Activation and Detoxification of N-2-Fluorenylacetamide in Man*

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SUMMARY

Five patients with various malignant diseases excreted rapidly, chiefly in the urine, isotope from a single oral tracer dose of N-2-fluorenyl-9-C14-acetamide. The urine contained 24-30 per cent of the dose of the detoxification product, N-(7-hydroxy-2-fluorenyl)acetamide, as the glucuronic acid conjugate. The other major metabolite was N-hydroxy-2-fluorenylacetamide, a proximate carcinogen derived from the compound administered. The amount of this compound varied from 4.4 to 13 per cent of the dose.

Certain carcinogenic chemicals, and also other drugs, may not be effective entities by themselves but are converted by metabolism into biologically active materials. Species vary in their capacity to perform this reaction. Thus, N-2-fluorenylacetamide (2-acetylaminofluorene, FAA) is definitely not carcinogenic in the guinea pig, because it is hydroxylated chiefly at the 7-position, followed by conjugation with glucuronic acid and sulfuric acid, and not on the nitrogen atom (9, 20). In the rat, and in other species in which this compound is carcinogenic, biochemical alteration of the molecule involves hydroxylation on other carbon atoms also, and significantly on the nitrogen atom. The last named reaction appears to be of paramount importance in producing a carcinogenic metabolite, N-hydroxy-2-fluorenylacetamide (8). Present concepts incriminate this compound, or its deacetylated product N-2-fluorenylhydroxylamine, as a proximate carcinogen, e.g., the agent actually eliciting the neoplastic transformation. Recent studies suggest that virtually all carcinogenic aromatic amines undergo this reaction (3, 4, 6, 10, 13). It was considered important, therefore, to establish whether man possessed the enzyme systems required to perform N-hydroxylation of FAA.

MATERIALS AND METHODS

Five patients were given a tracer dose of about 0.3 mg. of carbon-14-labeled N-2-fluorenylacetamide (approximately 10 x 10^4 counts/min or 10 µc.) by mouth in a gelatin capsule (Table 1). The patients were: (a) patient 1, a 58-year-old male with inoperable undifferentiated carcinoma of the lung; (b) patient 2, a 61-year-old male with mycosis fungoides; (c) patient 3, a 38-year-old male with multiple myeloma; (d) patient 4, a 58-year-old male with chronic myelogenous leukemia; and (e) patient 5, a 45-year-old male with inoperable teratocarcinoma of the testis. All five patients had essentially normal liver function tests.

Urine, blood, and stool samples were collected at regular intervals. Aliquots (100–200 µl.) of the urine were counted in triplicate in an automatic liquid scintillation counter set at optimal efficiency. Appropriate corrections for quenching (internal standards) and background were made. Samples were counted for a time sufficient to yield a standard error of 3 per cent or less. The scintillation mixture was composed of 700 ml. toluene, 300 ml. methanol, 3 gm. 2,5-diphenyloxazole (PPO), and 100 mg. 1,4-bis-(5-phenyloxazolyl)benzene (POPOP). Stools collected in tared 1-gallon paint cans were diluted with an equal weight of water and homogenized by shaking for 20 minutes on a commercial-type paint shaker. Aliquots of the homogenate were transferred to dialysis bags, dried, and subjected to a Schoniger combustion (11). In one case the blood was separated into serum and red cells and the activity of each fraction determined.

Urinary metabolites were classified as free, glucosiduronic acids, and sulfuric acid conjugates by sequential ether extractions (a) of the urine buffered to pH 7, (b) of the urine after incubation with bacterial β-glucuronidase, and (c) after acidification to pH 1 followed by refluxing 15 minutes and neutralization. The ether extracts from the various fractions were chromatographed on Whatman 3MM paper in the solvent system cyclohexane, tert.-butanol, acetic acid, and water (16:4:2:1) (21). The paper chromatograms were exposed to Royal Blue x-ray film for 2–4 weeks. The spots on the paper corresponding to radioactive areas on film were cut out and


1 We are grateful to Dr. M. B. Shimkin, Dr. C. G. Zubrod, and Dr. N. I. Berlin for assistance in the arrangements to pursue the present study.

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TABLE 1
EXCRETION OF RADIOACTIVITY FOLLOWING A SINGLE ORAL DOSE OF N-2-FLUOREN-9-C14-YLACETAMIDE BY FIVE MALE PATIENTS

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Urinary radioactivity (Per cent of dose)</th>
<th>Fecal radioactivity (Per cent of dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-12</td>
<td>49 46 64 59 74</td>
<td>2.3</td>
</tr>
<tr>
<td>12-24</td>
<td>28 9.7 10 22 23</td>
<td>2.3</td>
</tr>
<tr>
<td>24-48</td>
<td>21 12 9.2 7.0 9.8</td>
<td>0.6</td>
</tr>
<tr>
<td>48-72</td>
<td>5.5 3.6 2.3 1.1 1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>72-96</td>
<td>2.3 1.0 1.3 0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Total:*</td>
<td>106 72 87 90 109</td>
<td>5.2 9.9 7.1 6.4 2.7</td>
</tr>
</tbody>
</table>

* Recovery of isotope exceeds 100 per cent in a few cases because of the high multiplication factor between the aliquots of urine counted and the large total volume available which contained only small amounts of material.

counted. Thus, information on the relative quantities of the individual metabolites was obtained. Each series of chromatograms was controlled by reference to authentic standard compounds.

The ether extract of glucuronidase-hydrolyzed urine was also subjected to chromatography on a silicic acid column (21). The individual fractions from the column were chromatographed on paper to ascertain and confirm their identity. In addition, the first peak containing the N-hydroxy-FAA was examined by inverse isotope dilution analysis with carrier compound to secure absolute proof of its identity.

RESULTS

All five patients eliminated a substantial fraction (46-74 per cent) of the isotope from the tracer dose of labeled FAA in the urine in the first 12 hours (Table 1). The excretion pattern varied somewhat from individual to individual, but in any case only small portions of the dose appeared after the 2d day. Radioactivity in the stools accounted for only a small part of the dose, again with some variations. Thus, the bulk of the material was eliminated via the kidneys.

The radioactivity in the blood serum 12, 24, and 36 hours after the administration of the compound was relatively low (Table 2), indicating that only small amounts of the compound and its metabolites were in circulation at any one time.

The urinary metabolites consisted of 2.7-4.4 per cent free compounds, of 38-59 per cent glucosiduronic acids, and 3.1-9.3 per cent of sulfuric acid conjugates (Table 3). In one case, a second study involving one patient gave similar results for free compounds and sulfuric acid conjugates, but the glucuronic acid conjugates amounted to 63 per cent instead of 43 per cent (patient 4). However, there was an interval of 3 months between these two studies, and the diet and other experimental conditions could not be controlled. It would appear, nevertheless,
that the values given are a fair measure of the relative proportions of these metabolites.

Paper chromatograms of the ether extract of a urine hydrolyzed by \( \beta \)-glucuronidase exhibited eight distinct areas containing radioactivity. Two of these were well defined and contained appreciable amounts of radioactivity (Table 4). As judged by the mobility of these two areas in comparison to known standards, they were the \( N \)-hydroxy-FAA and 7-hydroxy-FAA, respectively. Quantitation of the radioactive spots in relation to the isotope on the entire fraction afforded a measure of the amount of metabolite present. Thus, in the four patients so evaluated \( N \)-hydroxy-FAA ranged from 4 to 14 per cent of the dose, and the 7-hydroxy derivative varied from 25 to 30 per cent of the dose in the fraction consisting of glucuronic acid conjugates.

In a second study on patient 4 after a 3-month interval, a value of 8 per cent of the dose for the \( N \)-hydroxy compound was found. Unfortunately, the other patients used in the first study were no longer available. Therefore, the accurate quantitative aspects of the excretion of various metabolites would have to receive further examination if this were a crucial point. In any case, the urine contained 2–7 times as much of the 7-hydroxy derivative as the \( N \)-hydroxy compound. The relative variation in the excretion of 7-hydroxy-FAA was less than with the \( N \)-hydroxylated compound.

Additional evidence for the nature and identity of the metabolites conjugated with glucuronic acid was obtained by column chromatography on silicic acid. Chart 1 shows a typical elution pattern obtained in several experiments. The metabolites in the ether extract were resolved into two main peaks. The first one had the mobility of \( N \)-hydroxy-FAA on the column and on subsequent paper chromatography of this fraction. It accounted for 21 per cent of the activity. The other main peak, with 66 per cent, corresponded to the 7-hydroxy derivative. Thus, the ratio of 7-hydroxy/\( N \)-hydroxy derivative was 3.1, of a similar order of magnitude to that found by paper chromatography—namely, 2.5 (Table 4). The more sensitive column technic also furnished some evidence for the presence of the 5-hydroxy derivative, which amounted to 3.7 per cent of the fraction.

An isotope dilution experiment was performed on peak 1 from a chromatographic experiment. Successive carrier recrystallization of 2.04 \( \times \) \( 10^4 \) counts/min with 200 mg. of unlabeled \( N \)-hydroxy-FAA gave products with specific activities of 943, 980, 1058, and 1071 counts/min/mg, thus demonstrating unambiguously that peak 1 contained only \( N \)-hydroxy-FAA.

## DISCUSSION

Our studies show that (a) a small number of patients suffering from a variety of malignant diseases excreted the isotope from a tracer dose of the labeled carcinogen \( N \)-2-fluorenylacetamide very rapidly and predominantly in the urine, (b) the urinary metabolites were chiefly in the form of glucosiduronic acids, and (c) the main components so observed were the detoxification product, the 7-hydroxy derivative, and the activation product, the \( N \)-hydroxy derivative of FAA.

Noteworthy is the fact that the bulk of the dose was eliminated via the kidneys and only a small amount in the stools. Of all the species studied only the guinea pig even approached this pattern (20). Previously we proposed the concept that in part the mode of excretion was related to the form of metabolites circulating in the host (15). It was felt that excretion in the feces would be high in species in which metabolites without the acetyl group were predominant. On the other hand the urinary route would be favored where the equilibrium lay on the side of the acetylated metabolites. If this were so, the conclusion that the circulating metabolites in humans were chiefly the acetyl derivatives would be justifiable. On the other hand, the excretory pattern seen may be a reflection of the small dose (0.3 mg.) administered. This amount might be readily cleared by the kidneys. Larger doses could exhibit a different urine/feaces ratio. However, experi-

## TABLE 4

<table>
<thead>
<tr>
<th>Patient</th>
<th>( N )-OH-FAA (%)</th>
<th>7-OH-FAA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>27</td>
</tr>
</tbody>
</table>

* Ascending chromatography on Whatman 3MM paper in cyclohexane solvent system (see text).

† \( N \)-Hydroxy-\( N \)-2-fluorenylacetamide is \( N \)-OH-FAA; \( N \)-(7-hydroxy-\( 2 \)-fluorenyl)acetamide is 7-OH-FAA.

**CHART 1.—Column Chromatographic separation (21) of an ether extract of urine from patient 3 after hydrolysis with \( \beta \)-glucuronidase. The ether extract was taken to dryness and the residue dissolved in a small volume of absolute ethanol. An aliquot (8.3 \( \times \) \( 10^6 \)) counts/min of this solution which constituted 21.5 per cent of the total fraction was added to a silicic acid column (2 X 26 cm.). Partition chromatography was performed with the solvent system cyclohexane, tert-butanol, acetic acid, water (16:4:2:1). Further details are given in Methods. The peak with the mobility of N-hydroxy-FAA was obtained by column chromatography on silicic acid. Chart 1 shows a typical elution pattern obtained in several experiments. The metabolites in the ether extract were resolved into two main peaks. The first one had the mobility of \( N \)-hydroxy-FAA on the column and on subsequent paper chromatography of this fraction. It accounted for 21 per cent of the activity. The other main peak, with 66 per cent, corresponded to the 7-hydroxy derivative. Thus, the ratio of 7-hydroxy/\( N \)-hydroxy derivative was 3.1, of a similar order of magnitude to that found by paper chromatography—namely, 2.5 (Table 4). The more sensitive column technic also furnished some evidence for the presence of the 5-hydroxy derivative, which amounted to 3.7 per cent of the fraction.

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TABLE 5

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
<th>Carcinogenicity*</th>
<th>Per Cent of Dose</th>
<th>Glucuronides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N-OH-FAA</td>
<td>7-OH-FAA</td>
<td></td>
</tr>
<tr>
<td>Guinea pig</td>
<td>(9)</td>
<td>-</td>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td>Steppe lemming</td>
<td>(2)</td>
<td>-</td>
<td>trace</td>
<td>42</td>
</tr>
<tr>
<td>Rat</td>
<td>(9)</td>
<td>+</td>
<td>0.3-1.5</td>
<td>19-27</td>
</tr>
<tr>
<td>Mouse</td>
<td>(9)</td>
<td>+</td>
<td>1.8-2.3</td>
<td>16-20</td>
</tr>
<tr>
<td>Rabbit</td>
<td>(7)</td>
<td>+</td>
<td>13-30</td>
<td>15-29</td>
</tr>
<tr>
<td>Hamster</td>
<td>(17)</td>
<td>+</td>
<td>15-20</td>
<td>35-39</td>
</tr>
<tr>
<td>Dog</td>
<td>(12)</td>
<td>+</td>
<td>5.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Cat</td>
<td>(18)</td>
<td>+</td>
<td>1.5</td>
<td>11</td>
</tr>
<tr>
<td>Monkey</td>
<td>(5)</td>
<td>?</td>
<td>1.8-2.7</td>
<td>9-18</td>
</tr>
<tr>
<td>Man</td>
<td>?</td>
<td></td>
<td>4-14</td>
<td>25-30</td>
</tr>
</tbody>
</table>

* Data on carcinogenicity are reviewed in References 15 and 19.
† See Table 4 for abbreviations.
‡ Personal communication from Dr. H. Pogosianz, Institute for Experimental and Clinical Oncology, U. S. S. R.

mements in rats give little indication of an alteration in the urine/feces ratio when the dose was varied over a 1000-fold range (16).

Similarly to most other species, except for cats, the patients eliminated metabolites predominantly as glucosiduronic acids, with only small amounts of unconjugated compounds or sulfuric acid esters. The important glucosiduronic acid fraction, in turn, contained chiefly the 7-hydroxy-FAA and the N-hydroxy-FAA, as was found in the monkey (5) and the rabbit (7).

Since the 7-hydroxy derivative of FAA is apparently not carcinogenic, (cf. 15) one can consider the metabolic step producing this compound as a sort of detoxification reaction. On the other hand, studies by Drs. J. and E. Miller and their associates (5, 8, 9, 12) have demonstrated clearly that production of the N-hydroxy derivative is a crucial metabolic step leading to a material intimately connected with the development of neoplasia from FAA. Bahné (1) and we (19) have used the word “pro-carcinogen” to denote a material like FAA which is probably inactive by itself but which is activated by conversion to a proximate carcinogen typified by N-hydroxy-FAA, or a material derived from the latter by further metabolism. Thus, the capability of the four patients in our study to perform this reaction suggests that man would respond positively if exposed for a sufficiently long time to a carcinogenic aromatic amine derivative such as FAA. Consideration should be given to the possibility that the four patients used here might have produced the N-hydroxy derivative by virtue of their disease. However, they were deliberately chosen with different syndromes so as to yield a fairly comprehensive picture.

It has been also shown that other aromatic amines such as 2-naphthylamine and benzidine were N-hydroxylated in human patients (13, 14). In the case of these compounds, there is adequate record of their carcinogenicity in man (2, 4). Table 5 shows that only two species—namely, the guinea pig and possibly the steppe-lemming, did not have the enzyme system to perform N-hydroxylation of FAA, and the chemical is not carcinogenic in these two species. In contrast, virtually all other species thus far examined, including man, can N-hydroxylate FAA to varying degrees. In the light of these facts there is a good probability that FAA and other aromatic amines which can be N-hydroxylated and are carcinogenic in a rodent or other small animals are probably carcinogenic in man. Thus, if a compound of this class has exhibited true carcinogenicity in some species it can be presumed to be hazardous to man, and the obvious conclusions regarding safe handling of such materials should be drawn.

Our studies, although based on small numbers, lead to the suggestion that the capability of individuals to N-hydroxylate carcinogenic aromatic amines is variable. If further and more extensive studies bear out this variability, it may be of interest to develop a biochemical method to select those persons exhibiting the lowest capacity of N-hydroxylation for employment in areas where exposure to such aromatic amines might occur.

REFERENCES

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