

## Microheterogeneity and structures of neutral glycans present in quail ovomucoid

Josefa María ALONSO, Patrick BOULENGUER, Jean-Michel WIERUSZESKI, Yves LEROY, Jean MONTREUIL and Bernard FOURNET

Laboratoire de Chimie Biologique de l'Université des Sciences et Techniques de Lille Flandres Artois (Unité 217 du Centre National de la Recherche Scientifique et Laboratoire Pilote du Ministère de l'Éducation Nationale) Villeneuve d'Ascq

(Received December 16, 1987/May 20, 1988) – EJB 87 1395

Anionic chromatography of oligosaccharide-alditols obtained by alkaline hydrolysis of quail ovomucoid glycopeptides resulted in the isolation of a major neutral fraction (83%) which was separated into 14 fractions by high-performance liquid chromatography on an alkylamine silica column. The structures of nine oligosaccharide-alditols were determined. By the identification of mannosido-, and mannotriosido-di-*N*-acetylchitobiose mixed with *N*-acetyl-lactosaminic and oligomannosidic-types oligosaccharides, we demonstrate the high microheterogeneity of the glycans presents in the neutral fraction of this glycoprotein and suggest that ovomucoid glycosylation uses several pathways.

Hen and quail ovomucoids are characterized by a high degree of microheterogeneity in their carbohydrate moieties [1–3]. Ovomucoid is known as a trypsin inhibitor [4]. In both cases the protein part possesses four glycosylation sites; the glycosylation sites 1, 2 and 4 have the same amino acid sequence around the asparagine residue. For the glycosylation site 3, the glycan is linked to Asn<sup>69</sup> in hen ovomucoid and Asn<sup>75</sup> in quail ovomucoid [5, 6].

Hen ovomucoid glycan structural studies were first reported by Wooley in 1942 [7] and subsequently completed by Kobata [2, 8] and Montreuil [9–12]. As to quail (*Coturnix coturnix*) ovomucoid, Hase et al. [3, 13] have studied glycans liberated by hydrazinolysis and coupled with 2-amino-pyridine. By using exoglycosidases, methylation analysis and Smith degradation, these authors described the primary structure of three oligosaccharides and deduced the structure of three other oligosaccharides from HPLC elution positions of native oligosaccharides and enzymatic degradation products.

In this paper we report the separation of 14 neutral oligosaccharide fractions and the complete structures of nine oligosaccharides using methylation and FAB-MS analysis and 400-MHz <sup>1</sup>H-NMR spectroscopy.

### EXPERIMENTAL PROCEDURES

#### *Glycoproteins, glycopeptides and oligosaccharides*

Quail ovomucoid was prepared according to Fredericq and Deutsch [14]. The glycopeptides were isolated after pronase hydrolysis of ovomucoid according to Monsigny et al. [15]. Oligosaccharides were released from glycopeptides by treatment with 1 M NaOH in the presence of 1 M KBH<sub>4</sub> for 6 h at 100°C as previously described [16]. The oligosaccharide-alditols were *N*-reacetylated according to Reading et al. [17]. The oligosaccharides were purified by gel filtration on a

Sephadex G-15 column (100 × 2.5 cm internal diameter, Pharmacia Fine Chemicals) using 0.5% acetic acid in water as eluant (30 ml/h); the volume of each fraction was 4 ml. The fractions were revealed with orcinol/sulfuric acid reagent [18] on silica gel plates (precoated silica gel 60; Merck, Darmstadt, FRG).

#### *Separation of oligosaccharide-alditols by HPLC*

Oligosaccharide-alditols obtained by alkaline degradation from quail ovomucoid glycopeptides were fractionated by HPLC on a 10-μm Micro Pak AX-10 column (50 × 0.8 cm internal diameter; Varian; Walnut Creek, CA, USA) [19], with a Spectra-Physics model 8700 liquid chromatograph equipped with a Spectro monitor D (LDC/Milton Roy) variable-wavelength detector connected to a model 4100 computing integrator.

For preparative chromatography, 20 mg oligosaccharide-alditols dissolved in 500 μl distilled water was subjected to HPLC using a gradient of 500 mM potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) as follows: elution with distilled water for 20 min, linear gradient to 2.5% KH<sub>2</sub>PO<sub>4</sub> (500 mM) for 10 min, isocratic elution for 15 min, linear gradient to 5% KH<sub>2</sub>PO<sub>4</sub> (500 mM) for 10 min, isocratic elution for 15 min and linear gradient to 40% KH<sub>2</sub>PO<sub>4</sub> (500 mM) for 60 min. The flow rate was 2 ml/min. The oligosaccharides were detected at 200 nm with a detector sensitivity of 0.5 and 16 integrator attenuation. The chart speed of the integrator was 0.5 cm/min.

Each fraction was purified by gel filtration on Sephadex G-15 with distilled water as eluent. The fractions were revealed as described previously.

HPLC separation of neutral oligosaccharide-alditols was performed on a 5-μm Zorbax-NH<sub>2</sub> column (25 × 0.94 cm internal diameter; Du Pont de Nemours and Co., Inc., Wilmington, DE, USA). The column was equilibrated with the initial solvent (acetonitrile/water, 65:35); 10 mg neutral oligosaccharide-alditols dissolved in 100 μl water was injected. After the injection, a linear gradient from initial conditions to acetonitrile/water (60:40) was applied for 40 min, followed by isocratic conditions during 20 min and by a linear gradient from acetonitrile/water (60:40) to acetonitrile/water (50:50)

Correspondence to B. Fournet, Laboratoire de Chimie Biologique de l'Université des Sciences et Techniques de Lille Flandres Artois (U.A. no. 217 du CNRS et Laboratoire Pilote du Ministère de l'Éducation Nationale), F-59655 Villeneuve d'Ascq Cedex, France

Abbreviations. FAB-MS, fast-atom-bombardment mass spectrometry; NeuAc, *N*-acetylneuraminic acid; GlcNAc-ol, *N*-acetylglucosaminitol.

for 30 min. The flow rate was 3 ml/min. The oligosaccharides were detected at 200 nm with a detector sensitivity of 1.0 and an integrator attenuation of 16. The chart speed of the integrator was 0.5 cm/min.

The fractions were purified by HPLC gel filtration on a column of TSK HW-40 (36 × 1.5 cm; Merck, Darmstadt). The oligosaccharides were detected with an ultraviolet detector (SP 8400) at 206 nm. The flow rate of 0.5% acetic acid in water was 1 ml/min.

#### *Molar composition of oligosaccharides*

The molar composition of oligosaccharides was determined by gas-liquid chromatography after methanolysis (0.5 M HCl/methanol; 24 h, 80°C) and pertrifluoroacetylation [20].

#### *Thin-layer chromatography of oligosaccharides*

TLC of neutral oligosaccharide-alditols was performed on silica gel plates (precoated Silica gel 60; Merck, Darmstadt) using ethanol/*n*-butanol/pyridine/acetic acid/water (100:10:10:3:30, by vol.) during 7 h according to Bayard et al. [21]. Oligosaccharides were revealed with orcinol/sulfuric acid reagent [18].

#### *Permethylation and mass spectrometry*

Permethylation of the oligosaccharide-alditols was performed according to Ciucanu and Kerek [22] with methyl iodide, solid base (NaOH) and methyl sulphoxide. The partially methylated and acetylated monosaccharides were identified by GLC-MS according to [23].

Fast atom bombardment [24, 25] was performed on a Kratos MS 50 RF high-resolution equipped with a DS 90 (DGDG/30) data system; permethylated oligosaccharide-alditols were dissolved in methanol (5 µg/µl). The copper tip was first loaded with 1 µl 0.1% sodium acetate in methanol; after drying, 3–4 µl thioglycerol and 0.5–1.0 µl sample solution was applied. The copper tip was bombarded with xenon atoms having a kinetic energy equivalent to 7.3 kV; the mass spectrometer was operated at 8 keV accelerating potential. Spectra were recorded in the positive-ion mode. The mass range 650–3200 Da was scanned at 10 s/decade with a mass resolution of 3500 Da. The mass values indicated are in most of the analysis lower by 1 Da than the calculated ones based on: C = 12.011; H = 1.008; O = 15.999; N = 14.007.

#### *400-MHz <sup>1</sup>H-NMR spectroscopy*

For <sup>1</sup>H-NMR analysis, the oligosaccharide-alditols were repeatedly exchanged in D<sub>2</sub>O (99.96% atom D, Aldrich). <sup>1</sup>H-NMR spectroscopic analysis was performed on a Bruker AM-400 WB spectrometer operating in the Fourier-transform mode at a probe temperature of 300 K. Chemical shifts are given in ppm relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (indirectly to acetone in D<sub>2</sub>O: δ = 2.225 ppm) [26].

## RESULTS

#### *Preparation of neutral oligosaccharide-alditols from quail ovomucoid*

Quail ovomucoid (5.8 g) was submitted to pronase digestion. The fractionation of glycopeptides on Sephadex G-25

yielded two fractions denoted A (613 mg) and B (141 mg). In comparison with the reported carbohydrate composition for quail ovomucoid (Gal:Man:GlcNAc:NeuNAc = 0.54:3:1.49:0.6) the glycopeptide fraction A possesses the same molar carbohydrate composition with a slight increase in galactose content (0.61 mol/3 mol Man). The glycopeptide fraction B shows more differences with decreased content of galactose (0.33 mol), *N*-acetylglucosamine (1.10 mol) and *N*-acetylneuraminic acid (0.02 mol) calculated relative to 3 mol mannose. After alkaline hydrolysis of the major fraction A (613 mg) and *N*-reacetylation, the oligosaccharide-alditols were purified on a Sephadex G-15 column yielding thus 391 mg oligosaccharide-alditols. The mixture of oligosaccharide-alditols was fractionated by HPLC (Fig. 1) on quaternary amine packings into one neutral (fraction A-I) and three acidic fractions (fractions A-II, A-III, A-IV). Results of the preparative chromatography of 340 mg total oligosaccharide-alditols and the carbohydrate composition are given in Table 1. The use of a Micro Pak AX-10 column provides quantitative recovery (95%) of the oligosaccharide-alditols. The neutral fraction (A-I) represents 82.8% of total oligosaccharide-alditols. Fractions A-II, A-III and A-IV (14.5%, 2.3% and 0.4%, respectively) have the same chromatographic mobilities as monosialylated, disialylated and trisialylated oligosaccharides [19] but with low quantities of sialic acid residues. These chromatographic mobilities can be explained by the presence of sulphate or phosphate groups.

The neutral fraction (A-I) from AX-10 chromatography of oligosaccharide-alditols was subjected to HPLC using acetonitrile/water and an alkylamine-modified silica column. The effective separation of 14 fractions was obtained in 60 min (Fig. 2). The yield of oligosaccharide-alditols was 77%. Each fraction was analyzed by TLC (Fig. 3) and the carbohydrate composition analyzed by GLC (Table 2).

These oligosaccharide-alditol fractions were studied by methylation analysis. The permethylated oligosaccharide-alditols were analyzed by FAB-MS and the methyl ether derivatives obtained after methanolysis of permethylated oligosaccharide-alditols were identified by GLC-MS (Table 3). All oligosaccharide-alditol fractions were subjected to 400-MHz <sup>1</sup>H-NMR spectroscopy (Table 4).

#### *Fraction A-I-1*

This fraction represents 2.6% of total neutral oligosaccharide-alditols. The methyl-ether composition (Table 3) obtained after methanolysis of permethyl-oligosaccharide-alditol A-I-1 shows that this fraction contains a mixture of oligosaccharides. The identification of two tri-*O*-methyl mannoses (2,3,4- and 2,4,6-tri-*O*-methyl mannose: total amount 0.90 mol/mol) and one 2,4-di-*O*-methyl mannose residue, suggest the presence of a mixture of mannobiosido-di-*N*-acetylchitobiositols and mannotriosido-di-*N*-acetylchitobiositol in the same proportion. As to the mannobiosido-di-*N*-acetylchitobiositols, the molar ratio of tri-*O*-methyl mannose derivatives allows us to conclude on the presence of 80% of Man(1–6)Man-GlcNAc-GlcNAc-ol and 20% of Man(1–3)Man-GlcNAc-GlcNAc-ol.

These results were confirmed by the FAB mass spectrum of the permethylated fraction A-I-1 by the molecular ions at *m/z* 961 (MH)<sup>+</sup> and 983 (M + Na)<sup>+</sup> corresponding to the (Man)<sub>2</sub>GlcNAc-GlcNAc-ol and the molecular ions at *m/z* 1165 (MH)<sup>+</sup> and 1187 (M + Na)<sup>+</sup> corresponding to the mannotriosido-di-*N*-acetylchitobiositol.

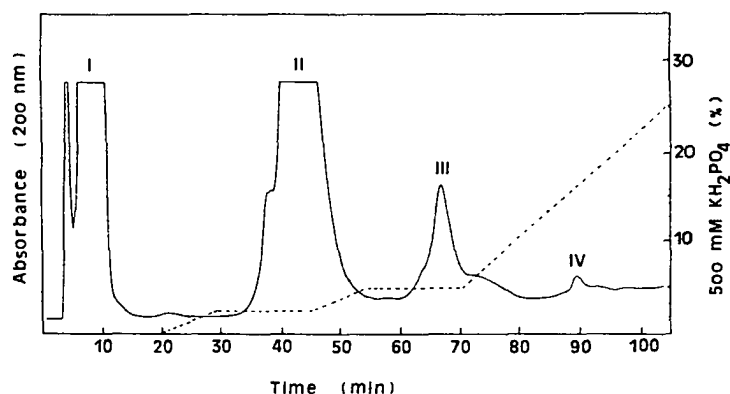


Fig. 1. Separation by HPLC on an AX-10 column of the oligosaccharide-alditols obtained by alkaline hydrolysis from quail ovomucoid glycopeptides (fraction A)

Table 1. Carbohydrate composition and masses of fractions obtained by AX-10 preparative chromatography of oligosaccharide-alditols liberated by alkaline hydrolysis from quail ovomucoid glycopeptides (fraction A)

Values are given relative to mannose = 3

| Fractions                                      | Molar ratio of |     |        |           |       | Mass   |
|------------------------------------------------|----------------|-----|--------|-----------|-------|--------|
|                                                | Gal            | Man | GlcNAc | GlcNAc-ol | NeuAc |        |
|                                                |                |     |        |           |       | mg     |
| Total oligosaccharide-alditols                 | 0.62           | 3   | 1.80   | 0.78      | 0.09  | 340.00 |
| Fraction A-I, neutral oligosaccharide-alditols | 0.57           | 3   | 1.70   | 0.76      | —     | 267.50 |
| Fraction A-II                                  | 1.20           | 3   | 2.75   | 0.87      | 0.63  | 47.00  |
| Fraction A-III                                 | 1.63           | 3   | 3.04   | 0.49      | 1.05  | 7.32   |
| Fraction A-IV                                  | 1.37           | 3   | 2.43   | 0.32      | 0.64  | 1.28   |
| Recovery                                       |                |     |        |           |       | 323.10 |

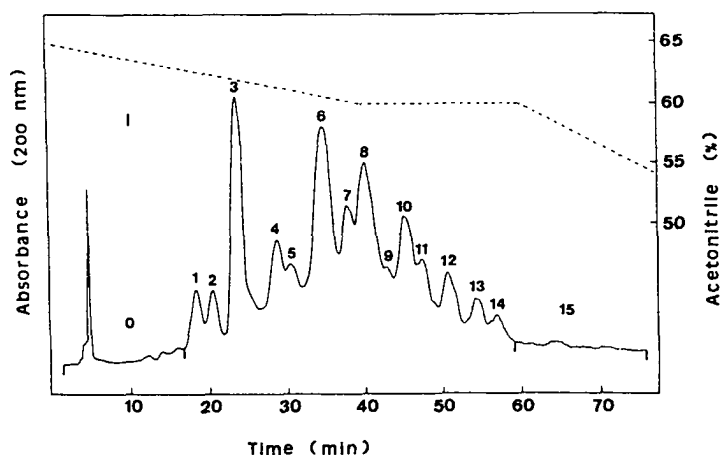


Fig. 2. Analysis of neutral oligosaccharide-alditols (fraction A-I) from quail ovomucoid by HPLC on a primary amine-bonded silica column (Zorbax-NH<sub>2</sub>)

In order to obtain independent proof of the structure of fraction A-I-1 the sample was subjected to 400-MHz <sup>1</sup>H-NMR spectroscopy. This fraction A-I-1 consisted of a mixture of two mannosido-di-*N*-acetylchitobiositols as revealed by the chemical shifts of H-1 of Man 3:  $\delta$  = 4.775 ppm and 4.782 and by the chemical shifts of H-2 of Man 3:  $\delta$  = 4.080 ppm and 4.252 ppm for the mannosido-di-*N*-acetylchitobiositols with Man 4' and Man 4 at a non-reducing terminal position respectively [26]. The NMR spectrum analysis shows also that these two mannosido-di-*N*-acetylchitobiositols are contaminated by a degraded product from

mannotrioso-di-*N*-acetylchitobiositol. The quantification of the various species in A-I-1 by integration of H-1 of Man 3, Man 4 and 4' is in accordance with the methylation data. Alkaline hydrolysis gave an heterogeneity on the GlcNAc 1. This was demonstrated by the effect on the chemical shifts of the *N*-acetyl proton of GlcNAc-1-ol (two signals at  $\delta$  = 2.056 ppm and  $\delta$  = 2.036 ppm) and by the chemical shift of the *N*-acetyl proton of GlcNAc 2 (one signal at  $\delta$  = 2.078 ppm). This heterogeneity can be assigned to an *N*-acetylmannosaminitol formed during alkaline hydrolysis as suggested by Austen and Marshall [27].

Table 2. Carbohydrate composition and masses of fractions obtained by HPLC chromatography on primary amine-bonded silica column of fraction A-I (neutral oligosaccharide-alditols)

Values are given relative to mannose = 3

| Fractions | Molar ratio of |     |        |           | Mass<br>mg |
|-----------|----------------|-----|--------|-----------|------------|
|           | Gal            | Man | GlcNAc | GlcNAc-ol |            |
| A-I       | 0.57           | 3   | 1.70   | 0.76      | 178.00     |
| A-I-0     | 0.63           | 3   | 1.72   | 0.82      | 5.60       |
| A-I-1     |                | 3   | 1.04   | 0.91      | 3.60       |
| A-I-2     |                | 3   | 0.82   | 0.38      | 5.00       |
| A-I-3     |                | 3   | 1.00   | 1.00      | 18.90      |
| A-I-4     | 0.21           | 3   | 1.88   | 0.86      | 7.10       |
| A-I-5     | 0.42           | 3   | 2.31   | 0.50      | 6.60       |
| A-I-6     | 0.57           | 3   | 2.08   | 0.81      | 25.90      |
| A-I-7     | 0.45           | 3   | 2.72   | 0.70      | 8.20       |
| A-I-8     | 0.76           | 3   | 2.15   | 0.77      | 17.80      |
| A-I-9     | 0.68           | 3   | 2.09   | 0.59      | 4.00       |
| A-I-10    | 1.01           | 3   | 2.37   | 0.69      | 10.30      |
| A-I-11    | 0.44           | 3   | 1.29   | 0.48      | 7.50       |
| A-I-12    | 1.41           | 3   | 2.77   | 0.72      | 6.50       |
| A-I-13    | 0.41           | 3   | 0.92   | 0.40      | 4.60       |
| A-I-14    | 1.77           | 3   | 3.27   | 0.70      | 3.70       |
| A-I-15    | 2.20           | 3   | 3.40   | 0.46      | 2.50       |
| Recovery  |                |     |        |           | 137.80     |

Based on the carbohydrate methylation, FAB-MS analysis and NMR data, the structure of oligosaccharide-alditols in fraction A-I-1 is proposed to be as shown in Fig. 5.

#### Fraction A-I-2

This fraction corresponds to the alkaline hydrolysis degradation products. The degradation of oligosaccharide-alditols can be observed by the low quantity of GlcNAc-ol (0.38 mol/mol, Table 2) and 1,3,5,6-tetra-*O*-methyl glucosaminitol (0.42 mol/mol, Table 3) obtained after methanolysis of permethyl fraction A-I-2. The methylation data suggests the presence of a trimannosido core in this fraction. By FAB mass spectrometry of the permethyl fraction A-I-2, we found two ions at  $m/z$  942 ( $M + Na$ )<sup>+</sup> and 1058 ( $M + Na$ )<sup>+</sup> corresponding to (Man)<sub>3</sub>GlcNAc-ol and (Man)<sub>3</sub>GlcNAc-GlcNAc-ol-129.

As compared to the NMR spectrum of fraction A-I-3 (Fig. 6), the fraction A-I-2 revealed the same chemical shifts for H-1 and H-2 protons of Man 3, Man 4 and Man 4' with values described for the mannotriosido-di-*N*-acetylchitobiositol [11]. As observed by carbohydrate analysis of the native fraction, methylation analysis of the permethylated fraction and FAB mass spectrometry, the NMR spectrum does not reveal the signals at  $\delta$  = 2.056 ppm and 2.036 ppm corresponding to GlcNAc-1-ol. In the same way we were not able to observe the signal at  $\delta$  = 4.242 ppm corresponding to the H-2 of GlcNAc-1-ol. The degraded GlcNAc-1-ol affects considerably the chemical shift of H-1 of GlcNAc 2 ( $\Delta\delta$  = -0.02 ppm) but have no effect on representative protons of Man 3, Man 4 and Man 4' (see Table 4).

On the basis of these results, we can conclude that a degraded mannotriosido-di-*N*-acetylchitobiositol is present on the GlcNAc-1-ol residue.

#### Fraction A-I-3

This fraction represents 14% of total neutral oligosaccharide-alditols. It is composed of a pure mannotriosido-di-*N*-

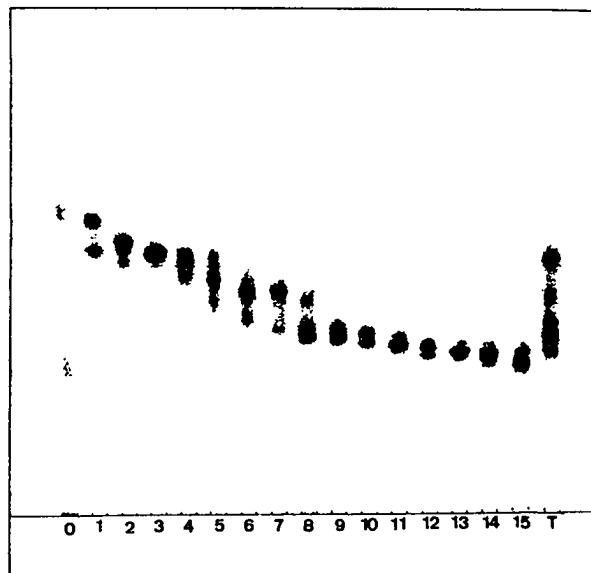


Fig. 3. Thin-layer chromatography on silica gel plates of the fractions obtained from neutral oligosaccharide-alditols (fraction A-I) by preparative chromatography on primary amine-bonded silica column. For thin-layer chromatographic conditions, see Experimental Procedures. T: total oligosaccharide-alditols in fraction A-I

acetylchitobiositol as confirmed by GLC molar carbohydrate composition (Table 2), methylation analysis (Table 3) and FAB-MS with the ion at  $m/z$  1188 ( $M + Na$ )<sup>+</sup> (Fig. 4).

NMR analysis of fraction A-I-3 shows data (Fig. 6 and Table 4) in agreement with the presence of a mannotriosido-di-*N*-acetylchitobiositol [11]. Nevertheless we observe the same perturbation for the *N*-acetyl proton of GlcNAc 1 with two signals at  $\delta$  = 2.056 ppm and  $\delta$  = 2.036 ppm corresponding to the degradation products from GlcNAc 1 produced during the alkaline hydrolysis of the GlcNAc-Asn linkage.

The structure of the oligosaccharide-alditol is given in Fig. 5.

#### Fraction A-I-4

This fraction represents 5.2% of total neutral oligosaccharide-alditols and possesses an additional GlcNAc residue in comparison with the mannotriosido-di-*N*-acetylchitobiositol fraction A-I-3. The identification of 0.2 residue galactose suggests that the fraction A-I-4 contains a slight contamination from fractions A-I-5 and A-I-6. The methylation data (Table 3) indicate a 1-2 linkage between the non-reducing glucosamine residue and the mannose 4 or 4'. The ion at  $m/z$  1432 ( $M + Na$ )<sup>+</sup> in the FAB-MS spectrum confirms the composition of the major oligosaccharide-alditol in fraction A-I-4 as GlcNAc(Man)<sub>3</sub>GlcNAc-GlcNAc-ol. In order to specify which mannose residue (4 or 4') was substituted by the additional GlcNAc residue, the fraction A-I-4 was subjected to 400-MHz <sup>1</sup>H-NMR spectroscopy.

As compared to the NMR spectrum of compound A-I-3, the oligosaccharide-alditols A-I-4 (Table 4) revealed an additional H-1 doublet at 4.551 ppm and also an additional *N*-acetyl signal at 2.049 ppm. These signals are attributed to the GlcNAc 5  $\beta$ 1-2-linked to Man 4 as demonstrated by downfield shift effects on H-1 and H-2 of Man 4 ( $\Delta\delta$  = +0.013 ppm and  $\Delta\delta$  = +0.121 ppm respectively). This ad-

Table 3. Molar ratios of monosaccharide methyl ethers present in the methanolysates of permethylated fractions A-I-1–14. Values are given relative to 2,4-di-*O*-methyl mannose = 1. *O*-Methyl is abbreviated as OMe, *N*-acetyl-*N*-methyl is abbreviated as NAcNMe

| Fraction | Molar ratio of partially methylated monosaccharides |                       |                   |                   |                   |                |                |                |                         |                      |                                 |
|----------|-----------------------------------------------------|-----------------------|-------------------|-------------------|-------------------|----------------|----------------|----------------|-------------------------|----------------------|---------------------------------|
|          | 2,3,4,6-tetra-OMe-Gal                               | 2,3,4,6-tetra-OMe-Man | 3,4,6-tri-OMe-Man | 2,3,4-tri-OMe-Man | 2,4,6-tri-OMe-Man | 2,4-di-OMe-Man | 3,4-di-OMe-Man | 2-mono-OMe-Man | 3,4,6-tri-OMe-GlcNAcNMe | 3,6-di-OMe-GlcNAcNMe | 1,3,5,6-tetra-OMe-Glc-ol NAcNMe |
| A-I-1    |                                                     | 2.50                  |                   | 0.75              | 0.15              | 1.0            |                |                |                         | 1.50                 | 1.53                            |
| A-I-2    |                                                     | 1.73                  |                   |                   |                   | 1.0            |                |                |                         | 0.85                 | 0.42                            |
| A-I-3    |                                                     | 1.94                  |                   |                   |                   | 1.0            |                |                |                         | 1.00                 | 1.10                            |
| A-I-4    | 0.20                                                | 0.82                  | 0.87              |                   |                   | 1.0            |                |                | 1.20                    | 1.15                 | 1.05                            |
| A-I-5    | 0.47                                                | 0.61                  | 1.10              |                   |                   | 1.0            |                |                | 0.70                    | 1.39                 | 0.40                            |
| A-I-6    | 0.85                                                | 1.00                  | 1.15              |                   |                   | 1.0            |                |                | 0.50                    | 1.51                 | 0.88                            |
| A-I-7    | 0.60                                                | 0.82                  | 1.90              |                   |                   | 1.0            |                | 0.89           | 2.77                    | 1.50                 | 0.92                            |
| A-I-8    | 0.70                                                | 0.82                  | 1.07              |                   |                   | 1.0            |                |                | 1.30                    | 1.13                 | 0.82                            |
| A-I-9    | 1.15                                                | 1.15                  | 1.49              |                   |                   | 1.0            | 0.43           | 0.50           | 1.70                    | 1.39                 | 0.72                            |
| A-I-10   | 1.10                                                | 0.57                  | 1.24              |                   |                   | 1.0            | 0.50           |                | 1.19                    | 1.42                 | 0.79                            |
| A-I-11   | 0.40                                                | 1.20                  | 1.03              |                   |                   | 1.0            |                |                | 0.45                    | 0.80                 | 0.60                            |
| A-I-12   | 1.16                                                | 0.45                  | 1.03              |                   |                   | 1.0            | 0.55           |                | 0.84                    | 1.38                 | 0.62                            |
| A-I-13   | 0.40                                                | 1.30                  | 1.20              |                   |                   | 1.0            |                |                | 0.30                    | 0.80                 | 0.60                            |
| A-I-14   | 1.20                                                | 0.40                  | 0.96              |                   |                   | 1.0            | 0.64           |                | 0.54                    | 1.04                 | 0.52                            |

ditional GlcNAc residue has not effect on other monosaccharide parameters [28].

On the basis of these data the structure of the oligosaccharide-alditol fraction A-I-4 is proposed in Fig. 5.

#### Fraction A-I-5

Like fraction A-I-2, the oligosaccharides obtained in fraction A-I-5 are alkaline hydrolysis degradation products. This fraction possesses the same carbohydrate composition as fraction A-I-6 except for the GlcNAc-ol residue (0.5 mol/mol in A-I-5, 0.81 mol/mol in A-I-6; Table 2). We could observe the same phenomena for the composition of methyl derivatives of glucosaminitol (Table 3). In the FAB-MS spectrum we observed the same modification of the terminal GlcNAc-ol residue by the ion at  $m/z$  1507 ( $M + Na$ )<sup>+</sup> corresponding to the oligosaccharide Gal-GlcNAc(Man)<sub>3</sub>GlcNAc-GlcNAc-ol – 129. The presence in the GLC pattern of permethylglucosamine and the identification in FAB-MS spectrum of an ion at  $m/z$  1548 ( $M + Na$ )<sup>+</sup> allowed us to conclude that the oligosaccharide (GlcNAc)<sub>2</sub>(Man)<sub>3</sub>GlcNAc-GlcNAc-ol was present in this fraction. We also observed in FAB-MS a minor ion at  $m/z$  1432 ( $M + Na$ )<sup>+</sup> corresponding to the oligosaccharide-alditol present in fraction A-I-4: GlcNAc(Man)<sub>3</sub>GlcNAc-GlcNAc-ol. This mixture was also observed by 400-MHz <sup>1</sup>H-NMR spectroscopy.

#### Fraction A-I-6

At 19% of total oligosaccharide-alditols, A-I-6 is the major fraction of neutral oligosaccharides from quail ovomucoid. This fraction contains a mixture of two peaks at  $m/z$  1636 ( $M + Na$ )<sup>+</sup> and  $m/z$  1677 ( $M + Na$ )<sup>+</sup> corresponding to Gal-GlcNAc(Man)<sub>3</sub>GlcNAc-GlcNAc-ol and (GlcNAc)<sub>2</sub>(Man)<sub>3</sub>GlcNAc-GlcNAc-ol in a ratio 85:15 respectively (Fig. 4). The methyl ether analysis (Table 3) confirms the proportion between these two oligosaccharide-alditols by the presence of permethylgalactose (0.85 mol/mol) from the non-reducing part of oligosaccharide-alditol with  $m/z$  1636 and of permethylglucosamine (0.5 mol/mol) from the non-reducing part of oligosaccharide-alditol with  $m/z$  1677. As shown by

the identification of permethylmannose, the oligosaccharide-alditol at  $m/z$  1636 possesses one mannose residue at the nonreducing end.

In order to obtain information for the lactosamine residue position (Man 4 or Man 4'), fraction A-I-6 was subjected to 400-MHz <sup>1</sup>H-NMR spectroscopy. The chemical shifts of the structural reporter group protons have been compiled in Table 4. As compared to the NMR data of fraction A-I-4, the oligosaccharide-alditol A-I-6 revealed an additional H-1 doublet at 4.466 ppm attributed to Gal 6 β1–4-linked to GlcNAc 5 as demonstrated by the modification of the chemical shift of the H-1 proton at  $\delta$  = 4.576 ppm ( $\Delta\delta$  = +0.025 ppm) [26]. This additional lactosamine residue is linked to the mannotriosido-di-*N*-acetylchitobiositol core by a β1–2-linkage to Man 4. Indeed the attachment of the lactosamine residue affects only the anomeric and H-2 protons of Man 4 ( $\delta$  = 5.119 ppm and  $\delta$  = 4.190 ppm respectively).

Based on chemical and spectroscopic data, the primary structure of oligosaccharide-alditol A-I-6 given in Fig. 5 consists as an extension of oligosaccharide-alditol A-I-4 with a Gal residue in β1–4 linkage to GlcNAc.

#### Fraction A-I-7

This fraction represents 6% of total neutral oligosaccharide-alditols. The results of methylation analysis (see Table 3) reveal the presence of 2,4-di-*O*-methyl mannose and 2-mono-*O*-methyl mannose. Assuming the presence of a mannotriose core structure, the identification of the aforementioned mannose methyl ether suggests the occurrence of one oligosaccharide-alditol with an intersecting GlcNAc residue β1–4-linked to Man 3. This result was confirmed by FAB-MS analysis with the identification of a major ion at  $m/z$  1923 ( $M + Na$ )<sup>+</sup> corresponding to (GlcNAc)<sub>3</sub>(Man)<sub>3</sub>GlcNAc-GlcNAc-ol (Fig. 4). The presence of other monosaccharide methyl ethers (Table 3) can be explained by the identification in the FAB-MS spectrum of minor ions at  $m/z$  1753 ( $M + Na$ )<sup>+</sup> and 1711 ( $M + Na$ )<sup>+</sup> corresponding to the oligosaccharides Gal(GlcNAc)<sub>2</sub>(Man)<sub>3</sub>GlcNAc-GlcNAc-ol – 129 and GlcNAc(Hex)<sub>5</sub>GlcNAc-GlcNAc-ol respectively.

The presence of the major oligosaccharide-alditol with an intersecting GlcNAc residue was confirmed by 400-MHz  $^1\text{H}$ -NMR spectroscopy. The resonance positions of the Man  $\underline{3}$ ,  $\underline{4}$  and  $\underline{4}'$  H-1 and H-2 protons in the spectrum of fraction A-I-7

(Table 4) clearly indicate the branching pattern to be intersected diantennary. The predominant differences in chemical shifts between the reporter group of fraction A-I-3 and A-I-7 are observed for H-1 of Man  $\underline{3}$ ,  $\underline{4}$  and  $\underline{4}'$  ( $\Delta\delta = -0.081$  ppm,

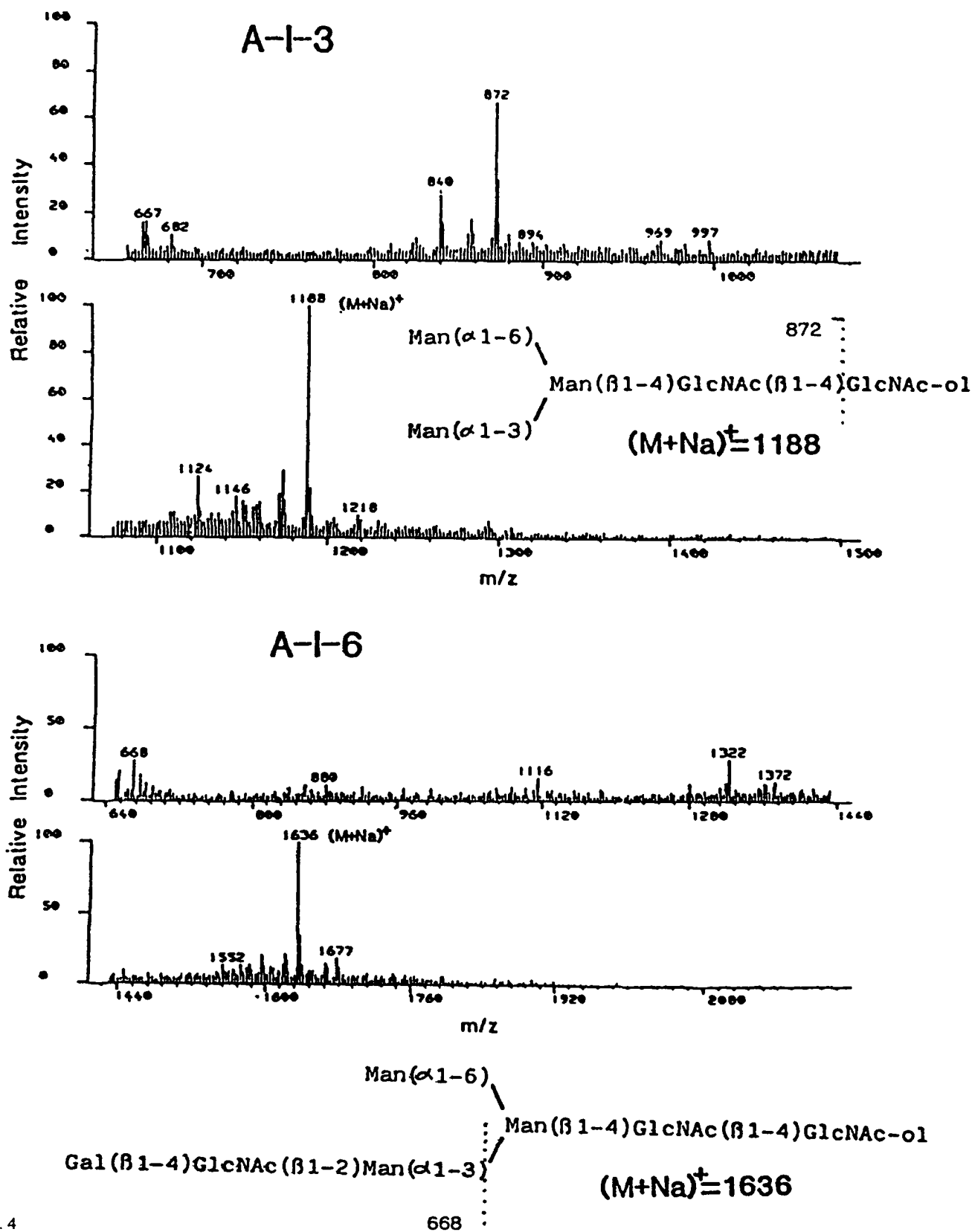


Fig. 4

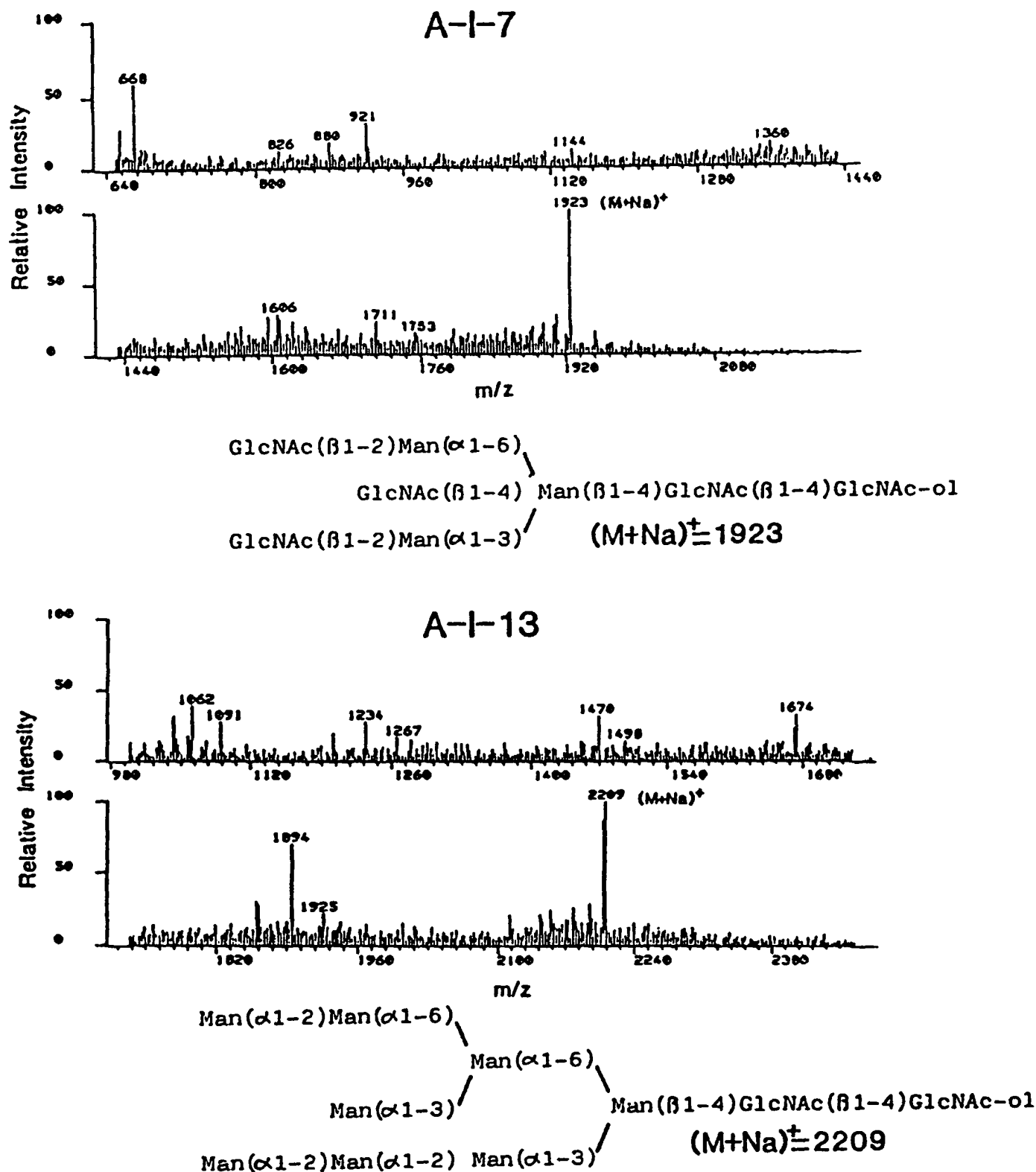


Fig. 4. FAB-MS spectra of permethylated oligosaccharide-alditol fractions A-I-3, A-I-6, A-I-7 and A-I-13

$\Delta\delta = -0.046$  ppm and  $\Delta\delta = +0.085$  ppm respectively) [29]. The additional intersecting GlcNAc residue (GlcNAc 9)  $\beta 1-4$ -linked to Man 3 with the H-1 proton at  $\delta = 4.466$  ppm and *N*-acetyl protons at  $\delta = 2.067$  ppm has no effects on chemical shifts of GlcNAc 5 and 5' H-1 and *N*-acetyl protons ( $\delta\text{H}-1 = 4.552$  ppm,  $\delta\text{N}$ -acetyl = 2.058 ppm and  $\delta\text{H}-1 = 4.545$  ppm,  $\delta\text{N}$ -acetyl = 2.048 ppm respectively).

The primary structure of intersected diantennary oligosaccharide-alditol A-I-7 is given in Fig. 5.

Fractions A-I-8, A-I-9, A-I-10, A-I-12 and A-I-14

This five fractions were composed of a very complex mixture of oligomannosidic, *N*-acetylglactosaminic and hybrid-

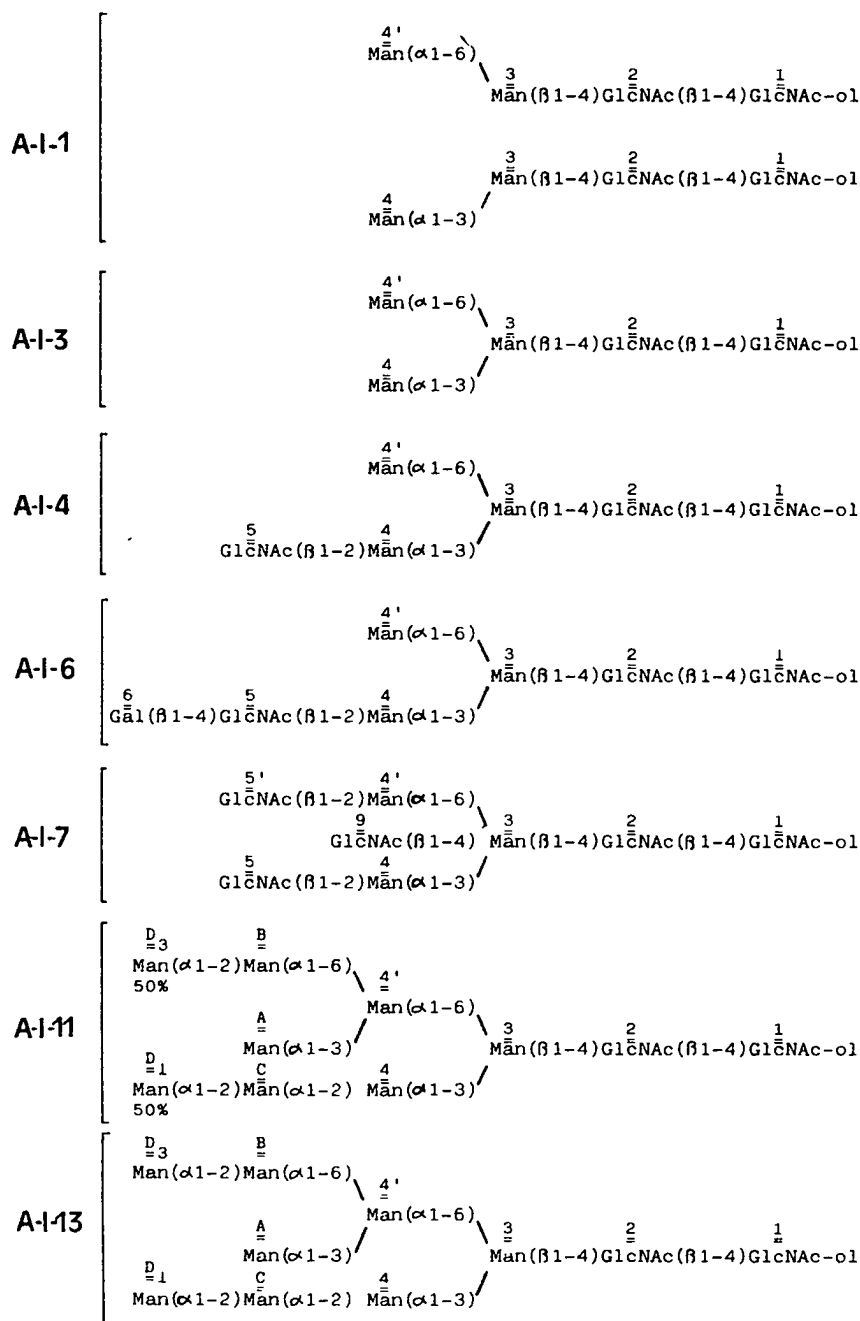


Fig. 5. Structures of the nine identified oligosaccharide-alditols isolated from quail ovomucoid

type oligosaccharide-alditols as is demonstrated by the high heterogeneity of methyl ethers (Table 3) obtained after methanolysis of permethylated fractions and also by FAB-MS analysis and 400-MHz  $^1\text{H}$ -NMR analysis.

#### Fraction A-I-11

With a high concentration of mannose with respect to GlcNAc residues (Table 2), this fraction (5% of total neutral oligosaccharides) was composed of a major oligomannosidic type oligosaccharide-alditol. The evidence for this structure was given by the FAB-MS analysis and the identification of the ion at  $m/z$  2004 ( $M + \text{Na}$ ) $^+$  corresponding to the oligosaccharide-alditol with  $(\text{Man})_7\text{GlcNAc-GlcNAc-ol}$ . The FAB-MS spectrum also shows an ion at  $m/z$  1689 ( $\text{MH}$ ) $^+$

indicating the presence in fraction A-I-11 of a hybrid-type oligosaccharide with  $\text{GlcNAc}(\text{Hex})_5\text{GlcNAc-GlcNAc-ol}$  - 129.

The 400-MHz  $^1\text{H}$ -NMR spectroscopy experiments yielded independent proof of the structure of the oligomannosidic-type oligosaccharide contained in fraction A-I-11. The NMR data shows that the oligosaccharide-alditol A-I-11 possesses an oligomannosidic-type structure with  $(\text{Man})_7(\text{GlcNAc})_2$  species with 50% of  $\text{Man } \underline{\text{D}}_3$  and 50% of  $\text{Man } \underline{\text{D}}_1$  (Table 4) [30].

This can be concluded from  $\text{Man } \underline{\text{4}} \text{ H-1}$  ( $\delta = 5.341$  ppm and  $\delta = 5.348$  ppm) proton shift which is characteristic for  $\text{Man } \underline{\text{C}}$  attachment and  $\text{Man } \underline{\text{4}}' \text{ H-1}$  ( $\delta = 4.869$  ppm), which suggests the presence of  $\text{Man } \underline{\text{A}}$  and  $\underline{\text{B}}$ .  $\text{Man } \underline{\text{A}} \text{ H-1}$  is detected at  $\delta = 5.092$  ppm. For the  $\text{Man } \underline{\text{B}} \text{ H-1}$  proton, two signals are observed at  $\delta = 4.908$  ppm when  $\text{Man } \underline{\text{B}}$  is in non-reducing

Table 4.  $^1\text{H}$ -chemical shifts of structural-reporter groups protons of constituent monosaccharides for the neutral oligosaccharide-alditols isolated from quail ovomucoid. Compounds are represented by a short symbolic notation: ● GlcNAc; ◆ Man; ■ Gal; ☐ modified GlcNAc-ol. X-ol corresponds to N-acetyl-D-mannosaminol (see text); n.p. = not present; n.d. = not determined

| Residue            | Reporter group | Shift of fraction (schematic structure shown) |       |       |             |             |             |             |             |  |  |  |  |  |
|--------------------|----------------|-----------------------------------------------|-------|-------|-------------|-------------|-------------|-------------|-------------|--|--|--|--|--|
|                    |                | A-I-1                                         | A-I-2 | A-I-3 | A-I-4       | A-I-6       | A-I-7       | A-I-11      | A-I-13      |  |  |  |  |  |
|                    |                |                                               |       |       |             |             |             |             |             |  |  |  |  |  |
|                    |                | ppm                                           |       |       |             |             |             |             |             |  |  |  |  |  |
| GlcNAc-1-ol        | H-2            | 4.243                                         | 4.243 | 4.242 | 4.243       | 4.242       | 4.242       | n.d.        | n.d.        |  |  |  |  |  |
| 1-ol/X-ol          | NAc            | 2.056/2.036                                   | n.p.  | n.p.  | 2.055/2.035 | 2.055/2.035 | 2.054/2.034 | 2.055/2.035 | 2.055/2.035 |  |  |  |  |  |
| GlcNAc 2           | H-1            | 4.641                                         | 4.641 | 4.639 | 4.639       | 4.637       | 4.635       | 4.629       | 4.629       |  |  |  |  |  |
|                    | NAc            | 2.078                                         | 2.078 | 2.076 | 2.077       | 2.076       | 2.078       | 2.063       | 2.063       |  |  |  |  |  |
| Man 3              | H-1            | 4.782                                         | 4.775 | 4.788 | 4.787       | 4.784       | 4.707       | 4.779       | 4.779       |  |  |  |  |  |
|                    | H-2            | 4.252                                         | 4.080 | 4.253 | 4.253       | 4.251       | 4.180       | 4.235       | 4.235       |  |  |  |  |  |
| Man 4              | H-1            | 5.104                                         | —     | 5.102 | 5.118       | 5.119       | 5.059       | 5.341       | 5.348       |  |  |  |  |  |
|                    | H-2            | 4.068                                         | —     | 4.067 | 4.189       | 4.190       | 4.248       | 4.090       | 4.090       |  |  |  |  |  |
| Man 4'             | H-1            | —                                             | 4.914 | 4.914 | 4.916       | 4.916       | 5.000       | 4.869       | 4.869       |  |  |  |  |  |
|                    | H-2            | —                                             | 3.969 | 3.974 | 3.975       | 3.975       | 4.147       | 4.146       | 4.146       |  |  |  |  |  |
| Man A              | H-1            | —                                             | —     | —     | —           | —           | —           | 5.092       | 5.092       |  |  |  |  |  |
|                    | H-2            | —                                             | —     | —     | —           | —           | —           | 4.072       | 4.072       |  |  |  |  |  |
| Man B              | H-1            | —                                             | —     | —     | —           | —           | —           | 4.908       | 5.142       |  |  |  |  |  |
|                    | H-2            | —                                             | —     | —     | —           | —           | —           | 3.986       | 4.025       |  |  |  |  |  |
| Man C              | H-1            | —                                             | —     | —     | —           | —           | —           | 5.302       | 5.055       |  |  |  |  |  |
|                    | H-2            | —                                             | —     | —     | —           | —           | —           | 4.072       | 4.105       |  |  |  |  |  |
| Man D <sub>1</sub> | H-1            | —                                             | —     | —     | —           | —           | —           | 5.042       | 5.042       |  |  |  |  |  |
|                    | H-2            | —                                             | —     | —     | —           | —           | —           | 4.063       | 4.064       |  |  |  |  |  |
| Man D <sub>3</sub> | H-1            | —                                             | —     | —     | —           | —           | —           | —           | —           |  |  |  |  |  |
|                    | H-2            | —                                             | —     | —     | —           | —           | —           | —           | —           |  |  |  |  |  |
| GlcNAc 5           | H-1            | —                                             | —     | —     | 4.551       | 4.576       | 4.552       | —           | —           |  |  |  |  |  |
|                    | NAc            | —                                             | —     | —     | 2.049       | 2.051       | 2.058       | —           | —           |  |  |  |  |  |
| GlcNAc 5'          | H-1            | —                                             | —     | —     | —           | —           | 4.545       | —           | —           |  |  |  |  |  |
|                    | NAc            | —                                             | —     | —     | —           | —           | 2.048       | —           | —           |  |  |  |  |  |
| Gal 6              | H-1            | —                                             | —     | —     | —           | 4.466       | —           | —           | —           |  |  |  |  |  |
| GlcNAc 9           | H-1            | —                                             | —     | —     | —           | —           | 4.466       | —           | —           |  |  |  |  |  |
|                    | NAc            | —                                             | —     | —     | —           | —           | 2.067       | —           | —           |  |  |  |  |  |

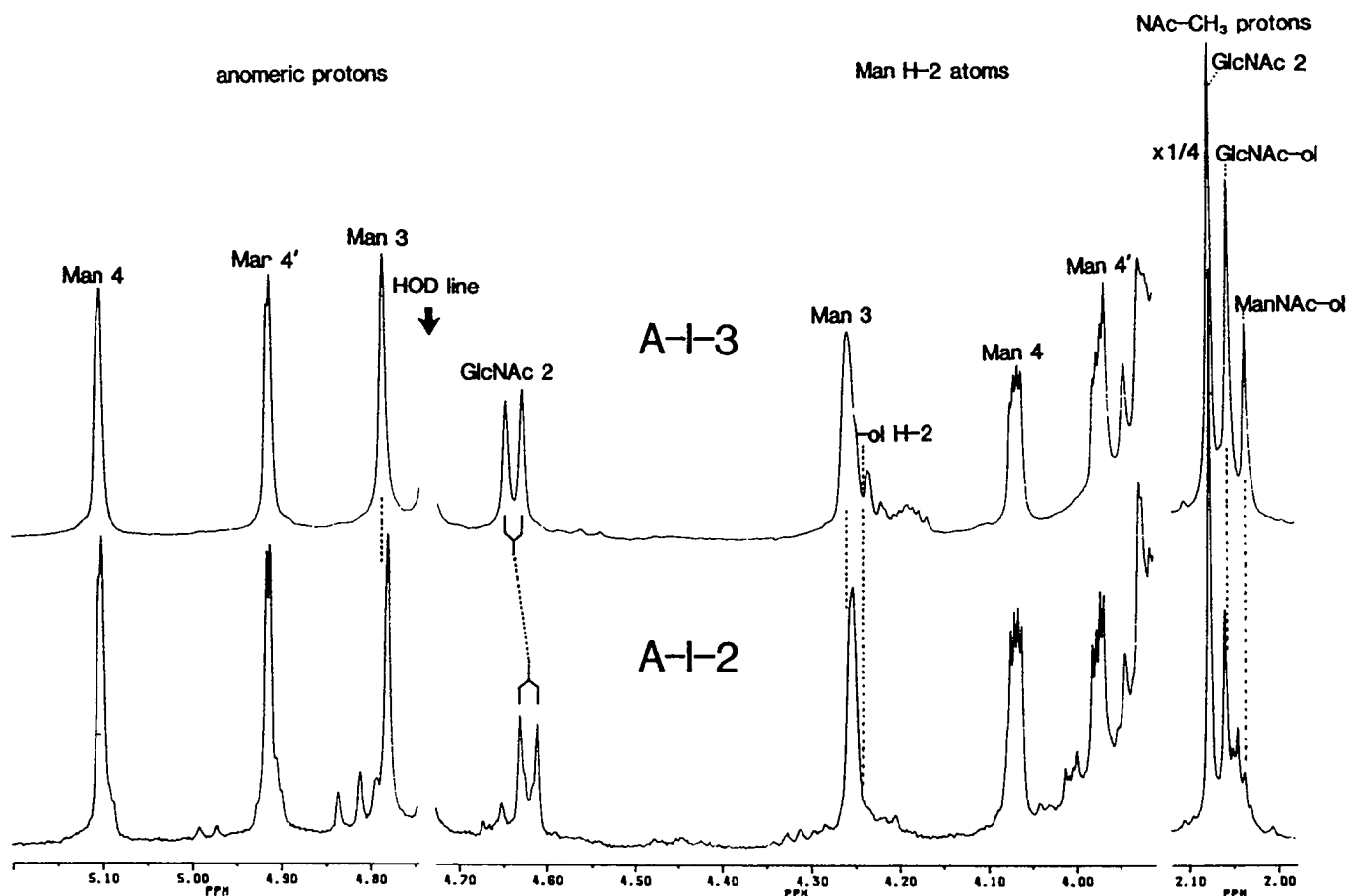


Fig. 6. Comparative study of the spectra of fraction A-I-2 and A-I-3 obtained by 400-MHz  $^1\text{H}$ -NMR spectroscopy

terminal position and at  $\delta = 5.142$  ppm when Man B is substituted with Man D<sub>3</sub>. From the intensity ratio of these two signals it was concluded that Man B is 50% substituted with Man D<sub>3</sub>. Concerning the Man C H-1 proton, two signals of equal intensity are present: a signal at  $\delta = 5.055$  ppm suggests a terminal Man C and a signal at  $\delta = 5.302$  ppm relates to Man C substituted by Man D<sub>1</sub>. The anomeric proton of Man D<sub>1</sub> and D<sub>3</sub> resonate at the same value  $\delta = 5.042$  ppm.

On the basis of these data, the primary structure of fraction A-I-11 was deduced (Fig. 5).

#### Fraction A-I-13

This fraction represents 3.5% of total neutral oligosaccharides. Like fraction A-I-11, fraction A-I-13 possesses a mixture of oligomannosidic and hybrid-type oligosaccharide-alditols as shown by methyl ether analysis (Table 3). With a major ion at  $m/z$  2209 ( $\text{M} + \text{Na}^+$ ), the FAB-MS spectrum shows evidence for the presence in this fraction of the oligosaccharide-alditol  $(\text{Man})_8\text{GlcNAc-GlcNAc-ol}$  (Fig. 4). There is also in the FAB-MS spectrum an ion at  $m/z$  1894 ( $\text{MH}^+$ ) corresponding to the hybrid-type oligosaccharide described in fraction A-I-11 with one additional hexose residue.

The structure of the oligomannosidic-type oligosaccharide-alditol was confirmed by 400-MHz  $^1\text{H}$ -NMR spectroscopy. The NMR data of fraction A-I-13 show clearly the presence of one oligomannosidic-type glycan with eight mannose residues. A signal at  $\delta = 5.086$  ppm indicates that the Man

A is in the terminal position. Signals at  $\delta = 5.142$  ppm and  $\delta = 5.302$  ppm are indicative for Man B H-1 and Man C H-1 substituted with Man D<sub>3</sub> and Man D<sub>1</sub> respectively.

On the basis of permethylation analysis, FAB-MS and 400-MHz  $^1\text{H}$ -NMR spectroscopy, the structure of fraction A-I-13 was proposed to be as shown in Fig. 5.

#### DISCUSSION

This work clearly reveals a high microheterogeneity in the glycan chains of quail ovomucoid. These glycans contain about 83% neutral oligosaccharides and 17% acidic oligosaccharides which have a peculiar sialic acid composition according to their chromatographic behaviour. These structures have not yet been determined. As to the neutral glycans, we describe new oligosaccharides not found by Hase et al. [3, 13]; particular, the identification, as in hen ovomucoid [2, 11], of an oligosaccharide with a bisecting *N*-acetylglucosamine residue linked to Man 3 by a  $\beta 1-4$  linkage (fraction A-I-7). Quail ovomucoid contains also high-mannose-type glycans with seven and eight mannose residues with classical primary structures.

Like Hase et al. [3, 13] in quail ovomucoid, Yamashita et al. [8] and Paz Parente et al. [11] in hen ovomucoid, we found the  $(\text{Man})_3(\text{GlcNAc})_2$  oligosaccharide in large amounts (14% of total neutral oligosaccharides). Beside the mannotrioso-di-*N*-acetylchitobiose we have detected a small proportion of  $(\text{Man})_2(\text{GlcNAc})_2$  oligosaccharide.

The presence, in quail ovomucoid, of oligosaccharides such as the mannotrioso-di-*N*-acetylchitobiose and the oligomannosidic-type oligosaccharides indicates that the sugar chains of quail ovomucoid may be synthesized by the pathway described by Kornfeld et al. for the class E Thy-1 negative mouse lymphoma cells [31] and by the classical biosynthetic pathway described by Gibson et al. [32].

The presence of the mannobiosido-di-*N*-acetylchitobiose could originate from the degradation of oligomannoside by processing mannosidases which have not yet been described; alternatively it could be due to a third biosynthetic pathway for the glycosylation of quail ovomucoid. This pathway would consist of direct mannosylation of the chitobiosyl-protein with GDP-Man as described by Hoflack et al. [33] for proteins in rat-spleen lymphocytes.

This investigation was supported by the *Centre National de la Recherche Scientifique* (Unité Associée n° 217, Directeur: Prof J. Montreuil), by the *Université des Sciences et Techniques de Lille Flandres Artois* and by a grant (to J.M.A. from the Spanish Education Minister. The authors are grateful to the *Conseil Régional de la Région Nord-Pas de Calais*, the *Centre National de la Recherche Scientifique*, the *Ministère de la Recherche et de la Technologie*, the *Ministère de l'Éducation Nationale*, the *Association de la Recherche sur le Cancer* and the *Institut National de la Santé et de la Recherche Médicale* for their contribution in the acquisition of the 400-MHz NMR apparatus and the Kratos MS 50 RF.

## REFERENCES

1. Paz Parente, J., Strecker, G., Leroy, Y., Montreuil, J. & Fournet, B. (1982) *J. Chromatogr.* **249**, 199–204.
2. Yamashita, K., Kamerling, J. P. & Kobata, A. (1982) *J. Biol. Chem.* **257**, 12809–12814.
3. Hase, S., Sugimoto, T., Takemoto, H., Ikenaka, T. & Schmid, K. (1986) *J. Biochem. (Tokyo)* **99**, 1725–1733.
4. Rhodes, M. B., Bennet, W. & Feeney, R. E. (1960) *J. Biol. Chem.* **235**, 1686–1693.
5. Beeley, J. G. (1976) *Biochem. J.* **159**, 335–345.
6. Kato, I., Schrode, J., Wilson, K. A. & Laskowski, M. Jr (1975) *Protides Biol. Fluids Proc. Colloq.* **23**, 235–243.
7. Stacey, M. & Woolley, J. M. (1942) *J. Chem. Soc.*, 550.
8. Yamashita, K., Kamerling, J. P. & Kobata, A. (1983) *J. Biol. Chem.* **258**, 3099–3106.
9. Bayard, B., Fournet, B., Bouquelet, S., Strecker, G., Spik, G. & Montreuil, J. (1972) *Carbohydr. Res.* **24**, 445–456.
10. Paz Parente, J., Wieruszkeski, J. M., Strecker, G., Montreuil, J., Fournet, B., Van Halbeek, H., Dorland, L. & Vliegthart, J. F. G. (1982) *J. Biol. Chem.* **257**, 13173–13176.
11. Paz Parente, J., Strecker, G., Leroy, Y., Montreuil, J., Fournet, B., Van Halbeek, H., Dorland, L. & Vliegthart, J. F. G. (1983) *FEBS Lett.* **152**, 145–152.
12. Egge, H., Peter-Katalinic, J., Paz Parente, J., Strecker, G., Montreuil, J. & Fournet, B. (1983) *FEBS Lett.* **156**, 357–362.
13. Hase, S., Okawa, K. & Ikenaka, T. (1982) *J. Biochem. (Tokyo)* **91**, 735–737.
14. Fredericq, E. & Deutsch, H. F. (1949) *J. Biol. Chem.* **181**, 499–510.
15. Monsigny, M., Adam-Chosson, A. & Montreuil, J. (1968) *Bull. Soc. Chim. Biol.* **50**, 857–874.
16. Lee, Y. C. & Scocca, J. R. (1972) *J. Biol. Chem.* **247**, 5753–5758.
17. Reading, C. L., Penhoet, E. & Ballou, C. (1978) *J. Biol. Chem.* **253**, 5600–5612.
18. Humbel, R. & Collaert, M. (1975) *Clin. Chim. Acta* **60**, 143–145.
19. Baenziger, J. U. & Natowicz, M. (1981) *Anal. Biochem.* **112**, 357–361.
20. Zanetta, J. P., Brekenridge, W. C. & Vincendon, G. (1972) *J. Chromatogr.* **69**, 291–304.
21. Bayard, B., Kerckaert, J. P., Roux, D. & Strecker, G. (1979) *Protides Biol. Fluids Proc. Colloq.* **27**, 153–156.
22. Ciucanu, I. & Kerek, F. (1984) *Carbohydr. Res.* **131**, 209–217.
23. Fournet, B., Strecker, G., Leroy, Y. & Montreuil, J. (1981) *Anal. Biochem.* **116**, 489–502.
24. Dell, A., Morris, H. R., Egge, H., Strecker, G. & Nicolai, H. (1983) *Carbohydr. Res.* **115**, 41–52.
25. Dell, A., Oates, J. E., Morris, H. R. & Egge, H. (1983) *Int. J. Mass. Spectrom. Ion. Phys.* **46**, 415–418.
26. Vliegthart, J. F. G., Dorland, L. & Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem.* **41**, 209–374.
27. Austen, B. M. & Marshall, R. D. (1973) in *Méthodologie de la structure et du métabolisme des glycoconjugués*, pp. 219–230, CNRS, Paris.
28. Van Kuik, A., Van Halbeek, H., Kamerling, J. P. & Vliegthart, J. F. G. (1986) *Eur. J. Biochem.* **159**, 297–301.
29. Strecker, G., Herlant-Peers, M. C., Fournet, B., Montreuil, J., Dorland, L., Haverkamp, J., Vliegthart, J. F. G. & Farriaux, J. P. (1977) *Eur. J. Biochem.* **81**, 165–171.
30. Neeser, J. R., Vedovo, S. D., Mutsaers, J. H. G. M. & Vliegthart, J. F. G. (1985) *Glycoconjugates J.* **2**, 355–364.
31. Kornfeld, S., Gregory, W. & Chapman, A. (1979) *J. Biol. Chem.* **254**, 11649–11654.
32. Gibson, R., Kornfeld, S. & Schlesinger, S. (1980) *Trends Biochem. Sci.* **5**, 290–293.
33. Hoflack, B., Debeire, Ph., Cacan, R., Montreuil, J. & Verbert, A. (1982) *Eur. J. Biochem.* **124**, 527–531.

Copyright of European Journal of Biochemistry is the property of Blackwell Publishing Limited and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.