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THE INHIBITION OF β-*N*-ACETYLHEXOSAMINIDASE BY LACTONES

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SUMMARY

β-*N*-Acetylhexosaminidase (2-acetamido-2-deoxy-β-hexoside acetamidodeoxyhexohydrolase, EC 3.2.1.52), from commercial bovine serum albumin, was inhibited by glucoheptonolactone, D- and L-gluconolactone, D-galactonolactone, D-fuconolactone, L-arabonolactone, D-ribonolactone, L-mannolactone and L-ascorbic acid. The kinetics of inhibition of the hydrolysis of 4-methylumbelliferyl-*N*-acetylglucosaminide (MU-GlcNAc) and 4-methylumbelliferyl-β-D-*N*-acetylgalactosaminide (MU-GalNAc) with both the purified enzyme and rat liver lysosomes was examined in detail. The lactones corresponding to both *N*-acetylamino sugars were competitive in nature. Employing MU-GlcNAc as substrate, both D-gluconolactone and L-ascorbic acid gave "mixed" type inhibitions. With MU-GalNAc as substrates, these two compounds were found to be non-competitive inhibitors.

INTRODUCTION

The presence of an enzyme which catalyzes the hydrolysis of β-*N*-acetylhexosaminidic linkages has been observed in most biological materials examined. These preparations do not possess high specificity for the configuration of the hydroxyl group at C-4 in the *N*-acetylamino sugar. The rate of hydrolysis of the *N*-acetylglucosaminide (GlcNAc) bond usually exceeds the rate of hydrolysis of the *N*-acetylgalactosaminide (GalNAc) bond[1–6]. This varies from 2.5 to 8 depending upon the preparations employed. Three distinct hexosaminidases have been reported present in rat and calf brain extracts. A preparation non-specific as to the nature of the β-*N*-acetylhexosaminide linkage in addition to one enriched in β-*N*-acetylgalactosaminide and one enriched in β-*N*-acetylglucosaminide were described[8]. This has been the only documentation of separate β-*N*-acetylhexosaminidases in mammalian tissues based upon differences in substrate specificity. Cellulose-acetate electrophoretic studies support this observation[9].

Abbreviations: GalNAc, *N*-acetylgalactosaminide; GlcNAc, *N*-acetylglucosaminide; MU-, 4-methylumbelliferone.

Evidence supporting a single protein catalyzing the hydrolysis of both GalNAc and GlcNAc β-glycosides comes from studies employing purified enzymes. Mammalian tissues possess at least 2 enzymatic components referred to as hexosaminidase "A" and hexosaminidase "B", which are readily separable either electrophoretically or column chromatographically[3]. The ratio of hydrolysis towards both β-*N*-acetylhexosaminides remains constant throughout these purifications. Furthermore, in the G_{M2} gangliosidosis, in which the detectable levels of hexosaminidases A and B are altered, activity towards GlcNAc and GalNAc glycosides are equally affected[10]. This evidence would suggest that a common protein is responsible for the hydrolysis of both substrates.

This laboratory reported the presence of β-*N*-acetylhexosaminidase in commercial bovine serum albumin preparations[11]. The purification of an enzyme component to apparent homogeneity, as judged by analytical polyacrylamide gel electrophoresis, and the inhibition of activity by certain structurally related carbohydrate compounds was reported[12].

The present study documents the inhibition of β-*N*-acetylhexosaminidase activity of both the highly purified enzyme from bovine serum and purified intact rat liver lysosomes by a variety of sugar acid lactones unrelated to these substrates.

MATERIALS AND METHODS

4-Methylumbelliferyl-β-D-*N*-acetylglucosaminide (MU-GlcNAc), 4-methylumbelliferyl-β-D-*N*-acetylgalactosaminide (MU-GalNAc), 2-acetamido-2-deoxy-gluconolactone (*N*-acetylglucosaminolactone, GlcNAc-lactone) and 2-acetamido-2-deoxy-galactonolactone (*N*-acetylgalactosaminolactone, GalNAc-lactone) were purchased from Koch-Light Limited (Colnbrook, England). D- and L-gluconolactone, L-mannonolactone, D-gluconoheptonolactone, D-ribonolactone, and L-arabonolactone were obtained from Pfansteihl Co. (Waukeegan, Ill.). L-Ascorbic acid was either from Fisher Scientific (Boston, Mass.) or G.B.I. (Grand Island, Mich.). D-Fuconolactone was prepared from D-fucose according to a published procedure[13].

The purified fraction from commercial bovine serum albumin was prepared as previously described[12]. Rat liver lysosomes were isolated according to the procedure of Ragab *et al.*[14].

The incubation mixtures contained 500 nmoles of substrate, 10 μmoles citrate–phosphate buffer (pH 4.5), 25 μmoles NaCl, 1 mg hexosaminidase-free bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), 0.2 μg–10 mg of protein, and sugar acid lactones where indicated, in a total volume of 0.2 ml and the tubes were shaken for 1 h at 37 °C. To terminate the reaction, 0.7 ml of 2.75 % trichloroacetic acid, 0.45 ml of 0.5 M KOH and 0.7 ml of glycine buffer (pH 10.2) were added. The quantity of 4-methylumbelliferone (MU) released was measured fluorimetrically in an Amino–Bowman spectrofluorimeter with the excitation wavelength at 366 nm and the emission wavelength at 466 nm. Each assay was carried out either in duplicate or in quadruplicate. K_m and K_i values were obtained by the double reciprocal plot procedure of Lineweaver and Burk[15]. Protein was quantitated with crystalline serum albumin as standard according to a published procedure[16].

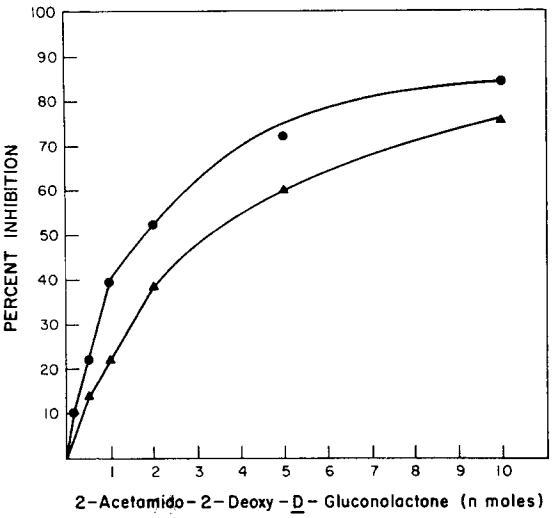


Fig. 1. Inhibition of MU-GlcNAc (●) and MU-GalNAc (▲) hydrolytic activity of purified rat liver lysosomes by 2-acetamido-2-deoxygluconolactone.

RESULTS

The ability of GlcNAc-lactone and GalNAc-lactone to inhibit the hydrolysis of both MU-GlcNAc and MU-GalNAc by the purified serum albumin enzyme preparation is shown in Figs 1 and 2. It is readily apparent that with both lactones the hydrolysis of MU-GlcNAc is more susceptible to inhibition than hydrolysis of MU-GalNAc. Approximately twice as much lactone was required to inhibit MU-GalNAc cleavage as compared to MU-GlcNAc cleavage. GalNAc-lactone was found to be a more

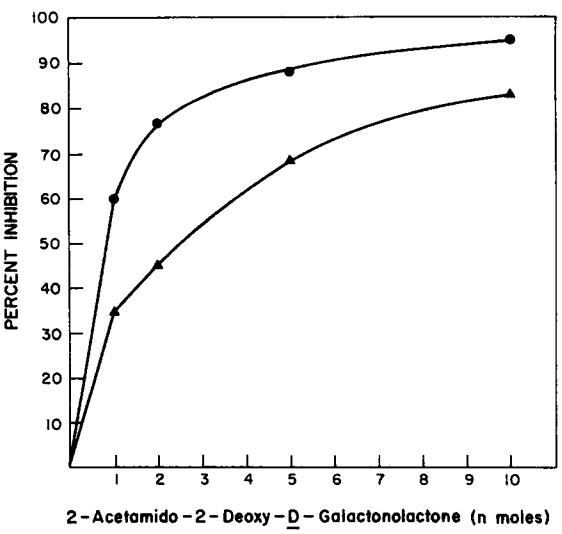


Fig. 2. Inhibition of MU-GlcNAc (●) and MU-GalNAc (▲) hydrolytic activity of rat liver lysosomes by 2-acetamido-2-deoxygalactonolactone.

effective inhibitor than GlcNAc-lactone for the hydrolytic activities with both substrates.

These observations were extended to an examination of the inhibitory properties of a variety of sugar acid lactones on MU-GlcNAc hydrolysis. The ability for several of these including glucoheptonolactone; D-glucono (1→5)-lactone; L-glucono-(1→5)-lactone; D-galactono(1→5)-lactone; D-fucono(1→5)-lactone; L-mannono-(1→4) lactone; L-arabono(1→4)-lactone; D-ribono(1→4)-lactone and L-ascorbic acid is presented in Fig. 3. These data indicate that all the lactones employed in these studies caused some degree of inhibition. The major common structural feature of these

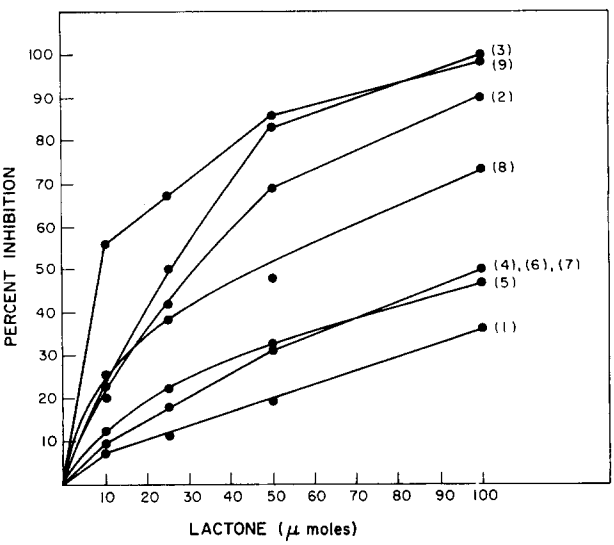


Fig. 3. Inhibition of MU-GlcNAc hydrolysis by β-*N*-acetylhexosaminidase of commercial bovine bovine serum albumin by sugar acid lactones. Curve (1) = glucoheptonolactone; Curve (2) = D-glucono(1→5)-lactone; Curve (3) = L-glucono(1→5)-lactone; Curve (4) = D-galactono(1→4)-lactone; Curve (5) = D-fucono(1→5)-lactone; Curve (6) = L-ariabono(1→4)-lactone; Curve (7) = D-ribono(1→4)-lactone; Curve (8) = L-mannono(1→4)-lactone; Curve (9) = L-ascorbic acid.

compounds is the presence of a lactone ring. The configuration of any given hydroxyl group in the molecule did not appear to specifically effect the inhibition (Curves 2 and 3). The presence of 5 or 6 carbon atoms or a primary hydroxyl group were not required for the suppression of activity (Curves 5, 6 and 7). L-Ascorbic acid, which possesses a double bond between carbon atoms 2 and 3, was also an effective inhibitor as was D-erythroascorbate (Sigma Chemical Co., St. Louis, Mo.).

This non-specific inhibition was examined in detail employing both the purified β-*N*-acetylhexosaminidase from bovine serum albumin and the purified rat liver lysosomes as enzyme sources with both MU-GalNAc and MU-GlcNAc as the substrates. *N*-Acetylglucosaminolactone and *N*-acetylgalactosaminolactone were used due to their structural relationship to the substrates. D-Gluconolactone and L-ascorbic acid were used because of their lack of structural relationship to the substrates. The kinetic data obtained from these studies are presented in Tables I and II. The major difference observed, aside from the absolute individual analytical values, was the

TABLE I

INHIBITION OF RAT LIVER LYSOSOMAL β-*N*-ACETYLHEXOSAMINIDASE BY DIFFERENT LACTONES

	Substrate:	*MU-GlcNAc*	*MU-GalNAc*
No additions, K_m		$4.3 \cdot 10^{-5}$ M	$1.6 \cdot 10^{-5}$ M
+ 5 nmoles GlcNAc-lactone, K_i		$1.5 \cdot 10^{-4}$ M (C)*	$4.0 \cdot 10^{-5}$ M (C)
+ 5 nmoles GalNAc-lactone, K_i		$3.1 \cdot 10^{-5}$ M (C)	$9.0 \cdot 10^{-5}$ M (C)
+ 10 μmoles D-gluconolactone, K_i		$8.6 \cdot 10^{-5}$ M (M)	$1.6 \cdot 10^{-5}$ M (N.C.)
+ 5 μmoles L-ascorbic acid, K_i		$1.2 \cdot 10^{-4}$ M (M)	$1.6 \cdot 10^{-5}$ M (N.C.)

* Letters in parenthesis: (C) = competitive type inhibition, (M) = mixed type inhibition (N.C.) = non-competitive inhibition.

type of inhibition observed with the different lactones. A summary of these observations is schematically presented in Fig. 4. The two acetylamino sugar lactones were found to be competitive inhibitors since the lines intersected at the ordinate, presumably due to their close relationship to the substrates MU-GlcNAc and MU-GalNAc. However, with MU-GalNAc as substrate, both D-gluconolactone and L-ascorbic acid acted as "non-competitive" inhibitors since the lines intersected at the abscissa.

TABLE II

INHIBITION OF PURIFIED SERUM β-*N*-ACETYLHEXOSAMINIDASE BY DIFFERENT LACTONES

	Substrate:	*MU-GlcNAc*	*MU-GalNAc*
No additions, K_m		$1.5 \cdot 10^{-4}$ M	$1.4 \cdot 10^{-5}$ M
+ 5 μmoles GlcNAc-lactone, K_i		$5.0 \cdot 10^{-4}$ M (C)*	—
+ 10 μmoles D-gluconolactone, K_i		$2.5 \cdot 10^{-4}$ M (M)	$1.4 \cdot 10^{-5}$ M (N.C.)
+ 5 μmoles L-ascorbic acid, K_i		$9.0 \cdot 10^{-5}$ M (M)	$1.4 \cdot 10^{-5}$ M (N.C.)

* Letters in parenthesis: (C) = competitive type inhibition, (M) = mixed type inhibition, (N.C.) = non-competitive type inhibition.

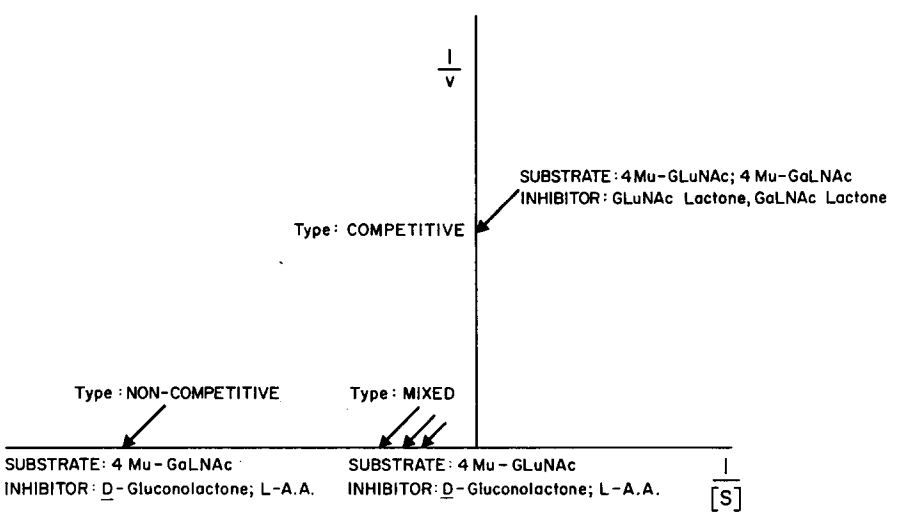


Fig. 4. Schematic representation of the types of inhibitions observed with MU-GlcNAc and MU-GalNAc by several lactones.

Employing MU-GlcNAc as substrate and these two lactones, a "mixed" type of inhibition was seen since the lines did not intersect at either the ordinate or the abscissa.

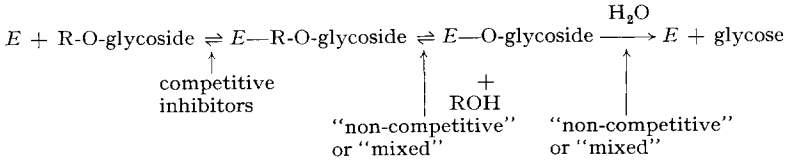
DISCUSSION

The accepted explanation of the aldonolactone inhibition of a specific glycosidase is believed to be due to the similarity in structure and configuration to the glycosyl group liberated by the enzyme. Inhibitions have been observed with β-glucuronidase by glucurono(1→4)-lactone[17], α- and β-glucosidase by glucono-(1→4)- and (1→5)-lactones[18], α- and β-mannosidase by mannono(1→4)-lactone, β-galactosidase by galactono(1→5)-lactone[19], β-glucosaminidase by 2-acetamido-2-deoxygluconolactone[20], β-D-fucosidase by D-fuconolactone[21].

In certain instances, these hydrolytic enzymes themselves do not exhibit absolute substrate specificities, and therefore, the aldonolactone inhibitions are not absolutely specific. β-*N*-Acetylhexosaminidase cleaves both *N*-acetylgalactosaminides and *N*-acetylglucosaminides. The lactones corresponding to both substrates inhibit both substrates[20]. The β-glucosidase of almond emulsin appears to possess β-galactosidase activity as well and is inhibited by both gluconolactone and galactonolactone[22]. Aldonolactone inhibition has been observed as being "competitive" in nature, implying a close structural relationship to the substrate being hydrolyzed.

The ability of both 2-acetamido-2-deoxygluconolactone and 2-acetamido-2-deoxygalactonolactone to inhibit the hydrolysis of both MUKGlcNAc and MU-GalNAc was shown in Figs 1 and 2. These are extremely potent inhibitors since the addition of 5 nmoles inhibitor in the presence of 500 nmoles of substrate caused 60–90 % reduction in enzymatic hydrolysis. The type of inhibition was found to be competitive (Tables I, II and Fig. 4).

At least 9 other sugar acid lactones, structurally unrelated to these substrates were found to inhibit (Fig. 3). However, in this case, greater quantities of materials were required. The sole common feature was the presence of a lactone ring structure. The nature of this inhibition was examined in greater detail with two of these compounds. D-Gluconolactone was selected as a model for a lactone related to a β-glucoside substrate for a lysosomal acid hydrolase, and due to its relatively high inhibitory power. L-Ascorbic acid was selected due to the absence of a known structurally related glycoside. The type of inhibition found with these two lactones differed with the substrate. "Non-competitive" inhibition was observed with MU-GalNAc and "mixed" type with MU-GlcNAc (Tables I, III and Fig. 3). It is difficult to explain these differences. Several reports on the ability of purified acid hydrolases to catalyze a transglycosylation have appeared[23–26]. One suggestion is that during the hydrolysis of a glycosidic bond by this group of enzymes, a glycosyl enzyme intermediate is formed, and then water is involved in the spontaneous hydrolysis of this linkage[26,28]. The lactones may, therefore, act at different steps in this sequence.



Nearly all of the experimental evidence available indicates an identity of *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activity. The difference in the nature of the inhibitions observed with D-gluconolactone and L-ascorbic acid for these two substrates, both with a purified protein and intact lysosomes, suggests that for certain properties there is a dissimilarity.

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