

## Enzyme Models. The Attachment of *N*-Methylhydroxamic Acid to Cyclohexaamylose. Its Reactivity and Specificity with Phenyl Esters

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The *N*-methylacetohydroxamic acid group has been introduced into cyclohexaamylose by the following sequence of reactions: (1) carboxymethylation of cyclohexaamylose by iodoacetic acid, (2) methylation of carboxymethylcyclohexaamylose with diazomethane, and (3) reaction of the carboxymethylcyclohexaamylose methyl ester with *N*-methylhydroxylamine to form the *N*-methylacetohydroxamic acid-substituted cyclohexaamylose. By employing purification procedures involving ion-exchange chromatography, the synthesis yielded a mono-substituted cyclohexaamylose-*N*-methylacetohydroxamic acid with selective modification of the C-2, C-3 hydroxyl group side of the cyclohexaamylose ring.

*p*-Nitrophenyl acetate and 2-hydroxy-5-nitro- $\alpha$ -toluenesulfonic acid sultone react 20- and 70-fold faster with cyclohexaamylose-*N*-methylacetohydroxamic acid than with *N*-methylmethoxyacetohydroxamic acid. Cyclohexaamylose-*N*-methylacetohydroxamic acid also displays a marked kinetic stereospecificity for *p*-nitro over *m*-nitrophenyl acetate (whereas cyclohexaamylose itself exhibits the reverse stereospecificity). These reactions were shown to be competitively inhibited by cyclohexanol. This evidence indicates that cyclohexaamylose-*N*-methylacetohydroxamic acid binds the substrate in a reversible complexation step prior to nucleophilic attack and thus is an enzyme model.

### INTRODUCTION

Earlier we reported that *N*-alkylhydroxamic acids catalyze ester hydrolysis, that an acyl intermediate is formed, and the catalysis can be enhanced by increasing the lability of the intermediate through the introduction of a second functionality into the catalyst that will assist deacylation intramolecularly (1). In addition we reported that a nitrogen macrocyclic *N*-methylhydroxamic acid liberates *p*-nitrophenol from *p*-nitrophenyl carboxylates faster than does the corresponding acyclic compound (2). Here we show that when the *small* *N*-methylacetohydroxamate group is introduced into the cyclohexaamylose ring, which has been shown to accelerate reactions both covalently and noncovalently (3-6), the reactivity of the hydroxamate ion is increased when compared to a small, acyclic *N*-methylhydroxamic acid. This effect is shown to be due to the formation of a cycloamylose-substrate complex. This reaction can serve as an enzyme

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model, for kinetic stereospecificity is observed between *p*-nitrophenyl acetate and *m*-nitrophenyl acetate (*para* is faster than *meta*, in opposition to reactions with cyclohexaamylose itself (3)).

## EXPERIMENTAL

**Materials.** *N*, *O*-Dimethoxyacetyl-*N*-methylhydroxylamine. Five grams (0.06 mole) of *N*-methylhydroxylamine hydrochloride and 20 g (0.185 mole) of methoxyacetyl chloride were stirred magnetically at 0°C while triethyl amine (18.2 g, 0.18 mole) was added dropwise. After addition, the mixture was stirred at 25°C. Then diethyl ether was added to the mixture with stirring and the triethylamine hydrochloride was separated. The ether was removed and the residue distilled, bp 84–85°C (0.3 mm). The product (1.5 g, 13% yield) was clear and odorless and initially gave no red color with FeCl<sub>3</sub> solution.

**Anal.** Calcd for C<sub>7</sub>H<sub>13</sub>O<sub>5</sub>N; C, 43.98; H, 6.86; N, 7.33. Found: C, 43.88; H, 6.75; N, 7.18. The nmr spectrum (CDCl<sub>3</sub>) consisted of five singlets at  $\delta$  3.35 (3H), 3.40 (3H), 3.50 (3H), 4.05 (2H), and 4.20 (2H).

*p*-Nitrophenyl acetate was twice recrystallized from absolute ethanol and twice from chloroform–hexane. It was a gift from Dr. Y. Nakagawa.

*m*-Nitrophenyl acetate was a gift of Dr. G. A. Clowes.

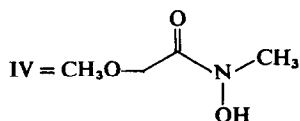
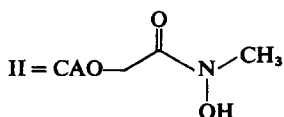
*m*- and *p*-*t*-Butylphenylchloroacetates were prepared by the reaction of *m*- and *p*-*t*-butylphenol with chloroacetyl chloride in benzene solution using triethylamine as catalyst. The *m*-*t*-butyl ester was distilled at 1.0 mm, bp 106–113, and the *p*-*t*-butyl ester was distilled at 2.0 mm, bp 120–122°C. The nmr spectrum of *m*-*t*-butylphenyl chloroacetate had peaks at  $\delta$  1.30 (9H, singlet, *t*-butyl protons), 4.50 (2H, singlet, methylene protons), 7.10 (4 H, multiplet, phenyl protons). The nmr spectrum of *p*-*t*-butylphenyl chloroacetate showed peaks at  $\delta$  1.45 (9H, singlet, *t*-butyl protons), 4.50 (2H, singlet, methylene protons), and 7.30 (4H, quartet, phenyl protons).

2. Hydroxy-5-nitro- $\alpha$ -toluene sulfonic acid sultone was a gift of Dr. E. T. Kaiser.

Cyclohexaamylose (I) was initially purchased from Pierce Chemical Company. Additional quantities were obtained from CPC International, Inc., Englewood Cliffs, N.J. The CPC material was contaminated with 15% of cycloheptaamylose, which was removed by bromobenzene precipitation from aqueous solution as previously described (7). The cyclohexaamylose used for synthesis and kinetic experiments was further purified by recrystallization from 60% 1-propanol–water, followed by recrystallization from water and drying in vacuo.

I = Cyclohexaamylose = CAO

III = CAO—CH<sub>2</sub>—COOH



Carboxymethylcyclohexaamylose was synthesized by the reaction of sodium iodoacetate with an excess of cyclohexaamylose monoanion in dimethylsulfoxide (DMSO) solution. A detailed description of this synthesis has been published elsewhere (3).

Unreacted cyclohexaamylose was separated from carboxylated product by chromatography on a DEAE-Sephadex A-25 column (formate counter ion). Material previously purified on a Sephadex G-10 column was applied to this column in 15 ml of water adjusted to pH 6. The column was first eluted with 350 ml of distilled water and then with 0.5 *N* aqueous formic acid. Unlike the native cyclohexaamylose, the cyclohexaamylose carboxylates do not form a blue color with methanolic iodine spray (9). (This finding suggests that the ring carboxyl group prevents formation of the extended cyclohexaamylose tunnel structures necessary for iodine color development (5, 9, 10).)

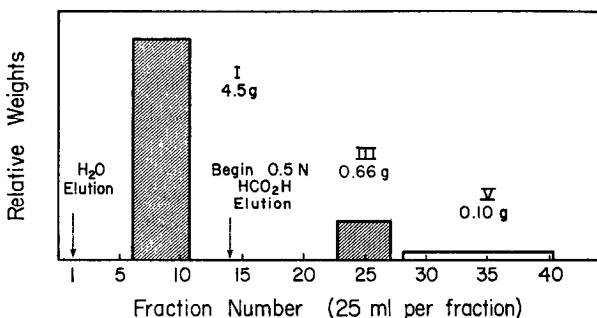


FIG. 1. Elution pattern of DEAE-Sephadex chromatography for separation of cyclohexaamylose, carboxymethyl-, and dicarboxymethylcyclohexaamylose.

The products, which were lyophilized, were dried in vacuo and titrated potentiometrically. On this basis the elution pattern in Fig. 1 was constructed. This figure shows the efficient chromatographic separation of cyclohexaamylose (I), carboxymethylcyclohexaamylose (III), and dicarboxymethylcyclohexaamylose (V).

The equivalent weight of the desired carboxymethylcyclohexaamylose was found to range between 1120 and 1060 g (MW = 1031) or 92–97% purity by weight based on carboxyl group content. The slightly high equivalent weights are probably due to hydration of the carboxymethyl derivative in air. (The equilibrium moisture content of cyclohexaamylose is 6% by weight at 25°C and 10–20% relative humidity or about 4 moles of water per mole of cyclohexaamylose (11). Even with extensive drying, cyclohexaamylose retains 2.5% (w/w) water (12).) This is in accord with the elemental analysis data for carboxymethylcyclohexaamylose below.

*Anal.* Calcd for  $C_{38}H_{62}O_{32}$ ; (MW = 1031), C, 44.27; H, 6.06. Calcd for  $C_{38}H_{62}O_{32} \cdot 4(H_2O)$ , (MW = 1103), C, 41.37; H, 6.40. Found: Sample (1); C, 40.74; H, 6.40. Sample (2); C, 41.76, H, 5.83.

The  $pK_a$  of III was determined by titration. From the pH at half neutralization:  $pK_a = 3.64 \pm 0.121$ , I = 0.001 *M*.

To record the nmr spectrum of carboxymethylcyclohexaamylose, a 100 mg sample was dissolved in 10 ml of 99.8%  $D_2O$ , lyophilized, and then redissolved in  $D_2O$ . The nmr

spectrum is shown in Fig. 2 (A). The HOD line was used as an internal standard with an assigned  $\delta$  of 4.70 relative to TMS (13). For comparison, the nmr spectrum of unmodified cyclohexaamylose is shown in Fig. 2 (B). Spectrum (B) is the same as previously reported (14, 15) for cyclohexaamylose in  $D_2O$  solution where the resonances

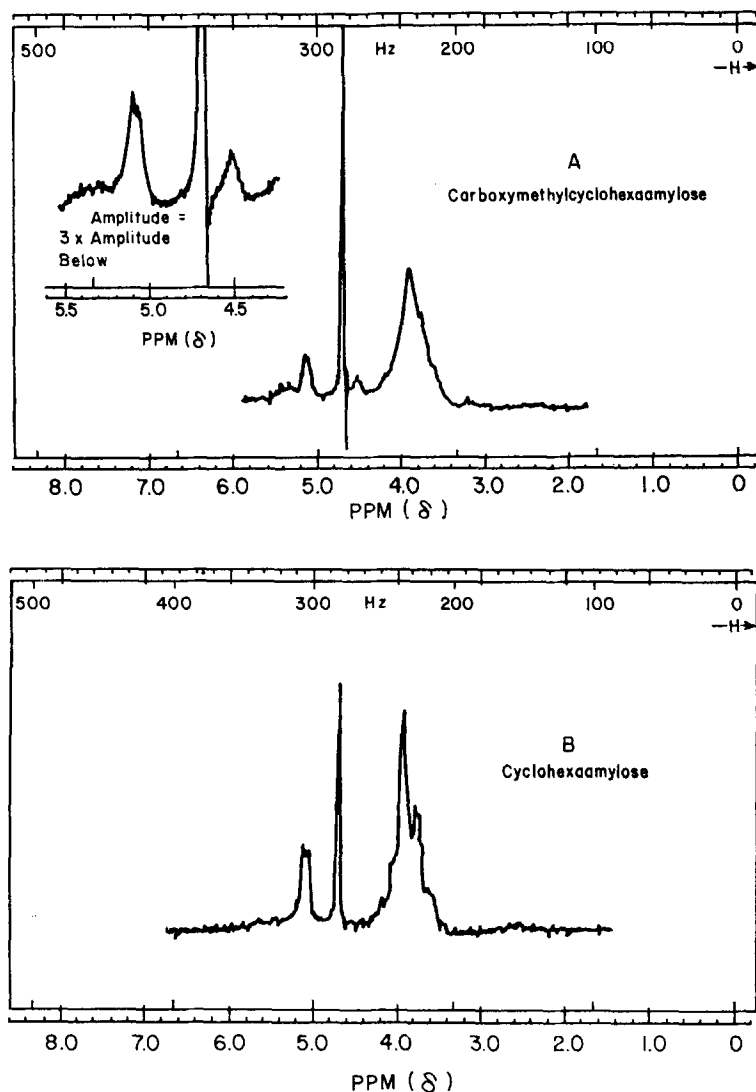


FIG. 2. 60-MHz nmr Spectra of carboxymethylcyclohexaamylose (A) and cyclohexaamylose (B).

have been assigned as follows:  $\delta 5.10$  (6H, doublet,  $J = 2.7$  Hz,  $C_1$ , anomeric protons),  $\delta 3.90$  and  $\delta 3.75$  (36H,  $C_2$ ,  $C_3$ ,  $C_4$ ,  $C_5$ , and  $C_6$  nonanomeric protons). The nmr spectrum of carboxymethylcyclohexaamylose, (B), resembles spectrum (A) in the  $C_1H$  and  $C_2-C_6H$  regions although there is diminished resolution of fine structures. However, there is an additional peak at  $\delta 4.50$  which can be assigned to the carboxymethyl

methylene protons and a poorly resolved small peak at  $\delta 5.43$ . The ratio of areas under the  $\delta 5.10$  and  $\delta 4.50$  resonances is 2.34/liter or about 5/2. Since there are six  $C_1$  anomeric protons and two carboxymethyl methylene protons, this ratio should be 6/2. The small relative area of the  $\delta 5.10$  resonance of the anomeric protons and the additional peak at  $\delta 5.43$  is probably due to a 0.33-ppm downfield shift of one anomeric proton which could result from deshielding of this proton by the carboxymethyl carbonyl group (16).

The infrared spectrum of carboxymethylcyclohexaamylose (KBr wafer) has bands at 2.95 (strong, OH), 3.40 (weak, shoulder, CH), 5.75 (strong, carboxyl C=O), 6.13 (weak, water), and 8.6–10  $\mu$ m (broad, C—O). The carboxymethyl derivative infrared spectrum is the same as unmodified cyclohexaamylose with the exception of the 5.75- $\mu$ m carbonyl stretch.

The 5.75- $\mu$ m carbonyl absorbance is lower than is usually observed for carboxylic acids (17). (The carbonyl stretch of methoxyacetic acid appears at 5.80  $\mu$  (18).) This suggests that carboxymethylcyclohexaamylose exists as a lactone in the solid state and there could be a facile equilibrium between the acid and lactone. An attempt was made to trap this presumed lactone by reaction with 5 *M* hydroxylamine at pH 4.5 and pH 3.0 in aqueous solution. However, no hydroxamic acid formation was detected at either pH upon reaction with  $1 \times 10^{-2}$  *M* carboxymethylcyclohexaamylose for 24 hr at 40°C. After 48 hr at 40°C and pH 3.0, hydroxamic acid was detected; however, the very slow rate of reaction indicates direct reaction of hydroxylamine with the carboxyl group and not with an intermediate lactone (19).

Carboxymethylcyclohexaamylose methyl ester was synthesized by the esterification of carboxymethylcyclohexaamylose with diazomethane in dimethylformamide solvent. Diazomethane was prepared from *p*-toluene sulfonylmethylnitrosamide (Diazald, Aldrich Chemical Co.) by the method outlined by Fieser and Fieser (20).

Carboxymethylcyclohexaamylose, 720 ng (0.70 mmole), was dried for 5 hr at 80°C and dissolved in 20 ml of anhydrous dimethylformamide. The solution was cooled to 10°C and 5 ml of 0.2 *M* diazomethane (1.0 mmole) was added dropwise. At the end of the addition, nitrogen evolution had ceased and the solution was pale yellow. Excess diazomethane was destroyed by treating the solution with acetic acid. The cycloamylose was precipitated with ether and the resulting suspension was centrifuged at 5000 rpm. The organic solvent was decanted and the precipitate was dissolved in water and lyophilized. After drying at 80°C for 24 hr, 0.56 g of a white amorphous solid was recovered which from pH measurements indicated complete esterification. The infrared spectrum of the carboxymethylcyclohexaamylose methyl ester is exactly the same as the parent acid.

Cyclohexaamylose-*N*-methylacetohydroxamic acid (II) was prepared by the reaction of carboxymethylcyclohexaamylose methyl ester with *N*-methylhydroxylamine in DMSO. *N*-Methylhydroxylamine hydrochloride, 0.83 g (10 mmole), was dissolved in 10 ml of anhydrous DMSO and cooled to 0°C. Then 0.48 g (10 mmole) of sodium methoxide was added and the sodium chloride precipitate was removed by filtration. After adding 0.56 g (0.54 mmole) of carboxymethylcyclohexaamylose methyl ester, the solution was heated at 70°C under a nitrogen atmosphere. Hydroxamic acid formation was followed by adding 15- $\mu$ l aliquots of the reaction solution to 3 ml of 5%  $FeCl_3$  in 0.1 *N* HCl and measuring the absorbance at 540 nm (21); this assay indicated that the

reaction was complete after 72 hr. The solution was then cooled and the cycloamylose was precipitated with 200 ml of acetone, collected by filtration, redissolved in water, and chromatographed on a  $2.5 \times 70$  cm G-10 Sephadex column. The column was eluted with distilled water and 25-ml fractions were collected. Fractions 7-9 contained hydroxamic acid ( $\text{FeCl}_3$  assay) and were free of sodium chloride; elution of sodium chloride and DMSO was detected in later fractions. Fractions 7-9 were lyophilized and 2.5 mg of the white solid product from each fraction was titrated in water with sodium hydroxide. The solid from fractions 7, 8, and 9 were found to be mixtures of the carboxymethyl and the hydroxamic acid cycloamylose derivatives. The formation of a large amount of carboxymethylcyclohexaamylose was probably due to hydrolysis of the *O*-acyl hydroxylamine which is known to be an intermediate in hydroxamic acid formation (22, 23).

An adequate separation of the carboxymethyl- from the hydroxamic acid-substituted cycloamylose was achieved by chromatography on a column of DEAE-Sephadex A-25 (formate form) and elution with distilled water. Hydroxamic acid fractions were lyophilized, dried at  $80^\circ\text{C}$  for 10 hr and titrated with 0.1 *N* base.

One inflection point was observed in the titration corresponding to an acid of  $\text{p}K \sim 8.3$  and an equivalent weight of 1360 g; no carboxymethylcyclohexaamylose was detected. The calculated molecular weight of cyclohexaamylose-*N*-methylacetohydroxamic acid is 1060 which indicates that the DEAE-Sephadex-purified material was 78% pure. As was found with carboxymethylcyclohexaamylose, this hydroxamic acid derivative hydrates rapidly in air, and about 6% of the 22% impurity is probably water based on the two analyses below. The additional 16% impurity was either unreacted methyl ester or more probably, from the absence of an ester carbonyl absorption in the IR, cycloamylose; all small molecular-weight molecules were separated on G-10 Sephadex.

*Anal.* Calcd for  $\text{C}_{39}\text{H}_{65}\text{O}_{32}\text{N}$ , (MW = 1060), C, 44.19; H, 6.18; N, 1.32. Calcd for  $\text{C}_{39}\text{H}_{65}\text{O}_{32}\text{N} \cdot 4(\text{H}_2\text{O})$ , (MW = 1132), C, 41.38; H, 6.50; N, 1.24. Found: Sample (1); C, 44.02; H, 6.36; N, 1.46, dried at  $100^\circ\text{C}$  for 5 hr by Micro-Tech Labs. and weighed in a dry box. Sample (2); C, 41.44; H, 5.85; N, 0.91. Sample (2) was dried for 10 hr at  $80^\circ\text{C}$  before being sent for analysis and was weighed in air by Micro-Tech Labs., Skokie, Ill.

The infrared spectrum of cyclohexaamylose-*N*-methylacetohydroxamic acid (KBr wafer) has peaks at 2.95 (strong, OH), 3.40 (weak, shoulder, C—H), 6.05 (Strong, hydroxamic acid, carbonyl) and 8.6–10 $\mu$  (broad, C—O).

*The periodate oxidation of carboxymethylcyclohexaamylose.* To distinguish between 6-O and 2-O or 3-O substitution, the oxidation of carboxymethylcyclohexaamylose by periodate ion was studied.

The oxidations were carried out at a periodate concentration 10% in excess of that required for complete oxidation and without added buffer (24).

The consumption of periodate was followed spectrophotometrically (25) at 300 nm. The oxidation solutions were stored in the dark between measurements. Plots of absorbance vs time are shown in Fig. 3 for the oxidation of carboxymethylcyclohexaamylose, A, cyclohexaamylose, B.

From the absorption spectrum of the periodate stock solution,  $\epsilon_{\text{IO}_4^-} = 137.3$  for sodium metaperiodate at 300 nm. Since the products of the oxidation,  $\text{NaIO}_3$  and

aldehydes, have negligible absorbance at 300 nm (25, 26) the theoretical final absorbance,  $Abs_f$ , for the oxidation of 1 mole of periodate per glycol residue in the cycloamylose is given by Eq. (1) where  $[IO_4^-]_i$  and  $[I]_i$  are the initial concentrations of periodate and carboxymethyl cyclohexaamylose, respectively. The dashed lines in Fig. 3 are the theoretical

$$Abs_f = \epsilon_{IO_4^-} \cdot [IO_4^-]_i - \epsilon_{IO_4^-} \cdot [I]_i \quad (1)$$

final absorbance values calculated from Eq. (1) for the consumption of 5 and 6 moles of periodate per mole of carboxymethylcyclohexaamylose and cyclohexaamylose, respectively. By comparison of the theoretical periodate consumption with the experimental curves, cyclohexaamylose contains the known six glycol residues while carboxymethylcyclohexaamylose clearly contains only five.

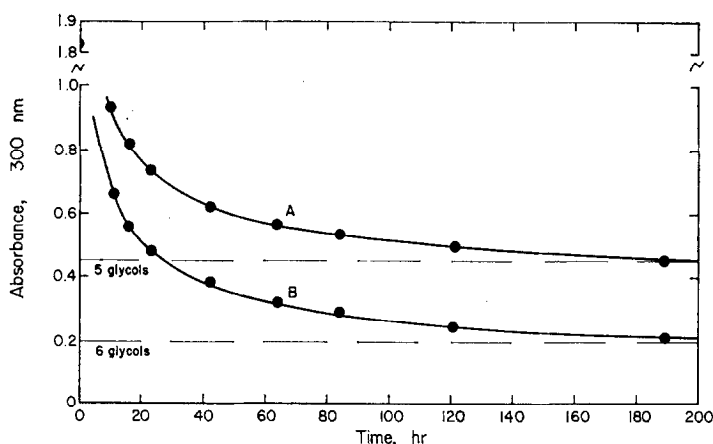


FIG. 3. The periodate oxidation of carboxymethylcyclohexaamylose (A) and cyclohexaamylose (B).

**Determination of dissociation constants of acids.** The  $pK_a$  of II and IV were determined using Eq. (2) and spectral data obtained for these hydroxamic acids at a series of pH values.

$$pK_a = pH + \log \frac{\epsilon_I - \epsilon}{\epsilon - \epsilon_p}, \quad (2)$$

where  $\epsilon_I$ ,  $\epsilon_p$ , and  $\epsilon$  are the molar absorptivities of the completely ionized form, the completely protonated form, and the compound at a pH near the  $pK_a$ , respectively (27, 28).

$\epsilon_I$  at 260 nm for II and IV were determined from spectra of these acids in 0.03 *N* sodium hydroxide,  $I = 0.2$  *M*, KCl.  $\epsilon_p$  for each acid was measured in 0.01 *N*,  $I = 0.2$  *M*, hydrochloric acid. The 0.01 *N* sodium hydroxide spectra of II and IV gave  $\lambda_{max} = 231$  nm and  $\epsilon_{max} = 7400$  for the II anion and  $\lambda_{max} = 232$  nm and  $\epsilon_{max} = 6980$  for the IV anion. The  $pK_a$  of each acid was calculated from Eq. (2) at four different pH values. The average  $pK_a$  values are recorded in Table 1.

### Kinetics

**Kinetics with II in excess over substrate.** II was dried at 80°C in vacuo and stored over  $P_2O_5$  before use.

TABLE I  
p*K*<sub>a</sub> DATA FOR  
CYCLOHEXAAMYLOSE-*N*-METHYLACETOHYDROXYAMIC ACID, II, AND  
*N*-METHYLMETHOXYACETOHYDROXYAMIC ACID, IV<sup>a</sup>

Compound	$\epsilon_1$	$\epsilon_p$	p <i>K</i> <sub>a</sub>
II <sup>b</sup>	2330	<100	8.50 ± 0.15
IV <sup>c</sup>	2290	30	8.51 ± 0.02

<sup>a</sup> 25°C, I = 0.2 *M*. All spectral data at 260 nm.

<sup>b</sup> (II) =  $4.5 \times 10^{-5}$  *M*.

<sup>c</sup> (IV) =  $3.18 \times 10^{-4}$  *M*, obtained from a completely hydrolyzed sample of *N*, *O*-dimethoxyacetyl-*N*-methylhydroxylamine.

Cycloamylose solutions were prepared by placing from 0.1–0.4 mg of II, weighed on a Cahn M-10 Electrobalance, in a 1.3-ml silica cuvette and adding 1 ml of the appropriate buffer. The solutions were used immediately after preparation.

Reactions were initiated by adding 5  $\mu$ l of an acetonitrile stock solution of the appropriate ester to the solution of II in the cuvette. The appearance of the phenol product, followed spectrophotometrically, was in all cases found to adhere strictly to pseudo-first-order kinetics over at least three half-lives. (Correlation coefficients of 0.998 or greater.)

The potentiometric titration of the II used in these experiments indicated that it was 78% pure by weight. To be certain that this II was not contaminated by reactive impurities which would invalidate the kinetic results, II was also titrated by a procedure based on the reactivity of II with a phenyl ester. (The application of such experiments in the determination of enzyme normality has been discussed (30).)

*p*-Nitrothiophenyl acetate was used as a titrant since *p*-nitrothiophenol has a p*K*<sub>a</sub> of 5.1 (32) and its anion is an excellent chromophore. Since II is highly reactive with *p*-nitrophenyl esters, *p*-nitrothiophenyl acetate can be considered to be a specific substrate of cyclohexaamylose.

When 10  $\mu$ l of a  $2.68 \times 10^{-2}$  *M* *p*-nitrothiophenyl acetate–acetonitrile solution (33) was added to 1 ml of a pH 7.95 solution ([II] =  $2.59 \times 10^{-5}$  *M*, [Ester] =  $2.44 \times 10^{-4}$  *M*), the release of *p*-nitrothiophenolate ion, followed at 400 nm was a biphasic curve. This type of biphasic, “burst,” kinetics has been described elsewhere (34). In ref. 34 it is shown that the intercept ( $\pi$ ) of the zero-order portion of the [P<sub>1</sub>] vs *t* curve at *t* = 0 (P<sub>1</sub> = *p*-nitrothiophenolate ion in this case) is given by Eq. 3.

$$\pi = \frac{(k_1[S]_0)^2 \cdot [II]_0}{(k_1[S]_0 + k_2)^2}, \quad (3)$$

where [S]<sub>0</sub> and [II]<sub>0</sub> are the initial ester and catalyst concentrations, respectively, and *k*<sub>1</sub> and *k*<sub>2</sub> are the rate constants for acylation and deacylation of the catalyst, respectively.

Since the rate of *p*-nitrothiophenolate ion appearance in the zero-order portion of the biphasic curve was the same as the buffer hydrolysis rate at pH 7.95, *k*<sub>1</sub>[S]<sub>0</sub> ≫ *k*<sub>2</sub> and by Eq. (3),  $\pi \approx [II]_0$ .



The average intercept at  $t = 0$  for several runs under the above experimental conditions was  $0.284 \pm 0.01$  absorbance units. From this value,  $\pi = [\text{II}]_0 = 2.14 \times 10^{-5} M$  using  $\epsilon = 1.325 \times 10^4$  at 400 nm for *p*-nitrothiophenolate ion (42). The equivalent weight of the reactive species was  $1300 \pm 50$  from spectrophotometry. Thus, this sample of II was 81 % pure (MW = 1060) on the basis of its reactivity with an ester substrate, in good agreement with 78 % purity based on potentiometric titration. Therefore, the molecular weight of II was taken as 1300 in the calculations of all the second-order rate constants determined with catalyst in excess.

The kinetics of the *p*-nitrothiophenyl acetate burst reactions with II were analyzed by the methods given before (34). For the reaction conditions given above, *p*-nitrothiophenolate ion appearance followed pseudo-first-order kinetics to 90 % completion with  $k_{\text{obs}} = 1.5 \times 10^{-2} \text{ sec}^{-1}$ . The observation of clean first-order kinetics gives evidence that II is the only molecule participating in this reaction.

*Kinetics with ester in excess over II.* The methods employed in these studies were for the most part the same as those described in the burst kinetic studies of the reaction of II with *p*-nitrothiophenyl acetate. However, the rapid spontaneous hydrolysis rate of 2-hydroxy-5-nitro- $\alpha$ -toluene sulfonic acid sultone at pH 7.95 caused appreciable curvature in the steady-state portion of the curve. To accurately correct for the contribution made by the spontaneous reaction to the burst portion of the curve, it was necessary to superimpose experimental curves for reactions in the presence and absence of II. The absorbance differences between these curves were then measured and plotted according to a first-order rate equation in the usual manner.

*The reaction of N-methylmethoxyacetohydroxamic acid IV with esters.* A  $9.53 \times 10^{-3} M$  stock solution of IV was prepared by dissolving *N,O*-dimethoxyacetyl-*N*-methylhydroxylamine in pH 7.95,  $I = 0.2 M$ ,  $0.005 M$  borate buffer. The *N, O*-dimethoxyacetyl-*N*-methylhydroxylamine rapidly hydrolyzed ( $k_{\text{obs}} = 2.04 \times 10^{-3} \text{ sec}^{-1}$ ,  $\tau_{1/2} = 5.7 \text{ min}$  at pH 7.95) to yield the stoichiometric amount of IV in this buffer and adjusted to pH 7.95 with sodium hydroxide.

Reaction solutions at different IV concentrations were prepared by diluting the stock solution with the pH 7.95 buffer. The normality of these solutions was checked by measuring their absorbance at 260 nm.

The reaction of esters with IV was initiated in 3-ml cuvettes by the usual techniques and followed spectrophotometrically. First-order rate plots were in all cases linear over at least three half-lives.

## RESULTS

*Kinetics in the presence of excess II and N-methylmethoxyacetohydroxamic acid (IV).* *p*-Nitrophenyl acetate and 2-hydroxy-5-nitro- $\alpha$ -toluenesulfonic acid sultone were reacted with a 15- to 30-fold excess of cyclohexaamylose-*N*-methylacetohydroxamic acid at pH 7.95,  $I = 0.2 M$ , 0.5 % acetonitrile. Pseudo-first-order rate constants,  $k_{\text{obs}}$ , for these reactions are recorded in Table II. The rate constants for the reactions of both *p*-nitrophenyl acetate and the nitrosultone as a function of II concentration are linear; therefore, the simple second-order rate equation,

$$k_{\text{obs}} = k_{\text{II}}[\text{II}] + k_{\text{obs}}^0 \quad (4)$$

describes  $k_{\text{obs}}$  as a function of [II]. ( $k_{\text{obs}}^0$  is the hydrolysis rate of the ester in buffer alone.) Second-order rate constants,  $k_{\text{II}}$ , were calculated by Eq. (4) from the slope (36) of the plots of  $k_{\text{obs}}$  vs [II] and are recorded in Table IV.

In order to compare the reactivity of II with that of a small *N*-methylhydroxamic acid, the esters in Table 2 were also reacted at pH 7.95 with *N*-methylmethoxyacetohydroxamic acid (IV). This hydroxamic acid has the same  $pK_a$  as II. Pseudo-first-order

TABLE 2  
PSEUDO-FIRST-ORDER RATE CONSTANTS FOR THE REACTION OF ESTERS WITH EXCESS II<sup>a</sup>

Ester	[Ester] $\times 10^6 M$	$\lambda$ (nm) <sup>b</sup>	[II] $\times 10^4 M$	$k_{\text{obs}}$ $\times 10^3 \text{ sec}^{-1}$
<i>p</i> -Nitrophenyl acetate	5.1	400	0.0	0.036 <sup>c</sup>
			0.82	4.27
			1.45	9.50
			1.86	11.3
			2.42	18.5
			3.89	29.4
<i>m</i> -Nitrophenyl acetate <sup>c</sup>	23.0	340	0.0	0.023 <sup>f</sup>
			3.54	2.55
2-Hydroxy-5- nitro- $\alpha$ - toluene- sulfonic acid sultone <sup>d</sup>	4.8	400	0.0	1.37
			0.82	5.97
			2.13	11.2
			4.51	21.5
<i>m-t</i> -Butylphenyl chloroacetate	49.0	275	0.0	0.90
			2.2	2.0
<i>p-t</i> -Butylphenyl chloroacetate	49.9	275	0.0	0.80
			3.0	1.9

<sup>a</sup> All reactions were run in pH = 7.95,  $I = 0.2$ , 0.05 *M* borate buffer, 0.49% (v/v) acetonitrile.

<sup>b</sup> Wavelength at which reaction was followed.

<sup>c</sup> Gift of Dr. G. A. Clowes.

<sup>d</sup> Gift of Dr. E. T. Kaiser.

<sup>e</sup> From initial rate.

<sup>f</sup> From  $k_{\text{obs}}^0$  data at pH 10.60<sup>35</sup> and Eq. (5).

rate constants were determined with 10- to 100-fold excess IV and the results are recorded in Table 3. Plots of the pseudo-first-order rate constants,  $k_{\text{obs}}$ , for these reactions as a function of *N*-methylmethoxyacetohydroxamic acid concentration are linear for the five esters in Table 3. Second-order rate constants,  $k_{\text{IV}}$ , were calculated from the equation  $k_{\text{obs}} = k_{\text{IV}}[\text{IV}] + k_{\text{obs}}^0$  and are shown in Table 4.  $k_{\text{IV}}$  for the chloroacetates in Table 4 were calculated from this equation using data at one IV concentration.

TABLE 3  
PSEUDO-FIRST-ORDER RATE CONSTANTS FOR THE REACTION OF ESTERS WITH  
*N*-METHYLMETHOXYACETOHYDROXAMIC ACID<sup>a</sup>

Ester	[Ester] $\times 10^5 M$	$\lambda$ (nm) <sup>b</sup>	[IV] $\times 10^3 M$	$k_{\text{obs}}$ $\times 10^3 \text{ sec}^{-1}$
<i>p</i> -Nitrophenyl acetate	7.8	400	0.0	0.04 <sup>c</sup>
			1.50	5.54
			3.75	13.7
			9.37	38.3
<i>m</i> -Nitrophenyl acetate	40.3	420	0.0	0.02 <sup>d</sup>
			1.51	1.81
			3.78	5.02
			9.45	12.3
2-Hydroxy-5- nitro- $\alpha$ - toluene- sulfonic acid sultone	9.44	400	0.0	1.37
			5.51	2.43
			3.78	3.62
			9.44	7.76
<i>m</i> - <i>t</i> -Butylphenyl chloroacetate	4.9	275	0.0	0.9
			1.50	5.3
<i>p</i> - <i>t</i> -Butylphenyl chloroacetate	5.0	275	0.0	0.8
			1.50	4.7

<sup>a</sup> pH 7.95,  $I = 0.2 M$ ,  $0.05 M$  borate buffer, 0.0–1.6% (v/v) acetonitrile, 25°C.

<sup>b</sup> Wavelength at which reaction was followed.

<sup>c</sup> See Table II.

<sup>d</sup> From  $k_{\text{obs}} = 6.0 \times 10^{-3} \text{ sec}^{-1}$  at pH 10.60 and Eq. (5).

Since II has 17 hydroxyls that are unmodified and which may react with esters, the reaction of native cyclohexaamylose with these five esters were also studied at pH 7.95. Second-order rate constants,  $k_{\text{obs}}$ , for these reactions are also given in Table 4.

The ratios of  $k_{\text{II}}$  to  $k_{\text{IV}}$  are presented in Table 4. It can be seen that the  $k_{\text{II}}/k_{\text{IV}}$  ratios vary greatly for the different esters studied. Thus, *p*-nitrophenyl acetate and the nitro-sultone, respectively, react 17 and 70 times faster with the cyclohexaamylose-substituted hydroxamic acid while much smaller difference in reactivity is observed for *m*-nitrophenyl acetate and the *t*-butylphenyl chloroacetates. With the exception of *m*-*t*-butylphenyl chloroacetate, II reacts much faster with these esters than does unsubstituted cyclohexaamylose at this pH.

*Cyclohexanol inhibition of the reaction of II with p-nitrophenyl acetate.* Since cyclohexaamylose is known to bind aromatic esters in aqueous solutions (3), the increased reactivity of II when compared to IV could be due to the formation of a II-ester complex.

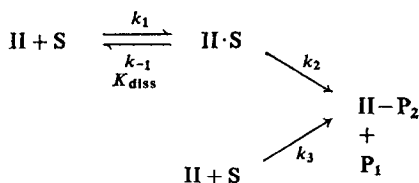
A kinetic scheme which accommodates both intermolecular and intracomplex reactions of II with ester (S) is presented in Scheme 1.

TABLE 4

KINETIC DATA FOR THE REACTION OF ESTERS WITH CYCLOHEXAAMYLOSE-*N*-METHYLACETOHYDROXAMIC ACID (II), *N*-METHYLMETHOXYACETOHYDROXAMIC ACID (IV), AND CYCLOHEXAAMYLOSE (I)<sup>a</sup>

Ester	$k_I$ $M^{-1} \text{ sec}^{-1}$	$k_{IV}$ $M^{-1} \text{ sec}^{-1}$	$k_{II}$ $M^{-1} \text{ sec}^{-1}$	$\frac{k_{II}}{k_{IV}}$
<i>p</i> -Nitrophenyl acetate	0.08	4.01	72.4	17.0
<i>m</i> -nitrophenyl acetate	0.9	1.30	7.3	5.6
2-Hydroxy-5-nitro- $\alpha$ -toluene-sulfonic acid sultone	0.25	0.65	45.2	70.0
<i>p</i> - <i>t</i> -Butylphenyl chloroacetate	0.06	2.6	7.0	6.27
<i>m</i> - <i>t</i> -Butylphenyl chloroacetate	46.0	29.0	(9.0)	<3.0

<sup>a</sup> pH = 7.95, I = 0.2 M, 0.05 M borate buffer, 25°C, acetonitrile concentration <1 %.



SCHEME 1

In this scheme,  $K_{\text{diss}}$  is the dissociation constant for the II-ester complex (II·S),  $k_2$  is the first-order rate constant for reaction within the complex to form phenol ( $\text{P}_1$ ) and acyl-II (II- $\text{P}_2$ ), and  $k_3$  is the second-order rate constant for product formation by intermolecular reaction of II with S.

When the concentration of II is much greater than that of the ester, Eq. (5) describes the concentration dependence of the observed pseudo-first-order rate constant for reactions that follow Scheme 1 (35, 37). If it is assumed that II·S cannot react with II but II·S can react with S,

$$k_{\text{obs}} = (k_2 + k_3 \cdot K_{\text{diss}}) \left( \frac{[\text{II}]}{K_{\text{diss}} + [\text{II}]} \right). \quad (5)$$

If [II] is increased such that  $[\text{II}] \gg K_{\text{diss}}$  and if  $k_2 \gg k_3 K_{\text{diss}}$  Eq. (6) predicts that  $k_{\text{obs}}$  will reach a constant value of  $k_2$  at high [II]. The observation of such a plateau in a

$k_{\text{obs}}$  vs  $[\text{II}]$  plot is usually termed "saturation." However, at low  $[\text{II}]$  where  $[\text{II}] \ll K_{\text{diss}}$ , Eq. (5) reduces to Eq. (6)

$$k_{\text{obs}} = (k_2/K_{\text{diss}})[\text{II}] + k_3[\text{II}] \quad (6)$$

and a linear dependence on  $[\text{II}]$  is expected, as found earlier.

Although the demonstration of saturation kinetics is the most direct method to show that a reaction adheres to Scheme 1, a useful indirect method is to demonstrate competitive inhibition. If an inert molecule, IN, which complexes with II is added to the II-S reaction mixture, IN will compete with S for II. Under the conditions of  $[\text{IN}] \gg [\text{II}]_0 \gg [\text{S}]$  and  $K_{\text{diss}} \gg [\text{II}]_0$ , Eq. (7) may be derived to describe the competitive inhibition by IN of a reaction which follows Scheme 1. (The derivation of this equation is shown in the appendix.)

$$\frac{k_{\text{obs}}}{[\text{II}]_0} = \left( \frac{k_2}{K_{\text{diss}}} \right) \frac{K_{\text{IN}}}{K_{\text{IN}} + [\text{IN}]_0} + k_3. \quad (7)$$

In Eq. (7),  $K_{\text{IN}}$  is the dissociation constant of the  $\text{II} \cdot \text{IN}$  complex.

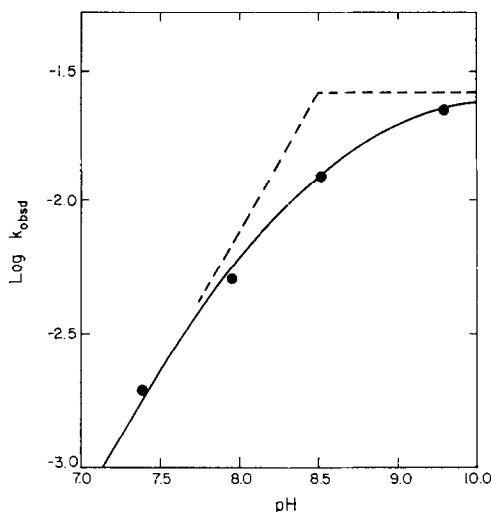


FIG. 4. pH- $\log k_{\text{obs}}$  profile for the reaction of II with *p*-nitrophenyl acetate. Intersection of dashed lines gives  $\text{p}K_a$ .

II was reacted with *p*-nitrophenyl acetate at pH 7.95,  $I = 0.2 M$ , in the presence of different concentrations of cyclohexanol inhibitor. Concentrations were selected such that  $[\text{IN}] \gg [\text{II}] \gg [\text{S}]_0$  and the kinetic results are shown in Table 5. As anticipated,  $k_{\text{obs}}$  decreases as  $[\text{IN}]$  increases.

According to Eq. (7), as  $[\text{IN}]$  becomes larger than  $K_{\text{IN}}$  ( $1/[\text{IN}]$  goes to zero),  $k_{\text{obs}}/[\text{II}]$  approaches  $k_3$ . Therefore, from a plot of  $k_{\text{obs}}/[\text{II}]_0$  vs  $1/[\text{IN}]$ ,  $k_3$  may be estimated by extrapolation to the intercept at  $1/[\text{IN}] = 0$ . From such a plot shown in Fig. 5,  $k_3$  is about  $3 M^{-1} \text{sec}^{-1}$ . Since  $k_{\text{II}} = k_2/K_{\text{diss}} + k_3 = 72 M^{-1} \text{sec}^{-1}$  for the reaction of II with *p*-nitrophenyl acetate at pH 7.95,  $k_2/K_s \sim 20 k_3$ . Hence the predominant pathway for this reaction appears to involve the formation of a  $\text{II} \cdot \text{S}$  complex.

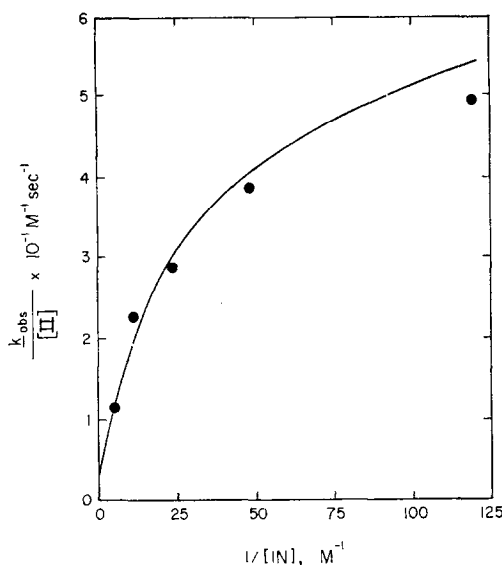


FIG. 5. The cyclohexanol inhibition of the reaction of *p*-nitrophenyl acetate with cyclohexaamylose-*N*-methylhydroxamic acid.

$K_{IN}$  for the cyclohexanol inhibition may be determined from Eq. (8) which is obtained by rearranging Eq. (7) and neglecting  $k_3$ .

$$\frac{k_2}{K_{diss}} \frac{1}{[II]_0} \frac{1}{k_{obs}} = 1 + \frac{[IN]}{K_{IN}} \quad (8)$$

By Eq. (8), a plot of  $(k_2/K_{diss})/(k_{obs}/[II]_0)$  vs  $[IN]$  should be linear and have a slope of  $1/K_{IN}$  and a unit intercept. The data in Table 5 were plotted in this fashion using a  $k_2/K_{diss} = 72 \text{ M}^{-1} \text{ sec}^{-1}$ , giving a slope of  $38 \text{ M}^{-1}$  and intercept of 1.15 as shown in Fig. 6. From the reciprocal of the slope,  $K_{IN} = 2.6 \times 10^{-2} \text{ M}$ . The curved line in Fig. 5 is the theoretical curve calculated from Eq. (7) using  $K_{IN} = 2.6 \times 10^{-2} \text{ M}$ ,  $k_3 = 3 \text{ M}^{-1} \text{ sec}^{-1}$ , and  $k_2/K_{diss} = 72 \text{ M}^{-1} \text{ sec}^{-1}$ .

TABLE 5

RATE CONSTANTS FOR THE REACTION OF *p*-NITROPHENYL ACETATE  
WITH II IN THE PRESENCE OF CYCLOHEXANOL INHIBITOR<sup>a</sup>

$[II]$ $\times 10^4 \text{ M}^0$	$[IN]^b$ $\times 10^2 \text{ M}$	$k_{obs}$ $\times 10^3 \text{ sec}^{-1}$	$\frac{k_{obs}}{[II]_0} \text{ M}^{-1} \text{ sec}^{-1}$
1.52	16.67	1.72	11.3
1.28	8.34	2.09	22.7
1.29	4.17	3.71	28.7
1.57	2.08	5.94	37.8
1.38	0.83	6.84	49.6

<sup>a</sup> pH = 7.95, I = 0.2 M, 0.05 M borate buffer, 0.5% acetonitrile,  
[*p*-nitrophenyl acetate] =  $5.07 \times 10^{-6} \text{ M}$ .

<sup>b</sup> Cyclohexanol concentration.

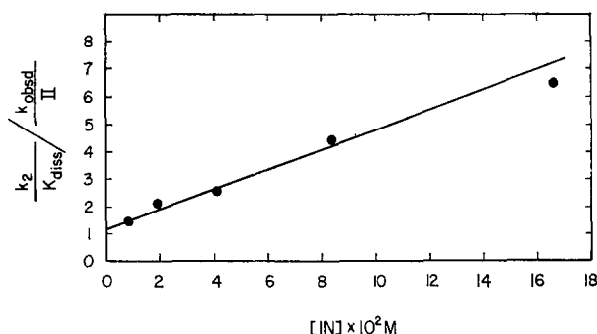


FIG. 6. Determination of  $K_{\text{IN}}$  for the cyclohexanol inhibition of the reaction of II with *p*-nitrophenyl acetate.

The pH-rate profile for the reaction of II with *p*-nitrophenyl acetate was determined with ester in excess. The results are shown in Fig. 4, where the points on the curve are the experimental results and the curved line is the theoretical, drawn from the equation

$$\log k_{\text{obs}} = \log (k_{\text{II}}^{\text{B}} \cdot [\text{S}]_0) + \log \left( \frac{K_{\text{app}}}{K_{\text{app}} + [\text{H}^+]} \right), \quad (9)$$

using  $\text{p}K_{\text{app}} = 8.50$  and  $k_{\text{II}}^{\text{B}} = 77 \text{ M}^{-1} \text{ sec}^{-1}$ . This  $\text{p}K_{\text{app}}$  determined kinetically is in very good agreement with the  $\text{p}K_{\text{a}} = 8.50 \pm .15$  which was determined for II spectrophotometrically.

## DISCUSSION

### 1. The Structure of II

The nmr, infrared spectra, and  $\text{p}K_{\text{a}}$  of carboxymethylcyclohexaamylose are all in accord with an alkoxy-substituted acetic acid. The periodate titration of carboxymethylcyclohexaamylose has shown that this cycloamylose derivative contains five glycol groups per molecule rather than six. The only structure consistent with these data is a cyclohexaamylose which is substituted on the C-2, C-3 secondary hydroxyl group side of the torus. Although the present data do not distinguish between substitution at the C-2 and C-3 oxygen of the glucopyranoside ring, other workers have found that in the preparation of dodecamethylcyclohexaamylose substitution of the secondary hydroxyls occurs selectively at the C-2 hydroxyls (38).

The  $\text{p}K_{\text{a}}$ , uv spectrum, elemental analysis, and extinction coefficient of II are all consistent with an alkoxy-substituted *N*-methylacetohydroxamic acid. In addition, it is highly unlikely that the transposition of the alkyl group would occur in the synthetic steps for the conversion of the acetic acid function to the *N*-methylacetohydroxamic acid group. Therefore, it will be assumed in the following discussion that the *N*-methylacetohydroxamic acid group of II is positioned at a C-2 oxygen.

### 2. The Reaction Pathway and the Origin of Specificity

The data presented in the Results section provide evidence for the existence of at least two intermediates in the reaction of *p*-nitrophenyl acetate with II. The competitive-type inhibition by cyclohexanol supports compulsory noncovalent complex

formation between the ester and the cycloamylose derivative. This is fully consistent with the kinetic and physical behavior of the cyclohexaamylose ring toward substrates of comparable size and hydrophobic character (35).

The II-S complex then reacts with the hydroxamate function in an intracomplex reaction with the simultaneous release of *p*-nitrophenolate ion. Evidence for the selective reaction of the hydroxamate group as opposed to acylation of a secondary hydroxyl is provided by the fact that: (a) The rate of the acylation reaction is strictly proportional to the concentration of the ionized hydroxamate function, cf. Fig. 4. (b) After the stoichiometric acylation of II, the molecule is catalytically inert whereas if acylation initially occurred at a hydroxyl group the hydroxamate function could be further acylated by an intermolecular process.

Concerning the mechanism of acylation, the pH dependence of the reaction, is in good agreement with a nucleophilic substitution at carbonyl carbon by the hydroxamate anion. This is in good agreement with the acylation mechanism of the small hydroxamic acid (*I*). The neighboring hydroxyl groups certainly do not act as general acid or base catalysts in the process since they have *pK* values higher than 11 and the reaction rate appears to become independent of pH at high pH.

Concerning the greater reactivity of II as compared to the small hydroxamic acid, IV, a definitive evaluation of this effect cannot be made until the  $k_{II}$  rate constants are separated into the individual  $k_2$  and  $K_{diss}$  terms. However, a reasonable value of  $K_{diss}$  for the II-*p*-nitrophenyl acetate complex is  $5 \times 10^{-2} M$  since: (a) II does appear to bind small molecules with greater facility than the nickel oximecycloamylose derivative studied by Breslow (39),  $K_{IN}$  for cyclohexanol complexation is  $2.6 \times 10^{-2} M$  and  $5-7 \times 10^{-2} M$  for II and Breslow's cycloamylose derivative, respectively. (b) II does not bind aromatic molecules with bulky substituent groups as evidenced by  $k_{II} < k_I$  in *m*-*t*-butylphenyl chloroacetate (cf. Table 4). (c)  $K_{diss} = 1 \times 10^{-2} M$  for the *I*-*p*-nitrophenyl acetate complex (35).

Assuming  $K_{diss} = 5 \times 10^{-2} M$  and from  $k_2/K_{diss} = 72 M^{-1} sec^{-1}$ ,  $k_2 = 3.6 sec^{-1}$  for the intracomplex reaction of II with *p*-nitrophenyl acetate. The "effective concentration" (40) of the II hydroxamate group in the vicinity of the complexed ester may then be evaluated from the ratio of the first-order rate constant for the intracomplex reaction to the second-order rate constant for the reaction with IV. From the data in Table 4,  $k_2/k_{IV} = 1 M$ . On the basis of this value, the increased reactivity of II can be fully accounted for by a locally increased concentration of the II hydroxamate group in the II-ester complex (40).

In its reactions with phenyl esters, II appears to exhibit a marked kinetic stereospecificity for *p*-substituted as opposed to *m*-substituted phenyl esters. This is the direct opposite of the stereospecificity observed for unmodified cyclohexaamylose which reacts selectively with *m*-substituted phenyl esters (37) and is particularly seen with the *t*-butylphenyl esters (Table 4).

The kinetic stereospecificity of II can be due to either different dissociation constants for the II-ester complexes or to different reaction rates within these complexes. The data of Bender and coworkers (35), however, indicate that  $K_{diss}$  is approximately the same for *m*- and *p*-substituted nitrophenyl esters. Assuming this to also be the case for II, it is possible to speculate that its kinetic specificity is due to the geometry of the II-ester complexes.



A scale molecular model of a II-*p*-nitrophenyl acetate complex was constructed by insertion of the nitro-substituent end of the ester into the cavity and positioning the hydroxamate group in proximity to the ester carbonyl. In this conformation the complex produces the ideal stereochemistry for the intracomplex reaction. However, this is not the case for the II-*m*-nitrophenyl acetate complex which upon rotation of the phenyl ring produces predominantly nonproductive orientations. The kinetic selectivity of II for *p*-nitro- over *m*-nitrophenyl acetate can be explained, therefore, by the closer average proximity of the *para* isomer carbonyl group to the nucleophilic hydroxamate group.

Since the complex formed by the insertion of the 2-hydroxy-5-nitro- $\alpha$ -toluenesulfonic acid sultone nitro group into the II cavity rotationally resembles the II-*m*-nitrophenyl acetate complex, the high reactivity of this molecule with II is surprising. This phenomenon may be due to a decreased reactivity of the intermediate formed upon reaction with II. As pointed out by Berg et al., the reactions of sultones with nucleophiles may be a readily reversible process (41). The low reactivity of this sultone with small hydroxamic acids could be due to the facility of the back reaction to expel the nucleophile and regenerate the sultone. The reverse reaction would be very slow in the case of the intermediate formed with II if the phenyl ring of this intermediate were complexed intramolecularly to the II cavity.

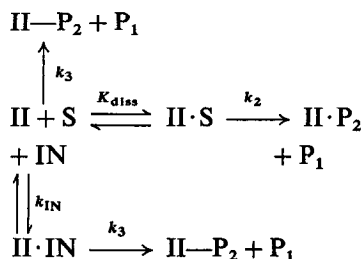
When the small *N*-methyl-acetohydroxamate group is introduced into the cyclohexaamylose ring the reactivity of the hydroxamate ion is increased 20- to 70-fold when compared to a small *N*-methylhydroxamic acid. This effect has been shown to be due to the formation of a cycloamylose-substrate complex. The observation of a kinetic stereospecificity for *p*-nitro over *m*-nitrophenyl acetate satisfies one important characteristic desired in an enzyme model. However, cyclohexaamylose incorporates only two of the mechanistic principles which are associated with the pathway of the proteolytic enzymes, namely, a covalent intermediate and substrate binding. Upon incorporation of the third mechanistic feature, internal catalysis (*I*), the prospects of observing large overall catalysis should be greatly enhanced. The work reported here clearly points the way to the ultimate goal of the synthesis of a simple yet catalytically functional model active site.

### ACKNOWLEDGMENT

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### APPENDIX

The scheme involving a competitive inhibitor is:



In the above scheme  $\text{II} \cdot \text{S}$  and  $\text{II} \cdot \text{IN}$  are complexes of 1:1 stoichiometry.  $\text{II} \cdot \text{IN}$  is assumed to react with S with the same intermolecular second-order rate constant,  $k_3$ , as  $\text{II}$ . For the initial conditions of  $[\text{IN}] \gg [\text{II}]_0 \gg [\text{S}]_0$ . And defining  $F_c$  as the fraction of S complexed,

$$F_c = \frac{[\text{II} \cdot \text{S}]}{[\text{S}][\text{II} \cdot \text{S}]}$$

and since  $[\text{II}]_0 \ll K_{\text{diss}}$ ,

$$F_c = \frac{[\text{II} \cdot \text{S}]}{[\text{S}]}.$$

Considering the pathways that lead to products

$$k_{\text{obs}} k_2 \cdot F_c k_3 [\text{II}]_0.$$

Using

$$K_{\text{diss}} = \frac{[\text{II}][\text{S}]}{[\text{II} \cdot \text{S}]} \quad \text{and} \quad K_{\text{IN}} = \frac{[\text{II}][\text{IN}]}{[\text{II} \cdot \text{IN}]}, \quad F_c = \frac{[\text{II}]_0}{K_{\text{diss}}} \cdot \frac{K_1}{K_1 + [\text{IN}]}.$$

Substituting into the expression for  $k_{\text{obs}}$

$$k_{\text{obs}} = \frac{k_2}{K_{\text{diss}}} \cdot \frac{K_{\text{IN}}}{K_{\text{IN}} + [\text{IN}]} \cdot [\text{II}]_0 + k_3 [\text{II}]_0.$$

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