The Necessity of Magnesium Cation for Acid Assistance of Aglycone Departure in Catalysis by *Escherichia coli* (*lacZ*) β -Galactosidase

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1. Removal of Mg^{2+} from *Escherichia coli* (*lacZ*) β -galactosidase slightly increases the rate of hydrolysis of galactosyl pyridinium salts, but decreases the rate of hydrolysis of aryl galactosides. 2. Fair correlation of $\log k_{cat}$ and $\log (k_{cat}/K_m)$ with the pK_a of aglycone is now observed for aryl galactosides, as well as for glycosyl pyridinium salts. 3. Degalactosylation of Mg^{2+} -free enzyme is the rate-limiting step in the hydrolysis of 2,4-dinitrophenyl galactoside. 4. α -Deuterium kinetic isotope effects for both sets of substrates are consistent with the rate-determining generation of a glycosyl cation. 5. The pH-independent, S_N1 hydrolysis of 3,4-dinitrophenyl galactoside has been measured: it is as fast as that of the galactosyl 3-chloropyridinium ion. 6. Hydrolysis of these two substrates by Mg^{2+} -free enzyme proceeds at very similar rates. 7. It is concluded that loss of both types of aglycone takes place, without acid catalysis, from the first ES complex of substrate and apoenzyme. 8. Data for galactosyl azide and thiopicrate confirm that neither charge nor change of atom is the cause of the differences in behaviour between aryl galactosides and galactosylpyridinium salts.

The *lacZ* β -galactosidase of *Escherichia coli* is a metallo-enzyme, bivalent metal cations, such as Mg^{2+} or Mn^{2+} , being required for maximal activity against 2-nitrophenyl galactoside (Tenu *et al.*, 1972). One Mg^{2+} cation per subunit of the tetrameric enzyme is bound (Case *et al.*, 1973). Most kinetic measurements have hitherto been made on the Mg^{2+} -saturated enzyme.

We have discovered a novel class of synthetic substrates for this enzyme, the β -D-galactopyranosylpyridinium salts (Sinnott & Withers, 1974). The assistance of an electrophile or acid to the departure of pyridine is structurally impossible, and α -deuterium kinetic isotope effects discount the direct participation of a nucleophile. These enzyme-catalysed unimolecular reactions are nonetheless 10^{10} – $10^{12.5}$ faster than the same S_N1 reactions in free solution (Jones *et al.*, 1977).

A short preliminary note that removal of Mg^{2+} slightly increased the activity of the enzyme towards the pyridinium salts has appeared (Sinnott *et al.*, 1975). The contrast in behaviour of these and conventional substrates extends to the molecular nature of the first step after formation of the initial complex of substrate and Mg^{2+} -enzyme. This is bond-breaking in the case of the pyridine salts (Sinnott & Withers, 1974), but a conformation change in the case of aryl galactosides (Sinnott & Souchard, 1973; Fink, 1977). Since Mg^{2+} and the cationic inhibitor

β -D-galactopyranosyltrimethylammonium bromide do not markedly influence each others binding to the enzyme, a direct electrophilic-catalytic role for Mg^{2+} seems unlikely (Case *et al.*, 1973). A hypothesis that accommodates these findings is that the conformation change is the Mg^{2+} -mediated correct placing of an acid-catalytic group. We now report experiments that verify this hypothesis.

Experimental

lacZ β -galactosidase

Enzyme from three sources was used. It was isolated from constitutive mutant 2E01 (Tenu *et al.*, 1971) or merodiploid strain A324-5 (Fowler, 1972), essentially by the method of Craven *et al.* (1965), or purchased from Boehringer, Uxbridge, Middx., U.K. (lot no. 7423216). No differences in normalized kinetic parameters were observed with enzyme from these sources.

Substrates

3,4-Dinitrophenyl β -D-galactopyranoside monohydrate, m.p. 176–180°C, $[\alpha]_D^{25} -33$ (c 1 in water) was made by Zemplén deacetylation of its tetra-O-acetyl derivative (Found: C, 39.1; H, 4.1; N, 7.3; $C_{12}H_{14}N_2O_{10} \cdot H_2O$ requires C, 39.6; H, 4.4, N, 7.7%). 3,4-Dinitrophenyl 2,3,4,6-tetra-O-acetyl- β -D-galactopyranoside, m.p. 145–147°C, $[\alpha]_D^{25} -9$ (c 1 in dichloro-

methane) was made in 50% yield from acetobromogalactose by the method of Latham *et al.* (1950) (Found: C, 46.6; H, 4.3; N, 5.1; $C_{20}H_{22}N_2O_{14}$ requires C, 46.7; H, 4.3, N, 5.5%).

2,4,6-Trinitrophenyl 1-thio- β -D-galactopyranoside. Qualitative observations on the β -galactosidase-catalysed hydrolysis of this compound have been reported (Wallenfels *et al.*, 1964), but no quantification of the enzymic process or characterization of the substrate has since appeared. Picrylation (Sinnott & Whiting, 1971) of 2,3,4,6-tetra-*O*-acetyl-1-thio- β -D-galactopyranose (Černý *et al.*, 1963) afforded 2,4,6-trinitrophenyl 2,3,4,6-tetra-*O*-acetyl-1-thio- β -D-galactopyranoside, m.p. 188–190°C, $[\alpha]_D^{25} +157$ (*c* 1.3 in chloroform) (Found: C, 41.5; H, 3.9; N, 7.1; S, 5.2; $C_{20}H_{21}O_{15}N_3S$ requires C, 41.7; H, 3.7; N, 7.3; S, 5.6%). Deacetylation with methanolic HCl (Ballardie *et al.*, 1973) yielded the *title compound*, m.p. 149–152°C (decomposition) (Found: C, 35.7; H, 3.4; N, 9.3; $C_{12}H_{13}N_3O_{11}S$ requires C, 35.4; H, 3.2; N, 10.3%). Other substrates with nitrogen aglycones (Sinnott & Withers, 1974) or with oxygen aglycones (Sinnott & Souchart, 1973) have been described.

Enzyme kinetic measurements

Mg^{2+} -free enzyme was obtained by dialysing the $(NH_4)_2SO_4$ slurry of the enzyme for 36 h at 4°C against a solution of final pH 7.0 (1000 vol.) containing 145 mM-NaCl, 33 mM-sodium phosphate and 10 mM-EDTA, which was changed twice. This medium was used for kinetic studies with apoenzyme. For holoenzyme 100 mM-sodium orthophosphate, pH 7.0, containing 1 mM- $MgCl_2$ was used. The spectrometer, calculation of results, wavelengths and absorption coefficients for measurement of isotope effects and Michaelis parameters have been described (Sinnott & Souchart, 1973; Sinnott & Withers, 1974) with the exception of 3,4-dinitrophenyl galactoside and galactosyl thiopicrate. Hydrolysis of 3,4-dinitrophenyl galactoside was followed by change of A_{400} , and that of galactosyl thiopicrate by change of A_{390} ; extinction coefficient changes were respectively 1.2×10^4 and 8.6×10^3 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$.

This last value was obtained by complete hydrolysis of the substrate with increasing amounts of enzyme until no further increase in maximal absorbance was apparent. Air oxidation of thiopicrate anion rapidly decreases the observed absorbance.

Spontaneous hydrolyses

These were measured as described previously (Jones *et al.*, 1977), but the reaction was monitored by the change of A_{400} .

Results and Discussion

Hydrolysis of galactosylpyridinium salts by apoenzyme

Michaelis–Menten parameters are given in Table 1,

Table 1. *Michaelis–Menten parameters for the hydrolysis of β -D-galactopyranosyl derivatives by Mg^{2+} -free Z-gene β -galactosidase*

Data are from experiments at 25.0°C in 10 mM-sodium EDTA / 33 mM-sodium phosphate buffer, pH 7.0, containing 0.145 M-NaCl. The k_{cat} values are relative to that for 2-nitrophenyl galactoside hydrolysed by Mg^{2+} -enzyme (1100).

Aglycone	K_m (mM)	k_{cat} (s^{-1})
3-Chloropyridine	0.85 ± 0.02	154
3-Bromopyridine	1.01 ± 0.05	163
4-Bromoisoquinoline	1.52 ± 0.02	73
Pyridine	2.73 ± 0.02	2.8
Isoquinoline	1.03 ± 0.11	1.07
3-Methylpyridine	2.32 ± 0.10	1.50
3,5-Dimethylpyridine	5.20 ± 0.20	0.98
Azide	2.40 ± 0.20	0.22
2,4-Dinitrophenol	0.34 ± 0.03	362
3,4-Dinitrophenol	0.32 ± 0.02	201
3,5-Dinitrophenol	0.33 ± 0.02	89
4-Nitrophenol	0.23 ± 0.01	26.5
2-Nitrophenol	0.70 ± 0.04	120
4-Cyanophenol	0.23 ± 0.01	6.0
3-Nitrophenol	0.53 ± 0.03	53.0
4-Bromophenol	0.44 ± 0.05	0.51
2-Naphthol	1.28 ± 0.12	3.22
Phenol	0.79 ± 0.09	2.15
3-Methylphenol	1.15 ± 0.11	1.77
3-t-Butylphenol	0.61 ± 0.06	3.72
3,4-Dinitrophenol	$0.18 \pm 0.01^*$	1289*
2,4,6-Trinitrothiophenol	0.122 ± 0.015	101
	$0.182 \pm 0.019^*$	340*

* For Mg^{2+} -enzyme, in 0.1 M-sodium phosphate buffer, pH 7.0.

and k_{cat} values, together with those for aryl galactosides and pertaining to Mg^{2+} -enzyme, are displayed in Fig. 1 as a function of the pK_a of aglycone. The removal of Mg^{2+} slightly increases k_{cat} for pyridinium salts, although K_m values are higher than for hydrolysis by Mg^{2+} -enzyme (Sinnott & Withers, 1974), as with other substrates.

k_{cat} values for hydrolysis of the pyridinium salts by apoenzyme, as for holoenzyme, substantially represent rates of C–N bond cleavage. This is shown by their simple dependence on pyridine pK_a (see Table 2 for ‘goodness of fit’ parameters) and an α -deuterium kinetic isotope effect (k_H/k_D) of 1.17 ± 0.03 for the unsubstituted salt. This isotope effect is the same, within experimental error, as that for its hydrolysis by holoenzyme (Sinnott & Withers, 1974) or for its spontaneous S_N1 hydrolysis (Jones *et al.*, 1977), and indicates the intermediacy of a galactosyl cation.

There is, however, apparently a significant difference in the value of the slope (β_{lg}) of the $\log k_{cat}$ against pK_a plot, that for apoenzyme being lower.

Fig. 1. $\log k_{\text{cat}}$ for the hydrolysis at 25°C and pH 7.0 of β -Gal-*p*-X by the *lacZ* β -galactosidase of *E. coli* as a function of the pK_a of HX

Triangles refer to pyridines, squares to phenols, filled symbols to Mg^{2+} -enzyme and open symbols to Mg^{2+} -free enzyme. Points pertaining to galactosides of *ortho*-substituted phenols are marked *o*, and arrowed points indicate that the degalactosylation rate has been reached. pK_a values for phenols are taken from the compendium of Kortum *et al.* (1961) and those for pyridines from that of Perrin (1972).

We originally attributed this to a 'Hammond postulate' effect on the basis of the expected decrease in selectivity towards leaving ability consequent upon the higher reactivity of the ES complex with apoenzyme (Sinnott *et al.*, 1975). It has since been shown that, for 2,4-dinitrophenyl galactoside hydrolysed by apo- as well as holo-enzyme, degalactosylation is rate-limiting (see under 'Hydrolysis of aryl galactosides by apoenzyme'). There is thus an error factor of 0.2–0.3 log units introduced by assuming k_{cat} , and rates of bond cleavage are identical for the two fastest substrates hydrolysed by apoenzyme. If this is accounted for, either from $k_{\text{cat}} = k_{+2}k_{+3}/(k_{+2} + k_{+3})$, or by correlating $\log k_{\text{cat}}/K_m$ with the pK_a of aglycone, a similar value of β_{lg} for apoenzyme to that for holoenzyme is obtained.

The similarity of k_{cat} and K_m values for the pyridinium salts with and without Mg^{2+} means that the presence of this cation does not alter any free-energy difference on the reaction profile for the enzymic

hydrolysis of these salts (Jones *et al.*, 1977) by more than 2RT. K_m values probably represent K_s for all salts except the 3-halopyridinium ions hydrolysed by apoenzyme (cf. Sinnott & Withers, 1974). The effect of removal of Mg^{2+} upon degalactosylation is likewise fairly small (4-fold decrease; see under 'Hydrolysis of aryl galactosides by apoenzyme'). The effect on the hydrolysis of aryl galactosides is more pronounced, decreases in k_{cat} of over two orders of magnitude being observed with the poorer leaving groups.

The simplest model that accommodates this contrasting behaviour is that Mg^{2+} is acting as an electrophilic catalyst. Other evidence, however, shows that this is not the case. First, direct n.m.r. spectroscopic measurements show that Mn^{2+} at least is too far from the aglycone (M. L. Sinnott & B. D. Sykes, unpublished work), confirming indications obtained previously (Case *et al.*, 1973). Secondly, Mg^{2+} is in any event a poor electrophile. Thirdly, removal of Mg^{2+} decreases k_{cat} for galactosyl azide from 44 s^{-1} (Sinnott, 1971) to 0.22 s^{-1} . Bond breaking is rate-limiting with holoenzyme for this substrate (Sinnott & Withers, 1974). Mg^{2+} , however, forms much stronger complexes with oxygen ligands than with nitrogen ligands, and would be expected to give much less assistance to the departure of azide than to the departure of a phenol. The data on galactosyl azide also show that the contrasting behaviour towards aryl galactosides and glycosylpyridinium ions, of holoenzyme and apoenzyme, arises from the susceptibility or otherwise of the aglycone to proton donation, and not from the elements (N or O) in the bond cleaved.

Hydrolysis of aryl galactosides by apoenzyme

The rate-limiting step in the hydrolysis of 2,4-dinitrophenyl galactoside is degalactosylation. This was shown by nucleophilic competition with methanol (Viratelle & Yon, 1973; Sinnott & Viratelle, 1973). If the kinetics of the enzyme are analysed by the simple two-step mechanism:

then the parameters governing the appearance of X are given by:

$$k_{\text{cat}} = \frac{k_{+2}(k_{+3} + k_{+4}[\text{methanol}])}{k_{+2} + k_{+3} + k_{+4}[\text{methanol}]}$$

and

$$K_m = \frac{k_{-1} + k_{-2}}{k_{+1}} \left(\frac{k_{+3} + k_{+4}[\text{methanol}]}{k_{+2} + k_{+3} + k_{+4}[\text{methanol}]} \right)$$

If $k_{+3} < k_{+2}$, then increases in both parameters should be observed with increasing methanol concentration. From Fig. 2 it is seen that this prediction is fulfilled for the hydrolysis of the 2,4-dinitrophenyl compound. No increase in $k_{cat.}$ is observed under these conditions with the (slower) 2-nitrophenyl galactoside (Viratelle & Yon, 1973).

Galactosylpyridinium salts are not susceptible to acid catalysis of their hydrolysis. Removal of Mg^{2+} has only a small effect. Neither do galactosylpyridinium salts experience a conformational change of the ES complex. Aryl galactosides, which are susceptible to acid catalysis of their hydrolysis, do experience such a conformational change. Their hydrolysis is much more influenced by Mg^{2+} . It is therefore a reasonable hypothesis that sensitivity to Mg^{2+} , the presence or absence of a conformational change and the operation of acid catalysis to aglycone departure have their origin in the same molecular events. The simplest pattern of such events is that the Mg^{2+} cation is required for the conformational change that is the correct placing of an acid-catalytic group. This proposal predicts four characteristics of the hydrolyses of aryl galactosides by apoenzyme. (i) A good correlation between the pK_a of aglycone and $\log k_{cat.}$ (ii) A value of β_{lg} for this correlation compatible with reaction without acid catalysis. (iii) A positive α -deuterium kinetic isotope effect for the hydrolysis of all aryl galactosides. (iv) The same absolute rate-enhancement of aryl galactoside hydrolysis as of galactosyl pyridinium ion hydrolysis. These four predictions are in fact fulfilled.

(i) *Correlation between phenol pK_a and $\log k_{cat.}$*
The correlation coefficients in Table 2 are uniformly

higher for hydrolysis of aryl galactosides catalysed by Mg^{2+} -free enzyme than for their hydrolysis by Mg^{2+} -enzyme. The correlations refer to the same compounds, except that the hydrolyses of 2,5-dinitrophenyl and 1-naphthyl galactosides by Mg^{2+} -free enzyme were not studied. These compounds are not in any event included in the two most informative

Fig. 2. Effect of methanol on the hydrolysis of 2,4-dinitrophenyl galactoside by apoenzyme at 25°C

For conditions see the text. Error bars on K_m values are least-squares standard deviations on the slopes of Eadie plots.

Table 2. Analysis of the dependence on aglycone pK_a of enzymic hydrolysis rate

Cation	Substrates-(number)	Omission	Parameter correlated with pK_a	Correlation coefficient	Slope
Mg^{2+}	Pyridinium salts (7)	None	$\log k_{cat.}$	0.997	0.93
None	(7)			0.990	0.72
Mg^{2+}	Pyridinium salts (7)	None	$\log (k_{cat.}/K_m)$	0.987	1.01
None	(7)			0.997	0.87
Mg^{2+}	Aryl galactosides (14)	None	$\log k_{cat.}$	0.686	0.20
None	(12)			0.887	0.44
Mg^{2+}	Aryl galactosides (8)	<i>ortho</i> -Substituted compounds and compounds for which degalactosylation is rate-limiting	$\log k_{cat.}$	0.034	0.02
None	(10)			0.865	0.48
Mg^{2+}	Aryl galactosides (14)	None	$\log (k_{cat.}/K_m)$	0.651	0.13
None	(12)			0.925	0.52
Mg^{2+}	Aryl galactosides (10)	<i>ortho</i> -Substituted compounds	$\log (k_{cat.}/K_m)$	0.651	0.14
None	(10)			0.923	0.60

correlations with phenol pK_a , those of $\log k_{cat.}$, omitting *ortho*-substituted compounds, and substrates for which degalactosylation is rate-limiting, and of $\log(k_{cat.}/K_m)$, omitting *ortho*-substituted compounds. The contrast between Mg^{2+} -free and Mg^{2+} -bound enzyme in these correlations is pronounced, confirming the subjective impression of Fig. 1. That $k_{cat.}/K_m$, as well as the value of $k_{cat.}$, for Mg^{2+} -free enzyme is determined by pK_a indicates that, not only is the bond-breaking step rate-limiting, it is also the first step after formation of the Michaelis complex, as for hydrolysis of the pyridinium salts.

Nonetheless, correlations are poorer than with the pyridinium salts. This difference may arise because aglycone rotation is possible about both glycone-oxygen and aglycone-oxygen bonds for aryl galactosides, but only about the carbon-nitrogen bond for pyridinium salts.

(ii) *Value of β_{lg} .* It is widely stated (e.g. Capon, 1971) that general acid catalysis of the departure of phenols from aryl acetals gives rise to a Hammett ρ value of approx. 1 [corresponding to a β_{lg} value of 0.45 (Barlin & Perrin, 1966)]. However, a detailed study of the general acid-catalysed hydrolysis of benzaldehyde methyl aryl acetals (Capon & Nimmo, 1975) has shown that Hammett plots are in fact curved.

Furthermore, the very fact that an enzymic reaction is so much faster than a model reaction will itself alter the value of β_{lg} . The Hammond postulate predicts that the more reactive a system the less selective it is; i.e., as reactions get absolutely faster, values of β_{lg} will tend to zero. This change is in the same direction as that brought about by partial proton donation.

Fortunately, an estimate of the size of this Hammond-postulate effect is available from a comparison of β_{lg} values for various enzyme hydrolyses and for the spontaneous hydrolyses of the galactosylpyridinium salts (1.26; Jones *et al.*, 1977). The β_{lg} value for the spontaneous S_N1 hydrolyses of aryl galactosides has been estimated as around 1.07 (Jones & Sinnott, 1977, and references therein). Therefore a value of β_{lg} derived from $\log(k_{cat.}/K_m)$ for aryl galactosides hydrolysed by Mg^{2+} -free enzyme of $1.07 \times 0.87/1.26$ (see Table 2) is predicted. The value (0.74) thus calculated is in fair agreement with the observed value (0.60). Because $k_{cat.}$ for 3-chloro- and 3-bromo-pyridinium salts approaches the degalactosylation rate, the apparent value of β_{lg} derived from $\log k_{cat.}$ for the pyridinium salts in the absence of Mg^{2+} does not truly represent that for breaking C-N bonds. However, the $k_{cat.}$ value of 3,4-dinitrophenyl galactoside also approaches the degalactosylation rate. The observed crude β_{lg} value for aryl galactosides (0.52) can then be compared roughly with a predicted crude β_{lg} value of $(1.07 \times 0.72/1.26 = 0.6)$.

The values of β_{lg} for the hydrolysis of the pyridinium salts were originally based on rather extended arguments held to support an S_N1 , rather than an S_N2 , mechanism for the enzyme hydrolysis of these salts. Recent data confirm the correctness of this argument. An unambiguous S_N2 displacement of substituted pyridines from their quaternary salts is characterized by a β_{lg} value of 0.39 (Berg *et al.*, 1976).

(iii) *α -Deuterium kinetic isotope effects.* Fig. 3 shows a plot of the α -deuterium kinetic isotope effects for a series of aryl galactosides hydrolysed by Mg^{2+} -free enzyme. The line is that predicted by a two-step mechanism for which $(k_{+2})_H/(k_{+2})_D = 1.09$ and $(k_{+3})_H/(k_{+3})_D = 1.21$, and all the points, within experimental error, conform to this relationship.

The isotope effect on degalactosylation of apo-enzyme is the same as that on holoenzyme, within experimental error (Sinnott & Souchart, 1973). Our rationalization of this effect in both cases is that the galactosyl-enzyme consists of an unreactive covalent α -galactosyl-enzyme in rapid equilibrium with a small proportion of ion-paired reactive species. The observed isotope effect is then the product of a direct isotope effect on the equilibrium and an inverse isotope effect on the hydrolysis of the ion-pair. Lewis & Wolfenden (1977) have recently shown that the magnitude of the α -deuterium equilibrium isotope effect for the hydration of acetaldehyde is of the right magnitude ($K_H/K_D = 1.37$; $K = [CH_3CHO]/[CH_3CH(OH)_2]$) for the model to be quantitatively, as well as qualitatively, plausible. The hydration of acetaldehyde is a good model for the reversible addition of an oxygen nucleophile such as carboxylate (Legler, 1973) to a glycosyl cation.

The isotope effect on the first step in the hydrolyses of aryl galactosides by apoenzyme indicates that

Fig. 3. α -Deuterium kinetic isotope effects on $k_{cat.}$ for hydrolysis of aryl galactosides by Mg^{2+} -free β -galactosidase

Error bars are standard deviations. The structure of the leaving group is shown next to the appropriate point.

this involves bond breaking, although its magnitude is not really informative as to whether the reaction involves the intermediacy of a glycosyl cation. The ring oxygen atom makes existing theoretical calculations of the magnitudes of these effects, based on hydrocarbon systems, inapplicable. Indeed, Lewis & Wolfenden (1977) have measured a significant difference in the α -deuterium equilibrium isotope effect for the addition of water and 2-mercaptoethanol to acetaldehyde.

There is chemical precedent, however, for thinking that the 9% α -deuterium kinetic isotope effect observed on k_{+2} is compatible with the departure of an anionic leaving group from a glycosyl cation in an S_N1 reaction, although some nucleophilic assistance cannot be discounted. Departure of 2,4-dinitrophenolate is associated with an α -deuterium kinetic isotope effect of 1.11 [calculated from Cocker & Sinnott (1976) assuming $T \ln(k_H/k_D) = \text{constant}$] as is departure of $H_2PO_4^-$ [calculated from Firsov *et al.* (1974) assuming $1.44 \ln(k_H/k_D) = \ln(k_H/k_T)$].

Mohr *et al.* (1973) observed an α -tritium kinetic isotope effect of 1.10 (at 50°C) on the spontaneous hydrolysis of 4-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside. This corresponds to an α -deuterium kinetic isotope effect of 1.08 at 25°C. These authors considered such an isotope effect too big for direct intramolecular displacement of 4-nitrophenolate by amide, and too small for a simple S_N1 departure of leaving group. They proposed rather that displacement of 4-nitrophenolate by amide took place in the reversibly formed intimate ion-pair of glycosyl cation and 4-nitrophenolate. It is always possible that such an $S_N2(ip)$ mechanism is operating with apoenzyme, the nucleophile being the counter-ionic group.

The α -deuterium kinetic isotope effect for 2-nitro-

phenyl galactoside is reliable only in the absence of Mg^{2+} . In its presence the observed α -deuterium kinetic isotope effect decreases with time. T.l.c. examination [cellulose plates in methanol/water (9:1, v/v) as eluent] of old solutions of this substrate reveal the presence of a slower-moving impurity if Mg^{2+} is present, but not in its absence. More impurity is formed from protiated than from deuterated substrate (Withers, 1977). These observations have a rational, if not unique, explanation in the known [see Döpp & Sailer (1975)] abstraction of adjacent alkyl hydrogen atoms by the nitro groups of *ortho*-nitroaryl compound; such a process on the anomeric hydrogen of 2-nitrophenyl galactoside will be subject to a primary isotope effect. The known (Angyal, 1974) complex-formation of Mg^{2+} by sugars then presumably occurs in such a way as to fix the glycoside in a favourable conformation for hydrogen transfer; alternatively traces of transition-metal cations could be removed by the EDTA necessary for work with apoenzyme and these traces could catalyse the hydrogen transfer. These data, however, mean that 2-nitrophenyl glycosides should not be used as standard substrates for the determination of glycosidase activities were more than about 10% accuracy is required.

(iv) *Absolute rate enhancement of the hydrolysis of 3,4-dinitrophenyl galactoside by apoenzyme.* The spontaneous pH-independent hydrolysis of 2,4-dinitrophenyl galactoside has been observed (Cocker & Sinnott, 1975), but k_{cat} for its hydrolysis by β -galactosidase represents decomposition, rather than formation, of the galactosyl-enzyme. Data on the pH-dependence of the non-enzymic hydrolysis of 3,4-dinitrophenyl galactoside are given in Table 3. The pH-independent rate, which extrapolates to $8 \times 10^{-9} s^{-1}$ at 25°C, is characterized by the near-

Table 3. First-order rates of hydrolysis of 3,4-dinitrophenyl galactoside in 1.0M- $NaClO_4$

pH of solution at 25°C	$10^6 \times k (s^{-1})$				
	5.0	6.0	7.0	8.0	9.0
Temperature (°C)					
59.3		1.04	1.26	1.54	
67.6			3.0	4.0	
72.6		5.6			
72.85	5.2	5.6			
77.1					
77.7			8.9	12.9	
80.5	19.9	14.7		17.4	
89.1	32.6				58.2
99.5	138		118	137	198
110.0	329	284	272	335	519
25	$0.0072 \pm 0.0044^*$	$0.0083 \pm 0.0007^*$	$0.0111 \pm 0.005^*$	$0.0155 \pm 0.0023^*$	$0.01 \pm 0.006^*$
$\Delta H^\ddagger$ (kcal)	28 ± 2	26.8 ± 0.3	26 ± 2	25.5 ± 0.5	27.6 ± 1.2
$\Delta S^\ddagger$ (cal·mol ⁻¹ ·K ⁻¹)	-1 ± 6	-4 ± 1	-5 ± 5	-7 ± 2	0 ± 4

* Extrapolated data.

zero entropy of activation diagnostic of an S_N1 reaction. This rate of $1 \times 10^{-8} \text{ s}^{-1}$ can thus be taken as representing the uncatalysed unimolecular fission of the C-O bond. The $k_{\text{cat.}}$ value of 201 s^{-1} thus represents an absolute rate enhancement of $10^{10.3}$.

Because of the differing β_{lg} values for enzymic and non-enzymic hydrolyses of the galactosylpyridinium salts, the absolute rate enhancement varies with the leaving-group ability of the pyridine. A fair comparison of the absolute rate enhancement observed with 3,4-dinitrophenyl galactoside with that achieved on a galactosylpyridinium salt requires therefore the galactosylpyridinium salt to be comparably labile to 3,4-dinitrophenyl galactoside. Galactosyl 3-chloropyridinium ion has a spontaneous S_N1 rate at 25°C of $7 \times 10^{-9} \text{ s}^{-1}$ (Jones *et al.*, 1977), and thus fulfils this criterion. The $k_{\text{cat.}}$ value for its hydrolysis by apoenzyme is 154 s^{-1} (Table 1), giving a rate acceleration identical with that brought about on the aryl galactoside.

Conclusion: the role of acid catalysis in hydrolysis by β -galactosidase

All four predictions of our model are fulfilled. We can thus now write the mechanism of this enzyme as:

The pathway via $\text{E} \cdot \beta\text{GalX}^*$ is taken by substrates susceptible to acid catalysis in the presence of Mg^{2+} . The identification of the conformation change between $\text{E} \cdot \beta\text{GalX}$ and $\text{E} \cdot \beta\text{GalX}^*$ as the correct placing of an acid-catalytic group means that, if X^- is a good enough leaving group, loss of aglycone from the first ES complex will take place even if, in principle, the departure of HX can be acid-assisted.

An active-site-directed irreversible inhibitor labels a catalytically inessential Met-500 residue (Sinnott & Smith, 1978), but the electrostatic effect of the ionization of the adjacent residue, Tyr-501, is reflected in the ratio of binding constants of the isosteric ligands β -D-galactopyranosylpyridinium ion and β -D-galactopyranosylbenzene (Withers, 1977). The phenolic hydroxy group of Tyr-501 is then a probable acid-catalytic group. Further, if the conformation change is associated with the motion of a tyrosyl side chain, then these two mechanistic features have precedents in the favoured mechanism

of carboxypeptidase (Jencks, 1975; Breslow & Wernick, 1976).

The rate enhancement achieved by acid catalysis will vary with the nature of the leaving group. Since $k_{\text{cat.}}$ is close to the bond-breaking rate for hydrolysis of galactosyl azide by Mg^{2+} -enzyme (Sinnott & Withers, 1974), and presumably represents it for hydrolysis by apoenzyme, the ratio (200) gives the rate acceleration attributable to acid catalysis of the departure of azide by (probably) Tyr-501. For this acid catalysis to operate it is not necessary for proton transfer from the phenol to the product azide ion to be complete. Such a process would be thermodynamically strongly disfavoured. Rather, for acid catalysis of the departure of acidic leaving groups such as azide, one need only envisage partial proton transfer (e.g. by hydrogen-bonding). Such effects have long been recognized in solvolysis chemistry. Even though toluene-*p*-sulphonic acid is deprotonated by sodium acetate in glacial acetic acid, S_N1 acetolyses of alkyl toluene-*p*-sulphonates are accelerated by partial proton donation to the leaving group by acetic acid (Winstein *et al.*, 1957; Bentley & Schleyer, 1976).

As the aglycone becomes more basic, the rate

acceleration attributable to acid catalysis will increase (cf. Capon & Nimmo, 1975). A very rough lower limit for its importance for the hydrolysis of methyl galactoside can be made; $k_{\text{cat.}}$ for its hydrolysis by Mg^{2+} -enzyme is 10^2 s^{-1} (Tenu *et al.*, 1971), but an *O*-glycoside from an aglycone of $\text{p}K_{\text{a}}$ 15–16 (Ballinger & Long, 1960) is estimated from Fig. 1 to have a $k_{\text{cat.}}$ for hydrolysis by apoenzyme of 10^{-3} s^{-1} . The resultant rate ratio (10^5) is a lower limit to the contribution of acid catalysis to the enzyme since $k_{\text{cat.}}$ for the hydrolysis of methyl galactoside may still represent the conformation change, but is close to the rate accelerations observed in many systems considered to be models for lysozyme (Dunn & Bruce, 1973).

The importance of proton transfer to sulphur will be much less than that to oxygen, and this importance will be still further decreased by conjugation of the sulphur atom with a strongly electron-withdrawing substituent (cf. Fife & Anderson, 1970). The

hydrolysis of galactosyl thiopicrate was therefore examined as a possible example of a neutral substrate for which acid catalysis of aglycone departure was of minimal importance. The ratio between k_{cat} for hydrolysis by Mg^{2+} -enzyme and k_{cat} for hydrolysis by apoenzyme for this substrate is indeed the lowest for any neutral substrate.

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