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SIALIC ACID-CONTAINING SUGAR CHAINS OF HEN OVALBUMIN AND OVOMUCOID*†

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ABSTRACT

The acidic oligosaccharide fractions released from hen ovalbumin and ovomucoid by hydrazinolysis contain both sialyloligosaccharides and sulfated oligosaccharides. The sialyloligosaccharides were converted into neutral oligosaccharides by sialidase digestion, and separated from sulfated oligosaccharides by paper electrophoresis. Structural studies of these neutral oligosaccharides showed that the oligosaccharides of ovalbumin have different structural characteristics from those of ovomucoid, *i.e.*, all sialyloligosaccharides from ovomucoid contain a pentasaccharide, α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-Glc*p*NAc-(1→4)-Glc*p*NAc, as a common core, and the smallest oligomannosyl core of the sugar chains from ovalbumin is α-D-Man*p*-(1→3)-α-D-Man*p*-(1→6)-[α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-Glc*p*NAc-(1→4)-Glc*p*NAc. By methanolysis followed by *N*-acetylation, sialyl oligosaccharides, free from sulfated oligosaccharides, were recovered quantitatively from the acidic fractions of ovalbumin and ovomucoid. Methylation analysis of these sialyloligosaccharide mixtures, before and after sialidase digestion, showed that all sialic acid of both glycoproteins occurs as a α-Sia-(2→3)-D-Gal*p* group.

INTRODUCTION

Ovalbumin and ovomucoid are two major glycoproteins of hen egg-white. The carbohydrate chains of these glycoproteins are all asparagine-linked and no mucin-type sugar chains are included. The oligosaccharides released from the two glycoproteins by hydrazinolysis can be separated into a neutral and two acidic frac-

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tions by paper electrophoresis[1,2]. Structures of the neutral oligosaccharides of both glycoproteins were elucidated previously[2-7]. These studies indicated that hen ovomucoid contains several sugar chains that are different from those of hen ovalbumin, although both glycoproteins are formed in the hen oviduct.

We have recently found that the acidic oligosaccharide fraction obtained from ovalbumin contains sulfated oligosaccharides as well as sialyl oligosaccharides[1]. The structures of the sulfated oligosaccharides were elucidated by the study of the sialidase-resistant, acidic oligosaccharides[1]. Subsequently, we obtained the intact sialyl oligosaccharides, free from the sulfated oligosaccharides, by methanolysis followed by *N*-acetylation. The intact sialyl oligosaccharides of ovomucoid, free from sialidase-resistant oligosaccharides, were also obtained by the same procedure. We describe herein the details of the isolation and structural study of the sialyl oligosaccharides obtained from hen ovalbumin and ovomucoid.

EXPERIMENTAL

Isolation of acidic oligosaccharide fractions from hen ovalbumin and hen ovomucoid. — The acidic oligosaccharide fraction of ovalbumin was obtained by hydrazinolysis of GP-F, isolated by Dowex 50 (H^+) column chromatography of ovalbumin glycopeptides as reported previously[4]. The major acidic fraction (A-1), which comprised >90% in molar ratio of total acidic oligosaccharides (Fig. 1a), was recovered from paper electrophoretogram by elution with water and used for the structural study described herein.

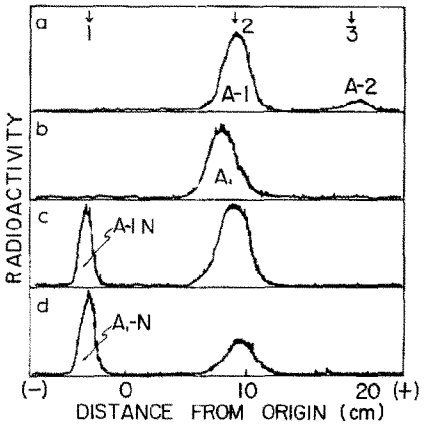
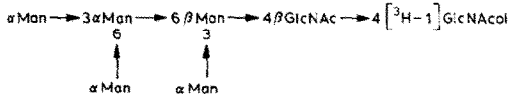


Fig. 1. Paper electrophoresis of oligosaccharides liberated from ovalbumin and ovomucoid and their sialidase digests. (a) Radioactive oligosaccharides liberated from ovalbumin GP-F by hydrazinolysis, followed by sodium borotritide reduction; (b) A_1 fraction obtained by paper electrophoresis of the radioactive oligosaccharides liberated from ovomucoid by hydrazinolysis, as reported previously[2]; (c) Fraction A-1 in (a) incubated with *Arthrobacter* sialidase (100 munits/50 μL for 18 h at 37°); (d) Fraction A_1 incubated with sialidase as in (c). Arrows at the top indicate the migrating positions of standard oligosaccharides: (1) lactitol; (2) compound **6**; and (3) compound **4**. Paper electrophoresis was performed in pyridine–acetate buffer, pH 5.4, at a potential of 80 V/cm for 1.5 h.



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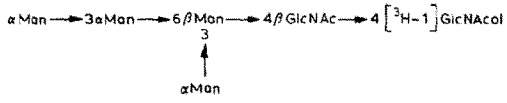

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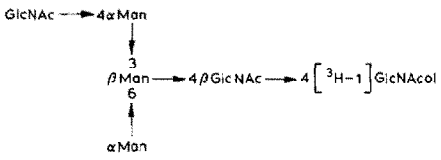

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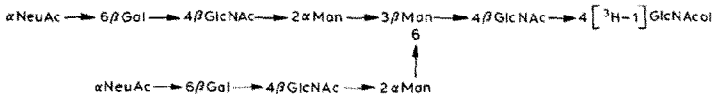

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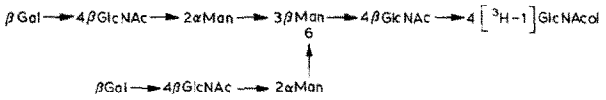

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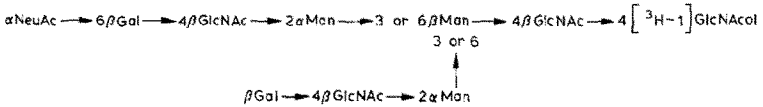

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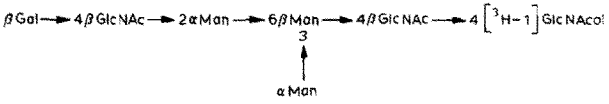

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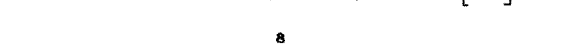

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* In this and in the following schemes, the D configurations, pyranose form, and glycosyl linkage at C-1 (C-2 for N-acetylneuraminic acid) are assumed.

The acidic oligosaccharide fraction of ovomucoid was obtained by paper electrophoresis of the oligosaccharide fraction, liberated from ovomucoid by hydrazinolysis, as reported previously[2]. The major acidic fraction (A_1), which also comprised >90% of the total acidic oligosaccharides (Fig. 1b), was recovered and used for the structural study described herein.

Oligosaccharides. — Reduced oligosaccharides **1** and **2** were obtained[4] from ovalbumin GP-V and GP-VI by hydrazinolysis[8] followed by sodium borotritide reduction; **3** was obtained as reported previously[9]; **4**, **5** and **6** were obtained from human ceruloplasmin[10], and **7** and **8** from human prothrombin[11].

Methanolysis. — Radioactive-labeled oligosaccharides were dissolved in dry methanol containing 50mM hydrogen chloride and kept for 4 h at room temperature. The solution was evaporated, and the residue was freed from hydrogen chloride by three evaporations with methanol. Since the reaction products were partially *N*-deacetylated, they were dissolved in a solution of sodium hydrogencarbonate and *N*-acetylated with acetic anhydride before paper electrophoretic analysis.

All other experimental procedures and materials were the same as described previously[7,12].

RESULTS

Isolation and fractionation of the neutral portions of sialic acid-containing oligosaccharides obtained from ovalbumin and ovomucoid. — By exhaustive sialidase treatment, ~30% of Fraction A-1 from ovalbumin and 70% of Fraction A_1 from ovomucoid were converted into neutral oligosaccharides (Figs. 1c and d, respectively). The sialidase-resistant acidic oligosaccharides from both samples were not converted into neutral oligosaccharide by the second, exhaustive sialidase digestion.

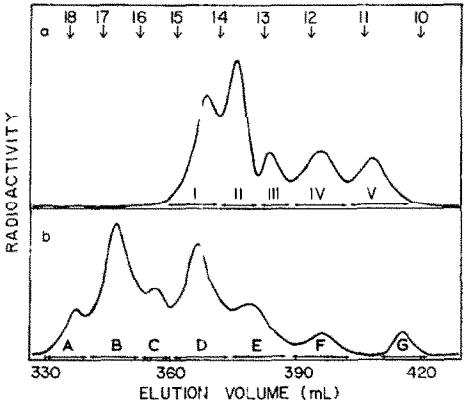


Fig. 2. Bio-Gel P-4 column chromatography of Fraction A-1N (a) and A_1N (b). The arrows at the top indicate the elution positions of D-glucose oligomers. The numbers indicate the number of D-glucose units.

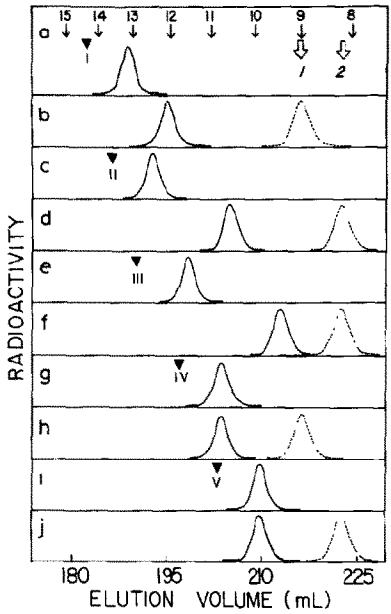
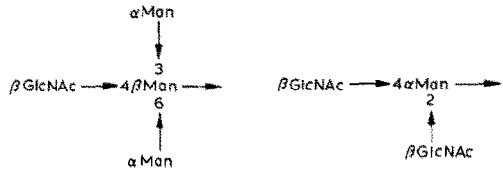


Fig. 3. Sequential exoglycosidase digestion of oligosaccharides **I–V**. The radioactive products were analyzed by Bio-Gel P-4 column chromatography. The black arrows are the same as in Fig. 2 and the white arrows (*1* and *2*) indicate the elution positions of authentic **1** and **2**, respectively. The black triangles indicate the elution positions of Oligosaccharides **I–V**. (a), (c), (e), (g), and (i): Oligosaccharides **I, II, III, IV,** and **V** incubated with diplococcal β-D-galactosidase (1.5 munits/40 μL), respectively. (b), (d), (f), (h), and (j): The radioactive peak in (a), (c), (e), (g), and (i) incubated either with diplococcal *N*-acetyl-β-D-hexosaminidase (10 munits/50 μL; solid line) or with jack bean *N*-acetyl-β-D-hexosaminidase (3.5 units/40 μL; dotted line), respectively. All enzymic digestions were performed for 18 h at 37°.

Bio-Gel P-4 column chromatography of the neutral oligosaccharide fractions obtained by sialidase digestion of the acidic oligosaccharides of ovalbumin and ovomucoid (Peak A-1N in Fig. 1c, and A_1N in Fig. 1d, respectively) gave quite distinct fractionation patterns. As shown in Fig. 2a, the sample from ovalbumin was separated into five peaks having elution positions of 14.3 (**I**), 13.6 (**II**), 12.9 (**III**), 11.8 (**IV**), and 10.9 (**V**) D-glucose units. On the other hand, the sample from ovomucoid was separated into seven peaks having elution positions of 17.6 (**A**), 16.5 (**B**), 15.5 (**C**), 14.5 (**D**), 13.4 (**E**), 11.7 (**F**), and 10.4 (**G**) glucose units (Fig. 2b).

Structural study of the neutral oligosaccharides obtained from the acidic oligosaccharide fraction of ovalbumin by sialidase treatment. — All five oligosaccharides (**I–V** in Fig. 2a) liberated a D-galactose residue by treatment with diplococcal β-D-galactosidase (Figs. 3a, c, e, f, and i, respectively). Subsequent treatment, by diplococcal *N*-acetyl-β-D-hexosaminidase, of the products from **I, II,** and **III**, released approximately one 2-acetamido-2-deoxy-D-glucose residue (solid lines

in Figs. 3b, d, and f, respectively)*. No 2-acetamido-2-deoxy-D-hexose was released from the products from **IV** and **V** by diplococcal β-*N*-acetylhexosaminidase digestion (solid lines in Figs. 3h and j). By jack-bean *N*-acetyl-β-D-hexosaminidase treatment, the degalactosylated **I** and **IV** were converted into an oligosaccharide having the same elution position as authentic **1** (dotted lines in Figs. 3b and h), and degalactosylated **II, III,** and **V** into an oligosaccharide having the same elution position as authentic **2** (dotted lines in Figs. 3d, f, and j). The structure of the product derived from **I** and **IV** was established by sequential digestion with jack bean α-D-mannosidase and β-D-mannosidase, methylation study, and periodate oxidation (data not shown) as **1**. By the same series of analyses, the structure of the product from **II, III,** and **V** was established as **2**. The results, so far described, indicated that Oligosaccharides I–V are a series of hybrid-type, sugar chains[4] having a β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc (*N*-acetyllactosaminyl) group** and various numbers of β-D-Glc*p*NAc residues linked to either a hepta- or hexa-saccharide core (**1** or **2**).



9 **10**

In the methylation analysis of Oligosaccharides I–V (see Table I), detection of 1 mole each of 2-*O*-methyl- and 3,6-di-*O*-methyl-mannitol for oligosaccharides **I** and **II** indicated the presence of group **9** and group **10** in these Oligosaccharides. Detection of ~1 mole each of 2-*O*-methyl- and 3,4,6-tri-*O*-methyl-mannitol for Oligosaccharide **III** indicated the presence of group **9** and a β-D-Glc*p*NAc-(1→2)-D-Man*p* group. As just described, one of the 2-acetamido-2-deoxy-D-glucopyranosyl residues of Oligosaccharides **I, II,** and **III** does occur as part of a β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc group. Detection of 2,3,6-tri-*O*-methylmannitol for Oligosaccharides **IV** and **V** indicated that the β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Man*p* group is included in these oligosaccharides.

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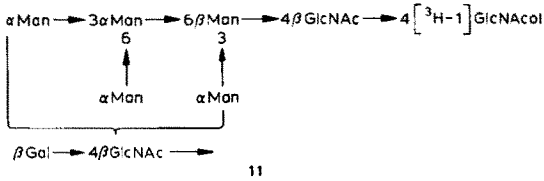
*As reported earlier[13], diplococcal β-*N*-acetylhexosaminidase cleaves the β-D-Glc*p*NAc-(1→2)-, but not the β-D-Glc*p*NAc-(1→4 or 6)-D-Man linkage. The β-D-Glc*p*NAc-(1→2)-D-Man linkage of the β-D-Glc*p*NAc-(1→4)-[β-D-Glc*p*NAc-(1→2)]-D-Man group is cleaved by the enzyme, but that of the β-D-Glc*p*NAc-(1→6)-[β-D-Glc*p*NAc-(1→2)]-D-Man group is not. The enzyme can remove only one 2-acetamido-2-deoxy-D-glucose residue from β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-[β-D-Glc*p*NAc-(1→4)]-[β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-D-[^{3}H-1]GlcNAc and convert it to β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)-[β-D-Glc*p*NAc-(1→4)]-[α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-D-[^{3}H-1]GlcNAc, indicating that the Glc*p*NAc-4 group cannot be removed by the enzyme, and that this group also sterically inhibits the enzyme action on the β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)-β-D-Man*p*-(1→ but not on the β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→ group.

**Diplococcal β-D-galactosidase hydrolyzes the β-D-Gal*p*-(1→4)-, but not the -(1→3)- and -(1→6)-D-Glc*p*NAc linkages[14].



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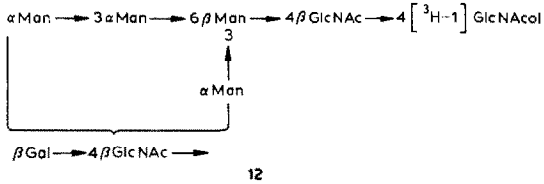

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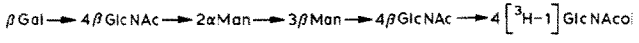

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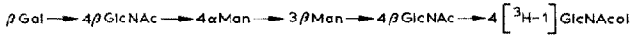

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In order to locate the D-mannopyranosyl residue to which the *N*-acetyllactosaminyl group is linked, the radioactive-labeled Peak A-1N (Fig. 1c) was incubated with a high concentration (3.5 units/40 μL) of jack-bean *N*-acetyl-β-D-hexosaminidase. All nonreducing 2-acetamido-2-deoxy-D-glucopyranosyl groups, including those linked to O-4 of the β-D-mannopyranosyl residue, are removed by this digestion*. When the incubation product was analyzed by Bio-Gel P-4 column chromatography, two radioactive-labeled peaks having elution positions of 11.8 and 10.8 glucose units were detected (Fig. 4a). Structures **11** and **12**, respectively, were attributed because the peaks were converted into a radioactive-labeled component (9.4 glucose units) by incubation with jack-bean α-D-mannosidase (Fig. 4b). The elution position of 9.4 glucose units is almost the same as that of authentic **8**, indicating that the *N*-acetyllactosaminyl groups of Oligosaccharides **I–V** are all linked to the α-D-mannopyranosyl group of the α-D-Man*p*-(1→3)-β-D-Man*p* branch**. That the radioactive-labeled component in Fig. 4b is a mixture of compounds **13** and **14** was confirmed by the following experiments. A D-galac-

*Although the 2-acetamido-2-deoxy-β-D-glucopyranosyl group linked to O-4 of the β-D-mannopyranosyl residue is resistant to jack-bean *N*-acetyl-β-D-hexosaminidase[15], it is removed when the enzyme concentration is raised[16] up to 3.5 units/40 μL.

**Jack-bean α-D-mannosidase cannot hydrolyze the α-(1→6)-linked D-mannosyl group from α-D-Man*p*-(1→6)-[R→α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-[^{3}H-1]GlcNAcol in which R represents sugar residues when the substrate concentration is <1 nmol/40 μL (ref. 17). However, the group is cleaved by the enzyme when the substrate concentration is raised up to 20 nmol/40 μL.

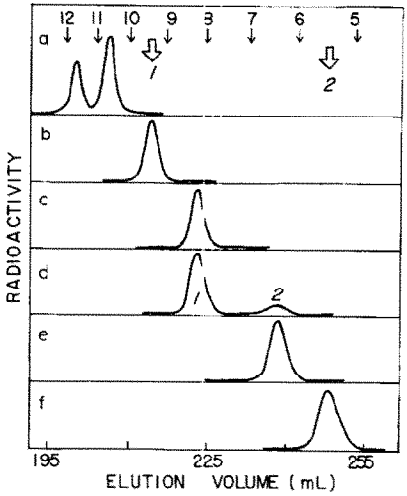
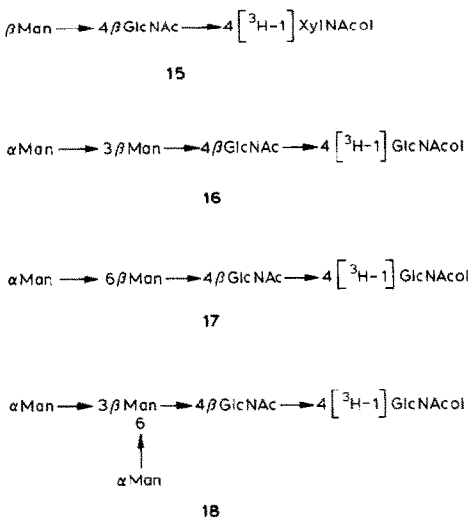


Fig. 4. Sequential exoglycosidase digestion and periodate oxidation of the radioactive-labelled components of A-1N. The radioactive products were analyzed by Bio-Gel P-4 column chromatography. The black arrows are the same as in Fig. 2, and the white arrows (*1* and *2*) indicate the elution positions of authentic **8** and **15**, respectively. (a) Component of A-1N (5.0×10^5 c.p.m., 25 nanomol) of Fig. 1c incubated with jack bean *N*-acetyl-β-D-hexosaminidase (3.5 units/40 μL); (b) the radioactive peaks in (a) incubated with jack-bean α-D-mannosidase (4.0 units/40 μL); (c) the radioactive peak in (b) incubated with diplococcal β-D-galactosidase (1.5 munits/40 μL); (d) the radioactive peak in (c) incubated with diplococcal *N*-acetyl-β-D-hexosaminidase (10 munits/50 μL); (e), the radioactive peak in (c) incubated with jack bean *N*-acetyl-β-D-hexosaminidase (0.5 units/40 μL); and (e) the radioactive peak in (e) subjected to periodate oxidation. All enzymic digestion were performed for 18 h at 37°.

topyranosyl group was removed from the compounds by diplococcal β-D-galactosidase digestion (Fig. 4c). Approximately 10% of the radioactive-labeled compounds of the peak in Fig. 4c released one 2-acetamido-2-deoxy-β-D-glucopyranosyl group by incubation with diplococcal *N*-acetyl-β-D-hexosaminidase (Fig. 4d). This result indicated that 10% of the oligosaccharides of peak A-1N illustrated in Fig. 1c contain a β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p* group. Since the ratio of 10% was the same as that of Oligosaccharide **III** to total oligosaccharides, the totality of the radioactive-labeled fragment having an elution position of 6.5 glucose units in Fig. 4d was produced from only Oligosaccharide **III**. Therefore, the *N*-acetyllactosaminyl group of Oligosaccharides **I, II, IV,** and **V** is linked at O-4 of the α-D-mannopyranosyl group of the α-D-Man*p*-(1→3)-β-D-Man*p* branch. That the *N*-acetyllactosaminyl groups of Oligosaccharides **I–V** are linked only to the α-D-mannopyranosyl residue of the α-D-Man*p*-(1→3)-β-D-Man*p* branch was further confirmed by the following experiments. All 2-acetamido-2-deoxy-β-D-glucopyranosyl groups of all the oligosaccharides of the peak illustrated in Fig. 4c were removed by jack-bean *N*-acetyl-β-D-hexosaminidase digestion (Fig. 4e). This radioactive component was completely converted into **15** by periodate oxidation (Fig. 4f). The trisaccharide **15** could be obtained from **16** but not from **17** and its α-D-mannosylated derivatives. On the basis of all the data described herein, structures **19–23** are proposed for Oligosaccharides **I–V**, respectively.



Structural study of the neutral oligosaccharides obtained from the acidic oligosaccharide fraction of ovomucoid by sialidase treatment. — By incubation with diplococcal β-D-galactosidase, 2 D-galactopyranosyl groups were removed from the compound of Peak **A** of Fig. 2b (Fig. 5a), whereas only 1 group was removed from the compounds of Peaks **B–G** (Figs. 5b–g, respectively). All seven degalactosyl peaks of Figs. 5a–g were converted into a radioactive-labeled oligosaccharide having the same elution position (7.3 glucose units) as authentic $(Man)_3 \rightarrow GlcNAc \rightarrow [^3H\text{-}1]GlcNAcol$ by incubation with a high concentration (3.5 units/40 μL) of jack-bean *N*-acetyl-β-D-hexosaminidase (data not shown). That the pentasaccharides derived from the seven fractions have structure **18** was confirmed by sequential digestion and methylation analysis (data not shown). Therefore, the structures of the oligosaccharides, in the seven fractions illustrated in Fig. 5a–g, may be written as **24**. Structural studies by sequential glycosidase digestion, methylation analysis, and periodate oxidation revealed that most of these oligosaccharides are identical with the neutral oligosaccharide fractions obtained from ovomucoid by hydrazinolysis[2,7]: Fractions **VI** and **VIII** are identical with **N-3b** and **N-5b**, respectively. Fraction **IX** is a mixture of **N-6a** and **N-6b**, and Fraction **X** is a mixture of **N-7a** and **N-7b**. Fractions **VII** and **XI** gave the same analytical results as the two degalactosylated oligosaccharides of **N-4** and **N-7a**. Since the detail of the structural studies of oligosaccharides in these fractions was reported previously[7], the data of structural analysis are not presented herein, and only structures **25–31** are presented.

Oligosaccharides from Peaks **A** and **B** (Fig. 2b) are di- and mono-β-D-galactopyranosyl derivatives of oligosaccharides from Fraction **VI**, and oligosaccharides from Peaks **C–G** are mono-β-D-galactopyranosyl derivatives of oligosaccharides from Fractions **VII–XI**, respectively. In order to locate the *N*-acetyllactosaminyl

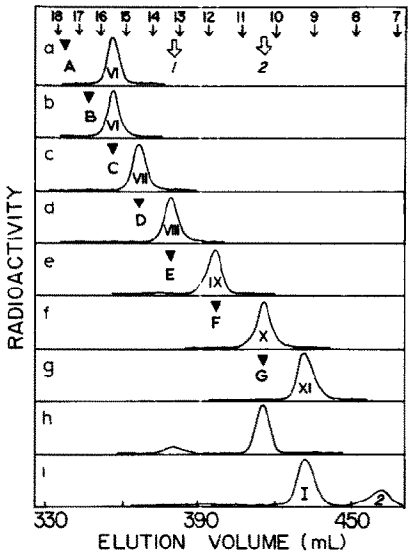
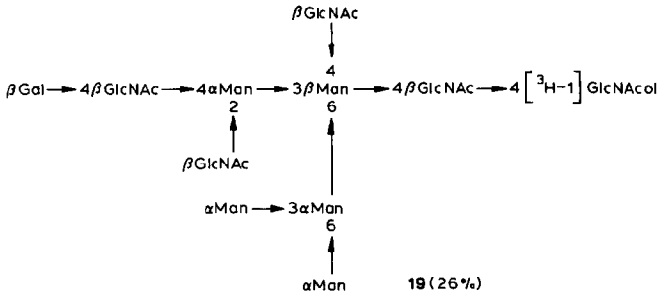
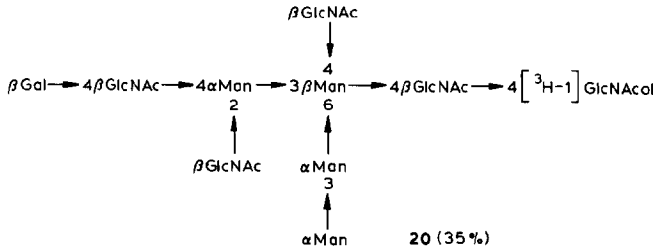


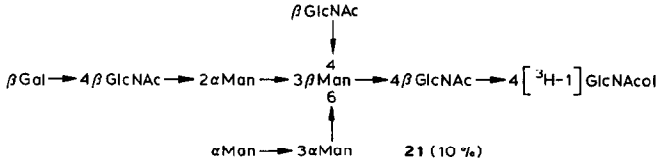
Fig. 5. Diplococcal β-D-galactosidase digestion of radioactive Peaks A to G in Fig. 2b and sequential exoglycosidase digestion of radioactive-labeled A_1N. The radioactive products were analyzed by Bio-Gel P-4 column chromatography. The black arrows at the top are the same as in Fig. 2, and the white arrows (*1* and *2*) indicate the elution positions of authentic **5** and **7**, respectively. The black triangles indicate the elution positions of Peaks A to G, respectively. (a)–(g): Peaks A–G incubated with diplococcal β-D-galactosidase (1.5 munits/40 μL), respectively; (h) radioactive-labeled A_1N incubated with jack bean *N*-acetyl-β-D-hexosaminidase (3.5 units/40 μL); (i) the major radioactive peak in (h) incubated with a mixture of diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase. The minor radioactive peak in (h) also gave the two peaks in (i) by the same treatment (data not shown).

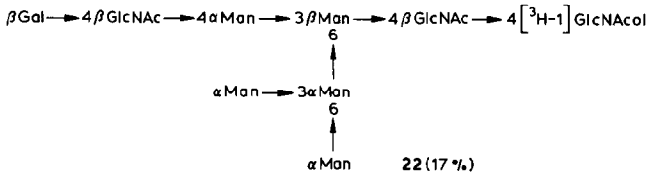
groups of these oligosaccharides, a mixture of radioactively labeled **A–G** was exhaustively digested with jack-bean *N*-acetyl-β-D-hexosaminidase. Analysis of the digestion product by Bio-Gel P-4 column chromatography showed a minor peak (13.2 glucose units) and a major peak (10.3 glucose units) (Fig. 5h). Since the elution position of the minor peak was the same as that of authentic **5**, it derived from Peak **A** only. Therefore, the major peak, which has the same elution position as authentic **7**, derived from Peaks **B–G**. The compounds from both peaks of Fig. 5h were converted into mixtures of **32** (Peak 1) and **18** (Peak 2) by incubation with a mixture of diplococcal β-D-galactosidase and diplococcal *N*-acetyl-β-D-hexosaminidase (Fig. 5i). These results indicated that the *N*-acetyllactosaminyl groups of the compounds from Peaks **A–G** are rather randomly located at O-2 and -4 of two α-D-mannopyranosyl residues of the trimannosyl core.

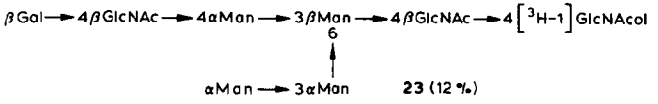
Structures of the sialic acid-containing oligosaccharides of ovalbumin and ovomucoid. — As is evident from Figs. 1c and d, the acidic oligosaccharide fractions obtained from ovalbumin and ovomucoid contain sialidase-resistant acidic oligosaccharides. We have recently confirmed that the sialidase-resistant acidic oligosaccharides of ovalbumin are sulfate-containing oligosaccharides[1]. In order to



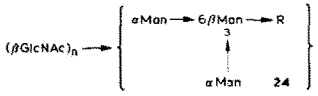


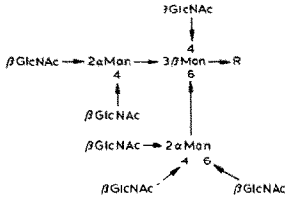


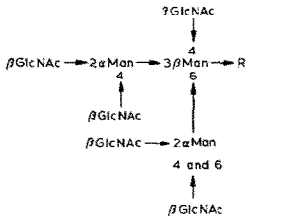


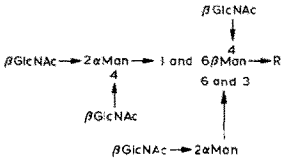


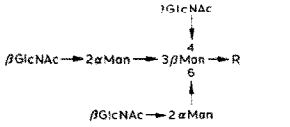
obtain a sialyloligosaccharides mixture free from the sulfated oligosaccharides, the methanolysis procedure described by Slomiany *et al.*[18] for the specific removal of the sulfate group from sulfate-containing glycolipid was attempted. For determining the optimal conditions for recovering intact, sialylated oligosaccharides quantitatively, authentic **4** ($1 \cdot 10^5$ c.p.m.) was methanolyzed, and the reaction product



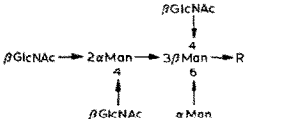


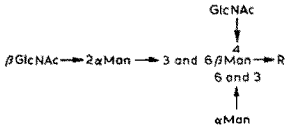


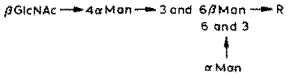




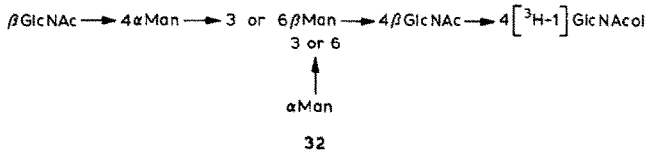
(28 + 29, 15%)







R = 4βGlcNAc → 4 [^{3}H-1]GlcNAcol



analyzed by paper electrophoresis. As shown in Fig. 6a, 70% of the radioactivity was recovered as an intact acidic oligosaccharide, and 30% was converted into an oligosaccharide having a less-acidic charge than the original oligosaccharide. This less-acidic oligosaccharide was considered to be an *N*-deacetylation product because all the radioactivity was detected as **4** after *N*-acetylation of the reaction mixture (Fig. 6b). Since the mobility of the degradation product indicated that only one acetamido group had been converted into a free amino group, *N*-deacetylation might have occurred only in the 2-acetamido-2-deoxy-D-glucitol residue. Based on these preliminary data, Fraction **A-1** (Fig. 1a) was methanolyzed and the product *N*-acetylated. When the reaction mixture was analyzed by paper electrophoresis, ~70% of the radioactively labeled component behaved as a neutral component (Fig. 6c), indicating that complete desulfation of the sulfate-containing oligosac-

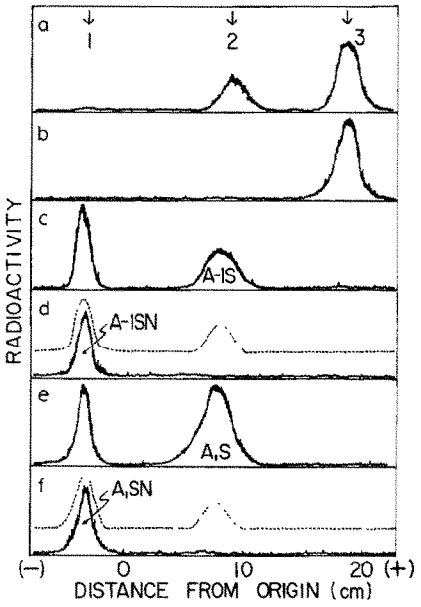


Fig. 6. Methanolysis of radioactive oligosaccharides. The radioactive product was analyzed by paper electrophoresis as in Fig. 1. Arrows at the top are the same as in Fig. 1: (a) authentic **4** subjected to methanolysis; (b) authentic **4** subjected to methanolysis followed by *N*-acetylation; (c) radioactive-labeled Fraction A-1 subjected to methanolysis followed by *N*-acetylation; (d) the radioactive-labeled Peak A-1S in (c) incubated with sialidase (50 munits/50 μL) at 37° either for 18 h (solid line) or for 6 h (dotted line); (e) radioactive-labeled Fraction A_1 subjected to methanolysis followed by *N*-acetylation; (f) the radioactive Peak A_1S in (e) incubated with sialidase (50 munits/50 μL) at 37° either for 18 h (solid line) or for 6 h (dotted line).

TABLE I

METHYLATION ANALYSIS OF SIALYLOLIGOSACCHARIDES AND THEIR PARTIAL DEGRADATION PRODUCTS RELEASED FROM OVALBUMIN AND OVOMUCOID

Partially methylated sugars	*Molar ratio*[a]								
	Ovalbumin							*Ovomucoid*	
	A-1SN	*A-1S*	*I*	*II*	*III*	*IV*	*V*	*A_1SN*	*A_1S*
Galactitol									
2,3,4,6-Tetra-*O*-methyl	*1.0*	0.0	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	trace[b]
(1,5-di-*O*-acetyl)									
2,4,6-Tri-*O*-methyl	0.0	*1.0*	0.0	0.0	0.0	0.0	0.0	0.1	*1.0*
(1,3,5-tri-*O*-acetyl)									
Mannitol									
2,3,4,6-Tetra-*O*-methyl	1.4	1.3	1.7	1.1	1.1	2.1	1.2	0.2	0.2
(1,5-di-*O*-acetyl)									
3,4,6-Tri-*O*-methyl	0.1	0.1	0.0	0.0	0.7	0.0	0.0	0.3	0.4
(1,2,5-tri-*O*-acetyl)									
2,4,6-Tri-*O*-methyl	0.6	0.5	trace	0.9	0.8	trace	1.0	0.0	0.0
(1,3,5-tri-*O*-acetyl)									
2,3,6-Tri-*O*-acetyl	0.4	0.3	0.0	0.0	trace	1.1	0.9	0.2	0.1
(1,4,5-tri-*O*-acetyl)									
3,6-Di-*O*-methyl	0.6	0.6	1.0	1.0	trace	0.0	0.0	0.6	0.7
(1,2,4,5-tetra-*O*-acetyl)									
2,4-Di-*O*-methyl	0.6	0.6	0.7	0.0	trace	1.7	1.1	0.1	0.1
(1,3,5,6-tetra-*O*-acetyl)									
3,4-Di-*O*-methyl	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2
(1,2,5,6-tetra-*O*-acetyl)									
2-Mono-*O*-methyl	0.6	0.7	1.0	1.1	0.8	0.0	0.0	1.0	0.9
(1,3,4,5,6-penta-*O*-acetyl)									
3-Mono-*O*-methyl	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.4
(1,2,4,5,6-penta-*O*-acetyl)									
2-Deoxy-2-N-methylacetamidoglucitol									
1,3,5,6-Tetra-*O*-methyl	0.6	0.7	0.6	0.7	0.6	0.5	0.6	0.7	0.8
(4-mono-*O*-acetyl)									
3,4,6-Tri-*O*-methyl	1.5	1.4	1.8	2.2	0.8	trace	0.0	3.7	3.5
(1,5-di-*O*-acetyl)									
3,6-Di-*O*-methyl	2.3	2.1	2.2	2.3	1.8	2.2	2.3	2.3	2.4
(1,4,5-tri-*O*-acetyl)									

[a]The values are given relative to the values of 2,3,4,6-tetra-or 2,4,6-tri-*O*-methylgalactitol (values in italic). [b]Less than 0.1.

charides had occurred, whereas the sialyloligosaccharide of this fraction remained intact. The acidic oligosaccharides (**A-1S**, Fig. 6c), which remained intact after methanolysis, were completely converted into neutral oligosaccharides by sialidase digestion (solid line, Fig. 6d). When Fraction **A-1S** was incubated with sialidase for a shorter time, whereby a part of the original sialyloligosaccharides still remained, no additional acidic oligosaccharide was detected between the neutral and the original acidic peaks (dotted line, Fig. 6d). This result indicated that the sialyloligosaccharides of Fraction **A-1S** contain only one sialyl group. Bio-Gel P-4 column chromatography of the neutral oligosaccharide fraction of Fig. 6d gave exactly the same fractionation pattern as Fig. 2a (data not shown). This evidence further confirmed that all sialyloligosaccharides from Fraction **A-1** were recovered intact after methanolysis.

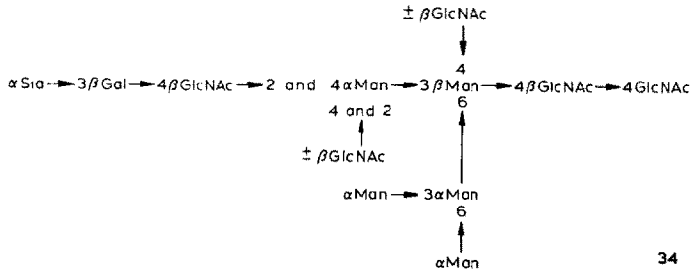
Approximately 30% of Fraction $\mathbf{A_1}$ (Fig. 1b) was also converted into neutral components by methanolysis, followed by *N*-acetylation (Fig. 6e). This result indicated that the sialidase-resistant oligosaccharides of Fraction $\mathbf{A_1}$ obtained from ovomucoid also contain a sulfate group. The acidic components resistant to methanolysis ($\mathbf{A_1S}$) were completely converted into neutral components by sialidase digestion (solid line, Fig. 6f). The neutral components gave exactly the same fractionation pattern as shown in Fig. 2b, indicating that all sialyloligosaccharides of Fraction $\mathbf{A_1}$ remained intact after methanolysis. Partial desialylation gave only neutral components, in addition to intact Fraction $\mathbf{A_1S}$ (dotted line, Fig. 6f), indicating that all sialyloligosaccharides of Fraction $\mathbf{A_1}$ also contain one sialyl group.

In order to locate the sialyl groups in oligosaccharides of Fractions **A-1S** and $\mathbf{A_1S}$, these fractions and their desialylation products (Fractions **A-1SN** and $\mathbf{A_1SN}$) were analyzed by methylation (see Table I), which indicated that the sialyl groups of all oligosaccharides of both fractions occur as an α-Sia-(2→3)-, and not as an α-Sia-(2→6)-D-Gal*p* group.

On the basis of the aforementioned results, structures **34** and **35** are proposed for the sialyloligosaccharides of ovalbumin and ovomucoid, respectively.

DISCUSSION

As discussed in a previous paper[2], ovalbumin and ovomucoid contain neutral sugar chains having different structures. All neutral sugar chains in ovomucoid contain the trimannosyl pentasaccharide α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc as a common core. On the other hand, the smallest oligomannosyl core of the neutral sugar chains of ovalbumin is α-D-Man*p*-(1→3)-α-D-Man*p*-(1→6)-[α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc. These characteristic structures were also found for the sialic acid-containing sugar chains of ovomucoid and ovalbumin, as reported herein. This observation strongly suggests that the acidic oligosaccharides, though in small proportion relation to the total oligosaccharides liberated from both glyco-



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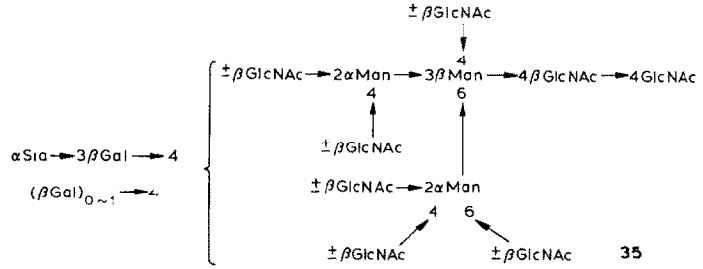

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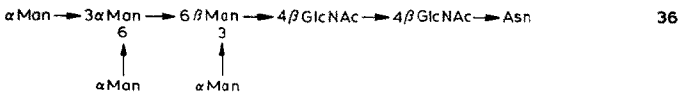

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proteins, are derived from ovalbumin and ovomucoid, and not from other contaminating glycoproteins.

Structurally, all of the sialic acid-containing sugar chains of ovalbumin belong to the hybrid-type sugar chains. An interesting finding is that two of these chains [sialyl derivatives of **IV** (**22**) and **V** (**23**)] contain the α-Sia-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Man*p*-(1→3) group. From the current knowledge of the biosynthesis of asparagine-linked sugar chains, the structures of these sugar chains are unexpected because the formation of the β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3) group by *N*-acetyl-β-D-glucosaminyltransferase I (ref. 19) is considered as a prerequisite for the removal of two α-D-mannopyranosyl groups from **36** to form the trimannosyl core[20], and for the addition of other 2-acetamido-2-deoxy-β-D-glucopyranosyl groups[21]. Interestingly, both sugar chains do not have the 2-acetamido-2-deoxy-β-D-glucopyranosyl group linked at O-4 of the β-D-mannopyranosyl residue, whereas all others contain this residue. The structural requirement is also observed for the sugar chains of ovomucoid; oligosaccharides of Fraction **XI**, which have the β-D-Glc*p*NAc-(1→4)-α-D-Man*p* group, lack the β-D-GlcNAc-4 group. How these peculiar sugar chains are formed is, therefore, an interesting subject for future study.

The linkage of all the sialyl groups of the sugar chains of both ovalbumin and

ovomucoid at O-3 of the β-D-galactopyranosyl residue is noteworthy. So far, both α-sialyl-(2→3)- and -(2→6)-β-D-Gal*p* linkages have been found in the sugar chains of glycoproteins*. Occurrence of the (2→3) linkage in asparagine-linked sugar chains, however, is rather limited as compared to the (2→6) linkage, and in many cases both (2→3) and (2→6) linkages coexist. Furthermore, there is evidence that the distribution of the (2→3) linkage might be regulated by the specificity of the α-(2→3)-sialyltransferase. Thus, the α-(2→3) group was found only on the β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Man group among the three *N*-acetyllactosamine outer units of the tribranched sugar chain of ceruloplasmin[10]. Interestingly, sialyl groups of human chorionic gonadotropin[23] and human placental β-glucocerebrosidase[24] occur only as the α-NeuAc-(2→3)-D-Gal group. These observations are in good accord with the observation reported by Van den Eijnden and Schiphorst[25] that human placenta preponderantly contains an α-(2→3)-sialyltransferase. Possibly, hen oviduct may also contain only an α-(2→3)-sialyltransferase.

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