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Specific Inactivation of Lysosomal Glycosidases in Living Fibroblasts by the Corresponding Glycosylmethyl-*p*-nitrophenyltriazenes

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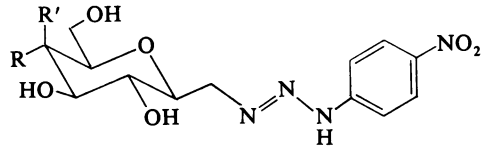
1. β-D-Galactopyranosylmethyl-*p*-nitrophenyltriazene can specifically inactivate lysosomal β-galactosidase in cultured human fibroblasts, without causing detectable changes in cell viability or the activities of other hydrolases. 2. β-D-Glucopyranosylmethyl-*p*-nitrophenyltriazene behaves similarly towards lysosomal β-glucosidase, but also inactivates some α-glucosidase. 3. Both β-galactosidase and β-glucosidase activities in triazene-treated confluent fibroblast cultures recover exponentially; if zero-order enzyme production is assumed, turnover times of 10 and 5 days respectively can be estimated.

Active-site-directed irreversible inhibitors have been used widely in enzymology and protein chemistry (Jakoby & Wilchek, 1977). The incorporation of 'overt' alkylating functionality (such as $-CO-CH_2Br$) into such molecules limits their usefulness in living systems: for successful application to these, the actual alkylating species must be generated in the active site of an enzyme, preferably by the catalytic process of the enzyme itself. 'Suicide substrates' (Abeles & Maycock, 1976) are required.

β-GalMNT has been shown to be a 'suicide substrate' for the *lacZ* β-galactosidase of *Escherichia coli* (Sinnott & Smith, 1976, 1978). Subsequent studies (P. J. Marshall, M. L. Sinnott, P. J. Smith & D. Widdows, unpublished work) indicated that the glycosylmethyl-*p*-nitrophenyltriazenes had wide applicability as active-site reagents for purified glycosidases, including the human lysosomal β-galactosidase. We now report the inactivation *in situ* in living human fibroblasts of lysosomal β-galactosidase, a soluble enzyme (Norden *et al.*, 1974), and β-glucosidase, a membrane-bound enzyme (Gatt & Rapport, 1966), by the corresponding glycosylmethyltriazenes, β-GalMNT and β-GlcMNT respectively.

Abbreviations used: β-GlcMNT, β-D-glucopyranosylmethyl-*p*-nitrophenyltriazene [3-(2,6-anhydro-1-deoxy-D-*glycero*-D-*gulo*-heptityl)-1-(4-nitrophenyl)triazene]; β-GalMNT, β-D-galactopyranosylmethyl-*p*-nitrophenyltriazene [3-(2,6-anhydro-1-deoxy-D-*glycero*-L-*manno*-heptityl)-1-(4-nitrophenyl)triazene].



R = H, R′ = OH for β-GalMNT
R = OH, R′ = H for β-GlcMNT

Since the triazenes were found to be specific and had no effect on the cell viability, they could be applied to estimate the lifetimes of lysosomal β-galactosidase and β-glucosidase in the living cell.

Materials and Methods

Cell culture

Normal human skin fibroblasts were cultured in 'standard medium', i.e. Ham's F10 medium supplemented with 10% (v/v) foetal calf serum and antibiotics (Gibco, Paisley, Scotland, U.K.). The fibroblasts were free of mycoplasma as judged by the method of Chen (1977).

Hydrolase assay

A rapid method for preparing cell-free enzyme extracts was used, which made harvesting cells with trypsin unnecessary. Monolayers of fibroblasts in 35 mm-diameter Petri dishes were washed four times with 0.9% (w/v) NaCl at room temperature and lysed *in situ* in 0.25% (w/v) Triton X-100 (200 μl) in

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water for 30min at 4°C with occasional gentle rocking. In this way more than 95% of each hydrolase tested could be solubilized and about 40% of cellular protein and less than 5% hydrolase activity was left bound to the dish. For some experiments cell homogenates in water were prepared by the conventional method of sonication (MSE 100W sonifier at 20kHz for 2 × 10s); these are referred to as homogenates and extracts of enzyme lysed *in situ* as cell lysates.

Enzyme activity, except neuraminidase, was assayed by incubating 10μl of cell lysate (or homogenate) with 20μl of the appropriate 4-methylumbelliferyl substrate (Koch-Light, Colnbrook, Bucks., U.K.) for 30min. at 37°C. The following substrate solutions were used to assay the corresponding hydrolases in cell lysates: 4-methylumbelliferyl-β-D-galactoside (1mM), 4-methylumbelliferyl-α-L-arabinoside (2.2mM) and 4-methylumbelliferyl-β-D-fucoside (1.5mM) in 0.1M-sodium acetate buffer, pH4.3, containing 0.1M-NaCl; 4-methylumbelliferyl-β-D-glucoside (5mM) and 4-methylumbelliferyl-β-D-xyloside (3.5mM) in McIlvaine's 0.2M-sodium phosphate/0.1M-citric acid buffer, pH5.8 (McIlvaine, 1921), containing 0.3% (w/v) sodium taurocholate; 4-methylumbelliferyl-α-D-galactoside (5mM) in 0.1M-sodium acetate buffer, pH4.5; 4-methylumbelliferyl-α-D-glucoside (2.2mM) in 0.2M-sodium acetate buffer, pH4.3; 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide (5mM) in 0.02M-sodium phosphate/0.012M-citric acid buffer, pH4.4; 4-methylumbelliferyl-α-D-mannoside (4mM) in 0.07M-sodium acetate buffer, pH4.3; 4-methylumbelliferyl-α-L-fucoside (2mM) in McIlvaine's 0.2M-phosphate/0.1M-citrate buffer, pH5.5; 4-methylumbelliferyl-β-D-glucuronide (2mM) in 0.1M-sodium acetate buffer, pH4.3.

Triton X-100 (0.2%, w/v) was included in substrate solutions when used to assay cell homogenates. After incubation, 500μl of 0.5M-Na_2CO_3/$NaHCO_3$ buffer, pH10.7, was added and the fluorescence was read at 448nm after excitation at 365nm.

Neuraminidase was assayed as described by Kleijer *et al.* (1979) by using 4-methylumbelliferyl-α-neuraminic acid which was a gift from Dr. J. S. O'Brien, La Jolla, CA, U.S.A. Galactosylcerebroside β-galactosidase was assayed as described by Suzuki *et al.* (1971).

Protein was determined as described by Lowry *et al.* (1951), except that 2% (w/v) sodium dodecyl sulphate was included in the Na_2CO_3/NaOH reagent.

Hydrolase activity is expressed in nmol of 4-methylumbelliferone/h per mg of protein.

Treatment of cells with β-GalMNT and β-GlcMNT

The inhibitors were freshly made before each experiment, as described by Sinnott & Smith (1978), dissolved in standard medium, and sterilized by filtration through a 0.22μm Millipore filter.

Cells (passage 15–21) were seeded at half-confluency in 35mm Petri dishes, incubated for 6 days under standard conditions and then incubated with standard medium containing inhibitor as indicated in the text. The hydrolase activities were then measured in cell lysates.

In turnover studies, conditions differed in that cells were seeded in medium containing 15% (v/v) foetal calf serum and after inhibitor treatment (0.2mM for 2h at 37°C) 6 days later were washed twice with standard medium and subsequently cultured in standard medium for various periods of time. This decrease in serum concentration was introduced in order to suppress further cell division.

Specific hydrolase activity in treated cultures is expressed as a percentage of that in untreated control cells lysed at the same time.

Results

Specific inactivation of lysosomal β-galactosidase by β-GalMNT, without other detectable changes in the cell

Fig. 1 shows the effect of treating cultured fibroblasts with various concentrations of β-GalMNT on the activity of intracellular α- and β-galactosidase, and α- and β-glucosidase. β-Galactosidase is inactivated by up to 99.5%, without

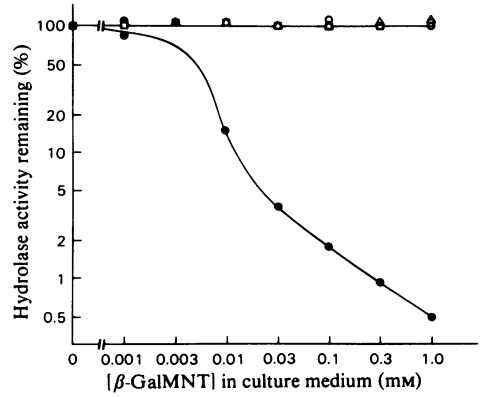

Fig. 1. *Inactivation of β-galactosidase in fibroblast cultures by different concentrations of β-GalMNT*
Cell cultures were incubated for 4h at 37°C, and residual glycosidase activity was measured as described in the Materials and Methods section: ●, β-galactosidase; □, β-glucosidase; ○, α-galactosidase; △, α-glucosidase.

detectable changes in the activities of the other three glycosidases.

The time course of this inactivation at various temperatures is shown in Fig. 2. These data remove a possible objection to those in Fig. 1, namely that the inactivation of the enzyme occurs after lysis and not during incubation of intact cells. It could have been argued that the hydrophobic *p*-nitrophenyl moiety of the label bound reversibly to the hydrophobic portions of the cell membrane, β-GalMNT being liberated during lysis and then inactivating the enzyme. If this were the case, the pronounced temperature-dependence of the inactivation would not be observed, since the cells from incubations at all three temperatures were washed and lysed at the same temperature.

On treatment of fibroblasts with β-GalMNT (0.2mM) at 37°C, β-galactosidase activity decays exponentially with a half-life of 7min until about 98.5% of the starting activity is lost, and then remains constant. This is not due to depletion of β-GalMNT, as addition of fresh β-GalMNT after 4h does not restore the fast inactivation initially observed.

Fig. 3 shows the growth capacity of cells treated for 2h with β-GalMNT. No difference in the increase of cellular protein of β-GalMNT-treated and control fibroblasts is detectable. After this β-GalMNT treatment the lactate dehydrogenase content of the cells is unaltered and no α-glucosidase, β-glucosidase, α-galactosidase or β-galactosidase could be detected in the culture medium. This indicates that neither cytoplasmic nor lysosomal constituents are aspecifically lost from the cells. Taken together, these results show that a β-GalMNT treatment for 2h is not toxic to the cells. After a 4h treatment the growth capacity is slightly altered: the initial growth capacity is unchanged but slightly lower cell densities are reached after one week in culture. An exposure of 16h to 0.2mM-β-GalMNT does not lead to loss of intracellular α-glucosidase, β-glucosidase or α-galactosidase activity. The capacity of these cells to attach after reseeding is unaltered but the cell density after a week in culture is about 20% lower than that of untreated control cells.

In the more extensive tests of the specificity of β-GalMNT, both on living cells and cell homogenates (Table 1), it is seen that this reagent has no effect on any hydrolase activity other than β-galactosidase, β-D-fucosidase, and α-L-arabinopyranosidase. It is probable that these three activities are associated with the same active site (β-D-galactopyranosides, β-D-fucopyranosides and α-L-arabinopyranosides differ structurally only in the substituent at C-5 of the pyranose ring), since Norden *et al.* (1974) have shown that purified lysosomal β-galactosidase from human liver will also hydrolyse β-D-fucopyranosides and α-L-arabinopyranosides. Confirmation that these three glycosidase activities do indeed arise from the catalytic action of a single active site comes from the data from denaturation experiments (Table 2). The three

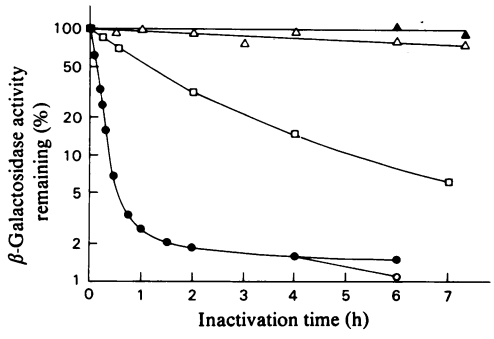

Fig. 2. *Inactivation of β-galactosidase in fibroblast cultures by β-GalMNT (0.2 mM) at various temperatures*
β-Galactosidase activity was measured as a function of time in cultures incubated at 37°C (●), 21°C (□), and 5°C (△). ▲, Untreated control cells at 5°C; O, a culture at 39°C to which had been added, after 4h, fresh β-GalMNT (0.2mM).

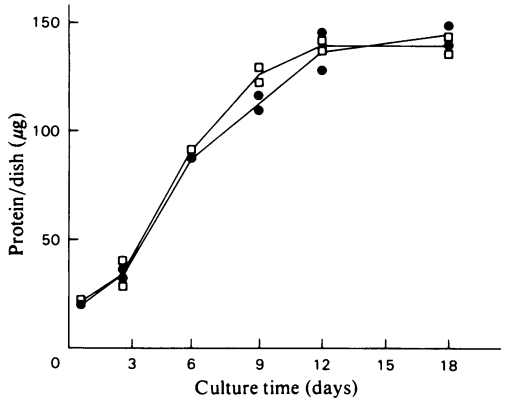

Fig. 3. *Effect of β-GalMNT treatment on subsequent growth capacity of fibroblasts*
Confluent fibroblast cultures in 35mm-Petri dishes were incubated in standard medium containing β-GalMNT (0.2mM) for 2h at 37°C, after which the cells were harvested quantitatively with trypsin. Aliquots (one-seventh) of each cell suspension were then reseeded in 35mm dishes and incubated. After various culture times, cells were dissolved in NaOH (1M) and cell protein was determined. ●, Cells pretreated with β-GalMNT; □, untreated control cells.

Table 1. *Specificity of hydrolase inactivation by β-GalMNT and β-GlcMNT in living cells and in cell homogenates*
n.d. = not determined

	Living cells				Cell homogenates		
		Percentage of control activity remaining after 2h at 37°C in the presence of:				Percentage of control activity remaining after 20min at 37°C in 20mM-sodium acetate buffer, pH 5.0, containing:	
Hydrolase	Activity of control (nmol/h per mg)	β-GalMNT (0.2mM)	β-GlcMNT (0.2mM)	*p*-Nitroaniline (0.2mM)	Activity of control (nmol/h per mg)	β-GalMNT (0.2mM)	*p*-Nitroaniline (0.2mM)
β-Galactosidase	1420	1.4	97	98	1160	2.5	100
β-D-Fucosidase	192	1.9	94	n.d.	132	3.1	104
α-L-Arabinopyranosidase	218	1.7	93	n.d.	147	2.3	100
β-Glucosidase	656	96	24	112	368	109	128
β-Xylosidase	15.7	116	21	n.d.	8.1	118	100
α-Galactosidase	114	92	102	96	55	96	93
α-Glucosidase	205	89	88	95	162	88	93
α-Mannosidase	190	131	72	n.d.	171	141	100
α-L-Fucosidase	269	97	98	n.d.	92	99	101
β-*N*-Acetylhexosaminidase	14500	86	107	n.d.	9290	91	82
β-Glucuronidase	204	81	94	n.d.	181	108	113
Neuraminidase	126	90	n.d.	n.d.	60	107	97
Aryl sulphatase	332	121	111	n.d.	278	99	117
Galactosylcerebroside β-galactosidase	5.4	76	n.d.	n.d.	6.4	81	94

Table 2. *Denaturation of β-Galactosidase, β-D-fucosidase and α-L-arabinopyranosidase in cell lysates*

The specific activity as a percentage of that at zero time is given; the specific activity at zero time (nmol/h per mg of protein) is given in parentheses. n.d. = not determined.

	Activity after denaturation at 37°C at pH 7.0 in absence of NaCl			Activity after denaturation at 52°C at pH 4.3 in 250mM-NaCl		
Denaturation time (min)	β-D-Galactosidase	β-D-Fucosidase	α-L-Arabinosidase	β-D-Galactosidase	β-D-Fucosidase	α-L-Arabinosidase
0	100	100	100	100	100	100
	(2560)	(290)	(320)	(2680)	(318)	(359)
5	24.3	23.5	23.5	n.d.	n.d.	n.d.
10	8.3	8.2	8.4	76	77	75
20	2.8	3.0	3.1	67	66	64
40	n.d.	n.d.	n.d.	53	52	50
60	n.d.	n.d.	n.d.	33	36	38

activities are lost in an exactly parallel manner under two widely differing sets of conditions.

The parallel losses of glycosidase activity in cell homogenates and in the living cells after inactivation by β-GalMNT (Table 1) indicate that any specific transport process to the lysosomes plays no significant role in the observed specificities of inactivation. Likewise, *p*-nitroaniline, one of the products of spontaneous decomposition of alkyl *p*-nitrophenyltriazenes (Vaughan & Stevens, 1978), has no effect on hydrolase activity (Table 1).

The above experiments show that conditions can be found where the only detectable effect of β-GalMNT on living human cells in culture is specifically to inactivate one lysosomal glycosidase: the enzyme (β-galactosidase) against which β-GalMNT was designed to act.

Specific inactivation of lysosomal β-glucosidase by β-GlcMNT

Despite the membrane-bound state of lysosomal β-glucosidase, β-GlcMNT specifically inactivates β-glucosidase in the living cell (Table 1 and Fig. 4). The initial rate of inactivation is some 7-fold lower than the rate of inactivation of β-galactosidase by β-GalMNT (Fig. 5), with the consequence that spontaneous decomposition of β-GlcMNT becomes significant. Addition of fresh β-GlcMNT after 3h leads to appreciable further inactivation of β-glucosidase; however, the initial rate of inactivation is not restored.

β-GlcMNT also destroys β-xylosidase activity in the cell (Fig. 4 and Table 1). However, this is again a consequence of the indifference of β-glucosidase to the substituent at C-5 of the pyranose ring of the substrate, rather than a lack of specificity of the

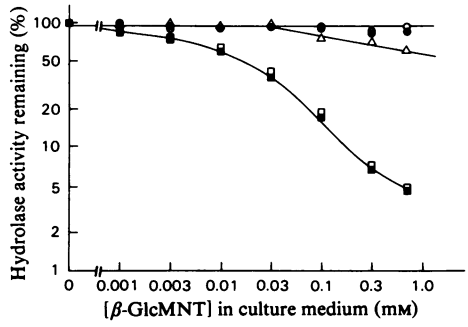

Fig. 4. *Inactivation of β-glucosidase and β-xylosidase in fibroblast cultures by different concentrations of β-GlcMNT*

Cell cultures were incubated for 4h at 37°C. □, β-Glucosidase; ■, β-xylosidase; ●, β-galactosidase; ○, α-galactosidase; △, α-glucosidase.

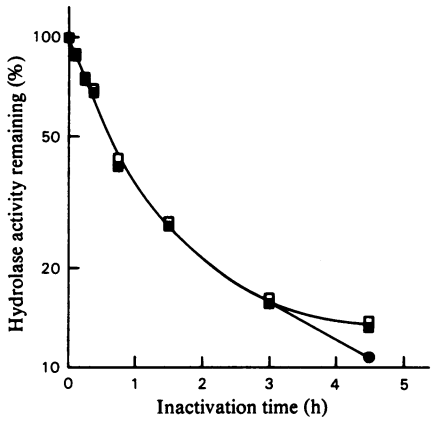

Fig. 5. *Time course of the inactivation of β-glucosidase and β-xylosidase in fibroblast cultures by β-GlcMNT (0.2 mM) at 37°C*

□, β-Glucosidase; ■, β-xylosidase; ●, β-glucosidase activity in one culture to which fresh β-GlcMNT (0.2 mM) had been added after 3 h.

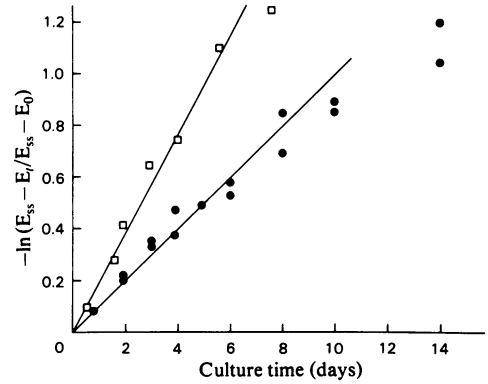

Fig. 6. *Restitution of β-galactosidase and β-glucosidase activity in confluent fibroblast cultures in which these enzymes had been inactivated by β-GalMNT and β-GlcMNT respectively*

For details see the Materials and Methods section. E_{ss}, steady-state enzyme activity in untreated control cells; E_0, residual enzyme activity immediately after treatment with triazene (for β-galactosidase, $E_0=0.017\times E_{ss}$ and for β-glucosidase $E_0=0.22\times E_{ss}$); E_t, enzyme activity at various times t after treatment with triazene. An exponential recovery to a final activity of E_{ss} would give a straight line passing through the origin. ●, β-Galactosidase; □, β-glucosidase.

reagent. This is shown in Fig. 5, where the destruction of β-xylosidase and β-glucosidase activities by β-GlcMNT in the cell proceeds in an exactly parallel fashion. The common identity of both hydrolase activities is also indicated by the observation that fibroblasts from patients with Gaucher disease are deficient in both β-glucosidase and β-xylosidase (results not shown).

Some slight but real failure of anomeric specificity is apparent in Fig. 4: significant loss of α-glucosidase activity is observed at high concentrations of β-GlcMNT.

Restitution of β-galactosidase and β-glucosidase activities after triazene inactivation

The unaltered viability of cells in which β-glucosidase and β-galactosidase activities have been destroyed by the respective triazenes enables the restitution of these enzymes in the cell to be studied. Fig. 6 indicates that this recovery is kinetically exponential, and proceeds to final activities close to those in untreated cells. On the simplest possible model for enzyme synthesis and degradation, namely constant rate of synthesis and first-order degradation (Price *et al.*, 1962), the reciprocal of the degradation rate constant can be identified with the turnover time of the enzyme. If this model is adopted, then the turnover time of β-galactosidase in one strain of fibroblasts is estimated to be 9.8 ± 1.4 days, and in another (results not shown), 13.3 ± 3.3 days. The turnover time of β-glucosidase is analogously estimated to be 5.1 ± 0.6 days.

Discussion

The results presented here clearly demonstrate that both β-GlcMNT and β-GalMNT selectively inactivate the corresponding hydrolase in living fibroblasts under physiological conditions without prejudice to the subsequent viability of the cells. Additionally it is apparent that, should specific transport proteins be involved in the passage of glycosylmethyltriazenes into lysosomes, these are not alkylated during this process. This conclusion is in accord with the observation that β-GalMNT is without effect on the non-catalytic *lac*-repressor protein of *Escherichia coli* (P. J. Marshall, M L. Sinnott, P. J. Smith & D. Widdows, unpublished work).

The clearly first-order inactivation of intracellular β-galactosidase and β-glucosidase implies that, in this respect, the lysosomes in which these enzymes are contained behave as homogeneous populations.

In view of the low β-GalMNT-resistant β-galactosidase activity (approx. 1% as measured with 4-methylumbelliferyl-galactoside), the inertness of galactosylcerebroside β-galactosidase to this reagent is also significant. Our results are consistent with the hypothesis that this latter enzyme has a low residual activity towards the synthetic fluorogenic galac-

toside. This is consistent with the observation (O. P. van Diggelen, H. Galjaard, M. L. Sinnott and P. J. Smith, unpublished work) that β-GalMNT inactivation of the 10–15% residual β-galactosidase activity in fibroblasts from patients with genetically determined β-galactosidase deficiencies [for reviews see Galjaard & Reuser (1977), Bootsma & Galjaard (1979) and Lowden & O'Brien (1979)] also leads to a residual 1% of β-GalMNT-resistant β-galactosidase. Should this prove to be the case, β-GalMNT inactivation of G_{M1} ganglioside β-galactosidase may lead to a rapid assay procedure for galactosylcerebroside β-galactosidase and thus facilitate the prenatal diagnosis of Krabbe's disease.

Our experimental evidence that both β-glucosidase and β-xylosidase activities reside in the same enzyme (glucocerebroside β-glucosidase) is in accord with the results of other workers (Ockermann, 1968; Ben-Yoseph *et al.*, 1979) and in disagreement with the findings of Chiao *et al.* (1979).

The triazenes were employed to estimate enzyme turnover in fibroblasts by adopting the zero-order synthesis/first-order degradation model (Price *et al.*, 1962). We are aware that regulatory mechanisms could invalidate the adopted model. However, the turnover times found (±10 days and ±5 days for β-galactosidase and β-glucosidase) are in the same range as those found for other lysosomal enzymes by the radioisotope method (Warburton & Wynn, 1976, 1977). This suggests that, if such regulatory mechanisms are present, they play no major role.

We expect β-GalMNT to be a very useful compound for characterizing the residual β-galactosidase activity in fibroblasts from patients with genetically determined β-galactosidase deficiencies [for reviews see Galjaard & Reuser (1977), Bootsma & Galjaard (1979) and Lowden & O'Brien (1979)]. It should be possible to establish the relationship between β-galactosidase degradation and this residual enzyme activity.

Furthermore, because glycosylmethyltriazenes are non-toxic towards the living cell, these compounds may have some potential application in the experimental induction of lysosomal storage diseases in animals.

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References

Abeles, R. H. & Maycock, A. L. (1976) *Accounts Chem. Res.* **9**, 313–319

Ben-Yoseph, Y., Fiddler, M. B., Rousson, R. & Nadler, R. (1979) *Biochim. Biophys. Acta* **568**, 386–394

Bootsma, D. & Galjaard, H. (1979) in *Models for the Study of Inborn Errors of Metabolism* (Hommes, F. A., ed.), pp. 241–256, Elsevier North Holland, Amsterdam

Chen, T. R. (1977) *Exp. Cell Res.* **104**, 255–262

Chiao, Y.-B., Peters, S. P., Diven, W. F., Lee, R. E. & Glew, R. H. (1979) *Metabolism* **28**, 56–62

Galjaard, H. & Reuser, A. J. J. (1977) in *The Cultured Cell and Inherited Metabolic Disease* (Harkness, R. A. & Cockburn, F., eds.) pp. 138–160, MTP Press, Lancaster

Gatt, S. & Rapport, M. (1966) *Biochim. Biophys. Acta* **113**, 567–576

Jakoby, W. B. & Wilchek, M. (1977) *Methods Enzymol.* **46**

Kleijer, W. J., Hoogeveen, A., Verheijen, F. W., Niermeijer, M. F., Galjaard, H., O'Brien, J. S. & Warner, T. G. (1979) *Clin. Genet.* **16**, 60–61

Lowden, J. A. & O'Brien, J. S. (1979) *Am. J. Hum. Genet.* **31**, 1–18

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

McIlvaine, T. C. (1921) *J. Biol. Chem.* **49**, 183–186

Norden, A. G. W., Tennant, L. L. & O'Brien, J. S. (1974) *J. Biol. Chem.* **249**, 7969–7976

Ockermann, P. A. (1968) *Biochim. Biophys. Acta* **165**, 59–62

Price, V. E., Sterling, W. R., Tarantola, V. A., Hartley, R. W. & Rechcigl, L. M. (1962) *J. Biol. Chem.* **237**, 3468–3475

Sinnott, M. L. & Smith, P. J. (1976) *J. Chem. Soc. Chem. Commun.* 223–224

Sinnott, M. L. & Smith, P. J. (1978) *Biochem. J.* **175**, 525–538

Suzuki, K., Suzuki, Y. & Eto, Y. (1971) in *Lipid Storage Diseases* (Bernsohn, J. & Grossman, H. J., eds.), pp. 111–136, Academic Press, London and New York

Vaughan, K. & Stevens, M. F. G. (1978) *Chem. Soc. Rev.* **7**, 377–397

Warburton, M. J. & Wynn, C. H. (1976) *Biochem. J.* **158**, 401–407

Warburton, M. J. & Wynn, C. H. (1977) *Biochem. J.* **162**, 201–203