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THE METABOLISM OF *N*-2-FLUORENYLACETAMIDE IN THE CAT: EVIDENCE FOR GLUCURONIC ACID CONJUGATES

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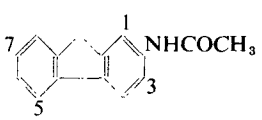
Abstract—The urine and feces of adult female cats contained 20% and 20–30%, respectively, of the isotope from a single dose of ^{14}C-labeled *N*-2-fluorenylacetamide (FAA) in a 2- to 5-day period. The urinary metabolites consisted of 5% unconjugated compounds, 60–80% sulfuric acid conjugates, and about 15% glucuronic acid conjugates. There was unambiguous evidence for the latter class of metabolites. The free compounds were composed chiefly of FAA and its 7-hydroxylated derivatives; the sulfuric acid fraction contained mainly the 7-hydroxy derivative and the glucuronic acid fraction the 5-, 7-, and N-hydroxy derivatives. Small percentages of a dose were found in the tissues examined, some in a protein-bound form.

ACCORDING to present concepts, cats are not considered to possess an appreciable ability to detoxify hydroxylated compounds by conjugation with glucuronic acid.[1, 2] The missing link appears to be a deficiency of glucuronyl transferase in cat liver.[3]



Since many species metabolize the carcinogen *N*-2-fluorenylacetamide (2-acetylaminofluorene, FAA) by hydroxylation on the ring and on the nitrogen, followed by conjugation of these hydroxylated products with glucuronic acid,[1–6] it seemed of interest to study the fate of this compound in the cat because of the presumed absence of this particular pathway. With FAA, cats exhibited tumor formation preferentially in the lung,[7, 8] in contrast to most other types of animals in which liver and mammary gland were the major sites affected.[4]

The present paper deals with the metabolic fate of FAA in female cats. It will be shown that ring and N-hydroxylation occurred, as in other species, but that the major fraction of the urinary metabolites was in the form of sulfuric acid conjugates. However, about 10–15% of the urinary radioactivity was in a form which not only was specifically hydrolysed by β-glucuronidase, but which was chromatographically proved to be glucuronides. Thus the cat may have limited ability to convert certain exogenous materials to glucuronic acid conjugates.

* With the capable technical assistance of E. Vanhorn and A. Parker.

MATERIALS AND METHODS

Three female cats obtained from the Animal Hospital of the National Institutes of Health and weighing 2·0, 2·0, and 3·4 kg, respectively, were injected intraperitoneally with *N*-2-fluoren-9-^{14}C-yl-acetamide as a suspension (Potter–Elvehjem glass homogenizer) in a 1% gum acacia solution. Two different specific activities at two dose levels were employed. The animals were housed in a stainless steel metabolism cage which permitted separate collection of urine and feces. The urine was allowed to run into a receiver immersed in ice in a Dewar flask. The feces were removed at intervals. Upon termination of an experiment the cages were washed and the washings counted. Food and water were freely available.

The animals were sacrificed under ether anesthesia by withdrawal of blood from the abdominal aorta, after which the cat was perfused with the pumping action of the heart by inserting a cannula into the portal vein, providing a flow of isotonic saline.

Blood was allowed to coagulate, and serum and red cells were separated by centrifugation. Red cells were washed by gentle resuspension in 0·9% sodium chloride solution. The liver, kidney, and lungs of the cats were removed. These organs were homogenized in a 0·2 M acetate buffer, pH 5. Aliquots of the homogenates were counted to give the total tissue activity. The remainder of the homogenates was worked up by published methods[9] to isolate the proteins which were washed and extracted with ethanol for the purpose of determining radioactivity bound to the proteins.

Classification of Urinary Metabolites[10, 11]

(1) A sample of urine diluted with an equal volume of water (1:1) was made 50% in ammonium sulfate. An extraction with ether–ethanol (3:1) was performed, and the extract was percolated through a column of alumina prepared in benzene. The initial eluate contained the free metabolites. After washing the column with ethanol followed by 50% aqueous ethanol, the sulfuric acid conjugates were removed. Glucuronic acid conjugates were subsequently displaced with sodium citrate buffer. Details of the procedure and evidence for the structure of the metabolites in the various fractions have been presented.[11]

(2) Samples of urine buffered to pH 7 were extracted five times with equal volumes of ether, thereby removing the free compounds. The aqueous phase was incubated in the presence of a small amount of chloroform with bacterial β-glucuronidase (type II, Sigma Chemical Co., St. Louis, Mo.) at 38° for 18 hr and extracted again with ether. The materials thus liberated were considered to have been glucuronic acid conjugates. The aqueous phase was next adjusted to pH 1 and refluxed for 20 min. After returning the pH to 7 the ether extracts contained materials formerly conjugated with sulfuric acid.

Aliquots of all fractions obtained by the two methods were chromatographed on Whatman 3MM paper by the ascending method in two solvent systems— (1) butanol:3% ammonium hydroxide (3:1); (2) cyclohexane: *tert*-butanol: acetic acid: water (16:4:2:1).[10] Appropriate reference compounds were chromatographed at the same time.[12] The chromatograms were exposed to Kodak Royal Blue X-ray film for suitable periods of time, usually 1–3 weeks. The developed film indicated the location of the radioactive metabolites which were cut out from the paper strip and counted in the toluene–methanol scintillation mixture for the determination of the relative

amounts of material present. Ultraviolet spectra and color tests were additional criteria to confirm the structure of the metabolites so obtained.

Determination of Radioactivity

Aqueous solutions such as urine were added to a scintillation counting mixture composed of 3 g/l 2,5-diphenyloxazole (PPO), 0·1 g/l 1,4-*bis*-2-(5-phenyloxazolyl)benzene (POPOP), 700 ml/l toluene, and 300 ml/l methanol. Counting was performed in a 10–100 V window in a liquid scintillation spectrometer set at optimal voltage. Tissue homogenates, dry proteins, and feces were dissolved in 3 ml Hyamine on a shaking water bath at 60° for 4 hr after which 12 ml of a scintillation mixture composed of 4 g/l PPO and 0.1 g/l POPOP in 1 l. toluene was added. In a few cases, aliquots of dry samples to be counted were suspended in a scintillation fluid containing Cab-o-sil and the PPO,POPOP:toluene system. Furthermore, Schoeninger-type combustion was employed for materials with a high quenching factor such as blood.[13] Corrections were made for background, quenching, and efficiency (internal standard).

RESULTS

Intraperitoneal injection of a small dose of labeled FAA resulted in the excretion of about 22 % of the dose in the urine and 28 % in the feces in a 48-hr period (Table 1). Larger doses were eliminated less speedily, and the fecal route seemed to be favored

TABLE 1. EXCRETION OF RADIOACTIVITY BY FEMALE CATS AFTER A SINGLE DOSE OF *N*-FLUOREN-9-^{14}C-YL=ACETAMIDE

Cat	1*	2†	3‡	1*	2†	3‡
Time (hr)		Urine			Feces	
0–22	16	0·4	2·3	28	0·3	19
22–48	5·8	22	7·7		18	
48–72		2·0	4·5		13	
72–96		0·3				
96–120						

* All data are expressed as per cent of dose. The dose was 14·1 mg or 4·79 × 10^8 cpm 2-FAA; the cat weighed 2·0 kg.

† The dose was 65·7 mg or 1·54 × 10^8 cpm 2-FAA; the cat weighed 2·0 kg.

‡ The dose was 102·8 mg or 2·40 × 10^8 cpm 2-FAA, but upon autopsy a depot with 5·6 × 10^7 cpm of unchanged material was found along and around the needle track through the skin. The data for cat 3 were corrected for unabsorbed material. The cat weighed 3·4 kg.

even more. In any case, the absorption of the injected material and the excretion proceeded relatively slowly as compared to a species such as the rat.[12] In cat 3 unabsorbed material was observed in the area of the injection.

Two different methods were used to study the nature of the urinary metabolites. The data obtained were in reasonably good agreement, thus supporting the reality of the classification (Table 2). All three cats studied gave a similar picture even though

the dose in the first cat was considerably lower than that in the other two. About 5% of the urinary radioactivity consisted of unconjugated compounds. The major part of the metabolites was in the form of sulfuric acid conjugates amounting to 60–80% of the urinary radioactivity. However, most interesting is the fact that both methods of analysis showed that about 15% of the urinary radioactivity was in the form of

TABLE 2. URINARY METABOLITES CLASSIFIED AS FREE, SULFURIC ACID ESTERS AND GLUCOSIDURONIC ACIDS

Cat		1	2	3
			Urinary isotope (%)	
Free compounds	a*	5	9·5	5
	b†	8	5	5
Sulfates	a*	61	56	56
	b†	78	79	75
Glucuronides	a*	13	13‡	14
	b†	8	15	20

* The classification was made on the basis of ether solubility of metabolites of buffered urine (free compounds), after action of β-glucuronidase, and after mild acid hydrolysis.[10]

† The classification was made by virtue of separation on an alumina column.[11]

‡ A control experiment involving ether extraction of a urine sample incubated with and without β-glucuronidase rendered 12 and 1·3% of the isotope extractable.

glucuronic acid conjugates. The bacterial β-glucuronidase used in the enzymatic hydrolysis of the urinary sample was a semipurified preparation and may have contained activities other than β-glucuronidase. However, our past experience with this kind of material suggests that it is nevertheless a fairly specific enzyme system with respect to the hydrolysis of β-glucosiduronic acids. Omission of the enzyme in a control experiment liberated but a fraction of the isotope in the sample studied. Furthermore, the alumina column method has yielded similar quantitative results. This technique was established as a rather clear-cut mode of separation with this series of metabolites.[11]

Examination of the paper chromatograms of a urine or of the aqueous phases after specific enzymatic or acid hydrolysis supports strongly the concept that glucuronic acid conjugates, sulfuric acid conjugates, and free compounds were present in the amounts indicated by the previous two methods. Thus there are two radioactive spots corresponding to the free compounds (Table 3). The two major areas correspond to the sulfuric acid conjugates of *N*-(7-hydroxy-2-fluorenyl)acetamide and 2-amino-7-fluorenol.[10, 14] In this same area can be found the glucuronide of the *N*-hydroxy-2-FAA, whereas the glucuronides of the 5- and 7-hydroxy derivative were clearly visible with a mobility of 0·065–0·13.[11, 14]

Further detailed information on the nature of the urinary metabolites was adduced by paper chromatography of the ether extracts of β-glucuronidase—incubated urine

TABLE 3. SEPARATION OF AQUEOUS URINARY METABOLITES BY PAPER CHROMATOGRAPHY

Spot number	Mobility × 100	Identity	Relative amounts (%)
3	6·5–13	*N*-(5- and 7-hydroxy-2-fluorenyl) acetamide glucuronide	7·7
8a	40 –46	*N*-(N-hydroxy-2-fluorenyl)acetamide glucuronide*	46
8b	40 –46	*N*-(7-hydroxy-2-fluorenyl)acetamide sulfate	
9	49 –55	2-Amino-7-fluorenol sulfate	15
11	81 –87	Unconjugated metabolites†	3·0
12	92 –98	Unconjugated metabolites†	3·3

Samples of urine chromatographed on Whatman 3MM paper in *sec*-butanol:3% ammonium hydroxide (3:1). Only the identified spots are shown, expressing the relative amount in terms of total activity chromatographed. The mobility is expressed from front to back of spot.

* After enzymatic hydrolysis of the glucuronide fraction, the ratio of spots 8 and 9 was changed from 46:15 to 29:15. The *N*-hydroxy-FAA glucuronide thus amounted to 17%.

†Resolved better in the cyclohexane solvent system (Table 4).

and of acid-hydrolysed urine. The paper chromatogram of the free compounds shows that unchanged FAA is the major component, next follows the 7-hydroxy derivative, then the 2-amino-7-fluorenol. Minor components consisted of the 5-hydroxy derivative and an unknown component which might be either the 3-hydroxy derivative of FAA or 2-fluorenamine (Table 4).

The major part of the sulfuric acid fraction was the 7-hydroxy derivative of FAA. The sulfate of 2-amino-7-fluorenol was present in smaller quantities.

The *N*-hydroxy-2-FAA was the main metabolite in the glucuronic acid fraction, amounting to 37%. Next in rank was the 5-hydroxy derivative while the 7-hydroxy derivative and 2-amino-7-fluorenol were present in small amounts.

Analysis of the liver, kidneys, and lungs for radioactivity showed decreasing amounts, in that order, at all time periods surveyed (Table 5). Of interest was the fact that some of the radioactivity was in the form of ethanol nonextractable tissue-bound

TABLE 4. PAPER CHROMATOGRAPHY OF ETHER-SOLUBLE METABOLITES

Compound*	Mobility × 100		Free	Sulfate	Glucuronide
	Reference	Metabolite	(% of fraction)		
7–OH–FA	0–3	0–3	18	29	14
7–OH–FAA	4–12	4–14	22	60	14
5–OH–FAA	10–25	10–22	11		22
FAA	57–72	60–81	33		
N–OH–FAA	80–91	68–89			37

The mobility was measured from front to back of spot. Only the significant, identified compounds are listed.

* 7–OH–FA is 2-amino-7-fluorenol (or 7-amino-2-fluorenol), 7–OH–FAA etc. are *N*-(7-hydroxy-2-fluorenyl)acetamide, etc.

form. Again, the liver had the highest amount, and the lung the smallest amount, with the kidney assuming an intermediate position. The total activity in the blood serum was rather similar to that found protein-bound to the lung. Incidentally, 12–20% of the isotope in the blood was found in the thoroughly washed red cells, whereas the remainder was transported by the blood serum.

TABLE 5. TISSUE RADIOACTIVITY IN FEMALE CATS AFTER A SINGLE DOSE OF *N*-2-FLUOREN-9-^{14}C-YLACETAMIDE

Time (hr)	Dose (mg/kg)	Liver (% of dose)	Kidney (% of dose)	Lung (% of dose)	Liver proteins (mμmole/g)†	Kidney proteins (mμmole/g)†	Lung proteins (mμmole/g)†	Blood* serum (mμmole/ml)
48	7	1·3	0·2	0·05	68	30	6·6	6·6
96	30	1·1	0·2	0·05	103	42	14	15
120	30	0·65	0·12	0·03	61	35	9·6	7·1

* 12–20% of the blood isotope was located in the red cells, the remainder in the serum.
† Calculated on the basis of the compound administered.

DISCUSSION

The studies show that in the cat, as in the dog,[6] the metabolites of FAA are excreted about equally in the stools and in the urine. In contrast, in other species such as the rat,[12, 15] hamster,[16] guinea pig,[12] and man[17] the materials are eliminated predominantly via the urine. Even though the experiments extended for as long as 5 days, not all the radioactivity was excreted in that period of time. In fact, recovery of the dose in these instances was lower than in our many other studies performed in this area. Cage washing accounted for only a small fraction of the missing radioactivity.

Numerous earlier approaches have provided evidence for the concept that cats do not eliminate detectable amounts of phenolic metabolites as glucosiduronic acids.[1, 2] Noteworthy, therefore, is the finding of a small but reliably established pathway of glucuronic acid conjugation. In the past it was believed that this particular route of detoxification of exogenous materials was not used by cats by virtue of a deficiency of glucuronyl transferase. Our sensitive and specific methods suggest that this deficiency in the cat is not absolute but only relative. This pathway is quantitively less important in cats than in most other species. It can be calculated that only about 3% of a dose of FAA appears in the urine conjugated with glucuronic acid, whereas at the other extreme guinea pigs eliminated 60–80% in this form. Incidentally, our earlier studies have demonstrated that of all the metabolites of FAA, only those compounds having a hydroxy group at the 7-position conjugate with sulfuric acid.[10, 11] The present results constitute no exception.

Current views on aromatic amine carcinogenesis indicate that these materials are carcinogenic by virtue of metabolic conversion to N-hydroxy derivatives.[18] Our studies show that about one-third of a single dose of FAA eliminated as glucosiduronic acid was accounted for by this crucial metabolite. It thus appears that only small total amounts of this important intermediate are present to initiate the carcinogenic

process unless, of course, this material or its deacetylated product, the hydroxylamine, circulate in some other form and are thus available.

On the basis of extensive experimentation, the conclusion was drawn that there is a relationship between protein-bound carcinogen and the eventual formation of tumors.[4, 9, 12, 18–21] Two reports,[7, 8] although they are in the form of abstracts, indicate that treatment of cats with FAA affects primarily the lung and only secondarily the liver. This predilection for the lung is not borne out quantititatively by the data on protein binding of radioactivity. Thus, although there are metabolites of FAA firmly bound to the tissue affected, there is no quantitative relationship. Further detailed analysis in a variety of species may elucidate the connection between tissue-bound metabolites and carcinogenesis.

REFERENCES

1. R. T. Williams, *Detoxication Mechanisms*, p. 284. John Wiley, New York (1959).
2. D. Robinson and R. T. Williams, *Biochem. J.* **68**, 23P (1958).
3. G. J. Dutton and C. G. Grieg, *Biochem. J.* **66**, 42P (1957).
4. J. H. Weisburger and E. K. Weisburger, *Clin. Pharmacol. Ther.* **4**, 110 (1963).
5. M. Enomoto, P. Lotlikar, J. A. Miller and E. C. Miller, *Cancer Res.* **22**, 1336 (1962).
6. L. A. Poirier, J. A. Miller and E. C. Miller, *Cancer Res.* **23**, 790 (1963).
7. H. E. Harding, *A.R. Brit. Emp. Cancer Campgn.* **24**, 116 (1947).
8. S. C. Skoryna, L. A. Rudis and D. R. Webster, *Cancer Res.* **11**, 280 (1951).
9. J. H. Weisburger, P. H. Grantham, H. P. Morris and E. K. Weisburger, *J. nat. Cancer Inst.* **27**, 153 (1961).
10. J. H. Weisburger, E. K. Weisburger, H. P. Morris and H. A. Sober, *J. nat. Cancer Inst.* **17**, 363 (1956).
11. J. H. Weisburger, P. H. Grantham, H. P. Morris and E. K. Weisburger, *Cancer Res.* **21**, 949 (1961).
12. E. K. Weisburger and J. H. Weisburger, *Advanc. Cancer Res.* **5**, 331 (1958).
13. V. T. Oliverio, C. Denham and J. D. Davidson, *Analyt. Biochem.* **4**, 188 (1962).
14. J. H. Weisburger, E. K. Weisburger and H. P. Morris, *Arch. Biochem.* **80**, 187 (1959).
15. J. W. Cramer, J. A. Miller and E. C. Miller, *J. biol. Chem.* **235**, 885 (1960).
16. J. H. Weisburger, P. H. Grantham and E. K. Weisburger. *Toxicol. appl. Pharm.*, in press.
17. J. H. Weisburger, N. H. Steigbigel, P. H. Grantham, E. C. Vanhorn and E. K. Weisburger, *Proc. Amer. Ass. Cancer Res.* **4**, 70 (1963); *Cancer Res.*, in press.
18. E. C. Miller, J. A. Miller and H. A. Hartmann, *Cancer Res.* **21**, 815 (1961).
19. C. W. Abell and C. Heidelberger, *Cancer Res.* **22**, 931 (1962).
20. U. S. Seal and H. R. Gutmann, *Arch. Biochem.* **95**, 1 (1961).
21. J. C. Arcos and M. Arcos, *Progr. Drug Res.* **4**, 407 (1962).