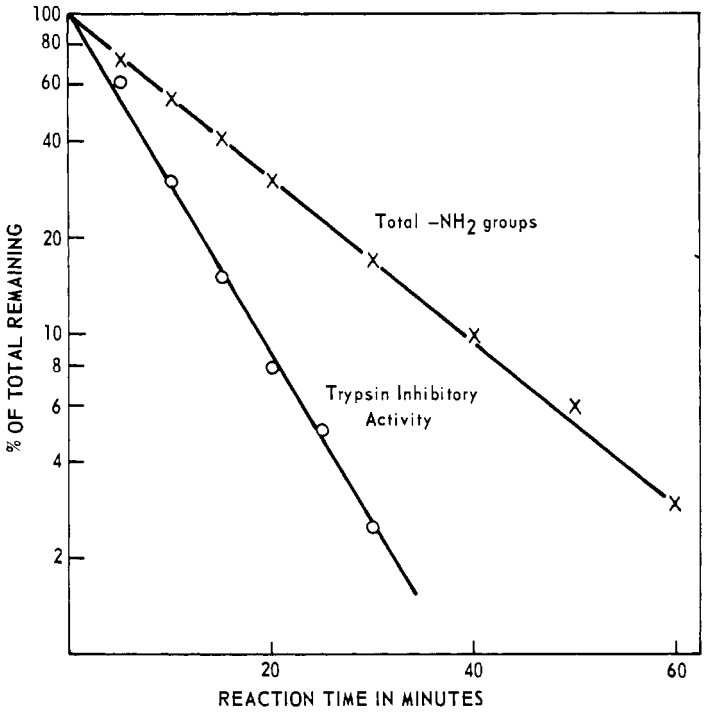

FIGURE 3: Semilogarithmic plot for loss of amino groups and loss of trypsin-inhibitory activity in bovine colostrum inhibitor by modification with trinitrobenzenesulfonic acid.

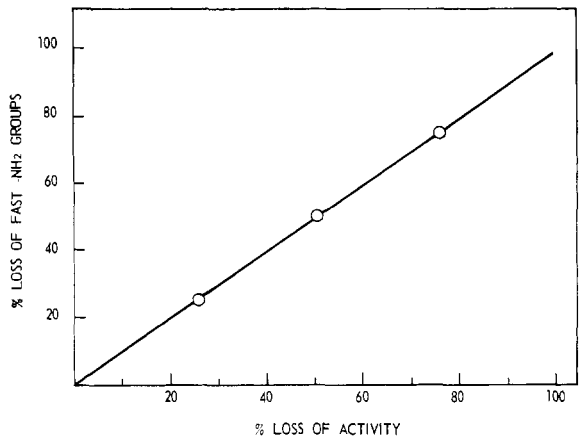

FIGURE 4: Linear dependence of trypsin-inhibitory activity of turkey ovomucoid on "fast"-reacting amino groups. These data are replotted from the data of Figure 2.

probable. The rates of inactivation for models a and b would be equal and double the rate of modification, respectively. In the case of model c the inactivation would be expected to yield a sigmoidal time-dependence curve. The rate of inactivation is approximately equal to the rate of loss for the "fast" amino groups in turkey and penguin ovomucoids (Table IV). This direct correspondence (Figure 4) fits model a and indicates that one particular amino group of the "fast"-reacting class is essential for inhibitory activity. The rates for cassowary ovomucoid indicated one, or possibly two, essential amino groups. In the case of bovine colostrum inhibitor, the rate constant for inactivation is approximately double that for modification of the three amino groups, which all react at the same rate (Figure 3). This result fits model b and implicates two amino groups at the active site. In line with the results obtained with the other inhibitors, it would appear that one of these two groups might be essential for inhibitory activity, while modification of the other group might cause some conformational change or steric interference leading indirectly to loss of activity.

The results with duck ovomucoid and lima bean inhibitor appear to be more complicated. Duck ovomucoid has been shown to inhibit two molecules of trypsin simultaneously (Rhodes *et al.*, 1960). The semilogarithmic plot of the results for duck ovomucoid shows a linear plot for loss of the trypsin-inhibitory activity. This indicates that the two sites are both being modified at the same rate. This rate, however, does not correspond to either the "fast"- or "slow"-reacting class of amino groups but is intermediary. In this case, the assumption of two distinct classes of reactive amino groups does not appear to be valid. That the required amino groups in duck ovomucoid are not as reactive as those in the other ovomucoids is supported by the results of carbamylation. The loss of trypsin-inhibitory activity caused by carbamylation of duck ovomucoid is much slower than that of the other ovomucoids. The semilogarithmic plot of the results for TNBS modification of lima bean inhibitor gave an almost continuous curve for the loss of total amino groups. A kinetic analysis of the curve, therefore, could not be carried out, since each of the three amino groups was apparently reacting at a distinctly different rate. However, it seems probable that the amino group required for activity is the one modified at the fastest rate. All the trypsin inhibitors in this study, with the exceptions of soybean trypsin inhibitor and chicken ovomucoid, have been shown to require amino groups for activity. Chicken ovomucoid, however, loses its trypsin-inhibitory activity upon modification of the arginine residues (Feinstein, 1966). Experiments are now under way to repeat this modification of arginine residues with soybean trypsin inhibitor.

The results of studies in this laboratory as well as others (Fraenkel-Conrat *et al.*, 1952; Finkenstadt and Laskowski, 1965; Ozawa and Laskowski, 1966) can be considered as showing that either lysines or arginines are required for the activities of protein trypsin inhibitors. The kinetic analyses in the present study indicate that either one or two of the "fast"-reacting amino groups, depending on the inhibitor, are essential for activity in four of the trypsin inhibitors studied. The possibility of identifying these particular residues is now being explored in our laboratory. Since catalytically inactive trypsin also forms competitive complexes with several of these inhibitors (Feinstein and Feeney, 1966), these particular residues must combine with the enzyme through mechanisms not requiring hydrolysis of their peptide bonds.

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Fluorescence Studies with Tryptophyl Peptides*

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ABSTRACT: The influence of amino and phenolic ionization on the fluorescence intensity of several series of tryptophan peptides has been evaluated. The effect of amino ionization falls off rather gradually in the series $(Gly)_{0-3}$-Trp. In the series, $NH_2(CH_2)_{2-8}CO$-Trp, fluorescence is quenched in the smaller molecules and augmented in the larger molecules with protonation of the amino group. Cyclization has been suggested to occur in the larger molecules in order to account for the reversal.

Quenching was observed in the series Trp-$(Gly)_{0-4}$-Tyr with ionization of the phenolic group. The efficiency of quenching decreases with increasing molecular size. Quenching is attributed to radiationless energy transfer between the tryptophan and ionized tyrosine residues.

Since the pioneering studies of Weber and his coworkers on the ultraviolet fluorescence of the aromatic amino acids (Weber, 1961; Teale and Weber, 1957; White, 1959), there has been evidence from his and other laboratories (Teale, 1960; Brand *et al.*, 1962; Steiner and Edelhoch, 1961, 1962, 1963a,b; Gally and Edelman 1964), that denaturation of proteins is usually accompanied by changes in the fluorescence parameters of tryptophan and/or tyrosine.

The indications are that a variety of functional groups in proteins may modify quantum yields of indole or phenol fluorescence. Of particular interest are the effects of amines, carboxyl groups (White, 1959; Cowgill, 1964; Weber, 1961), and the phenolate ion (Edelhoch *et al.*, 1963; Cowgill, 1963b).

In this report the influence of these functional groups on the fluorescence of indole in a number of simple model compounds will be described. It is hoped that these and future observations will make it possible to provide more definitive interpretations of the fluorescence changes observed when proteins undergo conformational or allosteric changes.

Methods

Computations

Apparent Equilibrium Constants of Phenolic Ionization. The apparent equilibrium constant (K_a) of the dissociation of the phenolic group in the L-tryptophyl-L-tyrosine series of compounds was obtained from a spectrophotometric titration curve. The data were plotted according to the law of mass action in the

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