Active-site-directed Irreversible Inhibition of *E. coli* β-Galactosidase by the 'Hot' Carbonium Ion Precursor, β-D-Galactopyranosylmethyl-*p*-nitrophenyltriazene

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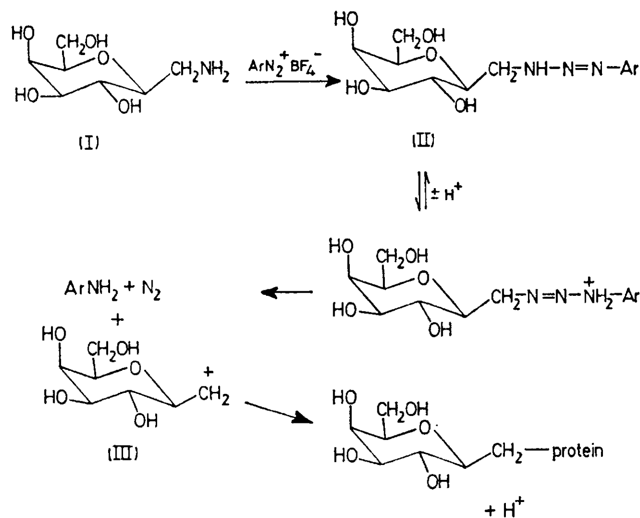
Summary The title compound deactivates Na^+- and Mg^{2+}-saturated *E. coli* β-galactosidase at 25 °C and pH 7·0 with a maximum rate of $(4{\cdot}02 \pm 0{\cdot}17) \times 10^{-3}\ s^{-1}$, its binding being governed by a K of $73 \pm 11\ \mu M$; the enzyme is protected against this irreversible inhibitor by the competitive inhibitor methyl 1-thio-β-D-galactopyranoside to an extent predictable from its kinetically-determined K_i, whilst removal of Mg^{2+} increases the maximum deactivation rate [to $(5{\cdot}7 \pm 0{\cdot}7) \times 10^{-2}\ s^{-1}$] and weakens the binding [$K = 207 \pm 47\ \mu M$], inhibition being quantitatively associated with attachment of the β-D-galactopyranosylmethyl group to the protein.

INVESTIGATIONS of liganding proteins, such as enzymes, are facilitated by the use of analogues of the substrate, which, when bound at the active site, there form a stable covalent bond to the protein. These 'affinity labels' thus both block the active site, rendering the protein inactive, and also, when radioactive, enable active-site residues to be determined. However, affinity labels of the α-halogenocarbonyl or epoxide type[1,2] require a suitably disposed active site group of significant nucleophilicity, whilst the highly energetic carbenes and nitrenes generated by photolysis are very nonspecific.[3] It therefore seemed that precursors of the 'hot' carbonium ions generated in deamination reactions of alkylamines might be 'affinity labels' of intermediate reactivity. A carbonium ion suitable for attacking residues at the active site of β-galactosidase would be the primary cation (III) from the deamination of 1-amino-2,6-anhydro-1-deoxy-D-*glycero*-L-*manno*-heptitol (I).[4]



Although White *et al.*[5] have, in parallel studies, successfully used *N*-nitrosamides to 'affinity label' chymotripsin and luciferase, the nitrosoamide route to the cation (III), which we independently explored, proved impracticable because of the efficacy of the C-3 hydroxy-group as an intramolecular nucleophile in the base-catalysed pathway for nitrosoamide rearrangement.[6] The triazene route[7] to the cation (III) however yielded a suitable inhibitor. Reaction of the amine (I) (2 mol) with arenediazonium tetrafluoroborate (1 mol) in the minimum volume of water at 22 °C for 30 min (when all the diazonium salt had been consumed), followed by filtration to remove pentazene by-product, and ether extraction to remove non-carbohydrate, organic material yielded a solution which slowly inhibited the enzyme. Extraction of the triazene from this solution with butan-1-ol, and evaporation of the butanol extracts at 22 °C yielded the triazene substantially pure. The triazenes decomposed on any attempt at chromatography (even Sephadex), and even slowly in water, this decomposition being strongly catalysed by phosphate buffer. The spontaneous decomposition was slowest for

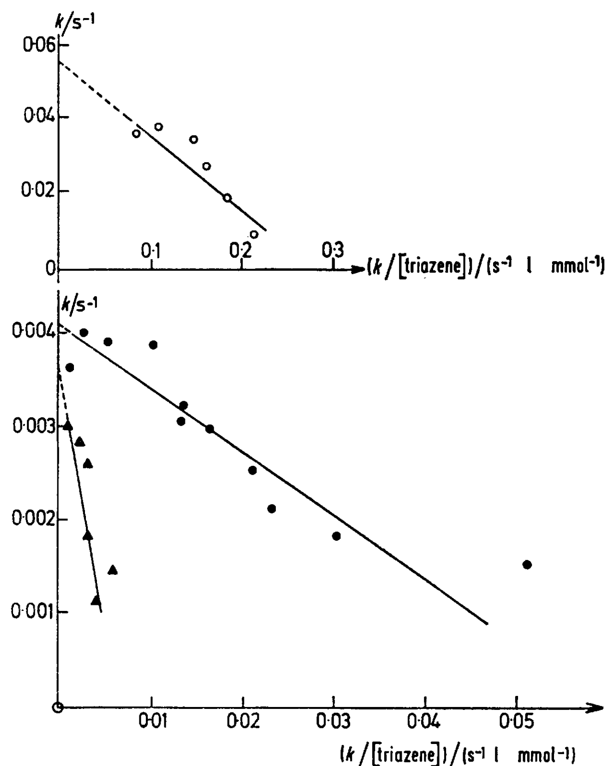


FIGURE. Hofstee plots for the inhibition of *E. coli* β-galactosidase by β-D-galactopyranosylmethyl-*p*-nitrophenyltriazene. (A) Mg^{2+}-free enzyme (dialysed against ethylenediaminetetraacetic acid) at 25 °C in 0·05 M Na_2HPO_4–NaH_2PO_4 buffer (pH 7). (B) Mg^{2+}-saturated enzyme, under the same conditions, but with the addition of 1 mM $MgCl_2$ (●) and also of 9·0 mM methyl 1-thio-β-D-galactopyranoside (▲). Lines shown are those calculated from a linear least-squares treatment of these plots, which also gave the derived parameters, and errors therein, quoted in the text.

the p-nitrophenyl triazene [half-life 9·5 h at 25 °C in distilled water in the presence of unchanged amine (I), between pH 8 and 9], and this was the inhibitor used in all quantitative work. The aromatic region of the ^1H n.m.r. spectrum resembled that of the (analytically pure) 1-(propyl)-3-(p-nitrophenyl)triazene and was different from that of the N-p-nitrophenyl derivative of the amine (I). The u.v. spectrum of the triazene (II) (Ar = p-nitrophenyl) was qualitatively identical with that of the crystalline 1-propyl model compound, and the extinction coefficients of both triazenes were assumed to be the same; in this way concentrations of the triazene (II) could be estimated. The assumption that the two primary alkyl p-nitrophenyltriazenes have the same extinction coefficients could lead to systematic error in our estimations of binding constants, but it is likely to be much smaller than the random error, since with the p-nitrophenyltriazene chromophore even the change in alkyl group from primary to secondary (octyl to 1-propylpentyl) causes only a 10% change in ϵ_{max}.[8]

The inhibition of Mg^{2+}-saturated β-galactosidase by the p-nitrophenyl triazene (II) was cleanly first-order only for the first *ca.* 20 min of reaction whereafter the deactivation decelerated. This deceleration could be accounted for quantitatively by the non-enzymic decomposition of the triazene [at a rate of $(7{\cdot}9 \pm 0{\cdot}13) \times 10^{-5}\,s^{-1}$] and the efficacy of its decomposition products as competitive inhibitors (with a 'weighted mean' K_i at 25 °C of 233 ± 44 μM). Triazene inhibition of Mg^{2+}-free β-galactosidase[9] was an order of magnitude faster than that of the Mg^{2+}-saturated enzyme, and, in accord with our interpretation of the deviations from first-order deactivation of the latter form of the enzyme, accurately first-order loss of activity of the Mg^{2+}-free enzyme was observed. In the Figure the rate constants (k) (calculated in the case of Mg^{2+}-saturated enzyme from the cleanly first-order portion of the reaction) are plotted against k/[triazene] (Hofstee plot), in the presence and absence of a known competitive inhibitor, methyl 1-thio-β-D-galactopyranoside. These data show, not only that p-nitrophenyl-triazene (II) is inhibiting the enzyme by a two-step process (binding preceding reaction), but also that the binding site for the deactivating process is the same as that for the catalytic process, since the K_i calculated for the thioglycoside (1·4 mM) corresponds, within experimental error, to that calculated from kinetic measurements (1·8 mM)[10].

Removal of Mg^{2+} increases the maximum deactivation rate 14 times [to $(5{\cdot}7 \pm 0{\cdot}7) \times 10^{-2}\,s^{-1}$], but weakens the binding (K = 207 ± 47 μM). The weakening of the binding is anticipated from the general increase in K_m values on removing Mg^{2+},[11] but k_{cat} values for oxygen substrates normally decrease, although those for the galactosyl pyridinium salts increase at certain pH values.[12]

That the observed inhibition arises from attachment of the carbonium ion (III) to the protein, and not from any diazonium ion, whether carried through the extraction procedure or arising from decomposition of the triazene, is shown by the following:

(i) No p-nitrophenol is formed in the decomposition of dilute aqueous solutions of the triazene.

(ii) Replacement of amine (I) in the triazene synthesis by propylamine, which gives an ether-soluble, water-insoluble triazene, gives, on taking up the evaporated butanol extracts in a volume of buffer solution corresponding to double the highest triazene concentration used in an inactivation experiment, a solution which inactivates the enzyme at a rate only barely distinguishable from that with buffer alone, and which can account for a maximum of 5% of the inactivation observed with triazene (II).

(iii) p-Nitrophenyl triazene (II), made from the amine (I) labelled with ^{14}C in the CH_2NH_2 group, reacts with β-galactosidase with incorporation of radioactivity into the protein [precipitated, with 0·5 mg/ml ovalbumin as carrier, by trichloracetic acid (4·5% w/v), and counted on glass fibre filter-papers]. With Mg^{2+}-free enzyme the correlation coefficient between radioactivity of the precipitate and enzymic activity just before precipitation is −0·975 with a triazene concentration of 0·133 mM and −0·988 at a triazene concentration of 0·66 mM.

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[1] B. L. Vallee and J. F. Riordan, *Ann. Rev. Biochem.*, 1969, **38**, 733.
[2] G. Legler, *Mol. Cell. Biochem.*, 1973, **2**, 31.
[3] J. R. Knowles, *Accounts Chem. Res.*, 1972, **5**, 155.
[4] B. Coxon and H. G. Fletcher, *J. Amer. Chem. Soc.*, 1964, **86**, 922.
[5] E. H. White, D. F. Roswell, I. R. Politzer, and B. R. Branchini, *J. Amer. Chem. Soc.*, 1975, **97**, 2290.
[6] R. Huisgen and J. Reinertshofer, *Annalen*, 1952, **575**, 174.
[7] E. H. White and H. Scherrer, *Tetrahedron Letters*, 1961, **21**, 758.
[8] R. M. Southam, D. Phil. Thesis, Oxford, 1965, pp. 110—113.
[9] J.-P. Tenu, O. M. Viratelle, and J. Yon, *European J. Biochem.*, 1972, **26**, 112.
[10] M. L. Sinnott, *Biochem. J.*, 1971, **125**, 717.
[11] O. M. Viratelle and J. M. Yon, *European J. Biochem.*, 1973, **33**, 110.
[12] M. L. Sinnott, O. M. Viratelle, and S. G. Withers, *Biochem. Soc. Trans.*, 1975, **3**, 1006.

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