Biosynthetic Preparation of the NO-Glucosiduronic Acid of N-Acetyl-N-Phenylhydroxylamine

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1. Sodium (N-acetyl-N-phenylhydroxylamine β-D-glucosid)uronate was isolated from the urine of rabbits receiving N-acetyl-N-phenylhydroxylamine. 2. Its chemical structure was confirmed by the correspondence of the infrared spectrum of its tri-O-acetyl methyl ester derivative with the tri-O-acetyl methyl ester derivative of an authentic specimen prepared by the Koenigs-Knorr synthesis.

Glucosiduronic acids of N-hydroxy aglycones (NO-glucosiduronic acids) have been encountered as metabolites in the urine in the case of the carcinogen, 2-fluorenylacetamide, from which the more harmful carcinogenic aglycone, N-hydroxy-2-fluorenylacetamide, could be liberated by β-glucuronidase added to the urine (Poirier, Miller & Miller, 1963; Miller, Cramer & Miller, 1960; Irving, 1962; Enomoto, Lottiker, Miller & Miller, 1962; Weisburger, Grantham & Weisburger, 1964). Clearly, glucuronic acid conjugates of this novel type merit investigation with regard to their chemical structure (Irving, 1965) and reactivity, biochemical origin and metabolic fate.

We have prepared biosynthetically the pure NO-glucosiduronic acid of N-acetyl-N-phenylhydroxylamine. Its O-acetyl methyl ester matched the properties of the corresponding derivative of the synthetic product.

EXPERIMENTAL AND RESULTS

Materials. N-Acetyl-N-phenylhydroxylamine was synthesized by the acetylation of N-phenylhydroxylamine (K and K Chemicals, Plainview, N.Y., U.S.A.) according to the method of Gupta & Sogani (1960). For column chromatography Whatman cellulose powder CF-II was used.

Paper chromatography. Ascending development was employed with Whatman no. 1 filter paper. The solvent system employed was butan-1-ol-acetic acid-water (4:1:5, by vol.). Synthetic products and metabolites were detected by spraying the following reagents on the chromatogram: (1) NaIO₄-benzidine for the detection of glucuronides (Mowery, 1967); (2) 1-0% FeCl₃ for the detection of N-acetyl-N-phenylhydroxylamine.

Thin-layer chromatography. For the O-acetyl methyl ester derivative of the glucosiduronic acid of N-acetyl-N-phenylhydroxylamine, thin-layer chromatography was carried out on silica gel H (E. Merck A.-G., Darmstadt, Germany) with ethyl acetate-benzene (1:1, v/v) as the solvent. The product was located as a black spot on heating the plate after spraying it with conc. H₂SO₄-ethanol (1:1, v/v).

Synthesis of methyl (N-acetyl-N-phenylhydroxylamine 2,3,4-tri-O-acetyl-β-D-glucopyranosyl)uronate. To a solution in a brown glass-stoppered bottle wrapped in tinfoil of 10-24g. of methyl (tri-O-acetyl-α-D-glucopyranosyl bromide)uronate and 2-0g. of N-acetyl-N-phenylhydroxylamine dissolved in 200ml. of benzene, 9-0g. of Ag₂CO₃ was added and the mixture was shaken in an automatic shaker at room temperature for 24hr. After filtering the precipitate, the resulting filtrate was evaporated under reduced pressure. The residue was crystallized from aq. ethanol, giving 1-2g. of white needle-shaped crystals, m.p. 157-158° (19-5% yield). (Found: C, 53-8; H, 5-3; N, 3-1. Calc. for $C_{41}H_{32}NO_{11}$: C, 54-0; H, 5-4; N, 3-0%.) The i.r. spectrum in Nujol showed $\lambda_{\text{max}}$ at 5-7, 5-9, 5-7, 7-7, 8-2, 9-2, 13-1 and 14-5µ. On thin-layer chromatography the $R_f$ value of this compound is 0-6.

Isolation of sodium (N-acetyl-N-phenylhydroxylamine β-D-glucosid)uronate from the urine of rabbits receiving N-acetyl-N-phenylhydroxylamine. N-Acetyl-N-phenylhydroxylamine in aqueous suspension with gum arabic was administered by stomach tube to 18 male rabbits, weighing about 3-0kg., at a dosage of 150mg./kg. body wt. They were starved for 36hr. after dosage and the 36hr. urine volume was collected in the presence of 5ml. of acetic acid/l. of urine at less than 15°. Under these conditions, urinary β-glucuronidase is inactive and the urine will not support bacterial growth.

The isolation was accomplished first by conventional lead salt technique (Kamil, Smith & Williams, 1951), and then by column chromatography. The isolation of the lead salt was performed at a temperature less than 10°. Lead acetate was added to the filtered urine (less than pH 4-0) until precipitation was complete. The precipitate was removed by centrifugation and discarded. The supernatant was brought to pH 7-5 with aq. NH₃ and saturated lead acetate was added in excess. The resulting precipitate was collected by centrifugation and washed
twice with water and once with methanol, and then made into a fine suspension in methanol. Lead was then removed by its conversion into insoluble PbS by saturation with H₂S. PbS was filtered off and the filtrate was evaporated to dryness under reduced pressure. The gummy residue (2.9 g.) was chromatographed on a cellulose (150 g.) column with butan-1-ol-ethanol-water (4:1:1, by vol.) as eluent. The fractions were concentrated under reduced pressure and a sample of each fraction was subjected to paper chromatography. The fractions, containing a product whose Rᵢ value on the paper chromatogram was approx. 0.6, were pooled and concentrated under reduced pressure. The product was dissolved in a small volume of water and the solution was brought to pH 5-2 with saturated NaHCO₃.

Next, the addition of acetone to this solution led to cristallization of the glucosiduronic acid at room temperature and the crystals were filtered and washed with cold acetone-water (9:1, v/v). The last traces of glucuronic acid contamination in the crystals were removed by repeated column chromatography on cellulose with butan-1-ol-ethanol-water (3:1:1, by vol.). The combined eluates containing the pure product were dissolved in a small amount of water, treated with activated charcoal for decolorization, filtered and acetone was gradually added to the filtrate until complete crystallization was achieved. After recrystallization from acetone-water (9:1, v/v), 700 mg. of white shiny needles were obtained, from the administration of 10 g. of N-acetyl-N-phenylhydroxylamine. The crystals decomposed at 158° [Found: C, 47.2; H, 4.7; N, 4.0. Calc. for C₁₄H₁₂ΝNaO₅ (this sample was dried over P₂O₅ for 2 days at room temp. in vacuo): C, 48.1; H, 4.8; N, 4.0%]. (K⁺)₂₁° = -84° (c 3 in water). The i.r. spectrum in Nujol showed λ_max at 2-9, 6-0, 7-5, 7-7, 9-5, 13-2 and 14-5 μ.

*Methylation and acetylation of sodium (N-acetyl-N-phenylhydroxylamine β-D-glucosid)uronate.* To an aqueous solution of 100 mg. of sodium salt of the glucosiduronic acid 5 ml. of Amberlite IR-120 (H⁺ form) was added and the mixture was stirred for a few minutes until the pH of the mixture was lowered to 2. The resin was filtered off and the filtrate was evaporated to dryness. The residue was dissolved in a small amount of ethanol and treated with an excess of diazomethane in ether. After standing in the refrigerator overnight, the mixture was evaporated under reduced pressure and was dried over P₂O₅ in vacuo. The dry residue (71 mg.) was dissolved in 1 ml. of acetic anhydride, and the solution was cooled to 0°. BF₃ was added, and after standing at room temperature for 1 hr. the reaction mixture was poured into 50-80 ml. of ice-water. The aqueous layer was decanted and the oily material extracted with CHCl₃. The CHCl₃ solution was washed with NaHCO₃ and water, dried over CaCl₂ and evaporated under reduced pressure. The residue was crystallized from aq. ethanol. The white needle-shaped crystals obtained had m.p. 156° not depressed by the synthetic acetyl methyl ester glucuronide of N-acetyl-N-phenylhydroxylamine. The i.r. spectrum of isolated derivative was identical with that of the synthetic compound (Fig. 1) (Found: C, 53.8; H, 5.4; N, 3.2. Calc. for C₂₁H₂₅N₀₁₁: C, 54.0; H, 5.4; N, 3.0%).

**DISCUSSION**

A glucosiduronic acid linked to nitrogen of the aglycone via an oxygen atom has been isolated from the urine of rabbits previously given N-acetyl-N-phenylhydroxylamine. The sodium salt of the glucosiduronic acid is soluble in water, slightly soluble in ethanol and acetone, and insoluble in benzene and chloroform. Its aqueous solution is stable in the refrigerator for several weeks, and at room temperature for 2 hr., provided that the pH is approx. 6-5. At pH 4-0, the substrate is stable for at least 24 hr. at room temperature. However, it is unstable in weak alkali (pH10-5) solution at room temperature. It undergoes complete hydrolysis by purified β-glucuronidase at pH4-0 and this reaction is greatly inhibited by saccharolactone (Ide, Kato, Green, Hirohata & Fishman, 1966).

In contrast with the stability (Williams, 1959) of acyl-O-glucosiduronic acids, we have observed a marked instability of the NO-glucosiduronic acid during its isolation from rabbit urine; hence the precautions for preserving urine, and the employment of low temperatures whenever possible. The glucosiduronic acid gum shows both aglycone and glucuronic acid as well as the metabolic conjugate on thin-layer chromatography. Moreover, aglycone could be recovered from the untreated urine, but it is
difficult to decide whether this was unchanged administered compound or a product of \( \beta \)-glucuronidase hydrolysis taking place in the bladder urine. It is therefore reasonable to assume that the amount of \( NO \)-glucosiduronic acid formed in the body was considerably greater than that which it was possible to isolate.

For the reason of the great difference in chemical properties between the \( NO \)-glucosiduronic acid and the \( O \)-glucosiduronic acid, and because a distinction should be made from the well-known \( N \)-glucosiduronic acids, we propose the use of the term '\( NO \)-glucosiduronic acid' to describe compounds such as the biosynthetic glucosiduronic acid of \( N \)-acetyl-\( N \)-phenylhydroxylamine.

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