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The β-Galactosidase-catalysed Hydrolyses of β-D-Galactopyranosyl Pyridinium Salts

RATE-LIMITING GENERATION OF AN ENZYME-BOUND GALACTOPYRANOSYL CATION IN A PROCESS DEPENDENT ONLY ON AGLYCONE ACIDITY

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1. β-D-Galactopyranosyl pyridinium salts are well-behaved substrates for the β-galactosidase of *Escherichia coli*, catalysis occurring by the interaction of the salt itself with the normal active site of the protein. 2. $\log k_{cat.}$ values for seven such salts show a linear relationship (correlation coefficient = −0.997) with the pK_a of the parent pyridine. 3. The β-D-galactopyranosyl derivatives of pyridine and 4-bromoisoquinoline exhibit α-deuterium kinetic isotope effects of 1.136±0.040 and 1.187±0.046 on their enzymic hydrolysis, indicating formation of a galactopyranosyl cation in the rate-limiting step. 4. This behaviour of the pyridinium salts contrasts with the behaviour of aryl galactosides and this contrast can be accommodated by the β-galactosidase mechanism of Sinnott & Souchard (1973). 5. The α-deuterium kinetic isotope effect for the hydrolysis of β-D-galactopyranosyl azide is 1.098±0.033; comparison of the $k_{cat.}$ value of the azide with that of a pyridinium salt of the same aglycone pK_a enables a maximum factor of 70 to be ascribed to the acceleration of the departure of azide by intracomplex general acid catalysis. 6. The possibility of the rate-limiting process in the glycosidase-catalysed hydrolysis of aryl glycosides being a conformation change is considered for a number of glycosidases where correlations of $k_{cat.}$ with aglycone acidity, reported in the literature, have been unsuccessful.

Investigations of the catalytic mechanism of glycosidases have previously centred around the nature of the glycosyl-enzyme intermediate and the processes leading to it. The role of induced conformational distortion in the sugar ring (e.g. Atkinson & Bruice, 1974) and of any proposed nucleophilic group in the enzyme active site (e.g. Bourne *et al.*, 1972) have been the subject of some debate, but the necessity of assistance by an acidic group to the departure of aglycone has been hitherto unquestioned (e.g. Capon, 1971), although the possibility of electrophilic (Lewis acid) catalysis rather than general (Brønsted) acid catalysis has been considered (Clark & Hay, 1973).

It was therefore surprising when a control experiment revealed that the β-D-galactopyranosyl pyridinium cation was a well-behaved substrate for the β-D-galactosidase of *Escherichia coli*. The bare fact of the behaviour of this salt as a substrate (reported as a preliminary communication; Sinnott, 1973) is here amplified by a study of the effects of substituents in the pyridine ring and α-deuterium substitution at C-1 of the pyranose ring, which provides substantial supporting evidence for the mechanism previously proposed for the enzyme by Sinnott & Souchard (1973).

This mechanism involves, for the hydrolysis of aryl β-D-galactopyranosides, a protein conformation change subsequent to substrate binding, and generation of a galactopyranosyl cation in the vicinity of a counterionic group (Fig. 1). This ion pair then rapidly and reversibly collapses to a great preponderance of covalent α-D-galactopyranosyl enzyme. Reaction with acceptor occurs through the ion-paired, rather than the covalent, form of the galactosyl-enzyme. The evidence on which this working hypothesis was based came from measurements of α-deuterium kinetic isotope effects (k_H/k_D) and Michaelis–Menten parameters for a series of aryl β-D-galactopyranosides. $k_{cat.}$ and K_m values showed no simple dependence on aglycone acidity; k_H/k_D values varied from 1.25 for rate-limiting degalactosylation to 1.00 for slow substrates. k_H/k_D ratios greater than about 1.1 are considered to be diagnostic of a change from 4- to 3-co-ordination of C-1 of the pyranose ring in the rate-limiting step: those near 1.0 are considered to indicate either an S_N2 reaction or the absence of bond breaking in the rate-limiting step (cf. Shiner, 1971). The k_H/k_D ratios for 'slow' aryl galactosides were considered to indicate a rate-limiting protein conformation change, rather than an S_N2 reaction,

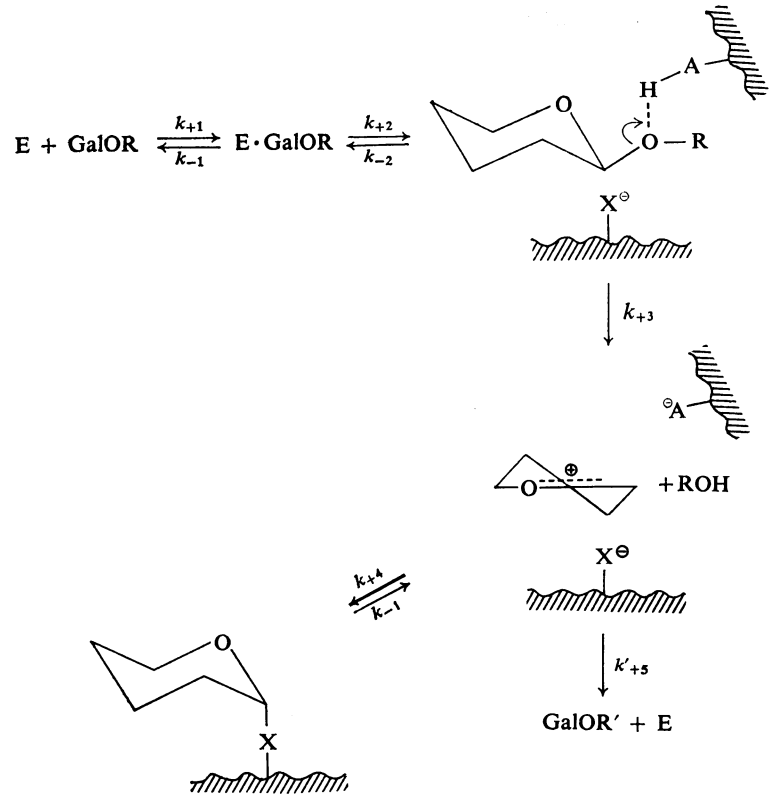


Fig. 1. *Mechanism of action of β-galactosidase proposed by Sinnott & Souchard* (1973)

Gal, β-D-galactopyranosyl; $k_{+4} \gg k_{-4}$. k'_{+5} is a complex quantity representing the reverse of the k_{+3}, k_{+2}, and k_{+1} step for the new galactopyranosyl derivative.

for the following two reasons. First, isotope effects for the reaction of methanol with the galactosyl-enzyme were compatible only with reaction via its ion-paired form: therefore hydrolysis of methyl β-D-galactopyranoside must, by the principle of microscopic reversibility, proceed via a galacto-pyranosyl cation. For a 'slow' aryl galactoside to proceed by an S_N2 reaction, there must be an unprecedented change in mechanism from S_N1 to S_N2 consequent on minor change of the leaving group from methoxide to phenoxide. Secondly, as had been pointed out previously by Viratelle & Yon (1973), the substantially random variation of $k_{cat.}$ values with aglycone acidity (Tenu *et al.*, 1971) indicates that the rate-limiting step is unlikely to be simple bond breaking.

The discovery of a new class of β-galactosidase substrates, the quaternary pyridinium salts, enables this mechanism to be subjected to a stringent test, for it is possible (in principle) to study Michaelis–Menten parameters as a function of the basicity of the parent pyridine, while at the same time measuring the α-deuterium kinetic isotope effect on the hydrolysis of representative examples of these salts. The stringency of this test arises in the first instance because the slowest of the pyridinium salt substrates has a $k_{cat.}$ value only 10^{-4} of the degalactosylation-rate of $1300\,s^{-1}$ (Sinnott & Viratelle, 1973). The conformation change we propose to be rate-limiting for 'slow' aryl galactosides must, for those dinitrophenyl galactosides the slow step in whose hydrolysis is degalactosylation, be at least ten times the degalactosylation rate (i.e. approx. $10^4\,s^{-1}$). Therefore if a conformation change, rather than bond breaking, is the rate-determining step in the hydrolysis of the slowest pyridinium salt, we must suppose that the rate of this conformation change changes by a factor of 10^5 solely as a consequence of a change in leaving group. This is implausible.

However, if bond breaking is rate-limiting with the pyridinium salts, then not only should $k_{cat.}$ for a series of (hetero-arene)-substituted pyridinium salts show a rational and unique dependence on the acidity of the parent pyridine, but also α-deuterium

kinetic isotope effects characteristic of the generation of a carbonium ion should be observed for the enzymic hydrolysis of these salts. Any other combination of results for the pyridinium compounds would either destroy the case, based on the results for aryl galactosides, for the mechanism of Fig. 1, or require additional and otherwise unsupported suppositions about the enzyme mechanism.

Materials and Methods

Tetra-O-acetyl-β-galactopyranosyl pyridinium bromides

These were readily made from those pyridines that are liquid at room temperature by the method of Fischer & Raske (1910). Acetobromogalactose (2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide) (5.0g) was dissolved in the pyridine (5.0ml) containing *m*-cresol (approx. 1ml) and left for 18h at 20°C. This method could be extended to pyridines with moderately low melting points by gently heating the mixture of pyridine, acetobromogalactose, and *m*-cresol until a homogeneous melt was obtained. Unchanged starting materials and *m*-cresol were removed by pouring the reaction mixture into ether (approx. 200ml); the quaternary salt and contaminating pyridinium hydrobromide were precipitated, usually as gums. Crystallization and recrystallization of the tetra-*O*-acetyl quaternary salts were best effected from mixed solvents, since the salt solubilities in various solvents seemed barely temperature-dependent. Useful solvent pairs (best solvent first) were acetone–carbon tetrachloride, water–acetone and dichloromethane–carbon tetrachloride: the latter is especially useful since contaminating pyridinium hydrobromides, unlike the quaternary salts, are insoluble in dichloromethane. Treatment of brown solutions of crude quaternary salts with decolourizing charcoal at room temperature often facilitated subsequent crystallization.

β-D-Galactopyranosyl pyridinium bromides

Deacetylation was achieved by heating 1–2% (w/v) solutions of the salt in aq. 2% (w/v) HBr for 20h at 40°C (Lemieux & Morgan, 1965). Evaporation of the solvent below 40°C yielded gums which could occasionally be crystallized. Trituration with ether (to remove HBr), followed by solution in ethanol or propan-2-ol, was the most productive procedure: addition of small quantities of ether or tetrahydrofuran was occasionally helpful. Recrystallization from ethanol was normally successful and appeared to be essential if β-galactosidase-inhibitory impurities were to be removed.

The crystallization behaviour of both acylated and deacylated compounds was largely unpredictable, and experimentation with a wide range of solvent systems was normally necessary before any success was achieved. Details and characterization data for the salts made are given in Table 1.

Acetobromo[1-^{2}H]galactose has been described (Sinnott & Souchard, 1973), and was used to make the various α-deuterated derivaties. The n.m.r. signal for the anomeric proton (approx. 2.4τ in carbon tetrachloride relative to tetramethylsilane) of the tetra-acetyl quaternary salts is well separated from all others: this is not the case for tetra-acetyl aryl β-D-galactopyranosides (Sinnott & Souchard, 1973). A lower maximum (of 5%) can thus now be placed on the proportion of protium at C-1 in the α-deuterated derivatives.

Other β-D-galactopyranosyl derivatives

The methyl 1-thio-β-D-galactopyranoside (m.p. 182–183°C), β-D-galactopyranosyl azide [m.p. 145–147°C (decomp.)], and 2-nitrophenyl-β-D-galactopyranoside (m.p. 200–203°C) used have been described (Sinnott, 1971), as has 4-nitrophenyl β-D-galactopyranoside (m.p. 176–177°C) (Sinnott & Souchard, 1973). β-D-[1-^{2}H]Galactopyranosyl azide [(m.p. 142–144°C (decomp.)] was made from acetobromo[1-^{2}H]galactose (Sinnott & Souchard, 1973) analogously to the undeuterated compound.

Buffer solutions

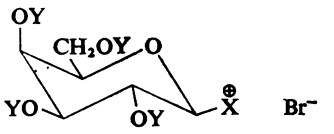
All enzyme experiments were performed in a medium obtained by mixing 0.1M solutions of NaH_2PO_4 and Na_2HPO_4 in doubly glass-distilled water until a pH value of 7.00 was registered at ambient temperature (22±2°C) on an EIL 23A pH-meter fitted with glass and calomel electrodes, and which had just been calibrated with standard buffer solution, pH7.00, purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Solid $MgCl_2$,-$6H_2O$ sufficient to bring the total Mg^{2+} concentration to 1.0mM was then added. All three inorganic reagents were of analytical reagent grade and purchased from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.

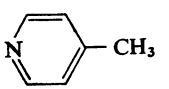
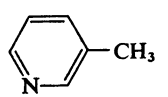
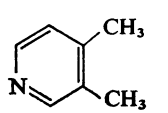
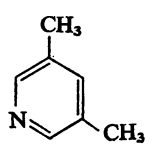
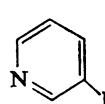
β-Galactosidase

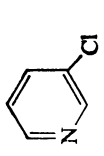
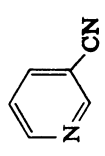
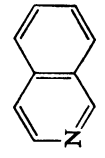
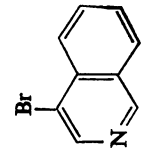
Crystalline enzyme, free of bovine serum albumin, from *E. coli* ML 308, lot nos. 7500408 and 7423216, was purchased as a suspension in 2.2M-$(NH_4)_2SO_4$ solution from Boehringer Corp. (London) Ltd., London W.5, U.K. The slurry (5mg/ml) was diluted appropriately with buffer solution, except for kinetic studies of those pyridinium salts with $k_{cat.}$ values less than $1s^{-1}$ when the large quantity of enzyme required would have resulted in the introduction of unacceptable quantities of $(NH_4)_2SO_4$. For these studies the enzyme crystals were centrifuged off and taken up in buffer solution. Low concentrations of $(NH_4)_2SO_4$ have negligible kinetic effect on β-galactosidase under the conditions of Na^+ and Mg^{2+} saturation used (Kuby & Lardy, 1953); we confirmed that $(NH_4)_2SO_4$ slightly (approx. 19%)

Table 1. *β*-D-*Galactopyranosyl pyridinium salts*

The general structure is:



Parent pyridine (X)	Y	Yield (%)	Melting point (°C) (decomp.)	[α]25 (c 1, in chloroform)	Empirical formula	Requires C	H	N	Br	Found C	H	N	Br	Anomeric proton [τ, J (Hz), [2H]chloroform]	Independent evidence for hydrate
	Ac	25	187–188	+22°	$C_{19}H_{24}BrNO_9$	46.55	4.9	2.85	—	46.15	4.9	2.95	—	2.40, 8.3	
	H	40	164–165	+141°	$C_{11}H_{16}BrNO_5$	41.0	4.95	4.35	—	41.4	5.25	4.45	—	—	
	H,[1-^{2}H]		164–165	(*c* 2 in water)											
	Ac	20	174–176	+1°	$C_{20}H_{26}BrNO_9,H_2O$	46.0	5.4	2.7	15.3	46.15	4.8	2.65	16.1	2.65, 8	N.m.r.: broad *s*, τ 75 2H; i.r. ν OH 3400 cm^{-1}
	H		(Could not be obtained crystalline)												
	Ac	25	117–119	+5°	$C_{20}H_{26}BrNO_9,H_2O$	46.0	5.4	2.7	15.3	44.25	5.15	3.1	19.6	2.55, 8	N.m.r.: broad *s*, τ 7.6, 2H; i.r. ν OH 3400 cm^{-1}
	H	25	141–142.5	—	$C_{12}H_{18}BrNO_5$	42.85	5.4	4.15	23.75	42.75	5.45	4.2	23.5	—	
	Ac	25	118–122	−13°	$C_{21}H_{28}BrNO_9,H_2O$	47.0	5.3	2.8	14.9	47.0	5.25	2.6	—	2.60, 8	N.m.r.: *s*, τ 7.8, augmented by water; i.r. ν OH 3400 cm^{-1}
	H		(Could not be obtained crystalline)												
	Ac	18	185–186	−12°	$C_{21}H_{28}BrNO_9$	48.65	5.45	2.7	15.1	48.65	5.3	2.7	15.55	2.55, 7.5	
	H	20	172–173	—	$C_{13}H_{20}BrNO_5$	44.6	5.75	4.0	22.8	44.05	5.95	4.5	—	—	
	Ac	22	141–147	−20°	$C_{19}H_{23}BrN_2O_9$	40.1	4.05	2.45	28.05	39.85	4.1	2.45	27.75	2.22, 8	
	H	20	118–120	—	$C_{11}H_{15}BrN_2O_5$	32.95	3.5	3.75	39.85	32.5	9.75	3.4	40.3	—	

	Ac	17	154–155	−9.3°	$C_{19}H_{23}BrClNO_9$	43.5	4.4	2.65	15.2	42.15	4.3	2.85	17.2	2.22, 8.2				
	H	15	124–125	—	$C_{11}H_{13}BrClNO_5$†	37.05	4.25	3.9	22.4	37.25	4.2	3.75	22.4	—				
	Ac*	40	161–163	−32° (*c* 1 in methanol)	$C_{20}H_{23}BrN_2O_9$	46.6	4.45	5.55	15.5	46.1	4.5	5.35	—	3.58, 8.6 (in $C^2H_3O^2H$)				
	H		(Could not be obtained crystalline)															
	Ac	20	140–141	−26°	$C_{23}H_{26}BrNO_9,H_2O$	49.4	5.0	2.5	14.2	49.6	5.05	2.55	14.3	2.45, 8	N.m.r.: 5 peaks in acetate region (τ 7.5–τ 7.8); i.r. ν OH 3400 cm^{-1}			
	H	42	157–159	—	$C_{15}H_{18}BrNO_5$	48.4	4.95	3.75	21.45	48.1	4.85	3.75	21.85	—				
	Ac	25	143–144	−36°	$C_{23}H_{26}BrNO9,H_2O$	43.35	4.25	2.2	25.05	43.0	4.1	2.25	25.2	2.58, 8	N.m.r.: broad *s*, τ 7.6; i.r. ν OH 3400 cm^{-1}			
	H	63	161–163	—	$C_{15}H_{17}BrN_2O_5$	39.9	3.8	3.1	35.4	40.5	3.95	3.25	36.1	—				
	H,[1-2H]12		161–163															

* Insoluble in chloroform and water.
† Requires Cl, 9.95; Found, Cl 10.2.

inhibited the hydrolysis of β-D-galactopyranosyl 4-bromoisoquinolinium bromide (0.4mM) at a concentration of 0.1M, three times the maximal concentration of $(NH_4)_2SO_4$ in our experiments.

Kinetic studies

These were done with a Unicam SP.1800 recording spectrophotometer system, with a cell block through which water maintained at 25.5± 0.1°C was circulated by a Tecam 'Tempunit' thermostatic pump. Isotope effects were measured at 25.0°C by direct comparison of protiated and deuterated substrates as described by Sinnott & Souchard (1973). Enzyme concentrations were calculated based on $k_{cat.}$ for 2-nitrophenyl β-D-galactopyranoside of $1000 s^{-1}$ and ε_{400} for 2-nitrophenol of 2.17×10^3 litre·mol^{-1}·cm^{-1} (Sinnott & Viratelle, 1973). Extinction-coefficient data for the pyridinium salts are given in Table 2.

The high absorbance of solutions of the pyridinium salts required for K_m determinations necessitated the use of short-path-length cells (1mm or 2mm); the consequential uncertainty in path-length was compensated for by the measurement of extinction coefficients, and the standardization of the enzyme, in the same cell as used for K_m and $k_{cat.}$ determinations.

Initial rates were estimated visually, and K_m and $k_{cat.}$ determined by computerized Eadie plots, as described by Sinnott & Viratelle (1973). If care was not taken in kinetic measurements, apparent non-Michaelian behaviour could be observed. To avoid the assignment of spurious mechanistic significance to these anomalies in any subsequent investigation, they are briefly described.

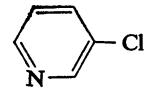
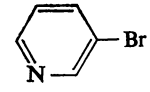
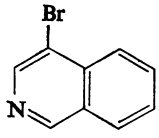
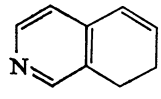
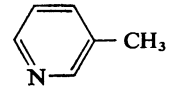
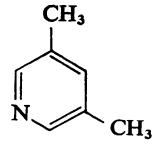
(i) '*Substrate inhibition*'. The hydrolysis of all the salts studied, except that derived from 4-bromoisoquinoline, could be followed only by a decrease in absorbance. Therefore care was necessary to ensure that with high sample absorbance values stray light did not cause apparent deviations from Beer's Law (cf. Cavalieri & Sable, 1973).

(ii) '*Transients*'. Apparent 'transients' of the order of 0.01 *E* unit (i.e. approx. 0.7% of total absorbance), observed on introduction of enzyme, are a temperature effect on the extinction coefficient of the salt. The amplitude and time-constant of the 'burst' are roughly independent of substrate nature and enzyme concentration; 'bursts' are not observed if the enzyme is carefully thermally equilibrated before introduction, but can be produced by the introduction of small quantities of cold water. The effect in the case of β-D-galactopyranosyl 4-bromoisoquinolinium bromide, in the hydrolysis of which an increase in absorbance is observed, is manifested as a 'lag'.

(iii) *Irreversible inhibition.* β-D-Galactopyranosyl 3-bromo- and 3-chloro-pyridinium salts, unless crystallized three times, contained impurities which

Table 2. *β-Galactosidase-catalysed hydrolyses of β-D-galactopyranosyl pyridinium salts at 25°C in 0.1 M-sodium phosphate buffer, pH 7.00, 1.0 mM in $MgCl_2$*

For details see the text.

Pyridine	$k_{cat.}$ (relative to 2-nitrophenyl β-D-galactopyranoside = $1000 s^{-1}$ ± approx. 10%)	K_m (mM)	Wavelength followed (nm)	Δε ($M^{-1} \cdot cm^{-1}$)
	110	1.40±0.11	292.5	−596
	90.4	1.15±0.15	298.0	−670
	58.1	0.49±0.04	308.0	+1163
	0.61* 0.67†	1.25±0.24 4.76±0.99†	275.0 275.0	−279 −279
	0.36	0.91±0.10	340.0	−3882
	0.22	1.93±0.17	273.0	−3173
	0.116	1.46±0.10	282.0	−3755

* The fivefold discrepancy between this value and that previously reported (Sinnott, 1973) arises from an arithmetical error.

† In the presence of 3.66 mM-methyl 1-thio-β-D-galactopyranoside.

slowly deactivated the enzyme. For this reason salts which could only be obtained amorphous were not studied; for example, the amorphous product from the deacetylation of 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl 3′-cyanopyridinium bromide was apparently not hydrolysed by the enzyme. The inhibition of the hydrolysis of 4-nitrophenyl β-D-galactopyranoside by β-D-galactopyranosyl pyridinium bromide was studied as described by Case *et al.* (1973) for β-D-galactopyranosyl trimethylammonium bromide. Some 72 initial rates, at eight glycoside concentrations from 408 to 28 μM, and at nine pyridinium salt concentrations, from 0 to 4.2 mM, were measured.

Products of the hydrolysis of β-D-galactopyranosyl pyridinium bromide

D-Galactose was determined by measuring the NADH formed on its oxidation by galactose dehydrogenase [lot no. 7081513, purchased from Boehringer Corp. (London) Ltd.] and NAD^+ spectrophotometrically (Wallenfels & Kurz, 1963). The reaction mixture was made up from buffer (2.75ml), NAD^+ (100μl, 10mg/ml in buffer), pyridinium salt (40μl, 16.6mM in buffer), β-galactosidase [100μl, 5mg/ml slurry in $(NH_4)_2SO_4$ solution] and β-galactose dehydrogenase [10μl, 5mg/ml slurry in $(NH_4)_2SO_4$ solution].

Pyridine was measured by extraction with spectroscopic-grade chloroform (1.00ml) of a mixture of 16.6mM salt in the buffer solution (0.45ml) and β-galactosidase suspension (50μl, 5mg/ml) which had been left for 18h at 22°C. The observed E_{259} of the chloroform solution was compared with that of a similar chloroform extract of the buffer solution containing pyridine (0.98mg/ml). Pyridine is about 90% extracted from the buffer by 1vol. of chloroform.

Results and Discussion

Occurrence of catalysis by interaction of pyridinium salt itself with the normal active site of the protein

The measured products of the hydrolysis of β-D-galactopyranosyl pyridinium bromide are D-galactose (88%) and pyridine (101%). All salts, even those derived from halopyridines, are stable indefinitely at room temperature in the buffer used, although β-D-galactopyranosyl 4-bromoisoquinolinium bromide seems slightly photosensitive. Michaelis–Menten kinetics are observed for all substrates; errors in the K_m values in Table 2 are standard deviations of the slopes of Eadie plots. The rate of hydrolysis of 1.0mM-β-D-galactopyranosyl 4-bromoisoquinolinium bromide was proportional to the enzyme concentration [despite the concomitant introduction of $(NH_4)_2SO_4$].

From the data in Table 2 it can be seen that methyl 1-thio-β-D-galactopyranoside, a competitive inhibitor of conventional substrates of the enzyme with a K_i value of 1.8mM (Sinnott, 1971), is also a competitive inhibitor of the hydrolysis of β-D-galactopyranosyl pyridinium bromide ($K_i = 1.3 \pm 0.5$mM). The inhibition of the hydrolysis of 4-nitrophenyl β-D-galactopyranoside by this salt is competitive; fitting the measured initial rates to an expression for mixed inhibition results in a physically meaningless non-competitive inhibition constant of −33mM, a competitive inhibition constant of 1.16mM, and a K_m for 4-nitrophenyl β-D-galactopyranoside of 34μM [cf. 28 ± 4μM obtained previously by Sinnott & Souchard (1973)]. The K_i value is

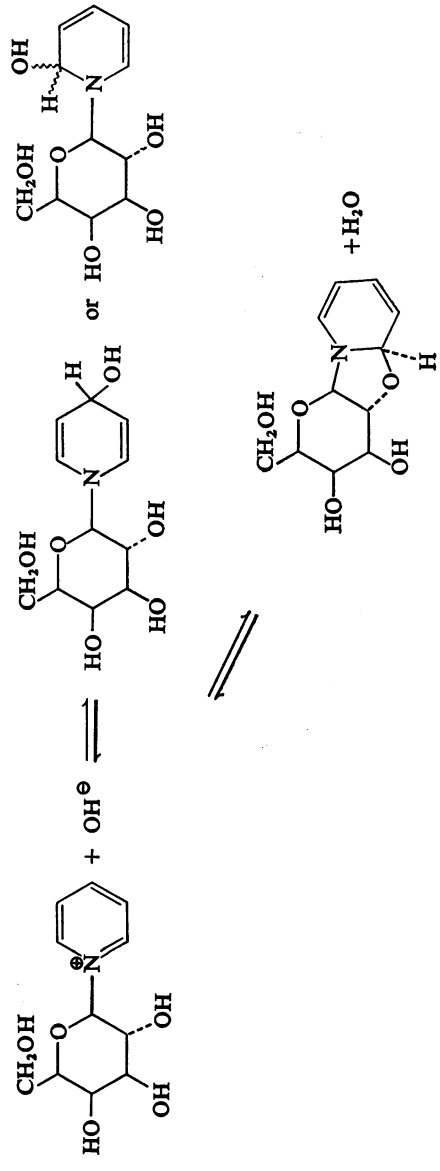


Fig. 2. *Possible pseudo-base forms of β-D-galactopyranosyl pyridinium hydroxide*

identical, within experimental error, with the K_m value for the hydrolysis of the pyridinium salt.

These data establish that hydrolysis is being effected at the active site of the enzyme. It is, however, not immediately apparent that the species being hydrolysed is the pyridinium salt itself, since quaternary pyridinium salts are in equilibrium with pseudo-base form, tricyclic analogues of which can be envisaged (Fig. 2) (e.g. Schofield, 1967). The pseudo-base form is favoured by electron-withdrawing substituents in the pyridine ring and high pH values.

At pH 12.1, β-D-galactopyranosyl 3-chloropyridinium bromide has the same $\lambda_{max.}$ (277nm) and 98% of the $\varepsilon_{max.}$ that it has at pH7; $\lambda_{max.}$ is unaltered, and $\varepsilon_{max.}$ decreases by a further 4%, at pH13, although a shoulder (at about 310nm) becomes discernible, possibly because of the ionization of the 2'-hydroxyl group of the pyranose ring. The aromatic chromophore is thus substantially unaltered even at pH13; a maximum pseudo-base proportion of 10% at pH12 and hence one of 10^{-4}% at pH7 can then be inferred.

This measurement makes enzymic reaction through the pseudo-base form improbable, since the rate of enzymic hydrolysis of the pseudo-base form of the β-D-galactopyranosyl 3-chloropyridinium cation would have to be ten times the degalactosylation rate. If it is assumed that, of a total pyridinium salt concentration of [S], a small fraction f of the pseudo-base is the true substrate, then the observed rate of disappearance of pyridinium salt under steady-state conditions, V, is given by:

$$V = \frac{k_{cat.}[E]_0 f[S]}{(f+K_m/K_i)\{[S]+K_m/(f+K_m/K_i)\}}$$

where K_m is the Michaelis constant, $k_{cat.}$ is the turnover number of the true substrate and K_i is the competitive inhibition constant of the salt itself. This last, K_i, term is necessary since β-D-galactopyranosyl trimethylammonium bromide, which cannot form a pseudo-base, is a competitive inhibitor of the enzyme (Case *et al.*, 1973); since $f \ll 1$, [S] is the effective inhibitor concentration.

Michaelis–Menten kinetics are still observed for such a reaction scheme, but the observed turnover number, $k'_{cat.}$, is $fk_{cat.}/(f+K_m/K_i)$, and the observed Michaelis constant, K'_m, is $K_m/(f+K_m/K_i)$. Order-of-magnitude estimates can, however, be made of the values of K_m, K_i and f. Our spectroscopic evidence establishes a maximum value of 10^{-6} for f at pH7; the minimum dissociation constant of an enzyme–inhibitor complex ever observed with a glycosidase is of the order of 10^{-7}M (e.g. Niwa *et al.*, 1970; Lai & Axelrod, 1973; Leaback, 1968). It would be unreasonable to suppose that the pseudo-base, which has no structural features which would make it bind especially tightly to the enzyme, has a K_m lower than 10^{-7}M. K_i would be anticipated to be of the same order as the dissociation constant of the β-D-galactopyranosyl trimethylammonium cation–β-galactosidase complex (1.4mM; Case *et al.*, 1973). These values indicate $K_m/K_i = 10^2 f$ (i.e. $K_m/K_i \gg f$); in these circumstances K'_m becomes equal to K_i, which can be ascribed the observed value of 1.4mM. $k'_{cat.}$ is $fk_{cat.}K_i/K_m$, or, assuming $f < 10^{-6}$, $K_m > 10^{-7}$M, and $K_i = 1.4$mM, $10^{-2}k_{cat.}$. But $k'_{cat.}$ for β-D-galactopyranosyl 3-chloropyridinium bromide is $122s^{-1}$, and the degalactosylation rate is $1300s^{-1}$. This argument is dependent on our assumptions about the magnitude of K_i only to the extent that $K_m/K_i \gg f$ so that K'_m, which is a measured quantity, is equal to K_i. Reaction via a pseudo-base is thus made improbable in the case in which it might have been most probable; we have therefore excluded reaction pathways for these substrates which would have invalidated the use of these salts to test the mechanism of Fig. 1.

Dependence of $k_{cat.}$ on pyridine pK_a only

Fig. 3 illustrates a plot of $\log k_{cat.}$ against pK_a for β-D-galactopyranosyl pyridinium salt hydrolysis: on the same axes are plotted the points for aryl galactoside hydrolysis, taken from Sinnott & Souchard (1973) with the neglect of *ortho*-substituted derivatives. The contrast is marked and is quantified by the correlation coefficients −0.997 and −0.05 respectively. Noteworthy is the predictability of the results for the isoquinolinium salts from those for the mononuclear compounds; the $k_{cat.}$ values for aryl galactosides, by contrast, seem to depend on aglycone shape rather than aglycone acidity: *meta* substituents, whether electron donating or withdrawing, uniformly accelerate the reaction. The K_m values (Table 2) are surprisingly constant, the mononuclear derivatives having K_m values in the range 1–2mM. The K_m values for aryl galactosides vary

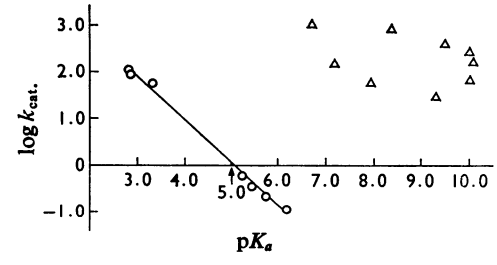


Fig. 3. *Variation of $k_{cat.}$ for two classes of β-D-galactopyranosyl derivatives with leaving-group acidity*

△, Aryl β-D-galactopyranosides; ○, β-D-galactopyranosyl pyridinium salts. $k_{cat.}$ values relative to that for 2-nitrophenyl β-D-galactopyranoside = 1000; 0.1M-NaH_2PO_4–Na_2HPO_4 (pH7.0) – 1.0mM-$MgCl_2$ buffer was used. Pyridine pK_a values are taken from Fischer *et al.* (1964*a*).

over a wider range, from 28μM for 4-nitrophenyl galactoside to 480μM for 3,5-dinitrophenyl galactoside (the latter value is probably a minimum value for K_s since degalactosylation is rate-limiting for this substrate). The K_m values for the isoquinolinium salts are slightly smaller than for the mononuclear derivatives; this is in accord with the hydrophobic nature of the aglycone-binding site (Jacob & Monod, 1961). There is some expectation that the K_m values for these pyridinium salt substrates represent K_s, since the observed $k_{cat.}$ values are well below the degalactosylation rate, and the enzymic hydrolysis of these salts can so far be adequately described by a two-step mechanism (see below).

The slope (α) of the log $k_{cat.}$–pK_a plot (–0.93±0.03) indicates that in the transition state of the rate-limiting process the nitrogen atom of the pyridine carries nearly as great a charge as the protonated pyridine. This will be substantially less than that in the quaternary salt, since strong hydrogen-bond donation by the protonated pyridine to water molecules will decrease the effective positive charge on the pyridine nitrogen. The high α value, however, accords with the rate-limiting process in the enzymic hydrolysis of these salts being the generation of a glycopyranosyl cation. Fischer *et al.* (1964*b*) observed that the Menshutkin reaction of ethyl iodide with a series of substituted pyridines in nitrobenzene gave an α value of 0.5; this indicated that in the transition state for the displacement of I^- by a pyridine molecule in an S_N2 reaction the charge on the nitrogen atom of the pyridine is substantially less than that of the protonated pyridine. The transition state for the S_N2 displacement of pyridine from an *N*-ethylpyridinium salt by I^- is, by the principle of microscopic reversibility, the same as that for the quaternization of pyridine by ethyl iodide, and so the data of Fischer *et al.* (1964*b*) would seem to indicate that displacement of a pyridine in an S_N2 reaction, at least by an anionic nucleophile, would involve much greater neutralization of the charge on nitrogen than is observed in the enzyme-catalysed hydrolysis of these β-D-galactopyranosyl pyridinium salts.

We consider that the data for the pyridinium salts displayed in Fig. 3 represent the first linear free-energy relationship that can give unequivocal information about the bond-breaking processes of a glycosidase; seven $k_{cat.}$ values, ranging over a factor of 1000, are correlated well by one line. The aglycone acidities could be better distributed (that they are not is due to crystallization difficulties), but the dependence of $k_{cat.}$ on aglycone acidity only is demonstrated. If the data for the slowest four pyridinium salt substrates only are used to calculate the least-squares line, $k_{cat.}$ for the fastest substrate, β-D-galactopyranosyl 3-chloropyridinium bromide, is thereby estimated to within a factor of 3.

Generation of an enzyme-bound galactopyranosyl cation in the rate-limiting step of the hydrolysis of β-D-galactopyranosyl pyridinium salts

The k_H/k_D ratio for β-D-galactopyranosyl 4-bromoisoquinolinium bromide hydrolysis is 1.187±0.046 (the error is a standard deviation of 8 k_H/k_D measurements with [S] = 9 K_m); that for β-D-galactopyranosyl pyridinium bromide is 1.136±0.040 (10 k_H/k_D measurements with [S] = 12 K_m). Although the precision of these data is not high, because of the opposing requirements of thorough mixing and accurate temperature control in the narrow-path-length cells dictated by the high absorbance of these salts, they demonstrate that the α-deuterium kinetic isotope effects for both salts lie within the range characteristic of the generation of glycopyranosyl cation. Thus Dahlquist *et al.* (1969) observed a value of 1.13±0.01 for the acid-catalysed hydrolysis of phenyl D-glucopyranoside, and we observed one of 1.08±0.03 for the acetolysis of 2,4-dinitrophenyl β-D-galactopyranoside (D. Cocker & M. L. Sinnott, unpublished work); in both these processes a glycopyranosyl cation is generated in the rate-limiting step (Capon, 1969; Cocker *et al.*, 1973). These two enzymic substrates have widely spaced $k_{cat.}$ values and contain no detectable inhibitory impurities.

So we have observed a combination of a good correlation of $k_{cat.}$ with aglycone acidity and α-deuterium kinetic isotope effects at extremes of substrate reactivity which are characteristic of the generation of a glycopyranosyl cation. This is the only combination of results which would have actively supported the mechanism of Fig. 1.

The β-D-galactopyranosyl-catalysed hydrolyses of β-D-galactopyranosyl pyridinium salts do not involve the significant action of an electrophile; the α-deuterium kinetic isotope effects indicate that a nucleophile is not involved at the transition state, therefore the entire catalytic action of the enzyme must come from distortion and media effects. Jencks (1969) has pointed out that methylations of a pyridine and a sulphide with *S*-adenosylmethionine cannot involve the action of acidic or basic catalytic groups on the respective enzymes, but we believe this is the first enzyme-catalysed dissociative reaction in which catalytic groups on the enzyme play no direct part. For the description of such dissociative processes, of course, the neologism 'orbital steering' is inapt, since there are no orbitals to steer.

Intra-complex general acid catalysis in galactosidase action: departure of azide

The observation of glycosidase-catalysed hydrolysis of glycosyl pyridinium cations discredits the traditional ascription of most of the catalytic action of glycosidases to some form of electrophilic pull (be it by a co-ordinated metal ion or by a proton)

on the aglycone, since the pyridinium salts possess no lone pair to which this pulling species can be co-ordinated. In addition to conventional alkyl and aryl glycosides, the more unusual glycosidase substrates such as glycosyl fluorides (von Hofsten, 1961; Barnett *et al.*, 1967*a*,*b*), azide (Sinnott, 1971), thiophenolate (Wallenfels *et al.*, 1964) or selenophenolate (Wagner & Metzner, 1965*a*,*b*) all possess a lone pair of electrons on the glycone-proximal atom of the aglycone, and the apparent ubiquity of this structural feature led to the supposition of its necessity for glycosidase substrates (Sinnott, 1971); the results with the pyridinium salts now show this supposition to have been incorrect.

Since, in the case of the β-galactosidase of *E. coli*, the necessary Mg^{2+} ion has been shown to play a conformational, rather than a catalytic, role (Case *et al.*, 1973) further consideration of the hypothesized 'pull' on the aglycone will be confined to general acid catalysis. General acid catalysis in the hydrolysis of acetals and ketals is usually regarded as contiguous with their specific acid-catalysed hydrolyses (e.g. Anderson & Capon, 1969), and such a process involving co-ordination to a lone pair of electrons on the aglycone moiety is impossible with the pyridinium salt substrates. There are, however, two other possible processes whereby a general acid could be envisaged as accelerating the departure of the pyridine moiety, but these have only the remotest of precedents. The first (Fig. 4*c*) involves an S_E2 (bimolecular electrophilic substitution) reaction on nitrogen, with the general acid as an approaching electrophile and the glycosyl cation as a leaving group. Such S_E2 processes are known, but on carbon [e.g. the acid protolyses of alkyl σ-bonded organometallic compounds (Ingold, 1969) and with much more favourable leaving groups (e.g. Hg^{2+}); the second (Fig. 4*d*) involves hydrogen-bonding to the π-electron cloud of the pyridinium ring; such bonding is well known with benzene derivatives (e.g. Brown & Brady, 1952), but their occurrence with the strongly electron-deficient pyridinium ring is problematical.

However, since even the most favoured process (Fig. 4*b*) contributes surprisingly little to the acceleration of the heterolysis of a glycosyl-nitrogen bond, it is unlikely that less well-documented processes such as in Figs. 4(*c*) and 4(*d*) cause any significant lowering of the barrier to bond cleavage.

An estimate of the importance of acid catalysis of type Fig. 4(*b*) is possible since β-D-galactopyranosyl azide is a substrate (Sinnott, 1971). This substrate possesses a lone pair of electrons on the glycone-proximal nitrogen atom, but in other respects the chemical change brought about by the enzyme is

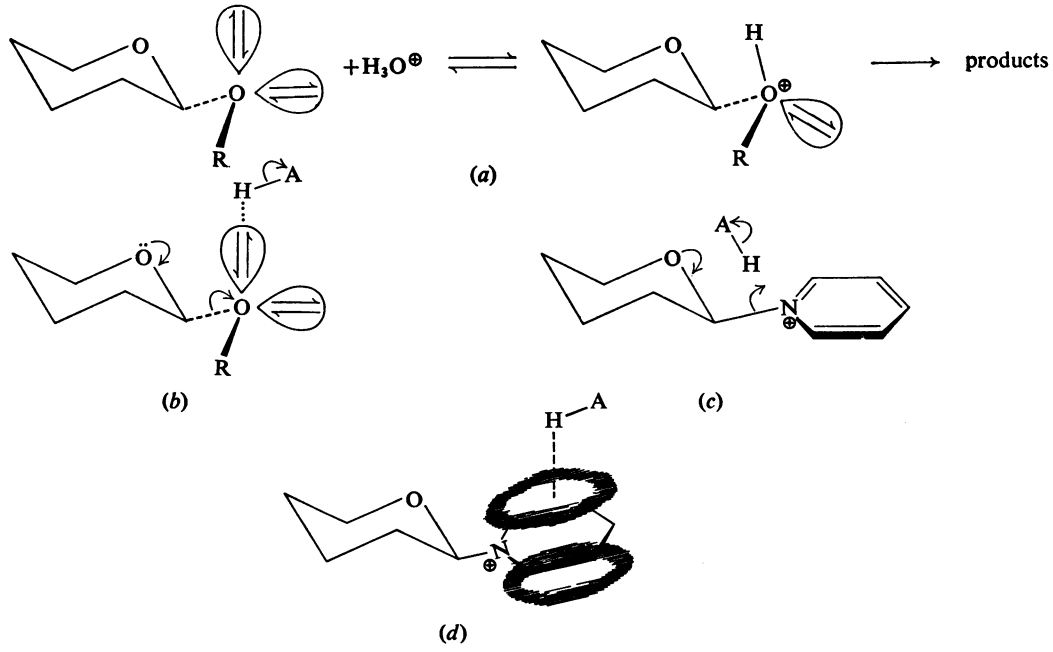


Fig. 4. *Possible modes of acid-catalysed assistance of the departure of aglycone*

(*a*) Specific acid catalysis; (*b*) general acid catalysis contiguous with specific acid catalysis; (*c*) S_E2 reaction on glycone-proximal aglycone atom; (*d*) H-bonding to the aromatic π cloud of a pyridine residue.

directly comparable with that brought about on the pyridinium salts: a glycosyl-nitrogen bond, constructed from a nitrogen sp^2 orbital, is cleaved. Therefore a comparison of the rates of bond cleavage of the azide and of a pyridinium salt of which the aglycone has the same pK_a as hydrazoic acid (4.6; Quintin, 1940) should give the estimate required. In the hydrolysis of the pyridinium salts $k_{cat.}$ represents bond breaking and so interpolation in Fig. 3 at $pK_a = 4.6$ gives the rate of C–N cleavage unassisted by acid catalysis.

In the absence of other evidence, however, it would be unjustified to assume that $k_{cat.}$ for the azide represented bond breaking. The α-deuterium kinetic isotope effect (k_H/k_D) of 1.098±0.033 (seven points, $[S] = 10K_m$) indicates that bond breaking is within a factor of about 4 of the $k_{cat.}$ value of $44 s^{-1}$; this factor of 4 would arise if the true $(k_H/k_D)_{cat.}$ ratio were 1.066 and the k_H/k_D ratio for the bond-breaking steps were the theoretical maximum for a simple process of 1.25, with a conformation change, similar to that proposed for aryl galactosides, being partly rate-limiting.

Combination of these two rate estimates results in a maximum factor of 70 being ascribed to the facilitation of C–N cleavage by intracomplex general acid catalysis. Even if due allowance is made for galactosyl azide being an unnatural substrate (and the $k_{cat.}$ value is similar to that for lactose, the natural substrate) (Wallenfels & Malhotra, 1961), this is surprisingly low.

The importance of intracomplex general acid catalysis to the departure of aglycone will of course depend on the chemical nature of the aglycone, generally becoming more important the worse the leaving group (e.g. Anderson & Capon, 1969). However, it is noteworthy that β-galactosidase is a less efficient general acid catalyst (to the departure of azide) than the *ortho*-carboxyl group of the salicyl glucopyranosides is to the departure of salicylate ion, which is accelerated by a factor of 10^3 (Capon *et al.*, 1969) compared with the *para*-substituted compound.

A substantially aqueous active site for β-galactosidase?

β-D-Galactopyranosyl trimethylammonium bromide has been shown to be an inhibitor of β-galactosidase, but it is not a substrate ($k_{cat.} < 0.005 s^{-1}$; Case *et al.*, 1973). This is in accord with the pK_a of trimethylamine (9.8, Arnett, 1973). However, there is a report that in the gas phase trimethylamine and pyridine have comparable basicities (Arnett, 1973). If this is indeed correct, then any mechanism of β-galactosidase which involved substantial desolvation of the substrate in the enzyme active site is made unlikely, for then pyridine and trimethylamine would be expected to have comparable leaving-group abilities.

Possible rate-limiting protein-conformation changes in the hydrolysis of aryl glycosides by other glycosidases

Our further evidence that the random variation of $k_{cat.}$ with aglycone acidity in the β-galactosidase-catalysed hydrolyses of aryl galactosides arises from a substrate-dependent protein conformation change raises the possibility that such a process may occur with other glycosidases. This is most likely for sweet almond β-glucosidase, since the data of Nath & Rydon (1954), taken as a whole, indicate a random variation of $k_{cat.}$ with aglycone acidity, and Dahlquist *et al.* (1969) have found an α-deuterium kinetic isotope effect (k_H/k_D) of 1.01±0.01 for the hydrolysis of phenyl β-D-glucopyranoside by this enzyme. Poor Hammett correlations have, however, also been found for brewer's yeast α-glucosidase (Hall *et al.*, 1962), α-maltosidase (Suetsugu *et al.*, 1971), mammalian β-glucuronidase (Watanabe & Suzuki, 1970; Wang & Touster, 1972), and fungal β-xylosidase (van Wijnendaele & De Bruyne, 1970). The non-linearity of such Hammett plots has occasionally unjustifiably been taken as indicative of a change in catalytic mechanism (see e.g. Ritchie & Sager, 1964; Hammett, 1970 for authoritative discussions of the interpretation of deviations from linear free-energy relationships).

Conclusion

The results described above give an estimate of the importance of intracomplex general acid catalysis in β-galactosidase action; they also provide support of our postulation of a protein-conformation change in the hydrolysis of aryl galactosides. The nature of this conformation change is not known, but protein-conformation changes subsequent to binding of substrates often have the function of placing catalytic groups in the correct orientation for catalysis. The simplest explanation for the absence of a rate-limiting protein-conformation change during the hydrolysis of the pyridinium salts is then that the conformation change for aryl galactoside hydrolysis is associated with the correct placing of the general acid catalytic group. This speculation receives circumstantial evidence from the rough constancy of the K_m values for the five mononuclear pyridinium salts; were the conformation change to take place, its rate would be expected to vary randomly with pyridine structure, by analogy with aryl galactosides. Bond breaking is the rate-limiting process in the pyridinium salt hydrolysis; therefore the conformation change would be a fast process preceding it. Its rate, then, would affect K_m rather than $k_{cat.}$, and K_m values would be expected to vary over a wider range than

they do. Occurrence of a conformation change would, however, be compatible with near constancy of K_m values if it were both rapid and reversible.

The α-deuterium kinetic isotope effects here reported support our proposal that a galactopyranosyl cation lies on the direct reaction pathway in β-galactosidase catalysis. The results for the pyridinium salts have little bearing on subsequent reactions of this cation, but it is gratifying that a post-rate-determining collapse of the glycosyl cation to an acylal is now considered most likely during lysozyme catalysis (Atkinson & Bruice, 1974).

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