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The Mechanism of Action of β-Galactosidase

EFFECT OF AGLYCONE NATURE AND α-DEUTERIUM SUBSTITUTION ON THE HYDROLYSIS OF ARYL GALACTOSIDES

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1. Steady-state kinetic parameters for the β-galactosidase-catalysed hydrolysis of 13 aryl β-D-galactopyranosides show no simple dependence on aglycone acidity. 2. α-Deuterium kinetic isotope effects (k_H/k_D) for seven of these substrates, measured under steady-state conditions with $[S] \gg K_m$, vary from 1.00 for poor substrates to 1.25 for hydrolysis of the galactosyl-enzyme. 3. Methanolysis of the galactosyl-enzyme in 1.5M-methanol increases k_H/k_D for degalactosylation, but leaves that for hydrolysis of 'slow' substrates unchanged. 4. These data are incompatible with a simple two-step mechanism. A scheme consisting of a conformation change, liberation of a galactopyranosyl cation in an intimate ion-pair, non-productive but preferential collapse of the ion-pair to a covalent species and reaction of the galactosyl enzyme through the ion-paired form is proposed. 5. This scheme is used to rationalize previously puzzling data about the enzyme mechanism.

The multistep nature of the mechanism of action of β-galactosidase, required by the stereochemistry of the catalysed reaction, has been confirmed by methanol-competition experiments, and substrates have been described for which both formation and hydrolysis of a galactosyl-enzyme is the limiting process under steady-state conditions (Sinnott & Viratelle, 1973, and references cited therein). α-Deuterium kinetic isotope effects have been widely used to throw light on the nature of the transition states and intermediates in nucleophilic substitution reactions at carbon atoms. With the availability of substrates whose steady-state rates represented different steps of the reaction therefore it became possible to study the nature of the galactosyl-enzyme by investigation of the transition states leading both to and from it.

The origin of α-deuterium kinetic isotope effects is considered to lie in the alteration of the C–H bending frequency consequent upon a change in co-ordination number. The C–H σ bond in tetrahedrally co-ordinated species is constructed from a carbon sp^3 orbital; in a trigonally co-ordinated species it is constructed from an sp^2 orbital. The greater *s* character results in a 'tighter' bond and hence a higher bending frequency (see, e.g., Wolfsberg, 1972). Therefore reactions which result in a decrease in co-ordination number ($sp^3 \rightarrow sp^2$), such as the generation of a carbonium ion, show k_H/k_D ratios of greater than unity (Shiner *et al.*, 1968). An increase in co-ordination number ($sp^2 \rightarrow sp^3$), as at certain centres during a cyclo-addition reaction (Brown & Cookson, 1965) or during the hydrolysis of a carbonium ion (Kirmse *et al.*, 1972), is associated with an inverse isotope effect. S_N2 reactions, which involve no change in co-ordination number, exhibit isotope effects near unity (Shiner *et al.*, 1970). The numerical values of these isotope effects (10–20%) make their measurement in (zero-order) enzymic reactions possible with conventional techniques.

Dahlquist *et al.* (1968, 1969) have made measurements of α-deuterium kinetic isotope effects on reactions at C-1 of glucopyranosyl derivatives. Acid-catalysed hydrolysis of phenyl β-D-glucopyranoside, which is considered to involve the generation of a glucopyranosyl cation, shows $k_H/k_D = 1.13$; the base-catalysed hydrolysis, supposedly involving an intramolecular S_N2 reaction, shows $k_H/k_D = 1.03$, whereas the lysozyme-catalysed hydrolysis of phenyl 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside shows $k_H/k_D = 1.11$, in accord with a 'carbonium ion' mechanism for this enzyme (Chipman & Sharon, 1969).

Methods and Materials

General methods

β-Galactosidase from *Escherichia coli*, 0.1M-sodium phosphate buffer, pH7.0, 1.0mM in $MgCl_2$, and 2,4- and 3,5-dinitrophenyl β-D-galactopyranosides have been described (Sinnott & Viratelle, 1973). Acetobromogalactose (2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide) and acetobromo[1-^{2}H]-galactose were made from the sugars by the method of Lemieux (1963), m.p. 84–85°C (lit. 84–85°C) and m.p. 76–78°C (before recrystallization) respectively. Phenyl β-D-galactopyranoside, lot no. 47467, was bought from Koch–Light Ltd., Colnbrook, Bucks., U.K.

Glycosides. These were made by condensation of the sodium salt of the phenol with (deuterated or undeuterated) acetobromogalactose in aqueous acetone followed by deacylation without isolation of the tetra-acetate. A solution of acetobromogalactose (2.5g) in acetone (15ml) was mixed with a solution of the phenol (10mmol) in 1.0M-NaOH (10ml) and left for 18h at 22°C. The acetone was evaporated (temp. <60°C) and the aqueous slurry was diluted with water and extracted with dichloromethane (3×50ml). The dichloromethane extracts were extracted with 2.0M-NaOH (3×100ml) and water, and were then dried ($MgSO_4$) and evaporated. The residual gums were dissolved in dry methanol (10ml) and were left for 12h at 22°C after the addition of 1.0M-sodium methoxide (1ml). After neutralization with a few drops of acetic acid, the methanol was evaporated. The glycoside crystallized on trituration with water and was repeatedly recrystallized from water or ethanol. Isolated yields in the range 20–50% (from acetobromogalactose) were obtained. *4-Cyanophenyl* and *3-(t-butyl)phenyl β-D-galactopyranosides* had m.p. 171–172°C and 145–146°C, $[\alpha]_D^{25}$ −61° (*c* 5 in water) and −15° (*c* 3 in water) ($C_{13}H_{15}NO_6$ requires C, 55.5; H, 5.35; N, 5.0%; $C_{16}H_{24}O_6,H_2O$ requires C, 58.2; H, 7.85%. Found: C, 55.4; H, 5.6; N, 5.1; and C, 59.0; H, 7.8%). Data on other (known) galactosides are given in Table 1.

[1-^2H]Galactose (*cf. Murray & Williams*, 1958). To a cooled solution of D-galactono-γ-lactone (10g) in deuterium oxide (>99.8%, 50g) were added 2.5% sodium amalgam (180g) and a solution of 85% deuterophosphoric acid (14ml) in dry ether (30ml) at such a rate that the temperature remained at 5–10°C and the pH between 3 and 4.

The mercury was removed by filtration, the filtrate was neutralized with solid calcium carbonate, and the calcium phosphate was removed by centrifugation. Unchanged lactone was hydrolysed by addition of 0.1M-NaOH, the pH was adjusted to 6 by addition of 28% phosphoric acid and the solution finally neutralized with solid calcium carbonate. After centrifugation, the combined calcium phosphate residues were leached with hot methanol (200ml) and this extract was added to the labelled galactose solution. This solution was evaporated *in vacuo* to a thick syrup, and three successive portions of water (200ml) were added and removed *in vacuo* to replace the exchangeable deuterium. The remaining syrup was seeded and left to crystallize in the presence of methanol (100ml). The product was washed with cold ethanol and ether, and recrystallized from methanol, yielding 5.3g (53%), m.p. 144–146°C.

Kinetic techniques

K_m and $k_{cat.}$ values. Enzyme concentrations of 0.3–30μg/ml and substrate concentrations of 0.2–10K_m were generally employed. Otherwise kinetic techniques were identical with those described for the effect of methanol on the enzymic hydrolysis of dinitrophenyl β-D-galactopyranoside (Sinnott & Viratelle, 1973). Extinction coefficients were generally determined by complete enzymic hydrolysis of the glycoside, which was in general far more readily purified than the parent phenol. When so determined they were independent of enzyme and substrate concentration. Table 2 gives details.

Isotope effects. Enzyme concentrations of approx. 0.2μg/ml, and substrate concentrations of ≥10K_m, in 0.1M-sodium phosphate buffer, pH7, with 1mM-Mg^{2+} were used. Conditions of enzyme saturation ([S]>10K_m) were used, and rate of appearance of aglycone was measured spectroscopically in the

Table 1. *Melting points of aryl β-D-galactopyranosides*

Aglycone	Melting point (°C) Deuterated	Undeuterated	Literature	Reference
2-Nitrophenol		200–203 (decomp.)	193–194	
3-Nitrophenol	177–178	181–182	178–179	
4-Nitrophenol	169–170	176–177	177–178	Csűrös *et al.* (1964)
3-Methylphenol		163–164	159–161	
4-Bromophenol	169–170	170–172	172–173	
1-Naphthol		201–203	202–203	Ryan & Mills (1901)
2-Naphthol	208–210 (ex water)	205–208 (ex water) 192–195 (ex EtOH)	190–192 (ex EtOH)	Tsou & Seligman (1952)
4-Cyanophenol	174–175	171–172	This paper	
3,5-Dinitrophenol*	112–115 (ex acetone)	114–115 (ex acetone)		Sinnott & Viratelle (1973)
2,4-Dinitrophenol	158–160	161–162		

* Enzyme experiments were performed with the poorly crystalline glycoside hydrate, obtained by recrystallizing the acetonide from water.

Table 2. *Molar extinction coefficients for β-D-galactopyranosides and parent aglycones*

Measurements were made in 0.1M-sodium phosphate buffer, pH7.0, 1.0mM in $MgCl_2$.

Aglycone	Wavelength (nm)	$10^{-3}\epsilon$ ($l \cdot mol^{-1} \cdot cm^{-1}$) Glycoside	Phenol
3-Nitrophenol	400	0.01	0.188
4-Nitrophenol	400	0	7.52
4-Cyanophenol	281	0.180	2.80
4-Bromophenol	288	0.44	1.12
Phenol	277	0.108	0.572
3-Methylphenol	279	0.083	0.848
3-t-Butylphenol	280	0.049	0.713
1-Naphthol	322	0.48	2.40
2-Naphthol	330	0.075	1.035

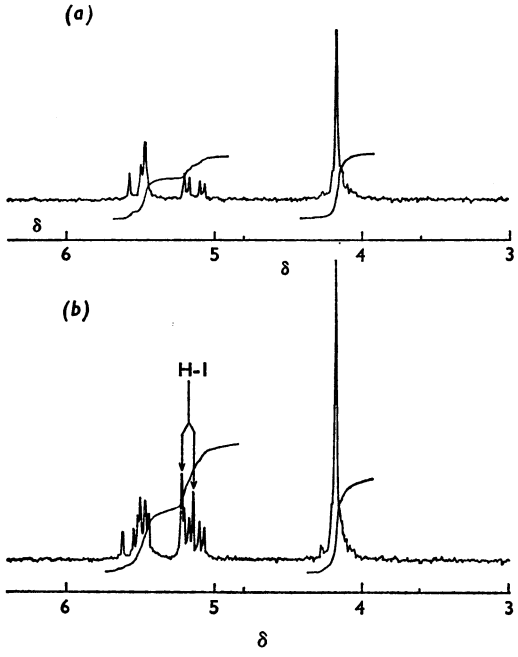

Fig. 1. *Proton-magnetic-resonance spectra for (a) 4-nitrophenyl 2′,3′,4′,6′-tetra-O-acetyl-β-D-[1-²H]-galactopyranoside and (b) its non-deuterated analogue*

Values of δ were obtained at 100MHz for the compounds in C^2HCl_3, relative to trimethylsilane.

Unicam SP.1800 system described previously. The protiated and deuterated substrate, in as small a volume as was compatible with enzyme saturation ($[S] \geqslant 10K_m$; this varied from 50μl to 500μl), was alternately added to a stock enzyme solution. Ratios of successive zero-order increases of extinction were measured; errors are standard errors on more than six points. Loss of enzymic activity over the time-course of the experiment was negligible and identical concentrations of protiated and deuterated substrates were used. In studies of the effect of methanol, substrates were dissolved in the requisite volume of methanol before addition to aqueous enzyme.

Results and Discussion

Fig. 1 illustrates the 100MHz proton n.m.r. spectrum of 4-nitrophenyl 2′,3′,4′,6′-tetra-*O*-acetyl-β-D-[1-²H]galactopyranoside and its protiated analogue, and confirms that the glycoside is effectively completely deuterated at C-1. The singlet at 4.18 δ is due to a fortuitous correspondence of the chemical shifts of the protons on C-5 and C-6, and thus represents three protons (Lemieux & Stevens, 1965). Integration therefore reveals that the region 5.0–5.6 δ is augmented by 1.0 protons in the lower spectrum, which are absent in the upper. This is associated with the appearance of the doublet at 5.18 δ, $J = 8$Hz in the lower spectrum (arrowed in Fig. 1). Such a large splitting is associated with a single, *trans*-diaxial, coupling, and must therefore be associated with the axial proton at C-1. The doublet of doublets centred on 5.13 δ, on which the H-1 doublet is superimposed, integrates for 1 proton; the splittings indicate one axial–axial and one axial–equatorial coupling, and this proton must therefore be on C-3. The 2-proton multiplet around 5.5 δ must therefore represent the protons on C-2 and C-4; in the deuterated compound these would be expected to be subject to one *trans*-diaxial coupling (C-2) and two axial–equatorial couplings (C-4) respectively. One would therefore predict a broad singlet for the proton on C-4 and a doublet for that on C-2. This is observed. Further, protiation at C-1 should greatly complicate this resonance without altering the integration; this is again observed. Such a detailed assignment of all the

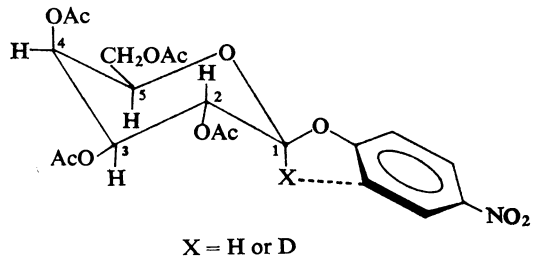

Fig. 2. *4-Nitrophenyl 2',3',4',6'-tetra-O-acetyl β-D-galactopyranoside and its C-1-deuterated analogue in the preferred 'chair' conformation*

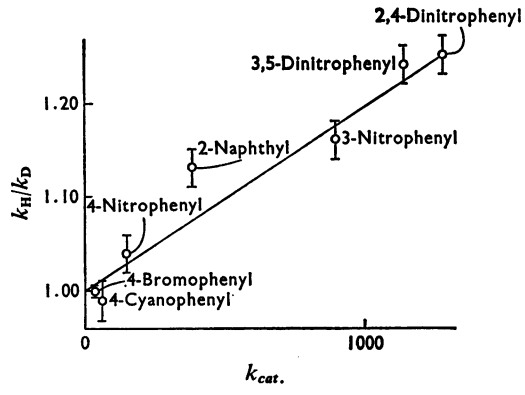

Fig. 3. *Isotope effects for aryl galactosides as a function of $k_{cat.}$.*

Vertical bars denote standard deviations.

ring protons is necessary since the anomeric proton is resonating at remarkably high field; it is normally the lowest-field resonating of the ring protons (Lemieux & Stevens, 1965). This may be partly due to acetylation of the hydroxyl groups on C-2, C-3, C-4 and C-6 in our compounds, but Dahlquist *et al.* (1969) reproduce without comment 220MHz n.m.r. spectra of protiated and partially C-1-deuterated phenyl β-D-glucopyranoside in which the anomeric proton is resonating at higher field than any protons but those on C-5. It seems probable therefore that some form of shielding by the aryl group is taking place (Fig. 2). Integration of the whole of the region 4.6 δ and of the acetate region (not shown) indicated a loss of 1.16 protons in the deuterated compound. Such integration is less reliable than the integration shown, but even 16% deuteration at C-2 would not affect measured isotope effects, since β-deuterium substitution only results in kinetic isotope effects greater than 2–3% if elimination or hydride-shift takes place (Tanida & Tsushima, 1971). Such is demonstrably rarely the case in reactions at C-1 of sugars. Since all deuterated glycosides were synthesized from identical preparations of acetobromogalactose, effectively complete deuteration at C-1 can be assumed.

Fig. 3 shows the kinetic isotope effect as a function of $k_{cat.}$. These data are incompatible with a two-step mechanism characterized by a unique value of k_H/k_D for each step. The line (Fig. 3) is that predicted for a two-step mechanism characterized by k_H/k_D for k_{+2} of 1.00, k_H/k_D for k_{+3} of 1.25 and a value of k_{+3} of 1300. It is seen that the point for 2-naphthyl galactoside lies off this line by three times the standard deviation of k_H/k_D for this compound. Considerations based on the value of k_H/k_D for degalactosylation also indicate that a more complex mechanism is required. The values of k_H/k_D for degalactosylation (i.e. for 3,5- or 2,4-dinitrophenyl β-D-galactopyranoside 1.24 ± 0.02 or 1.25 ± 0.02 respectively) are incompatible with a simple S_N2 reaction or the addition of water to a hypothetical C-1-trigonal species (e.g. a Schiff base).

A possible trivial cause of this result (the selective presence of inhibitory impurities, e.g. mercury compounds, in the deuterated substrates) was eliminated by doubling the substrate concentration and demonstrating that the isotope effect remained unchanged (at 1.24 ± 0.02 for 3,5-dinitrophenyl galactoside). If the galactosyl-enzyme were trigonally co-ordinated at C-1 an inverse isotope effect would be expected; if it were tetrahedral a k_H/k_D ratio near unity would be expected. In fact the value of k_H/k_D for degalactosylation indicates that the transition state for the addition of a nucleophilic solvent (e.g. water) to the galactosyl-enzyme has more trigonal character than the galactosyl-enzyme itself. Since the rate of degalactosylation of the enzyme is dependent on the concentration of acceptor (Sinnott & Viratelle, 1973), this transition state must contain an acceptor molecule. This paradox can only be explained satisfactorily on the postulation of an equilibrating galactosyl-enzyme, in which a small fraction of reactive, C-1-trigonal species is in rapid equilibrium with a preponderance of unreactive, C-1-tetrahedral species. [Postulation of an S_N2 transition state with substantial 'carbonium ion character', apart from being a restatement of, rather than a solution of, the paradox, is unlikely on two grounds. The value of k_H/k_D is high even for 'limiting' nucleophilic substitution reactions (Shiner *et al.*, 1968), and increases when a better nucleophile (methanol) is added.] The transition state for the addition of a nucleophile thus has more trigonal character than the galactosyl-enzyme as a whole. What is the nature of this equilibrating galactosyl-enzyme? Most glycosidases are considered to involve glycosyl cations as intermediates, by analogy with lysozyme and from the

Table 3. *Michaelis–Menten parameters for the β-galactosidase-catalysed hydrolysis of some aryl β-D-galactopyranosides*

Measurements were made in 0.1 M-sodium phosphate buffer, pH 7.0, 1.0 mM in $MgCl_2$, at 25.0°C. $k_{cat.}$ values are ±approx. 7%.

Aglycone	pK_a*	K_m (μM)	$k_{cat.}$ (rel. to 2-nitrophenol galactoside = 1000)
2,4-Dinitrophenol	3.96†	160±10	1290
3,5-Dinitrophenol	6.69†	480±20	1144
2,5-Dinitrophenol	5.22†	430±40	1084
2-Nitrophenol	7.17‡	100±7	(1000)
3-Nitrophenol	8.39	220±25	893
4-Nitrophenol	7.15	28±4	156
4-Cyanophenol	7.95	35±2	59
4-Bromophenol	9.34	43±3	30
Phenol	9.99	87±6	70
3-Methylphenol	10.09	104±9	169
3-t-Butylphenol	10.10§	72±9	259
2-Naphthol	9.51	260±40	379
1-Naphthol	9.34	110±7	52

* Barlin & Perrin (1966).
† Robinson *et al.* (1960).
‡ Kortüm *et al.* (1961).
§ Calculated from Barlin & Perrin (1966).

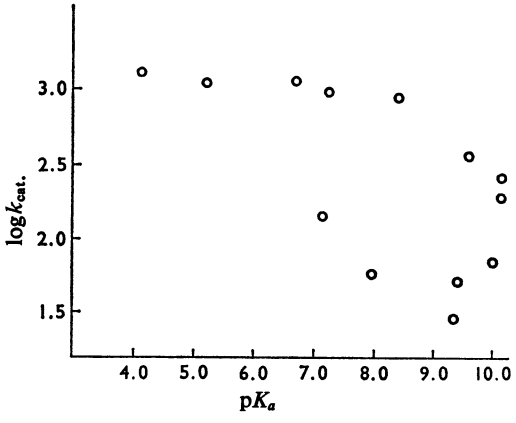

Fig. 4. *Values of $k_{cat.}$ for the hydrolysis of aryl galactosides as a function of aglycone pK_a*

The correlation coefficient is −0.64.

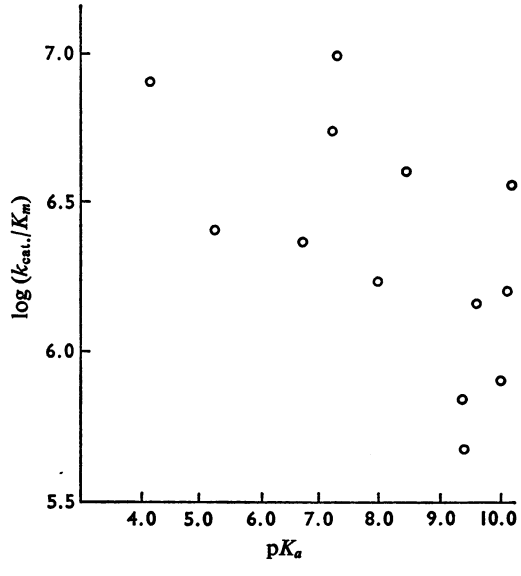

Fig. 5. *Values of $k_{cat.}/K_m$ for the hydrolysis of aryl galactosides as a function of aglycone pK_a*

The correlation coefficient is −0.61.

observation that the catalytic processes of many functionally similar enzymes (e.g. serine proteases) are virtually identical. Glycosyl cations are exceedingly reactive; they are of comparable stability to tertiary alkyl cations (Cocker *et al.*, 1973), which will amongst other things immediately *C*-alkylate activated aromatic compounds. It is therefore contrary to chemical expectation that the simple hydrolysis of a carbonium ion should be the slowest step in the overall catalytic sequence, with a rate of merely 1300 s^{-1}. The glycosyl cation on lysozyme is considered to be stabilized

electrostatically by the ionized Asp-52: however, two opposite charges separated by approx. 0.6nm are clearly unstable with respect to motion towards one another, and Bruice & Dunn, (T. C. Bruice & B. M. Dunn, personal communication; Dunn, 1970) have proposed that the glycosyl cation carboxylate ion-pair of this enzyme collapses to an acylal. Many features of β-galactosidase-catalysed hydrolyses become rationalizable on the equation of the C-1-trigonal ⇌ C-1-tetrahedral interconversion necessary to explain the isotope effects with the reversible collapse of an ion-pair. The moderate rate of hydrolysis of the galactosyl-enzyme is then a reflexion of the low proportion of reactive species. The paradox of a bimolecular nucleophilic substitution reaction with many 'S_N1-like characteristics' has been remarked upon in a number of allyl systems (which yield cations of comparable stability to glycosyl cations). Recent work (Sneen & Bradley, 1972; Sneen & Kay, 1972; Sneen & Carter, 1972) has elegantly and conclusively demonstrated that such reactions proceed by bimolecular nucleophilic attack on reversibly formed ion-pairs. The process we postulate for the hydrolysis of the galactosyl-enzyme therefore must be regarded as precedented and conventional.

The principle of microscopic reversibility, however, requires that the loss of aglycone from the glycoside be the reverse of the addition of water or a nucleophile to the galactosyl-enzyme. This means that the bond-breaking step for release of aglycone involves generation of a glycosyl cation, which should show a k_H/k_D ratio of approx. 1.13. This is not so for the 'slowest' substrates, which have isotope effects near 1.00. A further postulation of a rate-limiting change in the conformation of the enzyme–substrate complex for these substrates is necessary; for such a process an isotope effect of 1.00 would, of course, be predicted. There is considerable, if circumstantial, supporting evidence for this. An examination of Michaelis parameters for a series of aryl β-D-galactopyranosides (Table 3) reveals a dependence on aglycone shape rather than acidity; e.g. *meta* substituents, whether electron-donating or -attracting, indiscriminately accelerate the hydrolysis. Figs. 4 and 5 show $\log k_{cat.}$ and $\log(k_{cat.}/K_m)$ plotted against pK_a of the parent phenol; the points are badly scattered. Phenol acidities range over six powers of 10, and $k_{cat.}$ and $k_{cat.}/K_m$ over factors of 40 and 20 respectively. The low correlation coefficients are therefore meaningful and, coupled with the low gradients of the least-squares lines (−0.20±0.07 and −0.13±0.05, corresponding to Hammett values of 0.5±0.15 and 0.3±0.1 respectively), indicate that electronic effects cannot alone provide the explanation for the variation

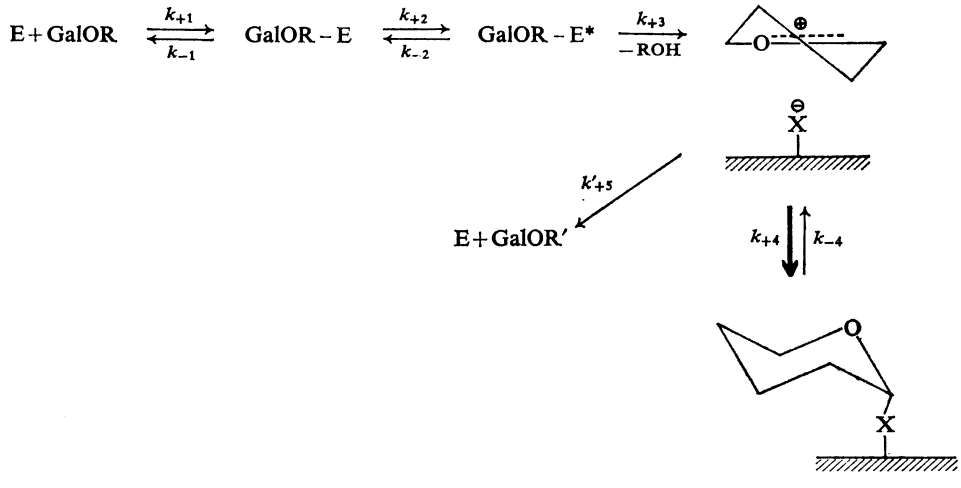

Fig. 6. *Postulated mechanism of action of β-galactosidase*

Gal = β-D-galactopyranosyl. k_2 represents a conformation change. $k_{+4} \gg k_{-4}$. k'_{+5} is a complex quantity representing the reverse of k_{+3}, k_{+2} and k_{+1} steps for the new galactopyranosyl derivative(s).

$$k'_{+5} = \Sigma k_{+5}[\mathrm{ROH}] \qquad \text{(R can be H)}$$

$$k_{cat.} = 1/\{(k_{+4}/k'_{+5}\,k_{-4}) + (1/k_{+2}) + [(k_{+2}+k_{-2})/k_{+2}\,k_{+3}]\}$$

$$K_m = \frac{k_{+2}+k_{-1}}{k_{+1}\,k_{+2}}\left[1\Big/\left(\frac{k_{+4}}{k'_{+5}\,k_{-4}} + \frac{1}{k_{+2}} + \frac{k_{+2}+k_{-2}}{k_{+2}\,k_{+3}}\right)\right]$$

of $k_{cat.}$ with aglycone structure. The pH-dependence of $k_{cat.}$ for 4-nitrophenyl galactoside shows a dependence on a pK of about 9, as does the rate of reaction of the enzyme with the affinity label *N*-(bromoacetyl)-β-D-galactopyranosylamine (Naider *et al.*, 1972; Sinnott & Viratelle, 1973). The susceptibility of the enzyme to the affinity label is considered to vary with protein conformation (Naider *et al.*, 1972). Protein-conformation changes in which the catalytic groups are placed in the correct orientation for facilitation of bond-fission are widely if not universally observed (e.g. Gutfreund, 1972, p. 186). Finally, the operation of a lysozyme-type mechanism would require conformation distortion of the pyranose ring: with lysozyme this is so pronounced that the contribution of a monosaccharide unit binding in the D (catalytic) subsite to the overall free energy of binding of an oligosaccharide is zero (Chipman & Sharon, 1969). Since β-galactosidase hydrolyses monosaccharide derivatives, this procedure is not available to it, and it may be that the distortion has to be introduced in a slow step subsequent to binding.

Fig. 6 therefore represents our postulated mechanism of action of β-galactosidase. Binding is followed by a conformation change; the catalytic step is generation of a galactopyranosyl cation (presumably by the operation of some form of general acid catalysis) in close proximity to a counter-ion. This ion-pair rapidly and reversibly collapses to an unreactive tetrahedral species; the addition of water or a nucleophile takes place to the galactosyl cation. This mechanism would predict that substrates with a high $k_{cat.}$ but with a poor leaving group (i.e. a leaving group with a high pK_a) might have the conformational step speeded up sufficiently that the liberation of the

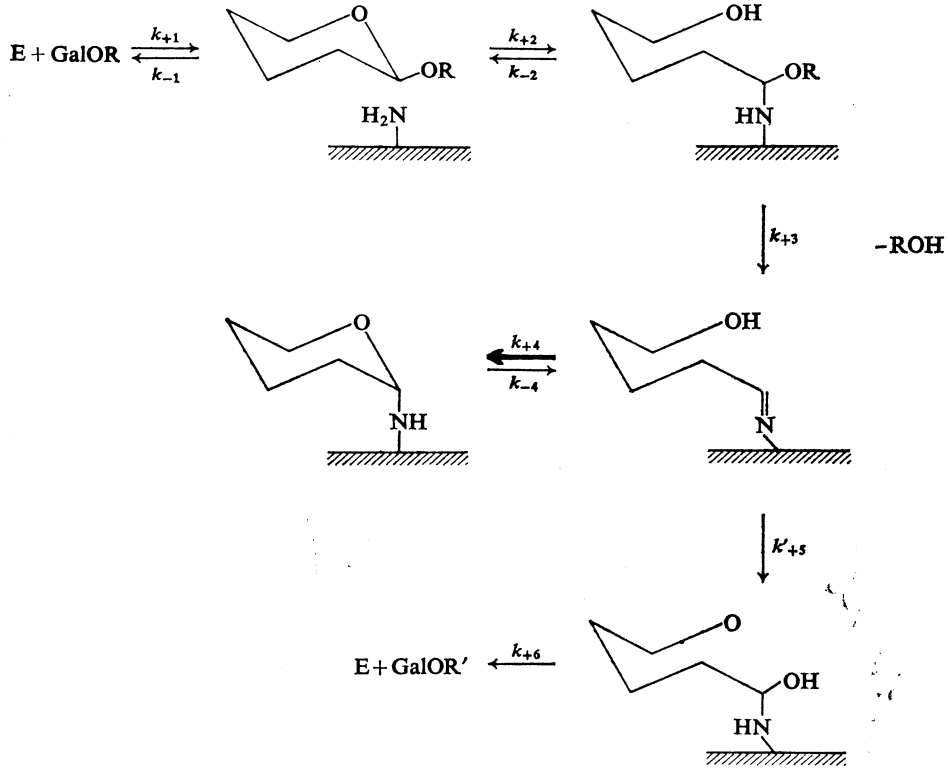

Fig. 7. *Minimum mechanism of action for β-galactosidase if neither a conformation change nor a breakdown of the extension of the principle of microscopic reversibility is assumed*

In this mechanism k_{+2} has to be rate-limiting for 'slow' substrates and $k_{-4}k_{+5}/k_{+4}$ for 'fast' ones. Gal = β-D-galactopyranosyl. $k_{+4} \gg k_{-4}$. $k'_{+5} = \Sigma k_{+5}[\mathrm{ROH}]$.

$$k_{cat.} = 1 \Big/ \left(\frac{k_{+4}}{k_{-4}k_{+5}} + \frac{1}{k_{+2}} + \frac{1}{k_{+6}} + \frac{k_{+2}+k_{-2}}{k_{+2}k_{+3}} \right)$$

aglycone becomes rate-limiting. Such a substrate would have a k_H/k_D ratio higher than that predicted by a two-step mechanism. These expectations are fulfilled by the data for 2-naphthyl β-D-galactopyranoside (Fig. 3).

The possible objection that Fig. 3 simply represents the variation of k_H/k_D for one process (liberation of aglycone) with leaving-group ability can be discounted. As the leaving-group ability, as measured by $k_{cat.}$, goes up so does k_H/k_D. This is the reverse of what is observed in acetal hydrolysis: as the reaction gets faster, the more reactant-like transition states are reflected by lower isotope effects (Bull *et al.*, 1971).

Objections to the use of methanol competition as a criterion for detection of kinetically important degalactosylation are met similarly. The 'curving-over' of plots of $k_{cat.}$ against [MeOH] is ascribed to a 'medium effect' (Sinnott & Viratelle, 1973), on the basis, among other things, of the increase of k_H/k_D for 3,5-dinitrophenyl galactoside in 1.5M-methanol to 1.34±0.04. The increase can be explained from the very fact that methanol is more nucleophilic than water towards the galactosyl-enzyme. For rate-limiting degalactosylation (see Fig. 6):

$$k_{cat.} = \frac{k_{-4}\,k_{+5}}{k_{+4}} \text{ i.e. } \frac{(k_{cat.})_H}{(k_{cat.})_D} = \left(\frac{k_{-4}}{k_{+4}}\right)_H \left(\frac{k_{+4}}{k_{-4}}\right)_D \left(\frac{k'_{5H}}{k'_{5D}}\right)$$

It is reasonable to suppose that $(k_{-4}/k_{+4})_H/(k_{+4}/k_{-4})_D$ remains constant on changing the medium to 1.5M-methanol; k'_{+5} is defined as Σk_{+5} [ROH]. k'_{5H}/k'_{5D} will be less than 1.00. However, the numerical value will be closer to unity for the addition of methanol than for the addition of water, since the better nucleophile will be added in a more reactant-like transition state (cf. do Amaral *et al.*, 1972). Therefore k'_{5H}/k'_{5D}, and hence $(k_{cat.})_H/(k_{cat.})_D$, will increase when the galactosyl-enzyme is reacting largely with methanol; this is definitely the case in 1.5M-methanol: on the assumption of a water concentration of 55M, k_{+5} (MeOH)/k_{+5} (HOH) is 121 (Sinnott & Viratelle, 1973): therefore in 1.5M-methanol the galactosyl-enzyme is reacting preferentially with methanol by a factor of 3.3. That this increase in k_H/k_D is an effect specific to the degalactosylation step is demonstrated by the isotope effects for 4-nitrophenyl β-D-galactopyranoside and 4-bromophenyl β-D-galactopyranoside in 1.5M-methanol. These remain constant, the values being 1.05±0.01 and 1.00±0.02 respectively.

The postulation of undefined 'conformational changes', without their direct observation, can become a seductive catch-all explanation for puzzling kinetic measurements. Nevertheless, any alternative mechanism for the enzyme must explain the low k_H/k_D for slow substrates and the high k_H/k_D for substrates for which degalactosylation is rate-limiting. There are three possible other explanations for these isotope effects, which do not invoke a conformational change.

(i) Liberation of aryl aglycones in an S_N2 reaction, followed by a reaction of water or methanol via a cation as previously postulated. Since water or methanol adds to the glycosyl cation, by the principle of microscopic reversibility the hydrolysis of methylgalactoside or the virtual reaction with galactose must go by an S_N1 pathway. Therefore this explanation requires that microscopic reversibility cannot be applied to species as different as water or methanol and phenols, and that an asymmetrical mechanism for the hydrolysis of aryl galactosides is possible. It does, however, require that substrates with poor leaving groups (water or methanol) react via S_N1-type reactions, whereas substrates with good leaving groups (phenols) react via S_N2-type reactions. This is contrary to chemical experience, in which a change from unimolecular to bimolecular departure of a leaving group is associated with increased reluctance of the leaving group to depart (cf. Ingold, 1969). Neither does it explain the isotope effect for 2-naphthyl galactoside. Objections to S_N2 reactions on acetals (see below) are reinforced by objections to the displacement of equatorial substituents in a six-membered ring by such a process (Fry *et al.*, 1970).

(ii) If non-productive binding of substrates is postulated, the irrational variation of kinetic parameters with aglycone nature can be rationalized. Further, the k_H/k_D ratios of poor substrates (1.00) can be rationalized if it is assumed, on the scheme below, that $k_{+1}K \ll k_{+3}, (k_{-4}/k_{+4})k_{+5}$, and $k_{-1} \ll k_{+2}$, when $k_{cat.} = k_{+1}K$ and $K_m = K$.

$$\text{E+GalOR} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{E-GalOR} \xrightarrow{k_{+3}} \underset{+\text{ROH}}{\text{E-Gal}} \xrightarrow{k_{+5}} \text{E+GalOH}, \qquad \text{E-Gal}^1 \underset{k_{+4}}{\overset{k_{-4}}{\rightleftharpoons}} \text{E-Gal}$$

$$\text{E+GalOR} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{E-GalOR}^1 \qquad K = k_{-2}/k_{+2}$$

$$k_{cat.} = \frac{(k_{+1}\,k_{+3})(k_{+4}\,k_{+5})K/k_{+4}}{k_{+1}\,k_{+3}K + k_{+1}(k_{-4}/k_{+4})k_5K + (k_{+3}+k_{-1})(k_{-4}/k_{+4})k_{+5}}$$

$$K_m = \frac{(k_{-4}\,k_{+5})(k_{+3}+k_{-1})K/k_{+4}}{k_{+1}\,k_{+3}K + k_{+1}\,[(k_{-4}\,k_{+5})/k_{+4}]K + (k_{+3}+k_{-1})[(k_{-4}\,k_{+5})/k_{+4}]}$$

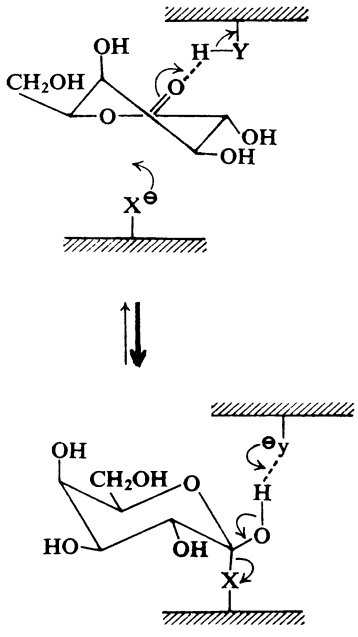

Fig. 8. *Process analogous to ion-pair collapse in explanation of the greater affinity of β-galactosidases for δ-galactonolactone than for thiogalactosides*

However, according to this argument, $k_{+1} = k_{cat.}/K_m$ for poor substrates; these values are ($M^{-1} \cdot s^{-1}$) 7×10^5, 1.7×10^6 and 6×10^6 for 4-bromo-, 4-cyano- and 4-nitro-phenyl galactosides respectively. These rates are below the normal range for enzyme–substrate combinations, 10^7–$10^8\ M^{-1} \cdot s^{-1}$ (Gutfreund, 1972, p. 159), and moreover themselves vary irrationally with substrate structure [according to this scheme, since $k_{cat.}/K_m = k_{+1}k_{+3}/(k_{+3}+k_{-1})$: thus for 2-nitrophenyl galactoside $k_{+1} > 10^7\ M^{-1} \cdot s^{-1}$].

Furthermore, the pattern of substrate-specificity of β-galactosidase, a hydrolase with a high specificity for one monosaccharide unit, and indifference to the aglycone (Wallenfels & Malhotra, 1961), is different from that of enzymes, where non-productive binding is observed. Thus lysozyme has a series of monosaccharide binding-sites (Chipman & Sharon, 1969), and chymotrypsin has an indiscriminate affinity for hydrophobic residues (Fastrez & Fersht, 1973).

(iii) A complex ring-opening mechanism is required if neither a conformation change nor non-productive binding nor a breakdown of the conventional extension of the principle of microscopic reversibility is assumed. Fig. 7 illustrates a minimal mechanism along these lines; an amino group is drawn as the most probable nucleophile capable of supporting a C-1-trigonal species, but various forms of acid catalysis are not shown. Apart from the inefficiency as a catalytic process of pyranose ring-opening, and the well-documented reluctance of acetals to undergo S_N2 reactions, this mechanism cannot account for the inhibition of β-galactosidase by D-galactonolactone. The inhibition of glycosidases by aldonolactones of the corresponding configuration has been very widely observed; inhibition of the β-galactosidase from limpet, barley and sweet-almond meal (Conchie *et al.*, 1968), and from various mammalian sources (Levvy *et al.*, 1962), has been reported. This inhibition, which is generally considerably more powerful than that caused by competitive inhibitors of the thioglycoside type, has been rationalized on the hypothesis that the lactone is isosteric with the (high-energy) glycosyl cation intermediate, and is thus a 'transition-state analogue' (Wolfenden, 1972). This idea can be accommodated and extended by the ion-pair mechanism, for the lactone can be envisaged as participating in a process similar to the ion-pair return (Fig. 8). Such a process, or any analogy to it, cannot occur with the ring-opening mechanism; indeed, a nucleophile so placed as to open the ring would be sterically incapable of attacking the carbonyl group of a lactone inhibitor.

The puzzle of the biological function of the (chemically non-productive) ion-pair return can be clarified if it is realized that the natural leaving group in many glycosidase reactions is another saccharide residue. This saccharide residue will have its own binding-site (see, e.g., Wallenfels & Malhotra, 1961) and thus its departure from the enzyme will be slower than diffusion-controlled. The highly reactive glycosyl cation would, in the absence of ion-pair return, have the possibility of glycosylating a different hydroxyl group of the departing saccharide residue. The immediate removal of the glycosyl cation by the ion-pair-return process thus may serve to avoid the formation of 'wrong' glycosidic linkages. For the β-galactosidase of *E. coli* itself, there is evidence that the process may be involved in a more subtle regulatory manner, for an initial stage in the induction of the enzymes of the *lac* operon by lactose is the conversion of lactose, with a β-(1→4)-linkage, into *allo*-lactose, with a β-(1→6)-linkage, by basal activities of β-galactosidase (e.g. Müller-Hill, 1971). Such considerations refute the potential objection to our mechanism, that ion-pair return is unlikely since it is biologically inefficient.

This work enables one to put forward a mechanism of action of β-galactosidase that does violence to received ideas in neither chemistry nor biology. It has at present, however, only the status of a working hypothesis, and further work is necessary before it can be regarded as established. However, the mechanism does explain the known features of the chemistry of β-galactosidase-catalysed hydrolysis.

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