Behaviour of the *NO*- β -D-Glucosiduronic Acid of *N*-Acetyl-*N*-phenylhydroxylamine as a Substrate for β -Glucuronidase

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1. Biosynthetic sodium (*N*-acetyl-*N*-phenylhydroxylamine *NO*- β -D-glucosid)-uronate is hydrolysed completely by purified mouse urinary β -glucuronidase into the products *N*-acetyl-*N*-phenylhydroxylamine and glucuronic acid. The hydrolysis is inhibited by saccharo-(1 $\rightarrow$ 4)-lactone. These results not only confirm the identity and purity of the substrate but also establish it as a substrate for β -glucuronidase. 2. Mammalian and bacterial β -glucuronidase preparations hydrolysed the substrate at a rate one-fifth of that for (phenolphthalein β -D-glucosid)uronic acid under the optimum conditions of hydrolysis for each source. 3. The pH optimum is 4.1 and the Michaelis constant, K_m , is 3.3×10^{-4} M with purified mouse urinary β -glucuronidase as the enzyme source acting on the *NO*- β -D-glucosiduronic acid. The aglycone after extraction into chloroform was quantitatively determined spectrophotometrically at its absorption maximum (256 m μ). 4. The hydrolysis was studied as a function of time and temperature. 5. From a consideration of the chemical and enzymic properties of this *NO*- β -D-glucosiduronic acid it is possible to suggest its catabolism *in vivo*.

It is known that the administration of a carcinogen such as 2-fluorenylacetamide leads to its hydroxylation to a compound whose carcinogenicity is greatly increased. This is excreted as *NO*-glucosiduronic acid in the urine (Poirier, Miller & Miller, 1963; Miller, Cramer & Miller, 1960; Irving, 1962; Enomoto, Lottiker, Miller & Miller, 1962; Weisburger, Grantham & Weisburger, 1964). This is presumed to be a substrate for β -glucuronidase, since β -glucuronidase treatment of the urine collected from animals given 2-fluorenylacetamide is a necessary step in the isolation of the corresponding *N*-hydroxy metabolite. However, no definitive studies have appeared on the nature of pure *NO*- β -D-glucosiduronic acids and on their behaviour as substrates for β -glucuronidase.

Our choice of *N*-acetyl-*N*-phenylhydroxylamine as the aglycone for the purpose of preparing the corresponding *NO*- β -D-glucosiduronic acid was made because the aglycone is easily prepared (Gupta & Sogani, 1960) from commercially available *N*-phenylhydroxylamine and because the compound is not known to be carcinogenic. Such a substrate could serve as a model *NO*- β -D-glucosiduronic acid for enzyme kinetic studies and perhaps

for investigations of metabolic fate without safety precautions as would be required for an extremely active carcinogen.

The isolation, identification and proof of structure of the sodium salt of (*N*-acetyl-*N*-phenylhydroxylamine β -D-glucosid)uronic acid has been reported by Kato, Ide, Hirohata & Fishman (1967). We now describe the experiments that characterize the action of β -glucuronidase on this conjugate.

MATERIALS AND METHODS

Materials. Sodium (*N*-acetyl-*N*-phenylhydroxylamine *NO*- β -D-glucosid)uronate was isolated and purified (m.p. 158°, decomp.) as described by Kato *et al.* (1967). (Phenolphthalein glucosid)uronic acid was prepared in our Laboratory (Fishman, 1963). Sodium (methyl β -D-glucosid)uronate (m.p. 265°, decomp.) was prepared by the method of Yoshida, Kato & Tsukamoto (1964). *N*-Acetyl-*N*-phenylhydroxylamine (m.p. 67°) 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).

Mouse urinary β -glucuronidase with a specific activity of 100 000 units/mg. of protein was prepared from the urine collected from testosterone-stimulated mice (Pettengill & Fishman, 1962). These mice also supplied the kidneys

from which tissue β -glucuronidase (specific activity 5000 units/mg. of protein) was prepared. Leech endo- β -glucuronidase (specific activity 131 units/mg. of protein) was prepared as described by Yuki & Fishman (1963). Commercially available β -glucuronidase preparations were Ketodase (ox liver, 5000 units/ml.) and Glusulase (*Helix pomatia*, 100000 units/ml.) from Warner-Chilcott General Diagnostics Division, Morris Plains, N.J., U.S.A., and Endo Laboratories, New York, N.Y., U.S.A., and type II *Escherichia coli* β -glucuronidase (40000 units/g.) from Sigma Chemical Co., St. Louis, Mo., U.S.A. One unit of β -glucuronidase activity is defined as that amount of enzyme which will liberate 1 μ g. of phenolphthalein from (phenolphthalein glucosid)uronic acid under standard conditions (Fishman, 1963).

Enzyme assay. Sodium (*N*-acetyl-*N*-phenylhydroxylamine NO- β -D-glucosid)uronate and a suitable enzyme dilution (final volume 1.0 ml.) were incubated in a 13 mm. $\times$ 100 mm. glass-stoppered test tube at the desired pH, time, temperature and conditions of substrate concentration indicated in the Table and Figures. To arrest the enzymic reaction, 0.1 ml. of saccharo-(1 $\rightarrow$ 4)-lactone (0.1 mM) (Levy, 1952) was added to the reaction mixture, which was then cooled rapidly in ice. The cold digest was adjusted to pH 6 with 0.1 N-NaOH and the final volume adjusted to 2 ml. with water. The aglycone was extracted into 4 ml. of CHCl_3 by mechanical shaking for 10 min. After centrifugation at 480 g for 20 min., the lower CHCl_3 layer was carefully aspirated with a transfer pipette (avoiding contamination with water) and transferred to a cuvette. The concentration of *N*-acetyl-*N*-phenylhydroxylamine in the CHCl_3 layer was then determined spectrophotometrically by reading the E_{256} value against a CHCl_3 extract of an unhydrolysed digest. The molecular extinction coefficient of *N*-acetyl-*N*-phenylhydroxylamine in CHCl_3 at 256 m μ is 7.86×10^5 cm./mole/l.

RESULTS

Properties of sodium (N-acetyl-N-phenylhydroxylamine NO- β -D-glucosid)uronate

Kato *et al.* (1967) have demonstrated the purity of this isolated compound with respect to elemental analysis and the identification of the infrared spectrum of its derivative by its correspondence with that of the synthetic compound. Instability

in alkali was characteristic of the sodium salt and of the derivative. Even at pH 8.0, the compound becomes unstable, as was noted after inadvertent use of an excess of sodium hydrogen carbonate in making the sodium salt.

The NO-glucosiduronic acid does not give a positive naphtharesorcinol reaction (Fishman & Green, 1955) except after it has been completely hydrolysed by β -glucuronidase to yield free glucuronic acid. On the other hand, the Dische (1947) carbazole reaction was fully positive for the unhydrolysed conjugate.

With regard to other chemical reactions, the sodium salt gives a reddish precipitate of cupric oxide with Fehling's (1858) solution but not with Barfoed's (1873) solution. These results confirm the instability in alkali solution and its stability in acid solution. The sodium salt is unstable in hot ethanol as judged by a change in R_F value on paper chromatography (Kato *et al.* 1967).

Hydrolysis of sodium (N-acetyl-N-phenylhydroxylamine NO- β -D-glucosid)uronate by mouse urinary β -glucuronidase

A 0.1 ml. sample of a stock substrate solution (final concentration 0.285 μ mole/ml.) plus 0.1 ml. of purified mouse urinary β -glucuronidase (500 units) and 0.8 ml. of 0.1 M-acetate buffer, pH 5.0, were combined in a 13 mm. $\times$ 100 mm. glass-stoppered tube and incubated for 15 hr. at 37°. The liberated *N*-acetyl-*N*-phenylhydroxylamine was extracted with chloroform, and the E_{256} value was measured in a Beckman model DU spectrophotometer. The reading was converted into μ g. of *N*-acetyl-*N*-phenylhydroxylamine from a calibration curve prepared with pure *N*-acetyl-*N*-phenylhydroxylamine. The liberated free glucuronic acid in the aqueous layer was determined by the Fishman & Green (1955) method: 0.274 μ mole of aglycone and 0.282 μ mole of free glucuronic acid were released, which are close to the theoretical amount (0.285 μ -mole) expected.

Table 1. *Comparison of extent of hydrolysis of (N-acetyl-N-phenylhydroxylamine NO- β -D-glucosid)-uronic acid with (phenolphthalein β -D-glucosid)uronic acid by several types of β -glucuronidase*

β -Glucuronidase preparation	pH (0.1 M-acetate buffer)	Aglycone (μ mole)		100 a/b
		Phenolphthalein (a)	N-Acetyl-N-phenylhydroxylamine (b)	
Mouse urinary	4.5	0.212	0.050	23.6
Mouse kidney	4.5	0.197	0.049	24.8
Ketodase	5.0	0.240	0.048	20.0
Glusulase	5.0	0.238	0.046	19.3
Leech (endo- β -glucuronidase)	5.5	0.000	0.000	—
Bacterial	6.0	0.288	0.055	18.4

Fig. 1. Velocity of liberation of product. The system consisted of 0.1 ml. of substrate solution (final concn. $0.57 \mu\text{mole/ml.}$), 0.1 ml. of mouse urinary β -glucuronidase (100 units) and 0.8 ml. of 0.1 M-acetate buffer, pH 4.1. Incubation was at 37° .

Fig. 2. Influence of pH on the velocity of hydrolysis. The system consisted of 0.1 ml. of substrate solution (final concn. $0.285 \mu\text{mole/ml.}$), 0.1 ml. of mouse urinary β -glucuronidase (100 units) and 0.8 ml. of 0.1 M-acetate buffer. Incubation was for 30 min. at 37° . The reaction was stopped by the addition of 0.1 ml. of 0.1 M-saccharo-(1 $\rightarrow$ 4)-lactone.

Hydrolysis of (N-acetyl-N-phenylhydroxylamine NO- β -D-glucosid)uronic acid by several types of β -glucuronidase

The hydrolysis of this compound by β -glucuronidase from a variety of sources was compared with that of (phenolphthalein β -D-glucosid)uronic acid (Table 1). Dilutions of these preparations were chosen so that they were equivalent on the basis of their ability to hydrolyse (phenolphthalein β -glucosid)uronic acid. Then each enzyme dilution (0.1 ml.) was tested separately with 0.1 ml. of the glucosiduronic acids of phenolphthalein and *N*-acetyl-*N*-phenylhydroxylamine at a final concentration of 0.5 mM. The final volume was made up to 1 ml. with 0.1 M-acetate buffer and incubated for 1 hr. at 37° . The liberated *N*-acetyl-*N*-phenylhydroxylamine and phenolphthalein were measured by the u.v. method as described above and by the Fishman (1963) method respectively.

It is clear that all the different preparations of β -glucuronidase except that from leech (which is specific only for endo- β -D-glucosiduronic acid linkages) can hydrolyse this substrate at about 20% the rate of hydrolysis of (phenolphthalein β -glucosid)uronic acid.

Conditions for hydrolysing (N-acetyl-N-phenylhydroxylamine NO- β -D-glucosid)uronic acid with purified mouse urinary β -glucuronidase

Time-course of hydrolysis. In an experiment to determine the time-course of hydrolysis, $0.57 \mu\text{mole}$ of the sodium salt of the *NO*- β -glucosiduronic acid

in 0.1 M-acetate buffer, pH 4.1, was hydrolysed by mouse urinary β -glucuronidase for times varying from 15 min. to 8 hr. As shown in Fig. 1, the rate is linear for at least 30 min. It is noted also that by 8 hr. close to 75% of the substrate had undergone hydrolysis.

Optimum pH. The pH optimum for the hydrolysis of sodium salt was determined at a substrate concentration of $0.57 \mu\text{mole/ml.}$ in 0.1 M-acetate buffer between pH 3 and 6 at 37° for 30 min. The pH-activity curve is given in Fig. 2 and exhibits a rather sharp peak at pH 4.0-4.1.

Inhibition by saccharolactone. A concentration of 0.1 mM-saccharo-(1 $\rightarrow$ 4)-lactone (Levy, 1952) produced 88% inhibition of the enzyme activity at 0.57 mM substrate concentration at pH 4.1.

Substrate concentration. The initial rate of hydrolysis as a function of substrate concentration is shown in Fig. 3, which surprisingly exhibits no inhibition by excess of substrate. From a reciprocal plot of substrate concentration, $[S]$, and initial velocity, v , according to the method of Lineweaver & Burk (1934), a value of $3.3 \times 10^{-4} \text{ M}$ was obtained for the Michaelis constant, K_m .

Effect of temperature. Fig. 4 shows the curve usually obtained when β -glucuronidase and substrate are incubated simultaneously at stepwise increasing temperatures. The maximum activity

Fig. 3. Relation of substrate concentration to initial velocity. The system consisted of 0.1 ml. of different concentrations of substrate solution, 0.1 ml. of mouse urinary β -glucuronidase (100 units) and 0.8 ml. of 0.1 M-acetate buffer, pH 4.1. Incubation was for 30 min. at 37°. Initial velocity, v , is expressed as μ moles of N -acetyl- N -phenylhydroxylamine liberated/min.

is at 60° and is in agreement with the literature (Pettengill & Fishman, 1962; Fishman, Nakajima, Anstiss & Green, 1964) on O -glucosiduronic acids. The increase in activity was 2.4-fold between 40° and 60°.

Enzymic transfer of the glucuronyl group from (N-acetyl-N-phenylhydroxylamine NO- β -D-glucosid)uronic acid to methanol

The study of the glucuronyl transfer reaction catalysed by β -glucuronidase (Fishman & Green, 1957; Tsukamoto, Kato, Yoshida & Tatsumi, 1964) required thin-layer chromatography. Thus the incubation mixture (5 ml.) contained 0.5 ml. of sodium salt (final concentration 0.5 μ mole/ml.), 0.5 ml. of mouse urinary β -glucuronidase (1000 units), 0.5 ml. of redistilled methanol and 3.5 ml. of 0.1 M-acetate buffer, pH 5.5. After incubation for 1 hr. at 37°, the mixture was boiled for 1 min., cooled and extracted with chloroform to remove the aglycone, and the water layer evaporated to dryness. The dry residue was redissolved in 0.2 ml. of 50% (v/v) ethanol and applied on a silica gel F₂₅₄ prepared chromatoplate (E. Merck A.-G., Darm-

Fig. 4. Influence of temperature. Digests were prepared and brought to the desired temperature before adding 100 units of mouse urinary β -glucuronidase. The substrate concentration was 0.57 μ mole/ml. in 0.1 M-acetate buffer, pH 4.1. Incubation was for 30 min.

stadt, Germany). The solvent system was ethyl acetate-acetic acid-water (5:3:1, by vol.). Compounds were detected as black spots by heating the plate at 125° for 15 min. after spraying with concentrated sulphuric acid-ethanol (1:1, v/v). A small spot, which had the same R_F value as that of an authentic sample of sodium (methyl glucosid)-uronate (Yoshida *et al.* 1964) (R_F 0.38), was obtained between the two large spots identified as unchanged (N -acetyl- N -phenylhydroxylamine β -D-glucosid)uronic acid (R_F 0.55) and free glucuronic acid (R_F 0.21). This spot gave a positive naphtha-resorcinol test after it had been eluted with water. Control digests lacking urinary β -glucuronidase showed only the unchanged donor compound with a very faint trace of free glucuronic acid, presumably due to slight decomposition of the NO -glucosid-uronic acid.

DISCUSSION

Biosynthetic NO -glucosiduronic acids represent a recently discovered group of metabolic conjugates of unique interest in the metabolism of carcinogens and drugs. The best known example of this conjugation is the NO -glucosiduronic acid of N -hydroxy-2-fluorenylacetamide, the aglycone being more carcinogenic than 2-fluorenylacetamide. This glucosiduronic acid was characterized by Irving (1965), who obtained the tri- O -acetyl methyl ester

derivative, which, however, was unstable on saponification.

The isolation of biosynthetic (*N*-acetyl-*N*-phenyl-hydroxylamine β -D-glucosid)uronic acid in reasonable yield by Kato *et al.* (1967) has made possible a study of this substance as a substrate for β -glucuronidase. The aglycone, although not carcinogenic, is a bone-marrow depressant (Wagner, Janata & Hykes, 1964).

The strongest evidence that this *NO*- β -D-glucosiduronic acid is a specific substrate for β -glucuronidase rests on its almost complete hydrolysis into products whose equimolar amounts match those expected from the quantity of substrate present. Not only was this hydrolysis accomplished with the β -glucuronidase of highest specific activity (100 000 units/mg. of protein), but also by a variety of less-pure β -glucuronidase preparations from the mouse, ox, *Helix pomatia* and *E. coli*. It is not hydrolysed by leech endo- β -glucuronidase.

The pH optimum, 4.1, is in the range of the optimum pH values reported for mouse urinary β -glucuronidase with (phenolphthalein glucosid)uronic acid (Pettengill & Fishman, 1962).

Two unusual chemical properties are noted for this *NO*-glucosiduronic acid, namely its marked instability in dilute alkali and its failure to react with naphtharesorcinol. In the latter case, it may be possible to detect the presence of *NO*-glucosiduronic acids by observing a higher value for hexuronic acid with the Dische reaction than with the naphtharesorcinol reaction of a substance that is alkali-sensitive.

It may be pointed out that the reverse situation, a higher naphtharesorcinol value than Dische value for hexuronic acid, is a standard means of evaluating the amount of iduronic acid in a mixture.

Before the discovery of the *NO*-glucosiduronic acids, a class of nitrogen-conjugated compounds, the *N*-glucosiduronic acids, was already known. These do occur naturally in the urine and may be formed non-enzymically as well as by enzymic means. They are not substrates for β -glucuronidase (Takanashi *et al.* 1964).

One may reasonably suggest the metabolic fate of *NO*-glucosiduronic acids synthesized in the liver. These are first excreted in the bile and then enter the intestinal lumen. Here conditions of alkalinity would destroy the N-O-C linkage. The intact conjugate would also be a substrate for the β -glucuronidase in the gastrointestinal juice and perhaps in the mucosa of the intestine. A reabsorbed mixture of aglycone and conjugate would enter the portal blood and would later conceivably be excreted by the kidney tubules into the urine, the conjugate more efficiently than the aglycone. In

the bladder urine, however, the acid condition and the presence of β -glucuronidase would favour cleavage of the *NO*-glucosiduronic acid. Consequently, one could predict that owing to metabolic hydrolysis in several body compartments the aglycone would be circulating in the body for a much longer period of time than would the aglycone of an *O*-glucosiduronic acid such as (pregnanediol glucosid)uronic acid, in which the glycosidic linkage is alkali-stable.

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