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Oligosaccharides accumulated in the Bovine β-Mannosidosis Kidney

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Summary: The phenotype of bovine β-mannosidosis (β-mannosidase deficiency), recently identified in Salers cattle, is similar to the caprine form of the disease (Abbitt *et al.*, 1991). This investigation was designed to characterize accumulated kidney oligosaccharides in bovine β-mannosidosis. Oligosaccharides were extracted from the kidney of an affected Salers calf and purified by chromatographic techniques. The amount of accumulating oligosaccharides in 1 g of wet tissue was about 21 μmol. Structures of derivatized oligosaccharides were characterized by high-performance liquid chromatography, mass spectrometry, methylation analysis and sequential exoglycosidase digestions. The major accumulating oligosaccharides were Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc. Oligosaccharides accumulating in minor amounts were Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAc, Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc. As in caprine β-mannosidosis, oligosaccharides with terminal β-mannose residues and cleaved as well as uncleaved chitobiose linkages were identified in bovine β-mannosidosis kidney. The accumulating oligosaccharides in tissue were thus identical in bovine and caprine β-mannosidosis; however, the source of the novel oligosaccharides remains to be determined.

Recently, β-mannosidosis, an inherited defect of glycoprotein catabolism associated with a deficiency of β-mannosidase (EC 3.2.1.25), was identified in the bovine (Salers) species (Abbitt *et al.*, 1991). The clinical, pathological, biochemical and physiological features of the bovine form of the disease were similar to those first characterized in goats (Hartley and Blakemore, 1973; Jones and Dawson, 1981; Jones and Laine, 1981; Jones *et al.*, 1983; Jones, 1989). Major stored and excreted oligosaccharides in caprine β-mannosidosis were Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc (Jones and Laine, 1981; Matsuura *et al.*, 1981, 1983). The human β-mannosidosis phenotype included mental retardation and uncatabolized Manβ1-4GlcNAc accumu-

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lation (Cooper *et al.*, 1988; Wenger *et al.*, 1986; Dorland *et al.*, 1988). Reflecting differences in human and ruminant glycoprotein catabolic pathways, the storage oligosaccharides from human β-mannosidosis have a single GlcNAc residue at the reducing terminus, while those in caprine β-mannosidosis have a single GlcNAc or a chitobiose linkage (Hancock *et al.*, 1986; Hancock and Dawson, 1987; Aronson and Kuranda, 1989). The role of the accumulated oligosaccharides in the pathogenesis of the disease, particularly the central nervous system dysmyelination and thyroid dysfunction observed in both ruminant species, has not been clarified (Boyer *et al.*, 1990a,b; Lovell *et al.*, 1991).

In this paper, we report the structures of the accumulating oligosaccharides in the bovine β-mannosidosis kidney. Preliminary studies of this work have appeared (Abbitt *et al.*, 1991).

MATERIALS AND METHODS

Bio-Gel P-2 was purchased from Bio-Rad Laboratories, Richmond, CA, USA. *p*-Aminobenzoic acid ethyl ester (ABEE) was purchased from Wako Pure Chemical Industries, Osaka, Japan and sodium cyanoborohydride from Nacalai Tesque Inc., Kyoto, Japan. Jack bean meal β-*N*-acetylhexosaminidase and α-mannosidase were purchased from Sigma Chemical Company, St Louis, MO, USA. Snail β-mannosidase and chitobiose were obtained from Seikagku Kogyo Company Ltd, Tokyo, Japan. Other oligosaccharide standards for chromatography were isolated from the tissues of goats with β-mannosidosis, as described previously (Matsuura and Jones, 1985). All other chemicals and organic solvents were analytical or HPLC grade and were used without further purification.

Extraction and isolation of oligosaccharides: Frozen kidney (2 g) from an affected and a normal calf was extracted separately using the methods as described by Matsuura and Jones (1985). The extract was applied to a Bio-Gel P-2 (100–200 mesh) column (1.0 cm × 100 cm) and the column was eluted with water containing 0.02% sodium azide and 1-ml fractions were collected. Each fraction was monitored by measuring hexose using the phenol–sulphuric acid method (Dubois *et al.*, 1956) and thin-layer chromatography as described previously (Matsuura and Jones, 1985).

Preparation of ABEE derivatives of oligosaccharides and analysis by HPLC: Oligosaccharides were labelled by reductive amination with ABEE using the method of Wang *et al.* (1984), as modified by Matsuura and Imaoka (1988). The ABEE derivatives were purified by ether extraction and subsequent chromatography using Pre-Sep C_{18} cartridges as described by Matsuura and Imaoka (1988) and analysed by HPLC with the use of a two-dimensional technique (Matsuura *et al.*, in press) using a TSKgel Amide-80 (TOSOH) and a Wakosil 5C18-200 octadecylsilyl (ODS) column. The Amide-80 column was eluted with a gradient from 15% to 45% of Solvent A (acetonitrile/water, 1:9 v/v) in Solvent B (acetonitrile/water, 9:1 v/v) over 60 min, at a flow rate of 1 ml/min at 40°C. The ODS column was eluted isocratically with

acetonitrile/100 mmol/L acetic acid (9:91 v/v) at a flow rate of 1 ml/min at 40°C. Elutions were monitored by absorbance at 304 nm.

Fast atom bombardment–mass spectrometry (FAB–MS): FAB–MS and FAB collisionally activated dissociation tandem mass spectrometry (FAB–CAD–MS/MS) of ABEE-oligosaccharidies was performed on a JEOL HX110 double-focusing mass spectrometer (di- and trisaccharide) or on a JEOL HX110/HX110 four-sector tandem mass spectrometer (tetra- and pentasaccharides) using methods described by Gage *et al.*, in press).

Methylation analysis and sequential exoglycosidase digestion: Methylation analysis and sequential exoglycosidase digestion of the oligosaccharides were performed according to procedures described previously (Matsuura and Jones, 1985; Ohta *et al.*, in press).

RESULTS

Figure 1 shows the elution profile of oligosaccharides accumulated in β-mannosidosis calf kidney on a Bio-Gel P-2 column. Oligosaccharides A, B, C1,C2 and D were

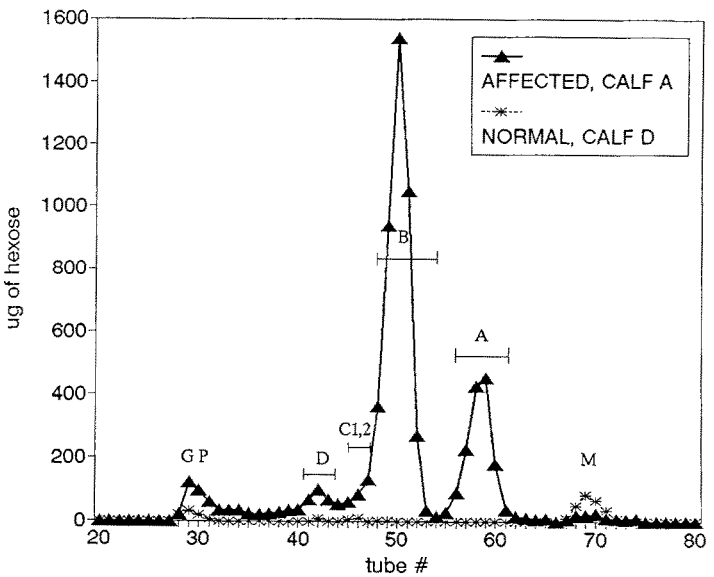

Figure 1 Bio-Gel P-2 column chromatography of oligosaccharides accumulated in the kidney of a calf with β-mannosidosis and a normal calf. Oligosaccharides were extracted from the kidney of a β-mannosidosis calf and applied to a Bio-Gel P-2 column (1.0 × 100 cm). The column was eluted with water containing 0.02% sodium azide. Fractions of 1 ml were collected and a portion of the eluate was measured for hexose by the phenol–sulphuric acid method. Oligosaccharides A, B, C1,C2 and D were pooled as indicated by horizontal arrow on the basis of TLC behaviour. In a separate run, these pooled fractions (32–80) were subjected to ABEE derivatization followed by HPLC

isolated and subjected to structural characterization. Fractionation of the ABEE derivatives of the pooled oligosaccharide fractions (Fractions 32–80 in Figure 1) by Pre-Sep C_{18} cartridge chromatography indicated that 99.5% of total ABEE-oligosaccharides from control kidney and 97.1% of the total ABEE-oligosaccharides from affected kidney were neutral oligosaccharides (Matsuura and Imaoka, 1988). The amounts of the neutral ABEE-oligosaccharides estimated by molecular absorptivity coefficient in control and affected kidney were 3.6 μmol/g wet kidney and 20.6 μmol/g wet kidney, respectively. When the neutral ABEE-oligosaccharides were analysed by HPLC with combined use of the Amide-80 (Figure 2) and ODS columns, five oligosaccharides which were not present in the normal animal were found in the affected animal. The chromatographic behaviours of the accumulated oligosaccharides were the same as those of oligosaccharides which accumulate in caprine β-mannosidosis. The percent molar ratio of oligosaccharide A:B:C1:C2:D was 29.5:66.5:0.9:2.0:1.1 on the basis of peak area.

Structure of oligosaccharide A: FAB–CAD–MS/MS studies revealed that the ABEE-oligosaccharide A was identical to that of the Manβ1-4GlcNAc disaccharide obtained

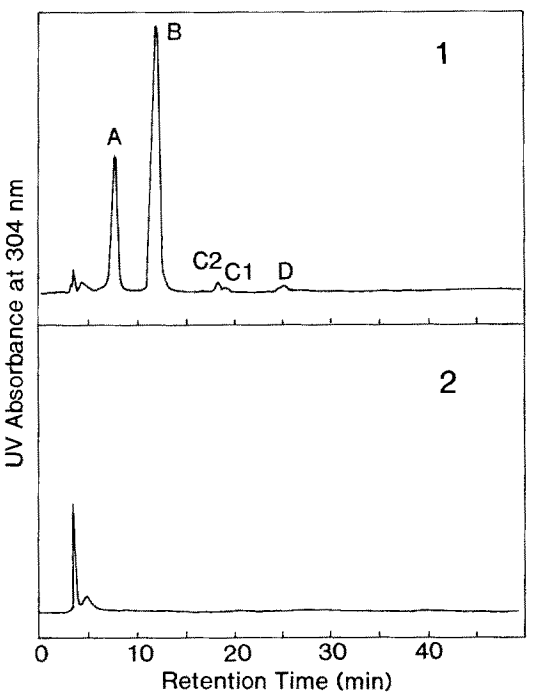

Figure 2 HPLC analysis of ABEE-oligosaccharides on a TSKgel Amide-80 column. The pooled oligosaccharide fraction obtained by Bio-Gel P-2 column chromatography (see Figure 1) was derivatized with ABEE and subjected to HPLC analysis on a TSK gel Amide-80 column. The column was eluted with the mixture of acetonitrile and water as described. (1) ABEE-oligosaccharides from the kidney of a calf affected with β-mannosidosis. (2) ABEE-oligosaccharides from the kidney of a normal calf

from β-mannosidosis-affected goat tissue (Gage *et al.*, in press). Methylation analysis of the reduced oligosaccharide A concurs with the structure (data not shown). When the ABEE-oligosaccharide A was digested with snail β-mannosidase and analysed by HPLC, it lost a mannose residue and gave a peak with the same mobility as authentic GlcNAc-ABEE. From these results, the structure of oligosaccharide A is proposed as Manβ1-4GlcNAc.

Structure of oligosaccharide B: FAB–CAD–MS/MS studies revealed that the ABEE-oligosaccharide B was identical to the Manβ1-4GlcNAcβ1-4GlcNAc trisaccharide obtained from β-mannosidosis-affected goat tissue (Gage *et al.*, in press). Methylation analysis of the reduced oligosaccharide B concurs with the structure (data not shown). When the ABEE-oligosaccharide B was sequentially digested with β-mannosidase and β-*N*-acetylhexosaminidase, it gave a peak with the same mobility as authentic GlcNAc-ABEE. From these results, the predominant oligosaccharide accumulated in the kidney is proposed as Manβ1-4GlcNAcβ1-4GlcNAc.

Structures of oligosaccharide C1 and C2: The oligosaccharide C1,C2 separated by Bio-Gel P-2 chromatography was derivatized with ABEE and subjected to FAB–MS and FAB–CAD–MS/MS analysis. As these data have not been presented previously they are summarized here. FAB–MS of the ABEE derivative of oligosaccharide C1,C2 displayed a prominent $[M + Na]^+$ ion at $m/z = 920$, suggesting that the oligosaccharide was an ABEE-tetrasaccharide composed of two Hex, and two HexNAc residues. The positive ion in FAB–CAD–MS/MS of the sodium adduct ion $[M + Na]^+$ at $m/z = 920$ provided evidence for the presence of two tetrasaccharide sequence isomers (Figure 3a). Product ions at $m/z = 758$ (Y_3) and $m/z = 740$ (Z_3), with the complements at $m/z = 185$ (B_1) and 201 (C_1), indicated that Hex is the non-reducing terminal sugar in both isomers. The absence of peaks at $m/z = 717$ (Y_3), 699 (Z_3) and 242 (C_1) demonstrated that a HexNAc could not be located at the non-reducing terminus in either compound. Additional fragment ions in the spectrum showed how the sequences of the two isomers differed. The presence of the tetrasaccharide isomer (C1) in this fraction is indicated by the glycosidic bond cleavage fragments present at $m/z = 555$ (Y_2) and 537 (Z_2), a loss of 203 from the Y_3 and Z_3 ions ($m/z = 758$ and 740, respectively). This was supported by the complementary fragments at $m/z = 388$ (B_2) and 404 (C_2). Thus, the sequence for this isomer is Hex-HexNAc from the non-reducing terminus. Differences of 162 mass units, from these sequence ions to a set of peaks $m/z = 393$ (Y_1), 566 (C_3), and 550 (B_3), indicates that the next sugar in the sequence is a hexose in this isomer. By inference from the $[M + Na]^+$ ion at $m/z = 920$, the reducing terminal sugar must be HexNAc, revealing a sequence of Hex-HexNAc-Hex-HexNAc for the C1 isomer. Ring cleavage fragments at m/z 786 ($^{1,5}X_3$), 583 ($^{1,5}X_2$), and 421 ($^{1,5}X_1$), support this sequence. Ring cleavage fragments at $m/z = 259$ ($^{3,5}A_2$), 462 ($^{3,5}A_3$) and 638 ($^{3}A_4$) are also pertinent in suggesting that the linkages again must be 1,4 or 1,6 positions (Orlando *et al.*, 1990, Gage *et al.*, in press).

The set of glycosidic bond cleavage fragments present at $m/z = 596$ (Y_2) and 578 (Z_2) along with the peaks at $m/z = 347$ (B_2) and 363 (C_2) provides evidence for an

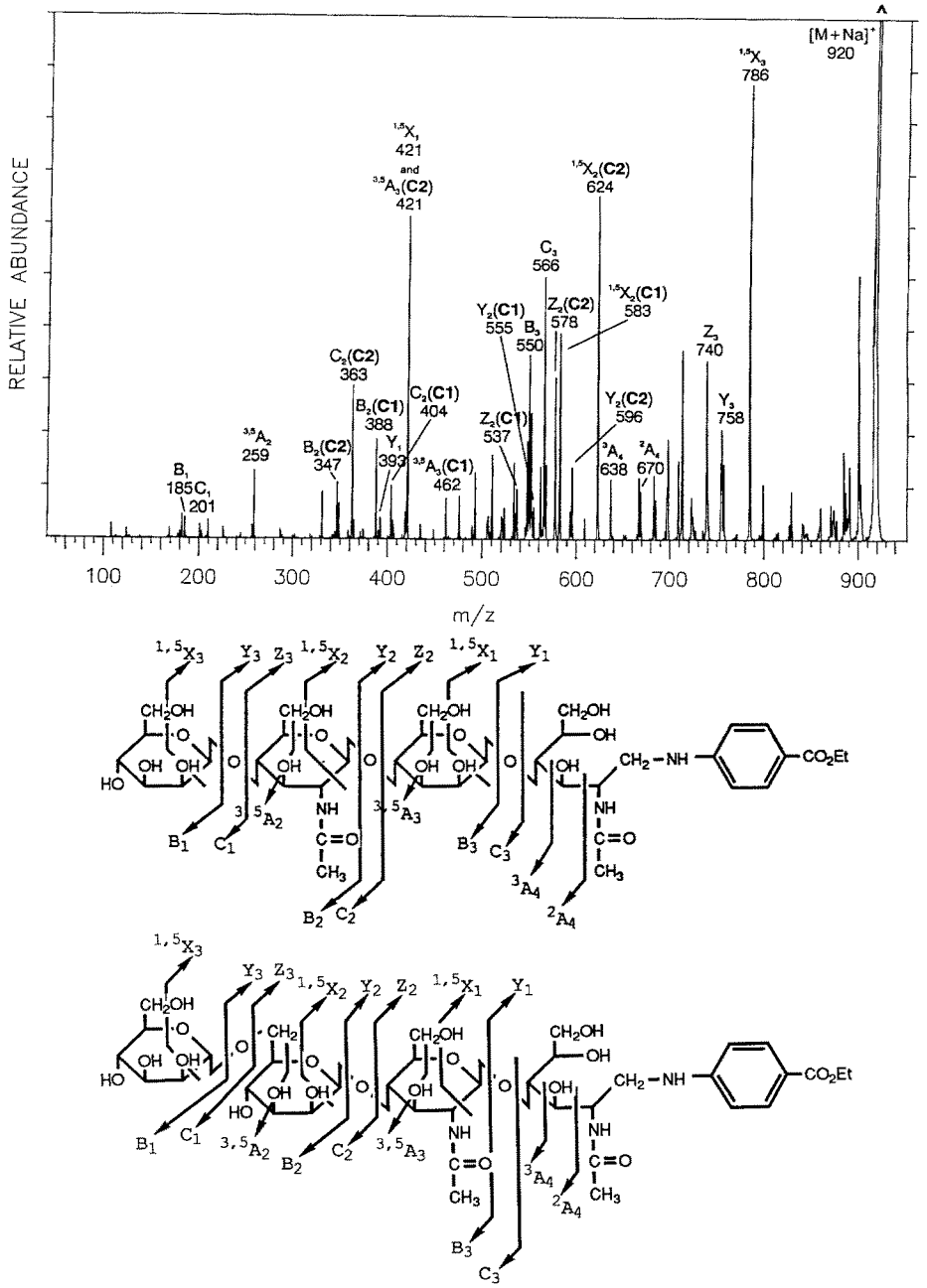

Figure 3 (a) FAB–CAD–MS/MS spectrum of the isobaric $[M + Na]^+$ ions from a mixed sample of the ABEE derivatives of the isomeric tetrasaccharides C1 and C2. The two sets of fragment ions, labelled according to the nomenclature of Domon and Costello (1988) indicate the presence of two sequence isomers. These two sets are designated in the spectrum (C1 and C2). The structures of the C1 and C2 isomer derivatives, Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAc-ABEE and Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-ABEE, are shown in Figures 3b and 3c, respectively. The bond cleavages indicated by the fragments in the spectrum are labelled. For clarity, fragments resulting from secondary losses of EtOH (−46) and COOEt (−72) are not labelled. Note that the sequences, as well as some of the linkages, of these two compounds can be determined from the fragmentation patterns. (Hydrogen transfers and sodium attachments involved in the formation of the fragments are not shown.) See the text for further discussion.

adjacent Hex in the second most abundant isomer (C2). In this compound, the difference of 203 mass units from the Y_2, B_2 and C_2 fragment ions to the set of peaks at $m/z = 393$ (Y_1), 566 (C_3), and 550 (B_3), along with the absence of peaks at $m/z = 434$ (Y_1), 509 (B_3), and 525 (C_3) indicated the third sugar in the sequence of this isomer must be HexNAc rather than another Hex. From the $[M + Na]^+$ ion at $m/z = 920$ it follows that the reducing terminal sugar must be HexNAc and therefore the sequence is Hex-Hex-HexNAc-HexNAc in this isomer (C2). Ring cleavage fragments at $m/z = 786$ ($^{1,5}X_3$), 624 ($^{1,5}X_2$), and 421 ($^{1,5}X_1$), support this sequence, while the ring cleavage fragments at $m/z = 259$ ($^{3,5}A_2$), 421 ($^{3,5}A_3$), and 638 ($^{3}A_4$) and the lack of other ions of this type suggest that all the linkages must be 1,4 or 1,6. Thus, even in a mixture, the sequences of the two isomers can be determined. Further characterization of the two isomers was provided by preparation of the partially methylated alditol acetates.

Methylation analysis of the reduced oligosaccharides C1,C2 mixture provided 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol; 1,4,5-tri-*O*-acetyl-2,3,6-tri-O-methyl-mannitol; 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol; 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol; 4-mono-*O*-acetyl-1,3,5,6-tetra-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol and 4-mono-*O*-acetyl-1,3,5,6-tetra-*O*-methyl-2-*N*-acetylacetamido-2-deoxyglucitol (data not shown). Methylation analysis of the ABEE derivatives of C1 and C2 isolated by HPLC provided 2,3,6-tri-*O*-methylmannitol and 2,3,4-tri-*O*-methylmannitol, respectively, as tri-*O*-methylmannitol. When the ABEE derivative of C1 was digested with snail β-mannosidase, it lost a mannose residue and gave a peak in the region of ABEE-trisaccharide. The trisaccharide-ABEE digested with β-*N*-acetylhexosaminidase gave a peak which co-migrated with authentic Manβ1-4GlcNAc-ABEE, which was further converted to GlcNAc-ABEE by β-mannosidase digestion. The ABEE derivative of C2 gave a peak at the elution position of authentic Manβ1-4GlcNAcβ1-4GlcNAc-ABEE after the treatment with jack bean α-mannosidase. The digest thus obtained was converted to GlcNAc-ABEE after sequential digestion with snail β-mannosidase and then jack bean β-*N*-acetylhexosaminidase. From these results, the structures of oligosaccharide C1 and C2 are proposed as Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAc and Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc, respectively.

Structure of oligosaccharide D: Positive FAB–CAD–MS/MS of the ABEE derivative of oligosaccharide D was identical to that of the Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-ABEE found in caprine β-mannosidosis tissue (Gage *et al.*, in press). Methylation analysis of the reduced oligosaccharide D concurs with the structure (data not shown). When the ABEE oligosaccharide D was digested with snail β-mannosidase, it lost a mannose residue and gave a peak in the region of tetrasaccharide. The digest was converted to an ABEE derivative with the same retention time as authentic Manβ1-4GlcNAcβ1-4GlcNAc-ABEE after β-*N*-acetylhexosaminidase digestion. When the trisaccharide-ABEE thus obtained was subjected to further sequential glycosidase digestion with β-mannosidase and then with β-*N*-acetylhexosaminidase, it gave a peak with the same mobility as that of

authentic GlcNAc-ABEE. Summarizing these results, the structure of oligosaccharide D is proposed as Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc.

DISCUSSION

Altogether, structural analysis indicates that the oligosaccharides accumulating in the bovine β-mannosidosis kidney are identical to those reported in the caprine β-mannosidosis kidney and thyroid gland (Jones and Laine, 1981; Matsuura and Jones, 1985; Matsuura *et al.*, 1981; Gage *et al.*, in press). Preliminary studies of urine from an affected calf also showed oligosaccharides (Abbitt *et al.*, 1991) identical to those characterized in the urine of the β-mannosidosis goat (Matsuura *et al.*, 1983). As expected in a β-mannosidase deficiency state, the terminal residue of the main accumulated compounds was a β-linked mannose residue. Although approximately twice as much accumulated oligosaccharide was detected in the affected bovine kidney when compared with affected caprine kidney, the oligosaccharide composition was similar. The greater accumulation in the bovine kidney was probably due to the significantly longer gestation in this species. An explanation for the accumulation of an oligosaccharide with an α-linked mannose at the non-reducing terminus awaits determination of the specificities of ruminant α-mannosidases.

The presence of compounds with a single GlcNAc and chitobiose linkage at the reducing terminus in affected bovine and caprine tissues is probably related to a similar pathway of glycoprotein catabolism for ruminants and to the presence of an endo-β-hexosaminidase as well as an amidohydrolase in the tissues of ruminant species (Hancock *et al.*, 1986). Alternative pathways of glycoprotein catabolism have been proposed, where removal of the reducing-end GlcNAc from oligosaccharides previously freed by an amidohydrolase is catalysed by the lysosomal glycosidase chitobiase in humans and rats and by β-hexosaminidase in other mammals such as dogs, cats, goats, sheep or cattle (Aronson and Kuranda, 1989). A chitobiase has not been found in the latter animals. This alternative explanation does not account for the significant amounts of accumulating oligosaccharides with a single GlcNAc at the reducing terminus identified in our studies of affected ruminant tissues.

It has been shown that the storage oligosaccharides in bovine α-mannosidosis have the structure of $(Man)_nGlcNAc$-GlcNAc (Abraham *et al.*, 1983). Oligosaccharides with a single GlcNAc at the reducing end were not detected, suggesting only amidohydrolase cleavage of the linkage between oligosaccharide and peptide. By contrast, oligosaccharides accumulated in bovine β-mannosidosis kidney have both a single GlcNAc and chitobiose at their reducing terminus. These probably require the action of an endo-β-hexosaminidase and an amidohydrolase, respectively (Hancock and Dawson, 1987). The reason for the apparent differences in the structures of the core region of the storage oligosaccharides in α- and β-mannosidosis is not understood. The most abundant urinary oligosaccharide in bovine α-mannosidosis was Manα1-6Manβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAc, suggesting the presence of unique core structures in bovine Asn-linked oligosaccharides (Lundblad *et al.*, 1975). Oligosaccharides having such core structures were not detected in our studies of

bovine or caprine β-mannosidosis kidney or urine (Jones and Laine, 1981; Matsuura *et al.*, 1981, 1983; Matsuura and Jones, 1985; Abbitt *et al.*, 1991).

Accumulation of previously reported tetra- and pentasaccharides which had not been found as common structural features of Asn-linked oligosaccharides of glycoproteins (Matsuura and Jones, 1985) was confirmed in bovine β-mannosidosis. Storage oligosaccharides having structures not related to those of Asn-linked oligosaccharides have also been reported in human β-mannosidosis (NeuAcα2-6Manβ1-4GlcNAc) (Van Pelt *et al.*, 1990) and in aspartylglucosaminuria (Galβ1-4GlcNAc-Asn and NeuAcα2-3/6Galβ1-4GlcNAc-Asn) (Beaudet and Thomas, 1989). Although a source was hypothesized for NeuAcα2-6Manβ1-4GlcNAc in human β-mannosidosis (Van Pelt *et al.*, 1990), the origin of unusual oligosaccharides in bovine and caprine β-mannosidosis (present in small amounts compared to di- and trisaccharide) has not been determined. The Asn-linked glycoproteins have been documented as the source of the accumulated di- and trisaccharides (Hancock *et al.*, 1986). The biological activity of the accumulated oligosaccharides and their role in the pathogenesis of the β-mannosidosis phenotype remain to be determined.

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BOOK REVIEW

Medical Ethics: Evolution, Rights and the Physician. H.A. Shenkin. Number 17 in the series *Episteme*. Kluwer Academic Publishers, Dordrecht, Netherlands, 1991, ISBN 0-7923-1031-4, 500 pp., Dfl.190/US$124/£65

This book is written by a (retired) American physician, with emphasis on the doctor–patient relationship. It is divided into two sections.

Part One covers philosophical and (socio)-biological topics, law and rights. In relation to medical practice, these are further discussed under the headings of Autonomy and Beneficence, Non-maleficence, Justice, Confidentiality, Patients' Right to Medical Care and the Right to Know. Dr. Shenkin aims to reconcile the two basic ethical approaches, the deontological (*what ought I do in this situation...*) and the consequentialist, which includes the utilitarian (*actions are to be judged by their consequences...*) by adding philosophical and biological arguments in favour of an evolutionary basis for ethics.

Part Two applies these considerations to very wide range of medical situations which raise ethical issues for medical practitioners, for their patients, and for society. Among these are a number which are immediately relevant to the members of the SSIEM. They include; testing for genetic disease, research on humans and on animals, biotechnology, the human genome, abortion.

References to the literature in English are well covered, but there are very few contributions from the Continent of Europe, which is a pity, since these countries with their different histories, traditions and legal systems have much to offer. This book takes us on from the SSIEM Symposium on medical ethics held in Sheffield, 1987.

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