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The Effect of Methanol and Dioxan on the Rates of the β-Galactosidase-Catalysed Hydrolyses of some β-D-Galactopyranosides: Rate-Limiting Degalactosylation

THE pH-DEPENDENCE OF GALACTOSYLATION AND DEGALACTOSYLATION

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1. The effect of methanol on the β-galactosidase-catalysed hydrolysis of some nitrophenyl β-D-galactopyranosides has been studied under steady-state conditions. 2. The initial fractional rate of increase of $k_{cat.}$ as a function of methanol concentration with 2,4- and 3,5-dinitrophenyl β-D-galactopyranosides, but not with the other substrates studied, indicated that degalactosylation of the enzyme was rate-limiting. 3. The decrease in $k_{cat.}$ at high methanol concentrations for these substrates is considered to arise from causes other than galactosylation becoming rate-limiting. 4. Both galactosylation and degalactosylation of the enzyme require protonation of a group of pK_a approx. 9.

The retention of configuration at C-1 of the galactopyranose ring during transgalactosylations catalysed by the β-galactosidase of *Escherichia coli* (Wallenfels & Malhotra, 1961; Wallenfels & Kurz, 1962) necessitates sequential liberation of products. The demonstration of a common intermediate in the enzymic hydrolysis of a series of aryl β-D-galactopyranosides (Stokes & Wilson, 1972) requires that one of the steps in the sequence be the hydrolysis of a galactosyl-enzyme. Viratelle *et al.* (1969) and Tenu *et al.* (1971) had shown, by using the technique of nucleophilic competition with methanol, that such a step was partly rate-limiting in the hydrolysis of the (hitherto) best substrates known, 2- and 3-nitrophenyl β-D-galactopyranosides. To study the degalactosylation step in any detail, it is necessary to have substrates for which the galactosylation of the enzyme is as fast as possible. An examination of the hydrolysis of β-D-galactopyranosides of more acidic chromogenic aglycones seemed promising, but as, when we started this work, 2,4-dinitrophenyl β-D-galactopyranoside was obtainable only with difficulty (Hengstenberg & Wallenfels, 1969), we first studied the 3,5- and 2,5-isomers; the parent aglycones have pK_a values of 6.7 and 5.2, compared with 4.1 for 2,4-dinitrophenol (Robinson *et al.*, 1960); later Dr. B. Capon and Mr. J. D. G. Sutherland informed us of a simple acid-catalysed deacylation of the tetra-acetate of the 2,4-dinitrophenyl glycoside, and so we also studied the 2,4-isomer.

Methods and Materials

Syntheses

Methyl and 2-nitrophenyl β-D-galactopyranosides. These were synthesized by reaction of the aglycone with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide in the presence of base, followed by catalytic deacylation with sodium methoxide (Conchie *et al.*, 1957), and were recrystallized from ethanol: m.p. 173–175°C and 202–203°C respectively.

3,5-Dinitrophenol hydrate. This was made by the method of Verkade & Witjens (1946).

Dinitrophenyl β-D-galactopyranosides. The tetra-acetates were made by condensation of the sodium salt of the phenol with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (Lemieux, 1963) in aqueous acetone (Conchie & Levvy, 1963); the 3,5-isomer was deacylated by the method of these authors. Greater care had to be exercised in the base-catalysed deacylation of the 2,5-isomer: a suspension (10mg/ml) of the tetra-acetate in 0.01M-sodium methoxide was shaken at 22°C until homogeneous, and was then kept for 2h at −5°C. Neutralization with acetic acid, evaporation below 25°C and trituration with water yielded *2,5-dinitrophenyl β-D-galactopyranoside.* The 2,4-isomer was deacylated under the acidic conditions used by B. Capon & J. D. G. Sutherland (personal communication): a suspension (16mg/ml) of the tetra-acetate in methanolic 3% HCl was shaken at 22°C until homogeneous,

Table 1. *Dinitrophenyl β-D-galactopyranosides and their tetra-acetates*

	Yield (%)	m.p. (decomp.) (°C)	$[\alpha]_D^{25}$ (°) (±2°)	Found (%)		
				C	H	N
Tetra-acetates:						
2,4-	14	181–182	+40, *c* 2, CH_2Cl_2			
2,5-	53	180–181	+60, *c* 1, CH_2Cl_2	46.7	4.4	5.5
3,5-	55	186–187	−17, *c* 3, CH_2Cl_2	47.0	4.4	5.9
Glycosides:						
2,4-	56	161–163	−143, *c* 1, acetone	40.8	4.1	7.7
2,5- (ex acetone)	50	158–160	−84, *c* 1, H_2O	44.55	5.35	6.7
3,5- (ex acetone)	64	114–115	−73, *c* 3, H_2O	43.75	5.05	7.15
3,5- (ex water)				39.7	4.05	7.6

and was then kept for 48h at 4°C. Dilution with 2vol. of chloroform, and precipitation with light petroleum, yielded the product. All three deacylated glycosides could be recrystallized conveniently by addition of ether to their acetone solutions: the most stable (3,5-) isomer could also be recrystallized from water. The 2,5- and 3,5-isomers formed solvates (an acetonide and an acetonide and a hydrate respectively), which were stable at 22°C/66.6Pa (cf. Hengstenberg & Wallenfels, 1969); use of a drying pistol was prohibited by the lability of these glycosides. Characterization data are given in Table 1: the tetra-acetates, $C_{20}H_{22}N_2O_{14}$, require C, 46.7; H, 4.3; N, 5.5; the glycosides, $C_{12}H_{14}N_2O_{10}$, require C, 41.6; H, 4.05; N, 8.1; $C_{12}H_{14}N_2O_{10},H_2O$ requires C, 39.55; H, 4.4; N, 7.7; $C_{12}H_{14}N_2O_{10},C_3H_6O$ requires C, 44.55; H, 4.95; N, 6.95%.

Materials

Solvents. Methanol was purified by distillation from magnesium methoxide and dioxan by distillation from $LiAlH_4$.

β-Galactosidase. This was purchased from the Boehringer Corp. Ltd., Ealing, Middx., U.K. (lot no. 7500408), or was prepared as described by Tenu *et al.* (1971).

Buffers. Buffer 1: 0.100M-sodium phosphate buffer, pH7.00, was made by mixing 0.100M solutions of AnalaR NaH_2PO_4 and Na_2HPO_4 until a pH of 7.00 was registered on a Radiometer pH-meter, calibrated with standard buffer; 1.0mM-$MgCl_2$ was added subsequently. Buffer solutions for pH studies were: 2-(*N*-morpholino)ethanesulphonic acid (from Calbiochem)–NaOH, range pH5.5–7.0; *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (Calbiochem)–NaOH, pH7.0; tetrasodium pyrophosphate (Merck)–HCl, range pH7.7–9.2; sodium hydrogen carbonate (Merck)–NaOH, range pH9.2–9.5. Buffer solutions contained 0.145M-NaCl and 1mM-$MgSO_4$, except for those of pH5.5–6.0, in which 10mM-Mg^{2+} was required for enzyme saturation (Tenu *et al.*, 1971).

Kinetic techniques

Effect of organic solvents on dinitrophenyl galactoside hydrolysis at pH7.0. Hydrolyses were followed in a Unicam SP.1800 double-beam spectrophotometer system, with a thermostat-controlled cell-block (25.0±0.1°C) by a Julabo Paratherm II circulating pump. Reactions were carried out in silica cells (1.00cm, 3.0ml), and were initiated by the addition of a stock solution (5.0μl from an S.G.E. microlitre syringe) of enzyme (protein concentration 0.100mg/ml). Cells were filled with thermally pre-equilibrated buffer and substrate solutions and, after addition of the appropriate quantity of organic solvent, were left in the cell-block for at least 5min before initiation of the reaction. Initial velocities were estimated visually and Michaelis–Menten parameters were determined by unweighted least-squares treatment of (nine-point) plots of v against $v/[S]$: error bars in Figs. 3 and 4 are standard deviations calculated from these plots.

The reaction was followed at 390nm, 440nm and 400nm for the 2,4-, 2,5- and 3,5-dinitrophenyl glycosides respectively. Extinction coefficients ($10^{-3}\epsilon$) were 11.6, 4.11 and 1.86 litre·mol^{-1}·cm^{-1} respectively [cf. Halford (1971) for the first value]. These values were independent of batch of buffer, spectrometer or whether the phenol was weighed out directly or produced by complete enzymic hydrolysis of the glycoside, to within a 5% range; they were not significantly altered by <6% (v/v) methanol. $k_{cat.}$ values are expressed relative to $k_{cat.}$ for 2-nitrophenyl β-D-galactopyranoside of 1000 ($10^{-3}\epsilon = 2.17$ litre·mol^{-1}·cm^{-1} at 400nm for 2-nitrophenol), but the dinitrophenyl glycosides were also standardized relative to each other. [If enzyme concentrations are estimated on the assumption of pure protein, a molecular weight per

active site of 135000 and a value of $E_{1cm}^{1mg/ml}$ of 2.1 at 280nm, $k_{cat.}$ values of up to $800s^{-1}$ for 2-nitrophenyl β-D-galactopyranoside are obtained (Tenu *et al.*, 1971); we estimated values of $600s^{-1}$ by this method.]

Effect of methanol on the enzymic hydrolysis of methyl β-D-galactopyranoside. The change in optical rotation at 346nm consequent upon the addition of 0.100mg of enzyme/ml to 0.118M-methyl β-D-galactopyranoside in the sodium phosphate medium described, containing various quantities of methanol, was followed in a cell (with jacket) of 1ml capacity and 10cm path-length in a Perkin–Elmer 114 photoelectric polarimeter. Analog output was obtained through an AEI pen-recorder, and water maintained at 25.0±0.1°C by a Tecam Tempunit pump was circulated through the jacket of the polarimeter cell. Linear changes in optical rotation were observed for up to 15min.

Effect of pH on the hydrolyses of 4-nitrophenyl galactoside and 3,5-dinitrophenyl galactoside. These hydrolyses were followed in a Cary 16 spectrophotometer with a thermostat-controlled cell-block (25.0±0.1°C) by a Haake cryostat, model FK. 4-Nitrophenyl galactoside hydrolysis was followed at 347.5nm ($\Delta\epsilon = 3270$). 3,5-Dinitrophenyl galactoside hydrolysis was followed at 362.5nm ($\Delta\epsilon = 1035$ at pH7; $\Delta\epsilon$ values at other pH values were calculated by comparison of the total absorbance change during the hydrolysis of substrate at pH7 and at other pH values).

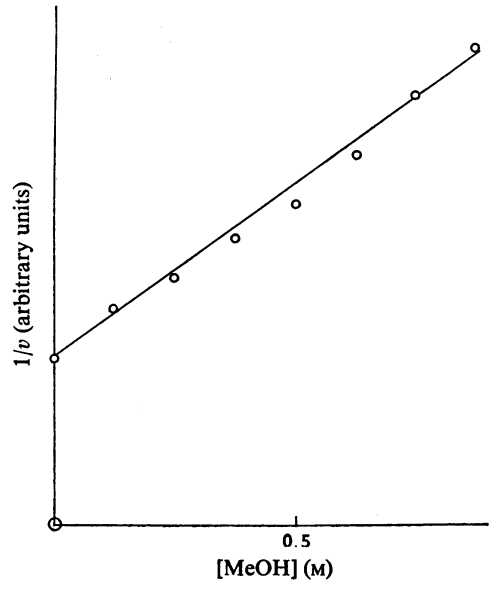


Fig. 1. *Effect of methanol on β-galactosidase-catalysed hydrolysis of methyl β-D-galactopyranoside*

Hydrolysis was done in buffer 1 (see the Methods and Materials section). $[S] \gg K_m$.

Results and Discussion

Relative affinity of the galactosyl-enzyme for water and methanol

Tenu *et al.* (1971) have obtained kinetic data for the β-galactosidase-catalysed hydrolysis of methyl β-D-galactopyranoside: $k_{cat.} = 61s^{-1}$, $K_m = 8$mM; since $k_{cat.} = 61s^{-1}$ is much less than the value for substrates with good leaving groups but the same glycone, degalactosylation of the enzyme cannot be rate-limiting for this substrate. The effect of methanol on its enzymic hydrolysis can be analysed according to the scheme of Viratelle *et al.* (1969):

$$E + GalOMe \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E-GalOMe \underset{k_{+4}[MeOH]}{\overset{k_{+2}}{\rightleftharpoons}} E-Gal \xrightarrow{k_{+3}} E + GalOH$$

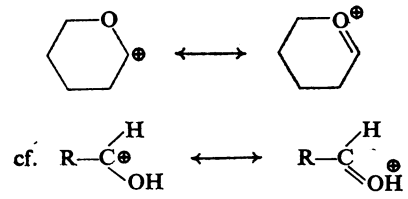
This analysis is not materially altered by the inclusion of other steps between Michaelis complex and galactosyl-enzyme. Since $k_3 \gg k_2$:

$$k'_{cat.} = \frac{k_{+2}k_{+3}}{k_{+3} + k_{+4}[MeOH]} \quad \text{(for appearance of galactose)}$$

$$K_m = \frac{k_{-1}k_{+3} + k_{-1}k_{+4}[MeOH] + k_{+2}k_{+3}}{k_{+1}(k_{+3} + k_{+4}[MeOH])}$$

Hence a plot of $1/k'_{cat.}$ versus [MeOH] should give a straight line of intercept $1/k_{+2}$ and gradient $k_{+4}/k_{+3}k_{+2}$; the ratio of gradient to intercept then gives k_{+4}/k_{+3}. If saturation concentrations of substrate are used (and we used $15K_m$) then $k'_{cat.}$ can be replaced by velocities (in arbitrary units). A k_{+2}-decreasing 'medium effect' (see below) at high methanol concentrations should cause an upward curvature. Fig. 1 demonstrates that linearity is observed, and a least-squares analysis yields the value $2.2 \pm 0.1 M^{-1}$ for k_{+4}/k_{+3}. This is in excellent agreement with the values obtained by Stokes & Wilson (1972) and Viratelle *et al.* (1969) by different techniques ($2.03 \pm 0.08 M^{-1}$ and $1.96 \pm 0.19 M^{-1}$ respectively).

If there are distinct binding and reaction steps for the reaction of the galactosyl-enzyme with water and methanol, then the quantity k_{+4}/k_{+3} is complex and not readily interpretable. The 121:1 preference of the galactosyl-enzyme for methanol is not necessarily caused by binding of methanol; although, if anything, methanol is less nucleophilic than water to saturated



Scheme 1. *Isoelectronic relation of a glycosyl cation with protonated aldehyde*

carbon in both S_N1 and S_N2 reactions (Streitweiser, 1962), it is about 10^2 times as reactive as water towards esters (Bender *et al.*, 1964), and Overend *et al.* (1962) observed capture of glycosyl cations by 0.1 M-glycoside to yield disaccharide to the extent of 5–10% during acid-catalysed hydrolysis of these compounds. It thus appears that alcohols are more reactive than water towards carbonyl-type carbon; a glycosyl cation is isoelectronic with a protonated aldehyde (Scheme 1). The greater nucleophilicity of methanol towards the galactosyl-enzyme is thus consistent with reaction through a galactosyl cation if preferential binding of methanol is not invoked.

Effect of methanol and dioxan on Michaelis parameters for the dinitrophenyl glycosides at the pH optimum

The effect of both methanol and dioxan is to increase K_m values, but the effect of methanol is much more marked (Figs. 3 and 4): the effect of methanol is to increase $k_{cat.}$ for the appearance of aglycone (Fig. 2), but dioxan decreases this quantity (Fig. 4). This is further confirmation of the conclusion of Viratelle *et al.* (1969) that methanol is acting as a nucleophilic solvent towards a galactosyl-enzyme. Stokes & Wilson (1972) cited the maxima observed by Shifrin & Hunn (1969) in the rates of hydrolysis of 2-nitrophenyl β-D-galactopyranoside at high (up to 40%, v/v) methanol concentrations and queried whether the rate enhancement in methanol was evidence for a galactosyl-enzyme. Their objections cannot account for the absence of a rate augmentation either with a non-nucleophilic solvent with a fast substrate, or with a nucleophilic solvent and a slow substrate. Fitting of the data for the variation of $k_{cat.}$ with [MeOH] to the theoretical expression for $k_{cat.}$ derived from Scheme 2, by using the algorithm Spiral (Jones, 1970), indicates that for the 2,4- and 3,5-dinitrophenyl galactosides $k_{+2} = 5k_{+3}$ and for the 2,5-isomer $k_{+2} = 3k_{+3}$. However, the non-linearity of the variation of $k_{cat.}$ with [MeOH] could have had two other causes: (i) specific binding of methanol to the galactosyl-enzyme (i.e. saturation of acceptor binding-sites at high methanol concentrations); (ii)

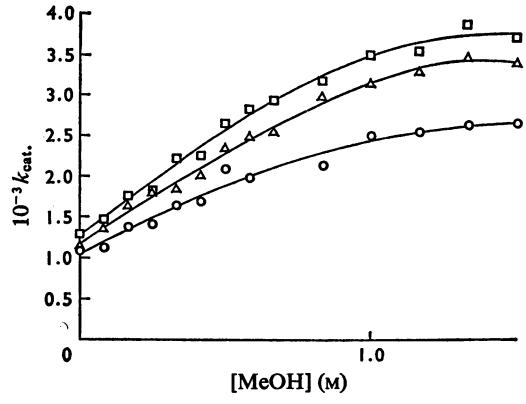


Fig. 2. *Effect of methanol on $k_{cat.}$ for the β-galactosidase-catalysed hydrolysis of dinitrophenyl β-D-galactopyranosides*

Buffer 1 (see the Methods and Materials section) was used. Results for 2,4- (□), 2,5- (○) and 3,5-dinitrophenyl (△) compounds are shown.

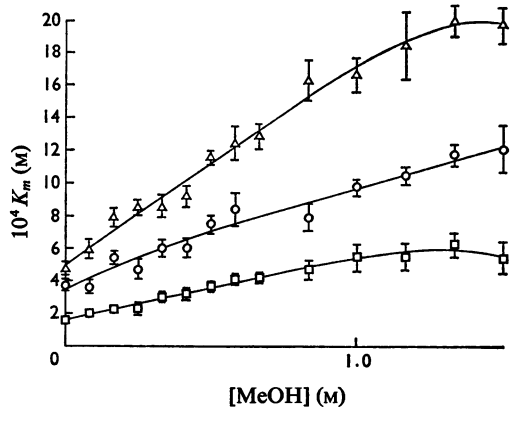


Fig. 3. *Effect of methanol on K_m for the β-galactosidase-catalysed hydrolysis of dinitrophenyl β-D-galactopyranosides*

Buffer 1 (see the Methods and Materials section) was used. Results for 2,4- (□), 2,5- (○) and 3,5-dinitrophenyl (△) compounds are shown. Vertical bars denote standard deviations.

various effects caused by changing the medium to up to 6% (v/v) methanol.

That observed effects are due largely to pH changes on addition of organic solvent is unlikely for two reasons: the pH–rate profile of both accessible steps of the reaction is flat in the region of pH7, and it was observed that 6% (v/v) methanol altered the

$$E + GalOR \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E - GalOR \xrightarrow{k_{+2}} \begin{cases} \xrightarrow{k_{+3}} E + GalOH \\ \xrightarrow[[MeOH]]{k_{+4}} E + GalOMe \end{cases}$$

$$k_{cat.} = \frac{k_{+2}(k_{+3} + k_{+4}[MeOH])}{k_{+2} + k_{+3} + k_{+4}[MeOH]};$$

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \cdot \frac{k_{+3} + k_{+4}[MeOH]}{k_{+2} + k_{+3} + k_{+4}[MeOH]}$$

Scheme 2. *Derivation of theoretical expression for $k_{cat.}$.*

apparent extinction coefficient of 2-nitrophenol, which is approximately half-ionized at this pH, by only about 2%.

A more sophisticated analysis of the variation of $k_{cat.}$ with [MeOH] will enable one to test for rate-limiting degalactosylation of the enzyme even in the presence of complications caused by (i) and, in certain circumstances, (ii) as well. Differentiation of the expression for $k_{cat.}$ in Scheme 2 yields an expression for the initial fractional rate (A) increase in $k_{cat.}$:

$$A \equiv \underset{[MeOH] \to 0}{\text{Lim.}} \left(\frac{1}{k_{cat.}} \cdot \frac{d(k_{cat.})}{d[MeOH]} \right) = \frac{k_{+2}}{k_{+2} + k_{+3}} \cdot \frac{k_{+4}}{k_{+3}}$$

The data in Fig. 2 were fitted by a least-squares procedure to empirical quartic equations, which are plotted through the experimental points in this figure and which yielded values for A of 2.12, 2.13 and 1.87 M^{-1} for the 2,4-, 3,5- and 2,5-dinitrophenyl galactosides respectively. These values suggest that degalactosylation of the enzyme is rate-limiting for the 2,4- and 3,5-isomers because the identity of their A values with the value of k_{+4}/k_{+3} measured from the hydrolysis of methyl galactoside means that $k_{+2} \gg k_{+3}$. This implies that by this technique complications caused by the 'medium effect' are avoided. Degalactosylation of the enzyme would not be rate-limiting for the 2,5- derivative, and this would be in accord with the smaller total increase in $k_{cat.}$ for the latter isomer. The difference in $k_{cat.}$ values for the 2,4- and 3,5-isomers could easily have arisen from the accumulation of small errors in $k_{cat.}$ and extinction coefficients.

α-Deuterium kinetic isotope effects indicate that the non-linearity of the variation of $k_{cat.}$ for the 3,5- and 2,4-dinitrophenyl galactosides with methanol concentration is not caused by a change in rate-limiting step; k_H/k_D ratios in the absence of methanol vary from 1.00±0.01 for very poor substrates to 1.24±0.02 for 3,5-dinitrophenyl galactoside and 1.25±0.02 for 2,4-dinitrophenyl galactoside (Sinnott & Souchard, 1973). It is reasonable to suppose that k_{+2} is associated with an isotope effect near 1.00, and k_{+3} with one of 1.25; a change of rate-limiting step from k_{+3} to k_{+2} should therefore be associated with a decrease in isotope effect. If the curvature of plots of $k_{cat.}$ against methanol concentration were caused by the tendency of $k_{cat.}$ to reach a limiting value of k_{+2} therefore, k_H/k_D for 3,5-dinitrophenyl galactoside should decrease in 1.5 M-methanol. In fact it increases (to 1.34±0.04), whereas for 4-nitrophenyl galactoside (for which $k_{+2} \ll k_{+3}$) it remains constant, being 1.04±0.02 in the absence of methanol and 1.05±0.01 in its presence.

Further evidence that the initial gradient technique does compensate for the 'medium effect' comes from considerations of solvent polarity. Data on the Winstein–Grunwald Y value in aqueous methanol (Fainberg & Winstein, 1956) confirm the intuitive expectation that, even for chemical reactions with highly polar transition states, up to aq. 6% (v/v) methanol is virtually identical with pure water. This

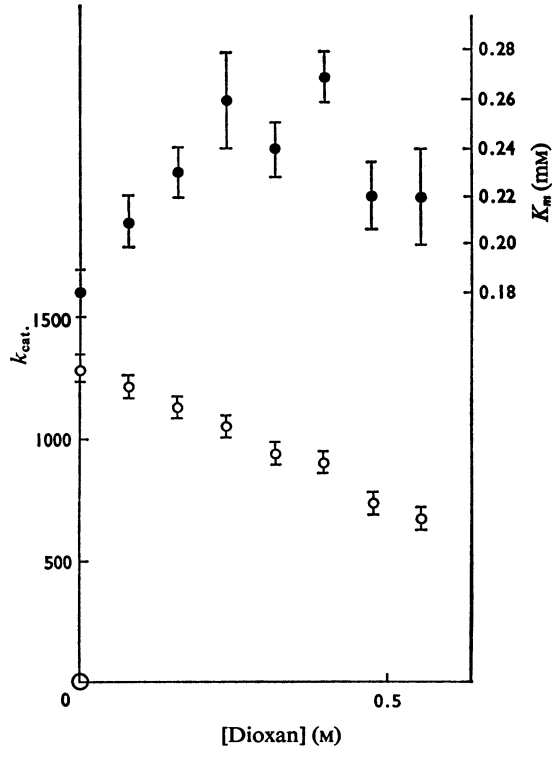


Fig. 4. *Effect of dioxan on the β-catalysed hydrolysis of 2,4-dinitrophenyl β-D-galactopyranoside*

Buffer 1 (see the Methods and Materials section) was used. ●, K_m; ○, $k_{cat.}$. Vertical bars denote standard deviations.

strongly indicates that the 'medium effect' operates through a change in protein conformation and therefore should be observed, uncomplicated by any nucleophilic competition, for substrates for which $k_{+2} \ll k_{+3}$. Indeed a decrease in $k_{cat.}$ for 4-nitrophenyl galactoside is observed (Viratelle & Yon, 1973); however, the initial rate of decrease is zero. This is the criterion for the 'initial gradient' method to compensate for the 'medium effect', and is in accord with the correspondence of A with k_{+4}/k_{+3} for 2,4- and 3,5-dinitrophenyl galactosides.

The effect of dioxan on K_m for 2,4-dinitrophenyl galactoside is to increase it by a factor of (maximally) 1.5 (Fig. 4); the effect of methanol is to increase K_m by about a factor of 4 for the 3,5- and 2,4-dinitrophenyl galactosides, and 3 for the 2,5-isomer. This can again be accounted for by nucleophilic competition, according to a simple two-step scheme:

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

and so, if $k_{+2} \gg k_{+3}, k_{+4}$, a linear variation of K_m with [MeOH] should be observed. As with the variation of $k_{cat.}$ with [MeOH], differentiation gives:

$$B \equiv \underset{[\text{MeOH}]\to 0}{\text{Lim.}}\left(\frac{1}{K_m}\cdot\frac{dK_m}{d[\text{MeOH}]}\right) = \frac{k_{+2}}{k_{+2}+k_{+3}}\cdot\frac{k_{+4}}{k_{+3}}$$

Values of B (M^{-1}) of 2.8, 2.75 and 2.65 for 3,5-, 2,4- and 2,5-dinitrophenyl galactosides respectively were obtained in a manner exactly analogous to A values. In view of the lower precision of K_m values, the doubt about what they represent in terms of individual rate constants, and the possibility of still further unforeseen effects of organic solvents (e.g. competitive binding to the active site), these values of B are not in conflict with our postulation of rate-limiting degalactosylation for the 3,5- and 2,4-isomers.

The 'initial gradient' treatment was also applied to data for 2- and 3-nitrophenyl β-D-galactopyranosides (Viratelle & Yon, 1973). Values of A of 1.81 and 1.85 and of B of 2.14 and 2.59 respectively were obtained; these are in accord with degalactosylation not being completely rate-limiting with these substrates. Only a

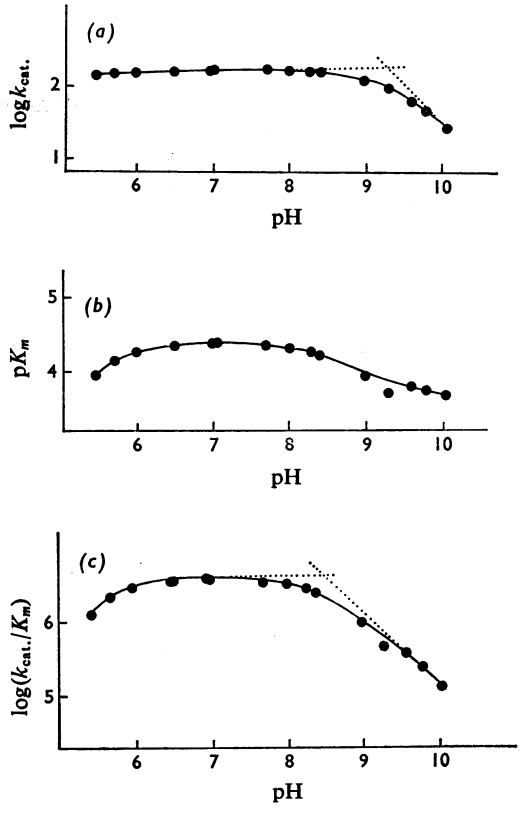


Fig. 5. *Effect of pH on the hydrolysis of* 4-*nitrophenyl galactoside*

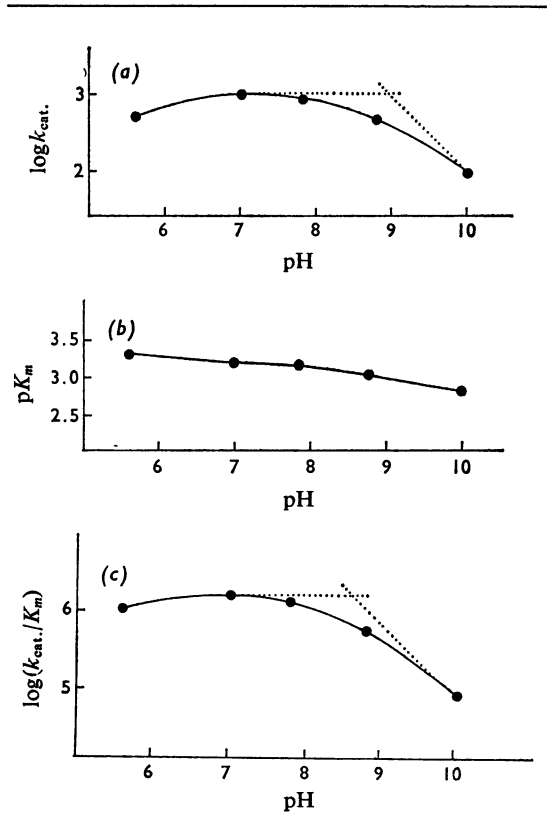


Fig. 6. *Effect of pH on the hydrolysis of* 3,5-*dinitrophenyl galactoside*

cubic expression was used, since a quartic produced spurious points of inflexion.

Figs. 5 and 6 show the effect of pH on the hydrolysis of 4-nitrophenyl galactoside and 3,5-dinitrophenyl galactoside. In the alkaline pH range, the $k_{cat.}$ values for both substrates show a dependence on a pK of about 9 (8.9 and 9.2 respectively). However, the 3,5-dinitrophenyl compound shows some indication of a decrease in $k_{cat.}$ in the acidic region.

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