# Active-site-directed Irreversible Inhibition of Glycosidases by the Corresponding Glycosylmethyl-(*p*-nitrophenyl)triazenes

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β-D-Galactopyranosylmethyl-(*p*-nitrophenyl)triazene is an active-site-directed irreversible inhibitor (ASDIN) of the *ebg*° and the *lacZ* β-galactosidases of *Escherichia coli*, of the β-galactosidase of human-liver lysosomes, and, less effectively, of the α-galactosidase of green coffee beans; it has no effect on the *lac* repressor of *E. coli*. β-D-Glucopyranosylmethyl-(*p*-nitrophenyl)triazene is an ASDIN of both β-glucosidase and β-galactosidase activities of sweet-almond β-glucosidase B; it has no effect on yeast α-glucosidase, the *lacZ* β-galactosidase of *Escherichia coli*, or glucoamylase from *Aspergillus niger*. β-D-Xylopyranosylmethyl-(*p*-nitrophenyl)triazene is an ASDIN of the β-xylosidase from *Penicillium wortmanni*, but has no effect on the β-xylosidase of *Bacillus pumilus*, except by virtue of its consumption of stabilising dithiothreitol. α-L-Arabinofuranosylmethyl-(*p*-nitrophenyl)triazene is an ASDIN for the extracellular α-L-arabinofuranosidase of *Monilinia fructigena* of more alkaline pH optimum at pH 7; the other isoenzyme is inert at this pH, and more acidic pHs destroy the reagent. It is concluded that glycosylmethyl(aryl)triazenes are ASDINs for glycosidases for which both substrate and product stereochemistries at the anomeric centre are the same as that of the reagent; that they can act, with lower effectiveness, on glycosidases for which both substrate and product stereochemistries are opposite to that of the reagent; and that they have no effect on glycosidases with opposite stereochemistries of substrate and product, or proteins of no catalytic function.

THE specific covalent labelling of groups at the active site of an enzyme can provide information about its catalytic mechanism,[1] and the ability selectively to inactivate it can be a useful tool in elucidating its role *in vivo*.[2] The functional group of the substrate-analogue that becomes attached to the protein can be overtly reactive (*e.g.* $-CO-CH_2Br$) or can be derived by the action of light or the catalytic process of the enzyme itself from a precursor bound non-covalently in the active site. There exists, however, a gap in the range of available reactivities of the labelling species, between highly selective alkylating agents such as halogenocarbonyl compounds or Michael acceptors, and the indiscriminate species such as carbenes and nitrenes produced in photoaffinity labelling. The ions produced in deamination reactions promised to fill this gap, as seen when ions were generated in the active site of chymotrypsin from appropriate nitrosoamides,[3] and in the active site of the *lacZ* β-galactosidase of *Escherichia coli* from a galactosylmethyl(aryl)triazene.[4]

We have investigated the latter reaction in detail.[5,6] The galactopyranosylmethyl moiety becomes attached exclusively to the sulphur atom of methionine 500 of both $Mg^{2+}$ and $Mg^{2+}$-free enzyme. The alkylating species (probably the diazonium ion [7,8]) is generated in the active site of $Mg^{2+}$-enzyme 4—5 times faster, and in the active site of $Mg^{2+}$-free enzyme 800 times faster, than in free solution, although it is captured by $Mg^{2+}$-enzyme with only 80% efficiency, and by $Mg^{2+}$-free enzyme with only 25% efficiency. The triazene is thus a ' suicide substrate '.[9] No random labelling of protein was observed, as is anticipated from this conclusion and the short lifetime of alkane diazonium ions.

Since glycosylmethylamines † are available in two steps from acylglycosyl halides,[10,11] and one system had been characterised in detail, it seemed worthwhile to test the generality of glycosylmethyl-(*p*-nitrophenyl)-triazenes as active-site-directed irreversible inhibitors (and hence most probably affinity labels) of glycosidases. We addressed ourselves to the following questions. (1) Is inhibition in any way dependent upon the biological source of the enzyme? (2) Will inactivation of an enzyme away from the optimum pH for catalysis be observed? The spontaneous decomposition of glycosylmethyl-(*p*-nitrophenyl)triazenes becomes inconveniently fast below pH 6, where many glycosidases have their pH optima. The decomposition of alkyl(aryl)triazenes is slowed down by electron-withdrawing groups in the aryl moiety, but the *p*-nitrophenyl compounds represent the limit of what is conveniently (and safely) attainable. (3) How sensitive are glycosidases to the glycosylmethyl-(aryl)triazenes derived from an inappropriate sugar? (4) Will glycopyranosylmethyltriazenes with equatorial methylene groups inhibit glycosidases which act on substrates with axial aglycones? (5) Will a triazene inactivate an enzyme which gives initial products of inverted anomeric configuration? (6) Will a suitable triazene inactivate a furanosidase? (7) Will inactivation of a glycosyl-binding protein which has no catalytic function occur?

Answers have come from the enzymes and inactivators shown in Table 1. Additionally, a binding protein (*lac* repressor) has been tested, and the cross-specificity of β-glucosidase and β-galactosidase towards triazenes of the *gluco*- and *galacto*-configurations has been investigated.

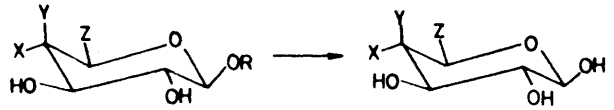
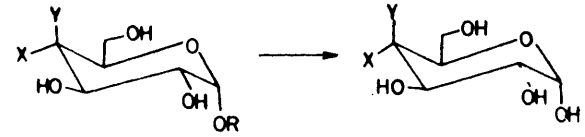
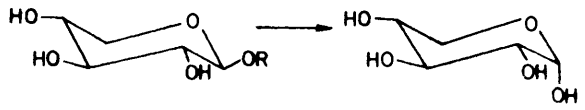
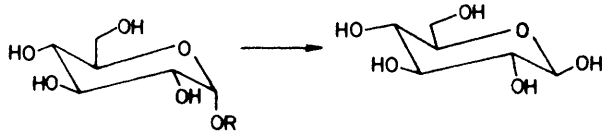
## RESULTS AND DISCUSSION

The criteria for the active-site-directed nature of an irreversible inhibition brought about by triazene adopted

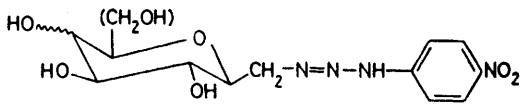
† I.U.P.A.C. names for the glycosylmethylamines employed in this study are 1-amino-2,6-anhydro-1-deoxy-D-*glycero*-L-*manno*-heptitol (β-D-galactopyranosylmethylamine), 1-amino-2,6-anhydro-1-deoxy-D-*glycero*-D-*gulo*-heptitol (β-D-glucopyranosylmethylamine), 1-amino-2,6-anhydro-1-deoxy-D-gulitol (β-D-xylopyranosylmethylamine), 1-amino-2,5-anhydro-1-deoxy-L-mannitol (α-L-arabinofuranosylmethylamine).

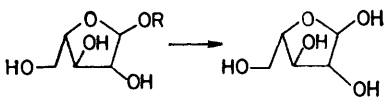
TABLE 1

Structures of substrates, active-site reagents, and stereochemical course of glycosidase-catalysed reactions

| Reaction catalysed | Designation |
|---|---|
|  | X = OH, Y, Z = H, β-Xylosidase (*Penicillium wortmanni*),[a] EC 3.2.1.37 |
| e ⟶ e | Z = $CH_2OH$, X = H, Y = OH, β-Galactosidase (product of the *lacZ* gene [b] and *ebg*[c] gene [c] of *E. coli*). Z = $CH_2OH$, X = H, Y = OH, β-Galactosidase (Human-liver lysosomes: see text) } EC 3.2.1.23. |
| | Z = $CH_2OH_2$, X = OH, Y = H, β-Glucosidase (sweet almonds, B isoenzyme),[d] EC 3.2.1.21. |
|  | X = H, Y = OH α-Galactosidase (Coffee beans),[e] EC 3.2.1.22. |
| a ⟶ a | X = OH, Y = H α-Glucosidase (Yeast),[d] EC 3.2.1.20. |
|  | c ⟶ a β-Xylosidase (*Bacillus pumilus*) [f] EC 3.2.1.37. |
|  | a ⟶ c Glucoamylase (*Aspergillus niger*),[g] EC 3.2.1.3. |

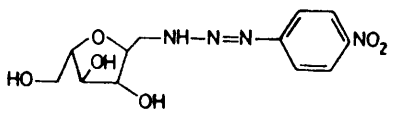
Active-site reagents for the above enzymes have the general formula





f(r) α-L-Arabinofuranosidases (*Monolinia fructigena*),[h] EC 3.2.1.55.

Active-site reagent



[a] Ref. 17. [b] K. Wallenfels and G. Kurz, *Biochem. Z.*, 1962, **335**, 559. [c] Ref. 12. [d] F. W. Parrish and E. T. Reese, *Carbohydrate Res.*, 1967, **3**, 424. [e] H. Carchon and C. K. De Bruyne, *Carbohydrate Res.*, 1975, **41**, 175. [f] H. Kersters-Hilderson, M. Claeyssens, and C. K. De Bruyne, *Carbohydrate Res.*, 1976, **47**, 269. [g] J. E. G. Barnett, *Biochem. J.*, 1971, **123**, 607. [h] Ref. 23.

in this paper are protection by a reversible inhibitor of catalysis and/or observation of saturation kinetics during the inactivation. In favourable circumstances, where inactivation is fast compared to triazene decomposition, and where the reversible inhibitor of catalysis is cleanly competitive, the kinetics of inactivation should obey equation (1) where Tr denotes triazene, and I denotes a

$$\text{(Activity)} = \text{(starting activity)} \times \exp.\ -\left\{\frac{k_{max.}[Tr]}{[Tr] + K\left(1 + \frac{[I]}{K_i}\right)}\right\}t \quad (1)$$

competitive inhibitor of catalysis for which the dissociation constant of the EI complex is $K_i$.* The dissociation constant of the non-covalent E—Tr complex is $K$ and the first-order rate constant with which it is converted into glycosylmethylated enzyme is $k_{max.}$. The results are summarised in Table 2.

*e* ⟶ *e Glycopyranosidases.*—The five enzymes of this class investigated were all inactivated by the corresponding triazene. The enzymes were obtained from a wide range of biological sources (bacterium, fungus, higher

* If the inactivation obeys this law, enzyme activity always decays exponentially to zero; the dependence of the observed rate constant on triazene concentration is hyperbolic, with a maximum rate constant for inactivation of $k_{max.}$ when all the enzyme active sites are occupied by triazene molecules. The triazene concentration to give inactivation at $k_{max.}/2$ is $K$, and this is increased by a factor of $(1 + [I]/K_i)$ in the presence of a competitive inhibitor of catalysis (see *e.g.* C. Walsh, 'Enzymatic *Reaction Mechanisms*,' W. H. Freeman, San Francisco, 1979, pp. 86—92).

TABLE 2

Active-site-directed irreversible inhibition of glycosidases by glycosylmethyl-(*p*-nitrophenyl)triazenes (at 25 °C unless otherwise stated)

| $R-CH_2-NH-N=N-p-C_6H_4NO_2$ | | Buffer | | | | | | |
|---|---|---|---|---|---|---|---|---|
| R | Enzyme | Cation | Anion | pH | Other solute | $10^5\ k_{max.}/s^{-1}$ | $K$/mmol $l^{-1}$ | $\log_{10}\{k_{max.}/s^{-1} / K/\text{mol}\ l^{-1}\}$ |
| β-D-Gal-*p* | $Mg^{2+}$-*lacZ* β-galactosidase | $Na^+$ | $H_2PO_4^-$ (0.05M) | 7.0 | 0.5mM-$MgCl_2$ | 40 ± 2 | 0.07 ± 1 | 0.76 |
| β-D-Gal-*p* | $Mg^{2+}$-free *lacZ* β-galactosidase | $Na^+$ | EDTA (0.05M) | 7.0 | | 970 ± 20 | 0.48 ± 0.02 | 1.31 |
| β-D-Gal-*p* | $Mg^{2+}$-*ebg*° β-galactosidase | $K^+$ | $H_2PO_4^-$ (0.125M) | 7.5 | 5 mM-$MgCl_2$ | 2.3 ± 0.1 | 0.19 ± 0.07 | −1.0 |
| β-D-Gal-*p* | $Mg^{2+}$-free *ebg*° β-galactosidase | $K^+$ | Citrate (0.1M) | 7.5 | | 14 ± 1 | 1.8 ± 0.3 | −1.1 |
| β-D-Gal-*p* | $Mg^{2+}$-free *ebg*° β-galactosidase | $K^+$ | Citrate (0.1M) | 7.5 | | 1.0 ± 0.1 | 3 ± 0.8 | −2.5 at 4 °C |
| β-D-Gal-*p* | Human lysosomal β-galactosidase | $Na^+$ | $CH_3CO_2^-$ (0.1M) | 6.0 | | 680 ± 60 | 0.018 ± 0.003 | 2.58 |
| β-D-Gal-*p* | Sweet almond β-glucosidase B | $Na^+$ | $CH_3CO_2^-$ (0.1M) | 6.0 | | | | *ca.* −3 to −4 |
| β-D-Glc-*p* | Sweet almond β-glucosidase B | $Na^+$ | $CH_3CO_2^-$ (0.1M) | 6.0 | | | | −1.2 |
| β-D-Xyl-*p* | β-Xylosidase (*P. wortmanni*) | $Na^+$ | EDTA (0.1M) | 7.0 | | | ~100 | 1.58 ± 0.06 [a] |
| α-L-Ara*f* | α-L-arabinofuranosidase I (*M. fructigena*) | $(CH_2OH)_3CNH_3^+$ (10mM) | $Cl^-$ | 7.0 | | | | < −2.8 |
| | α-L-arabinofuranosidase III (*M. fructigena*) | | | 7.0 | | | | −2 |
| β-D-Gal-*p* | α-Galactosidase (coffee) | $Na^+$ | $CH_3CO_2^-$ (0.1M) | 6.0 | | | | −1.8 |
| β-D-Glc-*p* | α-Glucosidase (yeast) | $Na^+$ | EDTA (0.1M) | 7.0 | | | | < −2.3 |
| β-D-Glc-*p* | Glucoamylase (*A. niger*) | $Na^+$ | EDTA (100mM) | 6.0 | | | | < −3.6 |
| | | $Na^+$ | EDTA (10mM) | 7.2 | | | | < −2.9 |
| β-D-Xyl-*p* | β-Xylosidase (*B. pumilus*) | $Na^+$ | $H_2PO_4^-$ (10 mM) | 7.2 | 1mM-EDTA, 1mM-dithiothreitol | | | < −2 |
| β-D-Gal-*p* | *lac* repressor | $(CH_2OH)_3CNH_3^+$ (0.1M) | $Cl^-$ | 8.0 (at 4 °C) | 0.1M-NaCl | < 1 at [Tr] = 1mM | | At 4 °C |

[a] From least-squares treatment of eight-point of $1/k_{obs.}$ against 1/[Tr]

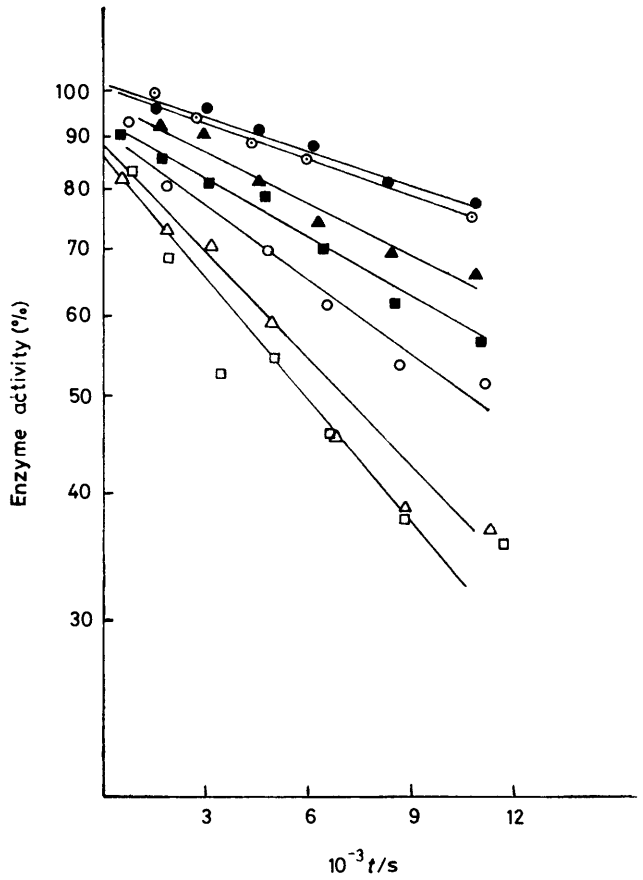



FIGURE 1 Loss of activity of $Mg^{2+}$-free *ebg*° β-galactosidase in the presence of various concentrations of *galacto*-triazene in 0.1M-potassium citrate buffer, pH 7.5, at 25 °C. Triazene concentrations (mM): ○, 0.27; ●, 0.41; ▲, 0.68; ■, 0.96; ○, 1.36; △, 2.7; □, 4.1. The lines were those whose gradients were used to calculate $k_{max.}$ and $K$

plant, and mammal), so source cannot be an important factor in determining the susceptibility of a glycosidase to the corresponding triazene.

ebg° β-*Galactosidase of* E. coli.—Problems of triazene decomposing at a rate comparable with enzyme inactivation were particularly acute with this enzyme, and protection of the $Mg^{2+}$-form of the enzyme by a competitive inhibitor of catalysis could be shown only qualitatively [β-D-galactopyranosyltrimethylammonium bromide (28 mM, $2 \times K_i$ [12]) protected against the *ca.* 15% inhibition produced by an initial triazene concentration of 0.2mM].

As with the *lacZ* β-galactosidase from the same organism, removal of $Mg^{2+}$, although lowering the catalytic effectiveness against *O*-glycosides,[12] increases the rate of inactivation by triazene, loss of activity being illustrated in Figure 1. Essentially complete (97.5%) inactivation can be achieved at 4 °C, indicating that $k_{max.}/K$ is governed by a lower activation energy than the spontaneous decomposition of the triazene.

lacZ β-*Galactosidase of* E. coli. *Further Studies.*—To test whether a triazene-based active site reagent can work at a pH removed from the optimal one for catalysis, we chose this enzyme, since the pH dependence of the hydrolysis of both *O*-[13] and *N*-glycosides,[14] and the site of triazene attack is known, and we had already done a study with $[Tr] \sim K$.[5] The effect of pH on $k_{max.}$ and $k_{max.}/K$ is shown in Figure 2. Both these rate parameters show a dependence on pH of the form shown in equation (2), the $pK_a$ being 7.9 for the ionisation governing the

$$\text{Observed rate parameter} = \frac{[\text{maximal rate parameter}]}{1 + K_a/[H^+]} \quad (2)$$

variation with pH of $k_{max.}$ and 6.9 for the variation of $k_{max.}/K$. One rationalisation of these data would be that decomposition of the triazene requires protonation of a single group of $pK_a$ 6.9 in the free enzyme and 7.9 in the EI complex. This pH dependence parallels that for catalysis of the hydrolysis of *o*-nitrophenylgalactoside where $pK_a$ values of 6.5 and 8.4 govern the dependence of $k_{cat.}/K_m$ and $k_{cat.}$, respectively.

This pH-dependence suggests that it might be possible to use a triazene as an active site reagent at a pH a couple of units more alkaline than the optimum for catalysis, provided the enzyme were particularly sensitive to this type of reagent at its pH-optimum.

β-*Galactosidase from Human-liver Lysosomes.*—Although the stereochemical course of the reaction catalysed

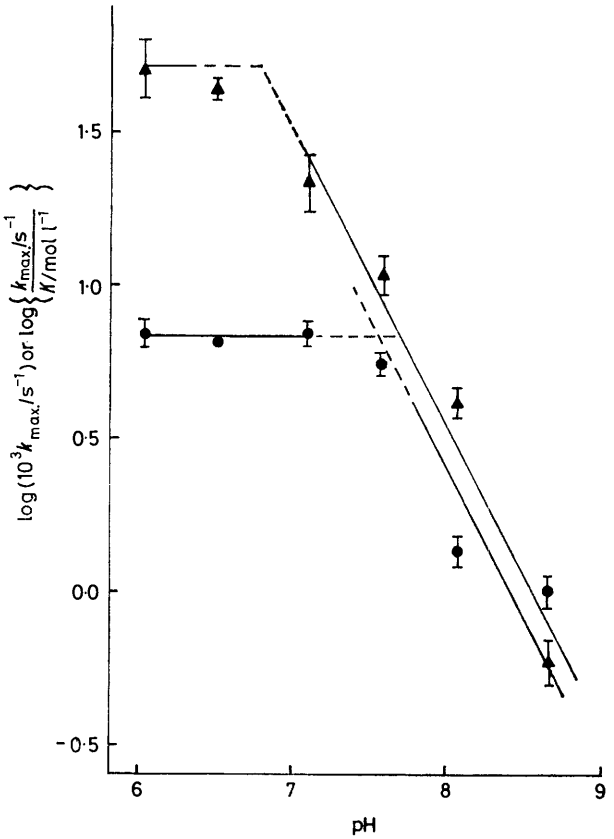



FIGURE 2 Effect of pH upon rate of inactivation of $Mg^{2+}$-free *lacZ* β-galactosidase by *galacto*-triazene, in sodium–EDTA buffers at 25 °C. ●, log $k_{max.}$; ▲, log $(k_{max.}/K)$. The lines are theoretical for one-proton ionisations of $pK_a$ 7.9 and 6.9, respectively

by this enzyme has not been directly determined, it can be assumed to be retention for the following reasons. The corresponding enzyme from cat liver has been shown to catalyse trans-glycosylation reactions.[15] Since this

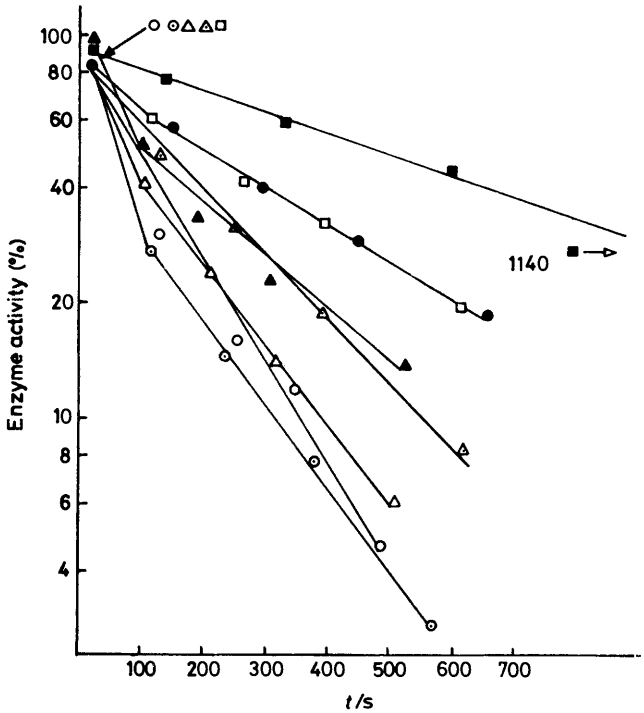



FIGURE 3 Inactivation of human-liver lysosomal β-galactosidase by various concentrations of *galacto*-triazene in 0.1M-sodium acetate–acetic acid buffer, pH 6.0, at 25 °C. Triazene concentrations: ⊙, 178μM; ○, 89μM; △, 44.5μM; ▲, 22.3μM; ◬, 17.8μM; ●, 8.9μM; ■, 4.45μM; □, 22.3μM triazene plus 10mM-methyl 1-thio-β-D-galactopyranoside. At the higher triazene concentrations, an initial burst of inactivation, probably due to adventitious diazonium hydroxide, is detectable; some lines are drawn through all the points, others through only the subsequent ones, to illustrate the (small) effect of this on reported kinetic parameters for the inactivation

enzyme cannot hydrolyse α-galactosides, microscopic reversibility requires the anomeric configuration of the transgalactosylation products to be the same as that of the substrate. It is unlikely that the catalytic pathway of closely related enzymes in humans and in cats will differ radically.

The optimal pH for catalysis by human-liver lysosomal β-galactosidase is around 4,[16] at which pH the spontaneous decomposition rate of the *galacto*-triazene can be estimated as of the order of $10^{-3}$ $s^{-1}$. Figure 3 shows the effect of incubating the enzyme with *galacto*-triazene at pH 6.0. Quantitative protection is observed: on the assumption that substrate and methyl 1-thio-β-D-galactopyranoside compete for the same site, a $K_i$ value of 9.5 mmol $l^{-1}$ is obtained, identical with that obtained from the time-courses in Figure 3. The inactivation, although 2 pH units away from the pH optimum for catalysis, is one of the fastest observed.

*β-Xylosidase from* Penicillium wortmanni.—The pH optimum for this enzyme is 3.3,[17] although activity is still 50% of the maximum at pH 6.0. At pH 7.0 the enzyme is rapidly inactivated by the *xylo*-triazene; so rapidly, in fact, that, because of the high $K$ value (*ca.* 0.1 mol $l^{-1}$) it was not possible to estimate $k_{max.}$ and $K$ separately.

The inactivation is active-site-directed since quantitative protection is observed, as is shown from the time-courses displayed in Figure 4. *p*-Nitrophenyl 1-thio-β-D-xylopyranoside is a competitive inhibitor at pH 7.0 with a $K_i$ value of 0.72 mmol $l^{-1}$. If it is assumed that [Tr] $\ll K$, and that triazene and inhibitor compete for the same site, the solid line of Figure 4 for inactivation in the presence of inhibitor is predicted from that in its absence.

*β-Glucosidase B from Sweet Almonds.*—This enzyme was inactivated only slowly by the *gluco*-triazene, making a full kinetic analysis impossible. Nonetheless, the time-courses displayed in Figure 5 show that the inactivation is active-site-directed, since efficient protection by a competitive inhibitor is observed. Increasing the pH to 7 decelerates inactivation more than triazene decomposition; pH 6 is optimal for catalysis.[18]

*Cross-specificity.*—β-Glucosidase from almonds hydrolyses both β-glucosides and β-galactosides. Whether this is caused by real indifference to glycone structure, or by the presence of two closely-related proteins each

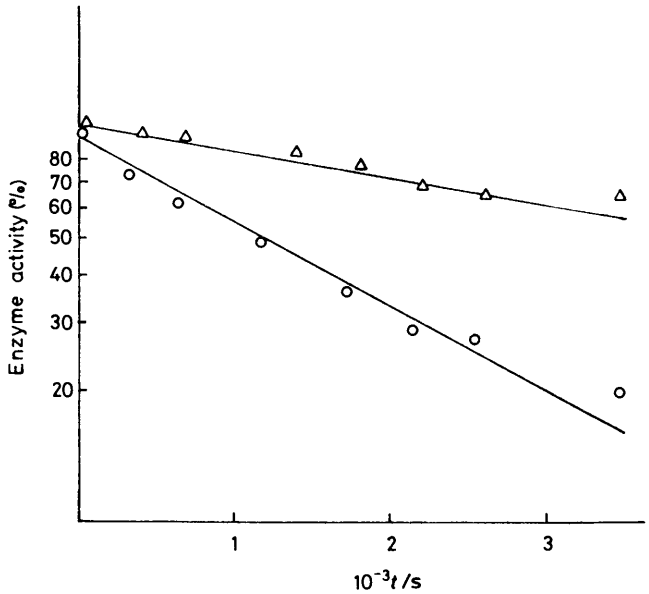



FIGURE 4 Inactivation of the β-xylosidase of *Penicillium wortmanni* by β-xylosylmethyl-(*p*-nitrophenyl)triazene (15.2μM) in the presence (△) and absence (○) of 1.60mM-*p*-nitrophenyl-1-thio-β-D-xylopyranoside. The inactivation medium was 100-mM-EDTA buffer, pH 7.0, at 25 °C. The theoretical line through the triangles is calculated assuming [Tr] $\ll K$, and that the thioxyloside and triazene compete for the same catalytic site; the line through the circles is that used to calculate the first-order rate constant for loss of enzymic activity at this triazene concentration

absolutely specific for glucosides or galactosides, possibly as a hetero-dimer, is not clear from the literature.[19] In the case of the B isoenzyme, the parallel loss of β-glucosidase and β-galactosidase activities on inactivation

with β-D-glucopyranosyl-(*p*-nitrophenyl)triazene shows that the two activities do indeed reside in the same site. On assay of activity with the appropriate 3,4-dinitrophenylglycoside at a concentration of $5 \times K_m$ ($K_m$ values being for the glucoside 0.61 mmol $l^{-1}$, and for the galactoside 0.54 mmol$^{-1}$), the β-galactosidase : β-glucosidase ratio was 0.401 for active enzyme, 0.414 for a 59% active sample produced by overnight incubation at pH 6.0 with 0.8mM-triazene, and 0.397 for a 30% active enzyme produced with 1.6mM-triazene under the same conditions. Although inactivation by the *galacto*-triazene is

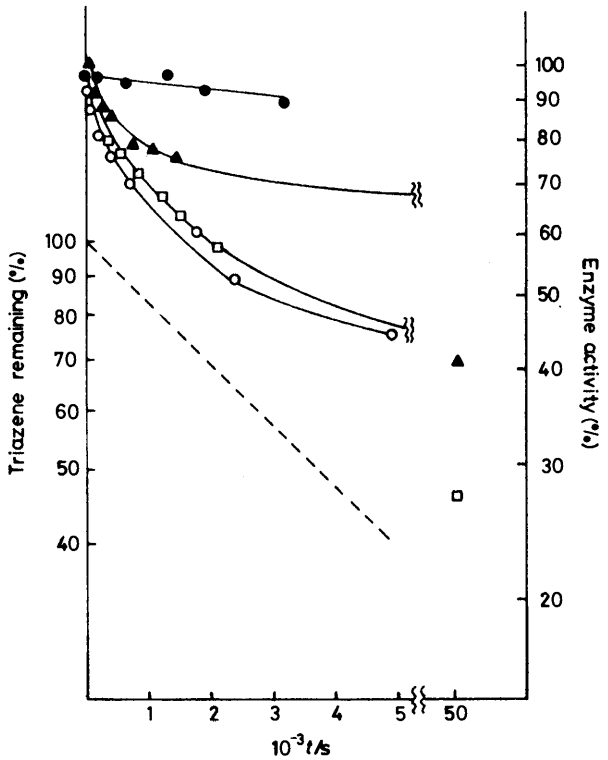



FIGURE 5 Inactivation of the β-glucosidase B from sweet almonds in 0.1M-sodium acetate–acetic acid buffer, pH 6.0 at 25 °C, in the presence of various concentrations of β-D-glucopyranosylmethyl-(*p*-nitrophenyl)triazene. Initial triazene concentrations: ○, 2mM; □, 1.7mM; ▲, 0.8mM; ●, 2mM plus 1.0mM D-glucono-1,5-lactone. The dotted line represents the percentage of triazene remaining, calculated from the measured first-order rate constant for spontaneous decomposition; the solid lines are merely illustrative

only barely detectable (20% inhibition overnight by 10 mM-reagent), this again causes no change in the β-glucosidase : β-galactosidase ratio.

Viratelle *et al.*[20] observed that the second-order rate constant, $k_{max.}/K$, for alkylation of the sulphur of a methionine residue in the active site of $Mg^{2+}$ *lacZ* β-galactosidase, was very similar to that for *N*-(2-bromoacetyl)-β-D-galactopyranosylamine and *N*-(2-bromoacetyl)-β-D-glucopyranosylamine, even though the enzyme has no mechanistically significant catalytic power towards β-glucosides. One model advanced by these authors was that the bromoacetylglycosylamines alkylated the methionine residue only when bound in the acceptor (leaving-group) site, the complex of enzyme with bromoacetylgalactosylamine bound in the galactose site being inert. This ' non-productive ' binding of the *galacto*-derivative was then reflected in a low $K$ and a low $k_{max.}$.

If this explanation is indeed correct, and if the methionine residue alkylated by the bromoacetylglycosylamines [21] is the same as the methionine 500 alkylated by the *galacto*-triazene, then similar behaviour should be observed with the *gluco*- and *galacto*-triazenes as with the *gluco*- and *galacto*-bromoacetylglycosylamines. In fact more than 98% of the activity of $Mg^{2+}$-free *lacZ* β-galactosidase remained after 66 min in the presence of a 3.3mM-concentration of the *gluco*-triazene. This time corresponds to 49 half-lives in the presence of the same concentration of *galacto*-triazene ($K$ 0.48 mmol $l^{-1}$). Towards this enzyme, there is therefore at least a 1 700-fold greater reactivity of the *galacto*-triazene compared to its *gluco*-analogue.

If the possibility of two types of affinity label, with electrophilic centres only *ca.* 2½ Å apart, alkylating two different active-site methionine residues can be discounted, then the inertness of *lacZ* β-galactosidase to the *gluco*-triazene means that the proposal that both bromoacetylglucosylamine and bromoacetylgalactosylamine only alkylate the enzyme when bound in the acceptor site cannot be correct. The alternative proposal, enhanced reactivity of the target methionine in the EI complex with the *gluco*-compound,[20] is more probably accurate.

Formation of a covalent link between label and protein is bound to distort the active site somewhat, and this distortion could very well be more severe for a label which closely resembles substrate than for one where the fit in the non-covalent EI complex is poor. In the present system, the *gluco*-compound binds so awkwardly that the subsequent nucleophilic displacement of $Br^-$ by the methionine sulphur of the enzyme is facilitated.

When expressed in terms of a reaction-co-ordinate diagram (Figure 6), the data of Viratelle *et al.*[20] illustrate an important limitation on all attempts to design reagents to specifically label one protein in a complex mixture, if this active site reagent operates by alkylating the protein in the rate-determining step of the inactivation. The limitation arises since the selectivity of the alkylation reaction then rests, not on the initial, non-covalent recognition of the label, but on the selectivity of the enzyme towards the transition state for its own alkylation. It is possible that intra-complex alkylation of a protein in a very tight non-covalent complex with an active-site reagent will be disfavoured precisely because the complementarity of enzyme and reagent is so good that formation of a covalent link would seriously disturb it. The situation is entirely analogous to enzymic catalysis, where low $K_s$ values can denote feeble catalysis.[22]

We attribute the fact that glycosylmethyl(aryl)triazenes are able to inactivate the target glycosidase specifically in living cells [2] to the rate-determining step

in their decomposition when enzyme-bound being prior to the alkylation reaction. Consider a notional free-energy profile for the inactivation of β-galactosidase by *gluco*- and *galacto*-triazenes if the decomposition of the *gluco*-compound in its particular EI complex is faster than the

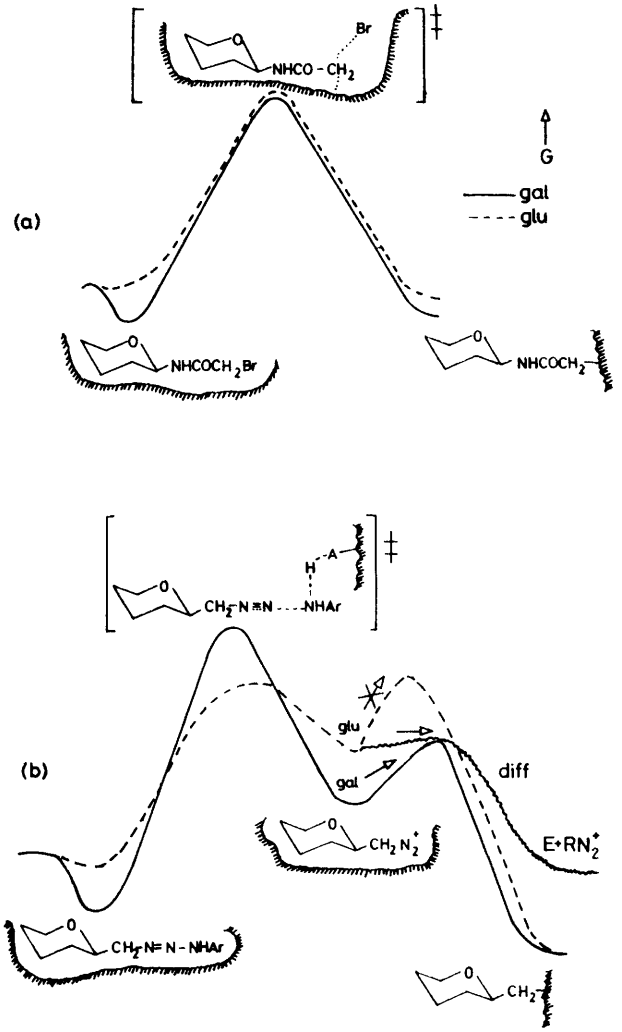



FIGURE 6 (*a*) Gibbs free-energy profile for the alkylation of *lacZ* β-galactosidase by *N*-bromoacetyl-β-D-glucopyranosylamine (– – – – –) and *N*-bromoacetyl-β-D-galactopyranosylamine (——). (*b*) Hypothetical free energy profile for reaction of, *e.g.*, β-D-glucopyranosylmethyl-(– – – – –) and β-D-galactopyranosyl-(——) (*p*-nitrophenyl)triazenes with a β-galactosidase where for some reason the catalysis of triazene decomposition is faster in the non-covalent EI complex with *gluco*-triazene rather than with *galacto*-triazene. The wavy line represents the common diffusional pathway of glucosylmethane- and galactosylmethane-diazonium ions to the active site

corresponding decomposition of the *galacto*-compound (Figure 6, bottom). Alkanediazonium ions are free, solvent-equilibrated intermediates in the decomposition of alkyl(aryl)triazenes,[8] and it is therefore likely that the glycosylmethanediazonium ions have meaningful lifetimes in enzyme-active sites.

It is reasonable to suppose that the binding constants of the *gluco*- and *galacto*-diazonium ions will be in a

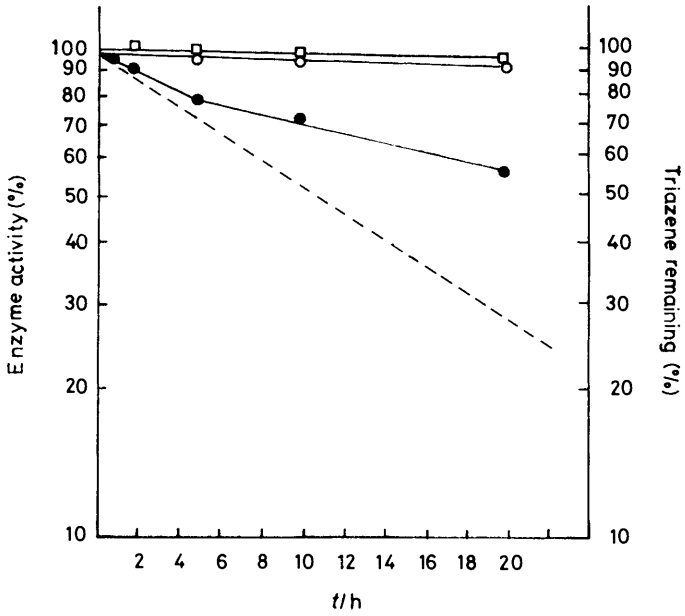



FIGURE 7 Inactivation of α-L-arabinofuranosidase III of *Monilinia fructigena* by *arabino*-triazene (1mM) at pH 7.0 and 25 °C in the presence of 10mM-$(CH_2OH)_3CNH_2$–HCl buffer ●. Points marked ○ refer to a control solution and □ to a reaction in the presence of 1mM-triazene and 1.5mM-L-arabino-1,4-lactone. The dotted line refers to the concentration of triazene remaining in the solution; the solid lines are merely illustrative

similar ratio to the binding constants of the *gluco*- and *galacto*-triazenes. However, the diffusional barrier to formation of these complexes will be the same. Therefore, the barrier to diffusion from the enzyme will be

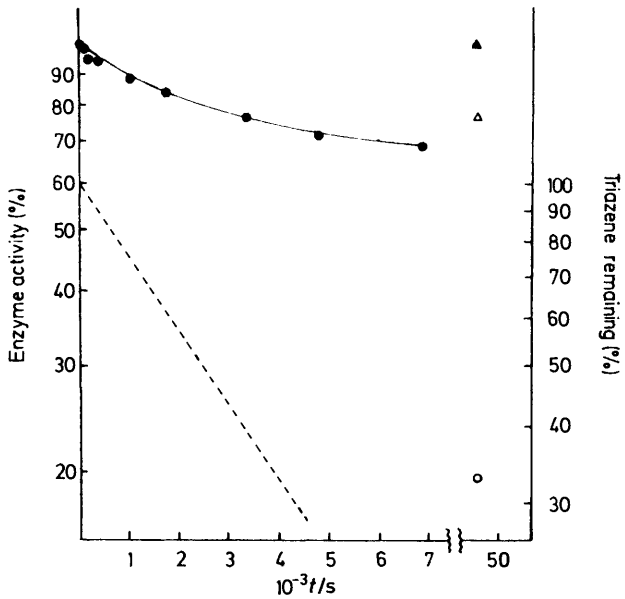



FIGURE 8 Inactivation of coffee-bean α-glucosidase in 0.1M-sodium acetate–acetic acid buffer, pH 6.0, at 25 °C, in the presence of 1.86mM β-D-galactopyranosylmethyl-(*p*-nitrophenyl)triazene (●). The points at 5 × $10^4$ s refer to control (▲), enzyme in the presence of 3.73mM-triazene (○), and enzyme in the presence of 3.73mM-triazene but protected with 50mM-D-galactose (△). The dotted line represents the percentage of triazene remaining, calculated from the spontaneous decomposition rate; the solid lines are merely illustrative

lower in the case of the *gluco*-cation than in the case of the *galacto*-cation. The specificity of alkylation will thus parallel the specificity of initial, non-covalent recognition; 'wrong' diazonium ions, even if formed faster than 'right' ones, leave the enzyme-active site before they alkylate it.

*f(r) Glycosidases.*—The fungus *Monilinia fructigena* secretes two extracellular α-L-arabinofuranosidases, one of pI 6.5 and pH-optimum of 5.5 (AF III), and one of pI 3.0 and pH-optimum of 4 (AF I).[22] No inactivation of AF I or AF III was observed when they were incubated with 1mM-α-L-arabinofuranosylmethyl-(*p*-nitrophenyl)-triazene (probably because of the lability of the reagent at these pH values) but at pH 7 active-site-directed irreversible inhibition of AF III was demonstrated (Figure 7). In principle then, reagents of this type work for retaining furanosidases. The failure to observe inactivation of AF I is probably a combination of the effects of working too far from the pH optimum for catalysis, and the spontaneous decomposition of the reagent generating a protecting agent. (Triazene decomposition products have an apparent $K_i$ of 0.05 mmol $l^{-1}$ against AF I and >2 mmol $l^{-1}$ against AF III at pH 5.)[23] These α-L-arabinofuranosidases behave towards the corresponding triazene much as e → e pyranosidases.

*a → a Glycosidases.*—Figure 8 shows that the *galacto*-triazene is a feeble inactivator of coffee-bean α-galactosidase, and that this inactivation is probably active-site-directed. Human-lysosomal α-glucosidase shows similar slight sensitivity to the *gluco*-triazene,[2] but the yeast enzyme is inert ($k_{obs.} < 5 \times 10^{-6}$ $s^{-1}$ in the presence of 2.6mM-triazene). This is not a consequence of the failure to capture the cation from triazene decomposition, since there was no detectable acceleration of the decomposition of the triazene with $[E]_0 \approx [Tr]$.

The catalytic pathway of a ⟶ a glycosidases probably involves a β-glycosyl enzyme intermediate. If such an enzyme were to bind the corresponding triazene, of opposite anomeric configuration to substrate, in the first place, then the electrophilic site of the triazene would be correctly situated to capture the group which normally became attached to the glycone in this β-glycosyl enzyme intermediate.

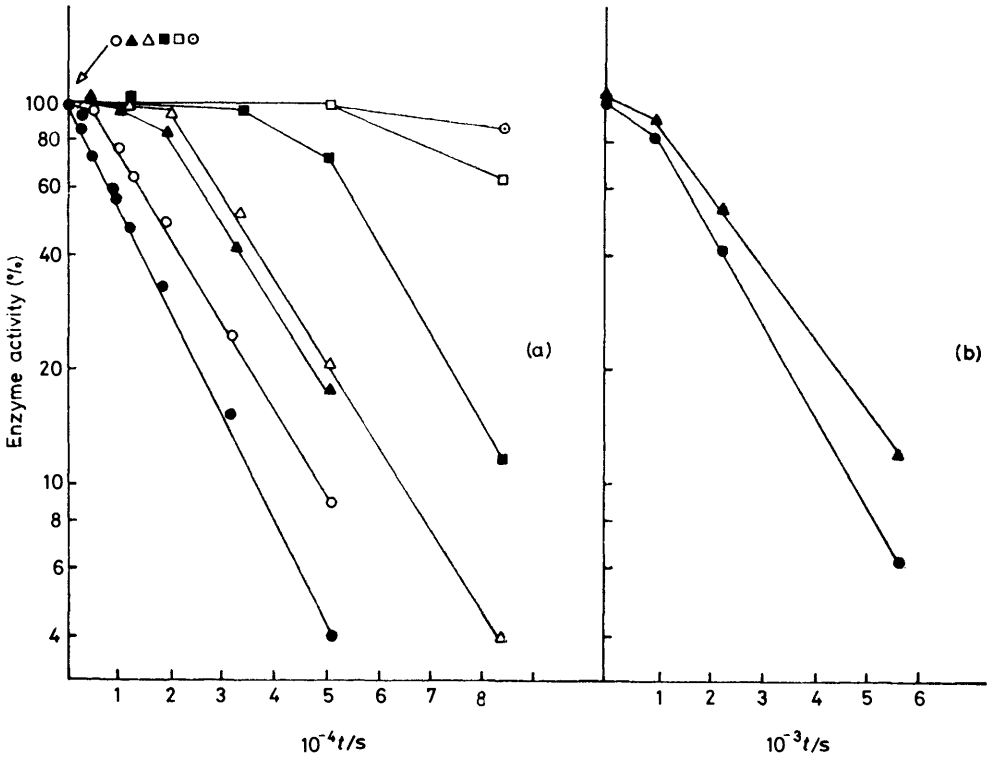



FIGURE 9 (*a*) Consequences of incubation of the β-xylosidase of *Bacillus pumilus* with various concentrations of β-D-xylopyranosylmethyl(*p*-nitrophenyl)triazene in 10mM-sodium phosphate buffer, pH 7.2, 1mM in EDTA and 1mM in dithiothreitol at 25 °C. Triazene concentrations (mM) ●, 7.27; ○, 3.63; ▲ 1.45; △, 0.73; ■, 0.36; □, 0.145; and ○, control. (*b*) Effect of D-ribose on apparent inactivation of *Bacillus pumilus* β-xylosidase by *xylo*-triazene (3.63mM): ●, no ribose; ▲, 16.7mM ribose

*Inverting (a ⟶ e and e ⟶ a) Glycosidases.*—Neither of the inverting enzymes tested was inactivated by the corresponding triazene. In the presence of 3.6mM-*gluco*-triazene, the activity of the gluco-amylase of *Aspergillus niger*, an a ⟶ e enzyme, decayed at rates less than $1 \times 10^{-6}$ $s^{-1}$ at pH 6.0 and $3 \times 10^{-6}$ $s^{-1}$ at pH 7.2. Likewise, the β-xylosidase of *Bacillus pumilus*, an e ⟶ a enzyme, was inert to the *xylo*-triazene. This enzyme contains an oxidisable cysteine residue,[24] and, as is conventional, is normally kept in the presence of a thiol such as mercaptoethanol or dithiothreitol. Our initial attempts to inactivate it produced the general

appearance of successful inhibition [Figure 9(a)], even to apparent weak protection by D-ribose, a reversible inhibitor of catalysis [25] [Figure 9(b)]. However, the inactivation is preceded by an induction time which depends on the concentration of triazene, and then proceeds at a constant rate.

That this apparent inactivation is in reality caused by a triazene-mediated oxidation of the protecting agent is shown by the following experiments: (i) increasing the dithiothreitol concentration increases the induction period: the enzyme is stable to 1mM-triazene in the presence of 11mM-dithiothreitol for 4 h; in this time in the presence of only 1mM-dithiothreitol it loses 38% of its activity; (ii) if solutions of dithiothreitol are maintained as for enzyme inactivation, then the groups titratable with 5,5'-dithiobis-(2-nitrobenzoic acid) decay faster in the presence of 0.6mM-*gluco*-triazene than in its absence; the decay is not first order. The apparent protection of enzyme by ribose is accounted for by the known [24] protection of an active-site thiol group against oxidation by reversible inhibitors of catalysis.

The presence of dithiothreitol alters neither the rate nor the chromophoric product of triazene decomposition: since radicals are possibly [26] involved in triazene tautomerisation, they could act as radical initiators of the air oxidation of dithiothreitol.

*Binding Proteins.*—A protein which binds β-D-galactopyranosides, but does not transform them, the *lac* repressor of *E. coli*, is not inactivated by the *galacto*-triazene ($k_{obs.} < 10^{-5}$ $s^{-1}$ at 4 °C in the presence of 1mM-triazene). This is in accord with the status of glycosylmethyl-(*p*-nitrophenyl)triazenes as ' suicide substrates ' rather than simple alkylating agents. Indeed, the ability of these compounds to work *in vivo* [2] is critically dependent on the triazenes *not* alkylating the sugar-transport proteins of the living cells.

*Conclusions.*—Although this investigation of the potential of the $-CH_2-N=N-NH-p-C_6H_4NO_2$ functionality as a source of covert electrophilicity in active-site reagents has been performed with glycosidases, this is a consequence of our interest in these enzymes, rather than their especial suitability for such studies. The triazene functionality could in principle be attached to a wide range of molecular structures to give active-site reagents for a wide range of enzymes, and the combination of inertness of the precursor of the immediate alkylating agent, and its short lifetime, make such reagents attractive for *in vivo* studies.

In the specific case of glycosidases, active-site reagents for those that work with retention of configuration at the anomeric centre can be obtained by replacing the aglycone of the substrate by the $CH_2-N=N-NH-p-C_6H_4NO_2$ group. Some optimisation of pH and temperature may however be necessary before unambiguous active-site-directed irreversible inhibition is seen.

## EXPERIMENTAL

*Glycosylmethyl-(p-nitrophenyl)triazenes.*—Reaction of β-D-glucopyranosylmethylamine,[10] β-D-galactopyranosylmethylamine,[11] β-D-xylopyranosylmethylamine, or α-L-arabinofuranosylmethylamine (2 mol) with *p*-nitrobenzenediazonium tetrafluoroborate (1 mol) in the minimum volume of ice-cold water, followed by filtration, extraction of the filtrate with ether and butan-1-ol, and evaporation of the butanol extract at 1 mmHg (no external heating) gave the triazenes [5] in 2—10% yield, as estimated from the u.v. absorbance at 362 nm. When this procedure was carried out with radio-labelled β-D-galactopyranosylmethylamine,[5] the triazene concentration estimated from radioactivity agreed with that estimated on the basis of u.v. absorbance. The *gluco*-compound, m.p. 124—126 °C (decomp.) and the *xylo*-compound, m.p. 88—98 °C (decomp.), crystallised on addition of water, but decomposed on attempted further characterisation.

β-D-*Xylopyranosylmethylamine.*—Reduction of 2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl cyanide,[27] essentially as in ref. 10, but with replacement of ether by tetrahydrofuran as reaction solvent, afforded the *glycosylmethylamine* as intensely hygroscopic crystals after repeated evaporations of anhydrous methanol. Recrystallisation from anhydrous methanol gave the pure product (53%), m.p. 82—90 °C (sealed tube), $[\alpha]_D^{25}$ − 37.6° (*c*, 1 in $H_2O$) (Found: C, 43.2; H, 8.7; N, 8.1. $C_6H_{13}NO_4$ requires C, 44.2; H, 8.0; N, 8.6%).

α-L-*Arabinofuranosylmethylamine.*—Reduction [10] of 2,3,5-tri-*O*-benzoyl-α-L-arabinofuranosyl cyanide (2.5 g) afforded a gum (0.77 g) which on drying over $P_2O_5$ for 2 days crystallised. Recrystallisation from anhydrous methanol gave a sample for analysis, which deliquesced on the Kofler hot-stage, $[\alpha]_D^{25}$ − 64.7° (Found: C, 44.3; H, 8.4; N, 7.3%). The material was homogeneous and ninhydrin-positive when analysed by t.l.c. (silica gel G; ethanol—1% aqueous ammonia as eluant).

2,3,5-*Tri*-O-*benzoyl*-α- (*and* -β-) L-*arabinofuranosyl Cyanides.*—2,3,5-Tri-*O*-benzoyl-α-L-arabinofuranosyl bromide [28] (10 g) and dry mercuric cyanide (20 g) were stirred in dry benzene (50 ml) at 22 °C for 24 h. Filtration, removal of residual mercuric salts by washing with 0.5M-KBr, drying ($MgSO_4$), and evaporation gave the crude product containing largely α- and β-cyanides. Integration of the characteristic [29] resonances of the anomeric protons [δ 5.06 (s) and 5.2 (d, *J* 5 Hz), respectively] indicated a 3 : 1 mixture of α : β cyanides to have been produced. Chromatography on silica gel with chloroform–toluene (1 : 1, v/v) as eluant gave, after recrystallisation from ethanol, the α-*cyanide* (3.4 g, 38%), m.p. 78—81 °C, $[\alpha]_D^{25}$ + 21° (*c*, 1.2 in $CHCl_3$) (Found: C, 68.65; H, 4.5; N, 2.9. $C_{27}H_{22}NO_7$ requires C, 68.8; H, 4.45; N, 2.95%). Preparative t.l.c. [silica gel; toluene–chloroform (2 : 1 v/v) as eluant] gave the pure β-*cyanide* from the later fractions of the column chromatogram. m.p. 132—133 °C, $[\alpha]_D^{25}$ + 51° (*c*, 1 in $CHCl_3$) (Found: C, 68.7; H, 4.7; N, 2.9%). The cyanide, rather than isocyanide, structure of both compounds is established by the absence of a ν(C≡N) absorption; this is quenched in aldosyl cyanides [11] but present in aldosyl isocyanides.[30]

p-*Nitrophenyl* 1-*thio*-β-D-*Xylopyranoside.*—2,3,4-Tri-*O*-acetyl-1-thio-β-D-xylopyranose [31] was heated under reflux for 1.2 h with *p*-fluoronitrobenzene (3.6 ml) in dry acetone (25 ml) in the presence of powdered anhydrous potassium carbonate (4.7 g). The solids were filtered off, and the filtrate was treated with charcoal and evaporated. The residue was recrystallised from ethanol to give p-*nitrophenyl* 2,3,4-*tri*-O-*acetyl*-1-*thio*-β-D-*xylopyranoside* (2.4 g, 17%), m.p. 158—163 °C, $[\alpha]_D^{28}$ − 76.5° (*c*, 1.0 in $CHCl_3$) (Found: C,

49.2; H, 5.0; N, 2.9; S, 7.3. $C_{17}H_{19}NO_9S$ requires C, 49.4; H, 4.6; N, 3.4; S, 7.7%). Zemplen deacetylation afforded the *title compound* (36%), m.p. 159—160 °C, $[\alpha]_D^{28}$ − 84.9° (*c*, 1.0 in MeOH) (Found: C, 45.6; H, 4.7; N, 4.8; S, 10.7. $C_{11}H_{13}NO_6S$ requires C, 46.0; H, 4.6; N, 4.9; S, 11.1%).

*Enzymes.*—Yeast α-glucosidase (Sigma Type III; lot no. 77C-0190), coffee-bean α-galactosidase (Sigma, lot no. 97C-0084), and glucoamylase (Sigma, lot no. 126C-0047) were commercial materials. Human-liver lysosomal β-galactosidase,[32] *lac* repressor of *E. coli*,[33] and α-L-arabinofuranosidases I and III [34] were the gifts of Professor D. Robinson (Queen Elizabeth College, London), Dr. K. Beyreuther (Universität zu Köln), and Dr. R. J. W. Byrde (Long Ashton Research Station, Bristol), respectively. Sweet almond β-glucosidase B was purified from commercial 'emulsin' (B.D.H.) by the procedure of Legler and Hasnain [18] (specific activity 640 U mg$^{-1}$ [lit.,[35] 630 U mg$^{-1}$], one band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate). *ebg*° β-Galactosidase from *E. coli* was isolated by literature [35] procedures [specific activity 5.5 U mg$^{-1}$ (lit., 10.6 U mg$^{-1}$)]: contamination with *lacZ*-gene product was negligible since the enzyme was completely inactivated by $Na^+$. $Mg^{2+}$-free enzyme was obtained by repeated dialysis against potassium citrate buffer.

*β-Xylosidases from* Penicillium wortmanni *and* Bacillus pumilus.—Both micro-organisms were obtained from Professor C. K. De Bruyne, Rijksuniversiteit te Gent, and were cultured as described by him.[17,25]

The culture-filtrate from *Penicillium wortmanni* [17] was concentrated and subjected to an ammonium sulphate fractionation, the bulk of the activity residing in the material precipitated at between 40 and 60% saturation. This precipitate, dissolved in 20mM-potassium phosphate buffer, pH 6.8 (20 ml), and dialysed against the same buffer (2 × 1 l) was applied to a column (2.5 × 20 cm) of DEAE-cellulose (Whatman DE-52), equilibrated with the same buffer. After passage of one column-volume of this buffer, a linear salt gradient (0—0.5M-NaCl) was applied. Those fractions defining a peak of protein corresponding to a peak of *p*-nitrophenyl β-xylosidase activity were pooled to give a material of specific activity 8.3 U mg$^{-1}$ (lit.,[17] 11.4 U mg$^{-1}$).

The $K_i$ value for *p*-nitrophenyl 1-thio-β-D-xylopyranoside was measured against *p*-nitrophenyl β-D-xylopyranoside ($K_m$ 0.41 ± 0.06 mmol l$^{-1}$).

Isolation of the β-xylosidase from *Bacillus pumilus* 12 was performed as reported [25] as far as the ammonium sulphate fractionation. Thereafter the protein was chromatographed successively on Whatman DE-52 cation-exchanger and Sephadex G-150 superfine. Dithiothreitol (1 mM) was added to maintain stability and homogeneity of the enzyme.

Assay conditions for all the above enzymes are given in Table 3.

TABLE 3

Assays performed spectrophotometrically in a Unicam SP 1800 with a cell-block thermostatted at 25 °C, unless otherwise stated

| Enzyme | Source | Substrate (concentration) | pH | Buffer cation | Buffer anion | Other solute |
|---|---|---|---|---|---|---|
| *lacZ* β-Galactosidase | *E. coli* | *p*-Nitrophenyl β-D-galactopyranoside (0.3mM) | 7.0 | $Na^+$ | $H_2PO_4^-$ (0.1M) | 1mM-$MgCl_2$ |
| *ebg*° β-Galactosidase | *E. coli* | *o*-Nitrophenyl β-D-galactopyranoside (5mM) | 7.5 | $K^+$ | $H_2PO_4^-$ (0.125M) | 5mM-$MgCl_2$ |
| Lysosomal β-Galactosidase | Human liver | 3,4-Dinitrophenyl β-D-galactopyranoside [a] (1.7mM) | 4.5 | $Na^+$ | $^-O_2CMe$ (0.05M) | 0.05M-NaCl |
| β-Xylosidase | *P. wortmanni* / *B. pumilus* | *p*-Nitrophenyl β-D-xylopyranoside (4mM) | 7.2 | $Na^+$ | $H_2PO_4^-$ (10mM) | 1mM-EDTA |
| β-Glucosidase | Sweet almonds | 3,4-Dinitrophenyl β-D-glucopyranoside [b] (3mM) | 6.0 | $Na^+$ | $^-O_2CMe$ | |
| α-L-Arabinofuranosidases I and III | *M. fructigena* | *p*-Nitrophenyl α-L-arabinofuranoside [c] (10mM) | 7.0 | $(CH_2OH)_3\overset{+}{C}HNH_3$ (10mM) | $Cl^-$ | |
| α-Galactosidase | Coffee beans | *o*-Nitrophenyl α-D-galactopyranoside (5mM) | 6.5 | $K^+$ | $H_2PO_4^-$ (0.1M) | |
| α-Glucosidase | Yeast | *p*-Nitrophenyl α-D-glucopyranoside (2mM) | 7.0 | $Na^+$ | $H_2PO_4^-$ (0.05M) | |
| Glucoamylase | *A. niger* | 'Instant Robin' cold-water starch (Reckitt and Colman) (0.8 mg ml$^{-1}$), filtered, at 35 °C | 4.8 | $Na^+$ | $^-O_2CMe$ (0.04M) | * |
| *lac* Repressor | *E. coli* | [$^{14}$C]-2′-propyl-1-thio-β-D-galactopyranoside (gift of Dr. K. Beyreuther), at 4 °C | 8.0 (at 4 °C) | $(CH_2OH)_3\overset{+}{C}HNH_3$ (0.1M) | $Cl^-$ | 0.1M-NaCl 0.3M-dithiothreitol. Assay by equilibrium dialysis [d] |

* Commercial glucose oxidase peroxidase kit (Sigma, lot No. 47C-0106); d(A500)/d$t$ ∝ [glucoamylase] up to [glucoamylase] = 5× concentration used in inactivation experiments.

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