Volume 335, number 2, 280–284 FEBS 13340 December 1993

A novel β-*N*-acetylglucosaminidase activity in hog gastric mucosal microsomes: Preferential hydrolysis of terminal GlcNAcβ1–3 linkages in GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc, but GlcNAcβ1–6 linkages in GlcNAcβ1–3(GlcNAcβ1–6)Gal

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Received 20 September 1993; revised version received 20 October 1993

Hog gastric mucosal microsomes contain β-*N*-acetylglucosaminidase activity which cleaves GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc at the terminal GlcNAcβ1–3Gal linkage faster than at the GlcNAcβ1–6Gal bond, producing mainly GlcNAcβ1–6Galβ1–4GlcNAc. In a marked contrast, GlcNAcβ1–3(GlcNAcβ1–6)Gal is cleaved primarily at the GlcNAcβ1–6Gal bond, while partial hydrolysis of GlcNAcβ1–3(GlcNAcβ1–6)Gal β1–4Glc reveals similar rates of cleavage for the (1–3) and (1–6) linkages. Our data support the notion that the terminal β1,6-linked GlcNAc unit of GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc may interact with the reducing end GlcNAc unit intramolecularly in water solution.

β-*N*-Acetylglucosaminidase; Linkage specificity; Oligo-*N*-acetyllactosaminoglycan; In vitro synthesis; Hog gastric mucosal microsome

1. INTRODUCTION

We have shown earlier that partial hydrolysis of GlcNAcβ1–3(GlcNAcβ1–6)Gal, GlcNAcβ1–3(GlcNAcβ1–6)Galβ1-OCH_3, GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4-Glc and GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc with jack bean β-*N*-acetylhexosaminidase (EC 3.2.1.30) leads to preferential cleavage of the GlcNAcβ1–6Gal linkage rather than the GlcNAcβ1–3Gal linkage [1]. The present study describes a novel β-*N*-acetylglucosaminidase activity from hog gastric mucosal microsomes, which is shown to cleave GlcNAcβ1–3-(GlcNAcβ1–6)Galβ1–4GlcNAc with relatively high preference for the GlcNAcβ1–3Gal-linkage, while GlcNAcβ1–3(GlcNAcβ1–6)Gal is cleaved via the opposite pathway, with a high preference for the GlcNAcβ1–6Gal bond.

2. MATERIALS AND METHODS

2.1. *Paper chromatography*

Chromatography on Whatman III Chr paper was carried out with *n*-butanol/ethanol/water 10:1:2 by vol. The radioactivity on the chromatograms was analyzed as described [2]. The mobilities of saccharides are presented in relation to that of lactose (R_{Lac}), maltotriose (R_{MT}), maltotetraose (R_{MTet}), and maltopentaose (R_{MP}), which were purchased from Sigma (St. Louis, MO, USA). The marker saccharides were visualized with silver nitrate–sodium hydroxide staining.

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Abbreviations: Gal, D-galactose; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; dpm, disintegrations per minute

2.2. *Saccharides*

The saccharides studied in the present experiments are shown in Table I. [U-^{14}C]Galβ1–4GlcNAc, GlcNAcβ1–3[U-^{14}C]Gal (**I**), GlcNAcβ1–6[U-^{14}C]Gal (**II**), GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc (**VII**), GlcNAcβ1–6[U-^{14}C]Galβ1–4GlcNAc (**VIII**), GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Gal (**III**) and GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**IX**) were constructed as described [3]. GlcNAcβ1–3-[6-^{3}H]Galβ1–4Glc (**IV**), GlcNAcβ1–6[6-^{3}H]Galβ1–4Glc (**V**), and GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4[6-^{3}H]Glc (**VI**) were obtained as described [1].

2.3. *Enzymic methods*

Hog gastric mucosal microsomes were prepared as described [4]. Two microsome preparations were studied, both of which were obtained from two hogs. Preparations contained 39 mg/ml and 48 mg/ml of total protein. Preparations were stored at −20°C until use.

Assays of β-*N*-acetylglucosaminidase activity were performed by incubating 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) in 200 μl of sodium citrate/phosphate buffer containing 0.5 mg/ml bovine serum albumin with 10 μl of microsomes for 20–60 min at 37°C. The reactions were stopped by addition of 800 μl 1 M sodium carbonate solution or, in the case of measuring the effect of Triton X-100, 800 μl 0.1 M NaOH (addition of 1 M sodium carbonate to reactions with Triton X-100 produced a cloudy solution). The quantity of released *p*-nitrophenol was determined by measuring the absorption at 405 nm; $\varepsilon = 18.6\ 1 \times mmol^{-1} \times cm^{-1}$. One unit is defined as the enzyme activity which hydrolyzes 1 μmol of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide/min at pH 4.5 at 37°C.

Cleavage of oligosaccharides with mucosal β-*N*-acetylglucosaminidase was conducted by incubating the substrate saccharides, 5 μmol sodium cacodylate (pH 7.0), 0.8 μmol sodium azide and 0.2 μmol

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EDTA with 100 μl of microsomes at 37°C for indicated periods of time. Bio-Gel P-2 chromatography was used to remove sucrose, a stabilizer in the microsome suspension [3]; aliquots from the fractions were assayed for radioactivity, and occasionally also for carbohydrate content with phenol-sulfuric acid method [5]. Peaks obtained from Bio-Gel P-2 chromatography were submitted to paper chromatography for final separation of the reaction products.

All enzymic treatments were terminated by heating for 5 min in a boiling water bath, and desalted by filtration through a column of Dowex AG-50 (H^+) and Dowex AG-1 (AcO^-) (Bio-Rad, CA, USA).

3. RESULTS

3.1. *Properties of β-N-acetylglucosaminidase in mucosal microsomes*

The enzyme preparation was found remarkably stable, as microsome preparations stored for 4 and 28 months at −20°C showed very similar activities (mU/mg protein) towards *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide. Moreover, two or three cycles of freezing and thawing did not reduce the activity. The pH optimum of the microsomal β-*N*-acetylglucosaminidase was found to be at pH 4.5; at pH 7, the activity was about 10% of that in the optimal pH. Addition of 0.1% Triton X-100 had no effect on the activity, but 1% Triton X-100 reduced the activity about 20% (data not shown).

Under optimal conditions the activity of the enzyme in the preparations studied was 12 mU/mg total protein.

3.2. *Incubation of radiolabeled GlcNAcβ1–3-(GlcNAcβ1–6)Gal (III) with mucosal microsomes*

The branched trisaccharide GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Gal (**III**) (3220 dpm, 5.4 pmol) was incubated for 68 h with hog gastric mucosal microsomes that contained β-*N*-acetylglucosaminidase activity. The reaction mixture was desalted and chromatographed on a column of Bio-Gel P-2. The resulting chromatogram revealed three radiolabeled saccharide peaks (Fig. 1A). Peaks labeled 1, 2 and 3 were taken to represent the original trisaccharide acceptor, the two isomeric GlcNAcβ[U-^{14}C]Gal disaccharides, and [U-^{14}C]galactose, respectively. This assumption was later confirmed when the putative tri- and disaccharide fractions were subjected to paper chromatography (see below). The relative amounts of the labeled products in the P-2 chromatogram suggested that 56% of the terminal GlcNAcβGal linkages had become cleaved during the reaction.

Peaks 1 and 2 were pooled together, desalted and analyzed by paper chromatography that separated the isomeric disaccharides as well (Fig. 2A). Peaks 1,2 and 3 were identified as the intact trisaccharide **III**, the disaccharide **II** and the disaccharide **I**, respectively, by their migration rate; the *R* values (see Table I) agree well with those of authentic markers. The yield of GlcNAcβ1–3[U-^{14}C]Gal (694 dpm) was approximately six times higher than that of GlcNAcβ1–6[U-^{14}C]Gal

Table I

The structures and paper chromatographic mobilities of key saccharides in the present experiments

No.	Saccharide	R_{Lac}	R_{MT}	R_{MTet}	R_{MP}
I	GlcNAcβ1 → $_3$Gal GlcNAcβ1 →	1.47	3.03		
II	GlcNAcβ1 → ^{6}Gal	0.96	1.98		
III	GlcNAcβ1 → $^6_3$Gal GlcNAcβ1 →	0.34	0.70	2.30	
IV	GlcNAcβ1 → $_3$Galβ1–4Glc GlcNAcβ1 →	0.42	0.88	2.76	
V	GlcNAcβ1 → ^{6}Galβ1–4Glc GlcNAcβ1 →	0.30	0.62	1.93	
VI	GlcNAcβ1 → $^6_3$Galβ1–4Glc GlcNAcβ1 →		0.26	0.83	2.67
VII	GlcNAcβ1 → $_3$Galβ1–4GlcNAc GlcNAcβ1 →	0.76	1.54		
VIII	GlcNAcβ1 → ^{6}Galβ1–4GlcNAc GlcNAcβ1 →	0.53	1.08	3.31	
IX	GlcNAcβ1 → $^6_3$Galβ1–4GlcNAc GlcNAcβ1 →	0.22	0.44	1.40	

R_{Lac}, R_{MT}, R_{MTet} and R_{MP} give the mobilities of the saccharides in relation to that of lactose, maltotriose, maltotetraose and maltopentaose, respectively.

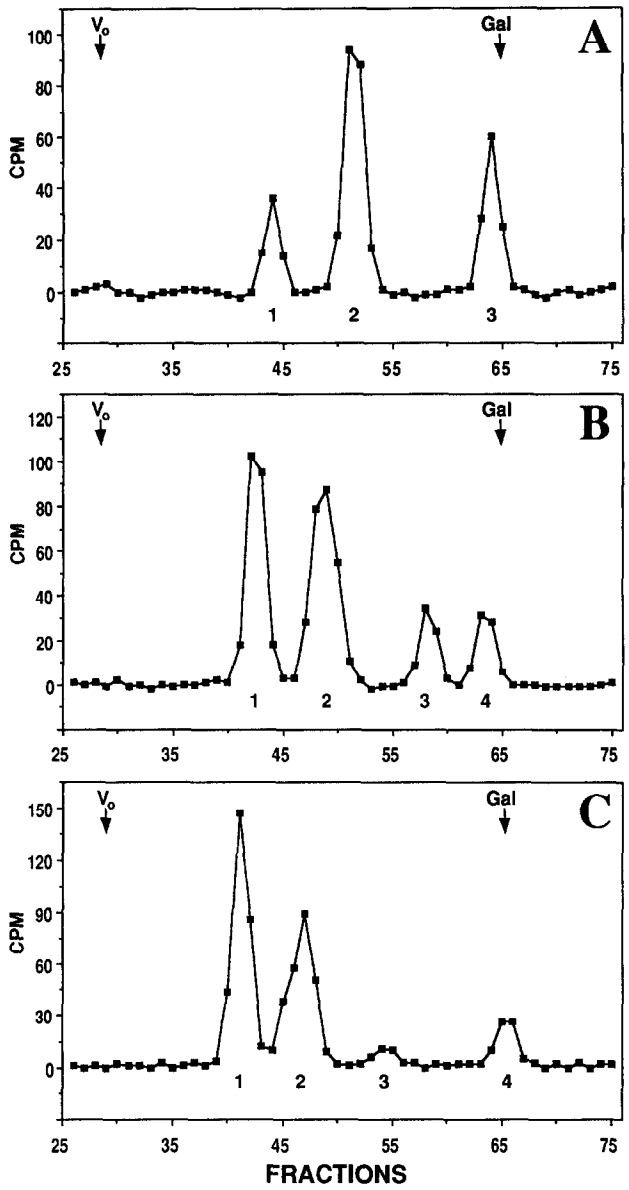

Fig. 1. Bio-Gel P-2 chromatograms of microsomal β-*N*-acetylglucosaminidase digests of the trisaccharide **III** (A), tetrasaccharide **VI** (B) and tetrasaccharide **IX** (C). All substrates were incubated with microsomes for 68 h. The arrows marked V_0 and Gal show the void volume of the column and the position of eluted galactose, respectively.

(112 dpm) showing that the GlcNAcβ1–6Gal linkage was hydrolyzed faster than the GlcNAcβ1–3Gal bond. By combining the data in Figs. 1A and 2A it was concluded that GlcNAcβ1–3Gal was obtained in a remarkably high yield, suggesting that most of the free galactose seen in Fig. 1A was liberated from the intermediary disaccharide GlcNAcβ1–6[U-^{14}C]Gal. In other words, the GlcNAcβ1–6Gal linkage appeared to be the much preferred cleavage site in the trisaccharide **III**, and likewise among the intermediary GlcNAcβGal disaccharides **I** and **II**.

3.3. *Incubation of radiolabeled GlcNAcβ1–3-(GlcNAcβ1–6)Galβ1–4Glc (VI) with mucosal microsomes*

The tetrasaccharide GlcNAcβ1–3(GlcNAcβ1–6)-Galβ1–4[6-^{3}H]Glc (**VI**) (37140 dpm, 6.8 pmol) was incubated for 68 h mucosal microsomes, and the reaction mixture was subjected to Bio-Gel P-2 chromatography. The chromatogram revealed four radiolabeled peaks which were eventually identified as the original tetrasaccharide, a mixture of two isomeric GlcNAcβGalβ1–4-[6-^{3}H]Glc trisaccharides, Galβ1–4[6-^{3}H]Glc and [6-^{3}H]-Glc, respectively (Fig. 1B). As judged from the relative amounts of the cleavage products, about 43% of the GlcNAcβGal linkages originally present in the substrate were hydrolyzed during the reaction.

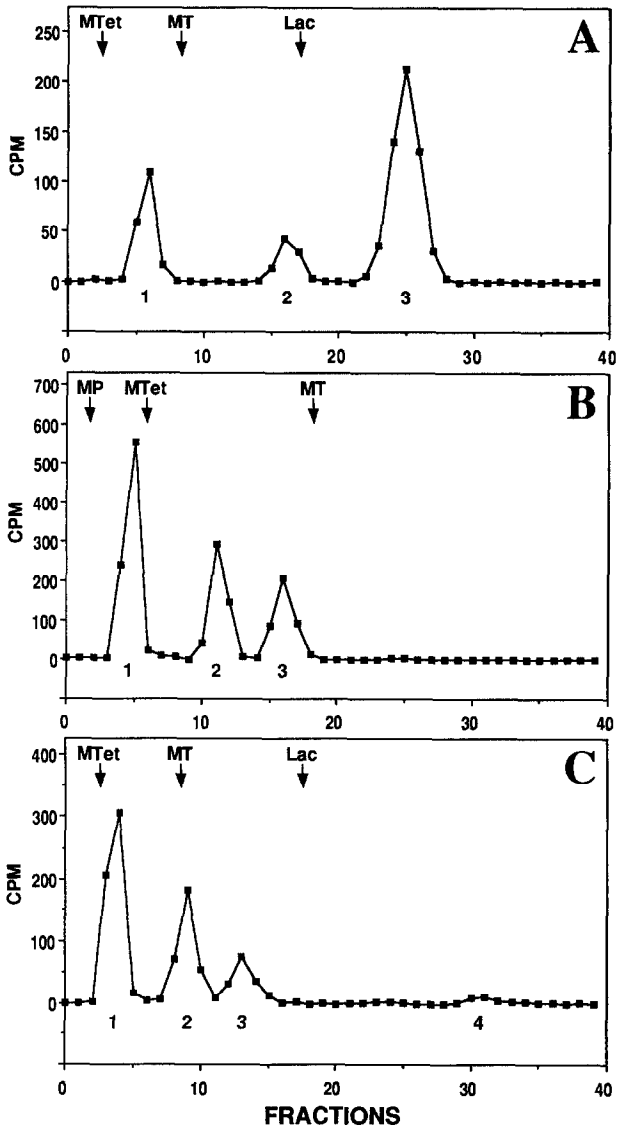

Fig. 2. Paper chromatography of pooled oligosaccharides from Bio-Gel P-2 chromatograms shown in Fig. 1 (A) Peaks 1 and 2 from Fig. 1A. (B) Peaks 1 and 2 from Fig. 1B. (C) Peaks 1, 2 and 3 from Fig. 1C. The arrows marked Lac, MT, MTet and MP show the position of lactose, maltotriose, maltotetraose and maltopentaose, respectively.

Because sucrose comigrates with Galβ1–4[6-^{3}H]Glc in the P-2 chromatogram of Fig. 1B, only the peaks of the intact tetrasaccharide and of the isomeric GlcNAcβGalβ1–4[6-^{3}H]Glc trisaccharides were pooled for subsequent analysis by paper chromatography. The chromatogram shown in Fig. 2B revealed three components, identified as the tetrasaccharide GlcNAcβ-1–3(GlcNAcβ1–6)Galβ1–4[6-^{3}H]Glc (**VI**) and the isomeric trisaccharides GlcNAcβ1–6Galβ1–4[6-^{3}H]Glc (**V**) and GlcNAcβ1–3Galβ1–4[6-^{3}H]Glc (**IV**) (peaks 1, 2 and 3, respectively), by their migration rate. The trisaccharides **IV** and **V** were found in the digest in a ratio of 1:1.2, suggesting that with this substrate the microsomal enzyme cleaved GlcNAcβ1–6Gal and GlcNAcβ1–3Gal linkages at nearly identical rates.

3.4. *Incubation of radiolabeled GlcNAcβ1–3-(GlcNAcβ1–6)Galβ1–4GlcNAc (IX) with mucosal microsomes*

The tetrasaccharide GlcNAcβ1–3(GlcNAcβ1–6)-[U-^{14}C]Galβ1–4GlcNAc (**IX**) (2950 dpm, 4.9 pmol) was incubated for 68 h with mucosal microsomes. Bio-Gel P-2 chromatography (Fig. 1C) revealed in the reaction mixture four radiolabeled peaks that were eventually identified as the intact tetrasaccharide, two isomeric trisaccharides GlcNAcβ[U-^{14}C]Galβ1–4GlcNAc, the disaccharide [U-^{14}C]Galβ1–4GlcNAc and [U-^{14}C]Gal, respectively. The relative amounts of the products indicated that 36% of the GlcNAcβGal-linkages had been cleaved. The di-, tri- and tetrasaccharides were pooled together, and subjected to paper chromatography (Fig. 2C). Peak 1 chromatographed like the intact tetrasaccharide **IX**, while peaks 2 and 3 migrated like the isomeric trisaccharides GlcNAcβ1–6[U-^{14}C]Galβ1–4GlcNAc (**VIII**) and GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc (**VII**), respectively. The small peak 4 was [U-^{14}C]-Galβ1–4GlcNAc. The result shows that the β-*N*-acetylglucosaminidase in mucosal microsomes, surprisingly, cleaved GlcNAcβ1–3Gal linkages faster than GlcNAcβ1–6Gal bonds.

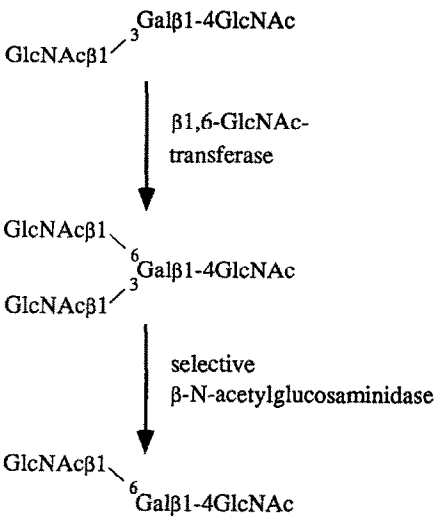

Fig. 3. Combined use of microsomal β1,6-GlcNAc transferase and β-*N*-acetylglucosaminidase to catalyze the conversion of GlcNAc-β1– 3Galβ1–4GlcNAc to GlcNAcβ1–6Galβ1–4GlcNAc.

In a longer incubation of the tetrasaccharide **IX** (139 h hydrolysis, 77% of GlcNAcβGal bonds cleaved), the yield of the trisaccharide **VIII** was six times higher than that of the trisaccharide **VII** (not shown). Because in the experiment with 68 h incubation time (Fig. 2C) the yield of **VIII** was about two times higher than that of **VII**, it seemed that even at the later stages of the incubation, when the intermediary trisaccharides were further de-*N*-acetylglucosaminylated, the cleavage of GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc was faster than that of GlcNAcβ1–6[U-^{14}C]Galβ1–4GlcNAc.

3.5. *Combined use of the β1,6-GlcNAc transferase and the β-N-acetylglucosaminidase activities in hog gastric mucosal microsomes for construction of GlcNAcβ1–6Galβ1–4GlcNAc from GlcNAcβ1–3-Galβ1–4GlcNAc*

Hog gastric mucosal microsomes are known to contain β1,6-GlcNAc transferase activity which has been used in several laboratories, including our own, for construction of branched saccharides containing the GlcNAcβ1–3(GlcNAcβ1–6)Gal-OR sequence [3,6]. It thus appeared possible to combine the transferase activity of the microsomes with the newly discovered glycosidase activity and use the microsomes as a catalyst in a two-step reaction leading from GlcNAcβ1–3Galβ1–4-GlcNAc to GlcNAcβ1–6Galβ1–4GlcNAc (see Fig. 3).

To test this possibility, the trisaccharide GlcNAcβ1–3-[U-^{14}C]Galβ1–4GlcNAc (**VII**) (2480 dpm, 7.2 pmol) was incubated with the mucosal microsomes for 73 h; in addition to the buffer, NaN_3 and EDTA, also 4.5 μmol UDP-GlcNAc, 0.2 μmol ATP and 3 μmol GlcNAc were present in the reaction mixture. The incubation mixture was worked up as above, yielding 517 dpm (1.5 pmol) of the trisaccharide GlcNAcβ1–6-[U- ^{14}C]Galβ1–4GlcNAc (**VIII**) in addition to 1006 dpm (2.9 pmol) of the tetrasaccharide **IX** and 70 dpm (0.2 pmol) of the trisaccharide **VII**.

4. DISCUSSION

In the present report we show that hog gastric mucosal microsomes contain β-*N*-acetylglucosaminidase activity that reveals a novel kind of interesting and useful substrate specificity. The branched tetrasaccharide GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (**IX**) was cleaved with high preference for the GlcNAcβ1–3Gal linkage, while the closely related branched trisaccharide GlcNAcβ1–3(GlcNAcβ1–6)Gal (**III**) was hydrolyzed with a high preference for the GlcNAcβ1–6Gal linkage. These data form an example of small oligosaccharides revealing surprisingly different behaviour despite closely similar overall structures. The structural differ-

ences between saccharides **III** and **IX** locate four and five bonds away from the linkages to be cleaved.

β-N-acetylhexosaminidase from *Diplococcus pneumoniae* has been shown to cleave GlcNAcβ1–Man isomers with high site specificity [7], but to our knowledge, no *β-N*-acetylglucosaminidase has been previously described to prefer GlcNAcβ1–3Gal linkages rather than GlcNAcβ1–6Gal linkages in any biantennary glycan.

The observed difference between the tetrasaccharide **IX** and the trisaccharide **III** could be due to particularly slow cleavage rate of the (1–6) linkage, or to particularly fast hydrolysis of the (1–3) linkage in the tetrasaccharide **IX**. Our data suggest that the cleavage of GlcNAcβ1–3Galβ1–4GlcNAc was faster than the hydrolysis of GlcNAcβ1–6Galβ1–4GlcNAc. Also, the hydrolysis rate of **IX** seemed slower as compared to **III** (see Fig. 1). These data suggest that the essential difference between the tetrasaccharide **IX** and the trisaccharide **III** is the slow hydrolysis of the (1–6) linkage in **IX**.

The sluggish reactivity of the β1,6-GlcNAc linkage in the tetrasaccharide **IX** and in the trisaccharide GlcNAcβ1–6Galβ1–4GlcNAc (**VIII**) suggests that the rotational freedom of the (1–6)-linked GlcNAc residue may be reduced in these oligosaccharides: We postulate that the (1–6)-linked GlcNAc unit may interact with the reducing end GlcNAc unit. The *N*-acetyl group of the reducing end GlcNAc appears to be involved in this interaction, which is more prominent in the tetrasaccharide **IX** than in the glucose analog **VI**. It will be interesting to try to see whether NMR data supporting this notion can be obtained. Several *N*-glycosidic type saccharides have in solution a conformation in which the α1,6-Man branch is folded back to the core [8].

The reducing end GlcNAc appears to curtail the relative reactivity of the (1–6)-linked branch of big I-type saccharides in several other enzymic reactions in a way analogous to the present one, but the effects previously observed were less striking (reviewed in [1]).

The present observations are also of practical use, because they provide an improved enzymic way of constructing linear saccharide sequences containing GlcNAcβ1–6Gal units: GlcNAcβ1–3Galβ1–4GlcNAc was converted with the gastric microsomes in a 'one pot reaction' into GlcNAcβ1–6Galβ1–4GlcNAc with a yield of 20% (see Fig. 3). The yield can certainly be improved, because the major product of the incubation was the branched tetrasaccharide **IX**.

Finally, the present data suggest that some of the linear sequences containing GlcNAcβ1–6Gal units found in naturally occurring structures [9–11] may have been derived by a combination of branching and selective de-*N*-acetylglucosaminylation events.

Acknowledgements: This work was supported in part by grants from the University of Helsinki and from the Academy of Finland to O.R., from the Orion Corporation Research Foundation to J.H., and from the Jenny and Antti Wihuri Foundation to A.S.

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