Determination of the Sialylation Pattern of Human Fibrinogen Glycopeptides with Fast Atom Bombardment†

R. Reid Townsend, David N. Heller, Catherine C. Fenselau, and Yuan Chuan Lee*

ABSTRACT: Sialylated biantennary glycopeptides from human fibrinogen were analyzed with fast atom bombardment mass spectrometry. The mass spectrometric conditions used in the positive mode showed predominantly molecular ions with no fragment ions due to the loss of sialic acid. Standard mixtures of glycopeptides with zero, one, and two sialic acid residues revealed a linear relationship between ion abundance and molar fraction. The desorption efficiency varied according to the number of sialic acid residues in these biantennary glycopeptides. The relative abundance of different molecular ion species differing only in amino acid content was in agreement with chemical analysis. Sensitivity, precision, and requirements for sample preparation were assessed. Both assignment of molecular weights and quantification of individual glycopeptide species from human fibrinogen were obtained. The glycopeptides from human fibrinogen were found to consist of a mixture of equal amounts of monosialylated and disialylated species with no asialoglycopeptides.

It is well recognized that total desialylation of most glycoproteins results in their removal from the circulation within minutes by the hepatic Gal/GalNAc[1] lectin [for review, see Harford & Ashwell (1982)]. It has been shown that partial desialylation of ceruloplasmin exposing two galactoses, on the average, results in its rapid plasma clearance (Ashwell & Morell, 1974). Studies with asialotriantennary glycopeptides (three Gal cluster) from bovine fetuin and asialobiantennary glycopeptides (two Gal cluster) from human immunoglobulin demonstrated that asialotriantennary but not biantennary glycopeptides were endocytosed by isolated rat hepatocytes (Baenziger & Fiete, 1980). The rapid clearance of human asialotransferrin, type 3 (Regoeczi et al., 1982), and asialotriantennary glycopeptides from human α_1-protease inhibitor (Townsend et al., 1984) by rat liver further implicates the asialotriantennary oligosaccharide as a major determinant in the clearance of infused asialoglycoproteins. However, glycoproteins such as type 1 transferrin (Wong et al., 1978) and human fibrinogen (Townsend et al., 1982) possessing only biantennary oligosaccharides are cleared over hours (Martinez et al., 1977; Regoeczi et al., 1979) at rates similar to those of the isolated native glycoprotein. Since isolated fibrinogen is partially desialylated (Townsend et al., 1982), it becomes important to characterize the sialylation pattern of isolated native fibrinogen and to eventually isolate and determine the clearance rate of fibrinogen molecules possessing a defined array of sialic acid residues. As an initial step, we have determined the distribution of sialic acid among the isolated biantennary glycopeptides of human fibrinogen.

The distribution of sialic acid on the oligosaccharide chain of a glycoprotein requires the identification and quantification of each oligosaccharide with or without sialic acid at each glycosylation site. Chromatographic separation of sialylated glycopeptides with quantitative recovery is inefficient and often unsatisfactory. Furthermore, each separated fraction must be individually characterized. Recent advances in fast atom bombardment (FAB) mass spectrometry enable detection of molecular ions of underivatized oligosaccharides of several thousand daltons (Kamerling et al., 1983; Dell & Ballou, 1983). We show that FAB mass spectrometry can also be utilized for the quantification of sialylated biantennary glycopeptides in a mixture. Using this approach, we found equal proportions of monosialylated and disialylated biantennary glycopeptides from human fibrinogen with no detectable asialo species.

Materials and Methods

Glycopeptide Preparation. The fibrinogen glycopeptides previously characterized (Townsend et al., 1982) were analyzed without chemical modification (Tables I and II).

To obtain glycopeptides with only Asn for standard mixture analysis (Figures 1 and 2), the above fibrinogen glycopeptides (50 μmol) were further digested with Pronase (5 mg) (Calbiochem-Behring) in 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.5 (digest buffer) (10 mL), containing 2 mM calcium chloride. The digestion was continued for 5 days at 37 °C under sterile conditions with daily additions of the same dose of Pronase. After Sephadex G-50 (2.5 × 200 cm) chromatography, the material was dissolved in the digest buffer containing 1 mM magnesium chloride and 5.5 units of aminopeptidase M (Boehringer-Mannheim). The digestion was allowed to proceed for 24 h at 37 °C; then another addition of enzyme was made and the incubation was continued for 24 h. The glycopeptides were purified by Sephadex G-50 chromatography and further digested with 40 units of carboxypeptidase Y (Boehringer-Mannheim) in acetate buffer (10 mL, pH 5.0) for 24 h. Another addition of carboxypeptidase was made, and the pH was raised to 6.0, followed by incubation for another 24 h. Since amino acid analysis of the glycopeptides at this stage revealed the presence of Glu, further digestion with aminopeptidase (48 h) was performed, which resulted in 90% of the glycopeptides containing only Asn. Monosialobiantennary

† From the Department of Biology and the McCollum Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218 (R.R.T. and Y.C.L.), and the Department of Pharmacology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 (D.N.H. and C.C.F.). *Received May 8, 1984.* Contribution No. 1269 from the McCollum-Pratt Institute. R.R.T. was supported by U.S. Public Health Service–National Institutes of Health New Investigators Award AM 31376 and by the Daland Fund of the American Philosophical Society. D.N.H. and C.C.F. were supported by National Science Foundation Grant PCM 8209954. Y.C.L. was supported by U.S. Public Health Service–National Institutes of Health Research Grant AM 9970 and National Science Foundation Research Grant PCM 8109855.

[1] Abbreviations: AS, asialobiantenary; DI, disialobiantennary; FAB, fast atom bombardment; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GP, glycopeptide; MO, monosialobiantennary.

0006-2960/84/0423-6389$01.50/0

(Asn-MO-GP) and disialobiantennary (Asn-DI-GP) glycopeptides were separated on a DEAE-Sephacel column (1.6 × 75 cm) equilibrated in 10 mM tris(hydroxymethyl)aminomethane buffer, pH 7.6, as previously described for the separation of colominic acid oligomers (Nomoto et al., 1982). Asialobiantennary glycopeptide (Asn-AS-GP) was prepared from Asn-MO-GP by treatment with neuraminidase as previously described (Townsend et al., 1982). All glycopeptides analyzed by mass spectrometry were desalted on a Sephadex G-25 column (1.6 × 100 cm) equilibrated in a buffer (pH 4.7) of 50 mM pyridine and 50 mM acetic acid and on a Dowex 50W-X8 column (1.5 × 20 cm) (pyridinium form), both linked in series. The concentrations of the stock glycopeptide solutions were based on the Gal content as determined by automated liquid chromatography (Lee, 1972). Despite this vigorous desalting technique a small proportion of cationized molecular ions were detected (Figure 1).

Positive Ion Fast Atom Bombardment Mass Spectrometry. Mass spectra were measured on an MS-50 mass spectrometer (Kratos, Manchester, England) equipped with a 23-kG magnet. The instrument was operated at an accelerating potential of 8 kV. Samples were placed on a 2 mm diameter copper FAB probe tip and were irradiated by a xenon atom beam with a translational energy of about 8 kV. The mass range was scanned from m/z 2731 to m/z 1691 at a scan rate of 30 s/decade. Peak profiles were collected by a DATAGENERAL-NOVA3 computer, and all scans that produced molecular ions visible on the oscilloscope were averaged and then centroided off line before mass assignment by the DS-55 data system. The data accumulated represented ions produced immediately after beginning irradiation until just before the total ion current showed a marked dropoff. One microliter of the glycopeptide solution (1–5 mM) in water was added to 1 μL of distilled monothioglycerol (Sigma Chemical Co.) on the surface of the probe, and then the solution was stirred with the micropipet tip.

The uncorrected ion abundance for each glycopeptide was obtained by summing the intensities of each isotope in the clusters corresponding to MH^+ and MNa^+ ions (and also MK^+ when present). When a significant level of chemical background was present, an average background intensity was estimated from the peaks 8 amu on either side of each molecular ion cluster. This value was manually subtracted from the intensity of each peak in the cluster.

Six replicates of a 10:1 mixture of Asn-AS-GP and Asn-MO-GP were carried out to assess the precision of this method. With the results reported in terms of the percent Asn-MO-GP, the standard deviation was ±4%, indicating that measurements can be assigned a 95% confidence limit of ±10%. The lower limit for quantification by the above protocol was 500 pmol of glycopeptide.

Results and Discussion

Fast atom bombardment mass spectrometry has recently been utilized for the analysis of complex carbohydrates [for review, see Reinhold & Carr (1983)]. Both molecular weight assignment from molecular ion species detected in both the positive and negative ion mode and structural information from fragmentation ions detected in the negative mode have been reported for glycopeptides and oligosaccharides (Kamerling et al., 1983; Dell & Ballou, 1983).

Mass spectrometric analysis of positive ions desorbed by fast atom bombardment of previously characterized (Townsend et al., 1982) human fibrinogen glycopeptides revealed six molecular ion species (Table I). The molecular ions of sialylated species and those due to incomplete removal of Glu and Gly, known to be on the amino side of Asn (Doolittle, 1979), are evident (Table I). The assigned masses are consistent with the reported molecular formulas (Townsend et al., 1982). The proportions of the molecular ion intensities are in agreement with the amino acid composition and the known sequence around the glycosylation sites (Doolittle, 1979). However, the molecular ions from the monosialylated species were 2–3 times more abundant than those from the disialylated species (Table I). The sialic acid/Gal ratio computed from Table I was found to be lower (0.65) as compared to that determined by sialic acid content of the total mixture (0.75) or from methylation analysis (0.80) (Townsend et al., 1982). Since the apparent paucity of detected disialylated species might be explained by their reduced desorption efficiency, as has been shown with other types of compounds using FAB mass spectrometry (Fenselau, 1984), the relationship between the molar fraction of asialobiantennary and sialylated biantennary glycopeptides in a mixture and the relative abundance of molecular ions was examined. Asn-DI-GP, Asn-MO-GP, and Asn-AS-GP were prepared after extensive proteolytic digestions (see Materials and Methods) since the presence of Glu prevents resolution of the sialylated glycopeptides by anion-exchange chromatography. The spectra of these biantennary glycopeptides containing only the amino acid Asn are shown in Figure 1. The presence of only Asn in these glycopeptides was confirmed by amino acid analysis. The assigned masses are in agreement with the indicated formulas shown in Figure 1. Positive mode FAB spectra of biantennary glycopeptides showed predominantly molecular ions and no fragment ions due to the loss of sialic acid at interfering m/z values (Figure 1). Negative mode FAB spectra showed extensive fragmentation at most of the glycosidic bonds as has been reported (Kamerling et al., 1983). These less sialylated molecular species make negative FAB inappropriate for the measurement of a mixture of sialylated glycopeptide species.

Table I: Comparison of Relative Molecular Ion Abundance and Molar Fraction of Glycopeptides from Fibrinogen

glycopeptide	m/z (MH^+)	molecular ion abundance[a]	molar fraction[b]
Asn-DI-GP	2338	0.154	
Asn-MO-GP	2047	0.422	
subtotal		0.576	0.626
Glu-Asn-DI-GP	2467	0.114	
Glu-Asn-MO-GP	2176	0.213	
subtotal		0.327	0.312
Gly-Glu-Asn-DI-GP	2524	0.033	
Gly-Glu-Asn-MO-GP	2233	0.062	
subtotal		0.095	0.066

[a] Fraction of the total molecular ion intensities. [b] Based on the amino acid analysis and known sequence (Doolittle, 1979).

We examined the linearity of the dose–response relationship by analyzing a series of binary mixtures consisting of known concentrations of either Asn-AS-GP (prepared by neuraminidase treatment of Asn-MO-GP) and Asn-MO-GP or Asn-MO-GP and Asn-DI-GP. The results showed (Figure 2) that a linear relationship exists between the molar ratios of each glycopeptide pair (from 0.025 to 40) and the intensity ratios of molecular ion peaks. The slope was found to be 0.45 in the Asn-MO-GP/Asn-AS-GP mixture and 0.38 in the Asn-MO-GP/Asn-DI-GP mixture, indicating that the presence of each additional sialic acid reduced the ionization efficiency 2–3-fold in these compounds. The peak intensities of Asn-DI-GP were multiplied by 2.6 to obtain the corrected ion intensity, and the molar fraction of each glycopeptide was determined as described in Table II. As expected, the pro-

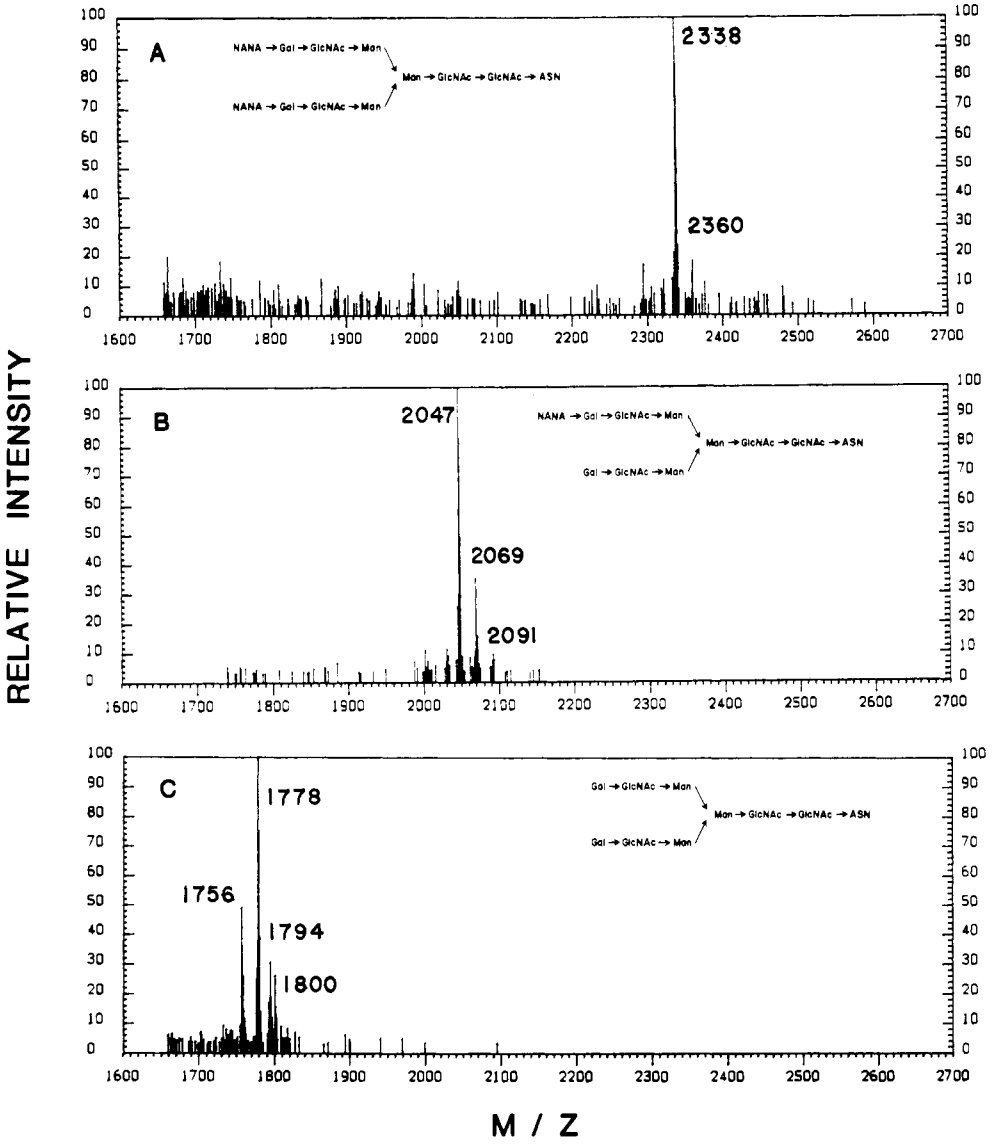

FIGURE 1: Positive mode fast atom bombardment mass spectra of biantennary glycopeptides. The spectra were obtained and the data reduced as described under Materials and Methods. Panels A, B, and C show the spectra of Asn-DI-GP, Asn-MO-GP, and Asn-AS-GP, respectively. The peaks at m/z 2091 (panel B) and m/z 1800 (panel C) correspond to $(M + 2Na^+ - H)^+$ ions.

portions of species with different amino acid composition determined by mass spectrometry remained in good agreement with those determined by amino acid analysis (Table II). The proportion of Asn-MO-GP to Asn-DI-GP after such correction is now approximately 1 (Table II). The sialic acid/Gal ratio is 0.79, which is in good agreement with values obtained by other methods (0.75–0.80) (Townsend et al., 1982). Thus, this positive ion FAB technique can provide not only molecular weight determination but also direct quantification of sialylated glycopeptides.

An interesting question is whether the sialylation pattern we have found reflects that of circulating fibrinogen. This is the case only if desialylation does not occur during isolation. The absence of totally desialylated species in the isolated glycopeptides argues against desialylation occurring after plasma procurement. Further, we have found that only 10% of Asn-DI-GP is converted to Asn-MO-GP after storage at −20 °C as an aqueous solution over 6 months with four thawing–freezing cycles. Fibrinogen circulates in the plasma at a concentration of approximately 6–12 μM. Since fibrinogen possesses four glycosylation sites (Doolittle, 1979), our results show that the concentrations of both monosialylated and disialylated oligosaccharides are 12–24 μM. Partially

Table II: Quantification of Sialylated Fibrinogen Glycopeptides

glycopeptide	molar fraction[a]	glycopeptide	molar fraction[a]
Asn-DI-GP	0.272	Asn-MO-GP	0.284
Glu-Asn-DI-GP	0.200	Glu-Asn-MO-GP	0.141
Gly-Glu-Asn-DI-GP	0.058	Gly-Glu-Asn-MO-GP	0.042
subtotal	0.53	subtotal	0.47

[a] The molar fraction was calculated from the equations

$$X_{MO} = \frac{I_{MO}}{\sum_i (I_{MO})_i + 2.6\sum_i (I_{DI})_i} \qquad X_{DI} = \frac{I_{DI}}{\sum_i (I_{MO})_i + 2.6\sum_i (I_{DI})_i}$$

where X_{MO} and X_{DI} are the molar fractions of each indicated monosialylated and disialylated glycopeptide, respectively. I_{MO} and I_{DI} are the ion intensities ($MH^+ + MNa^+$ and MK^+, when present) from each monosialylated and disialylated glycopeptide.

sialylated fibrinogen may be part of the reported Gal-terminated glycoconjugates in human plasma (Stockert et al., 1982). Asialobiantennary oligosaccharide in human fibrinogen was not detected. Since the FAB technique allows detection of as little as 2.5% Asn-AS-GP in a mixture (Figure 2), the maximum amount of asialobiantennary oligosaccharide in the plasma is 0.15–0.3 μM, which is a 3–7 times lower concentration than the estimated dissociation constant (1 μM) of

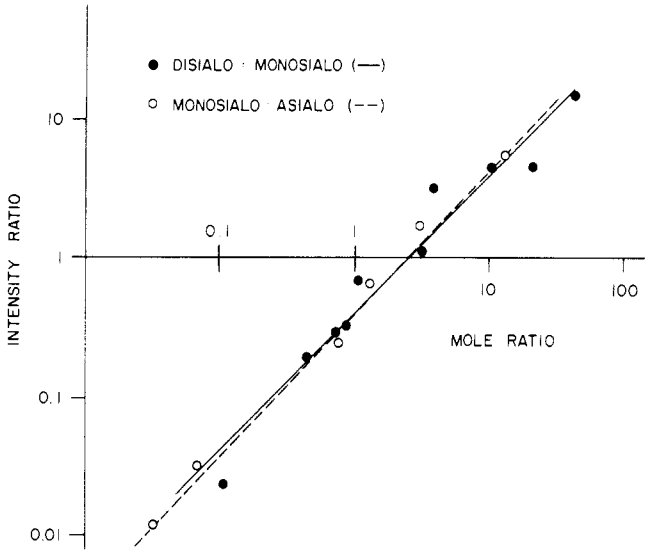

FIGURE 2: Quantification of standard binary glycopeptide mixtures. Standard mixtures of Asn-AS-GP/Asn-MO-GP and Asn-MO-GP/Asn-DI-GP were prepared and subjected to FAB mass spectrometry as described under Materials and Methods. The intensities of the MH^+ and cationized molecular ions were summed for each glycopeptide type and the indicated ratios calculated. The molar concentration for each glycopeptide was based on the determination of Gal as described under Materials and Methods. After linear regression analysis, the correlation coefficient for the Asn-MO-GP/Asn-AS-GP (○) mixture was 0.998 with a slope (- - -) of 0.45 and the correlation coefficient for the Asn-DI-GP/Asn-MO-GP (●) mixture was 0.974 with a slope (—) of 0.38. The data points are plotted on a log scale.

asialobiantennary glycopeptide for the Gal/GalNAc lectin on the surface of isolated hepatocytes (Lee et al., 1983) and, thus, most of the asialofibrinogen would be bound. If a catabolic sequence of stepwise removal of sialic acid residues from completely sialylated fibrinogen to totally desialylated fibrinogen is assumed, the absence of detectable AS-GP-Asn can also be interpreted as the result of plasma removal of asialobiantennary oligosaccharide at a faster rate than its production. It is also possible that asialobiantennary oligosaccharide may not be produced in vivo and clearance by the Gal/GalNAc lectin may be mediated by binding of single or multiple monosialylated biantennary oligosaccharides.

The presence of both Asn-DI-GP and Asn-MO-GP in equal amounts raises the question of the distribution of the monosialylated and the disialylated oligosaccharides at the four glycosylation points (Doolittle, 1979) on the fibrinogen molecule. Either disialylated or monosialylated oligosaccharides can be attached at each of the four glycosylation sites. Even if we assume that all four sites are indistinguishable, there are still five different modes of sialylation, various combinations of which will result in the observed Asn-MO-GP/Asn-DI-GP ratio of about 1. FAB analysis of glycopeptides after specific proteolysis of fibrinogen could determine whether desialylation was random between the two types of polypeptide chains ($B\beta$ and γ). Fibrinogen molecules with certain arrays of partially sialylated oligosaccharides may be cleared more efficiently from the plasma. If such is the case, then the circulating fibrinogen pool should contain an enrichment of "slow uptake forms".

We have demonstrated that FAB mass spectrometry can produce molecular ions from each component of a glycopeptide mixture and the relative abundance of the molecular ions can be related to its molar fraction if differential desorption is accounted for by standard mixture analysis. Using this approach, we have been able to quantify the sialylated biantennary glycopeptide species from human fibrinogen. This technique should be applicable for the identification and quantification of other sialylated complex carbohydrates.

Acknowledgments

We thank Gi Hyung Lee for assistance in the preparation of purified glycopeptides and Mark R. Hardy for help with computer plotting.

References

Ashwell, G., & Morell, A. G. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 41*, 99–128.

Baenziger, J. B., & Fiete, D. (1980) *Cell (Cambridge, Mass.) 22*, 611–620.

Dell, A., & Ballou, C. E. (1983) *Biomed. Mass Spectrom. 10*, 50–56.

Doolittle, R. F. (1979) in *The Chemistry and Physiology of the Human Plasma Proteins* (Bing, D. H., Ed.) pp 77–95, Pergamon Press, Oxford, England.

Fenselau, C. C. (1984) *J. Nat. Prod. 47*, 215–225.

Harford, J., & Ashwell, G. (1982) in *The Glycoconjugates* (Horowitz, M. I., Ed.) Vol. IV, Part B, pp 27–52, Academic Press, New York.

Kamerling, J. P., Heerma, W., Vliegenthart, J. F. G., Green, B. N., Lewis, I. A. S., Strecker, G., & Spik, G. (1983) *Biomed. Mass Spectrom. 10*, 420–425.

Lee, Y. C. (1972) *Methods Enzymol. 28*, 702–713.

Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., Arnarp, J., Haraldsson, M., & Lönn, H. (1983) *J. Biol. Chem. 258*, 199–202.

Martinez, J., Palascak, J., & Peters, C. (1977) *J. Lab. Clin. Med. 89*, 367–377.

Nomoto, H., Iwasaki, M., Endo, T., Inoue, S., Inoue, Y., & Matsumura, G. (1982) *Arch. Biochem. Biophys. 218*, 335–341.

Regoeczi, E., Taylor, P., Debanne, M. T., Marz, L., & Hatton, M. W. C. (1979) *Biochem. J. 184*, 399–407.

Regoeczi, E., Chindemi, P. A., Debanne, M. T., & Hatton, M. W. C. (1982) *J. Biol. Chem. 257*, 5431–5436.

Reinhold, V. N., & Carr, S. A. (1983) *Mass Spectrom. Rev. 2*, 153–221.

Stockert, R. J., Kressner, M. S., Collins, J. C., Sternlieb, I., & Morell, A. G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6229–6231.

Townsend, R. R., Hilliker, E., Li, Y.-T., Laine, R. A., Bell, W. R., & Lee, Y. C. (1982) *J. Biol. Chem. 257*, 9704–9710.

Townsend, R. R., Wall, D. A., Hubbard, A. L., & Lee, Y. C. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 466–470.

Wong, K.-L., Debanne, M. T., Hatton, M. W. C., & Regeoczi, E. (1978) *Int. J. Pept. Protein Res. 12*, 27–37.