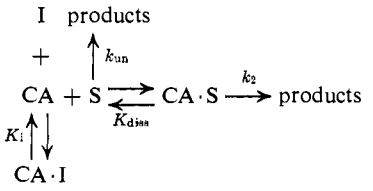
**Determination of Dissociation Constants by Inhibition Methods.** In the reaction scheme




$K_i$ is the dissociation constant of the 1:1 cycloamylose–inhibitor complex CA·I, $k_{un}$ is the spontaneous reaction rate constant of the substrate S, $K_{diss}$ is the dissociation constant of the 1:1 cycloamylose–substrate complex CA·S (determined by independent kinetic methods as already described), $k_2$ is the catalyzed reaction rate constant of the complexed substrate, and $[S]_0$ and $[CA]_0$ the initial stoichiometric concentrations of substrate and cycloamylose, respectively. The fraction $F_c$ of complexed substrate will be[22] $(k_{obsd} - k_{un})/(k_2 - k_{un})$. Then

$$K_{diss} = [S][CA]/[CA\cdot S] = [S_0 - CA\cdot S][CA]/[CA\cdot S]$$
$$= [(1 - F_c)/F_c][CA]$$
$$= [(1 - F_c)/F_c][CA_0 - CA\cdot I - CA\cdot S]$$

but since $[A_0 + CA\cdot I] >> [CA\cdot S]$ this becomes

$$= [(1 - F_c)/F_c](CA_0 - [CA][I]/K_i)$$
$$= [(1 - F_c)/F_c]\left(CA_0 - \frac{[I][CA\cdot S]}{[S]}\frac{K_{diss}}{K_i}\right)$$
$$= [(1/F_c) - 1]\left[\frac{[CA_0 - [I]K_{diss}}{K_i}\left(\frac{F_c}{1 - F_c}\right)\right]$$
$$= [(1/F_c) - 1]CA_0 - ([I]K_{diss}/K_i)$$

so that on rearranging

$$[I] = [(1/F_c) - 1][CA_0]K_i/K_{diss} - K_i$$
$$= \left(\frac{1/(k_{obsd} - k_{un})}{(k_2 - k_{un})} - 1\right)\frac{[CA_0]K_i}{K_{diss}} - K_i$$
$$= \left(\frac{k_2 - k_{obsd}}{k_{obsd} - k_{un}}\right)\frac{[CA_0]K_i}{K_{diss}} - K_i$$

Thus, by plotting the inhibitor concentration *vs.* $(k_2 - k_{obsd}/(k_{obsd} - k_{un})$ an approximate straight line is obtained with intercept $-K_i$ and slope $([CA_0]\cdot K_i)/K_{diss}$.

# The Mechanism of the Cycloamylose-Accelerated Cleavage of Phenyl Esters[1-3]

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**Abstract:** The release of phenols from a number of *meta*-substituted phenyl benzoates is accelerated in alkaline solution by cyclohexaamylose and cycloheptaamylose. The acyl portion is transferred to a hydroxyl group of the amylose, forming a cycloamylose benzoate which undergoes hydrolysis *via* a subsequent reaction at a rate independent of the nature of the phenolic group. Cyclohexaamylose benzoate was separated from phenol and unreacted *m*-nitrophenyl benzoate by gel filtration chromatography and was found to undergo hydrolysis at the same rate as the intermediate formed *in situ* during the acceleration of phenol release from a variety of *meta*-substituted phenyl benzoates (substituents on the phenyl group). The hydrolysis of cycloamylose benzoates (deacylation) conforms to a Hammett relationship with $\rho = 1.6$. The pH dependence of the initial rate acceleration (acylation) and of the deacylation reaction agrees with a dependence on a group of $pK_a = 12.1$. Heptamesylcycloheptaamylose (primary hydroxyl groups blocked) causes as large an acceleration of phenyl ester cleavage as native cycloheptaamylose, but dodecamethylcyclohexaamylose (primary hydroxyl and half of secondary hydroxyl groups blocked) causes a small inhibition of the hydrolysis. This is consistent with a proposed mechanism involving nucleophilic participation by an alkoxide ion derived from the secondary hydroxyl groups of the cycloamylose. The hydrolysis of cycloamylose benzoates occurs ~20 times more rapidly than would be predicted on the basis of simple steric and electronic considerations so that the deacylation step may be subject to general acid or general base catalysis by vicinal hydroxyl groups of the cycloamylose. The cycloamylose pathway of binding, acylation, and deacylation is formally similar to the pathway of chymotrypsin-catalyzed hydrolysis of esters. Comparisons of chymotrypsin catalyses and cycloamylose reactions are made, including a comparison of the second-order rate constants of these substances with substrates.

The cycloamyloses cause a markedly stereoselective acceleration of the release of phenols from substituted phenyl acetates, the rate accelerations with *meta*-substituted esters being larger than with the corresponding *para*-substituted esters.[3] For example, 0.01 *M* cycloheptaamylose causes a 250-fold increase

(1) This research was supported by a grant from the National Science Foundation.

(2) A preliminary account of this work has been published: M. L. Bender, R. L. VanEtten, and G. A. Clowes, *J. Am. Chem. Soc.*, **88**, 2319 (1966).

(3) Accompanying article: R. L. VanEtten, J. F. Sebastian, G. A. Clowes, and M. L. Bender, *ibid.*, **89**, 3242 (1967).

(4) National Institutes of Health Postdoctoral Research Fellow.

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in the rate of phenol release from *m-t*-butylphenyl acetate but only a 2.2-fold increase for the *para* analog. The rate effects are mediated by a cycloamylose–ester complex in solution, and the reaction system exhibits many characteristics of enzyme-catalyzed reactions.[3] Because the rate effects are independent of the stabilities of the respective complexes it appeared unlikely that the rate effects were due to an unusual medium effect such as has been observed[6] for the hydrolysis of alkyl hydrogen sulfate esters. Moreover, the stereospecificity of the rate accelerations can be readily interpreted only on the basis of an interaction of the secondary hydroxyl groups of the cycloamylose (derived from carbon atoms 2 and 3 of the individual glucose residues) with the carbonyl carbon of the ester. The carbonyl carbon of *para*-substituted phenyl esters can readily be placed in proximity to the primary hydroxyl groups (derived from carbon atom 6 of the glucose residues) of cyclooctaamylose whereas it cannot be placed in proximity to the secondary hydroxyl groups of cyclohexa- and -heptaamylose. Cyclooctaamylose causes a large acceleration of phenol release from both *m*- and *p-t*-butylphenyl acetate consistent with the fact that the cavity is so large that the carbonyl group of either ester may be placed in proximity to the secondary hydroxyl groups of the cycloamylose when the aromatic ring and the *t*-butyl group are included in the cavity.

The specific mechanism by which the cycloamylose hydroxyl groups accelerate the release of phenols from phenyl esters is not obvious since participation may occur by nucleophilic, general acid, or general base mechanisms.[7] Thus it is important to attempt to distinguish between these possible mechanisms. The isolation of cyclohexaamylose phosphate from heterogeneous alkaline reaction mixtures (where the cycloamyloses cause an acceleration of phenol release from diphenyl pyrophosphate) has been reported.[8] This observation suggested that the rate accelerations observed in the course of the present work might be the result of nucleophilic catalysis. The present investigation of the hydrolysis of substituted phenyl benzoates and of the pH dependence of the rate accelerations was therefore undertaken in order to determine the mechanisms by which the cycloamyloses exert their effects on phenyl ester hydrolysis reactions.

## Experimental Section

All melting points and boiling points are uncorrected. Aqueous solutions were made up with distilled or double-distilled water and reagent grade chemicals. Eastman Spectro grade acetonitrile was employed for stock solutions of esters and for reaction solvents. Elemental analyses were carried out by Micro-Tech Laboratories, Skokie, Ill. A Radiometer 4c pH meter was used for the determination of pH values.

Cyclohexaamylose and cycloheptaamylose were obtained and purified as already described.[3] Dodecamethylcyclohexaamylose was very kindly provided by Dr. Hermann Schlenk.[9] The preparation of hexamesylcyclohexaamylose and heptamesylcycloheptaamylose has been described.[10] The hexa(6-O-mesyl)cyclohexaamylose[11] was purified by recrystallization from methanol. The ultraviolet absorption spectrum of this compound revealed an absorption band ($\lambda_{max}$ 259 m$\mu$) which was attributed to residual pyridine. Efforts to completely remove the impurity by dilute acid wash (pH 2.2) and vacuum drying were unsuccessful. The amount of pyridine was calculated to be less than 5% (by weight) based on the optical extinction coefficient of pyridine (*ca.* 3600 in water). The infrared spectrum (potassium bromide disk) indicated the presence of covalent sulfonate (bands at 1345 and 1180 $cm^{-1}$) consistent with the literature value.[12]

*Anal.* Calcd for $(C_7H_{12}O_7S)_6$: C, 35.01; H, 5.04; S, 13.33. Found: C, 35.09; H, 4.95; S, 13.52.

Hepta(6-O-mesyl)cycloheptaamylose also showed a small absorption band attributed to residual pyridine (less than 2% by weight). The infrared spectrum showed bands at 1340 and 1165 $cm^{-1}$ which were attributed to covalent sulfonate.

*Anal.* Calcd for $(C_7H_{12}O_7S)_7$: C, 35.01; H, 5.04; S, 13.33. Found: C, 34.92; H, 5.19; S, 13.61, 14.02.

The mesyl compounds were relatively insoluble in water so that reactions in the presence of these compounds were carried out in 20.5% acetonitrile solution (v/v). The reported values of the pH are the observed values obtained for the aqueous acetonitrile solutions.

**Benzoate** esters were prepared by the Schotten–Baumann procedure using the appropriate benzoyl chloride (25 mmoles) and sodium phenolate (25 mmoles) in 40 ml of water. Solid esters were recrystallized from 95% ethanol and dried under vacuum.

*m-t*-Butylphenyl benzoate was obtained as a liquid; vacuum distillation through a 1 × 10 cm vacuum jacketed column gave the ester, bp 186–188° (11 mm).

*Anal.* Calcd for $C_{17}H_{18}O_2$: C, 80.30; H, 7.12. Found: C, 80.12; H, 7.27.

*m*-Chlorophenyl benzoate after recrystallization was obtained as colorless crystals, mp 69–70° (lit.[13] mp 71–72°).

*m*-Nitrophenyl benzoate was obtained as colorless needles, mp 91–92° (lit.[14] mp 95°).

*m*-Chlorophenyl *p*-chlorobenzoate was obtained as colorless needles, mp 99–99.5° (lit.[15] 101.5°).

*Anal.* Calcd for $C_{13}H_8Cl_2O_2$: C, 58.42; H, 3.00; Cl, 26.59. Found: C, 58.52; H, 3.30; Cl, 26.06.

*m*-Chlorophenyl *m*-chlorobenzoate was obtained as colorless needles, mp 65–66°.

*Anal.* Calcd for $C_{13}H_8Cl_2O_2$: C, 58.42; H, 3.00; Cl, 26.59. Found: C, 58.67; H, 3.04; Cl, 26.40.

*m*-Chlorophenyl *p*-nitrobenzoate was obtained by reaction of *m*-chlorophenol (1.3 g, 10 mmoles) with *p*-nitrobenzoyl chloride (1.9 g, 10 mmoles) in dry pyridine (10 ml). The reaction mixture was heated to boiling for 5 min and then diluted with 200 ml of 5% hydrochloric acid. The resulting solid was separated, washed successively with several portions of dilute acid, dilute alkali, and water, and then dried under vaccum. The solid was then recrystallized twice from ethanol, being obtained as colorless needles, mp 96–97° (lit.[16] mp 101°).

*Anal.* Calcd for $C_{13}H_8ClNO_4$: Cl, 12.79. Found: Cl, 13.03.

**Reaction Kinetics of Phenyl Benzoates.** The reactions of substituted phenyl benzoates in alkaline solution were studied spectrophotometrically using a Cary Model 14 recording spectrophotometer equipped with a thermostated cell holder and cell compartment, and 0–1.0 and 0–0.1 absorbance unit slide wires. The general experimental techniques have been described.[3] The final concentrations of benzoate esters were 1.6–2.0 × $10^{-5}$ *M* while that of added acetonitrile was 0.5% (v/v) unless otherwise stated. The reactions of the phenyl benzoates shown in Table I occurred in two steps in the presence of 0.01 *M* cycloamyloses (see Results),

(6) B. D. Batts, *J. Chem. Soc., Phys. Org.*, 547 (1966).

(7) For examples, see B. Capon, *Quart. Rev.* (London), **18**, 45 (1965), especially pp 58–62.

(8) N. Hennrich and F. Cramer, *J. Am. Chem. Soc.*, **87**, 1121 (1965).

(9) J. Staerk and H. Schlenk, Jr., Abstracts, 149th National Meeting of the American Chemical Society, Detroit, Mich., 1965, p 11C; see also R. Kuhn and H. Trischmann, *Chem. Ber.*, **96**, 284 (1963).

(10) W. Lautsch, R. Wiechert, and H. Lehmann, *Kolloid-Z.*, **135**, 134 (1954).

(11) The location of the mesyl groups on the primary hydroxyl groups of the cycloamylose (that is, the C-6 hydroxyl groups of the individual glucose residues) following this method[10] of preparation has been established by Drs. F. Parrish and L. Long, Jr. (private communication). For a contrasting example of the reactivity of mesyl chloride with both primary and secondary hydroxyl groups see R. C. Chalk, D. H. Ball, and L. Long, Jr., *J. Org. Chem.*, **31**, 1509 (1966).

(12) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," John Wiley and Sons, Inc., New York, N. Y., 1958, p 364.

(13) W. J. Wohlleben, *Ber.*, **42**, 4371 (1909).

(14) F. M. Beringer, A. Brierley, M. Drexler, E. M. Grundler, and C. C. Lumpkin, *J. Am. Chem. Soc.*, **74**, 2708 (1952).

(15) M. Neeman, A. Modiano, and Y. Shor, *J. Org. Chem.*, **21**, 671 (1956).

(16) R. S. Tadkod, P. B. Sattur, N. S. Kulkarni, and K. S. Nargund, *J. Karnatak Univ.*, **2**, 29 (1957); *Chem. Abstr.*, **53**, 8063*a* (1957).

a rapid release of phenol (or phenoxide ion) followed by a slower disappearance of benzoate ester. The appearance of phenol was followed to completion by observing the increase in absorbance at 290 or 390 mμ. After the absorbance reached a constant value the spectrophotometer was adjusted to permit the observation of benzoate ester disappearance at 245 mμ (or 260 mμ for *p*-nitrobenzoate disappearance). Because carbonate buffer absorbs strongly at this wavelength, the slit width of the spectrophotometer was large. The infinite absorbance values were more sensitive to experimental errors and variations in instrumental operation because of the small over-all absorbance change and slowness of the reactions. The estimated error for the phenol appearance reactions is ±3%, while that for benzoate disappearance reactions is ±10%.

The hydrolysis rate of *m*-*t*-butylphenyl benzoate in the absence of cyclohexaamylose was determined by extrapolation of data obtained in 10% (v/v) acetonitrile solution (apparent pH 12.75) on the basis of the known pH dependence of *m*-tolyl acetate hydrolysis and the hydrolysis rate in the acetonitrile solution. The estimated error of this rate constant is ±15% while that of the other spontaneous hydrolysis rate constants is ±5%.

A quantitative estimate of the amount of cycloamylose benzoate formed during the course of the acylation of cycloamyloses by reaction with phenyl benzoates was made as follows. The observed change in absorbance during the alkaline hydrolysis of $2.5 \times 10^{-5}$ *M* methyl benzoate was 0.09 absorbance unit at 245 mμ. During the cycloheptaamylose-accelerated hydrolysis of *m*-nitrophenyl benzoate the average change in absorbance at 245 mμ (observed after completion of the initial phenol release reaction) was 0.06 absorbance unit, while in the presence of cyclohexaamylose the absorbance change in the second step of the reaction was 0.084 absorbance unit. Assuming that the extinction coefficients of benzoate esters are not greatly perturbed in the presence of cycloamyloses (spectral perturbations of these compounds are relatively small[3]) then the minimum amount of cycloheptaamylose benzoate produced is ~70%, while the minimum amount of cyclohexaamylose benzoate is >90%. Since the disappearance of benzoate ester was not followed until completion of the phenol appearance reaction it appears reasonable to conclude that the acylcycloamylose intermediate is produced in stoichiometric amounts during the course of the initial (phenol release) rate acceleration.

**Gel Filtration Chromatography.** In order to effect a separation of cycloamylose benzoate and reaction products the following experiment was carried out. Cyclohexaamylose (0.10 g, $1.0 \times 10^{-4}$ mole) was dissolved in 3 ml of pH 11 carbonate buffer. Fine crystals of *m*-nitrophenyl benzoate (0.026 g, $1.0 \times 10^{-4}$ mole) were added with 0.2 ml of acetonitrile. The reaction mixture was shaken vigorously for 5 min, brought to pH 3 with a few drops of 10% hydrochloric acid, and filtered through a small glass wool plug to remove undissolved ester. The resulting solution was chromatographed using a 1 × 23 cm column of Sephadex G-10 gel. The column was developed by eluting with distilled water. Consecutive 3-ml fractions were tested for the presence of cyclohexaamylose by consecutively spotting and drying four drops on filter paper and then spraying with 2% methanolic iodine solution. Cyclohexaamylose was found to be present in the first four fractions taken after passage of the void volume, consistent with the molecular exclusion limits of Sephadex G-10. Only traces of cyclohexaamylose were found in the subsequent fractions. After 15 fractions were collected, a 5-ml portion of pH 10.6 10% acetonitrile solution carbonate buffer was passed through the column causing a distinct yellow band to appear *ca.* one-quarter of the way down the column. No *m*-nitrophenol or unreacted ester was present in any of the earlier fractions, as judged by spectral examination of the neutral and the strongly basic solutions (made basic with excess sodium hydroxide).

The ultraviolet absorption spectra of fractions 2-4 showed $\lambda_{max}$ at 233, 274, and 280 mμ (shoulder) and were practically superimposable on the spectrum of ethyl benzoate or methyl benzoate.

## Results

**Hydrolyses of Phenyl Benzoates.** The alkaline hydrolysis of substituted phenyl benzoates in buffered alkaline solution follow first-order kinetics, the rate constants varying in the manner expected on the basis of the electronic effects of the substituents. As anticipated from data obtained for phenyl acetates[3] the release of the *meta*-substituted phenolic portion of the ester is considerably accelerated in the presence of 0.01 *M* cycloamyloses relative to the rate of alkaline hydrolysis at the same hydroxide ion concentration (Table I).

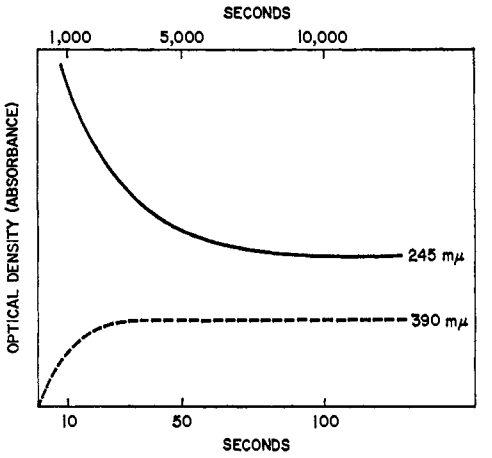



Figure 1. Hydrolysis of *m*-nitrophenyl benzoate at pH 10.6 in the presence of 0.01 *M* cyclohexaamylose; lower curve, change in optical density at 390 mμ due to *m*-nitrophenoxide ion appearance as a function of time (lower time scale); upper curve, subsequent reaction followed at 245 mμ (benzoate ester disappearance) as a function of time (upper time scale). For reaction conditions see Table I and the Experimental Section.

**Table I.** Rate Constants of the Cycloamylose-Catalyzed Reactions of Some Aryl Benzoates[a]

| Ester | Alkaline hydrolysis | Rate constants, $10^{-4}$ sec$^{-1}$ 0.01 *M* cycloheptaamylose 290 or 390 mμ | 245 or 260 mμ | 290 or 390 mμ | 245 mμ |
|---|---|---|---|---|---|
| *m*-Nitrophenyl benzoate | 15.4 | 1400 | 4.6 | 250 | 3.3 |
| *m*-Chlorophenyl benzoate | 5.5 | 390 | 4.6 | 22 | 2.7 |
| *m*-*t*-Butylphenyl benzoate | 1.2[b] | 140 | 4.4 | | |
| *m*-Chlorophenyl *p*-chlorobenzoate | | 810 | 10.6 | 75 | 5.3 |
| *m*-Chlorophenyl *m*-chlorobenzoate | | 1140 | 16.6 | 107 | 6.5 |
| *m*-Chlorophenyl *p*-nitrobenzoate | 163 | >1500 | 75[c] | | |

[a] pH 10.6, 25°, 0.5% (v/v) acetonitrile–water solution, $I = 0.2$, carbonate buffer, [ester] ≃ $1.6–2.0 \times 10^{-5}$ *M*. [b] Obtained by extrapolation of data obtained for reaction in 10% (v/v) acetonitrile–water, pH 12.75, and comparison with similar data obtained for hydrolysis of *m*-tolyl acetate. [c] Determined at 260 mμ.

Most importantly, there is a significant change in the over-all reaction pathway in the presence of cycloamyloses. Figure 1 shows the time course of the reaction of $2 \times 10^{-5}$ *M* *m*-nitrophenyl benzoate in the presence of 0.01 *M* cyclohexaamylose at constant pH (10.6). The release of phenol (as measured by phenoxide ion appearance at 390 mμ where there is no significant absorption by the ester or by benzoate ion) occurs very rapidly, reaching completion in 30 sec (bottom curve, Figure 1). Upon completion of this reaction the spectrophotometer wavelength can be changed to 245 mμ, permitting the observation of a second rate process which occurs much more slowly (top curve, Figure 1) than does the liberation of the phenol.

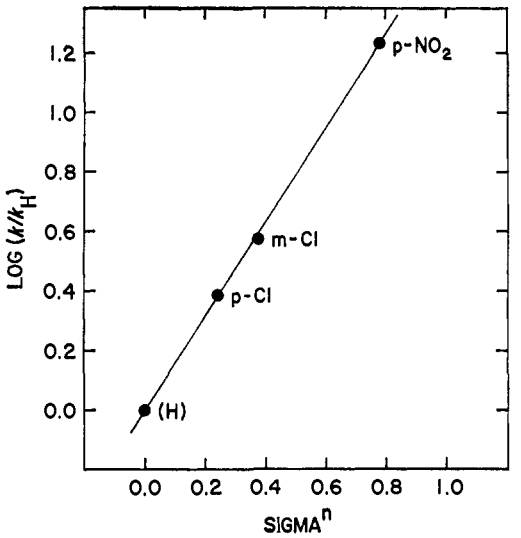



Figure 2. Hammett plot for deacylation of cyclohexaamylose benzoates at pH 10.6. The log of the relative hydrolysis rate of cyclohexaamylose benzoates (Table I) is graphed as a function of the Hammett substituent constant $\sigma^n$.

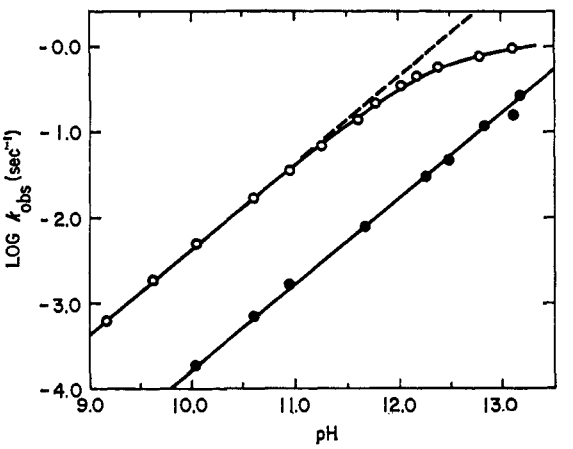



Figure 3. Pseudo-first-order rate constant for release of the phenol from *m*-tolyl acetate as a function of pH; lower curve, spontaneous hydrolysis; upper curve, rate constant in the presence of $5 \times 10^{-3}$ *M* cyclohexaamylose (data of Table II).

The appearance of phenols during reactions of a number of *m*-phenyl-substituted benzoate esters occurs some 8–300 times more rapidly than does the subsequent rate process observed at 245 mμ (Table I). Significantly, although the rates of phenol release from *m*-*t*-butyl-, *m*-chloro-, and *m*-nitrophenyl benzoates in the presence of cyclohexaamylose differ by an order of magnitude, the rates of the subsequent reactions followed at 245 mμ are the same within experimental error. Similarly, although the rate of appearance of the phenol from *m*-nitrophenyl benzoate in the presence of cycloheptaamylose is more than an order of magnitude greater than that from *m*-chlorophenyl benzoate, the rates of the subsequent reactions are essentially the same. These results indicate that the same intermediate is formed during the course of the phenol release reaction and that this common intermediate then undergoes hydrolysis at a rate which is independent of the nature of the phenolic portion of the original ester. The observed identical rates of reaction for the subsequent rate process demand the interpretation that a common intermediate is formed whose decomposition is the rate-determining step. This interpretation together with the results of gel filtration chromatography (below) is consistent only with the interpretation that the intermediate is a cycloamylose benzoate.

In order to provide further support for this interpretation the effect of substitution of the benzoate portion of the ester was examined. The *m*-chlorophenyl esters of *p*-chloro-, *m*-chloro-, and *p*-nitrobenzoic acids were hydrolyzed in the presence of excess cycloamyloses. After completion of the rapid phenol release reaction the subsequent benzoate ester disappearance was followed at 245–260 mμ. The rate of the benzoate ester disappearance was increased by an amount consistent with the electronic effects of the substituent group on the benzoate ester (Table I, Figure 2). In fact the hydrolysis of cyclohexaamylose benzoates conforms to a Hammett relationship with $\rho = 1.6$, suggesting the absence of significant steric effects. The hydrolysis of cycloamylose benzoates was not dependent on cycloamylose concentrations up to $10^{-2}$ *M*. Cyclohexaamylose benzoate undergoes hydrolysis somewhat more rapidly than does cycloheptaamylose benzoate; this may be a consequence of the greater steric hindrance by the amylose residue derived from seven glucose molecules.

In an attempt to obtain a sample of cyclohexaamylose benzoate from a solution similar to a reaction mixture, equivalent amounts of *m*-nitrophenyl benzoate and cyclohexaamylose were mixed together in an alkaline aqueous acetonitrile solution, resulting in a rapid appearance of *m*-nitrophenoxide ion. After a few minutes this mixture was neutralized and chromatographed on Sephadex G-10 (see Experimental Section), which resulted in the complete separation of both the phenol and the unreacted ester from the fractions containing the cyclohexaamylose. The ultraviolet absorption spectrum of the recovered cycloamylose fraction showed the presence of a material having a spectrum identical with that of alkyl benzoate esters such as ethyl benzoate. The cycloamylose benzoate had $\lambda_{max}$ 233, 274, and 280 mμ (sh) in water. When the material was dissolved in pH 10.6 carbonate buffer it was found to undergo hydrolysis with a rate constant of $4.6 \times 10^{-4}$ $sec^{-1}$, identical with that of the cyclohexaamylose benzoate formed *in situ* (Table I). On the basis of the observed differences in optical extinction coefficients between alkyl benzoate esters and benzoate ion it was calculated that the yield (calculated on the basis of the ester used) of cycloamylose benzoate formed *in situ* approached 100% for reactions of esters such as *m*-nitrophenyl benzoate where there is a large differential between the rate of the phenol release reaction and the subsequent deacylation (see Experimental Section).

**pH Dependence of the Cycloamylose Rate Effects.** If the intermediate cycloamylose benzoates are produced by a nucleophilic displacement reaction by an alkoxide ion derived from the secondary hydroxyl groups of the cycloamylose it would be reasonable to expect a first-order dependence on hydroxide ion of the rate of phenol release ($k_2$) and on the rate of the subsequent deacylation reaction ($k_3$). To this end the pH dependence of both the acylation and deacylation reactions was examined (Table II). In Figure 3 is shown the pH dependence of the rate of phenol release from *m*-tolyl acetate in the presence of $5 \times 10^{-3}$ *M* cyclohexaamylose

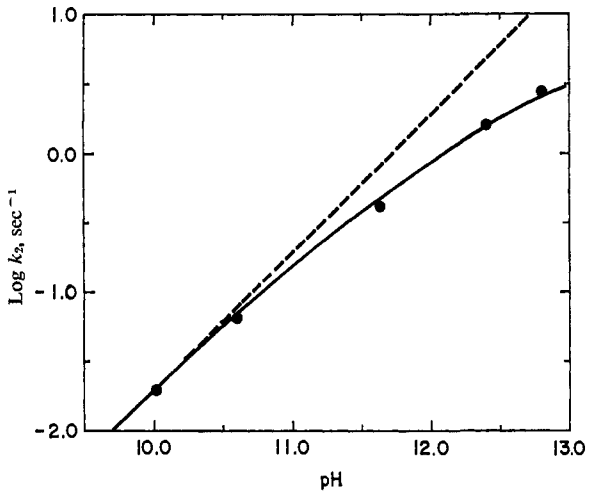



Figure 4. Maximal rates of acylation of cyclohexaamylose by *m*-tolyl acetate graphed as a function of pH (data of Table III).

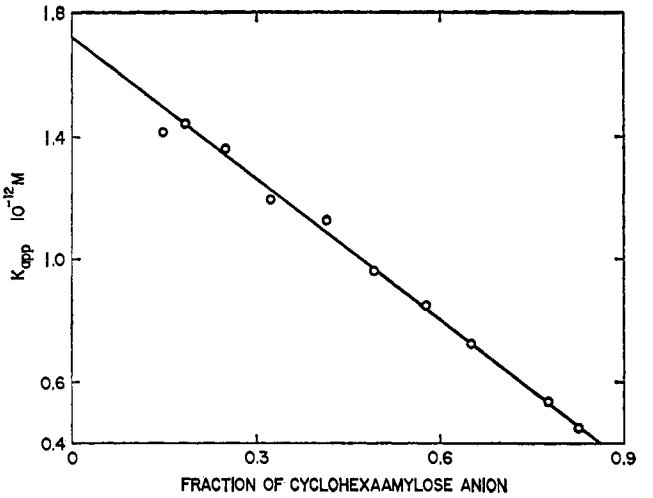



Figure 5. Calculation of the kinetic p$K_a$ value for acylation of cyclohexaamylose with *m*-tolyl acetate. The apparent ionization constant $K_{app}$ for cyclohexaamylose is plotted *vs.* the fraction of cyclohexaamylose ionized (data estimated from Figure 4; see Appendix).

(top curve) as well as the pH dependence of the hydrolysis reaction in the absence of cycloamylose (bottom curve). The basic hydrolysis reaction of the ester exhibits the expected first-order dependence on hydroxide ion, as does the accelerated reaction below pH 11. The acylation of cyclohexaamylose by *m*-tolyl acetate occurs some 22 times more rapidly than the alkaline hydrolysis reaction in the pH range 9–11.

**Table II.** Rate of Phenol Release from *m*-Tolyl Acetate in the Absence and Presence of Cyclohexaamylose as a Function of pH

| pH | Buffer[a] | $k_{un}$ (spontaneous) | $k_{obsd}$ (cycloamylose added)[b] |
|---|---|---|---|
| 9.17 | Sodium carbonate | ... | $6.23 \times 10^{-4}$ |
| 9.62 | | ... | $1.86 \times 10^{-3}$ |
| 10.04 | | $1.84 \times 10^{-4}$ | $4.97 \times 10^{-3}$ |
| 10.60 | | $6.96 \times 10^{-4}$ | $1.51 \times 10^{-2}$ |
| 10.95 | | $1.60 \times 10^{-3}$ | $3.72 \times 10^{-2}$ |
| 11.26 | Sodium–potassium | ... | $7.00 \times 10^{-2}$ |
| 11.61 | phosphate | $7.84 \times 10^{-3}$ | ... |
| 11.67 | | ... | $1.39 \times 10^{-1}$ |
| 11.77 | | ... | $2.03 \times 10^{-1}$ |
| 12.03 | Potassium hydroxide | ... | $3.48 \times 10^{-1}$ |
| 12.18 | | ... | $4.47 \times 10^{-1}$ |
| 12.27 | | $3.00 \times 10^{-2}$ | ... |
| 12.39 | | ... | $5.69 \times 10^{-1}$ |
| 12.49 | | $4.50 \times 10^{-2}$ | ... |
| 12.80 | | ... | $6.71 \times 10^{-1}$ |
| 12.84 | | $1.16 \times 10^{-1}$ | ... |
| 13.12 | | $1.57 \times 10^{-1}$ | $9.39 \times 10^{-1}$ |
| 13.18 | | $2.58 \times 10^{-1}$ | ... |

[a] Ionic strength = 0.2 throughout, with 0.5% (v/v) added acetonitrile, 25 ± 0.2°. As many be seen from Figure 3, there appear to be no specific effects observed when changing from one to the other of these buffer systems. [b] Cyclohexaamylose concentration $5.0 \times 10^{-3}$ *M*. (The units of *k* are sec$^{-1}$.)

However, above pH 11 the rate acceleration begins to decrease until at pH 13.1 there is observed only a sixfold rate acceleration. One interpretation of this result is that the cycloamylose molecule has become largely ionized so that further increases in hydroxide ion concentration will not result in a corresponding increase in cycloamylose alkoxide ion concentration; the hydroxide ion reaction will continue to increase in rate even after there are no longer any of the catalytically active hydroxyl groups left to ionize. An alternative interpretation is that the dissociation constant of the cyclohexaamylose–*m*-tolyl acetate complex is pH dependent. Thus, the observed rate effect might decrease because less of the ester is complexed at higher pH. To eliminate this possibility the maximal rates of acylation (phenol release, $k_2$) were determined as a function of pH (Table III). These data for *m*-tolyl acetate are shown in Figure 4. Again the rate conforms to a first-order dependence on hydroxide ion below pH 11 but begins to fall off at higher pH. When the data of Figure 4 are transformed to a plot of $K_{app}$ *vs.* fraction of hydrogen ions released (see Appendix), the p*K* of the catalytically active group is estimated to be 11.8 ± 0.4 (Figure 5). When the rate of deacylation of cyclohexaamylose benzoate (prepared *in situ* by reaction with *m*-nitrophenyl benzoate) is determined as a function of pH (Table IV), a curve is obtained (Figure 6) which is similar to that obtained for the pH dependence of acylation. The pH dependence of deacylation also exhibits a first-order dependence of hydroxide ion below pH 11 but the rate increases less rapidly above this point. The data shown in this figure were used to calculate the p*K* of the ionizing group as shown in Figure 7 (see Appendix); this was found to be 12.3 ± 0.2. Thus, the rate effects caused by the cycloamyloses are dependent on an ionizable group with a p*K* = 12.1 which

**Table III.** Maximal Rates of Acylation of Cyclohexaamylose by *m*-Tolyl Acetate in the Presence of Cyclohexaamylose at Various pH Values[a]

| pH | $k_2$, sec$^{-1}$ [b] |
|---|---|
| 10.01 | $1.95 \pm 0.33 \times 10^{-2}$ |
| 10.60 | $6.58 \pm 1.97 \times 10^{-2}$ |
| 11.63 | $4.18 \pm 1.20 \times 10^{-1}$ |
| 12.40 | $1.6 \pm 1.0$ |
| 12.80 | $2.8 \pm 0.6$ |

[a] Ionic strength = 0.2 with 0.5% (v/v) added acetonitrile, 25.0 ± 0.1°; for buffers see Table II. [b] The maximal rate of acylation is determined by calculating the rate of phenol release in the presence of an infinite amount of cyclohexaamylose; for details see ref 3.

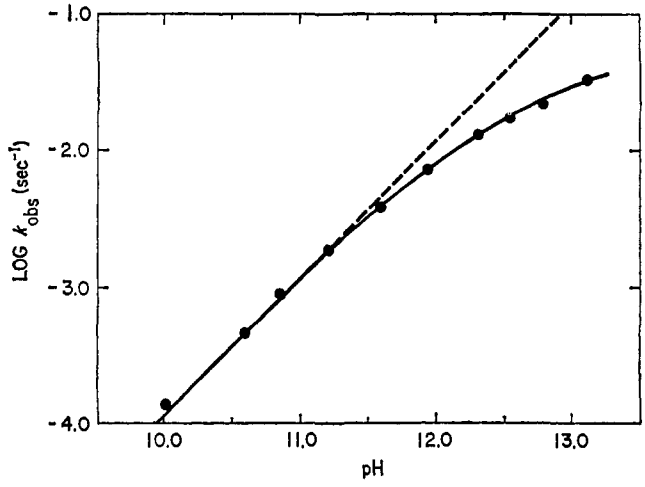



Figure 6. Pseudo-first-order rate constants for hydrolysis of cyclohexaamylose benzoate as a function of pH. The ester was formed *in situ* by acylation of the cyclohexaamylose with *m*-nitrophenyl benzoate (data of Table IV).

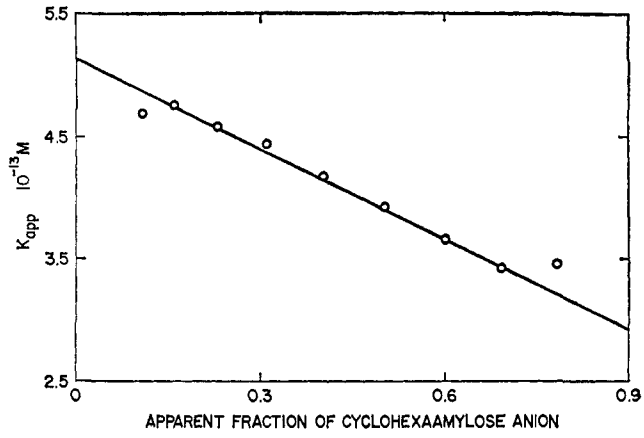



Figure 7. Calculation of the kinetic p$K_a$ of cyclohexaamylose from data obtained for the hydrolysis of cyclohexaamylose benzoate. The apparent ionization constant $K_{app}$ is plotted *vs.* the fraction of cyclohexaamylose ionized (data estimated from Figure 6; see Appendix).

is assigned to one of the two kinds of secondary hydroxyl groups (see Discussion).

**Table IV.** Hydrolysis Rate of Cyclohexaamylose Benzoate as a Function of pH[a]

| pH | Buffer[b] | $k_{obsd}$, sec$^{-1}$ | [Cyclohexaamylose], *M* |
|---|---|---|---|
| 10.01 | Sodium carbonate | $1.36 \times 10^{-4}$ | $1.00 \times 10^{-2}$ |
| 10.60 | | $4.58 \times 10^{-4}$ [c] | $1.00 \times 10^{-2}$ |
| 10.85 | | $8.67 \times 10^{-4}$ | $1.00 \times 10^{-2}$ |
| 11.20 | Sodium–potassium phosphate | $1.84 \times 10^{-3}$ | $1.00 \times 10^{-2}$ |
| 11.22 | | $1.99 \times 10^{-3}$ | $5.00 \times 10^{-3}$ |
| 11.56 | | $3.52 \times 10^{-3}$ | $5.00 \times 10^{-3}$ |
| 11.58 | | $3.80 \times 10^{-3}$ | $1.00 \times 10^{-2}$ |
| 11.93 | Potassium hydroxide–chloride | $7.33 \times 10^{-3}$ | $1.00 \times 10^{-2}$ |
| 12.31 | | $1.34 \times 10^{-2}$ | $1.00 \times 10^{-2}$ |
| 12.55 | | $1.75 \times 10^{-2}$ | $1.00 \times 10^{-2}$ |
| 12.57 | | $1.88 \times 10^{-2}$ | $5.00 \times 10^{-3}$ |
| 12.63 | | $2.02 \times 10^{-2}$ | $1.00 \times 10^{-3}$ |
| 12.63 | | $2.08 \times 10^{-2}$ | $5.00 \times 10^{-4}$ |
| 12.63 | | $1.98 \times 10^{-2}$ | $1.00 \times 10^{-4}$ |
| 12.78 | | $2.26 \times 10^{-2}$ | $1.00 \times 10^{-2}$ |
| 13.11 | | $3.33 \times 10^{-2}$ | $1.00 \times 10^{-2}$ |

[a] The cyclohexaamylose benzoate was prepared *in situ* by acylation of the cycloamylose with $2.5 \times 10^{-5}$ *M* *m*-nitrophenyl benzoate. [b] Ionic strength = 0.2 with 0.5% (v/v) added acetonitrile, 25.0 ± 0.1°. [c] Data of Table I.

**Effects of Blocking Cycloamylose Hydroxyl Groups.** Since the hydroxyl groups of the cycloamyloses are almost certainly the catalytically active groups, the effect of substituting particular hydroxyl groups was examined in an attempt to identify which of the hydroxyl groups were required to produce large accelerations of the phenol release reaction. When both the 6 and 3 positions of the glucose residues of cyclohexaamylose are blocked by conversion to methoxyl groups[9] the resulting dodecamethylcyclohexaamylose is catalytically inactive. Indeed, the alkaline hydrolysis of *m*-nitro- and *m*-*t*-butylphenyl acetates is slightly inhibited (Table V) although these compounds show a large acceleration of the phenol release reaction with cyclohexaamylose. The observed rate reductions must be interpreted as the result of formation of a cycloamylose complex which protects the ester from reaction with hydroxide ion in solution.[3] It is not the result of a medium effect since methyl glucoside produces a small rate acceleration.[3]

**Table V.** Rate Effects on Phenyl Acetate Hydrolysis of 1% Dodecamethylcyclohexaamylose[a]

| Phenyl acetate | Hydroxide ion rate constant $k_{un}$, sec$^{-1}$ | Rate with 1% dodecamethylcyclohexaamylose $k_{obsd}$, sec$^{-1}$ | Rate effect $k_{obsd}/k_{un}$ |
|---|---|---|---|
| *m*-Nitro | $7.01 \times 10^{-3}$ | $4.54 \times 10^{-3}$ | 0.64 |
| *m*-*t*-Butyl | $7.60 \times 10^{-4}$ | $4.61 \times 10^{-4}$ | 0.61 |

[a] In pH 10.7 carbonate buffer with $I$ = 0.2, 0.5% (v/v) added acetonitrile, 25°.

However, when only the primary (C-6) hydroxyl groups are blocked the rate accelerations observed for the cleavage of phenyl esters are at least as large as those produced by cyclohexa- and -heptaamylose. Hexamesylcyclohexaamylose and heptamesylcycloheptaamylose were prepared[10,11] and their effects on the rate of reaction of *m*-*t*-butylphenyl acetate were determined in aqueous acetonitrile solutions (see Experimental Section). Since the extent of complex formation was expected to be less in the presence of large amounts of acetonitrile[3] the dissociation constants of the cycloheptaamylose–*m*-*t*-butylphenyl acetate complex and the corresponding mesyl system were determined using methods previously described.[3] The values of $k_2$ (the first-order rate constant of the fully complexed ester) as well as the values of $K_{diss}$ (the dissociation constants of the complexes) are given in Table VI. As anticipated, the complex is less stable in the presence of large amounts of acetonitrile, the value of $K_{diss}$ increasing from $1 \times 10^{-4}$ *M* in 0.5% acetonitrile to $2 \times 10^{-3}$ *M* in 20.5% solution. This is similar to results obtained for the effect of acetonitrile on the *m*-chlorophenyl acetate–cyclohexaamylose complex.[3] Most importantly the maximal rate effects $k_2/k_{un}$ for the mesyl compound and for unchanged cycloheptaamylose are nearly the same. Thus, block-

ing the primary hydroxyl groups by formation of the mesyl derivative results in no diminution of the rate effect whereas blocking the primary and one-half of the secondary hydroxyl groups by formation of the dodecamethyl derivative results in a complete loss of the rate-accelerating effect.[17]

Table VI. Maximal Rate Accelerations and Dissociation Constants of Cycloamylose-*m*-*t*-Butylphenyl Acetate Complexes[a]

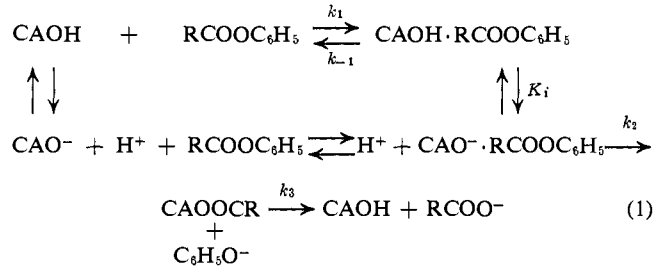
| Cycloamylose | Ester hydrolysis rate constant, $sec^{-1}$ | $k_2$, $sec^{-1}$ | $k_2/k_{un}$ | $K_{diss}$, $M$ |
|---|---|---|---|---|
| Cycloheptaamylose[b] | $3.1 \times 10^{-4}$ | 0.286 | 940 | $2.3 \pm 1.2 \times 10^{-3}$ |
| Hepta(6-O-mesyl)cycloheptaamylose[c,d] | $3.1 \times 10^{-4}$ | 0.475 | 1550 | $3.1 \pm 0.9 \times 10^{-3}$ |

[a] In 20.5% acetonitrile, pH 10.6 carbonate buffer, $I = 0.2$. All determinations were made at 25.0 ± 0.2°. Error limits were calculated[3] from the experimental data by application of Student's t test at the 95% probability level. The ester concentration was $3.6 \times 10^{-5}$ *M*. [b] The cycloheptaammylose concentration was varied from $4.7 \times 10^{-4}$ to $4.7 \times 10^{-3}$ *M*. [c] The heptamesylcycloheptaamylose concentration was varied from $1.9 \times 10^{-4}$ to $3.5 \times 10^{-3}$ *M*. [d] Since mesyl esters are reasonably reactive in the presence of hydroxide ion [S. Hartman and R. E. Robertson, *Can. J. Chem.*, **38**, 2033 (1960)] the mesylcycloamylose solutions were used within 30 min after preparation.

## Discussion

**The Reaction Pathway.** These kinetic and isolation studies prove that the large accelerations in the cleavage of *meta*-substituted phenyl esters in alkaline solution in the presence of cycloamyloses are the result of a nucleophilic reaction of an alkoxide ion derived from either the C-2 or C-3 secondary hydroxyl groups of the cycloamyloses. The ester hydrolysis reaction shows the expected first-order dependence on hydroxide ion as does the reaction with the cycloamyloses in the pH range before an appreciable fraction of the cycloamylose has become ionized (*cf.* Figure 3). Since the ester cleavage reaction exhibits an apparent first-order dependence on ester concentration at a given pH, the hydrolysis reactions follow second-order kinetics overall. On the basis of minimal estimates of the percentage of acyl cycloamyloses produced during the course of some of the ester hydrolyses, it is clear that the nucleophilic pathway is by far the major pathway of the reaction; general acid or general base catalysis by a hydroxyl group may make only a small contribution, if any, to the hydrolytic process.

The following reaction scheme describes the reactions of phenyl esters in the presence of cycloamyloses. The phenyl ester undergoes a rapid reversible association[3,18] with the cycloamylose, CAOH, or with the




(1)

partially ionized form, $CAO^-$. An alkoxide ion derived from the secondary hydroxyl groups of the cycloamylose may react with an included ester molecule to liberate the phenoxide ion in a reaction characterized by a rate constant $k_2$ which is equal to the observed rate constant[3] of the phenol release reaction of the fully complexed ester. This reaction, which results in the acylation of the amylose, is markedly dependent on the stereochemistry of the phenyl group substituents. Since previous studies[3] showed that *meta* substituents on the phenyl ring resulted in large rate effects the present study employed *meta*-substituted phenyl benzoates. As expected with these compounds, the cycloamyloses cause large increases in the rate of phenol release compared to the rate in buffered alkaline solution (Table I).

The rate effects of cycloamyloses on the hydrolysis of alkyl benzoate esters contrast sharply with their effects on reactions of phenyl esters. Complex formation with cycloheptaamylose apparently completely inhibits the alkaline hydrolysis of ethyl *p*-aminobenzoate.[19] This effect can be explained in two ways. The unreactivity of the complexed ester can be explained on the basis of nonproductive binding[3] in which the carbonyl carbon atom of the ester molecule is located in the cycloamylose cavity at some distance from the secondary hydroxyl groups. If, for example, the ester was complexed in such a way that the carbonyl group was positioned near the primary hydroxyl groups of the cycloamylose, it would be well protected from reaction in solution. Alternatively, the unreactivity of alkyl benzoate esters in the cycloamylose complexes may be due to an unfavorable partitioning of the tetrahedral intermediate.[20] For example, addition of an alkoxide ion of the cycloamylose to the carbonyl group of methyl benzoate would lead to the formation of a tetrahedral intermediate having either cycloamylose alkoxide ion or methoxide ion as potential leaving groups. Because of the greater acidity of the cycloamylose compared to methanol, it may be expected that the tetrahedral intermediate would preferentially revert to reactants.

It is interesting to find that the reactive hydroxyl group of cyclohexaamylose has a kinetic p$K$ of 12.1. Although this p$K$ at first appears low for an aliphatic alcohol there are related examples in the carbohydrate field. On the basis of changes in intrinsic viscosity and optical rotation of starch solutions, Rao and Foster postulated that modifications of the polymer chain configuration occur as the result of the ionization of

(17) Large increases in the rate of phenol releases were seen with hexamesylcyclohexaamylose also, but this compound was too insoluble (relative to the value of the amylose–ester dissociation constant) to permit an accurate determination of the maximal rate effect.

(18) The rate of formation of the complex is expected to be very rapid for relatively unhindered and uncharged ester molecules [F. Cramer, W. Saenger, and H.-C. Spatz, *J. Am. Chem. Soc.*, **89**, 14 (1967)]. No evidence was observed in the course of the present studies that the rate of complex formation was slow (*i.e.*, within one or two orders of magnitude) compared to the rates of the cleavage reactions. Thus, even the reaction of *m*-nitrophenyl benzoate or *m*-*t*-butylphenyl acetate in the presence of excess cyclohexaamylose exhibited good first-order kinetics.

(19) J. L. Lach and T.-F. Chin, *J. Pharm. Sci.*, **53**, 924 (1964). We have made qualitatively similar observations for the alkaline hydrolysis of methyl benzoate, ethyl benzoate, ethyl cinnamate, and methyl *m*-chlorobenzoate.

(20) M. L. Bender and B. W. Turnquest, *J. Am. Chem. Soc.*, **79**, 1657 (1957).

the amylose hydroxyl groups at high pH.[21] These authors concluded that the pH dependence of the optical rotation changes was consistent with a p$K$ of 12 or slightly higher. Such a value is in agreement with the results of the present study. More recently, studies on the thermodynamics of ionization of carbohydrate derivatives[22] showed that the p$K$ of the (two adjacent) secondary hydroxyl groups of the ribose moiety of adenosine is 12.35. The relatively acidic hydroxyl groups of the cycloamylose are probably the result of a specific interaction involving the C-2 and C-3 secondary hydroxyl groups, just as in ribose. The existence of a hydrogen bond in the crystalline state between these hydroxyl groups has been noted.[23] The enhanced acidity of these hydroxyl groups may be due to the combined inductive effects of the relatively electronegative oxygen atoms and also to stabilization of the alkoxide ion by means of an intramolecular hydrogen bond to a neighboring hydroxyl group. Other workers are attempting to distinguish between these effects for ribose compounds.[22]

Since the facile methylation of ribose derivatives by diazomethane in the presence of base has been reported[24] it is tempting to speculate that such a reaction could be exhibited by the cycloamyloses. Noting that only one of the two types of secondary hydroxyl groups of cyclohexaamyloses is methylated in certain methylation reactions,[9] it is possible that this is the same group of p$K$ = 12.1 determined in the present work. From the methylation data of Staerk and Schlenk,[9] leading to dodecamethylcyclohexaamylose, the reactive secondary hydroxyl groups would appear to be the C-3 hydroxyl groups (numbering the carbons of the individual glucose units). This conclusion is consistent with the fact that the dodecamethyl derivative causes no rate enhancement of the phenyl ester cleavage reaction. While it is difficult to assess steric hindrance accurately from an examination of molecular models, it appears that the presence of a methyl group on either the C-2 or C-3 hydroxyl group would not completely hinder the approach of the remaining hydroxyl (oxygen atom) to the carbonyl carbon of an included ester molecule. Thus, either the introduction of the first methyl group radically changes the reactivity of the other secondary hydroxyl groups or, more likely, the C-2 and C-3 hydroxyl groups differ significantly in their reactivity and acidity.

The deacylation step, $k_3$, in mechanism 1 depends on the electronic nature of the substituents on the benzoic acid portion of the ester but does not appear to be sensitive to differing steric patterns of *meta* and *para* substitution (Figure 2). The pseudo-first-order rate constant for the alkaline hydrolysis of cyclohexaamylose benzoate at pH 10.6 is $4.6 \times 10^{-4}$ sec$^{-1}$ (Table I), corresponding to a second-order rate constant of 1.2 $M^{-1}$ sec$^{-1}$. The secondary ester isopropyl benzoate is a reasonable model for cyclohexaamylose benzoate with respect to linkage and steric hindrance; the second-order rate constant for the alkaline hydrolysis of isopropyl benzoate is $0.6 \times 10^{-2}$ $M^{-1}$ sec$^{-1}$.[25] Thus, cyclohexaamylose benzoate hydrolyzes 200 times more rapidly than does isopropyl benzoate. The difference in p$K$ between cyclohexaamylose and isopropyl alcohol may account for a factor of 10 in rate[26] but it still appears that cyclohexaamylose benzoate hydrolyzes some 20 times more rapidly than would be expected on the basis of steric and electronic effects. This rate difference is presumably the result of general acid or general base catalysis of the deacylation step by adjacent hydroxyl group(s). Both types of catalysis would exhibit the leveling off in the rate of the deacylation reaction at high pH when the alkoxide ion is ionized (Figure 6). If, however, isopropyl benzoate is not a proper model for the steric hindrance to be expected in cyclohexaamylose benzoate, then it is possible that the latter ester does not hydrolyze unusually rapidly. In this event the leveling off in the rate of deacylation at high pH could be explained by electrostatic repulsion of partly ionized cycloamylose and hydroxide ion.

**Comparisons with Chymotrypsin.** The cycloamylose system may be compared with chymotrypsin catalysis in a number of respects. Chymotrypsin reactions are typically true catalyses. However, although cycloamylose reactions often show large increases in the rate of phenol release (the acylation reaction), there is true catalysis of over-all hydrolysis for only some of the esters studied. Of the esters listed in Table I, the over-all hydrolysis of *m-t*-butylphenyl benzoate is catalyzed by cyclohexaamylose, since the rate constant for alkaline hydrolysis of the ester at pH 10.6 is approximately four times less than the rate constant for hydrolysis of cyclohexaamylose benzoate. For the other esters of Table I, however, the deacylation rate constant is less than the rate constant for direct alkaline hydrolysis of the ester. The failure of the cycloamylose to catalyze the over-all hydrolysis of these esters is due to the relatively slow deacylation reaction, which is catalyzed by no more than 20-fold by adjacent hydroxyl groups, although the acylation reaction can be accelerated by many times that figure. In principle, there is no reason why more catalytically active general base or nucleophilic catalysts could not be introduced into the cycloamylose molecule, and thus make the analogy between cycloamylose and chymotrypsin more nearly complete.[27]

Both cycloamylose and chymotrypsin catalyze the hydrolysis of esters by the reaction scheme shown in eq 1. The reactant and catalyst form a complex in a rapid and reversible process described by a dissociation constant $K_{diss} = k_{-1}/k_1$. The similarity of the values of $K_{diss}$ for chymotrypsin and for the cycloamyloses has been previously mentioned.[3] Both catalysts produce large rate effects in the acylation step. The magnitude of the rate effect depends on the stereochemistry of the substrate whereas the value of the dissociation constant of the respective complex is independent of the stereochemistry of the substrate.[3] However, the value of $K_{diss}$ in the two systems does de-

(21) V. S. R. Rao and J. F. Foster, *Biopolymers*, **1**, 527 (1963); but see also V. S. R. Rao and J. F. Foster, *J. Phys. Chem.*, **69**, 636 (1965).

(22) R. M. Izatt, J. H. Rytting, L. D. Hansen, and J. J. Christensen, *J. Am. Chem. Soc.*, **88**, 2641 (1966); J. J. Christensen, J. H. Rytting, and R. M. Izatt, *ibid.*, **88**, 5105 (1966).

(23) A. Hybl, R. E. Rundle, and D. E. Williams, *ibid.*, **87**, 2779 (1965).

(24) T. A. Khwaja and R. K. Robins, *ibid.*, **88**, 3640 (1966).

(25) M. L. Bender, *ibid.*, **73**, 1626 (1951).

(26) J. F. Kirsch and W. P. Jencks, *ibid.*, **88**, 833, 837 (1964).

(27) The introduction of imidazole and amino groups onto the cycloamylose ring has recently been reported [F. Cramer and G. Mackensen, *Angew. Chem. Intern. Ed. Engl.*, **5**, 601 (1966)]. Rate effects of 3–4 were observed (relative to the effects produced by noncovalently bonded nitrogen bases and cycloamyloses) in the hydrolysis of *p*-nitrophenyl acetate at pH ~7.

pend on the type of substituent groups in the substrate, both the cycloamyloses and chymotrypsin preferentially binding apolar substrates. This is in contrast to the situation in, for example, trypsin, which preferentially binds cationic substrates.

Both chymotrypsin and the cycloamyloses have an aliphatic alcohol group at the active site which acts as an acyl group acceptor during the catalysis of ester hydrolysis.[28] Whereas the acylation step in reactions with the cycloamyloses depends on a hydroxyl group with a p$K$ of 12, the acylation step in chymotrypsin-catalyzed reactions depends on a group with a p$K$ of 7, presumably the imidazole group of a histidine residue.[29] The imidazole group is also active in the deacylation step. Mention has already been made of the apparent 20-fold intramolecular catalysis of the deacylation of cyclohexaamylose benzoate. Catalysis of the deacylation step is thus a feature of both the cycloamylose and chymotrypsin reaction systems but the much larger catalysis of chymotrypsin deacylations leads to a large over-all catalysis of hydrolysis. In addition, the deacylation step in chymotrypsin-catalyzed reactions is highly stereospecific, but this is not the case for the deacylation of the cycloamyloses.

In the acylation reactions the reaction rates of chymotrypsin and of the cycloamyloses are very similar. It is possible to calculate the second-order rate constants for reaction of substrates with the cycloamyloses in the following way. For the reaction scheme

$$C + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} X \xrightarrow{k_2} P$$

the assumption of a preequilibrium and the condition that $C_0 >> E_0$ leads to the Michaelis–Menten equation $dP/dt = k_2[C]_0[E]_0/(K_{diss} + [C]_0)$. If the condition is imposed that $(C)_0 << K_{diss}$, then a second-order rate constant can be determined from $dP/dt = k_2[(C)_0 \cdot (E)_0]/K_{diss}$. The values of the second-order rate constants calculated in this manner using the values of $k_2$(lim) (the maximal rate constant of acylation of the fully complexed ester in the pH region where the cycloamylose is completely ionized[30]) and the dissociation constants of the respective complexes[3] are shown in Table VII. Also shown in the table are corresponding values of $k_{cat}(lim)/K_m(app)$ $(= k_2(lim)/K_s)$[28] for chymotrypsin reactions.

The second-order rate constants of Table VII show one method of comparing cycloamylose or chymotrypsin reactions with hydroxide ion reactions. The values of Table VII state the relative rates when equal amounts of cycloamylose or hydroxide ion catalysts are used, or when equal amounts of chymotrypsin or hydroxide ion catalysts are used. On this basis, the cycloamylose reactions are clearly superior to the hydroxide ion reactions, as are the chymotrypsin reactions. In fact, the rate enhancements of the cycloamyloses with respect to hydroxide ion are approximately as great as the rate enhancements of chymotrypsin with respect to hydroxide ion. Does this mean that cycloamyloses are identical kinetically with chymotrypsin? The answer to this question is no, because $k_2$(lim) is determined at *ca.* pH 13 for the cycloamylose while it is determined at *ca.* pH 8 for chymotrypsin. This comparison of Table VII is, however, a true indication of the effect of complexing on the rates of reaction. In that sense, cycloamyloses compare very well with chymotrypsin. If some method makes it possible to reduce the pH at which the cycloamylose acts without losing any of its other properties, it might be possible to come closer to chymotrypsin reactivity.

**Table VII.** Second-Order Rate Constants for Reactions of Phenyl Esters with Cycloamyloses and of Substrates with Chymotrypsin

| Reactants | Rate constant, $M^{-1}$ sec$^{-1}$ | Catalyzed rate/ hydroxide ion rate |
|---|---|---|
| (1) Cyclohexaamylose + *m-t*-butylphenyl acetate[a] | $2.1 \times 10^3$ | |
| Hydroxide ion + *m-t*-butylphenyl acetate | 1.2 | |
| | | $1.8 \times 10^3$ |
| (2) Cycloheptaamylose + *m-t*-butylphenyl acetate[a] | $3.1 \times 10^4$ | |
| Hydroxide ion + *m-t*-butylphenyl acetate | 1.2 | |
| | | $2.6 \times 10^4$ |
| (3) Chymotrypsin + acetyltryptophanamide[b] | 12.6 | |
| Hydroxide ion + acetyltryptophanamide[c] | $3 \times 10^{-4}$ | |
| | | $4 \times 10^4$ |
| (4) Chymotrypsin + acetyltryptophan ethyl ester[b] | $4 \times 10^5$ | |
| Hydroxide ion + acetyltryptophan ethyl ester[d] | 0.6 | |
| | | $10^6$ |
| (5) Chymotrypsin + acetyltyrosine ethyl ester[e] | $1.2 \times 10^4$ | |
| Hydroxide ion + acetyltyrosine ethyl ester[e] | 0.45 | |
| | | $3 \times 10^4$ |

[a] Data for the cycloamylose reactions were obtained by division of the limiting value[30] of $k_2$ (see text) by $K_{diss}$ (ref 5). [b] M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *J. Am. Chem. Soc.*, **86**, 3680 (1964). [c] M. L. Bender, F. J. Kézdy, and C. R. Gunter, *ibid.*, **86**, 3714 (1964). [d] D. J. Whelan and M. L. Bender, unpublished work. [e] H. Gutfreund, "An Introduction to the Study of Enzymes," John Wiley and Sons, Inc., New York, N. Y., 1965, p 296.

Cycloamylose-catalyzed and chymotrypsin-catalyzed hydrolyses of phenyl esters exhibit many similarities. Both catalyses occur by a similar pathway involving a rapid association to form complexes. The ester substrates then react with the catalyst to form an acylated intermediate, which subsequently undergoes hydrolysis in a slow step. The dissociation constants of binding of the two systems are similar, complex formation being best with apolar substrates. Increasing ionic strength of the solution tends to favor complex formation, while added apolar solvents decrease the stability of the complexes. Both systems are subject to competitive in-

(28) M. L. Bender and F. J. Kezdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964), and references cited therein.

(29) For a recent study of the pH dependence of chymotrypsin-catalyzed reactions and references to previous work see M. L. Bender, M. J. Gibian, and D. J. Whelan, *Proc. Natl. Acad. Sci. U. S.*, **56**, 833 (1966).

(30) At pH 10.6, assuming that both cyclohexaamylose and cycloheptaamylose have a p$K$ of 12.1, the ratio of ionized to un-ionized cycloamylose is $3.1 \times 10^{-2}$. The limiting rate of acylation of cyclohexaamylose by *m-t*-butylphenyl acetate assuming complete ionization will be $(12.9 \times 10^{-2}\ \text{sec}^{-1})/(3.1 \times 10^{-2})$ where $12.9 \times 10^{-2}$ sec$^{-1}$ is the calculated value of $k_2$ at pH 10.6.[3] The limiting values of $k_2$ calculated in this manner for the acylation of cyclohexaamylose and cycloheptaamylose by *m-t*-butylphenyl acetate are 4.2 and 4.0 sec$^{-1}$, respectively. No correction has been made for the fact that there are 6 or 7 equivalent hydroxyl groups in the cycloamylose; this correction would make the calculated values proportionately larger.

hibition by added organic molecules. The maximal rate effects produced in the two systems are unrelated to the *stabilities* of the complexes, but depend on their *stereochemistry*. The specific stereochemical relationships between the cycloamyloses and their substrates are well described on the basis of the three-dimensional structure of these materials. A similar description of enzyme–substrate interactions should be possible.

## Appendix

**Calculation of the Ionization Constant of Cyclohexaamylose from Kinetic Data.** For the compound $AH_n$ capable of successive ionizations

$$AH_n \rightleftharpoons AH_{n-1} + H^+ \qquad K_1$$

$$AH_{n-1} \rightleftharpoons AH_{n-2} + H^+ \qquad K_2, \text{ etc.}$$

Because the successive ionizations and reactivities of the cycloamylose alkoxide ions are probably perturbed by preceding ionizations it is desired to determine the value of $K_1$, the first ionization constant, as opposed to $K_{app}$, where

$$K_{app} = \frac{[H^+][\text{no. of moles of charges on molecules}]}{[\text{no. of moles of undissociated molecules}]} = \frac{[H^+]([AH_{n-1}] + 2[AH_{n-2}] + \cdots\cdots n[AH_{n-n}])}{[AH_n]} = \frac{[H^+]\sum_{x=1}^{n} x[AH_{n-x}]}{[AH_n]}$$

In the limit of single, kinetically productive ionizations then $K_{app}$ will equal $K_1$.

Figures 3 and 4 show, in addition to a plot of values observed in the presence of cycloamylose $k_c$, a second line based on extrapolation from the observed data at low pH and low fraction of ionization; the extrapolated rate constant at any pH may be designated $k_{ex}$. At any pH the fraction $k_c/k_{ex}$ is equal to the fraction of undissociated amylose $AH_n$. That is

$$k_c/k_{ex} = [AH_n]/\sum_{x=1}^{n} x[AH]_{n-x}$$

$$1 - k_c/k_{ex} = \frac{\sum_{x=1}^{n} x[AH_{n-x}]}{[AH_n]}$$

Since also

$$K_{app} = [H^+]\sum_{x=1}^{n} x[AH_{n-x}]/[AH_n]$$

it is possible to calculate $K_1$ by constructing a graph of $K_{app}$ *vs.* fraction of ionized cycloamylose. Extrapolation to low fractions of ionization gives $K_1$. The appropriate values of $1 - k_c/k_{ex}$ were taken from Figures 4 or 6, multiplied by the respective values of the pH to give $K_{app}$, and these values plotted in Figures 5 and 7, respectively. Extrapolation to low fractions of ionization gives the ionization constants. The kinetic $pK_a$ value based on the acylation rate data is 11.8, while that for the deacylation rate data is 12.3. The uncertainty in these values is probably $\pm 10\%$ for the deacylation rate data and $\pm 30\%$ for the acylation rate data.

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