

Structure of the Carbohydrate Units of IgE Immunoglobulin

II. SEQUENCE OF THE SIALIC ACID-CONTAINING GLYCOPEPTIDES*

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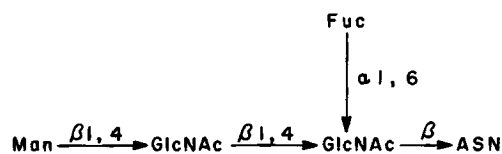
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SUMMARY

In the preceding paper (BAENZIGER, J., KORNFELD, S., AND KOCHWA, S. (1974) *J. Biol. Chem.* 249, 1889) it was shown that an IgE myeloma protein has 4 oligosaccharide units per heavy chain, 1 of which contains only mannose and *N*-acetylglucosamine while the other 3 contain sialic acid, fucose, galactose, mannose, and *N*-acetylglucosamine in the ratio of 1 to 2:1:2:3:4. The data in this paper establish the structure of the three sialic acid-containing glycopeptides, designated B-1, B-2, and B-3. Glycopeptides B-2 and B-3 are branched structures containing two nonreducing termini with the sequence sialic acid $\xrightarrow{\alpha 2,6}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,2}$ Man $\xrightarrow{\alpha}$, while B-1 has one nonreducing terminus with the above structure and another with the sequence Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,2}$ Man $\xrightarrow{\alpha}$. The core of all three B-glycopeptides is identical and has the sequence:



The branch of B-1 terminating in sialic acid originates from position 3 of the β -linked mannose in the core while the branch terminating in galactose originates from position 6 of this mannose.

In the preceding paper we demonstrated that an IgE myeloma protein contains 4 oligosaccharide units per heavy chain, 1 of which contained only mannose and *N*-acetylglucosamine while the other 3 contained sialic acid, galactose, fucose, mannose, and

N-acetylglucosamine (1). The complete sequence of the high mannose oligosaccharide chain was established. We now present the structural studies leading to the complete sequencing of the sialic acid-containing glycopeptides B-1, B-2, and B-3 isolated from this IgE myeloma protein. These oligosaccharide units of IgE appear to be very closely related to the sialic acid-containing glycopeptides of IgG (2), IgM (3), and IgA¹ (4).

METHODS

The methods used in characterizing the sialic acid-containing glycopeptides of IgE have been presented in the accompanying paper (1) with the following exceptions and additions.

Cleavage of Glucosamine-Asparagine Linkage—The glucosamine-asparagine bonds of glycopeptides B-1, B-2, and B-3 were cleaved by treatment with 1 M NaOH-1 M NaBH₄ for 4 to 6 hours at 100° (5). The resultant oligosaccharide with glucosaminitol at the reducing terminus was desalted by passage over Bio-Gel P-2 (18 × 0.9 cm) in water. The reduced oligosaccharide was treated with acetic anhydride in dilute sodium bicarbonate to insure complete *N*-acetylation of the glucosamine residues (6). The oligosaccharide was then split into two portions, one of which was hydrolyzed directly in 4 N HCl for 4 hours at 100° while the other was subjected to periodate oxidation prior to the acid hydrolysis (1).

Preparation of 4 N Aqueous HCl Hydrolysates for Gas-Liquid Chromatographic Analysis—Unlike the high mannose glycopeptide, the sialic acid-containing glycopeptides had asparagine as their single amino acid residue. This appeared to result in resistance of the *N*-acetylglucosamine-asparagine bond to hydrolysis with methanolic HCl. It was found that hydrolysis in 4 N HCl for 4 hours at 100° in evacuated tubes gave reproducible values for both neutral and amino sugars which were in good agreement with the values obtained for neutral sugars using methanolysis in 1.5 N methanolic HCl. The 4 N HCl hydrolysates were prepared for gas-liquid chromatographic analysis by lyophilization, acetylation of the amino sugars in 0.2 ml of pyridine-acetic anhydride (1:1 v/v), and conversion to the methylglycosides by incubation in 0.5 N methanolic HCl for 1 hour at 65° before trimethylsilylation.

When it was necessary to determine glucosaminitol as well as

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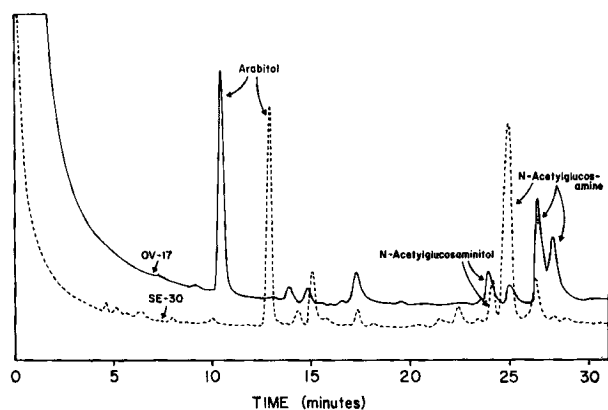


FIG. 1. Gas-liquid chromatographic separation of *N*-acetylglucosamine and *N*-acetylglucosaminitol. The trimethylsilyl derivatives were prepared and separated on columns of OV-17 and SE-30. The details of the procedure are described under "Methods" and in Ref. 1. The scan shown was that obtained for glycopeptide B-2 following treatment with 1 M NaOH, 1 M NaBH₄, 100°, for 4 hours (Table V, Experiment 4a).

glucosamine, the neutral and amino sugars were separated by passage of the dried hydrolysates over Dowex AG 50 (H⁺) in water followed by 1 N HCl to elute the deacetylated amino sugars and amino sugar alcohols. The fraction which eluted with H₂O (containing the neutral sugars and the internal standard) was worked up as above. The fraction eluted with 1 N HCl was taken to dryness after addition of arabitol as the internal standard. The glucosaminitol and glucosamine were re-*N*-acetylated in dilutesodium bicarbonate with acetic anhydride (6), and sodium was removed by passage over Dowex AG 50 (H⁺) in water. The *N*-acetylated amino sugars and amino sugar alcohols were then directly trimethylsilylated for gas-liquid chromatographic analysis. This procedure was necessary because the pyridine-acetic anhydride method of acetylation did not result in *N*-acetylation of the alcohol form of glucosamine or galactosamine. Direct trimethylsilylation of the nonacetylated glucosaminitol resulted in a derivative which emerged in the same location as glucose on both OV-17 and SE-30. This was undesirable since small amounts of glucose were often present in these samples. Direct trimethylsilylation of *N*-acetylated glucosamine and glucosaminitol resulted in derivatives which separated from each other and from glucose, allowing absolute identification because of their differing mobilities on OV-17 and SE-30 (Fig. 1).

RESULTS

Total Composition—The total compositions and recoveries of glycopeptides B-1, B-2, and B-3 have been presented in the previous paper (Table III, Ref. 1). The three B-glycopeptides differ only in their content of sialic acid and amino acids. In addition to 1 or 2 sialic acid residues, each has fucose, galactose, mannose, and *N*-acetylglucosamine in the molar proportions of 1:2:3:4, respectively. Although B-1 and B-3 have less than a full residue of asparagine, no *N*-acetylglucosaminitol was produced when these glycopeptides were treated with NaBH₄ under mild conditions (Table V, Experiment 2). This would imply that all of the *N*-acetylglucosamine residues at the reducing termini are indeed linked to an asparagine residue and the deficiency of asparagine in the amino acid determination has some other explanation.

Molecular Weight Estimation—Estimation of the molecular weights of glycopeptides B-2 and B-3 by exclusion chromatogra-

TABLE I
Relative proportions of methylated sugars in glycopeptides B-1, B-2, and B-3

Glycopeptide	Fucose	Galactose		Mannose		<i>N</i> -Methyl- <i>N</i> -acetylglucosamine	
	2, 3, 4-Trimethyl	2, 3, 4, 6-Tetra-methyl	2, 3, 4-Tri-methyl	3, 4, 6-Tri-methyl	2, 4-Di-methyl	3, 6-Dimethyl	3-Mono-methyl
B-1 ^a	0.9	1.1	1.1	1.8	1.2	N.D. ^b	N.D.
B-2 ^a	1.0	0	1.5	1.9	1.1	N.D.	N.D.
	0.5	0	2.1	2.0	1.0	2.7	0.4
B-3 ^a	0.8	0	1.7	1.9	1.1	N.D.	N.D.

^a Determined by setting total area for mannose = 3.00.

^b N.D., not determined.

phy was presented in Fig. 3 and Table IV of the previous paper (1). The calculated molecular weight of 2440 and the estimated weights of 2460 and 2340 for B-2 and B-3, respectively, are in excellent agreement.

Methylation—The results of permethylation of the B-glycopeptides are presented in Table I. All three glycopeptides have 3,4,6-trimethylmannose and 2,4-dimethylmannose in the approximate ratio of 2:1, respectively, as well as close to a full residue of terminal 3,4,6-trimethylfucose. As expected glycopeptide B-1, which has only 1 residue of sialic acid, differs from B-2 and B-3 in that it has both terminal 2,3,4,6-tetramethylgalactose and 2,3,4-trimethylgalactose in the ratio of 1:1 while B-2 and B-3 have only 2,3,4-trimethylgalactose (approximately 2 residues per oligosaccharide unit). In the case of B-2, which was also analyzed for permethylated amino sugars, the only two species of *N*-methyl-*N*-acetylglucosamine which were found were the 3,6-dimethyl and the 3-monomethyl derivatives. The low yield of the 3-monomethylglucosamine may reflect a resistance of the glucosamine-asparagine bond to the hydrolysis conditions used.

The results of periodate oxidation of the intact glycopeptides (Table V, Experiment 3) are in good agreement with the methylation data since the fucose residue, both galactose residues, and 2 of the 3 mannose residues are destroyed while all of the *N*-acetylglucosamine residues survive periodate oxidation. The terminal sialic acid residues are also destroyed by this procedure.

In order for the reader to better follow the subsequent experiments an outline of the scheme used for determining the structures of the B-type glycopeptides is presented in Fig. 2. Also shown in the figure is the proposed structure of the B-glycopeptides.

Sequence of Outer Branches of B-Glycopeptides—The results of serial enzymatic degradations followed by periodate oxidation of the reisolated products at various stages of degradation are presented in Tables II, III, and IV for glycopeptides B-1, B-2, and B-3, respectively. Sequential enzymatic degradations of glycopeptide B-1 (Table II) indicate the presence of a branched structure with two nonreducing termini, one of which is terminated by sialic acid and has the sequence: sialic acid $\xrightarrow{\alpha}$ galactose $\xrightarrow{\beta}$ *N*-acetylglucosamine $\xrightarrow{\beta}$ mannose $\xrightarrow{\alpha}$ X and the other of which is terminated by galactose and has the sequence: galactose $\xrightarrow{\beta}$ *N*-acetylglucosamine $\xrightarrow{\beta}$ mannose $\xrightarrow{\alpha}$ X. Essentially identical results were obtained for glycopeptides B-2 and B-3 (Tables III and IV) except for the fact that both nonreducing termini have sialic acid as the terminal residue. For all three glyco-

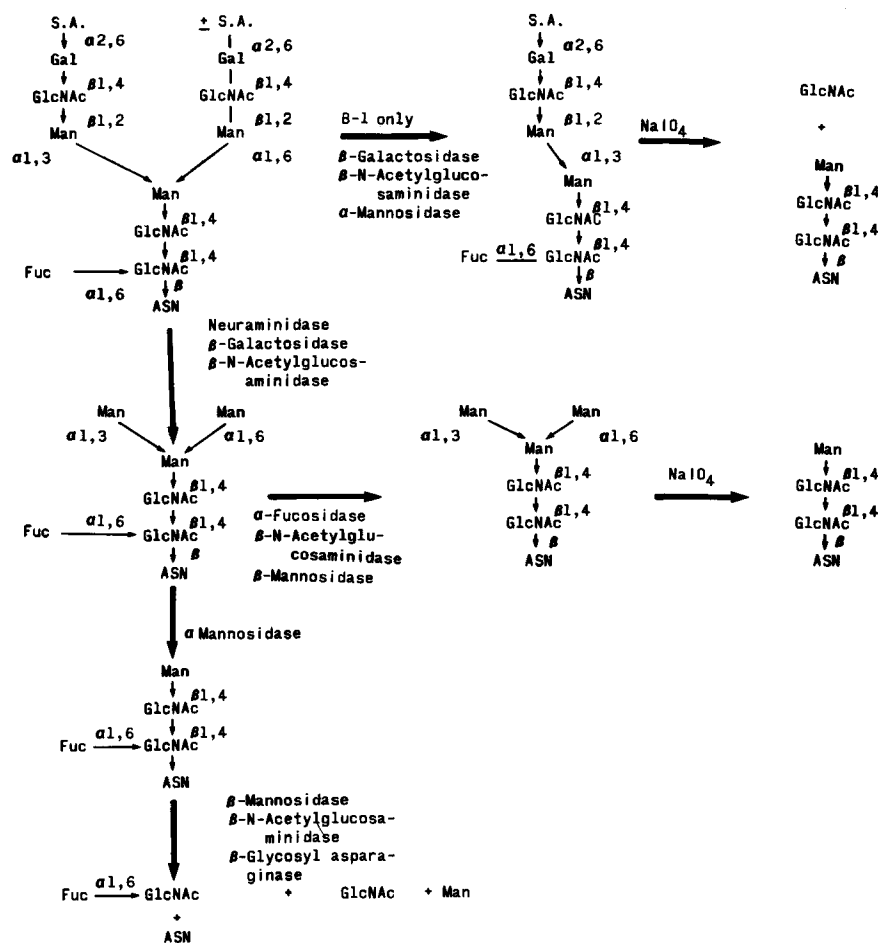
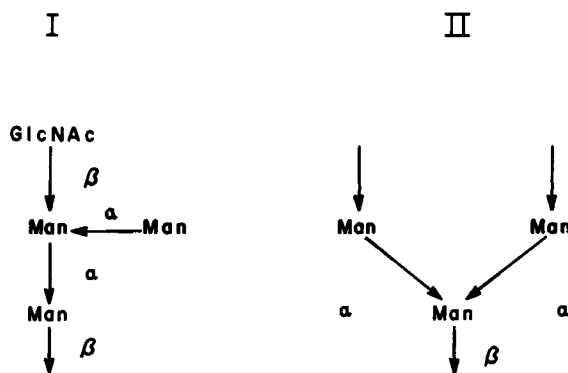


FIG. 2. Scheme for characterization of B-glycopeptides of IgE.

peptides removal of both branches enzymatically resulted in a core composed of mannose, *N*-acetylglucosamine, and fucose in the ratio 1:2:1. Most of the remaining mannose residue was released by β -mannosidase from either bromelain or rat epididymides (Table II, Experiments 2, and 5; Table III, Experiment 5; Table IV, Experiments 1 and 5).

Nature of Branch Point—On the basis of the methylation data which revealed the presence of a 2,4-dimethylmannose derivative, it appeared that a disubstituted mannose acted as the branch point. However, two possible structures could exist:

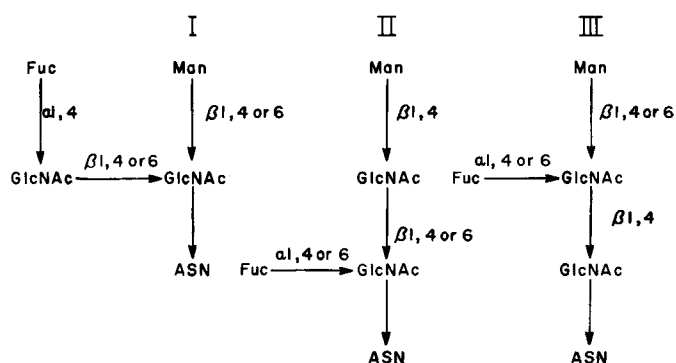


Possibility I is ruled out by the following experiment performed on all three glycopeptides. The glycopeptides were subjected to periodate oxidation followed by NaBH_4 reduction and mild acid hydrolysis to release reduced fragments (8). As noted before, the periodate step destroys 2 of the 3 mannose residues (Table V).

The resultant cores were reisolated by passage over Bio-Gel P-2. Analysis of the cores revealed that they contained 1 mannose residue which could be released with β -mannosidase treatment. This shows that it is the β -linked mannose which is resistant to periodate oxidation and must therefore serve as the branch point in all three B-glycopeptides.

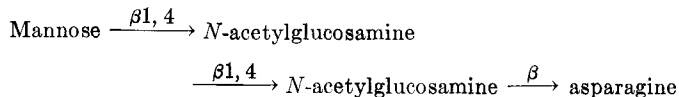
Since glycopeptide B-1 has only a single sialic acid it was possible to investigate the question of whether the sialic acid is distributed randomly on the two branches arising from positions 3 and 6 of the β -linked mannose or if the sialic acid occurs exclusively on one of the two termini. Glycopeptide B-1 was treated with β -galactosidase, β -N-acetylglucosaminidase, α -mannosidase, and α -fucosidase (Table II, Experiment 7) to produce a product which contained sialic acid, galactose, and mannose in the ratio of 1:1:2, respectively (the *N*-acetylglucosamine value was not as high as expected due to the hydrolysis conditions as discussed under "Methods"). When this reisolated material (see Fig. 2) was treated with periodate, the sialic acid and galactose residues were destroyed, indicating that the sialic acid was exclusively present on the terminus arising from position 3 of the branched mannose residue in the core.

Structure of Core—All three B-type glycopeptides appeared to have a core composed of 1 β -linked mannose, 1 terminal α -linked fucose, and 2 *N*-acetylglucosamine residues. The amino sugar methylation data (Table I, B-2) indicated that 1 of the 2 *N*-acetylglucosamine residues was substituted at position 4 only and the other at both positions 4 and 6. Therefore, three general core structures were possible.



In order to distinguish among these structures the following experiments were performed. An enzymatically produced glycopeptide (Tables II, III, IV, Experiment 6) containing only mannose and *N*-acetylglucosamine in the ratio of 3:2 (all of the fucose, galactose, and outer *N*-acetylglucosamine residues having been removed) was oxidized with sodium metaperiodate. Both of the *N*-acetylglucosamine residues survived the periodate oxidation, while 2 of the 3 mannose residues were destroyed, thus ruling out Possibility I since removal of the fucose residue would have exposed a periodate-sensitive *N*-acetylglucosamine. In addition,

this experiment shows that the fucose must be linked to position 6 of either the inner or outer *N*-acetylglucosamine. Therefore, the core, exclusive of the fucose, must have the sequence:



When a glycosidase-produced core consisting of mannose, *N*-acetylglucosamine, and fucose (ratio 1:2:1, respectively) was treated with a rat epididymal enzyme preparation containing β -mannosidase, β -*N*-acetylglucosaminidase, and β -aspartyl-*N*-acetylglucosamine amidohydrolase but not α -fucosidase (Tables II, III, and IV, Experiment 5), 0.5 residue of mannose and a full residue of *N*-acetylglucosamine were released. This result is most consistent with the fucose residue being linked 1,6 to the inner *N*-acetylglucosamine. Thus, β -*N*-acetylglucosaminidase would release approximately half of the outer *N*-acetylglucosamine residue following removal of the mannose by the β -mannosidase while removal of the asparagine by the β -aspartyl-*N*-acetylglucosamine amidohydrolase would result in the formation of the disaccharide fucose $\xrightarrow{\alpha 1,6}$ *N*-acetylglucosamine (equal to 0.5 residue). This disaccharide would account for the additional

TABLE II
Effect of glycosidases on glycopeptide B-1

The released galactose and mannose were assayed enzymatically (2), and the released sialic acid, GlcNAc, and fucose were assayed colorimetrically (2). The composition of the reisolated cores was analyzed by gas-liquid chromatography.

Experiment	Residues released					Experiment	Residues released				
	Sialic acid	Gal	Man	GlcNAc	Fuc		Sialic acid	Gal	Man	GlcNAc	Fuc
B-1 intact ^a	1.1	2.0	3.0	4.2	1.0	dohydrolase (rat epididymal Preparation I).....			0.5	1.0	0 ^c
1. β -Galactosidase (J. B.) ^b		1.0		0		6. Neuraminidase.....	1.0				
Then α -mannosidase.....			0			Then β -galactosidase (<i>Escherichia coli</i>).....		1.6			
Then β - <i>N</i> -acetylglucosaminidase (J. B.).....				0.6		Then β -galactosidase + β - <i>N</i> -acetylglucosaminidase (rat epididymal Preparation I).....		0.5	0	1.5	
2. β - <i>N</i> -Acetylglucosaminidase.....				0		Then α -fucosidase + β -galactosidase + β - <i>N</i> -acetylglucosaminidase (rat epididymal Preparation II).....			0	0.4	0.5
Then neuraminidase.....	1.0			0		Repeat above.....				0.2	0.7
Then β - <i>N</i> -acetylglucosaminidase.....				0		Reisolated glycopeptide ^a	0	0	3.4	2.1	0
Then β -galactosidase + β - <i>N</i> -acetylglucosaminidase (J. B.).....		1.6		1.6		Reisolated glycopeptide after NaIO ₄ ^a	0	0	1.4	2.0	0
Then α -mannosidase + β - <i>N</i> -acetylglucosaminidase.....			1.5	0		7. β -Galactosidase (<i>E. coli</i>).....		0.9			
Then β -mannosidase (bromelain).....			0.4			Then β -galactosidase + β - <i>N</i> -acetylglucosaminidase + α -fucosidase + β -mannosidase (rat epididymal Preparations I + II) + α -mannosidase.....		0.1	0.8	0.9	0.6
3. α -Mannosidase.....			0			Repeat above.....		0	0.1	0.1	0.2
Then neuraminidase.....	1.0					Reisolated glycopeptide ^a	1.0	1.0	2.0	2.0 ^d	0
Then α -mannosidase.....			0			Reisolated glycopeptide after NaIO ₄	0	0	1.1	1.6 ^d	0
4. Neuraminidase.....	0.9										
Then α -galactosidase (ficin).....		0									
5. Neuraminidase.....	1.1										
Then β -galactosidase + β - <i>N</i> -acetylglucosaminidase + α -mannosidase (J. B.).....		2.1	1.6	1.3							
Repeat above.....		0.1	0.5	0.5							
Then β -mannosidase + β - <i>N</i> -acetylglucosaminidase + β -aspartyl- <i>N</i> -acetylglucosamine ami-											

^a In this case the number of residues represents total composition.

^b J. B., Jack Bean.

^c No free fucose was found after the digest was desalted on Amberlite MB-3 and subjected to descending paper chromatography in 1-butanol-ethanol-water (10:1:2). The sample and guide strips containing mannose and fucose in amounts equal to that in the digest were developed using the silver nitrate sugar dip method (7).

^d This value is low (2 residues instead of the expected 3) because of incomplete hydrolysis in methanolic HCl (see "Methods").

TABLE III

Effect of glycosidases on glycopeptide B-2

Experiment	Residues released				
	Sialic acid	Gal	Man	GlcNAc	Fuc
B-2 Intact ^a	2.0	2.1	3.0	4.0	1.0
1. β -Galactosidase (J. B.) ^b	<0.2				
Then neuraminidase.....	1.6				
Then β -galactosidase (J. B.).....		1.6		0.6	
Then β -galactosidase + β -N-acetylglucosaminidase (J. B.).....		0.4		1.5	
2. β -N-Acetylglucosaminidase (J. B.).....				0	
Then neuraminidase.....	2.0				
Then β -N-acetylglucosaminidase.....				0	
Then β -galactosidase (J. B.).....		1.9		0.3	
Then β -N-acetylglucosaminidase.....				2.0	
Then α -mannosidase + β -N-acetylglucosaminidase.....			1.9	0	
3. α -Mannosidase.....			0		
Then neuraminidase.....	2.0				
Then α -mannosidase.....			0		
Then α + β -galactosidase + β -N-acetylglucosaminidase (ficin).....		2.0		1.6	
Then β -N-acetylglucosaminidase + α -mannosidase.....			2.1	0.4	
4. Neuraminidase.....	1.9				
Then α -galactosidase (ficin) β -N-acetylglucosaminidase (ficin).....		0		0	
5. Neuraminidase.....	1.9				
Then β -galactosidase (J. B.) + β -N-acetylglucosaminidase + α -mannosidase.....		2.0	1.6	1.2	
Repeat above.....		0.1	0.1	0.2	
Then β -mannosidase + β -N-acetylglucosaminidase + β -aspartyl-N-acetylglucosamine amidohydrolase ((rat epididymal Preparation I).....			0.5	0.9	0 ^c
6. Neuraminidase.....	1.9				
Then β -galactosidase + β -N-acetylglucosaminidase + β -mannosidase (rat epididymal Preparation I).....		2.1	0	1.3	0
Repeat above.....		<0.1		0.8	<0.3
Then α -fucosidase + β -N-acetylglucosaminidase + β -mannosidase (rat epididymal Preparation II).....			0	<0.1	0.6
Repeat above.....				<0.1	0.6
Reisolated glycopeptide ^a	0	0	2.8	1.9	0
Reisolated glycopeptide after NaIO ₄ ^a	0	0	1.0	2.0	0

^a In this case the number of residues represents total composition.

^b J. B. = Jack Bean

^c See Table II.

TABLE IV

Effect of glycosidases on glycopeptide B-3

Experiment	Residues released				
	Sialic acid	Gal	Man	GlcNAc	Fuc
B-3 intact ^b	2.0	2.0	3.0	3.9	1.0
1. β -Galactosidase (J. B.) ^b	<0.2				
Then neuraminidase.....	1.6				
Then β -galactosidase (J. B.).....		1.5		0.5	
Then β -galactosidase + β -N-acetylglucosaminidase (J. B.).....		0.5		1.0	
Repeat above.....	<0.2			0.8	
Then α -mannosidase.....			1.9		
Then β -mannosidase (bromelain).....			0.7		
2. β -N-Acetylglucosaminidase.....				0	
Then neuraminidase.....	2.3				
Then β -N-acetylglucosaminidase.....				0	
Then β -galactosidase (J. B.).....		1.5		0.2	
Then α -mannosidase.....			0.3		
3. α -Mannosidase.....			0		
Then neuraminidase.....	2.0				
Then α -mannosidase.....			0		
4. Neuraminidase.....	1.9				
Then α -galactosidase (ficin) + β -N-acetylglucosaminidase (ficin).....		0		0	
5. Neuraminidase.....	1.9				
Then β -galactosidase (J. B.) + β -N-acetylglucosaminidase + α -mannosidase.....		1.9	2.0	1.4	
Repeat above.....		0.1	0.1	0.4	
Then β -mannosidase + β -N-acetylglucosaminidase + β -aspartyl-N-acetylglucosamine amidohydrolase (rat epididymal Preparation I).....			0.3	0.6	0 ^c
6. Neuraminidase.....	1.8				
Then β -galactosidase + β -N-acetylglucosaminidase + β -mannosidase (rat epididymal Preparation I).....		1.9	0	1.3	
Repeat above.....		0.2		0.5	
Then α -fucosidase + β -N-acetylglucosaminidase + β -mannosidase (rat epididymal Preparation II).....				<0.1	0.4
Repeat above.....				0.2	0.6
Reisolated glycopeptide ^a	0	0	3.0	2.0	0
Reisolated glycopeptide after NaIO ₄ ^a	0	0	1.2	1.7	0

^a In this case the number of residues represents total composition.

^b Jack Bean.

^c See Table II.

half-residue of *N*-acetylglucosamine detected with the Morgan-Elson amino sugar color test since in this assay *N*-acetylglucosamine substituted at position 6 gives a color yield of 100% as compared to free *N*-acetylglucosamine (9). The failure to detect free fucose in this experiment supports this explanation. The formation of any Man $\beta(1 \rightarrow 4)$ GlcNAc $\beta(1 \rightarrow 4)$ [fuc $\alpha(1 \rightarrow 6)] \rightarrow$ GlcNAc would not be detected since amino sugars substituted

Effect of NaOH, NaBH₄, and NaIO₄ on B-glycopeptides

been reported that pronase digestion can result in the cleavage of the *N*-acetylglucosamine-asparagine bond leading to the exposure of the reducing function of the *N*-acetylglucosamine residue (11). To test this possibility the glycopeptides were treated with NaBH₄ to convert any *N*-acetylglucosamine with a free reducing group to its alditol derivative. No *N*-acetylglucosaminitol could be detected following this procedure, implying that in fact glycopeptides B-1 and B-3 probably have a full residue of asparagine and that destruction or incomplete hydrolysis under the conditions used may be the reason for finding fractional residues of asparagine.

It is of considerable interest that the single sialic acid residue found in glycopeptide B-1 is not randomly distributed but is instead located exclusively on the branch originating from position 3 of the core mannose residue. This indicates that the sialyltransferase can distinguish the two branches of the oligosaccharide chain from each other and, if B-1 is a result of incompleteness of B-2 and B-3, there is an ordered addition of the sialic acid residues to these glycopeptides. On the other hand, the fully sialylated B-2 and B-3 glycopeptides may originate from different locations on the heavy chain than the monosialylated B-1 glycopeptide, implying that the activity of the sialyltransferases is affected both by the structure of the oligosaccharide chains in the receptor molecule and by the local environment of the polypeptide chain where the oligosaccharide unit is located.

Similarly, the different core structures of the high mannose glycopeptide and the B-glycopeptides indicate that very early in the process of elongation of the oligosaccharide chains, namely

after the 1st glucosamine residue has been attached, the glycosyltransferases must display exquisite specificity in their ability to recognize the "right" acceptor substrate. In the case of the high mannose glycopeptide a mannose residue must attach, and in the case of the B-glycopeptides either an *N*-acetylglucosamine or fucose residue must attach. Therefore, these enzymes must recognize some aspect of the polypeptide chain in addition to the GlcNAc $\xrightarrow{\beta}$ Asn acceptor. Knowledge as to whether any of the other enzymes involved in synthesizing these molecules must also recognize aspects of the polypeptide chain will have to await direct studies of the involved biosynthetic enzymes.

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