Model Catalysts Which Simulate Penicillinase. V. The Cycloheptaamylose-Catalyzed Hydrolysis of Penicillins[1,2]

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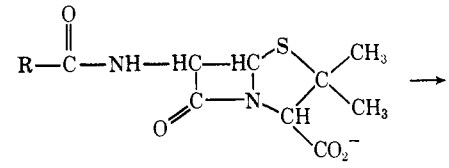
Abstract: Under mildly alkaline conditions β-lactam cleavage of penicillins (I) was catalyzed by cycloheptaamylose (BCD). In the presence of an excess of BCD the rate of cleavage of penicillin β-lactam exhibited Michaelis–Menten-like kinetics, and values were obtained for the apparent dissociation constants ($K_{m_{app}}$) and first-order rate constants for loss of β-lactam (k_2) for the penicillin–BCD intracomplexes. BCD accelerated β-lactam cleavage (20–90-fold) when compared to alkaline hydrolysis under the same conditions. Variation of the penicillin side chain produced considerable specificity in substrate–BCD binding. $K_{m_{app}}$ values were much lower (7.5×10^{-2}–3.85×10^{-3} *M*), corresponding to stronger complexing, than for previously reported models and closer to those for actual penicillin–penicillinase complexes. The presence of a bulky group adjacent to the point of attachment of the side chain to the penicillin nucleus was shown to be important in increasing hydrophobic binding within the BCD cavity. In the presence of either an excess of BCD (apparent first-order loss of β-lactam) or of an initial excess of penicillin (zero-order loss of β-lactam at saturation) the rate of loss of penicillin was faster than the rate of formation of penicilloic acid (**2**) (final product), indicating the formation of an intermediate, which exhibited properties of a penicilloic acid ester (penicilloyl BCD).

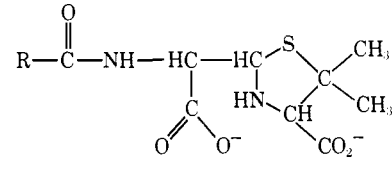
The ability of the cycloamyloses[3-5] (cyclic α-1,4-linked D-glucose polymers containing six, seven, or eight glucose residues per molecule) and their derivatives[6] to catalyze a number of chemical reactions has led to their description as enzyme models. Their catalytic properties have been described[3,4] in terms of the formation of cycloamylose–substrate inclusion complexes within the hydrophobic cavity and subsequent catalysis by either the hydroxy or other appropriate catalytic groups located around the circumference of the cavity. Some degree of stereospecificity has been observed in the cycloamylose-catalyzed hydrolysis of substituted phenyl acetates and benzoates,[3,4] depending on the nature of the fit of the substrate within the cavity and the consequent orientation of the ester carbonyl relative to the catalytic secondary alkoxide ion. The possible importance of specificity of binding in enzyme-catalyzed processes was emphasized recently by Bender and coworkers[7] who reported large differences in the relative rates of intramolecular carboxylate ion catalyzed hydrolysis of mono-*p*-carboxyphenyl esters of 3-substituted glutaric acids in the presence of cycloheptaamylose. In all cases cycloheptaamylose inhibited the reaction and, since cycloheptaamylose was shown to be involved *only* as a binding site, the authors suggested that their results supported "the idea that specificity in enzymic reactions may result from the geometry of binding the substrates to the enzymes, rather than resulting from the catalytic reactions themselves."

These observations suggested to us that the cycloamyloses might serve as simple models[8] for penicillinase (β-lactamase) in the investigation of the mechanism of action of the enzyme. The reaction catalyzed by penicillinase is shown in eq 1. Relatively little is



1a–k

(1)



2a–k

R = side chain (see Table I)

known about either the mechanism of action or nature of the active site of penicillinase.[9] Moreover, the manner in which the β-lactam of resistant penicillins is protected remains undetermined. Although the nature of the side chain is important in determining stability of penicillins toward penicillinases,[10] the apparent bulkiness of the side chain (and hence steric hindrance as such) seldom appears to be related to susceptibility

(1) For a preliminary account of a part of this work, see D. E. Tutt and M. A. Schwartz, *Chem. Commun.*, 113 (1970).

(2) Part IV: R. D. Kinget and M. A. Schwartz, *J. Pharm. Sci.*, **58**, 1102 (1969).

(3) R. L. VanEtten, J. F. Sebastian, G. A. Clowes, and M. L. Bender, *J. Amer. Chem. Soc.*, **89**, 3242 (1967).

(4) R. L. VanEtten, G. A. Clowes, J. F. Sebastian, and M. L. Bender, *ibid.*, **89**, 3253 (1967).

(5) (a) F. Cramer and W. Kampe, *ibid.*, **87**, 1115 (1965); (b) N. Henrich and F. Cramer, *ibid.*, **87**, 1121 (1965); (c) T. Chin, P. Chung, and J. L. Lach, *J. Pharm. Sci.*, **57**, 44 (1968); (d) R. Breslow and P. Campbell, *J. Amer. Chem. Soc.*, **91**, 3085 (1969).

(6) (a) F. Cramer and G. Mackensen, *Angew. Chem., Int. Ed. Engl.*, **5**, 601 (1966); (b) R. Breslow and L. E. Overman, *J. Amer. Chem. Soc.*, **92**, 1075 (1970); (c) F. Cramer and H. Hettler, *Naturwissenschaften*, **24**, 625 (1967).

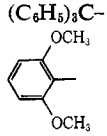
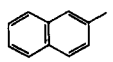
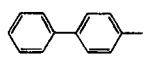
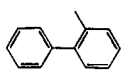
(7) D. L. VanderJagt, F. L. Killian, and M. L. Bender, *J. Amer. Chem. Soc.*, **92**, 1016 (1970).

(8) For a review of other model systems, see M. A. Schwartz, *J. Pharm. Sci.*, **54**, 1308 (1965); M. A. Schwartz and G. R. Pflug, *ibid.*, **56**, 1459 (1967); R. D. Kinget and M. A. Schwartz, *ibid.*, **57**, 1916 (1968); and ref 2.

(9) For a review of the biochemistry and function of penicillinase, see N. Citri and M. R. Pollock, *Advan. Enzymol.*, **28**, 237 (1966).

(10) R. H. Depue, A. G. Moat, and A. Bondi, *Arch. Biochem. Biophys.*, **107**, 374 (1964).

Table I. Variation of the First-Order Rate Constant, k_2, and Apparent Dissociation Constant, $K_{m_{app}}$, of Penicillin–BCD Complexes, and of the Apparent First-Order Rate Constant, k_{hyd}, for Hydrolysis of Penicillins (1) as a Function of Side Chain, R[a]

No.	Penicillin[b] side chain, R	k_2, min^{-1}	k_{hyd},[c] min^{-1}	k_2/k_{hyd}	$10^3 K_{m_{app}}$,[c] M
1a	H_3C-	0.151[d]	0.0041 ± 0.0001	37[d]	33 ± 3[d]
1b	$H_3C(CH_2)_4-$	0.230	0.0035 ± 0.0001	66	41 ± 8
1c	$H_3C(CH_2)_5-$	0.179	0.0038 ± 0.0002	47	21 ± 3
1d	$C_6H_5CH_2-$	0.324	0.0042 ± 0.0001	77	43 ± 4[d]
1e	$(C_6H_5)_2CH-$	0.161[d]	0.0047 ± 0.0002	34[d]	4.7 ± 0.2[d]
1f	$(C_6H_5)_3C-$	0.624[d]	0.016 ± 0.001	40[d]	3.85 ± 0.01[d]
1g		0.074	0.0035 ± 0.0001	21	12.8 ± 0.08
1h		0.109	0.0035 ± 0.0001	31	16 ± 6
1i		0.427	0.0048 ± 0.0002	89	75 ± 19
1j		0.263	0.0049 ± 0.0002	54	38 ± 6
1k		0.200	0.0032 ± 0.0002	63	13.2 ± 0.1

[a] At pH 10.24, 31.5°, μ = 1.0 M in water. [b] See eq 1 for the structure of penicillins. [c] The precision of these measurements is expressed as ± standard deviation of the mean. [d] These data, which differ slightly from previously published values,[1] have been corrected as a result of recalculation of the purity of BCD based on optical rotation measurements.

to hydrolysis.[11] Doyle and coworkers[12] investigated a series of penicillins prepared from 6-aminopenicillanic acid and 2,6-disubstituted aromatic or heteroaromatic carboxylic acid chlorides. Almost any pair of 2,6 substituents conferred stability to penicillinase, and the authors concluded that steric effects were of primary importance in determining stability to penicillinase. However, the lack of correlation between the apparent "bulkiness" of the side chain and the K_m and V_{max} values of penicillins led Citri and Pollock[11] to rule out steric hindrance as the major factor in reducing the susceptibility of penicillins to penicillinase-catalyzed hydrolysis. Citri and Garber[13] interpreted the steric effect in terms of the binding of penicillin at the active site, in which steric interaction of the side chain with the *flexible* active site determines the conformation, and hence catalytic activity, of the active site.

We have investigated the effect of variation of the side chain of penicillins (**1a–k**, Table I) in their hydrolysis catalyzed by cycloheptaamylose (BCD),[14] in which the latter acts as a simple model for penicillinase.

Results

Alkaline Hydrolysis of Penicillins at pH 10.24. The hydrolysis of penicillins, **1**, was measured under conditions similar to those employed in the BCD-catalyzed reactions, but in the absence of BCD. The rate of loss of β-lactam under the reaction conditions was relatively slow, $t_{1/2} \sim 1$ hr, and, consequently, alkaline hydrolysis was measured only to about 30% completion. The apparent first-order rate constants (k_{hyd}) were calculated from the slopes of plots of the logarithm of residual penicillin concentration as a function of time, assuming[16] zero absorbance for the penicillin assay solution of an aliquot of reaction mixture taken at t_∞. The results are shown in Table I.

Hydrolysis of Penicillins in the Presence of an Excess of BCD. The rate of β-lactam cleavage of penicillins, **1**, in the presence of an excess of BCD (0.0040–0.021 M) was measured under mildly alkaline conditions (see Experimental Section). The loss of penicillin obeyed an apparent first-order rate law [often to 93% loss of penicillin; *e.g.*, triphenylmethylpenicillin (**1f**), 4.0×10^{-3} M BCD; Figure 1]. In contrast, the rate of formation of final product, penicilloic acid (**2f**), obeyed no simple rate law, and was always *slower* than the rate of loss of penicillin. The acid liberated in the *overall* reaction corresponded to 100% conversion of penicillin to penicilloic acid, which was determined by (a) total sodium hydroxide titrant added by the pH-stat and (b) a penicilloic acid specific assay[17] on the reaction mixture at t_∞. These results suggested the presence of an intermediate in the reaction pathway. The intermediate produced ultraviolet spectral changes on treatment with mercuric chloride at neutral pH characteristic of a penicilloic acid ester,[17] and was assumed to be a penicilloyl cycloamylose.

The variation of the apparent first-order rate constants (k_{obsd}) for loss of penicillin in the presence of an *excess* of BCD as a function of BCD concentration revealed a complex dependence of k_{obsd} on (BCD) [*e.g.*, triphenylmethylpenicillin (**1f**): Figure 2]. The

(11) See p 261 of ref 9.
(12) F. P. Doyle, A. A. W. Long, J. H. C. Nayler, and E. R. Stove, *Nature (London)*, **192**, 1183 (1961).
(13) N. Citri and N. Garber, *J. Pharm. Pharmacol.*, **14**, 784 (1962).
(14) Cycloheptaamylose, which contains seven glucose units per molecule[15] and has a cavity diameter of 7–8 Å,[5a] has also been referred to as β-Schardinger dextrin and β-cyclodextrin.[15] BCD (from β-cyclodextrin) will be used as an abbreviation to denote cycloheptaamylose.
(15) D. French, *Advan. Carbohyd. Chem.*, **12**, 189 (1957).
(16) M. W. Brandriss, E. L. Denny, M. A. Huber, and H. G. Steinman, "Antimicrobial Agents and Chemotherapy—1962," American Society for Microbiology, Ann Arbor, Mich., p 626.
(17) (a) C. H. Schneider and A. L. deWeck, *Helv. Chim. Acta*, **49**, 1689 (1966); (b) M. A. Schwartz and A. J. Delduce, *J. Pharm. Sci.*, **58**, 1137 (1969).

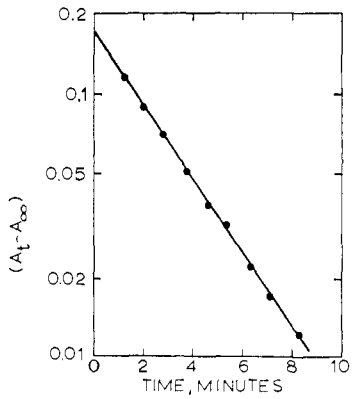


Figure 1. Apparent first-order loss of β-lactam for triphenylmethylpenicillin (**1f**; 3.0×10^{-4} *M*) in the presence of an excess of BCD (4.0×10^{-3} *M*) at pH 10.24, μ = 1.0 *M*, 31.5° in water to at least 93% loss of penicillin.

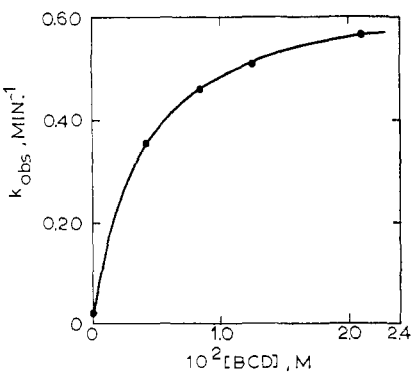


Figure 2. Variation of the apparent first-order rate constant (k_{obsd}) for cleavage of the β-lactam of triphenylmethylpenicillin (**1f**) as a function of the concentration of an excess of BCD under mildly alkaline conditions (see Experimental Section).

circles in Figure 2 represent the experimental results and the line was calculated using eq 3 and the appropriate data in Table I. Plots[18,19] of ($k_{obsd} - k_{hyd}$) *vs.* ($k_{obsd} - k_{hyd}$)/(BCD) for each penicillin afforded straight lines [*e.g.*, benzylpenicillin (**1d**) and diphenylmethylpenicillin (**1e**); Figure 3] demonstrating the formation of a penicillin–BCD intracomplex and adherence to Michaelis–Menten-like kinetics.[20] Straight line fits to plots of ($k_{obsd} - k_{hyd}$) *vs.* ($k_{obsd} - k_{hyd}$)/[BCD] were shown to be very significant (p = 0.05) using the method of least squares for two dependent variables.[21] In the presence of an *excess* of BCD the rate expression and k_{obsd} for *loss* of penicillin are given by eq 2 and 3, respectively, where P = penicillin,

$$\text{rate} = \frac{k_2(\text{BCD})}{(\text{BCD}) + K_{m_{app}}}(\text{P}) + k_{hyd}(\text{P}) \qquad (2)$$

$$k_{obsd} = \frac{k_2(\text{BCD})}{(\text{BCD}) + K_{m_{app}}} + k_{hyd} \qquad (3)$$

$$K_{m_{app}} = \frac{(\text{P})(\text{BCD})}{(\text{P---BCD})} \qquad (4)$$

PA = penicilloic acid, P---BCD = inclusion complex, P—BCD = covalent intermediate (penicilloyl–BCD), k_2 is the first-order rate constant for β-lactam cleavage of complexed penicillin, and $K_{m_{app}}$ is the apparent dissociation constant of the penicillin–BCD complex (eq 4). The data are shown in Table I. A mechanism, which would account for the observed results, is given by eq 5 and 6. It should be emphasized that, for such a mechanism, the rate of loss of substrate (P) in the presence of an *excess* of catalyst (BCD) would obey an apparent first-order rate law given by eq 3. The ratios k_2/k_{hyd} for each penicillin, which

$$\text{P} + \text{BCD} \underset{K_{m_{app}}}{\rightleftharpoons} \text{P---BCD} \xrightarrow{k_2} \text{P—BCD} \xrightarrow{k_3} \text{PA} + \text{BCD} \qquad (5)$$

$$\text{P} + \text{OH}^- \xrightarrow{k_{hyd}} \text{PA} \qquad (6)$$

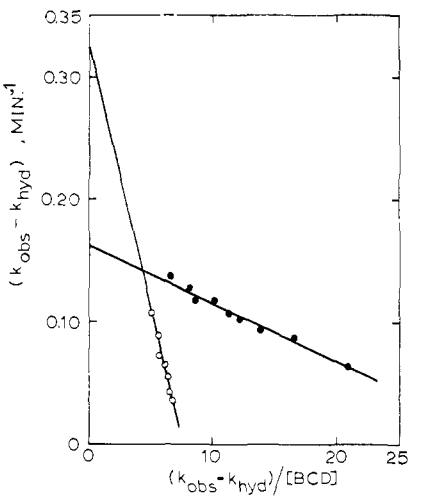


Figure 3. Linear plots of ($k_{obsd} - k_{hyd}$) *vs.* ($k_{obsd} - k_{hyd}$)/(BCD) for benzylpenicillin (**1d**, open circles) and diphenylmethylpenicillin (**1e**, full circles) for loss of β-lactam in the presence of an excess of BCD, demonstrating adherence to Michaelis–Menten-like kinetics.

have been used as a measure of the relative catalytic activity of BCD for the various penicillin–BCD complexes, are also shown in Table I. Such a comparison would incorporate any inherent differences in the susceptibility of the β-lactam carbonyl groups to nucleophilic attack.

Hydrolysis of Penicillin Catalyzed by BCD in the Presence of an Initial Excess of Penicillin. Both the rates of loss of penicillin and formation of penicilloic acid were measured in the presence of BCD and of an initial *excess* of penicillin under the conditions used previously [*e.g.*, 0.021 *M* BCD, and 0.080 *M* benzylpenicillin (**1d**)]. The rate of loss of penicillin was again faster than the rate of formation of penicilloic acid, although no simple rate law was obeyed by *either* process. Careful measurement of the rate of formation of penicilloic acid as a function of time revealed the presence of an initial induction period[22] during the first 5 min of the reaction prior to the maximal rate of formation of penicilloic acid. This observation is consistent with previous results demonstrating the presence of an intermediate (penicilloyl BCD) in the re-

(18) G. S. Eadie, *J. Biol. Chem.*, **146**, 85 (1942).

(19) Dowd and Riggs have demonstrated the so-called Eadie plot to be statistically preferable when the magnitude of the error to which ($k_{obsd} - k_{hyd}$) is subject is not known: J. E. Dowd and D. S. Riggs, *J. Biol. Chem.*, **240**, 863 (1965).

(20) M. Dixon and E. C. Webb, "Enzymes," 2nd ed, Academic Press, New York, N. Y., 1964, p 63.

(21) J. Topping, "Errors of Observation and Their Treatment," 3rd ed, Reinhold, New York, N. Y., 1962, p 104.

(22) K. J. Laidler, "Reaction Kinetics," Vol. 1, Pergamon Press, Oxford, England, 1963, p 24.

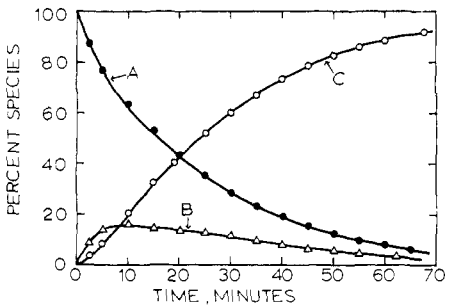


Figure 4. Variation of the concentration of benzylpenicillin (**1d**; curve A, full circles) and benzylpenicilloic acid (**2d**; curve C, open circles), expressed as per cent initial penicillin concentration, as a function of time in the presence of an *initial* excess of penicillin (0.021 *M* BCD and 0.080 *M* penicillin) under the reaction conditions (see Experimental Section). Curve B was obtained using eq 7, and corresponds to the variation of intermediate (penicilloyl BCD) concentration, expressed as per cent initial penicillin concentration, as a function of time.

action pathway. The variations of benzylpenicillin and benzylpenicilloic acid concentration (expressed as per cent initial or final concentration, respectively) as a function of time are shown in Figure 4. Curve A corresponds to per cent penicillin (% P) and curve C corresponds to per cent penicilloic acid (% PA) as a function of time, both determined experimentally. Curve B was obtained using eq 7 and corresponds to the calculated concentration of intermediate (P–BCD) as a function of time, in which (P–BCD) is expressed as per cent initial penicillin (or final penicilloic acid) concentration. Thus, it was calculated that, using

$$(\text{P–BCD}) = 100 - (\%\ \text{P}) - (\%\ \text{PA}) \qquad (7)$$

initial concentrations of 0.080 *M* benzylpenicillin (**1d**) and 0.021 *M* BCD under the reaction conditions, after 10 min the maximum concentration of penicilloyl BCD was 0.0135 *M* corresponding to 64% BCD[23] covalently bound to penicillin (Figure 4).

Hydrolysis of Penicillin in the Presence of an Excess of Methyl α-D-Glucopyranoside. The hydrolysis of benzylpenicillin (**1d**) in the presence of an *excess* of methyl α-D-glucopyranoside was investigated to determine whether the rate enhancements observed for penicillin loss in the presence of an excess of BCD should be attributed to the structural[15] and stereochemical[24] properties of BCD or merely to the presence of a high concentration of glucoside. The apparent first-order rate constant for loss of penicillin in the presence of 0.070 *M* methyl α-D-glucopyranoside was only threefold larger than that for alkaline hydrolysis under comparable conditions. A plot of the apparent first-order rate constants for loss of penicillin as a function of methyl α-D-glucopyranoside concentration (0–0.14 *M*) revealed a linear relationship [shown statistically to be highly significant ($p = 0.05$)]. These results demonstrate that methyl α-D-glucopyranoside is a poor catalyst (relative to BCD) for the hydrolysis of penicillins and that the overall second-order reaction does not involve any significant penicillin–methyl α-D-glucopyranoside complex formation within the concentration range studied.

(23) See Table I, footnote *d*.

(24) B. Casu, M. Reggiani, G. G. Gallo, and A. Vigevani, *Tetrahedron*, 22, 3061 (1966), and references therein.

Discussion

Under mildly alkaline conditions and in the presence of an *excess* of cycloheptaamylose (BCD) (3.0×10^{-4} *M* penicillin at zero time, 0.0040–0.021 *M* BCD, pH 10.24, $\mu = 1.0$ *M*, 31.5°, solvent water) the rate of β-lactam cleavage of penicillins (**1**) was accelerated (20–90-fold) when compared (k_2/k_{hyd}) to alkaline hydrolysis under the same conditions. In contrast, treatment of penicillin with an *excess* of methyl α-D-glucopyranoside (0.070 *M*) under comparable conditions produced only a twofold increase in the rate of β-lactam cleavage when compared to alkaline hydrolysis. Adherence to Michaelis–Menten-like kinetics for *loss* of penicillin in the presence of an *excess* of BCD demonstrated the initial formation of a penicillin–BCD complex in the reaction pathway eq 5, and variation of the penicillin side chain revealed considerable specificity of substrate–BCD binding. $K_{m_{app}}$ (7.5×10^{-2}–3.85×10^{-3} *M*) was much lower (corresponding to stronger complexing) than for previously reported penicillinase models[8] and closer to values reported for actual penicillinase–penicillin complexes.[9] The absence of any significant complex formation between penicillin and methyl α-D-glucopyranoside was demonstrated by a *linear* dependence of the observed apparent first-order rate constants for loss of penicillin as a function of methyl α-D-glucopyranoside concentration (0–0.14 *M*, corresponding to the equivalent concentration range[25] 0–0.02 *M* BCD). Thus, the formation of penicillin–BCD inclusion complexes and rate accelerations observed for loss of penicillin in the presence of BCD may be attributed to the unusual properties[24] of BCD.

The presence of a covalent intermediate (penicilloyl BCD) in the reaction pathway was demonstrated by (a) the observation that, in the presence of either an excess of BCD or of an initial excess of penicillin, the rate of loss of penicillin was always faster than the rate of formation of final products, penicilloic acid, (b) the existence of an initial induction period[22] in the rate of formation of penicilloic acid in the presence of an initial excess of penicillin, and (c) an assay[17] which is specific for penicilloyl derivatives (amides and esters) and penicilloic acid, and able to distinguish between them.

The mechanism given by eq 5 for the BCD-catalyzed hydrolysis of penicillins is similar to mechanisms proposed[3,4,7] for other BCD-catalyzed processes in which BCD acts as a simple enzyme model, and BCD may now be described as a simple model for penicillinase. We believe this to be the first model for penicillinase which exhibits both strong and specific binding of the penicillin side chain. Moleuclar models of penicillin–BCD complexes, in which the side chain (R) is included within the cycloamylose cavity, show the β-lactam to be quite flexible (the carbonyl carbon of the β-lactam being four atoms removed from the side chain) but accessible to the hydroxy groups. In this situation orientation of the labile group in the complex (and hence catalytic specificity as measured by k_2/k_{hyd}) would be affected minimally (four- to fivefold differences observed) by stereochemical requirements of hydrophobic bonding of the side chain. For example, variation of the bulkiness of the side chain

(25) Based on the molar concentration of glucose units in BCD.

in the series benzyl-, diphenylmethyl-, and triphenylmethylpenicillin (**1d**, **1e**, and **1f**, respectively) produced only a twofold decrease in the maximal rate acceleration (k_2/k_{hyd}) for β-lactam cleavage of BCD-complexed penicillin. In contrast, substrate–BCD complexation *increased* elevenfold in the same series (Table I). However, the greatest increase in binding (over ninefold) was observed for the addition of a second phenyl group to the side chain (from benzylpenicillin to diphenylmethylpenicillin, **1d** to **1e**). Addition of a third phenyl group (triphenylmethylpenicillin, **1f**) produced only a further 1.2-fold increase in binding. The use of molecular models shows that the side chain of benzylpenicillin (**1d**) may pass unhindered *through* the cavity until the phenyl ring is partly outside the hydrophobic region.

In contrast, the second phenyl group in diphenylmethylpenicillin (**1e**) provides steric hindrance, which prevents the side chain from passing through the cavity, *fixes* the other phenyl group within the BCD cavity, and thus increases hydrophobic binding. A further phenyl group in the penicillin side chain (triphenylmethylpenicillin, **1f**) would not be anticipated to contribute substantially to this effect. Furthermore, comparison of $K_{m_{app}}$ values for 1- and 2-naphthylpenicillins (**1h** and **1i**, respectively) and for 2- and 4-biphenylpenicillins (**1k** and **1j**, respectively) reveals a similar effect in which penicillins containing bulky ortho-substituted side chains (**1h** and **1k**) exhibit greater binding (about threefold) than the corresponding para-substituted side chains (**1i** and **1j**) (see Table I). These observations may be relevant to the interpretation of steric effects in the penicillinase-catalyzed hydrolysis of penicillins.[9] Data for *both* bacillary and staphylococcal penicillinase-catalyzed hydrolysis of methicillin (2,6-dimethoxyphenyl side chain) and benzylpenicillin (benzyl side chain) show the maximal rate of hydrolysis of methicillin, a penicillinase-resistant penicillin, to be much less than for benzylpenicillin by a factor of 10^2–10^4. The corresponding apparent dissociation constants for the enzyme–substrate complexes indicate that methicillin binds stronger than benzylpenicillin to bacillary penicillinases, but weaker to staphylococcal penicillinases.[9] In this respect also BCD simulates the enzyme, since the maximal rate acceleration for methicillin (**1g**, Table I: $k_2/k_{hyd} = 21$) was the *lowest* observed in the series of penicillins studied. However, the maximal rate acceleration for ancillin (**1k**, Table I: $k_2/k_{hyd} = 63$) was larger than would be expected since ancillin is also resistant to penicillinases.

We conclude that BCD, which simulates the action of certain hydrolytic enzymes,[3,4,6c] behaves as a simple model for penicillinase in that BCD accelerates β-lactam cleavage of penicillins (as compared to alkaline hydrolysis under the same conditions) and exhibits specificity of penicillin side chain complexation. This model, which incorporates minimal features of the active site of penicillinase, will serve as a basis for further studies using suitably constituted derivatives.

Experimental Section

Materials. Methylpenicillin sodium salt (**1a**),[2] diphenylmethylpenicillin potassium salt (**1e**),[2] and *n*-pentylpenicillin potassium salt (**1b**)[26] were prepared according to the literature. 6-Aminopenicillanic acid, benzylpenicillin potassium salt (**1d**), and methicillin sodium salt (**1g**) were provided by Bristol Laboratories and ancillin sodium salt (**1k**) was provided by Smith Kline and French Laboratories. These penicillins were used without further purification. Acid chlorides were commercially available, except triphenylacetyl chloride[27] and 4-phenylbenzoyl chloride,[28] which were prepared from the corresponding acid and thionyl chloride according to the literature. Cycloheptaamylose (BCD) was kindly supplied by Corn Products Co., and its purity determined by optical rotation measurements in aqueous solution (lit.[15] $[\alpha]^{20}D$ +162°). Commercially available methyl α-D-glucopyranoside was used without further purification; 30% solutions in *n*-butyl alcohol of sodium 2-ethylhexanoate and potassium 2-ethylhexanoate were prepared according to the literature.[29]

Triphenylmethylpenicillin Sodium Salt (1f). A solution of triphenylacetyl chloride (9.2 g; 0.030 mol) in anhydrous acetone (180 ml) was added dropwise with stirring to a solution of 6-aminopenicillanic acid (5.4 g; 0.025 mol) in 250 ml of 3% aqueous sodium bicarbonate and 75 ml of acetone at room temperature. Stirring was continued for a further 3 hr at room temperature. The reaction mixture was washed with diethyl ether (600 ml) in two portions, and the ether was discarded. The aqueous solution was filtered and allowed to crystallize at −10°. The crystalline solid, which was removed by centrifugation, was recrystallized from 20% acetone–water at −10°: yield 4.8 g (38%); mp 114–116.5° (lit.[30] 110–120°). The infrared spectrum showed characteristic absorption bands at 1770 (β-lactam C=O), 1660 (secondary amide C=O), and 1600 cm^{-1} (carboxylate C=O).

4-Phenylphenylpenicillin Sodium Salt (1j). A suspension of 4-phenylbenzoyl chloride (2.70 g; 0.0125 mol) in anhydrous acetone (60 ml) was added dropwise with stirring to a solution of 6-aminopenicillanic acid (4.32 g; 0.020 mol) in 100 ml of 3% aqueous sodium bicarbonate solution and 40 ml of acetone at 5°. Stirring was continued for a further 2 hr at room temperature. The reaction mixture was cooled to 5° and washed with diethyl ether (300 ml) in two portions, and the ether discarded. The aqueous layer was covered with ether (250 ml) and acidified to pH 2 with 3 *N* aqueous phosphoric acid. The mixture was shaken, the ether layer removed, and the aqueous layer extracted with further ether (360 ml) in two portions. The ethereal extracts were combined, washed twice with cold water (200 ml), dried (Na_2SO_4), diluted to 1000 ml with anhydrous ether, and cooled to 5°. A 30% solution (10 ml) of sodium 2-ethylhexanoate (0.013 mol) in *n*-butyl alcohol was added dropwise with stirring to produce a white precipitate which was filtered, washed with ether, and dried before recrystallization from *n*-butyl alcohol–water: yield 4.45 g (81%); mp 219–221° dec. The infrared spectrum showed characteristic bands at 1775 (β-lactam C=O), 1659 (secondary amide C=O), and 1600 cm^{-1} (carboxylate C=O). The presence of 1.00 ± 0.04 molecules of water of crystallization was determined using the method of Karl Fischer.[31] *Anal.* Calcd for $C_{21}H_{19}N_2O_4SNa \cdot H_2O$: C, 57.77; H, 4.85; N, 6.42. Found: C, 58.96; H, 4.73; N, 6.34.

***n*-Nonylpenicillin Potassium Salt (1e).** A solution of *n*-decanoyl chloride (11.5 g, 0.060 mol) in anhydrous acetone (180 ml) was added dropwise with stirring to a solution of 6-aminopenicillanic acid (13.0 g, 0.060 mol) in 300 ml of 3% aqueous sodium bicarbonate solution and 120 ml of acetone at 5°. Stirring was continued for a further 3 hr at 5°. The reaction mixture was washed with diethyl ether (1000 ml) in two portions, and the ether was discarded. The aqueous layer was extracted with more ether (1000 ml) in two portions, and the ethereal solutions were combined, washed twice with cold water (600 ml), dried (Na_2SO_4), diluted to 3600 ml with further anhydrous ether, and cooled to 5°. A 30% aqueous solution (16 ml) of potassium 2-ethylhexanoate (0.060 mol) in *n*-butyl alcohol was added dropwise with stirring to yield a white precipitate which was filtered, washed with ether, dried, and recrystallized from *n*-butyl alcohol–water: yield 5.7 g (23%); mp 188–190° dec. The infrared spectrum contained characteristic bands at 1790 (β-lactam

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C=O), 1670 (secondary amide C=O), and 1600 cm^{-1} (carboxylate C=O). The absence of water of crystallization was demonstrated by the Karl Fischer method. *Anal.* Calcd for $C_{18}H_{20}N_2O_4SK$: C, 52.90; H, 7.16; N, 6.86. Found: C, 51.90; H, 7.27; N, 6.59.

1-Naphthylpenicillin Potassium Salt (1h). A solution of 1-naphthoyl chloride (5.73 g; 0.030 mol) in anhydrous acetone (90 ml) was added dropwise with stirring to a solution of 6-aminopenicillanic acid (6.48 g, 0.030 mol) in 150 ml of 3% aqueous sodium bicarbonate solution and 60 ml of acetone at room temperature, and allowed to stand for a further 2 hr at room temperature. The solution was cooled to 5° and washed with diethyl ether (500 ml) in two portions, and the ether was discarded. The aqueous layer was covered with ether (300 ml), acidified to pH 2 with 3 *N* aqueous phosphoric acid, and shaken, and the ether layer was removed. The aqueous solution was treated with more ether (500 ml) in two portions, and the ether solutions were combined, washed twice with water (300 ml), dried (Na_2SO_4), diluted to 1500 ml with anhydrous ether, and cooled to 5°. A 30% solution (18.6 ml) of potassium 2-ethylhexanoate (0.030 mol) in *n*-butyl alcohol was added dropwise with stirring to the ether solution to yield a white precipitate, which was filtered, washed with ether, dried, and recrystallized from *n*-butyl alcohol–water: yield 8.3 g (65%); mp 182–186° dec. The infrared spectrum exhibited characteristic band wt 1785 (β-lactam C=O), 1660 (secondary amide C=O), and 1600 cm^{-1} (carboxylate C=O). Karl Fischer analysis indicated the presence of 1.12 ± 0.03 molecules of water of crystallization per molecule of penicillin. *Anal.* Calcd for $C_{19}H_{17}N_2O_4SK \cdot H_2O$: C, 53.51; H, 4.49; N, 6.57. Found: C, 52.18; H, 4.20; N, 6.27.

2-Naphthylpenicillin Potassium Salt (1i). 2-Naphthylpenicillin potassium salt was prepared by treatment of a solution of 6-aminopenicillanic acid (2.16 g; 0.010 mol) in 50 ml of 3% aqueous sodium bicarbonate solution and 20 ml of acetone with a solution of 2-naphthoyl chloride (1.91 g; 0.010 mol) in a similar manner to that used for 1-naphthylpenicillin potassium salt: yield 1.7 g (40%); mp 199–201° dec. The infrared spectrum showed characteristic bands at 1768 (β-lactam C=O), 1646 (secondary amide C=O), and 1600 cm^{-1} (carboxylate C=O). The presence of 0.87 ± 0.01 molecule of water of crystallization per molecule of penicillin was determined by the Karl Fischer method. *Anal.* Calcd for $C_{19}H_{17}N_2O_4SK \cdot H_2O$: C, 53.51; H, 4.49; N, 6.57. Found: C, 51.49; H, 4.17; N, 6.19.

Solvents. Water, which was used as solvent for kinetic runs and some penicillin assays, was deionized and distilled under nitrogen in all-glass apparatus. Ethanol, *n*-butyl alcohol, and *n*-propyl alcohol were used without further purification.

Apparatus. A Radiometer pH-stat system (TTT-1c pH-stat, SBU 1c syringe buret fitted with a jacketed reaction cell, and SBR 2c titrigraph) was used for (a) maintenance of pH in the kinetic reaction solution and (b) measurement of the rate of formation of penicilloic acid as a function of time (an alternate method to the penmaldate assay for penicilloic acid). Constant temperature was maintained for kinetic runs (31.5 ± 0.1°) and for certain assay procedures (40.0 ± 0.1°; see Table II) using Bronwill circulating water pumps. Ultraviolet spectroscopic measurements were made using a Hitachi Perkin-Elmer 139 spectrophotometer (residual penicillin assay), a Beckman-Gilford recording quartz spectrophotometer (for the penamaldate assay), and a Beckman DB-G double-beam spectrophotometer fitted with a Sargent SR recorder (for λ_{max} determination in assays). Stoppered silica cells were used for all ultraviolet measurements. Infrared spectra of penicillins were measured as KBr disks using a Beckman IR 8 double-beam spectrophotometer. Water of crystallization in the penicillin salts was determined by the method of Karl Fischer[31] using a Luft A-KF titration assembly and Luft Model 77 controller. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. An Olivetti Programma 101 was used for calculation of rate constants, statistical significance of data, and regression lines.

Kinetic Procedure. All kinetic experiments were at pH 10.24 and 31.5° in water under an atmosphere of nitrogen, and at a calculated ionic strength of 1.0 *M* (with KCl). pH was maintained using a Radiometer TTT-1 pH-stat. The rates of loss of penicillin and formation of products were measured (a) in the absence of BCD (k_{hyd}), (b) in the presence of an excess of BCD (k_{obsd}) (*e.g.*, 3.0×10^{-4} *M* penicillin at zero time, 0.040–0.021 *M* BCD), (c) in the presence of an initial excess of penicillin (*e.g.*, 0.021 *M* BCD and 0.080*M* penicillin at zero time), and (d) in the presence of an excess of methyl α-D-glucopyranoside (0.070 and 0.14 *M*) to determine the effect of glucoside concentration on the rate of loss of penicillin (3.0×10^{-4} *M* penicillin at zero time).

In a given run an aliquot portion of the appropriate penicillin solution was mixed with an aliquot portion of the proper salt–sugar (BCD or methyl α-D-glucopyranoside) solution preequilibrated at 31.5° and pH 10.24. The time of mixing was reckoned as zero. Aliquot portions of the reaction mixture were removed at various time intervals and assayed for residual penicillin (see "Assay for Residual Penicillin" in this section) or for penicilloyl derivatives (see "Assay for Penicilloyl Derivatives. Penmaldate Assay" in this section). The rate of alkaline hydrolysis of the penicillins was measured to at least 30% completion using the assay for residual penicillin.[16] In the calculation of rate constants (k_{obsd}) for *loss* of penicillin in the presence of an *excess* of BCD the method of Guggenheim[32] was used. Straight-line fits to plots of ($k_{obsd} - k_{hyd}$) *vs.* ($k_{obsd} - k_{hyd}$)/(BCD) (*e.g.*, Figure 3) were shown to be statistically significant at the 95% confidence limits. The best straight-line fits to the data were obtained using the method of inter)-class correlation,[21] where both variables are distributed normally, and values of k_2 (intercept) and $K_{m_{app}}$ (slope = $-K_{m_{app}}$) were obtained (see eq 3). Values of k_2 and $K_{m_{app}}$ thus calculated (Table I) were used to obtain the theoretical curves for the variation of k_{obsd} as a function of *excess* BCD concentration (*e.g.*, solid line in Figure 2).

Assay for Residual Penicillin. In the determination of penicillin remaining as a function of time, aliquot portions of the reaction solution were assayed[16] by conversion of the penicillin to penicillenic acid (chromophore, λ_{max} 322–365 nm) in acid solution (pH 2.0–2.9) containing mercuric chloride (8.0×10^{-3} *M*). The particular conditions used for each penicillin assay, which depended on the relative stabilities of penicillin and penicillenic acid in acid solution (pH and temperature), and on the solubility of the penicillenic acid (solvent), are shown in Table II. Glycine–hydrochloric acid (0.2 *M*) was used as buffer for pH 2.2–2.9 and hydrochloric acid was used for pH 2.0. The variation of absorbance at t_{max} (corresponding to maximum penicillenic acid concentration) as a function of *initial* penicillin concentration was shown to obey Beer's law for all penicillenic acids studied spectrophotometrically.

Table II. Conditions Employed in the Assay for Residual Penicillin

Penicillin[a]	pH[b]	t_{max},[b] min	Temp, °C	Wavelength, nm	Solvent[c]
1a	2.9	70	25	323	1
1b	2.9	70	25	325	1
1c	2.9	80	25	325	2
1d	2.7	100	25	325	1
1e	2.0	100	25	325	1
1f	2.0	350	25	328	2
1g	2.2	80	25	363	3
1h	2.2	130	40	345	3
1i	2.7	200	25	328	1
1j	2.7	180	40	365	3
1k	2.7	60	40	362	3

[a] See Table I. [b] t_{max} may vary slightly depending on the actual pH of an assay solution. [c] The solvent systems employed were 1, water; 2, 19% ethanol–water; 3, 20% *n*-propyl alcohol–water.

Assay for Penicilloyl Derivatives. Penmaldate Assay. The variation of the concentrations of penicilloic acid and penicilloyl cycloamylose as a function of time was determined using an assay procedure[17] (treatment of aliquot portions of the reaction solution with mercuric chloride at pH 7) which is specific for penicilloic acid and penicilloyl derivatives (amides and esters), and able to distinguish between them.

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