1974). The biodegradative ornithine decarboxylase has the highest pH optimum of the biodegradative enzymes, consistent with an evolutionary trend downward from the high value characteristic of the biosynthetic decarboxylases. Second, on routine screening of many strains of *E. coli* for amino acid decarboxylases, we found that all contained the biosynthetic ornithine decarboxylase and nearly all possessed the biodegradative lysine and arginine decarboxylases. On the other hand, only about one out of ten produced the biodegradative ornithine decarboxylase (D. Applebaum, unpublished results). Therefore, we would suggest that the two ornithine decarboxylases have only recently diverged in selected strains of *E. coli*, and that the newly derived biodegradative enzyme has been left with a vestigial regulatory mechanism in the nucleotide activation.

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Active Site Specific Inactivation of Chymotrypsin by Cyclohexyl Isocyanate Formed during Degradation of the Carcinostatic 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea†

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ABSTRACT: Prolonged incubation of 1-(2-chloroethyl)-3-([1-^{14}C]cyclohexyl)-1-nitrosourea with chymotrypsin resulted in covalent modification and concomitant inactivation of chymotrypsin via degradation of the nitrosourea to form cyclohexyl isocyanate. Cyclohexyl isocyanate was shown to be an active-site-specific inactivator of chymotrypsin. A cyclohexyl isocyanate to enzyme molar ratio of 0.63 was required to produce 50% enzyme inactivation, thus demonstrating the high specificity of inactivation. At 2.38×10^{-4} M chymotrypsin this near stoichiometric inactivation was not significantly affected by the presence of 1, 5, and 10 mM L-lysine. Degradation of an excess of 1-(2-chloroethyl)-3-([1-^{14}C]-cyclohexyl)-1-nitrosourea in the presence of enzyme yielded 1.11 ± 0.07 mol of covalently bound [^{14}C]cyclohexyl moiety per mol of enzyme inactivated. Short-term incubation demonstrated that the nitrosourea neither inhibited nor protected the enzyme from cyclohexyl isocyanate inactivation. Treatment of chymotrypsin with less than stoichiometric amounts of cyclohexyl isocyanate or titration of the active-site serine with phenylmethanesulfonyl fluoride followed by in situ degradation of excess 1-(2-chloroethyl)-3-([1-^{14}C]cyclohexyl)-1-nitrosourea resulted in a decreased amount of covalently bound ^{14}C proportional to the extent of inactivation by these reagents prior to ^{14}C labeling. These results strongly suggest that cyclohexyl isocyanate, whether added directly or generated by CCNU degradation, reacted almost exclusively with the active site of the enzyme. The extent of this inactivation indicates that 70% of the CCNU degraded in such a manner as to form cyclohexyl isocyanate.

Interest in the antitumor activity of certain nitrosoureas is being directed towards the alkylating and carbamylating intermediates formed during chemical degradation of the nitrosoureas (Montgomery et al., 1967, 1975; Reed et al., 1975; Colvin et al., 1974). Reed et al. (1975) have reported that alkylation by CCNU[1] may occur via a 2-chloroethyl carbonium ion. In vivo, alkylation of thiols occurs producing thiodiacetic acid as a major urinary product after administration of CCNU (Reed and May, 1975).

Wheeler et al. (1975) have emphasized the carbamylation of both the ϵ-amino group of lysine and the α-amino groups of amino acids, peptides, and proteins by nitrosoureas in vitro.

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[1] Abbreviations used are: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; [*chx*-1-^{14}C]CCNU, [^{14}C]CCNU labeled in its cyclohexyl moiety; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; DNBS, 2,4-dinitrobenzenesulfonic acid; Dnp, 2,4-dinitrophenyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; HPLC, high-pressure liquid chromatography.

The binding of ^{14}C with proteins during degradation of [*chx*-1-^{14}C]CCNU has been shown to occur both in vivo and in vitro (Cheng et al., 1972; Schmall et al., 1973). Chromatographic evidence from in vitro experiments with proteins indicates cyclohexyl carbamylation of the lysine residue can occur to form N^6-cyclohexylcarbamyllysine (Schmall et al., 1973).

Brown and Wold (1973a,b) have shown that butyl isocyanate and octyl isocyanate are active-site-directed inactivators of elastase and chymotrypsin, respectively. The inactivation, which results from carbamylation of a serine hydroxyl, is highly dependent upon specific recognition of the proper alkyl-chain structure by the hydrophobic binding pocket adjacent to the serine hydroxyl at the catalytic site of the enzyme (Brown, 1975).

This report describes the selective carbamylation by cyclohexyl isocyanate that results in a near stoichiometric inactivation of chymotrypsin. CCNU was found to inactivate the enzyme only after it had degraded to form cyclohexyl isocyanate. The presence of competing nucleophiles, particularly L-lysine, had no significant effect on the stoichiometry of cyclohexyl isocyanate inactivation of chymotrypsin. The rapidity of formation of the cyclohexyl isocyanate–enzyme complex appears to account for the exclusive carbamylation of the enzyme in the presence of lysine and other nucleophiles.

Materials and Methods

α-Chymotrypsin, three times crystallized, was obtained from Worthington Biochemical Corp. All reagents and solvents were reagent grade and commercially available. Cyclohexyl isocyanate and *n*-octyl isocyanate, from Eastman, were redistilled and maintained anhydrous under nitrogen prior to use. CCNU and [*chx*-1-^{14}C]CCNU were supplied by the National Cancer Institute. Purity was greater than 98% as determined by high-pressure liquid chromatography (May et al., 1975).

General Conditions for Chymotrypsin Incubations. Conditions used for chymotrypsin incubations throughout this study, unless otherwise noted, were 0.1 M Tris-HCl (pH 7.7) at 25 °C, and will be referred to as buffered solutions. All CCNU and cyclohexyl isocyanate additions were made as aliquots in anhydrous acetone (5–40 mM). Controls containing acetone, enzyme, and buffer were run with all enzyme incubations. Acetone concentrations of 2% (v/v) or less did not result in enzyme inactivation.

Assays. Chymotrypsin activity was measured spectrophotometrically by following the increase in absorbance at 256 nm due to the hydrolysis of BTEE according to the method of Hummel (1959). The protein concentrations were routinely determined either by the absorbance at 280 nm using $E_{280}^{1\%} = 20.4$ (Morimoto and Kegeles, 1967), or by the method of Lowry et al. (1951), and confirmed by dry weight. Radioactivity was determined with a Packard 2425 liquid scintillation spectrometer using Triton N-101–xylene fluor. Counting efficiency was determined with [^{14}C]toluene internal standards. The degradation of isocyanates in buffered solution was followed by the conversion of the isocyanate to the corresponding benzylurea with benzylamine as described by Brown and Wold (1973a). The isocyanate half-lives were calculated from these data.

Determination of Half-Life for CCNU Degradation. CCNU (0.100 μmol) was added to buffered solutions containing 0 or 0.095 μmol of chymotrypsin (4.75×10^{-5} M). The decrease in the absorbance of CCNU at 230 nm (due to loss of the nitroso group) with time was followed using a Cary 15 spectrophotometer and the half-life was determined from these data.

Degradation of CCNU and Cyclohexyl Isocyanate and the Concurrent Formation of Cyclohexylamine. CCNU (0.25 μmol) was added to either 0.5 mL of 0.1 M Tris-HCl (pH 7.7), 0.5 mL of 0.1 M $NaPO_4$ buffer (pH 7.7), or 0.5 mL of glass distilled water, and incubated for 21–22 h at 25 °C. Cyclohexyl isocyanate (0.40 μmol) was similarily treated. All incubation mixtures were reacted with 2,4-dinitrobenzenesulfonic acid (DNBS) according to a procedure of Smith and Jepson (1967). 2,4-dinitrophenylcyclohexylamine was synthesized by the same method, recrystallized, and used as a standard. The extracted 2,4-dinitrophenyl (DNP) derivatives were subjected to high-pressure liquid chromatography (HPLC) on a 4 × 250 mm LiChrosorb column, 5-μm particle size, using a Model 3500 Spectra Physics liquid chromatograph equipped with a Model 770 spectrophotometric detector. The solvent was 2,2,4-trimethylpentane–dichloromethane–2-propanol (930:63:0.8), the flow rate 1.8 mL/min, and the elution time of 2,4-dinitrophenylcyclohexylamine was 380 s. The 2,4-dinitrophenylcyclohexylamine peak was quantitated using a Spectra Physics Autolab System I computing integrator.

Titration of Chymotrypsin with Cyclohexyl Isocyanate. Chymotrypsin was titrated by the sequential addition of small increments of cyclohexyl isocyanate in anhydrous acetone to the same buffered solutions containing from 10^{-6} to 10^{-4} M enzyme according to the procedure of Brown and Wold (1973a). Similar titrations were also performed on solutions containing 0.238 μmol of enzyme (2.38×10^{-4} M) and 0, 1, 5, or 10 mM L-lysine hydrochloride.

Incubation of Chymotrypsin with CCNU. CCNU (0.250 μmol) was added to a buffered solution containing 0.238 μmol of chymotrypsin (2.38×10^{-4} M). The reaction was incubated at 25 °C for a period of time equivalent to six CCNU half-lives (21–22 h). The enzyme was assayed at 0 time and 21–22 h using a dilution of sufficient magnitude (500-fold) to reverse any effects of a noncovalently bound inhibitor.

Inactivation of Chymotrypsin with Cyclohexyl Isocyanate in the Presence of CCNU. Chymotrypsin (10^{-5}–10^{-4} M) was titrated with cyclohexyl isocyanate in the absence and presence of up to a tenfold excess of CCNU. Controls for each incubation contained the same enzyme and CCNU concentration but no added cyclohexyl isocyanate. The controls were assayed at times corresponding to cyclohexyl isocyanate titration assay points (0, 7, 14, 21, and 28 min).

[chx-1-^{14}C]CCNU Labeling of Chymotrypsin. [*chx*-1-^{14}C]CCNU (0.850 μmol) was added to buffered solutions containing 0.476 μmol of chymotrypsin (2.38×10^{-4} M). The incubations were allowed to proceed at 25 °C for 21–22 h. The enzyme activity was assayed at 0 min and 21 h. Aliquots (0.50 mL) were subjected to column chromatography on a 1.5 × 60 cm Sephadex G-25 column, eluted with 0.01 M Tris-HCl (pH 7.7), and/or dialyzed against 8 M urea in 0.1 M Tris-HCl (pH 7.7) at 4 °C overnight, followed by extensive dialysis in 0.01 M Tris-HCl (pH 7.7) at 4 °C. Some samples were dialyzed against 5 M urea in 0.1 M ammonium bicarbonate (pH 9.0) according to the procedure used by Woolley et al. (1976). The samples were then assayed for radioactivity and protein to determine the moles bound of [^{14}C]cyclohexyl moiety/mole of enzyme.

[chx-1-^{14}C]CCNU Labeling of Partially Inactivated Chymotrypsin. Buffered solutions containing 0.476 μmol of chymotrypsin (2.38×10^{-4} M) were titrated with varying amounts of cyclohexyl isocyanate. The solutions were incubated for 20 min at which time 0.825 μmol of [*chx*-1-^{14}C]-

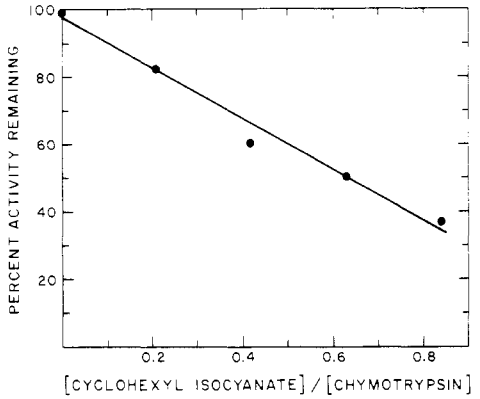

FIGURE 1: Titration of chymotrypsin (2.38×10^{-4} M in 0.1 M Tris-HCl, pH 7.7) by the sequential addition of small increments of 0.01 M cyclohexyl isocyanate in anhydrous acetone.

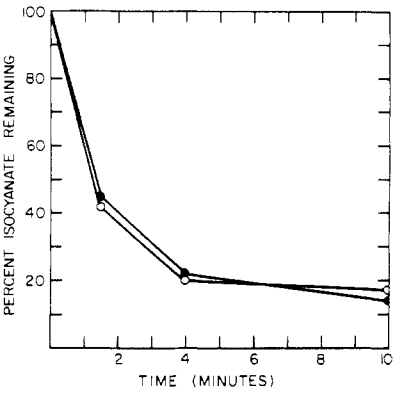

FIGURE 2: Degradation of octyl isocyanate (●), and cyclohexyl isocyanate (○) in 0.1 M Tris-HCl, pH 7.7, at 25 °C.

TABLE I: Titration of Chymotrypsin with Cyclohexyl Isocyanate. Cyclohexyl Isocyanate to Chymotrypsin Mole Ratio for 50% Inactivation.

Enzyme Concn (M)	Cyclohexyl Isocyanate/Chymotrypsin
4.76×10^{-6}	7.96
1.19×10^{-5}	4.20
2.38×10^{-5}	3.25
4.76×10^{-5}	1.29
1.10×10^{-4}	0.90
2.38×10^{-4}	0.63

CCNU was added. The reactions were allowed to proceed for 21–22 h at 25 °C. Enzymatic activity was determined prior to cyclohexyl isocyanate addition, 20 min after cyclohexyl isocyanate addition, and at 21–22 h. Aliquots (0.50 mL) were dialyzed against 8 M urea in 0.1 M Tris-HCl (pH 7.7) at 4 °C and then extensively in 0.1 M Tris-HCl until dialysis buffer contained only background radioactivity. The amounts of ^{14}C label and of protein were then determined as described above, and the cyclohexyl/chymotrypsin molar ratio was calculated. A similar experiment was performed using $PhCH_2SO_2F$ to inactivate the enzyme prior to ^{14}C labeling.

[chx-1-^{14}C]CCNU Labeling of Chymotrypsin in the Presence of L-*Lysine.* [*chx*-1-^{14}C]CCNU (0.850 μmol) was added to buffered solutions containing 0.476 μmol of chymotrypsin (2.38×10^{-4} M) and 0, 2, 10, and 20 μmol of L-lysine hydrochloride (0, 1, 5, and 10 mM). The reaction mixtures were treated and analyzed as described above.

Results

Cyclohexyl Isocyanate Inactivation of Chymotrypsin. Brown and Wold (1973a) have shown that *n*-alkyl isocyanates are active-site-directed inactivators of chymotrypsin. Results of this study demonstrate that cyclohexyl isocyanate reacts with chymotrypsin to inactivate the enzyme rapidly and irreversibly. A typical titration of chymotrypsin with cyclohexyl isocyanate is shown in Figure 1. As the enzyme concentration was increased (Table I), the molar ratio of cyclohexyl isocyanate added to chymotrypsin to achieve 50% enzyme inactivation became nearly mole/mole. For example, at 2.38×10^{-4} M chymotrypsin the cyclohexyl isocyanate to enzyme ratio for 50% inactivation was 0.63. This enzyme concentration effect is to be expected for a second-order reaction that must compete with another rapid reaction which in this instance is that between cyclohexyl isocyanate and Tris ions. The lack of reaction with water was concluded from the absence of cyclohexylamine formation in Tris buffer.

Cyclohexylamine Formation during Cyclohexyl Isocyanate Degradation. CCNU degrades via a free cyclohexyl isocyanate which is then capable of reacting with nucleophiles. Thus, it was deemed important to investigate the stability and reactivity of cyclohexyl isocyanate in aqueous solution. As Figure 2 indicates, the half-life of cyclohexyl isocyanate and octyl isocyanate are both approximately 1 min, as are the half-lives of several other alkyl isocyanates (Brown and Wold, 1973a).

Degradation of cyclohexyl isocyanate in 0.1 M Tris-HCl (pH 7.7) resulted in no detectable cyclohexylamine formation. Cyclohexyl isocyanate reacting with water would be expected to give a carbamic acid which decomposes to form cyclohexylamine. When degradation was allowed to proceed in 0.1 M phosphate buffer (pH 7.7) or in glass distilled water, the amount of cyclohexylamine formed was 44 ± 10% (± SE) and 72 ± 8% (± SE), respectively, of the theoretical yield. Thus, it appears that Tris and phosphate ions are carbamylated to an extent that is reflected in the relative nucleophilicity of these ions. The surprising degree to which chymotrypsin was inactivated by cyclohexyl isocyanate, even in the presence of competitive Tris ions (Tris/chymotrypsin ratio was 420), indicates the rapidity of "productive" binding of cyclohexyl isocyanate to the enzyme at a complimentary binding pocket adjacent to the active site.

Inactivation of Chymotrypsin during Incubation with CCNU, Quantitation of Cyclohexyl Isocyanate Formation. Since CCNU degrades to form cyclohexyl isocyanate, one might expect inactivation of chymotrypsin and that the extent of this inactivation would be proportional to the amount of CCNU degradation and thus to the amount of free isocyanate formed. The half-life of CCNU in buffered solution in the absence or presence of enzyme was the same, 3.6 h. Incubation of chymotrypsin with CCNU for 21–22 h resulted in near stoichiometric inactivation of the enzyme. It was noted that the length of the incubation determined the amount of inactivation, whereas direct addition of cyclohexyl isocyanate resulted in almost immediate enzyme inactivation. As will be discussed in greater detail in a following section, evaluation of the enzyme inactivation data was based on the assumption that any cyclohexyl isocyanate formed during CCNU degradation has an opportunity equivalent to that of added cyclohexyl isocyanate to inactivate chymotrypsin. Consequently, the extent of enzyme inactivation observed indicates that 70 ± 12% (±SE) of the CCNU degraded via a cyclohexyl isocyanate intermediate. When CCNU was allowed to degrade in

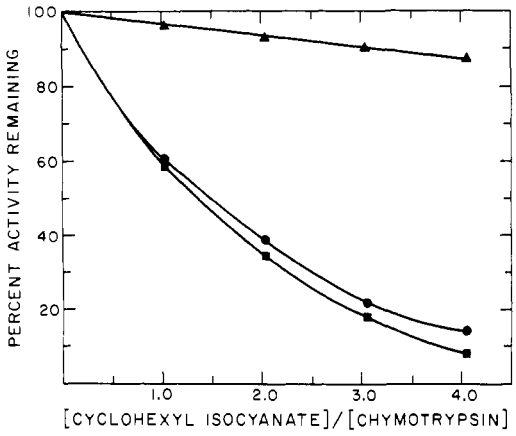

FIGURE 3: Titration of 4.76×10^{-5} M chymotrypsin with 0.01 M cyclohexyl isocyanate in the presence of 1.25×10^{-4} M CCNU (■), and absence of CCNU (●). A control to measure the extent of enzyme inactivation by 1.25×10^{-4} M CCNU was performed under similar conditions (▲). The control was assayed at time intervals (0, 7, 14, 21, and 28 min) corresponding to times of titration samplings. The reaction conditions and procedure are further described in the text.

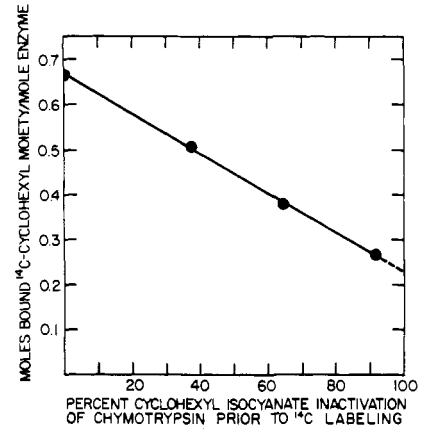

FIGURE 4: [*chx*-1-^{14}C]CCNU labeling of cyclohexyl isocyanate inactivated chymotrypsin. Reaction mixtures containing 2.38×10^{-4} M chymotrypsin were titrated to varying extents of enzyme inactivation with cyclohexyl isocyanate. The reaction mixtures were then incubated with 8.0×10^{-4} M [*chx*-1-^{14}C]CCNU for 21.6 h.

glass distilled water for 21 h followed by the quantitation of cyclohexylamine, 74 ± 6% (± SE) of the theoretical cyclohexylamine yield was found. These results demonstrate that CCNU degrades in such a manner as to generate a free cyclohexyl isocyanate intermediate and that the extent of this cyclohexyl isocyanate formation is at least 70% of the maximum possible yield.

Titration of Chymotrypsin with Cyclohexyl Isocyanate in the Presence of CCNU. Interpretation of the above results is based on two assumptions: (1) that undegraded CCNU does not effectively protect the enzyme from cyclohexyl isocyanate inactivation, and (2) that undegraded CCNU does not inactivate the enzyme. The following experiment was performed to substantiate these assumptions.

Incubations containing from 10^{-5} to 10^{-4} M enzyme were titrated with cyclohexyl isocyanate in the absence and presence of up to a tenfold excess of CCNU. Since the cyclohexyl isocyanate/chymotrypsin ratio required for 50% enzyme inactivation is an indication of the effectiveness of cyclohexyl isocyanate to inactivate the enzyme, any significant protection by CCNU would be evidenced by an increase in this ratio. As shown in Figure 3, this ratio did not increase but was observed to decrease slightly. This result can be explained on the basis of (a) the small calculated quantity of the cyclohexyl isocyanate formed from CCNU during the incubation period and (b) the observed amount of inactivation in the CCNU control (▲, Figure 3). These data indicate that CCNU must first degrade to form the isocyanate which then inactivates the enzyme, and that undegraded CCNU does not effectively protect the enzyme from cyclohexyl isocyanate inactivation under conditions used throughout this study.

Inactivation and Labeling of Chymotrypsin by [chx-1-^{14}C]CCNU. [*chx*-1-^{14}C]CCNU was used to accurately determine the extent of specific covalent modification of chymotrypsin by CCNU. [*chx*-1-^{14}C]CCNU was allowed to degrade in the presence of 2.38×10^{-4} M chymotrypsin (CCNU/enzyme = 1.3) in buffered solution at 25 °C for 21 h, followed by measurement of enzymatic activity and removal of noncovalently bound ^{14}C. It was noted that Sephadex G-25 chromatography was not sufficient to remove all noncovalently bound [^{14}C]cyclohexyl moiety. When the column fractions containing label and protein were dialyzed overnight against 8 M urea in 0.1 M Tris-HCl (pH 7.7) at 4 °C, 30% of the ^{14}C label was subsequently removed. The remaining label was found to be stable to dialysis against 5 M urea in 0.1 M ammonium bicarbonate (pH 9.0) at 4 °C and assumed to represent covalently bound [^{14}C]cyclohexyl moiety. At these concentrations (3.12×10^{-4} M CCNU and 2.38×10^{-4} M chymotrypsin), 90% of the total enzyme was inactivated. The appropriate controls indicated that 60% of the total enzyme was inactivated by CCNU and 30% of the enzyme was inactivated by acetone (5%, v/v) during the 21–22-h incubation period. Brown and Wold (1973a) observed a similar degree of acetone inactivation and demonstrated that only active enzyme reacted specifically with octyl isocyanate. The molar ratio of bound [^{14}C]cyclohexyl moiety to total enzyme in seven experiments was 0.70 ± 0.05. The ratio of bound label to CCNU-inactivated enzyme (total inactivated enzyme less acetone inactivation) was 1.11 ± 0.07. This stoichiometry is in agreement with that obtained during added cyclohexyl isocyanate inactivation of chymotrypsin and indicates up to 20% nonspecific carbamylation under these conditions.

^{14}C Labeling with [chx-1-^{14}C]CCNU of Chymotrypsin Partially Inactivated by Cyclohexyl Isocyanate and Phenylmethanesulfonyl Fluoride. The rapid and stoichiometric inactivation of the chymotrypsin by cyclohexyl isocyanate strongly indicates that it covalently binds to the active site of the enzyme, which exhibits a high specificity for the cyclohexyl moiety. It was possible to demonstrate further that either cyclohexyl isocyanate formed during CCNU degradation or directly added cyclohexyl isocyanate both display a similar high degree of active-site specificity during their inactivation of chymotrypsin. When [*chx*-1-^{14}C]CCNU was incubated with chymotrypsin which had been previously inactivated to varying extents by unlabeled cyclohexyl isocyanate, the decrease in bound [^{14}C]cyclohexyl moiety was found to be proportional to the extent of cyclohexyl isocyanate inactivation prior to ^{14}C labeling (Figure 4). Linear-regression analysis of these values and extrapolation to 100% inactivation with cyclohexyl isocyanate prior to addition of [*chx*-1-^{14}C]CCNU resulted in a value of 0.23 mol of [^{14}C]cyclohexyl moiety bound per mol of enzyme. A similar experiment was performed using $PhCH_2SO_2F$ to inactivate the enzyme prior to labeling with [*chx*-1-^{14}C]CCNU. The decrease in the bound [^{14}C]cyclohexyl moiety was again observed to be proportional to the extent of enzyme inactivation prior to labeling. Enzyme incu-

TABLE II: [*chx*-1-^{14}C]CCNU Labeling of Chymotrypsin in the Presence of L-lysine.[a]

L-Lysine Concn (mM)	Percent			[^{14}C]Cyclohexyl Moiety Bound/ mol of Total Enzyme	[^{14}C]Cyclohexyl Moiety Bound/mol of CCNU Inactivated Enzyme
	Total Enzyme Inactivation[b]	Acetone Control Inactivation[b]	[^{14}C]CCNU Inactivated Enzyme[b]		
0	90	26	64	0.70	1.10
1	89	24	65	0.61	0.93
5	88	27	62	0.65	1.04
10	85	21	64	0.60	0.94

[a] [*chx*-1-^{14}C]CCNU (0.850 μmol) was added to buffered solutions containing 0.476 μmol of chymotrypsin (2.38×10^{-4} M) and 0, 2, 10, and 20 μmol of L-lysine hydrochloride. The solutions were incubated for 21 h and then treated and analyzed as described in the text. [b] Values given as percent of total enzyme.

bations which had been 100% inactivated by $PhCH_2SO_2F$ and subsequently incubated with [*chx*-1-^{14}C]CCNU contained 0.23 mol of bound [^{14}C]cyclohexyl moiety per mol of enzyme. This value and the extrapolation in Figure 4 suggest that approximately 20% of the covalently bound label was not at the active site. This extent of nonspecific ^{14}C labeling is not unexpected, since the added [*chx*-1-^{14}C]CCNU to enzyme ratio was 1.8 in these incubations. Brown and Wold (1973a) also observed 20% nonspecifically bound [^{14}C]butyl isocyanate with chymotrypsin. $PhCH_2SO_2F$ has been demonstrated by Gold and Fahrney (1964) to react exclusively with the active-site serine of chymotrypsin. Thus, these results indicate that both cyclohexyl isocyanate and $PhCH_2SO_2F$ protect the active site from covalent labeling by [*chx*-1-^{14}C]CCNU and that cyclohexyl isocyanate formed during CCNU degradation inactivated the enzyme by carbamylation of the active site. The involvement of the active-site serine is indicated by its role in catalysis and the stable nature of the observed covalent modification. Due to the lability of carbamylimidazole above pH 6 (Stark, 1965), the carbamylation of the active-site histidine seems unlikely. Further support for the involvement of the active-site serine is given by the isolation of [^{14}C]butylcarbamylserine from chymotrypsin after treatment with [^{14}C]butyl isocyanate (Brown and Wold, 1973b).

Chymotrypsin Inactivation by Cyclohexyl Isocyanate in the Presence of L-Lysine. Wheeler and co-workers (1974) have utilized the extent to which L-lysine is carbamylated by various nitrosoureas as an indication of the carbamylation potential of those nitrosoureas that are capable of degrading via isocyanates. Since chymotrypsin has shown its capability to compete for cyclohexyl isocyanate in a nucleophilic environment, it was of interest to determine the extent to which the presence of L-lysine could prevent enzyme inactivation by cyclohexyl isocyanate. Chymotrypsin was titrated as previously described by the sequential addition of small amounts of cyclohexyl isocyanate in the presence of 0, 1, 5, and 10 mM L-lysine. Figure 4 summarizes the results of these incubations which show that the presence of L-lysine had no significant effect on inactivation by cyclohexyl isocyanate. Only 10 mM L-lysine had a slight effect on enzyme inactivation. This effect was not manifested until 60% of the enzyme had been inactivated and it resulted in only a 10% decrease in the extent of total enzyme inactivated. It must be emphasized that when 60% of the enzyme is inactivated the ratio of L-lysine to active enzyme is approximately 70. These data demonstrate that the binding of cyclohexyl isocyanate to the hydrophobic pocket of the enzyme and the subsequent carbamylation of chymotrypsin must be specific and very rapid in order to prevent any carbamylation of L-lysine.

[chx-1-^{14}C]CCNU Labeling of Chymotrypsin in the Presence of L-Lysine. [*chx*-1-^{14}C]CCNU labeling of the enzyme in the presence of L-lysine further demonstrated that the same high specificity and rapidity of binding exists for cyclohexyl isocyanate generated by the degradation of CCNU. When 4.0×10^{-4} M [*chx*-1-^{14}C]CCNU was allowed to degrade for 21 h in the presence of 2.38×10^{-4} M chymotrypsin and 0, 1, 5, and 10 mM L-lysine, no significant alteration was observed in the extent of enzyme inactivation (Table II). The L-lysine appears to decrease the amount of nonspecifically bound label but does not compete well enough to affect the active-site-directed labeling of the enzyme.

Discussion

Cyclohexyl isocyanate has been shown to be an active-site-directed reagent that is capable of stoichiometric and irreversible inactivation of chymotrypsin. It has been demonstrated that CCNU degrades in such a manner as to form cyclohexyl isocyanate with a 70% yield, as evidenced by active-site-specific inactivation of chymotrypsin and by cyclohexylamine yield in the absence of enzyme and any reactive nucleophile.

Brown and Wold (1973a) have postulated that octyl isocyanate inactivation of chymotrypsin proceeds in a two-step manner. The first step entails the binding of the *n*-alkyl side chain analogous to an enzyme–substrate complex formation. The second step has been demonstrated to involve the covalent linkage of the isocyanate group to the active-site serine hydroxyl group (Brown and Wold, 1973b). Carbamylation of chymotrypsin by cyclohexyl isocyanate appears to proceed in a similar manner, as indicated by: (a) the almost identical enzyme titration data obtained with octyl isocyanate (Brown and Wold, 1973a) and cyclohexyl isocyanate (Table I), (b) the near stoichiometric inactivation and stable ^{14}C labeling of chymotrypsin by [*chx*-1-^{14}C]CCNU (ratio of 1.11 ± 0.07), and (c) the proportional decrease in ^{14}C labeling after $PhCH_2SO_2F$ modification of the active-site serine.

The high degree of "productive" binding of the cyclohexyl side chain to chymotrypsin was expected from previous studies on the hydrophobic pocket of this enzyme by other workers, especially the x-ray diffraction study of Brown (1975). He demonstrated that the limiting binding efficiency should be with *n*-alkyl chains of 8 to 9 carbons. It was of interest to determine whether the nucleophilic environment of chymotrypsin in solution could alter the effectiveness of cyclohexyl isocyanate as an active-site-directed reagent. Thus, "productive" binding of the cyclohexyl side chain to the hydrophobic pocket of chymotrypsin was measured against the rapid reaction of the cyclohexyl isocyanate moiety with competing nucleophiles. Surprisingly, when using L-lysine as a competing nucleophile,

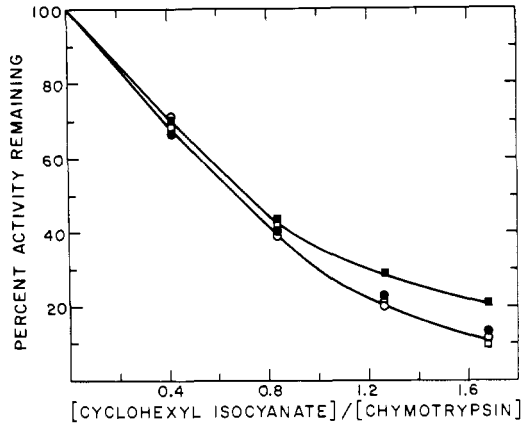

FIGURE 5: Chymotrypsin (2.38×10^{-4} M) was titrated with cyclohexyl isocyanate as described in the text (●). Similar titrations were carried out in the presence of 1 mM (□), 5 mM (○), and 10 mM (■) L-lysine hydrochloride.

a 70-fold excess of lysine (Figure 5) was required to demonstrate any decrease in the stoichiometric inactivation of chymotrypsin (2.38×10^{-4} M).

It might be assumed that degradation of cyclohexyl isocyanate in 0.1 M Tris buffer (pH 7.7) would lead to the formation of cyclohexylamine as the major degradation product due to the reaction of the isocyanate moiety with water and/or hydroxyl ion. However, this was not the case, since there was a complete absence of cyclohexylamine in Tris-buffered incubation mixtures as determined by DNBS-derivatization and HPLC analysis. In contrast, a 72% yield of cyclohexylamine was observed in glass distilled water and a 44% yield in 0.1 M phosphate buffer. Therefore, the role of competing nucleophiles is of considerable importance in determining the carbamylation products formed during the degradation of CCNU and other nitrosoureas.

Wheeler et al. (1975) incubated amino acids, peptides, and proteins in Tris and phosphate buffers with either [*chx*-1-^{14}C]CCNU, unlabeled CCNU, or cyclohexyl isocyanate. They observed carbamylation as evidenced by ^{14}C labeling and chromatographic R_f values of the reaction products. Since yields were not given, it is not possible to determine the degree to which the buffer ions successfully competed for reaction with cyclohexyl isocyanate. Thus, it was of interest to quantitate the extent of chymotrypsin inactivation by [^{14}C]cyclohexyl isocyanate formed during [*chx*-1-^{14}C]CCNU degradation. Again the effectiveness of "productive" binding of the CCNU generated cyclohexyl isocyanate with the enzyme-binding pocket was such that L-lysine concentration of up to 10 mM had essentially no effect on the stoichiometric ^{14}C labeling and inactivation of chymotrypsin (2.38×10^{-4} M).

These studies indicate that even in a concentrated nucleophilic environment chymotrypsin successfully competes for the reactive cyclohexyl isocyanate generated by CCNU degradation. This ability to compete successfully involved the rapid and "productive" binding afforded the enzyme by its hydrophobic binding pocket. A similar selective reaction with isocyanates has been shown to occur with transglutaminase (Gross et al., 1975) and alcohol dehydrogenase (Twu and Wold, 1973). The speculation that these and other such enzyme-binding sites exist in vivo seems reasonable and indicates that the carbamylating activity of nitrosourea-derived isocyanates may be more specific than previously thought. Thus, the structure of the isocyanates formed in vivo from CCNU and its monooxygenated metabolites (May et al., 1975) should be considered when attempting to elucidate the mechanism of the action of CCNU. These studies also indicate that attention should be directed toward the carbamylation of other amino acid side chains, analogous to the "activated" serine-195 of chymotrypsin, in addition to the carbamylation of lysine and N-terminal amino groups. Further work is now in progress to investigate the extent of protease inactivation and active-site-directed inactivation of other enzymes by those nitrosoureas currently being utilized as promising antineoplastic agents.

Acknowledgment

The authors are grateful to Dr. H. E. May for his criticism and advice during the preparation of this manuscript.

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