Catalytic Consequences of Experimental Evolution. Part 1. Catalysis by the Wild-type Second β-Galactosidase (*ebg*^{0}) of *Escherichia coli*: a Comparison with the *lacZ* Enzyme

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β-D-Galactopyranose is the initial product of the hydrolysis of β-D-galactopyranosyl fluoride by *ebg*^{0} enzyme. Transfer to methanol of a β-D-galactopyranosyl residue from either *m*-nitrophenol or 3-bromopyridine is seven times more favourable than to water. Breakage of the aglycone–glycone bond limits the rate of, and is the first irreversible step in, the hydrolysis of both aryl galactopyranosides and galactosylpyridinium ions. In the transition state for this process the anomeric carbon atom is *ca.* 1/3 of the way towards sp^2 hybridisation, the fissile bond is largely broken, and a proton is transferred a little way towards the aglycone oxygen of the leaving group.

In addition to serving as a vehicle for now classic work on the regulation of protein biosynthesis in bacteria,[1] the tetrameric β-galactosidase coded for by the *lacZ* gene of the gut bacterium *Escherichia coli* has been the subject of intensive mechanistic [2-6] and protein-chemical [7] investigations. The *lacZ* enzyme however is not the only β-galactosidase produced by this bacterium: a second, less catalytically effective enzyme was discovered in 1973.[8] This enzyme, referred to as *ebg*, is coded for by the *ebgA* gene at 66.5 min on the *E. coli* chromosome, compared with 7.9 min for *lacZ*,[8] these figures being based on a total chromosome length of 100 min.[9] Since it has been suggested that the *E. coli* chromosome arose from two successive doublings of an ancestral chromosome,[10] there is a possibility that the *ebg* and *lacZ* genes are related. This paper reports mechanistic studies on the wild-type *ebg* enzyme (*ebg*^{0}),† similar to those carried out on the *lacZ* enzyme,[5] which make possible a functional comparison between the two enzymes. A further reason for study of this enzyme is that it has been the subject of extensive work on experimental evolution by Hall and his co-workers.[11,12] The catalytic consequences of this evolution, in terms of the structures of transition states and the relative energies of intermediates, are described in subsequent papers. Some biochemical investigations on an *ebg* evolvant have been previously reported.[13]

Results and Discussion

A *Determination of Conditions Optimal for Catalysis.*—The pH optimum of *ebg* enzymes is known [12,13] to be 7.5 if nitrophenyl galactosides are used as substrates. These enzymes are also known to be poisoned irreversibly by Na^+: [12,13] loss of activity on addition to assay buffers containing Na^+ is therefore a useful check that catalytic activity is not due to contaminating *lacZ* enzyme, which is normally assayed in Na^+-based buffers.[2,3,5]

The very labile *ebg*^{0} enzyme loses activity against *p*-nitrophenyl galactoside in the presence of residual contaminants in AnalaR potassium phosphate or ammonium sulphate. Activity is regained on dialysis against metal-complexing agents such as dithiothreitol or potassium citrate. Therefore, in the absence of other complexing agents, 25μM-2,2′-bipyridyl was added to all buffers: no further trouble was encountered. We did not observe the ' lag ' before the steady state hydrolysis, reported by Arraj and Campbell,[13] which may be related to similar contamination problems.

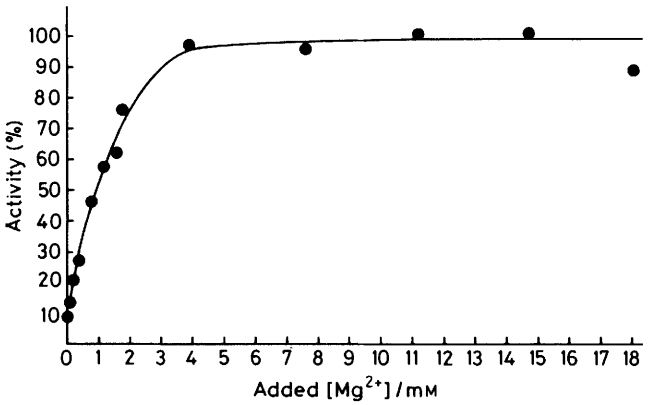


Figure 1. Dependence of enzyme activity on Mg^{2+} concentration. Enzyme had been dialysed against 4 × 500 volumes of 0.125M-potassium phosphate buffer, pH 7.5, containing 1mM-dithiothreitol. Enzyme activity against 5mM-*p*-nitrophenyl galactoside at 25 °C, in 0.125M-potassium phosphate, 5mM-potassium citrate, pH 7.5, and varying concentrations of Mg^{2+}, was measured

Like the *lacZ* enzyme,[3] *ebg*^{0} enzyme requires Mg^{2+} for maximal activity against *O*-glycosides.[13] Exhaustive dialysis against 0.125M-potassium citrate buffer, pH 7.5, yields an enzyme of 4—8% residual activity against 5mM-*p*-nitrophenyl galactoside. The effect of various concentrations of added $MgCl_2$ on the activity of Mg^{2+}-depleted enzyme is displayed in Figure 1. Because of the presence of 5mM-potassium citrate buffer in the assay medium as well as phosphate dianion, which will also complex Mg^{2+}, the scale on the abcissca overestimates free Mg^{2+} concentration (p*K* for dissociation of the Mg^{2+}–citrate complex is 3.85).[14] In the absence of complexing agents, therefore, the enzyme is saturated with Mg^{2+} at a concentration of 5mM.

For most experiments, then, the standard medium was 0.125M-potassium phosphate, pH 7.5, 5mM-$MgCl_2$, 25μM-2,2′-bipyridyl.

B *Initial Products of Enzyme Action.*—Glycosyl fluorides are good, non-chromophoric substrates for glycosidases. This makes them the substrates of choice for determination of the initial products of glycosidase action,[15] since liberation of F^- can be followed electrochemically, and enzymic hydrolysis can be made comparably fast to mutarotation of the reducing sugar product, with accessible concentrations of enzyme.

† ' *ebg* ' is an acronym for *e*volved *b*eta *g*alactosidase, something of a misnomer since the ancestral enzyme, *ebg*^{0}, is not evolved in the laboratory.

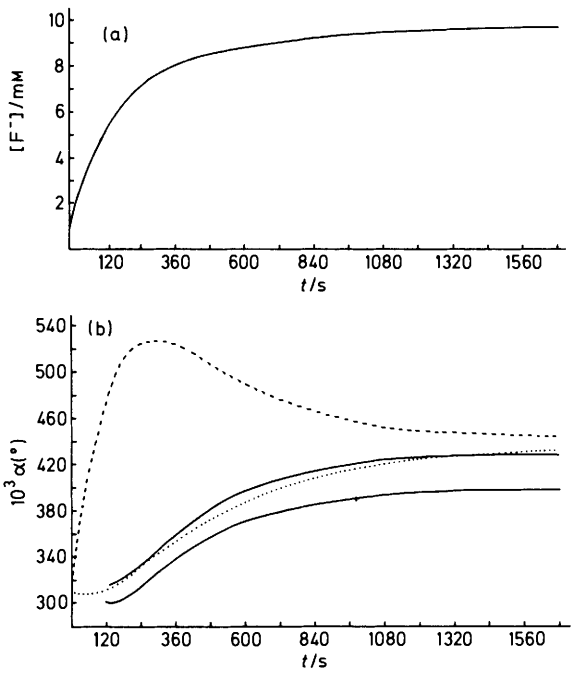
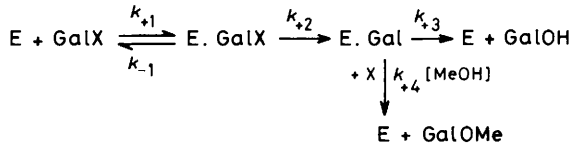


Figure 2. (a) Time-course of an enzymic hydrolysis of 10mM-β-D-galactopyranosyl fluoride, measured by F^- liberation, 0.125M-potassium phosphate buffer, pH 7.5, 5.0mM-$MgCl_2$, 25μM-2,2′-bipyridyl at 25 °C. (b) Optical rotation changes observed with two identical reactions: ---- predicted changes for inversion; •••• predicted changes for retention

Figure 2 displays the time course of *ebg*^{0}-catalysed hydrolyses of β-D-galactopyranosyl fluoride, as monitored electrochemically, using a fluoride-ion electrode, and polarimetrically. Also displayed are the time courses of optical activity, calculated from the experimental curve for fluoride-ion liberation, on the assumption of retention and of inversion during catalysis and measured mutarotation rates. The mutarotation of galactose is complex,[16] but under the conditions of the reaction departure from a first-order process with a rate constant of 3.47×10^{-3} s^{-1} could not be observed, probably because the deviation from first-order mutarotation occurs only during the initial stages.

The hydrolysis of β-D-galactopyranosyl fluoride by *ebg*^{0} clearly proceeds with retention of configuration. That this enzyme, like the *lacZ* enzyme, is a retaining one was confirmed by the finding that the enzyme has transferase activity (microscopic reversibility requires an inverting transferase to act on both anomeric glycosides). In the presence of methanol (1.0M) *m*-nitrophenyl β-galactoside gives 13% methyl β-galactopyranoside and β-galactosyl-3-bromopyridinium bromide gives 15% methyl β-galactopyranoside: no methyl α-galactopyranoside is formed. The preference for methanol (7 : 1 on a molar basis) is, within experimental error, independent of the aglycone. This is a necessary (but not a sufficient) condition for the intermediacy of a discrete glycosyl-enzyme. Such an intermediate is made likely by the retention of configuration in the product.[17] The simplest possible kinetic mechanism involving a glycosyl-enzyme is that given in the Scheme, according to which equations (1)—(3) hold where $d[X]/dt = k_{cat}[E]_0[S]/(K_m + [S])$, $[E]_0$ being the total concentration of active sites and [S] that of substrate.

If the Scheme is correct, then as the leaving group ability of X is increased, k_{cat} will reach a plateau value of k_{+3} the pseudounimolecular rate constant for reaction of water with



Scheme

$$k_{cat} = k_{+2}k_{+3}/(k_{+2} + k_{+3}) \quad (1)$$

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \frac{k_{+3}}{k_{+3} + k_{+2}} \quad (2)$$

$$k_{cat}/K_m = k_{+1}k_{+2}/(k_{-1} + k_{+2}) \quad (3)$$

galactosyl-enzyme, but k_{cat}/K_m will continue to increase. This is observed with aryl galactosides with the evolvants *ebg*^{a} [18] and *ebg*^{b}; [19] below a pK_a value of *ca.* 9 for the aglycone k_{cat} is invariant but k_{cat}/K_m varies with a β_{lg} of −0.3 to −0.4. The buildup of galactosyl-enzyme has been observed directly with *ebg*^{b}, and rate-limiting degalactosylation in the hydrolysis of 3,4-dinitrophenyl galactoside by *ebg*^{a} and *ebg*^{b} has been demonstrated by nucleophilic competition with methanol.[20] The kinetic mechanism of the enzyme is therefore described by the Scheme.

C *Structure–Reactivity Parameters and α-Deuterium Kinetic Isotope Effects.*—Michaelis–Menten parameters for a series of nine aryl β-galactosides and seven β-galactopyranosyl-pyridinium salts are given in the Table, as are those for a representative α-L-arabinopyranoside. k_{cat} Values are absolute, calculated from the specific activity of the most active sample of the enzyme and a molecular weight per active site of 150 000. The essential correctness of this proceeding is confirmed by the amplitude of the ' burst ' observed with *ebg*^{b}.[20]

Before mechanistic deductions can be made from the contrast between *O*- and *N*-glycosides, it is necessary to establish that they are both being transformed at the same active site. Evidence for this, in addition to similar selectivity of transfer to methanol and water, is as follows.

(i) The K_I value for the competitive inhibitor β-D-galactopyranosyltrimethylammonium bromide is the same, within experimental error, when measured against *p*-nitrophenyl galactoside ($12 \pm 2 \times 10^{-3}$ mol l^{-1}) or galactosyl-3-bromopyridinium bromide ($14 \pm 1 \times 10^{-3}$ mol l^{-1}). A similar experiment with methyl 1-thio-β-D-galactopyranoside gave K_I values of $80 \pm 9 \times 10^{-3}$ and $125 \pm 16 \times 10^{-3}$ mol l^{-1}, respectively, but this discrepancy is readily accounted for by the concentrated solutions (up to 1.5% w/v) necessary for the measurement.

(ii) The K_I value for β-D-galactopyranosyl-3-methylpyridinium bromide, measured as a competitive inhibitor of *p*-nitrophenyl galactoside hydrolysis ($1.12 \pm 0.04 \times 10^{-3}$ mol l^{-1}) is the same as the K_m for its own hydrolysis.

Given, then, that the pyridinium salts are hydrolysed at the same active site as aryl galactosides, it is immediately apparent from the Table, and from the K_I values of galactosyltrimethylammonium bromide and methyl thiogalactoside, that this active site binds positively charged species much more tightly than neutral molecules. Arraj and Campbell noted this phenomenon with their evolvant.[13] Its presence in the wild-type enzyme, therefore, means that it is not a consequence of the evolutionary process. K_m values for pyridinium salts are roughly similar to those obtained with the *lacZ* enzyme, but K_m values for aryl galactosides are 2—3 orders of magnitude

Table. Michaelis–Menten parameters for hydrolysis of aryl galactoside sand β-galactopyranosylpyridiniums by Mg^{2+}–*ebg*^{0} enzyme at 25 °C and pH 7.5

Galactoside substituent (pyridinium salts as bromides)	pK_a [b]	k_{cat}/s^{-1}	$10^3\ K_m$/mol l^{-1}	$10^{-3}\ k_{cat}\ K_m^{-1}$/ 1 $mol^{-1}\ s^{-1}$
3-Chloropyridine	2.81	5.2	0.85 ± 0.08	6.7
3-Bromopyridine	2.85	5.5	0.59 ± 0.09	8.7
3-Bromopyridine		1.75	0.35 ± 0.05 [a]	3.6
4-Bromoisoquinoline	3.31	1.75	0.34 ± 0.03	3.5
4-Bromoisoquinoline		0.99	0.45 ± 0.08 [a]	1.83
Pyridine	5.22	0.029	0.84 ± 0.02	0.025
Isoquinoline	5.40	0.012	0.86 ± 0.02	0.015
3-Methylpyridine	5.70	0.031	1.18 ± 0.09	0.026
3,5-Dimethylpyridine	6.14	0.029	1.32 ± 0.16	0.020
3,4-Dinitrophenol	5.42	186	9.7 ± 1.4	18.3
3,5-Dinitrophenol	6.69	147	7.5 ± 1.4	14.4
4-Nitrophenol	7.15	66	25 ± 3	2.45
4-Nitrophenol		1.76	17.8 ± 0.8 [a]	0.096
2-Nitrophenol	7.17	157	59 ± 2	2.56
4-Cyanophenol	7.95	34	38 ± 1	0.875
3-Nitrophenol	8.39	51	30 ± 3	1.92
4-Bromophenol	9.34	16	150 ± 9.3	0.107
2-Naphthol	9.51	0.64	5.7 ± 0.9	0.132
Phenol	9.99	38	156 ± 24	0.265
3-Methylphenol	10.09	2.55	9.6 ± 2.6	0.266
2-Nitrophenyl α-L-arabinopyranoside				0.0171

[a] For Mg^{2+}-depleted enzyme, obtained by three-fold dialysis against 1 000 volumes of 0.125M-potassium citrate, pH 7.5. [b] For sources of literature pK_a values, see refs. 5*c*, 5*e*, and 6*b*.

bigger.[5] Like the *lacZ* enzyme, *ebg*^{0} will hydrolyse an α-L-arabinopyranoside, albeit at a slower rate.[5]

The dependence of the rate of hydrolysis of the pyridinium salts on leaving group pK_a is described by equations (4) and (5). The good correlation coefficients indicate that a process

$$\log (10^{3}\ k_{cat}/K_m) = (3.38 \pm 0.40) - (0.893 \pm 0.085)\ pK_a\ (r\ -0.978) \quad (4)$$

$$\log k_{cat} = (2.89 \pm 0.45) - (0.798 \pm 0.097)pK_a\ (r\ -0.965) \quad (5)$$

strongly dependent on the pK_a of the leaving pyridine is both the rate-determining step (*i.e.* determines k_{cat}), and, since it also determines k_{cat}/K_m, is the first irreversible step. The existence of an α-deuterium kinetic isotope effect on k_{cat} for the 4-bromoisoquinolinium salt of $k_H/k_D = 1.110 \pm 0.035$ is evidence that this process is bond-breaking.

The sensitivity of the bond-breaking process to aglycone acidity (β_{lg} values) can provide an estimate of the degree to which the C–N bond has broken in the transition state. The data of Arnett and Reich [21] on the effect of substituents on the position of equilibrium for the reaction of pyridines with methyl iodide in acetonitrile can be converted to a β_{lg} of -1.47 ± 0.22 using aqueous pK_a values. It is reasonable to assume that this represents an upper limit for this and similar reactions in the more polar solvent, water. A lower limit comes from the β_{lg} value for spontaneous hydrolysis of β-D-galactopyranosylpyridinium ions in water (-1.26 ± 0.13): [6] at the transition state the C–N bond cannot be more than completely broken.

The β_{lg} values for the pyridinium salt hydrolyses by *ebg*^{0} enzyme imply, then, that at the transition state the C–N bond is half to two-thirds broken. If the reactions were merely enzyme-catalysed S_N1 reactions, then the α-deuterium kinetic isotope effect should show that the anomeric carbon is half to two-thirds of the way to sp^2 hybridisation. A value of *ca.* 1.33 for the equilibrium isotope effect for the addition of a pyridine to a glycosyl cation seems reasonable in the light of an equilibrium isotope effect of 1.37 ± 0.03 for the hydration of acetaldehyde,[22] and relative (deuterium) fractionation factors of 1.15 for $>CHNH_3^+$ and 1.18 for $>CHOH$.[23] On this basis the anomeric carbon is only *ca.* 1/3 of the way to sp^2 hybridisation. Some degree of nucleophilic assistance is involved. A transition state similar to that for the hydrolysis of *N*-methoxymethyl-*NN*-dimethylanilinium ions, where the presence of a nucleophile is enforced by the short lifetime of $CH_3OCH_2^+$, is indicated by the similarity of the β_{lg} value (-0.87) and α-deuterium kinetic isotope effect (1.11 per D).[24]

The dependence of the rate of hydrolysis of the nine aryl galactosides without *ortho* substituents on the phenol pK_a is described by equations (6) and (7). The good correlation of

$$\log (10^{-3}\ k_{cat}/K_m) = (4.76 \pm 0.73) - (0.39 \pm 0.06)pK_a\ (r\ -0.926) \quad (6)$$

$$\log k_{cat} = (4.49 \pm 1.05) - (0.37 \pm 0.12)pK_a\ (r\ -0.75) \quad (7)$$

$\log (k_{cat}/K_m)$ with aglycone pK_a indicates that the first irreversible step in the enzyme turnover sequence, as with the pyridinium salts, is breakage of the glycone–aglycone bond. Confirmation of this comes from an α-deuterium kinetic isotope effect of 1.13 ± 0.03 on k_{cat}/K_m for *p*-nitrophenyl galactoside. The worse correlation of k_{cat} with aglycone pK_a could come from any of the following phenomena, singly or in combination: (i) experimental error in separation of k_{cat} and K_m, arising from K_m values in many cases above the aqueous solubilities of the substrate; (ii) non-productive binding (possibly through indiscriminate hydrophobic interactions of the aglycone) of substrate to the active site; (iii) degalactosylation becoming rate limiting for the galactosides of more acidic aglycones.

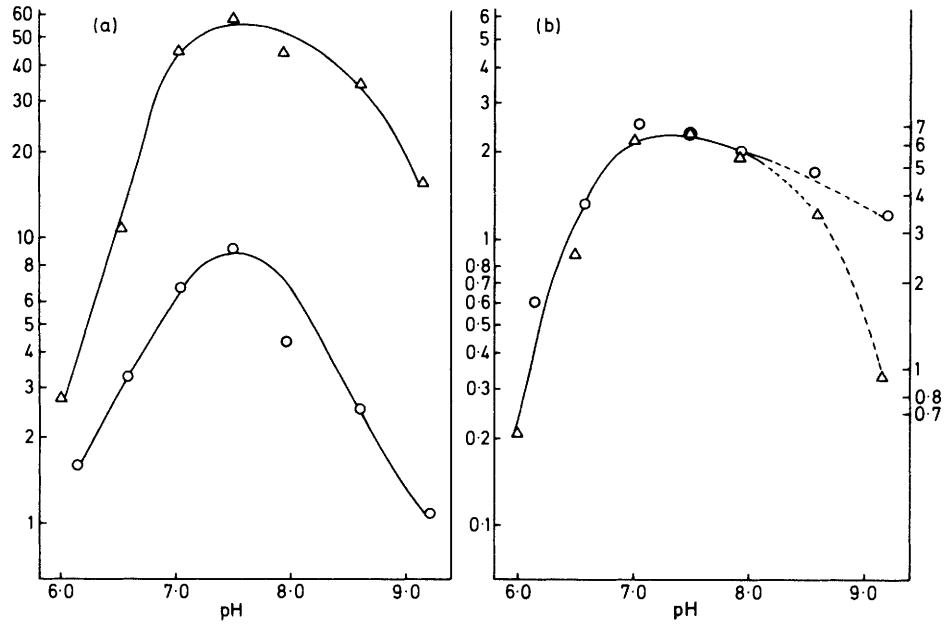


Figure 3. Kinetic parameters for *p*-nitrophenyl β-D-galactopyranoside (△) and β-D-galactopyranosyl-3-bromopyridinium ion (○) as a function of pH: (a) k_{cat}/s^{-1} (plotted logarithmically): (b) $k_{cat}K_m^{-1}/l\ mol^{-1}\ s^{-1}$, plotted logarithmically on normalised scales. The size of the symbols is a rough guide to the error. The right-hand ordinate rule refers to β-D-galactopyranosyl-3-bromopyridinium ion- the left to *p*-nitrophenyl β-D-galactopyranoside

Be this as it may, it is clear that a lower limit of 186 s^{-1} can be put on the degalactosylation of galactosyl-*ebg*^{0}.

Interpretation of the β_{lg} value in the case of *O*-glycosides is complicated because it is affected by two parameters, the degree of proton transfer to the oxygen and the degree of C–O bond cleavage. Absolute rate enhancements provide clues as to the importance of the first. Galactosyl-3-chloropyridinium ion and 3,4-dinitrophenyl galactoside hydrolyse in a pH-independent manner at a rate of $1 \times 10^{-8}\ s^{-1}$ at 25°.[6] Absolute rate enhancements of 10^9 and $10^{10.5}$ therefore, are observed with *ebg*^{0} in the hydrolysis of these two compounds, *i.e.* acidic catalysis is not of crucial importance in the catalytic action of this enzyme.

α-Deuterium kinetic isotope effects and β_{lg} values are the same for *ebg*^{0} catalysed hydrolyses of pyridinium salts and the 'water' reaction of *N*-methoxymethyl-*NN*-dimethylanolinium ions,[24] and the α-deuterium kinetic isotope effect for *ebg*^{0} catalysed hydrolysis of *p*-nitrophenyl galactoside (1.13 ± 0.03) is the same as that for the 'water' reaction of methoxymethyl 2,4-dinitrophenolate (1.11).[25] It is therefore reasonable to attribute the difference in β_{lg} value for oxygen leaving groups, between the S_N2 reactions of water and methoxymethyl derivatives (−0.82),[26] and *ebg*^{0}-catalysed hydrolyses of aryl galactosides, to proton transfer in the latter case. This increase of β_{lg}, from −0.82 to −0.48, corresponds to the proton being *ca.* 1/3 transferred at the transition state. Such a transition state would predict a definite solvent deuterium isotope effect directly attributable to the proton being transferred. Fortunately the properties of *ebg*^{0} enzyme are such as to allow this to be detected.

D *pH Dependence of Kinetic Parameters. Deuterium Solvent Isotope Effects.*—The pH variations of log k_{cat} and log k_{cat}/K_m for β-D-galactopyranosyl-3-bromopyridinium ion and *p*-nitrophenyl β-D-galactopyranoside are shown in Figures 3a and b, respectively. These variations cannot be simply interpreted,[27] particularly as it is probable that the pyridinium salt reacts through a 'non-bottleneck' mechanism,[2d] but the informative feature is that the pH variation of k_{cat}/K_m for the two substrates is qualitatively similar, and quantitatively identical within 0.5 pH units of the pH optimum. This greatly simplifies the interpretation of solvent deuterium kinetic isotope effects on the enzyme reaction.

Normally this is complicated by the many sites at which deuterium substitution can take place and the subtlety of the consequences of this substitution,[28] one of them being solvent isotope effects on the pK_a values of ionising groups. These are usually *ca.* 0.5 p*K* units for OH and NH acids, less for SH acids.[29,30] However, Figure 3b indicates that the pH optimum would have to be shifted by a full pH unit in D_2O if a differential effect were to be observed on the two substrates: even so this differential effect would only be *ca.* 0.1 log units.

Therefore, on the assumption that perturbations of protein conformation, consequent upon H–D exchange, affect the nucleophilic and non-covalent contributions to the hydrolysis of *O*- and *N*-glycosides equally, and from the demonstration that significantly different responses to isotope effects on ionisations would require these to be implausibly large, the ratio of the solvent deuterium isotope effect on k_{cat}/K_m for *p*-nitrophenyl galactoside (k_{H_2O}/k_{D_2O} 2.39 ± 0.12) to that for the pyridinium salt (k_{H_2O}/k_{D_2O} 1.66 ± 0.09) represents the effect due to proton transfer in the transition state for *O*-glycoside hydrolysis (1.44 ± 0.11). This effect is entirely in accord with the picture, from structure–reactivity correlations, of a transition state in which the proton is *ca.* 1/3 transferred. (Proton transfer cannot take place to the departing pyridine.)

One interpretation of the divergence of the log k_{cat}/K_m–pH profiles at high pH is that the acid catalytic group starts to ionise, in the free enzyme, around pH 9. The β-D-galactopyranosylmethyl residue of the affinity label, β-D-galactopyranosylmethyl-*p*-nitrophenyltriazene, becomes attached to the OH group of serine or threonine.[31] Since, with this label, the electrophilic site is at exactly the atom to which, in the substrate, acid catalysis is applied, identification of this residue as the acid catalyst is the simplest assumption.

The immediate problem, if this identification is made, of why an aliphatic hydroxy-group ionises at pH *ca.* 9, and is a

detectable acid catalyst, may have its resolution in the role of Mg^{2+}. Mg^{2+}-depletion of the enzyme has little effect on pyridinium salt hydrolysis, but drastically slows down that of *p*-nitrophenyl galactoside (Table). A direct role for Mg^{2+} is unlikely, from the solvent isotope effect, since co-ordination of the substrate, rather than water, to the metal, should, by analogy with the behaviour of the Zn^{2+} ion in yeast alcohol dehydrogenase, result in an inverse solvent isotope effect.[30] A possible role for Mg^{2+} is co-ordination to the acid catalytic aliphatic hydroxy-group to increase its acidity.

E *A Functional Comparison of* lacZ *and* ebg^0 *Enzymes.*—Both enzymes hydrolyse a wide variety of β-galactopyranosyl (and related) derivatives with retention of configuration at the anomeric centre. The catalytic effectiveness of *ebg*^{0} against pyridinium salts, indeed, is only an order of magnitude less that that of *lacZ* enzyme, whether measured against k_{cat} or k_{cat}/K_m. It is against *O*-glycosides that the enzyme's catalytic feebleness is manifest.

k_{cat}/K_m values are *ca.* 10^3 times higher for the *lacZ* enzyme. k_{cat} Values are roughly an order of magnitude less, but it is difficult to interpret this since with aryl galactosides hydrolysed by *lacZ* it is not clear which step is rate determining: the correlation coefficient between log (k_{cat}/K_m) and pK_a, for example, is −0.65 and the α-deuterium kinetic isotope effects for slow substrates approach 1.00. If it is assumed that it is in the application of acid catalysis to aglycone departure that the ineffectiveness of *ebg*^{0} enzyme lies, then the main functional differences apart from the feeble affinity for neutral molecules, are explained. The pH–rate profiles for pyridinium salts and aryl galactosides, hydrolysed by *lacZ*, are very different, whereas for *ebg*^{0} they are qualitatively similar, ionisation of the acid catalyst only being tentatively detected at alkaline pH. The affinity label becomes attached to a serine or threonine residue, rather than to methionine, and *ebg*^{0} enzyme is less sensitive to the agent than the *lacZ* [32] enzyme. However, the Mg^{2+} ion appears to be associated with the operation of acid catalysis in both enzymes.

If then *ebg*^{0} β-galactosidase resembles *lacZ* β-galactosidase functionally in most respects, except for the application of acid catalysis, then the idea of the two enzymes being related [12] is entirely plausible. It also makes it likely that, placed under selection pressure, *ebg*^{0} will evolve towards more effective application of acid catalysis. This is indeed observed.[19]

Experimental

Enzyme Isolation.—The enzyme was extracted from cultures of *E. coli* K12 strain 1B1, constitutive for *ebg*^{0} enzyme synthesis [genotype HfrC *spc lacZ* (deletion in 4 680) *ebgA*^{0}, *ebgR*$^-$], the strain being kindly supplied by Dr. B. G. Hall, University of Connecticut, Storrs. The strain was grown up, and the enzyme isolated, essentially as described by him,[12] except that the ion-exchange chromatography was carried out on Sephadex A50 (rather than hydroxyapatite) and the gel filtration on Sephadex G200 (rather than Biogel A 1.5M). Activity was obtained in a single peak in both chromatograms, the final activity being 51% of the initial and the specific activity 90% of Hall's.[12] Microdensitometer traces of Coomassie blue-stained slabs of polyacrylamide gel on which the protein had been subjected to electrophoresis in the presence of sodium dodecyl sulphate showed that one stained band accounted for >70% of stainable material.

Substrates, Inhibitors, and Buffers.—Apart from β-D-galactopyranosyl fluoride,[33] all the substrates and inhibitors have been described.[5,6] AnalaR reagents were used to make up buffers in doubly glass-distilled water, and the pH was measured with a newly calibrated Radiometer PHM 62 pH meter: for comparison with H_2O buffers at pH 7.50, D_2O buffers were made up to a pH meter reading of 7.10. Kinetic measurements normally pertain to 0.125M-potassium phosphate buffer, pH 7.50, 5.0mM in $MgCl_2$, 25μM in 2,2′-bipyridyl: those on Mg^{2+}-depleted enzyme to 0.125M-potassium citrate, pH 7.50. For the measurement of pH rate profiles solutions contained 0.15M-KCl, 5mM-$MgCl_2$, and 25μM-2,2′-bipyridyl, in addition to the buffering component: pH 6—7.5, 31.25mM-potassium phosphate; pH 7.95, 31.25mM-imidazole; pH 8.6—9.2, 31.25mM-glycine. Binding of Mg^{2+} and H^+ to *lacZ* enzyme is competitive,[3] so it was confirmed that addition of extra Mg^{2+} to the most acidic buffer had no kinetic consequences.

Kinetic Techniques.—Extinction changes were monitored in a Unicam SP 8-200, SP 1800, or SP 1700 spectrophotometer, the first being fitted with the manufacturer's thermostatting system, the others with a Tecam Tempunit or Techne C-100 circulator, respectively. Optical rotation changes at 334 nm were monitored in a Perkin-Elmer 241MC polarimeter, using jacketted cells through which thermostatted water was circulated from a Julabo Paratherm thermostat. Fluoride ion changes were monitored by a Corning fluoride-ion electrode and EIL calomel reference electrode, connected to a Philips PW 9414 ion meter, in a jacketted vessel through which water, thermostatted by an MLW thermostatting pump, was circulated. Because of possible complexation between fluoride ion and the Mg^{2+} necessary for full enzymic activity, calibration curves were constructed for F^- in the assay buffer.

k_{cat}/K_m Values were estimated from initial velocities by linear least squares treatment of double reciprocal plots. Individual k_{cat} and K_m values were estimated by iterative fitting of the data directly to $V = V_{max.}[S]/(K_m + [S])$, using a program for an HP9845A computer written by Dr. P. J. England. A similar program was used for K_I determinations, where the initial rate data was fitted iteratively to $V = V_{max.}[S]/([S] + K_m\{1 + [I]/K_I\})$. If saturating concentrations of substrates with high K_m values were called for by this approach, K_I values were calculated from the gradient of a plot of $1/V$ against [I].

Measurement of α-deuterium kinetic isotope effects on $V_{max.}$ has been described.[5,6] That on $V_{max.}/K_m$ for *p*-nitrophenyl galactoside hydrolysis was obtained by plotting the reciprocal of the absolute absorbance at 347.5 nm of a solution of protiated or deuteriated substrate against the reciprocal of its rate of change on addition of a standard quantity of enzyme: linear least-squares treatment of plots for protiated and deuteriated substrates gave the isotope effect. The solvent isotope effect for this compound was measured by monitoring absorbance at 347.5 nm, the isosbestic point between *p*-nitrophenol and *p*-nitrophenolate.

Molar optical rotations of α- and β-D-galactopyranose (91.4 and 34.7°, respectively) were obtained by extrapolating the mutarotation curve to zero time. To calculate time courses of optical rotation, predicted for retention and inversion, from the time course for liberation of F^- a program for an HP97 calculator was written which divided the F^- liberation curve into 12 s intervals. Optical rotation changes in each interval were calculated from equations (8)—(10) and summed over the time course of the reaction.

$$\delta[\alpha\text{-GalOH}] = \delta[F^-] - 4.164 \times 10^{-2}[\alpha\text{-GalOH}] \quad (8)$$

$$\delta[\beta\text{-GalOH}] = \delta[F^-] - 4.164 \times 10^{-2}[\beta\text{-GalOH}] \quad (9)$$

$$\delta[\text{Equilibrium mixture of anomers}] = 4.164 \times 10^{-2}\,[\alpha\text{- or }\beta\text{-GalOH}] \quad (10)$$

Estimation of Transfer to Methanol.—Substrate (5mM) was dissolved in standard assay buffer (5 ml) and methanol was added to a total concentration of 1M. Enzyme was added and the solutions were left at 25 °C until complete liberation of aglycone had occurred; they were then extracted with ether to remove aglycone, shell-frozen, freeze-dried, and left in a vacuum desiccator over P_2O_5 for 2—3 days. The saccharides were converted to their *O*-trimethylsilyl ethers by addition of a 10 : 4 : 2 (v/v) mixture of dry pyridine, hexamethyldisilazane, and trimethylchlorosilane (0.2 ml). After 1 h the solutions were analysed by g.l.c. on a Pye 104 chromatograph, on a 1.25 m column of 5% SE30 on Supasorb at 162 °C. The carrier gas was nitrogen, flowing at 40 ml min^{-1}. Peaks were identified by derivatization and analysis of genuine samples of α- and β-D-galactopyranose and methyl α- and β-galactopyranoside.

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