Isolation of Neuraminlactose from Human Male Urine

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Earlier studies have shown that approximately 30 % of the neuraminic acid of human male urine is bound to a fraction consisting of small-molecular compounds (mol.wt. 500—1500).[1,2] The bulk of these mucosaccharides can be separated as a group from the high-molecular-weight urinary substances as well as from the still lower-molecular-weight carbohydrates by gel filtration on Sephadex G-25. Further chromatography of this mixture on activated carbon and on anion exchange column revealed that in addition of neutral oligosaccharides several neuraminic-acid-containing subfractions were present.[3] A procedure for the purification and preparative isolation of these neuraminic-acid-containing mucosaccharides has been developed.[4] With this method, at least five sialic-acid-containing compounds could be isolated in a chromatographically homogeneous form. The present paper concerns the chemical characterization of one of these compounds, a trisaccharide identical with neuramin(2→3)lactose, hitherto isolated only from milk.

Experimental. Hexose, hexosamine, and neuraminic acid were determined by the methods presented in earlier papers.[1,2] Reducing sugar was assayed by the modified method of Somogyi and Nelson.[5] α-Aminonitrogen was analysed according to Rubinstein and Pryce[6] and acetyl groups by the method of Ludowieg and Dorfman.[7] Descending paper chromatography was carried out using the following solvent systems: (a) ethyl acetate-pyridine-water (10:4:3), (b) ethyl acetate-pyridine-acetic acid-water (5:5:1:4), (c) ethyl acetate-propanol-water (1:7:2), (d) ethyl acetate-acetic acid-water (10:5:6). Sugars were routinely detected by alkaline silver nitrate according to Trevelyan *et al.*[8]

Isolation procedure. Normal male human urine was shaken with activated carbon. After washing with water, oligosaccharides were eluted with 50 % ethanol. The eluate was concentrated to a small volume in a rotary evaporator. Seven volumes of ethanol were added (final ethanol concentration 87 %) and the precipitate formed was separated by centrifugation. The supernatant was removed and the ethanol evaporated with a stream of nitrogen under reduced pressure. The residue still contained noncarbohydrate substances, including pigments and peptides, and was therefore subjected to gel filtration on a Sephadex G-25 column equilibrated with 0.5 mM EDTA. Neuraminic acid-containing fractions eluted from the column were pooled and the pool was passed through a Dowex-50 (H^+) column to remove basic compounds for further characterization. The eluate, containing neutral and acidic oligosaccharides, was chromatographed on a Dowex-1X8 ($HCOO^-$) column, using a formic acid gradient. This procedure separated the neutral oligosaccharides from the acidic ones, the latter being retained by the resin. The neuraminic acid-containing compounds emerged from the column in 0.3—0.5 N HCOOH as three slightly overlapping fractions, designated I, II, and III, as can be seen in Fig. 1. Detailed analyses of fraction III indicated that it consists of neuraminlactose. Fractions I and

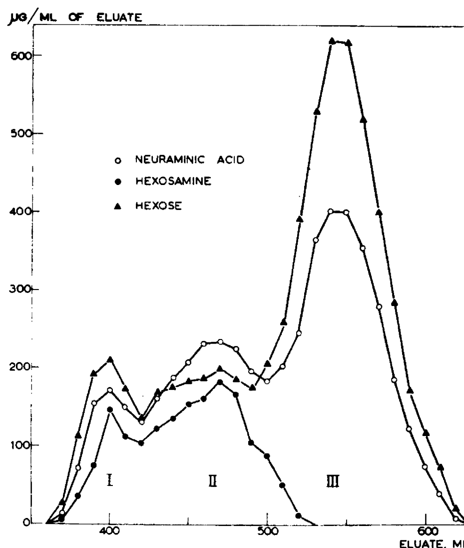

Fig. 1. Elution curves for hexose, hexosamine and neuraminic acid obtained when neuraminic acid-containing oligosaccharides isolated by the methods described in the text were subjected to chromatography on Dowex-1X8 column (1.5 × 30 cm). Elution was performed with a linear formic acid gradient from 0 to 0.7 N acid (800 ml). Fractions of 10 ml were collected.

II have not yet been studied extensively but preliminary experiments have shown that they are still heterogeneous. Three other fractions separated during the isolation of neuraminlactose, *viz.* material eluted with water from the anion exchange column, material retained by the cation exchange column, and material precipitated by 87 % ethanol, have not yet been analysed extensively.

Fraction III was finally purified by preparative paper chromatography on Whatman 3MM sheets to remove contaminants of other oligosaccharides. A single band was obtained, however, with the mobility of the authentic neuramin(2→3)lactose (Sigma Chemical Co.) used as reference. The corresponding area was separated and the carbohydrates eluted with water onto a small Dowex-1 column to remove contaminants released from paper. The oligosaccharide was recovered with dilute formic acid. The eluate was treated several times with ethyl ether to remove formic acid and the aqueous phase lyophilized. The white powder obtained was used for the following studies.

Identification procedure. On high-voltage electrophoresis in 0.05 M borate and on paper chromatography in acidic, basic and neutral solvents systems (a, b, c, d), the material isolated gave a single spot with the mobility of authentic neuramin(2→3)lactose. Chemical analysis showed that the molar ratio neuraminic acid : hexose : reducing sugar was 1.00:2.09:0.86. Approximately the same ratio was obtained for authentic neuraminlactose. Paper chromatography carried out after mild acid hydrolysis (0.05 N HCl, 80°, 1 h) or after treatment with viral neuraminidase revealed the presence of N-acetyl-neuraminic acid and lactose. After a more drastic hydrolysis (2 N HCl, 100°, 2 h) two spots with the respective mobilities of glucose and galactose could be seen (Fig. 2). The identity of the hydrolysis products of these experiments was confirmed by high-voltage electrophoresis in 0.05 M borate. Acetyl determinations indicated that one mole of acetyl was present per mole of the isolated trisaccharide.

All paper chromatographic analyses suggested that the trisaccharide isolated from five different urine samples was neuramin-(2→3)-lactose(R_{lactose} 0.68 in solvent b). There was only occasionally a very faint spot with the mobility of neuramin(2→6)lactose on paper chromatography of fraction III indicating that this isomer can only be present in very small amounts if at all in normal human male urine. Final evidence for the 2→3-linkage between neuraminic acid and galactose was obtained from periodate oxidation experiments. The trisaccharide was oxidized for 4 days at 4° in the dark with excess of periodate. The reaction mixture was transferred onto a small carbon-Celite column. Salts were removed with water and carbohydrates were eluted with 30 % ethanol. Approximately one half of the original hexose was unchanged in the eluate and paper chromatography after acid hydrolysis revealed the presence of galactose but not of glucose. The resistance of galactose to periodate is understandable, provided neuraminic acid is linked to carbon-3 of galactose.

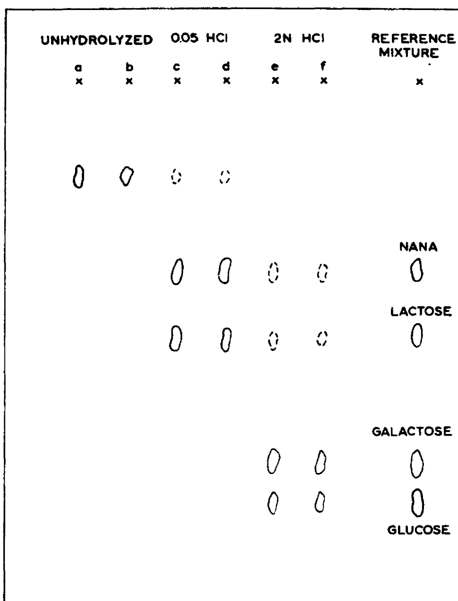

Fig. 2. Paper chromatography of urinary neuraminlactose and its hydrolysis products. Whatman paper No. 1. Solvent: ethyl acetate-pyridine-water (10:4:3). Descending system for 20 h. Samples a, c, and e are runs from urinary neuraminlactose prior to and after hydrolysis. Samples b, d, and f are, respectively, runs from authentic neuraminlactose. For details see text.

Discussion. The identity of the isolated trisaccharide with N-acetyl-neuramin-(2→3)lactose seems to be well established from the results described above. Hitherto, this trisaccharide has been found only in milk [9,10] and milk-synthesizing

tissues[11,12] of various mammals. The urinary excretion of the oligosaccharide seems to be quite constant, ranging from 20 to 50 mg/24 h in normal men. The origin of neuraminlactose in human male urine is unknown. The only compounds known to contain the carbohydrate sequence of neuraminlactose are the gangliosides.[13] Consequently, the catabolism of these glycolipids might give rise to the formation of free neuraminlactose. On the basis of preliminary observations, the concentration of neuraminic-acid-containing oligosaccharides is markedly increased in the urine of lactating women. This finding is in good agreement with the observations of Date, who isolated milk-typical neutral oligosaccharides from female urine during lactation.[14,15]

The chemical characterization of the other substances isolated is in progress and will be reported separately.

1. Miettinen, T. A. *Scand. J. Clin. Lab. Invest.* **14** (1962) 380.
2. Miettinen, T. A. *Clin. Chim. Acta* **8** (1963) 693.
3. Miettinen, T. A. and Huttunen, J. K. *Acta Chem. Scand.* **18** (1964) 579.
4. Huttunen, J. K. *Abstr. 2nd Meeting Fed. Europ. Biochem. Soc. Meeting Edition*, Vienna 1965, p. 93.
5. Nelson, N. *J. Biol. Chem.* **153** (1944) 375.
6. Rubinstein, H. M. and Pryce, J. D. *J. Clin. Pathol.* **12** (1959) 80.
7. Ludowieg, J. and Dorfman, A. *Biochim. Biophys. Acta* **38** (1960) 212.
8. Trevelyan, W. E., Procter, D. E. and Harrison, J. S. *Nature* **166** (1950) 444.
9. Kuhn, R. and Brossmer, R. *Chem. Ber.* **89** (1956) 2013.
10. Mayron, L. W. and Tokes, Z. A. *Biochim. Biophys. Acta* **45** (1960) 601.
11. Trucco, R. E. and Caputto, R. *J. Biol. Chem.* **206** (1954) 901.
12. Heyworth, R. and Bacon, J. S. D. *Biochem. J.* **66** (1957) 41.
13. Kuhn, R. and Wiegandt, H. *Chem. Ber.* **96** (1963) 867.
14. Date, J. W. *Scand. J. Clin. Lab. Invest.* **16** (1965) 597.
15. Date, J. W. *Ibid.* **16** (1965) 604.

Received June 18, 1965.

Studies of β-Glucuronidase Activity in Bile and Liver of Developing Chick Embryos and Chicks

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The β-glucuronidase activity was first described by Masamune in 1934.[1] Since 1948 when Fishman [2] described a colorimetric method for the determination of β-glucuronidase activity in serum many reports have been published about the nature and significance of the enzyme. β-Glucuronidase has been shown to catalyze the hydrolysis of β-glucuronides as well as the transfer of glucuronyl groups to acceptor alcohols.[3,4] As substrates of the enzyme can act, *e.g.*, β-glucuronides of steroid hormones,[5,6] bilirubin,[7,8] phenolphthalein,[2] menthol, and borneol. The enzyme does not hydrolyse either α-glucuronides or α- and β-glucosides. The β-glucuronidase has a wide distribution. It is found to occur in bacteria,[9] plants,[10] fish,[11] and in most tissues of mammals and other animals.[2,12]

In the course of a study on the bile pigment metabolism [13] in developing chick embryos and chicks it was noted that a considerable amount of bilirubin and biliverdin could occur in the gall bladders as the unconjugated pigments. In a report [14] concerning the β-glucuronidase activity in bile, the bile of healthy humans does not contain β-glucuronidase. Therefore it was of interest to check if the same holds true in developing chick embryos and chicks or if the presence of unconjugated pigments could be partly explained by a β-glucuronidase activity. For comparison the liver activity values were determined at the same time.

To obtain chick-embryo bile and liver, fertile White Leghorn eggs were incubated at 37.5 ± 0.5°C in a relative humidity of 65 ± 5 %. After a desired incubation time the embryos were removed from the eggs with a forceps and freed from adherent membranes. The gall bladders and livers were quickly removed and homogenised in a Potter-Elvehjem type homogeniser in tubes kept in ice cooled water, the bladders in 0.5 ml of water for 20 sec and the livers in two volumes of 0.154 M KCl