Endo-*N*-acetyl- β -D-glucosaminidase activity in rat liver

Studies on substrate specificity, enzyme inhibition, subcellular localization and partial purification

Jan J. W. LISMAN, Cor J. VAN DER WAL and Bernard OVERDIJK

Department of Medical Chemistry, Van der Boechorststraat 7, 1007 MC Amsterdam, The Netherlands

(Received 10 December 1984/6 March 1985; accepted 1 April 1985)

1. Endo-*N*-acetyl- β -D-glucosaminidase (EC 3.2.1.96, endoglucosaminidase) has been partially purified (520-fold with respect to the cytoplasmic activity) by using concanavalin A-Sepharose, CM-Sephadex and Bio-Gel P-150 chromatography. 2. From the influence of exogenous glycopeptides on the endoglucosaminidase activity it can be concluded that this activity consists of one enzyme hydrolysing both *N*-acetyl-lactosaminic-type and oligomannosidic-type substrates. 3. Glycoproteins present in the homogenate inhibit the endoglucosaminidase activity. 4. On re-examination of the subcellular distribution of endoglucosaminidase (after removal of inhibiting glycoproteins from the respective subcellular fractions), its cytoplasmic localization was confirmed.

The first discovery of a mammalian endo-*N*-acetyl- β -D-glucosaminidase (EC 3.2.1.96, endoglucosaminidase) was reported by Nishigaki *et al.* (1974). The enzyme activity was found to be present in rat and pig organs. Since then a few publications have appeared (Pierce *et al.*, 1979, 1980) concerning its cytoplasmic localization in rat liver. More recently endoglucosaminidase activity has also been demonstrated in human tissues (Tachibana *et al.*, 1981; Overdijk *et al.*, 1981).

In these mammalian tissues endoglucosaminidase was active towards two types of substrate, both *N*-glycosidic ones: the oligomannosidic type and the *N*-acetyl-lactosaminic type. The tentative conclusion from preliminary work was that these two substrates were hydrolysed by different enzyme proteins (Pierce *et al.*, 1980; Overdijk *et al.*, 1981). Tachibana *et al.* (1982) have studied the substrate specificity of endoglucosaminidase from rat liver in more detail. Their results from cross-inhibition experiments indicated that a single enzyme cleaves both types of substrate.

The suggestion has been made that endoglucosaminidase in human tissues is involved in the catabolism of asparagine-linked oligosaccharides (Nishigaki *et al.*, 1974; Montreuil, 1975, 1980). This was based on the common characteristic of the oligosaccharides that are accumulated in the tissues and urine of patients with lysosomal storage diseases. At their reducing ends these oligosaccharide chains are each terminated by one *N*-acetylglucosamine residue. However, the cytosolic

localization of endoglucosaminidase seems to be in contradiction to the above concept of the catabolic role for this enzyme, since substrates that are within the lysosomes but cannot be degraded because of a deficiency of an exoglycosidase would have to be translocated to the cytoplasm.

In order to be able to study the properties of the enzyme in more detail, we attempted to purify it. In the course of that work we investigated the influence of glycopeptides and glycoproteins on the endoglucosaminidase activity from rat liver. In the light of these results we judged it necessary to re-examine the subcellular localization of endoglucosaminidase. Finally, we introduced a modified assay procedure for the enzyme.

Experimental

Materials

The following materials were purchased from the suppliers indicated: 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside and NADH (disodium salt) (Koch-Light Laboratories, Colnbrook, Bucks., U.K.); 2-(iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (J. T. Baker Chemicals, Deventer, The Netherlands); ovalbumin (grade V) and human serotransferrin (Sigma Chemical Co., St. Louis, MO, U.S.A.); [14 C]- and [3 H]-acetic anhydride (New England Nuclear, Boston, MA, U.S.A.); Pronase (Calbiochem-Behring, San Diego, CA, U.S.A.); concanavalin A-Sepharose, DEAE-Sephacel

and CM-Sephadex C-50 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Preparation of glycoasparagine substrates

The glycopeptides GP-IIIa and GP-V (GlcNAc₂Man₅GlcNAc₂Asn and Man₅GlcNAc₂Asn, oligomannosidic-type substrates), derived from ovalbumin by Pronase digestion, were prepared as described by Tai *et al.* (1977). The biantennary asialo-glycoasparagine of human serotransferrin (ASTF-glycopeptide, Gal₂GlcNAc₂Man₃GlcNAc₂Asn, an *N*-acetyl-lactosaminic-type substrate) was isolated as described by Spik & Montreuil (1969). The glycopeptides GP-IIIa and GP-V were radiolabelled by [¹⁴C]-acetylation, whereas the ASTF-glycopeptide was radiolabelled by [³H]- or [¹⁴C]-acetylation by the method of Koide & Muramatsu (1975).

Endoglucosaminidase assay

The incubation mixture consisted of 1 nmol of glycoasparagine substrate in 10 μ l of distilled water, 10 μ l of enzyme preparation, and 10 μ l of 0.1 M-sodium phosphate buffer, pH 6.5, containing 30 mM-dithiothreitol. After incubation for an appropriate time at 37°C, the reaction mixture was applied on 500 μ l of concanavalin A-Sepharose, packed in a disposable-pipette tip of 1 ml. The liberated radioactive GlcNAc-Asn, which does not bind to the column material, was washed off from the column with 1 ml of 0.1 M-sodium phosphate buffer, pH 7.0, containing 1.0 M-NaCl. The radioactivity of the break-through was measured.

One unit of enzyme activity liberates 1 nmol of GlcNAc-Asn/h at 37°C.

Partial purification of endoglucosaminidase

All procedures were performed at 0–4°C unless otherwise stated. A 25% (w/v) homogenate of rat liver in 0.25 M-sucrose was centrifuged at 10000g for 20 min. The supernatant was centrifuged at 100000g for 1 h. The final supernatant (cytoplasm) was used as a crude enzyme preparation for studying kinetic properties of endoglucosaminidase.

A typical purification procedure was then continued as follows. The cytoplasmic fraction (60 ml) was dialysed against 50 mM-Tris/0.5 M-NaCl/6 mM-2-mercaptoethanol, adjusted to pH 7.4 with conc. HCl (buffer I). The dialysis residue (80 ml) was applied to a column (10 cm $\times$ 1.2 cm diam.) of concanavalin A-Sepharose, equilibrated in the above buffer, at a flow rate of 20 ml/h. The unretained material was washed off with 20 ml of buffer I. The resulting break-through (100 ml) was dialysed against 50 mM-sodium phosphate buffer, pH 7.0, containing 6 mM-2-mercaptoethanol (buffer II). Subsequently the enzyme sample

was applied to a CM-Sephadex C-50 column (10 cm $\times$ 1.2 cm diam.) at a flow rate of 20 ml/h. Unretained material was washed off with 30 ml of the sodium phosphate buffer. Elution was continued with a linear gradient composed of 10 ml of starting phosphate buffer and 10 ml of this buffer containing 0.5 M-NaCl (flow rate 20 ml/h). The fractions of this eluate containing endoglucosaminidase activity were dialysed against buffer II and concentrated with the aid of an Amicon concentrator, fitted with a PM-10 filter, to 1 ml. This enzyme preparation was applied to a Bio-Gel P-150 (100–200 mesh) column (110 cm $\times$ 1 cm diam.) at a flow rate of 3.8 ml/h. Fractions of 0.95 ml volume were collected.

Influence of exogenous glycopeptides and glycoproteins on endoglucosaminidase activity

Ovalbumin or serotransferrin was added as an exogenous effector to reaction mixtures of endoglucosaminidase and radioactive substrates. This was also done with unlabelled glycopeptides from these glycoproteins. The details of these experiments are given in the Results and discussion section and in the legend to Fig. 2.

The influence of endogenous glycoproteins and glycopeptides was studied by mixing various amounts of a 100000g supernatant and of a resuspended 100000g pellet, both prepared from a rat liver homogenate. The details of these experiments are also given in the Results and discussion section.

The calculation of the glycan concentrations was as follows: the carbohydrate contents of asialotransferrin and ovalbumin were taken to be 4% and 10% respectively, and the *M_r* values of the respective glycopeptides were calculated from the formulae given by Tachibana *et al.* (1982).

Subcellular fractionation

Homogenization and centrifugation of rat liver tissue was performed as described by de Duve *et al.* (1955). The microsomal and cytoplasmic fractions were obtained by centrifugation of the 250000g-min supernatant for 60 min at 100000g.

Marker enzymes for the respective subcellular fractions and their assays were as follows: for mitochondria, succinate dehydrogenase (EC 1.3.99.1) (Pennington, 1961); for lysosomes, *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30) (Overdijk *et al.*, 1975); for microsomal fraction, glucose-6-phosphatase (EC 3.1.3.9); for cytoplasm, lactate dehydrogenase (EC 1.1.1.27) (Lisman *et al.*, 1979).

Endoglucosaminidase activity (with [¹⁴C]-labelled glycopeptide GP-V as substrate) was assayed in these fractions before and after removal of endogenous concanavalin A-binding glycopeptides and glycoproteins. This latter was achieved

by the following procedure. The subcellular fractions were first dialysed against a 50mM-Tris solution containing 1.0M-NaCl and 1% (v/v) Triton X-100, adjusted to pH 7.0 with conc. HCl. The dialysed fractions were centrifuged at 100000g for 1 h. Portions (3ml) of the supernatants were chromatographed on concanavalin A-Sepharose (1ml of gel in a disposable-pipette tip of 5ml) in the same buffer. Endoglucosaminidase activity was measured in the break-through of the concanavalin A-Sepharose columns.

Results and discussion

Endoglucosaminidase assay

Several methods have been described in which the radioactive GlcNAc-Asn liberated by the action of endoglucosaminidase is separated from the substrate: (1) descending paper chromatography (Fischer & Nebel, 1955); (2) high-voltage paper electrophoresis (Nishigaki *et al.*, 1974); (3) h.p.l.c., with use of dansyl-asparaginyl-oligosaccharides (Iwase *et al.*, 1981); (4) batchwise binding to concanavalin A-Sepharose of unchanged substrate (Tkacz, 1981; Li *et al.*, 1981).

The use of concanavalin A-Sepharose in our assay procedure was based on the last-mentioned method. However, this batchwise binding procedure for the isolation of liberated GlcNAc-Asn gave no reproducible results in our hands.

The endoglucosaminidase activity was measured in the crude enzyme preparation (a 100000g supernatant; see the Experimental section) and in diluted suspensions of it after incubation for various times. Linearity for 60min was only achieved with a 10-fold diluted suspension (Fig. 1). On the basis of these data the enzyme solutions used in the following experiments were diluted in such a way that a linear reaction velocity was obtained. In this respect we were not able to reproduce the results reported by Tachibana *et al.* (1981). They found a constant reaction rate during 9h with glycopeptide GP-V as substrate and a homogenate of human fibroblasts, although 76% of the added substrate (0.5nmol) was cleaved.

Concanavalin A affinity of endoglucosaminidase

In order to test whether endoglucosaminidase is a glycoprotein or not, the affinity of this enzyme for concanavalin A was tested. After concanavalin A-Sepharose chromatography of a crude enzyme preparation 87% of the recovered endoglucosaminidase activity (with ^{14}C -labelled glycopeptide GP-V as substrate) was found in the break-through, and 13% of the activity was retained on the column and could be eluted with α -methyl mannoside.

Although the percentage of endoglucosaminidase bound to the lectin is low, affinity for

Fig. 1. Linearity of endoglucosaminidase activity with time. In these experiments a 100000g supernatant (Δ) of a rat liver homogenate was used as enzyme source. Dilutions of it were made with 0.25M-sucrose: 40% ($\circ$), 20% ($\square$) and 10% ($\bullet$) supernatant. ^{14}C -labelled glycopeptide GP-V was used as substrate.

concanavalin A cannot be ruled out completely. However, this low percentage of binding may be the result of indirect binding of endoglucosaminidase via a glycoprotein substrate bound to concanavalin A.

Influence of exogenous glycopeptides on endoglucosaminidase activity

To test whether endoglucosaminidase consists of one enzyme form that is active towards both *N*-acetyl-lactosaminic-type and oligomannosidic-type substrates or whether each of these substrates is hydrolysed by different enzymes, the following experiments were carried out.

In one experiment 1 nmol of ^{14}C -labelled glycopeptide GP-IIIa and 1 nmol of ^3H -labelled ASTF-glycopeptide were added together to the enzyme assay system. The enzymic conversion of each of these individual substrates was determined in separate experiments. The crude cytoplasmic fraction was used as enzyme source. It appeared that the hydrolysis of both glycopeptides was inhibited with respect to the controls that contained only one glycopeptide. This preliminary result was substantiated further.

To a standard assay system for the determination of the endoglucosaminidase activity, with ^{14}C -labelled glycopeptide GP-IIIa or ^{14}C -labelled glycopeptide GP-V as substrate and an appropriate dilution of the crude cytoplasmic fraction as enzyme source, various amounts of unlabelled ASTF-glycopeptide (0–20nmol of glycan) were added. The results of these incubations are shown

in Fig. 2(a). The ASTF-glycopeptide inhibited the hydrolysis of both substrates, but to different extents. The degradation of glycopeptide GP-IIIa and of glycopeptide GP-V was inhibited by 55% and 85% respectively at 20 nmol of ASTF-glycopeptide.

With ^{14}C -labelled ASTF-glycopeptide as substrate and glycopeptide GP-IIIa as effector, the inhibition of the endoglucosaminidase activity increased to 45% of 20 nmol of ovalbumin glycopeptide (Fig. 2b).

These results strongly suggest that the endoglucosaminidase activity consists of one enzyme that hydrolyses both *N*-acetyl-lactosaminic-type and oligomannosidic-type substrates. This confirms the study by Tachibana *et al.* (1982), but it is in contradiction to the results reported by El Battari *et al.* (1983). These latter authors obtained results that favour the presence of different enzyme forms of endoglucosaminidase, each with its own substrate specificity. Their results showed that the enzyme activities towards both types of substrates differed in pH optimum, kinetic parameters K_m and V_{max} , and sensitivity towards ZnCl_2 .

Influence of exogenous (glyco)proteins on endoglucosaminidase activity

In an analogous way to the one described for glycopeptides, we studied the influence of serotransferrin or ovalbumin on the endoglucosaminidase activity with glycopeptides GP-IIIa and GP-V or ASTF-glycopeptide as substrate respectively.

The hydrolysis of glycopeptide GP-IIIa was stimulated slightly at low concentrations of serotransferrin (Fig. 3a). At higher concentrations serotransferrin inhibited the enzyme activity. The hydrolysis of glycopeptide GP-V was stimulated by serotransferrin (Fig. 3b). This stimulation was not specific, because the same effect was obtained with bovine serum albumin.

Our results and those reported by Tachibana *et al.* (1982) exclude the existence of different enzymes for the hydrolysis of glycopeptides GP-IIIa and GP-V, and so that cannot explain the differential effect of serotransferrin. An explanation in terms of competitive inhibition is improbable for the following reason. Although glycopeptide GP-IIIa contains a bisecting *N*-acetylglucosamine residue whereas glycopeptide GP-V does not, both structures have an identical orientation of their $\alpha 1 \rightarrow 6$ -linked branch ($\omega = -60^\circ$) (Brisson & Carver, 1983). We do not know whether the sialylation and/or the attachment of the glycan to the protein influence the spatial arrangement of the $\alpha 1 \rightarrow 6$ -linked branch in serotransferrin. In the asialo-glycopeptide of this substance the orientation of this branch can vary between $\omega = -60^\circ$ and 180° . Nevertheless it is hard to see that any of these orientations could help to explain the differential effect shown in Figs. 3(a) and 3(b), the more so since the effects of the ASTF-glycopeptide on the hydrolysis of glycopeptides GP-IIIa and GP-V are qualitatively the same (Fig. 2a). It is therefore more probable that the protein part of the

Fig. 2. *Influence of exogenous glycopeptides on endoglucosaminidase activity*

The enzyme assays were carried out with the crude enzyme preparation, appropriately diluted, in order to obtain a constant reaction rate. (a) Influence of ASTF-glycopeptide on the hydrolysis of ^{14}C -labelled glycopeptide GP-IIIa (●) or ^{14}C -labelled glycopeptide GP-V (○). Various amounts of unlabelled ASTF-glycopeptide (0–20 nmol of glycan) were added. The control value (= 100% activity) for the hydrolysis of glycopeptide GP-IIIa was 14 munits and for that of glycopeptide GP-V was 50 munits. (b) Influence of glycopeptide GP-IIIa on the hydrolysis of ^{14}C -labelled ASTF-glycopeptide. Various amounts of unlabelled glycopeptide GP-IIIa (0–20 nmol of glycan) were added. The control value (= 100% activity) for the hydrolysis of ASTF-glycopeptide was 0.8 munit.

Fig. 3. Influence of exogenous (glyco)proteins on endoglucosaminidase activity

(a) Influence of human serotransferrin (○—○) and bovine serum albumin (○—○) on the hydrolysis of glycopeptide GP-V. (b) Influence of human serotransferrin on the hydrolysis of glycopeptide GP-IIIa. (c) Influence of ovalbumin on the hydrolysis of ASTF-glycopeptide.

serotransferrin, like albumin, causes the stimulation of the hydrolysis of glycopeptide GP-V. This kind of stimulation is apparently less effective in the hydrolysis of glycopeptide GP-IIIa.

With ASTF-glycopeptide as substrate the endoglucosaminidase activity was inhibited by ovalbumin (Fig. 3c). The inhibiting effect of the glycan moieties of ovalbumin apparently prevails over the stimulating effect of the protein part of the molecules.

Influence of endogenous (glyco)proteins on endoglucosaminidase

Because of the observed influence of exogenous (glyco)proteins on the endoglucosaminidase

activity, we decided to study to what extent endogenous (glyco)proteins in the enzyme preparation influence the enzymic activity. For that purpose two sets of experiments were performed.

In a first experiment a rat liver homogenate was prepared in 0.25M-sucrose and centrifuged at 100000g for 1 h. The pellet was resuspended to the original homogenate volume. The endoglucosaminidase activity was determined in the supernatant and in the pellet suspension, both at various dilutions. It appeared that on dilution the enzymic activity did not decrease linearly but was higher than its theoretical value (Table 1).

In a second experiment various amounts of supernatant and pellet suspension were mixed. The

Table 1. *Influence of endogenous (glyco)proteins on endoglucosaminidase activity*

A homogenate of rat liver (25%, w/v, in 0.25M-sucrose) was centrifuged at 100000g for 1 h. The pellet was resuspended to the original homogenate volume with 0.25M-sucrose. This solution had 100% of the theoretical pellet activity (4375 d.p.m.). The endoglucosaminidase activity was determined in the supernatant and in the pellet suspension, at various dilutions made in 0.25M-sucrose as indicated by the theoretical values for the supernatant and the pellet activities. Next, the following mixtures of suspensions were used for the enzyme assay: (1) 10 µl of supernatant; (2) 7.5 µl of supernatant + 2.5 µl of pellet suspension; (3) 5.0 µl of supernatant + 5.0 µl of pellet suspension; (4) 2.5 µl of supernatant + 7.5 µl of pellet suspension.

	Supernatant activity			Pellet activity			Supernatant + pellet activity	
	Theoretical (%)	Actual (d.p.m.)	(%)	Theoretical (%)	Actual (d.p.m.)	(%)	Theoretical (d.p.m.)	Actual (d.p.m.)
(1)	100	4954	100	0	0	0	4954	4954
(2)	75	4447	90	25	2932	67	7379	5434
(3)	50	3544	72	50	3628	83	7171	4217
(4)	25	2959	60	75	4028	92	6987	3708

Table 2. *Purification of endoglucosaminidase*

The experimental details of the purification procedure are described in the Experimental section. ¹⁴C-labelled glycopeptide GP-V was used as substrate.

	Activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Cytoplasm	66	1128	0.059	1	100
Concanavalin A-Sepharose break-through	33	928	0.036	0.6	50
CM-Sephadex	55	14	3.9	67	83
Bio-Gel P-150	14	0.45	31	520	21

enzymic activity in these mixtures was determined, and the results are given in Table 1. The actual value of the enzymic activity in the mixture was always lower than its theoretical value. This influence was more pronounced at higher proportions of the pellet suspension.

From these experiments it was concluded that endogenous (glyco)proteins inhibit the endoglucosaminidase activity. These results prompted us to re-examine the subcellular localization of endoglucosaminidase, especially since glycoproteins are not equally distributed among subcellular fractions.

Re-examination of the subcellular localization

A rat liver homogenate (25%, w/v, in sucrose) was separated by differential centrifugation into the following fractions: nuclei, mitochondria, lysosomes, microsomal fraction and cytoplasm. The endoglucosaminidase activity was measured in the original subcellular fractions and in the break-through after concanavalin A-Sepharose chromatography of the same fractions. Before removal of endogenous glycoproteins, the subcellular distribution of the marker enzymes and of endoglucosaminidase was comparable with the results obtained

by Pierce *et al.* (1979). After chromatography of the fractions on concanavalin A-Sepharose the distribution pattern of the endoglucosaminidase activity did not change. The enzymic activity of endoglucosaminidase in the lysosomal fraction was too low (<1% of the total activity, both before and after concanavalin A-Sepharose chromatography) to permit calculation. The relative specific activity of the endoglucosaminidase activity in the cytoplasmic fraction was 2.2. After concanavalin A-Sepharose chromatography this relative specific activity decreased to 1.8, owing to loss of enzymic activity.

The above results demonstrate that the finding of a cytoplasmic localization of endoglucosaminidase in rat liver, as originally reported by Pierce *et al.* (1979, 1980), is not due to an artifact. Artifacts might have been due to the influence of the glycoproteins, present in the respective subcellular fractions, on the endoglucosaminidase activity.

Purification of endoglucosaminidase

The results of the partial purification of endoglucosaminidase from rat liver are summarized in Table 2. The purification factors were calculated with respect to the cytoplasmic fraction. The fact

that the yield after CM-Sephadex chromatography (83%) is higher than that after concanavalin A-Sephadex chromatography (50%) may be due to removal of inhibitory glycoproteins that were not bound by concanavalin A-Sephadex.

Attempts to improve the purification with affinity-chromatography methods were unsuccessful. These involved (1) coupling of glycopeptide GP-IIIa to CH-Sephadex and (2) coupling of ovalbumin to CNBr-activated Sephadex. Both gels showed no affinity for endoglucosaminidase, either at pH 8.0 or at pH 6.5.

The main problem still to be solved is: what is the function of endoglucosaminidase in the normal catabolic pathway of glycoproteins and in cases of lysosomal storage disorders. In this respect some preliminary experiments were carried out with fibroblasts cultured in the absence and in the presence of swainsonine. Swainsonine inhibits α -mannosidase, so inducing a situation of mannosidosis with a concomitant accumulation of high-mannose oligosaccharides (Dorling *et al.*, 1983). In order to catabolize these glycoproteins an induction in the synthesis of endoglucosaminidase might be expected. However, this supposed induction was not observed by us (unpublished work).

We thank Dr. B. Winchester for his gift of swainsonine.

References

- Brisson, J.-R. & Carver, J. P. (1983) *Can. J. Biochem. Cell Biol.* **61**, 1067–1078
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Dorling, P., Huxtable, C., Cenci di Bello, I. & Winchester, B. (1983) *Biochem. Soc. Trans.* **11**, 717–718
- El Battari, A., Appourchaux, P., Montreuil, J. & Spik, G. (1983) *Proc. Int. Symp. Glycoconjugates 7th* 480
- Fischer, F. G. & Nebel, H. G. (1955) *Hoppe-Seyler's Z. Physiol. Chem.* **302**, 10–18
- Iwase, H., Moriga, T., Li, Y.-T. & Li, S.-C. (1981) *Anal. Biochem.* **113**, 93–95
- Koide, N. & Muramatsu, T. (1975) *J. Biol. Chem.* **249**, 4897–4904
- Li, S.-C., Asakawa, M., Hirabayashi, Y. & Li, Y.-T. (1981) *Biochim. Biophys. Acta* **660**, 278–283
- Lisman, J. J. W., De Haan, J. & Overdijk, B. (1979) *Biochem. J.* **178**, 79–87
- Montreuil, J. (1975) *Pure Appl. Chem.* **42**, 431–477
- Montreuil, J. (1980) *Adv. Carbohydr. Chem. Biochem.* **37**, 157–223
- Nishigaki, M., Muramatsu, T. & Kobata, A. (1974) *Biochem. Biophys. Res. Commun.* **59**, 638–645
- Overdijk, B., Van der Kroef, W. M. J., Veltkamp, W. A. & Hooghwinkel, G. J. M. (1975) *Biochem. J.* **151**, 257–261
- Overdijk, B., Van der Kroef, W. M. J., Lisman, J. J. W., Pierce, R. J., Montreuil, J. & Spik, G. (1981) *FEBS Lett.* **128**, 364–366
- Pennington, R. J. (1961) *Biochem. J.* **80**, 649–654
- Pierce, R. J., Spik, G. & Montreuil, J. (1979) *Biochem. J.* **180**, 673–676
- Pierce, R. J., Spik, G. & Montreuil, J. (1980) *Biochem. J.* **185**, 261–264
- Spik, G. & Montreuil, J. (1969) *Bull. Soc. Chim. Biol.* **51**, 1271–1285
- Tachibana, Y., Yamashita, K., Kawaguchi, M., Arashima, S.-i. & Kobata, A. (1981) *J. Biochem. (Tokyo)* **90**, 1291–1296
- Tachibana, Y., Yamashita, K. & Kobata, A. (1982) *Arch. Biochem. Biophys.* **214**, 199–210
- Tai, T., Yamashita, K. & Kobata, A. (1977) *Biochem. Biophys. Res. Commun.* **78**, 434–441
- Tkacz, J. S. (1978) *Anal. Biochem.* **84**, 49–55