Glycobiology vol. 6 no. 2 pp. 217–230, 1996

The hypothetical N-glycan charge: a number that characterizes protein glycosylation

Peter Hermentin[1,2,3], Reinhild Witzel, Ernst-Jürgen Kanzy, Gaston Diderrich, Dieter Hoffmann, Hubert Metzner, Jürgen Vorlop and Heinz Haupt

Research Laboratories of Behringwerke AG, P.O. Box 1140, D-35001 Marburg, Germany

[1]Dedicated to Professor Dr. Raymond Lemieux, The University of Alberta, Edmonton, Alberta, Canada, on the occasion of his 75th birthday

[2]A preliminary report was presented at the XIIIth International Symposium on Glycoconjugates, August 20–26, 1995, Seattle, WA, USA; Abstracts of Papers (*Glycoconjugate J.* (1995) **12**, 407)

[3]To whom correspondence should be addressed

The production of recombinant glycoprotein therapeutics requires characterization of glycosylation with respect to the lot-to-lot consistency. Here we introduce the 'hypothetical N-glycan charge *Z*' as a parameter that allows to characterize the protein glycosylation in a simple, however, efficient manner.

The hypothetical N-glycan charge of a given glycoprotein is deduced from the N-glycan mapping profile obtained via HPAE-PAD. In HPAEC, N-glycans are clearly separated according to their charge, i.e., their number of sialic acid residues, providing distinct regions for neutral structures as well as for the mono- di-, tri, and tetrasialylated N-glycans (Hermentin *et al.*, 1992a).

***Z* is defined as the sum of the products of the respective areas (*A*) in the asialo, monosialo, disialo, trisialo, tetrasialo, and pentasialo region, each multiplied by the corresponding charge:**

$$Z = A_{(as)^*}\,0 + A_{(MS)^*}\,1 + A_{(DiS)^*}\,2 + A_{(TriS)^*}\,3 + A_{(TetraS)^*}\,4\,[+ A_{(PentaS)^*}\,5]$$

Thus, a glycoprotein with mostly C4–4* structures will provide $Z \cong 400$ (e.g., rhu EPO (CHO), $Z = 361$), a glycoprotein carrying largely C3–3* structures will amount to $Z \cong 300$ (e.g., bovine fetuin, $Z = 290$), a glycoprotein with mostly C2–2* structures will have $Z \cong 200$ (e.g., human serum transferrin, $Z = 207$, or human plasma AT III, $Z = 180$), and a glycoprotein carrying only high-mannose type or trunkated structures will provide $Z \cong 0$ (e.g., bovine pancreas ribonuclease B, $Z = 15$, and hen ovomucoid, $Z = 15$, respectively).

The determination of *Z* was validated in multiple repetitive experiments and proved to be highly accurate and reliable. *Z* may therefore be regarded as a new and characteristic parameter for protein N-glycosylation.

Key words: high-performance anion-exchange chromatography (HPAEC)/pulsed amperometric detection (PAD)/HPAE-PAD/human plasma/recombinant expression/CHO/BHK/interleukin 4-receptor/erythropoietin/fetuin/transferrin/thyroglobulin/antithrombin/ribonuclease/ovomucoid/orosomucoid/α_1-acid glycoprotein/fibrinogen/α_1T-glycoprotein/α_1-antitrypsin/α_1-antichymotrypsin/β_2-glycoprotein I/thyroxin-binding globulin/α_1B-glycoprotein/8Sα_3-glycoprotein/haptoglobin/hydrazinolysis/PNGase F/consistency/clearance/*in vivo* half-life

Introduction

In aiming for parameters that ensure the lot-to-lot consistency of (recombinant) glycoproteins, we have defined a theoretical number that reflects the composition of the N-linked carbohydrates. These are known to normally consist in bi-, tri-, and tetraantennary structures, the antennae of which usually terminate with negatively charged sialic acid residues attached to β-linked galactose. These sialic acid residues play an important role with respect to the *in vivo* half-life of a (therapeutic) glycoprotein and thus influence its biological efficacy.

In order to guarantee the lot-to-lot consistency of a (recombinant) glycoprotein with respect to its *in vivo* half-life as well as its biological efficacy, parameters have to be defined that ensure the integrity of the N-glycan pattern as well as the degree of sialylation and reflect the carbohydrate microheterogeneity. The routine sialic acid determination of (recombinant) glycoproteins is part of registration works as well as of stability studies. It enables to calculate the degree of sialylation and allows to judge the *in vivo* clearance rate that has to be expected.

Although the sialic acid content—expressed as mol Neu5Ac per mol of glycoprotein (peptide bone)—is a very useful figure, its accuracy is hampered by (i) the accuracy of the sialic acid determination and (ii) the accuracy of the protein determination. In fact, the protein determination of glycoproteins is not an easy task and may vary dramatically due to the carbohydrate content as well as from method to method (such as, e. g., UV determination, Lowry assay, BCA assay, amino acid determination). Thus, the degree of sialylation will vary according to the method used. In addition, the coefficients of variation of each determination (i. e., the sialic acid, the monosaccharide component and the protein determination) will add to the uncertainty of the degree of sialylation (mol Neu5Ac/mol Gal).

We have now found that a number '*Z*' that reflects the hypothetical N-glycan charge is a much better and more reliable parameter to ensure the biological safety as well as the therapeutical efficacy of (recombinant) glycoproteins. This parameter is usually measurable with a degree of variation of less than 3% and reflects not only the negative charge carried by sialic acid residues but also the charge introduced by sulfate (and phosphate) groups.

The hypothetical N-glycan charge of the glycoprotein is deduced from the N-glycan mapping profile obtained via

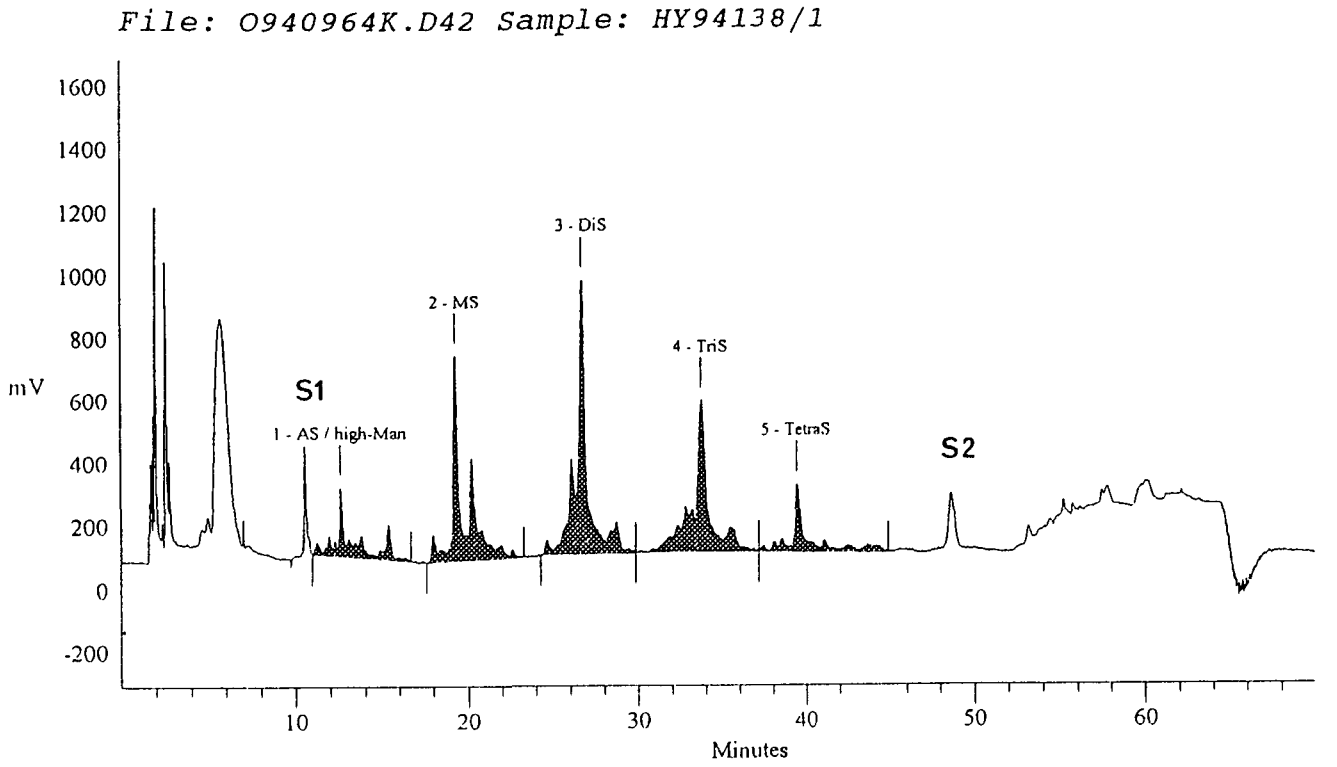


Fig. 1. HPAE-PAD mapping profile of rhu IL-4R (CHO) (lot no. E4-930914). S1, S2, internal standards.

HPAE-PAD. In HPAEC, N-glycans are first separated according to their charge, exhibiting retention times in the range of 13–18 min (asialo, as), 20–23 min (monosialo, MS), 27–32 min (DiS), 34–38 min (TriS), and 40–43 min (TetraS structures), using the validated gradient 'S' for sialylated N-glycans (Hermentin *et al.*, 1992a). As the response factors of the distinct N-glycans may be regarded as comparable (Lee, 1990), the area under the curve (A) in the monosialo region is equivalent to the charge carried by the monosialylated N-glycan structures appearing in the monosialo region of the chromatogram. The area in the disialo region, multiplied by 2, is equivalent to the charge carried by the disialylated N-glycan structures; the area in the trisialo region, multiplied by 3, is equivalent to the charge carried by the trisialylated N-glycan structures; the area in the tetrasialo region, multiplied by 4, is equivalent to the charge carried by the tetrasialylated N-glycan structures; and the area in the pentasialo region, multiplied by 5, is equivalent to the charge carried by the pentasialylated N-glycan structures. In analogy, the area in the asialo region reflects the amount of neutral structures, carrying a net-charge of zero.

'Z' is defined as the sum of the products of the various areas, multiplied by the respective charge:

$$Z = A_{(\text{asialo})}\cdot 0 + A_{(\text{MS})}\cdot 1 + A_{(\text{DiS})}\cdot 2 + A_{(\text{TriS})}\cdot 3 + A_{(\text{TetraS})}\cdot 4\,[+ A_{(\text{pentaS})}\cdot 5]$$

$$Z = \Sigma A_{(i)}\cdot (i)$$

where i is 0 in the asialo region, 1 in the monosialo (MS) region, 2 in the disialo (DiS) region, 3 in the trisialo (TriS) region, 4 in the tetrasialo (TetraS) region, and 5 in the pentasialo (PentaS) region.

Results and Discussion

Rhu IL-4R (Idzerda *et al.*, 1990; Enssle *et al.*, 1995) and rmur IL-4R (Mosley *et al.*, 1989), expressed in CHO and BHK cells, respectively, are research and developmental products of Behringwerke AG and therefore used as model glycoproteins. In the course of this investigation, the IL-4R production was optimized with respect to clone expression, culture conditions and mode of fermentation, taking Z as one of the parameters used for the assessment of protein glycosylation (unpublished data). Thereby, Z turned out to be one of the most predictive and reliable parameters of protein glycosylation available to date, enabling to describe the glycosylation status of the respective glycoprotein in a simple number. The reproducibility and reliability of Z determination was ascertained by extensive validation experiments, some of which are communicated in this report. Consequently, the determination of Z was extended to a number of human serum as well as non-human glycoproteins, the Z values of which are also communicated. It is suggested that the hypothetical N-glycan charge Z of a glycoprotein may be regarded as a new and very helpful parameter that enables judgement of the protein glycosylation in a simple but efficient manner.

Validation experiments
Z and the validity of automated hydrazinolysis versus PNGase F release, using rhu IL-4R (CHO)

The repetitive automated hydrazinolysis of rhu IL-4R (lot E4–930914) with 1.0 mg of sample per reactor provided $Z = 201 \pm 2.8$ (CV = 1.4%) ($n = 18$) (for a representative

Table I. Calculation of Z, based on the HPAE-PAD mapping profile of rhu IL-4R (CHO) of Figure 1

File no.	Peak group no.	RT of highest peak (min)	Area of integration (peak group)	Percentage of peak group area (%)	Multiplication factor	Charge number share
0940964K.D42	1	12.57	Asialo/high Mannose	9.70	0	0
	2	19.23	Monosialo (MS)	23.31	1	23.31
	3	26.69	Disialo (DiS)	31.81	2	63.62
	4	33.73	Trisialo (TriS)	25.38	3	76.14
	5	39.44	Tetrasialo (TetraS)	9.81	4	39.24
			Total peak area	100.01	Z = sum of shares =	202

mapping profile see Figure 1; the corresponding calculation of Z is outlined in Table I), whereas $Z = 194 \pm 4.5$ (CV = 2.3%) ($n = 18$) was obtained, using 0.5 mg of rhu IL-4R (Table II). These values proved to be comparable with the Z values derived via PNGase F-digestions in the presence or absence of CHAPS, with and without the previous digestion of the glycoprotein with trypsin or Lys C, providing an average of $Z = 204.5 \pm 3.4$ (1.7%) (Table II).

Rhu IL-4R from the second cell line (B11–930406), produced under identical culture and work-up conditions, provided $Z = 235 \pm 2.6$ (CV = 1.1%) ($n = 10$) upon hydrazinolysis and $Z = 243$ after PNGase F treatment in the presence or absence of 0.5% CHAPS (Table II).

The various Z values proved to be very similar, irrespective of whether the N-glycan pool was gained via automated hydrazinolysis or via PNGase F treatment. It is noteworthy that the hydrazinolysis-derived glycan pool had to be desalted prior to Z determination.

The differences in the Z values obtained for the two clones (Table II), although produced under identical production and work-up conditions, clearly indicated differences in the respective clone-specific glycosylation.

Z and the PNGase F digestion of AGP

AGP is known to resist PNGase F digestion, unless the conditions are carefully optimized (Nuck *et al.*, 1990). In order to see whether the incomplete digestion of AGP could be proven via Z, AGP was digested with PNGase F for a period of 48 h, applying the conditions described for rhu EPO, and compared to the hydrazinolysis-derived and trypsin/PNGase F-derived N-glycan pool (Figure 2). Whereas the hydrazinolysis-derived N-glycan pool (Figure 2, run a) and the trypsin/PNGase F-derived N-glycan pool (Figure 2, run c) provided $Z = 289$, each, the hypothetical N-glycan charge was clearly reduced when AGP was digested by PNGase F without any detergent (i.e., EPO conditions) ($Z = 248$; Figure 2, run b). Moreover, the HPAE-PAD mapping profile suggested difficulties for the enzyme in cleaving the tetraantennary tetrasialylated (C4–4*) structures (Figure 2, run b). Thus, the chromatogram revealed C3–235300 (the triantennary disialylated isomer[s]) as the highest peak, and the peaks in the C4–4* region were clearly reduced, whereas the ratio of the C3–3* and C4–3* structures (Hermentin *et al.*, 1992b) appeared 'normal', i.e., comparable with the hydrazinolysis-derived mapping profile ($Z = 289$; Figure 2, run a) as well as with the tryptic/PNGase F-derived mapping profile ($Z = 289$; Figure 2, run c).

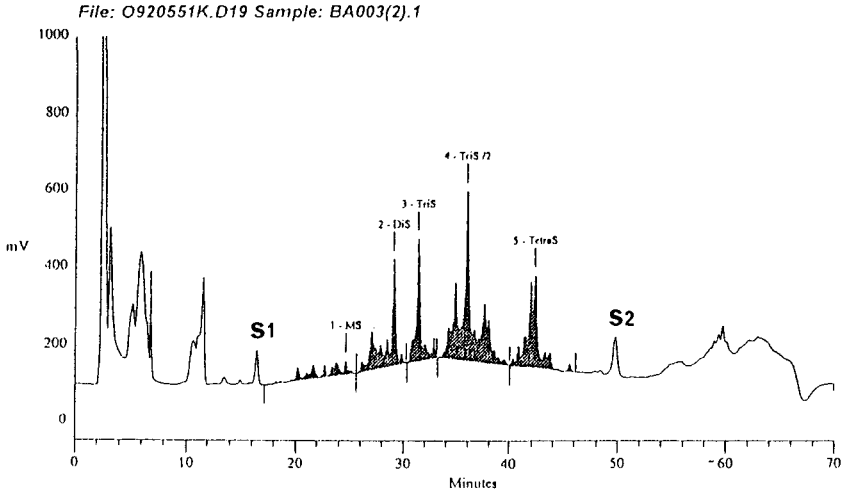
Z and the consistency control of protein glycosylation
Z and the comparison of various AGP-derived N-glycan pools

Various samples of AGP-derived N-glycan pools have previously been compared on the basis of their respective HPAE-PAD mapping profiles, in order to evaluate the loss of terminal sialic acid, due to different N-glycan release, work-up and storing conditions (Hermentin *et al.*, 1992b, Figure 5). As reported, approximately 40 distinct peaks and free Neu5Ac (~3 mol%) could be detected in a hydrazinolysis-derived N-glycan pool (a 50 mg batch; Hermentin *et al.*, 1992b, Figure 5, run b), whereas only about 30 distinct peaks were detected in a PNGase F-derived N-glycan pool, while the amount of free sialic acid was negligible (Hermentin *et al.*, 1992b, Figure 5, run e). The amount of free Neu5Ac detected by HPAE-PAD in the various preparations decreased in the order of run a (LB-001; OGS) > run b (50 mg hydrazinolysis batch) > run c (1000 mg hydrazinolysis batch) > run d (2 mg batch, GlycoPrep 1000) > run e (PNGase F-derived N-glycan pool, obtained after tryptic AGP digest). Concomitant with

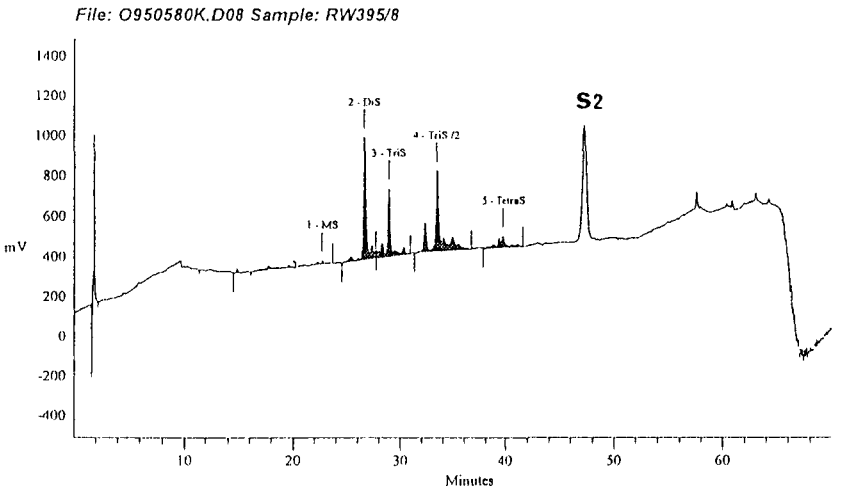
Table II. Validation of Z determination, using rhu IL-4R (CHO) as model glycoprotein

Lot no.	Origin of glycan pool	Z single	Z average	SD	CV	No. of expts.	No. of runs
E4-930914	GlycoPrep (1.0 mg/reactor)		201	±2.8	1.4%	6	18
E4-930914	GlycoPrep (0.5 mg/reactor)		194	±4.5	2.3%	6	18
E4-930914	PNGase F (without CHAPS)	208					
E4-930914	PNGase F (with 0.5% CHAPS)	206					
E4-930914	PNGase F (trypsin/with 0.5% CHAPS)	200	204.5	±3.4	1.7%	4	4
E4-930914	PNGase F (Lys C/with 0.5% CHAPS)	204					
B11-930406	GlycoPrep (0.5 mg/reactor)		235	±2.6	1.1%	5	10
B11-930406	PNGase F (without CHAPS)	241					
B11-930406	PNGase F (with 0.5% CHAPS)	246	243.5	±3.5	1.5%	2	2

a



b



c

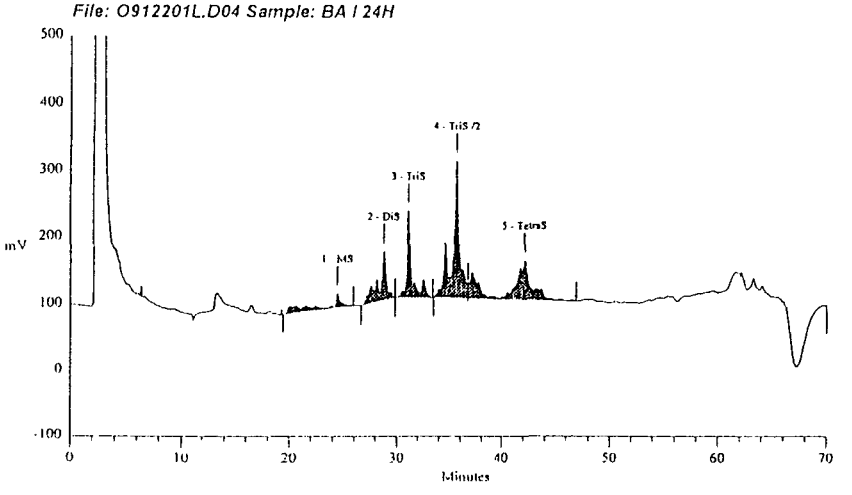


Table III. Analysis of lot-to-lot consistency of rhu IL-4R (CHO) produced in a pilot plant

	rhu IL-4R purified via mAb*				rhu IL-4R purified via conventional chromatographic steps			
	Neu5Ac (mol/moL)		Z		Neu5Ac (mol/mol)		Z	
Harvest no.	Individual results	Average Stdev CV (%)	Individual results	Average Stdev CV (%)	Individual results	Average Stdev CV (%)	Individual results	Average Stdev CV (%)
R007	7.3		150		9.6		189	
R008	6.8		143		9.6		167	
R009	7.1		148		10.4		175	
R010	9.9		140		10.4		167	
R011	6.6		135		10.2		181	
R012	7.7	6.9	145	138.7	10.1	10.3	175	176.3
R013	6.3	1.2	135	6.9	11.3	0.5	172	7.2
R014	5.6	17.4	129	5.0	9.9	5.1	187	4.1
R015	5.9	(n = 11)	133	(n = 11)	10.8	(n = 11)	176	(n = 11)
R016	6.1		133		10.7		172	
R017	6.1		135		10.7		178	

*. Small scale for analytical investigations.

the decrease of free Neu5Ac, going from run a to run d, the HPAE-PAD chromatograms increasingly resembled the PNGase F-derived N-glycan pool (run e), with the closest resemblance achieved with the GlycoPrep 1000 (run d) (Hermentin *et al.*, 1992b). At that time, a comparison of these various N-glycan pools was not possible without the presentation of the corresponding mapping profiles (Hermentin *et al.*, 1992b, Figure 5).

In order to prove the usefulness of the hypothetical N-glycan charge for the appropriate characterization of N-glycosylation, Z was retrospectively calculated for each of these samples from the respective HPAE-PAD mapping profiles (Hermentin *et al.*, 1992b, Figure 5), and these results are summarized as follows: Z = 248 for run a (LB-001, OGS), Z = 262 for run b (50 mg hydrazinolysis batch), Z = 276 for run c (1000 mg hydrazinolysis batch), Z = 285 for run d (2 mg batch, GlycoPrep 1000), and Z = 289 for run e (trypsin/PNGase F-derived N-glycan pool). Thus, the increase of Z clearly corresponded with the decrease in the loss of sialic acid (going from run a to run d). The GlycoPrep-derived N-glycan pool (Hermentin *et al.*, 1992b, Figure 3, run d; Z = 285) exhibited Z almost identical to the trypsin/PNGase F-derived N-glycan pool (Hermentin *et al.*, 1992b, Figure 3, run e; Z = 289), suggesting their virtual identity.

Z and the lot-to-lot consistency of rhu IL-4R

Samples of 11 successive harvests of rhu IL-4R (CHO) production in a pilot plant (R007-R017) were worked up, employing conventional chromatographic steps (unpublished data) as well as small scale monoclonal antibody affinity chromatography, and were analyzed with respect to sialic acid content and Z determination. As can be seen from Table III, the mAb-purified materials provided an average sialic acid content of 6.9 ± 1.2 mol/mol (CV = 17.4%; n = 11) and a hypothetical N-glycan charge of Z = 138,7 ± 6.9 (CV = 5,0%; n = 13). Due to its higher accuracy, the Z determination appeared to be more reliable than the sialic acid content determination, most likely as Z is independent of the protein determination. As the validation experiments have shown that the determination of Z is highly accurate, with CV < 3% (Table II), the higher variation of Z for the pilot plant production/mAb purification (CV = 5.0%; Table III) is due to slight, but tolerable, inconsistencies of the 11 production lots (R007–R017).

The conventional chromatographic purification process was designed such that it should remove the lower-charged glycoforms, which were expected to increase the clearance rate of the material in the liver (compare the results of rmur IL-4R, summarized in Table IV). Indeed, the chromatographic purification provided higher sialic acid contents (average: 10.3 ± 0.5 mol/mol; CV = 5.1%; n = 11) and higher Z values (average: Z = 176.3 ± 7.2; CV = 4.1%; n = 11), as was intended. Again, the Z determination appeared to be slightly more accurate than the sialic acid content determination. The variation of Z was slightly reduced to CV = 4.1% (versus CV < 3% during validation), proving the high lot-to-lot consistency of the purified rhu IL-4R materials.

Z and the glycoprotein clearance in vivo, exemplified with rmur IL-4R (BHK)

A preparation of rmur IL4R (BHK) was passed over a column of Q-Sepharose FF and collected in 5 fractions (Figure 3). The isoelectric focusing of the fractions revealed dramatic differences in the patterns of the glycoforms for fractions Q1 to Q4, while fractions Q4 and Q5 proved to be indistinguishable (Figure 4). The relevant

Fig. 2. HPAE-PAD mapping profiles of the α_1-acid glycoprotein N-glycan pool. (**a**) Automated hydrazinolysis. (**b**) PNGase F release using 'EPO conditions'. (**c**) Tryptic digestion prior to PNGase F release (taken from Hermentin *et al.*, 1992b).

Table IV. *In vivo* clearance and analytical data of rmur IL-4R (BHK)-Q-sepharose fractions

Starting material: fraction	Neu5Ac (μg/mg)	Neu5Ac/Gal (mol/mol)	Man/Gal (mol/mol)	Z	AUD(5-30) (μg/ml*min)	CI(5-30) (ml/min)
	78.5	0.54	1.28	n.d.	42.7	0.23
Q1	13.6	0.20	3.02	147	0.8	10.00
Q2	38.2	0.42	1.99	191	5.3	2.00
Q3	82.9	0.62	1.24	238	33.6	0.29
Q4	109.5	0.67	1.04	248	63.7	0.16
Q5	108.6	0.63	0.98	n.d.	85.9	0.12

n.d., Not determined.

analytical data of these fractions are summarized in Table IV. The sialic acid content was found close to zero in fraction Q1 (13.6 μg/mg) and increased up to fraction Q4 (109.5 μg/mg), while it remained constant in fraction Q5 (108.6 μg/mg).

In theory, the Neu5Ac/Gal ratio (i.e., the degree of sialylation) should reflect the *in vivo* clearance via the Gal receptors of the liver, whereas the Man/Gal ratio should permit to predict the clearance behavior via the high-mannose receptors present in the liver and on macrophages. The degree of sialylation therefore should allow prediction of the *in vivo* half-life of glycoproteins, relative to an appropriate standard. However, as the degree of sialylation is determined from two independent assays, the Neu5Ac and Gal determination, respectively, its coefficient of variation is dependent from the CV values of both tests. Hence, a more reliable parameter would be appreciated that would allow to predict the clearance behavior of glycoproteins in a more accurate manner. As may be seen from Table IV, the hypothetical N-glycan charge proved to be more predictable in that regard.

The degree of sialylation (Neu5Ac/Gal; mol/mol) was found to be lowest in fraction Q1 (0.20) and increased steadily up to fraction Q4 (0.67), whereas it slightly decreased again in fraction Q5 (0.63). The ratio of Man/Gal (mol/mol) was used to judge the ratio between high mannose and complex type structures. The Man/Gal ratio proved to be highest in fraction Q1 (3.02 mol/mol), reveal-

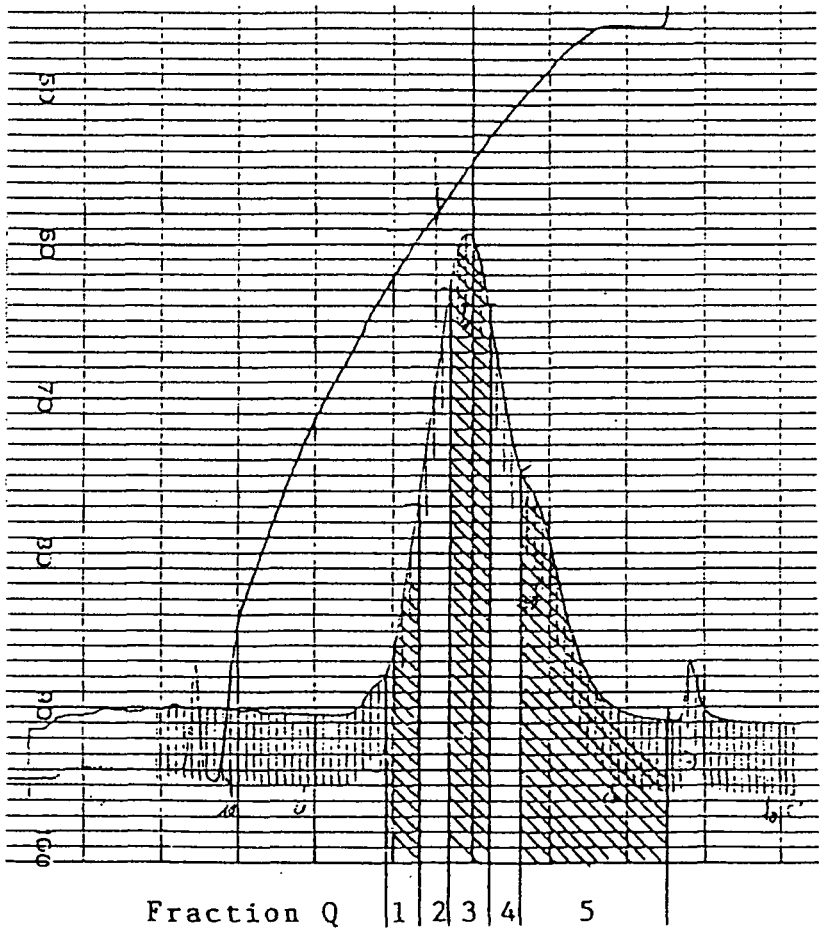


Fig. 3. Fractionation on Q-Sepharose FF of rmur IL-4R (BHK) (lot no. 018PP).

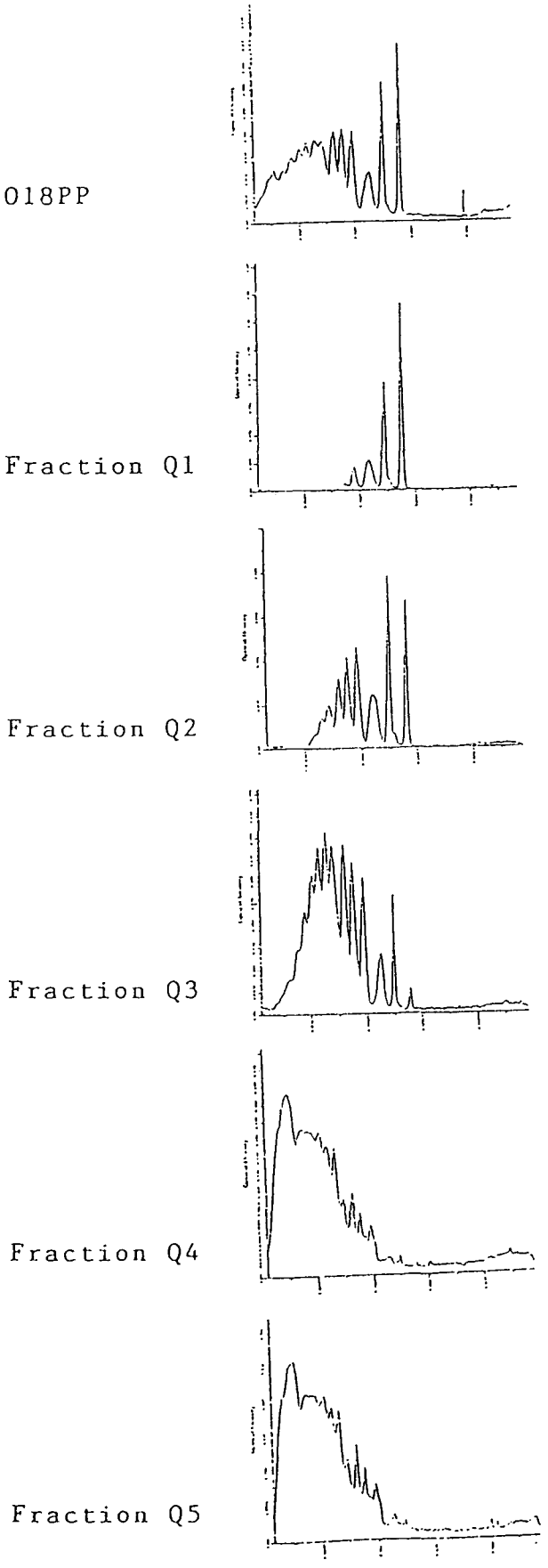


Fig. 4. IEF scans of Q-Sepharose FF fractionated rmur IL-4R (BHK) (lot no. 018PP).

ing a significant amount of high mannose-type structures. It decreased to 1.04 mol/mol in fraction Q4 and remained comparable in fraction Q5 (0.98 mol/mol), which reflected, at an average, the presence of triantennary complex type structures and the absence of high-mannose type structures. As the degree of both the asialo and high mannose type structures goes into the calculation of Z (Eq. 1), the hypothetical N-glycan charge should reflect the contributions to the clearance of both. Therefore, Z should better reflect the clearance behaviour of a glycoprotein *in vivo* than any other of the analytical parameters summarized in Table IV.

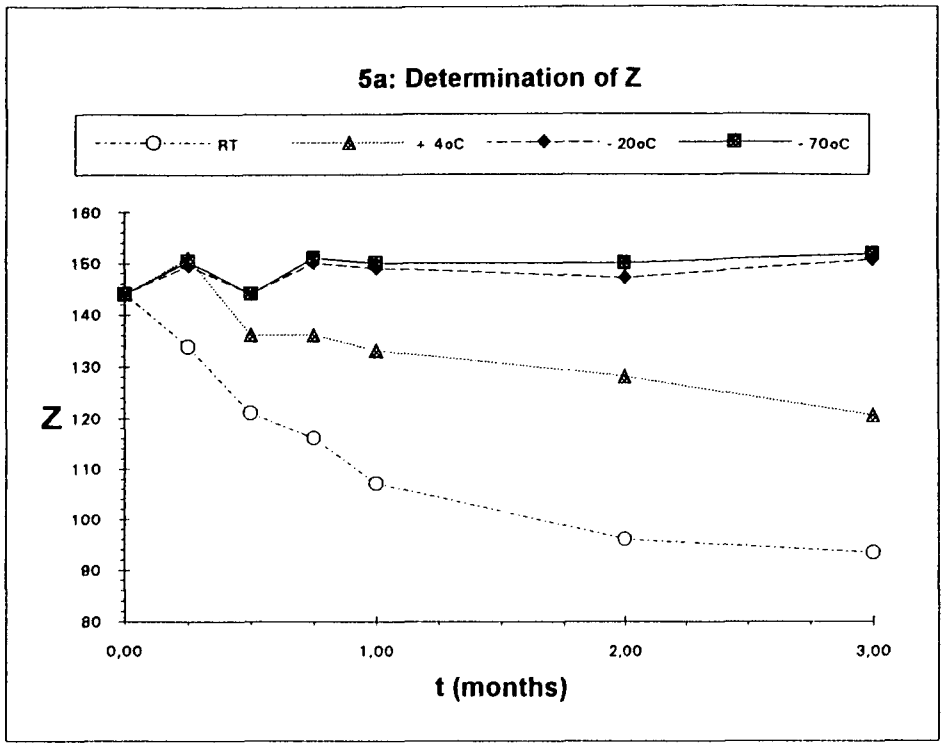
As expected, Z proved to be smallest in fraction Q1 (Z = 147), and increased to fraction Q4 (Z = 248). Similarly, the *in vivo* clearance in mice, expressed as AUD values (Table IV), was found to be smallest in fraction Q1 (AUD = 0.8) and increased to fraction Q4 (AUD = 63.7) and Q5 (AUD = 85.9), revealing the instant removal of the Q1 material from the circulation via the galactose and mannose receptors available in the mouse system. Note: Z could not be determined for fraction Q5 due to the lack of sufficient amounts of material. In comparison with the other analytical data, the AUD value of fraction Q5 appeared too high; however, the measurement could not be repeated for the same reason. Moreover, it needs to be mentioned that the AUD values were determined from a single mouse each, for animal protection reasons, which makes the AUD values somewhat questionable. Therefore, Z determination may be regarded as an analytical alternative that should enable doing without the highly costly and contested *in vivo* clearance studies.

Z and the IL-4R stability upon storage

In order to determine how long culture supernatants of the rhu IL-4R (CHO) fermentation could be stored prior to work-up, aliquots of the culture supernatant of the pilot plant were kept at RT, +4°C, −20°C, and −70°C for periods of up to 3 months. After incubation the samples were purified by immunoaffinity chromatography, and the stability of IL-4R was assessed, among other parameters (not shown), via sialic acid and Z determination. As can be seen from Figure 5a, the hypothetical N-glycan charge appeared to be constant for aliquots stored at −70°C or even −20°C, however, decreased upon storage at RT or +4°C, which clearly indicated the loss of charge upon storage without freezing. Indeed, the sialic acid content dropped analogously (Figure 5b), indicating that the decrease of Z was attributable to the loss of sialic acid. However, the sialic acid results appeared to be less reliable and less accurate, suggesting the superior reproducibility and accuracy of Z determination.

Z determination of distinct glycoproteins rhu EPO (from CHO and BHK cells)

The carbohydrate analysis of rhu EPO, originally described by Sasaki *et al.* (1987) and Takeuchi *et al.* (1988) for rhu EPO (CHO) and by Tsuda *et al.* (1988) for rhu EPO (BHK), has recently been extended by studies of Hokke *et al.* (1995) and Watson *et al.* (1994) (for CHO-EPO) and Nimtz *et al.* (1993) (for BHK-EPO). As rhu EPO is known



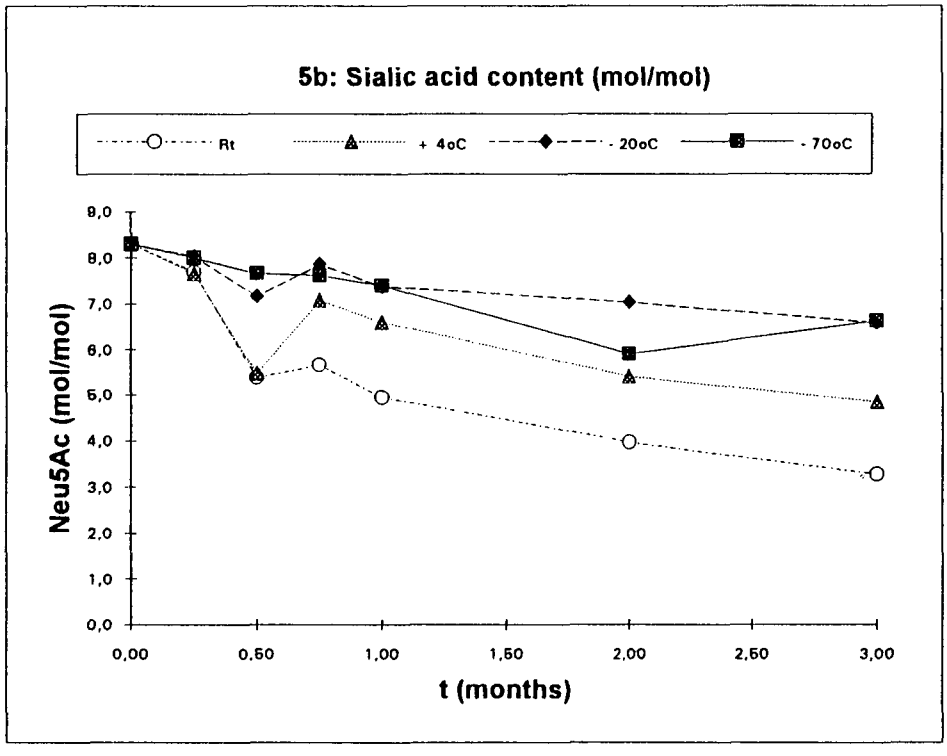


Fig. 5. Stability of cell culture supernatants of rhu IL-4R (CHO) at RT, +4°C, −20°C, and −70°C . (**a**) Determination of Z. (**b**) Sialic acid content (mol/mol).

to consist of mainly tetraantennary structures with 0–3 LacNAc repeats (Takeuchi and Kobata, 1991), *Z* should amount to a number between 300 and 400.

Indeed, the hypothetical N-glycan charge of CHO-EPO (Boehringer Mannheim) was determined to $Z = 361 \pm 2$ (CV = 0.6%) ($n = 6$; three different experiments with 2 HPAE-PAD runs, each), and the N-glycan charge of BHK-EPO (Merckle) was determined to $Z = 323 \pm 2$ (CV = 0.7%) ($n = 4$; four different lots; four different experiments; one HPAE-PAD run, each) (Table V). Thus, the smaller *Z* value of the BHK-EPO from Merckle clearly reflected the greater share of undersialylated N-glycans: 34% of the

Table V. Hypothetical N-glycan charge Z of various glycoproteins

Glycoprotein	Origin of glycoprotein	Origin of glycan pool	Z	Rem.
rhu EPO (CHO)	Boehringer Mannheim	PNGase F	361	
rhu EPO (CHO)	Amgen	PNGase F	367	a
rhu EPO (CHO)	Organon Teknika	PNGase F/SDS	286	b
rhu EPO (BHK)	Merckle	PNGase F	323	
Fetal calf serum fetuin	Sigma	Large scale hydrazinolysis	256	c
Bovine fetuin	Sigma	GlycoPrep	290	d
Bovine fetuin			267	e
Bovine pancreas ribonuclease B		OGS	15	f
Hen ovomucoid		OGS	15	
Pig thyroglobulin		OGS	82	
Human alpha-1-acid glycoprotein	BW AG	GlycoPrep	289	g
Human serotransferrin		OGS	207	
Human antithrombin III	BW AG	GlycoPrep	180	
Human fibrinogen		OGS	184	
Alpha-1-T glycoprotein	BW AG	GlycoPrep	187	
Alpha-1-antitrypsin	BW AG	GlycoPrep	190	
Alpha-1-antichymotrypsin	BW AG	GlycoPrep	236	
β-2-glycoprotein-I	BW AG	GlycoPrep	185	
TBG glycoprotein	BW AG	GlycoPrep	208	
Alpha-1-B glycoprotein	BW AG	GlycoPrep	194	
Alpha-2-HS glycoprotein	BW AG	GlycoPrep	158	
8S-alpha-3 glycoprotein	BW AG	GlycoPrep	145	
Haptoglobulin	BW AG	GlycoPrep	197	

[a] Z was calculated from the data provided by Watson et al. (1994).
[b] Z was calculated from the data provided by Hokke et al. (1993).
[c] Glycan pool desalted.
[d] Glycan pool not desalted.
[e] Z was calculated from the data provided by Green et al. (1988).
[f] OGS, Oxford GlycoSystems.
[g] BW AG, Behringwerke AG.

N-glycans were missing one and 12% of the N-glycans were missing two terminal sialic acid residues; the structures consisted in 40.9 % tetrasialylated, 35.0 % trisialylated and 21.1 % disialylated structures (Nimtz *et al.*, 1993). These data from the literature allowed calculation of the N-glycan charge to $Z = 311$, which is in good agreement (deviation <4%) with the N-glycan charge determined according to Equation 1, i.e., $Z = 323$, using the same rhu EPO (BHK) from Merckle.

On the other hand, in the CHO-EPO from Amgen the major (>95%) di-, tri- and tetraantennary structures were fully sialylated (Watson *et al.*, 1994). Their separation according to charge of the PNGase F-released N-glycans (using a Glycopak DEAE column) allowed to calculate the N-glycan charge to $Z = 367$, which is in excellent agreement with the glycan charge of the EPO (CHO) from Boehringer Mannheim, used in this study ($Z = 361$).

In contrast, in the study of Hokke *et al.* (1995), which used CHO-EPO from Organon Teknika, 18–20% of the N-glycans were missing one and 3% of the N-glycans were missing two sialic acid residues. Their structural analysis enabled to calculate the N-glycan charge to $Z = 286$, which proved to be significantly smaller than the CHO-EPO from Amgen ($Z = 367$) and Boehringer ($Z = 361$) as well as the BHK-EPO from Merckle ($Z = 323$). This undersialylation observed for the CHO-EPO from Organon ($Z = 286$) could be attributable to differences in the distinct cell line used, the production process, and the respective work-up conditions. Most important, however, it may be speculated that it should result in a reduced half-life (due to clearance via asialo receptors of the liver) and, hence, in a reduced bioactivity.

Thus, the N-glycan charge appears as a very helpful number that enables judgement of the glycosylation status of different EPO preparations, and the same should hold true for other glycoproteins.

Bovine serum fetuin

For bovine serum fetuin, Green *et al.* (1988) have assigned 23 distinct N-linked oligosaccharides, 3% of which were mono, 35% were di-, 54% were tri-, and 8% were tetrasialylated structures. From these data, a hypothetical N-glycan charge of $Z = 267$ may be anticipated. Uncertainties of course arise from the occurrence on fetuin of O-glycans (Spiro and Bhoyroo, 1974), which show up in the HPAE-PAD chromatogram as the 'peeled' disaccharide Neu5Acα 2,3Gal (Hermentin *et al.*, 1994) as a peak at 22.5 min (in the monosialo-region) (see Hermentin and Witzel, 1993; Figure 9, run f). In a distinct set of experiments the large-scale (0.5 g) hydrazinolysis-derived glycan pool of fetal calf serum fetuin was desalted via Sephadex G-25, which resulted in the removal of most of the O-glycans (Hermentin *et al.*, 1994). When the integration of the residual 'degraded O-glycan'-peak in the HPAE-PAD chromatogram (at 22.5 min) was suppressed via the software, the hypothetical N-glycan charge was determined to $Z = 256$ (Table V), which was only 4% less then the one calculated from the data provided by Green *et al.* (1988) (i. e., $Z = 267$).

In a different experiment, the automated hydrazinolysis-derived glycan pool of bovine (instead of fetal calf) serum fetuin was mapped via HPAE-PAD without prior desalting, and Z was calculated according to Equation 1, taking the 'degraded O-glycan'-peak as monosialylated

glycan, which provided $Z = 290$ (Table V). It is obvious that special care has to be taken when using the hydrazinolysis-derived glycan pools of glycoproteins carrying O-glycans. In addition, the determination of Z for bovine fetuin is hampered by the occurrence of antennary Galβ1,3GlcNAc (instead of Galβ1,4GlcNAc), concomitant with the presence of Neu5Ac attached to antennary GlcNAc (see Green *et al.*, 1988, and references cited therein), which complicates the Z calculation. However, in the case of fetuin, where the groups of glycan peaks are clearly separated (see Hermentin and Witzel, 1993; Figure 9, run f), Z may be calculated without any difficulties.

Hen ovomucoid

The carbohydrate moieties of hen ovomucoid were reported to consist almost exclusively in bisected trunkated complex type N-glycan structures (see, e.g., Yamashita *et al.*, 1982, 1983). Therefore, Z should be expected to be close to zero. Indeed, the mapping profile of the chicken ovomucoid N-glycan pool LB013 (OGS) (see Hermentin and Witzel, 1993; Figure 9, run b) provided $Z = 15$ (Table V), which reflects the absence of charged structures.

Bovine pancreas ribonulease B

The complete structural characterization of the oligosaccharides from bovine pancreas ribonulease B has recently been described (Liang *et al.*, 1980; for a recent study, see Fu *et al.*, 1994, and references cited therein), revealing a unique nature of high mannose type structures. The mapping profile of the bovine pancreas ribonulease B N-glycan pool LB009 (OGS) (see Hermentin and Witzel, 1993; Figure 9, run a) provided $Z = 15$ (Table V), due to small spikes in the monosialylated region, which could be impurities of the hydrazinolysis-derived glycan pool.

Porcine thyroglobulin

The determination of Z for the carbohydrate chains of porcine thyroglobulin (Yamamoto *et al.*, 1981) is made more difficult by the occurrence of monosialylated sulfated N-glycans and by the fact that 8% of the major structures carry Neu5Gc instead of Neu5Ac (de Waard *et al.*, 1991). The hypothetical N-glycan charge was determined from the mapping profile of glycan pool LB-011 (OGS) (see Hermentin and Witzel, 1993; Figure 9, run c), which proved to be devoid of any tri- and tetrasialylated structures (apart from a few spikes which could be regarded as impurities or artefacts), yielding $Z = 82$ (Table V).

Human serum glycoproteins

Most of the human serum glycoproteins examined (Table V) have thus far not been analyzed with respect to their distinct N-glycan structures. Some of their features may be gained from reviews of Baenziger (1984), Schwick and Haupt (1984), Haupt (1990), and Turner (1992).

α_1-Acid glycoprotein

The major N-glycan structures of α_1-acid glycoprotein have been elucidated (after desialylation) by Fournet *et al.* (1978) and Yoshima *et al.* (1981) as being mainly tri- and tetraantennary structures with and without antennary fucose, and additional minor components have recently been assigned by comparison with structures contained in an HPAE-PAD and a HPCE mapping database (Hermentin *et al.*, 1992b, 1994). Due to the occurrence of branch fucosylation, which makes the fucosylated N-glycans elute about 4 min faster (Hermentin *et al.*, 1992b), the calculation of Z for AGP proved to be more sophisticated than with the other glycoproteins of Table V. Thus, tetrasialylated C4–4* structures, upon the introduction of antennary fucose, will appear in or near the group of peaks of the trisialylated C4–3*/C3–3* structures, which again, upon fucosylation, will shift versus the group of peaks of the disialylated C4–2*/C3–2*/C2–2* structures (Hermentin *et al.*, 1992b). Therefore, the AGP-derived N-glycan pool did not result in the clearly separated groups of peaks normally seen. Bearing these AGP-related complications in mind, the integration of the groups of peaks was chosen such that the most valid integration was achieved rather than to reflect the de facto charge carried by the respective N-glycan peaks. Thus, the hypothetical N-glycan charge of AGP lot 281184 was determined to $Z = 289$ (Table V). As the glycosylation of AGP is known to change during inflammation (De Graaf *et al.*, 1993), it is predicted that the Z values of AGP of individuals may be regarded as numbers that correlate with the status of the inflammation process.

Human fibrinogen

According to Townsend *et al.* (1982), human fibrinogen contains four biantennary oligosaccharide chains which are partially desialylated. These data should amount to $Z \sim 200$, and $Z = 184$ was in fact obtained for the human fibrinogen N-glycan pool LB012 from OGS (Table V). However, the mapping pattern (see Hermentin and Witzel, 1993; Figure 9, run e) looked much more complex than should be expected from the data of Townsend *et al.* (1982), which needs further evaluation.

Human serum transferrin

Similarly, human serum transferrin is known to carry mostly the C2–224300.02 glycan structure (Spik *et al.*, 1975), and hence a glycan charge in the range of 200 should again be expected. Indeed, the mapping profile of the transferrin N-glycan pool LB010 (OGS) revealed C2–224300.02 as the predominant structure (see Hermentin and Witzel, 1993; Figure 9, run d), and the glycan charge was calculated to $Z = 207$ (Table V). In contrast to AT III, the occurrence of traces of tri- and tetrasialylated structures was also observed, elevating Z to >200. As tetraantennary structures have thus far not been described for human serum transferrin, the spikes detected in the tetrasialylated region of the mapping profile (Hermentin and Witzel, 1993) may be regarded as artefacts of the distinct transferrin N-glycan pool used (LB010, OGS). The glycosylation of transferrin is known to change in alcohol abuse (Turner, 1992; De Jong *et al.*, 1995; Landberg *et al.*, 1995). It is predicted that the Z values of transferrin of individuals will correlate with the status of alcohol abuse.

Human antithrombin III

Human antithrombin III is known to carry only biantennary complex-type N-glycans, of which 70% are disialyated and 30% are monosialyated structures (Mizuochi *et al.*, 1980; Franzén *et al.*, 1980). These data should amount to a hypothetical N-glycan charge of $Z = 170$. Indeed, $Z = 170$ was determined from the AT III N-glycan pool obtained via large-scale hydrazinolysis (performed at OGS), whereas $Z = 180$ was obtained after automated hydrazinolysis, using the GlycoPrep 1000 (Table V).

Additional human serum glycoproteins

Various other human serum glycoproteins, such as α_1-T glycoprotein, α_1-antitrypsin, α_1-antichymotrypsin, β_2-glycoprotein-I, TBG-glycoprotein, α_1-B glycoprotein, α_2-HS glycoprotein, 8S-α_3 glycoprotein, and haptoglobulin (for further information see the reviews of Baenziger, 1984; Schwick and Haupt, 1984; Haupt, 1990), have been subjected to automated hydrazinolysis, and the respective Z values are summarized in Table V.

With the exception of AGP ($Z = 289$), α_1-antichymotrypsin ($Z = 236$), TBG-glycoprotein ($Z = 208$) and serotransferrin ($Z = 207$), the human serum glycoproteins measured so far proved to exhibit $Z < 200$, with Z ranging between $Z = 145$ (8S-α_3 glycoprotein) and $Z = 197$ (haptoglobulin). The corresponding HPAE-PAD mapping profiles (not shown) revealed the absence of peaks in the tetrasialylated region for α_1-T glycoprotein, α_1-antitrypsin, β_2-glycoprotein-I, TBG-glycoprotein, α_1-B glycoprotein, α_2-HS glycoprotein, and 8S-α_3 glycoprotein. Moreover, for these glycoproteins, the disialyated (DiS) region proved to be in the range between 50% (α_2-HS glycoprotein) and 75% (α_1-antitrypsin), suggesting the disialylated biantennary (C2–2*) structure(s) as the dominate N-glycan(s) of most of the human serum glycoproteins.

General discussion

The demand for reproducible, fast, and facile carbohydrate analysis is increasing steadily. However, the more detailed assignment of protein N-glycosylation comprised, up till now, a costly and time-consuming structural analysis, which required expertise and infrastructure for methylation analysis/GC-MS, FAB-MS, and high resolution ^{1}H-NMR spectroscopy. We have recently pointed out that an alternative appears attractive that should allow reduction of these highly costly and time-consuming demands of carbohydrate structural analysis by using standard separation techniques and affordable hardware (Hermentin *et al.*, 1992a, 1994). High-pH anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) fulfills the criteria for a quick, reliable, and cheap routine standard separation technique that enables the lot-to-lot consistency control of recombinant glycoproteins (Conradt *et al.*, 1991) as well as the structural assignment of N-glycans by mere comparison of retention times (Hermentin *et al.* 1992a,b).

In HPAE-PAD, the separation is achieved under alkaline conditions by the use of an anion exchange column, which separates the isolated N-glycans primarily according to charge, i.e., the number of sialic acid residues (Lee, 1990; Conradt *et al.*, 1991; Townsend and Hardy, 1991; Hermentin *et al.*, 1992a), and renders this technique especially suitable for Z determination. The peaks of N-glycans of a given charge are mostly comprised in distinct groups of peaks that are well separated from each other (Figure 1). This enables to assign the N-glycan composition according to neutral (asialo), mono-, di-, tri-, and tetrasialo-structures, i.e., N-glycans with zero to four negatively charged sialic acid residues (Conradt *et al.*, 1991; Hermentin *et al.*, 1992a; Hermentin and Witzel, 1993).

Particularly helpful is the use of two internal standards of which the first one (S1 = e.g., LNnT or LNFP-V) elutes before, and the second one [S2 = (Neu5Ac)3] elutes after the distinct peaks of the N-glycan pool. Thus, the N-glycans detected between the two standards normally amount to the total peak area of the glycan pool, which is set equal to 100%. Similarly, the areas of the groups of peaks of a distinct charge are calculated via the chromatography software and expressed as the percentage of the total peak area.

Here we show that the mapping profiles of protein N-glycan pools obtained via HPAE-PAD can be used to calculate the 'hypothetical N-glycan charge Z,' which may be regarded as a new and helpful number that characterizes protein N-glycosylation. Z allows one to compare the glycosylation of various glycoproteins, to check the lot-to-lot consistency of recombinant glycoproteins, and to predict their *in vivo* clearance rates, relative to an appropriate standard preparation, and thus enables, to a certain extent, evaluation of their respective therapeutic efficacy and safety. This again may reduce the need for the very costly and time-consuming and quite inaccurate *in vivo* bio-assays, a reduction that will also protect animals.

Thus, the hypothetical N-glycan charge Z of a glycoprotein may be regarded as a new and very helpful parameter that enables evaluation of the protein glycosylation in a simple, however, efficient manner.

Similarly, it is predicted that the hypothetical N-glycan charge can be used as a diagnostic tool in those cases where the glycosylation varies with the status of the disease, e.g., disease-related inflammations (α_1-acid glycoprotein; see De Graaf *et al.*, 1993; Mackiewicz and Mackiewicz, 1995), hepatocellular carcinoma (α-fetoprotein; see Aoyagi, 1995), ovarian cancer (α_1-proteinase inhibitor and haptoglobin; see Turner *et al.*, 1995), colorectal cancer (see Shahangian *et al.*, 1991), cerebrospinal fluid leakage and alcohol abuse (transferrin; see De Jong *et al.*, 1995), in Tamm-Horsfall-related diseases (see Hard *et al.*, 1992), or in cancers where circulating blood group related carbohydrate antigens are regarded as tumor markers (see Shahangian *et al.*, 1991; Orntoft and Bech, 1995) (for a comprehensive review, see Turner, 1992).

Materials and methods

Chemicals

The chemicals used for automated hydrazinolysis were purchased from Oxford GlycoSystems, Abingdon, England.

The internal standards LNnT and LNFP-V were also purchased from Oxford GlycoSystems, Abingdon, England. LNnT was also kindly provided by Dr. Ed McGuire, NEOSE Pharmaceuticals, Inc., Horsham, PA, USA). $(Neu5Ac)_3$ was prepared from colominic acid (Sigma) as previously described (Hermentin *et al.*, 1992a)

Glycoproteins

Rhu IL-4R (CHO) (lots E4–930914 and B11–930406) and rmur IL-4R (BHK) (lot 018PP) were developmental products of Behringwerke AG. Rhu EPO (BHK) and rhu EPO (CHO) were from Merckle AG (Ulm, Germany) and Boehringer Mannheim GmbH (Penzberg, Germany), respectively. The following human serum glycoproteins were products of Behringwerke AG (Marburg, Germany): AGP (lot no. 281184), antithrombin III (lot no. 244711), α_1T-glycoprotein (lot no. 120891), α_1-antitrypsin (lot no. 030179), α_1-antichymotrypsin (lot no. 041181), β_2-glycoprotein I (lot no. 768), TBG-glycoprotein (lot no. 81641), α_1B-glycoprotein (lot no. 3525), α_2HS-glycoprotein (lot no. 369/L6), 8Sα_3-glycoprotein (lot no. 270792/1A), haptoglobin (lot no. 3525). Bovine serum fetuin and fetal calf serum fetuin were from Sigma.

Hydrazinolysis-derived N-glycan pools

Hydrazinolysis was performed as previously described (Hermentin, 1993; Hermentin *et al.*, 1994), using an automated glycan release and recovery apparatus (GlycoPrep 1000, Oxford GlycoSystems, Abingdon, England). The N-glycan pools were desalted via a column of Sephadex G-25 superfine (Pharmacia) (column size 21 × 1 cm).

The following hydrazinolysis-derived glycan pools were purchased from Oxford GlycoSystems for measurements by HPAE-PAD: ribonuclease B from bovine pancreas (LB-009), human serumtransferrin (LB-010), porcine thyroglobulin (LB-011), human fibrinogen (LB-012), chicken ovomucoid (LB-013).

PNGase F-derived N-glycan pools

EPO. The liberation by PNGase F of the N-glycans of rhu EPO was performed as described by Nimtz *et al.* (1993).

AGP. In a first series of experiments, AGP was digested with trypsin prior to digestion with PNGase F, as previously described (Hermentin *et al.*, 1992b). In a second series of experiments, AGP was directly digested with PNGase F, applying the conditions used for rhu EPO, in order to achieve incomplete digestion.

IL-4R. The liberation by PNGase F of the N-glycans of rhu IL-4R (CHO) (500 μg) was achieved after reduction with dithioerythrol (25 μl of aq. 0.3 M DTE; 10 min at 70°C). Excess of DTE was removed by concentration/washing (with digestion buffer) in Centricon tubes (10,000 D, Amicon). The concentrated material was digested with PNGase F (Boehringer Mannheim, 5 units) in 50 mM sodium phosphate buffer pH 7.6 (500 μl), 48 h, 37°C (Eppendorf vials), with or without the presence of 0.5% CHAPS. The mixture was desalted via Sephadex G-25 superfine (Pharmacia) (column size 21 × 1 cm).

Monosaccharide and sialic acid determination

Monosaccharides were hydrolyzed in 2 N trifluoroacetic acid at 100°C for 4 h and measured via HPAE-PAD in analogy to the procedure described by Hardy *et al.* (1988). Neu5Ac was determined via HPAE-PAD as earlier described (Hermentin and Seidat, 1991).

Mapping by HPAE-PAD

The glycan pools were measured by HPAE-PAD, using the set-up and the optimized standard gradient 'S' for sialylated glycans previously described (Hermentin *et al.*, 1992a).

Determination of Z

The hypothetical N-glycan charge of the glycoproteins was gained by (i) liberating the N-glycan-pool of the glycoprotein via hydrazinolysis or PNGase F; (ii) measuring the N-glycan pool via HPAE-PAD; (iii) calculating the percentage of the areas (A) of the groups of peaks, separated by charge; (iv) multiplying the area% of the peak-groups in the neutral (asialo-, as), monosialo- (MS), disialo-(DiS), trisialo- (TriS), tetrasialo- (TetraS) and pentasialo (PentaS) region by zero (asialo), 1 (MS), 2 (DiS), 3 (TriS), 4 (TetraS), and 5 (PentaS), respectively; and (v) summarizing the respective products.

Thus, Z was defined as the sum of the products of the respective areas (A) in the asialo (as), monosialo (MS), disialo (DiS), trisialo (TriS), tetrasialo (TetraS), and pentasialo (PentaS) region, each calculated as the percentage of the total peak area set equal to 100%, and each multiplied by the corresponding charge:

$$Z = A_{(as)} \cdot 0 + A_{(MS)} \cdot 1 + A_{(DiS)} \cdot 2 + A_{(TriS)} \cdot 3 + A_{(TetraS)} \cdot 4\,[+ A_{(PentaS)} \cdot 5]$$

Validation experiments

Z and the validity of automated hydrazinolysis, using rhu IL-4R (CHO). Rhu IL-4R (CHO), produced in two different cell lines (E4 and B11) under identical culture and work-up conditions (lots no. E4–930914 and B11–930406, respectively; unpublished results), was subjected to automated hydrazinolysis in the presence of LNnT or LNFP-V as internal standard in 3 × 6 distinct experiments, using 0.5 mg of glycoprotein per hydrazinolysis reactor, each. Alternatively, E4–930914 was measured in six distinct experiments, using 1.0 mg of glycoprotein per reactor. Each N-glycan pool was desalted via Sephadex G-25 superfine and measured via HPAE-PAD three times at three different days. A reference chromatogram is shown in Figure 1; the determination of Z is outlined in Table I.

Z and the validity of PNGase F digestions, using rhu IL-4R (CHO)

The two samples of rhu IL-4R (Lots no. E4–930914 and B11–930406) were digested with PNGase F (see *PNGase F-derived N-glycan pools*), in the presence or absence of 0.5% CHAPS. Lot E4–930914 was further used to compare the PNGase F digest after previous digestion with trypsin or Lys C (Table II), applying the conditions previously described for the tryptic digestion of AGP (Hermentin *et al.*, 1992b).

Z and the PNGase F digestion of AGP. AGP was digested with PNGase F for a period of 48 h, applying the conditions described for rhu EPO (Nimtz *et al.*, 1993), in order to achieve incomplete N-glycan release. Z was compared with the value obtained via hydrazinolysis as well as with the value obtained via PNGase F digestion of a tryptic AGP digest (Hermentin *et al.*, 1992b) (Figure 2).

Z and the consistency control of protein glycosylation

Z and the comparison of various AGP-derived N-glycan pools. Two manual "large scale" hydrazinolysis-derived N-glycan pools (from 50 mg and 1000 mg of AGP, lot no. 281184), an automated hydrazinolysis (GlycoPrep 1000)-derived N-glycan pool, and a fourth hydrazinolysis-derived N-glycan pool of AGP, purchased from Oxford GlycoSystems (Cat. No. LB-001; derived from AGP, produced at Sigma), were previously compared with the PNGase F-derived N-glycan pool of AGP, by comparison of their respective HPAE-PAD chromatograms (Hermentin *et al.*, 1992b). These HPAE-PAD chromatograms have now been reprocessed (about 3 years later) in order to enable their comparison via Z determination.

Z and the lot-to-lot consistency of rhu IL-4R. Samples of culture supernatants of rhu IL-4R (CHO) production in a pilot plant (unpublished data) were purified on a column of immobilized monoclonal anti-rhu IL-4R antibody (mAb 971/134-Sepharose 4B). After extensive washing with PBS pH 7.2 + 0.5% Triton X-100 and PBS pH 7.2, rhu IL-4R was eluted with 0.1 mol/litre glycine-HCl pH 2.5. The eluate was neutralized immediately with 2 mol/litre Tris–HCl pH 8.0, concentrated by ultrafiltration, and dialyzed against PBS pH 7.2. Alternatively, the cell culture supernatants were worked-up via a series of chromatographic steps (unpublished procedure). The isolates were routinely analyzed with respect to the lot-to-lot consistency of the protein glycosylation, using Neu5Ac and Z determination as the appropriate parameters. The results are summarized in Table III.

Z and the glycoprotein clearance in vivo, *exemplified with rmur IL-4R (BHK)*

Purification of rmur IL-4R (BHK). Rmur IL-4R (BHK) was purified from culture supernatant by immunoaffinity chromatography on immobilized monoclonal anti-rmur IL-4R antibody (mAb M1-Sepharose 4B). After extensive washing with PBS pH 7.2 + 0.5% Triton X-100 and PBS pH 7.2, rmur IL-4R was eluted with 0.1 mol/litre dibasic sodium phosphate-NaOH pH 11.5. The eluate was neutralized immediately with 1 mol/litre monobasic sodium phosphate-NaOH pH 5.5, concentrated by ultrafiltration and dialyzed against PBS pH 7.2.

Fractionation of rmur IL-4R by anion-exchange chromatography and

analysis of the fractions. Rmur IL-4r (BHK) (21 mg; lot 018PP) in 20 mmol/litre Tris–HCl pH 7.5 (buffer A) was loaded on a column (1 × 10 cm) of Q-Sepharose FF equilibrated with buffer A. Protein was eluted with a linear gradient from 50 mmol/litre to 300 mmol/litre sodium chloride in buffer A at a linear flow rate of 76 ml/h; 1 ml fractions were collected and pooled as shown in Figure 3. The sialic acid content, expressed as Neu5Ac/peptide bone (mol/mol), the degree of sialylation, expressed as Neu5Ac/Gal (mol/mol), as well as the presence of high-mannose type structures, expressed as Man/Gal (mol/mol), was determined for each fraction and compared with the respective *Z* value, which is summarized in Table IV. Horizontal IEF of the samples (40 μl; 5–7 mg/ml) was performed in slab polyacrylamide gels (T5C5.1) with a gradient from pH 3.5 to 6.0 (Pharmacia Ampholines 3.5–5.0 and 4.0–6.0, 1 + 1). After IEF the gel was stained with Serva-Blue-G and scanned, using a Molecular Dynamics (Sunnyvale, CA 94086) MD 300A imaging system equipped with the Discovery One software from PDI (Protein and DNA ImageWare Systems, Huntington Station, NY) (Figure 4).

In vivo *clearance studies with rmur IL-4R.* The rate of clearance of rmur IL-4R (BHK) was determined as follows. Female BALB/C mice received an intravenous bolus injection of IL-4R (10 μg, 0.2 μg/kg) via the tail vein. Five, 10 and 30 min after application, blood was withdrawn by puncture of the retroorbital vein complex, and serum was prepared. Thereafter the concentration of IL-4R in serum samples was measured by an ELISA assay (unpublished data). The area under the serum concentration versus time data (AUD_{5-30}) was calculated by application of the trapezoidal rule (Ritschel, 1983), and the initial clearance of IL-4R (CI_{5-30}) was calculated according to the formula: CI_{5-30} = Dose/AUD_{5-30}, which is summarized in Table IV.

Z and the IL-4R stability upon storage

Culture supernatants containing rhu IL-4R (CHO) were stored at various temperatures (RT, +4°C, −20°C, and −70°C) over a period of 3 months. Samples were taken at day zero and after 1, 2, 3, 4, 8, and 12 weeks of storage and affinity-purified on immobilized monoclonal anti-rhu IL-4R antibody (mAb 971/134-Sepharose 4B). After extensive washing with PBS pH 7.2 + 0.5% Triton X-100 and PBS pH 7.2, rhu IL-4R was eluted with 0.1 mol/litre glycine-HCl pH 2.5. The eluate was neutralized immediately with 2 mol/litre Tris–HCl pH 8.0, concentrated by ultrafiltration and dialyzed against PBS pH 7.2, in order to enable sialic acid as well as (after automated hydrazinolysis) *Z* determination. The results are summarized in Figure 5.

Z determination of distinct glycoproteins

The glycan pools isolated from various glycoproteins via hydrazinolysis or PNGase F treatment, as summarized in Table V, were separated via HPAE-PAD, using the standard gradient 'S' previously described (Hermentin *et al.*, 1992a). The human serum glycoproteins were subjected to automated hydrazinolysis in amounts of 2 mg per reactor each and were mapped via HPAE-PAD without any further desalting. The various glycan pools that were purchased from OGS were dissolved in water and analogously measured by HPAE-PAD without any prior desalting. The mapping profiles of these OGS samples, which have been communicated previously (Hermentin and Witzel, 1993), were recalculated for *Z* determination (Table V).

Acknowledgements

We would like to thank Wiegand Lang, Roland Köhler, Uschi Knödler, and Dr. Karlheinz Enßle for their cooperation in the IL-4R project.

Abbreviations

rhu, recombinant human; rmur, recombinant murine; IL-4R, interleukin 4-receptor; AGP, α_1-acid glycoprotein; EPO, erythropoietin; CHO, Chinese hamster ovary; BHK baby hamster kidney; AT III, antithrombin III, TBG, thyroxin-binding globulin, HPAE-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; PBS, phosphate buffered saline; mAb, monoclonal antibody; IEF isoelectric focusing.

References

Aoyagi,Y. (1995) Carbohydrate-based measurements on alpha-fetoprotein in the early diagnosis of hepatocellular carcinoma. *Glycoconjugate J.*, **12,** 194–199.

Baenziger,J.U. (1984) The oligosaccharides of plasma glycoproteins: synthesis, structure, and function. In Putnam,F.W. (ed.), *The Plasma Proteins*, 2nd ed., Academic Press, New York. Vol IV, pp. 271–315.

Conradt,H.S., Schaper,K., Proppe, C. and Nimtz,M. (1991) Mapping of N-linked oligosaccharides from human recombinant glycoproteins: quality control of glycosylated therapeutic polypeptides. In Spier,R.E., Griffiths,J.B. and Meignier,B. (eds.), *Production of Biologicals from Animal Cells in Culture*, Butterworth-Heinemann Ltd., London, pp. 775–782.

De Graaf,T.W., Van der Stelt,M.E., Anbergen,M.G. and van Dijk,W. (1993) Inflammation-induced expression of sialyl Lewis X-containing glycan structures on $_1$-acid glycoprotein (orosomucoid) in human sera. *J. Exp. Med.*, **177,** 657–666.

De Jong,G., Feelders,R., van Noort,W.L. and van Eijk (1995) Transferrin microheterogeneity as a probe in normal and disease states. *Glycoconjugate J.*, **12,** 219–226.

De Waard,P., Koorevaar,A., Kamerling,J.P. and Vliegenthart,J.F.G. (1991) Structure determination by ^{1}H NMR spectroscopy of (sulfated) sialylated N-linked carbohydrate chains released from porcine thyroglobulin by peptide-N^4-(N-acetyl-β-glucosaminyl)asparagine amidase-F. *J. Biol. Chem.*, **266,** 4237–4243.

Enssle,K., Enders,B., Kurrle,R., Lauffer,L., Schorlemmer,H.-U., Dickneite,G., Kanzy,E.J. and Seiler,F.R. (1995) Biology of natural and recombinant soluble interleukin-4 receptor. *Behring Inst. Mitt.*, **96,** 103–117.

Fournet,B., Montreuil,J., Strecker,G., Dorland,L, Haverkamp,J., Vliegenthart,J. F. G.,Binette, J. P. and Schmid,K. (1978) Determination of the primary structures of 16 asialo-carbohydrate units derived from human plasma α_1-acid glycoprotein by 360-MHz ^{1}H NMR spectroscopy and permethylation analysis. *Biochemistry,* **17,** 5206–5214.

Franzén,L.-E., Svensson,S. and Larm,O. (1980) Structural studies on the carbohydrate portion of human antithrombin III. *J. Biol. Chem.*, **255,** 5090–5093.

Fu,D., Chen,L. and O'Neill,R.A. (1994) A detailed structural characterization of ribonuclease B oligosaccharides by ^{1}H NMR spectroscopy and mass spectrometry. *Cabohydr. Res.*, **261,** 173-186.

Green,E.D., Adelt,G., Baenziger,J.U., Wilson,S. and Van Halbeek,H. (1988) The asparagine-linked oligosaccharides on bovine fetuin: structural analysis of N-glycanase-released oligosaccharides by 500-megahertz ^{1}H NMR spectroscopy. *J. Biol. Chem.*, **263,** 18253–18268.

Hard,K., van Zadelhoff,G., Moonen,P., Kamerling,J.P. and Vliegenthart,J.F.G. (1992) The Asn-linked carbohydrate chains of human Tamm-Horsfall glycoprotein of one male. Novel sulfated and novel N-acetylgalactosamine-containing N-linked carbohydrate chains. *Eur. J. Biochem.*, **209,** 895–915.

Hardy,M.R.,Townsend,R.R. and Lee,Y.C. (1988) Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. *Anal. Biochem.*, **170,** 54–62.

Haupt,H. (1990) Chemie und klinische Bedeutung der Human-Plasmaproteine. *Behring Inst. Mitt.*, **86,** 1–66.

Hermentin,P. and Seidat,J. (1991) Micro-scale analysis of N-acetylneuraminic acid. Protein glycosylation: cellular, biotechnological and analytical aspects. *GBF Monogr,* **15,** 185–188.

Hermentin,P. and Witzel,R. (1993) Strategie zum Mapping von N-Glycanen mittels HPAE-PAD. In Schneider, H.-J. (ed.), *Kapillarelektrophorese-Chromatographie, Würzburger Kolloquium.* Bertsch Verlag, Straubing, pp. 137–148.

Hermentin,P., Witzel,R., Vliegenthart,J.F.G., Kamerling,J.P., Nimtz,M. and Conradt,H.S. (1992a) A strategy for the mapping of N-glycans by high-pH anion-exchange chromatography with pulsed amperometric detection. *Anal. Biochem.*, **203,** 281–289.

Hermentin,P., Witzel,R., Doenges,R., Bauer,R., Haupt,H., Patel,T., Parekh,R.B. and Brazel,D. (1992b) The mapping by high-pH anion-exchange chromatography with pulsed amperometric detection and capillary electrophoresis of the carbohydrate moieties of human plasma α_1-acid glycoprotein. *Anal. Biochem.*, **206,** 419–429.

Hermentin,P., Doenges,R., Witzel,R., Hokke,C.H., Vliegenthart,J.F.G.,

Kamerling,J.P., Conradt,H.S., Nimtz,M. and Brazel,D. (1994) A strategy for the mapping of N-glycans by high-performance capillary electrophoresis. *Anal. Biochem.*, **221,** 29–41.

Hokke,C.H., Bergwerff,A.A., van Dedem,G.W.K., Kamerling,J.P. and Vliegenthart,J.F.G. (1995) Structural analysis of the sialylated N- and O-linked carbohydrate chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells. Sialylation patterns and branch location of dimeric N-acetyllactosamine units. *Eur. J. Biochem.*, **228,** 981–1008.

Idzerda,R.L., March,C.J., Mosley,B., Lyman,S.D., Vanden Bos,T., Gimpel,S.D., Din,W.S., Grabstein,K.H., Widmer,M.B., Park,L.S., Cosman,D. and Beckmann,M.P. (1990) Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily. *J. Exp. Med.*, **171,** 861–873.

Landberg,E., Pahlsson,P., Lundblad,A., Arnetorp,A. and Jeppsson,J.-O. (1995) Carbohydrate composition of serum transferrin isoforms from patients with high alcohol consumption. *Biochem. Biophys. Res. Commun.*, **210,** 267–274.

Lee,Y.C. (1990) High-performance anion-exchange chromatography for carbohydrate analysis. *Anal. Biochem.* **189,** 151–162.

Liang,C.-J., Yamashita,K. and Kobata,A. (1980) Structural study of the carbohydrate moiety of bovine pancreatic ribonuclease B. *J. Biochem.*, **88,** 51–58.

Mackiewicz,A. and Mackiewicz,K. (1995) Glycoforms of serum α_1-acid glycoprotein as markers of inflammation and cancer. *Glycoconjugate J.*, **12,** 241–247.

Mizuochi,T., Fujii,J., Kurachi,J. and Kobata,A. (1980) Structural studies of the carbohydrate moiety of human antithrombin III. *Arch. Biochem. Biophys.*, **203,** 458–465.

Mosley,B., Beckmann,M.P., March,C.J., Idzerda,R.L., Gimpel,S.D., Vanden Bos,T., Friend,D., Alpert,A., Anderson,D., Jackson,J., Wignall,J.M., Smith,C., Gallis,B., Sims,J.E., Urdal,D., Widmer,M.B., Cosman,D. and Park,L.S. (1989) The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms. *Cell,* **59,** 335–348.

Nimtz,M., Martin,W., Wray,V., Klöppel,K.-D., Augustin,J. and Conradt,H.S. (1993) Structures of sialylated oligosaccharides of human erythropoietin expressed in recombinant BHK-21 cells. *Eur. J. Biochem.*, **213,** 39–56.

Nuck,R., Zimmermann,M., Sauvageot,D., Josic,D. and Reutter,W. (1990) Optimized deglycosylation of glycoproteins by peptide-N^4-(N-acetyl-β-glucosaminyl)-asparagine amidase from *Flavobacterium meningosepticum*. *Glycoconjugate J.*, **7,** 279–286.

Orntoft,T.F. and Bech,E. (1995) Circulating blood group related carbohydrate antigens as tumour markers. *Glycoconjugate J.*, **12,** 200–205.

Ritschel,W.A. (1983) Introduction to pharmacokinetics. In: Ritschel,W.A. (ed.), Graphic approach to clinical pharmacokinetics. J.R. Prous Publishers, Barcelona, pp. 1–18.

Sasaki,H., Bothner,B., Dell,A. and Fukuda,M. (1987) Carbohydrate structure of erythropoietin expressed in chinese hamster ovary cells by a human erythropoietin cDNA. *J. Biol. Chem.*, **262,** 12059–12076.

Schwick,H.G. and Haupt,H. (1984) Human plasma proteins of unknown function. In Putnam,F.W. (ed.), *The Plasma Proteins*, 2nd ed., Academic Press, New York. Vol IV, pp. 167–220.

Shahangian,S., Fritsche,H.A., Hughes,J.I., Foemmel,R.S. and Katopodis,N. (1991) Plasma protein-bound sialic acid in patients with colorectal polyps of known histology. *Clin. Chem.*, **37,** 200-204.

Spik,G., Bayard,B., Fournet,B., Strecker,G., Bouquelet,S. and Montreuil,J. (1975) Studies on glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferrin. *FEBS Lett.*, **50,** 296–299.

Spiro,R.G. and Bhoyroo,V.D. (1974) Structure of the O-glycosidically linked carbohydrate units of fetuin. *J. Biol. Chem.*, **249,** 5704–5717.

Takeuchi,M. and Kobata,A. (1991) Structures and functional roles of the sugar chains of human erythropoietins. *Glycobiology*, **1,** 337–346.

Takeuchi,M., Takasaki,S., Miyazaki,H., Kato,T., Hoshi,S., Kochibe,N. and Kobata,A. (1988) Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant chinese hamster ovary cells. *J. Biol. Chem.*, **263,** 3657–3663.

Townsend,R.R. and Hardy,M.R. (1991) Analysis of glycoprotein oligosaccharides using high-pH anion exchange chromatography. *Glycobiology*, **1,** 139–147.

Townsend,R.R., Hilliker,E., Li,Y.-T., Laine,R.A., Bell,W.R. and Lee,Y.C. (1982) Carbohydrate structure of human fibrinogen: use of 300-MHz ^{1}H-NMR to characterize glycosidase-treated glycopeptides. *J. Biol. Chem.*, **257,** 9704–9710.

Tsuda,E., Goto,M., Murakami,A., Akai,K., Ueda,M., Kawanishi,G., Takahashi,N., Sasaki,R., Chiba,H., Ishihara,H., Mori,M., Tejima,S., Endo,S. and Arata,Y. (1988) Comparative structural study of N-linked oligosaccharides of urinary and recombinant erythropoietins. *Biochemistry,* **27,** 5646-5654.

Turner,G.A. (1992) N-Glycosylation of serum glycoproteins in disease and its investigation using lectins. *Clin. Chim. Acta,* **208,** 149–171.

Turner,G.A, Goodarzi,M.T. and Thompson,S. (1995) Glycosylation of alpha-1-proteinase inhibitor and haptoglobin in ovarian cancer: evidence for two different mechanisms. *Glycoconjugate J.,* **12,** 211–218.

Watson,E., Bhide,A. and van Halbeek,H. (1994) Structure determination of the intact major sialylated oligosaccharide chains of recombinant human erythropoietin expressed in chinese hamster ovary cells. *Glycobiology,* **4,** 227–237.

Yamamoto,K., Tsuji,T., Irimura,T., and Osawa,T. (1981) The structure of carbohydrate unit B of porcine thyroglobulin. *J. Biochem.*, **195,** 701–713.

Yamashita,K., Kamerling,J.P. and Kobata,A. (1982) Structural study of the carbohydrate moiety of hen ovomucoid: occurrence of a series of pentaantennary complex-type asparagine-linked sugar chains. *J. Biol. Chem.*, **257,** 12809–12814.

Yamashita,K., Kamerling,J.P. and Kobata,A. (1983) Structural studies of the sugar chains of hen ovomucoid: evidence indicating that they are formed mainly by the alternative biosynthetic pathway of asparagine-linked sugar chains. *J. Biol. Chem.*, **258,** 3099–3106.

Yoshima,H., Matsumoto,A., Mizuochi,T., Kawasaki,T. and Kobata,A. (1981) Comparative study of the carbohydrate moieties of rat and human plasma α_1-acid glycoproteins. *J. Biol. Chem.*, **256,** 8476–8484.

Received on September 15, 1995; revised on November 13, 1995; accepted on November 15, 1995