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Structures of asparagine linked oligosaccharides of immunoglobulins (IgY) isolated from egg-yolk of Japanese quail

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Structures of the Asn linked oligosaccharides of quail egg-yolk immunoglobulin (IgY) were determined in this study. Asn linked oligosaccharides were cleaved from IgY by hydrazinolysis and labelled with *p*-aminobenzoic acid ethyl ester (ABEE) after *N*-acetylation. The ABEE labelled oligosaccharides were then fractionated by a combination of Concanavalin A–agarose column chromatography and anion exchange, normal phase and reversed phase HPLC before their structures were determined by sequential exoglycosidase digestion, methylation analysis, HPLC, and 500 MHz ^{1}H-NMR spectroscopy. Quail IgY contained only neutral oligosaccharides of the following categories: the glucosylated oligomannose type (0.6%, Glcα1-3Glcα1-3Man$_9$GlcNAc$_2$; 35.6%, Glcα1-3Man$_{7-9}$GlcNAc$_2$). oligomannose type (15.0%, with the structure Man$_{5-9}$GlcNAc$_2$) and biantennary complex type with core structures of -Manα1-3(-Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc (9.9%), -Manα1-3(GlcNAcβ1-4)(-Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc (25.1%) and -Manα1-3(GlcNAcβ1-4)(-Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc (11.4%). Although never found in mammalian proteins, glucosylated oligosaccharides (Glc$_1$Man$_{7-9}$GlcNAc$_2$) have been located previously in hen IgY.

Keywords: Asn-linked oligosaccharide, quail egg-yolk, immunoglobulin, *p*-aminobenzoic acid ethyl ester, HPLC, glucosylated oligomannose-type oligosaccharide

Abbreviations: IgG, IgM, IgA, IgY, immunoglobulin G, M, A and Y, respectively; ABEE, *p*-aminobenzoic acid ethyl ester.

Introduction

It is of interest to study the immune response, immunoglobulin structures and immunoglobulin genes of avian species because they are submammalian vertebrates and have evolved on a line divergent from that leading to mammals. These studies have been performed primarily on the chicken [1]. Chicken IgG differs in many important aspects from any mammalian IgG [1]. Both mammalian and avian IgG are known to contain Asn linked oligosaccharides. However, although the structures and functions of oligosaccharides of mammalian IgG have been investigated extensively [2–7], little is known about avian IgG. Thus, we decided to investigate the structures of oligosaccharides from avian immunoglobulins and the first study was on immunoglobulin isolated from egg-yolk (IgY) [8]. IgY is the immunoglobulin G transferred from the serum into egg-yolk [9, 10]. IgY is indistinguishable from serum IgG in many aspects [11], and its content in egg-yolk is the same or occasionally higher than the serum IgG level [9]. The structures of oligosaccharides in hen IgY differed markedly from those of any mammalian IgG [8]. In addition, hen IgY contained unusual monoglucosylated oligosaccharides with Glc$_1$Man$_{7-9}$GlcNAc$_2$ structures. The presence of these oligosaccharides in mature glycoprotein is unique, and their biosynthetic pathways are of much interest, as these substances are distributed commonly in IgG of avian species.

In the present study, we have attempted to elucidate the structures of oligosaccharides of IgG from another member of the Phasianidae family, the Japanese quail, to examine the distribution of unusual glucosylated oligosaccharides in IgG of avian species. Some properties of quail IgG are similar to those of chicken IgG, such as a sedimentation coefficient of about 7S [12], a molecular weight of 170 000–180 000 Da [13], some free H and L chains are found on reduction in alkaline buffer [12], Fab and Fc

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fragments are formed following papain digestion [14], and they aggregate in high salt containing buffer [15]. Furthermore, IgG from these birds share H and L chain isotypic antigenic determinants [16].

IgY from quail egg yolk was isolated in a manner similar to that reported previously for hen IgY [17], and the oligosaccharide structures were elucidated.

Materials and methods

Chemicals and enzymes

ABEE was purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium cyanoborohydride, α-methyl mannoside and ^2H_2O were purchased from Nacalai Tesque Inc. (Kyoto, Japan). *Aspergillus saitoi* α-mannosidase I and *Tetrahymena pyriformis* α-glucosidase were purified by the method of Amano and Kobata [18] and Banno and Nozawa [19], respectively. Jack bean meal β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase, as well as bovine kidney α-*L*-fucosidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Standard oligosaccharides

Glcα1-3Man$_9$GlcNAcβ1-4GlcNAc-ABEE (Glc$_1$Man$_9$GlcNAc$_2$-ABEE) was obtained from hen IgY [8]. Glcα1-3Manα1-2Manα1-2Manα1-2Manα1-3[Manα1-3(Manα1-6)-Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-ABEE (Glc$_1$-Man$_7$GlcNAc$_2$-ABEE) and Glcα1-3Manα1-2Manα1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-ABEE (Glc$_1$-Man$_4$GlcNAc$_2$-ABEE) were obtained from Glc$_1$Man$_9$GlcNAc$_2$-ABEE by digestion with *A. saitoi* α-mannosidase I and jack beam α-mannosidase, respectively. Manα1-2Manα1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-ABEE (Man$_4$GlcNAc$_2$-ABEE) was prepared from Glc$_1$Man$_4$GlcNAc$_2$-ABEE by digestion with *T. pyriformis* α-glucosidase. Other standards ABEE-oligosaccharides were prepared as described previously [20].

Preparation of quail IgY

Quail IgY was purified to apparent homogeneity from quail egg-yolk in a similar manner to that reported for the isolation of hen IgY [17].

Liberation of asparagine linked oligosaccharides from quail IgY and preparation of ABEE derivatives

Oligosaccharides were liberated from quail IgY (350 mg) by hydrazinolysis/re-*N*-acetylaction [21]. The liberated oligosaccharides were derivatized with ABEE by reductive amination, and the ABEE-oligosaccharides were purified by chromatography first on a PRE-SEP C_{18} cartridge (Tessek, Mountain View, CA, USA) followed by a Bio-Gel P-4 column (200–400 mesh, 1.0 cm × 45 cm) as described previously [8, 21].

Concanavalin A (Con A)–agarose affinity chromatography

The Con A–agarose column (10 ml) was equilibrated with 0.05M sodium phosphate buffer (pH 7.2) containing 0.15M NaCl (PBS buffer). The sample was dissolved in PBS buffer, and applied to the column before stepwise elution with PBS; the PBS contained 10 mM, and then 100 mM methyl-α-mannoside. Fractions of 2 ml were collected. Elution of oligosaccharides was monitored by UV absorbance at 304 nm. The fractions containing ABEE-oligosaccharides were pooled and desalted on a Bio-Gel P-4 column.

High performance liquid chromatography

HPLC analyses were performed with a Shimadzu LC-6A liquid chromatograph. Fractionation of ABEE-oligosaccharides according to their ionic charges was performed by anion-exchange HPLC equipped with a TSKgel DEAE-5PW column (0.75 cm × 7.5 cm, Tosoh Co Ltd) under reported conditions [8]. Fractionation of neutral ABEE-oligosaccharides was performed on a TSKgel Amide-80 column (0.46 cm × 25 cm, Tosoh) and/or a Wakosil 5C18-200 column (0.4 cm × 25 cm, Wako) as previously described [8, 20]. Structural analyses of ABEE-oligosaccharides by HPLC were performed with the use of a 2-dimensional mapping (2D-map) technique that we have developed recently [20]. In this method, a set of elution positions of each ABEE-oligosaccharide on Amide-80 and ODS HPLC is mapped 2-dimensionally and compared with those for standard ABEE-oligosaccharides.

Analytical methods

Carbohydrate compositions expressed as alditol acetate derivatives, were determined by gas chromatography [22]. Separation was performed on a DB-5 fused silica capillary column (0.32 mm × 25 m, J&W Scientific, USA) using a Hewlett-Packard 5840A gas chromatograph. Sialic acid was determined by the periodate–resorcinol reaction [23].

Methylation of ABEE-oligosaccharides was carried out by the method of Ciucanu and Kerek [24]. The methylated sample was converted to partially methylated alditol acetate before analysis by gas chromatography/mass spectrometry [8]. Because partially methylated 2-*N*-methylacetamido-2-deoxyglucitol originating from pre-ABEE-derivatized *N*-acetylglucosamine located at the reducing terminal could not be detected by the present method, an alternative analysis was employed. Briefly, a portion of the permethylated ABEE-oligosaccharide was subjected to methanolysis in 2.5M methanolic HCl at 85 °C for 16 h. The reaction mixture was evaporated to dryness under a nitrogen stream, and the residue was acetylated with a pyridine:acetic anhydride (1:1 by vol) mixture at 80 °C for 30 min. The *p*-aminobenzoic acid methyl ester derivative of partially methylated 2-*N*-methylacetamido-2-deoxyglucitol thus obtained was analysed by gas chromatography on a DB-5 capillary column. The column temperature was

programmed from 50 °C to 170 °C at 20 °C min^{-1}, and then to 300 °C at 7 °C min^{-1}.

Gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12% slab gels [25]. Proteins and carbohydrates were stained with Coomassie Blue R-250, and the periodate Schiff reagent [26], respectively.

Glycosidase digestion

Digestions of ABEE-oligosaccharides with jack bean β-galactosidase, jack bean β-*N*-acetylhexosaminidase, jack beam α-mannosidase, bovine kidney α-L-fucosidase, and *A. saitoi* α-mannosidase I were carried out as described previously [8]. *T. pyriformis* α-glucosidase digestion was performed with 50 milliunits of the enzyme in 100 µl 0.1M sodium acetate buffer, pH 4.5, containing 10 µg swainsonine at 37 °C for 18 h. The digestion was terminated by heating the reaction mixture for 3 min, and the products were purified by PRE-SEP C_{18} cartridge chromatography as described previously [8]. The reaction products at each step were analysed by Amide-80 HPLC or 2D-map technique.

500 MHz proton NMR spectrometry

ABEE-oligosaccharides were repeatedly treated with ^2H_2O (99.96% ^2H) through intermediate lyophilization. 500 MHz ^1H-NMR spectra were obtained with a JEOL FX-500S FT NMR spectrometer operating in the Fourier transform mode at a probe temperature of 25 °C. Chemical shifts (δ) relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate were actually measured by reference to internal acetone (δ = 2.225 ppm in ^2H_2O at 25 °C).

Results

Characterization of quail egg-yolk immunoglobulin

The quail IgY was dissociated into heavy and light chains with M_r 64 kDa and 28 kDa, respectively, on SDS-PAGE (Fig. 1). Staining with Schiff reagent revealed that oligosaccharides were present mainly on the heavy chain (data not shown).

The IgY preparation (1 mg) contained 7.3 nmol fucose, 102.3 nmol mannose, 8.1 nmol galactose, 15.0 nmol glucose and 68.2 nmol *N*-acetylglucosamine, indicating the occurrence of only Asn linked oligosaccharides in quail IgY. No sialic acid was detected by the periodate–resorcinol reaction [23].

Liberation and fractionation of oligosaccharides

After hydrazinolysis, re-*N*-acetylation and ABEE-derivatization, the ABEE-oligosaccharides obtained from quail IgY were subjected to anion-exchange HPLC, and only a neutral oligosaccharide (denoted fraction N) was obtained (Fig. 2).

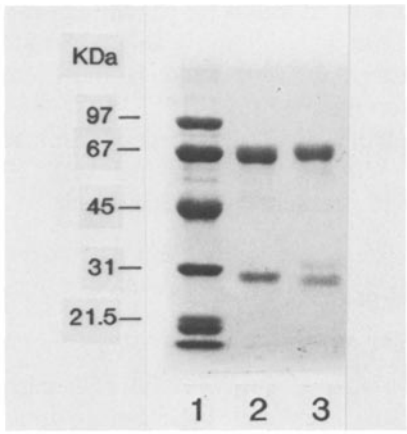


Figure 1. SDS-PAGE of quail IgY. Electrophoresis was performed as described in the Materials and methods section. After electrophoresis, the gels were stained for protein bands with Coomassie brilliant blue. Lanes 1, 2 and 3 represent standard, hen egg-yolk IgY and quail egg-yolk IgY, respectively.

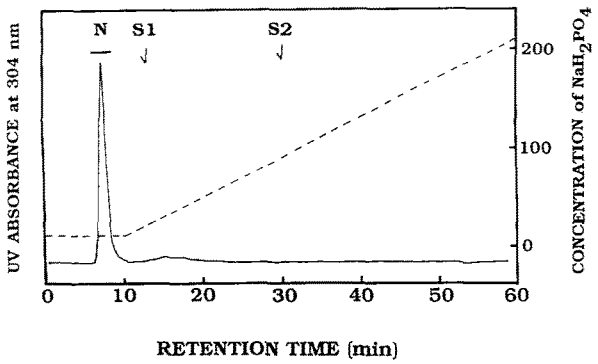


Figure 2. Ion-exchange HPLC of ABEE-oligosaccharides of quail IgY. ABEE derivatives of Asn linked oligosaccharides from IgY were applied to a column of TSKgel DEAE-5PW (0.75 cm × 7.5 cm). The column was eluted isocratically with 10 mM NaH_2PO_4 for 10 min and then with a linear gradient from the same buffer to 170 mM NaH_2PO_4 for 40 min at a flow rate 0.5 ml min^{-1}. The fraction indicated by a bar was pooled. Elution positions of fractions N, SI and SII corresponded to those of ABEE-derivatives of neutral, monosialyl and disialyl oligosaccharides, respectively.

Fraction N was pooled and further fractionated on a Con A–agarose column into two fractions: one was eluted with equilibration buffer and 10 mM methyl-α-mannoside (fraction NC), whereas the other was eluted with 100 mM methyl-α-mannoside (fraction NM). The elution profile from the Con A–agarose column [27] suggests that fraction NC (45.5% of total ABEE-oligosaccharides) probably contained complex-type and fraction NM (54.5% of total ABEE-oligosaccharides) oligomannose-type oligosaccharides.

Analysis of fraction NM

The methylation analysis showed terminal, C-2 substituted and C-3 and C-6 disubstituted mannose typical for

Table 1. Methylation analysis of fraction NM and NC of quail IgY

Partially methylated alditol acetate	*Molar ratio*[a]	
	NM	*NC*
Fucitol		
2,3,4-Tri-*O*-methyl-(1,5-di-*O*-acetyl)	–	0.26
Galactitol		
2,3,4,6-Tetra-*O*-methyl-(1,5-di-*O*-acetyl)	trace[b]	0.36
Glucitol		
2,3,4,6-Tetra-*O*-methyl-(1,5-di-*O*-acetyl)	0.92	–
2,4,6-Tri-*O*-methyl-(1,3,5-tri-*O*-acetyl)	trace[b]	–
Mannitol		
2,3,4,6-Tetra-*O*-methyl-(1,5-di-*O*-acetyl)	2.08	trace[b]
3,4,6-Tri-*O*-methyl-(1,2,5-tri-*O*-acetyl)	3.50	1.43
2,4,6-Tri-*O*-methyl-(1,3,5-tri-*O*-acetyl)	1.15	–
2,4-Di-*O*-methyl-(1,3,5,6-tetra-*O*-acetyl)	2.00	0.28
2-Mono-*O*-methyl-(1,3,4,5,6-penta-*O*-acetyl)	–	0.72
2-*N*-Methylacetamido-2-deoxyglucitol		
3,4,6-Tri-*O*-methyl-(1,5-di-*O*-acetyl)	trace[b]	2.57
3,6-Di-*O*-methyl-(1,4,5-tri-*O*-acetyl)	1.00	1.29
3,5,6-Tri-*O*-methyl-(4-mono-*O*-acetyl)[c]	+[d]	+[d]
3,5-Di-*O*-methyl-(4,6-di-*O*-acetyl)[c]	–	+[d]

[a] The amount of 2,4-di-*O*-methyl-mannitol is set to 2.0 for NM and the sum of 2,4-di-*O*-methylmannitol and 2-mono-*O*-methylmannitol is set to 1.0 for NC.
[b] Less than 0.1.
[c] The reducing terminal *N*-acetylglucosamine residue was detected as the *p*-aminobenzoic acid methyl ester derivative of partially methylated 2-*N*-metylacetamido-2-deoxyglucitol (see the Materials and methods section).
[d] +, present but unable to quantify.

oligomannose type oligosaccharides (Table 1). Terminal and C-3 substituted glucose indicated the presence of mono- and diglycosylated oligomannose type oligosaccharides. In addition, terminal galactose and *N*-acetylglucosamine suggested the presence of a small amount of complex-type oligosaccharides.

The NM fraction was further fractionated by Amide-80 HPLC, which separates oligosaccharides basically according to their size. Eight subfractions designated as NM-a to NM-h were obtained (Fig. 3). These subfractions were subjected to sequential exoglycosidase digestion and 500 MHz ^{1}H-NMR spectroscopy to elucidate the structures. However, the amounts of ABEE-oligosaccharides in NM-a, -c and -d were too low for ^{1}H-NMR analysis.

The elution position of **NM-a** on Amide-80 HPLC corresponded to that for authentic $Man_5GlcNAc_2$-ABEE. The elution position of NM-a was unaffected after digestion with *A. saitoi* α-mannosidase I. However, following digestin with jack bean α-mannosidase, a product eluting at the same position as authentic Manβ1-4GlcNAcβ1-4GlcNAc-ABEE ($Man_1GlcNAc_2$-ABEE) was obtained, corresponding to the release of four mannose residues (Fig. 4). These results indicate that fraction NM-a contained oligomannose

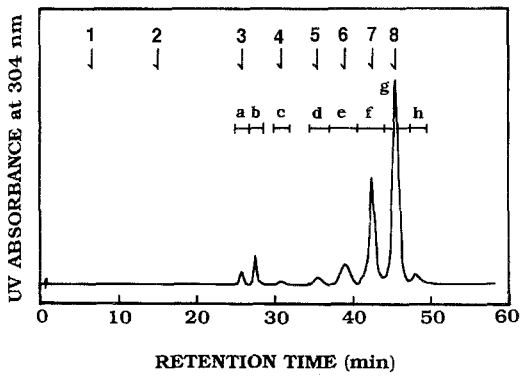


Figure 3. Amide-80 HPLC of ABEE-oligosaccharides in fraction NM of quail IgY. The fraction NM was obtained from total ABEE-oligosaccharides of quail IgY by Con-A–agarose column chromatography. HPLC was performed with a TSKgel Amide-80 column (0.46 cm × 25 cm). The column was equilibrated with a mixture of solvent A (acetonitrile:water, 9:1 by vol): solvent B (acetonitrile:water, 1:9 by vol), 80:20, and after injection of the sample, elution was performed using a linear gradient to a ratio of solvent A:B, ratio of 50:50 for 60 min at a flow rate of 1.0 ml min^{-1} at 40 °C. A total of eight fractions (NM-a to -h) indicated by bars were collected. Arrows (↓) indicate the elution positions of authentic ABEE-oligosaccharides: (1) $Man_1GlcNAc_2$-ABEE; (2) $Man_3GlcNAc_2$-ABEE; (3) $Man_5GlcNAc_2$-ABEE; (4) $Man_6GlcNAc_2$-ABEE; (5) $Man_7GlcNAc_2$-ABEE (6) $Man_8GlcNAc_2$-ABEE; (7) $Man_9GlcNAc_2$-ABEE; and (8) $Glc_1Man_9GlcNAc_2$-ABEE.

type oligosaccharide containing four α-mannosyl residues (Table 2).

The elution position of **NM-b** did not correspond to any authentic oligomannose type oligosaccharides but rather to a biantennary oligosaccharide with a galactose residue at the nonreducing terminal. This suggestion was supported by methylation analysis (Table 1) and Con A–agarose column chromatography. Analytical data of this fraction will be shown below, together with those for other complex-type oligosaccharides.

Subfraction **NM-c** eluted at the same posiion as authentic $Man_6GlcNAc_2$-ABEE on the Amide-80 HPLC (Fig. 3). Further analyses on this fraction could not be performed because of insufficient sample.

Subfraction **NM-d** eluted at a position corresponding to that for authentic $Man_7GlcNAc_2$-ABEE (Fig. 3). After digestion with *A. saitoi* α-mannosidase I, NM-d illustrated a single peak with the same mobility as authentic $Man_5GlcNAc_2$-ABEE, corresponding to the release of two mannose residues (Fig. 4). The residual ABEE-oligosaccharide released four mannose residues by digestion with jack bean α-mannosidase to produce $Man_1GlcNAc_2$-ABEE. As a result, we conclude that fraction NM-d is an oligomannose-type oligosaccharide with six α-mannosyl residues (Table 2).

Subfraction **NM-e** eluted at a position identical to authentic $Man_8GlcNAc_2$-ABEE (Fig. 3). When NM-e was

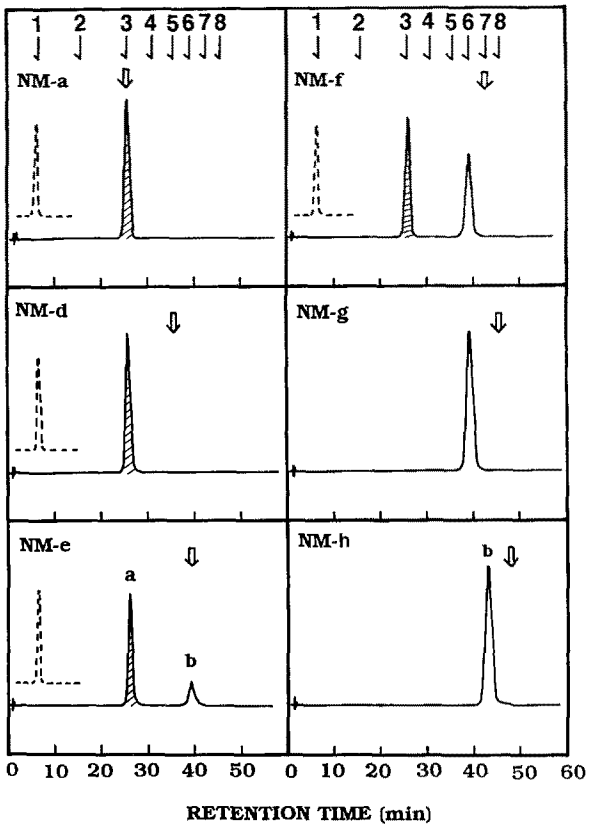


Figure 4. HPLC analysis of the digested products of ABEE-oligosaccharides in NM-a, -d, -e, -f, -g and -h by *A. saitoi* α-mannosidase I. Digested products were subjected to HPLC on a TSKgel Amide-80 column under the conditions described in Fig. 3. When hatched peak was subjected to jack bean α-mannosidase digestion, a peak corresponding to $Man_1GlcNAc_2$-ABEE (peak shown by dotted line) was demonstrated. Arrows (⇩) are similar to those in Fig. 3. Open arrows (⇓) indicate the elution position of untreated ABEE-oligosaccharides.

digested with *A. saitoi* α-mannosidase I, approximately 81% of the ABEE-oligosaccharides were hydrolysed to yield a peak with the same mobility as authentic $Man_5GlcNAc_2$-ABEE (release of three mannose residues), whereas about 19% was resistant to the enzyme (Fig. 4). They were separately pooled and subjected to further exoglycosidase digestions. The subfraction in peak a (Fig. 4) was converted to $Man_1GlcNAc_2$-ABEE by digestion with jack bean α-mannosidase (Fig. 4). The ABEE-oligosaccharide resistant to α-mannosidase I (peak b in Fig. 4) was converted to an ABEE-oligosaccharide with the same mobility as authentic $Glc_1Man_4GlcNAc_2$-ABEE by digestion with jack bean α-mannosidase, corresponding to the release of three mannose residues. The product released one glucose residue by digestion with *T. pyriformis* α-glucosidase. The derived product, which showed the same mobility as authentic $Man_4GlcNAc_2$-ABEE, released three mannose residues when incubated with jack bean α-mannosidase (Fig. 5a). The final product was eluted on Amide-80 HPLC and appeared at the position of $Man_1GlcNAc_2$-ABEE.

From the chemical shifts of H-1 and H-2 for mannose residues [28, 29], NMR analysis (Table 3) revealed the presence of oligomannose type structures in this fraction. The anomeric proton signals for Man-A′ (Man-A without Man-D_2) at $\delta = 5.082$ ppm and Man-B′ (Man-B without

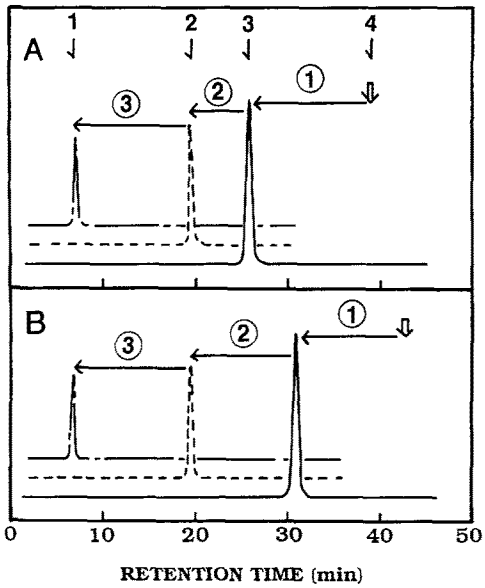
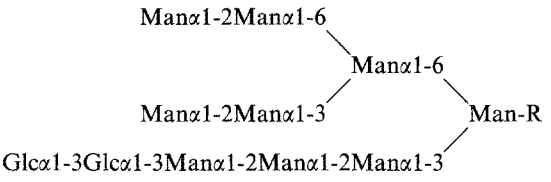
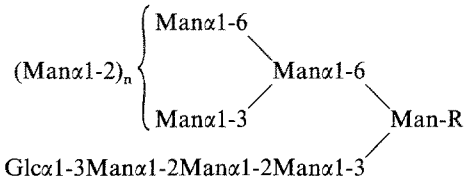
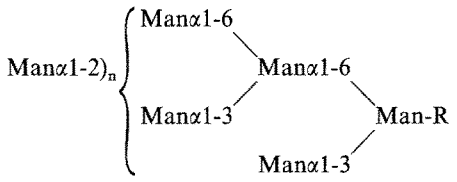
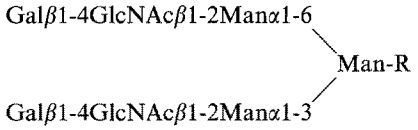
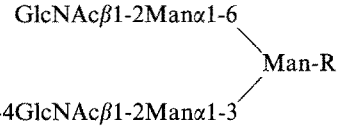
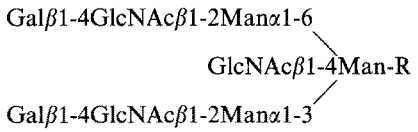
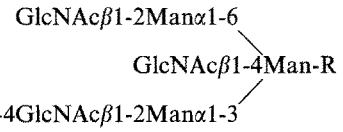
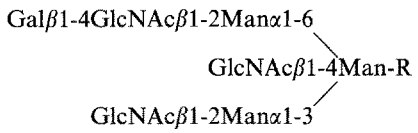
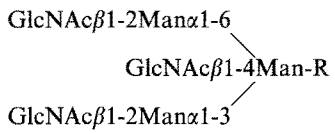
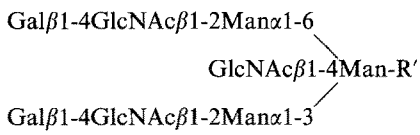


Figure 5. Sequential exoglycosidase digestion of the ABEE-oligosaccharide component (a) NM-e-b and (b) NM-h-b in Fig. 4. These components were sequentially digested with: ①: jack bean α-mannosidase; ②: *Tetrahymena pyriformis* α-glucosidase; and ③: jack bean α-mannosidase. Products at each step were analysed by TSKgel Amide-80 HPLC under the conditions described in Fig. 3. Arrows (⇩) indicate elution positions of authentic ABEE-oligosaccharides: (1) $Man_1GlcNAc_2$-ABEE; (2) $Man_4GlcNAc_2$-ABEE; (3) $Glc_1Man_4GlcNAc_2$-ABEE; and (4) $Glc_1Man_7GlcNAc_2$-ABEE. Open arrows (⇓) locate the elution positions of untreated ABEE-oligosaccharides.

Table 2. Proposed structures of Asn linked oligosaccharides of quail IgY.

Structures[a]	*Fraction No.*[b]	*Relative amount (%)*
	NM-h	0.6
	NM-g (n = 2)	29.4
	NM-f (n = 1)	5.2
	NM-e (n = 0)	1.0
	NM-f (n = 4)	9.1
	NM-e (n = 3)	4.0
	NM-d (n = 2)	0.8
	NM-c (n = 1)	0.1
	NM-a (n = 0)	1.0
	NC-a	6.6
	NM-b	3.3
	NC-b	0.6
	NC-c	1.9
	NC-d	4.0
	NC-e	18.6
	NC-f	1.3

(*continued*)

Table 2. *Continued.*

Structures[a]	*Fraction No.*[b]	*Relative amount (%)*
GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Man-R′ Galβ1-4GlcNAcβ1-2Manα1-3	NC-g	0.8
Galβ1-4GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Man-R′ GlcNAcβ1-2Manα1-3	NC-h	1.6
GlcNAcβ1-2Manα1-6 GlcNAcβ1-4Man-R′ GlcNAcβ1-2Manα1-3	NC-i	7.7
Uncharacterized oligosaccharides[c]		2.4

[a] R = GlcNAcβ1-4GlcNAc, R′ = -GlcNAcβ1-4(Fucα1-6) GlcNAc.
[b] See Figs 3 and 6.
[c] Structures could not be determined due to limited availability.

Man-D_2) at δ = 5.082 ppm and Man-B′ (Man-B without Man-D_3) at δ = 4.908 ppm together with those for Man-A (δ = 5.405 ppm) and Man-B (δ = 5.140 ppm) indicated that NM-e contained the structure without one or both Man-D_2 and Man-D_3 (the numbering of the sugar residues refers to the structure above Table 3). In addition to these signals, a minor signal for H-2 of Man-D1 was obtained, indicating that this mannose was 3-O-substituted by a terminal glucose [30, 31]. The signal at δ = 5.256 ppm was from the terminal glucose (Glc-a) [30, 31].

These results indicated that subfraction NM-e contained $Man_8GlcNAc_2$-ABEE (81%) and $Glc_1Man_7GlcNAc_2$-ABEE (19%) with the structures shown in Table 2.

The elution position of **NM-f** corresponded to that of $Man_9GlcNAc_2$-ABEE (Fig. 3). When NM-f was digested with *A. saitoi* α-mannosidase I, two peaks with the same mobility as $Glc_1Man_7GlcNAc_2$-ABEE (release of one mannose residue) and $Man_5GlcNAc_2$-ABEE (release of four mannose residues) in the ratio of 36:64 (Fig. 4) were demonstrated. Sequential exoglycosidase digestion of the former with jack beam α-mannosidase, *T. pyriformis* α-glucosidase and jack bean α-mannosidase produced the same result as those described for component b in Fig. 5a. The latter was converted to $Man_1GlcNAc_2$-ABEE with jack bean α-mannosidase (Fig. 4).

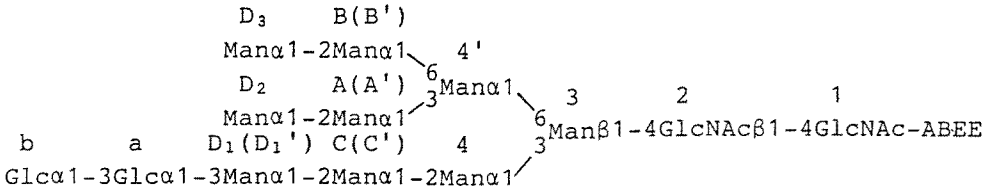
^{1}H-NMR analysis (Table 3) revealed the presence of $Glc_1Man_8GlcNAc_2$-ABEE in fraction NM-f. This assignment was based on the chemical shifts for H-1 of Glc-a (δ = 5.256 ppm), H-2 of Man-D_1 (δ = 4.236 ppm), H-1 of Man A′ (δ = 5.082 ppm) and H-1 of Man-B′ (δ = 4.908 ppm). The signals at δ = 5.402 ppm, 4.143 ppm and 5.309 ppm, corresponding, respectively, to H-1 of mannose residues -A, -B and -C, confirm the presence of $Man_9GlcNAc_2$-ABEE.

These results thus indicated that fraction NM-f contained $Glc_1Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ with the structures shown in Table 2 in the ratio of 36:64.

The chromatographic behaviour (Fig. 3) and ^{1}H-NMR spectrum (Table 3) of subfraction **NM-g** resembled those of authentic $Glc_1Man_9GlcNAc_2$-ABEE [8]. When NM-g was digested with *A. saitoi* α-mannosidase I, a single peak with the same mobility as $Glc_1Man_7GlcNAc_2$-ABEE was obtained, corresponding to the release of four mannose residues (Fig. 4). Sequential exoglycosidase digestion of the product with jack bean α-mannosidase, *T. pyriformis* α-glucosidase and jack bean α-mannosidase produced the same result as those described for NM-e (Fig. 5). On the basis of these results, the oligosaccharide in NM-g was identified (Table 2).

The elution profile of **NM-h** from the Amide-80 column suggested that the oligosaccharide had an additional hexose residue compared with $Glc_1Man_9GlcNAc_2$-ABEE (Fig. 3). When fraction NM-h was sequentially digested with *A. saitoi* α-mannosidase I (Fig. 4) and jack bean α-mannosidase (Fig. 5), peak shifts corresponding to the release of two mannose followed by three mannose residues were indicated. The resultant product released two glucose residues by *T. pyriformis* α-glucosidase (Fig. 5). The ABEE-oligosaccharide obtained after α-glucosidase digestion showed the same mobility as $Man_4GlcNAc_2$-ABEE, and was converted to $Man_1GlcNAc_2$-ABEE by digestion with jack bean α-mannosidase (Fig. 5). Based on these results and methylation analysis, fraction NM-h contained $Glc_2Man_9GlcNAc_2$-ABEE (Table 2). To complete structural determination, the ABEE-oligosaccharide was analysed by ^{1}H-NMR (Table 3). The spectrum was closely similar to that of $Glc_1Man_9GlcNAc_2$-ABEE, except a downfield shift

Table 3. ^{1}H-Chemical shifts of structural reporter-groups for the ABEE derivatives of oligosaccharides.



Reporter group	*Residue*	*Chemical shifts*[a] *(ppm) in*				
		NM-h $Glc_2Man_9GN_2$*-ABEE*	*NM-g* $GlcMan_9GN_2$*-ABEE*	*Nm-f* $GlcMan_8GN_2$*-ABEE* Man_9GN_2*-ABEE*	*NM-e* $GlcMan_7GN_2$*-ABEE* Man_8GN_2*-ABEE*	$GlcMan_9GN_2$*-ABEE*[d]
H-1	GlcNAc-2	4.618	4.618	4.618	4.618	4.619
	Man-3	ND[b]	4.762	ND[b]	ND[b]	4.762
	Man-4	5.334	5.337	5.335	5.335	5.337
	Man-4*	ND[b]	4.867	4.866	4.861	4.867
	Man-A, (-A')[c]	5.401	5.406	5.402, (5.082)	5.405, (5.082)	5.403
	Man-B, (-B')[c]	5.140	5.146	5.143, (4.908)	5.140, (4.908)	5.144
	Man-C	5.305	5.311	5.309	5.310	5.309
	Man-D_1, (-D'_1)[c]	5.040	5.036	5.037, (5.052)	5.039, (5.054)	5.039
	Man-D_2	5.056	5.051	5.052	5.054	5.045
	Man-D_3	5.040	5.036	5.037	5.039	5.039
	Glc-a	5.273	5.257	5.256	5.256	5.258
	Glc-b	5.358	–	–	–	–
H-2	GlcNAc-1	4.342	4.336	4.338	4.338	4.342
	Man-3	4.216	4.217	4.215	4.215	4.216
	Man-4	4.091	4.092	4.092	4.091	4.091
	Man-4'	4.152	4.152	4.150	4.151	4.152
	Man-A, (-A')[c]	4.108	4.109	4.106, (4.068)	4.109, (4.069)	4.112
	Man-B, (-B')[c]	4.016	4.012	4.010, (3.990)	4.017, (3.988)	4.016
	Man-C	4.108	4.109	4.106	4.109	4.112
	Man-D_1, (-D'_1)[c]	4.239	4.238	4.236, (4.071)	4.238, (4.069)	4.239
	Man-D_2	4.070	4.075	4.071	4.069	4.070
	Man-D_3	4.070	4.075	4.071	4.069	4.070
NAc	GlcNAc-1	1.906	1.907	1.903	1.909	1.906
	GlcNAc-2	2.055	2.056	2.055	2.057	2.057

[a] Data were obtained at 500 MHz in ^2H_2O solution at 25 °C.
[b] Value could not be determined merely by spectrum inspection.
[c] A', B' and D'_1 represent mannose and glucose residues without Man-D_2, Man-D_3 and Glc-a, respectively.
[d] Reference oligosaccharide prepared from hen IgY.

($\Delta\delta = +0.016$ ppm) in the H-1 signal of Glc-a and an additional H-1 signal with $J = 4.0$ Hz detected at $\delta = 5.358$ ppm were observed. The downfield shift indicated that Glc-a was 3-O-substituted by a glucose [30, 31]. The signal at $\delta = 5.358$ ppm was from the terminal glucose [30, 31].

Analysis of fraction NC

The methylation analysis of fraction NC produced C-3 and C-6 disubstituted and C-3, C-4 and C-6 trisubstituted mannose as mannose residue at the branching point, indicating the presence of biantennary oligosaccharides with or without bisecting *N*-acetylglucosamine. The relative amount of terminal and C-4 substituted *N*-acetylglucosamine suggested that non or partially galactosylated biantennary oligosaccharides were predominant in this fraction. *N*-Acetylglucosamine at the reducing end was C-4 substituted and C-4 and C-6 disubstituted, indicating the presence both non-fucosylated and fucosylated oligosaccharides.

On further fractionation of the NC fraction by ODS/HPLC, nine subfractions (denoted by NC-a to NC-i) were separated and were categorized into the following three groups; group 1 (NC-a), corresponding to non-bisected and non-fucosylated biantennary oligosaccharides), group 2 (NC-b to NC-e) corresponding to bisected and non-fucosylated biantennary oligosaccharides), and group 3 (NC-f to NC-i) corresponding to bisected and fucosylated biantennary oligosaccharides [20].

Each subfraction was evaluated by the 2D-map technique, sequential exoglycosidase digestion and/or ^{1}H-NMR analysis. Fraction NM-b was eluted in the region of group 1 (elution position shown in Fig. 6 as dotted line). Analytical data for NM-b are as follows.

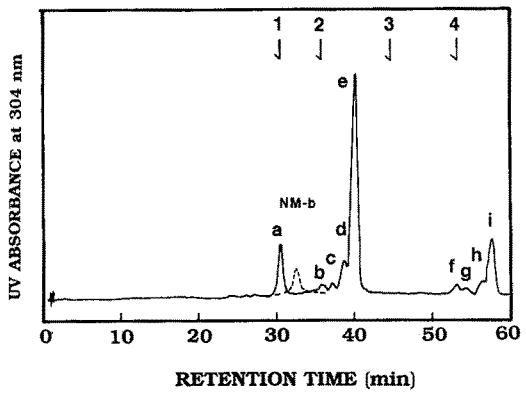


Figure 6. ODS/HPLC analysis of the ABEE-oligosaccharides in fraction NC of quail IgY. Fraction NC was obtained from total ABEE-oligosaccharides of quail IgY by Con A–agarose column chromatography. ABEE-oligosaccharides were subjected to HPLC on a Wakosil 5C18-200 (0.4 cm × 25 cm) column. The column was eluted with a linear gradient from 9% acetonitrile in the 100 mM acetic acid to 11% acetonitrile in the acetic acid for 60 min at a flow rate of 0.8 ml min^{-1} at 40 °C. A total of nine fractions (NC-a to -i) were collected. The dotted line represents the elution position of NM-b in Fig. 3. Arrows (↓) indicate the elution positions of standard ABEE-oligosaccharides: (1) $Gal_2GlcNAc_2Man_3GlcNAc_2$-ABEE; (2) $Gal_2GlcNAc_2Man_2GlcNAcManGlcNAc_2$-ABEE; (3) $Gal_2GlcNAc_2Man_3GlcNAcFucGlcNAc$-ABEE; and (4) $Gal_2GlcNAc_2Man_2GlcNAcManGlcNAcFucGlcNAc$-ABEE.

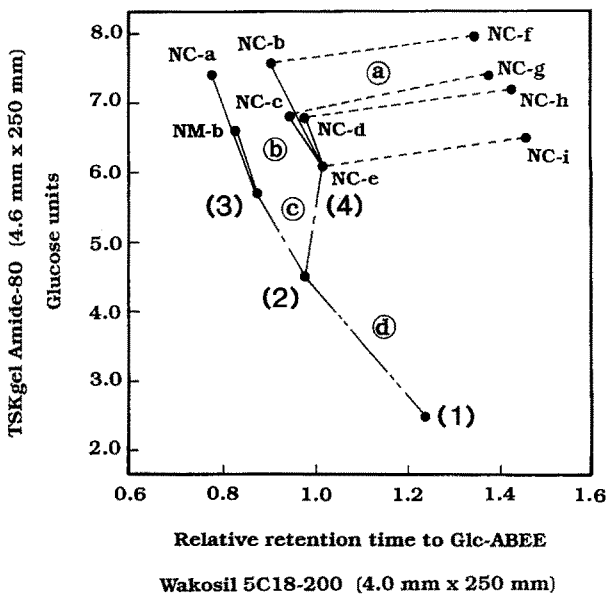


Figure 7. Two-dimensional map of the ABEE-oligosaccharides in NC-a to -i, and NM-b and products of their sequential exoglycosidase digestion. Each ABEE-oligosaccharide was analysed by HPLC on TSKgel Amide-80 and Wakosil 5C18-200 columns under the conditions described in Fig. 3 and Fig. 6. Elution positions of ABEE-oligosaccharides on Amide-80 and ODS columns were expressed as the number of glucose units and relative retention time to glucose-ABEE, respectively. Digestions were performed with bovine kidney α-L-fucosidase (----- ⓐ), jack bean β-galactosidase (——— ⓑ), jack bean β-*N*-acetylhexosaminidase (--- ⓒ) and jack bean α-mannosidase (---- ⓓ). Numerals indicate the elution positions of standard ABEE-oligosaccharides (1) $Man_1GlcNAc_2$-ABEE; (2) $Man_3GlcNAc_2$-ABEE; (3) $GlcNAc_2Man_3GlcNAc_2$-ABEE; and (4) $GlcNAc_2$○ $Man_2GlcNAcManGlcNAc_2$-ABEE.

Group 1 (NM-b and Nc-a). The elution positions of **NM-b** and **NC-a** on the 2D-map corresponded to those for authentic monogalactosyl (located on Manα1-3 branch) and digalactosyl biantennary oligosaccharide, respectively (Fig. 7). Both subfractions were further analysed by ^{1}H-NMR. The biantennary complex type structure was inferred from the characteristic signals for H-1 and *N*-acetylmethyl protons of GlcNAc-2, H-1 of Man-3, H-1 of Man-4 and H-1 of Man4′ [3, 8, 29]. The single galactose residue present in NM-b was located at the nonreducing terminal on the Manα1-3 branch. This assignment is based on the chemical shift values for H-1 of Gal-6 (δ = 4.466 ppm), Man-4 (δ = 5.113 ppm) and Mam-4′ (δ = 4.914 ppm) [3, 29]. The chemical shifts of various structural-reporter group protons of NC-a coincided completely with those of the authentic digalactosyl biantennary oligosaccharide (Table 4) [3, 8, 29].

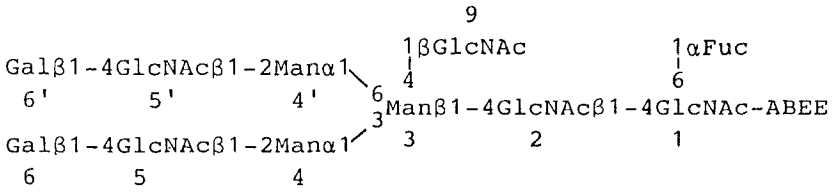
Degalactosylation of NM-b and NC-a showed peak shifts corresponding to respective release of one and two galactose residues, implying an ABEE-oligosaccharide with the same mobility as $GlcNAc_2Man_3GlcNAc_2$-ABEE. Further sequential digestion with β-*N*-acetylhexosaminidase and α-mannosidase gave peak shifts corresponding to the release of two *N*-acetylglucosamine followed by two mannose residues (Fig. 7).

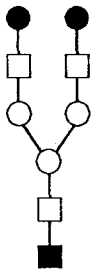
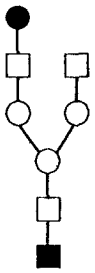
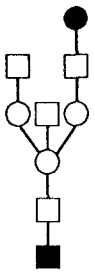
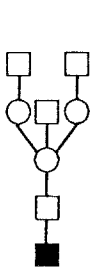
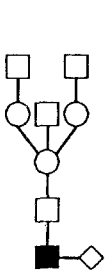
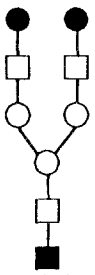
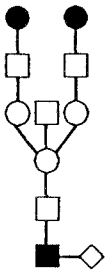
Thus, the structures of the oligosaccharides NM-b and NC-a were proposed as shown in Table 2.

Group 2 (NC-b to NC-e). The positions of **NC-b** to **NC-e** on a 2D-map corresponded to the ABEE-derivatives of bisected and non-fucosylated biantennary oligosaccharides with two, one (on Manα1-3 branch), one (on Manα1-6 branch) and no galactose residue, respectively [20]. This finding (or postulation) was substantiated by the results of sequential exoglycosidase digestion. After β-galactosidase digestion, their peak-shifts corresponded to the release of two, one, one and no galactose residues, respectively, and a product with the same mobility as authentic $GlcNAc_2Man_2GlcNAcManGlcNAc_2$-ABEE was obtained (Fig. 7). Subsequent digestions of the product with β-*N*-acetylhexosaminidase and α-mannosidase produced an ABEE-oligosaccharide with the same mobility as authentic $Man_1GlcNAc_2$-ABEE.

To complete the structural determination, NC-d and NC-e were each subjected to analysis by ^{1}H-NMR spectroscopy (Table 4). The presence of a bisecting β-GlcNAc residue in both NC-c and NC-e manifested itself by the characteristic signals for H-1 and *N*-acetylmethyl protons of GlcNAc-9 and by large upfield shifts of H-1 signals of Man-3 ($\Delta\delta$ = −0.07 ppm) and Man-4 ($\Delta\delta$ = −0.06 ppm) compared with NC-a or NM-b [3, 29].

Table 4. ^{1}H-chemical shifts of structural reporter-groups for the ABEE derivatives of oligosaccharides.



Reporter group	*Residue*	*Chemical shifts*[a] *(ppm) in*						
		Nc-a[b]	*NM-b*	*NC-d*	*NC-e*	*NC-i*	*R1*[c]	*R2*[d]
								
H-1	GlcNAc-2	4.627	4.622	4.623	4.618	4.686	4.625	4.680
	Man-3	4.762	4.760	4.689	4.686	4.692	4.762	4.690
	Man-4	5.117	5.113	5.058	5.056	5.058	5.117	5.052
	Man-4′	4.925	4.914	5.011	4.998	4.994	4.925	4.998
	GlcNAc-5	4.576	4.572	4.553	4.554	4.555	4.576	4.565
	GlcNAc-5′	4.576	4.550	4.571	4.536	4.537	4.576	4.565
	Gal-6	4.468	4.466	–	–	–	4.467	4.463
	Gal-6′	4.468	–	4.471	–	–	4.467	4.463
	GlcNAc-9	–	–	4.462	4.464	4.462	–	4.463
	Fuc	–	–	–	–	ND[d]	–	4.849
NAc	GlcNAc-1	1.906	1.907	1.900	1.900	1.933	1.907	1.938
	GlcNAc-2	2.067	2.065	2.072	2.066	2.069	2.068	2.074
	GlcNAc-5	2.052	2.052	2.058	2.061	2.052	2.052	2.056
	GlcNAc-5′	2.043	2.046	2.032	2.041	2.043	2.043	2.033
	GlcNAc-9	–	–	2.069	2.066	2.061	–	2.066
Me	Fuc	–	–	–	–	1.115	–	1.120

[a] Data were obtained at 500 MHz in ^2H_2O solution at 25 °C.
[b] Structures are represented as follows: ●, Gal; □, GlcNAc; ○, Man; ◇, Fuc; and ■, GlcNAc-ABEE.
[c] R1 and R2 correspond to reference compounds obtained from human chorionic gonadotropin and hen IgY, respectively [8, 39].
[d] Value could not be determined.

In the ^{1}H-NMR spectrum of NC-e, signals for H-1 or GlcNAc-5 and GlcNAc-5′ were noted at $\delta = 4.554$ ppm and $\delta = 4.536$ ppm, respectively. However, signals for H-1 of galactose were not indicated. Thus, the structure of ABEE-oligosaccharide in NC-e was confirmed as a bisected and non-fucosylated biantennary oligosaccharide containing no galactose residue (Table 2). In the ^{1}H-NMR spectrum of NC-d, a signal attributed to H-1 of β-Gal residue was observed at $\delta = 4.471$ ppm. The location of the galactose residue was determined to be the Manα1-6 branch based on the upfield shifts of H-1 signals for Man-4′ and GlcNAc-5′ compared with those in NC-e. Thus, the ABEE-oligosaccharide in NC-d was proposed as a bisected and non-fucosylated biantennary oligosaccharde with a galactose of the Manα1-6 branch (Table 2).

Group 3 (NC-f to NC-i). The positions of ABEE-oligosaccharides in **NC-f** to **NC-i** on the 2D-map corresponded to the ABEE-derivatives of bisected and fucosylated biantennary oligosaccharides with two, one (on Manα1-3 branch), one (on Manα1-6 branch) and no galactose residues, respectively [20]. Defucosylation of these oligosaccharides shifted their retention times to coincide with peaks NC-b, NC-c, NC-d and NC-e, respectively. Results of subsequent sequential exoglycosidase digestion were similar to those of NC-b to NC-e.

The structure of ABEE-oligosaccharide in NC-i was confirmed by ^{1}H-NMR analysis (Table 4). Chemical shifts of various structural-reporter group protons of NC-i matched completely those of the authentic bisected and fucosylated biantennary oligosaccharide that was devoid of galactose residues. From these results, the structures of oligosaccharide in NC-f to NC-i were proposed (Table 2).

Discussion

The oligosacccharide structures of Japanese quail IgY have been determined and are summarized in Table 2. The results obtained in this study show that the quail IgY contains three types of oligosaccharides, mono- (35.6%) and diglucosylated (0.6%) oligomannose type oligosaccharides with the structure $Glc_1Man_{7-9}GlcNAc_2$ and $Glc_2Man_9GlcNAc_2$; oligomannose type oligosaccharides with the structures $Man_{5-9}GlcNAc_2$ (15.0%); and complex-type biantennary oligosaccharides (46.4%) with or without a bisecting *N*-acetylglucosamine residue, a fucose residue attached to reducing terminal *N*-acetylglucosamine and nonreducing terminal galactose residues.

In a previous study [8], we have reported that hen IgY contains unusual monoglucosylated oligomannose type oligosaccharides with the same structures as those from quail IgY. However, diglucosylated oligosaccharide found in quail IgY has not been detected in hen IgY. Among the monoglucosylated oligosaccharides, $Glc_1Man_9GlcNAc_2$ has been found in oligosaccharides of mature glycoproteins [31, 32], but not $Glc_1Man_{7-8}GlcNAc_2$. Glucosylated oligomannose type oligosaccharides are the key intermediates in the biosynthesis and processing of Asn-linked oligosaccharides. In the common oligosaccharide processing pathway, soon after a transfer of lipid-linked $Glc_3Man_9GlcNAc_2$ to nascent polypeptides, the three glucose residues are quickly eliminated and then trimming of the α(1-2) linked mannose residues occurs [33]. Recently, two alternative Asn linked oligosaccharide processing routes involving the transient formation of $Glc_1Man_{7-9}GlcNAc_2$ have been postulated [34, 35]. One involves the formation of glucosylated oligosaccharides by glucosylation of protein-linked $Man_{7-9}GlcNAc_2$ [34], and the other by successive deglucosylation and demannosylation of protein-linked $Glc_3Man_9GlcNAc_2$ [35]. The glucose residues are quickly degraded by α-glucosidase II [34] or endo-α-mannosidase [35]. The reason(s) for the presence of monoglucosylated oligosaccharides in the mature glycoprotein remains to be determined.

Although the structures of oligomannose type oligosaccharides of quail IgY were similar to those of hen IgY, the amounts were different.

Complex-type oligosaccharides of both quail and hen IgY displayed exclusively biantennary structures. However, there were several differences among them. Two-thirds of the complex-type oligosaccharides of hen IgY are mono- or disialylated, but none of the oligosaccharides in quail IgY was sialylated. Bisected biantennary oligosaccharides predominated in hen and quail IgY. Although these compounds were wholly (96%) fucosylated in hen IgY, those of quail IgY were either fucosylated (26%) or non-fucosylated (75%).

Fucosylated biantennary oligosaccharides without a bisecting *N*-acetylglucosamine were either absent or found only in trace amounts in these birds.

Both quail and hen IgY contained biantennary oligosaccharides with two, one and no galactose residues. However, the ratios of these oligosaccharides were 1:1.4:3 in quail IgY and 7:1.5:1 in hen IgY. The galactose residue in monogalactosyl oligosaccharides in quail IgY was distributed in both the Manα1-3 and Manα1-6 branch, and the oligosaccharide with a galactose on the Manα1-6 predominated over that with a galactose on the Manα1-3 branch. In contrast, the galactose residue was exclusively distributed on the Manα1-6 branch in hen IgY.

It is of interest to compare the structures of peptides and oligosaccharides of avian IgG with those of mammalian immunoglobulins. On the basis of nucleotide sequence determination of chicken immunoglobulin cDNA, the chicken Cγ differs from any mammalian Cγ and is closely related to the mammalian Cε and Cμ in length and the presence of four CH domains [36]. Moreover, the distribution of cysteine in the CH1 and CH2 domains of chicken Cγ resembles that of mammalian Cε. As such, Parvari *et al.* [36] propose that chicken Cγ is the ancestor of the mammalian Cε and Cγ subclasses. Thus, our findings further advocate that oligosaccharide patterns of hen and quail IgY differ from those of any mammalian IgG [2–7], although apparent resemblance to those of mammalian IgM [37] and IgE [38] are observed. It is presumed that the protein architecture of immunoglobulins affects their oligosaccharide patterns.

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