The $N$- and Ring-Hydroxylation of 2-Acetylaminofluorene and the Failure To Detect $N$-Acetylation of 2-Aminofluorene in the Dog*

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SUMMARY

$N$-Hydroxy-2-acetylaminofluorene ($N$-hydroxy-AAF), in conjugated form, was identified as a urinary metabolite of 2-acetylaminofluorene (AAF) in male mongrel dogs. This metabolite was isolated and characterized in crystalline form. 7-Hydroxy-AAF, in conjugated form, and AAF were also found in the urine of dogs fed AAF; no 1-, 3-, or 5-hydroxy-AAF was detected. The ingestion of $N$-hydroxy-AAF led to the urinary excretion of the same metabolites; however, none of these acetylated metabolites was detected in the urine of dogs fed 2-aminofluorene, $N$-hydroxy-2-aminofluorene, 1-hydroxy-AAF, or 3-hydroxy-AAF. Dietary supplementation with calcium pantothenate and riboflavin and an attempt to induce acetylase activity by feeding 2-aminofluorene for several days did not lead to the urinary excretion of any recognizable acetylated urinary metabolites of 2-aminofluorene. Furthermore, under similar conditions the specific activities of the acetylated urinary metabolites of 2-(acetyl-1'-C¹⁴)aminofluorene fed in mixtures with unlabeled 2-aminofluorene were not appreciably different from the specific activity of the ingested acetyl-labeled AAF. In a dog fed a single dose of AAF-9-C¹⁴ 63 per cent of the C¹⁴ was excreted in the feces, and 19 per cent of the C¹⁴ was found in the urine during the next 5 days. Approximately 3 per cent of an oral dose of AAF was found as 7-hydroxy-AAF in the feces collected during the 1st day.

No $N$-hydroxy amides were detected in the urine of dogs after ingestion of the following amides: acetanilide and its p-vinyl, p-fluoro, and p-ethoxy derivatives; trans 4-acetylaminostilbene; 2-propionylaminofluorene; and 2-n-butyrylaminofluorene. Administration of 2-acetylaminonaphthalene to a dog led to the urinary excretion of very small amounts of this amide and its $N$-hydroxy metabolite.

The synthesis of $N$-hydroxy-2-aminofluorene, a new compound, is described.

The versatile carcinogenic activity of 2-acetylaminofluorene (AAF) has been demonstrated by the variety of species and tissues in which this compound can induce malignant tumors (34). In the dog AAF has been found to be a carcinogen for the urinary bladder (26) and the liver (3, 18).

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26. Other aromatic amides and amines such as 2-aminonaphthalene (5, 16), 4,4'-diaminobiphenyl (benzidine) (30, 33'), 4-aminobiphenyl (11, 33) and 4-acylamidobiphenyl (18) also exhibit carcinogenic activity in the urinary bladder of the dog. Likewise several of these carcinogens—viz., 2-aminonaphthalene, 4,4'-diaminobiphenyl, and 4-aminobiphenyl—produce tumors of the urinary bladder in humans following industrial exposure (7, 14, 21, 32). Thus, studies on the metabolism of carcinogenic aromatic amines and amides in the human body are clearly indicated. Further studies on the metabolism of these compounds are in progress.

1 The article by Walpole et al. (33) contains further data on the carcinogenicity of benzidine in the dogs of Spitz et al. (30).
dog may provide clues to the mechanism by which these compounds induce bladder tumors in both species.\textsuperscript{3}

In the rat the carcinogenic action of AAF (22) and of 4-acetylanobiphenyl (25) appears to proceed through the N-hydroxylation of the parent amide. The dog is known to N-hydroxylate 4-acetylanobiphenyl (25), and both the dog (6, 31) and the human (31) N-hydroxy-2-aminonaphthalene. This report presents data on: (a) the N- and ring-hydroxylation of AAF and related compounds in the dog and (b) our failure to demonstrate the N-acetylation of 2-aminofluorene in this species.

**MATERIALS AND METHODS**

**Compounds.**—Except where noted the melting points (°C.) are uncorrected. The respective melting points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected. The respective melt points (°C.) are uncorrected.
light yellow microcrystalline mass separated readily from the paper, and it was relatively stable to oxygen as long as it was kept in the dark in the refrigerator. The yield was usually about 0.6 gm. The substance had an indefinite melting point; it sintered and partly melted with decomposition at 170°–175° C. in a capillary tube in air or in nitrogen. Recrystallization of this compound from various solvents under N₂ did not yield material with a sharper melting point. Elementary analysis⁷ gave the following data:

Calculated for C₁₉H₁₁NO: C, 79.25; H, 5.95; N, 7.12.

Found: C, 78.75; H, 5.59; N, 6.85.

The hydroxylamine reacted with benzaldehyde in absolute ethanol to give a nitron which melted at 200°–202° C. Elementary analysis⁷ of this nitron gave the following values:

Calculated for C₂₀H₁₈NO:
C, 84.22; H, 5.26; N, 4.91.

Found:
C, 84.69; H, 5.55; N, 4.69.

Analyses of the hydroxylamine by titration with TiCl₃ (15) gave values of 94–98 per cent of theory. Usually 0.2 mmoles of the compound was reacted in 30 ml. of dimethylformamide with 40 ml. of 0.04 N TiCl₃ at room temperature for 15 minutes. The reaction mixture was stirred magnetically. The solution was then back-titrated with standard 0.04 N ferric ammonium sulfate. Ten ml. of 10 per cent ammonium thiocyanate was added as an indicator near the end of the titration. The titration solution, indicator solution, and reaction mixture were kept free of oxygen by a stream of N₂. The fenric ion solution was standardized by analysis with excess titanous ion and back-titration with standard 0.02 N K₂Cr₂O₇.

For enzymatic release of the conjugated metabolites and the paper partition chromatography of the metabolites were performed essentially according to the methods previously published (8, 23). The 24-hour urine samples were diluted to 1 liter with distilled water and brought to pH 6 with a few drops of glacial acetic acid. An 18-ml. aliquot was incubated for 15 hours at 37° with 12 mg. of a bacterial β-glucuronidase preparation and 15 mg. of Takadiastase in the presence of 0.25 ml. of chloroform. The mixture was then extracted with 60 ml. of peroxide-free ethyl ether, and the ether in turn was successively extracted with 15 ml. each of 10 per cent Na₂CO₃ (17), water, 0.5 N HCl, and water. The ether was evaporated with a stream of N₂, and the residue dissolved in 0.5 ml. of ether and distributed between three paper strips for chromatography. Quantitative estimations of the per cent excretion of each metabolite were obtained from the spectra of the eluates of the chromatograms. When AAF, N-hydroxy-AAF, and 7-hydroxy-AAF were added to normal urine prior to incubation, the compounds could be recovered after chromatography to the extent of 95, 55, and 87 per cent, respectively. The urinary excretions reported here are corrected for these recoveries.

Isolation of N-hydroxy-AAF from urine.—Two dogs were each fed 1 gm. of AAF over a period of 4 days, and the urine was collected and pooled. After enzymatic hydrolysis of the conjugate, the isolation of the N-hydroxy-AAF was performed essentially according to the cupric chelate procedure of Enomoto et al. (13). After decomposition of the cupric chelate in ethanol by H₂S and removal of the cupric sulfide by centrifugation, the ethanolic solution was taken to dryness in vacuo in a flash evaporator. The N-hydroxy-AAF was quantitatively transferred to a centrifuge tube with about 2 ml. of ethanol, and the product was precipitated by the addition of 6 ml. of n-hexane. The N-hydroxy-AAF was then recrystallized once
from ethanol-hexane and 3 times from ethanol-water and finally dried over P₂O₅ in vacuo.

**Urinary metabolites of 2-(acetyl-1'-C⁴)aminofluorene.**—The specific radioactivities of the urinary metabolites of 2-(acetyl-1'-C⁴)aminofluorene were determined in four experiments. In one experiment only acetyl-labeled AAF was administered. In the other experiments 2-aminofluorene was also fed, and in two experiments daily supplements of calcium pantothenate (20 mg/kg/day) and riboflavin (2.5 mg/kg/day) were fed. In the first three experiments the doses of the carcinogens were divided into three equal portions and fed at 8-hour intervals. The urines were collected for 24 hours after the first portion, diluted with an equal volume of water, and brought to pH 6. As many as fourteen 18-ml. aliquots of the diluted urines were incubated with the enzymes, and the metabolites were extracted and chromatographed on paper as described above. This provided the large number of strips needed for the location of the metabolites and for the accumulation of enough of the metabolites for the spectral and radioactivity measurements. All the paper strips used for chromatography in these experiments were pre-washed by descending chromatography with the upper (organic) phase of the solvent system to remove contaminants that absorb ultraviolet light. After chromatography of the extracts three strips from each experiment were sprayed with the indicator reagents (8) to locate the N-hydroxy-AAF and 7-hydroxy-AAF zones. The positions of these zones and of AAF on the remaining strips from which the metabolites were to be eluted were confirmed by visual observation under ultraviolet light (∼254 μm).

The portions of the chromatographic strips containing the N-hydroxy-AAF and AAF (the zones containing these compounds partly overlap) or the 7-hydroxy-AAF were cut out for elution. Groups of three to six pieces were placed in glass-stoppered test tubes, moistened with 0.3 ml. of ethanol, and allowed to stand overnight at 5°C. The next day 4–6 ml. (depending on the number of strips) of ethyl ether were added to each tube, and the contents were gently shaken. The eluates from like tubes were combined, each tube of strips was washed once or twice with 3–5 ml. of ethyl ether, and the washings were added to the eluates. The separation of the N-hydroxy-AAF and AAF was accomplished as follows: The combined eluates were extracted 3 times in a small separatory funnel with 5-ml. portions of 0.5 N NaOH. The combined alkaline extracts were extracted twice with 15-ml. portions of ether, and the ether layers were added to the original ether solutions. This combined ether solution containing the AAF was then washed successively with 10 ml. of 0.5 N NaOH and 15 ml. of water. The alkaline solution containing the N-hydroxy-AAF was brought to pH 6 by the addition of 15 ml. of 0.5 N HCl and 7 ml. of 1 M pH 6 sodium acetate buffer. This solution was then extracted twice with 20 ml. of ether, and the ether extracts containing the N-hydroxy-AAF were combined and washed once with 20 ml. of water.

For the determination of the specific radioactivities of the metabolites each of the ethereal extracts was evaporated to dryness with a stream of N₂, and the residue was dissolved in 0.5 ml. of ether. The concentrates were applied to three pre-washed paper strips for chromatography in the same solvent system. The central portion of each chromatographed zone (the most intensely absorbing region viewed under ultraviolet light) from the three strips was eluted into 5.0 ml. of absolute ethanol. The ultraviolet absorption spectra of the solutions were recorded with a Beckman DB spectrophotometer, and the concentrations of the metabolites were determined from the spectra. Immediately afterwards, 100–250 μl. of each solution was plated in triplicate on aluminum planchets. The radioactivities of these samples were determined from duplicate 5-minute readings in a gas-flow end-window Geiger counter.

A fourth experiment with acetyl-labeled AAF was performed to investigate the possibility of induction of an acetylase system in the dog by the administration of 2-aminofluorene for several days. A dog was fed supplements of calcium pantothenate and riboflavin for 12 days. On days 7–11 100 mg. of 2-aminofluorene (two 50-mg. capsules given 6 hours apart) was administered daily. On day 11 the urine was collected for 24 hours and examined carefully for acetylated metabolites. On day 12 a mixture of 2-(acetyl-1'-C⁴)aminofluorene and 2-aminofluorene (34 and 136 mg., respectively) was fed, and the urine was again collected for 24 hours. The 24-hour urine sample collected on the 11th day was treated as follows. It was diluted with distilled water to 500 ml., and the pH was adjusted to 6 with acetic acid. Fifty ml. of the diluted urine was then added to each of ten 125-ml. Erlenmeyer flasks. To each flask was added 0.5 ml. chloroform, 25 ml. of 1.0 M pH 6 sodium acetate buffer, and 2 ml. of an enzyme solution containing 30 mg. of Takadiastase and 36 mg. of bacterial β-glucuronidase. The flasks were incubated at 37°C for 15 hours. After the incubation the contents of pairs of flasks were combined and extracted with 200 ml. of peroxide-free ether. The aqueous layer was discarded. The ether layer was
successively extracted with 50 ml. of 10 per cent sodium carbonate, 50 ml. water, 50 ml. 0.5 M HCl, and 50 ml. of water. The ether extracts were then combined and taken to dryness in vacuo in a flash evaporator. The residue was removed from the flask by three successive 50-ml. rinses with ether. The ether washes were combined in a separatory funnel and washed once with 40 ml. of water. The ether layer was then divided equally between two pear-shaped flasks, and the ether was removed with a stream of N2. The residue from each flask was spotted on three paper strips. After chromatography three strips were sprayed with the indicator reagents, and the appropriate fractions of the remaining three were eluted for spectral analysis as described above. The urine collected after the ingestion of acetyl-labeled AAF and 2-aminofluorene was treated in essentially the same manner. However, the final ether extract was divided into six portions for drying and application to eighteen paper strips. Prior to the final spectral and radioactivity measurements a third chromatographic purification of the metabolites was performed.

Distribution of the metabolites of AAF-9-C14 in urine and feces.—Three experiments were performed to determine the urinary and fecal distribution of the radioactivity from ingested AAF-9-C14 in the dog. Single doses of 150 mg. of AAF-9-C14 with specific radioactivities of 6—7 × 106 counts/min/mg were employed. The excreta were collected for 1, 3, or 5 days in these trials. Appropriate aliquots of the daily urine samples were diluted with ethanol, and 100—200 μl. of the diluted urines were plated in triplicate on aluminum planchets for radioactivity measurements. The daily fecal samples were homogenized in a Waring Blender with sufficient water or 20 per cent ethanol to give suspensions which could be accurately sampled volumetrically. In a search for fecal metabolites aliquots of a water suspension were brought to pH 6 with acetic acid and incubated with enzymes, extracted, and the extracts chromatographed as described above. Other aliquots of the fecal suspensions were further diluted with ethanol, and triplicate 100-μl. volumes were plated for radioactivity determinations.

RESULTS

Observations with paper chromatograms.—The ingestion of AAF by the dog led to the excretion of AAF, N-hydroxy-AAF, and 7-hydroxy-AAF in the urine at average levels of 0.7, 5.2, and 0.7 per cent, respectively, of the dose (Table 1) during the first day following ingestion. N-Hydroxy-AAF was easily detected on the chromatograms by its capacity to form a yellow color slowly when sprayed with an acidic solution of p-dimethylaminobenzaldehyde; it also reduced the Folin-Ciocalteu phenol reagent to give a blue color. The RF (0.74—0.82) and absorption spectrum of this zone were identical with those of synthetic N-hydroxy-AAF. The 7-hydroxy-AAF was detected with the Folin-Ciocalteu reagent and identified by the correspondence of its RF and spectrum with those of the authentic compound. Likewise, the AAF zone was identified by its RF and spectrum. Neither N-hydroxy-AAF nor 7-hydroxy-AAF was observed in the urine prior to incubation with β-glucuronidase and Takadiastase; thus, these metabolites were excreted as conjugates. When N-hydroxy-AAF was fed to the dog, 12.3 per cent of the dose was recovered in the urine as N-hydroxy-AAF, 1.2 per cent as AAF, and 0.3 per cent as 7-hydroxy-AAF. No 1-, 3-, or 5-hydroxy-AAF could be detected in the urine following the ingestion of AAF or of N-hydroxy-AAF either by spraying the paper strips with the phenol and diazo reagents (8) or by spectral examination of eluates of the strips. Any of these urinary metabolites should have been readily detected on the strips by visual observation under ultraviolet light and with the indicator reagents if they had been excreted in excess of 0.3 per cent of the highest doses of AAF or N-hydroxy-AAF. The elution and spectrophotometric analysis of the chromatographic zones in which these metabolites occurred could have measured as little as 0.2 per cent of these doses. Even when 150 mg. of 1- or 3-hydroxy-AAF was fed to the dog the chromatographic strips showed no recognizable metabolites. Likewise, no recognizable N-acetylated urinary metabolites could be observed following the ingestion of 2-aminofluorene or N-hydroxy-2-aminofluorene by the dog.

When 500 mg. of 2-acetylamino-naphthalene was fed, a urinary metabolite was detected that gave a positive test for a hydroxamic acid with the acidic p-dimethylaminobenzaldehyde reagent and had the same RF (0.75—0.86) as synthetic N-hydroxy-2-acetylaminonaphthalene.8 This metabolite was soluble in dilute NaOH and possessed an absorption spectrum in ethanol similar to that of the synthetic hydroxamic acid (maximum at 248 mμ, inflection at 254 mμ) and distinct from that of 2-acetylaminonaphthalene (maximum at 248 mμ and 250 mμ). The spectral analyses indicated that approximately 0.1 per cent of the dose was excreted as 2-acetylaminonaphthalene and as its N-hydroxy derivative. No N-hydroxy-2-acetylamino-naphthalene was detected.

8 We are indebted to Dr. Raymond R. Brown, Cancer Research Division, Department of Surgery, University of Wisconsin, for a sample of this compound.
naphthalene was detected in the urine after a dose of only 131 mg. of the amide. Likewise, no N-hydroxy amides were detected by chromatographic analysis of the urine of dogs fed the following amides (highest doses tested in parentheses): acetanilide (500 mg.), p-vinylacetanilide (225 mg.), p-fluoroacetanilide (150 mg.), p-ethoxyacetanilide (500 mg.), trans 4-acetylaminostilbene (100 mg.), 2-propionylaminofluorene (300 mg.), and 2-n-butyrylaminofluorene (200 mg.). Our inability to detect the N-hydroxy derivatives in the urine does not preclude the formation of these compounds in vivo. These tests were carried out before the removal of various interfering substances in normal urine by extraction of the ether extract of urine with sodium carbonate was reported (17). Hence, although no phenolic metabolites were observed with the Folin-Ciocalteu and diazo reagents, some may have been obscured by the normal reducing substances present on the lower half of the chromatograms.

**Isolation of N-hydroxy-AAF.**—Direct proof of the formation of N-hydroxy-AAF from AAF by the dog was provided by the isolation of approximately 30 mg. of the hydroxamic acid in crystalline form from urine by the cupric chelate method. The melting point of the recrystallized material was 146°–147° C., uncorrected, which is identical with that of synthetic N-hydroxy-AAF. An approximately 1:1 mixture of the isolated product and synthetic N-hydroxy-AAF gave no depression of the melting point. The following elementary analysis was obtained:

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Huffman Microanalytical Laboratories, Wheatridge, Colorado.
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### TABLE 1

**THE URINARY METABOLITES OF 2-ACETYLAMINOFLUORENE (AAF) AND CERTAIN OF ITS DERIVATIVES IN THE DOG DURING THE FIRST DAY FOLLOWING INGESTION**

<table>
<thead>
<tr>
<th>Compound Ingested</th>
<th>No. Trials</th>
<th>Dog Nos.</th>
<th>Total Dose (mg.)</th>
<th>N-Hydroxy-AAF</th>
<th>1-Hydroxy-AAF</th>
<th>3-Hydroxy-AAF</th>
<th>5-Hydroxy-AAF</th>
<th>7-Hydroxy-AAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAF</td>
<td>18</td>
<td>1-5</td>
<td>57–300†</td>
<td>0.7±0.4 †</td>
<td>5.2±2.7</td>
<td>–</td>
<td>–</td>
<td>0.7±0.4</td>
</tr>
<tr>
<td>N-Hydroxy-AAF</td>
<td>2</td>
<td>1,5</td>
<td>300(1) 225(3)</td>
<td>1.2±0.6</td>
<td>12.3±0.7</td>
<td>–</td>
<td>–</td>
<td>0.3‡</td>
</tr>
<tr>
<td>1-Hydroxy-AAF</td>
<td>2</td>
<td>1, 4</td>
<td>54(1) 150(3)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3-Hydroxy-AAF</td>
<td>1</td>
<td>3</td>
<td>150(9)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2-Aminofluorene</td>
<td>1</td>
<td>3</td>
<td>150(3)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>N-Hydroxy-2-aminofluorene</td>
<td>1</td>
<td>3</td>
<td>150(3)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* The numbers in parentheses are the number of equal portions in which the dose was administered.
† The doses of AAF were administered in one, two, or three equal portions.
‡ Standard deviation.
§ – = not detected.
¶ Analysis of second trial. In the first trial the presence or absence of 7-hydroxy-AAF could not be determined because of contaminating reducing agents on the chromatographic strips.
∥ Several attempts were made to feed 2-aminofluorene in doses of 150–300 mg. given in one or three portions. In general, if a dog received 200 mg. within a period of 3 hours, the dose was regurgitated.
** Analysis of urine from dog fed 100 mg. of 2-aminofluorene per day for 5 days prior to administration of 2-(acetyl-1'-Cl')-aminofluorene (Table 2, Experiment 4). The urine was collected for 24 hours after the fifth dose of 2-aminofluorene and the entire urine sample was used for chromatographic analysis. No metabolites were detected with the spray reagents, and spectral analysis of the material from one-half of the urine sample showed that less than 0.04 per cent of the dose could have been excreted as each acetylated metabolite (see "Studies on N-acetylation of 2-aminofluorene" under "Results").
Studies on N-acetylation of 2-aminofluorene.—

The absence of N-acetylated metabolites on the chromatograms of the urine from the dog fed 2-aminofluorene raised the question whether the dog could acetylate 2-aminofluorene or its hydroxy metabolites. Although the data in the literature (39) have generally indicated that the dog does not appear to acetylate aromatic amines, Allison et al. (1) reported the presence of AAF in the urine of dogs fed 2-aminofluorene. Accordingly, this problem has been further investigated in four experiments in which the specific activities of ingested 2-(acetyl-1'-C14)aminofluorene and of its urinary metabolites were compared.

In Experiment 1 only 2-(acetyl-1'-C14)aminofluorene was administered. 2-Aminofluorene has been found as a urinary metabolite of AAF in the dog (1, 26, 35), and the assumption was made that there would be sufficient deacetylation of the acetyl-labeled AAF in vivo (94) to provide an acceptor for unlabeled acetyl groups from the body pool. In the second experiment 2-aminofluorene and 2-(acetyl-1'-C14)aminofluorene were administered in a molar ratio of 2:1 to ensure that sufficient 2-aminofluorene was present in the body to accept unlabeled acetyl groups. Experiment 3 was carried out in a manner similar to that of Allison and Wase (9) in an attempt to enhance acetylation by the administration of supplements of calcium pantothenate and riboflavin for 9 days prior to the administration of a mixture of 2-aminofluorene and acetyl-labeled AAF in a molar ratio of 5:1. The results of these experiments are summarized in Table 2. In no instance was there a significant decrease in the specific radioactivities of the N-acetylated urinary metabolites as compared with the activity of the acetyl-labeled AAF fed. In Experiment 1, in which the administered acetyl-labeled AAF had a relatively low specific activity, the probable errors of the specific activity measurements would not have permitted detection of the replacement of less than 5 per cent of the acetyl groups of AAF or any of its metabolites. The probable errors of the measurements in Experiments 2 and 3 were sufficient to mask possible acetylations11 of the 2-aminofluorene of only approximately 1.9 and 0.8 per cent, respectively.

**Equation (II)** was derived from equation (I) by application of the equation (III) for the probable error $p$ (9):

$$p = \sqrt{\left(\frac{\partial Y}{\partial x}\right)^2 r_1^2 + \left(\frac{\partial Y}{\partial z}\right)^2 r_2^2 + \left(\frac{\partial Y}{\partial w}\right)^2 r_3^2 + \ldots}$$  

where $\partial Y/\partial x$, $\partial Y/\partial z$, $\partial Y/\partial w$, ... are the probable errors respectively in $x$, $z$, $w$, ... .

In Experiment 1 the extent of replacement of the labeled acetyl groups of the ingested AAF by unlabeled acetylation was determined as follows:

$$\text{Percent replacement} = 100 \left[ 1 - \frac{s.a.\text{-met.}}{s.a.\text{-ing.}} \right],$$

where $s.a.\text{-met.} = $ specific activity of metabolite and $s.a.\text{-ing.} = $ specific activity of the ingested AAF.

In Experiments 2 to 4 the extent of acetylation was expressed as the per cent acetylation of the ingested 2-aminofluorene and it was calculated as follows:

$$\text{Percent acetylation} = 100 \left[ \frac{1 - \frac{s.a.\text{-met.}}{s.a.\text{-ing.}}}{\frac{s.a.\text{-met.}}{s.a.\text{-ing.}}} \right],$$

where $s.a.\text{-met.} = $ fraction of acetylated metabolite retaining labeled acetyl group, $s.a.\text{-ing.} = $ fraction of acetylated metabolite possessing unlabeled acetyl groups, and $M = $ molar ratio of ingested 2-aminofluorene to ingested 2-(acetyl-1'-C14)aminofluorene.

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1 The specific activity (s.a.) of each metabolite was calculated from equation (I):

$$s.a. = \frac{A B}{C D},$$  

where $A = $ counts/min/planchet, $B = $ absorption coefficient of metabolite, $C = $ volume of eluate plated per planchet, and $D = $ optical density of the eluate at wavelength selected for determination of $B$. The probable error ($p$) of the specific activity was determined from equation (II):

$$p = \frac{1}{CD} \sqrt{A^2 b^2 + B^2 a^2 + \frac{A^2 B^2 c^2}{C^2} + \frac{A^2 B^2 d^2}{D^2}},$$

where $a = $ The probable error in the value for the counts/min/planchet; it was determined from the standard deviation in the determination of the counts/min/planchet, $b = $ The probable error in $B$; it was estimated to be 2.5 per cent, $c = $ The probable error in the volume (C) plated on each planchet; in Experiments 1 and 2, in which different micropipettes were used for each metabolite, it was estimated as 2 per cent; in Experiment 3, in which the same micropipette was used in all platings, it was estimated to be 1 per cent, $d = $ The probable error in the optical density (D) of the eluate; it was estimated to be 1.5 per cent.

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C, 75.3; H, 5.49; N, 5.85.

Found:

C, 75.0; H, 5.0; N, 5.30.
The analyses in the experiments described above were conducted on urine samples collected for 24 hours after the ingestion of the compounds. It was conceivable, however, that the ingestion of 2-aminofluorene by the dog might induce the synthesis of an acetylase for aromatic amines and that this might require more than 24 hours to achieve a level of enzyme activity sufficient to alter the specific activities of the urinary metabolites. Therefore a fourth experiment was performed in which daily supplements of riboflavin and calcium pantothenate were given for 12 days, and, on days 7–11, 100 mg of 2-aminofluorene was fed daily. On day 12 a mixture of 2-aminofluorene and acetyl-labeled AAF (molar ratio of 5:1) was administered. Once again no notable decreases were found in the specific activities of the urinary metabolites (Table 2). The probable errors of the specific activities of the metabolites were about the same as in Experiment 3 and indicated again that less than 1–2 per cent of the ingested 2-aminofluorene could have been acetylated. Chromatographic and spectral analysis of the whole urine sample, collected on the last day of ingestion of 2-aminofluorene without any AAF, showed that less than about 0.04 per cent of the dose of 2-aminofluorene appeared in the urine in the form of each of the known N-acetylated metabolites of AAF. This calculation was made on the assumption that the small optical densities of the eluates of the paper strips actually represented N-acetylated metabolites. In fact, however, no AAF or any other acetylated metabolite was detected visually on the chromatograms or by spectrophotometric examination of the eluates of the chromatograms obtained from each day for 3 days following the ingestion of AAF. The usual three metabolites were easily detected on the 1st and 2nd days. No N-hydroxy-AAF and only very small amounts of AAF and 7-hydroxy-AAF could be found in the urine collected on the 3rd day. Within 5 days following the ingestion of AAF (Experiment 7) only 81 per cent of the radioactivity of the ingested AAF-9-C¹⁴ had been excreted in the combined urine and feces. Metabolites were still being excreted during the 5th day, and on that day the urine and feces contained 0.1 and 0.8 per cent, respectively, of the radioactivity administered.

**DISCUSSION**

The observations recorded in this paper extend the previous findings on the capacity of the dog to N-hydroxylate aromatic amines and amides. The dog is now known to N-hydroxylate 2-aminonaph-
Likewise no 5-hydroxy-AAF and only relatively low amounts of 7-hydroxy-AAF (in agreement with previous reports [26, 35]) were found in the urine of dogs fed AAF. N-Hydroxy-AAF is the major known urinary metabolite of AAF in the dog. Other investigators (1, 26, 35) have reported that 1—2 per cent of a dose of AAF is excreted in the urine as 2-aminofluorene. These known urinary metabolites account for more than one-half of the radioactivity excreted in the urine within the first 24 hours after ingestion of AAF-9-C'4. In contrast to the relatively high urinary excretion of metabolites by the rat (36) the dog excretes only 19—22 per cent of the radioactivity from AM?-9-C'4 in the urine within 3—5 days. The major share of the C'4 is excreted in the feces, and, except for a small amount of 7-hydroxy-AM?, the nature of the fecal metabolites is unknown. Weisburger and Weisburger (34) have suggested that the fecal metabolites in the rat may include oxidation and polymerization products of aminofluorenols.

Of special interest in the present study was our failure to detect the N-acetylation of 2-aminofluorene in the dog either by chromatographic and spectrophotometric analysis or by dilution of the isotope in the urinary metabolites of acetyl-labeled AM? administered with 2-aminofluorene. The summary of previous literature by Williams (39), our recent experience with 4-aminobiphenyl (25), and the present findings all indicate that, if the dog can N-acetylate aromatic amines and amides, it can do so only to a small extent which is below the limits of detectability of the analytical methods employed. However, Allison et al. (1) have reported the presumptive N-acetylation of 2-aminofluorene in the dog. They observed an "ether soluble conjugated amine" in the urine of dogs fed 2-aminofluorene and found under various conditions that this conjugate appeared to represent 0.09—1.5 per cent (calculated as AM?) of the dose. The higher excretions were obtained in dogs whose diet was supplemented with calcium pantothenate and riboflavin. The urinary conjugate was concluded to be AM? on the basis of its absorption spectrum, but it was not chromatographed or isolated. Allison et al. (1) found that under various conditions low percentages (0.2—2.0) of doses of AAF fed to dogs were excreted in the urine as AAF. If it is assumed that a similarly low excretion of AAF

require a comparison of the carcinogenicity of each of the above amines and amides with the activity of the corresponding N-hydroxy metabolite in the dog. Such a comparison of the activities of 4-aminobiphenyl and the corresponding hydroxylamine is now in progress in this laboratory.

Although the dog is able to metabolize aromatic amines to o-hydroxy derivatives (39), neither 1- nor 3-hydroxy-AAF could be detected as a urinary metabolite of AAF. However, since neither compound could be detected in the urine even after a large amount of 1- or 3-hydroxy-AAF was fed, no conclusions with regard to the o-hydroxylation of AAF in the dog can be drawn from these data.
occurred from any AAF formed in vivo from 2-aminofluorene, calculations from the data of Allison et al. suggest that as much as 17–49 per cent of the administered 2-aminofluorene may have been N-acetylated by their dogs. If acetylations of this magnitude had occurred in our experiments, large dilutions of isotope in the metabolites derived from 2-(acetyl-1-C¹⁴)aminofluorene should have been observed when it was administered to dogs with 2-aminofluorene. Similarly, urinary excretions of AAF of the magnitude reported by Allison et al. should have been readily detected chromatographically and spectrophotometrically in our studies. A possible explanation of the discrepancy may reside in the previous treatment of the dogs used in the experiments of Allison et al. In their experiments the 2-aminofluorene was fed 7 days after a dose of AAF. There is no mention of control determinations for the possible presence of ether-soluble conjugated amine in the urine collected from the dogs immediately before the administration of 2-aminofluorene. Possibly enough previously stored AAF appeared in the urine following the administration of the 2-aminofluorene to account for their findings. This suggestion is consistent with our finding that even after 5 days only 81 per cent of the radioactivity from a dose of AAF-9-C¹⁴ in the dog was accounted for in the urine and feces; on the 5th day small amounts of radioactivity were still being excreted in the urine and feces. In our experiments with 2-aminofluorene the dogs had not received AAF or any of its derivatives for 6–15 weeks previously.

The studies in the dog with acetyl-labeled AAF also show that the metabolites N-hydroxy-AAF and 7-hydroxy-AAF either are formed directly from AAF or arise via derivatives of AAF which retain the acetyl group. The same conclusion was drawn previously on the metabolism of labeled AAF in the rat (23).

Because the dog is either unable to N-acetylate aromatic amines or does so very poorly it could be a useful species for studies on the role of the N-acetyl group in carcinogenesis with these compounds and their N-acetyl derivatives. Among the aromatic amines or their derivatives known to produce bladder tumors in the dog are the following: 2-aminonaphthalene (5, 16), 4,4′-diaminobiphenyl (benzidine) (30, 33), 4-aminobiphenyl (11, 33), AAF (26), 4-acetylaminobiphenyl (18), 4-nitrobiphenyl (10), 2-aminoazotoluene (28), and 4-dimethylaminoazobenzene (28). With the exception of one of the azo dyes only the two acetylated amines, AAF and 4-acetylaminobiphenyl, have also induced tumors of the liver in the dog. The dog N-hydroxylates both of these amides and 2-aminonaphthalene. These observations and the fact that 2-aminonaphthalene, 4-aminobiphenyl, and 4-nitrobiphenyl induce tumors in the urinary bladder of the dog are consistent with the possibility that one of the proximate carcinogens in the bladder may be a hydroxylamine, whereas in the liver an N-acetyl hydroxylamine may be required for carcino genesis. However, the final proximate carcinogen may be the same regardless of the tissue involved, and the differences cited above may reside in differences in metabolic pathways or rates of metabolism in various tissues. Further studies on the metabolism and carcinogenic properties of aromatic amines and amides in the dog are desirable.

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