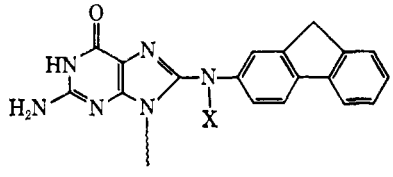
Synthesis of the O-Glucuronide of N-2-Fluorenylhydroxylamine. Reaction with Nucleic Acids and with Guanosine 5'-Monophosphate*

Charles C. Irving and Laretta T. Russell

ABSTRACT: O-Glucuronides of arylhydroxylamines have not been previously reported in the literature. The facile removal of the N-acetyl group from sodium (N-acetyl-N-2-fluorenylhydroxylamine β-D-glucosid)uronate has led to the synthesis of sodium (N-2-fluorenylhydroxylamine β-D-glucosid)uronate. This latter glucuronide is unstable in aqueous solutions, decomposing completely within several minutes; the products of the decomposition have not yet been characterized. Sodium (N-2-fluoren-9-^{14}C-ylhydroxylamine β-D-glucosid)-uronate has been synthesized and the compound reacts with RNA and DNA *in vitro*, resulting in the covalent binding of radioactivity to the nucleic acids. This glucuronide reacts with guanosine 5'-monophosphate to give 8-(N-2-fluorenylamino)guanosine 5'-monophosphate, but does not react with the 5'-monophosphates of uridine, cytidine, or adenosine. It is proposed that the O-glucuronide of N-2-fluorenylhydroxylamine might be formed as an intermediate metabolite of the carcinogen N-acetyl-N-2-fluorenylhydroxylamine, thus accounting, at least in part, for the observed binding of this carcinogen to rat liver DNA *in vivo*.

There have been a number of reports indicating that the carcinogens 2-acetylaminofluorene and N-acetyl-N-2-fluorenylhydroxylamine are bound in covalent linkage to rat liver RNA and DNA *in vivo* (reviewed by Miller and Miller, 1969a,b; for more recent studies, see also Agarwal and Weinstein, 1970, DeBaun *et al.*, 1970a,b, Epstein *et al.*, 1969, Irving and Veazey, 1969, Irving *et al.*, 1969a,b, and Kriek, 1969). The covalent binding of these carcinogens involves the attachment of the nitrogen of a 2-acetylaminofluorene moiety (Ia) or a 2-aminofluorene moiety (Ib) to the 8-carbon



Ia, X = $COCH_3$
Ib, X = H

of guanine residues in the nucleic acids. Recent studies on mechanisms of biochemical activation of 2-acetylaminofluorene and N-acetyl-N-2-fluorenylhydroxylamine which lead to the binding of these carcinogens to nucleic acids *in vivo* have resulted in the conclusion that several metabolites may be involved. Thus, N-2-fluorenylhydroxylamine (King and Phillips, 1969; Kriek, 1969), the O-sulfonate conjugate of N-acetyl-N-2-fluorenylhydroxylamine (DeBaun *et al.*, 1970a,b; King and Phillips, 1969), and the O-glucuronide of N-acetyl-N-2-fluorenylhydroxylamine (Irving *et al.*, 1969a,b; Irving, 1970) have each been implicated as reactive metabolites of these carcinogens.

We now wish to report the synthesis and characterization of an additional potential metabolite of N-acetyl-N-2-fluorenylhydroxylamine which might be formed as a transient intermediate, leading to the covalent attachment of a residue of 2-aminofluorene to nucleic acids *in vivo*. The easy removal of the N-acetyl group from N-GlO-AAF[1] (IIa) with dilute alkali has made possible the synthesis of N-GlO-AF (IIb). This type of glucuronide (IIb) has not previously been reported in the literature. Compound IIb, which is quite unstable in aqueous systems, reacts with guanine residues of RNA and DNA *in vitro* at a rate much faster than that previously reported for IIa (Irving *et al.*, 1969a,b). It is suggested that IIb might be formed as an intermediate in the reaction of IIa with nucleic acids *in vivo* and *in vitro*.

Experimental Procedures

Materials. The following were obtained from commercial sources: yeast tRNA (Calbiochem), calf thymus DNA (Worthington), GMP (Sigma), and AMP, CMP, and UMP (Pabst). Guanosine-^{14}C(U) 5'-monophosphate, which was obtained from New England Nuclear Corp., was diluted with unlabeled GMP to give a product with a specific radioactivity of 0.05–1.5 mCi/mmole. The actual specific radioactivity of the GMP-^{14}C used in the experiments was calculated from data obtained by determining the concentration of

* From the Cancer Research Laboratory, Veterans Administration Hospital, and the Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee 38104. *Received March 6, 1970.* Supported by Part I Designated Research Funds of the U. S. Veterans Administration and by U. S. Public Health Service Research Grant CA-05490 from the National Cancer Institute.

[1] Abbreviations used are: N-GlO-AAF, sodium (N-acetyl-N-2-fluorenylhydroxylamine β-D-glucosid)uronate; N-GlO-AF, sodium (N-2-fluorenylhydroxylamine β-D-glucosid)uronate; GMP-AAF, 8-(N-2-fluorenylacetamido)guanosine 5'-monophosphate; GMP-AF, 8-(N-2-fluorenylamino)guanosine 5'-monophosphate; Guo-AAF, 8-(N-2-fluorenylacetamido)guanosine; Guo-AF, 8-(N-2-fluorenylamino)guanosine.

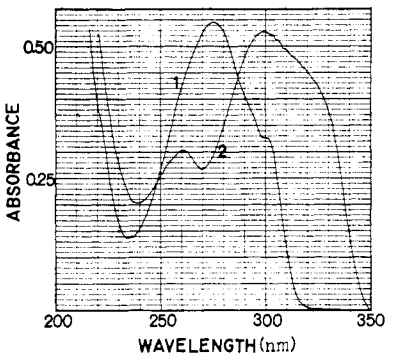


FIGURE 1: Ultraviolet absorption spectra of 8-(*N*-2-fluorenylacetamido)guanosine 5′-monophosphate (spectrum 1; ϵ_{276} 37,300) and 8-(*N*-2-fluorenylamino)guanosine 5′-monophosphate (spectrum 2; ϵ_{300} 29,600). Each compound was dissolved in 0.1 M ammonium bicarbonate (pH 7.8).

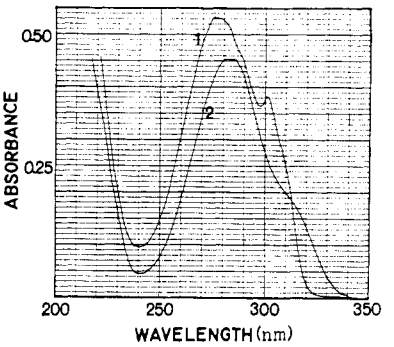


FIGURE 2: Ultraviolet absorption spectra of sodium (*N*-acetyl-*N*-2-fluorenylhydroxylamine β-D-glucosid)uronate (IIa; spectrum 1; ϵ_{275} 22,200) and sodium (*N*-2-fluorenylhydroxylamine β-D-glucosid)-uronate (IIb; spectrum 2; ϵ_{284} 21,900) in methanol.

GMP from the absorbance at 252 mμ (ε 13,700; Dawson *et al.*, 1959) and counting an aliquot of the solution.

Compound IIa was prepared biosynthetically by feeding rabbits *N*-acetyl-*N*-2-fluorenylhydroxylamine and isolating the glucuronide from urine (Hill and Irving, 1967); it was obtained as the crystalline sodium salt. The 9-^{14}C compound was also prepared biosynthetically (Hill and Irving, 1967; Irving *et al.*, 1969b).

GMP-AAF was synthesized by reaction of *N*,*O*-diacetyl-*N*-2-fluorenylhydroxylamine with GMP (Kriek, 1968). GMP-AF was obtained by deacetylation of GMP-AAF in 0.01 N NaOH at 100° for 30 min (Kriek, 1968). The nucleotide derivatives were purified by column chromatography on Sephadex G-25 at 0–5° using 0.1 M ammonium bicarbonate (pH 7.8) for elution. Each compound gave a single ultraviolet-absorbing spot when subjected to thin-layer chromatography on cellulose F with 2-propanol–ammonium hydroxide–water, 6:3:1 (R_F GMP-AAF, 0.49; R_F GMP-AF, 0.12). The ultraviolet spectra of these products are shown in Figure 1.

Ethyl acetate was purified by refluxing with acetic anhydride and a trace of sulfuric acid, followed by washing with solid anhydrous K_2CO_3 and distillation (Vogel, 1956). Methanol was redistilled twice. A solution of sodium methoxide was prepared fresh by dissolving 1 g of clean sodium in 100 ml of methanol. The concentration of sodium methoxide was determined by titration with 0.1 N HCl.

Methods. SODIUM (*N*-2-FLUORENYLHYDROXYLAMINE β-D-GLUCOSID)URONATE. Compound IIa (0.26 mmole) was dissolved in 5 ml of methanol and 0.70 ml of 0.38 M sodium methoxide in methanol was added. The solution was allowed to stand at 25° for 2 hr, during which time the product began to crystallize. The mixture was allowed to stand further at 2–4° overnight. The product was collected by centrifugation and was washed in the centrifuge tube three times with ethyl acetate. After drying at room temperature *in vacuo*, 79 mg of IIb (0.20 mmole; 77% yield) was obtained. *Anal.* Calcd for $C_{19}H_{18}NNaO_7$ (395.4): C, 57.72; H, 4.59; N, 3.54. Found: C, 57.47; H, 4.71; N, 3.28. The infrared spectrum of IIb revealed the disappearance of the amide I band at 1665 cm^{-1} which was prominent in IIa. Compound IIb had an absorption maximum in methanol at 284 mμ (ε 21,900; Figure 2, **2**). The disappearance of the absorption maximum at 302 mμ which was present in IIa (Figure 2, **1**) was also consistent with the removal of the *N*-acetyl group (Hill and Irving, 1967).

Compound IIb could also be prepared from the triacetyl methyl ester derivative of IIa. Since this latter derivative can be synthesized (Irving, 1965), this afforded a means whereby IIb could be synthesized chemically, *i.e.*, by a procedure not involving the biosynthesis of IIa. Briefly, the procedure was the same as above except that water (5% by volume) was added to the reaction mixture before placing it at 2–4° overnight.

Compound IIb was a cream-colored solid which showed no change in color when kept under vacuum over calcium chloride for several days, but darkened rapidly when exposed to a moist atmosphere. Compound IIb was soluble in water, decomposing rapidly to give a reddish-brown colored solution. Although the half-life of IIb in aqueous solutions has not been determined, it must be on the order of several minutes or less as estimated from the reactivity with GMP *vs.* time (see Results).

9-^{14}C-Labeled IIb, specific radioactivity 0.45 mCi/mmole, was synthesized from 9-^{14}C-labeled IIa by treatment with sodium methoxide in methanol as above.

Reaction of 9-^{14}C-Labeled IIb with Nucleic Acids. A solution of yeast tRNA or heat-denatured calf thymus DNA in 0.5 ml of 0.10 M NaCl–0.02 M Tris-HCl (pH 7.7, 25°) was added to 1.5 mg of solid 9-^{14}C-labeled IIb. After stirring at 25° for about 5 min, the mixture was left at 38° for 90 min. The nucleic acids were precipitated by the addition of 3 volumes of cold ethanol, then dissolved and reprecipitated four times. The nucleic acids were then dissolved in 1 ml of 0.10 M NaCl–0.02 M Tris-HCl (pH 7.7) and extracted twice with equal volumes of water-saturated phenol. The aqueous phase was extracted with ether, and the nucleic acids were precipitated by the addition of 3 volumes of cold ethanol. In one experiment, the tRNA was purified by chromatography on a column of Sephadex G-25 (0.9 × 60 cm) instead of by phenol extraction. Elution was carried out with 0.10 M NaCl–0.02 M Tris-HCl (pH 7.7).

Reaction of IIb with GMP. To 0.5 mg (1.3 μmole) of solid IIb was added 0.2 ml of a solution of GMP-^{14}C in 0.1 M Tris-HCl (pH 7.7, 25°). The molar ratio of GMP-^{14}C to IIb is given in the tables. The mixtures were stirred at 25°

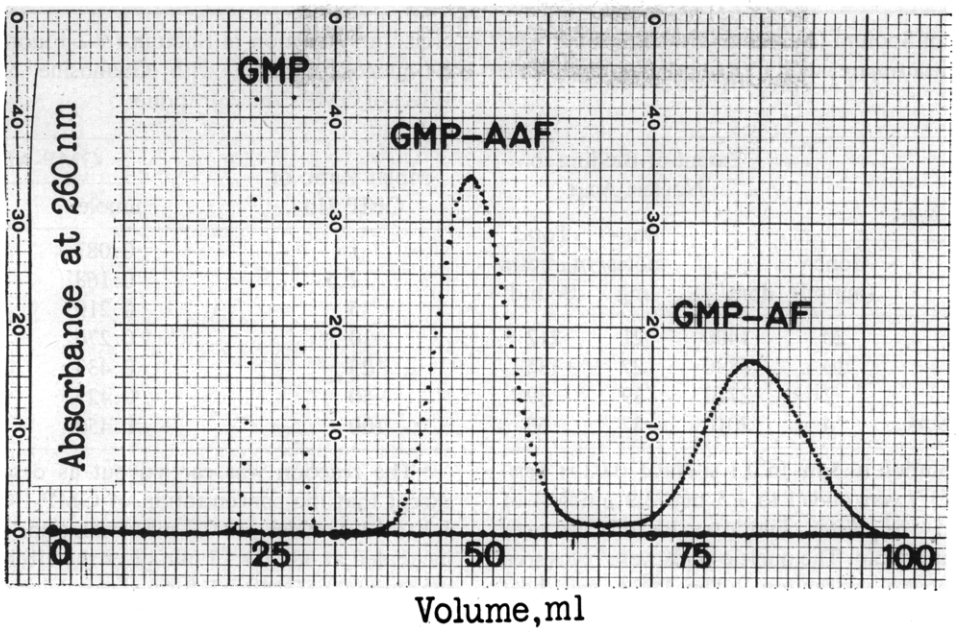


FIGURE 3: Separation of guanosine 5′-monophosphate (GMP), 8-(*N*-2-fluorenylacetamido)guanosine 5′-monophosphate (GMP-AAF), and 8-(*N*-2-fluorenylamino)guanosine 5′-monophosphate (GMP-AF) by column chromatography on Sephadex G-25. Elution was carried out with 0.1 M ammonium bicarbonate–2 M urea (pH 9). See text for details.

for several minutes until IIb had completely dissolved. After incubation at 38° the mixtures were diluted to 2 ml with 0.1 M ammonium bicarbonate–2 M urea (pH 9) and a 50-μl aliquot was counted. The remainder of the solution was transferred to a column of Sephadex G-25 (0.9 × 60 cm) which had previously been equilibrated with 0.1 M ammonium bicarbonate–2 M urea (pH 9) (Kriek, 1968). The column was washed with the same buffer at a flow rate of 20 ml/hr. Approximately 120 fractions of 1 ml each were collected directly into counting vials. Each fraction was dissolved in 10 ml of Triton X-100 counting solution and the amount of radioactivity was determined. Comparative studies with IIa were carried out under identical conditions. The elution pattern was established by cochromatography of authentic GMP, GMP-AAF, and GMP-AF under identical conditions (Figure 3). Unreacted GMP was eluted in fractions 20–30, GMP-AAF was eluted in fractions 35–60, and GMP-AF in fractions 70–100. The yield of GMP-AAF or GMP-AF obtained from reaction of IIa or IIb was calculated from the radioactivity in the fractions in which the carriers were eluted. The recovery of radioactivity applied to the columns was greater than 98%. In initial experiments, aliquots of fractions were subjected to thin-layer chromatography on cellulose F in 2-propanol–ammonium hydroxide–water (6:3:1) in order to further substantiate the identity of the product (Kriek, 1968).

Radioactivity Determinations. Radioactivity was determined by liquid scintillation counting in a Packard Model 3375 liquid scintillation spectrometer. Efficiency of counting was determined by the addition of toluene-^{14}C as an internal standard. Aqueous samples containing nucleic acids or the nucleotide derivatives in the presence of high salt concentration were counted in the Triton X-100 system originally described by Benson (1966) and later modified by Williams (1968). The counting solution contained 7.0 g of 2,5-diphenyloxazole, 0.2 g of 1,4-bis-2-(5-phenyloxazolyl)benzene, 350 ml of Triton X-100 (purified for liquid scintillation counting, Packard), and 650 ml of toluene.

Results

Reaction of 9-^{14}C-Labeled IIb with Nucleic Acids. Incubation of 9-^{14}C-labeled IIb with either yeast tRNA or heat-denatured DNA led to the binding of radioactivity to the nucleic acids (Table I). There did not appear to be any significant difference between the extent of binding of radioactivity to tRNA and DNA. The specific radioactivity of the tRNA decreased as the concentration of nucleic acid was increased in the reaction mixture. However, as to be expected, the total amount of radioactivity bound was greater at the higher concentrations of tRNA. At the highest concentration of tRNA studied (20 mg/ml) approximately 5% of the radioactivity added was bound to the nucleic acid.

In contrast to results obtained previously on the reaction of nucleic acids with IIa (Irving *et al.*, 1969a), constant specific radioactivity of the nucleic acids isolated from the reaction mixture with IIb could not be attained by solution and reprecipitation of the nucleic acids. The nucleic acids still contained some highly colored radioactive material which could be removed by phenol extraction or by chromatography on Sephadex G-25. For example, the tRNA from the reaction mixture containing 2 mg of tRNA/ml (Table I) had a specific activity of 34,800 dpm/A_{260} after solution and reprecipitation four times. After purification by phenol extraction, the specific radioactivity was 2710 dpm/A_{260} and after chromatography on Sephadex G-25 the specific activity was 2790 dpm/A_{260}.

Reaction of IIb with GMP. As noted under Experimental Procedures IIb decomposed rapidly in aqueous systems yielding a highly colored solution. Therefore the reaction

TABLE I: Reaction of Sodium (*N*-2-Fluoren-9-^{14}C-ylhydroxylamine β-D-glucosid)uronate with Yeast tRNA and Calf Thymus DNA.[a]

Nucleic Acid		Extent of Binding to Nucleic Acid		
Source	Concn (mg/ml)	dpm/A_{260}	mμmoles/mg	Total mμmoles Bound
Yeast tRNA	20	1480	21	212
Yeast tRNA	10	1890	27	135
Yeast tRNA	2	2710	39	39
Calf thymus DNA[b]	4	2010	32	65

[a] Nucleic acid solution (0.5 ml in 0.1 M NaCl–0.02 M Tris-HCl (pH 7.7) at 25°) was added to 1.5 mg (3.9 μmoles) of solid 9-^{14}C-labeled IIb. After solution, the mixture was incubated at 38° for 90 min. The nucleic acids were recovered and purified by phenol extraction. [b] The DNA was heat denatured at 100° for 10 min, and cooled rapidly in an ice bath.

TABLE III: Reaction of Sodium (*N*-2-Fluorenylhydroxylamine β-D-glucosid)uronate with Guanosine 5′-Monophosphate: Effect of GMP Concentration.[a]

Molar Ratio of GMP:IIb	GMP-AF Formed	
	μmole	% Yield
1	0.087	6.8
2.5	0.168	12.7
5	0.219	16.7
10	0.276	22.6
25	0.433	34.1
50	0.429	34.1
100	0.450	35.4

[a] The reaction was carried out as described in Table II except that the concentration of GMP-^{14}C was varied from 1.3 to 130 μmoles per 0.2 ml of solution. The solutions were incubated at 38° for 15 min after the IIb had dissolved, then the amount of GMP-AF formed was determined.

of IIb with nucleic acids or with GMP could only be detected if the solution of the nucleic acid or GMP were added to the solid IIb. In the reaction of IIb with GMP, a maximum yield of the product, GMP-AF, was obtained when the mixture was analyzed just after solution of the IIb was complete (Table II). The yield was not increased by continued incubation of the reaction mixture at 38°. Addition of the GMP immediately after dissolving IIb in buffer yielded no GMP-AF (Table II).

Increasing the concentration of GMP from 6.5 to 162 mM resulted in an increase in the amount of GMP-AF formed in the reaction of IIb with GMP (Table III). Above 162 mM GMP (molar ratio GMP:IIb = 25 in Table III), the yield of GMP-AF was not increased further.

Competition Studies with AMP, CMP, and UMP. At GMP concentrations which gave maximum yields of GMP-AF in the reaction with IIb, there was no decrease in the yield of GMP-AF when either AMP, CMP, or UMP was added to the system at concentrations equal to the GMP concentration (Table IV). The results of this experiment do not eliminate the possibility of a very low extent of reaction of IIb with AMP, CMP, or UMP. On the other hand, reaction of IIb with these nucleotides is not significant in terms of the extent of the reaction with GMP. Compound IIa and synthetic esters of *N*-acetyl-*N*-2-fluorenylhydroxylamine do not react with cytosine, thymine, or uracil derivatives

TABLE II: Reaction of Sodium (*N*-2-Fluorenylhydroxylamine β-D-glucosid)uronate with Guanosine 5′-Monophosphate: Effect of Time.[a]

Time (min) after Addition of GMP	GMP-AF Formed	
	μmole	% Yield
5	0.416	31.5
30	0.439	33.2
120	0.438	32.4
180	0.446	33.8
Compound IIb dissolved in buffer 5 min prior to addition of GMP	0	0

[a] A solution containing 27.5 μmoles of GMP-^{14}C in 0.2 ml of 0.1 M Tris-HCl (pH 7.7, 25°) was added to 1.3 μmoles of solid IIb. About 3–4 min were required for IIb to dissolve completely. The solution was then incubated at 38° and the amount of GMP-AF present at the times indicated was determined.

TABLE IV: Lack of Competition of Adenosine 5′-Monophosphate, Cytidine 5′-Monophosphate, and Uridine 5′-Monophosphate in the Reaction of Sodium *N*-(2-Fluorenylhydroxylamine β-D-glucosid)uronate with Guanosine 5′-Monophosphate.[a]

Additions	GMP-AF Formed	
	μmole	% Yield
GMP	0.356	28.5
GMP + AMP	0.354	27.8
GMP + CMP	0.374	29.9
GMP + UMP	0.356	28.0

[a] A solution containing 32 μmoles of GMP-^{14}C and, where indicated, 32 μmoles of AMP, CMP, or UMP, in 0.2 ml of 0.1 M Tris-HCl, pH 7.7 (25°), was added to 0.5 mg (1.3 μmoles) of solid IIb. Following incubation at 38° for 2 hr, the amount of GMP-AF formed was determined.

TABLE V: Comparison of the Extent of Reaction of Sodium (*N*-2-Fluorenylhydroxylamine β-D-glucosid)uronate and Sodium (*N*-Acetyl-*N*-fluorenylhydroxylamine β-D-glucosid)uronate with Guanosine 5′-Monophosphate.[a]

Glucuronide Used in Reaction	GMP-AF Formed		GMP-AAF Formed	
	μmole	% Yield	μmole	% Yield
N-GlO-AF	0.413	30.1		
N-GlO-AAF	0.082	5.8	0.039	2.8

[a] The reaction was carried out as described in the footnote in Table II. The molar ratio of GMP-^{14}C to IIb or IIa was 22:1. The amount of product was determined after incubation at 38° for 22 hr.

TABLE VI: Effect of Concentration of Sodium (*N*-Acetyl-*N*-2-fluoren-9-^{14}C-ylhydroxylamine β-D-glucosid)uronate on Its Reaction with Yeast tRNA.[a]

Concn of ^{14}C-Labeled IIa (μmole/ml)	Extent of Binding to Nucleic Acid	
	dpm/A_{260}	mμmole/mg
0.18	1910	5.8
0.45	4300	10.7
0.90	7040	17.5
1.80	10,820	26.9
4.50	15,070	37.4

[a] The reaction mixture contained 1 mg of yeast tRNA in 1 ml of 0.10 M NaCl–0.01 M Tris-HCl (pH 7.7, 25°) containing dextran sulfate (100 μg/ml). ^{14}C-Labeled IIa (specific radioactivity 2.60 mCi/mmole) was added at the concentrations indicated. The samples were incubated at 37° in air for 23 hr and the radioactivity bound to the RNA was determined as previously described (Irving *et al.*, 1969a).

but have been shown to react to a limited degree with adenine residues (Miller and Miller, 1969b).

Comparison of Extent of Reaction of GMP with IIb and IIa. Compound IIa reacts with guanosine yielding a mixture of GuO-AF and GuO-AAF in a ratio of about 2 moles of GuO-AF:1 mole of GuO-AAF (Miller *et al.*, 1968). Compound IIa also reacts with GMP to give GMP-AF and GMP-AAF in a molar ratio of 2:1 (Table V). Because of the instability of *N*-GlO-AF and of the very slow rate of reaction of IIa with GMP and with nucleic acids, it seems meaningless to attempt to compare the rates of reactions of these two related glucuronides with nucleic acids. Compound IIb reacts with GMP and with nucleic acids very rapidly and because of the instability of this glucuronide the reaction is complete in a few minutes (Tables I and II). The yield of product is dependent upon the concentrations of GMP and nucleic acid used in the reaction mixture. On the other hand, IIa reacts slowly with guanosine (Miller *et al.*, 1968), with GMP (these studies, Table V), and with nucleic acids (Irving *et al.*, 1969a), the reaction continuing at a rate linear with time for several days at least. The extent of binding of acetylaminofluorene and aminofluorene residues on reaction of IIa with nucleic acids is markedly dependent upon pH (Irving *et al.*, 1969a,b) and on the concentration of IIa in the reaction mixture (Table VI).

Discussion

The synthesis of an *O*-glucuronide of an arylhydroxylamine has not been described previously in the literature. In several instances the presence of conjugates of arylhydroxylamines in urine following administration of aromatic amines has been reported. However there was no substantial evidence that these conjugates were, in fact, *O*-glucuronides. Kiese *et al.* (1966) described a conjugate of *N*-2-fluorenylhydroxylamine which was excreted in the urine of guinea pigs after administration of 2-aminofluorene. Since *N*-2-fluorenylhydroxylamine was liberated by treatment of the urine with β-glucuronidase, it was implied that the conjugate was IIb. Weisburger *et al.* (1966) have also suggested that small amounts of this glucuronide were excreted in the urine of rats after administration of 2-aminofluorene. It seems doubtful that these investigators actually detected IIb since our studies with authentic IIb show that the compound is extremely unstable in aqueous solutions. Boyland (1963) claimed to have isolated the *O*-glucuronide of 2-naphthylhydroxylamine as a metabolite of 2-naphthylamine from rat and dog urine. However, in a more detailed study published later, no evidence was found for the presence of this glucuronide in urine after administration of 2-naphthylamine or 2-naphthylhydroxylamine to the dog, guinea pig, hamster, rabbit, or rat (Boyland and Manson, 1966).

Several years ago we reported the synthesis and characterization of IIa (Hill and Irving, 1967). This novel type of glucuronide (C–O–N linkage) was shown to be alkali labile. Studies on the mechanism of the instability under alkaline conditions suggested migration of the *N*-acetyl group of the aglycon to the 2′-hydroxyl group of the glucuronic acid moiety. The 2′-*O*-acetyl ester is readily hydrolyzed, yielding *N*-GlO-AF as the product as indicated by the present studies (Figure 4).

Compound IIb, which has now been synthesized and characterized, is very unstable in aqueous solutions. The products of the decomposition have not been characterized. The instability of IIb in aqueous systems is not due to the hydrolysis of this glucuronide to yield *N*-2-fluorenylhydroxylamine (C. C. Irving, unpublished experiments). Like synthetic esters of *N*-acetylarylhydroxylamines (Miller and Miller, 1969a,b; Irving, 1970), IIb reacts with several nucleophilic components of biological macromolecules. The glucuronide reacts with nucleic acids and has been shown to react with guanosine 5′-monophosphate to yield 8-(*N*-2-fluorenylamino)-guanosine 5′-phosphate. Compound IIb also reacts with methionine, yielding 3-methylmercapto-2-aminofluorene, and with tryptophan to yield a product which has not yet been completely characterized (Irving and Russell, 1969; Irving, 1970). These reactions are not contingent upon hydrolysis of IIb to yield *N*-2-fluorenylhydroxylamine, since the latter

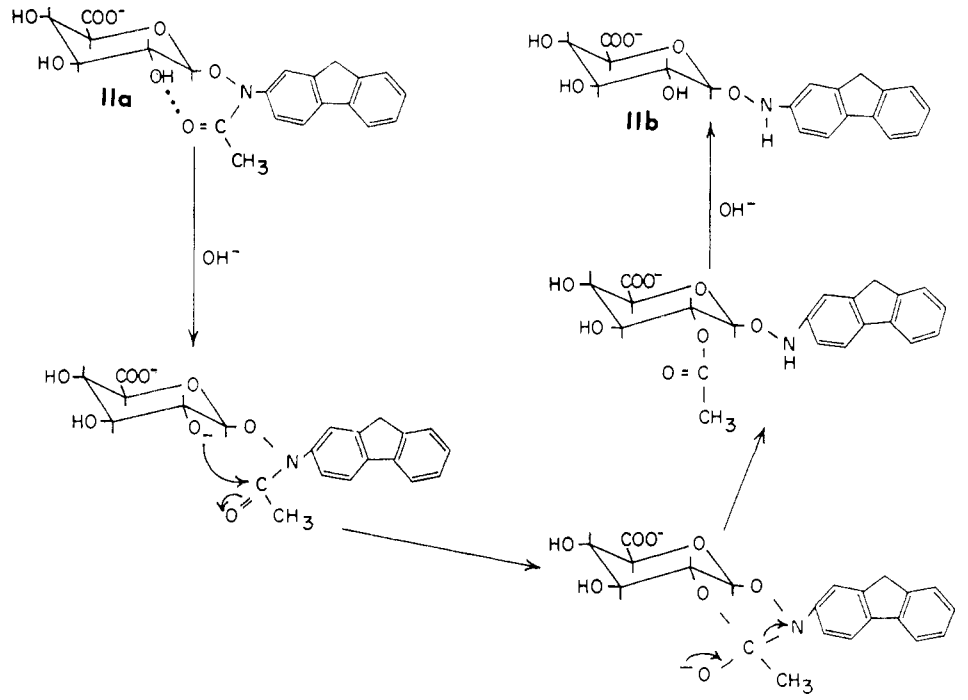


FIGURE 4: Proposed mechanism for the formation of IIb from IIa.

compound shows little reactivity at neutral pH (Miller *et al.*, 1968).

After administration of 2-acetylaminofluorene or *N*-acetyl-*N*-2-fluorenylhydroxylamine to the rat, a major fraction of the fluorene residues bound to liver DNA do not possess the *N*-acetyl group (Irving *et al.*, 1969a,b; Kriek, 1968, 1969). Several interpretations have been offered to account for the lack of the *N*-acetyl group on the fluorene moieties bound to rat liver DNA (Irving *et al.*, 1969b). One alternative involves the reaction of a metabolite not containing the *N*-acetyl group, such as *N*-2-fluorenylhydroxylamine or IIb, or perhaps some unknown ester of *N*-2-fluorenylhydroxylamine. We feel that IIb formed transiently from IIa may account for most of the binding of *N*-acetyl-*N*-2-fluorenylhydroxylamine to rat liver DNA *in vivo*. This belief is based upon presumptive evidence which is discussed elsewhere (Irving *et al.*, 1969b) and is substantiated by the present work in which IIb has been synthesized and shown to react with guanine residues of nucleic acids.

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