ELSEVIER

Carbohydrate Research 292 (1996) 1–9

CARBOHYDRATE RESEARCH

The resolution of the neutral *N*-linked oligosaccharides of IgG by high pH anion-exchange chromatography

John M. McGuire *, Maureen Douglas, Kevin D. Smith

Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow G1 1XW, UK

Received 5 March 1996; accepted in revised form 12 July 1996

Abstract

The introduction of high pH anion-exchange chromatography (HPAEC) has represented a major development in the qualitative analysis of glycoprotein derived oligosaccharides. When coupled with pulsed amperometric detection, the technique permits the detection of picomole quantities of heterogeneous mixtures of oligosaccharide without the need for derivatisation. The applications of HPAEC have generally been limited to the analysis of sialylated oligosaccharides, however, it is now possible to analyse heterogenous mixtures of neutral oligosaccharides with the latest systems. We have used such a system to separate completely a panel of seven commercially available neutral *N*-linked oligosaccharides and found the influence of monosaccharide substitution on elution position to be identical to that for sialylated structures. A standard monosialylated *N*-linked oligosaccharide was modified by sequential digestion with specific exoglycosidases to produce a monogalactosylated, diantennary oligosaccharide which is commercially unavailable. This standard's elution position was confirmed by HPAEC. The technique was applied to the identification of neutral *N*-linked oligosaccharides released from human immunoglobulin G using the enzyme peptide-*N*-glycosidase F. © 1996 Elsevier Science Ltd.

Keywords: High pH anion-exchange chromatography; Neutral oligosaccharides; Immunoglobulin G

1. Introduction

One of the most significant developments in the chromatography of *N*-linked oligosaccharides has been the emergence of anion-exchange separation using quaternary

* Corresponding author.

PII S0008-6215(96)00185-1

ammonium groups bonded onto pellicular resins (CarboPAc PA-1 or PA-100) at high pH elution. The use of strong alkali exploits the weakly acidic property of the hydroxyl groups of monosaccharides (and thus oligosaccharides) at pH > 12. In particular, the oxyanions of oligosaccharides can be chromatographed without pre-derivatisation or the use of additives in the mobile phase, on the basis of molecular size, charge, and monosaccharide composition and linkage [1–4]. Excellent separations of the oxyanion derivatives of isomers and structurally closely related neutral and acidic oligosaccharides have been achieved [1–15]. The spherical pellicular resin contributes towards the high resolution of the technique by locating all the ionic groups on the surface through the elimination of diffusion [3]. The technique is further enhanced by using pulsed amperometric detection (PAD) which allows the detection of minute quantities of oligosaccharides due to sensitivity in the picomole range. It utilises the principle of triple amperometry to overcome the problem of electrode contamination by the oxidised product of carbohydrates associated with normal amperometry. Oligosaccharides are oxidised on the PAD electrode surface at the detection potential specified in the amperometric waveform which thereafter employs successive anodic and cathodic polarisations to clean and thus reactivate the electrode surface.

A major application of HPAEC is in the qualitative determination of the *N*-linked oligosaccharides present on a glycoprotein as a whole or at a particular glycosylation site. It is particularly useful in the resolution of mixtures of complex sialylated structures. The initial separation, and thus the degree to which an individual structure is retained on the column, is determined by the number of charges (normally sialic acids) per chain; an increased negative charge is correlated with an increased retention time. Thereafter, separation occurs within each charge band on the basis of size (a disialylated, diantennary chain will be retained to a lesser extent than a disialylated, triantennary chain) and then, for similarly sized structures, in terms of isomeric differences normally due to differing intra-chain linkages. The involvement of one or more hydroxyl groups of a monosaccharide in glycosidic bonds to other monosaccharides will alter its ionisation and thus two chains with identical monosaccharide sequences but different linkages can be separated. A complex oligosaccharide chain containing a lactosamine branch with a $\beta(1 \rightarrow 4)$ linkage between galactose (Gal) and *N*-acetylglucosamine (GlcNAc) will elute before a chain with a Gal$\beta(1 \rightarrow 3)$GlcNAc lactosamine branch [6]. A similar effect occurs with the linkages between sialic acids [8]; the greater the proportion of $\alpha(2 \rightarrow 3)$ to $\alpha(2 \rightarrow 6)$ linkages the longer the elution time [8]. Additionally, elution times are dependent upon the presence or absence of peripheral monosaccharides: the presence of fucosylation causes a chain to elute earlier than its non-fucosylated counterpart [6,7,10,12] while the addition of a bisecting GlcNAc increases its retention [15].

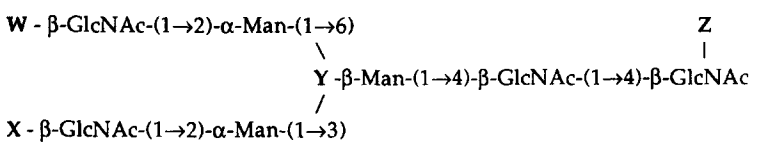
Until recently the superiority of HPAEC over other chromatography systems for the resolution of oligosaccharides was confined to sialylated structures; the characterisation of neutral oligosaccharides had generally been achieved using RP-HPLC with pyridylaminated (PA) [16] and, more recently, 2-aminobenzamide labelled (2-AB) [17] oligosaccharides. However, the latest HPAEC systems now make it possible to resolve asialylated complex chains [11,18] such as those present on immunoglobulin (Ig) G. Here we maximise the chromatographic separation of a panel of neutral diantennary

N-linked oligosaccharides by HPAEC and apply it to the identification of neutral oligosaccharides released enzymatically from Sigma IgG and IgG isolated from human sera.

2. Results and discussion

A previous study [11] utilised the latest HPAEC system to separate an IgG oligosaccharide library into its constitutive neutral, mono- and di-sialylated oligosaccharide components using Gradient 1. This gradient however was unsuccessful at resolving the panel of diantennary oligosaccharide standards, with coelution of several structures observed (Table 1). In an attempt to maximise the resolution of these oligosaccharides the sodium acetate (Gradient 2) and sodium hydroxide concentrations (Gradient 3) were altered, however, coelution of certain structures still occurred (Table 1). The optimal separation which successfully resolved each oligosaccharide to baseline was achieved with an isocratic mobile phase of 250 mM NaOH and 5 mM NaOAc (Fig. 1, Table 1) with excellent reproducibility when repeated on the same or different days. The range of coefficients of variation for retention time ratio was 1.38–2.30% for a total of 10 runs with *N*-acetylneuraminic acid used as the internal standard. To date the most successful chromatographic resolution of mixtures of neutral *N*-linked oligosaccharide chains has been accomplished by Takahashi et al. [16] by the fluorescent detection of PA derivatives after separation on a reversed phase ODS column. While the resolution

Table 1
Retention times of neutral diantennary *N*-linked oligosaccharides used in the HPAEC analysis.



Structure	Identity of substituents				Retention time on different HPAEC gradients (min) [a]			
	W	X	Y	Z	1	2	3	4
A	–	–	–	α-Fuc(1 → 6)	10.5	9.5	6.9	12.8
B	–	–	–	–	11.9	11.6	8.2	15.1
C	–	–	β-GlcNAc(1 → 4)	α-Fuc(1 → 6)	12.4	12.6	8.8	16.8
D	β-Gal-(1 → 4)	β-Gal-(1 → 4)	–	α-Fuc(1 → 6)	12.4	12.6	9.8	21.8
E	β-Gal-(1 → 4)	β-Gal-(1 → 4)	–	–	13.8	14.8	11.8	25.8
F	β-Gal-(1 → 4)	β-Gal-(1 → 4)	β-GlcNAc(1 → 4)	α-Fuc(1 → 6)	13.8	15.4	11.8	28.2
G	β-Gal-(1 → 4)	β-Gal-(1 → 4)	β-GlcNAc(1 → 4)	–	14.7	16.9	13.5	30.9
H	β-Gal-(1 → 4)	–	–	–	nd	nd	nd	20.25

[a] See Materials and Methods for elution conditions.
nd: Not determined.

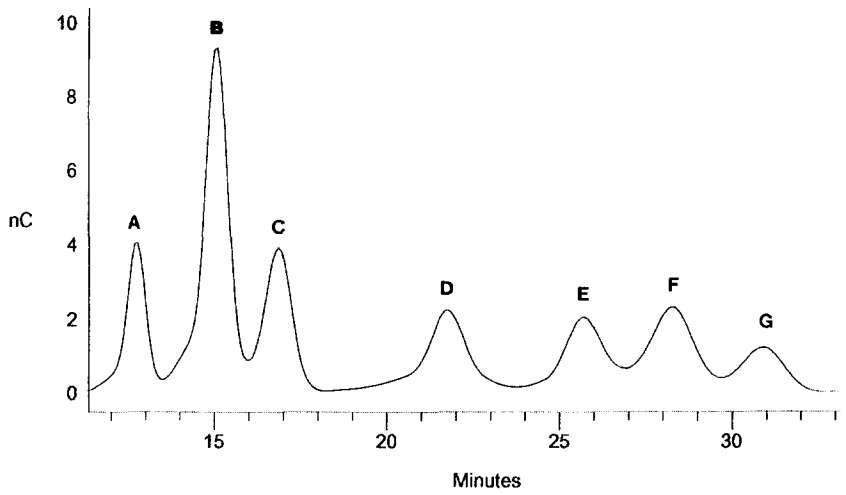


Fig. 1. Separation of a panel of neutral diantennary *N*-linked oligosaccharides by HPAEC by Gradient 4. See Table 1 for oligosaccharide structures and Materials and Methods for elution conditions.

obtained using our HPAEC gradient is not an improvement on the established method, the working sensitivity is equivalent to fluorescence detection and it represents the best separation of diantennary *N*-linked oligosaccharides by other chromatographic means, with the added advantage that no derivatisation step is required.

The resolution of asparagine linked oligosaccharides is essentially a relationship between the structure of the chain and its retention time on the pellicular CarboPac column. The majority of research into the aforementioned relationship has concentrated upon the behaviour of sialylated complex *N*-linked structures, however, this paper demonstrates that similar criteria can be applied to the resolution of neutral complex *N*-linked chains. From Table 1 it can be seen that peripheral monosaccharide substitution has an effect on elution position identical to that reported for sialylated *N*-linked structures. The larger the oligosaccharide, the greater the retention time and hence agalactosylated structures elute before digalactosylated structures. This was further confirmed by the fact that the monogalactosylated oligosaccharide produced from the sequential exoglycosidase digestion of a monosialylated standard had an elution position intermediate to that of the a- and di-galactosylated oligosaccharides (Table 1). The addition of a bisecting GlcNAc residue also increases retention time while the presence of fucosylation results in a decrease in retention time. This reduction in retention time of both a- and di-galactosylated structures following core fucosylation may be the result of a reduction in net charge through the masking of another monosaccharide constituent which decreases interaction with, and therefore retention, by the column.

IgG contains on average 2.8 *N*-linked oligosaccharide diantennary chains per molecule of glycoprotein. Of these oligosaccharides, 2.0 are located at Asn-297, the conserved glycosylation site located on the heavy chains in the CH_2 region in the Fc domain [19]. Unusually, the oligosaccharides are predominantly neutral with only approximately 30% of chains sialylated. The majority of these neutral oligosaccharides are located on the Fc

portion of the molecule [19] and studies have shown that glycosylation at this site effects function [20] and may play a role in the pathogenesis of rheumatoid arthritis [21]. Studies have shown extensive interactions between Fc oligosaccharides and the polypeptide backbone. These contacts occur with specific amino acids and result in an oligosaccharide moiety which is essentially buried within the three dimensional structure adopted by the CH_2 and CH_3 domains [22] resulting in a level of inaccessibility of the native molecule to a variety of structural probes [23]. The final part of this study attempted to utilise the elution conditions developed above to identify the neutral oligosaccharides released from human IgG using the enzyme peptide-*N*-glycosidase F

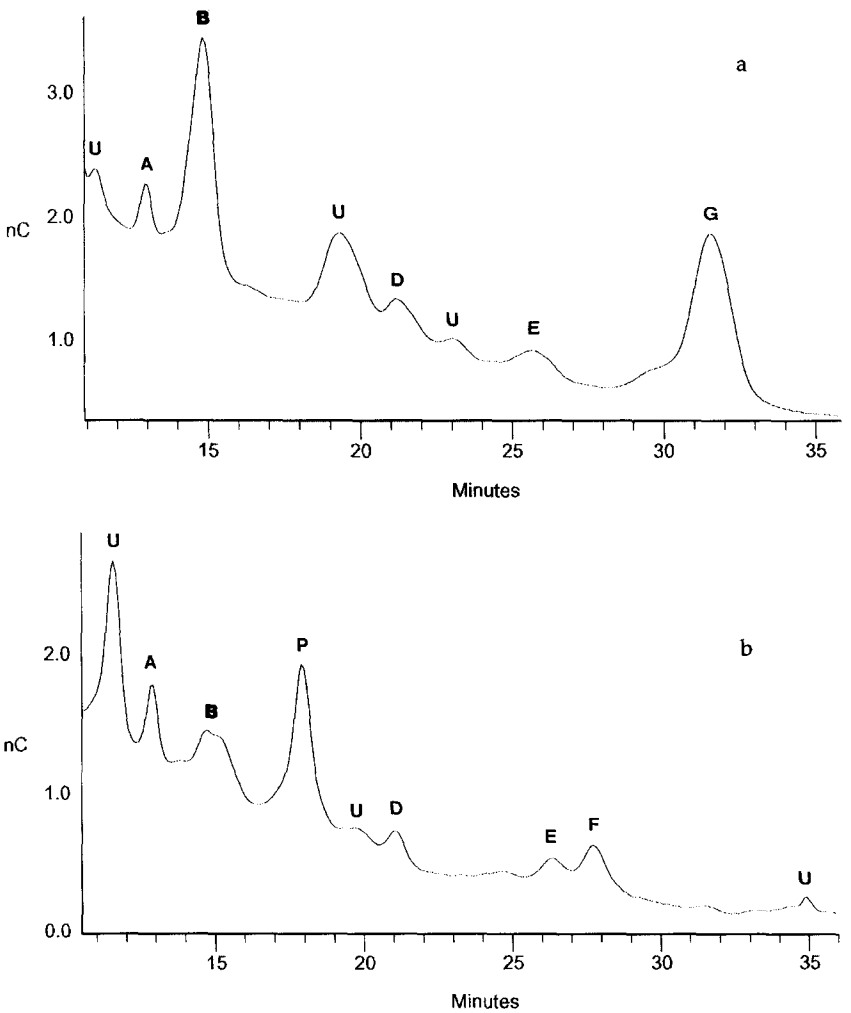


Fig. 2. HPAEC profiles for neutral *N*-linked oligosaccharides released from native (a) and trypsin/chymotrypsin treated (b) Sigma IgG (250 μg) on Gradient 4. Peaks were identified using *N*-acetylneuraminic acid as an internal standard.

(PNGaseF). The effect of disruption of the tertiary structure by pre-treatment of IgG with protease enzymes was also studied.

The enzyme PNGaseF was used to remove oligosaccharides from both native IgG and that which had been pre-treated with trypsin and chymotrypsin to disrupt the tertiary structure. It can be seen from Fig. 2a that treatment of native IgG with PNGaseF releases neutral *N*-linked oligosaccharides with both a- and di-galactosylated structures identified. Release of neutral *N*-linked oligosaccharides from IgG pre-treated with protease enzymes is also observed (Fig. 2b). However, when the oligosaccharides released from both are compared qualitative differences can be observed. The same

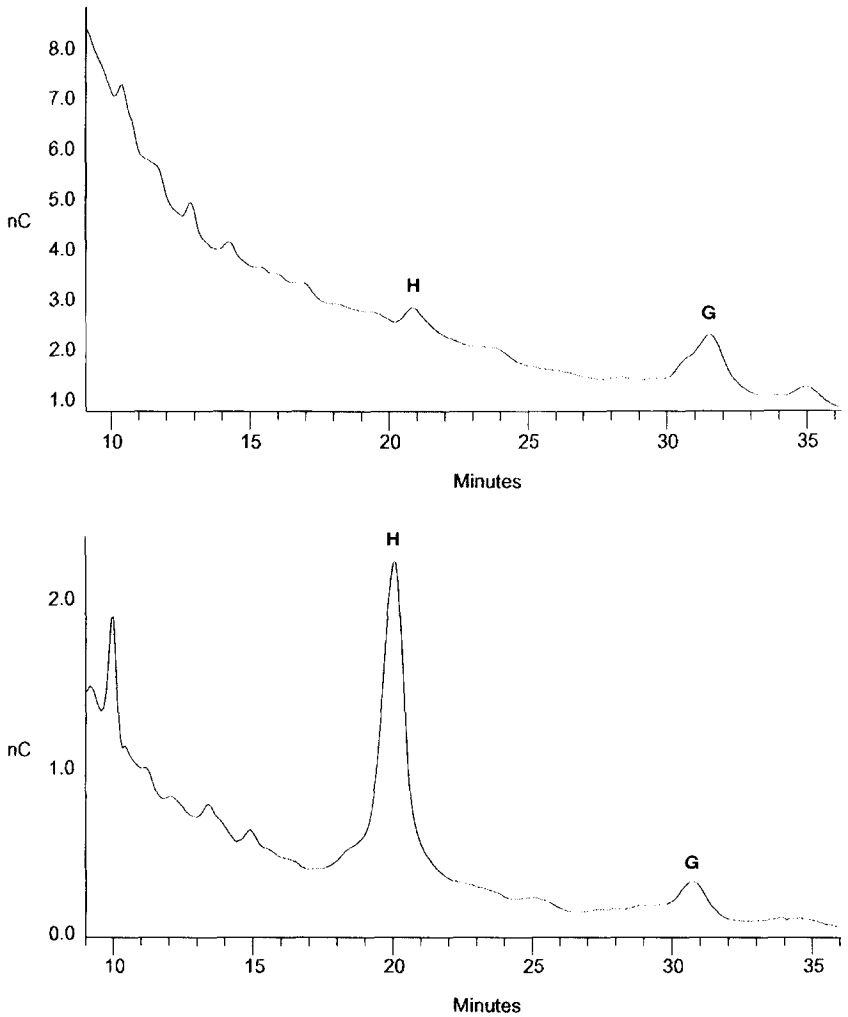


Fig. 3. HPAEC profiles for neutral *N*-linked oligosaccharides released from native (a) and trypsin/chymotrypsin treated (b) IgG (60 μg) isolated from human serum on Gradient 4. Peaks were identified as previously.

population of agalactosylated oligosaccharides is released from native and pre-treated IgG. A similar population of digalactosylated oligosaccharides is released from both, although surprisingly, a digalactosylated, diantennary oligosaccharide was released from native IgG and not from that which had been protease treated. However, the major qualitative differences lie in the release of monogalactosylated structures which are, like agalactosylated oligosaccharides, known to predominate in the Fc region [19]. Structure P is released from pre-treated IgG but not from native and the elution position of this structure is such that it is suspected to be a monogalactosylated, fucosylated diantennary oligosaccharide. This effect was also shown when the oligosaccharides released from IgG isolated from human serum were studied (Fig. 3). It is difficult to speculate as to the identity of a- and di-galactosylated oligosaccharides released due to the small quantity of IgG used in the study. However, the increase in release of a monogalactosylated diantennary oligosaccharide upon pretreatment with protease enzymes is clearly shown. Given that both a- and mono-galactosylated structures are known to predominate in the Fc region [19], it would appear that agalactosylated oligosaccharides can be released from native Fc while monogalactosylated oligosaccharides require disruption of the tertiary structure before enzymatic release can occur.

A recent study which analysed the effect of reduced outer arm galactosylation on Fc oligosaccharide mobility within the CH_2 domain may explain these observed results [21]. This study observed that agalactosylated oligosaccharides have a reduced number of contacts with the polypeptide backbone such that they have a greatly increased mobility within the CH_2 domain. This increased mobility in which the agalactosylated oligosaccharides may 'pop out' of their restricted pocket may allow increased access to PNGaseF and subsequent release of these oligosaccharides. However, monogalactosylated structures still interact extensively with the polypeptide backbone and thus disruption of the tertiary structure may be a prerequisite for release of these oligosaccharides with PNGaseF. The similarities in the population of digalactosylated oligosaccharides released, and the fact that bisected structures were identified, suggests that they may originate from the Fab region.

In summary, the separation of a panel of neutral *N*-linked diantennary oligosaccharides was optimised using HPAEC and the elution conditions used in the identification of neutral oligosaccharides released, using PNGaseF, from human IgG. The release of agalactosylated oligosaccharides from native IgG was observed, while monogalactosylated structures required disruption of tertiary structure with protease enzymes before release could be achieved. These release characteristics may be explained by the relative mobility of a- and mono-galactosylated oligosaccharides within the three-dimensional structure of the CH_2 domain.

3. Experimental

Materials: standard oligosaccharides and enzymes.—The IgG oligosaccharide library, panel of diantennary *N*-linked oligosaccharides, β-galactosidase (bovine testes), sialidase (*Arthrobacterium ureafaciens*) and PNGaseF (*Flavobacterium meningosepticum*) were purchased from Oxford GlycoSystems (Abingdon, UK). Trypsin and chymotrypsin were purchased from Sigma (Poole, UK).

Equipment.—High pH anion-exchange chromatography was performed using a DX-500 system supplied by Dionex (Camberley, UK). The DX-500 system consisted of a GP40 gradient pump, ED40 electrochemical detector and an anion self-regenerating suppressor controlled by a Vtech 486SX 25 (Viglen, UK) using PeakNet software.

HPAEC analysis of N-*linked oligosaccharides.*—For all analyses, the oligosaccharides were applied to a CarboPac PA-100 guard column and CarboPac PA-100 analytical column (25 × 0.4 cm i.d.) equilibrated with an eluent mixture, corresponding to the initial conditions of the separation gradient, at a flow rate of 1 mL/min at room temperature. The chromatography eluents were prepared using NaOH and NaOAc purchased from BDH (Poole, UK) and Sigma (Poole, UK), respectively and HPLC grade water purchased from Rathburn (Murrayburn, UK). Detection was by a pulsed electrochemical detector using the following pulse potentials and durations: time = 0 s, E = +0.05 V; 0.29 s, +0.05 V; 0.49 s, +0.05 V; 0.50 s, 0.05 V; 0.51 s, +0.6 V; 0.6 s, +0.6 V; 0.61 s, −0.6 V; 0.65 s, −0.6 V; 0.66 s, +0.05 V. In Gradient 1 the column was equilibrated in 10% solvent A (1 M NaOH)/2% solvent B (1 M NaOAc)/88% solvent C (HPLC water). This solvent elution was continued for 3 min after which a linear gradient of 10%A/2%B/88%C to 10%A/14%B/76%C was achieved over 57 min. The limit solvent was continued for a further 5 min before the column was regenerated by moving to 50%A/50%C in 1 min and holding for a further 10 min. Thereafter, the initial conditions were resumed. In Gradient 2 the column was equilibrated in 10% solvent A (1 M NaOH)/2% solvent B (1 M NaOAc)/88% solvent C (HPLC water). This solvent elution was continued for 3 min after which a linear gradient of 10%A/2%B/88%C to 10%A/4%B/86%C was achieved over 32 min. The limit solvent was continued for a further 5 min before the column was regenerated as with Gradient 1 and initial conditions were resumed. Gradient 3 was identical to Gradient 2 except 250 mM NaOH (25% A) was used. In Gradient 4 the column was eluted isocratically with 25% A/0.5%B/74.5% C for 40 min and the column was regenerated as with Gradient 1 and initial conditions were resumed.

Release of oligosaccharides.—Oligosaccharides were released from human IgG by two methods:

(i) Native IgG was incubated with 2U of PNGaseF for 24 h at 37 °C.

(ii) Native IgG was incubated with trypsin and chymotrypsin (1%) in sodium hydrogen carbonate buffer pH 8.0 for 24 h as described in [24], lyophillised and incubated with PNGaseF as above.

The released oligosaccharides were purified by precipitation in ice-cooled ethanol and, after lyophillisation, reconstituted in 50 μL water for HPAEC analysis.

Degalactosylation and desialylation.—The sequential digestion of the monosialylated diantennary oligosaccharide with β-galactosidase and sialidase to produce the gal(1) standard was carried out as detailed in the protocol supplied by Oxford Glycosystems. Monosialylated substrate, 6 μg, at a concentration of 40 μg/mL, was incubated in 100 mM citrate/phosphate buffer, pH 3.5, with β-galactosidase, 2 U/mL, at 37 °C for 24 h. The oligosaccharides were purified as above and an aliquot was withdrawn for HPAEC analysis. The remaining oligosaccharides were incubated with sialidase, 2U/ml, at 37 °C for 24 h and the oligosaccharides purified as described above.

IgG sample.—The human normal IgG was isolated by DEAE chromatography [23] and kindly donated by Angela Bond and Frank Hay, St. George's Hospital Medical School, London.

Acknowledgements

J.M.'s work was supported by a BBSRC Quota Award.

References

[1] R.R. Townsend and M.R. Hardy, *Glycobiology*, 1 (1991) 139–147.
[2] M.W. Spellman, *Anal. Chem.*, 62 (1990) 1714–1722.
[3] Y.C. Lee, *Anal. Biochem.*, 189 (1990) 151–162.
[4] M.R. Hardy, *Meth. Enzymol.*, 179 (1989) 76–82.
[5] M.R. Hardy and R.R. Townsend, *Meth. Enzymol.*, 230 (1994) 208–225.
[6] M.R. Hardy and R.R. Townsend, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 3289–3293.
[7] M.R. Hardy and R.R.Townsend, *Carbohydr. Res.*, 188 (1989) 1–7.
[8] R.R. Townsend, M.R. Hardy, D.A. Cumming, J.P. Carver, and B. Bendiak, *Anal. Biochem.*, 182 (1989) 1–8.
[9] R.R. Townsend, M.R. Hardy, and Y.C. Lee, *Meth. Enzymol.*, 179 (1989) 65–76.
[10] G. Pfeiffer, H. Geyer, I. Kalsner, P. Wendorf, and H. Geyer, *Biomed. Chromatogr.*, 4 (1990) 193–199.
[11] J.M. McGuire, M.A. Elliott, H.G. Elliott, and K.D. Smith, *Carbohydr. Res.*, 270 (1995) 63–69.
[12] L.J. Basa and M.W. Spellman, *J. Chrom.*, 499 (1990) 205–220.
[13] K.D. Smith, M.J. Davies, and E.F. Hounsell, *Meth. Mol. Biol.*, 32 (1993) 143–155.
[14] M.R. Hardy, R.R. Townsend, and Y.C. Lee, *Anal. Biochem.*, 170 (1988) 54–62.
[15] P. Hermentin, R. Witzel, J.F.G. Vliegenthart, J.P. Kamerling, Nimitz, and H.S. Conradt, *Anal. Biochem.*, 203 (1992) 281–289.
[16] N. Tomiya, J. Awaya, M. Kurono, S. Endo, Y. Arata, and N. Takahashi, *Anal. Biochem.*, 171 (1988) 73–90.
[17] R. Parekh and A. Ventom, *Amer. Biotech. Lab.* 13 (1995) 9–10.
[18] G.A. Cooper and J.S. Rohrer, *Anal. Biochem.*, 226 (1995) 182–184.
[19] T.W. Rademacher, R.B. Parekh, and R.A. Dwek, *Ann. Rev. Biochem.*, 57 (1988) 785–838.
[20] J. Lund, N. Takahashi, J.D. Pound, M. Goodhall, H. Nakagawa, and R. Jefferis, *FASEB J.*, 9 (1995) 115–119.
[21] R. Malhotra, M.R. Wormald, P.M. Rudd, P.B. Fischer, R.A. Dwek, and R.B. Sim, *Nature (Medicine)*, 1 (1995) 237–243.
[22] J. Deisenhoffer, *Biochemistry*, 20 (1981) 2361–2370.
[23] A. Bond, M. Jones, and F.C. Hay, *J. Immunological Methods*, 166 (1993) 27–33.
[24] J. Lund, N Takahashi, H. Nakagawa, M. Goodhall, T. Bentley, S.A. Hindley, R. Tyler, and R. Jefferis, *Mol. Immunol.*, 39 (1993) 741–748.