Alkyl Isocyanates as Active-Site-Specific Reagents for Serine Proteases. Reaction Properties†

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ABSTRACT: Alkyl isocyanates have been found to inhibit serine proteases with a degree of specificity which strongly suggests a derivatization of the active site which depends on specific recognition of the proper alkyl chain by each enzyme. Thus, chymotrypsin is inactivated by stoichiometric reaction with octyl isocyanate and near stoichiometric reaction with butyl isocyanate; elastase does not react with octyl isocyanate, but is inactivated by stoichiometric reaction with butyl isocyanate; and trypsin is not affected by either reagent. Using chymotrypsin as the model, it has been shown that the inactivation reaction requires an intact unprotected active site (chymotrypsinogen and inactive forms of chymotrypsin produced by protonation or by treatment with acetone do not react, and indole protects the active enzyme against inactivation) and also that the carbamoylated enzyme derivative has an altered active site (loss of all-or-none activity in the titration with *N*-trans-cinnamoylimidazole, and altered reactivity of one of the two histidines in chymotrypsin). The selectivity observed in the reaction of the alkyl isocyanates with the three proteases is consistent with their known substrate specificities, and can also be explained on the basis of the structure of the substrate binding pockets observed in the three-dimensional models derived from X-ray diffraction analyses.

The pancreatic serine proteases chymotrypsin, trypsin, and elastase have been extensively studied during the last few decades. The three enzymes have been found to have very similar properties in their roles as catalysts for the hydrolysis of amides (and esters), and differ only in their substrate specificity; chymotrypsin recognizes large hydrophobic side chains, trypsin the positively charged side chains of lysine and arginine, and elastase small, aliphatic side chains in the position immediately to the N-terminal side of the peptide bond which is cleaved. The complete covalent structures of all three enzymes have been determined (Hartley, 1964; Walsh and Neurath, 1964; Hartley *et al.*, 1965; Shotton and Hartley, 1970) and more recently complete three-dimensional models have been constructed from high-resolution crystallographic analyses of derivatives of the three enzymes (Sigler *et al.*, 1968; Stroud *et al.*, 1972; Watson *et al.*, 1970). A comparison of the three structures reveals a remarkable degree of homology, the three catalytic sites are essentially identical, all containing the active-site serine which is acylated during the catalytic process and the participating side chains of histidine, aspartic acid, and another serine in very similar geometrical arrangements. Adjacent to the catalytic site all three enzymes contain a very pronounced cavity or pocket, and the dimensions and chemical makeup of the three individual pockets have been proposed to match perfectly with the substrate specificity of the three enzymes (Shotton and Watson, 1970). Thus, the chymotrypsin binding pocket is hydrophobic and open, the trypsin binding pocket is similar to that of chymotrypsin but uniquely contains a negatively charged aspartic acid residue in the bottom (Stroud *et al.*, 1972), and the elastase binding pocket, also similar to the other two, contains a bulky valine residue toward the front of the cavity, giving this enzyme a much shallower binding pocket (Shotten and Watson, 1970).

This very attractive structure–function model of these three well-characterized, homologous enzymes rests heavily on information derived from the studies of the crystals of inactive derivatives of the enzymes, and it is of obvious interest to be able to test the model by studies on the active enzymes in solution. In an attempt to perform such a test, we have reacted the serine proteases with a homologous series of alkyl isocyanates. The finding that these reagents behaved as highly specific active-site reagents for some of the serine proteases is not very surprising in view of the highly reactive serine residue in these enzymes. However, the finding that the size of the alkyl group determined the specificity of a given reagent toward a certain enzyme was more surprising and suggested to us that these reagents might represent an experimental tool by which the binding pocket model could be tested for the proteases in dilute aqueous solution. Initial experiments were conducted with ethyl, propyl, butyl, and octyl isocyanates and with hexamethylene diisocyanate, but because of the unpleasant properties of the lower homologues in particular, the bulk of the work to be reported was restricted to butyl isocyanate and octyl isocyanate as representatives of a "short" and a "long" side chain, respectively. Most of the detailed studies were done only with chymotrypsin, but the findings are judged to be relevant to the other enzymes as well.

A preliminary report of alkyl isocyanate specificity has appeared (Brown and Wold, 1971). It is the purpose of the present series of papers (1) to document the usefulness of alkyl isocyanates as active-site-specific reagents for chymotrypsin and elastase, (2) to present the evidence that the active-site serine residue is specifically derivatized in the reaction (Brown and Wold, 1973), and (3) to establish the actual location of the alkyl group in relation to the binding pocket by crystallographic analyses (work in progress).

Experimental Section

Materials and Assay Procedures. The materials and the standard assay procedures used for determination of either

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TABLE I: Reagents, Assays, and Methods Employed in This Study.

Material (Source)	Purity	Assay Substrate (Source)	Ref
α-Chymotrypsin (Pentex)	Twice-crystallized lots 41 and 43	Bz-L-TyrOEt (Sigma)	*a*
		Dimethyl-casein	*b*
		Transcinnamoylimidazole (Aldrich)	*c*
Elastase (Worthington)	Electrophoretically pure	Elastin (Worthington)	*d*
	Twice crystallized	*N*-Ac-L-Ala-L-Ala-L-AlaOMe (Cyclo)	*e*
Trypsin (Pentex)	Twice crystallized	Tos-L-ArgOMe (Sigma)	*a*
Carboxypeptidase A (Worthington)	Three-times-crystallized lot 6139	Hippuryl-L-phenylalanine (Mann)	*f*
Carboxypeptidase B (Worthington)	Dip-F-treated lot 91 A	Hippuryl-L-arginine (Mann)	*g*
Papain (Worthington)	Twice crystallized	Bz-L-ArgOEt (Sigma)	*h*
Pepsin (Worthington)	Twice-crystallized lot 622	Equine hemoglobin (Pentex)	*i*
n-Butyl isocyanate (K & K Labs)	95–99%	Benzylamine (Eastman)	*j*
n-Octyl isocyanate (K & K Labs)	95–99%		
Hexamethylene diisocyanate (Aldrich)	98%		
[^{14}C]Butyl isocyanate (New England)	3.25 Ci/mol		
	99^{+}% pure lot 544–046 as determined by radioactivity trace of mass trace of gas chromatography data supplied by New England Nuclear.		

[a] Hummel (1959); Worthington Biochemical Corp. (1972). [b] Lin *et al.* (1969). [c] Schonbaum *et al.* (1961). [d] Schneider *et al.* (1962). [e] Gertler and Hofmann (1970). [f] Folk and Schirmer (1963); Worthington Biochemical Corp. (1972). [g] Folk *et al.* (1960); Worthington Biochemical Corp. (1972). [h] Whitaker and Bender (1965); Worthington Biochemical Corp. (1972). [i] Anson (1938); Worthington Biochemical Corp. (1972).

activity or purity of enzymes and reagents are listed in Table I. Assay procedures not found in the literature are described in detail below.

ASSAY OF ISOCYANATE CONCENTRATION. Owing to the rapid hydrolysis rate and low solubility of alkyl isocyanates in aqueous solvents, all stock solutions and dilutions of the isocyanates were made in anhydrous acetone. In order to determine the actual concentration of isocyanate used in each protein reaction, a procedure was developed for the quantitative titration of the isocyanates based on their reaction with benzylamine in chloroform, extraction of the unreacted benzylamine into aqueous acid solution, and spectrophotometric determination of the unreacted benzylamine as its hydrochloride.

To a series of stoppered test tubes containing 0.5 ml of chloroform was added 5 μl of 1.0 M isocyanate (stock solution in acetone) and graduated quantities (0–100 μl) of 8.7×10^{-2} M benzylamine (stock solution in chloroform). The mixtures were reacted at room temperature for 15 min with shaking. Three milliliters of 0.1 M HCl was then added and the tubes were shaken for 15 min to ensure complete extraction of the unreacted benzylamine. The aqueous layers were collected using Whatman silicone-treated 1-PS phase-separation paper, and their absorbance was measured at 266 nm. A plot of the absorbance *vs.* micromoles of benzylamine added gave the equivalents of isocyanate present in the reaction mixture. This procedure was found to be reproducible to ±1%.

TITRATION OF ISOCYANATES IN AQUEOUS SOLUTION. To a constantly stirring solution (20 ml) of 0.08 M Tris-HCl (pH 7.7), containing 0.1 M $CaCl_2$, was added 200 μl of 5.1×10^{-2} M isocyanate (in acetone). At selected time intervals, 2.0-ml aliquots were removed and added immediately to 5 ml of 1.0×10^{-2} M benzylamine in chloroform. The mixture was stirred vigorously for 1 min on a Vortex mixer and transferred to a separatory funnel containing 50 ml of 0.1 M HCl. This mixture was shaken for 1 min and the optical density of the chloroform layer was measured at 258 nm. In this method, the isocyanate available for reaction with benzylamine after different lengths of exposure to aqueous buffer was determined as the chloroform soluble *N*-alkyl-*N'*-benzylurea derivative.

TITRATION OF ENZYMES WITH ISOCYANATES. To a buffered solution (0.1 M Tris-HCl, pH 7.7) containing enzyme (10^{-6}–10^{-4} M) was added an aliquot of isocyanate (0.25–10 mg/ml in anhydrous acetone). After 15 min of constant stirring at room temperature, an aliquot of the reaction mixture was removed for enzyme activity assay and whenever applicable, for determination of radioactivity. A second aliquot of isocyanate solution was then added. After 15 min, another assay aliquot was removed and the preceding procedure was repeated until the desired end point was reached. The final concentration of acetone in the reaction mixture never exceeded 2% (v/v). Radioactivity was determined with a Beckman LS-133 liquid scintillation spectrometer after gel filtration to remove excess reagent and reagent products from the protein derivative. Reagents tested 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.

Results

Characterization of the Alkyl Isocyanates. The stability of alkyl isocyanates in aqueous solvents has been studied (Saunders and Slocombe, 1948); however, it was felt important to determine the stability of the isocyanates under the exact conditions used in this work. The decomposition of isocyanates in the presence of water should be the result of the hydrolysis to carbon dioxide and the corresponding amine (eq 1), and the subsequent reaction of the amine with a second

$$RN{=}C{=}O + HOH \longrightarrow RNH_2 + CO_2 \quad (1)$$

mole of isocyanate to form symmetrical ureas (eq 2). The

$$RN{=}C{=}O + RNH_2 \longrightarrow RNHCNHR \quad (2)$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\quad \| \quad (O)$$

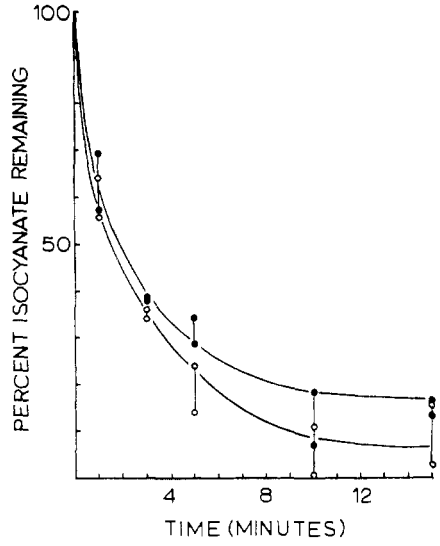

FIGURE 1: The rate of decomposition of octyl isocyanate (○) and butyl isocyanate (●) in aqueous media under the conditions used for the protein reactions (0.08 M Tris-HCl buffer, pH 7.7, containing 0.1 M $CaCl_2$).

results from our studies (Figure 1) show that the half-life of the isocyanates under our reaction conditions is approximately one minute and that there is no significant difference between the rates of decomposition of octyl and butyl isocyanate.

Studies on the Isocyanate–Protease Reaction. SPECIFICITY. The results of the titration of a variety of enzymes with octyl and butyl isocyanate under similar conditions of enzyme concentration, ionic strength, and temperature are given in Table II. Under these conditions octyl isocyanate at a molar ratio of 50:1 (reagent:enzyme) had little or no effect on trypsin, elastase, pepsin, or carboxypeptidases A and B, while chymotrypsin and papain[1] were totally inactivated. Butyl isocyanate under identical conditions, similarly had no effect on trypsin but inactivated both chymotrypsin and elastase with a greater efficiency toward elastase than toward chymotrypsin.

KINETICS. The rate of inactivation of chymotrypsin by octyl isocyanate and elastase by butyl isocyanate is too rapid to measure by normal sampling techniques. A search for special spectral properties of the product which would make it possible to follow the reaction directly in the spectrophotometer was negative, and a detailed study of the reaction kinetics was therefore not attempted. However, knowing the rate of hydrolysis of the isocyanates (Figure 1) it is possible to obtain an indirect measure of the rate of the protein reaction relative to the competing hydrolysis reaction. When chymotrypsin and elastase, at different concentrations, were titrated with either octyl or butyl isocyanate the results in Table III were obtained, showing that as the enzyme concentration increases from 1.9×10^{-6} to 4×10^{-4} M the inactivation reaction becomes increasingly more efficient. This is consistent with the expected second-order reaction which successfully competes with the hydrolysis reaction. The fact that at the higher enzyme concentrations the reaction with the enzyme predominates (50% inactivation with 0.6 mol of isocyanate/mol of enzyme)

[1] The reaction of papain will not be discussed further in this paper. The finding that papain was inactivated at any pH below 5.7 has subsequently been followed up in this laboratory and will be reported in a future publication.

TABLE II: Effect of Octyl and Butyl Isocyanate on Proteases.

Enzyme	Enzyme Concn (M × 10^6)	Reaction pH	% Act. Remaining at Reagent to Enzyme Molar Ratio of 50:1	
			Octyl Isocyanate	Butyl Isocyanate
Chymotrypsin	1.9	7.6	0	19
Trypsin	2.0	7.6	85	85
Elastase	1.9	7.6	94	5
Carboxypeptidase A	6.4	7.6	90	
Carboxypeptidase B	3.0	7.6	100	
Pepsin	2.3	2.0	100	
Papain	4.8	3.0	0	

demonstrates that the protease–isocyanate reactions are very fast. The results further emphasize the specificity of octyl isocyanate for chymotrypsin and butyl isocyanate for elastase.

STOICHIOMETRY. All the titration experiments indicated that the protease–isocyanate reaction involved a single reaction site and that loss of activity was associated with the modification of that single site. However, this point is sufficiently important for the argument that the alkyl isocyanates are active-site-specific reagents, that it was deemed essential to determine directly the relationship of reagent incorporation (not merely reagent added, as in the previous experiments) to loss of activity. This was accomplished using [^{14}C]butyl isocyanate (labeled in the carbonyl carbon), and the resulting plot of activity loss *vs.* reagent incorporation (Figure 2) demonstrates the exact stoichiometry of one for both enzymes. The deviation from linearity in the curve for chymotrypsin under conditions where elastase could be titrated directly to the end point is consistent with the lower specificity of butyl isocyanate for chymotrypsin.

Evidence for the Direct Involvement of the Active Site of Chymotrypsin in the Reaction with Alkyl Isocyanates. The conclusion that the alkyl isocyanates indeed act as active-site-specific reagents for the serine proteases, should be based on solid evidence that an intact, operational active site is required for the specific inactivation reaction. There are several approaches through which such evidence can be obtained, and some of these were used to explore the inactivation of chymotrypsin.

REACTION OF ZYMOGEN. It was found early in this work that the inactive zymogen, chymotrypsinogen, could be treated with alkyl isocyanates under condition were chymotrypsin in parallel experiments was completely inactivated, and then be activated with trypsin to give 100% yield of active chymotrypsin. Thus, the specific inactivation reaction does not occur with the zymogen.

pH DEPENDENCE. It is well known that low pH, protonated forms of chymotrypsin are enzymatically inactive. Are they also inactive in the reaction with octyl isocyanate? Chymotrypsin (2×10^{-6} M) was reacted with octyl isocyanate (1.6×10^{-5} M) at variable pH values, and after incubation for 15 min, the chymotrypsin activity was assayed at pH 7.6 using benzoyl-L-tyrosine ethyl ester as substrate. In order to interpret the results in terms of protein reactivity, it was necessary to establish that the competing reaction, the hy-

TABLE III: Effect of Enzyme Concentration on the Reaction of Chymotrypsin and Elastase with Octyl and Butyl Isocyanate.

Enzyme Concn (M)	Reagent to Enzyme Ratio Required for 50% Inactivation			
	Chymotrypsin		Elastase	
	Butyl	Octyl	Butyl	Octyl
1.9×10^{-6}	16	2.0	3.6	>100
8.0×10^{-5}	1.4	0.6		
4.0×10^{-4}	0.6	0.6	0.6	>100

drolysis of the isocyanate was independent of pH in the range studied. To this end, three samples of octyl isocyanate were incubated in aqueous solution at pH 3, 5, and 7 for exactly 1 min and then added to an excess of chymotrypsin at pH 7.6. The extent of inactivation of chymotrypsin was identical for all three samples, showing that the rate of hydrolysis of octyl isocyanate is identical at the three pH values investigated. (Above pH 8 a very significant increase in the rate of hydrolysis was observed.)

The data for the inactivation of chymotrypsin as a function of pH fit a theoretical titration curve for a functional group with a pK of 5.6 (Figure 3). It is not clear what the nature of this group or function in chymotrypsin is. We had expected to obtain some correlation with the considerably higher pK value (6.4–6.8) characteristic of the acylation step in the catalytic function. Although we have no explanation for this quantitative discrepancy at present, we feel that the general similarity between the pH effects on the catalytic activity and the carbamoylation of chymotrypsin strongly suggest that only the active (deprotonated) form of the enzyme reacts with the isocyanate.

EFFECT OF ACETONE. A second, similar type of experiment was based on the unique reversible inactivation of chymotrypsin by increasing concentrations of acetone (eq 3). The

$$\text{active chymotrypsin (E)} \underset{-\text{acetone}}{\overset{+\text{acetone}}{\rightleftharpoons}} \text{inactive chymotrypsin (E*)} \quad (3)$$

activity assay of chymotrypsin with dimethyl-casein as substrate gives a direct measure of E present at different concentrations of acetone. If an intact active site is required for the isocyanate reaction, octyl isocyanate should react only with E and not with E*. Assuming that the rate of interconversion of E and E* is slow relative to the isocyanate reaction with E, it should be possible to inactivate all E present at any acetone concentration by treatment with octyl isocyanate, and subsequently measure E* present in the original reaction mixture as the amount of active enzyme recovered after removal of the acetone. In order to minimize the conversion of E* to E during the reaction with octyl isocyanate, the reaction was allowed to proceed for only 2 min. The results of this experiment are given in Figure 4, and the best explanation for the complementarity of the two curves seems to be that only active enzyme (E) reacts specifically with the reagent. This can be illustrated using the data at 10% acetone (dotted line in Figure 4). The rate assay (solid circles) shows that 70% of the chymotrypsin is in the active form E in 10% acetone. After

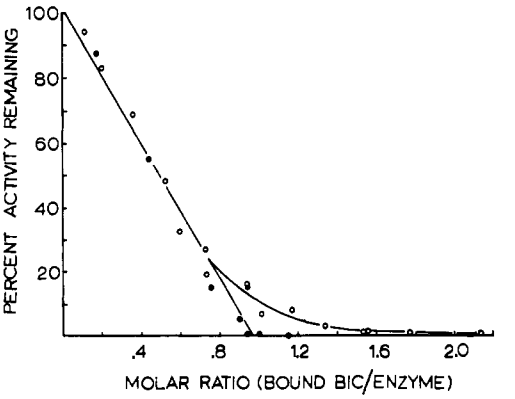

FIGURE 2: Inactivation of chymotrypsin (○) and elastase (●) as a function of incorporation of [^{14}C]butyl isocyanate (BIC). The concentration of both enzymes was 4×10^{-4} M. The reaction conditions and procedures are described in the text.

reaction with octyl isocyanate in 10% acetone, and measurement of activity remaining after removal of acetone (open circles), about 30% of the original activity was recovered, showing that 70% of the enzyme had been inactivated by octyl isocyanate.

KINETIC ANALYSIS OF PARTIALLY INACTIVATED ENZYME. Routinely, the inactivation of chymotrypsin was measured as the decrease in the direct initial rate assay at nonsaturating substrate concentrations. Since the rates measured in this manner could reflect changes in either catalytic turnover or in substrate affinity, it was deemed important to carry out a kinetic analysis of partially inactivated enzyme. When a sample of octylcarbamoyl-enzyme which by the initial rate assay had been 93.3% inactivated was subjected to substrate kinetic analysis, the K_m of the remaining activity was found to be identical to that of the fully active enzyme control, while the V_{max} was decreased to 5.5%. These findings were confirmed by the all-or-none assay (Schonbaum *et al.*, 1961), which simply quantitates the available active sites by acylation with *N*-trans-cinnamoylimidazole. Titration of the above inactivated sample showed that 6.3% of the original active sites remained

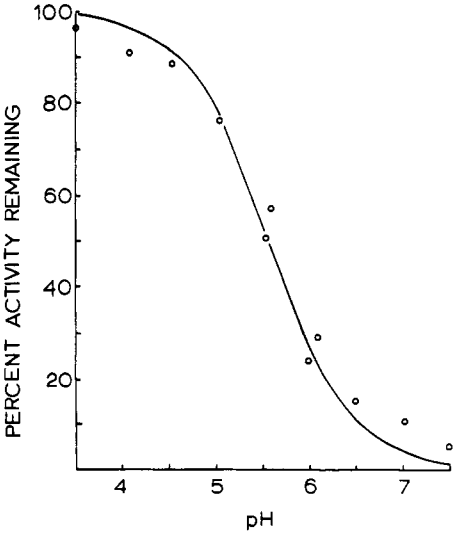

FIGURE 3: The effect of pH on the inactivation of chymotrypsin by an 8:1 molar excess of octyl isocyanate. The solid line represents the theoretical titration curve for a group with an apparent pK of 5.6.

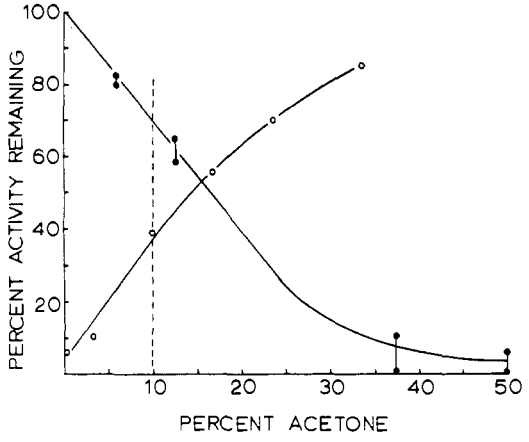

FIGURE 4: The effect of acetone on the reaction of chymotrypsin with octyl isocyanate. Chymotrypsin (3.2×10^{-7} M) in 0.1 M phosphate buffer (pH 7.5) was assayed in the presence of increasing concentrations of acetone using dimethyl-casein as substrate (●). Solutions of $4–8 \times 10^{-5}$ M chymotrypsin in 0.01 M Tris-HCl buffer (pH 7.5), containing increasing concentrations of acetone were reacted with a 1.1:1 molar ratio of octyl isocyanate to chymotrypsin After 2 min, each reaction mixture was diluted by a factor of 30 and the amount of unreacted chymotrypsin was estimated in terms of activity remaining using Bz-L-TyrOEt as substrate (○).

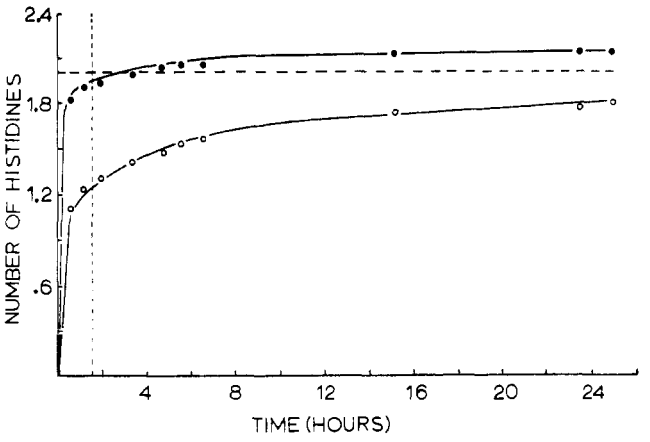

FIGURE 5: The titration of histidine residues in native chymotrypsin (●) and octylcarbamoyl-chymotrypsin (91% inactivated, ○) with diazo-1*H*-tetrazole. The proteins were diluted to 1.25×10^{-5} M in 1 M $KHCO_3$, pH 8.8. Two additions of diazo-1*H*-tetrazole (0.2 M) were made at 10-min intervals and the optical density was read at 480 and 550 nm in the Cary Model 15 spectrophotometer. Using the equation from Sokolovsky and Vallee (1966), the number of histidines were calculated as a function of time. The horizontal dashed line represents the theoretical number of histidines in chymotrypsin and the vertical dashed line represents the incubation time (90 min) recommended in the procedure of Sokolovsky and Vallee (1966).

after reaction with octyl isocyanate. These experiments show that the incorporation of the alkylcarbamoyl group leads to total inactivation of each site involved.

EFFECT OF CARBAMOYLATION WITH OCTYL ISOCYANATE ON THE REACTIVITY OF HISTIDINE IN CHYMOTRYPSIN. Chymotrypsin contains only two histines, one of which (histidine-57) is an essential component of the catalytic site. When a sample of native enzyme and a sample of totally octyl isocyanate inactivated chymotrypsin were titrated in parallel with the histidine-specific reagent diazonium-1*H*-tetrazole according to the method of Sokolovsky and Vallee (1966), the results in Figure 5 were obtained, showing that the reactivity of one of the two histidines had been significantly decreased in the inactivated enzyme. Since, as will be shown in the companion paper (Brown and Wold, 1973), the carbamoylation of the enzyme involves the active-site serine, the most reasonable interpretation of these findings is that the octylcarbamoyl-serine derivative sterically blocks the access of one of the histidines to the tetrazole reagent. We assume that the blocked residue is histidine-57, but we have no direct evidence to back up this assumption. A similar protection of histidine-57 against reaction with tosylamidophenyethyl chloromethyl ketone after derivatization of serine-195 with diisopropyl fluorophosphate has been reported, however (Schoellman and Shaw, 1963).

EFFECT OF INDOLE AS A PROTECTIVE AGENT AGAINST INACTIVATION. Indole is known to be a competitive inhibitor of chymotrypsin with a K_i of 0.8×10^{-3} M (Wallace *et al.*, 1963). If isocyanates bind in the active site, indole should protect chymotrypsin from the inactivation by isocyanates. Chymotrypsin was titrated with octyl isocyanate in the presence and absence of indole, and the data in Figure 6 show that indole gives almost complete protection at a molar ratio of 10^4:1 (indole:chymotrypsin). Finding this protection is especially meaningful since X-ray crystallographic work has provided direct evidence that the actual site of indole binding is the binding pocket (Steitz *et al.*, 1969). In connection with the indole protection it must be mentioned that we also tested several alkyl derivatives as protecting agents. Butyl alcohol at 10^4:1, octyl alcohol at 10^3:1, octylamine at 10^3:1, and hexyl cyanide at 10^4:1 gave no significant protection of chymotrypsin against inactivation by octyl isocyanate.

Discussion

Whereas aromatic isocyanates and isothiocyanates are quite common protein reagents (Edman, 1956; Schick and Singer, 1961; Fasold, 1965), the aliphatic isocyanates have found only limited use, probably because of their lability and lack of specificity. Ozawa (1967) studied the reaction of hexamethylene diisocyanate with the ϵ-amino group of lysine and with ribonuclease and chymotrypsin. He found in these studies that in 44% acetone, a 50:1 molar excess of hexamethylene diisocyanate gave complete inactivation of chymotrypsin. In view of the effect of acetone found in our work (Figure 4), the requirement for the large excess of reagent is not surprising. We have repeated Ozawa's (1967) experiments with octyl isocyanate and found that the molar ratio (reagent to enzyme) of 50:1 gave 90% inactivation in 44% acetone. In the absence of acetone hexamethylene diisocyanate was found to be as effective as octyl isocyanate, giving complete inactivation at a molar ratio of very nearly 1:1.

The results presented in this work indicate that the alkyl isocyanates represent very useful protein reagents. Because of the identical chemical properties of the reactive group of these readily available members of the homologous series of isocyanates, and because of their rapid reaction with functional groups in proteins under mild conditions, these compounds do indeed offer some unique possibilities as chemical probes in the study of enzyme structure and function. A large number of enzymes use substrates containing simple aliphatic side chains, *e.g.*, alcohols, fatty acids or esters, and amines. In all these cases the alkyl isocyanates should represent potential active-site reagents. As an example of this proposal it can be mentioned that as a result of the work reported here, and

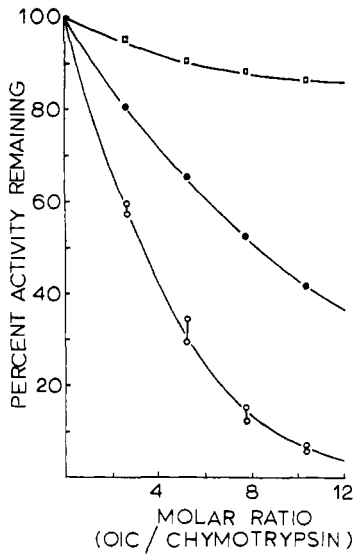

FIGURE 6: The effect of indole on the reaction of chymotrypsin with octyl isocyanate (OIC). Chymotrypsin (1×10^{-6} M) was incubated in 0.08 M Tris-HCl buffer (pH 7.8) plus 0.1 M $CaCl_2$ containing no indole (○), 10^{-3} M indole (●), and 10^{-2} M indole (□). After 15 min, these mixtures were titrated with appropriate aliquots of a solution of 1.29×10^{-2} M octyl isocyanate in acetone. Chymotryptic activity was measured by rate assay using Bz-L-TyrOEt as substrate.

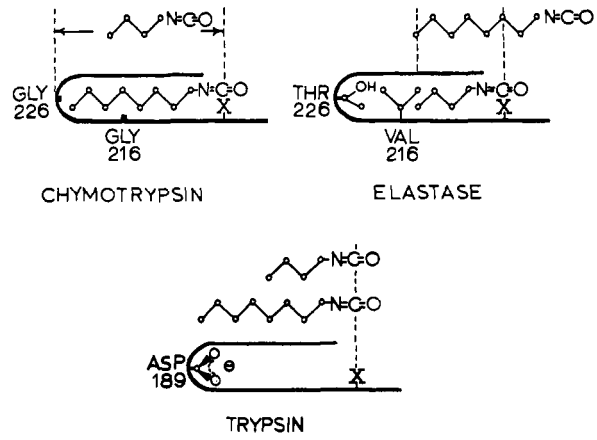

FIGURE 7: Schematic representations of the proposed interaction of the alkyl isocyanates with the binding pockets of trypsin, chymotrypsin, and elastase based on the models derived by Shotten and Watson (1970) and Stroud *et al.* (1972).

after establishing that the isocyanates form moderately stable derivatives with sulfhydryl groups, it was predicted that butyl isocyanate, as an analog of butanol, should be an active-site reagent for alcohol dehydrogenase. This prediction has now been confirmed (Twu and Wold, 1973).

With respect to the proteases, the significant finding in this work is the fact that the alkyl isocyanates show such a high degree of specificity in their reaction with the three proteases. The catalytic apparatus of trypsin, chymotrypsin, and elastase are essentially identical and since the active-site serine residue appears to be equally reactive in all three enzymes, a simple random reaction with alkyl isocyanate should not be expected to distinguish between the three enzymes. The most reasonable explanation for the reagent specificity must therefore be that the alkyl group is specifically recognized by the individual enzyme, and that the inactivation requires the formation of an enzyme–reagent complex that gives proper alignment of the two reactive groups (serine in the enzyme and the isocyanate in the reagent). The requirement for an intact active site documented for chymotrypsin is consistent with this explanation. In Figure 7 we have illustrated in a very schematic form the binding pockets for the three proteases. Assuming that the specificity is determined by reagent binding in these binding pockets, and the fact that indole protected chymotrypsin against inactivation by octyl isocyanate is consistent with that assumption, it is possible to explain most of our data. Octyl isocyanate is a specific reagent for chymotrypsin because this enzyme–reagent complex gives perfect alignment of the reactive groups and therefore, high probability of covalent-bond formation. In the butyl isocyanate–chymotrypsin complex, the alignment is more random. However, since the isocyanate is now in a hydrophobic environment, the hydrolysis rate could be reduced and the probability of covalent-bond formation might still be high. If we accept the argument that specific binding is a prerequisite for inactivation, and the fact that the rates of hydrolysis of octyl and butyl isocyanates are equal, as illustrated in Figure 1, then it should be possible to assess the relative affinity of butyl and octyl isocyanate for chymotrypsin from our data as the ratio of the concentrations of butyl isocyanate and octyl isocyanate required for 50% inactivation. Using the data in Table III at an enzyme concentration of 1.9×10^{-6} M, we determined this ratio to be 8:1. This is in excellent agreement with the results of Antonov *et al.* (1970), who found a K_i ratio of 10:1 for the inhibition of chymotrypsin by boronic acids with *n*-butyl and *n*-octyl chains, respectively.

The binding pocket of elastase is very similar to that of chymotrypsin but, because of the steric interference of the valine and threonine side chains, the binding pocket of elastase is shallower. Thus, only butyl isocyanate can bind with proper alignment for covalent bond formation. The longer octyl side chain probably also binds but because of poor alignment, has virtually zero probability of covalent-bond formation.

The negative results obtained with trypsin are also important for the argument. If the binding to trypsin requires a positive charge, none of the alkyl isocyanates should bind. Unless one also assumes other differences in the trypsin binding pocket such as in the extent of hydration, for example, it is difficult to explain the lack of effect of the shorter chain isocyanates on this enzyme. It is relevant to this model that hexamethylene diisocyanate has been found to inactivate trypsin at very nearly stoichiometric concentration. In this case we would like to propose that the hydrolysis of the diisocyanate leads to the formation of the monoamine–monoisocyanate intermediate, and that this is the real inhibitor of trypsin. It may be possible to check this hypothesis by X-ray crystallography studies of the inactive derivative, but it will also be important to attempt to prepare a compound which contains a protected, unreactive positive charge in addition to the isocyanate function, and test this compound as a specific trypsin inhibitor.

The companion paper (Brown and Wold, 1973) presents the experimental evidence which shows that residue X in Figure 7 is indeed the active-site serine. The evidence discussed above that the alkyl chain is located in the binding pocket, is clearly indirect, however, and it will be essential to prove this point before any direct comparison of solid state and solution properties of the proteases can be carried out in a meaningful manner. The reason for concern about this point is centered

about the question of whether there is more than one binding site which affects substrate binding in chymotrypsin (Jones *et al.*, 1965; Hofstee, 1957). One important test which could resolve this question and prove the binding pocket hypothesis, would be the direct observation by X-ray diffraction analysis of the actual position of the alkyl chain in the enzyme derivatives. The work to obtain this information has been undertaken. Octylcarbamoyl-chymotrypsin has been crystallized, and the crystallographic analysis is in progress.

References

Anson, M. L. (1938), *J. Gen. Physiol. 22*, 79.

Antonov, V. K., Ivanina, T. V., Berezin, I. V., and Martinek, K. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 23.

Brown, W. E., and Wold, F. (1971), *Science 174*, 608.

Brown, W. E., and Wold, F. (1973), *Biochemistry 12*, 835.

Edman, P. (1965), *Acta Chem. Scand. 10*, 761.

Fasold, H. (1965), *Biochem. Z. 342*, 295.

Folk, J. E., Piez, K. A., Carroll, W. R., and Gladner, J. (1960), *J. Biol. Chem. 235*, 2272.

Folk, J. E., and Schirmer, E. W. (1963), *J. Biol. Chem. 238*, 3884.

Gertler, A., and Hofmann, T. (1970), *Can. J. Biochem. 48*, 384.

Hartley, B. S. (1964), *Nature (London) 201*, 1284.

Hartley, B. S., Brown, J. R., Kauffman, D. L., and Smillie, L. B. (1965), *Nature (London) 207*, 1157.

Hofstee, B. H. J. (1957), *Biochem. Biophys. Acta 24*, 211.

Hummel, B. C. W. (1959), *Can. J. Biochem. Physiol. 37*, 1393.

Jones, J. B., Kunitake, T., Niemann, C., and Hein, G. E. (1965), *J. Amer. Chem. Soc. 87*, 1777.

Lin, Y., Means, G. E., and Feeney, R. E. (1969), *J. Biol. Chem. 244*, 789.

Ozawa, H. (1967), *J. Biochem. (Tokyo) 62*, 419.

Saunders, J. H., and Slocombe, R. J. (1948), *Chem. Rev. 43*, 203.

Schick, A. F., and Singer, S. J. (1961), *J. Biol. Chem. 236*, 2477.

Schneider, I. J., Tindel, S., Shapiro, D., and State, D. (1962), *J. Lab. Clin. Med. 60*, 514.

Schoellman, G., and Shaw, E. (1963), *Biochemistry 2*, 252.

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

Shotton, D. M., and Hartley, B. S. (1970), *Nature (London) 225*, 802.

Shotton, D. M., and Watson, H. C. (1970), *Nature (London) 225*, 811.

Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol. 35*, 143.

Sokolovsky, M., and Vallee, B. L. (1966), *Biochemistry 5*, 3579.

Steitz, T. A., Henderson, R., and Blow, D. M. (1969), *J. Mol. Biol. 46*, 337.

Stroud, R. M., Kay, L. M., and Dickerson, R. E. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36*, 125.

Twu, J.-S., and Wold, F. (1973), *Biochemistry* (in press).

Wallace, B. A., Kurtz, A. N., and Niemann, C. (1963), *Biochemistry 2*, 824.

Walsh, K., and Neurath, H. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 884.

Watson, H. C., Shotton, D. M., Cox, J. M., and Muirhead, H. (1970), *Nature (London) 225*, 806.

Whitaker, J. R., and Bender, M. L. (1965), *J. Amer. Chem. Soc. 87*, 2728.

Worthington Biochemical Corp. (1972), Worthington Enzyme Manual, Freehold, N. J.