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THE METABOLISM OF *N*-2-FLUORENYLHYDROXYLAMINE IN MALE AND FEMALE RATS

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Abstract—Because of its importance as a proximate carcinogen derived from *N*-2-fluorenylacetamide, the metabolic fate of *N*-2-fluorenylhydroxylamine was studied with isotopic techniques. In a 24-hr period after i.v. injection, adult female rats excreted 51 and 9·7 per cent of the dose in urine and feces, males 45 and 3·7 per cent. Liver and kidneys had 9·6 and 0·5 per cent (males), 8·3 and 0·4 per cent (females); liver and kidney proteins had 210 and 118 mμmoles/g (males), 137 and 118 mμmoles/g (females). The urinary metabolites in male rats consisted of sulfuric acid esters, glucosiduronic acids, and unconjugated compounds in decreasing order of magnitude; with females more glucosiduronic acids were found. The metabolites identified were the acetylated derivatives *N*-hydroxy-*N*-2-fluorenylacetamide and 3-, 5-, and 7-hydroxy-*N*-2-fluorenylacetamide, and also 2-amino-7-fluorenol, present in differing amounts in males and females. After zero 6 and 24 hr, increasing amounts of isotope were bound to serum proteins. At 24 hr, erthyrocytes were extensively labeled.

Thus, highly reactive *N*-2-fluorenylhydroxylamine, as evidenced by tissue and serum binding, underwent acetylation followed by the metabolic transformation characteristic of the product, *N*-hydroxy-*N*-2-fluorenylacetamide. An isomerization of the hydroxylamine and a subsequent acetylation may also have occurred.

RECENT investigations of the metabolism of carcinogenic aromatic acylamides, such as *N*-2-fluorenylacetamide (FAA), have implicated the *N*-hydroxy derivatives as being closer to the proximate carcinogen.[1–4] However, the further transformations of these *N*-hydroxy compounds remain to be elucidated, especially since biochemical studies indicate that dehydroxylation[4, 5] or deacylation, possibly followed by oxidation,[6] do occur with *N*-hydroxy-*N*-2-fluorenylacetamide (N—OH—FAA). The metabolism and mechanism of transport of one of these possible intermediates, *N*-hydroxy-2-fluorenyl amine (*N*-2-fluorenylhydroxylamine, N—OH—FA), was therefore examined. Since N—OH—FA is fairly unstable, it was administered intravenously to overcome the possibility that alteration would occur if it were given orally or intraperitoneally. Additionally, this mode would simulate more closely the actual physiological condition in which N—OH—FA is formed in the cell.

MATERIALS AND METHODS

9-^{14}C-Labeled 2-nitrofluorene (Tracerlab, Waltham, Mass.) was reduced to N—OH—FA by the method[4, 7] of Miller *et al.** Because of its relative instability,

* We are grateful to Drs. J. A. Miller and E. C. Miller for information on their improved method.

accentuated by self-irradiation from the isotope, the product was analyzed, stored at −20°, and used within less than 3 days. The isotopic and unlabeled compounds for use as chromatographic standards were described in previous reports.[6, 8, 9]

Fischer 344-strain rats were purchased from the A. R. Schmidt Co., Madison, Wis. Males weighed 250–290 g; females 150–190 g. The labeled compound was injected into the saphenous vein as a fine suspension in 1% gum acacia-isotonic saline. The animals were kept in stainless steel metabolism cages; urine and feces were collected separately. The rats were sacrificed after 24 hr by withdrawing blood from the abdominal aorta under light ether anesthesia, followed by perfusion through the portal vein with isotonic saline solution. The liver and kidney were excised, cooled, and homogenized (1 g/4 ml) in 0·2 M Tris buffer, pH 7·4, and the protein was precipitated by addition of 4 volumes of ethanol and washed according to the procedures of Gutmann *et al.*[10]

The metabolites in urine were determined by our previously published methods[5, 8, 9] employing ether extraction before and after enzymic or acid hydrolysis, and paper or column chromatography for separation and identification. Quantitative data after paper chromatography were secured by cutting out the radioactive spots, localized by autoradiography, and counting the strip. Reference compounds were run with each chromatogram; color tests or ultraviolet spectroscopy aided in confirming the identity of metabolites.

For the serum studies, blood was withdrawn from the abdominal aorta by the usual procedures, zero (2–3 min in practice), 6, or 24 hr after i.v. injection of the labeled compound. The serum was isolated by centrifugation, and an aliquot was counted. The red cells were washed by careful resuspension in 0·9% NaCl solution and centrifugation. They were lysed, and the proteins precipitated with 4 volumes ethanol, washed,[10] and counted.

Serum samples were applied to columns (1·3 × 40 cm) of Sephadex G-25 prepared in 1 M NaCl solution. The elution of material absorbing at 280 mμ was monitored with a Vanguard automatic ultraviolet analyzer. Fractions of 6 ml were collected, and aliquots were counted. The ^{14}C-elution curve was matched with the u.v. absorbance curve, yielding three fractions. Fraction I, containing macromolecular material was eluted between 30 and 90 ml; a minor intermediate fraction II between 90 and 125 ml; and fraction III with the retarded smaller molecular species, between 140 and 270 ml. Related experiments indicated that a portion of the high-molecular weight fraction I contained low-molecular weight carcinogen metabolites (especially at the zero-time period), which were loosely bound, being dialyzable, or more simply ether-extractable. Thus, fraction I was extracted with ether to determine the amount of such materials.

Carbon-14 was measured on a liquid scintillation counter, with a toluene, methanol, PPO, and POPOP mixture for liquids. Solids such as proteins or homogenates were digested in Hyamine prior to counting.[5]

RESULTS

Distribution of radioactivity. In the 24-hr experimental period after a single i.v. injection of N-OH-FA, male rats excreted somewhat less of the dose (50 per cent) than females (60 per cent), but in turn had somewhat higher tissue and blood levels (Table 1).

TABLE 1. DISTRIBUTION OF RADIOACTIVITY 24 HOURS AFTER INTRAVENOUS INJECTION OF *N*-2-FLUOREN-9-^{14}C-YLHYDROXYLAMINE INTO RATS*

Sex	Urine				Feces	Liver	Kidneys	Liver protein	Kidney protein	Blood serum	Red cells
	Total ^{14}C	Metabolites									
		Free	Glucuronides	Sulfates							
	(% of dose)							(mμmole/g)†			
Male	45	2·3	13 14·5‡	20 21‡	3·7	9·6	0·5	210	118	7·3	170
Female	51	1·2	23 22‡	15 18‡	9·7	8·3	0·4	137	118	3·4	

* Six male and six female rats were used. The average body weight for males was 278 g, for females 170 g. The dose was 10 mg/kg body weight. Each male received a dose of 2·78 mg (14·1 μmoles) with $1·74 \times 10^6$ counts/min; each female 1·70 mg (8·6 μmoles) with $1·07 \times 10^6$ counts/min. The data are averages of individual results.

† Data for blood serum and red cells expressed as mμmoles/ml original blood sample; for liver and kidney proteins as mμmoles/g dry protein.

‡ Values obtained by chromatography on DEAE-cellulose of whole urine.[5]

Erythrocytes bound sizable amounts of radioactivity. On a dry weight basis about 1 μmole/g was located therein. The blood serum level was much lower; in females it was about half that in males, as was the amount of isotope tightly bound with liver proteins. Kidney proteins on the other hand were labeled to a similar extent in male and female rats.

Urinary metabolites. Similar data were obtained by two different techniques: (1) solvent extraction after specific enzymic and acid hydrolysis of urine; (2) chromatography of the total urine on DEAE-cellulose. A small portion of the urinary radioactivity was present as free unconjugated metabolites and sizable amounts as glucosiduronic acids and sulfuric acid esters. Males and females exhibited a contrary pattern. Conjugation with glucuronic acid was low in the males and high in the females, whereas the reverse held for sulfuric acid conjugates.

The individual metabolites in the free fraction, and sulfuric acid conjugate fraction after acid hydrolysis were identified and quantitated by paper chromatography. The generally more complex glucuronides were resolved first by column chromatography followed by paper chromatography of the individual fractions (Fig. 1 and Table 2). A major metabolite excreted as free compound as well as in conjugated form was N-(7-hydroxy-2-fluorenyl)acetamide (7-OH—FAA). It was present in larger amounts in males than in females, chiefly because of the high sulfate ester content of the urine of male animals. Next in quantitative importance was the 5-hydroxy derivative. N—OH—FAA, as glucuronide, was present in sizable amounts in females, but the urine of males contained much less of this compound. The column fraction II (Fig. 1), when chromatographed on paper, contained not only a compound identified as 3—OH—FAA by mobility on paper and u.v. spectroscopy but had also a second band with a mobility of 0·32–0·39. Chromatography of authentic N—OH—FA under these conditions gives three spots, the main one at the origin probably representing evidence of decomposition, the other two with the mobilities of 2-fluorenamine (0·32–0·40) and of 2-nitrosofluorene (0·84–0·94). Thus, the presence of a small amount

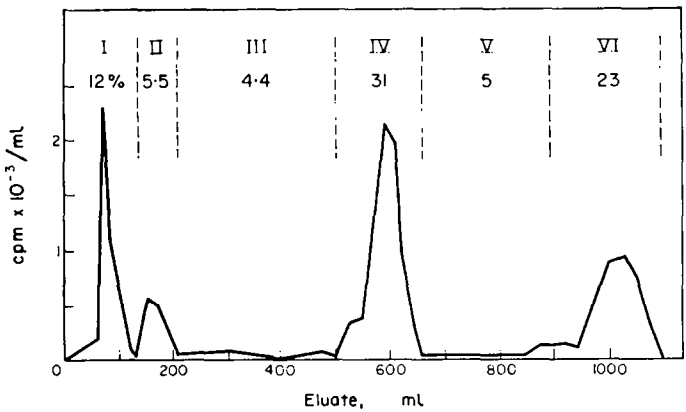


FIG. 1. Chromatographic resolution of the ether-soluble urinary metabolites of *N*-2-fluorenylhydroxylamine after enzymic hydrolysis of the glucosiduronic acids. An ethanol solution of the sample was applied to a 2 × 26 cm column of silicic acid and fractions eluted by percolation of a solvent system of cyclohexane:*tert.* butanol:acetic acid:water (16:4:2:1, v/v). Fractions of 16 ml/hr were collected and aliquots counted. Appropriate tubes were combined, concentrated, and rechromatographed on paper together with appropriate standards. Fraction I was *N*-hydroxy-*N*-2-fluorenylacetamide; II, 3-hydroxy-FAA and also a material with mobility on paper of 0·32–0·39 (see text); IV, 5-hydroxy-FAA; VI, 7-hydroxy-FAA.

of material with this mobility in the hydrolyzed glucosiduronic acid fraction suggests that some N—OH—FA glucuronide was present. Quantitation is inaccurate, however, because of the instability of the compound. Irving[11] surmised the possible presence of a glucuronide of N-2-fluorenylhydroxylamine formed by the action of a 10,000*g* rabbit liver supernatant on N—OH—FAA.

TABLE 2. URINARY METABOLITES OF *N*-2-FLUORENYLHYDROXYLAMINE IN RATS

Identity*	Mobility† × 100	Free compounds		Glucuronides		Sulfates	
		♂	♀	♂	♀	♂	♀
				(% of dose)			
7—OH—FA	1–4·5			0·9	2·3	2·8	2·7
7—OH—FAA	13–21	1·0	0·36	6·0	8·5	12	6·6
5—OH—FAA	24–33	0·4	0·18	3·9	7·8	1·2	1·2
	32–39			0·14	0·43		
3—OH—FAA	48–56	0·07	0·06	0·2	0·66	0·8	0·98
N—OH—FAA	62–71	0·22	0·16	0·59	2·5	0·4	0·58

* 7—OH—2—FAA is *N*-(7-hydroxy-2-fluorenyl)acetamide; 7—OH—FA is 2-amino-7-fluorenol.

† Mobility measured from front to back of spot in our standard chromatographic system run on strips 7 mm wide (Whatman 3MM paper; solvent, cyclohexane:*tert.* butanol:acetic acid:water, 16:4:2:1). The metabolites of standard compounds, determined simultaneously, were identical with those of the corresponding metabolites. The free compounds and sulfuric acid conjugates (after hydrolysis) were estimated directly on the paper chromatograms. The glucosiduronic acid derivatives (after enzymic hydrolysis) were first resolved on a silicic acid column, and the quantitative data shown were secured thus (see Fig. 1). The qualitative identification was performed by paper chromatography of each column fraction, yielding the classification in the table.

Transport in blood. The compound and its metabolites were rapidly cleared from blood after the intravenous injection (Table 3). Fractionation on Sephadex at zero time indicated that about 50 per cent of the radioactivity was associated with the macromolecular components of blood serum, and half of that again was in a loosely bound ether-extractable form. Six and 24 hr later virtually all the radioactivity migrated with the macromolecular fraction, and very little was loosely bound. The specific activity of the serum protein was highest at zero time, decreased about half from 0 to 6 hr, and then leveled off. Considerable radioactivity, about 0·1 μmole/ml blood, was also associated with the red cells (see also Table 1).

TABLE 3. DEMONSTRATION OF BINDING OF RADIOACTIVITY FROM N—OH—FA—^{14}C TO SERUM PROTEINS BY GEL FILTRATION*

	Hours after intravenous dose		
	0	6	24
Weight of rats (g)	256	263	262
Dose (mg)	3	3	3
Dose (counts/min × 10^{-7})	5·89	5·89	5·89
Blood (% of dose)	10·6	4·5	5·8
Serum (counts/min × 10^{-4}/ml blood)	7·5	1·6	1·3
Serum (mμmoles/ml blood)	19	4·1	3·3
Sephadex fraction I† (% of serum)	46	95	98
Sephadex fraction II (% of serum)	13	5·2	2·3
Sephadex fraction III (% of serum)	41		
Fraction of I ether-soluble (%)	49	7·8	4·8
^{14}C on fraction I serum protein (mμmoles/g)	156	79	62
Conc. of serum proteins (mg/ml)	104	108	133

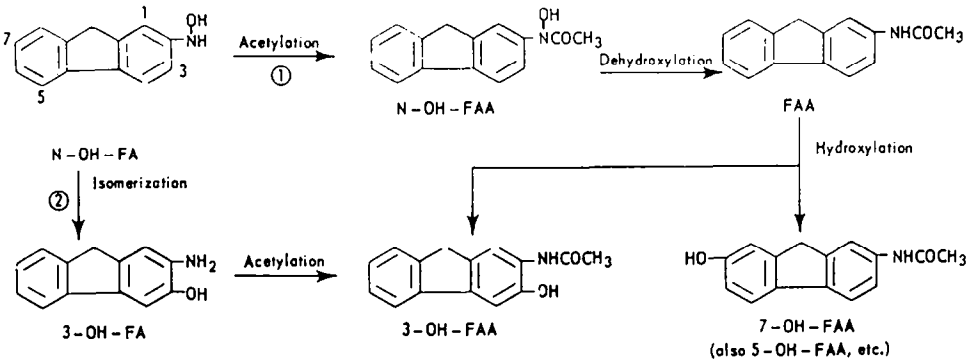
* Each series represents average data on two male young adult rats. Gel filtration procedure is described in text.
† See text.

DISCUSSION

In our earlier studies of the metabolism of N—OH—FAA we found specific differences between male and female rats.[5] These consisted in a faster rate of excretion of the compound, the presence of more metabolites as glucosiduronic acids and less as sulfuric acids, the excretion of more N—OH—FAA and less ring-hydroxylated FAA in female rats, and finally higher tissue levels in males. Our experiments now reported with the deacetylated form of N—OH—FAA, namely N—OH—FA, correspond exactly to this pattern.

The metabolites of N—OH—FA identified in urine were mostly in the form of acetyl derivatives. Miller *et al.*[12] have presented similar evidence. Just as the aromatic amines themselves are excreted chiefly as acetamides in most species except the dog,[7] there appears to be the same type of acetylating mechanism involving acetyl coenzyme A operating on N—OH—FA. The question arises whether the ring-hydroxylated metabolites of N—OH—FA excreted were (1) the result of acetylation, *N*-dehydroxylation, followed by hydroxylation on a ring carbon atom, or (2) whether migration of the hydroxyl from the nitrogen to a ring carbon was succeeded by acetylation of the resulting C-hydroxyfluorenamine. Miller and Miller[13] have presented evidence that N—OH—FAA can rearrange to 1-OH—FAA and to a slight extent

to 3-OH—FAA. Booth and Boyland[14] have also described an isomerase performing this reaction. However, the major 5- and 7-hydroxy derivatives probably do not arise from a direct rearrangement but would more likely result from direct enzymic hydroxylation of FAA (scheme 1).[13] This point is favored also by the occurrence of appreciable amounts of N—OH—FAA, obviously resulting from the direct acetylation of the hydroxylamine, the first proposed step 1 of scheme 1.



Because of the presence in many tissues of deacylases converting the acetyl derivative to the hydroxylamine, it seems logical that the acetyl derivative serve as a transport form for the more reactive, locally liberated hydroxylamine. Our present data with the hydroxylamine are in agreement with this concept. Because of the increased reactivity of the hydroxylamine we have resorted to intravenous injection of the compound. Even so, we have after 24 hr found similar levels of metabolites bound to liver proteins with the hydroxylamine and its acetyl derivative[15] (210 and 196 mμmoles/g respectively). In contrast however, zero-time binding to serum proteins and especially to erythrocytes was high with N—OH—FA and low with the acetyl derivative (manuscript in preparation). After 1 hr both compounds gave similar levels, perhaps as a result of deacetylation of N—OH—FAA. These observations suggest that the binding is limited by the availability of appropriate receptor sites. In long-term studies with 2-naphthylamine, red cells contained more activity than plasma,[16] perhaps because of metabolic conversion to 2-naphthylhydroxylamine.[2] Crick and Jackson[17] reported the specific localization in erythrocytes and firm binding to hemoglobin of *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene.

We have demonstrated that N—OH—FAA and FAA gave significant amounts of bound metabolites associated with the albumin fraction of serum. This likely is also true for N—OH—FA. Similar results were reported for FAA by Bahl and Gutmann[18] and for 3'-methyl-4-dimethylaminoazobenzene by Dijkstra and Joubert.[19] Also, Avigan[20] noted that FAA was found in the higher-density hydrophilic serum proteins, whereas carcinogenic hydrocarbons tended to migrate with the serum lipoproteins.

Miller and associates[12] have reported that fluorenylhydroxylamine as well as nitrosofluorene caused tumors at the point of subcutaneous injection, suggesting that these compounds are close to the active proximate or ultimate carcinogen. Goodall[21] and Miller *et al.*[22] and Poirier *et al.*[23] have also reported that the acetyl derivative either

as such or as the copper chelate did produce local cancers. In view of the probable presence of deacylases in the subcutaneous tissue the acetyl derivative can be converted there to the hydroxylamine. Also, the relative instability of this latter compound to oxidative conditions would readily yield decomposition products at or near the site of injection, whereas the enzyme system leading to the more stable acetyl derivative is located virtually exclusively in liver.

The data reported here are consistent with the concept that N—OH—FAA is an active intermediate in the carcinogenic process from FAA, and that it is further metabolized to even more reactive compounds such as N—OH—FA which in turn readily affect, after enzyme-mediated or spontaneous chemical interaction, a number of host receptors.* Determination of the latter remains the important problem for the future.

REFERENCES

1. E. C. Miller, J. A. Miller and H. A. Hartmann, *Cancer Res.* **21**, 815 (1961).
2. E. Boyland, C. E. Dukes and P. L. Grover, *Br. J. Cancer* **17**, 79 (1963).
3. R. W. Baldwin and W. R. D. Smith, *Br. J. Cancer* **19**, 433 (1965).
4. P. D. Lotlikar, E. C. Miller, J. A. Miller and A. Margreth, *Cancer Res.* **25**, 1743 (1965).
5. E. K. Weisburger, P. H. Grantham and J. H. Weisburger, *Biochemistry* **3**, 808 (1964).
6. P. H. Grantham, E. K. Weisburger and J. H. Weisburger, *Biochim. biophys. Acta* **107**, 414 (1965).
7. L. A. Poirier, J. A. Miller and E. C. Miller, *Cancer Res.* **23**, 790 (1963).
8. J. H. Weisburger, E. K. Weisburger, H. P. Morris and H. A. Sober, *J. nat. Cancer Inst.* **17**, 363 (1956).
9. J. H. Weisburger, P. H. Grantham, H. P. Morris and E. K. Weisburger, *Cancer Res.* **21**, 949 (1961).
10. H. R. Gutmann, U. S. Seal and C. C. Irving, *Cancer Res.* **20**, 1072 (1960).
11. C. C. Irving, *J. biol. Chem.* **239**, 1589 (1964).
12. E. C. Miller, C. W. Cooke, P. D. Lotlikar and J. A. Miller, *Proc. Am. Ass. Cancer Res.* **5**, 45 (1964).
13. E. C. Miller and J. A. Miller, *Biochim. biophys. Acta* **40**, 380 (1960).
14. J. Booth and E. Boyland, *Biochem. J.* **91**, 362 (1964).
15. Y. Shirasu, P. H. Grantham, R. S. Yamamoto and J. H. Weisburger, *Cancer Res.* **26**, 600 (1966).
16. A. F. Henson, A. R. Somerville, M. E. Farquharson and M. W. Goldblatt, *Biochem. J.* **58**, 383 (1954).
17. J. Crick and H. Jackson, *Br. J. Pharmac.* **8**, 87 (1953).
18. O. P. Bahl and H. R. Gutmann, *Biochim. biophys. Acta* **90**, 391 (1964).
19. J. Dijkstra and F. J. Joubert, *Br. J. Cancer* **15**, 168 (1961).
20. J. Avigan, *Cancer Res.* **19**, 831 (1959).
21. C. M. Goodall, *Nature Lond.*, In press (1966).
22. J. A. Miller, M. Enomoto and E. C. Miller, *Cancer Res.* **22**, 1381 (1962).
23. M. M. Poirier, J. A. Miller and E. C. Miller, *Cancer Res.* **25**, 527 (1965).
24. E. Kriek, *Biochem. biophys. Res. Commun.* **20**, 793 (1965).

* One such possible target has been identified recently in model experiments by Kriek[24] who demonstrated that *N*-2-fluorenylhydroxylamine reacted readily with yeast-s-RNA and with calf thymus DNA.