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Absence of endo-β-*N*-acetylglucosaminidase activity in the kidneys of sheep, cattle and pig

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The kidneys of man, sheep, cattle and pig were all found to contain 1-aspartamido-β-acetylglucosamine amidohydrolase activity. However, among these, only human kidney was found to contain endo-β-*N*-acetylglucosaminidase activity. The absence of this enzyme in the kidneys of sheep and cattle explains why the oligosaccharides accumulated in, and excreted by, sheep and cattle afflicted with disorders of glycoprotein catabolism (i.e. α-mannosidosis and β-mannosidosis) contain two *N*-acetylglucosamine residues at the reducing terminus instead of one, as is the case for human patients afflicted with similar disorders.

INTRODUCTION

It has been widely recognized that patients with disorders of glycoprotein catabolism accumulate and excrete oligosaccharides with only one GlcNAc residue at the reducing terminus (Montreuil, 1980). However, cattle and sheep afflicted with similar disorders (i.e. natural or induced α-mannosidosis and β-mannosidosis) accumulate and excrete oligosaccharides which contain two GlcNAc residues at the reducing terminus (Abraham *et al.*, 1983; Daniel *et al.*, 1984; Hancock *et al.*, 1986; Jones & Laine, 1980). Although the above facts suggest the possible absence of E-β-GNase activity in cattle and sheep, no direct evidence has yet been provided to show that these animals are indeed devoid of E-β-GNase activity. Here we show that the kidneys of pig, cattle and sheep contain Asn-GNase, but not E-β-GNase, whereas human kidney contains both activities.

EXPERIMENTAL

Materials

Asparaginyl-glycopeptide D ($Man_6GlcNAc_2$-Asn) from OVD was prepared as described by Huang *et al.* (1970). The biantennary *N*-acetyl-lactosamine-type asparaginyl-glycopeptide was isolated from human fibrinogen (Townsend *et al.*, 1982). Owing to the difficulty in obtaining the fibrinogen glycopeptide fully sialylated, the glycopeptide was treated with clostridial neuraminidase to produce the AFG ($Gal_2GlcNAc_2Man_3GlcNAc_2$-Asn). The structure of OVD and AFG have been well characterized (Van Halbeek *et al.*, 1985; Townsend *et al.*, 1982). Asn-GNase was isolated from human liver as described by Dugal & Stromme (1977). Human kidneys obtained from autopsy were kept at −70 °C before use. Fresh kidneys from sheep, cattle and pig were obtained from a local slaughterhouse. The following were obtained from commercial sources: silica-gel 60 precoated thin-layer plates, Merck; Endo-F devoid of peptide *N*-glycanase activity, New England Nuclear; swainsonine, Boehringer-Mannheim; D-galactono-γ-lactone, Pierce; *Clostridium perfringens* neuraminidase type X, Sigma; and Sephadex G-200, Pharmacia.

Enzyme assay

OVD was used as the substrate for the assay of the E-β-GNase activity towards asparaginyl-glycopeptides with the high-mannose-type sugar chain, and AFG was used as the substrate for the activity towards glycopeptides with the *N*-acetyl-lactosamine-type sugar chain. The assay was performed in a 1.5 ml polypropylene tube. The reaction mixture contained the following components in 50 μl: OVD or AFG, 10 nmol; swainsonine, 2.5 nmol, or D-galactono-γ-lactone, 0.75 μmol; sodium citrate, pH 4.0, 1 μmol; and an appropriate amount of the fractionated proteins. Swainsonine (Dorling *et al.*, 1980) was used to inhibit the peeling of mannose residues from OVD by α-mannosidase, whereas D-galactono-γ-lactone was used to inhibit the peeling of galactose residues from AFG by β-galactosidase. The mixture was incubated at 37 °C for 17 h. The assay of Endo-F was performed in 50 mM-sodium acetate buffer, pH 4.0 (Plummer *et al.*, 1984). The assay of Asn-GNase activity was performed in 50 mM-sodium phosphate buffer, pH 7.5. After incubation, the tube was heated for 3 min in a bath of boiling water to terminate the reaction and then centrifuged in a microcentrifuge at 12000 rev./min (*r* 8.0 cm) to remove the precipitated protein. A 10 μl portion of the supernatant was analysed by t.l.c., with butan-1-ol/acetic acid/water (1:1:1, by vol.) as the developing solvent. To reveal glycopeptides and oligosaccharides, the plate was sprayed with diphenylamine reagent (Harris & McWilliam, 1954) and heated at 110 °C for 15 min.

Fractionation of kidney extracts

The following method was used to fractionate the kidney extracts of man, sheep, cattle and pig. Unless otherwise indicated, the procedure was carried out at a

Abbreviations used: GlcNAc, *N*-acetylglucosamine; E-β-GNase, endo-β-*N*-acetylglucosaminidase; Asn-GNase, 1-aspartamido-β-acetylglucosamine amidohydrolase; OVD, ovalbumin; AFG, asialo-(fibrinogen glycopeptide); Endo-F, E-β-GNase isolated from *Flavobacterium meningosepticum*.

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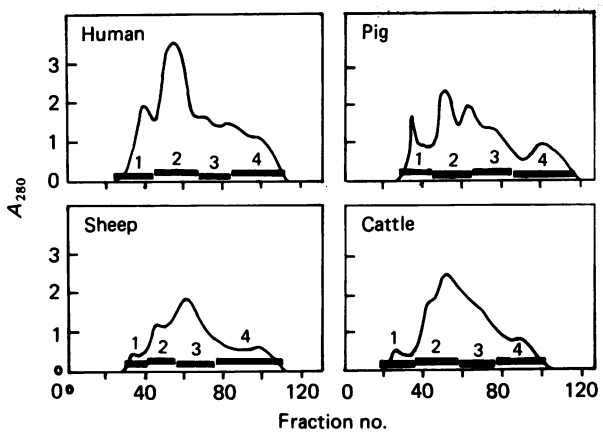

Fig. 1. Fractionation of crude protein extracts derived from the kidneys of man, pig, sheep and cattle by Sephadex G-200 filtration

Detailed conditions are described in the text.

temperature between 0 and 5 °C. Centrifugation was routinely carried out at 13000 *g* for 20 min using a Sorvall RC5C refrigerated centrifuge. In a typical protocol, 70 g of frozen kidney was homogenized with 250 ml of 20 mM-sodium phosphate buffer, pH 7.0, in a Waring Blendor and centrifuged to obtain a crude extract. The pH of the crude extract was adjusted to pH 4.5 with a saturated citric acid solution. After being left for 2 h, the precipitate was removed by centrifugation. The supernatant was adjusted to 75% saturation with solid $(NH_4)_2SO_4$. The precipitated protein (1.5 g) was collected the next day by centrifugation (13000 *g*, 20 min) and dissolved in 25 ml of 50 mM-sodium phosphate buffer, pH 7.0. This crude preparation was applied to a column (5 cm × 80 cm) of Sephadex G-200 that had been equilibrated with the same buffer. The column was eluted with the same buffer at the flow rate of 30 ml/h, and 15 ml fractions were collected. Fig. 1 shows the elution profiles of the comparable crude preparations derived from the kidneys of man, cattle, pig and sheep. In all cases the effluent from the Sephadex G-200 column was divided into four fractions, as indicated by the horizontal bars. These fractions were separately pooled and the proteins in these fractions were precipitated by the addition of $(NH_4)_2SO_4$ to 75% saturation. The precipitated proteins were collected by centrifugation dissolved in a minimum amount of 50 mM-sodium phosphate buffer, pH 7.0, and exhaustively dialysed against 50 mM-sodium citrate buffer, pH 4.0. A portion (200 μg of protein) of each fraction was subsequently used to assay for the ability to liberate oligosaccharides from OVD or AFG.

RESULTS

Hydrolysis of OVD by the four fractions derived from the kidneys of man, sheep, cattle and pig

As shown in Fig. 2, only fraction 3 (lane 5) derived from human kidney could hydrolyse OVD to produce an oligosaccharide with a mobility on t.l.c. identical with

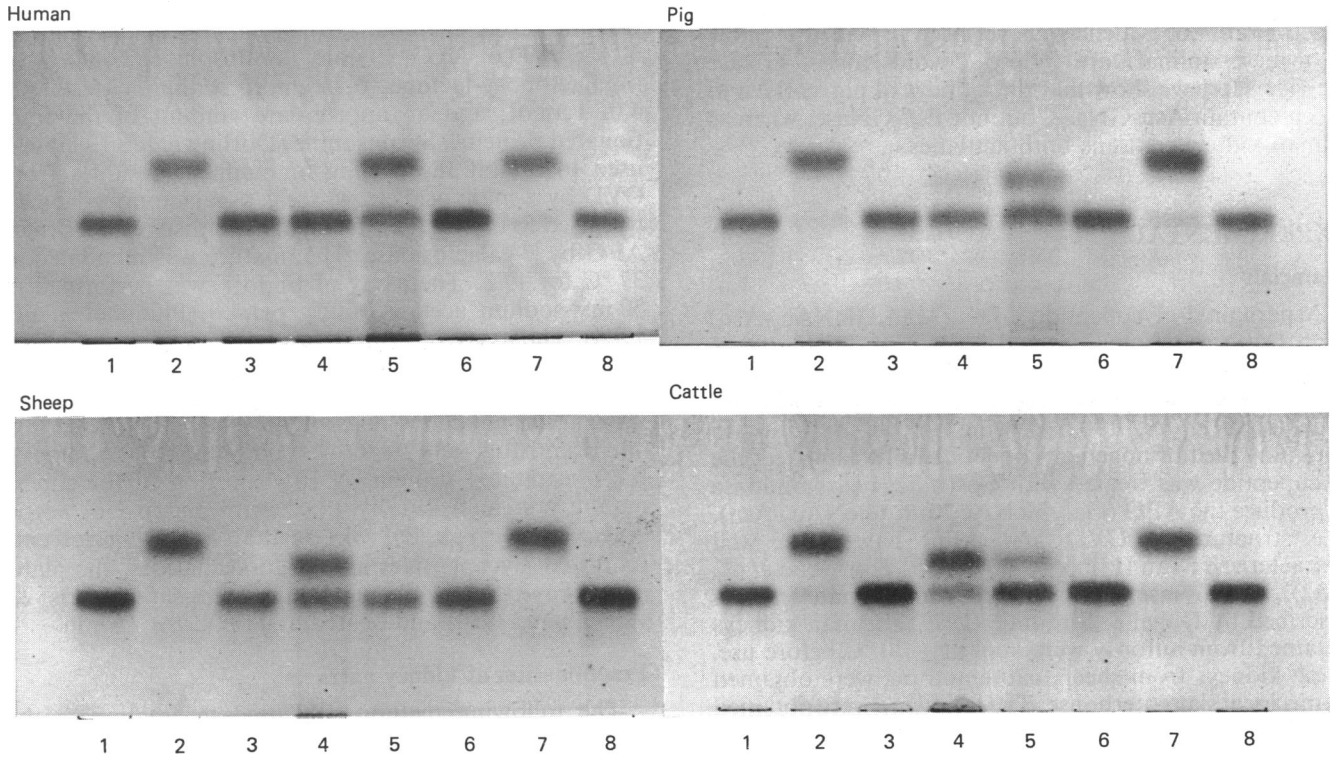

Fig. 2. Thin-layer chromatograms showing the release of oligosaccharides from OVD by the four protein fractions derived from the Sephadex G-200 filtration shown in Fig. 1

Lanes 1 and 8, OVD; lanes 2 and 7, OVD+0.8 unit of Endo-F; lane 3, OVD+fraction 1; lane 4, OVD+fraction 2; lane 5, OVD+fraction 3; lane 6, OVD+fraction 4. In each experiment, 200 μg of protein was incubated with OVD at 37 °C for 17 h. Detailed assay conditions are described in the text.

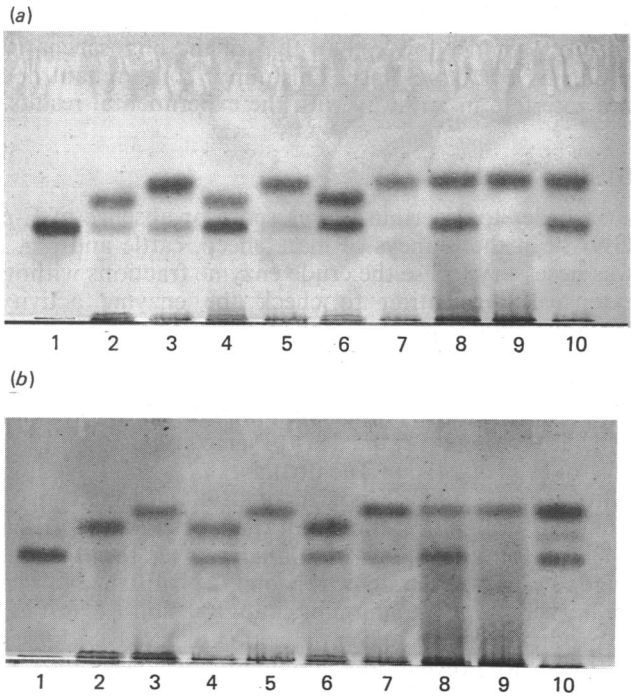

Fig. 3. Thin-layer chromatograms showing the effect of Endo-F on the oligosaccharides produced from OVD (*a*) and AFG (*b*) by the kidney fractions shown in Figs. 2 and 3

Endo-F was added to the incubation mixtures shown in Figs. 2 and 3 to hydrolyse the oligosaccharides released by the kidney fractions. (*a*): Lane 1, OVD; lane 2, OVD + fraction 3 from pig kidney; lane 3, lane 2 + 0.8 unit of Endo-F; lane 4, OVD + fraction 2 from sheep kidney; lane 5, lane 4 + 0.8 unit of Endo-F; lane 6, OVD + fraction 2 from cattle kidney; lane 7, lane 6 + 0.8 unit of Endo-F; lane 8, OVD + fraction 3 from human kidney; lane 9, lane 8 + 0.8 unit of Endo-F; lane 10, OVD + 0.2 unit of Endo-F. (*b*): Lane 1, AFG; lane 2, AFG + fraction 3 from pig kidney; lane 3, lane 2 + 0.8 unit of Endo-F; lane 4, AFG + fraction 2 from sheep kidney; lane 5, lane 4 + 0.8 unit of Endo-F; lane 6, AFG + fraction 2 from cattle kidney; lane 7, lane 6 + 0.8 unit of Endo-F; lane 8, AFG + fraction 3 from human kidney; lane 9, lane 8 + 0.8 unit of Endo-F; lane 10, AFG + 0.2 unit of Endo-F. In all cases, incubations were carried out at 37 °C for 17 h.

that of the oligosaccharide produced by Endo-F (lanes 2 and 7), which served as the standard product (Man_6-GlcNAc) of E-β-GNase activity. No fraction from the kidneys of sheep, cattle and pig was able to produce this oligosaccharide. Instead, fraction 2 of sheep (lane 4) and fractions 2 and 3 (lanes 4 and 5) of cattle and pig produced an oligosaccharide with a mobility on t.l.c. slightly lower than that of the oligosaccharide produced by Endo-F from OVD (lanes 2 and 7). As shown in Fig. 3(*a*), this oligosaccharide was not produced by E-β-GNase, but was the result of the action of Asn-GNase, since Endo-F could further convert this oligosaccharide into the fast-moving oligosaccharide (lanes 3, 5 and 7) with a mobility on t.l.c. identical with that of the

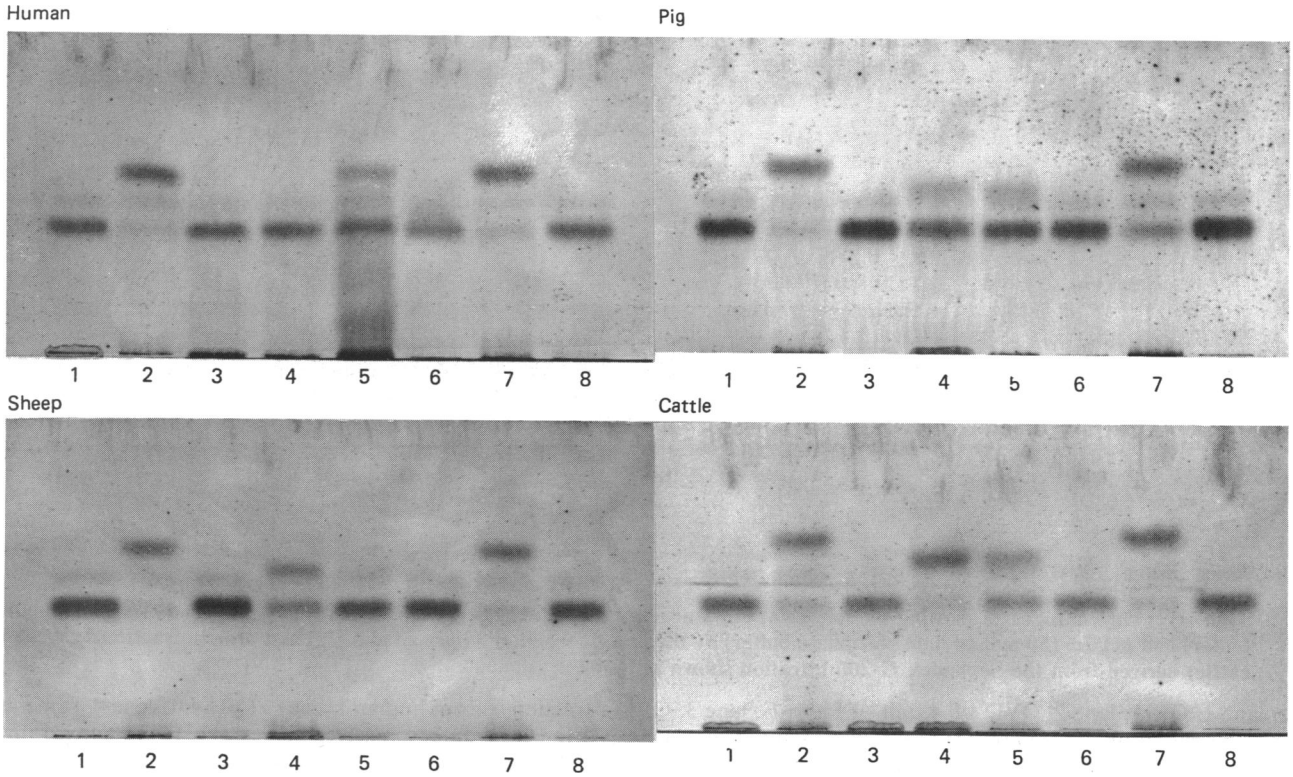

Fig. 4. Thin-layer chromatograms showing the release of the oligosaccharides from AFG by the four protein fractions derived from the Sephadex G-200 filtration shown in Fig. 1

Lanes 1 and 8, AFG; lanes 2 and 7, AFG + 0.8 unit of Endo-F; lane 3, AFG + fraction 1; lane 4, AFG + fraction 2; lane 5, AFG + fraction 3; lane 6, AFG + fraction 4. The incubation conditions and assay conditions are identical with those described in Fig. 2.

oligosaccharide (one GlcNAc residue at the reducing end) produced by Endo-F from OVD (lane 10).

Hydrolysis of AFG by the four fractions derived from the kidneys of man, sheep, cattle and pig

Hydrolysis of AFG by the four fractions prepared from the kidneys of man, sheep, cattle, and pig is shown in Fig. 4. Again, only fraction 3 (lane 5) derived from human kidney could produce an oligosaccharide with a mobility on t.l.c. identical with that of the oligosaccharide produced by Endo-F (lanes 2 and 7). As in the case of OVD, fraction 2 (lane 4) of sheep and fractions 2 and 3 (lanes 4 and 5) of cattle and pig contained the activity which liberated an oligosaccharide with a mobility on t.l.c. slightly lower than that of the oligosaccharide produced by Endo-F from AFG (lanes 2 and 7). As shown in Fig. 3(*b*), Endo-F could further convert this oligosaccharide (lanes 3, 5 and 7) into the fast-moving oligosaccharide with a mobility on t.l.c. identical with that of the oligosaccharide (one GlcNAc residue at the reducing end) produced directly from AFG by Endo-F (lane 10). Therefore this oligosaccharide must be the product of the action of Asn-GNase which contains two GlcNAc residues at the reducing end. As shown in Fig. 4, lanes 1 and 8, the AFG preparation used in the present study appears to contain a minor contaminant having a mobility slightly lower than that of the oligosaccharide produced by Asn-GNase. This minor contaminant does not interfere in any way with the experimental results.

DISCUSSION

In order to ascertain the presence or absence of E-β-GNAse in the kidneys of man, sheep, cattle and pig, it was necessary to use the crude enzyme fractions without extensive purification to check the enzyme activity. Previous studies on mammalian E-β-GNase were based on the detection of the released GlcNAcAsn, since the amino group in asparagine can be easily labelled by either dansylation or acetylation with [^{14}C]acetic anhydride (Nishigaki *et al.*, 1974; Pierce *et al.*, 1979, 1980; Overdijk *et al.*, 1981; Tachibana *et al.*, 1981, 1982; El Battari *et al.*, 1983; Cook *et al.*, 1984; Lisman *et al.*, 1985). Although the analysis of the labelled GlcNAcAsn is much simpler than the analysis of the released oligosaccharide, the detection of GlcNAcAsn may not totally represent the action of endoglycosidases, as it can be produced via the sequential action of exoglycosidases. In order to prevent any ambiguity, we used t.l.c. to identify the production of the oligosaccharide from OVD and AFG. Since we assayed the E-β-GNase activity in

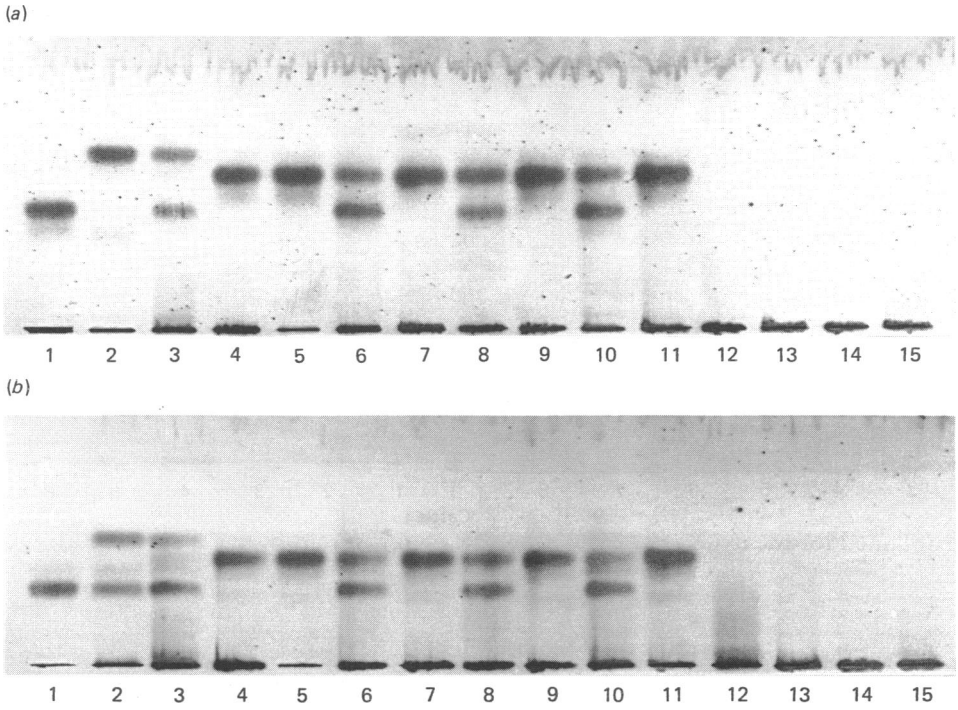

Fig. 5. Thin-layer chromatograms showing the release of oligosaccharides from OVD (*a*) and AFG (*b*) at pH 4.0 (50 mM-sodium citrate buffer) and pH 7.5 (50 mM-sodium phosphate buffer) by the protein fraction 3 (human and pig) and protein fraction 2 (sheep and cattle) derived from the Sephadex G-200 filtration shown in Fig. 1

(*a*) lane 1, OVD; lane 2, OVD + 0.4 unit of Endo-F; lane 3, OVD + fraction 3 from human kidney at pH 4.0; lane 4, same as lane 3, except at pH 7.5; lane 5, OVD + 5 μg of Asn-GNase; lane 6, OVD + fraction 2 from sheep kidney at pH 4.0; lane 7, same as lane 6, except at pH 7.5; lane 8, OVD + fraction 2 from cattle kidney at pH 4.0; lane 9, same as 8, except at pH 7.5; lane 10, OVD + fraction 3 from pig kidney at pH 4.0; lane 11, same as lane 10, except at pH 7.5; lane 12, fraction 3 from human kidney; lane 13, fraction 2 from sheep kidney; lane 14, fraction 2 from cattle kidney; lane 15, fraction 3 from pig kidney. Lanes 12–14 were incubated at pH 4.0. Although not shown, when incubated at pH 7.5, no oligosaccharides were released from these fractions. (*b*) all the conditions were the same for panel (*a*), except that AFG was used instead of OVD. In all cases, incubations were carried out at 37 °C for 17 h. The incubation conditions and assay conditions are identical with those described in Fig. 2.

the crude protein preparations fractionated by Sephadex G-200 filtration, it was essential to use swainsonine and D-galactono-β-lactone to inhibit the activities of α-mannosidase and β-galactosidase respectively. Under our assay conditions (after Sephadex G-200 filtration), no release of mannose from OVD or galactose from AFG suggests the effective inhibition of α-mannosidase and β-galactosidase. At pH 4.0, only fraction 3 (see Fig. 1) derived from human kidney was found to contain E-β-GNase activity. Under the same conditions, no E-β-GNase activity was detected in any of the fractions derived from the kidneys of sheep, cattle and pig. Instead, the kidneys of these three animals contained Asn-GNase activity (Fig. 3). In order to exclude the possibility that the kidneys of these three animals might have an E-β-GNase with neutral optimal pH, we incubated OVD and AFG separately with fraction 3 derived from human and pig kidneys and fraction 2 derived from sheep and cattle kidneys at both pH 4.0 and pH 7.5 (Fig. 5). For the oligosaccharide standards, we used Endo-F to generate $Man_6GlcNAc$ and $Gal_2GlcNAc_2Man_3GlcNAc$ and we used human hepatic Asn-GNase to generate $Man_6GlcNAc_2$ and $Gal_2GlcNAc_2Man_3GlcNAc_2$ from OVD and AFG respectively. Fig. 5(*a*) shows that, at pH 4.0, fraction 3 from human kidney cleaved OVD to produce an oligosaccharide with a t.l.c. mobility identical with that of the oligosaccharide standard ($Man_6GlcNAc$). At pH 7.5, this same fraction produced an oligosaccharide with a mobility on t.l.c. identical with that of the oligosaccharide standard $Man_6GlcNAc_2$. These results indicate the presence of both E-β-GNase and Asn-GNase activities in human kidney. The reason for not finding the Asn-GNase product at pH 4.0 in human kidney is probably due to the fact that the oligosaccharide produced by Asn-GNase was immediately further cleaved by E-β-GNase. In other words, $Man_6GlcNAc$ and $Gal_2GlcNAc_2Man_3GlcNAc$ can be derived from the asparaginyl-glycopeptide either by E-β-GNase alone or by the action of Asn-GNase first, then by E-β-GNase (Baussant *et al.*, 1986; Kuranda & Aronson, 1986). Since E-β-GNase is inactive at neutral pH, only the Asn-GNase product can be detected at pH 7.5. Unlike human kidney, at both pH 4.0 and pH 7.5, fractions from pig, sheep and cattle produced from OVD only an oligosaccharide with a mobility on t.l.c. identical with that of the oligosaccharide produced by human hepatic Asn-GNase. Exactly the same results were obtained for the hydrolysis of AFG at these two pH values (Fig. 5*b*). These results clearly indicate that kidneys of pig, sheep and cattle contain only Asn-GNase activity.

The results presented here provide the first direct evidence that the kidneys of sheep, cattle and pig are devoid of E-β-GNase activity for both high-mannose type and *N*-acetyl-lactosamine-type asparaginyl-glycopeptides. The absence of E-β-GNase in sheep and cattle fully explains why the major oligosaccharides accumulated in, and excreted by, these animals with disorders of glycoprotein catabolism contain two GlcNAc residues at the reducing end instead of only one GlcNAc residue, as is the case of human patients afflicted with similar disorders.

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REFERENCES

Abraham, D., Blakemore, W. F., Jolly, R. D., Sidebotham, R. & Winchester, B. (1983) Biochem. J. **215**, 573–579
Baussant, T., Strecker, G., Wieruszeski, J.-M., Montreuil, J. & Michalski, J.-C. (1986) Eur. J. Biochem. **159**, 381–385
Cook, N. J., Zanetta, J. P. & Vincendon, G. (1984) J. Chromatogr. **292**, 479–484
Daniel, P. F., Warren, C. D. & James, L. F. (1984) Biochem. J. **221**, 601–607
Dorling, P. R., Huxtable, C. R. & Colegate, S. M. (1980) Biochem. J. **191**, 649–651
Dugal, B. & Stromme, J. (1977) Biochem. J. **165**, 497–502
El Battari, A., Appourchaux, P., Montreuil, J. & Spik, G. (1983) Int. Glycoconjugate Symp. 7th, Lund, Sweden, 480
Hancock, L. W., Jones, M. Z. & Dawson, G. (1986) Biochem. J. **234**, 175–183
Harris, G. & McWilliam, I. C. (1954) Chem. Ind. (London) 249
Huang, C. C., Mayer, H. E., Jr. & Montgomery, R. (1970) Carbohydr. Res. **13**, 127–137
Jones, M. Z. & Laine, R. A. (1980) J. Biol. Chem. **256**, 5181–5184
Kuranda, M. J. & Aronson, N. N. (1986) J. Biol. Chem. **261**, 5803–5809
Lisman, J. J. W., van der Wal, C. J. & Overdijk, B. (1985) Biochem. J. **229**, 379–385
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, M. J., Lisman, J. J. W., Pierce, R. J., Montreuil, J. & Spik, G. (1981) FEBS Lett. **128**, 364–366
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
Plummer, T. H., Jr., Elder, J. H., Alexander, S., Phelan, A. W. & Tarentino, A. L. (1984) J. Biol. Chem. **259**, 10700–10704
Tachibana, Y., Yamashita, K., Kawaguchi, M., Arashima, S. & Kobata, A. (1981) J. Biochem. (Tokyo) **90**, 1291–1296
Tachibana, Y., Yamashita, K. & Kobata, A. (1982) Arch. Biochem. Biophys. **214**, 199–210
Townsend, R. R., Hilliker, E., Li, Y.-T., Laine, R. A., Bell, W. R. & Lee, Y. C. (1982) J. Biol. Chem. **257**, 9704–9710
Van Halbeek, H., Vliegenthart, J. F., Iwase, H., Li, S.-C. & Li, Y.-T. (1985) Glycoconjugate J. **2**, 235–253

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