Studies on the Interactions of Spin-Labeled Substrates with Chymotrypsin and with Cycloamyloses

Kathleen Flohr,[1] R. M. Paton,[2] and E. T. Kaiser*[3]

Contribution from the Department of Chemistry, University of Chicago, Chicago, Illinois 60637. *Received July 18, 1974*

Abstract: Stereospecificity in the interactions of several spin-labeled substrates with α-chymotrypsin and with the chymotrypsin models, cyclohexaamylose and cycloheptaamylose, has been studied. The cyclohexaamylose and cycloheptaamylose complexes of 2,2,6,6-tetramethyl-4-oxopiperidinyl-1-oxy (I) in aqueous solutions have been observed using esr spectroscopy. On binding to cycloheptaamylose, the nitroxide function of I moves to a relatively hydrophobic environment, and on binding to either cycloamylose, the nitroxide loses some of its freedom of rotation. The dissociation constant for the cyclohexaamylose complex of I is greater than the constant for the cycloheptaamylose complex of I, consistent with the observation that a molecular model of I can fit into the cavity of a model of cycloheptaamylose but cannot fit completely into the cavity of a model of cyclohexaamylose. In the catalysis of the hydrolysis of the asymmetric compound 3-carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *m*-nitrophenyl ester (IIc) by cyclohexaamylose enantiomeric specificity has been found in the acylation step. However, it was not observed in either formation of the "Michaelis complex" or in the hydrolysis of the acylcycloamylose intermediate. No differences have been found in the esr spectra of solutions of trapped acylcyclohexaamylose intermediates derived from (+)-IIc and (±)-IIc. The nitroxide function in the acylcycloamylose intermediate is less free to rotate than in the "Michaelis complex" and is not included in the cycloamylose cavity. The k_3 values at pH 8.6 for hydrolysis of the acylcycloamyloses derived from either enantiomer of IIc are the same. In the hydrolysis of the asymmetric compound 3-carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *p*-nitrophenyl ester (IId) catalyzed by α-chymotrypsin enantiomeric specificity has been found only in the catalytic steps and not in the binding step. No differences have been found in esr spectra of solutions of α-chymotrypsin complexes of (+)- and (−)-IId at pH 2.2, and the dissociation constants for the chymotrypsin complexes of either enantiomer at pH 7.0 are the same. On binding to α-chymotrypsin at pH 2.2, the nitroxide function of IId loses some of its freedom to rotate. The k_2 value for acylation of α-chymotrypsin by bound (+)-IId at pH 7.0 is (9.1 ± 1.1) times larger than the corresponding value for (−)-IId. The k_3 value at pH 7.0 for the hydrolysis of the acyl enzyme derived from (+)-IId is (21 ± 3) times larger than the corresponding value for the acyl enzyme derived from (−)-IId. No differences have been found in esr spectra of solutions of acyl enzyme intermediates derived from (+)-IId and (−)-IId. The nitroxide groups in the acyl enzymes are not free to rotate with respect to the enzyme. In summary, the enantiomeric specificity found in the chymotrypsin-catalyzed hydrolysis of IId is only slightly greater than that found in the hydrolysis of the closely related ester IIc catalyzed by the model enzyme cyclohexaamylose.

A large body of evidence indicates that chymotrypsin catalyzes the hydrolysis of derivatives of carboxylic acids by the pathway of eq 1 where E is the enzyme, S is the substrate, ES is the noncovalent Michaelis complex, ES′ is the acyl enzyme intermediate, P_1 is the product alcohol or amine, and P_2 is the product carboxylic acid.[4] In an effort to understand the mode of action of enzymes better, model enzymes consisting of molecules much smaller and/or simpler in structure than enzymes have been studied. A group of compounds which closely mimic the action of chymotrypsin are the cycloamyloses, α-1,4-linked oligomers of D-glucose formed during the degradation of starch by an amylase of *Bacillus macerans*. The pathway by which the cycloamyloses catalyze the hydrolysis of esters in aqueous solutions is identical with that shown above for chymotrypsin, except that for the cycloamylose reaction, E is the cycloamylose, ES is the inclusion compound or "Michaelis complex" formed by the catalyst and the ester, and ES′ is an acylcycloamylose intermediate.[5]

$$E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} ES' \xrightarrow[H_2O]{k_3} E + P_2 + P_1 \qquad (1)$$

Specificity in binding and in catalysis is observed in chymotrypsin- and in cycloamylose-catalyzed reactions. For both chymotrypsin- and cycloamylose-catalyzed reactions it has been shown that the reactions of substrates which bind well are not necessarily catalyzed well.[5,6] In the cases of many specific substrates of chymotrypsin, the D and L isomers of a substrate bind to the enzyme equally well, but the k_2 and k_3 values for the L isomer are much larger than for the D isomer. There is very little information in the literature on values of k_2, k_3, and K_s for the D and L isomers of asymmetric nonspecific substrates of chymotrypsin, and until a recent report from our laboratory appeared,[7] there was no case in which k_2, k_3, and K_s had all been determined for both enantiomers of an asymmetric substrate, the hydrolysis of which is stereospecifically hydrolyzed by a cycloamylose.

Preliminary accounts of our findings on the kinetics of the hydrolysis of asymmetric nonspecific substrates catalyzed by α-chymotrypsin[8] and of comparative studies on the reactions of closely related substrates with cycloamyloses[7,9] have been published by us. Spin-labeled substrates were employed in our work because their use allowed us not only to carry out the necessary kinetic experiments but also to determine the polarity and rigidity of the environment of the spin-label probe. In the present article our observations on the binding of 2,2,6,6-tetramethyl-4-oxopiperidinyl-1-oxy (I), a relatively simple spin-labeled species, to cyclohexaamylose and cycloheptaamylose are reported. Additionally, a full account is given here of our studies of the interactions of the enantiomers of the *m*- and *p*-nitrophenyl esters, IIc and IId, of 3-carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy with cyclohexaamylose and α-chymotrypsin, respectively. Finally, results on the cycloamylose-catalyzed hydrolysis of the *m*-nitrophenyl ester of the unsaturated acid 3-carboxy-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy (IIIc) are briefly summarized.

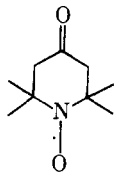
Experimental Section

Materials. 2,2,6,6-Tetramethyl-4-oxopiperidine hydrochloride was purchased from the Aldrich Chemical Co. (±)-3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine was obtained from Frinton Laboratories, mp 113–120° (lit.[10] mp 129–130°), and from Eastman, mp 128–130°. 3-Carbamoyl-2,2,5,5-tetramethylpyrroline was purchased from Frinton. Some of the (±)-3-carboxy-2,2,5,5-tetra-

Table I. Nitrophenyl Esters of 3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy and 3-Carboxy-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy

Compd	Crystn solvent	Mp, °C	Analyses	[α]D, deg
(±)-IIc	*n*-Hexane	95.5–96.5	C, 58.63,[a] H, 6.19, N, 9.12	
(+)-IIc	*n*-Hexane	46–49	C, 58.27,[a] H, 6.18, N, 9.22	+29 ± 3 (*c* 0.05–0.13, CH_3CN)
(±)-IId	*n*-Hexane	77–78[b]		
(+)-IId	Ether	84.5–86.0		+35 ± 2 (*c* 0.15, CH_3CN)
(−)-IId	Acetone–*n*-hexane	84–85	C, 58.48,[a] H, 6.27, N, 9.21	−36 ± 2 (*c* 0.12, CH_3CN)
IIIc	Benzene–chloroform	106–107	C, 58.89,[c] H, 5.68, N, 9.01	

[a] Calcd for $C_{15}H_{19}N_2O_5$: C, 58.51; H, 6.16; N, 9.01. [b] Lit.[16] mp 77.1–77.7°. [c] Calcd for $C_{15}H_{17}N_2O_5$: C, 59.01; H, 5.61; N, 9.18.



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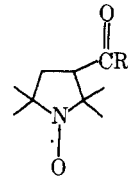
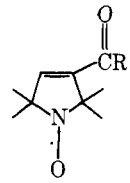


IIa, R = $-NH_2$
b, R = $-OH$
c, R = $-O\text{-}C_6H_4\text{-}m\text{-}NO_2$
d, R = $-O\text{-}C_6H_4\text{-}p\text{-}NO_2$



IIIa, R = $-NH_2$
b, R = $-OH$
c, R = $-O\text{-}C_6H_4\text{-}m\text{-}NO_2$

methylpyrrolidinyl-1-oxy used was obtained from Eastman, mp 194–196° (lit.[11] mp 193°).

Cyclohexaamylose was obtained from Eastman Organic Chemicals and from Corn Products Co. (85% pure, Lot 7317) and was purified by the method of French, *et al.*[12] Cycloheptaamylose was obtained from Corn Products Co. and was also purified as described by French, *et al.*[12] The two sources of crystalline α-chymotrypsin used in these experiments were the Armour Pharmaceutical Co. and the Worthington Biochemical Corp. (three times crystallized, salt-free enzyme).

Brucine sulfate was purchased from Eastman Organic Chemicals. Both D(+)- and L(−)-α-methylbenzylamine were purchased from the Aldrich Chemical Co.

Reagent grade acetonitrile from the J. T. Baker Chemical Co. was refluxed over P_2O_5 and distilled before use. α-Methyl glucoside was purchased from Matheson Coleman and Bell.

N-Acetyl-L-tyrosine ethyl ester was obtained from Dr. John Heidema. *N-trans*-Cinnamoylimidazole was obtained from Eastman Organic Chemicals and recrystallized three times from cyclohexane before use: mp 133–134° (lit.[13] mp 133–133.5°). Methyl *p*-nitrobenzenesulfonate was obtained from Eastman Organic Chemicals and recrystallized from a mixture of petroleum ether and ethyl ether: mp 90.5–91.5° (lit.[14] mp 91.5–92.5°).

Syntheses. 2,2,6,6-Tetramethyl-4-oxopiperidinyl-1-oxy (I). Compound I was prepared by oxidation of 2,2,6,6-tetramethyl-4-oxopiperidine, using hydrogen peroxide, EDTA, and sodium tungstate in aqueous solution.[15] The product was purified by column chromatography on silica gel, using benzene as eluent. After vacuum sublimation, the melting point was 36.5–38.5° (lit.[15] mp 36.5°).

Anal. Calcd for $C_9H_{16}NO_2$: C, 63.50; H, 9.47; N, 8.23. Found: C, 63.38; H, 9.54; N, 8.12.

The product was further analyzed by thin-layer chromatography on silica gel using chloroform for development; spots were visualized after staining with iodine. Freshly prepared product showed only one spot with R_f 0.8, but, over a period of weeks, a second spot, with R_f 0.4, appeared.

(±)-3-Carbamoyl-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (IIa). Compound IIa was prepared from (±)-3-carbamoyl-2,2,5,5-tetramethylpyrrolidine by the method of Rozantsev and Krinitzkaya:[11] mp 167–169° (lit.[11] mp 174°).

(±)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (IIb). (±)-IIa was hydrolyzed to (±)-IIb by refluxing in 10% sodium hydroxide solution. (±)-IIb was extracted from the acidified reaction mixture with ether and recrystallized from benzene: mp 194–195° (lit.[11] mp 193°).

3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy (IIIa). Compound IIIa was prepared from (±)-3-carbamoyl-2,2,5,5-tetramethylpyrroline by the method of Rozantsev and Krinitzkaya:[11] mp 203–204° (lit.[11] mp 203–204°). Mr. S. Louie carried out this reaction as well as the conversion of IIIa to IIIb.

3-Carboxy-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy (IIIb). Compound IIIa was hydrolyzed to give IIIb: mp 208–210° (lit.[11] 210–211°).

Nitrophenyl Esters of Acids (+)-, (−)-, and (±)-IIb and Acid IIIb. Nitrophenyl esters of acids (+)-, (−)-, and (±)-IIb and of acid IIIb were prepared by mixing approximately equimolar amounts of acid, nitrophenol, and dicyclohexylcarbodiimide in ethyl acetate at 0° and then allowing the reaction mixture to stand at room temperature overnight.[16] The reaction mixture was then cooled to 0°, the white solid which formed was filtered, and the product recovered by evaporation of solvent from the filtrate. In some cases, before purification by column chromatography, the crude product was dissolved in ether, washed with 5% potassium hydroxide, washed with water, and dried over anhydrous sodium sulfate; the partially purified product was recovered by evaporation of the ether. All of the ester products were finally purified by column chromatography on silica gel, using carbon tetrachloride, benzene, and chloroform as eluents. When the product was not optically active, it was next recrystallized from an appropriate solvent (see Table I). When the product was expected to be optically active, it was crystallized in an appropriate solvent, and then, without separating the crystals from the mother liquor, the solvent was removed from the crystallization mixture by blowing nitrogen over it; this crystallization procedure was followed in order to avoid changing the optical activity of the product. The ester products were dried under vacuum.

Table II. Resolution of 3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy

Isomer	Resolving agent	Mp, °C	[α]D (*c* 0.05–0.68, EtOH), deg
(+)	Brucine	206.5–208.5	78 ± 3
(+)	L(−)-α-Methylbenzylamine	202.5–205.0	79 ± 1
(−)	D(+)-α-Methylbenzylamine	205–207	−83 ± 2

Resolution of 3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (IIb). Compound IIb was resolved by making a salt of (±)-IIb with an optically active amine and separating the two diastereomers of the salt formed by fractional crystallization (see Table II). Each salt was prepared from equimolar amounts of (±)-IIb and the resolving agent; the solvent used for the recrystallizations was acetone. Either of two different resolving agents, brucine or L(−)-α-methylbenzylamine, was used to prepare (+)-IIb. The (−) isomer of IIb was prepared using D(+)-α-methylbenzylamine. After two recrystallizations of the salt formed between IIb and any of the re-

solving agents used, further recrystallization did not result in any further change in the melting point or specific rotation of the acid extracted from the salt. Acid IIb was extracted from the recrystallized brucine or α-methylbenzylamine salt by adjusting an aqueous solution of the salt to pH 4 and then extracting with ether. The yield of (−)-IIb from a resolution starting with 2.0 g of (±)-IIb and an equimolar amount of D(+)-α-methylbenzylamine was 0.18 g [9% of the starting material or 18% of the theoretical yield of (−)-IIb].

Partial Resolution of 3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *m*-Nitrophenyl Ester (IIc) by Partial Hydrolysis with Cyclohexaamylose. A mixture of 1.73 g (1.77 mmol) of cyclohexaamylose in 760 ml of pH 8.48, 0.05 *M* Tris–HCl buffer and 0.0537 g (0.18 mmol) of (±)-IIc in 1 ml of CH_3CN was stirred for 10 min at room temperature. The reaction was stopped by the addition of dilute HCl, lowering the pH to 2. Unreacted *m*-nitrophenyl ester was recovered from the reaction mixture by extracting with ether. The recovered ester was purified by column chromatography on silica gel, using benzene and chloroform as eluents: mp 92–94°; $[\alpha]_D$ (*c* 0.12, CH_3CN) −6 ± 2°.

Preparation of Solutions of the Cycloamylose Esters of (±)- or (+)-IIb. Solutions of acylcycloamylose were prepared by the method of Van Etten, *et al.*[5] Cycloamylose and either (+)- or (±)-IIc were allowed to react in a basic buffer solution, the reaction was slowed by lowering the pH, and acylcycloamylose was separated from unreacted IIc, *m*-nitrophenol, and IIb by gel filtration on either Sephadex G-10 or G-15 equilibrated with pH 5.8, 0.07 *M* phosphate buffer.

Preparation of Solutions of the Chymotrypsin Ester of (+)- or (−)-IIb. Solutions of the chymotrypsin ester of (+)- or (−)-IIb were prepared by reacting excess (+)- or (−)-IId, respectively, with chymotrypsin ($1.6–4.3 \times 10^{-4}$ *M*) in pH 4.5–4.7, 0.1 *M* acetate or phosphate buffer containing 2–16% (v/v) acetonitrile, at 25°, for 5–16 hr in the case of (−)-IId or for 1.0–2.5 hr in the case of (+)-IId. The solution was freed of unreacted IId, IIb, and *p*-nitrophenol either by dialysis[16] or by gel filtration on Sephadex G-15.

Methyl Chymotrypsin. Chymotrypsin methylated at the 3 position of the imidazole ring of histidine-57 was prepared by reaction of chymotrypsin (purified by CM Sephadex C-50 chromatography) with methyl *p*-nitrobenzenesulfonate.[14] After methylation, the activity of the enzyme, determined by rate assay with *N*-acetyl-L-tyrosine ethyl ester as substrate, dropped to 0.04% of the activity before methylation.

Methods. All melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Micro-Tech Laboratories, Inc., Skokie, Ill. A Radiometer 4c pH meter was used to measure pH values. For measurement of pK_a values, a Radiometer Titrator 11, Titrigraph syringe burette recorder, and syringe burette of 0.5-ml capacity were used. Viscosities of solutions were determined using a Cannon-Fenske Ostwald-type viscometer.

Optical rotations were measured with a Perkin-Elmer polarimeter, using a cell with a 10-cm path length. Uv-visible spectrophotometry was done with either a Cary 15 spectrophotometer, a Gilford spectrophotometer with a Beckman DU monochromator, or an Aminco stopped-flow spectrophotometric system. Electron spin resonance spectra were taken with a Varian E-3 spectrometer.

Equilibrium Dialysis. Dialysis tubing was obtained from A. Daigger and Co. and was prepared by boiling in 2% sodium bicarbonate solution for 30 min, rinsing with deionized water, boiling in 10 m*M* EDTA solution for 30 min, and then boiling in water for 30 min. For determination of dissociation constants, dialysis cells from TechniLab Instruments were used.

Analysis of Chymotrypsin Solutions. Chymotrypsin solutions were analyzed either by active site titration with *N-trans*-cinnamoylimidazole[13] or by measurement of the enzyme-catalyzed rate of hydrolysis of *N*-acetyl-L-tyrosine ethyl ester.

Kinetics of Hydrolysis of Cycloamylose or Chymotrypsin Esters of (±)-, (+)-, or (−)-IIb Studied by Esr Spectroscopy. The pH of a solution of cycloamylose or chymotrypsin ester of IIb, prepared at low pH where the ester was stable, was raised to a pH at which hydrolysis would occur. The kinetics of the hydrolysis were then followed by recording the high-field line of the esr spectrum of IIb in the solution as a function of time. The pseudo-first-order rate constant for the hydrolysis was calculated from a plot of $\log\{(h_{-1})_\infty - (h_{-1})_t\}$ *vs.* time, where $(h_{-1})_\infty$ is the height of the high-field line at the conclusion of the hydrolysis reaction and $(h_{-1})_t$ is the height of the high-field line at any time *t*.

Table III. Viscosities of Aqueous Solutions of Cycloheptaamylose and of α-Methyl Glucoside at 25.1°

Cycloheptaamylose solutions	
10^4[Cycloheptaamylose], *M*	Viscosity, mP
0	8.93
9.5	8.99
59.5	9.29
99.8	9.46
α-Methyl glucoside solutions	
10^4[α-Methyl glucoside], *M*	Viscosity, mP
0	8.95
100	8.99
482	9.05
3330	9.91

Results

Viscosities of Cycloheptaamylose and α-Methyl Glucoside Solutions. Effect of Viscosity on the Esr Spectrum of (±)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *m*-Nitrophenyl Ester (IIc). Viscosity values for aqueous solutions of cycloheptaamylose and α-methyl glucoside at 25.1° and pH 5.8 are listed in Table III. In order to test the effect of increasing viscosity on the esr spectrum of IIc, esr spectra of solutions of IIc containing varying concentrations of α-methyl glucoside were recorded. The esr spectrum of an aqueous solution of (±)-IIc (9.9×10^{-5} *M*) at pH 5.8 with 0.5% (v/v) CH_3CN did not change significantly as the α-methyl glucoside concentration was raised from zero to 0.33 *M*, *i.e.*, as the viscosity was increased from 8.95 to 9.91 mP.

Titration of the Ionizable Group(s) of (±)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (IIb) over the Range pH 3–11. When 5 ml of a 1.34×10^{-2} *M* aqueous solution of (±)-IIb (6.7×10^{-5} mol) was brought to pH 11 by the addition of concentrated NaOH solution and then titrated with 1.0 *N* HCl at 24.3°, no titratable group with a pK_a between 11 and 5 was found. A group of $pK_a = 4$ was titrated with approximately 5.3×10^{-5} mol of acid, *i.e.*, with approximately 1 equiv of acid. The group with $pK_a = 4$ is presumably the carboxyl group of (±)-IIb.

Dependence of the Esr Spectrum of 2,2,6,6-Tetramethyl-4-oxopiperidinyl-1-oxy (I) on pH. The nitrogen hyperfine coupling constant and the line shape of the esr spectrum of a 10^{-4} *M* solution of I in 0.07 *M* phosphate buffer did not change as the pH was raised from 6.0 to 8.3, suggesting that the p*K* of the nitroxide function does not lie between 6.0 and 8.3.

Binding of 2,2,6,6-Tetramethyl-4-oxopiperidinyl-1-oxy (I) to Cyclohexaamylose and to Cycloheptaamylose. The effect of increasing concentration of cycloamylose on the isotropic nitrogen hyperfine coupling constants, a_N, and ratios, *R*, of middle to high-field line height, in esr spectra of solutions of I (10^{-4} *M*) in 0.07 *M*, pH 5.75 phosphate buffer containing 0.5% (v/v) CH_3CN, is shown in Table IV. Increases in viscosities of solutions with cycloheptaamylose concentrations comparable to those listed in Table IV have been shown to have no significant effect on the esr spectrum of a nitroxide (see above). Thus, the change in the esr spectrum of I observed with increasing cycloamylose concentration is due to formation of a cycloamylose complex of I.

The dissociation constant for the cycloheptaamylose complex of I, estimated from the dependence of the heights of high-field and middle lines in the esr spectrum of I on cycloamylose concentration,[17] is 6×10^{-3} *M*. The rotational correlation time[18] of I in aqueous solution at pH 5.75 and

Table IV. Esr Spectral Data from Solutions of 2,2,6,6-Tetramethyl-4-oxopiperidinyl-1-oxy and Cycloamylose[a]

Cyclohexaamylose solutions		
10^3[Cyclohexaamylose], M	$R^{b,c}$	a_N,[d] G
0	1.12	16.28 ± 0.05
1.85	1.15	16.28
3.94	1.17	16.25
8.67	1.30	16.25
15.38	1.40	16.22
19.70	1.48	16.22
Cycloheptaamylose solutions		
10^3[Cycloheptaamylose], M	$R^{e,e}$	a_N,[d] G
0	1.10	16.19 ± 0.05
1.74	1.32	16.18
4.52	1.73	16.15
6.46	2.04	16.13
7.53	2.25	16.09
10.10	2.63	16.10
13.00	3.16	16.05
15.82	3.22	15.99
18.91	3.36	15.88

[a] 0.07 M phosphate buffer, pH 5.75, 0.5% (v/v) CH_3CN. [b] 1.21 × 10^{-4} M I. [c] R is the ratio of the height of the middle line to the height of the high-field line in the esr spectrum of the solution. [d] a_N is the isotropic nitrogen hyperfine coupling constant, calculated by dividing the separation between the high- and low-field lines in the esr spectrum by two. [e] 9.61 × 10^{-5} M I.

Table V. Esr Spectral Data from Solutions of (±)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (IIb) and Cycloheptaamylose[a]

10^4[Cycloheptaamylose], M	R^b	a_N,[c] G
0	1.05	16.32 ± 0.05
9.3	1.14	16.32
30.0	1.30	16.24
82.4	1.63	16.15
153.4	1.90	16.01
219.6	1.94	15.97

[a] Aqueous solution, pH 5.75, 9.9 × 10^{-5} M IIb. [b] R is the ratio of the height of the middle line to the height of the high-field line in the esr spectrum of the solution. [c] Isotropic nitrogen hyperfine coupling constant.

25° in the absence of cycloamylose is 1.7 × 10^{-11} sec; a_N for I under the same conditions is 16.19 ± 0.05 G. In the presence of 15.82 × 10^{-3} M cycloheptaamylose, *i.e.*, nearly fully complexed I, the rotational correlation time is 2.8 × 10^{-10} sec, and a_N is 15.99.

A dissociation constant for the cyclohexaamylose complex of I could not be calculated because saturation of I with cyclohexaamylose was not approached.

Binding of (±)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (IIb) to Cycloheptaamylose. The effect of increasing concentration of cycloheptaamylose on the esr spectrum of a solution of (±)-IIb is shown in Table V.

Kinetics of Release of *m*-Nitrophenolate from 3-Carboxy-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy *m*-Nitrophenyl Ester (IIIc) at pH 9.7 Catalyzed by 0.01 *M* Cycloamylose. The release of *m*-nitrophenolate from 3-carboxy-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy *m*-nitrophenyl ester at 25° in pH 9.69, 0.05 M carbonate buffer containing 1.2% (v/v) CH_3CN was followed by measuring the absorbance, A, of the solution at 392 nm. The initial concentration of *m*-nitrophenyl ester in each reaction mixture was 1.10 × 10^{-4} M. Plots of log ($A_\infty - A$) *vs.* time for the reaction[19] in the absence of cycloamylose or in the presence of 0.996 × 10^{-2} M cyclohexaamylose or 0.98 × 10^{-2} M cycloheptaamylose are all straight lines, as expected for either the spontaneous or the cycloamylose-catalyzed reaction of an ester without an asymmetric center. The pseudo-first-order rate constant for the reaction in the absence of cycloamylose is 1.93 × 10^{-4} sec^{-1}. In the presence of 0.996 × 10^{-2} M cyclohexaamylose, the pseudo-first-order rate constant is 25.8 × 10^{-4} sec^{-1}, 13 times the spontaneous rate constant; in the presence of 0.98 × 10^{-2} M cycloheptaamylose, it is 6.70 × 10^{-4} sec^{-1}, 3.5 times the constant for the spontaneous reaction.

Cycloheptaamylose-Catalyzed Hydrolysis of (±)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *m*-Nitrophenyl Ester (IIc). Kinetics of Release of *m*-Nitrophenolate from (±)-IIc Catalyzed by Excess Cycloheptaamylose at pH 9.7. A plot of log ($A_\infty - A$) *vs.* time for the appearance of *m*-nitrophenolate followed at 400 nm, in a solution of (±)-IIc (initial concentration 9.9 × 10^{-5} M) in pH 9.7, 0.05 M carbonate buffer with 0.5% (v/v) CH_3CN at 25°, is a straight line from which a pseudo-first-order rate constant of 23.5 × 10^{-4} sec^{-1} can be calculated (see Table VI). Similar plots for the same reaction in the presence of added cycloheptaamylose are somewhat curved, presumably due to different rates of reaction of the (+) and (−) isomers of IIc; the deviation from linearity is small, and approximate average first-order rate constants can be calculated from the plots. From pseudo-first-order rate constants determined at varying concentrations of cycloheptaamylose in pH 9.7 carbonate buffer, values of K_s = (7.5 ± 0.6) × 10^{-4} M and k_2 = 6.9 × 10^{-3} sec^{-1} were calculated.

Binding of (±)-IIc to Cycloheptaamylose at pH 5.75 Observed by Esr Spectroscopy. When the cycloheptaamylose concentration of a solution of (±)-IIc (1 × 10^{-4} M) in pH 5.75, 0.07 M phosphate buffer with 0.5% (v/v) CH_3CN, was raised from zero to 4 × 10^{-3} M, the rotational correlation time calculated from the esr spectrum of the solution changed from 0.35 × 10^{-10} sec to 3.34 × 10^{-10} sec, presumably due to a complex formation between (±)-IIc and cycloheptaamylose. From the dependence of the line heights in the spectrum of (±)-IIc on cycloheptaamylose concentration, a value for K_s for the cycloheptaamylose-(±)-IIc complex of (6 ± 2) × 10^{-4} M can be calculated. On raising the cycloheptaamylose concentration from 0 to 4 × 10^{-3} M, the isotropic nitrogen hyperfine coupling constant for IIc fell from 16.16 to 15.75 G.

Characterization of the Cycloheptaamylose Ester of (±)-IIb by Esr Spectroscopy. Parameters calculated from the esr spectrum of a solution of the cycloheptaamylose ester of (±)-IIb in pH 5.75, 0.07 M phosphate buffer are listed in Table VII. The isotropic nitrogen hyperfine coupling constant is nearly the same for the cycloheptaamylose and *m*-nitrophenyl esters of (±)-IIb.

Kinetics of Hydrolysis of the Cycloheptaamylose Ester of (±)-IIb Followed by Esr Spectroscopy. When the pH of a solution of the cycloheptaamylose ester of (±)-IIb was raised from 5.8 to 9.6, a gradual increase in the height of the high-field line in the esr spectrum of the pH 9.6, 0.05 M carbonate buffer solution was observed as the more rapidly tumbling acid (±)-IIb was formed. From the time dependence of the increase in height of the high-field line, a rate constant, k_3, of 3.2 × 10^{-5} sec^{-1} was calculated (see Table VI).

Cyclohexaamylose-Catalyzed Hydrolysis of (+)- and (±)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *m*-Nitrophenyl Ester (IIc). Kinetics of Release of *m*-Nitrophenolate from (±)-IIc Catalyzed by Excess Cyclohexaamylose at pH 8.6. A plot of log ($A_\infty - A$) *vs.* time for the appearance of *m*-nitrophenolate, followed at 392 nm, in a solution of (±)-IIc (initial concentration 1.02 × 10^{-4} M), in pH 8.6, 0.05 M Tris-HCl buffer containing 0.5% (v/v) CH_3CN, at 25°, is a straight line. The pseudo-first-order rate constant

Table VI. Kinetic Parameters for Spontaneous and Cycloamylose-Catalyzed Reactions of (+)- and (±)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *m*-Nitrophenyl Ester (IIc)

Catalyst	Substrate	k_0, sec^{-1}	Kinetic parameters		
			K_s, M	k_2, sec^{-1}	k_3, sec^{-1}
None	(±)-IIc	23.5×10^{-4} [a] 2.4×10^{-4} [b] 2.2×10^{-4} [b]			
Cycloheptaamylose	(±)-IIc		$(7.5 \pm 0.6) \times 10^{-4}$ [a] $(6 \pm 2) \times 10^{-4}$ [d]	6.9×10^{-3} [a]	3.2×10^{-5} [c]
Cyclohexaamylose	(±)-IIc				
	Fast-reacting isomer		$(1.3 \pm 0.2) \times 10^{-2}$ [b]	$(22 \pm 2) \times 10^{-3}$ [b]	8×10^{-6} [e] 7×10^{-6} [f]
	Slow-reacting isomer		$(1.3 \pm 0.5) \times 10^{-2}$ [b]	$(3.2 \pm 0.6) \times 10^{-3}$ [b]	8×10^{-6} [e] 7×10^{-6} [f]
	(+)-IIc		$(2.0 \pm 0.2) \times 10^{-2}$ [b]	$(25 \pm 2) \times 10^{-3}$ [b]	8×10^{-6} [g]

[a] pH 9.7, 0.05 M carbonate buffer, 0.5% (v/v) CH_3CN, K_s determined kinetically, 25°. [b] pH 8.6, 0.05 M Tris-HCl buffer, 0.5% (v/v) CH_3CN, K_s determined kinetically, 25°. [c] pH 9.6, 0.05 M carbonate buffer. [d] pH 5.75 aqueous solution, 0.5% CH_3CN, 23–25°, K_s determined from dependence of esr spectrum on cycloheptaamylose concentration. [e] Value for k_3 determined at pH 9.62 in 0.05 M carbonate buffer and adjusted to pH 8.6, assuming a linear dependence of the rate constant on hydroxide ion concentration, *i.e.*, (rate constant at pH 8.6) = (rate constant at pH 9.62) × $(10^{-9.62}/10^{-8.6})$. [f] Value for k_3 determined at pH 9.77 in mixed carbonate–phosphate buffer and adjusted to pH 8.6, assuming a linear dependence on hydroxide ion concentration. [g] Value for k_3 determined at pH 9.75 in 0.05 M carbonate buffer and adjusted to pH 8.6, assuming a linear dependence on hydroxide ion concentration.

Table VII. Esr Spectral Data for Esters of 3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (IIb)

Ester	R[a]	a_N, G[b]	$10^{10}\tau_c$, sec[c]
Cycloheptaamylose ester of (±)-IIb[d]	2.40	16.02 ± 0.05	5
Cyclohexaamylose ester of (±)-IIb[d]	1.80	16.23	3
Cyclohexaamylose ester of (+)-IIb[d]	1.79	16.24	3
m-Nitrophenyl ester of (±)-IIb[e]	1.12	16.16	0.4

[a] R is the ratio of middle to high-field line heights in the esr spectrum of the nitroxide. [b] Isotropic nitrogen hyperfine coupling constant. [c] Rotational correlation time. [d] pH 5.8, 0.07 M phosphate buffer. [e] pH 5.0, 0.1 M acetate buffer, 0.5% (v/v) CH_3CN.

Table VIII. Dependence of the Ratio, R, of the Middle to the High-Field Line Height in Esr Spectra of Solutions of (±)- and (+)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *m*-Nitrophenyl Ester (IIc) on the Concentration of Cyclohexaamylose[a]

10^4[Cyclohexaamylose], M	R for (±)-IIc	R for (+)-IIc
0	1.12 ± 0.2	1.12
109.7	1.23	1.25
123.0	1.26	1.26
149.0	1.27	1.30
175.6	1.30	1.31
210.0	1.33	1.34
277.0	1.37	1.40

[a] pH 5.0, 0.1 M acetate buffer, 0.5% (v/v) CH_3CN, 10^{-4} M *m*-nitrophenyl ester.

for the reaction is 2.35×10^{-4} sec^{-1}. A similar plot for the same reaction in the presence of excess cyclohexaamylose is a curve due to different rates of reaction for the (+) and (−) isomers of IIc. From each such curved plot, two pseudo-first-order rate constants, k_f and k_s, can be calculated.[17] From measurement of k_f and k_s at varying concentrations of excess cyclohexaamylose, values of K_s and k_2 for the fast and slowly reacting isomers of IIc can be calculated;[20] such values are listed in Table VI. The values of K_s for the fast and slowly reacting isomers of IIc are nearly equal, but k_2 for the fast reacting isomer is 6.9 ± 1.5 times larger than k_2 for the slowly reacting isomer.

Kinetics of Release of *m*-Nitrophenolate from (+)-IIc Catalyzed by Excess Cyclohexaamylose at pH 8.6. Plots of log $(A_\infty - A)$ *vs.* time for the appearance of *m*-nitrophenolate, followed at 392 nm, in solutions of (+)-IIc (initial concentration $1.0–1.8 \times 10^{-4}$ M) in pH 8.6, 0.05 M Tris-HCl buffer containing 0.5% (v/v) CH_3CN at 25°, in the absence or presence of cyclohexaamylose ($22–196 \times 10^{-3}$ M), are straight lines. Pseudo-first-order rate constants calculated from such plots were used to calculate the values of K_s and k_2 listed in Table VI. These values are in good agreement with K_s and k_2 values calculated for the fast reacting isomer of IIc.

Binding of (+)- and (±)-IIc to Cyclohexaamylose at pH 5.0 Observed by Esr Spectroscopy. Esr spectra of solutions of (+)- or (±)-IIc (10^{-4} M) in pH 5.0, 0.1 M acetate buffer containing 0.5% (v/v) CH_3CN, with different amounts of excess cyclohexaamylose, were recorded immediately after preparation of the solutions. (During the time required for recording of an esr spectrum, no appreciable hydrolysis of IIc occurred.) The dependence of R, the ratio of middle to high-field line heights in the esr spectra of such solutions, on cyclohexaamylose concentration is shown in Table VIII. The change in esr spectrum cannot be accounted for by an increase in viscosity with increasing cyclohexaamylose concentration and is therefore presumably due to complex formation between IIc and cyclohexaamylose. Extrapolating to infinite cyclohexaamylose concentration,[17] values of R = 2.5 ± 1.2 for the cyclohexaamylose complex of (±)-IIc and R = 2.2 ± 0.6 for the complex of (+)-IIc can be calculated. For neither (±)- nor (+)-IIc did the isotropic nitrogen hyperfine coupling constant change as the cyclohexaamylose concentration was raised from 0 to 2.77×10^{-2} M. No significant differences were observed between (±)- and (+)-IIc in binding to cyclohexaamylose as studied by esr spectroscopy.

Characterization of the Cyclohexaamylose Esters of (±)- and (+)-IIb by Esr Spectroscopy. Parameters calculated from esr spectra of solutions of the cyclohexaamylose esters of (±)- and (+)-IIb are listed in Table VII. No difference was observed between the esr spectrum of the cyclohexaamylose ester of (+)-IIb and that of (±)-IIb.

Kinetics of Hydrolysis of the Cyclohexaamylose Esters of (+)- and (±)-IIb Followed by Esr Spectroscopy. Pseudo-first-order rate constants, k_3, for the hydrolysis of the cyclohexaamylose esters of (+)- and (±)-IIb in carbonate buffer were determined from straight-line plots of log $\{(h_{-1})_\infty - (h_{-1})\}$ *vs.* time, where h_{-1} is equal to the height of the high-field line in an esr spectrum of a solution of ester at time t and $(h_{-1})_\infty$ is the height of the high-field line

Table IX. Pseudo-First-Order Rate Constants for the Release of *m*-Nitrophenol from (±)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *m*-Nitrophenyl Ester (IIc) Catalyzed by Excess Chymotrypsin at pH 6.7[a,b]

10^4[Chymotrypsin], *M*	10^3k_f, sec^{-1}	10^4k_s, sec^{-1}	k_f/k_s
1.98	3.68	3.70	9.9
3.01	4.14	3.88	10.7

[a] pH 6.66–6.72, 0.07 *M* phosphate buffer, 0.1% (v/v) CH_3CN, 25°, 2.94×10^{-5} *M*, initial *m*-nitrophenyl ester concentration. [b] Release of *m*-nitrophenol was followed by measuring the absorbance of the reaction mixture at 330 nm.

when the ester hydrolysis reaction is complete.[17] The cyclohexaamylose ester of (±)-IIb, prepared as described in the Experimental Section, was shown to be an approximately equimolar mixture of the cyclohexaamylose esters of (+)- and (−)-IIb by measuring the optical rotation of the acid IIb recovered from the reaction mixture at the conclusion of the hydrolysis reaction. As can be seen from Table VI, no significant difference was found in values for k_3 for the cyclohexaamylose esters of (+)-IIb and of (±)-IIb.

Kinetics of Release of *m*-Nitrophenol from (±)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *m*-Nitrophenyl Ester (IIc) at pH 6.7 Catalyzed by Excess Chymotrypsin. Plots of log $(A_\infty - A)$ *vs.* time for the appearance of *m*-nitrophenol, followed at 330 nm, in solutions of (±)-IIc (initial concentration 2.94×10^{-5} *M*) in pH 6.7, 0.07 *M* phosphate buffer with 0.1% (v/v) CH_3CN and $2\text{–}3 \times 10^{-4}$ *M* chymotrypsin are curves. Two pseudo-first-order rate constants, k_f and k_s, one for each isomer of IIc, were calculated from each such curve; the rate constants are listed in Table IX.

Chymotrypsin-Catalyzed Hydrolysis of (+)- and (−)-3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *p*-Nitrophenyl Ester (IId). Kinetics of Release of *p*-Nitrophenolate from (+)- and (−)-IId Catalyzed by Excess Chymotrypsin. Plots of log $(A_\infty - A)$ *vs.* time for the release of *p*-nitrophenolate followed at 390 nm, in solutions of (+)- or (−)-IId (initial concentration $0.189\text{–}4.68 \times 10^{-5}$ *M*) in pH 7.0, 0.13 *M* phosphate buffer, adjusted to ionic strength 0.5 by addition of NaCl, with 0.5–1.0% (v/v) CH_3CN, at 25°, in the absence or presence of chymotrypsin ($1.72\text{–}9.83 \times 10^{-4}$ *M*) are straight lines. Similar plots for the release of *p*-nitrophenol, followed at 340 nm, in solutions of (+)- or (−)-IId (initial concentration $0.796\text{–}3.90 \times 10^{-5}$ *M*) in pH 5.9, 0.13 *M* phosphate buffer adjusted to ionic strength 0.5 by addition of NaCl, with 0.5–3.0% CH_3CN, at 25°, containing excess chymotrypsin ($1.9\text{–}7.3 \times 10^{-4}$ *M*) are also straight lines. Pseudo-first-order rate constants were calculated from the above-mentioned plots, and, from the chymotrypsin concentration dependence of the rate constants, the kinetic parameters listed in Table X were determined.[20] At pH 7.0 values of K_s for the two isomers of IId do not differ significantly, but k_2 for the reaction of (+)-IId is (9.1 ± 1.1) times larger than k_2 for the reaction of (−)-IId. At pH 5.9, values of K_s for the two isomers of IId also do not differ significantly, but k_2 for (+)-IId is (11 ± 2) times larger than k_2 for (−)-IId. On changing the pH from 7.0 to 5.9, K_s for neither isomer changed significantly, and the degree of stereospecificity exercised by the enzyme in the k_2 step remained constant.

Determination of Dissociation Constants for the Chymotrypsin Complexes of (+)- and (−)-IId at pH 2.2. Dissociation constants, K_s, for chymotrypsin complexes of (+)- and (−)-IId were determined by equilibrium dialysis in 0.05 *M* KCl, pH 2.2–2.3 HCl solution containing 1% (v/v) CH_3CN; at this low pH, no appreciable release of *p*-nitrophenol from IId was observed in the time required for equilibration at 0° for (+)-IId and at 0–15° for (−)-IId. The K_s values, listed in Table X, were calculated as described by Klotz, *et al.*,[21] assuming that the number of molecules of IId capable of binding to each enzyme molecule is one. The K_s values determined at pH 2.2 are about ten times greater than the values determined at pH 6–7; *i.e.*, binding of IId to chymotrypsin is less favorable at the lower pH. No significant difference was observed between values for (+)- and (−)-IId.

Binding of (+)- and (−)-IId to Chymotrypsin at pH 2.2 Observed by Esr Spectroscopy. The effect of increasing chymotrypsin concentration on esr spectra of solutions of (+)- or (−)-IId in 0.05 *M* KCl, pH 2.2–2.3 HCl solution with 0.5–2.5% (v/v) CH_3CN, is shown in Table XI. The isotropic nitrogen hyperfine coupling constant of IId did not change on addition of chymotrypsin to the solution, but the rotational correlation time increased slightly. The increase in rotational correlation time with increase in enzyme concentration is not entirely due to increased viscosity of the solution and may be due to immobilization of some of the IId in a noncovalent complex with chymotrypsin.

Effect of Methylation of Histidine-57 of Chymotrypsin on Binding of (+)-IId, (−)-IId, and *p*-Nitrophenol to the Enzyme at pH 5.9. The ability of methyl chymotrypsin to bind (+)- or (−)-IId was tested by dialyzing a 1-ml sample of 5.96×10^{-4} *M* methyl chymotrypsin in pH 5.96, 0.13 *M* phosphate buffer with ionic strength adjusted to 0.5 by addition of NaCl, against a 1-ml sample of either 1.92×10^{-4} *M* (+)-IId or 2.04×10^{-4} *M* (−)-IId in the same buffer, containing 2% (v/v) CH_3CN, for 12 hr at 23–24°. At the conclusion of the dialysis, uv spectra of the dialyzed solutions indicated that considerable release of *p*-nitrophenol from the *p*-nitrophenyl ester had taken place in solutions from cells containing a mixture of methyl chymotrypsin and IId and that the *p*-nitrophenol concentration on the enzyme-containing side of the cell was higher than on the enzyme-free side of the cell. From the absorbance of the dialyzed solutions at 340 nm, it was estimated that up to 20% of the theoretical amount of *p*-nitrophenol had been released during the dialysis. Disregarding the problem of *p*-nitrophenol release, an approximate lower limit for the dissociation constant for the methyl chymotrypsin complex of (+)- or (−)-IId can be calculated; for the complex of (+)-IId the value calculated is 2.1×10^{-3} *M*, and for (−)-IId, the value is 2.6×10^{-3} *M*.

The ability of methyl chymotrypsin to bind *p*-nitrophenol was tested by dialyzing 1 ml of 5.96×10^{-4} *M* methyl chymotrypsin against 1 ml of 1.6×10^{-4} *M* *p*-nitrophenol in pH 5.9, 0.13 *M* phosphate buffer with ionic strength 0.5, containing 0.4% (v/v) CH_3CN. A parallel experiment was done in which 1 ml of 6.10×10^{-4} *M* unmodified chymotrypsin in the pH 5.9 buffer was dialyzed against 1 ml of the *p*-nitrophenol solution. The dissociation constant calculated for the methyl chymotrypsin complex of *p*-nitrophenol is 3×10^{-4} *M*; for the chymotrypsin complex, the value is 4×10^{-4} *M*.

Characterization of the Chymotrypsin Esters of (+)- and (−)-IIb by Esr Spectroscopy. Solutions of the chymotrypsin esters of (+)- and (−)-IIb have esr spectra characteristic of quite strongly immobilized spin labels. An esr spectrum of the chymotrypsin ester of (−)-IIb in 0.1 *M* NaCl, pH 3.06 HCl solution, is shown in Figure 1; the sharp lines in the spectrum are due to free (−)-IIb formed by slow deacylation of the acyl enzyme at low pH.

Part of the experiment of Shimshick and McConnell[22] done with acyl enzyme derived from (±)-IId and chymotrypsin was repeated using acyl enzyme derived from re-

Table X. Kinetic Parameters for Spontaneous and Chymotrypsin-Catalyzed Reactions of (+)- and (−)-IId

Catalyst	Substrate	pH	Temp, °C	k_0, sec^{-1}	Kinetic parameters[a] 10^4K_s, M	10^3k_2, sec^{-1}	10^4k_3, sec^{-1}
None[b]	(+)-IId	7.0	25	1.03×10^{-5}			
None[b]	(−)-IId	7.0	25	1.24×10^{-5}			
Chymotrypsin[b]	(+)-IId	7.0	25		4.1 ± 0.4	370 ± 20	
Chymotrypsin[b]	(−)-IId	7.0	25		5.1 ± 1.2	41 ± 5	
Chymotrypsin[b]	(+)-IId	5.9	25		4.8 ± 1.1	60 ± 7	
Chymotrypsin[b]	(−)-IId	5.9	25		2.7 ± 0.9	5.3 ± 0.7	
Chymotrypsin[c]	(+)-IId	2.2	0		64 ± 20		
Chymotrypsin[c]	(−)-IId	2.2	0		69 ± 6		
Chymotrypsin[c]	(−)-IId	2.2	15		45 ± 10		
Chymotryspin[d]	(−)-IId	2.2	25		34		
Chymotrypsin[e]	(+)-IId	7.0	25				45 ± 10
Chymotrypsin[e]	(−)-IId	7.0	25				2.3 ± 0.2
Chymotrypsin[f]	(+)-IId	7.0	25				52 ± 2
Chymotrypsin[f]	(−)-IId	7.0	25				2.5 ± 0.3

[a] Values determined kinetically, using uv-visible spectroscopy, except where otherwise indicated. [b] 0.13 M phosphate buffer, ionic strength adjusted to 0.5 by addition of NaCl, 0.5–3.5% (v/v) CH_3CN. [c] 0.05 M KCl, pH 2.2–2.3 HCl solution with 1% (v/v) CH_3CN; K_s values determined by equilibrium dialysis. [d] K_s value obtained by extrapolation to 25° using values obtained at 0 and 15°, assuming log K_s is proportional to $1/T$. [e] pH 6.9–7.3, 0.13 M phosphate buffer with ionic strength adjusted to 0.5 by addition of NaCl; k_3 values determined using esr spectroscopy. [f] pH 7.02–7.06, 0.13 M phosphate buffer with ionic strength adjusted to 0.5 by addition of NaCl, 0.9–3.5% (v/v) CH_3CN.

Table XI. Dependence of Esr Spectra of Solutions of (+)- and (−)-IId at pH 2.2 on the Concentration of Chymotrypsin

10^4[Chymotrypsin], M	η/η_{H_2O}[a]	(+)-IId[b] R[c]	(+)-IId[b] $10^{10}\tau_c$,[d] sec	(−)-IId[b] R	(−)-IId[b] $10^{10}\tau_c$,[d] sec
0	0.98	1.13	0.4	1.12	0.4
2.84	1.02	1.12	0.5	1.13	0.5
8.14	1.09	1.21	0.8	1.18	0.7
11.4	1.17	1.23	0.9	1.22	0.8
14.0				1.24	1.1
22.8	1.38	1.36	1.4	1.36	1.3

[a] η is the viscosity of the solution; η_{H_2O} is the viscosity of water; chymotrypsin solutions were made in 0.05 M KCl, pH 2.2–2.3 HCl solution, 25°. [b] 5–19 $\times 10^{-5}$ M IId in 0.05 M KCl, pH 2.2–2.3 HCl solution, containing 0.5–2.5% CH_3CN, 25°. [c] R is the ratio of the middle to the high-field height in the esr spectrum of the solution. [d] τ_c is the rotational correlation time.

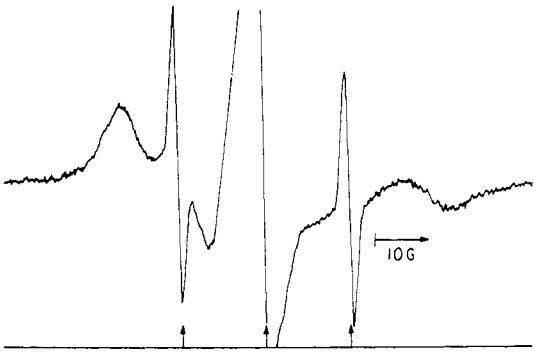


Figure 1. Esr spectrum of a solution of the α-chymotrypsin ester of (−)-IIb in 0.1 M NaCl, pH 3.06 HCl solution. The three sharp lines in the spectrum (vertical arrows) are due to (−)-IIb formed by slow deacylation of the acyl enzyme at this pH.

solved IId. Viscosity values for solutions of acyl enzyme in 0.1 M, pH 3.06 HCl solution were determined at 25.9°, assuming a value of 0.870 cP for the viscosity of water.[23] The viscosity of the solution of the chymotrypsin ester of (+)-IIb was 0.909 cP, and $(T/\eta)^{2/3}$ was 1026°K/P. The viscosity of the solution of the chymotrypsin ester of (−)-IIb was 0.914 cP and $(T/\eta)^{2/3}$ was 1023°K/P. The value for $2T'$, the separation between the high- and low-field extrema, in the esr spectrum of the chymotrypsin ester of (+)-IIb is 61.8–62.0 G and $(H_h - H_0)$, the separation of the high-field line of the acyl enzyme spectrum from the high-field line of the free acid spectrum, is 19.1–19.5 G, corresponding to a rotational correlation time of approximately 10–12 nsec for the acyl enzyme. For the solution of the chymotrypsin ester of (−)-IIb, $2T'$ = 61.4–62.8 G and $(H_h - H_0)$ = 18.0–20.2 G, corresponding to a rotational correlation time of 8–17 nsec.

Kinetics of the Hydrolysis of the Chymotrypsin Esters of (+)- and (−)-IIb Followed by Esr Spectroscopy. Pseudo-first-order rate constants, k_3, for the hydrolysis of the chymotrypsin ester of (+)- or (−)-IIb in pH 6.9–7.3, 0.13 M phosphate buffer with ionic strength adjusted to 0.5 by addition of NaCl, at 25°, were determined from the increase in height of the high-field line in the esr spectrum of IIb with time. Values are listed in Table X.

Kinetics of the Chymotrypsin-Catalyzed Release of *p*-Nitrophenolate from Excess (+)- or (−)-IId at pH 7. When chymotrypsin (4.98–5.45 $\times 10^{-6}$ M) was added to a solution containing excess (+)- or (−)-IId (4.60–13.9 $\times 10^{-5}$ M) in pH 7.02–7.06, 0.13 M phosphate buffer with ionic strength adjusted to 0.5 by addition of NaCl, with 0.9–3.5% (v/v) CH_3CN, and the reaction followed by measuring the absorbance of the solution at 400 nm, an initial "burst" of *p*-nitrophenolate was observed, followed by a slower steady-state zero-order reaction. Values of k_3 listed in Table X were calculated as described by Bender, *et al.*[24] The k_3 value for the hydrolysis of the chymotrypsin ester of (+)-IIb is (21 ± 3) times larger than the value for the ester of (−)-IIb.

Discussion

Binding of Spin-Labeled Compounds I and (±)-IIb to Cycloamyloses. The cyclohexaamylose and cycloheptaamylose complexes of compound I and the cycloheptaamylose complex of compound (±)-IIb have been observed using esr spectroscopy. The decrease in isotropic nitrogen hyperfine coupling constant, a_N, of I and (±)-IIb with increasing cycloheptaamylose concentration, shown in Tables IV and V, is consistent with the movement of the nitroxide to an environment less polar than water on formation of the cycloheptaamylose complex.[25] The dissociation constant for the cyclohexaamylose complex of I is greater than the dissociation constant for the cycloheptaamylose complex of I; examination of molecular models of cyclohexaamylose, cycloheptaamylose, and I shows that the entire nitroxide molecule can fit into the cavity of cycloheptaamylose but cannot completely fit into the cyclohexaamylose cavity. Thus, greater interaction between the nonpolar compound I and

the relatively hydrophobic interior of the cycloamylose ring is possible in the case of cycloheptaamylose than in the case of cyclohexaamylose. The rotational correlation times for I and (±)-IIb increased on binding to the cycloamyloses, indicating that the nitroxide loses some of its freedom to rotate in the cycloamylose complex.

pK_a Values of the Nitroxide Groups. Little is known about the pK_a of the nitroxide group, the effect of protonation on the esr spectrum of the nitroxide function in aqueous solution, and the effect of protonation on the stability of the group. Nitroxides are unstable in anhydrous HCl, concentrated H_2SO_4, or 20% aqueous CF_3CO_2H.[26] They form immonium chlorides and hydroxylamine hydrochlorides quickly in 6 *N* HCl and slowly in 0.1 *N* HCl.[27] Protonation of the nitroxide 2,2,6,6-tetramethylpiperidinyl-1-oxy without destruction of the paramagnetic center was accomplished by Hoffman and Eames,[26b] using a Lewis acid in CH_2Cl_2 containing a trace of water. Protonation caused a shift in the *g* value of the esr spectrum of the compound and an increase in the isotropic nitrogen hyperfine coupling constant. Berliner and McConnell[16] found the esr spectrum of (±)-IIb in aqueous solution to be insensitive to pH over the range from 3.5 to 6.8. Rozantsev and Gintsberg[28] have reported that the pK_a of I in aqueous solution at 25° is 7.40, but Neiman, *et al.*,[29] have reported a polarographic study in which I remained unprotonated at pH 2.

We have found that the esr spectrum of I in aqueous solution is not sensitive to pH over the range from pH 6.0 to 8.3, suggesting that the pK_a of the nitroxide function does not lie between 6.0 and 8.3. Compound (±)-IIb was found to have no acid-titratable group between pH 11 and 5; thus, in (±)-IIb, the pK_a of the nitroxide group does not lie between 5 and 11.

Cycloamylose-Catalyzed Reactions of Spin-Labeled Substrates. Intermediates in the cyclohexa- and cycloheptaamylose catalyzed hydrolyses of nitrophenyl esters of IIb have been trapped and characterized by esr spectroscopy. Values for k_2, k_3, and K_s for each of these hydrolysis reactions have been determined.

The release of *m*-nitrophenolate from compound (±)-IIc in pH 9.7 carbonate buffer is catalyzed by cycloheptaamylose. K_s for the reaction, determined kinetically, is 7.5×10^{-4} *M* and k_2 is 6.9×10^{-3} sec^{-1}. The noncovalent "Michaelis" complex was trapped by mixing cycloheptaamylose and substrate (±)-IIc at low pH (pH 5.8), where the k_2 step is very slow but binding between substrate and catalyst can occur. On binding to cycloheptaamylose in the noncovalent complex, (±)-IIc was found to lose some of its rotational mobility; the rotational correlation time increased from 0.35×10^{-10} sec in free solution to 3.3×10^{-10} sec. The isotropic nitrogen hyperfine coupling constant for (±)-IIc fell from 16.16 G in free solution to 15.75 G in the cycloheptaamylose complex, consistent with movement of the nitroxide function to an environment less polar than water.

The acylcycloheptaamylose intermediate in the cycloheptaamylose-catalyzed hydrolysis of (±)-IIc was trapped by allowing (±)-IIc and cycloheptaamylose to react at pH 9.6 and then lowering the pH to 5.8 where the acylcycloamylose is stable. From the esr spectrum of a solution of acylcycloamylose prepared as described in the Experimental Section, a rotational correlation time of 5×10^{-10} sec was determined, indicating that the nitroxide group in the acylcycloheptaamylose has less rotational freedom than it has either in free solution or in the noncovalent "Michaelis" complex. The a_N value for the nitroxide in the acylcycloheptaamylose is the same as for the nitroxide of (±)-IIc in free solution, suggesting that the nitroxide group in the acylcycloheptaamylose is not included in the cycloamylose cavity. The k_3 value for the acylcycloheptaamylose at pH 9.6 is 3.2×10^{-5} sec^{-1}. This value is lower than the rate constant for the spontaneous hydrolysis of (±)-IIc at the same pH; thus, the overall hydrolysis of (±)-IIc is negatively catalyzed by cycloheptaamylose.

Although the hydrolysis of IIc, an asymmetric compound, is stereospecifically catalyzed by cyclohexaamylose, enantiomeric specificity is seen only in the acylation (k_2) step and not in either the formation of the Michaelis complex or in the hydrolysis of the acylcycloamylose intermediate (k_3 step). Kinetically determined values of K_s, listed in Table VI, for dissociation of the cyclohexaamylose complexes of the two enantiomers of IIc at pH 8.6 are identical. The value of K_s determined for the fast-reacting isomer of (±)-IIc agrees reasonably well with the value determined for (+)-IIc.

The value of k_2 for the reaction of (+)-IIc with cyclohexaamylose is 6.9 times larger than the value of k_2 for the reaction of (−)-IIc [*i.e.*, for the slowly reacting isomer of (±)-IIc]. This is the largest known asymmetric catalytic effect for a model enzyme-catalyzed hydrolysis of an ester of a carboxylic acid. Larger effects are observed in reactions of asymmetric phosphorus compounds with cyclohexaamylose,[30] but in these cases reaction occurs at the asymmetric center of the phosphorus compound, and a larger effect is therefore expected.[31]

The noncovalent cyclohexaamylose complexes ("Michaelis" complexes) of (±)-IIc and (+)-IIc were trapped by mixing either (±)- or (+)-IIc with a pH 5 solution of the cycloamylose. Binding of either isomer of IIc to cyclohexaamylose causes the esr spectrum of the nitroxide solution to change from that of a rapidly tumbling molecule to that of a weakly immobilized molecule. The isotropic nitrogen hyperfine coupling constant, a_N, of IIc does not change on binding to cyclohexaamylose. The lack of change of a_N on binding to cyclohexaamylose and the similarity of K_s values for cyclohexamylose complexes of several different *m*-nitrophenyl esters[5,32] suggest that the nitroxide group of IIc in the cyclohexaamylose complex is not included in the cycloamylose cavity and that the primary binding interaction is between cycloamylose and the nitrophenyl moiety of the ester.

Since the Michaelis complex of cyclohexaamylose with (+)-IIc is inherently more reactive than the complex with (−)-IIc, one might hope to see differences in the esr spectra, *i.e.*, differences in degree of immobilization or differences in a_N values, between the complexes of (+)-IIc and (±)-IIc, despite the similarity of the K_s values for the two isomers of IIc. No such differences have been observed. One possible explanation for the absence of differences is that there is a large amount of nonproductive binding of both (+)- and (−)-IIc to cyclohexaamylose, that the principal mode of binding observed by the esr technique is therefore nonproductive, and that the rotational correlation times and a_N values for both isomers of IIc in the nonproductive complexes are the same.

The acylcycloamylose intermediates in the reactions of (+)-IIc and (±)-IIc with cyclohexaamylose were trapped by reacting IIc with cyclohexaamylose at pH 10 and then lowering the pH to 5.8, where the acylcycloamylose is stable. No differences were found in rotational correlation times or in a_N values determined from the esr spectra of solutions of acylcyclohexaamylose derived from (±)-IIc and from (+)-IIc. The isotropic nitrogen hyperfine coupling constant of a solution of the acylcycloamylose is not significantly different from that of ester IIc in solution, indicating that the nitroxide function of the acylcyclohexaamylose is probably not included in the cycloamylose cavity.

No difference was found in the values of k_3 for the cycloamyloses derived from the two enantiomers of IIc. Since

k_3 at pH 8.6 is less than the rate constant for the spontaneous hydrolysis of IIc, the overall hydrolysis of IIc is retarded by cyclohexaamylose.

Chymotrypsin-Catalyzed Reactions of Spin-Labeled Substrates. The hydrolysis of the asymmetric substrate IId, 3-carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *p*-nitrophenyl ester, is stereospecifically catalyzed by α-chymotrypsin. However, kinetically determined values of K_s, listed in Table X, at pH 7.0 for the dissociation of the chymotrypsin complexes of the two enantiomers of IId are not significantly different, and they are similar to those measured at pH 5.9.

Neither method used for trapping of the Michaelis complex of α-chymotrypsin and (+)- or (−)-IId proved to be satisfactory. Either N-methylation of histidine-57 of α-chymotrypsin together with lowering of the pH to 5.9 or simple lowering of the pH to 2 stopped the k_2 reaction of either isomer of IId reasonably well, but both procedures interfered with the binding of the substrate to the enzyme and caused a large increase in K_s, determined by equilibrium dialysis.

The increase in rotational correlation time of (+)- or (−)-IId with increasing concentration of chymotrypsin at pH 2.2-2.3, shown in Table XI, is partially due to binding of the ester to the enzyme in a complex in which the nitroxide is less free to rotate than when free in solution but more free to rotate than the nitroxide group of the acyl enzyme. The lack of change in isotropic nitrogen hyperfine coupling constant, a_N, with increasing α-chymotrypsin concentration may mean that the nitroxide function remains in a polar environment when the ester binds to the enzyme, or it may be due to the fact that chymotrypsin concentrations high enough to approach saturation of the substrate with the enzyme could not be attained and, thus, the presence of free ester in the solutions interfered with detection of a change in a_N for the bound ester. No differences were observed between spectra of (+)- and (−)-IId in pH 2.2-2.3 solutions of comparable enzyme concentration.

The k_2 step of the chymotrypsin-catalyzed hydrolysis of IId is stereospecific. At pH 7.0, k_2 for (+)-IId is (9.1 ± 1.1) times larger than k_2 for (−)-IId (see Table X). At pH 5.9, the ratio of k_2 values is 11 ± 2. This degree of enantiomeric specificity is much smaller than that found in the chymotrypsin-catalyzed hydrolyses of specific substrates.[5]

The k_3 step of the chymotrypsin-catalyzed hydrolysis of IId is also stereospecific. At pH 7.0, k_3 for the hydrolysis of the acyl enzyme derived from (+)-IId is (21 ± 3) times larger than the corresponding value for the acyl enzyme derived from (−)-IId (see Table X). The stereospecificity in the k_3 step of the reaction of IId is much less than that found in the reactions of specific substrates. For example, k_3 for *N*-acetyl-L-tryptophanyl chymotrypsin is 2×10^4 times larger than k_3 for *N*-acetyl-D-tryptophanyl chymotrypsin.[33] The rate constant, k_3, for the rate-determining step in the overall hydrolysis of either isomer of IId is greater than k_0 at pH 7.0 and catalysis of the overall hydrolysis of IId by chymotrypsin does occur.

The acyl enzyme intermediates in the reactions of (+)- and (−)-IId with α-chymotrypsin were trapped by reacting the ester with the enzyme at pH 4.5-4.7 and then separating unreacted IId, nitrophenol, and the acid product IIb from the solution either by dialysis or by gel filtration. Esr spectra of solutions of the chymotrypsin esters of (+)- and (−)-IIb are identical (see Figure 1). The values for $(H_h - H_0)$ relative to $(T/\eta)^{2/3}$ from these spectra agree well with the literature values for the acyl enzyme derived from racemic IId.[22] These esr spectra are characteristic of immobilized but not quite "strongly immobilized" radicals.[16]

The enantiomeric specificity in the α-chymotrypsin-catalyzed release of *m*-nitrophenol from the *m*-nitrophenyl ester IIc, judged from the ratios of the pseudo-first-order rate constants shown in Table IX, is of the same order of magnitude as the enantiomeric specificity in the reaction of the *p*-nitrophenyl ester IId at comparable enzyme concentration.

In summary, no enantiomeric specificity has been found in the formation of the Michaelis complex in either the chymotrypsin-catalyzed hydrolysis of IId or the cyclohexaamylose-catalyzed hydrolysis of IIc. In both cases, enantiomeric specificity is found in the k_2 step. In the cyclohexaamylose reaction at pH 8.6, catalysis of the overall hydrolysis of IIc does not occur, but in the chymotrypsin reaction at pH 7.0, catalysis of the overall hydrolysis of IId does occur. In neither the cyclohexaamylose reaction nor in the chymotrypsin reaction were differences between corresponding intermediates derived from the (+) and (−) isomers of substrates observed using the method of esr spectroscopy. The absence of differences between esr spectra of acyl enzymes does not constitute good evidence against the importance of immobilization in the specificity of chymotrypsin-catalyzed reactions because the degree of enantiomeric specificity for IId in the k_3 step is very small, corresponding to a difference in energies of activation of only 1.8 kcal/mol. The acyl nitroxide groups of the chymotrypsin esters of (+)- and (−)-IIb have practically no mobility with respect to the enzyme;[22] this result gives no support to the idea that the acyl groups of poor substrates (as the acyl groups in both isomers of IId are) are free to rotate in the acyl enzyme species while those of good substrates are not. The differences in entropies of activation previously observed between good and poor substrates of chymotrypsin in the k_3 step[34] might be primarily due to differences in the entropies of the transition states and not to differences in the entropies of the ground states; or they may be largely solvent effects. Unfortunately, the mobility of a spin label in a transition state, unlike that in a ground state, is not susceptible to study by esr spectroscopy. The single most interesting conclusion which can be drawn from the study of the cyclohexaamylose-catalyzed hydrolysis of IIc and the chymotrypsin-catalyzed hydrolysis of IId is that the enantiomeric specificity seen in the latter (enzymatic) reaction is only slightly greater than that observed in the former (model) reaction.

References and Notes

(1) U.S. Public Health Service Medical Scientist Trainee.
(2) Department of Chemistry, University of Edinburgh, Edinburgh, Scotland.
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Theory of Weak Molecular Complexes. III. Observation Equations for Multiple Equilibria and an Application to Protein Charge-Transfer Titrations[1]

David A. Deranleau

Contribution from the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received August 8, 1974*

Abstract: The classical treatment of multi-site titration analysis is extended to include the specific experimental method used in the investigation of a binding system by means of a set of general *observation equations*. The observation equations are formulated for both statistical and intrinsic binding parameters and reduce to the classical equations in the special case of *direct* experimental methods, where the number of free and/or bound adsorbate molecules can be uniquely counted. For *indirect* experimental methods such as light absorption, an intensive parameter (*e.g.*, extinction coefficient) appears in the equations and cannot be factored from the sums unless independent information concerning the relationship of this parameter to the number of bound adsorbate molecules is available. Thus, in the general case of an indirect method, it is not possible to deduce the order of the reaction from titration experiments utilizing the intensive parameter alone. Multiple-site protein charge-transfer complexes are used as examples of an indirect titration method, and approximate treatments of the binding data are considered for these systems. Association constants and extinction coefficients obtained from 1:1 homomorphic model complexes of the protein donor amino acids with a suitable acceptor can be used to interpret the protein data and to extract information concerning both the number and kind of sites available provided that the protein data are analyzed in terms of intrinsic association constants and extinction coefficients. The present communication gives the mathematical basis for such an interpretation, as well as for less specific interpretations based on the classical straight-line technique for charge-transfer systems.

The analysis of data from studies of weak molecular complexes between donors and acceptors has been considered extensively by several authors,[2] most notably by Person.[3] A few years ago the present author attempted to give a theoretical explanation of Person's limit criteria for complexes in which complete saturation could not be reached and, following an earlier treatment by Weber,[4] extended the limit criteria on the basis of the amount of information obtained in a binding experiment.[5] In addition, the effects of second-order interactions on the calculated values of the extinction coefficients and association constants of charge-transfer complexes were examined.[6] More recently, we have been studying protein surface topography by probing for exposed aromatic amino acid donor residues with a suitable charge-transfer acceptor (1-methyl-3-carbamidopyridinium chloride).[7-9] The simple one- and two-site binding models previously considered do not provide an adequate description of these systems; each protein examined so far appears to have more than a single binding site for the probe, and, furthermore, some proteins have more than one type of site available for complexation (*e.g.*, both tryptophan and tyrosine side chains). In such cases the observed charge-transfer spectrum is composed of several nonidentical overlapping bands, each of which depends on the type of residue forming the complex and on the degree of saturation of the individual donor residues. The overlapping spectral distributions can be used to advantage in identifying the specific types of donors involved in the complex, but because of the very weak binding of the probe, it is not possible to obtain saturation of any of the donor sites. The inability to attain saturation requires the use of extrapolation techniques and other approximations to arrive at quantitative estimates of the extent of interaction and the number of residues participating in the binding.

Since the two-site model previously considered for charge-transfer complexes is inadequate for the description of multiple charge-transfer complexes on proteins, it was necessary to consider generalized equations for multiple equilibria. Such generalized equations are well known for methods in which the number of free and/or bound adsorbate molecules can be counted directly (*e.g.*, equilibrium dialysis), but general equations have apparently not been considered where an indirect method such as light absorption is used to estimate the extent of interaction. The key to a general analysis for any given experimental technique lies in the formulation of the relevant *observation equation*—the equation relating the desired molecular parameters to the experimental variable used to study the interaction.