The Glycoasparagines in Urine of a Patient with Aspartylglycosaminuria

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The isolation and analysis of 2-acetamido-*N*-(4'-L-aspartyl)-2-deoxy- β -D-glucopyranosylamine and 13 higher-molecular-weight derivatives from the urine of a patient with aspartylglycosaminuria is described. Some structural information is presented for nine of these compounds.

Aspartylglycosaminuria is an inborn error of metabolism associated with mental retardation and characterized by the excretion of large quantities of 2-acetamido-*N*-(4'-L-aspartyl)-2-deoxy- β -D-glucopyranosylamine (GlcNAc-Asn) in the urine (Jenner & Pollitt, 1967). The disease is apparently caused by a deficiency of 2-acetamido-*N*-(4'-L-aspartyl)-2-deoxy- β -D-glucosylamine amidohydrolase (EC 3.5.1.26) (Pollitt & Jenner, 1969; Palo *et al.*, 1971). A considerable number of cases are now known and some of the clinical features are suggestive of a lysosomal storage disease (Pollitt *et al.*, 1968; Pollitt & Jenner, 1971; Autio, 1972). Enlarged lysosomes have been demonstrated by electron microscopy in brain, liver and kidney (Palo *et al.*, 1971).

The urines of patients with this disease contain a number of compounds which are related to GlcNAc-Asn, but have additional carbohydrate residues. These compounds are termed glycoasparagines and some aspects of their composition have been reported (Pollitt & Pretty, 1972). It was thought that the higher-molecular-weight compounds might be responsible for the storage aspects of the disease, but this does not appear to be so for liver and brain (Palo *et al.*, 1973). In the present paper, the isolation of GlcNAc-Asn and 13 other glycoasparagines from the urine of a patient with aspartylglycosaminuria is described. Structures or partial structures are suggested for nine of these glycoasparagines and their possible origins are discussed.

Experimental

Isolation of compounds

Urine (1 litre) collected from the female patient described by Pollitt *et al.* (1968) and deep frozen until use, was concentrated under reduced pressure, partially desalted by ethanol precipitation (Westall, 1952), applied in a volume of 50ml to a column of

Sephadex G-15 (5cm $\times$ 60cm) and eluted with distilled water. Fractions of 10ml were collected. Samples from each fraction were subjected to high-voltage electrophoresis at pH2.0 [formic acid–acetic acid–water (19:10:971, by vol.)] and at pH5.3 [pyridine–acetic acid–water (5:2:493, by vol.)]. The compounds were detected by using a ninhydrin–collidine–acetic acid reagent (von Arx & Neher, 1963); the asparagine derivatives gave a characteristic yellow–brown colour.

The neutral glycoasparagines (fractions 46–60) were further separated on a column (100cm $\times$ 1cm) of Zeo-Karb 225 (2% cross-linked; 200–400 mesh; pyridine form). Elution was effected with a solution of 0.1M-pyridine adjusted to pH2.0 with HCl; 10ml fractions were collected and portions of these were examined by high-voltage electrophoresis in the formic acid–acetic acid–water buffer described above at pH2.0. Appropriate fractions were pooled, concentrated and either analysed directly or after further purification by high-voltage electrophoresis at pH2.0. The GlcNAc-Asn-containing fractions were also decreased to small volume, and the buffer was removed by a column of Sephadex G-15 (2cm $\times$ 20cm) eluted with water. Addition of ethanol to the concentrated effluent gave solid GlcNAc-Asn, which was recrystallized from aqueous ethanol.

The acidic compounds were isolated from pooled fractions 10–45 off the Sephadex G-15 column by preparative high-voltage electrophoresis at pH5.3. The compounds were eluted from the paper with water–ethanol (3:1, v/v).

Analytical methods

Hexose was determined by the orcinol method (Winzler, 1955; Francois *et al.*, 1962), *N*-acetylglucosamine by the Morgan–Elson reaction (Reissig *et al.*, 1955), total *N*-acetylneuraminic acid by the resorcinol method of Svennerholm (1957), free *N*-acetylneuraminic acid by the thiobarbituric acid method

(Warren, 1959) and reducing sugar by the method of Park & Johnson (1949). Sulphate was determined by the method of Lloyd (1959) after hydrolysis of the glycoasparagine in 1 M-HCl at 100°C for 6 h. The sugar compositions of the glycoasparagines were determined by methanolysis followed by g.l.c. of the trimethylsilyl ethers of the methyl glycosides as described by Clamp *et al.* (1967). For confirmation the glycoasparagines were sometimes hydrolysed rather than methanolysed before trimethylsilylation. The molar ratio of aspartic acid to glucosamine was determined on a Locarte amino acid analyser after hydrolysing the glycoasparagines for 4 h at 100°C in 2 M-HCl.

Structure determination

α -D-Mannosidase was prepared from jack-bean meal by the method of Snaith & Levvy (1968), omitting stage 6. The preparation was free of β -D-galactosidase, *N*-acetyl- β -D-glucosaminidase and β -D-mannosidase activity. *N*-Acetyl- β -D-glucosaminidase was partially purified from rat epididymis (Carlsen & Pierce, 1972) and was free from *N*-acetyl- α -D-glucosaminidase but contained a trace of α -D-mannosidase and β -D-galactosidase. 2-Acetamido-*N*-(4'-L-aspartyl)-2-deoxy- β -D-glucosylamine amidohydrolase was prepared from rat liver (Conchie & Strachan, 1969) and contained a trace of β -D-galactosidase. The enzyme was used in the presence of 2.3 mM-phenylthio- β -D-galactoside as a β -D-galactosidase inhibitor unless otherwise stated. The progress of hydrolysis of these enzymes was followed by the release of reducing sugar or of *N*-acetylglucosamine. Neuraminidase (from *Clostridium perfringens*) was obtained from Sigma Chemical Co., 3500 de Kalb St., St. Louis, Mo. 63118, U.S.A. and its action monitored by determination of free *N*-acetylneuraminic acid. The galactose oxidase system contained D-galactose oxidase (Sigma Chemical Co., type 1, from *Dactylium dendroides*), *o*-dianisidine and horseradish peroxidase (Avigad *et al.*, 1962). Quantitative periodate oxidations were monitored by the production of iodate (Eibl & Lands, 1970) after 20, 40 and 60 min reaction, and the quantity of glycoasparagine taken was determined by the Winzler (1955) orcinol method. For preparative periodate oxidations the glycoasparagine (1.5–3 mg) in 0.025 M-sodium acetate buffer pH 5.0 (1 ml) reacted with 0.25 M-sodium periodate (0.5 ml) at 4°C in the dark for 24 h. The excess of periodate was destroyed by the addition of ethylene glycol (0.1 ml) and a solution of NaBH₄ (50 mg in 1 ml of H₂O) was then added. After 24 h at 4°C the excess of borohydride was destroyed with acetic acid (0.1 ml) and the mixture was desalted on a column of Sephadex G-15. The reaction product was methanolysed for determination of the reaction products by g.l.c. Alter-

natively, the oxidized-reduced product was partially hydrolysed (pH 1; 37°C; 16 h). The products were desalted on Sephadex G-15 and submitted to a further cycle of periodate oxidation (Smith degradation).

Results

Isolation of GlcNAc-Asn

The combination of Sephadex and ion-exchange chromatography followed by recrystallization as a routine gave high-purity samples of GlcNAc-Asn from the patient's urine. The isolated compound had m.p. 237–239°C (Koffler block) with a change in crystal form at 218°C. Literature melting points for this compound range from 211° to 266°C depending on the degree of hydration (Marshall & Neuberger, 1972). A synthetic sample of GlcNAc-Asn (kindly provided by Professor A. Neuberger) gave m.p. 230–235°C on the Koffler block and had identical electrophoretic mobility with the isolated compound from urine at pH 2.0 and 8.6 [sodium diethylbarbiturate (10.3 g) and diethylbarbituric acid (1.84 g) in water (1 litre)]. Elemental analysis corresponded to the monohydrate (Found: C, 40.6; H, 6.4; N, 11.5. Calc. for C₁₂H₂₃N₃O₉: C, 40.8; H, 6.6; N, 11.9%). Other properties of the compound from urine have been described previously (Jenner & Pollitt, 1967).

Neutral glycoasparagines

The distribution of the glycoasparagines from the Sephadex G-15 fractionation of urine on electrophoresis at pH 2 and 5.3 is shown in Fig. 1. At pH 2.0 GlcNAc-Asn moved towards the cathode accompanied by a series of glycoasparagines containing only neutral additional carbohydrate residues. These neutral glycoasparagines formed a regular series of increasing molecular weight and decreasing electrophoretic mobility. These were designated N₁–N₅ according to the number of neutral carbohydrate residues attached to the GlcNAc-Asn residue. On prolonged electrophoresis N₅ was resolved into a slower-moving N_{5a} and a faster-moving N_{5b}. These components all showed virtually zero electrophoretic mobility at pH 5.3. On the Zeo-Karb 225 column the centres of the elution zones were: for N₅, 130 ml; N₄, 155 ml; N₃, 175 ml; N₂, 195 ml; N₁, 220 ml; GlcNAc-Asn, 270 ml. The various acidic compounds eluted between 90 and 160 ml.

Acidic glycoasparagines

The pH 2.0 electrophoretogram showed a considerable quantity of glycoasparagine material moving with the electroendosmotic flow or migrating

Fig. 1. Electrophoresis of Sephadex fractions

Portions of each fraction from desalted urine were applied along the origin (O) and subjected to electrophoresis (a) at pH 2.0 and (b) at pH 5.3. Only the glycoasparagine spots are shown and the position indicated is that of the centre of each elution zone which in fact covers several fractions for each compound. For further details see the text.

towards the anode. This represented acidic compounds which could be well separated by electrophoresis at pH 5.3. From the relationship between molecular weight and electrophoretic mobility, three series were defined. The first contained compounds with one additional sialic acid residue and according to the number of additional neutral sugar residues these were designated AI₁–AI₅, AI₃ not being detectable. AI₂ was resolved at pH 2 into AI_{2a} and AI_{2b}. Compounds with two sialic acid residues per molecule were termed AII compounds, again with a subscript indicating the number of neutral carbohydrate residues. Three sulphated compounds, SAI_{6a}, SIA_{6b} and SAI₄ were present; SAI₆ divided into two components on prolonged electrophoresis at pH 2, the a component being the slower.

Sugar analyses

The compositions of 13 of the glycoasparagines have been determined and are summarized in Table 1. Mobilities of some other components are described in Fig. 1. In some cases they were probably still mixtures and did not give clear-cut results on analysis. Compounds AI_{2a} and AI_{2b} are positional isomers and this is probably true of SAI_{6a} and SAI_{6b}.

Structural analyses

Compound N₁ consumed 1.7 mol of periodate/mol, the *N*-acetylglucosamine residue being unaffected.

After incubation with the amidohydrolase the product was Morgan–Elson-negative, suggesting that there was substitution at C-4 of the *N*-acetylglucosamine residue. Incubation with the amidohydrolase preparation in the absence of the β -D-galactosidase inhibitor released free D-galactose. The structure of the compound is thus β -D-Gal-(1 $\rightarrow$ 4)- β -D-GlcNAc-Asn. The experiments confirm also that the amidohydrolase preparation contains some β -D-galactosidase activity.

Compound N₂ consumed between 3 and 4 mol of periodate/mol (the end-point being difficult to determine because of continuing slow oxidation) with the destruction of the fucose and galactose residues and the production of propan-1,2-diol and glycerol but no threitol. Treatment of compound N₂ with 1 M-acetic acid at 90°C for 1 h released fucose but no other monosaccharide. Both compound N₂ and the N₂ from which fucose had been removed were Morgan–Elson-negative after treatment with the amidohydrolase. The defucosylated compound N₂ on incubation with this enzyme in the absence of the β -D-galactosidase inhibitor gave free D-galactose. The structure of compound N₂ is therefore either L-Fuc-(1 $\rightarrow$ 2 or 6)- β -D-Gal-(1 $\rightarrow$ 4)- β -D-GlcNAc-Asn or β -D-Gal-(1 $\rightarrow$ 4)-[L-Fuc-(1 $\rightarrow$ 3 or 6)-] β -D-GlcNAc-Asn.

Compound N_{3a} consumed 2.6 mol of periodate/mol with the destruction of both mannose residues and with the production of glycerol but no erythritol.

Table 1. *Composition of isolated glycoasparagines*

For analytical methods and naming of compounds see the text and Fig. 1.

Glycoasparagine	Asp	GlcNAc	Man	Gal	Fuc	Glc	<i>N</i> -Acetylneuraminic acid	SO ₄ ²⁻
N ₁	1.0	1.1	—	1.0	—	—	—	—
N ₂	1.0	1.3	—	1.3	1.0	—	—	—
N _{3a}	1.0	2.3	2.3	—	—	—	—	—
N _{3b}	1.0	1.0	2.2	1.0	—	—	—	—
AI _{2a}	1.0	1.2	—	2.1	—	—	1.1	—
AI _{2b}	1.0	1.2	—	1.9	—	—	1.0	—
AI ₄	1.0	2.0	—	3.0	—	—	1.0	—
AI ₅	1.0	2.0	—	3.6	—	—	0.9	—
SAI _{6a}	1.0	2.0	—	3.2	0.8	1.1	1.2	1.0
SAI _{6b}	1.0	2.0	—	3.3	0.9	1.2	1.2	1.5
AI ₁	1.0	0.9	—	0.9	—	—	1.8	—
AI ₂	1.0	1.0	—	2.2	—	—	2.2	—
SAI ₄	1.0	0.8	—	2.1	—	1.9	2.0	1.3

Sequential periodate oxidation (Smith degradation) gave successively products with two and then one *N*-acetylglucosamine residues per aspartate residue. Incubation with α -D-mannosidase released mannose and incubation with α -D-mannosidase and *N*-acetyl- β -D-glucosaminidase released both mannose and *N*-acetylglucosamine. Treatment with the amidohydrolase gave a Morgan–Elson-negative product. These results suggest that compound N_{3a} has the structure α -D-Man-(1 $\rightarrow$ 2)- α -D-Man-(1 $\rightarrow$ 3 or 4)- β -D-GlcNAc-(1 $\rightarrow$ 4)- β -D-GlcNAc-Asn.

Compound N_{3b} consumed 4.6mol of periodate/mol, leaving only *N*-acetylglucosamine intact. Glycerol was produced but no erythritol or threitol. Incubation of compound N_{3b} with α -D-mannosidase liberated mannose. After incubation with the amidohydrolase no Morgan–Elson-positive material was produced, but after incubation with both amidohydrolase and β -D-galactosidase (no inhibitor) a positive Morgan–Elson reaction was produced. The release of galactose but no mannose, by this latter incubation was confirmed by g.l.c. The probable structure of compound N_{3b} is α -D-Man-(1 $\rightarrow$ 2)- α -D-Man-(1 $\rightarrow$ 3 or 6)-[β -D-Gal-(1 $\rightarrow$ 4)-] β -D-GlcNAc-Asn.

Compound AI_{2a} consumed 4.3mol of periodate/mol with the destruction of the *N*-acetylneuraminic acid and one of the galactose residues. On periodate oxidation of the desialylated compound (prepared by the action of neuraminidase) both galactose residues were destroyed. Incubation with the amidohydrolase gave a Morgan–Elson-negative product. These results do not permit the assignment of a unique structure. The most likely structures are α -AcNeu-(2 $\rightarrow$ 3)-D-Gal-(1 $\rightarrow$ 2)-D-Gal-(1 $\rightarrow$ 4)- β -D-GlcNAc-Asn and α -AcNeu-(2 $\rightarrow$ 3)-D-Gal-(1 $\rightarrow$ 3 or 4 or 6)-[D-Gal-(1 $\rightarrow$ 3 or 4 or 6)]- β -D-GlcNAc-Asn with one of the Gal-GlcNAc linkages being (1 $\rightarrow$ 4).

Compound AI_{2a} gave a positive D-galactose oxidase reaction, but even after incubation for 48h one galactose residue remained. Under similar conditions both galactose residues of the desialylated compound were destroyed.

Compound AI_{2b}, isomeric with compound AI_{2a}, consumed 6.2mol of periodate/mol leaving only the *N*-acetylglucosamine residue intact. Neuraminidase released the *N*-acetylneuraminic acid and then galactose was released on incubation with the amidohydrolase- β -D-galactosidase mixture. Incubation of compound AI_{2b} with the amidohydrolase with the β -galactosidase inhibitor gave a Morgan–Elson-positive product. Compound AI_{2b} was not oxidized by the galactose oxidase system but after removal of the neuraminic acid (neuraminidase) a positive reaction was obtained. These results suggest the structure α -AcNeu-(2 $\rightarrow$ 6)- β -D-Gal-(1 $\rightarrow$ 6)- β -D-Gal-(1 $\rightarrow$ 3)- β -D-GlcNAc-Asn.

Compound AI₄ rapidly consumed 4.1mol of periodate/mol (20min) and more slowly consumed a further 1mol (1h). The initial reaction destroyed the sialic acid residue and one of the three galactose residues.

Mild hydrolysis of the reduced product followed by a further cycle of oxidation and reduction resulted in the loss of one *N*-acetylglucosamine residue. Periodate oxidation of the desialylated compound AI₄ (neuraminidase) also destroyed one galactose residue only. Compound AI₄ gave a positive reaction with the galactose oxidase system and after incubation with the amidohydrolase was Morgan–Elson-positive. Incubation of the desialylated compound AI₄ with *N*-acetyl- β -D-glucosaminidase (containing β -D-galactosidase) released *N*-acetylglucosamine and galactose. On the basis of the consumption of 4mol of periodate, these results define the structure except for the

position of the *N*-acetylneuraminic acid group which can be in any position except 2, 3 or 4 of the terminal galactose or 4 of the *N*-acetylglucosamine linked to the asparagine residue, i.e. [α -AcNeu2 $\rightarrow$] β -D-Gal-(1 $\rightarrow$ 3 or 4)- β -D-GlcNAc-(1 $\rightarrow$ 3)-D-Gal-(1 $\rightarrow$ 3)- β -D-GlcNAc-Asn.

Compound AII₁ consumed 2.6 mol of periodate/mol with the destruction of both *N*-acetylneuraminic acid residues. G.l.c. of the hydrolysed product showed galactose but no peak corresponding to a C₈ fragment from neuraminic acid (see below). Compound AII₁ gave a positive reaction with the galactose oxidase system and after hydrolysis with the amidohydrolase a positive Morgan-Elson reaction. The structure suggested by these reactions is AcNeu-(2 $\rightarrow$ 9)-AcNeu-(2 $\rightarrow$ 3)-D-Gal-(1 $\rightarrow$ 3)- β -D-GlcNAc-Asn.

Compound AII₂ consumed 2.8 mol of periodate/mol, with the destruction of both the *N*-acetylneuraminic acid residues. After methanolysis a substance with a retention time on g.l.c. of 1.4 relative to mannitol was found. This presumably represents the C₈ residue [5-acetamido-3,5-dideoxy-D-galacto-octulosonic acid (Suttajit & Winzler, 1971; McLean *et al.*, 1971)] from the sialic acid (compare with AII₁ and the AI compounds). Periodate oxidation of desialylated compound AII₂ destroyed one of the galactose residues; no 'C₈'-compound peak was present on the gas chromatogram. Compound AII₂ gave a positive reaction with the galactose oxidase system and after incubation with the amidohydrolase was Morgan-Elson-negative. The suggested structure is α -AcNeu-(2 $\rightarrow$ 7)- α -AcNeu-(2 $\rightarrow$ 3)-D-Gal-(1 $\rightarrow$ 3)-D-Gal-(1 $\rightarrow$ 4)- β -D-GlcNAc-Asn.

Discussion

Most of the detailed structures suggested here for the glycoasparagines in urine are tentative, since they rely largely on the periodate-oxidation results. The pitfalls in this method are well known, and some independent form of confirmation, for example by methylation analysis, is desirable.

The presence of glucose in compounds SAI_{6a}, SAI_{6b} and SAI₄ calls for some comment as it could have arisen as a contaminant from the filter paper used in electrophoresis. However, it was present only in the sulphated compounds, was in reasonable proportions to the other sugars, and was not present in blank areas of the paper adjacent to the glycoasparagine spots. Thus there are reasonable grounds for regarding its presence as authentic.

Galactose oxidase did not react with a 3-sialylated galactosyl compound isolated by Thomas & Winzler (1969), or with galactose residues substituted in the 2- or 3-position by a nonulosonic acid residue derived from the deamination of neuraminic acid (Isemura & Schmid, 1971), though 3-*O*-methyl

galactose reacts faster than galactose itself (Schlegel *et al.*, 1968). The behaviour of compound AI_{2a} is in keeping with the finding of Thomas & Winzler (1969) but the positive reaction given by both the AII compounds is anomalous. It appears that further substitution of the *N*-acetylneuraminic acid residue removes its inhibitory effect on the reaction.

The information so far available does not allow us to draw any definite conclusions as to the origin of the glycoasparagines in urine in aspartylglycosaminuria.

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