Active-Site-Directed Inhibition of α-Chymotrypsin by Deaminatively Produced Carbonium Ions: An Example of Suicide or Enzyme-Activated-Substrate Inhibition

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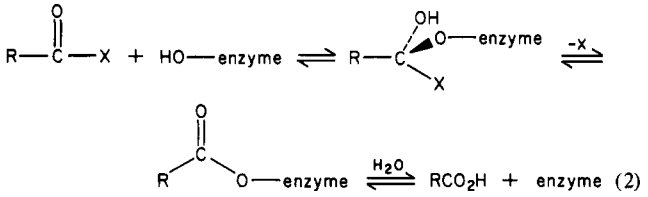
Abstract: *N*-Nitroso amides derived from phenylalanine and alanine were utilized as inhibitors of α-chymotrypsin. During the enzyme-catalyzed hydrolysis of these substrates, carbonium ions capable of alkylating nucleophilic groups are released in the active site. Nitroso lactams were also tested as substrates since they produce carbonium ions while still tethered to the enzyme at the acyl-enzyme stage. Kinetic studies indicated that at substrate/α-chymotrypsin ratios of 40:1 the acyclic substrates caused the following percent inhibition of α-chymotrypsin activity (substrate, percent inhibition): D-**1a**, 100; L-**1a**, <9; D-1C, 100; L-**1c**, 9. Nitroso lactams **2** and **3**, in substrate/enzyme ratios of 54:1 and 20:1, respectively, caused 91 and 97% inhibition of α-chymotrypsin activity. At low (<6:1) substrate/enzyme ratios, the inhibition of nitroso lactam **3** was partially reversible. The extents of inhibition were decreased by the competitive inhibitor *N*-acetyl-L-tryptophan, indicating that the inhibitor substrates were acting at the active site. Radioactive analogues of D- and L-**1a** and of **3** (^{14}C) provided evidence that the inhibition was irreversible, since ~1.0 mol of the benzyl group of D-**1a** and ~1.6 mol of the aryl moiety in the case of **3** remained bound to the inhibited enzyme after dialysis or Sephadex G-25 chromatography (no alkylation occurred with L-**1a**). The enzymatic hydrolyses of the L isomers of phenylalanine substrates (**1a,b**) were faster than those of the D enantiomers, whereas in the alanine series (**1c**) the rate ratio was reversed. A model based on "reverse" binding of the two aromatic groups of substrates D-**1a** and D-**1c** in the enzyme active site is proposed to explain the hydrolysis rates and the preferential inhibition of α-chymotrypsin by the D antipodes.

The high reactivity of carbonium ions derived from aliphatic amines via the corresponding diazonium ions and ion pairs[1,2] (eq 1) suggested that such ions could be used to alkylate nucleophilic

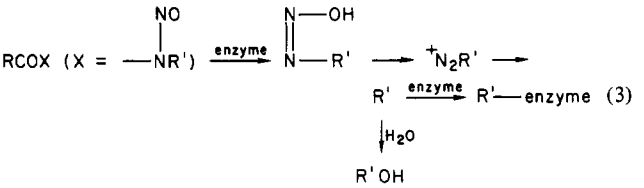
$$RNH_2 \xrightarrow{NO^+} RN_2^+ \xrightarrow{-N_2} R^+ \xrightarrow{\text{enzyme}} R\text{—enzyme} \quad (1)$$

groups on enzymes. We have utilized this approach to inhibit α-chymotrypsin[3-5] and now present details concerning this method of enzyme inhibition; the method shows promise for the mapping of the active sites of enzymes.[6]

α-Chymotrypsin catalyzes the hydrolysis of amides, esters, and other derivatives of carboxylic acids, as well as related derivatives of inorganic acids.[7] The catalysis is especially efficient for derivatives in which the acid moiety is furnished by an aromatic acid. The mechanism of action of α-chymotrypsin is believed to involve nucleophilic addition of the OH group of serine-195—at the active site—to the carbonyl group of the substrate (eq 2). Departure



of the leaving group X^- generates an enzyme ester (the "acyl-enzyme"), which normally is rapidly hydrolyzed to regenerate chymotrypsin.[8] Based on our previous studies of deamination chemistry, the nitrosoamino group was chosen as the leaving group X^- (eq 2 and 3) because of its potential for forming carbonium



ions. This grouping is the conjugate base of a strong acid, and thus the rate of hydrolysis of the RCOX compound by the enzyme was expected to be high. Furthermore, a sequence of fast reactions is known to convert the nitrosoamino group X^- into carbonium ions.[9,10] Lastly, deaminatively formed carbonium ions are extremely reactive entities[2] and they are capable of alkylating basic functional groups such as –OH, $-SCH_3$, –SS–, and aromatic rings on amino acid side chains,[11] as well as the amide NH and C=O functionalities of the protein backbone itself. Inhibition of the enzyme should result from such alkylation.[12]

In the enzyme inhibition described in the present work, a substrate innocuous to the enzyme is converted into a chemically active species by the catalytic apparatus of the enzyme; the subsequent reactions of this active species, which has been generated in the active site, with various functional groups of the

(1) White, E. H.; Woodcock, J. J. In "The Chemistry of the Amino Group"; Patai, S., Ed.; Wiley: New York, Chapter 8.
(2) White, E. H.; McGirk, R. H.; Aufdermarsh, C. A., Jr.; Tiwari, H. P.; Todd, M. J. *J. Am. Chem. Soc.* **1973**, *95*, 8107.
(3) White, E. H.; Roswell, D. F.; Politzer, I. R.; Branchini, B. R. *J. Am. Chem. Soc.* **1975**, *97*, 2290.
(4) White, E. H.; Jelinski, L. W.; Perks, H. M.; Burrows, E. P.; Roswell, D. F. *J. Am. Chem. Soc.* **1977**, *99*, 3171.
(5) White, E. H.; Roswell, D. F.; Politzer, I. R.; and Branchini, B. R. *Methods Enzymol.* **1977**, *46*, 216.
(6) White, E. H.; Perks, H. M.; Roswell, D. F. *J. Am. Chem. Soc.* **1978**, *100*, 7421.
(7) Bruice, T. C.; Benkovic, S. J. In "Bio-orgainic Mechanisms"; W. A. Benjamin: New York, 1966; Vol. I, Chapter 2. Zeffren, E.; Hall, P. L. In "The Study of Enzyme Mechanisms"; Wiley: New York, 1973.
(8) Bender, M. L. "Mechanisms of Homogeneous Catalysis from Protons to Proteins"; Wiley: New York, 1971.
(9) White, E. H.; Field, K. W. *J. Am. Chem. Soc.* **1975**, *97*, 2148.
(10) Moss, R. A.; Reger, D. W.; Emery, E. M. *J. Am. Chem. Soc.* **1970**, *92*, 1366.
(11) Alkylation of such groups and insertion into CH bonds by carbenes has been demonstrated in photoaffinity labeling by Westheimer; the carbenes were generated by the irradiation of α-diazoacyl derivatives of the enzymes: Shafer, J.; Baronowsky, P.; Laursen, R.; Finn, F.; Westheimer, F. H. *J. Biol. Chem.*, **1966**, *241*, 421. Singh, A.; Thornton, E. R.; Westheimer, F. H. *J. Biol. Chem.* **1962**, *237*, PC3006. Hexter, C. H.; Westheimer, F. H.; *ibid.* **1971**, *246*, 3928, 3934. Vaughan, R. J.; Westheimer, F. H. *J. Am. Chem. Soc.* **1969**, *91*, 217.
(12) Schoellman, G.; Shaw, E. *Biochemistry* **1963**, *2*, 252.

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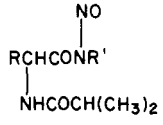
protein result in inhibition of the enzyme. This type of inhibition has been termed "suicide",[13] "k_{cat}",[14] or "Trojan Horse" inhibition;[15] we suggest the more descriptive term enzyme-activated-substrate inhibition (EASI) for the process.

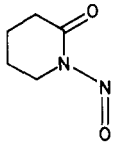
The EASI method has been the subject of intense study[16-23] largely because the chemical events leading to inhibition by this approach are more likely to be localized in the active site than when active-site-directed substrates (or affinity labels), which are intrinsically reactive molecules, are used. Most of the inhibitors that have been used in the above studies are direct descendents of the compounds used in the pioneering research of Bloch[24-26] in the sense that conjugated allenes or related systems are formed in the activation step, and subsequent inhibition of the enzyme occurs through alkylation of a nucleophilic center of the enzyme in a Michael-type reaction.[27] The EASI method described in the present work utilizes carbonium ions to alkylate the enzyme and, thus, it differs fundamentally from the "Michael-type" akylation approach.

Results and Discussion

N-Nitroso amides **1a-d**, based on phenylalanine and alanine,



1a (D and L), R = $C_6H_5CH_2$; R′ = $^{12}CH_2C_6H_5$ or $^{14}CH_2C_6H_5$
1b (D and L), R = $C_6H_5CH_2$; R′ = CH_2CH_3
1c (D and L), R = CH_3; R′ = $CH_2C_6H_5$
1d (L), R = $C_6H_5CH_2$; R′ = CH_3



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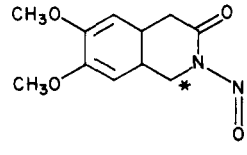


3, * = $^{12}CH_2$ or $^{14}CH_2$

N-nitrosovalerolactam (**2**), and *N*-nitroso-1,4-dihydro-6,7-dimethoxy-3(2*H*)-isoquinolinone (**3**), were examined as potential irreversible inhibitors of α-chymotrypsin. The nitroso lactams seemed especially attractive since they offered the advantage that diffusion of the carbonium ion out of the active site would be blocked as a result of the ion being an integral part of the acyl-enzymes (eq 2, Scheme I). Attention was initially focused on benzyl and benzyl-type carbonium ions since they lack β-hydrogens and thus are not destroyed by fast conversions into alkenes;[28] further, they were deemed to be active enough to react with typical nucleophiles on proteins. The bulky isobutyryl group was chosen as the acyl group in **1** in order to direct mononitrosation

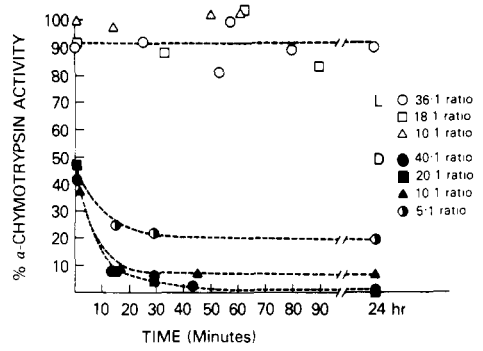
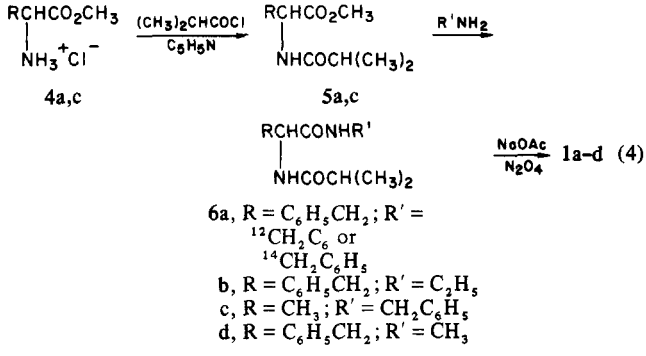


Figure 1. Percent α-chymotrypsin activity as a function of time after the addition of nitroso benzyl amide substrates D- and L-**1a**. The ratios are mole ratios of substrate to enzyme. Lines are drawn through the data points for the 36:1 run for the L isomer and for the 40:1, 10:1, and 5:1 runs for the D isomer.

to the *N*-benzyl amide moiety.

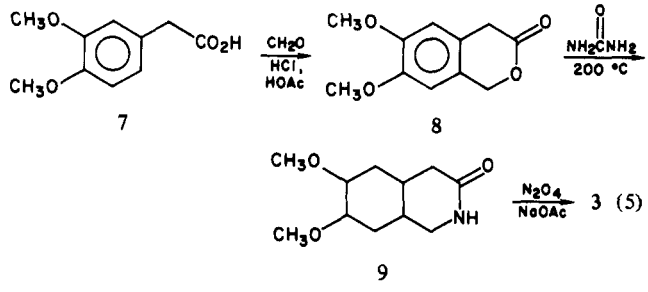
Syntheses. The *N*-nitroso amides were prepared by standard procedures (eq 4) using mild conditions to avoid racemization and



4a,c 5a,c 6a, R = $C_6H_5CH_2$; R′ = $^{12}CH_2C_6$ or $^{14}CH_2C_6H_5$
b, R = $C_6H_5CH_2$; R′ = C_2H_5
c, R = CH_3; R′ = $CH_2C_6H_5$
d, R = $C_6H_5CH_2$; R′ = CH_3

(4)

dinitrosation. Nitroso lactam **3** was prepared as shown in eq 5.



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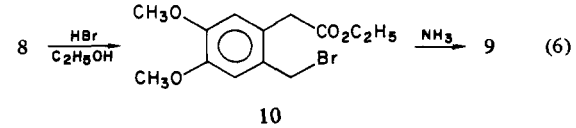
(5)

The synthesis of lactam **9** (mp 198–200 °C) using urea and lactone **8** is considerably shorter than the synthesis reported by McCorkindale and McCulloch.[29] Urea is commonly used to convert carboxylic acids into amides,[30] but we are unaware of any other applications of this approach to lactones. We have also synthesized **9** by the action of ammonia on the bromomethyl compound (**10**) prepared from the lactone **8** with hydrogen bromide in ethanol (eq 6), but we recommend the urea approach because of its directness and yields.



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(6)

Rosen and Popp[31] have reported that the same bromomethyl compound on treatment with hydrazine appeared to yield lactam **9** (mp 227–228 °C, with an elemental analysis correct for structure **9**). They also obtained the same material from the reaction of

(13) Abeles, R. H.; Maycock, A. L. *Acc. Chem. Res.* **1976**, *9*, 313.
(14) Rando, R. R. *Science* **1974**, *185*, 320.
(15) Miesowicz, F. M.; Bloch, K. *J. Biol. Chem.* **1979**, *254*, 5868–5877.
(16) Bechet, J. J.; Dupaix, A.; Yon, J.; Wakselman, J.; Robert, J. C.; Wilkas, M. *Eur. J. Biochem.* **1973**, *35*, 527.
(17) Bechet, J. J.; Dupaix, A.; Raucous, C.; Bonamy, A. M. *Biochimie* **1977**, *59*, 241.
(18) Marcotte, P.; Walsh, C. *Biochemistry* **1976**, *15*, 3070.
(19) Jung, M. J.; Metcalf, B. W.; Lippert, B.; Casara, P. *Biochemistry* **1978**, *17*, 2628.
(20) Soper, T. S.; Manning, J. M. *Biochemistry* **1978**, *17*, 3377.
(21) Metcalf, B. W.; Bey, P.; Danzin, C.; Jung, M. J.; Casara, P.; Vevert, J. P. *J. Am. Chem. Soc.* **1978**, *100*, 2551–2553.
(22) Seiler, M.; Jung, M. J.; Koch-Weser, J. "Enzyme-Activated Irreversible Inhibitors"; Elsevier/North-Holland Biomedical Press: New York, 1978.
(23) Jakoby, W. B.; Wilchek, M.; Eds. *Methods Enzymol.* 1977, *66*.
(24) Endo, K.; Helmkamp, C. M.; Bloch, K. *J. Biol. Chem.* **1970**, *245*, 4923.
(25) Bloch, K. *Enzymes* 1971, *5*, 441.
(26) Morisaki, M.; Bloch, K. *Biochemistry* **1972**, *11*, 309.
(27) Bergmann, E. D.; Ginsburg, D.; Pappo, R. *Org. React.* **1959**, *10*, 179.
(28) White, E. H.; Tiwari, H. P.; Todd, M. J. *J. Am. Chem. Soc.* **1968**, *90*, 4734–4736.
(29) McCorkindale, N. J.; McCulloch, A. N. *Tetrahedron* **1971**, *27*, 4653.
(30) Guthrie, J. L.; Rabjohn, N. "Organic Syntheses"; Wiley: New York, 1963; Collect. Vol. IV, p 513.
(31) Rosen, G.; Popp, F. D. *Can. J. Chem.* **1969**, *47*, 864.

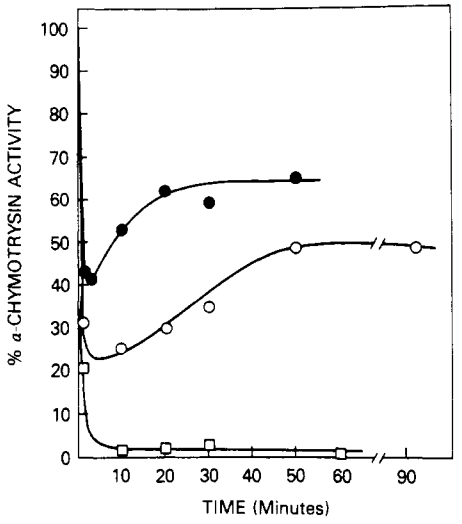


Figure 2. Effect of nitroso lactam **3** (final concentration 1.2×10^{-3} M) on α-chymotrypsin activity. The ratio of **3** to enzyme in 2.5-mL solutions of 0.05 M sodium phosphate buffer, pH 7.9 (4% acetonitrile), was as follows: closed circles, 3; open circles, 6; squares, 26.

Table I. Nitroso Amide Inhibition of α-Chymotrypsin

	% inhibition[a,b]				
substrate	substr/enz: 40:1	20:1	10:1	3000: 10:1[c]	other
D-**1a**	100	99	93	15	
L-**1a**	9	0	0		
D-**1b**					2[d]
L-**1b**					9[e]
D-**1c**	100	99	95	14	
L-**1c**	9				
2		84			91[d]
3		97		25[f]	98[g]
phenyldiazomethane					

[a] *N*-Benzoyl-L-tyrosine ethyl ester (BTEE) assay (Hummel, 1959), pH 7.9, 0.05 M phosphate buffer (<1% CH_3CN), 25 °C. Percent inhibition determined within 30 min after mixing substrate and enzyme, except for footnote *f* (time = 2 h). [b] Final enzyme concentration $\simeq 1 \times 10^{-5}$ M; operational normality determined by cinnamoylimidazole titration.[63] [c] *N*-acetyl-L-tryptophan/nitroso amide/enzyme ratio. [d] Substrate/enzyme = 54:1. [e] Substrate/enzyme = 120:1. [f] *N*-Ac-Trp/substrate/enzyme = 4000:10:1. [g] Substrate/enzyme = 26:1.

hydrazine with lactone **8**, followed by treatment of the product with hot 10% hydrochloric acid. We have obtained material with a melting point of 228–229 °C in trial runs utilizing the two approaches of Rosen and Popp, but the infrared spectra indicate that this product is a mixture which does not appear to contain lactam **9**.

Inhibition Studies. In view of the reported specificity of α-chymotrypsin for the L isomer of amino acids, the first substrate synthesized for this study, L-**1a**, was based on L-phenylalanine. In our early trials, L-**1a** led to only modest inhibition of the enzyme even when employed in a large molar excess.[3] The inhibition initially observed for the early samples of the nitroso amide prepared from L-phenylalanine (L-**1a**) was eventually traced to contamination by its D antipode, with racemization during nitrosation of amide **6a** being the source of the D isomer. The pure L isomer prepared under milder reaction conditions that avoided racemization did not inhibit the enzyme irreversibly, although it was rapidly hydrolyzed in a reaction catalyzed by the enzyme. The enantiomer (D-**1a**), on the other hand, proved to be a potent, active-site-directed, irreversible inhibitor of α-chymotrypsin. Substrate D-**1a** (and also substrates D-**1c**, **2** and **3**) caused essentially complete loss of catalytic activity of α-chymotrypsin (Table I, Figures 1 and 2).

Table II. Half-lives of Hydrolysis of Nitroso Amide Substrates with and without α-Chymotrypsin

		half-life, s	
substrate	substr/enz	with α-chymotrypsin (4.5×10^{-6} M)[a]	in buffer alone[a]
D-**1a**	1	~17[c]	~600[b]
L-**1a**	1	1.5[c]	~600[b]
D-**1b**	4	~54[d]	~600[b]
L-**1b**	1	1.4[c]	~600[b]
D-**1c**	10	~78[e]	265
L-**1c**	10	126[e]	265
L-**1d**	170	~60[f]	360
3	10	~120[g]	~420

[a] Medium = 0.05 M, pH 7.9, phosphate buffer (25 °C); rates for enzymatic hydrolysis uncorrected for irreversible inhibition by substrates **D-1a,c** and **3**. Buffer rates were measured at the same wavelengths as enzyme-catalyzed rates. [b] Estimates, since the substrates were not very soluble in the buffer and produced turbid solutions. [c] Recorded on a storage oscilloscope using stopped-flow techniques. Hydrolysis monitored by following the disappearance of the absorption band at 243 nm (log ε 3.81). [d] Measured using a conventional UV spectrometer by monitoring the disappearance of the absorption band at 242 nm (log ε 3.83). [e] Conditions as in footnote *d* (log ε 243 nm = 3.76). [f] [Enz] = 7×10^{-7} M for this run. [g] Followed at 420 nm (log ε 2.03) (30 °C).

The time of inhibition, as measured by stop–flow techniques, proved to be on the order of seconds. At the high concentrations of reagents used in the preparatory runs, however, precipitation of the inhibitor shifted the rate-limiting step to the rate of dissolution of the inhibitor, and 30-min inhibition periods were utilized. In later trials, a slow, constant addition of the inhibitor to the enzyme led to full inhibition within 5–10 min. *N*-Nitrosovalerolactam **2** was a less effective inhibitor of the enzyme than D-**1a**, and the pure L isomers L-**1a–c** did not lead to appreciable inhibition nor did D-**1b** or phenyldiazomethane (Table I). However, all of the nitrosoamides were hydrolyzed by α-chymotrypsin at a faster rate than by buffer alone (Table II). γ-Globulin did not accelerate the hydrolysis of nitroso amide L-**1a**; hence, the rate acceleration observed with α-chymotrypsin is not a general protein effect.

The disappearance of the *N*-nitroso absorption bands in the ultraviolet and visible regions of the spectrum were used to monitor the turnover of the substrates by the enzyme. The speed of the reactions of the nitroso amides with α-chymotrypsin (and presumably with other proteases), the irreversibility of the hydrolysis, and the ready monitoring of the hydrolysis at ~240 nm, as well as in a normally transparent region of the spectrum at 420 nm, recommend the nitroso amides for use in the assaying of enzyme activity and for active-site titrations.[32]

Elimination of the possibility that side products from buffer decomposition of the substrate D-**1a** were responsible for inhibition was achieved by the addition of fully hydrolyzed inhibitor to an α-chymotrypsin solution so that the final substrate/enzyme concentration was 44:1. Subsequent BTEE assay showed minimal loss of activity (~15%). The only products of the enzymatic hydrolysis of nitroso amide L-**1a** detectable by HPLC (Corasil II column) were benzyl alcohol and *N*-isobutyrylphenylalanine. No irreversible inhibition of α-chymotrypsin was observed with a 300-fold excess of *N'*-isobutyryl-*N*-benzylphenylalaninamide (L + DL), a 1000-fold excess of benzyl alcohol, or a 110-fold excess of sodium nitrite. The amide precursor of **1a** (**6a**) was not hydrolyzed by α-chymotrypsin in the times used for the nitroso amide. Hence, neither it nor HNO_2 (a possible hydrolysis product of the nitroso amide) could be acting to inhibit the enzyme. Similarly, a fully decomposed solution of nitroso lactam **3** caused

(32) Bender, M. L.; Begué-Cantón, M. L., Blakeley, R. L.; Brubacker, L. J.; Feder, J.; Gunter, C. R.; Kézdy, F. J.; Killheffer, J. V., Jr.; Marshall, T. H.; Miller, C. G.; Roeske, R. W.; Stoops, J. K. *J. Am. Chem. Soc.* **1966**, *88*, 5890–5913.

only an ca. 5% inhibition when added to the enzyme in a 110:1 molar ratio.

Although phenyldiazomethane was observed as a stable intermediate in the base-catalyzed decomposition of *N*-nitroso *N*-benzyl amide D-**1a** in acetonitrile, no phenyldiazomethane was detected upon decomposition of D-**1a** in 0.05% aqueous potassium hydroxide (the half-life of phenyldiazomethane is greater than 90 min under the latter conditions). Phenyldiazomethane was not extracted into pentane in a two-phase reaction of the *N*-nitroso *N*-benzyl amide D-**1a** with α-chymotrypsin in 0.05 M, pH 7.9, sodium phosphate buffer. Using stopped-flow techniques, the buildup of a transient intermediate with the UV maxima of phenyldiazomethane could not be detected (under these conditions, the half-life of phenyldiazomethane was ~7 s). Lastly, solutions of phenyldiazomethane did not inhibit the enzyme when added in a 110:1 substrate/enzyme molar ratio (Table I).

Competitive inhibition experiments with *N*-acetyl-L-tryptophan (*N*-Ac-Trp)[33] provided evidence that inhibition of α-chymotrypsin by D-**1a**, D-**1c**, and nitroso lactam **3** was active-site directed. Instead of the 93% inhibition observed for D-**1a** with a substrate/enzyme ratio of 10:1, only 15% inhibition was observed when *N*-Ac-Trp was added so that the ratio of *N*-Ac-Trp/inhibitor/enzyme was 3000:10:1. Similar competitive inhibition was seen for compound D-**1c** and for nitroso lactam **3** (Table I).

Relative Rates. Rigorous measurement of K_m and V_{max} was not possible for substrates D- and L-**1a** and D- and L-**1c** because of the insolubility of these compounds in aqueous buffers and because the D isomers inhibit the enzyme irreversibly. However, an estimate for V_{max} for L-**1a** was made by extrapolation of data points obtained in the narrow range where the Michaelis–Menten requirement that [S] > [E] was satisfied and where turbidity was not overwhelming. A plot of the initial reaction velocity vs. substrate concentration afforded an estimate of ca. 5×10^{-6} M s^{-1} for V_{max} for substrate L-**1a**, and ca. 10 mM for K_m. The latter value is similar to those reported for the α-chymotryptic hydrolysis of various amides.[34,35]

Estimates for the half-lives of substrates **1a–c** and **3** in the presence of α-chymotrypsin could be made at low substrate/enzyme molar ratios (where inhibition was low), and the values gave insight into their relative turnover rates. In the phenylalanine series (**1a,b**), the L substrates were hydrolyzed faster than their enantiomers; in alanine series **1c**, however, the rate ratio favored the D antipode (Table II).

Evidence for Irreversible Inhibition. Samples of nearly completely inactivated enzyme (>90%) obtained with substrate/enzyme ratios of 20:1 and 10:1 for nitroso benzyl amide D-**1a** and 26:1 for nitroso lactam **3** (Table I) were dialyzed exhaustively for 28 h at 4 °C against pH 7.9 sodium phosphate buffer (five bath changes, ~100-fold dilution each). In all of these trials, no activity (<2%) was regenerated and uninhibited control samples retained their complete activity (>98%).

Evidence for covalent attachment of the benzyl group of nitrosobenzyl amide D-**1a** to α-chymotrypsin was obtained by Sephadex G-25 chromatography of the enzyme inhibited in a 8.8:1 ratio with D-**1a** labeled in the $-N(NO)CH_2^-$ carbon with ^{14}C. Coincidence of the peak for protein absorbance and the radioactivity peak showed that the benzyl group of the inhibitor was either tightly associated with or covalently bound to α-chymotrypsin. After chromatography, an average α-chymotrypsin molecule contained 0.86 benzyl group. When α-chymotrypsin was inhibited with the nitrosobenzylamide D-**1a** in a 15:1 substrate/enzyme ratio and the product was dialyzed exhaustively, 0.97 benzyl group was irreversibly bound per enzyme molecule. A similar experiment, in which α-chymotrypsin was treated with radioactive L-**1a** in a 9.5:1 ratio, showed that no radioactivity remained bound to the enzyme (Table III); thus, random or surface alkylation of chymotrypsin by the nitroso amides is unlikely.

Table III. Incorporation of ^{14}C-Labeled Groups from Nitroso Amides **1** and **3** into α-Chymotrypsin

substrate	[α-chymotrypsin],[a] × 10^5 M	substr/enz	^{14}C groups per enz molecule[b]
D-**1a**	1.04	8.8	0.86[c]
	5.0	15	0.97
	1–4	9–18	1.0 ± 0.1[d]
L-**1a**	1.16	9.5	<0.01
3	2–7	25–29	1.4–1.9
	40	25	2.6

[a] Final concentration. [b] Values corrected for remaining active enzyme (generally 5–10%). [c] Ratio determined after purification of the protein by Sephadex G-25 chromatography. [d] Cited number = rounded-off value (considering experimental errors) of 0.97 ± 0.05, which is the arithmetical average of 11 determinations (method, ratio of benzyl groups to enzyme): gel filtration, 0.88, 0.97; dialysis vs. 0.001 M hydrochloric acid, 0.92, 0.97, 0.89, 0.97; dialysis vs. 0.05 M, pH 7.9, sodium phosphate buffer, 1.1, 0.99, 1.05; dialysis vs. water, 1.07, 0.95. In a separate experiment in which the initial concentrations of enzyme (affinity purified) and inhibitor were 1.37×10^{-5} and 3.96×10^{-4} M, respectively, a ratio of 1.06 mol of benzyl groups/enzyme was determined by dialysis of the inhibited enzyme vs. 0.001 M HCl.

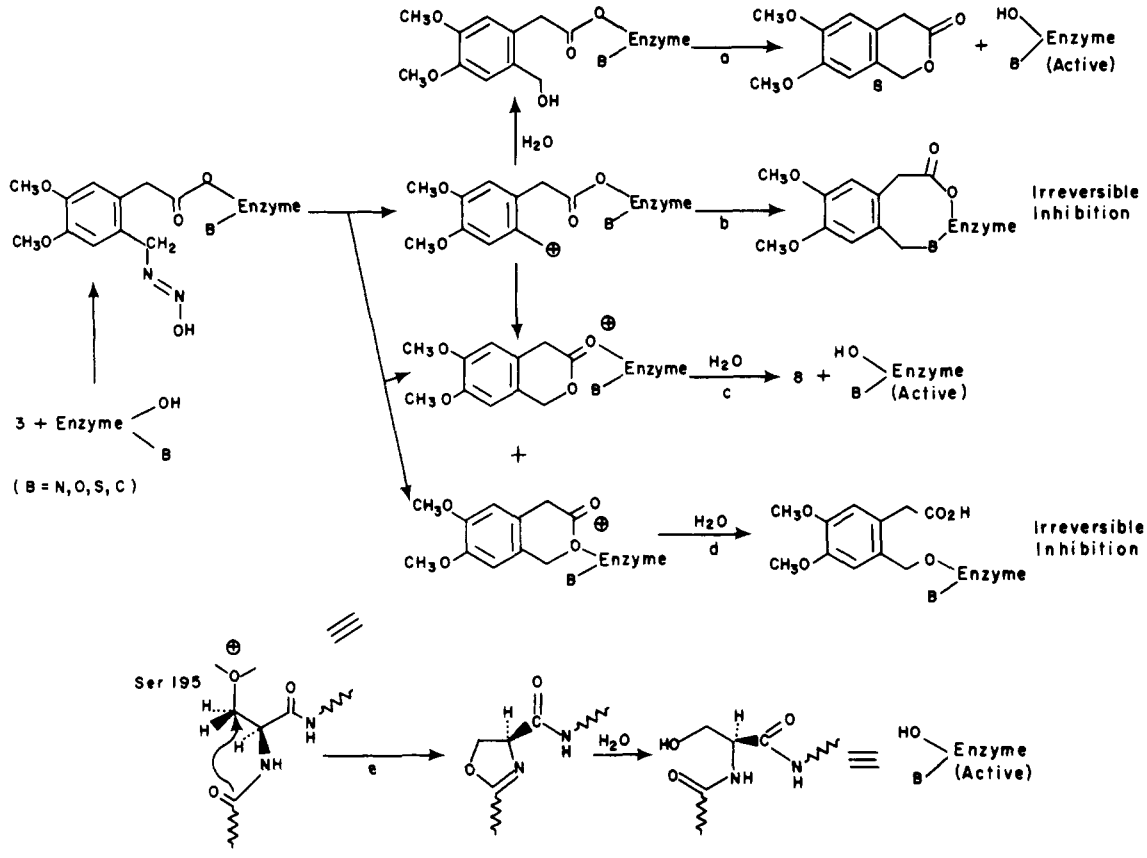
Measurements of the incorporation of ^{14}C-labeled nitroso lactam **3** into α-chymotrypsin showed that an average value of 1.6 mol of **3** was covalently bound when an excess (ca. 25- to 30-fold) of inhibitor **3** was added to the enzyme (ca. 2×10^{-5} M) (Table III). However, when a similar excess of **3** was added to more concentrated enzyme solutions ($3.4–4.0 \times 10^{-4}$ M), the amount of **3** incorporated per mole of enzyme rose to 2.6 equiv (Table III); at the higher concentrations, alkylation of a neighboring enzyme molecule may be occurring.[36,37]

Enzyme samples inactivated by D-**1a** (^{14}C/enzyme = 1) and by nitroso lactam **3** (^{14}C/enzyme = 1.6) had amino acid compositions, after hydrolysis with 6 N hydrochloric acid, identical (within experimental error) with that of the native enzyme; about one-half of the ^{14}C was retained in the amino acids. The amino acid composition for the control (native enzyme) was in very good agreement with published values.[38] The above facts indicate that either several alkylated amino acids, stable to 6 N hydrochloric acid,[6] are formed or that a residue present in large numbers (e.g., serine) had been modified and was not detected within the experimental error of the technique.[39] Identification of the sites of benzylation is in progress.[6]

Special Features of the Inhibition of α-Chymotrypsin by Nitroso Lactam 3. An unexpected facet of the inhibition by **3**, not seen with the acyclic nitroso amides, was a rapid regeneration of part of the enzymatic activity when a low ratio of inhibitor to enzyme was used. For example, when α-chymotrypsin was incubated with three- and sixfold excesses of nitroso lactam **3**, a decrease in enzyme activity to about 43 and 23%, respectively, of the original value was seen initially (~1 min). Activity was slowly regenerated, however, and it leveled off within 1 h to constant values of about 65 and 50% of the original activity (Figure 2). Nearly total (~98%) loss of enzyme activity was achieved when a 26-fold excess of compound **3** to enzyme was used, with no observed regeneration of activity within 1 h (open squares, Figure 2). Neither exhaustive dialysis nor treatment of the fully inhibited enzyme with hydroxylamine[40] restored the enzymatic activity to a value of more than 6% of the initial activity.

(33) Kézdy, F. J.; Clement, G. E.; Bender, M. L. *J. Am. Chem. Soc.* **1964**, *86*, 3690.
(34) Lehninger, A. L. "Biochemistry"; Worth Publishers: New York, 1970; p 154.
(35) Philipp, M.; Pollack, R. M.; Bender, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 517–520.
(36) Hexter, C. S.; Westheimer, F. H. *J. Biol. Chem.* **1971**, *246*, 3928–3933.
(37) Shafer, J.; Baronowsky, P.; Laursen, R.; Finn, F.; Westheimer, F. H. *J. Biol. Chem.* **1966**, *241*, 421–427.
(38) Nakagawa, Y.; Bender, M. L. *Biochemistry* **1970**, *9*, 259.
(39) Lawson, W. B.; Schramm, H. J. *Biochemistry* **1965**, *4*, 377.
(40) Dixon, G. H.; Dreyer, W. J.; Neurath, H. *J. Am. Chem. Soc.* **1956**, *78*, 4810.
(41) Tobias, P.; Heidema, J. H.; Lo, K. W.; Kaiser, E. T.; Kézdy, F. J. *J. Am. Chem. Soc.* **1969**, *91*, 202–203.

Scheme I. Inhibition of α-Chymotrypsin by Nitroso Lactam 3 and Partial Regeneration of Activity



An outline of the more reasonable reaction courses that might be followed by nitroso lactam **3** is given in Scheme I. Path a represents the hydrolysis of an ester of chymotrypsin. This reaction cannot account for the slow regeneration of activity (Figure 2) since, based on the following observations, hydrolysis of the acyl-enzyme derived from **3** must be a fast reaction. When 10 equiv of **3** is added to α-chymotrypsin (10^{-5} M at pH 7.9 in 0.05 M phosphate buffer at 30 °C), 2 molar equiv of **3** (corrected for buffer hydrolysis) is hydrolyzed in the 1st min of reaction. Thus, turnover is occurring rapidly. Similar results were obtained from a run with a substrate/enzyme ratio of 26. Paths b and d represent modes of irreversible inhibition of the enzyme. Path c represents a possible mode of regeneration of enzyme activity through hydrolysis of a carbonyl *O*-alkyl derivative of lactone **8**. Path e, involving a neighboring group reaction, is also a possibility for the reversible portion of the inhibition; similar imidate intermediates have been proposed for reactions of phenylmethanesulfonic acid esters of chymotrypsin[42] and also for the cyanogen bromide cleavage of methionine-containing proteins.[43]

N-Nitrosovalerolactam **2** also rapidly inactivated α-chymotrypsin (Table I), but large molar excesses of **2** over chymotrypsin were required (54:1). Carbonium ions may not be involved in this case; because of the relatively high energy of primary alkyl carbonium ions, the diazonium ion precursor has a relatively long lifetime and a displacement of nitrogen by a suitably placed nucleophile may be the reaction mode. Alkylations of this type have been suggested for the inhibition of enzymes by diazo ketones,[44] carbonium ions from which would also be relatively high in energy.

Stereochemical Conclusions. The current four-subsite view of the active site of α-chymotrypsin includes a hydrophobic aromatic binding cleft (*ar*), a pocket for the acylamino group (*am*), a region in which the cleaved amide or ester bond is positioned (*n*), and

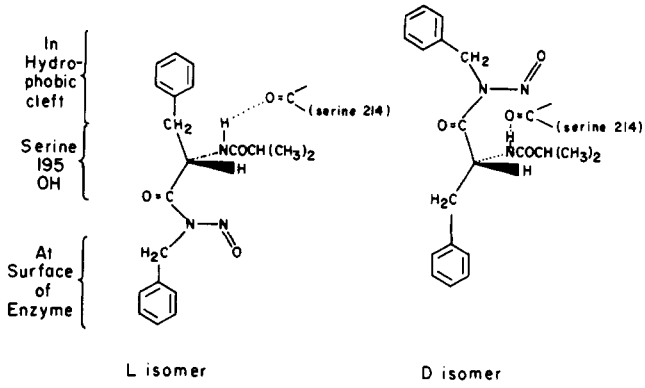


Figure 3. Schematic representation of the way a typical L substrate binds at the active site of α-chymotrypsin and the way that a D substrate possessing two aromatic groups can bind by reversing the positions of said groups.

a sterically restrictive area for the proton attached to the chiral center (*h*).[7] Binding stabilization is achieved by hydrogen bonding between the acylamino NH and the carbonyl group of serine-214 located near the *am* subsite and by a positioning of the aromatic side chain of the substrate in the hydrophobic *ar* region. The scissile substrate bond in the *n* subsite is then positioned near the active serine-195. These interactions dictate the normal L substrate specificity of α-chymotrypsin.

In this study we have shown that our L substrates, which do not inhibit α-chymotrypsin, are turned over by the enzyme. The D substrates (i.e., the *N*-benzyl *N*-nitroso amides D-**1a** and D-**1c**) are turned over by the enzyme but, in addition, they cause irreversible inhibition of the enzyme. In order to explain this unusual preferential inhibition, we must consider, in addition to the chirality of the active site, the location of the active site with respect to the surface of the enzyme and also the multiplicity of aromatic residues in the substrate.

In Figure 3, an L amino acid substrate (nitroso benzyl amide L-**1a**) is shown positioned schematically in the active site of α-

(42) Gold, A. M.; Fahrney, D. *Biochemistry* **1964**, *3*, 783.
(43) Gross, E. *Methods Enzymol.* 1967, *11*, 238.
(44) French, T. C.; Dawid, I. B.; Buchanan, J. M. *J. Biol. Chem.* **1963**, *238*, 2186.

chymotrypsin[45] with the functional groups in the proper subsites. From inspection of a molecular model of γ-chymotrypsin[46] (α- and γ-chymotrypsins have very similar active sites),[47] we conclude that the cleaved portion of the substrate (eq 3) would be released at the surface of the enzyme. In the case of L-**1a**, the benzyl carbonium ion formed from the cleaved portion would be in a position to be scavenged by the medium to produce benzyl alcohol; thus, alkylation of the enzyme might well not occur. When the D isomer is placed inside the active site of the model in the same fashion as outlined for the L isomer (that is, with the phenylalanyl aromatic and carboxyl moieties placed in the subsites in the normal way), the isobutyrylamino group encounters serious steric hindrance in the *h* subsite and the hydrogen bond to serine-214 is lost. Nitrosobenzylamide D-**1a** bears two aromatic groups and these are approximately equidistant from the carbonyl group ultimately attacked by serine-195. Molecular models show that if the D isomer binds in the manner shown in Figure 3 with the second aromatic residue in the *ar* subsite, the hydrogen bond to serine-214 can be maintained and the *h* subsite accommodates the hydrogen atom in the normal fashion. In this mode the cleaved portion (the benzyl carbonium ion) is imbedded in the *ar* hydrophobic cleft of the active site in a position to alkylate residues in or near the active site.

The view that compounds D-**1a** and D-**1c** are effective inhibitors because they bind in this "reverse" manner is supported by the rate data. Alanine substrates with non-aryl leaving groups are hydrolyzed by α-chymotrypsin with the normal enantiomeric specificity.[7,48] For D- and L-**1c**, however, the normal specficity is reversed and D-**1c** is hydrolyzed several times more rapidly than its L antipode (Table II).[49] Further, substrate D-**1a** (the nitrosobenzyl amide) is hydrolyzed approximately three times more rapidly than D-**1b** (the nitroso ethyl amide).

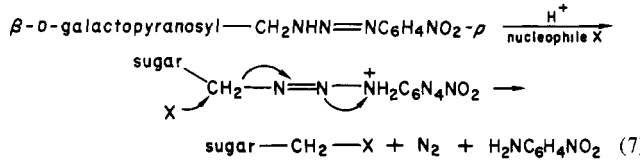
The observation that compounds D-**1b** and D-**1d** do not inhibit α-chymotrypsin irreversibly can be accounted for by the ocurrence of binding modes that would lead to the release of active species near the enzyme surface or by the formation of relatively long-lived species such as the corresponding diazoalkanes or diazohydroxides (favored by the high energy of the carbonium ion products formed in nitrogen-loss pathways).

The use of both enantiomers in enzymatic alkylation reactions has been reported in several instances. The L isomer of TPCK is a potent inhibitor of α-chymotrypsin,[12] whereas the D isomer has been reported to have no effect.[50] The reverse is true for the methyl ester of iodoacetyltryptophan.[51] Intimate knowledge of the reaction mechanism involved will be required before reliable predictions can be made concerning the activity of optical antipodes.[52]

Related Examples. Ethyl *N*-[L(−)-1-phenylethyl]-*N*-nitrosocarbamate has recently been used to inhibit hog liver esterase,[53] presumably, the mode of inhibition is closely related to that of the nitroso amides outlined in the present work.

Deaminatively formed carbonium ions are also available from other sources, e.g., triazenes.[54-56] Triazenes based on secondary and tertiary carbinamines react in acidic media to produce the corresponding carbonium ions, but the primary carbinamine analogues most probably carry out alkylations via displacement reactions involving either the protonated triazene or the corresponding diazonium ion.[9]

An example of the use of triazenes to inhibit enzymes was reported by Sinnott and Smith;[57] *Escherichia coli* galactosidase was found to be inhibited by a galactoside methyltriazene (eq 7).



(7)

The authors proposed that a carbonium ion derived from the triazene was the active species, but a displacement reaction would appear to be more likely (eq 7) in view of the high energy of the putative primary carbonium ion (see above). Sinnott and Smith identified the sulfur of methionine-500 as the site of alkylation (X in eq 7). This result is reminiscent of the alkylation of the thiol group of cysteine by the α-diazocarbonyl compounds azaserine and 6-diazo-5-oxo-L-norleucine (DON)[44,58] and by the diazoalkyl analogue of TPCK.[59] These alkylations at sulfur are consistent with the known high reactivity of sulfur in displacement reactions.[60] In other cases of enzyme inhibition involving displacement reactions, steric requirements, binding requirements, or the proximity of catalytic sites may force alkylation of a less nucleophilic center, e.g., oxygen, as in the inhibition of pepsin by diazoacetylphenylalanine methyl ester.[61]

The above references suggest that diazonium ions and precursors from primary systems and from α-diazocarbonyl compounds alkylate nucleophilic centers of an enzyme or protein in a rather discriminate way. In contrast, resonance-stabilized primary systems, as used in the present work (and presumably secondary and tertiary alkyl analogues), will form carbonium ions that react in a more random fashion and are thus more suitable for the mapping of active sites.

The major conclusions of the present study are that carbonium ions can be generated enzymatically, they are capable of alkylating the enzyme, and irreversible inhibition may occur in the process.

Experimental Section

General. Chemical shifts in the ^1H NMR spectra are reported in parts per million downfield from internal tetramethylsilane. Polarimetric measurements were obtained from a Perkin-Elmer 141 polarimeter at the sodium D line. Radioactivity was measured with a Beckman LS-133 liquid scintillation system or a Packard Tri-Carb liquid scintillation spectrometer using Bray's solution,[62] PCS solubilizer (Amershaw/Searle), or Aquasol (New England Nuclear).

Chemicals. α-Chymotrypsin (EC 3.4.4.5, salt free, three times recrystallized, Aldrich Chemical Co.) was either purified by CM-Sephadex C-50 chromatography according to the method of Nakagawa and Bender[38a] or it was used without further purification. Equivalent results were obtained in either case. In one ^{14}C-labeling experiment, the enzyme was purified by affinity chromatography.[63a] The active α-chymotrypsin concentration was determined by titration with *N-trans*-cinnamoylimidazole.[63b] Enzyme activity was assayed by measuring the change in

(45) Blow, D. M. *Acc. Chem. Res.* **1976**, *9*, 145.
(46) We thank Dr. David R. Davies of the National Institute of Arthritis, Metabolism, and Digestive Diseases for the use of this model.
(47) Segal, D. M.; Cohen, G. H.; Davies, D. R.; Powers, J. C.; Wilcox, P. E. *Cold Spring Harbor Symp. Quant. Biol.* **1971**, *36*, 85–90.
(48) Bow, D. M. *Enzymes* **1971**, *3*, 185.
(49) A trend in this direction had been noted earlier for the rate of chymotrypsin-catalyzed hydrolysis of the p-nitrophenyl esters of *N*-acetyltryptophan compared, for example, to the rate for the same ester of 2-(acetylamino)-4-methylpentanoic acid (Ingles, D. W.; Knowles, J. R. *Biochem. J.* **1967**, *104*, 369; **1968**, *108*, 561).
(50) Brown, J. R.; Hartley, B. S., "Abstracts of the First Meeting of the Federation of European Biochemical Societies"; Academic Press: London, 1964; p 25. Botvinik, M. M.; Novodarova, G. N.; D'yakov, V. L. *Khim. Prir. Soedin* **1968**, *4*(2), 116–120; *Chem. Abstr.* **1968**, *69*, 49384*s*.
(51) Gundlach, G.; Turba, F. *Biochem. Z.* **1962**, *335*, 573.
(52) Other examples of the stereochemical aspects of irreversible and reversible inhibition are given by Baker, B. R. "Design of Active-Site-Directed Irreversible Enzyme Inhibitors"; Wiley: New York, 1967; pp 138–142; ref 7, pp 225–228; and ref 8, pp 179–186.
(53) Gold, B.; Linder, W. B. *J. Am. Chem. Soc.* **1979**, *101*, 6772–6773.
(54) White, E. H.; Scherrer, H. *Tetrahedron Lett.* **1961**, 758.
(55) White, E. H.; Baum. A. A.; Eitel, D. E. *Org. Synth.* **1968**, *48*, 102.
(56) White, E. H.; Maskill, H.; Woodcock, D. J.; Schroeder, M. A. *Tetrahedron Lett.* **1969**, 1713.
(57) Sinnott, M. L.; Smith, P. J. *Biochem. J.* **1978**, *175*, 525.
(58) David, I. B.; French, T. C.; Buchanan, J. M. *J. Biol. Chem.* **1963**, *238*, 2186.
(59) Leary, R.; Larsen, D.; Watanabe, H.; Shaw, E. *Biochemistry* **1977**, *16*, 5857.
(60) Hendrickson, J. B.; Cram. D. J.; Hammond, G. S. "Organic Chemistry"; McGraw-Hill: New York, 1970; p 393.
(61) Bayliss, R. S.; Knowles, J. R.; Wybrandt, G. B. *Biochem. J.* **1969**, *113*, 377.
(62) Bray, G. A. *Anal. Biochem.* **1960**, *1*, 279.
(63) (a) "Affinity Chromatography; Principles and Methods"; Pharmacia Fine Chemicals: Uppsala, Sweden, 1977; p 23. (b) Schonbaum, G. R.; Zerner, B.; Bender, M. L. *J. Biol. Chem.* **1961**, *236*, 2930.

absorbance at 256 nm during the hydrolysis of benzoyl-L-tyrosine ethyl ester (BTEE).[64] CM-Sephadex C-50 and *N*-acetyl-L-tryptophan were Sigma Chemical Co. products and benzoyl-L-tyrosine ethyl ester, *N*-*trans*-cinnamoylimidazole, and hydrocinnamic acid were obtained from the Aldrich Chemical Co. Phenyldiazomethane was prepared according to Closs and Moss.[65] [^{14}C]Formaldehyde, [1-^{14}C]benzoic acid, and [*methylene*-^{14}C]benzylamine hydrochloride were obtained from ICN Pharmaceuticals, Inc.

Nitroso Amide Substrates. Nitroso amides **1a–c**, **2**, and **3** can be stored indefinitely at −20 °C in a dry atmosphere. They are stable in nonpolar solvents for hours at 25 °C, but they decompose rapidly in alcohols or in aqueous media (vide infra). These compounds may be carcinogenic based on the structural relationship to known carcinogens, such as *N*-nitrosomethylurea, and precautions should be taken against exposure. It appears, however, that activity is absent, or much reduced, in nitroso amides of the higher molecular weight amines.[66] Compounds of this type can be mutagenic.[67]

D-Phenylalanine Methyl Ester Hydrochloride (D-4a). D-Phenylalanine (2.50 g, 15.1 mmol) was suspended in a mixture of 130 mL of 2,2-dimethoxypropane and 13 mL of concentrated hydrochloric acid in a vessel bearing a drying tube. After 48 h at room temperature (with occasional mixing), the reaction mixture was evaporated to dryness under aspirator pressure to yield a red-black residue. After three recrystallizations from methanol/ether, 2.78 g (85%) of fine needles was obtained: mp 158–159.5 °C (lit.[68] 158 °C for the L form); IR (KBr), 1742 and 1240 cm^{-1}; NMR (D_2O, internal acetone reference, values converted to parts per million from Me_4Si) δ 3.17 (dd, 2 H, *J* = 4.25 and 1.25 Hz), 3.71 (s, 3 H), 4.30 (t, H, *J* = 4.25 Hz), 7.1–7.35 (m, 5 H); $[\alpha]^{24}_D$ −37.0° (*c* 2, 95% EtOH).

***N*-Isobutyryl-D-phenylalanine Methyl Ester (D-5a).** Methyl ester D-**4a** (8.29 g, 38.4 mmol) was suspended in 250 mL of benzene and the mixture was cooled to 5 °C. Dry pyridine (12 mL, 148 mmol) was added, followed by the dropwise addition of isobutyryl chloride (8 mL, 76.8 mmol) in 50 mL of benzene over 50 min. Stirring and cooling were continued for an hour. The reaction mixture was washed with 100 mL of 3% hydrochloric acid, and the aqueous phases were extracted with two portions of benzene. The combined benzene layers were washed with 100 mL of 5% aqueous sodium carbonate and the solution was dried (sodium sulfate) and evaporated to afford 9.97 g of crude product. Recrystallization from hot hexane gave 6.36 g (67%) of long white needles: mp 66.5–68 °C; IR (KBr) 3300, 1730, 1640 cm^{-1}; NMR ($CDCl_3$) δ 1.08 (d, *J* = 8 Hz, 6 H), 2.33 (septet, *J* = 8 Hz, 1 H), 3.12 (dd, *J* ≃ 2 and 8 Hz, 2 H), 3.75 (s, 3 H), 4.90 (m, 1 H), 7.0–7.4 (m, 5 H); $[\alpha]^{26}_D$ −9.39° (*c* 2.09, CH_3CN). TLC on silica gel (benzene/methanol, 9:1) gave a single spot. Anal. ($C_{14}H_{19}NO_3$) C, H, N.

***N*-Isobutyryl-L-phenylalanine Methyl Ester (L-5a)** was synthesized as described for its antipode in 58% yield starting with L-**4a**: mp 67.5–68.5 °C; $[\alpha]^{21}_D$ +9.29° (*c* 1.91, CH_3CN). Anal. ($C_{14}H_{19}NO_3$) C, H, N.

***N*-Benzyl-*N'*-isobutyryl-D-phenylalaninamide (D-6a).** Methyl ester D-**5a** (1.0 g, 4.02 mmol) was dissolved in 1.2 mL of benzene containing benzylamine (0.44 mL, 4.02 mmol). The solution was degassed and sealed in glass under oil-pump vacuum; the tube was heated at 115 °C for 20 h. After evaporation, the resulting product was recrystallized from acetone/hexane to afford 0.816 g (63%) of white needles: mp 175–177 °C; IR (KBr) 1630, 1532 and 693 cm^{-1}; NMR ($CDCl_3$) δ 1.00 (d, *J* = 7 Hz, 6 H), 2.28 (septet, *J* = 7 Hz, 1 H), 3.07 (d, *J* = 7 Hz, 2 H), 4.27 (m, 2 H), 4.78 (m, 1 H), 6.95–7.30 (m, 10 H); $[\alpha]^{26}_D$ +11.7° (*c* 1.73, $CHCl_3$). TLC on silica gel (benzene/methanol, 9:1) gave a single spot. Anal. ($C_{20}H_{24}N_2O_2$) C, H, N.

***N*-Benzyl-*N'*-isobutyryl-L-phenylalaninamide (L-6a)** was prepared in 63% yield as described for D-**6a**: mp 180–180.5 °C; $[\alpha]^{25}_D$ −9.7° (*c* 1.65, $CHCl_3$). Anal. ($C_{20}H_{24}N_2O_2$) C, H, N.

***N*-Nitroso-*N*-benzyl-*N'*-isobutyryl-D-phenylalaninamide (D-1a).** Compound D-**6a** (1.0 g, 3.1 mmol) was dissolved in 100 mL of methylene chloride and the solution was cooled to 0 °C in an ice bath. Anhydrous sodium acetate (1.77 g, 21.6 mmol) was added, followed by liquid dinitrogen tetroxide (0.78 mL, 12.4 mmol), which was introduced slowly under the surface of the stirred solution. After stirring for 25 min, the solution was filtered, washed with 100 mL of 3% aqueous sodium bicarbonate, and dried over anhydrous sodium sulfate. After the solvent was evaporated under vacuum at room temperature, the solid residue obtained was dissolved in chloroform and chromatographed on silica gel (benzene/chloroform, 9:1). The fast-moving yellow band was collected and the solvent was removed under vacuum to give 0.72 g (2 mmol, 65%) of yellow crystals. Two recrystallizations (chloroform/hexane, 1:3) were required to obtain material with constant optical rotation: mp 109.5–110 °C; IR (KBr) 1731, 1645, 1501, 1373, 1240, 1057, 689 cm^{-1}; UV (CH_3CN) λ_{max} 241 nm (log ε 3.81); vis (CH_3CN) λ_{max} 428 nm (ε 94), 409 (88), 394 (sh); $[\alpha]^{24}_D$ −48.32° (*c* 0.84, CH_3CN). Anal. ($C_{20}H_{23}N_3O_3$) C, H, N.

***N*-Nitroso-*N*-benzyl-*N'*-isobutyryl-L-phenylalaninamide (L-1a)** was prepared as described for D-**1a**, except that the dinitrogen tetroxide (16.8 mL) was added as a solution in 1 mL of cold methylene chloride. After chromatography, a yield of 51% was obtained. Recrystallization (chloroform/hexane, 1:3) gave yellow crystals: mp 109.5–110 °C; $[\alpha]^{22.5}_D$ +47.06° (*c* 0.68 CH_3CN).

Nitrosation for prolonged periods or at high temperatures led to racemization; prior to this discovery, specific rotations of about 20° were obtained for L-**1a**.

***N*-Methyl-*N'*-isobutyryl-L-phenylalaninamide (L-6d).** *N*-Isobutyryl-L-phenylalanine methyl ester L-**6a** (0.98 g, 3.95 mmol) in 4.3 mL of benzene and methylamine (∼1 mL, ∼25 mmol) were heated in an evacuated, sealed tube at 50 °C for 16 h. A precipitate was observed after 4 h. The tube was opened and warmed to remove excess methylamine, and the residue was extracted with methanol. The solution was then taken to dryness in vacuo. The material obtained was recrystallized from acetone to yield amide L-**6d** (0.85 g, 3.4 mmol, 87%): mp 119–119.5 °C; IR (KBr) 1660 (sh), 1640, 1540 cm^{-1}; NMR ($CDCl_3$) δ 1.08 (6 H, d), 2.3 (1 H, m), 2.7 (3 H, d), 3.02 (2 H, d), 4.65 (1 H, m), 6.22 (2 H, s), 7.22 (5 H, m).

***N*-Nitroso-*N*-methyl-*N'*-isobutyryl-L-phenylalaninamide (L-1d)** was prepared by nitrosation of the corresponding amide as described for compound D-**1a**. The crude product was recrystallized from methylene chloride–cyclohexane at 5 °C to give a yellow solid (1.89 g, 6.85 mmol, 85%): mp 138 °C; IR (KBr) 1730, 1650, 1550, 1505 cm^{-1}; UV (CH_2Cl_2) 427 nm (ε 155), 408 (152), 392 (sh) (104.5); NMR ($CDCl_3$) δ 7.5 (5 H, m), 6.2 (2 H, m), 3.15 (2 H, m), 3.03 (3 H, s), 2.4 (1 H, m), 1.05 (6 H, d). Anal. ($C_{14}H_{19}N_3O_3$) C, H, N.

***N*-Ethyl-*N'*-isobutyryl-L-phenylalaninamide (L-6b).** *N'*-Isobutyryl-phenylalanine methyl ester L-**5a** (0.956 g, 3.84 mmol) in 1.5 mL of benzene and ethylamine (1 mL) in a glass tube were degassed; the tube was sealed under oil-pump vacuum and heated at 50 °C for 6 h. After cooling, the crystalline product was isolated and recrystallized from hot acetone to give 0.75 g (75%) of white needles: mp 193–194 °C; IR (KBr) 1636, 1540 cm^{-1}; NMR ($CDCl_3$) δ 1.00 (t, *J* = 7 Hz, 3 H), 1.10 (d, *J* = 7 Hz, 6 H), 2.40 (septet, *J* = 7 Hz, 1 H), 3.11 (br dd, 2 H), 3.2 (m, 2 H), 4.68 (m, 1 H), 7.29 (s, 5 H); $[\alpha]^{25}_D$ −5.3° (*c* 1.37, $CHCl_3$). Anal. ($C_{15}H_{22}N_2O_2$) C, H, N.

***N*-Ethyl-*N'*-isobutyrl-D-phenylalaninamide (D-6b)** was prepared from D-**6a** as described above for L-**6b**. The material was isolated in 65% yield: mp 185–186 °C; $[\alpha]^{25}_D$ +3.3° (*c* 2.12, $CHCl_3$).

***N*-Nitroso-*N*-ethyl-*N'*-isobutyryl-D-phenylalaninamide (D-1b)** was prepared using the method described for compound D-**1a**. The oily product was chromatographed on silica gel (benzene/chloroform, 9:1). The fast-moving yellow band was collected, the solvent was removed by evaporation, and the solid residue was recrystallized from chloroform/hexane (1:3) to give a 41% yield of yellow crystals: mp 95.8–96.2 °C dec; UV (CH_3CN) λ_{max} 242 (ε 6836); vis (CH_3CN) λ_{max} 428 (ε 106), 409 (103), 394 (shoulder), $[\alpha]^{21}_D$ −47.65° (c 1.47, CH_3CN). Anal. ($C_{15}H_{61}N_3O_3$) C, H, N.

***N*-Nitroso-*N*-ethyl-*N'*-isobutyryl-L-phenylalaninamide (L-1b)** was prepared from L-**6b** using the method described for D-**1b** in 22% yield: mp 94–96 °C; $[\alpha]^{26}_D$ +46.98° (*c* 1.27, CH_3CN).

***N*-Isobutyryl-D-alanine Methyl Ester (D-5c).** D-Alanine methyl ester, prepared from D-alanine using the method described for compound **4a** (in 79% yield) was treated with isobutyryl chloride, as described for the phenylalanine derivative D-**5a**, to afford 81% of the amide: mp 55.5–57.0 °C (from hexane/benzene); IR (nujol) 1740, 1645 cm^{-1}. Anal. ($C_8H_{15}NO_3$) C, H, N.

***N*-Isobutyryl-L-alanine Ethyl Ester (L-5c)** was prepared from L-alanine ethyl ester hydrochloride by the procedure outlined for D-**5a** (19% yield after crystallization from hexane): mp 50.0-50.5 °C; IR (KBr) 1720, 1640 cm^{-1}. Anal. ($C_9H_{17}NO_3$) C, H, N.

***N*-Benzyl-*N'*-isobutyryl-D-alaninamide (D-6c)** was synthesized via the procedure described for compound D-**6a**. The product was recrystallized from acetone (23 and 40% yields): mp 176–176.5 °C, IR (KBr) 1635, 1545 cm^{-1}; NMR ($CDCl_3$) δ 1.04 (dd, *J* = 5 and 5 Hz, 6 H), 1.36 (d, *J* = 7.5 Hz, 3 H), 2.31 (m, 1 H), 4.31 (d, *J* = 6.5 Hz, 2 H), 4.56 (m, 1 H), 6.46 (br s, 1 H), 1.17 (s, 5 H), 7.34 (br s, 1 H); $[\alpha]^{23}_D$ +51.9° (*c* 1.03, $CHCl_3$).

***N*-Benzyl-*N'*-isobutyryl-L-alaninamide (L-6c)** was synthesized from L-**5c** in 27% yield in an analogous procedure to that used for D-**6c**: mp 175.6–176.3 °C; $[\alpha]^{24}_D$ −46.5° (*c* 2.09, $CHCl_3$).

(64) Hummel, B. C. W. *Can. J. Biochem. Physiol.* **1959**, *37*, 1393.
(65) Closs, G. L.; Moss, R. A. *J. Am. Chem. Soc.* **1964**, *36*, 4042.
(66) Weisburger, E., private communication, 1976.
(67) Brundrett, R. B.; Colvin, M.; White, E. H.; McKee, J.; Hartmann, P. E.; Brown, D. L. *Cancer Res.* **1979**, *39*, 1328–1333.
(68) Curtius, H.; Müller, E. *Chem. Ber.* **1904**, *37*, 1266.

***N*-Nitroso-*N*-benzyl-*N'*-isobutyryl-D-alaninamide (D-1c).** Compound D-**6c** (92.0 mg, 0.371 mmol) was dissolved in 20 mL of methylene chloride and the solution was cooled to −23 °C in a dry ice/carbon tetrachloride slush. Anhydrous sodium acetate (0.213 g, 2.6 mmol) was added, followed by gaseous dinitrogen tetroxide (30 mL, 1.11 mol), which was introduced slowly under the surface of the stirred mixture. The mixture was warmed to −5 °C and stirred for 20 min. Filtration yielded a yellow solution, which was washed with 50 mL of 3% aqueous sodium bicarbonate. The solution was dried (anhydrous sodium sulfate) and evaporated in vacuo to afford compound D-**1c** in 53–70% yields: mp 95.5–96 °C dec (recrystallization from $CHCl_3/C_6H_{14}$ raised the melting point to 105 °C dec); IR (KBr) 1740, 1650 cm^{-1}, UV (CH_3CN) λ_{max} 241 nm (ϵ 5733); vis (CH_3CN) λ_{max} 425 nm (ϵ 61), 408 (63), 393 (43); NMR ($CDCl_3$) δ 1.20 (d, J = 6.5 Hz, 6 H), 1.51 (d, J = 8 Hz, 3 H), 2.52 (m, 1 H), 5.03 (s, 2 H), 5.97 (m, 1 H), 6.48 (br s, 1 H), 7.42 (s, 5 H); $[\alpha]^{26}_D$ −8.9° (c = 1.01, CH_3CN). Anal. ($C_{14}H_{19}N_3O_3$) C, H, N.

***N*-Nitroso-*N*-benzyl-*N'*-isobutyryl-L-alaninamide (L-1c)** was prepared from L-**6c** in 26% yield by the procedure described for D-**1c**: mp 94–95 °C, $[\alpha]^{23}_D$ +11.4° (c 0.675, CH_3CN).

***N*-Nitroso-*N*-[*methylene*-^{14}C]benzyl-*N'*-isobutylryl-D- and -L-phenylalaninamide (Radioactive D-1a)** was prepared from [*methylene*-^{14}C]-benzylamine hydrochloride using the procedures described for nonradioactive D-**1a**. Activity = 8.5×10^6 cpm/mg. Radiochemical purity was ensured by co-crystallization with nonradioactive D-**1a** to constant activity. In some cases, the labeled benzylamine was prepared from [*carboxy*-^{14}C]benzoic acid; this compound was converted in sequence into benzoyl chloride and benzamide, and the amide was reduced to [*methylene*-^{14}C]benzylamine with lithium aluminum hydride in refluxing tetrahydrofuran.[69] The L isomer of ^{14}C-labeled **1a** was prepared by the procedure described for labeled D-**1a**; activity = 2.33×10^6 cpm/mg.

***N*-Nitrosovalerolactam (2)** was prepared from valerolactam by the method of Huisgen and Reinertshofer.[70]

6,7-Dimethoxy-3-isochromanone (8) was prepared from homoveratic acid and formaldehyde in 66% yield by the method of Finkelstein and Brossi.[71]

1,4-Dihydro-6,7-dimethoxy-3(2*H*)-isoquinolinone (9) was prepared in 42% yield from 6,7-dimethoxy-3-isochromanone (**8**) and urea.[5] It was also prepared from ethyl 2-(bromomethyl)-4,5-dimethoxyphenylacetate (**10**), which was in turn synthesized from isochromanone **8** as described by Finkelstein and Brossi.[71] The total sample of bromomethyl compound **10**, prepared from 4.2 g (20 mmol) of isochromanone **8**, was dissolved in 75 mL of absolute ethanol and a moderate stream of gaseous ammonia was passed through the solution for 10 min; a cooling bath was required to maintain a temperature of 25 °C. Upon cooling the solution in a refrigerator overnight, crystals separated weighing 0.85 g (4 mmol, 20%). Recrystallization from ethanol yielded crystals of **9** melting at 199–207 °C; physical data showed this material to be identical with that obtained by the urea approach.[5]

The reaction of the bromomethyl compound **10** with hydrazine[31] and the reaction of isochromanone **8** with hydrazine, followed by treatment with hot 10% hydrochloric acid,[31] yielded a product melting at 227–228 °C, which appeared by TLC to be a mixture of compounds: IR ($CHCl_3$) 1710, 1680, 1625 cm^{-1}; NMR ($CDCl_3$) δ 3.75 (m), 3.90 (D), 4.35 (m), 4.60 (m), 4.95 (m), 6.65 (s). Lactam **9** could not be isolated from this mixture.

***N*-Nitroso-1,4-dihydro-6,7-dimethoxy-3(2*H*)-isoquinolinone (3)** was prepared as described by White et al.[5] The isotopically substituted compound, labeled at the $-NCH_2-$ position with ^{14}C, was prepared by the method used for the nonradioactive nitroso lactam **3**, except that ^{14}C-labeled formaldehyde was used in the preparation of the lactone precursor **8**; activity = 4.65×10^4 cpm/mg. Anal. ($C_{11}H_{12}N_2O_4$) C, H.

Inhibition of α-Chymotrypsin with ^{14}C-Labeled Acyclic Substrates and Measurement of Ratio of Bound Inhibitor. α-Chymotrypsin (0.94 g) was dissolved in 50 mL of 0.05 M, pH 7.9, sodium phosphate buffer; *N*-*trans*-cinnamoylimidazole active-site titration[63] of this solution gave the concentration of active enzyme (5.75×10^{-4} M). The solution was diluted to 1 L with additional pH 7.9 phosphate buffer and 10 mL of 2.88×10^{-2} M ^{14}C-labeled *N*-nitroso-*N*-benzyl-*N'*-isobutyryl-D-phenylalaninamide (radioactive D-**1a**) in acetonitrile was added dropwise with rapid stirring (inhibitor/enzyme ratio = 10; the radioactivity of the acetonitrile solution of the inhibitor was 3.24×10^6 cpm/mL). The final solution was turbid. After 50 min, α-chymotrypsin activity was measured by the BTEE method[64] and compared to a control, which had been removed before the addition of the inhibitor; 90% inhibition was found.

This solution was lyophilized, at which point 69% of the total radioactivity was found in the lyophilization trap. The lyophilizate in ~40 mL of water was dialyzed against 500 mL of water at 4 °C. Six bath changes were performed over a 42-h period. After dialysis, the contents of the dialysis bag had a total activity of 3.48×10^6 cpm and 10.7% of the original counts were present. This corresponds to 1.07 molecules of inhibitor per molecule of α-chymotrypsin based on the original concentration of active enzyme.

When the contents of the dialysis bag were lyophilized, an additional 11% of the radioactivity was collected in the traps. Correcting for the amount of active enzyme remaining led to a final ratio of 0.95 molecule of inhibitor bound per molecule of α-chymotrypsin. Other values and conditions used for this measurement are listed in Table III.

Sephadex G-25 purification of the inhibited enzyme was used in one case. α-Chymotrypsin in 0.005 M, pH 7.9, sodium phosphate buffer (42.3 mL of a 10^{-5} M solution) was inhibited with 1.37 mg of ^{14}C-labeled D-**1a** in 2.0 mL of acetonitrile. The final molar ratio of inhibitor to enzyme was 8.8:1. Duplicate BTEE analyses indicated that the enzymatic activity was 9.3% after incubation at 25 °C for 33 min. The inhibited enzyme solution was lyophilized; the product was then dissolved in 1 mL of 0.005 M ammonium acetate and loaded onto a 2 × 28 cm column packed with 26.5 g of Sephadex G-25 (50–150 mesh) in 0.005 M ammonium acetate. The flow rate was adjusted to 1 mL/min. Ammonium acetate (0.005 M) was used as the eluent and fractions were taken every minute. UV absorption at 280 nm indicated that fractions 25 through 31 contained the enzyme. The counts of fractions 25–31 showed that 0.6 mol of inhibitor was bound per mol of enzyme, and the BTEE assay indicated that the enzyme was ~30% active. Correction for the amount of active enzyme remaining indicated that 0.86 mol of the [^{14}C]benzyl group was bound per inhibited enzyme molecule.

Inhibition of α-Chymotrypsin with Nitroso Lactam 3. A 4.0×10^{-4} M solution of α-chymotrypsin in 10 mL of 0.05 M sodium phosphate buffer, pH 7.9, was incubated with ^{14}C-labeled nitroso lactam in acetonitrile (the label was at the NCH_2 position; activity = 1.1×10^{10} cpm/mol). The final concentration of compound **3** was 1.0×10^{-2} M, the ratio of **3**/enzyme was 25, and the final acetonitrile concentration was 5%. The enzyme–inhibitor solution was incubated for 1 h at 25 °C; analysis[64] of a 50-μL aliquot indicated that the enzyme was inhibited by 97%. The enzyme–inhibitor solution was dialyzed against distilled water at 4 °C for 56 h with ten bath changes (300 mL each); see also Tables I and III.

Kinetic Measurements. Half-lives for substrate hydrolysis in the presence of α-chymotrypsin were measured by monitoring the decrease in the 242-nm absorption band by stopped-flow (Durrum D110 and Hewlett Packard 198A storage oscilliscope) for compounds D-**1a**, L-**1a**, and L-**1b**. These half-lives were confirmed in some cases by measuring the decrease in the absorption bands at ~420 nm. Half-lives for compounds D-**1c**, L-**1c**, and D-**1b** were measured with a Varian UV spectrometer by monitoring the decrease in the 242-nm absorption band. Relative rate measurements were determined using the stopped-flow apparatus by measuring the initial slope of the decay of the absorption band at 242 nm for time periods which were much less than the half-lives.

Estimates of V_{max} and K_m for L-**1a** came from measurements of the decay of the absorption band at 242 nm during the first 20 s after mixing (stopped flow apparatus). The enzyme concentration was set at 1.0×10^{-7} M, and the substrate concentration was varied by 10 increments over the range 6.4×10^{-5} to 6.3×10^{-6} M. K_m and V_{max} were determined from a Lineweaver–Burk plot of the initial reaction velocity vs. substrate concentration. These results were confirmed by monitoring the decrease of the visible absorption band at 420 nm.

Amino Acid Analysis. Inhibited α-chymotrypsin solutions were adjusted to 6 N in hydrochloric acid in Pyrex tubes and the contents were degassed (~0.1 mmHg) with two freeze–pump–thaw cycles. After sealing under vacuum, the tubes were heated at 110 °C for 22 h. The hydrolysates were lyophilized and dissolved in pH 2.2 diluter buffer (Pierce Chemical Co.) for analysis. For amino acid analyses of the product when **3** was used as the inhibitor, a 29:1 ratio with the enzyme ([enz] = 6.6×10^{-5} M) was used and the protein (97% inhibited) had a ^{14}C incorporation of 1.6 residues per enzyme molecule.

Chromatography on Aminex Q-15S of the Hydrolysate of α-Chymotrypsin Inhibited with ^{14}C-Labeled Nitroso Lactam 3. A sample of α-chymotrypsin (~30 mg) which had incorporated approximately 2.6 mol of nitroso lactam **3** (Table III) from inhibition with a 25-fold molar excess of the ^{14}C-labeled inhibitor ([enz] = 4×10^{-4} M) was exhaustively dialyzed against 3 L of water (10 changes, 300 mL each) at 4 °C over a 56-h period. The sample was then lyophilized and hydrolyzed with 6 M HCl. The hydrolysate was chromatographed on an Aminex Q-15S column (0.6 × 125 cm) in a Technicon automatic amino acid analyzer. Elution was accomplished at 0.5 mL/min at 60 °C with a citrate buffer linear gradient from pH 2.9 to 5.0. Fractions (6.1 mL) were collected

(69) Moffett, R. B. "Organic Synthesis"; Wiley: New York, 1963; Collect Vol. IV, p 354.

(70) Huisgen, R.; Reinertshofer, J. *Justus Liebigs Ann. Chem.* **1952**, *575*, 197.

(71) Finkelstein, J.; Brossi, A. *J. Heterocycl. Chem.* **1967**, *4*, 315.

and aliquots of each (0.6 mL) were withdrawn, dissolved in PCS solubilizer (Amersham/Searle), and assayed for radioactivity. Seven areas of radioactivity were observed. The fractions comprising each peak and the percent of total recovered radioactivity in each were as follows: 8–14, 2%; 21–25, 4%; 28–32, 8%; 33–41, 16%; 54–59, 2.5%; 75–82, 21.5%; 83–92, 46%.

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Micellar Solubilization of Biopolymers in Organic Solvents. 5. Activity and Conformation of α-Chymotrypsin in Isooctane–AOT Reverse Micelles

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Abstract: The enzymatic activity of α-chymotrypsin solubilized in reverse micelles formed in isooctane by bis(2-ethylhexyl)sodium sulfosuccinate and water (0.6–2.5% v:v) has been investigated with the use of *N*-glutaryl-L-phenylalanine *p*-nitroanilide as the substrate. The enzyme obeys Michaelis–Menten kinetics in the investigated concentration range, with K_m values which are considerably higher than those in bulk water (when concentrations are referred to as water pools). Under certain conditions, there is an enhanced turnover number (up to a factor of 6) in micelles with respect to the aqueous solution. The pH profile of the enzyme activity in the hydrocarbon micellar solution is different from that in water, being shifted to higher pH values and the more so the lower the water content. This appears to occur via a complex mechanism in which most likely the p*K* of the enzyme's active groups is modified by the peculiar nature of the solvent in the water pools. Fluorescence studies show only minor changes of the protein emission with respect to aqueous solutions, which is taken as a confirmation of the enzyme confinement within the water pool of the reverse micelle. Circular dichroism studies show significant changes in both the near- and far-UV regions, and the marked intensification of the ellipticity in the 230-nm region at lower water concentration is interpreted as arising from an increase in the content of helical structure. This is attended by a parallel increase of the enzymatic activity. Furthermore, under conditions of low water content (0.6–1% v:v) the enzyme's stability is greater than in aqueous solution. Thus, it appears that in micelles with low water content the enzyme assumes a more rigid conformation and has a higher stability, a higher turnover number, and at the same time a lower kinetic affinity for the substrate. These structure and activity changes of the enzyme are discussed in terms of the size and structure of the micellar aggregate.

It has been shown independently by Martinek et al.,[1] by Menger and Yamada,[2] by Douzou et al.,[3] and by our group[4] that hydrophilic enzymes can be solubilized in hydrocarbon solvents with the help of ionic surfactants and a small amount of water. According to a tentative structural model recently proposed,[4c] the protein is confined in the water pool of the reverse micelles formed by the surfactant molecules, and a layer of water separates and protects the protein surface from the inner surface of the surfactant layer. Such enzymatic systems may be technologically interesting, e.g., for the catalytic transformation of lipophylic substrates. Aside from this practical aspect, a number of questions arise which are of general interest both for the micelle field and for protein chemistry. One such question concerns the relation between the enzyme's reactivity and the enzyme's environment provided by the micellar core. The relevance of this question will be apparent in this paper, as it will be shown that under certain conditions the enzyme in the hydrocarbon micellar solution has a higher turnover number than that in bulk water. Another question of general interest concerns the nature of structural changes induced in the protein upon its confinement in the water pool. We will show here that, in parallel with the case of lysozyme,[4d] marked changes in the main chain's conformation can occur without impairing the enzyme's activity. Finally, one should recognize that the reactivity in a micellar system containing the enzyme and the substrate is based upon the encounter among different micellar species: i.e., there is a mechanism of mass transport across microinterfaces, a problem which is of interest in several areas of chemistry, including membrane chemistry.

At the present state, our knowledge of these micellar aggregates is very limited, and in this paper we address only part of these questions. In particular, we describe the properties of α-chymotrypsin solubilized in isooctane with bis(2-ethylhexyl)sodium sulfosuccinate (AOT), and we will try to correlate the enzymatic activity with the structure of the micellar aggregate and with the conformation of the protein. An analogous paper on the activity and stucture of lysozyme in reverse micelles is presented elsewhere.[4d]

Experimental Section

Materials. Bis(2-ethylhexyl)sodium sulfosuccinate (AOT) was obtained from Serva, purified as previously described[4b] and stored over P_2O_5 in an evacuated desiccator. A 50 mM solution of purified AOT in isooctane had generally an absorbance less than 0.03 at 280 nm.

Isooctane puriss was purchased from Fluka. α-Chymotrypsin was obtained from Boehringer, *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA) from Serva and *N-trans*-cinnamoylimidazole from Sigma. Deionized water was used throughout.

Preparation of Enzyme and Substrate Micellar Solutions. The enzyme and substrate micellar solutions were prepared by injecting with a mi-

* Dedicated to Professor Piero Pino on the occasion of his 60th birthday.

(1) Martinek, K.; Levashov, A. V.; Klyachko, N. L.; Berenzin, I. V. *Dokl. Akad. Nauk SSSR* **1977**, *236*, 920–923.
(2) Menger, F. M.; Yamada, K. *J. Am. Chem. Soc.* **1979**, *101*, 6731–6734.
(3) Douzou, P.; Keh, E.; Balny, C. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 681–684.
(4) (a) Luisi, P. L.; Bonner, F.; Pellegrini, A.; Wiget, P.; Wolf, R. *Helv. Chim. Acta* **1979**, *62*, 740–753. (b) Wolf, R.; Luisi, P. L. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 209–217. (c) Bonner, F. J.; Wolf, R.; Luisi, P. L. *J. Solid-Phase Biochem.* **1980**, *5*, 255–268. (d) Grandi, C.; Smith, R.; Luisi, P. L. *J. Biol. Chem.* **1981**, *256*, 837–843. (e) Smith, R., and Luisi, P. L. *Helv. Chim. Acta* **1980**, *63*, 2302–2311. (f) Meier, P.; Luisi, P. L. *J. Solid-Phase Biochem.* **1980**, *5*, 269–282. (g) Luisi, P. L.; Meier, P., Wolf, R. *Enzyme Eng.* **1980**, *5*, 369–371.

0002-7863/81/1503-4239$01.25/0