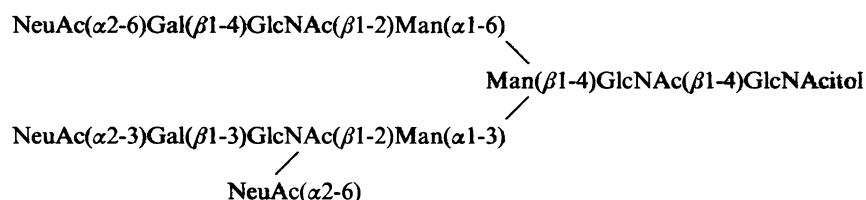


Comparative study of asparagine-linked glycans of plasma T-kininogen in normal rats and during acute inflammation

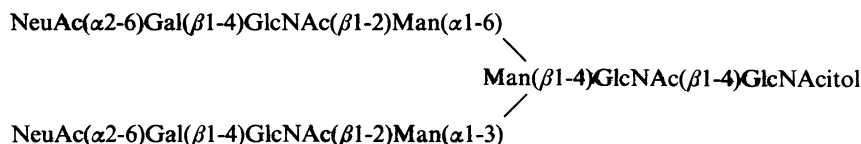
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Rat T-kininogen has been separated into two molecular variants by affinity chromatography on concanavalin A (ConA): a ConA-reactive (ConA⁺) and a ConA-non-reactive (ConA⁻) fraction, from which carbohydrate chains were quantitatively released by hydrazinolysis. On the basis of high-resolution 400 MHz ¹H-n.m.r. spectroscopy of the re-*N*-acetylated hydrazinolysates, the carbohydrate structures of the two ConA molecular variants of rat T-kininogen were established. The ConA-non-reactive species contains a single type of carbohydrate chain with the following structure:



The ConA-reactive fraction contains the same structure and the following additional one:



The relative abundance of the two molecular forms is profoundly affected during inflammation (ratio ConA⁺/ConA⁻: 44 % in normal and 95 % in inflamed T-kininogen), but no structural modification of the carbohydrate chains was observed.

INTRODUCTION

Kininogens are multidomain and multifunctional glycoproteins containing the sequence of the vasodilator peptide bradykinin in their structure. In rat, three different kininogen species have been identified. High-*M_r* kininogen and low-*M_r* kininogen are both able to generate bradykinin when hydrolysed by plasma or glandular-type kallikreins [1–3]. The third kininogen species, called T-kininogen (T-Kg), which so far has only been found in the rat, is able to liberate isoleucyl-seryl-bradykinin or T-kinin when hydrolysed *in vitro* by trypsin, cathepsin D or serine esterases, but is insensitive to kallikreins. However, the putative role of T-Kg as a kinin precursor *in vivo* remains a matter of debate. T-Kg is also a potent inhibitor of thiol proteinases [4]. Analysis of sequences obtained by cDNA cloning has revealed that T-Kg is identical with rat major acute-phase protein [5,6], which was itself previously shown to be physicochemically and immunochemically identical with α₁-cysteine-proteinase inhibitor [4]. T-Kg indeed increases markedly in plasma during all types of experimental inflammation [7] and after limited surgical trauma [8]. There are two closely related genes corresponding to T-Kg. All rat kininogens are therefore closely related structurally and share a large similar sequence beginning at the *N*-terminal part of the molecule and terminating

shortly after the bradykinin sequence [9]. All rat kininogen species are synthesized in the liver as shown by the presence of corresponding mRNAs in this organ [6].

The total carbohydrate content of T-Kg is estimated to be 15 % [4], but no information is available on the nature of the glycans. A detailed knowledge of the structure of the carbohydrate chains is essential since it may influence important biological properties such as stability and immunological regulation [10]. Moreover, a possible involvement of carbohydrate chains in the inflammation phenomenon could be put forward. Differences observed in the ConA affinity pattern of normal and acute inflammation proteins [10] prompted us to investigate the nature of the oligosaccharide chains.

In the present study, both normal and inflamed rat plasma T-Kg were divided into two variants by means of ConA affinity chromatography (ConA⁻ and ConA⁺), and glycans were released from each variant by the hydrazinolysis technique. The structures of the oligosaccharide chains were then determined by 400 MHz ¹H-n.m.r. spectroscopic analysis.

MATERIALS AND METHODS

Purification of normal and inflamed rat plasma T-Kg

For purification of inflamed rat plasma T-Kg, five male Wistar

Abbreviations used: ConA, concanavalin A; T-Kg, T-kininogen.

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rats weighing 300–350 g (I.F.F.A. Credo, Saint Germain/L'Abresle, France) were treated with turpentine (0.5 ml/100 g body wt., by the subcutaneous route), and 24 h later they were anaesthetized with diethyl ether and blood was withdrawn from the aorta. After coagulation overnight at room temperature, the serum was used for T-Kg purification by a simplified procedure described by Gauthier *et al.* [11] consisting in hydroxyapatite (DNA grade; Bio-Rad Laboratories) chromatography followed by gel filtration on AcA 34 (I.B.F., Clichy, France). Purified T-Kg was dialysed against distilled water, adjusted to pH 7.5 with aq. NH_3 and freeze-dried.

For purification of normal rat plasma, 15 male Wistar rats, not treated with turpentine, were used, and T-Kg was purified by the same procedure.

In the two cases, T-Kg was homogeneous on SDS/PAGE, and overall recovery was higher than 90%. Contamination by kallikrein-sensitive low- M_r kininogen was less than 3% as shown by h.p.l.c. analysis of kinin after trypsin hydrolysis of T-Kg performed as previously described [12].

ConA affinity chromatography

For analytical ConA chromatography, 400 μl of freeze-dried normal or inflamed rat plasma T-Kg was dissolved in 1 ml of 50 mM-Tris/HCl buffer, pH 7.8, containing 150 mM-NaCl, 1 mM- CaCl_2 , 1 mM- MnCl_2 and 0.01% NaN_3 (ConA buffer) containing 10 mg of BSA (Sigma Chemical Co.) in order to prevent non-specific adsorption. This mixture was submitted to affinity chromatography on a ConA-Ultrogel (I.B.F.) column (1.5 cm \times 11.5 cm) at a flow rate of 20 ml/h. Fractions (2 ml) were collected in polyethylene tubes containing 1 mg of BSA. The ConA-bound T-Kg was eluted by a linear gradient of 0–250 mM-methyl α -glucoside (Sigma Chemical Co.). T-Kg was assayed by direct radioimmunoassay as described previously [12].

For preparative ConA chromatography, 5 mg of each T-Kg was dissolved in ConA buffer alone and fractionated by the same procedure. Unbound and bound fractions were separately pooled, dialysed against distilled water adjusted to pH 7.5 with aq. NH_3 and freeze-dried before isolation of glycans.

Glycopeptidase F digestion of T-Kg

Normal or inflamed rat plasma T-Kg (10 μg) was incubated with 2 units of glycopeptidase F (Boehringer) in 30 μl of 0.15 M-phosphate buffer, pH 7.5, containing 20 mM-EDTA and 1% (v/v) 2-mercaptoethanol. After 24 h incubation at 37 $^\circ\text{C}$, the reaction was stopped by boiling for 5 min, after addition of 30 μl of PAGE sample buffer.

Samples were analysed by SDS/PAGE in reducing conditions according to the method of Laemmli [13] on 10% (w/v) gels. M_r markers were from Pharmacia. Proteins were stained with Coomassie Blue R-250 (Kodak).

Isolation of hydrazinolysis-released oligosaccharide from T-Kg

Each ConA affinity variant, both from normal and inflamed rat plasma T-Kg, was submitted to hydrazinolysis [14]. Re-*N*-acetylation was carried out in saturated NaHCO_3 with acetic anhydride [15]. The re-*N*-acetylated oligosaccharides were desalted on a Bio-Gel P-2 column (1 cm \times 50 cm) (Bio-Rad Laboratories, Richmond, CA, U.S.A.); monitoring of the oligosaccharide was by measuring u.v. absorption at 206 nm. Before structural investigations, in an effort to prepare relatively homogeneous fractions, oligosaccharides released by hydrazinolysis were separated by using anion-exchange h.p.l.c. on a Micropak AX-10 μm column (50 cm \times 0.8 cm internal diameter; Varian) as described by Paz-Parente *et al.* [16].

Briefly, disialylated compounds were eluted isocratically with a mixture of water/500 mM- KH_2PO_4 (pH 4.0) (19:1, v/v), and

trisialylated compounds were eluted with a mixture of water/500 mM- KH_2PO_4 (pH 4.0) (17:3, v/v). The eluate (flow rate 1 ml/min) was monitored at 206 nm. After the chromatography, fractions were desalted on a Bio-Gel P-2 (200–400 mesh; Bio-Rad Laboratories) column (1 cm \times 50 cm) with distilled water as eluent.

Sugar analysis

Carbohydrate composition of the native protein and released oligosaccharides was determined by g.l.c. according to the method of Zanetta *et al.* [17].

^1H -n.m.r. spectroscopy

For the n.m.r. analysis, solutions of purified oligosaccharides were exchanged several times in $^2\text{H}_2\text{O}$ with intermediate freeze-drying. ^1H -n.m.r. spectral analysis of the compounds in $^2\text{H}_2\text{O}$ (99.95%; Commissariat à l'Énergie Atomique, Saclay, France) was carried out on a Bruker AM-400 WB spectrometer operating in the Fourier-transform mode at a probe temperature of 300 K. Chemical shifts are given relative to acetone in $^2\text{H}_2\text{O}$ ($\delta = 2.225$ p.p.m.). N.m.r. spectra were interpreted by comparison with n.m.r. data from the literature [18,19] with an accuracy of 0.004 p.p.m.

RESULTS

ConA affinity chromatography

The ConA chromatography profiles of normal and inflamed rat plasma T-Kgs are presented in Fig. 1. The protein is distributed in two peaks, the first one corresponding to the unbound fraction and the second one to the ConA-reactive fraction.

For normal rat plasma T-Kg, the ConA-reactive fraction

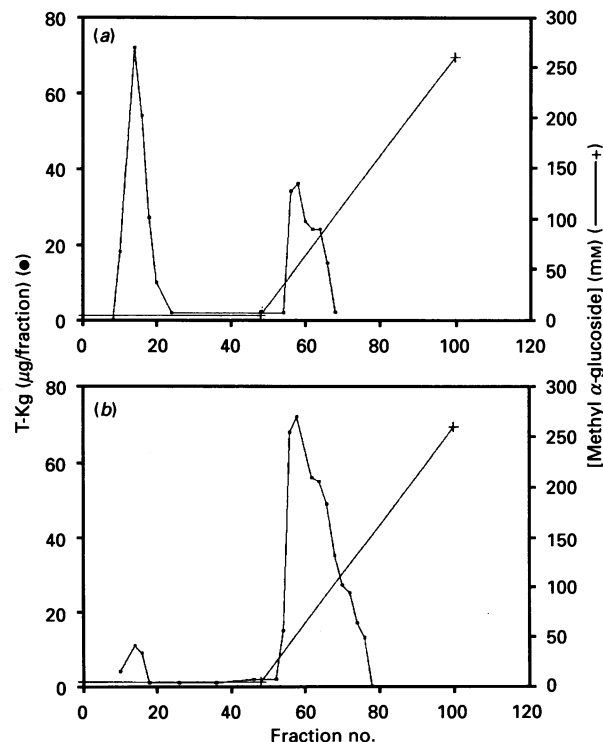


Fig. 1. Elution profile of (a) normal and (b) inflamed rat T-Kg on ConA Ultrogel column (1.5 cm \times 11.5 cm)

For experimental details see the Materials and methods section.

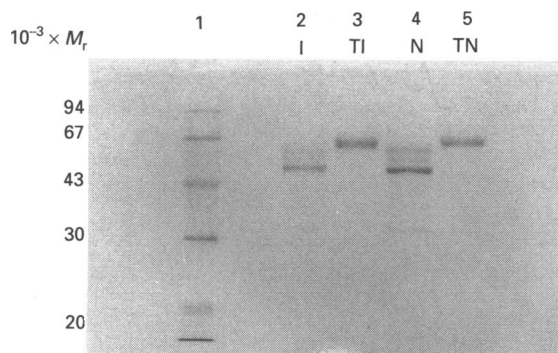


Fig. 2. SDS/PAGE of T-Kgs after glycopeptidase F treatment

Lane 1, M_r calibration standards; lane 2, inflamed rat plasma T-Kg digested with glycopeptidase F; lane 3, native inflamed rat plasma T-Kg; lane 4, normal rat plasma T-Kg digested with glycopeptidase F; lane 5, native normal rat plasma T-Kg.

represented 44% of total protein. For inflamed rat plasma T-Kg, the ratio of unbound and ConA-reactive fraction is profoundly affected, the latter representing 95% of total T-Kg.

For the two forms of T-Kg, the ConA-reactive fraction is eluted with about 50 mM-methyl α -glucoside.

Glycopeptidase F digestion of T-Kgs

As shown in Fig. 2, SDS/PAGE of the two T-Kg species before and after glycopeptidase F treatment revealed that the two native proteins had an apparent M_r of 64000. After glycopeptidase F treatment, we observed similar patterns for normal and inflamed rat plasma T-Kg, the two proteins exhibiting an apparent M_r of 49000. Since T-Kg contains only glycopeptidase F-sensitive *N*-glycans, as attested by the monosaccharide molar composition, the apparent M_r of total oligosaccharide chains is 15000, representing 23% of the T-Kg M_r .

In addition, for normal rat plasma T-Kg treated with glycopeptidase F, we observed two minor intermediate bands of M_r 59000 and 54000, which probably result from incomplete T-Kg glycan liberation.

Molar composition

Normal T-Kg contained 18% of carbohydrates identified as mannose, galactose, *N*-acetylglucosamine and *N*-acetylneuraminic acid in the proportions 3.0:1.8:3.5:2.8 respectively (mannose is taken as 3.0, and *N*-acetylglucosamine is corrected for the amount of asparagine-linked *N*-acetylglucosamine that is not cleaved during methanolysis). Inflamed T-Kg contained 16% of carbohydrates with similar values for mannose, galactose and *N*-acetylglucosamine, but with a slight decrease in the proportion of sialic acid to 2.2.

Fractionation and characterization of T-Kg carbohydrate chain

The glycan chains of each ConA variant from normal and inflamed T-Kgs were released by hydrazinolysis followed by quantitative re-*N*-acetylation.

Each of the ConA fractions was further fractionated by h.p.l.c. as described in the Materials and methods section. As indicated in Fig. 3, the glycans from the ConA-reactive form of normal T-Kg were resolved into two peaks called FI and FII corresponding to di- and tri-sialylated compounds respectively. The ConA-non-reactive form gives a single peak called F'II corresponding to a trisialylated oligosaccharide. Similar profiles

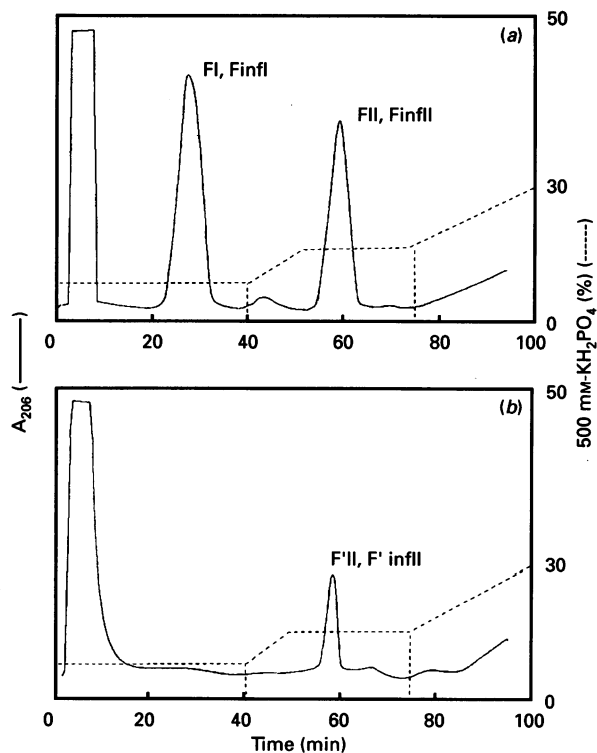


Fig. 3. H.p.l.c. analysis on AX-10 μ m column of oligosaccharides released by hydrazinolysis from the ConA⁺ (a) and ConA⁻ (b) fraction from both normal and inflamed rat T-Kgs

were obtained for the inflamed protein leading to fractions FinfI, FinfII and F'infII.

N.m.r. analysis

The n.m.r. spectral parameters for disialyloligosaccharide-alditol present in fractions FI and FinfI show close similarities to those acquired for the sialo biantennary glycan structure derived from human transferrin (see Table 1) [20,21]. The structural reporter group signals of Man-3, -4 and -4' demonstrate the presence of the mannotriose core unit and its biantennary type of branching. The chemical shifts of NeuAc H-3_{ax} and H-3_{eq} point to the presence of *N*-acetylneuraminic acid in α -2-6-linkage to galactose. The occurrence of *N*-acetylneuraminic acid in both branches is revealed by the resonance positions of the signal for Gal-6 and -6' H-1 and Man-4 and Man-4' H-1 atom resonances [18]. For the trisialylated oligosaccharide-alditol (fractions FII, F'II, FinfII and F'infII), the presence of signals at δ 5.110 p.p.m. (H-1 Man-4) and δ 2.733 p.p.m. [H-3_{eq}, NeuAc(α 2-6)], δ 2.038 (NAc GlcNAc-5) and δ 4.507 p.p.m. (H-1 Gal-6) indicates the glycan structure to contain the following sequence: NeuAc(α 2-3)Gal(β 1-3)[NeuAc(α 2-6)]GlcNAc(β 1-2)Man(α 1-3)..., previously characterized in rat haemopexin [19], bovine glycoproteins [22] and rat serotransferrin [5], for which identical n.m.r. spectral parameters were established.

Consequently, the structures of the different glycans of normal and inflamed rat T-Kg may be established as illustrated in Fig. 4. Whereas the ConA-form appears to possess a single type of glycan (F'II, F'infII), the ConA⁺ form possesses two types of glycan (FI, FinfI, FII, FinfII).

DISCUSSION

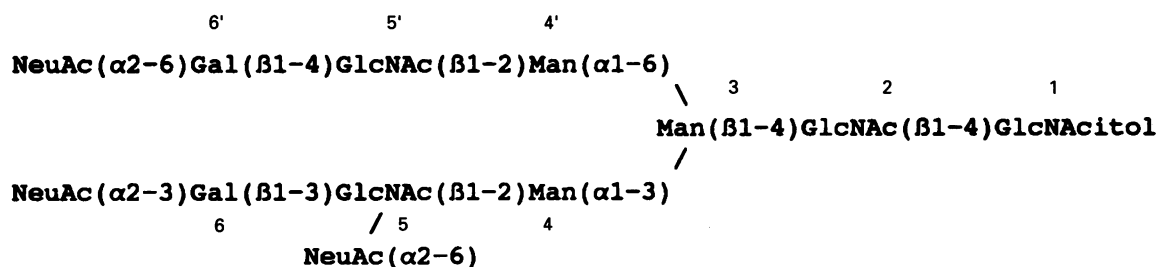
During turpentine-induced inflammation in rat, we observed an increase in ConA-reactive T-Kg; meanwhile the unbound

Table 1. 400 MHz ^1H -n.m.r. chemical shifts of structural reporter groups of constituent monosaccharides for the glycans derived from ConA^+ and ConA^- species of rat T-Kg

Chemical shifts are given at 27 °C in p.p.m. downfield relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate as explained in the Materials and methods section. The structures represented by shorthand notation in the Table are GlcNAc (●), Man (◆), Gal (■), NeuAc(α 2-6) (○) and NeuAc(α 2-3) (△). For numbering of monosaccharides see Fig. 4. $^4\text{Gal-6}$ denotes Gal linked (β 1→4) to GlcNAc-5; $^3\text{Gal-6}$ denotes Gal linked (β 1→3) to GlcNAc-5; $^3\text{NeuAc}$ denotes NeuAc linked (α 2→3) to $^3\text{Gal-6}$; $^6\text{NeuAc}$ denotes NeuAc linked (α 2→6) to $^4\text{Gal-6}$. Abbreviation: N.D., not determined.

Reporter group	Residue	Chemical shift in	
		FI-FinFI	FII-F'II-FinFII F'inFII
H-1	GlcBAC-o1	—	—
	GlcBAC-2	4.620	4.620
	Man-3	4.77	4.77
	Man-4	5.133	5.110
	Man-4'	4.947	4.947
	GlcNAc-5	4.605	4.582
	GlcNAc-5'	4.605	4.605
	$^4\text{Gal-6}$	4.445	—
	$^3\text{Gal-6}$	—	4.507
	$^4\text{Gal-6}$	4.445	4.445
H-2	Man-3	4.25	4.25
	Man-4	4.20	4.20
	Man-4'	4.11	4.11
H-3 _{ax.}	$^6\text{NeuAc}$	1.717	1.717
	$^3\text{NeuAc}$	—	1.785
	$^6\text{NeuAc}'$	1.717	1.717
H-3 _{eq.}	$^6\text{NeuAc}$	2.666	2.733
	$^3\text{NeuAc}$	—	2.760
	$^6\text{NeuAc}'$	2.672	2.672
NAc	GlcNAc-o1	N.D.	N.D.
	GlcNAc-2	2.083	2.083
	GlcNAc-5	2.066	2.038
	GlcNAc-5'	2.063	2.063
	NeuAc	2.030	2.030

FII, F'II, FinFII, F'inFII



FI, FinFI

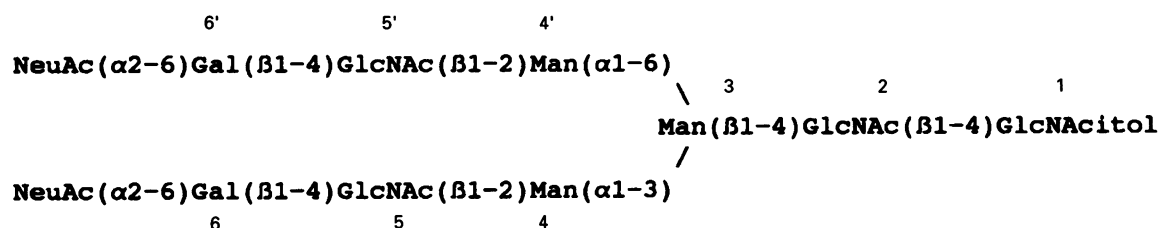


Fig. 4. Proposed structures of the glycans from normal and inflamed rat T-Kg

fraction was considerably diminished. Koj *et al.* [23] have reported such an increase in ConA-reactive plasma α -1 acute-phase protein (identical with T-Kg) in turpentine-induced inflammation in rat using crossed immunoaffinity electrophoresis. However, these authors did not observe a diminution of the unbound fraction. This discrepancy may eventually be explained by differences in the techniques used. Similar results were obtained by Nicollet *et al.* [24], who also observed an increase in the ConA-reactive form of human α -1 acid glycoprotein isolated from plasma of patients with acute inflammation. After digestion with glycopeptidase F, both normal and inflamed rat plasma T-Kg yield a major product with an apparent M_r of 49000. Our results are in good agreement with those of Anderson *et al.* [25], who have reported an M_r of 48000 for the non-glycosylated form of rat α -1 major acute-phase protein, which was proved to be identical with T-Kg. Analysis of the carbohydrate chains of normal and inflamed rat plasma T-Kg points provide evidence that the acute inflammation process does not alter the structure of the glycan moiety of the protein. The only difference was in the ratio of ConA⁻/ConA⁺ forms. The decrease observed for the ConA⁻ trisialylated form could be attributed to regulation of the transcription of the gene coding for one protein variant, occurring during inflammation as an answer to the environmental changes, rather than to a post-transcriptional modification of the glycosylation pathway. This observation may be related to the evidence presented by Furuto-Kato *et al.* [6] for the existence of two kinds of mRNAs for TI and TII kininogens in rat liver. It would be of interest to investigate whether a correlation exists between the two forms of T-Kg polypeptide and the two glycan variants.

Recently, Enjoji *et al.* [26] have reported the purification of TI and TII kininogen from inflamed rat plasma. These authors observed an increase in TII kininogen concentration during inflammation.

From a structural point of view, the most notable feature is found for the ConA⁻ form which possesses the unit NeuAc-(α 2-3)Gal(β 1-3)[NeuAc(α 2-6)]GlcNAc, which was previously described for rat plasma haemopexin [19] and more recently for rat serotransferrin [27]. It should also be mentioned that the ConA⁺ form possesses two kinds of glycan chain. This observation does not correlate with a previous hypothesis advanced by Bayard & Kerckaert [28], who have proposed that single polypeptides possess complex N-linked glycans of uniform structure.

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REFERENCES

- Okamoto, H. & Greenbaum, L. M. (1983) *Life Sci.* **32**, 2007–2013
- Barlas, A., Gao, X. & Greenbaum, L. M. (1987) *FEBS Lett.* **218**, 266–270
- Gutman, N., Moreau, T., Alhenc-Gelas, F., Baussant, T., El Moujahed, A., Akpona, S. & Gauthier, F. (1988) *Eur. J. Biochem.* **171**, 577–582
- Esnard, F. & Gauthier, F. (1983) *J. Biol. Chem.* **258**, 12443–12447
- Anderson, K. P. & Heath, E. (1985) *J. Biol. Chem.* **260**, 12065–12071
- Furuto Kato, S., Matsumoto, A., Kitamura, N. & Nakanishi, S. (1985) *J. Biol. Chem.* **260**, 12054–12059
- Barlas, A., Okamoto, H. & Greenbaum, L. M. (1985) *Biochem. Biophys. Res. Commun.* **129**, 280–286
- Suzuki, H., Alhenc-Gelas, F., Bouhnik, J., Corvol, P. & Menard, J. (1988) *Endocrinology (Baltimore)* **122**, 2809–2815
- Kitagawa, H., Kitamura, N., Hayashi, H., Miata, T. & Nakanishi, S. (1987) *J. Biol. Chem.* **262**, 2190–2198
- Hatton, M. W. C., März, L. & Regoczi, E. (1983) *Trends Biochem. Sci.* **8**, 287–291
- Gauthier, F., Moreau, T. & Esnard, F. (1986) *Gene Biol. (Portugal)* **11**, 93–99
- Bouhnik, J., Baussant, T., Savoie, F., Alhenc-Gelas, F., Gauthier, F. & Esnard, F. (1987) *Biochem. Biophys. Res. Commun.* **144**, 1090–1097
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Bayard, B. & Fournet, B. (1975) *Carbohydr. Res.* **46**, 75–86
- Michalski, J. C., Peter-Katalinic, J., Egge, H., Paz-Parente, J., Montreuil, J. & Strecker, G. (1984) *Carbohydr. Res.* **134**, 177–189
- Paz-Parente, J., Leroy, Y., Montreuil, J. & Fournet, B. (1984) *J. Chromatogr.* **288**, 147–155
- Zanetta, J. P., Breckenridge, W. C. & Vincendon, G. (1972) *J. Chromatogr.* **69**, 291–304
- Vliegthart, J. F. G., Dorland, L. & Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem.* **41**, 209–374
- Bernard, N., Engler, R., Strecker, G., Montreuil, J., Van Halbeek, H. & Vliegthart, J. F. G. (1984) *Glycoconjugate J.* **1**, 123–140
- Spik, G., Bayard, B., Fournet, B., Bouquet, S. & Montreuil, J. (1975) *FEBS Lett.* **50**, 296–299
- Spik, G., Coddeville, B. & Montreuil, J. (1988) *Biochimie* **70**, 1459–1469
- Mizuochi, T., Yamashita, K., Fujikawa, K., Kisiel, W. & Kobata, A. (1979) *J. Biol. Chem.* **254**, 6419–6425
- Koj, A., Dubin, A., Kasperczyk, H., Bereta, J. & Gordan, A. H. (1982) *Biochem. J.* **206**, 545–553
- Nicollet, I., Lebreton, J. P., Fontanne, M. & Hiron, M. (1981) *Biochim. Biophys. Acta* **668**, 235–245
- Anderson, K. P., Martin, A. D. & Heath, E. C. (1984) *Arch. Biochem. Biophys.* **233**, 624–635
- Enjoji, K., Kato, H., Hayashi, I., Oh-Ishi, S. & Iwanaga, S. (1988) *J. Biol. Chem.* **263**, 973–979
- Spik, G., Coddeville, B., Strecker, G., Montreuil, J., Recoeci, E., Chindemi, P. A. & Rudolph, J. R. (1991) *Eur. J. Biochem.* **195**, 397–405
- Bayard, B. & Kerckaert, J. P. (1980) *Biochem. Biophys. Res. Commun.* **95**, 774–784

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