

## *p*-Nitrophenyl Carbamates as Active-Site-Specific Reagents for Serine Proteases†

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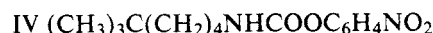
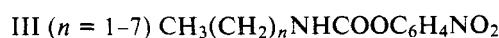
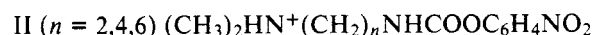
**ABSTRACT:** A series of *p*-nitrophenyl *N*-R-carbamates has been prepared, in which R represents either (I) aminoalkyl, (II) dimethylaminoalkyl, or (III) alkyl groups of different chain length. The 13 reagents have been tested as active-site-specific reagents for trypsin, chymotrypsin, and elastase, and the observed inactivation of the three enzymes is consistent with their established substrate specificity. Thus, trypsin was specifically inactivated by the positively charged reagents (I), where R is aminopentyl or aminoheptyl, while chymotrypsin and elastase were inactivated by the uncharged reagents (III), chymotrypsin when R is hexyl or butyl, and elastase when R is butyl or propyl. In addition to the specific inactivation predicted from the established properties of the three proteases, some unexpected affinity labeling patterns were observed. The positively charged reagents (I and II) where R is either aminoheptyl or dimethylaminohexyl were found to be quite ef-

fective inactivating agents for chymotrypsin, and, since the inactivation was enhanced rather than impaired by the presence of indole, a compound which is known to bind to the primary substrate-binding site, this finding is taken as new evidence for the secondary binding site specific for positively charged side chains in chymotrypsin. Similarly, trypsin was found to be inactivated by the reagents in group III, regardless of the length of branching of the alkyl chain in the R group. Since alkyl isocyanates with similar alkyl groups have no effect on trypsin, this finding was taken as independent evidence for a *p*-nitrophenyl-binding site in trypsin. A similar, specific inactivation by the *p*-nitrophenyl *N*-alkylcarbamates (III), but not by alkyl isocyanates, was also observed with chymotrypsinogen, providing additional support for the proposed existence of a *p*-nitrophenyl-binding site in this zymogen.

Based on the results of extensive kinetic studies and x-ray diffraction analyses, the three serine proteases trypsin, chymotrypsin, and elastase have been well characterized, and a convincing model correlating the three-dimensional structure to the mechanism of catalytic activity has been derived for the three enzymes. The catalytic apparatus made up of the uniquely nucleophilic serine residue, activated through the charge relay Ser-His-Asp, is common to all three enzymes, accounting for the similar mechanism of peptide (or ester) bond hydrolysis catalyzed by the enzymes. The unique specificity of each enzyme can be explained in terms of the structural makeup of the substrate-binding site juxtaposed to the active serine residue (Sigler et al., 1968; Watson et al., 1970; Shotton and Watson, 1970; Stroud et al., 1972). In chymotrypsin, the three-dimensional density map reveals an open, hydrophobic cavity of the proper dimensions to accommodate the aromatic or long-chain aliphatic residues (Tyr, Phe, Leu, Ile); in elastase, a homologous cavity is made shallow and more restricted by certain amino acid replacements, accounting for the specificity of elastase for short-chain amino acids (Ala); in trypsin, the binding cavity has a more hydrophilic interior and contains a carboxylate anion which presumably provides electrostatic affinity for positively charged side chains (Lys and Arg) which are the specificity determinants for that enzyme. The predictive value of this general model of both the common and the unique properties characterizing the three serine proteases has been substantiated by experimental tests of the solution properties of the three enzymes: One such test was the prediction that a series of reagents (R-X) containing a single electrophilic functional group (X), but differing in the

nature of the R group, should provide active-site-specific reagents for the three proteases in a predictable manner. Thus, it was predicted that short-chain alkyl isocyanates should react preferentially with elastase, long-chain alkyl isocyanates specifically with chymotrypsin, and long-chain aminoalkyl isocyanates with trypsin. Because of the difficulties in preparing the latter reagent, only the first two could be tested and were found to behave according to prediction (Brown and Wold, 1973a,b).

In this paper, we report a complete test of the binding-pocket model through the comparison of the affinity labeling pattern of the three proteases using a different family of reagents, namely, *p*-nitrophenyl *N*-alkylcarbamates. The reactive group, the *p*-nitrophenyl carbamate, has been shown to hydrolyze via an isocyanate intermediate (Bender and Homer, 1965) and the use of *N*-alkylnitrophenyl carbamates as enzyme active-site reagents has been suggested by Gross et al. (1975) in their studies of transglutaminase. The following families of compounds were prepared



and according to the model members of I and II should specifically inhibit trypsin, short-chain members of III elastase, and long-chain members of III and IV chymotrypsin.

### Experimental Section

**Materials and Assay Procedures.** Trypsin (Miles-Seravac, Batch 482, two times crystallized) and  $\alpha$ -chymotrypsin (Pentex, Lot 43, two times crystallized) were assayed according to the procedure of Hummel (1959) using *p*-tosyl-L-arginine

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methyl ester hydrochloride (Sigma) and benzoyl-L-tyrosine ethyl ester (Sigma) as substrates, respectively. Elastase (Sigma, Lot 123C-8230) was assayed according to Schneider et al. (1962) by quantitating the amount of amino acids and polypeptides liberated from elastin by incubation with the enzyme. Chymotrypsinogen was purchased from Worthington Biochemical Corp. (Lot CGC 8HA).

**Titration of Enzymes with Reagents.** To a  $10^{-4}$  M solution of the desired enzyme in 0.1 M Tris-HCl, pH 7.0, was added an aliquot of a stock solution of reagent ( $\sim 5 \times 10^{-3}$  M, compounds III and IV in acetone and compounds I and II in  $10^{-3}$  M HCl). After stirring for 15 min, an aliquot of the reaction mixture was removed and assayed for enzyme activity. A second aliquot of the reagent stock solution was added. Again, after 15 min, an aliquot was removed from the reaction mixture and assayed. The above procedure was repeated until the desired end point was obtained. Compounds used as active-site protective agents were mixed in the buffer solution and preincubated with the enzyme for at least 15 min before the titration was begun.

**Reaction of Chymotrypsinogen.** Stock solutions of *n*-octyl isocyanate and compounds II and III were prepared and the concentrations of the reagents were determined (see Brown and Wold (1973) and below). An aliquot of a reagent stock solution was added to a 1 mL of solution of 0.1 M Tris-HCl, pH 7.0, containing 2.5 mg of chymotrypsinogen A and the reaction was allowed to proceed for 24 h. Chymotrypsinogen solutions with the appropriate amounts of acetone or  $10^{-3}$  M HCl were used as controls. The zymogen was separated from reagent and side products by gel filtration in a Sephadex G-25 column ( $10 \times 1$  cm), and the resulting protein peaks were lyophilized. The desalted zymogen was carefully weighed and dissolved in 0.1 M Tris-HCl, pH 7.0, to yield a final concentration of 2.5 mg/mL of chymotrypsinogen. To this solution was added an aliquot of a  $10^{-3}$  M trypsin solution in  $10^{-3}$  M HCl so that the final chymotrypsinogen/trypsin ratio was 100/1. The solution was allowed to incubate for 2 h and was then assayed for chymotrypsin activity.

**Preparation of Reagents.** All reagents were synthesized by the condensation of *p*-nitrophenyl chloroformate (Aldrich Chemical Co.) and the corresponding amine. The general procedures used in the preparation of compounds I, II, III, and IV are outlined below.

**Compound I.** To 1 mmol of the *n*-alkyldiamine dissolved and stirring in anhydrous benzene at  $10^\circ\text{C}$  was added quickly 0.95 mmol of *p*-nitrophenyl chloroformate dissolved in anhydrous benzene. The reaction was allowed to proceed for 5 min, during which a precipitate formed. The precipitate was collected by filtration and washed three times with anhydrous ethyl ether. The resulting compound I was contaminated with either or both the free or protonated diamine and polymers, as evidenced by paper chromatography and high-voltage paper electrophoresis. Prior to use, the contaminated compound I was dissolved in  $10^{-3}$  M HCl and the polymeric, insoluble material was filtered off. The acid solution was washed two times with ethyl ether to extract any residual *p*-nitrophenol. Paper chromatography and high-voltage paper electrophoresis of this acid solution showed compound I to be contaminated only by small amounts of the starting diamine. The yield of compound I was on the order of 5 to 15%.

**Compound II.** To 1 mmol of the *N,N*-dimethyl-*n*-alkyldiamine dissolved and stirring in anhydrous benzene at room temperature was added dropwise 1.2 mmol of *p*-nitrophenyl chloroformate dissolved in anhydrous benzene. The reaction mixture was stirred for 3 h and the resulting precipitate was

collected by filtration. The solid was washed three times with anhydrous ethyl ether and then dried under vacuum overnight. The colorless product, compound II, was free of both *p*-nitrophenol and diamine, as evidenced by paper chromatography and high-voltage paper electrophoresis. The yields ranged from 85 to 95%. Compound II was stored in a desiccator under vacuum.

**Compounds III and IV.** To 1 mmol of the *n*-alkylamine dissolved and stirring in anhydrous benzene was added dropwise 1.2 mmol of *p*-nitrophenyl chloroformate dissolved in anhydrous benzene. The reaction mixture was left stirring overnight and was taken to dryness on a rotary evaporator. The resulting solid was dissolved in ethyl ether and filtered; the ether solution was washed two times with 0.1 M HCl, two times with 5% sodium bicarbonate solution, two times with 0.1 M HCl, and dried over anhydrous magnesium sulfate for 30 min. The ether solution was finally filtered and taken to dryness. The resulting colorless solid, III, was free of *p*-nitrophenol and unreacted *n*-alkylamine, as evidenced by paper chromatography and high-voltage paper electrophoresis. Yields varied from 60 to 95%, depending on the *n*-alkylamine used. In general, as the length of the alkyl chain increased, the yields increased. In addition, the longer-chain derivatives were more stable to hydrolysis and could be stored under vacuum for longer periods of time than their shorter-chain analogues.

**Determination of Reagent Concentrations.** Stock solutions of compound III and IV in acetone and compounds I and II in  $10^{-3}$  M HCl were prepared so that the reagent concentrations were in the order of  $5 \times 10^{-3}$  M. The solutions of I and II were washed with ethyl ether immediately prior to use. Aliquots of the stock solutions were hydrolyzed in 0.1 M NaOH and the absorption at 400 nm was recorded. The absorbance at 400 nm of the *p*-nitrophenylate ion was determined to be  $18\,350 \pm 50$ , in excellent agreement with the value established by Kezdy and Bender (1962). The exact concentrations of the reagent stock solutions could thus be determined from the release of *p*-nitrophenol.

**Reagent Half-Life Determinations.** An aliquot of reagent stock solution was added to 3 mL of a buffer at the desired pH, ionic strength, and in some instances protein content. The increase in absorbance was monitored at 400 nm on a Cary 15 spectrophotometer, and the half-life was read directly from a plot of  $(A_\infty - A)$  against time.

## Results

The effect of the different *p*-nitrophenyl carbamate derivatives in inactivating the three proteases is summarized in Table I. In the previous studies,  $10^{-4}$  M chymotrypsin was 50% inactivated at a molar ratio of octyl isocyanate/enzyme of 0.6 and  $10^{-4}$  M elastase at a molar ratio of butyl isocyanate/enzyme of 0.8–0.9 (Brown and Wold, 1973a). Based on these observations and for the sake of easy discussion, we arbitrarily set the criteria for primary-site affinity labeling of the proteases as 50% inactivation at a reagent/enzyme ratio equal to or less than one. Using this arbitrary scale, we conclude that the series of compounds tested include active-site-specific reagents for each of the three enzymes. Thus, the aminoalkyl derivatives with five and seven methylene groups specifically inactivate trypsin, the alkyl derivative with three methylene groups (four carbons) inactivates elastase and chymotrypsin, and the alkyl derivative with five methylene groups (six carbons) inactivates chymotrypsin. This pattern of specificity is clearly consistent with the current model of the relative binding-site topography of the three proteases, and confirms the basic hypothesis that these groups of compounds should provide such active-site-

TABLE I: Effect of N-Substituted Nitrophenyl Carbamates as Active-Site-Specific Reagents of Serine Proteases.<sup>a</sup>

Reagent	Designation	$t_{1/2}^c$ (min)	Reagent/enzyme ratio required for 50% inact. <sup>b</sup>		
			Chymotrypsin	Trypsin	Elastase
I $\text{H}_3\text{N}^+(\text{CH}_2)_n\text{NHCOOC}_6\text{H}_4\text{NO}_2$	$n = 2$ I-2	3	<100*	<100*	
	$n = 5$ I-5	11	11	0.65	
	$n = 7$ I-7	17.5	3	0.55	
II $(\text{CH}_3)_2\text{N}^+\text{H}(\text{CH}_2)_n\text{NHCOOC}_6\text{H}_4\text{NO}_2$	$n = 2$ II-2	1.5(0.12)	<100*	<100*	85*
	$n = 4$ II-4	9.7(0.5)	22	25	<100*
	$n = 6$ II-6	15.5(0.8)	6	20	<100*
III $\text{CH}_3(\text{CH}_2)_n\text{NHCOOC}_6\text{H}_4\text{NO}_2$	$n = 1$ III-1				6
	$n = 2$ III-2				1.3
	$n = 3$ III-3	23.5	0.95	3.4	0.9
	$n = 4$ III-4				4.0
	$n = 5$ III-5	41.2	0.85	3.2	7.4
	$n = 7$ III-7	46.3(7.5)	4.5	2.1	
IV $(\text{CH}_3)_3\text{C}(\text{CH}_2)_4\text{NHCOOC}_6\text{H}_4\text{NO}_2$ Octyl isocyanate <sup>d</sup>	IV	1	16*	2.1	
			0.6	<100	<100

<sup>a</sup> All studies were carried out with  $10^{-4}$  M enzyme in 0.1 M Tris buffer, pH 7.0, containing 0.01 M  $\text{CaCl}_2$ . The enzyme was incubated with the reagent for 15 min before the activity was determined. <sup>b</sup> The 50% inactivation values were obtained from titration curves of activity remaining against increasing reagent concentration. Each titration curve contained 3–5 data points, and in most instances the reagent concentration range spanned the 50% inactivation. Those values obtained by extrapolation, rather than interpolation, are marked with an asterisk. <sup>c</sup> The reagent half-life was determined under the same conditions as those used in the inactivation experiments. (The values in parentheses are half-times at pH 8.) <sup>d</sup> Data from Brown and Wold (1973a).

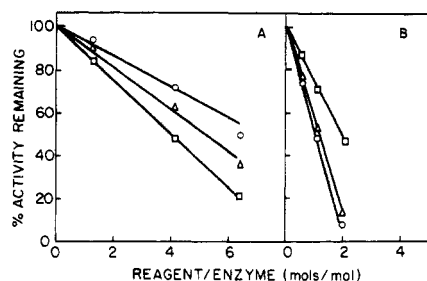


FIGURE 1: The effect of indole on the inactivation of  $\alpha$ -chymotrypsin by reagents II-6 (A) and III-5 (B). The indole concentrations were 0 (○);  $10^{-3}$  M (Δ);  $10^{-2}$  M (□); and the standard set of reaction conditions ( $10^{-4}$  M enzyme in 0.1 M Tris buffer, pH 7.0, containing 0.01 M  $\text{CaCl}_2$ ) were used throughout.

taining the same functional group, give a wide spectrum of inactivation efficiencies with the three enzymes validates the conclusion regarding the specificity of given reagents for a given enzyme. The elastase inactivation merits special consideration. From x-ray crystallography and substrate specificity data, it has been concluded that short-chain aliphatic side chains give optimal fit in the active site of elastase. The data in Table I unequivocally establish that, based on the criterion of inactivation, the optimal chain length is four carbons. With shorter as well as longer chains, the reagent efficiency decreases.

The stability of the different reagents is also reported in Table I. If the rate of the nonproductive competing reactions (primarily hydrolysis) should become significantly greater than that of the reaction with the protein, the observed relative inactivation efficiency of a family of compounds could merely reflect differences in effective concentration rather than specificity of interaction. Since the half-lives of all the compounds used are longer than the 1–2-min half-lives observed for the alkyl isocyanates under the same conditions (Brown and Wold, 1973a) and since the inactivation process was found to be very fast compared to the hydrolysis, we conclude that the

specific reagents. The fact that the 13 compounds, all con-effect of reagent stability at most should require a minor correction in the reagent to enzyme ratios for compounds I-2 and II-2.

The data in Table I contain additional information about the binding of the reagents to the active-site region of the three proteases. Two aspects appeared to warrant further studies, the relatively efficient inactivation of chymotrypsin by the reagents containing the charged aminoalkyl or dimethylaminoalkyl groups (I and II), and the inactivation of trypsin by the reagents containing the alkyl groups (III and IV).

The poor inactivation of trypsin with the dimethylaminoalkyl derivatives, II-4 and II-6, was not unexpected in view of the fact that *N*<sup>ε</sup>-methyllysine methyl ester is well known to be a poorer substrate for trypsin than is lysine methyl ester (Bensiton and Deneault, 1966; Gorecki and Shalitin, 1967). The finding that compounds I-7 and II-6 were such effective inhibitors of chymotrypsin had not been expected, however. Even a relatively low-affinity interaction of the positively charged side chains of these reagents with the putative hydrophobic binding pocket of chymotrypsin would be in conflict with both the general model of the protease binding sites and the known specificity of chymotrypsin. In order to explore this phenomenon further, we tested the effect of indole, a compound known to occupy the regular binding pocket of chymotrypsin (Steitz et al., 1969), on the inactivation of the enzyme by compounds II-6 and III-5. The results are given in Figure 1. Whereas indole competed with compound III-5 for the binding pocket, in the case of compound II-6 it actually enhanced the inactivation. The only reasonable explanation of this observation is that the positively charged reagents inactivate chymotrypsin through an interaction of the side chain with a secondary binding site, and that either the affinity for this site or the reactivity of the active-site serine residue is enhanced when the primary binding pocket is occupied. This explanation appears to be in agreement with the recent report that bis-quaternary amine compounds act as allosteric activators of chymotrypsin action on regular aromatic substrates (Erlanger

TABLE II: The Effect of N-Substituted Nitrophenyl Carbamates in Inactivating Chymotrypsinogen A.<sup>a</sup>

Reagent	mol of reagent mol of zymogen	Act. Recovered after Trypsin Activation (%)
None		100
II-6	30	100
III-1	5	30
III-2	5	46
III-3	5	32
III-5	5	38
III-7	5	27
IV	5	30
<i>n</i> -Octyl isocyanate	5	98

<sup>a</sup> Standard reaction conditions ( $10^{-4}$  M zymogen in 0.1 M Tris buffer, pH 7, 0.01 M  $\text{CaCl}_2$ ) were used. After 24-h incubation, the zymogen was activated with trypsin (see text) and activity was determined.

et al., 1976). In the studies by Erlanger et al. (1976) it was shown that the effect of the effectors was on  $k_{\text{cat}}$  rather than on  $K_m$ .

The inactivation of trypsin by all the members of group III and IV reagents appears to reflect binding of the *p*-nitrophenyl group rather than the alkyl side chain. This conclusion is based on the observations that octyl isocyanate does not inactivate trypsin and that III-3, III-5, III-7, and IV all are essentially equally effective inactivating agents in spite of their different alkyl groups (Table I). Again, the existence of such a secondary aromatic binding site is consistent with observations in the literature (Trowbridge et al., 1963; Howard and Mehl, 1965; Heidberg et al. 1967; Sanborn and Hein, 1968; Hartmann and Holler, 1970). Furthermore, the evidence in the literature also suggests that such a binding site may be present in both chymotrypsin and chymotrypsinogen (Gertler et al., 1974) and in trypsinogen (Robinson et al., 1973). In order to assess this latter possibility, we treated chymotrypsinogen with several of the carbamates and determined the activity after activation with trypsin. The results are given in Table II. Consistent with previous findings (Brown and Wold, 1973a), octyl isocyanate had no effect on the zymogen, nor did the positively charged derivative II-6. All the alkyl carbamates, however, apparently reacted with the zymogen to give a partially inactivated chymotrypsin after activation with trypsin. As was the case for the inactivation of trypsin with the group III and IV reagents, the degree of inactivation appears to be independent of the nature of the alkyl group, and we therefore conclude that the affinity determinant in all cases is the common *p*-nitrophenyl group.

In an attempt to establish the nature of the binding sites involved in the binding of aminoalkyl (I-7) and alkyl (III-7) groups to trypsin, guanidinium chloride (up to 0.1 M) was added to the reaction mixture as a competitive inhibitor, assumed to interact specifically with the primary binding site. Only a very minor degree of competition was observed, but it is interesting to note that it was the same for both reagents. Gorecki and Shalitin (1967) have shown that cationic compounds will act as competitive inhibitors of the action of trypsin on both neutral and positively charged substrates. The relatively small effect in this case probably reflects the contribution of the secondary binding of the *p*-nitrophenyl group. It should be pointed out that neither of the competitive inhibitors tested in this work, indole and guanidinium chloride, had any effect on the half-life of the reagents involved.

## Discussion

Two aspects of the reported results should be significant; first, the easy preparation of a whole family of *p*-nitrophenyl carbamates and their suitability as electrophilic protein reagents, and, second, the conclusions that can be drawn about the active-site topology of the three proteases.

Gross et al. (1975) have suggested the use of *p*-nitrophenyl carbamates as readily prepared and stored precursors of the corresponding isocyanates, and they prepared potential active-site-directed inactivators of transglutaminase by the method of Bender and Homer (1965). The same procedure was used in this work to prepare a large number of homologous alkylnitrophenyl carbamates. The preparation of the three aminoalkyl derivatives (I) is clearly not as direct as one might wish, but the procedure represents the optimal one in our hands for producing the set of unstable and reactive products. The yield, as expected, was very poor but, since the starting materials are readily available, sufficient amounts of the desired products can be prepared. The preparation of the dimethylaminoalkyl derivatives (II) and the alkyl derivatives (III, IV) presents no difficulties, and these reagents were prepared in good yields. The results in Table I illustrate one important feature in the general strategy of affinity labeling, namely, the availability of a relatively large number of homologous reagents with the same functional group and differing only in the affinity-determining group. This enables one to compare reactivity directly and each individual reagent becomes an internal control for the next one. The wide spectrum of observed inactivation efficiencies of the different reagents with the three proteases should, for example, eliminate the possibility that a given reagent's unique effect is due to its unique solubility or reactivity, rather than to its affinity for the enzyme. Thus, the observation that IV is a relatively poor active-site-specific reagent for chymotrypsin could be explained by its low solubility. However, since its reactivity with trypsin is essentially the same as that of other more soluble reagents, that explanation becomes untenable.

We conclude from these observations that *p*-nitrophenyl carbamates should represent a very attractive group of both monofunctional and polyfunctional protein reagents, easy to prepare from readily available starting materials, and easy to store and handle.

The conclusions regarding the topology of the affinity sites in the three proteases are primarily confirmatory. According to the current models of serine proteases, the Ser-His-Asp charge relay exists in both the enzymes and the zymogens, and the conclusion that the difference in activity between the enzymes and the corresponding zymogens must reflect the availability of substrate binding sites adjacent to the catalytic apparatus (Gertler et al., 1974; Kerr et al., 1975) has been convincingly supported by the x-ray diffraction maps showing blocked or closed binding pockets in the zymogens. There is also a good deal of work showing that, in addition to the primary binding sites, numerous subsites are also involved in the binding and positioning of the protease substrates (see, for example, Atlas et al., 1970), and, with the recent findings of Erlanger et al. (1976), it appears clear that cooperative interaction of the different sites must be considered as well.

The results presented in this paper are in agreement with all aspects of this current view of the serine proteases. The primary, strong inactivation of the three enzymes by specific members of the series of reagents tested is consistent with the established properties of the primary binding sites which are present in the enzymes but not in the zymogens. Chymotrypsin

interacts optimally with the *N*-alkyl-*p*-nitrophenyl carbamate with six (or seven) carbons in the alkyl chain; the eight-carbon chain derivative is definitely a poorer inactivation reagent. Although octyl isocyanate is found to be an efficient active-site-specific reagent in previous work (Brown and Wold, 1973a), Brown (1975) has actually shown by x-ray crystallography that the alkyl chain in the crystallized, inactive derivative is too long for optimal fit in the binding cavity. The fact that indole is a competitive inhibitor of III-5 inactivation is taken as direct evidence that the primary binding pocket is involved. As predicted, trypsin reacts optimally with the aminoalkyl derivatives with five and seven carbons in the alkyl chain (I-5, I-7) and elastase with reagents with short alkyl chains, III-3 giving the optimal inactivation. Based on the established peptide substrate specificity of elastase (Gertler and Hofmann, 1970), one might have expected a shorter side chain to give optimal effect on this enzyme. However, in a comparison of catalytic efficiency on a series of *p*-nitrophenyl fatty acid esters, Marshall and Akgun (1971) found acyl side chains with three and four carbons to give optimal "fit".

The inactivation pattern observed in this work also supports the existence of secondary binding sites. Thus, the fact that all the reagents in groups III and IV inactivated chymotrypsinogen and trypsin with equal effectiveness while octyl isocyanate had no effect provides evidence for a *p*-nitrophenyl-binding site. Similarly, the observation of the indole-induced inactivation of chymotrypsin by I-7 and II-6 demonstrates the existence of a secondary site with high affinity for alkyl chains with a terminal positive charge and illustrates the cooperative interaction between this site and the primary (indole) binding site in chymotrypsin.

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