Short Communications

A Crystalline Glycopeptide from Normal Human Urine

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There is abundant evidence that glycosaminoglycans are linked covalently to the protein in tissues (Mathews & Lozaityte, 1958; Muir, 1958). This fact suggested that isolation and characterization of glycosaminoglycan-peptides, particularly uronic acid-containing glycopeptides in normal urine, will assist in the understanding of the normal metabolism of glycosaminoglycans in tissues. The present study was undertaken to isolate and characterize glycopeptides containing uronic acid in normal human urine. Hakomori, Kawanchi & Ishimoda (1962) isolated three glycopeptides from urine, but none contained any uronic acid. One of them resembled a glycopeptide isolated from aorta connective tissue. Cherian & Radhakrishnan (1966) have isolated from human urine a uronic acid-containing glycopeptide, the amino acid composition of which suggested that it was derived from collagen.

Materials and methods. Sephadex G-50, Sephadex G-25 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Dowex 1 (X8; 200-400 mesh) resin was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A. A.R.-grade reagents were used throughout this study and all organic solvents were redistilled before use.

Combined urine from male laboratory workers (24-40 years old) was collected with toluene and chloroform as preservatives. The urine was stored at 4° (not more than 48 hr.) until processing and was then concentrated at 40° under reduced pressure to one-fifth of its original volume. The concentrated urine was dialysed at 4° against running tap water for 24 hr. Any precipitate formed was discarded. The dialysed supernatant was treated with alkaline cetyltrimethylammonium bromide and potassium thiocyanate solution. The precipitate formed during the treatment was discarded. The treated urine supernatant was concentrated at 40° under reduced pressure to one-eightieth of its original volume. The concentrated urine supernatant was dialysed at 4° against four

changes of deionized distilled water for 20 hr. The non-diffusible material of the dialysis bag was further concentrated at 40° under reduced pressure to one-hundredth of the original volume and was chromatographed on a Sephadex G-50 column (53 cm. $\times$ 4.5 cm.). The column was eluted with 0.1M-NaCl. The fractions eluted between 750 and 1020 ml. were pooled and concentrated at 40° under reduced pressure to one-fifth of the volume. The concentrated material was then chromatographed on a Sephadex G-25 column (52 cm. $\times$ 4.5 cm.). The column was eluted with deionized distilled water. The fractions eluted between 350 and 720 ml. were pooled and concentrated to one-tenth of the volume. This concentrated material was further chromatographed on a column (200 ml. bed volume) of DEAE-Sephadex A-50 (acetate form) followed by chromatography on a column (200 ml. bed volume) of Dowex 1 (X8; acetate form). The active material was eluted from the columns with 0.02 M-acetic acid. The progress of the column elutions in all the above steps was followed by uronic acid determinations by the method of Bitter & Muir (1962). The acetic acid eluate from the Dowex 1 column was concentrated to a syrupy liquid at 40° under reduced pressure. The syrup was diluted with a minimum amount of deionized distilled water and left at -18°. Fine needle-shaped crystals, which appeared after 5 days, were collected by centrifugation at 50000 g-min. The crystals were dissolved in a minimum amount of deionized distilled water for recrystallization at -18° (Fig. 1). A total of 35 mg. of crystalline glycopeptide was obtained from 100 l. of urine.

Results. The following results were obtained on the limited amount of crystalline material thus obtained. The constituents of the glycopeptide were analysed after acid hydrolysis under appropriate conditions in sealed tubes. Only five amino acids, namely serine, glycine, aspartic acid, glutamic acid and alanine, were detected by two-dimensional paper chromatography and the identity of the amino acids was further confirmed by analysis with

Fig. 1. Crystals of the glycopeptide. Magnification $\times 144$.

the Beckman Amino Acid Analyzer. Uronic acid was identified as glucuronic acid by the method of Davidson (1966). Galactosamine and glucosamine were identified on paper with the solvent system described by Mukherjee & Sri Ram (1964). The amino sugars were further characterized by the phosphorylation reaction with ATP and yeast hexokinase (Singh & Bachhawat, 1968). The reaction products were identified by the method of Gatt & Berman (1966) after chromatography on a column (10 ml. bed volume) of Dowex 1 (X8; acetate form). Galactosamine was not phosphorylated under the assay conditions and hence was eluted from the column with distilled water in the first 2–5 ml. of eluate. Galactose and xylose were identified by paper chromatography with two solvent systems (Mukherjee & Sri Ram, 1964; Spiro, 1960). The identity of galactose was further confirmed by the galactose oxidase method [Worthington Manual: *Enzyme Reagent* (1966), Worthington Biochemical Corp., Freehold, N.J., U.S.A.]. Sulphate was found to be absent from the glycopeptide (Dodgson & Price, 1962).

The identity of the following constituents of the glycopeptide have thus been firmly established. Amino acids: serine, glycine, aspartic acid, glutamic acid and alanine; glucuronic acid; amino sugars: galactosamine and glucosamine; neutral sugars: galactose and xylose. Other observations indicated that the glucuronic acid:neutral sugar:amino sugar molar proportions were 1:10:1.

Discussion. To my knowledge this is the first glycopeptide that has been crystallized from urine. The urinary glycopeptide reported here is non-diffusible in the initial stages of fractionation, but after Sephadex G-50 chromatography it becomes diffusible. It is reasonable therefore to assume that the glycopeptide was not covalently linked with other proteins since no enzymic or drastic hydrolytic methods were employed for the isolation of the glycopeptide. The nature of the linkage that gives the non-diffusible property to the glycopeptide in the earlier stages of fractionation is unknown. The presence of low-molecular-weight glycopeptides containing uronic acid in human urine was previously demonstrated by Sephadex G-25 chromatography (Lundbald & Berggård, 1962), but none of these glycopeptides was characterized. The chemical composition of the isolated glycopeptide has some characteristics in common with that of the linkage region of the protein-glycosaminoglycans isolated from tissues.

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