S_N1 Hydrolyses of Glycosyl Pyridinium Salts, and Quantification of the Main Source of Catalytic Power of *E. coli*(*lacZ*)-β-Galactosidase

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The hydrolysis of the 3-chloro-1-(β-D-galactopyranosyl) pyridinium ion is independent of pH between pH 2 and 8; this hydrolysis and those of another four pyridine-substituted cations exhibit positive entropies of activation. Rates at 25 °C are between 10^{-10} and $10^{-12.5}$ times the k_{cat} values for the β-galactosidase-catalysed hydrolyses of the same compounds: for the 3-chloropyridinium salt $\Delta H^{\ddagger}$ is lowered by 21 kcal mol^{-1} and $\Delta S^{\ddagger}$ by 25 cal mol^{-1} K^{-1}. The α-deuterium kinetic isotope effect for the spontaneous hydrolysis of the β-D-galactopyranosylpyridinium ion is the same as that for its enzymic hydrolysis, indicating that both processes involve a galactosyl cation. The whole of the catalytic effect of the enzyme towards these substrates must therefore arise from non-covalent interactions with parts of the substrate other than the bond being cleaved. Arguments are presented that such interactions have comparable importance in the hydrolyses of oxygen glycosides.

THE source of the rate enhancements brought about by enzymes is a subject of intensive current investigation; unfortunately, many complications intervene even before a kinetic parameter obtained for an enzyme reaction can be directly compared with one for a non-enzymic reaction in solution. Reactions taking place in enzyme active sites are only rarely observed in solution without significant changes in the number and disposition of participating atoms, and even more rarely do steady-state k_{cat} values represent rates of bond-breaking in the enzyme–substrate (ES) complex. Even where rates for comparable processes can be compared, the resulting ratio of rate constants usually has dimensions, and so the derived magnitude of the decrease in the free-energy barrier to reaction that takes place when the substrate binds to the enzyme depends in an arbitrary way upon the thermodynamic standard state chosen.

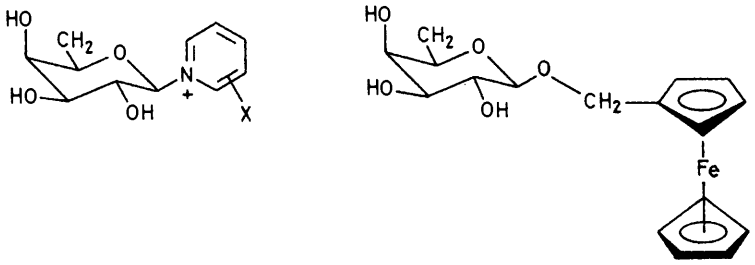
We have discovered an enzyme–substrate combination where these complications are not a problem. The β-galactosidase of *E. coli* hydrolyses β-D-galactopyranosylpyridinium salts, and k_{cat} values represent rates of carbon–nitrogen bond cleavage in the ES complex.[1] The structure of these substrates precludes assistance by acid or electrophile to the departure of aglycone (Scheme 1), and α-deuterium kinetic isotope effects for their enzymic hydrolysis, on conventional interpretation, discount any direct participation of a nucleophile. These hydrolyses are indeed enzyme-catalysed S_N1 reactions. The idea of reaction brought about by an enzyme without the direct intervention of catalytic groups was—despite the pinpointing of a couple of such reactions by Jencks [2]—at the time somewhat unorthodox. However, a recent authoritative

[1] M. L. Sinnott and S. G. Withers, *Biochem. J.*, 1974, **143**, 751.

[2] W. P. Jencks, 'Catalysis in Chemistry and Enzymology,' McGraw-Hill, New York, 1969, p. 83.

review [3] considers mechanisms for destabilisation of the bound substrate relative to the transition state in some detail, and states: 'The binding energy from interactions with parts of the substrate other than the reacting group can provide the driving force for destabilisation that is relieved in the transition state and causes a rate acceleration.'

For this reason the absolute magnitude of the rate enhancement brought about upon the galactosyl-



SCHEME 1

pyridinium salts by β-galactosidase was of interest. The ratio of the k_{cat} value for the enzymic hydrolysis to the rate-constant for the spontaneous, S_N1 reaction would of course be dimensionless, and a direct measure of the lowering of the free-energy barrier to C–N heterolysis could therefore be obtained. We now report kinetic data for the S_N1 hydrolysis of a number of glycosylpyridinium salts, and confirm that the rate-enhancement during enzyme catalysis is large enough for meaningful conclusions to be drawn about the enzyme mechanism. Experiments indicating that the large contribution of substrate destabilisation to catalysis is not in some way uniquely associated with the pyridinium salt substrates are also described.

EXPERIMENTAL

(A) *Materials.*—The β-D-galactopyranosylpyridinium bromides have been described elsewhere,[1] and α-L-arabinopyranosylpyridinium bromide is described in the following paper. β-D-Galactopyranosyl[U-^{2}H]pyridinium bromide, m.p. 155°, was made from >99% deuteriated pyridine. Methoxymethylferrocene, n_D^{20} 1.601 0 (lit.,[4] 1.599 6), was made by heating hydroxymethylferrocene in methanol with a few drops of perchloric acid, neutralisation with sodium carbonate, and distillation under water-pump vacuum. β-Galactosidase from *Escherichia coli* ML 308 (lot no. 7423216) was purchased from Boehringer, Ltd.

Ferrocenylmethyl β-D-Galactopyranoside.—This was made like the glucoside,[5] but with silver carbonate rather than silver oxide as the Koenigs–Knorr catalyst. Hydroxymethylferrocene (1.0 g), anhydrous calcium sulphate (5.0 g), silver carbonate (5.0 g), and glass balls (6.0 g) were shaken in calcium-hydride-dried dichloromethane (12 ml) for 20 min, and then 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (1.2 g) was added and shaking was continued. The calcium sulphate had been activated by ignition at 1.0 Torr for 30 min, and other solid reagents had been dried for 24 h over phosphorus pentaoxide at 20 °C and 15 Torr. After 24 h the mixture was filtered, the solids were washed with dichloromethane, and the total filtrate was evaporated. The residue was taken up in ethanol (15 ml). The crystals which slowly appeared were recrystallised from ethanol to afford 2,3,4,6-*tetra*-O-*acetylferrocenylmethyl* β-D-*galactopyranoside* (0.2 g, 13%), m.p. 136—137 °C, $[\alpha]_D^{20}$ +1° (*c* 0.5 in Me_2CO) (Found: C, 54.95; H, 5.5. $C_{25}H_{30}FeO_{10}$ requires C, 54.95; H, 5.4%). Zemplén deacetylation gave, after recrystallisation from water, the *glycoside*, m.p. 114—116 °C, $[\alpha]_D^{20}$ +14° (*c* 0.3 in H_2O) (Found: C, 53.15; H, 6.0. $C_{17}H_{22}FeO_6$ requires C, 54.0; H, 5.8%).

(B) *Methods.—Measurements of non-enzymic hydrolysis rates.* All reactions were monitored by following change in u.v. absorbance at a wavelength where it was maximal (aglycone 3-chloropyridine, 276 nm, 3-bromopyridine, 290 nm, 4-bromoisoquinoline, 350 nm, pyridine, 260 nm, and isoquinoline, 340 nm). All rate constants refer to solutions 1.0M in potassium chloride, the various acid and neutral buffer species (at a concentration of 0.05M) being mixed at 22 °C until the required pH was registered on an EIL pH meter calibrated previously with B.D.H. standard buffer solutions. The buffer systems were, at pH 2.0, hydrogen chloride, at pH 3.0 hydrogen chloride–ethylenediaminetetra-acetic acid (EDTA), at pH 4.0 and 5.0 sodium acetate–acetic acid, at pH 6.0, 7.0, and 8.0 disodium hydrogen phosphate–sodium dihydrogen phosphate, at pH 9.0 and 10.6 sodium hydroxide–EDTA, at pH 11.5 sodium dihydrogen phosphate–sodium hydroxide, and above this sodium hydroxide alone.

Above 100 °C rates were measured by withdrawing sealed ampoules at suitable intervals from a Tamson TEB 45 thermostatic oil-bath, which maintained the temperature constant to within ±0.2 °C, and measuring the absorbance at 25 °C with a Unicam SP 1800 or SP 800 spectrophotometer. Rates were calculated by using experimental infinity values, one of which, when checked, differed from the theoretical one by 0.5%. Rates at 100 °C were measured similarly, but using a steam-bath. Reactions at below 80 °C were followed continuously in 1 cm cells in the thermostatted cell-block of a Unicam SP 1700 spectrophotometer, water from a Julabo Paratherm U4 circulating thermostat being pumped through the block. The difference in temperature between the bath and the solvolysis medium was measured by a copper–constantan thermocouple. It was found necessary to provide an outer casing of expanded polystyrene for the block in order that the temperatures in cells in various positions should be within *ca.* 0.2—0.3 °C of one another. Later work using modified cells with a thermocouple pocket that enabled the tem-

[3] W. P. Jencks, *Adv. in Enzymology*, 1975, **43**, 219.

[4] E. G. Perevalova, Yu. A. Ustynyuk, L. A. Ustynyuk, and A. N. Nesmeyanov, *Izvest. Akad. Nauk S.S.S.R.*, 1963, **11**, 1977.

[5] A. N. de Belder, E. J. Bourne, and J. B. Pridham, *J. Chem. Soc.*, 1961, 4464.

perature of the liquid whose absorbance was actually being followed to be measured indicated that the serious systematic temperature errors previously encountered in this system [6] had been rectified by the lagging. Rate constants were calculated by the Guggenheim [7] method.

Measurement of secondary deuterium isotope effects on the hydrolysis of β-D-*galactopyranosylpyridinium ion.* Pairs of ampoules containing protiated and deuteriated substrate, placed next to one another, were withdrawn simultaneously from a steam-bath, and the absorbances of both solutions were measured. In the case of α-deuterium kinetic isotope effects, the k_H/k_D value for each pair of ampoules removed was calculated directly from equation (i), and starting

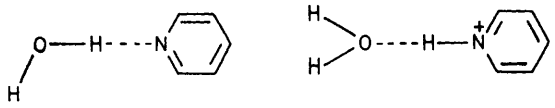
$$k_H/k_D = \log\left\{\frac{a_0(H) - a_\infty(H)}{a_t(H) - a_\infty(H)}\right\} \Big/ \log\left\{\frac{a_0(D) - a_\infty(D)}{a_t(D) - a_\infty(D)}\right\} \quad \text{(i)}$$

concentrations of protiated and deuteriated salts were as close to each other as possible. The error quoted is the standard deviation on 13 such measurements from two independent runs.

Because of the isotope effect on the extinction coefficient of both pyridinium salt and free pyridine when the pyridine nucleus was deuteriated, with the consequent danger of large systematic errors, the leaving group isotope effect was calculated from a simple first-order plot (using an experimental infinity reading) of both substrates. The quoted error is the standard deviation of the quotients of the gradients of first-order plots from three independent runs.

*Temperature dependence of the rate of enzymic hydrolysis of 3-chloro-*1-(β-D-*galactopyranosyl)pyridinium ion.* The hydrolysis was carried out in 2.0 mm path-length cells containing 0.1M-sodium phosphate buffer solution, pH 6.5, containing 1.0mM-magnesium chloride. The reaction was followed in a Unicam SP 1800 spectrophotometer fitted with a cell block through which water was pumped from a bath maintained at a constant temperature by a Tecam Tempunit; no correction was applied for the difference between cell- and bath-temperature, as working temperatures were within 12 °C of ambient. At 272.5 nm, the wavelength at which the reaction was followed, the free pyridine did not absorb, but the salt had an extinction coefficient of 435 l mol^{-1} cm^{-1} at 25 °C. The extinction coefficient was temperature dependent, with a temperature coefficient of +2.9 l mol^{-1} cm^{-1} °C^{-1}. Values of K_m and $V_{max.}$ were obtained by least-squares treatment of six-point plots of V against $V/[S]$: k_{cat} values are expressed relative to one for 2-nitrophenyl β-D-galactopyranoside at pH 7.0 and 25 °C of 1 000 s^{-1}. Error bars in Figure 2 are $\delta V_{max.}/2.3\ V_{max.}$, where $\delta V_{max.}$ is the least-squares-derived standard deviation in $V_{max.}$, and are included solely as an aid to judgement of the linearity of the Arrhenius plot; actual k_{cat} values will be uniformly subject to substantial additional temperature-independent systematic errors.

Analysis of the products of aqueous methanolysis of ferrocenylmethyl β-D-*galactopyranoside.* Five ampoules, each containing the glycoside (5 mg) were set up. To the first was added 0.1M-sodium phosphate buffer, pH 7.0, 1.0mM in magnesium chloride (0.95 ml); to the second, buffer and enzyme (50 μl) as a 5 mg ml^{-1} slurry in 2M-ammonium sulphate; to the third, buffer, enzyme, and methanol (50 μl); to the fourth, water (0.95 ml) and aqueous N-sulphuric acid (50 μl); and to the fifth, water, sulphuric acid, and methanol (50 μl). After the acidic solutions had been left for 1.5 h at 22 °C, and been neutralised with solid sodium carbonate, and the neutral solutions had been left for 3 days at 22 °C, the products from the aglycone were extracted with ether, the ether was evaporated off in a stream of nitrogen,



SCHEME 2

and a few drops of *NO*-bis(trimethylsilyl)acetamide were added. The resultant solutions were analysed by g.l.c. on a 2 m Geo-100-packed column of *ca.* 2 700 theoretical plates at 140 °C, on which methoxymethylferrocene and trimethylsilyloxymethylferrocene had retention times in the ratio 1 : 1.1.

The products of the spontaneous hydrolysis of β-D-galactopyranosylpyridinium cations were analysed in the same way as the products of enzymic hydrolysis.[1]

Measurement of the secondary deuterium kinetic isotope effect on the enzymic hydrolysis of β-D-*galactopyranosyl-*[U-^{2}H]*pyridinium bromide.* This was achieved with substrate at a concentration of 10 K_m at 276 nm, in the same manner as that of α-deuterium kinetic isotope effects;[1,8] the apparent k_H/k_D value (6 points) was 1.36 ± 0.02. However, the extinction coefficients of protiated and deuteriated compounds differ, being respectively (l mol^{-1} cm^{-1}) 336.5 and 265.1 for the salts and 22.8 and 16.7 for the free pyridine. This gives a true k_H/k_D value of 1.08.

RESULTS AND DISCUSSION

(A) *Spontaneous Hydrolyses of Glycosylpyridinium Salts.*—In Table 1 are given the first-order rate constants for the hydrolysis of 3-chloropyridinium bromide in a series of buffer solutions of various pH values. Extrapolation of these data to 25 °C and addition of high-pH data obtained directly at 25 °C gives the pH–rate profile of Figure 1. This consists of a region where the hydrolysis is independent of pH and a region where it is base-catalysed. Least-squares treatment of the rates above

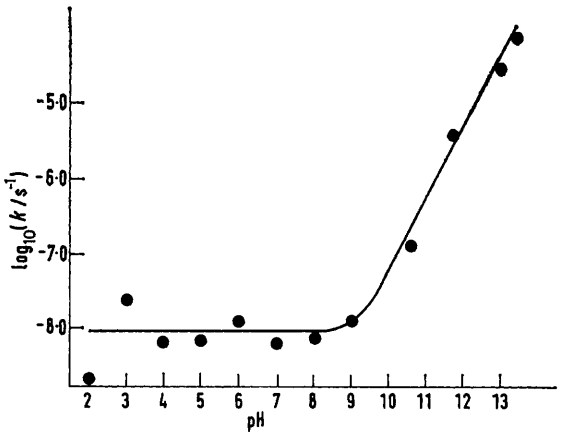


FIGURE 1 pH–Rate profile at 25 °C in 1.0M-KCl for the non-enzymic hydrolysis of the 3-chloro-1-(β-D-galactopyranosyl)-pyridinium ion

[6] D. Cocker and M. L. Sinnott, *J.C.S. Perkin II*, 1976, 618.
[7] E. A. Guggenheim, *Phil. Mag.*, 1926, **7**(2), 538.
[8] M. L. Sinnott and I. J. L. Souchard, *Biochem. J.*, 1973, **133**, 89.

pH 10.0 gives a gradient of the log k *vs.* pH plot of 0.94 ± 0.11, indicating a specific base catalysed process. This process results in the liberation of 3-chloropyridine and probably proceeds *via* participation of the ionised 2'-hydroxyl group in a normal pathway for the base-catalysed hydrolysis of glycosyl derivatives possessing a *trans*-disposition of this hydroxy-group with respect to the leaving group.[9] However, it is known that the quaternary glycosyl salts of more basic pyridines are degraded by base *via* nucleophilic attack on the pyridine ring.[10]

The preferred pathway for hydrolysis of the 3-chloro-1-(β-D-galactopyranosyl)pyridinium ion between pH 2.0 and 8.0 is independent of pH; it thus conforms to one of the classic criteria for an S_N1 reaction, independence of the rate from lyate ion concentration. The low precision of the rates at pH 2.0 and 3.0 arises because the free pyridine (pK_a 2.8) is now substantially protonated. That the other classic criterion (near-zero or positive entropy of activation) is also obeyed is shown from the data in Tables 1 and 2.[11] The entropy of activation is in fact strongly positive, indicating, perhaps, the decrease in solvent ordering around the transition state anticipated from the greater dispersal of charge in this species. The positive entropy of activation for an S_N1 hydrolysis involving the departure of a neutral leaving group is in any event precedented: $\Delta S^{\ddagger}$ for the hydrolysis of dimethyl-t-butylsulphonium bromide is +18 cal K^{-1} mol^{-1}.[12]

TABLE 1

pH Dependence of the hydrolysis of the 3-chloro-1-(β-D-galactopyranosyl)pyridinium ion

pH at 25°C	T/°C	$10^4\ k$/s^{-1}	$\Delta H^{\ddagger}$/ kcal mol^{-1}	$\Delta S^{\ddagger}$/ cal mol^{-1} K^{-1}
2.0 *	100.4	11.5 ± 2.22	38.8 ± 1.8	29.7 ± 5.3
	70.8	0.13 ± 0.01		
3.0 *	100.8	9.6 ± 0.363	39.1 ± 0.4	30.2 ± 1.2
	72.1	0.12 ± 0.002		
4.0	100.8	9.4 ± 0.301	34.6 ± 0.4	18 ± 1
	70.7	0.16 ± 0.003		
5.0	100.2	9.1 ± 0.425	33.9 ± 1.6	18 ± 4
	71.0	0.193 ± 0.001		
	72.2	0.189 ± 0.002		
6.0	(see Table 2)			
7.0	100.6	10.5 ± 0.366	34.5 ± 1.4	20 ± 4
	71.4	0.21 ± 0.002		
	72.4	0.20 ± 0.001		
8.0	100.5	11.6 ± 0.372	33.9 ± 1.4	19 ± 4
	70.7	0.22 ± 0.005		
9.0	100.3	16.4 ± 0.124	34.4 ± 0.2	18.8 ± 0.6
	70.8	0.31 ± 0.002		

* pK_a of 3-chloropyridine = 2.8: data at these pH values are less accurate because of small extinction changes.

In Table 2 are gathered data for the hydrolyses of five β-D-galactopyranosylpyridinium salts at pH 6.0. This pH is sufficiently basic that the free pyridines are substantially deprotonated—and hence have a u.v. absorption markedly different from that of the glycosyl salts—but it is still 2 units away from the pH at which the base-catalysed pathway made a significant contribution to the hydrolysis of the 3-chloropyridinium salt. There are two further reasons for confidence that base-catalysed pathways are making no significant contribution to the hydrolyses of the other pyridinium salts we studied. First, the entropies of activation are similar to that for the 3-chloropyridinium salt, whereas entropies of activation for the base-catalysed hydrolyses of aryl galactosides are negative.[13] Secondly, the products from the glycosyl salts of basic pyridines are not pyridine degradation products; and an 88% yield of pyridine was obtained from the hydrolysis of β-D-galactopyranosylpyridinium bromide. However, the sugar was degraded under the vigorous reaction conditions used (14 days at 110 °C and pH 6) and only a

TABLE 2

Hydrolysis of β-D-galactopyranosyl salts at pH 6.0 †

Aglycone	T/°C	$10^6\ k$/s^{-1}	E_a/ kcal mol^{-1}	$\Delta S^{\ddagger}$/ cal mol^{-1} K^{-1}
3-Chloropyridine	25.0	0.014 *		
	63.8$_3$	8.34	32.3	13.8
		7.97	±0.2	±0.7
		8.32		
	72.5$_8$	27.5		
		29.1		
		28.3		
	72.9	31.8		
	81.8$_8$	101, 102, 104		
	100.25	966		
3-Bromopyridine	25.0	0.013 8*		
	48.9$_5$	0.740		
		0.750		
		0.753		
	58.8	3.99	31.6	11.6
		4.11	±0.4	±1.1
		3.63		
	70.6	17.87		
		17.82		
	100.0	71.4		
		82.9		
4-Bromoisoquinoline	25.0	0.001 9 *		
	70.6	3.38		
		3.85		
	77.4	12.15	33.5	14
	100.8	24.1	±0.7	±2
		19.0		
	110.0	67.1		
		62.7		
Pyridine	25.0	0.000 027 *		
	110.0	18.6		
		12.2	35	11
		16.4	±2	±5
	137.5	357		
		451		
Isoquinoline	25.0	0.000 003 3 *		
	110	8.41	38.8	19
		8.66	±1.3	±3
	137.5	30.6		
		24.4		
α-L-Arabino-pyranosyl-pyridinium ion	25.0	0.000 092 *		
	100.0	13.28	34	9.7
	110.0	31.95	±2	±5.2
	120.5	142.53		
	137.5	852.07		

* Extrapolated from data at other temperatures. † Measured pH at 22 °C.

[9] B. Capon, *Chem. Rev.*, 1969, **69**, 407.
[10] P. Karrer, A. Widmer, and J. Straub, *Helv. Chim. Acta*, 1924, **7**, 519.
[11] G. Kohnstam, *Adv. Phys. Org. Chem.*, 1967, **5**, 1.
[12] K. A. Cooper, E. D. Hughes, C. K. Ingold, G. A. Maw, and B. J. MacNulty, *J. Chem. Soc.*, 1948, 2049.
[13] R. C. Gasman and D. C. Johnson, *J. Org. Chem.*, 1966, **31**, 1830.

21.1% yield of galactose was obtained, the solution having gone brown. However, from the faster reacting 3-chloropyridinium salt, an 83.1% yield was obtained after 2.3 h at 100 °C and pH 6.

The effect on rate of removing the hydroxymethyl group at C-5 to give α-L-arabinopyranosylpyridinium bromide is the same as in other reactions in which glycopyranosyl cations are generated. Thus, at 25 °C, the α-L-arabinopyranosyl *vs.* β-D-galactopyranosyl rate ratio is 3.4 : 1 for the S_N1 hydrolysis of the pyridinium salts and 2.6 : 1 for the S_N1 hydrolysis of the 2,4-dinitrophenyl glycosides,[14] although the possible error on the first ratio is large. However, the limited data available on the base-catalysed hydrolyses of glycosides appear to indicate that removal of the C-5 hydroxymethyl group has a similar effect on this process.[15]

If the data in Table 2 are extrapolated to give rates at 25 °C, then a linear free energy relationship can be shown to exist between the hydrolysis of the β-D-galactopyranosylpyridinium salts and protonation of the pyridine. The gradient of the log k *vs.* pK_a plot is -1.26 ± 0.12 and its correlation coefficient is -0.987. That the gradient is steeper than unity indicates that there is a bigger change in charge on nitrogen in going from the quaternary glycosyl salt to the transition state for its hydrolysis, than there is in completely protonating the pyridine in water. A plausible explanation for this apparent paradox lies in the probability that in aqueous solution the pyridines in both forms are hydrogen-bonded in ways that will effectively reduce the difference in charge on nitrogen (Scheme 2). This interpretation receives support from recent work on the gas-phase basicities of pyridines:[16] for a series of 3- and 4-substituted pyridines, the plot of gas-phase proton affinity against standard free energy of aqueous protonation had a gradient of 2.3, *i.e.* there was a considerable attenuation of substituent effects in aqueous solution.

The α-deuterium kinetic isotope effect for the hydrolysis of the β-D-galactopyranosylpyridinium ion, $k_H/k_D = 1.13 \pm 0.04$ at 100 °C (corresponding, if the isotope effect is 'classical' and has its origin solely in the differing energies of activation, to one of 1.16 at 25 °C) is somewhat below the range commonly regarded as indicating the generation of a free (secondary) carbocation.[17] However, the validity of detailed arguments derived from the precise values of α-deuterium kinetic isotope effects has been questioned even in simple secondary cases,[18] and reactions resulting in the generation of α-oxocarbocations often show low isotope effects.[19] On empirical grounds this isotope effect is typical of those observed in reactions involving glycopyranosyl ions, such as the acid-catalysed hydrolysis of phenyl β-D-glucopyranoside (1.13 at 50 °C [20]).

(B) *Comparison of Spontaneous and β-Galactosidase-catalysed Hydrolyses.*—The k_{cat} value for the enzymic hydrolysis of the 3-chloro-1-(β-D-galactopyranosyl)-pyridinium ion [1] is $10^{2.0}$ s^{-1} at pH 7.0 and 25 °C; its spontaneous hydrolysis rate is 0.7×10^{-8} s^{-1}. This acceleration, by a factor of $10^{10.2}$, corresponds to a lowering of the free-energy barrier to C–N heterolysis of 13.8 kcal mol^{-1} on formation of the ES complex. In solution this barrier has a height of 28.5 kcal mol^{-1}, so it is halved in the Michaelis complex. The magnitude of this rate enhancement stills any residual doubts that because of the unnatural nature of the galactosyl-pyridinium salts their β-galactosidase-catalysed hydrolysis does not yield valid information on the enzyme mechanism. Unnatural or not, β-galactosidase displays towards these substrates a greater catalytic efficiency than an enzyme occupying a crucial position in primary metabolism does towards its natural substrate. Triose phosphate isomerase, an enzyme considered to have reached evolutionary perfection, accelerates the rate of enolisation of dihydroxyacetone phosphate by a factor of 10^9.[21]

Moreover, the catalytic efficiency of β-galactosidase towards the pyridinium salts increases with the basicity of the parent pyridine. This is a consequence of the more marked dependence of non-enzymic hydrolysis on aglycone pK_a. For the pyridinium salts, k_{cat} represents the bond-breaking step, and so plots of log k_{cat} against aglycone pK_a are linear, and have gradients ($-\beta$) of -0.93 and -0.73 for the Mg^{2+}–enzyme and for the Mg^{2+}-free enzyme,[22] respectively. Therefore the Mg^{2+}–enzyme accelerates the hydrolysis of the isoquinoline salt by a factor of 10^{12} and the Mg^{2+}-free enzyme by a factor of $10^{12.5}$. That the β value for the spontaneous hydrolyses should be significantly greater than those for the enzymic hydrolyses follows from their much greater rates. The Hammond postulate predicts that, as the reaction becomes faster, the transition state will become more reactant-like. A more reactant-like transition state implies more positive charge on the pyridine nitrogen. The change in charge between ground and transition states is thus less and is manifested in a smaller β value.

The observation of the same α-deuterium kinetic isotope effect for the hydrolysis of a pyridinium salt which is, by other criteria, unambiguously unimolecular, as for the enzymic hydrolyses of the pyridinium salts ($k_H/k_D = 1.187 \pm 0.046$ [1] for the 4-bromoisoquinolinium salt and 1.136 ± 0.040 and 1.17 ± 0.03 for the unsubstituted salt with Mg^{2+} – [1] and Mg^{2+}-free [22] enzyme, respectively) confirms our interpretation of these latter effects as indicating that the generation of a galactosyl

[14] D. Cocker and M. L. Sinnott, *J.C.S. Perkin II*, 1975, 1391.
[15] B. N. Stepanenko and O. G. Serdyuk, *Doklady Akad. Nauk S.S.S.R.*, 1964, **154**, 877 (English trans. p. 137).
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[17] V. J. Shiner and R. D. Fisher, *J. Amer. Chem. Soc.*, 1971, **93**, 2553.
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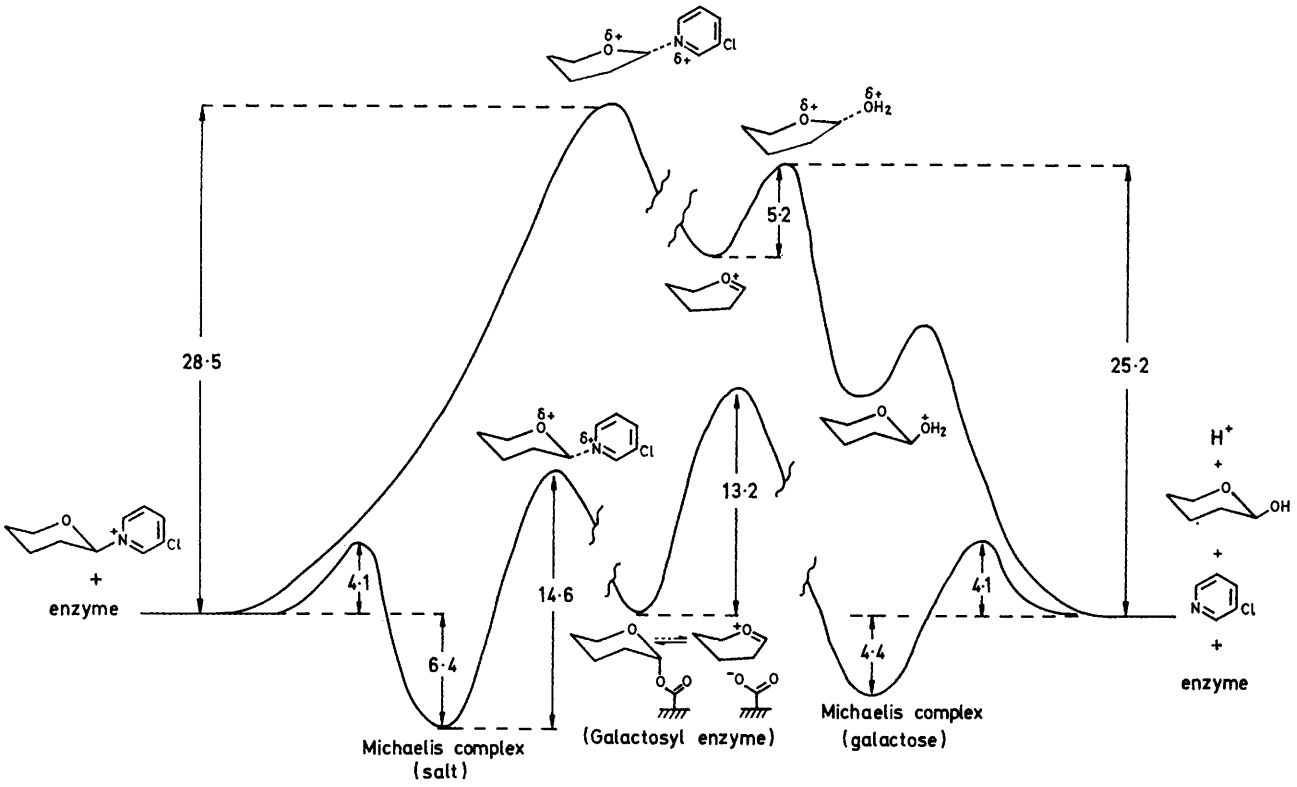
[21] A. Hall and J. R. Knowles, *Biochemistry*, 1975, **14**, 4348.
[22] M. L. Sinnott, O. M. Viratelle, and S. G. Withers, *Biochem. Soc. Trans.*, 1975, **3**, 1005.

cation, rather than a direct displacement is involved in C–N cleavage. · The enzyme must therefore bring about the entire rate enhancement of between 10^{10} and $10^{12.5}$ times solely by substrate destabilisation. Included in this term would be not only conformational distortion of the pyranose ring but also desolvation and various electrostatic effects. Scheme 3 illustrates the energetics of enzymic and non-enzymic reactions in the case of the 3-chloro-1-(β-D-galactopyranosyl)pyridinium ion: the standard state is mole fraction 1.0. Since the free-energy difference between free and bound substrate is dependent on this standard state, no meaningful distinction can be made between destabilisation of the bound substrate and stabilisation of the enzymic transition state. Extension of the profile beyond the first enzymic transition state requires two pieces of information which are not yet available: the equilibrium constant for the hydrolysis of this substrate, and the rate of the reaction of bound β-D-galactopyranose to give the galactosyl enzyme. However, the barrier to hydrolysis of the galactosyl enzyme is known fairly accurately,[23] and the free energy of binding of β-D-galactopyranose is known approximately from competitive inhibition data:[24] these two values are incorporated in Scheme 3. Binding of the pyridinium salt and of galactose is assumed to be diffusion-controlled, with a rate of 10^8 l mol^{-1} s^{-1}. In the reaction profile for the non-enzymic hydrolysis, the energy barrier to collapse of the galactosyl cation is presumed to be the same as that for the collapse of the diphenylmethyl cation, an ion of comparable stability,[25,26] and the barrier between cation and free sugar is assumed to be the same as that for the hydrolysis of methyl β-D-galactopyranoside in 1.0M-hydrogen chloride.[27] The overall rate of acid-catalysed hydrolysis of non-bulky alkyl glycosides is insensitive to the nature of the alkyl group,[9] and so extrapolation to the virtual reaction of galactose itself seems justified. The proton-



SCHEME 3 Reaction profile for the uncatalysed (top) and β-galactosidase-catalysed (bottom) hydrolysis of the 3-chloro-1-(β-D-galactopyranosyl)pyridinium ion at 25 °C; numbers are differences in Gibbs free energy (kcal mol^{-1}), for a standard state of unit mole fraction

ated sugar is shown as a discrete intermediate, but its position is arbitrary, as a reliable estimate of its pK_a is not available.

The energy profiles of Scheme 3 are concerned with Gibbs free energy: in an attempt to dissect the lowering of the first barrier to reaction into enthalpic and entropic terms, the temperature variation of the rate of enzymic hydrolysis of this substrate was studied. The hazards of a simple approach to the effect of temperature on enzymic rates have been documented by Talsky,[28] the three greatest being temperature-dependence of an essential ionisation, change in rate-limiting step, and protein conformation changes. To avoid the first two dangers the 3-chloropyridinium salt was studied at pH

[23] M. L. Sinnott and O. M. Viratelle, *Biochem. J.*, 1973, **133**, 81.
[24] O. M. Viratelle, Thèse du Troisieme Cycle, Faculté des Sciences d'Orsay, Université de Paris, 1970.
[25] D. Cocker, L. E. Jukes, and M. L. Sinnott, *J.C.S. Perkin II*, 1973, 190.
[26] S. Winstein, *Quart. Rev.*, 1969, **23**, 141.
[27] C. K. De Bruyne and G. van der Groen, *Carbohydrate Res.*, 1972, **25**, 59.
[28] G. Talsky, *Angew. Chem. Internat. Edn.*, 1971, **10**, 548.

6.5 in sodium phosphate buffer. Phosphate has a low heat of ionisation, and in any case this is the only pH region in which both k_{cat} and K_m are reasonably insensitive to pH.[22] At this pH also the hydrolysis of the pyridinium salt is about 15 times slower than that of the galactosyl-enzyme.[8] The Arrhenius plot in Figure 2

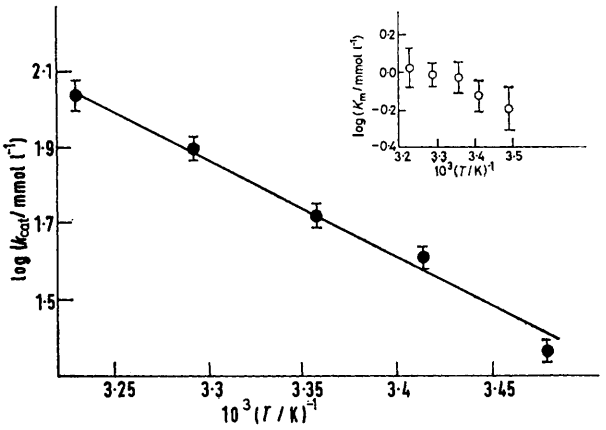


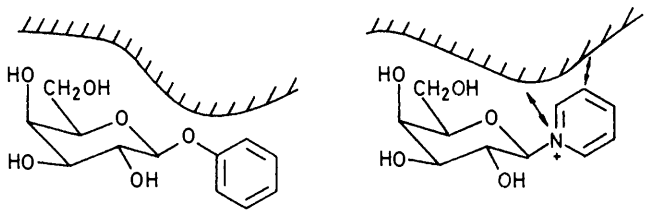
FIGURE 2 Effect of temperature on the β-galactosidase-catalysed hydrolysis of the 3-chloro-1-(β-D-galactopyranosyl)pyridinium ion; data in the inset indicate an enthalpy of binding of *ca.* 4 kcal mol^{-1}

shows that, at least over the temperature range studied, the enzymic hydrolysis is behaving classically, the derived parameters being $\Delta H^{\ddagger} = 11.6 \pm 0.8$ kcal mol^{-1}; $\Delta S^{\ddagger} = -11.8 \pm 2.7$ cal mol^{-1} K^{-1}. The temperature dependence of K_m was so slight that a precise estimate of the heat of binding could not be obtained (for these pyridinium salt substrates $K_m = K_s$ [1]).

If the simple approach to the temperature dependence of k_{cat} is justified, then the activation parameters thereby derived stand in striking contrast to those for the uncatalysed hydrolysis (Table 2). A lowering of $\Delta H^{\ddagger}$ is to be expected, but a lowering by 20.7 kcal mol^{-1} is dramatic. More surprisingly the enzyme appears to be working against itself entropically, the entropy of activation being 25 cal mol^{-1} K^{-1} more negative when the S_N1 reaction is carried out in the ES complex than when it is carried out in solution. A plausible picture of the origin of this large fall in entropy of activation can be arrived at if the ideas of a barrier to reaction in solution arising both from an intrinsic barrier to chemical change and an energy required to reorganise the solvent around the reacted species are applied. Such a picture is applied to proton transfer and to solvolysis reactions to acetic acid. On this picture the free-energy barrier to the S_N1 reaction of the pyridinium salt in solution will arise both from the strength of the C–N bond and from the structure of the solvent cage around the cation. As the C–N bond is broken, there is dispersal of charge, and a consequent disordering of the solvent shell, resulting in a positive entropy of activation. In the ES complex, however, the extent of the solvent shell around the pyridinium salt is necessarily considerably reduced, if not entirely eliminated. There must thus be less disordering of the solvent as the C–N bond is broken.

(C) *Extent of Substrate Destabilisation not Uniquely Associated with Pyridinium Salt Substrates.*—The k_{cat} value for the β-D-galactopyranosyl[U-^{2}H]pyridinium salt is *ca.* 8% smaller than that of the protiated salt. Since there is a 4% increase [29] in the basicity of the deuteriated pyridine, which would be reflected in a 4% lowering of k_{cat}, further factors must only produce an isotope effect of *ca.* 4%. Such small isotope effects are encountered whenever there is a change in environment of the deuteriated species, and are attributed to small changes in vibration and rotation frequencies. Thus, similar effects of about 0.8% per deuterium are found in vapour-pressure equilibria [30] and partition chromatography.[31]

The smallness of this isotope effect, however, enables a possible objection to the extrapolation of results from the pyridinium salts to oxygen substrates to be discounted. It could be argued that since the aglycones of glycosylpyridinium salts lie closer to the glycone residue for which the enzyme has specificity than the aglycones of oxygen glycosides, steric clashes with some groups on the enzyme are possible with the pyridinium salts but not with oxygen glycosides. These clashes with, for example, an acid-catalytic group, could result in a far greater contribution of steric acceleration to the departure of the pyridine than to that of an alcohol or phenol (Scheme 4).



SCHEME 4

However, were this the case a large secondary deuterium isotope effect would be expected. Unimolecular departure of axial $-NMe_3^+$ in the steroid series is associated with a secondary isotope effect [for $-N(CD_3)_3^+$] of $k_H/k_D = 1.71$ at 70 °C, or 1.85_5 at 25 °C.[32] In an *E2* reaction, where this leaving group is unhindered, the isotope effect can be calculated from data of the same authors [32] to be *ca.* 2% per deuterium atom, compared with *ca.* 9% per deuterium atom where the leaving group is very hindered. This additional large effect is best explained on the basis of the C–D bond being shorter than the C–H bond (because of the greater amplitude of the ground-state vibration of the C–H bond).[33] In systems where the departure of the

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[31] N. Tanaka and E. R. Thornton, *J. Amer. Chem. Soc.*, 1976, **98**, 1617.
[32] G. H. Cooper, J. S. Bartlett, A. M. Farid, S. Jones, D. J. Mabbott, J. McKenna, J. M. McKenna, and D. G. Orchard, *J.C.S. Chem. Comm.*, 1974, 950.
[33] W. A. Van Hook, 'Isotope Effects in Chemical Reactions,' ed. C. J. Collins and N. S. Bowman, Van Nostrand, New York, 1970, p. 56.

leaving group is subject to steric acceleration, therefore, the smaller (deuteriated) leaving group leaves more slowly.

Indeed, the isotope effect on the spontaneous hydrolysis of β-D-galactopyranosylpyridinium ion is significantly higher than that on its enzymic hydrolysis, being 1.157 ± 0.019 at 100 °C, or 1.20 at 25 °C. This is much bigger than the isotope effect on the pK_a, and can be used to support, in a non-rigorous and intuitive fashion, the picture that best explained the differing entropies of activation of enzymic and non-enzymic hydrolyses, *viz.* that in solution the cation is tightly solvated and highly constrained by the water molecules, but that when the cation is bound to the enzyme this constraint by solvent is ameliorated.

Further indication that a rate enhancement of 10^{10}—$10^{12.5}$ times is not uniquely associated with the pyridinium salts comes from failure to observe any products from alkyl–oxygen fission in the enzymic hydrolysis of ferrocenylmethyl β-D-galactopyranoside (Scheme 1). The β-D-glucopyranoside of this aglycone is hydrolysed in acid 10^5—10^6 times faster than methyl β-D-glucopyranoside,[9] and this hydrolysis is associated with complete alkyl–oxygen fission.[34] Steric acceleration to the departure of the aglycone [25] is unlikely to provide any contribution to the enhanced rate of this compound, as the aglycone is primary. Therefore one can estimate that alkyl–oxygen fission is favoured over glycosyl–oxygen fission in the exocyclically protonated glycoside by a factor of 10^5—10^6. Because of the greater ease of formation of the galactosyl cation [14] this factor must be reduced to *ca.* 10^5 for the ferrocenylmethyl galactoside. When this compound is hydrolysed in aqueous 5% (v/v) methanol by acid, a 20% yield of methoxymethylferrocene is produced: with the same concentration of methanol, <0.1% methoxymethylferrocene is produced by β-galactosidase. Therefore <0.5% alkyl–oxygen fission takes place during β-galactosidase-catalysed hydrolysis. If the exocyclic oxygen atom is completely protonated in the ES complex, glycosyl–oxygen fission is accelerated, by means other than acid catalysis, by a factor of $>10^{7.3}$ over reaction in solution. On conventional models of glycosidase action, of course, the aglycone is only partially protonated at the transition state; this reduction in the reactivity of the species would be expected to lead to an increased discrimination between the two modes of fission, and so the figure of $10^{7.3}$ is a lower limit on the non-acidic enzymic acceleration of glycosyl–oxygen fission on two counts.

Methanolysis of galactosyl-β-galactosidase is associated with α-deuterium kinetic isotope effects which are most plausibly associated with nucleophilic attack by methanol on the ion-paired form of the galactosyl-enzyme.[8] Microscopic reversibility then requires enzymic hydrolysis of methyl β-D-galactopyranoside to proceed *via* a galactosyl cation.* Since the nucleophilic properties of the hydroxy-group of ferrocenylmethanol will resemble those of the hydroxy-group of methanol, it is a small extrapolation to say that hydrolysis of ferrocenylmethyl galactoside also proceeds through an enzyme-bound galactosyl cation. Substrate destabilisation would then account for a rate enhancement of at least $10^{7.3}$ times, even for an alkyl galactoside.

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* The enzyme exhibits simple Michaelian kinetics even in M-methanol, and so is unlikely to be a ' one-way ' enzyme with a rate approaching zero as equilibrium is reached.

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