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Biosynthesis and Studies of the Alkaline Sensitivity of the *NO*-Glucuronide of the Carcinogen *N*-2-Fluorenylacethydroxamic Acid*

Jim T. Hill† and Charles C. Irving

ABSTRACT: Because of its occurrence as a major metabolite of the carcinogen *N*-2-fluorenylacetamide in susceptible species, the *NO*-glucuronide of *N*-2-fluorenylacethydroxamic acid was needed for studies of its reactivity and carcinogenic properties. The desired product has been isolated from the urine of rabbits fed *N*-2-fluorenylacetamide and has been crystallized and characterized as sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate. This unusual type of glucuronide (C–O–N linkage) is alkali labile. Studies on the mechanism of the instability under alkaline conditions suggest migration of the *N*-acetyl group of the aglycon to the 2′-hydroxyl group of the glucuronic acid moiety, followed by hydrolysis of the glycosidic linkage to yield initially *N*-2-fluorenylhydroxylamine and, as an end product, 2,2′-bisazoxyfluorene.

Sodium (*N*-acetyl-*N*-2-fluoren[9-^{14}C]ylhydroxylamine β-D-glucosid)uronate has also been prepared biosynthetically.

A general metabolic reaction of aromatic amides and amines (reviewed in Miller and Miller, 1966), *N*-hydroxylation, initially discovered during studies on the metabolism of the carcinogen *N*-2-fluorenylacetamide (Cramer *et al.*, 1960), leads to the excretion of a novel type of alkali-labile glucuronide. The structure of the glucuronide of *N*-2-fluorenylacethydroxamic acid has been proven (Irving, 1965). Recently, Kato *et al.* (1967) described the isolation of the alkali-labile glucuronide of *N*-phenylacethydroxamic acid from rabbit urine and proposed the use of the term *NO*-glucosiduronic acid to describe this type of glucuronide.

We have now isolated and characterized the sodium salt of the *NO*-glucuronide of *N*-2-fluorenylacethydroxamic acid and studied the kinetics of the alkaline hydrolysis of this compound. Sodium (*N*-acetyl-*N*-2-fluoren[9-^{14}C]ylhydroxylamine β-D-glucosid)uronate has also been prepared biosynthetically from *N*-2-fluoren-[9-^{14}C]ylacethydroxamic acid.

Experimental and Results

Materials. *N*-2-Fluorenylhydroxylamine was synthesized using the procedure of Poirier *et al.* (1963):

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$\lambda_{max}^{50\% \text{ ethanol}}$ 280 mμ (ε 20,000), $A_{280}:A_{302}$ = 1.92. The high specific activity *N*-2-fluoren[9-^{14}C]ylacethydroxyamic acid (11.3 mc/mmole) was made by the acetylation (Irving, 1966) of *N*-2-fluoren[9-^{14}C]ylhydroxylamine. *N*-Acetyl-*N*-2-fluorenylhydroxylamine 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside was prepared by the procedure previously described (Irving, 1965) for the synthesis of methyl (*N*-acetyl-*N*-2-fluorenylhydroxylamine 2,3,4-tri-*O*-acetyl-β-D-glucosid)uronate[1] except tetra-*O*-acetyl-α-D-glucopyranosyl bromide was used instead of methyl(tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate in the reaction with the potassium salt of *N*-2-fluorenylacethydroxamic acid. Bacterial β-glucuronidase (type II) was purchased from Sigma Chemical Co., St. Louis, Mo.

Methods. Samples were analyzed for metabolites of *N*-2-fluorenylacetamide and *N*-2-fluorenylacethydroxamic acid by methods previously used (Irving, 1962). Thin layer chromatography of glucuronide conjugates was carried out on cellulose (MN 300 F_{254}, Brinkmann Instruments, Inc., Westbury, N. Y.) in (A) isopropyl alcohol–water–formic acid (40:9:1, v/v) or (B) *sec*-butyl alcohol–3% NH_4OH (3:1) and on DEAE-cellulose (MN 300 G/DEAE, Brinkmann) in (C) isopropyl alcohol–water–formic acid (40:9:1) or (D) ethyl alcohol–0.5 M sodium acetate (pH 5, 3:1). Radioactivity determinations were made by liquid scintillation counting. For column chromatography, DEAE-cellulose (Eastman) was washed in succession with 0.5 N NaOH, water, 0.1 N acetic acid, and then thoroughly with water. Columns were packed using 0.02 M ammonium acetate (pH 5.0), then washed with several liters of water prior to application of the sample. Effluents from columns were collected in a refrigerated fraction collector at 5° and fractions were stored at 2–3° until processed further. During evaporation of samples under reduced pressure, temperatures were kept at less than 40°. Syrups obtained at intermediate stages of the isolation were stored at −20°.

Isolation of Sodium (N-Acetyl-N-2-fluorenylhydroxylamine β-D-glucosid)uronate. *N*-2-Fluorenylacetamide, packed into gelatin capsules, was administered orally to male New Zealand rabbits (1.5–2.0 kg) at a dose level of 100 mg/day per rabbit. The animals were fed Purina rabbit chow and water *ad libitum*. Urine, collected in a receiver cooled in an ice bath, was pooled daily, adjusted to pH 6 with acetic acid, and stored at −20°. Approximately 4 l. of urine (6–8-days pooled volume from six rabbits) was used for each run of the isolation procedure.

The conventional lead salt technique (Kamil *et al.*, 1951) for isolation of the crude glucuronide fraction was not used because of a loss of about 10% of the desired product in the pH 4 precipitate. Instead, the urine was adjusted to pH 8.0, centrifuged at 0°, and solid normal lead acetate (40 g/l. of urine) was added to the clear supernatant while the suspension was maintained at pH 8.0 (meter) by the addition of NH_4OH. The resulting precipitate was collected by centrifugation and was washed once with water, then suspended in 2 l. of methanol. Lead was removed by treatment with H_2S in the usual manner. The filtrate was evaporated under reduced pressure and the dark-colored syrup which was obtained was dissolved in water (800 ml) and filtered. Ammonium sulfate (500 g/l.) was added and the solution was extracted six times with an equal volume of ether–ethanol (3:1, v/v). The combined extracts were dried with Na_2SO_4, the solvent was removed under reduced pressure, and the syrup obtained was dissolved in water (500 ml). The solution was adjusted to pH 5.0, filtered, and applied to a column of DEAE-cellulose (4 × 60 cm; 100 g of DEAE-cellulose). The column was first washed with 1 l. of water, then a linear gradient (7-l. total volume) of ammonium acetate buffer (pH 5.0) from 0.02 (3.5 l.) to 0.40 M (3.5 l.) was started. The flow rate was maintained at 300 ml/hr and the A_{275} of the effluent was recorded using a Model 2000 multiple-sample absorbance recorder (Gilford Instruments, Oberlin, Ohio). A typical elution profile is shown in Figure 1. Fractions of the effluent containing the desired product were pooled and crude material was recovered as a syrup from these fractions by the lead salt technique

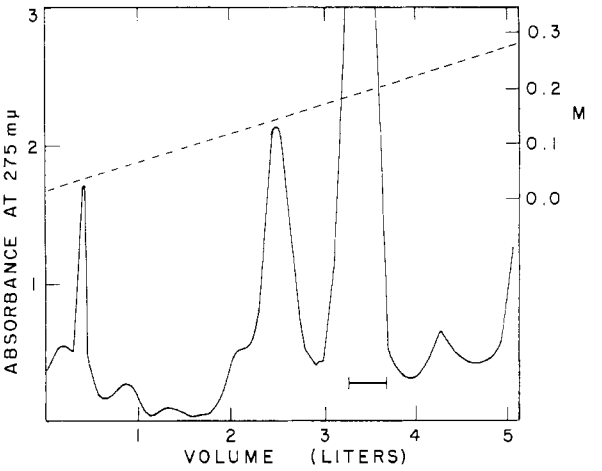

FIGURE 1: DEAE-cellulose chromatography of the crude glucuronide syrup obtained from rabbit urine after administration of *N*-2-fluorenylacetamide. A_{275} (solid line) was measured in a flow cell having a light path of 2 mm. The dotted line represents the concentration of the eluent, ammonium acetate buffer (pH 5.0); the gradient was started at zero volume on the abscissa. The glucuronide of *N*-2-fluorenylacethydroxamic acid was eluted in the fractions indicated (|——).

[1] Recent studies of the nuclear magnetic resonance spectrum of this compound (prepared synthetically) indicated that it was a mixture of the methyl and ethyl esters. The reaction of methyl-(tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate with the potassium salt of *N*-2-fluorenylacethydroxamic acid was carried out in absolute ethanol under slightly alkaline conditions. Apparently, some transesterification occurred under these conditions. On the other hand, the product prepared biosynthetically (Irving, 1965) was the methyl ester derivative.

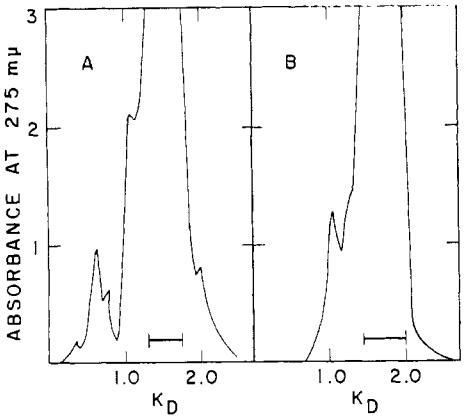

FIGURE 2: Chromatographical studies on Sephadex G-10. (A) Sephadex G-10 chromatography of syrup obtained from DEAE-cellulose chromatography of crude glucuronide fraction. A_{275} was measured in a flow cell having a light path of 2 mm. K_D is the partition coefficient between the stationary phase and the mobile phase. The void volume (V_0) of the column (represented by $K_D = 0$) was 180 ml; the flow rate was 30 ml/hr. Fractions indicated by the horizontal bar (|——|) were pooled and freeze dried. (B) Rechromatography on Sephadex G-10 of the amorphous solid obtained in part A. Same conditions as in part A.

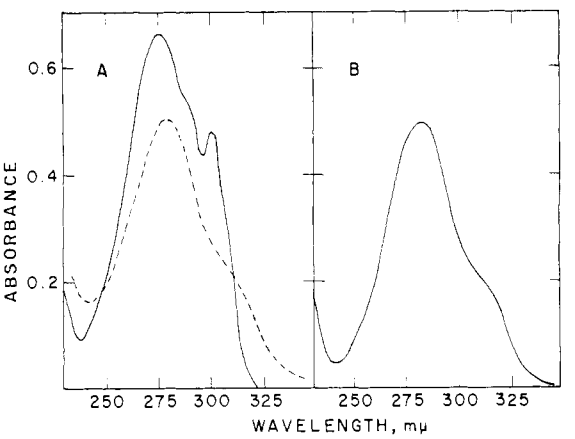

FIGURE 3: Ultraviolet absorption spectral studies. (A) Ultraviolet absorption spectra of sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate (solid line; concentration 2.71 × 10^{-5} M in 50% ethanol) and its alkaline hydrolysis product (dashed line). The spectrum of the product was obtained 20 min after making the solution of the glucuronide 0.02 M with respect to NaOH. (B) Spectrum of authentic *N*-2-fluorenylhydroxylamine; concentration, 2.50 × 10^{-5} M in 50% ethanol.

described above. Many attempts to crystallize the product from this syrup were unsuccessful.

An amorphous solid was obtained by chromatography of the syrup from the DEAE-cellulose chromatography on Sephadex G-10. The syrup obtained from 8 l. of urine was dissolved in water (10 ml) and excess solid $NaHCO_3$ was added in 50-mg portions (until frothing ceased). The solution was filtered and applied immediately to a column (2.5 × 100 cm) of Sephadex G-10 (250 g) which had been packed and previously washed thoroughly with deionized water. Elution was carried out using deionized water (Figure 2A). The product was adsorbed somewhat to the Sephadex G-10 as evidenced by a K_D value of 1.2–1.3. Fractions containing the product (Figure 2A) were pooled and the solution was freeze dried to yield an amorphous solid (0.8–1.3 g). The solid was dissolved in water (5 ml), treated with 50–75 mg of solid $NaHCO_3$, and rechromatographed on Sephadex G-10 (Figure 2B), yielding 0.75–1.2 g of amorphous solid.

The product was crystallized by dissolving the amorphous material in water (200 mg/ml) and then adding boiling ethanol followed by boiling ethyl acetate in the ratio of 1:5:10 (water–ethanol–ethyl acetate). Recrystallization from the same solvent system gave 0.5–0.7 g of pure sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate. The crystals decomposed at 195–196°; some preparations showed a prior melting at 156–160° followed by resolidification, then decomposition at 195–196°; $[\alpha]_D^{26}$ −96° (*c* 1, water); λ_{max}^{water} (Figure 3A) 275 mμ (ε 22,200) and 302 mμ (ε 16,300); λ_{max}^{KBr} 2.9, 6.0, 6.2, 7.1, 9.0–9.5, 12.8, and 13.4 μ. For analysis, the crystals were dried over night *in vacuo* at 78° over P_2O_5. *Anal.* Calcd for $C_{21}H_{20}NNaO_8$ (437.4): C, 57.7; H, 4.61; N, 3.20. Found: C, 57.8; H, 4.68; N, 2.98.

Biosynthesis of Sodium (N-Acetyl-N-2-fluoren[9-^{14}C]-ylhydroxylamine β-D-Glucosid)uronate. A suspension of 91 mg of *N*-2-fluoren[9-^{14}C]ylacethydroxamic acid (specific radioactivity, 11.3 mc/mmole) in 5 ml of 0.9% NaCl was injected intraperitoneally into a male rabbit (2.7 kg) and the urine was collected for 24 hr as above. The urine contained 52% of the radioactivity injected and approximately 50 mg of the glucuronide conjugate of the compound injected. Carrier sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate (103 mg) was added to the urine and the glucuronide was isolated as described above except that the procedure was scaled down. C ystalline sodium (*N*-acetyl-*N*-2-fluoren[9-^{14}C]ylhydroxylamine β-D-glucosid)uronate (89 mg; specific radioactivity, 3.63 mc/mmole) was obtained with an over-all radiochemical yield of 17% from the aglycon injected. The glucuronide was dissolved in water at a concentration of 10 mg/ml and stored at −20°. The product gave single radioactive spots upon thin layer chromatography in four different solvent systems (see Methods): A, B, C, and D, with R_F values of 0.66, 0.61, 0.41, and 0.47, respectively. Upon hydrolysis of the product with bacterial β-glucuronidase, a quantitative yield of *N*-2-fluoren[9-^{14}C]ylacethydroxamic acid (specific radioactivity, 3.67 mc/mmole) was obtained.

Kinetic Studies of the Alkaline Hydrolysis of Sodium (N-Acetyl-N-2-fluorenylhydroxylamine β-D-Glucosid)-

uronate and Related Compounds. The ultraviolet spectra of sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate and of the product of the action of dilute alkali (final concentration 0.02 M) on the glucuronide are shown in Figure 3A; the spectrum of authentic *N*-2-fluorenylhydroxylamine is shown in Figure 3B. The rate of alkaline hydrolysis of the glucuronide in 0.01 M NaOH in 50% ethanol was determined by measuring the disappearance of absorbance at 302 mμ upon addition of the alkali to a solution of the glucuronide. The concentration of glucuronide at time (*t*) was calculated (Irving, 1960) from the molar extinction coefficients of the glucuronide and the product at 302 mμ. Results of the kinetic studies of the alkaline degradation of sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate ($k = 10.9 \times 10^{-4}$ sec^{-1}) are shown in Figure 4. The rates of alkaline hydrolysis of methyl (*N*-acetyl-*N*-2-fluorenylhydroxylamine 2,3,4-tri-*O*-acetyl-β-D-glucosid)uronate ($k = 6.7 \times 10^{-4}$ sec^{-1}) and of *N*-acetyl-*N*-2-fluorenylhydroxylamine 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside ($k = 17.1 \times 10^{-4}$ sec^{-1}) were also determined in 0.01 M NaOH by measuring the rate of disappearance of A_{302} as described above. Upon addition of alkali to solutions of the acetylated glycoside derivatives, there was a lag phase of several minutes before the disappearance of A_{302} began (Figure 4). This was in contrast to data obtained with sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate, with which the A_{302} started to decrease immediately upon addition of the alkali.

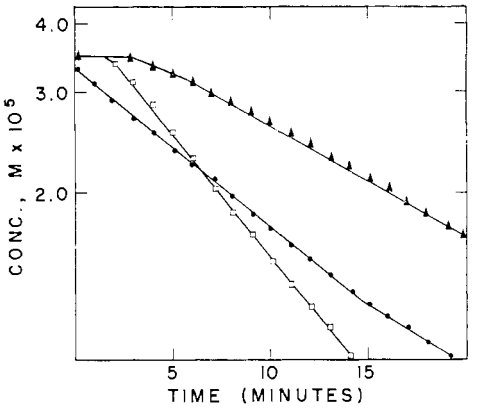

FIGURE 4: Alkaline hydrolysis (0.01 M NaOH in 50% ethanol) of sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate (●—●), methyl (*N*-acetyl-*N*-2-fluorenylhydroxylamine 2,3,4-tri-*O*-acetyl-β-D-glucosid)uronate (▲—▲), and *N*-acetyl-*N*-2-fluorenylhydroxylamine 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (□—□). First-order rate constants were calculated from the plot of the log of the concentration of the glycoside *vs.* time, using the interval between 6 and 11 min after the addition of alkali.

Discussion

The structure of the glucuronide of *N*-2-fluorenylacethydroxamic acid had been confirmed previously by comparison of the triacetyl methyl ester derivative, obtained by methylation and acetylation of the glucuronide gum isolated from rabbit urine after feeding *N*-2-fluorenylacetamide, with an authentic sample (Irving, 1965). Attempts to hydrolyze the triacetyl methyl ester derivative to obtain the free glucuronide of *N*-2-fluorenylacethydroxamic acid failed (Irving, 1965). We have now isolated the sodium salt of this *NO*-glucuronide from rabbit urine collected after administration of either *N*-2-fluorenylacetamide or *N*-2-fluorenylacethydroxamic acid.

Sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate is stable in solution below pH 8, particularly if kept at 0–5° or below, but decomposes at more alkaline pH values. The *NO*-glucuronide of *N*-phenylacethydroxamic acid has also been reported to be unstable in dilute alkali (Kato *et al.*, 1967). If *NO*-glucuronides of this structure are considered to be of the ester type, since they are glucuronides of substituted hydroxamic acids, it might not be too surprising that they are alkali labile. Alkaline hydrolysis of ester-type glucuronides ordinarily yields the aglycon as a product. However, the product of the action of dilute alkali on sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate is *not* the aglycon, *N*-2-fluorenylacethydroxamic acid. Since the aglycon is known to be stable in dilute alkali for the brief periods used in the kinetic studies (Figure 4), some mechanism other than simple alkaline hydrolysis to yield the aglycon must be postulated in order to explain the lability of the *NO*-glucuronide of *N*-2-fluorenylacethydroxamic acid. The previous observation that the end product of the alkaline decomposition was azoxyfluorene (Irving, 1965) suggested that *N*-2-fluorenylhydroxylamine might be an intermediate. This appeared to be confirmed by the marked similarity of the ultraviolet absorption spectra of the reaction mixture upon the addition of alkali and of authentic *N*-2-fluorenylhydroxylamine (Figure 3).

A minor sharp absorption band or shoulder at 300–302 mμ is characteristic of nitrogen-substituted derivatives of *N*-2-fluorenylacetamide, *e.g.*, *N*-2-fluorenylacethydroxamic acid, $\lambda_{max}^{ethanol}$ 290 mμ and 302 mμ; *N*-acetoxy-*N*-2-fluorenylacetamide, $\lambda_{max}^{ethanol}$ 276 mμ and 301 mμ; 8-(*N*-2-fluorenylacetamido)guanosine (Kriek *et al.*, 1967), $\lambda_{max}^{isopropyl\ alcohol-H_2O}$ 275 mμ and 300 mμ; and sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate (this paper). Addition of alkali to a solution of sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate resulted in the removal of the *N*-acetyl group, as evidenced by the immediate decrease in A_{302} (Figure 4). The removal of the *N*-acetyl group probably proceeds by the mechanism outlined in Figure 5. In the preferred chair conformation of the glucuronide, with all substituents in equatorial positions, the 2′-hydroxyl group is the only one in sufficient proximity to be involved in migration of the *N*-acetyl group. In other, less stable conformations the 3′-hydroxyl group could be involved. With the

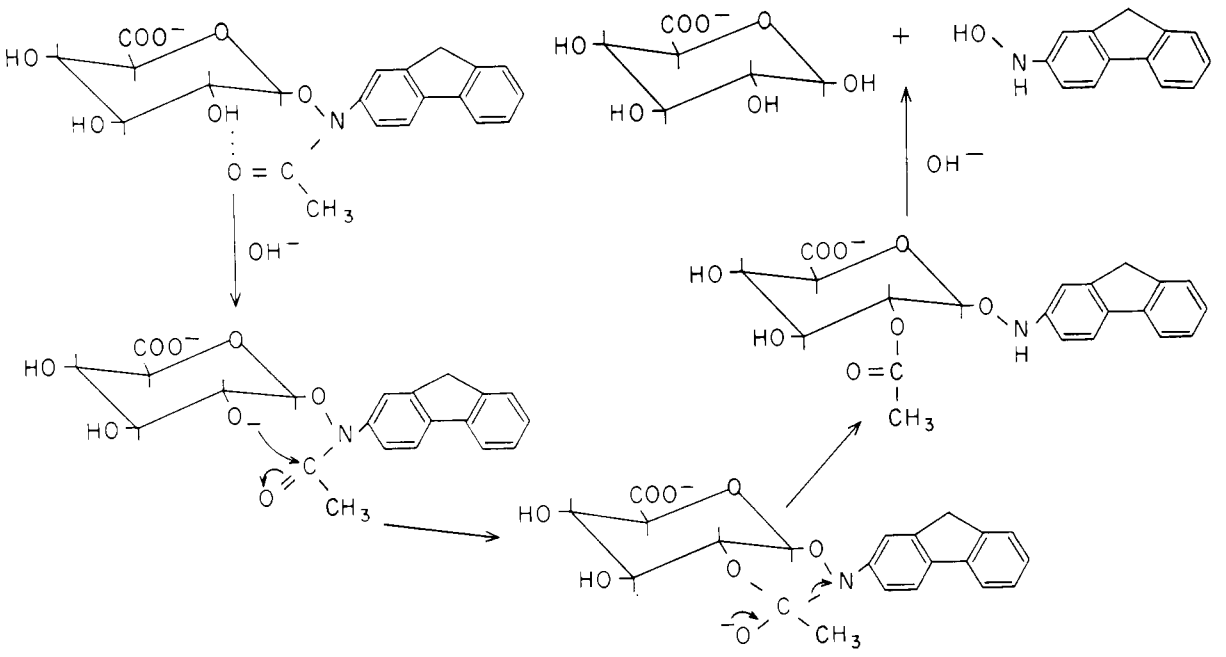

FIGURE 5: Proposed mechanism for the formation of *N*-2-fluorenylhydroxylamine from sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate by the action of dilute alkali. The evidence for the involvement of the 2′-hydroxyl group was obtained from an examination of a molecular model of the glucuronide. The glucuronide is possibly stabilized by hydrogen bonding between the 2′-hydroxyl group and the carbonyl oxygen of the *N*-acetyl group.

oxygen-acetylated glycoside derivatives, methyl (*N*-acetyl-*N*-2-fluorenylhydroxylamine 2,3,4-tri-*O*-acetyl-β-D-glucosid)uronate and *N*-acetyl-*N*-2-fluorenylhydroxylamine 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside, which also have sharp minor absorption bands at 302 mμ, there was a lag in the decrease in A_{302} upon addition of alkali (Figure 4). The lag period observed with the oxygen-acetylated derivatives was probably due to the time required for the deesterification of the *O*-acetyl groups involved in the migration of the *N*-acetyl group. In addition to the possibilities discussed with respect to the glucuronide, the 6′-hydroxyl group of the glucoside (following deesterification of the *O*-acetyl groups) could be involved in the migration of the *N*-acetyl group, particularly in the case of the less stable conformations. This may account for the higher rate constant observed in the alkaline hydrolysis of the *N*-acetyl-*N*-2-fluorenylhydroxylamine 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside.

The initial product of the action of alkali on sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate is, thus, probably the 2′-*O*-acetyl-*NO*-glucuronide of *N*-2-fluorenylhydroxylamine. Attempts to isolate this intermediate or its deacetylated product, the *NO*-glucuronide of *N*-2-fluorenylhydroxylamine, have not yet been successful, since they appear to readily hydrolyze to give *N*-2-fluorenylhydroxylamine, which is unstable, yielding azoxyfluorene as an end product. The instability of the proposed intermediates precludes the unequivocal identification of the product represented by the spectrum in Figure 3A (dashed line). However, the product must be either the *NO*-glucuronide of *N*-2-fluorenylhydroxylamine, *N*-2-fluorenylhydroxylamine, or a mixture of the two.

One of the reasons for synthesizing the *NO*-glucuronide of *N*-2-fluorenylacethydroxamic acid was to study its reactivity and metabolic fate (Irving *et al.*, 1967a–d). Preliminary studies have indicated that sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate reacts *in vitro* with methionine, tryptophan, and guanosine (Lotlikar *et al.*, 1967). More recent data (Irving *et al.*, 1967a) have demonstrated that sodium (*N*-acetyl-*N*-2-fluoren[9-^{14}C]ylhydroxylamine β-D-glucosid)uronate also reacts *in vitro* with RNA and DNA at a low rate at pH 7–7.5, but at a rate sufficient to account for the levels of binding to RNA and DNA which are observed *in vivo* after administration of either *N*-2-fluorenylacetamide or *N*-2-fluorenylacethydroxamic acid.

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Enzymatic Synthesis of Streptidine from *scyllo*-Inosamine*

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ABSTRACT: Cell-free preparations from post-log-phase mycelia of *Streptomyces bikiniensis* ATCC 11062 catalyzed the conversion of *scyllo*-inosamine (aminodeoxy-*scyllo*-inositol) to streptidine (all *trans*-1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane). Evidence has been obtained consistent with the following biosynthetic pathway: myoinositol $\xrightarrow{C}$ *scyllo*-inosose $\underset{D1}{\rightleftarrows}$ *scyllo*-inosamine $\xrightarrow{D2}$ *scyllo*-inosamine-P $\underset{E}{\rightleftarrows}$ *N*-amidino-*scyllo*-inosamine-P $\xrightarrow{F1}$ *N*-amidino-*scyllo*-inosamine $\xrightarrow{F2}$ *N*-amidino-3-keto-*scyllo*-inosamine $\underset{F3}{\rightleftarrows}$ *N*-amidinostreptamine $\xrightarrow{F4}$ *N*-amidinostreptamine-P $\underset{G}{\rightleftarrows}$ streptidine-P $\xrightarrow{W}$ streptidine. A free inosamine, apparently of the *scyllo* configuration, was detected for the first time in a biological system, in mycelia of *Streptomyces griseus* ATCC 12475 fed myo[^{14}C]-inositol. Enzymatic activities detected for the first time included: (i) *scyllo*-inosamine kinase (D2), which required adenosine 5′-triphosphate and Mg^{2+} and did not react with physiological concentrations of myoinosamine-2, DL-myoinosamine-4, or neoinosamine-2; (ii) a kinase which phosphorylates 2-deoxystreptamine and streptamine (1,3-diamino-1,3-dideoxy-*scyllo*-inositol) with adenosine 5′-triphosphate; (iii) *N*-amidinostreptamine–pyruvate transaminase (F3), which also reacts with α-ketoglutarate; and (iv) *scyllo*-inosamine–pyruvate transaminase (D1), which also reacts with α-ketoglutarate. In the forward direction of reaction F3, L-alanine and L-glutamate can serve as amino donors, but D-alanine, D-glutamate, L-glutamine, L-aspartate, and glycine are inactive. We conclude that amino groups of both inosamines and inosadiamines can be derived by transamination of nonphosphorylated precursors with certain amino acids.

A number of inosamine derivatives have been implicated in the biosynthesis of streptidine from myoinositol[1] (Walker and Walker, 1966, 1967a,b). The principal uncertainties in the biosynthetic sequences proposed earlier involved (i) configurations and state of phosphorylation of early intermediates in the pathway, and (ii) mechanisms by which amino groups were substituted for hydroxyl groups on the cyclitol ring. In this paper evidence will be presented consistent with all *trans-scyllo* configurations of early intermediates, and formation of cyclitol amino groups by transamination of nonphosphorylated inosose derivatives with L-alanine or L-glutamate. Phosphorylation steps appear to be required primarily for formation of the guanidino moieties of streptidine by transamidination of the amino groups of suitable phosphorylated-inosamine precursors. Our findings and hypotheses have been summarized in Scheme I.

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[1] Trivial names and abbreviations used: streptidine, all *trans*-1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane; *scyllo*-inosamine, aminodeoxy-*scyllo*-inositol; DL-myoinosamine-4, DL-4-amino-4-deoxymyoinositol; neoinosamine-2,2-amino-2-deoxy-neoinositol; streptamine, 1,3-diamino-1,3-dideoxy-*scyllo*-inositol; ATP, adenosine 5′-triphosphate; NAD^+ and $NADP^+$, oxidized nicotinamide–adenine dinucleotide and its phosphate; orn, ornithine; pyr, pyruvate; KG, α-ketolgutarate.

Results

Detection of Inosamine Derivatives in Mycelia Fed Myo[^{14}C]inositol. When myo[^{14}C]inositol was fed to mycelia of *Streptomyces griseus* ATCC 12475