a single amorphous compound which gave a mass spectrum identical with that of 17-methoxy-N-methylaspidofractinine (**16**), previously obtained from the ketone **13**.

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Acceleration of Phenyl Ester Cleavage by Cycloamyloses. A Model for Enzymatic Specificity[1,2]

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Abstract: The cycloamyloses cause a markedly stereoselective acceleration of phenol release from a variety of substituted phenyl acetates in alkaline solution. Unlike methyl glucoside or glucose which produces small uniform rate effects, both cyclohexaamylose and cycloheptaamylose cause large, nonuniform increases in the rate of phenol release from *meta*-substituted phenyl acetates; phenol release from *para*-substituted phenyl acetates is only slightly enhanced. Rate effects due to cyclooctaamylose are smaller and much less stereoselective. The rate accelerations are independent of the electronic nature of the substituents. The cycloamylose system exhibits many characteristics of enzyme-catalyzed reactions, including saturation, competitive inhibition, and nonproductive binding. Dissociation constants of cycloamylose complexes with a variety of guest molecules were obtained using kinetic, spectroscopic, and competitive inhibition methods and are in experimental agreement. The maximal rate effects are independent of the stabilities of the cycloamylose–substrate complexes, as in enzymatic catalysis. The rate accelerations are entirely explained by considering the region of the cycloamylose torus and the secondary hydroxyl groups to be the active site of the cycloamylose. In the complex the *meta* substituents on the phenyl ring fix the carbonyl carbon atom of the ester in close proximity to the secondary hydroxyl groups of the cycloamylose whereas *para* substituents prevent this approximation. The reaction system constitutes a striking model for the lock and key theory of enzymatic specificity proposed by Emil Fischer.

As an aid to the understanding of the mechanism of enzyme action it would be useful to have a relatively simple model system which exhibits some of the important characteristics of enzyme-catalyzed reactions. Such a model system should exhibit substantial catalytic effects which vary in a predictable manner depending upon the substrate. The catalysis should be ascribable to a known reactive group or groups and the structure and geometry of the catalyst should be known. The catalytic effects should be the result of prior complexation of the catalyst and substrate. It must be possible to accurately determine both the stability of the catalyst–substrate complex and its inherent reactivity since there is no necessary relationship between these two factors (for example, although chymotrypsin binds N-acetyl-D-tryptophanamide somewhat better that it binds the L isomer,[4] the deacylation rates of N-acetyl-D- and -L-tryptophanyl chymotrypsins differ by a factor of 1.6×10^4 in favor of the L isomer[5]). Stereochemical aspects of both the binding and the subsequent catalysis should be readily explainable on the basis of the geometry of the catalyst and substrate. Other features of enzymic catalysis which should be sought for in a model system include competitive inhibition and nonproductive binding. In addition, it would be preferable if the catalysis proceeded by a mechanism similar to those so far known for enzyme-catalyzed reactions.

It appeared possible that a system incorporating the cycloamyloses[6–9] might permit the development of such a model. As the name implies,[10] the cycloamyloses are cyclic α-1,4-linked D-glucose polymers and have 6, 7, or 8 glucose residues per molecule.[11] X-Ray crystallographic studies have firmly established the structure[7]

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

(2) A preliminary report of this research has been published: M. L. Bender, R. L. VanEtten, G. A. Clowes, and J. F. Sebastian, *J. Am. Chem. Soc.*, **88**, 2318 (1966).

(3) National Institutes of Health Postdoctoral Research Fellow.

(4) The K_s for acetyl-L-tryptophanamide is 5.0×10^{-3} M [R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, 77, 1886 (1955)] while the K_i for acetyl-D-tryptophanamide is 2.3×10^{-3} M [R. J. Foster, H. J. Shine, and C. Niemann, *ibid.*, 77, 2378 (1955)].

(5) D. W. Ingles, J. R. Knowles, and J. A. Tomlinson, *Biochem. Biophys. Res. Commun.*, 23, 619 (1966). Qualitatively similar observations may be made for K_s, K_i, and k_2 values of amide substrates but because reactions of the D isomers are so slow it is not possible to obtain an accurate ratio which describes the kinetic specificity of the k_2 step for chymotrypsin-catalyzed hydrolyses. Conversely, the k_2 step for chymotrypsin-catalyzed hydrolyses of esters related to specific substrates is too fast to permit a similar measurement.

(6) F. Cramer, "Einschlussverbindungen," Springer-Verlag, Heidelberg, 1954.

(7) D. French, *Advan. Carbohydrate Chem.*, 12, 189 (1957).

(8) J. A. Thoma and L. Stewart in "Starch: Chemistry and Technology," Vol. I, R. L. Whistler and E. F. Paschall, Ed., Academic Press Inc., New York, N. Y., 1965, p 209.

(9) F. R. Senti and S. Erlander in "Nonstoichiometric Compounds," L. Mandelcorn, Ed., Academic Press Inc., New York, N. Y., 1964, p 588.

(10) The names α-, β-, and γ-Schardinger dextrins and cyclodextrins became established early in the literature, the prefixes referring to cyclohexa-, -hepta-, and -octaamylose, respectively. Now that the structures of these molecules have been unequivocally established it seems appropriate to use the more descriptive amylose names.

(11) The structure of the homologous cyclononaamylose also appears to have been established: D. French, A. O. Pulley, J. A. Effenberger, M. A. Rougvie, and M. Abdullah, *Arch. Biochem., Biophys.*, **111**, 153 (1965).

and stereochemistry[12] of the cycloamyloses. They are doughnut-shaped molecules with the glucose units in the C-1 conformation. The primary hydroxyl groups (carbon 6 of the glucose unit) are located on one side of the torus while the secondary hydroxyls (carbons 2 and 3 of the glucose units) are located on the other side of the torus. The interior of the cavity contains a ring of C–H groups, a ring of glycosidic oxygens, and another ring of C–H groups. As a consequence the interior of the cycloamylose torus is relatively hydrophobic[13] when compared to water.

The cycloamyloses form solid inclusion complexes and complexes in solution with a variety of molecules and ions.[6–9] This characteristic has led to their utilization as enzyme models. For example, the cycloamyloses have been shown to act as asymmetric catalysts in the saponification of mandelic acid esters, although the rate effects and optical yields were small.[14] The cycloamyloses catalyze the decarboxylation of substituted cyanoacetic acids, accelerations of up to 15-fold being observed.[15] In neither of these studies was the relationship between complexing and catalysis determined. Cycloheptaamylose was found to inhibit the basic hydrolysis of ethyl *p*-aminobenzoate, the complexed ester being completely unreactive (within experimental error) in 0.04 *N* barium hydroxide solution.[16] The extent of inhibition due to added cycloheptaamylose was consistent with the formation of a 1:1 complex[17] having a dissociation constant of 2.34×10^{-3} *M*. Most pertinent to the present investigation is the work of Hennrich and Cramer where it was found that the cycloamyloses accelerate the decomposition of diaryl phosphates in heterogeneous alkaline mixtures with concomitant phosphorylation of the amylose.[18] The acceleration of the release of phenol from di-*p*-chlorophenyl pyrophosphate was *ca.* 200-fold with cycloheptaamylose, smaller accelerations being observed with cyclohexaamylose and cyclooctaamylose. Furthermore, the acceleration was reduced when phenolic products were present in the reaction mixture. Unfortunately, limitations of the experimental system made it impossible to investigate the relationship between the stability of the complex and its reactivity. The relative importance of steric and electronic effects on reactivity and on complexation could not be determined. The present investigation of the effects of cycloamyloses on the alkaline hydrolysis reactions of phenyl esters was therefore undertaken in an effort to determine these critically important facts.

Experimental Section

Cycloamyloses were initially obtained as a gift from Professor Dexter French. Subsequently cyclohexaamylose was purchased from Applied Science Laboratories. At the present time both cyclohexaamylose and cycloheptaamylose are available from Pierce Chemical Co. (Rockford, Ill.). Cyclooctaamylose was obtained as the pure crystalline material from Professor French. Cycloheptaamylose was purified by recrystallization from water and dried at 110° for 12 hr. *Anal.* Calcd for $C_{42}H_{70}O_{35}$: C, 44.44; H, 6.22. Found: C, 44.11; H, 6.29.

Cyclohexaamylose was purified by recrystallization from 1-propanol–water[7] followed by recrystallization from water and drying under vacuum at 80° for 12 hr. *Anal.* Calcd for $C_{36}H_{60}O_{30}$: C, 44.44; H, 6.22. Found: C, 44.16, 44.44; H, 6.32, 6.35.

Both the cyclohexaamylose and cycloheptaamylose were found to be pure and free from cross-contamination as judged by paper chromatography on Whatman No. 1 paper (descending chromatography, 25°, solvent: 1-butanol–dimethylformamide–water, 2:1:1, v/v/v) using 1% alcoholic iodine as a developing spray. The R_f values of cyclohexa- and -heptaamylose were 0.23 and 0.20, respectively.

The specific rotation of aqueous cyclohexaamylose was $[\alpha]^{25}{}_D$ 150.2 ± 4.20° (concentration varied from 0.0104 to 0.0286 g/ml), in agreement with the literature value[19] of 150.5 ± 0.5°. No trend was observed in the specific rotation values as the cyclohexaamylose concentration was varied. A Rudolph Model 200S/655 manual photoelectric spectropolarimeter equipped with an electrical null reading device was used to determine the optical rotations.

The molecular weight of cyclohexaamylose in water at 37° was determined using a Mechrolab Model 301A vapor pressure osmometer with sucrose as a standard. Three samples at concentrations of 58, 132, and 50 g/l. gave molecular weights of 1120, 1090, and 1110, respectively (calculated 972). Since raffinose pentahydrate also gave a somewhat high value of 630 (calculated 594), it is possible that there is some systematic error involved; hydration of the cycloamylose would also lead to high observed values. Both the molecular weight data and the optical rotation data indicate that in aqueous solution cyclohexaamylose is only slightly associated (if at all).

Esters. Phenyl acetates were purchased from commercial sources (Distillation Products Industries or Aldrich Organic Chem. Co.) or were synthesized generally by the method of Spasov.[20] The physical properties of the esters prepared as well as the wavelengths used to follow the reactions are shown in Table I. *m*-Carboxyphenyl acetate was prepared by reaction of acetic anhydride with *m*-carboxyphenol in pyridine followed by recrystallization from chloroform. *p*-Carboxyphenyl acetate was synthesized by the method of Renson and Huls[21] and recrystallized from chloroform. *p*-Carboxyphenyl 2-methylpropionate was prepared in 10% yield by reaction of *p*-carboxyphenol with isobutyryl chloride in pyridine followed by recrystallization from chloroform, mp 181.5–183°. *Anal.* Calcd for $C_{11}H_{12}O_4$: C, 63.46; H, 5.77. Found: C,

Table I. Phenyl Acetates Used as Substrates

Acetate	Bp, °C (mm), or mp, °C	Wavelength,[a] mμ
3,5-Dimethylphenyl	143 (54)	295
3,4,5-Trimethylphenyl	58.5–59.5	285–300
m-Ethylphenyl	127.5 (31)	280–298
m-*t*-Butylphenyl	160–161 (61)	290–295
p-*t*-Butylphenyl	157 (50)	300
p-Methoxyphenyl	160 (55)	315
p-Bromophenyl	154 (45)	305
m-Chlorophenyl	105–109 (15–16)	299–305
p-Chlorophenyl	143 (55)	305
p-Cyanophenyl	56–57	290
m-Carboxyphenyl	131–132.5	330
p-Carboxyphenyl	191–192.5	280–308

[a] Phenyl acetate and the tolyl acetates were assayed at 298–305 mμ, *m*-nitrophenyl acetate at 390 mμ, *p*-nitrophenyl acetate at 400 mμ, and *o*-nitrophenyl acetate at 470 mμ. (These wavelengths proved to be experimentally convenient but are not necessarily optimal.)

(12) A. Hybl, R. E. Rundle, and D. E. Williams, *J. Am. Chem. Soc.*, **87**, 2779 (1965); a detailed description of the structure of a cyclohexaamylose–potassium acetate complex is given.

(13) The term hydrophobic is used here in the sense of relative to water and should not be taken to mean hydrocarbon or to imply any particular mechanism as an explanation of the apolar bonding involved in complex formation; see also ref 8.

(14) F. Cramer and W. Dietsche, *Ber.*, **92**, 1739 (1959).

(15) F. Cramer and W. Kampe, *J. Am. Chem. Soc.*, **87**, 1115 (1965).

(16) J. L. Lach and T. F. Chin, *J. Pharm. Sci.*, **53**, 924 (1964).

(17) Strong evidence in favor of 1:1 inclusion complexes for a variety of guest molecules with cycloamyloses has been presented recently: F. Cramer, W. Saenger, and H.-C. Spatz, *J. Am. Chem. Soc.*, **89**, 14 (1967).

(18) N. Hennrich and F. Cramer, *ibid.*, **87**, 1121 (1965).

(19) D. French, M. L. Levine, J. H. Pazur, and E. Norberg, *J. Am. Chem. Soc.*, 71, 353 (1949).

(20) A. Spasov, *Ann. Univ. Sofia, II, Fac. Phys. Math., Livre 2*, **35**, 289 (1938–1939); *Chem. Abstr.*, **34**, 2343 (1940).

(21) M. Renson and R. Huls, *Bull. Soc. Chim. Belges*, **61**, 599 (1952).

63.17; H, 5.85. p-Carboxyphenyl 3,3-dimethylbutyrate was prepared in 25% yield by reaction of p-carboxyphenol with t-butylacetyl chloride (Aldrich Chemical Co.) in pyridine followed by recrystallization from chloroform, mp 195–197°. *Anal.* Calcd for $C_{13}H_{16}O_4$: C, 66.10; H, 6.78. Found: C, 65.86; H, 6.72. Reactions of this and the previous compound were followed at 280 mμ.

Kinetics of Hydrolysis of Phenyl Acetates. The reactions were carried out by following the appearance of phenol spectrophotometrically using a Cary Model 14 recording spectrophotometer equipped with a thermostated cell compartment, thermostated cell holder, and 0–1 and 0–0.1 absorbance slide wires. The reaction medium, generally 3.00 ml of pH 10.6 sodium carbonate buffer, was placed in a stoppered 1-cm silica cell and thermostated for 15 min. The reaction was initiated by the addition of 15 μl of a stock solution of the ester in acetonitrile (Eastman Spectro Grade) using a plastic flat-tipped stirring rod (Calbiochem). The final ester concentration was 1×10^{-4} M and the acetonitrile concentration was 0.5% (v/v). When fast reactions were being followed, the time necessary to complete the mixing of the reactants and begin recording the spectral changes did not exceed 6 sec. The pH determined using a Radiometer 4c pH meter did not change during the course of the reaction. The hydrolysis of the phenyl acetates as determined in these buffered alkaline solutions followed (pseudo) first-order kinetics. Times were determined from the recorded chart divisions by calibration using a Precision Scientific Time-It electric clock and are accurate to ±1%. The infinite absorbance values were obtained after at least eight half-lives and reactions were recorded through 70% of completion. The rate constants were calculated by electronic computation of the linear least-squares slope of the line formed by the set of points [time, log (absorbance − infinite absorbance)] and division of the slope by −0.4343. The resulting points were checked by examination of a computer-produced graph of the first-order plot in order to eliminate transcription errors. The reported rate constants are averages of two or three determinations which agreed within 3%. The spectra of the products corresponded to the spectra obtained by addition of pure phenol to the buffered cycloamylose solutions.

Determination of Dissociation Constants by Kinetic Methods. The observed first-order rate constants for hydrolysis of phenyl acetates (as measured by phenol release) in the absence (k_{un}) and presence (k_{obsd}) of added cycloamylose were determined. From 5 to 12 points were obtained at cycloamylose concentrations spanning (whenever possible) the value of K_{diss} (the dissociation constant of the cycloamylose–ester complex calculated assuming a 1:1 stoichiometry). The values of $-K_{diss}$ and k_2 (the maximal catalyzed rate due to decomposition of the fully complexed ester) were obtained as the slope and Y intercept of the line[22,23] formed by plotting $k_{obsd} - k_{un}$ against $(k_{obsd} - k_{un})$/[cycloamylose]. Calculations were carried out using Eadie-type plots rather than the Lineweaver–Burk plot previously used[2] because the Eadie plot is statistically preferable.[24] The slope and intercept were obtained by electronic computation of the least-squares line. This appeared adequate since in an Eadie plot it is relatively easy to choose concentrations so that the experimental points are evenly distributed along the line. Unlike the usual situation in enzyme kinetics, the cycloamylose here is present in large excess over the substrate concentration and may be accurately determined by weight. The range of cycloamylose concentrations (depending on the value of K_{diss}) varied from 6×10^{-4} to 4×10^{-2} M. The error limits of K_{diss} were calculated from the experimental data by application of Student's t test for 95% probability of fit to the least-square line. The error in k_2 may be expected to parallel the error in K_{diss}. Graphical estimates of error limits made by drawing extreme lines through Lineweaver–Burk plots were significantly less than the computed values shown in Table IV.

Determination of Dissociation Constant by Spectrophotometric Methods. The increase in absorbance of the aromatic chromophore in the presence of varying amounts of cycloamylose were determined using a Cary Model 14 recording spectrophotometer equipped with a thermostated cell compartment and a 0–0.1, 0.1–0.2 slide wire. To minimize hydrolysis of esters and suppress phenol ionization a pH 2.2 hydrochloric acid–potassium chloride buffer (I = 0.06) was employed. Since the cycloamyloses are relatively unstable in acid solution the solutions were used within 1 hr of preparation. The substrate concentration was held constant at 10^{-4} M. The reference compartment contained phenol or ester and cycloamylose solutions contained in separate 1-cm silica cells. The sample compartment contained a cell with buffer and a cell with combined cycloamylose and phenol or ester. The difference spectrum of 4.4×10^{-2} M glucose plus 5.8×10^{-4} M phenol *vs.* phenol was identical with the difference spectrum of phenol *vs.* phenol.

In order to calculate the cycloamylose–substrate dissociation constants a linear relationship was employed[25] relating the observed spectral changes to the added concentrations of cycloamylose and ester (see Appendix). The slope $1/\Delta\epsilon$ and the intercept $K_{diss}/\Delta\epsilon$ were obtained by electronic computation of the linear least-squares line through the experimental points. The validity of the method of data treatment was checked by using synthetic data available in[26] the literature as well as by redetermining the dissociation constant of the methyl orange–cyclohexaamylose complex (see footnotes d and e to Table VI).

Determination of Inhibition Constants. The dissociation constants of cycloamylose–inhibitor complexes (K_i) were determined by measuring the rate of m-nitrophenyl acetate hydrolysis in the presence of a fixed amount of cycloamylose and varying concentrations of added inhibitor. The concentration of the ester was 9.9×10^{-5} M and the cycloamylose was 3.6×10^{-3} M; the inhibitor concentrations were chosen, when possible, to span the value of K_i.

One milliliter of 1.08×10^{-2} M cycloamylose solution in pH 10.05 sodium carbonate buffer was mixed with 2 ml of a sodium carbonate buffer solution of the inhibitor, the pH was adjusted if necessary to 10.05, and the mixture in a 1-cm quartz cell was thermostated for 15 min at 25.0 ± 0.1° in a Cary Model 14 spectrophotometer. The ester was added in 15 μl of acetonitrile (Eastman Spectro Grade) and the appearance of phenoxide ion was followed at 390 mμ.

Added inhibitor at the highest concentrations used had only small effects on the rate of the uncatalyzed reaction. The pH of the reaction mixture did not vary by more than 0.04 unit during the course of the reaction. The values of k_2 and K_{diss} for the cycloamylose-catalyzed decomposition of m-nitrophenyl acetate were previously determined (Table IV). The inhibitor concentration [I] was plotted as a function of $(k_2 - k_{obsd})/(k_{obsd} - k_{un})$. The Y intercept is $-K_i$ and the slope is [cycloamylose] $\times$ K_i/K_{diss} (see Appendix). A sample plot is shown in Figure 5.

Results

Kinetics of Hydrolysis of Phenyl Acetates in the Presence of Glucose and Methyl Glucoside. The pseudo-first-order rate constants for the alkaline hydrolysis of a variety of substituted phenyl acetates are shown in Table II. The hydroxide ion reaction rates vary in the manner expected from the electronic character of the substituents. Data for the various *meta*- and *para*-substituted phenyl acetate hydrolysis rates gives an excellent fit to a Hammett-type relationship. The line formed by plotting log (k/k_H) *vs.* the substituent constant[27] σ^n gives a slightly better correlation coefficient that the corresponding plot using ordinary σ values (footnote c to Table II), indicating that there is not a significant charge delocalization in the aromatic ring in the transition state.

No unusual effects are observed when 0.06 M α-methyl glucoside is added to the reaction mixture, there being only a 10–20% acceleration of the hydrolysis rates (Table II). Addition of 0.06 M glucose to the reaction mixture causes a two- to threefold increase in the hydrolysis rates. The larger effect due to glucose is presumably the result of reaction with the hemiacetal

(22) A. K. Colter, S. S. Wang, G. H. Megerle, and P. S. Ossip, *J. Am. Chem. Soc.*, **86**, 3106 (1964).
(23) G. S. Eadie, *J. Biol. Chem.*, **146**, 85 (1942).
(24) J. E. Dowd and D. S. Rigg, *ibid.*, **240**, 863 (1965).
(25) F. J. C. Rossotti and H. Rossotti, "The Determination of Stability Constants," McGraw-Hill Book Co., Inc., New York, N. Y., 1961, p 276.
(26) G. D. Johnson and R. E. Bowen, *J. Am. Chem. Soc.*, **87**, 1655 (1965).
(27) H. van Bekkum, P. E. Verkade, and B. M. Wepster, *Rec. Trav. Chim.*, **78**, 815 (1959).

Table II. Hydrolysis of Phenyl Acetates in the Presence of Glucose and Methyl Glucoside[a]

Phenyl acetate substituent	Hydroxide ion rate, 10^{-3} sec^{-1}	Rate with 0.06 *M* α-methyl glucoside, 10^{-3} sec^{-1}	Rate with 0.06 *M* glucose, 10^{-3} sec^{-1}	Substituent constants[b,c] σ	σ^n
H	0.804	0.879	2.39	(1)	(1)
p-OMe	0.749	0.802	2.14	−0.268	−0.111
p-*t*-Bu	0.607	0.710	1.87	−0.197	
m-*t*-Bu	0.490	0.582	1.51	−0.10	
p-Cl	1.52	1.69	5.31	0.227	0.238
m-Cl	1.91	2.08	7.07	0.373	
p-CN	4.78	5.12	19.0	0.660	0.674
m-NO_2	4.64	5.16	19.7	0.710	
p-NO_2	6.94	7.36	26.8	0.778	0.778

[a] Pseudo-first-order rate constants obtained using pH 10.60 sodium carbonate buffer with 0.5% (v/v) acetonitrile added; [ester] $\simeq 1 \times 10^{-4}$ *M*. [b] Taken from ref 27. [c] The correlation coefficients obtained for the least-squares fit to the line formed by plotting log (k/k_H) *vs.* σ for the uncatalyzed, methyl glucoside, and glucose reaction mixtures were 0.972, 0.977, and 0.980; for the plot *vs.* σ^n they were 0.987, 0.989, and 0.992, respectively. The ρ values obtained for the three reaction systems using σ^n values were 1.10, 1.07, and 1.24.

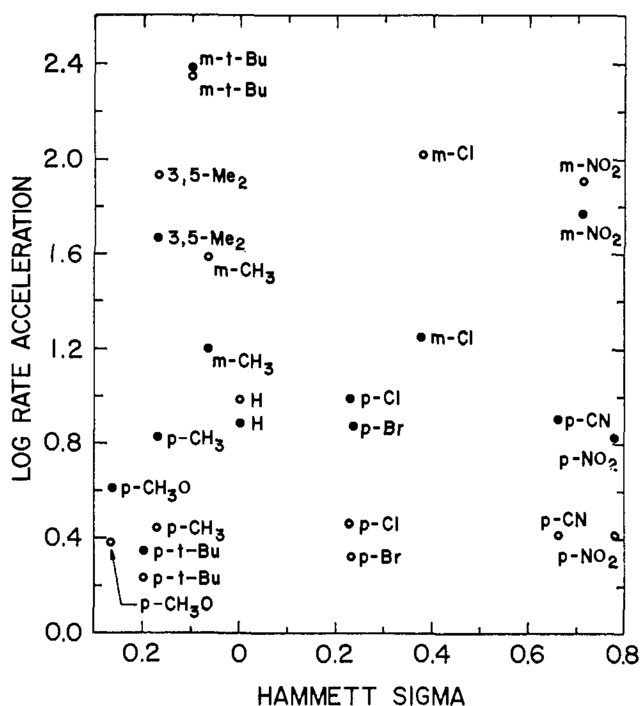


Figure 1. Graph of the logarithm of the acceleration of the rate of phenol release due to 1×10^{-2} *M* cycloamylose *vs.* the Hammett substituent constant σ: ●, added cycloheptaamylose; ○, added cyclohexaamylose (for experimental conditions see Table III).

alkoxide ion derived from glucose. This is consistent with the fact that the Hammett ρ value for the glucose-catalyzed reaction is 1.24 as compared with 1.10 for the pure hydroxide ion reaction (Table II). The alkoxide ion derived from glucose is more sensitive than hydroxide ion to the electronic nature of the substituted carbonyl group.

Kinetics of Hydrolysis of Phenyl Acetates in the Presence of Cycloamyloses. The hydrolysis rates of a variety of substituted phenyl esters in the absence (k_{un}) and presence (k_{obsd}) of 0.01 *M* added cycloamyloses are shown in Table III. Depending on the structure of chlorophenyl acetate occurs 113 times more rapidly in the presence of 0.01 *M* cyclohexaamylose. The acceleration k_{obsd}/k_{un} bears no apparent relationship to the electronic character of the substituents. A Hammett-type plot of log (k_{obsd}/k_{un}) *vs.* the substituent constant σ shows a tremendous scatter (Figure 1).

Table III. Hydrolysis Rates of Phenyl Acetates at pH 10.60 in the Absence and Presence of Cycloamyloses[a]

Acetate ester	Hydroxide ion rate, k_{un}, 10^{-4} sec^{-1}	[Cyclohexaamylose (0.01 *M*)] k_{obsd}, 10^{-2} sec^{-1}	k_{obsd}/k_{un}	[Cycloheptaamylose (0.01 *M*)] k_{obsd}, 10^{-2} sec^{-1}	k_{obsd}/k_{un}	[Cyclooctaamylose (0.01 *M*)] k_{obsd}, 10^{-2} sec^{-1}	k_{obsd}/k_{un}
Phenyl	8.04	0.779	9.7	0.609	7.6		
o-Tolyl	3.84	0.294	7.7	0.266	6.9		
m-Tolyl	6.96	2.70	39	1.14	16		
p-Tolyl	6.64	0.187	3.8	0.443	6.7		
3,5-Dimethylphenyl	5.80	4.97	86	2.64	46		
3,4,5-Trimethylphenyl	4.31	0.911	21	3.15	73		
m-*t*-Butylphenyl	4.90	11.1	226	12.1	250	2.65	54
p-*t*-Butylphenyl	6.07	0.102	1.7	0.135	2.2	2.51	41
p-Methoxyphenyl	7.49	0.174	2.3	0.298	4.0		
p-Bromophenyl	16.4	0.347	2.1	1.23	7.5		
m-Chlorophenyl	19.1	21.5	113	3.49	18	1.48	7.8
p-Chlorophenyl	15.2	0.453	3.0	1.55	10	1.34	8.8
p-Cyanophenyl	47.8	1.20	2.5	3.61	7.6		
o-Nitrophenyl	53.2	5.39	10.1				
m-Nitrophenyl	46.4	47.9	103	25.0	54	4.65	10.0
p-Nitrophenyl	69.4	1.79	2.6	4.66	6.7	4.33	6.2

[a] The pseudo-first-order rate constants were determined by following the release of phenol spectrophotometrically: see Experimental Section. All determinations were done at 25.0 ± 0.2° using pH 10.60 sodium carbonate buffer, $I = 0.2$, with 0.5% (v/v) added acetonitrile.

the ester, marked accelerations of the phenol release may occur as the result of addition of the cycloamyloses to the reaction mixture. Thus, *m*-*t*-butylphenyl acetate hydrolyzes 240 times more rapidly in the presence of 0.01 *M* cycloheptaamylose, and the hydrolysis of *m*-

However, there does appear to be a relationship between the stereochemistry of substitution of the phenyl acetates and the degree of acceleration of the hydrolysis reaction. Figure 1 suggests that phenyl acetates having substituents in the position *meta* to the acetoxy group

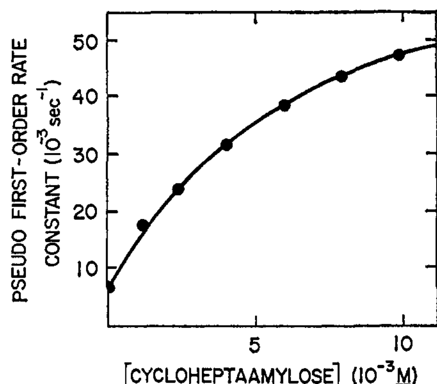


Figure 2. The pseudo-first-order rate constant for release of phenol from *p*-nitrophenyl acetate at pH 10.6 plotted as a function of added cycloheptaamylose; 0.5% (v/v) acetonitrile–water, 25°, [*p*-nitrophenyl acetate] $\simeq 1 \times 10^{-4}$ *M*.

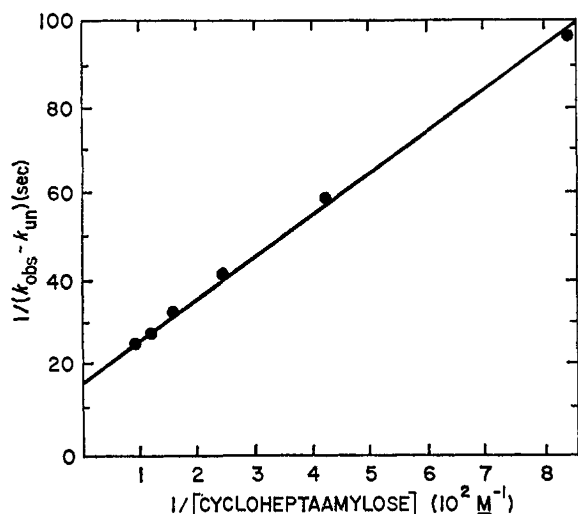


Figure 3. $1/(k_{obsd} - k_{un})$ for *p*-nitrophenyl acetate decomposition is plotted *vs.* the reciprocal of the cycloheptaamylose concentration (data from Figure 2).

exhibit the largest rate accelerations. For example, although *m*-chlorophenyl acetate hydrolysis shows a 113-fold acceleration in the presence of cyclohexaamylose, the *para* isomer shows only a threefold acceleration; likewise *m*-*t*-butylphenyl acetate shows a 230-fold acceleration whereas the *para* isomer shows only a 1.7-fold rate enhancement. Similar observations may be made for the effects of added cycloheptaamylose on reaction of substituted phenyl acetates, there being a remarkable stereoselective enhancement of the phenol release reaction favoring the *meta*-substituted compounds.

Because the cycloamyloses form inclusion complexes in solution with a variety of organic and inorganic substances[6–9] it appeared likely that the rate increases shown in the data of Table III involved a cycloamylose–ester complex. Such being the case, it is uncertain whether the apparent geometric specificity evident in the accelerations is due to differences in the reactivity of the complexes (or in both). In an effort to resolve this problem the concentration dependence of the catalytic action was examined. The effect of varying (excess) cycloheptaamylose concentration on the pseudo-first-order rate constant for *p*-nitrophenyl acetate hydrolysis (as measured by phenol release) is shown in Figure 2. The rate accelerations approach a

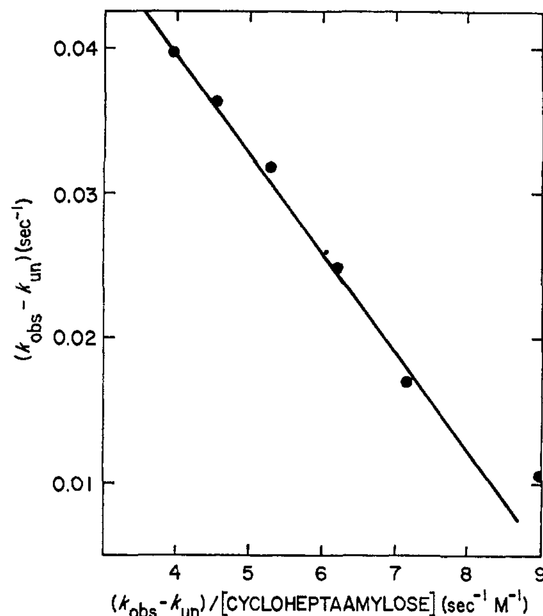


Figure 4. $(k_{obsd} - k_{un})$ for *p*-nitrophenyl acetate decomposition is plotted as a function of $(k_{obsd} - k_{un})$/[cycloheptaamylose] (data from Figure 2).

maximum saturation value similar to the behavior observed in enzyme kinetics. The data were treated by a variant of Michaelis–Menten kinetics previously employed for investigation of reactions involving complex formation. By plotting the *p*-nitrophenyl acetate data in the form of $1/(k_{obsd} - k_{un})$ *vs.* the reciprocal of the cycloamylose concentration a straight line is obtained (Figure 3) having a slope of K_{diss}/k_2 and a *Y* intercept equal to $1/k_2$, where K_{diss} is the dissociation constant of the complex and k_2 is the rate constant for reaction of the entirely complexed ester ($k_{obsd} - k_{un}$ at infinite cycloamylose concentration).[28]

In order to permit a more accurate evaluation of the various values of K_{diss} and k_2, a statistically preferable form[24] of Michaelis–Menten kinetics due to Eadie[23] was employed. As shown in Figure 4 for the *p*-nitrophenyl acetate data, by plotting $k_{obsd} - k_{un}$ against $(k_{obsd} - k_{un})$/[cycloamylose] a straight line is obtained with slope $-K_{diss}$ and a *Y* intercept of k_2.

Dissociation constants and maximal rate accelerations obtained in this manner (by electronic computation; see Experimental Section) are presented in Table IV. It is of importance to compare the dissociation constants and the rate effects observed with some of the isomeric phenyl acetates. *p*-Nitrophenyl acetate forms a more stable complex with both cyclohexaamylose and cycloheptaamylose than does *m*-nitrophenyl acetate, but the maximal rate accelerations k_2/k_{un} are much greater for the *meta* isomer. Similarly, although the stability of the *m*-*t*-butylphenyl acetate–cyclohexaamylose complex differs by only a factor of 3 from that of the *para* isomer, the release of phenol from the *meta* isomer is accelerated some 230 times more than from the *para* isomer. Qualitatively similar observations may be made for the reaction of *o*-, *m*-, and *p*-tolyl acetates in the presence of cyclohexaamylose. Thus, the

(28) This method of data treatment is in effect the Lineweaver–Burk plot of 1/velocity *vs.* 1/substrate concentration which is frequently employed in studies of the kinetics of enzyme-catalyzed reactions: H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

Table IV. Maximal Rate Accelerations and Dissociation Constants of Cycloamylose–Phenyl Acetate Complexes[a]

Acetate	Hydroxide ion rate, k_{un}, 10^{-4} sec^{-1}	Cyclohexaamylose k_2, 10^{-2} sec^{-1}	Cyclohexaamylose k_2/k_{un}	Cyclohexaamylose K_{diss}, 10^{-2} M	Cycloheptaamylose k_2, 10^{-2} sec^{-1}	Cycloheptaamylose k_2/k_{un}	Cycloheptaamylose K_{diss}, 10^{-3} M
Phenyl	8.04	2.19	27	2.2 ± 0.7			
o-Tolyl	3.84	0.72	19	1.9 ± 0.5			
m-Tolyl	6.96	6.58	95	1.7 ± 0.5			
p-Tolyl	6.64	0.22	3.3	1.1 ± 0.7			
3,5-Dimethylphenyl	5.80	11.5	200	1.5 ± 0.4	4.86	84	8.8 ± 1.4
3,4,5-Trimethylphenyl	1.04[b]				1.29[b]	124[b]	5.0 ± 1.1[b]
m-Ethylphenyl	5.49 (1.42)[b]	13.3	242	1.07 ± 0.14	1.26[b]	89[b]	2.2 ± 0.4[b]
m-*t*-Butylphenyl	4.90	12.9	260	0.20 ± 0.08	12.2	250	0.13 ± 0.03[c]
p-*t*-Butylphenyl	6.07	0.067	1.1	0.65 ± 0.39			
m-Chlorophenyl	19.1 (5.05)[b]	7.89[b]	156[b]	0.56 ± 0.03[b]	4.50	24	3.5 ± 0.9
m-Chlorophenyl	4.99[d]	6.88[d]	138[d]	0.42 ± 0.05[d]			
m-Chlorophenyl	9.08[e]	14.9[e]	164[e]	4.2 ± 1.0[e]			
m-Nitrophenyl	46.4 (14.0)[b]	42.5[b]	300[b]	1.9 ± 0.4[b]	44.4	96	8.0 ± 1.8
p-Nitrophenyl	69.4	2.43	3.4	1.2 ± 0.4	6.34	9.1	6.1 ± 1.3
m-Carboxyphenyl	8.15	5.55	68	10.5 ± 3.1			
p-Carboxyphenyl	12.5	0.67	5.3	15.0 ± 9.0			

[a] In pH 10.60 carbonate buffer, I = 0.2, 0.5% acetonitrile-water unless otherwise noted. All determinations at 25.0 ± 0.1°. Error limits for K_{diss} were calculated from the experimental data by application of Student's t test at the 95% probability level. The error in k_2 may be expected to parallel the error in K_{diss}. [b] In pH 10.01 carbonate buffer, I = 0.2, 0.5% acetonitrile-water. [c] Determined by graphically estimating the cycloheptaamylose concentration necessary to result in the half-maximal velocity from a plot of k_{obsd} *vs.* [cycloheptaamylose]; [ester] $\simeq 2 \times 10^{-5}$ M. [d] In pH 10.01 carbonate buffer, I = 2.0 (by addition of potassium chloride). 0.5% acetonitrile-water. [e] In pH 10.58 carbonate buffer, I = 0.2, 10% (v/v) (1.9 M) acetonitrile–water.

stereospecific rate accelerations are unrelated to the strength of binding. The sensitivity of k_2 to the stereochemistry of the substrate is strikingly similar to enzyme-catalyzed reactions (see Discussion).

Competitive Inhibition of Cycloamylose Catalysis. In order to determine the stability of complexes of the cycloamyloses with a variety of unreactive molecules as well as to provide further information about the nature of the reactive complex, the competitive inhibition of the cyclohexaamylose-catalyzed decomposition reaction of *m*-nitrophenyl acetate was examined. As anticipated, the rate effects of the cycloamyloses were decreased upon addition of a variety of organic compounds to the reaction mixture. By determining the extent of inhibition as a function of added inhibitor concentration it was possible to obtain an inhibition constant K_i which is the dissociation constant of the cycloamylose–inhibitor complex (see Experimental Section). The treatment of the data is exemplified by Figure 5. Application of this method made possible the determination of the inhibition constants for the anions shown in Table V. From these data it is apparent that small anions such as acetate and propionate do not bind well to cyclohexaamylose. Anions derived from acids containing larger relatively hydrophobic groups such as adamantane or cyclohexane form relatively stable complexes (see Discussion).

Table V. Dissociation Constants of Cyclohexaamylose Complexes Determined by Competitive Inhibition of the *m*-Nitrophenyl Acetate Hydrolysis Reaction[a]

Acid anion inhibitor[b]	K_i, M[c]
Acetate	>1.0
Propionate	$5.7 \pm 2.0 \times 10^{-1}$
Isobutyrate	$2.2 \pm 0.3 \times 10^{-1}$
Pivalate	$2.0 \pm 0.6 \times 10^{-1}$
Benzoate	$8.1 \pm 1.0 \times 10^{-2}$
p-Toluenesulfonate	$6.0 \pm 2.0 \times 10^{-2}$
p-Benzoylbenzoate	$4.0 \pm 0.5 \times 10^{-2}$
Cyclohexanecarboxylate	$1.9 \pm 0.3 \times 10^{-2}$
p-Phenylbenzoate	$1.7 \pm 0.2 \times 10^{-2}$
m-Chlorobenzoate	$1.2 \pm 0.2 \times 10^{-2}$
Adamantanecarboxylate[d]	$7.0 \pm 2.0 \times 10^{-3}$
m-Chlorocinnamate	$7.0 \pm 1.0 \times 10^{-3}$
p-Chlorobenzoate	$6.0 \pm 2.0 \times 10^{-3}$
p-Chlorocinnamate	$5.1 \pm 1.0 \times 10^{-3}$

[a] All determinations were made at 25.0 ± 0.1° using pH 10.0 sodium carbonate buffer, I = 0.2 (but when K_i was $>10^{-2}$ M then I > 0.2), 0.5% acetonitrile. [b] No unusual effects are observed with cationic inhibitors; for example, tetraethylammonium bromide gives $K_i = 3.3 \pm 0.6 \times 10^{-2}$ M. [c] Calculated assuming a 1:1 stoichiometry in the complex; it is possible that molecules such as *p*-benzoylbenzoate form higher order complexes, as has been observed for methyl orange[17] (where the dissociation constants differ by $\sim 10^2$). The error limits were obtained by drawing extreme lines through the experimental points. [d] K_i for inhibition of cycloheptaamylose catalysis is $7 \pm 4 \times 10^{-4}$ M.

Determination of Cycloamylose–Substrate Dissociation Constants by Spectrophotometric Methods. The formation of inclusion complexes with cycloamyloses is known to lead to perturbations of the ultraviolet spectra of a variety of organic molecules ranging from organic dyes[29] such as marine blue, methyl orange, and crystal violet to compounds such as N-acetyltyrosine ethyl ester.[30] Thus, it appeared possible to observe significant changes in the ultraviolet absorbtion spectra of aromatic molecules related to ester substrates occurring as the result of complex formation. This was indeed the case. An example of the type of observed spectral change is shown in Figure 6. Addition of 2.5×10^{-2} M cyclohexaamylose to an aqueous solution of 1×10^{-4} M *p*-*t*-butylphenol causes a spectral change almost identical with that observed when the phenol is dissolved in dioxane instead of water. This result is similar to observations made for cycloamylose complexes of iodine[8] and N-acetyltyrosine ethyl ester.[8,30] Glucose, at these concentration levels, has no significant effect on the ultraviolet spectrum. The similarity of the aqueous cyclohexaamylose solution spectrum to the

(29) W. Lautsch, W. Broser, W. Biedermann, and H. Gnichtel, *J. Polymer Sci.*, 17, 479 (1955).
(30) T. L. Warrington and M. Laskowski, Jr., Abstracts, 145th National Meeting of the American Chemical Society, New York, N. Y., 1963, p 76C; see also ref 8. For a study of the binding of benzoic acids to cyclohexaamylose, see B. Casu and L. Rava, *Ric. Sci. Rend.*, [8] 36, 733 (1966).

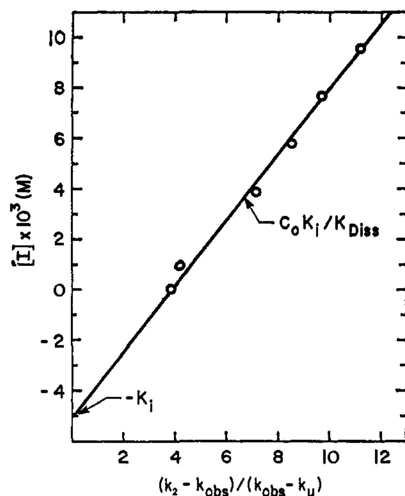


Figure 5. Added inhibitor concentration [I] graphed as a function of $(k_2 - k_{obsd})/(k_{obsd} - k_{un})$ for inhibition by *p*-chlorocinnamate ion of the acceleration of *m*-nitrophenyl acetate decomposition by cyclohexaamylose (experimental conditions given in Table V).

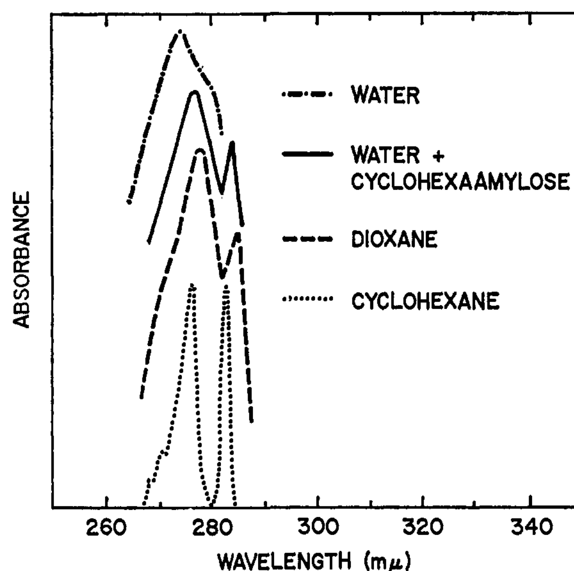


Figure 6. Ultraviolet absorption spectrum of *p*-*t*-butylphenol in various solvents (the absorbance values are arbitrarily shifted vertically for purposes of clarity); [cyclohexaamylose] = 2.5 × 10^{-2} *M;* [phenol] ≃ 1 × 10^{-4} *M.*

dioxane solution spectrum suggests that the aromatic chromophore is largely included in the cycloamylose cavity (see Discussion).

The absorbancy changes resulting from the addition of varying amounts of cycloamyloses to the aqueous solutions of the substrates were treated by plotting[25] $[A][B]/\Delta Abs$ against [A] + [S], where [A] and [S] are the initial stoichiometric concentrations of cycloamylose and substrate and ΔAbs is the spectral perturbation observed as the difference spectrum. Under the experimental conditions employed the product [A][S] was maintained much larger than $[C]^2$ where [C] is the concentration of the complex. Examples of such determinations are shown in Figure 7. The dissociation constants of some cyclohexaamylose complexes determined in this manner are shown in Table VI. Of greatest interest is the agreement between dissociation constants obtained by spectrophotometric, kinetic, and inhibition methods. For *m*-chlorophenyl acetate the comparable values are 5.6 × 10^{-3} *M* (kinetic) and 4.7 × 10^{-3} *M* (spectral), while for 3,5-dimethylphenyl acetate they are 1.5 × 10^{-2} *M* and 1.3 × 10^{-2} *M*, respectively.[31] The spectrophotometric and competitive inhibition methods give the same value for the dissociation constant of the cyclohexaamylose complex of *p*-chlorocinnamate ion.

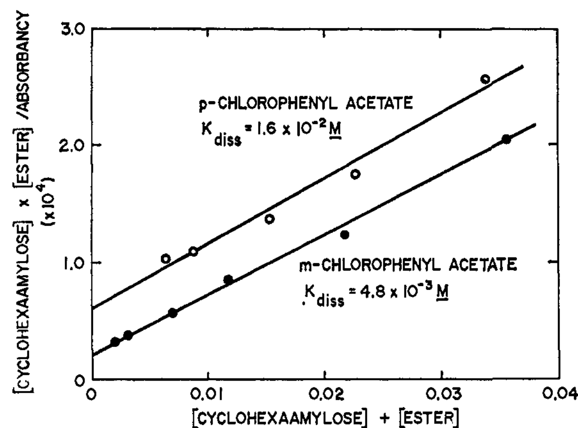


Figure 7. Determination of cyclohexaamylose–substrate dissociation constants by spectrophotometric methods by plotting ([cyclohexaamylose][ester])/absorbance as a function of [cyclohexaamylose] + [ester]; experimental conditions are given in Table VI.

Table VI. Dissociation Constants of Cyclohexaamylose Complexes Determined by Spectrophotometric Methods[a]

Substrate	Wavelength, mμ[b]	K_{diss}, *M*
Phenol	273, 280	5.3 × 10^{-2}
3,5-Dimethylphenol	235, 280	1.6 × 10^{-2}
3,5-Dimethylphenyl acetate	235	1.3 × 10^{-2}
m-Chlorophenyl acetate	230, 235	4.7 × 10^{-3}
p-Chlorophenyl acetate	269, 276	1.6 × 10^{-2}
m-*t*-Butylphenol	230, 282	3.4 × 10^{-2}
p-*t*-Butylphenol	278, 285	1.2 × 10^{-2}
2-Naphthol	290, 335	3.1 × 10^{-2}
p-Chlorocinnamate[c]	290, 301	5.1 × 10^{-3}
Methyl orange[d,e]	510, 520	2.2 × 10^{-4}

[a] Determined using pH 2.2 potassium chloride–hydrochloric acid buffer, I = 0.06 unless otherwise noted. All determinations were made at 25.0 ± 0.1°. [b] Where more than one wavelength is listed the reported dissociation constant is the average of two determinations which agreed to within 10%. [c] Determined using pH 10.05 sodium carbonate buffer, I = 0.2. [d] Determined using pH 6.75, 0.1 *M* sodium and potassium phosphate buffer. At cyclohexaamylose concentrations >5 × 10^{-3} *M*, additional spectral perturbations began to appear suggesting the formation of higher order complexes having a much larger dissociation constant; see also ref 17. [e] Reported 1.12 × 10^{-4} *M:* W. Broser and W. Lautsch. *Z. Naturforsch.*, **8b**, 711 (1953).

Temperature Dependence of Cycloheptaamylose–Substrate Dissociation Constants. The kinetic dissociation constants of some cycloheptaamylose–substrate complexes were determined at temperatures between 15 and 55° and the experimental results are shown in Table VII. In order to calculate the thermodynamic changes accompanying the dissociation process the least-squares slope was computed of the line formed by plotting ln K_{diss} against the reciprocal of the absolute temperature (Figure 8). From the relationship $d[\ln (K_{eq})]/d(1/T) = -\Delta H°/R$ and the assumption that

(31) 2-Naphthol forms a relatively stable complex with cyclohexaamylose (K_{diss} = 3.1 × 10^{-2} *M*) although an azo dye with a 2,8-disubstituted naphthol group apparently does not.[17]

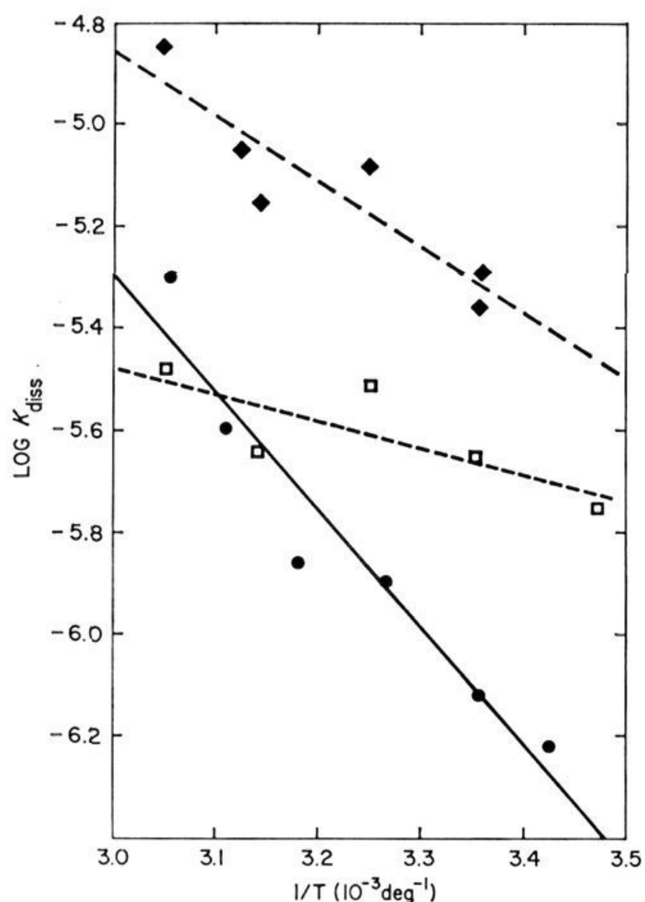


Figure 8. Plot of ln K_{diss} *vs.* the reciprocal of the absolute temperature. The data is that of Table VII for dissociation of cycloheptaamylose–substrate complexes: ●, *m*-ethylphenyl acetate; □, *m*-chlorophenyl acetate; ◆, 3,4,5-trimethylphenyl acetate.

Table VII. Temperature Dependence of Some Cycloheptaamylose–Substrate Dissociation Constants[a]

Ester	Temp, °C	K_{diss},[b] 10^{-3} *M*
m-Chlorophenyl acetate	15.3	3.2 ± 0.7
	25.0	3.5 ± 0.9[c]
	34.8	4.0 ± 0.4
	45.1	3.5 ± 0.5
	55.0	4.3 ± 1.0
3,4,5-Trimethyl-phenyl acetate	25.0	5.0 ± 1.1
	25.1	4.7 ± 0.6
	34.8	6.2 ± 0.4
	45.3	5.8 ± 0.4
	47.1	6.4 ± 1.7
	55.0	7.8 ± 0.3
m-Ethylphenyl acetate	18.8	2.00 ± 0.44
	25.0	2.16 ± 0.36
	32.8	2.74 ± 0.56
	41.1	2.85 ± 0.37
	48.2	3.70 ± 0.53
	54.2	4.97 ± 0.68

[a] At pH 10.01 (25°), $I = 0.2$ sodium carbonate buffer with 0.5% acetonitrile–water unless otherwise noted. [b] Error limits computed from the experimental data by application of Student's t test at the 95% probability level. [c] At pH 10.60 (25°), other conditions as in *a*.

the heat capacities of reactants and products are not significantly different (entirely justified here since the average error in K_{diss} is ~20%) one obtains the enthalpy change $\Delta H° = -R$(slope) and $\Delta S° = R$(intercept). The computed line was used to calculate an averaged value of $\Delta F°_{298}$.

Discussion

The cycloamyloses cause a markedly stereoselective acceleration of the phenol release from substituted phenyl acetates in alkaline solution. Unlike methyl glucoside or glucose, which produces small, uniform rate

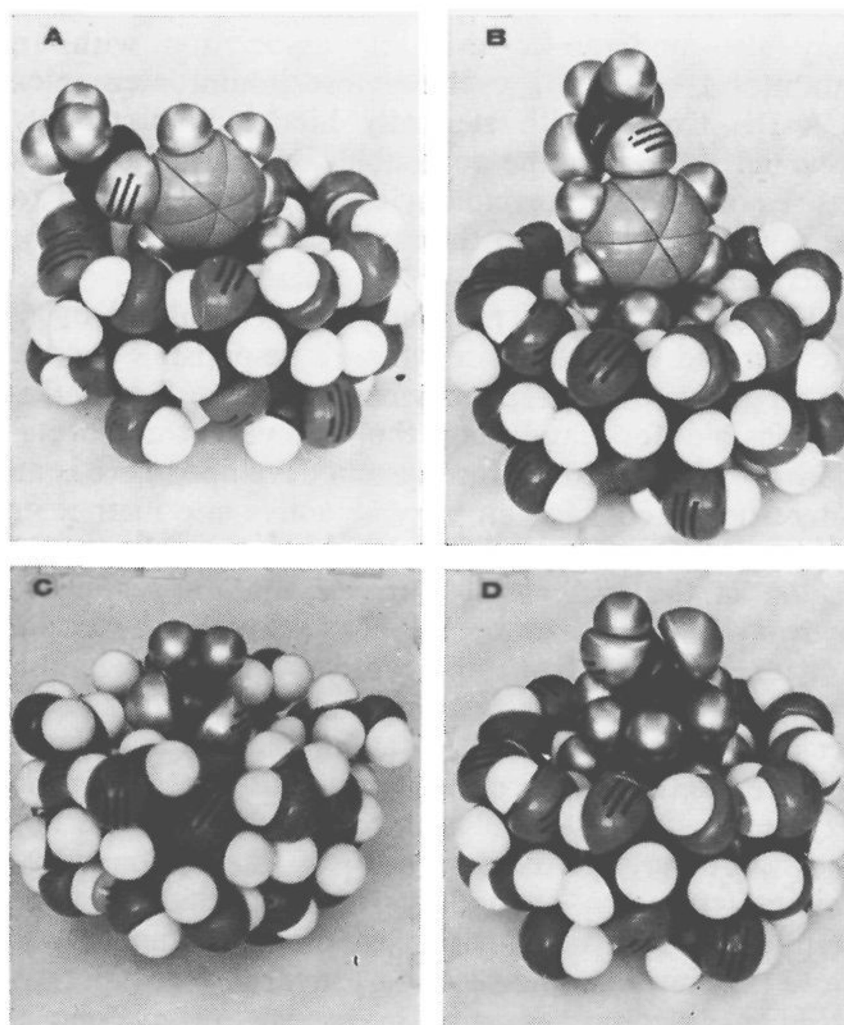
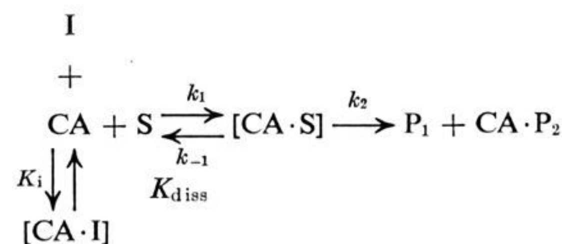


Figure 9. Scale molecular models of cyclohexaamylose complexes: (A) *m-t*-butylphenyl acetate complex with the *t*-butyl group inserted into the cavity from the secondary hydroxyl side; (B) *p-t*-butylphenyl acetate complex constructed as in A; (C) *p-t*-butylphenyl acetate complex constructed by inserting the acetyl group through the cavity until it protrudes from the primary hydroxyl side of the cyclohexaamylose; (D) adamantanecarboxylic acid complex. The oxygen and hydrogen atoms of the guest molecule are coated with metallic paint for greater contrast (the slots in the oxygen atoms are for hydrogen bonds).[35]

effects, both cyclohexaamylose and cycloheptaamylose cause large, nonuniform effects, the rate of phenol release from *meta*-substituted phenyl acetates being greatly enhanced whereas phenol release from the corresponding *para* isomers is only slightly enhanced. The rate effects due to cyclooctaamylose are also large but are much less stereoselective. The stereoselectivity is exerted independently of the stability of the complex and is thus similar to observations made for enzyme-catalyzed reactions (see introductory section). The cycloamylose system also shows spectral changes on binding, competitive inhibition, nonproductive binding, and saturation of the substrate with cycloamylose.[32] A minimal reaction scheme which accommodates these facts is shown below. Here, cycloamylose (CA) and



ester (S) undergo a reversible association to form a cycloamylose–substrate complex [CA·S] which may undergo a reaction with rate constant k_2 to form phenol P_1 and product(s)[33] CA·P_2. The cycloamylose

(32) For an example of the saturation of a substrate with an enzyme see F. J. Kézdy and M. L. Bender, *Biochemistry*, **1**, 1097 (1962).
(33) Accompanying article: R. L. VanEtten, G. A. Clowes, J. F. Sebastian, and M. L. Bender, *J. Am. Chem. Soc.*, **89**, 3253 (1967).

may also undergo a reversible association with an inhibitor I to form a cycloamylose–inhibitor complex [CA·I]. Except with sterically hindered or strongly solvated molecules the reversible association to form a 1:1 complex with cycloamyloses may be expected to be rapid compared[17] to the rates of chemical reactions described here.

The catalytic activity of enzymes is localized in an area termed the active site which is responsible for the binding of substrates, coenzymes, and inhibitors, and is responsible for catalyzing the chemical transformations.[34] The cycloamylose system developed here is an interesting model for an enzyme active site in that its properties are completely explained by considering the region of the cycloamylose cavity and the secondary hydroxyl[33] groups to be the active site of the cycloamylose torus. Most importantly, such a model readily explains the stereoselective rate accelerations. A scale[35] molecular model of a complex of *m-t*-butylphenyl acetate with cyclohexaamylose is shown in Figure 9A. This model was constructed by inserting the hydrophobic *t*-butyl group into the cycloamylose cavity from the secondary hydroxyl side.[36] This results in fixing the position of the ester function in close proximity to the secondary hydroxyl groups. A striking difference is seen in models of cyclohexaamylose complexes of *p-t*-butylphenyl acetate. If the *t*-butyl group is inserted first into the secondary side, then the ester function is located at some distance from the hydroxyl groups (Figure 9B). However, if the acetyl portion is inserted into the cavity from the secondary side until it protrudes from the primary hydroxyl side (which cannot be done for the *meta* isomer), then the aromatic ring and most of the *t*-butyl group are included in the cycloamylose cavity (Figure 9C). The ester function may be partially shielded from nucleophilic attack by hydroxide ion in solution, which would be consistent with the fact that the basic hydrolysis of ethyl *p*-aminobenzoate is inhibited by complexation with cycloheptaamylose.[16] In this model the ester function is located in close proximity to the primary hydroxyl groups. This model together with the unreactivity of the *para* isomer suggests that the interaction which leads to increases in the rate of phenol release involves the secondary hydroxyl groups; this has, in fact, been established.[33]

These models of the cycloamylose–substrate complexes also explain why spectral changes may be observed when some organic substrates form cycloamylose complexes. In cyclohexaamylose complexes, the aromatic chromophore of *m-t*-butylphenyl acetate is not greatly removed from the aqueous environment (Figure 9A); the model is thus consistent with the fact that the spectrum of *m-t*-butylphenol plus cyclohexaamylose in water shows only a small spectral perturbation compared to the spectrum of the phenol in water. However, *p-t*-butylphenol plus cyclohexaamylose shows a marked spectral change resembling the spectrum of the phenol in dioxane solution (Figure 6); this perturbation is consistent with the inclusion of the aromatic chromophore in the ether-like cycloamylose cavity. These arguments indicate that *p-t*-butylphenyl acetate is probably included in the cycloamylose active site with the ester function near the primary hydroxyl groups and the *t*-butyl group near the secondary hydroxyl groups, as shown in Figure 9C.

Differences are observed in the stereoselectivity of cyclooctaamylose and the two smaller amyloses (Table III). These differences are also seen in the values of K_{diss} and k_2 for cyclooctaamylose-accelerated reactions of *t*-butylphenyl acetate as seen in Table VIII. The selectivity ratio (*meta* rate acceleration/*para* rate acceleration) for *t*-butylphenyl acetate hydrolysis in the presence of cyclohexaamylose is 240 (Table IV) while with cyclooctaamylose it is only 1.7 (Table VIII). This is readily explained on consideration of the size of the respective active sites of the amyloses. In sharp contrast to cyclohexaamylose, the cyclooctaamylose cavity is so large that the *t*-butyl group of *para-t*-butylphenyl acetate may be placed in the cavity and the ester function may still be moved freely so as to orient it adjacent to the secondary hydroxyl groups. Since the cyclohexaamylose cavity is the smallest and most restrictive of the three it might be expected to be the most sensitive to the size of *para* substituents which prevent aromatic substrates from tipping in the cavity so as to orient the ester function in proximity to the secondary hydroxyl groups. This is in agreement with the observation that in the hydrolysis of *p*-nitrophenyl acetate, the maximal rate effect due to cyclohexaamylose is 3.4 while the effect due to cycloheptaamylose is 9.1 (Table IV). If there is no substituent on the phenyl ring then the acceleration due to cyclohexaamylose rises to 27 (Table IV). Thus the specificities exhibited by the *para*-substituted esters are negative specificities.

Table VIII. Maximal Rate Accelerations and Dissociation Constants for Cyclooctaamylose Complexes

Acetate substrate[a]	k_2, sec^{-1}	k_2/k_{un}	K_{diss}, *M*
m-t-Butylphenyl	4.7×10^{-2}	87	$9.9 \pm 3.6 \times 10^{-3}$
p-t-Butylphenyl	3.5×10^{-2}	55	$4.0 \pm 0.7 \times 10^{-3}$

[a] For reaction conditions see footnote *a*, Table IV.

The differing rate effects which result from a change in the geometry of the active site have their parallel in enzyme chemistry. Chymotrypsin, trypsin, and subtilisin all catalyze hydrolysis reactions by similar mechanisms and yet they exhibit very different specificities.[37] Changes in the nature of the groups which control binding as well as in the stereochemistry of the active site readily account for differing specificities of enzymes despite seemingly identical mechanisms. Not surprisingly, enzyme-catalyzed reactions may also show

(34) A useful introductory discussion of enzyme chemistry including the active site may be found in H. R. Mahler and E. H. Cordes, "Biological Chemistry," Harper and Row, New York, N. Y., 1966, Chapter 7.

(35) The structure of cyclohexaamylose appears to be reasonably well represented by Corey–Pauling–Koltun scale molecular models [described in W. L. Koltun, *Biopolymers*, 3, 665 (1965)], since the diameter of the cavity as determined by crystallographic measurements[12] varies from 4.7 to 5.1 A (measured between hydrogen atoms on C-3 of the glucose residues) while the scale model yields a distance of 5.3 A.

(36) The secondary hydroxyl groups are on a relatively rigid carbon chain while the primary hydroxyl groups can rotate so as to partially block that part of the cavity. This further accentuates the "V" shaped nature of the cycloamylose cavity in which the open side is surrounded by the secondary hydroxyl groups.

(37) M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, 34, 49 (1965); M. L. Bender and C. G. Miller, unpublished work.

differences in reaction rates and stabilities of the enzyme–substrate complexes depending on the position of substituents on an aromatic substrate.[38,39] The relative inhibitory properties toward cholinesterase of a series of alkyl-substituted phenyl N-methylcarbamates[39] exhibit an interesting parallel with data obtained for the cyclohexaamylose-catalyzed decomposition of phenyl acetates. Table IX illustrates how the seemingly unusual behavior of the enzymic system is paralleled by the cycloamylose system (it is not intended to imply that there is necessarily any actual similarity of the active sites).

Table IX. Parallel Effects of the Inhibition of Cholinesterase[39] and the Decomposition of Phenyl Esters in the Presence of Cyclohexaamylose

Alkyl group	Relative inhibition due to alkylphenyl N-methylcarbamates[a]	k_2/K_{diss} for accelerations by cyclohexaamylose[b]
H	(1)	(1)
o-Methyl	1.4	0.4
p-Methyl	2.0	0.2
m-Methyl	14	3.9
3,5-Dimethyl	33	7.7
m-Ethyl	42	12
m-*t*-Butyl	500	64

[a] The mechanism of inhibiton of cholinesterase by substituted carbamates probably involves acylation of the enzyme [R. D. O'Brien, *Exptl. Med. Surg.*, **23**, 117 (1965)] so that the "affinity" discussed by Metcalf and Fukuto[39] is probably k_2/K_s where k_2 is the acylation rate and K_s is the dissociation constant of the cholinesterase–carbamate complex. [b] Taken from Table IV; see footnote *a*.

For catalysis involving prior inclusion it is reasonable to expect that the limited number of active sites (here equal presumably to the number of cycloamylose molecules) could be saturated or entirely occupied by substrate molecules.[32] This is in accord with the experimental data (*cf.* Figure 2). It is also strikingly illustrated by the fact that for the hydrolysis of *m*-*t*-butylphenyl acetate in the presence of cycloheptaamylose, the ester is almost completely complexed at cycloamylose concentrations greater than $\sim 6 \times 10^{-4}$ *M*, virtually the same hydrolysis rate constant (as measured by phenol release) being obtained even as the cycloamylose concentration is increased to 10^{-2} *M*.

The presence of discrete catalytic sites such as in an inclusion complex also predicts that the addition of otherwise unreactive molecules to the reaction mixture might reduce the rate accelerations caused by cycloamyloses by acting as competitive inhibitors for the active site. This has been quantitatively demonstrated here for the cycloamyloses, added organic molecules reducing the rate acceleration observed for release of phenol from *m*-nitrophenyl acetate (Table V).[40]

Further support for the hypothesis that the active site of the cycloamylose is the region of the cavity and the secondary hydroxyls as well as information about the nature of forces involved in the complex may be obtained from consideration of experiments carried out with carboxyl-substituted phenyl esters. Table IV gives the maximal rate accelerations and dissociation constants determined for the reactions of *m*- and *p*-carboxyphenyl acetates in the presence of cyclohexaamylose. As previously noted, the *meta* isomer shows a large rate acceleration (68-fold) compared to the *para* isomer (fivefold). If the ester function of the *para* isomer is made increasingly hydrophobic by alkyl substitution then it is possible to bring about an inhibition of the hydrolysis reaction (Table X). This must be the result of including the ester function in the cycloamylose cavity. It was anticipated that the hydrophilic carboxylate anion would not be readily included in the cavity as a consequence of its solvation requirements. This latter hypothesis is consistent with the fact that substitution of a *p*-nitro group for the structurally similar carboxylate anion results in a 12-fold increase in the stability of the cyclohexaamylose complex (Table IV). Thus, at least two factors contribute to the formation of nonproductive complexes: steric effects and electrostatic effects.

Table X. Rate Accelerations and Dissociation Constants of Cyclohexaamylose Complexes of Carboxyl-Substituted Phenyl Esters

p-Carboxyphenyl ester	k_2, 10^{-3} sec^{-1}	k_2/k_{un}	K_{diss}, 10^{-3} *M*
Acetate[a]	6.7	5.3	150 ± 90
2-Methylpropionate[a]	0.44	0.68	12 ± 4
3,3-Dimethylbutyrate[b]	0.089	0.19	1.1 ± 0.2

[a] In pH 10.60 sodium carbonate buffer, $I = 0.2$, with 0.5% (v/v) added acetonitrile, $25.0 \pm 0.1°$. [b] In pH 11.22 sodium phosphate, $I = 0.2$, with 0.5% (v/v) added acetonitrile, $25.0 \pm 0.1°$.

Large hydrophobic substituents on guest molecules lead to the formation of relatively stable cycloamylose complexes. In an attempt to gain some understanding of the nature of the forces which lead to complex formation, the temperature dependencies of some cycloheptaamylose–substrate dissociation constants were examined using kinetic methods (Table VII). A plot of ln K_{diss} *vs.* 1/absolute temperature is shown in Figure 8. From the slope and intercept of the least-squares line (see Experimental Section) the thermodynamic quantities associated with the dissociation process were calculated (Table XI). The dissociation constant of the *m*-chlorophenyl acetate–cyclohepta-

Table XI. Thermodynamic Changes for the Dissociation of Cycloheptaamylose Complexes[a]

Substrate	$\Delta F°_{298}$, kcal/mole	$\Delta H°$, kcal/mole	$\Delta S°$, gibbs/mole
m-Chlorophenyl acetate	3.4 ± 1	1 ± 1	-8 ± 3
3,4,5-Trimethylphenyl acetate	3.1 ± 1	2.5 ± 1	-2 ± 3
m-Ethylphenyl acetate	3.6 ± 0.7	4.6 ± 0.7	$+3 \pm 2$

[a] The dissociation constants were determined kinetically (Experimental Section) using 7–10 points; for conditions see the footnotes to Table VII. The error limits given above were obtained by application of Student's t test to the experimental data of Table VII.

(38) *Cf.* R. M. Epand and I. B. Wilson, *J. Biol. Chem.*, **240**, 1104 (1965); H. F. Bundy and C. L. Moore, *Biochemistry*, **5**, 808 (1966).
(39) R. L. Metcalf and T. R. Fukuto, *J. Agr. Food. Chem.*, **13**, 220 (1965).
(40) This was qualitatively demonstrated by Hennrich and Cramer who showed that the presence of phenol in the reaction mixture reduced the acceleration of the alkaline decomposition of diphenyl pyrophosphate.[18]

amylose is not very temperature dependent: the binding is due primarily to a favorable entropy change. However, the binding of *m*-ethylphenyl acetate is due to a favorable enthalpy change.[41] 3,4,5-Trimethylphenyl acetate exhibits intermediate behavior. Discussions and experimental data relating to hydrophobic binding indicate that a favorable entropy change is often involved.[42–44] One reason[45] for the sizable enthalpy change observed in the binding of *m*-ethylphenyl acetate is probably that water molecules in the cavity cannot form their full complement of hydrogen bonds as a result of steric restrictions. The data for *m*-chlorophenyl acetate is difficult to interpret on this basis but may be related to the fact that the aromatic chloro substituent is capable of forming hydrogen bonds[46] with water molecules which would be freed on complexation, leaving only a net entropy effect. The hypothesis that exclusion of water from the region of the cavity is an important factor in binding to the cycloamyloses is consistent with the fact that complexes may be readily formed with guest molecules which are too large to be completely included. Thus, for example, adamantanecarboxylate which cannot enter the cavity of cyclohexaamylose (Figure 9D) still forms a relatively stable complex, K_{diss} being 7×10^{-3} *M* (Table V). The inhibitor molecule is able to interact with a portion of the cyclohexaamylose cavity slightly past the region of the secondary hydroxyl groups. However, when the substrate is able to entirely exclude water from the region of the cavity, quite stable complexes may be formed. Cycloheptaamylose complexes of *m*-*t*-butylphenyl acetate and of adamantanecarboxylate, for example, have dissociation constants of 1×10^{-4} *M* and 7×10^{-4} *M*, respectively. Substrates which fill the cavity completely or have large hydrophobic areas which can interact with the ether-like interior of the cavity form the most stable cycloamylose complexes, so that it would appear reasonable to expect a relationship between the free energy of complex formation and the molecular volume of the guest. In fact, there is an approximate linear relationship between the logarithm of the dissociation constant and the value of the parachor of the guest, the parachor being taken as a measure of molecular volume.[47] Thus, for the cyclohexaamylose dissociation constants reported here (excluding only the carboxyphenyl esters) a correlation coefficient of 0.79 is found for the least-squares-fitted line. As might be expected for the series of structurally similar substrates and inhibitors considered here, nearly identical correlations were obtained with molecular weight and with molar refraction.[48]

(41) Similar observations have been made for the binding of aromatic amino acid derivatives to cycloheptaamylose: M. Laskowski, Jr., and T. L. Warrington, quoted in ref 8.
(42) F. M. Richards, *Ann. Rev., Biochem.*, **32**, 269 (1963).
(43) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1959).
(44) A. Wishnia and T. W. Pinder, *Biochemistry*, **5**, 1534 (1966).
(45) The observed enthalpy change is also consistent with interpretations based on hydrotactic bonding as opposed to hydrophobic bonding: I. M. Klotz, *Federation Proc., Suppl. 15*, S24 (1965).
(46) C. M. Huggins, G. C. Pimentel, and J. N. Shoolery, *J. Phys. Chem.*, **60**, 1311 (1956); E. Krakower and L. W. Reeves, *Trans. Faraday Soc.*, **59**, 2528 (1963).
(47) O. R. Quayle, *Chem. Rev.*, **53**, 439 (1953). Electronic effects on the stabilities of cyclohexaamylose complexes of approximately isosteric *para*-substituted benzoic acids have been demonstrated: B. Casu and L. Rava, *Ric. Sci. Rend.*, [8] **36**, 733 (1966).
(48) S. S. Batsanov, "Refractometry and Chemical Structures," Consultants Bureau, New York, N. Y., 1961.

An alternative explanation for the large enthalpy contribution to the stability of the cycloamylose complexes is, of course, that van der Waal's forces are of primary importance in stabilizing the complex. While dispersion force interactions undoubtedly make a contribution to the apolar binding this explanation is not completely satisfactory because only small portions of some substrate molecules can interact with the curved cycloamylose cavity at a time. The nature of the forces important in the complexes may be revealed by X-ray crystallographic studies.[49]

Conclusion

In connection with his pioneering research in enzyme chemistry Emil Fischer proposed that a complementary molecular configuration must exist between enzymes and their substrates if reaction is to occur, and that this configurational relationship could be likened to that between a lock and key.[50,51] He furthermore predicted that simpler asymmetric systems should be capable of stereospecific effects like those seen in enzymic catalysis.[50] The cycloamylose system developed here represents an excellent model for enzymic catalysis, involving as it does complex formation, stereospecific rate accelerations, competitive inhibition, saturation, and other effects. The model is readily interpreted in terms of the lock and key theory of enzymic catalysis.

Appendix

Determination of Dissociation Constants by Spectrophotometric Methods.[25] Let *A* and *S* be the initial stoichiometric concentrations of the cycloamylose and substrate, having molar extinction coefficients of ϵ_a and ϵ_s, respectively. The perturbation introduced by formation of a complex may be represented by the term ΔAbs, which is related to the total optical absorbancy *Abs* by the relationship $\Delta Abs = Abs - \epsilon_a A - \epsilon_s S$. The equilibrium concentration of the complex *C* (assuming a 1:1 stoichiometry) is given by

$$C = \Delta Abs/\Delta\epsilon \qquad (1)$$

where $\Delta\epsilon = \epsilon_c - \epsilon_a - \epsilon_s$. The formation constant K_f is given by $K_f = C/[(A - C)(S - C)]$ which on expansion becomes $C/K_f = AS - SC - AC + C^2$. Assuming that $AS >> C^2$ this becomes $C/K_f = AS - SC - AC$, or

$$C = AS/(A + S + 1/K_f) \qquad (2)$$

On combining (1) and (2) and making the substitution $K_{diss} = 1/K_f$ the following is obtained

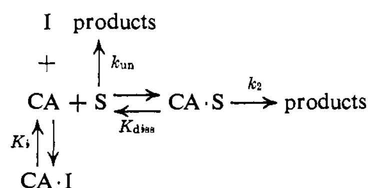
$$AS/(A + S + K_{diss}) = \Delta Abs/\Delta\epsilon$$

$$AS/\Delta Abs = K_{diss}/\Delta\epsilon + (A + S)/\Delta\epsilon$$

Since $\Delta\epsilon$ and K_{diss} are constants this equation represents a straight line when $[A][S]/\Delta Abs$ is plotted against the sum of the stoichiometric concentrations $[A] + [S]$.

(49) An extensive investigation of the crystal and molecular structure of a variety of 1:1 crystalline cycloamylose complexes is in progress: J. A. Hamilton and R. L. VanEtten, unpublished work.
(50) E. Fischer, *Ber.*, **27**, 2985 (1894).
(51) E. Fischer, *Z. Physiol. Chem.*, **26**, 60 (1898).

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

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

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.
(5) Department of Chemistry, Purdue University, Lafayette, Ind. 47907.