The N-Hydroxylation of 4-Acetylaminobiphenyl by the Rat and Dog and the Strong Carcinogenicity of N-Hydroxy-4-acetylaminobiphenyl in the Rat

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SUMMARY

N-Hydroxy-4-acetylaminobiphenyl has been identified as a urinary metabolite of the carcinogens 4-acetylaminobiphenyl and 4-aminobiphenyl in the rat. 4-Acetylaminobiphenyl, but not 4-aminobiphenyl, yielded the same metabolite in the dog. The N-hydroxy metabolite was excreted as a conjugate which was easily cleaved by a bacterial β-glucuronidase preparation but not by Takadiastase. The identity of the metabolite was established by its isolation in crystalline form and comparison with the synthetic compound. In opposition to the situation previously observed with 2-acetylaminofluorene in the rat, N-hydroxy-4-acetylaminobiphenyl was found in gradually decreasing amounts in the urine as the feeding of 4-acetylaminobiphenyl to rats progressed over several months. Upon oral administration to adult female rats N-hydroxy-4-acetylaminobiphenyl was as active a mammary carcinogen as 4-acetylaminobiphenyl and also induced more ear-duct carcinomas and forestomach papillomas than the parent amide. Upon repeated intraperitoneal injection into young female rats N-hydroxy-4-acetylaminobiphenyl was more active than 4-acetylaminobiphenyl as a mammary carcinogen. Dietary 3-methylcholanthrene had no marked effect on the N-hydroxylation of 4-acetylaminobiphenyl or on the carcinogenicity of this amide and its N-hydroxy metabolite toward the mammary gland. N-Hydroxy-4-acetylaminobiphenyl appears to be one of the proximate agents in carcinogenesis by 4-acetylaminobiphenyl in the rat.

Recent studies from this laboratory have demonstrated that the versatile carcinogens 2-acetylaminofluorene and 2-aminofluorene are metabolized to N-hydroxy-2-acetylaminofluorene in the rat (9, 17) and that this metabolite is more carcinogenic than the parent compounds (15). Similar studies have now been carried out with the analogous compounds 4-acetylaminobiphenyl and 4-aminobiphenyl as an initial test of the general importance of N-hydroxylation in the metabolism and carcinogenicity of aromatic amines. In the rat 4-acetylaminobiphenyl is as potent a mammary carcinogen as 2-acetylaminofluorene, but it is far less active than the latter agent at other sites such as the liver (16, 20). 4-Aminobiphenyl is of special interest, since it is a carcinogen for the urinary bladder of the human (11) and the dog (10, 26); in

1 Chemical Abstracts nomenclature: 4'-phenylacetanilide for 4-acetylaminobiphenyl; 4-biphenylamine for 4-aminobiphenyl; N-hydroxy-4'-phenylacetanilide for N-hydroxy-4-acetylaminobiphenyl.
2 Bousier et al. (2) misread our data (15, 16) on the carcinogenicity of 4-acetylaminobiphenyl in stating that we "obtained a number of fibroadenomas of the breast during a period of twelve months" with this compound. In our experiments 4-acetylaminobiphenyl induced high incidences of mammary adenocarcinomas by 6 months.

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the rat parenteral administration of this amine produced tumors at several sites including the intestinal tract (25). This report concerns the metabolism of these compounds to N-hydroxy-4-acetylaminobiphenyl in the rat, the metabolism of 4-acetylaminobiphenyl to the N-hydroxy derivative in the dog, and the carcinogenicity of this metabolite in the rat.

MATERIALS AND METHODS

Animals and diets.—Weanling female (50–60 gm.) and young, adult male and female (180–200 gm.) albino rats were used in these experiments. They were kept in screen-bottomed cages and given food and water ad libitum. A grain diet (20), customarily used in this laboratory for the induction of tumors with 2-acetylaminofluorene (16, 20), was employed in the carcinogenicity tests. For the metabolic studies the rats were fed an 18 per cent casein semi-purified diet (19) which contained, in addition to the supplements listed in the reference, 20 mg 2-methyl-1,4-naphthoquinone/kg diet. The urine from rats fed the grain diet contained substances which interfered with the chromatography of the urine extracts (17). Throughout the experiments terramycin was given in the drinking water (75 µg/ml) one week each month to control respiratory infection. Metabolism studies were also carried out with two male and one female mongrel dogs weighing 6.5–7.5 kg. and less than 1 year old. The dogs were housed in heavy wire mesh cages which were washed daily. During the experimental period commercial dry dog meals (Gains, Fromm) were fed. Food and water were given ad libitum, and the dogs were weighed during administration of the compound in order to adjust the dose to body weight.

Administration of compounds and collection of urines.—When 4-acetylaminobiphenyl, 4-aminobiphenyl, and N-hydroxy-4-acetylaminobiphenyl were given in the diet to rats the compounds were added to the above diets as 10 gin. of a glucose suspension were prepared fresh on each day of use. These compounds were given in the diet to rats the compounds were added at a level of 0.003 per cent in acetone solution as described in the latter reference. When dog urine was collected under toluene or into sodium fluoride solution the dog urines were not analyzed immediately they were frozen and stored at -15°.

Examination of the rats.—The rats were weighed at least twice a month during feeding of the compounds. When the compounds were injected the animals were weighed weekly in order to determine the dose for each rat for the subsequent week. The rats were palpated for tumors every 2d week after the 6th week in the experiments with young rats, and after the 12th week in the experiments with adult rats. During autopsy close inspection was made of the lungs, liver, kidneys, stomach, intestines, and urinary bladder. The car- duct glands and the mammary glands were examined carefully after removal of the overlying skin. Laparotomies were performed in some of the experiments in order to examine the liver during the course of the experiment. All tumors were fixed in neutral 10 per cent formalin, sectioned at 6µ, and stained with hematoxylin and eosin for histological study.

Determination of urinary metabolites.—The paper partition chromatography of the urinary metabolites of 4-acetylaminobiphenyl, 4-aminobiphenyl, and N-hydroxy-4-acetylaminobiphenyl was performed essentially as described by Cramer et al. (9) for the metabolites of 2-acetylaminofluorene. The color tests and spectrophotometry on these metabolites were also carried out as described in the latter reference. When dog urine was analyzed, the 24-hour collection was diluted to 1 liter, and the 18-ml. aliquots were treated enzymatically in the same manner as were the rat urines. In addition to the routine release of metabolites by β-glucuronidase and Takadiastase, certain experiments were carried out to release metabolites by alkaline treatment of the dog urines. For this purpose one-twentieth of the 24-hour
urine collection was buffered at pH 6, extracted with ether, and then the urine was made alkaline (> pH 12) with 4 N NaOH. The alkali-released metabolites were then extracted immediately into ether, the ether taken to near dryness, and the ether extracts spotted on paper chromatography strips.

Isolation of urinary metabolites.—The isolation of N-hydroxy-4-acetylaminobiphenyl from the urines of both rats and dogs was accomplished as described by Cramer et al. (9) for the isolation of N-hydroxy-2-acetylaminofluorene. Liter quantities of urine were incubated with β-glucuronidase (bacterial, Sigma) and Takadiastase preparations and extracted with ether; the residues from these extracts were partition-chromatographed on silicic acid columns. The column eluates were read in the Beckman DU spectrophotometer at 278 mg, which is the absorption maximum of N-hydroxy-4-acetylaminobiphenyl in this solvent mixture (9). For further identification absorption spectra from 350 to 250 mg in ethanol were obtained with the Beckman DK-1 recording spectrophotometer. The tubes containing the N-hydroxy compound were extracted with alkali, and the compound was transferred to ether after neutralization. The residue from the ether extracts was dissolved in a minimal volume of warm benzene, filtered, and the crude crystals were dried to constant weight at 60° C. over P2O5 in vacuo for elementary analysis.

Synthesis of compounds.—4-Aminobiphenyl was obtained commercially (Eastman) and purified by distillation in vacuo from zinc dust and recrystallized from ethanol-water (m.p., 51.5°—52.5° C. uncorr.). 4-Acetylaminobiphenyl was prepared from 4-aminobiphenyl by acetylation in benzene with acetic anhydride (20) and recrystallized from ethanol (m.p., 171°—173° uncorr.). N-Hydroxy-4-acetylaminobiphenyl is a new compound. It was prepared at first by the procedure initially used for the synthesis of N-hydroxy-2-acetylaminofluorene (9). Later preparations were made according to an improved procedure for synthesis of the latter compound (15). In essence the improved procedure involved the catalytic hydrogenation of 4-nitrobiphenyl (Eastman, Light) in 8-gm. batches in an ethyl acetate solution containing palladium-charcoal, triethylamine, and excess acetic anhydride. The N, O-diacyl derivative formed was then hydrolyzed to the N-hydroxy compound by ammonium hydroxide. The crude product was re-crystallized from benzene-hexane (m.p., 144°—145° uncorr.). The yield was approximately 14% per cent of theoretical.

Anal.—Calcd. for C14H13N02: C, 73.99; H, 5.76; N, 6.16.

Found: C, 73.79; H, 5.77; N, 6.03.

RESULTS

Observations with paper chromatograms.—From our previous experience on the N-hydroxylation of 2-acetylaminofluorene in the rat, the N-hydroxylation of 4-acetylaminobiphenyl was readily detected in both the rat and dog. Urines were collected either from adult rats of both sexes fed 0.04—0.08 per cent of 4-acetylaminobiphenyl in the diet for 2 weeks or from dogs given 4-acetylaminobiphenyl orally once or repeatedly by capsule. These urines were then treated with β-glucuronidase and Takadiastase preparations and extracted with ether. Paper chromatography (9) of these extracts yielded a zone with the properties expected of N-hydroxy-4-acetylaminobiphenyl. This material was soluble in alkali, it reduced the Folin-Ciocalteu phenol reagent, it reacted slowly with acidic p-dimethylaminobenzaldehyde reagent to form a yellow color, it failed to couple with diazotized 7-nitro-2-aminofluorene, and it had the same RF (0.83—0.86) and ultraviolet absorption spectrum as synthetic N-hydroxy-4-acetylaminobiphenyl. No free N-hydroxy metabolite was detected in fresh urine from either rats or dogs fed 4-acetylaminobiphenyl. The conjugated N-hydroxyl metabolite present in these urines was easily cleaved with the bacterial β-glucuronidase preparations, whereas Takadiastase liberated only traces of this derivative. 4-Acetylaminobiphenyl was detected on the paper chromatograms as a dark area by examination of the strips under UV light. It had an RF of 0.83—0.86, did not react with any of the reagents mentioned above, and was easily separated from the N-hydroxy metabolite by extraction from alkaline solution. When 4-aminobiphenyl was fed to rats under identical conditions, it yielded the same urinary metabolites as the N-acetyl derivative;4 evidently it was readily N-acetylated in this species.

1 Huffman Microanalytical Laboratories, Wheatridge, Colo.

4 Dr. S. Laham, Occupational Health Division, Department of National Health and Welfare, Canada, has kindly informed us that he has isolated from the urine of rats fed 4-aminobiphenyl a compound to which he has tentatively assigned the structure of N-hydroxy-4-acetylaminobiphenyl.
However, when 4-aminobiphenyl was given to the dog, identical treatment of the urine did not reveal N-hydroxy-4-acetylaminobiphenyl as a metabolite. Our other findings with 4-aminobiphenyl administration in the dog have been inconsistent. Alkaline treatment of the urine of one male dog after each of three doses of 4-aminobiphenyl released a metabolite which had an $R_f$ value similar to that of freshly prepared synthetic N-hydroxy-4-aminobiphenyl. Both the metabolite and the synthetic compound reacted quickly with the acidic p-dimethylaminobenzaldehyde reagent to form a yellow nitrore; they both reduced the phenol reagent; and they both failed to couple with diazotized 7-nitro-2-aminofluorene. However, all later attempts to reproduce these findings on the release of this metabolite by alkali from the urine of the same dog or of the other dogs failed. Since N-hydroxy-4-aminobiphenyl is unstable, an attempt was made to induce the dog to acetylate the $N$-hydroxy metabolite of 4-aminobiphenyl in vivo by giving excess calcium pantothenate and riboflavin daily for 9 days. No N-hydroxy-4-acylaminobiphenyl was detected in the dog urine even after this period. The dog is considered to have a poor ability to acetylate aromatic amines (29, p. 437).

Several other zones with $R_f$'s of 0.12–0.22, 0.26–0.35, 0.52–0.58, and 0.64–0.69 were noted on the paper strips from urines of rats receiving either 4-acylaminobiphenyl or 4-aminobiphenyl. All these zones reduced phenol reagent and coupled with the diazo reagent. These zones were presumably phenolic metabolites; their $R_f$'s resembled those of the phenolic metabolites of 2-acylamino fluorone (17, 28). It seems likely that ring-hydroxylation in the 3-, 2', and 4'-positions of 4-acylaminobiphenyl had occurred. These would be analogous with the 1- and 3-, the 5-, and the 7-positions of 2-acylamino fluorone, respectively. These presumed phenolic metabolites of 4-acylaminobiphenyl were not examined further.

Isolation of the $N$-hydroxy metabolite.—Proof of structure of the presumptive $N$-hydroxy metabolite of 4-acylaminobiphenyl detected by paper chromatography was obtained by isolation of the metabolite in crystalline form after column chromatography. Six 1-liter batches of urine were collected from adult male and female rats fed 0.08 per cent of 4-acylaminobiphenyl for 1–9 weeks. These urines were treated as described above in "Materials and Methods," and yields of 5–20 mg. of crystalline product per liter were obtained. The same metabolite was isolated from six pooled daily urine collections from a dog given 150 mg. of 4-acylaminobiphenyl per day. Approximately 130 mg. of the metabolite was obtained as a concentrate and 61 mg. of the crystalline $N$-hydroxy metabolite was actually isolated. Elementary analyses yielded satisfactory data for the metabolite from each species.

**Anal.**—Calcd. for $C_{14}H_{12}NO_2$: C, 73.99; H, 5.76; N, 6.16

Found (rat): C, 73.93; H, 5.72; N, 6.23

Found (dog): C, 74.11; H, 6.01; N, 5.97

The melting point of the metabolites was 145°–146° C. uncorr., and this was unchanged upon admixture with synthetic $N$-hydroxy-4-acylaminobiphenyl. The UV spectrum ($log a_m = 4.21$ at 278 m$\mu$ in ethanol), the $R_f$, and all of the previously mentioned chemical properties of the metabolites were identical with those of the synthetic compound.

Factors affecting the urinary excretion of $N$-hydroxy-4-acylaminobiphenyl.—Dr. Alfredo Margreth in this laboratory observed previously (18) that the ability of normal rats to excrete the $N$-hydroxy metabolite of 2-acylamino fluorone following single doses of this amide decreased with age. This effect appeared to be related to the amount of growing liver, since adult rats with regenerating livers induced by partial hepatectomy or by previous damage with hepatotoxic agents excreted high levels of the $N$-hydroxy metabolite of the amide; the excretion decreased to normal levels when the livers in these animals approached the normal state. However, continuous feeding of 2-acylamino fluorone to rats produced a steady increase in the urinary output of the $N$-hydroxy metabolite until a maximum high level was reached after many weeks (17). This effect is probably related to the continuous liver regeneration caused by continuous dosage with this hepatotoxic amide.

Quantitative estimations of the urinary excretion of $N$-hydroxy-4-acylaminobiphenyl by rats given 4-acylaminobiphenyl under several conditions revealed similarities and differences from the results obtained with 2-acylamino fluorone. As has been observed with 2-acylamino fluorone, normal rats excreted less of a test dose of 4-acylaminobiphenyl as the $N$-hydroxy metabolite as they grew older (Table 1). Likewise, induced liver growth in adult rats as a result of previous damage by 2-acylamino fluorone produced an increase in the urinary output of the $N$-hydroxy metabolite (Table 1). Unlike the case with 2-acylamino fluorone, continuous feeding of either 4-acylamino fluorone or its $N$-hydroxy metabolite to adult male rats for several weeks led to a gradual

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decrease in the urinary excretion of N-hydroxy-4-acetylaminobiphenyl (Chart 1). For comparison the previously observed increase (17) in the excretion of N-hydroxy-2-acetylaminoﬂuorene during the continued administration of 2-acetylaminoﬂuorene to rats is shown in this chart. Adult female rats fed 4-acetyaminobiphenyl or N-hydroxy-4-acetyaminobiphenyl with or without a low level of 3-methylcholanthrene in the diet also excreted less of the N-hydroxy metabolite as the feeding of the biphenyl derivatives progressed (Table 2). Dietary methylcholanthrene produces a large inhibition of the excretion of the N-hydroxy metabolite of 2-acetylaminoﬂuorene (17). These metabolic effects appear to correlate with the capacity of dietary methylcholanthrene to inhibit the carcinogenicity of 2-acetylaminoﬂuorene (14), whereas this hydrocarbon has little or no effect on the carcinogenicity of N-hydroxy-2-acetylaminoﬂuorene (15). The relative ineffectiveness of the hydrocarbon on the carcinogenicity of 4-acetyaminobiphenyl and N-hydroxy-4-acetyaminobiphenyl is discussed be-

**TABLE 1**

**URINARY EXCRETION OF N-HYDROXY-4-ACETYLAMINOBIPHENYL (N-HO-AABP) BY MALE RATS GIVEN INJECTIONS INTRAPERITONEALLY OF SINGLE DOSES OF 4-ACETYLAMINOBIPHENYL (AABP)**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. rats</th>
<th>Av. wt. (g.m.)</th>
<th>Liver, per cent body wt.</th>
<th>Urinary N-HO-AABP* (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weanlings</td>
<td>8</td>
<td>46</td>
<td>2.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Young adults</td>
<td>4</td>
<td>188</td>
<td>3.0</td>
<td>0.5</td>
</tr>
<tr>
<td>(4 mo. old)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adults</td>
<td>4</td>
<td>470</td>
<td>2.7</td>
<td>0.2</td>
</tr>
<tr>
<td>(5 mo. old)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adults</td>
<td>4</td>
<td>372</td>
<td>7.5</td>
<td>1.6</td>
</tr>
<tr>
<td>with damaged,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>regenerating</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liver†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Urine collected during the 24 hours following a single intraperitoneal injection of 4 mg AABP/100 gm/body weight in 0.5 ml. suspension medium (1.75 per cent gum acacia in 0.9 per cent saline). Data are corrected for the differences in molecular weights.
† These rats were fed 0.08 per cent 2-acetylaminoﬂuorene in the diet for 10 weeks and then placed on the basal diet for 1 week before injection.

**TABLE 2**

**URINARY EXCRETION OF N-HYDROXY-4-ACETYLAMINOBIPHENYL (N-HO-AABP) BY ADULT FEMALE RATS FED 4-ACETYLAMINOBIPHENYL (AABP) OR ITS N-HYDROXY METABOLITE WITH OR WITHOUT 3-METHYLCHOLANTHRENE (MC)**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Urinary excretion of N-HO-AABP* (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 wk.</td>
</tr>
<tr>
<td>AABP</td>
<td>3.9</td>
</tr>
<tr>
<td>AABP+MC</td>
<td>2.6</td>
</tr>
<tr>
<td>N-HO-AABP</td>
<td>5.0</td>
</tr>
<tr>
<td>N-HO-AABP+MC</td>
<td>4.9</td>
</tr>
</tbody>
</table>

* Each group consisted of four rats fed either 0.04 per cent AABP or 0.003 per cent N-HO-AABP with or without 0.003 per cent MC. Data were obtained from 24-hour urine collections and are corrected for the differences in molecular weights.

**Chart 1.**—The urinary excretion of N-hydroxy-4-acetylaminoﬂuorene (N-HO-AABP) when it or 4-acetylaminoﬂuorene (AABP) was fed to rats. The data for 2-acetylaminoﬂuorene (AAF) (17) are given for comparison. Each point represents the average excretion for 24 hours for four adult male rats. Data are corrected for the differences in molecular weights.

* Comparison of the data in Chart 1 for male rats fed 4-acetylaminoﬂuorene with the data in Table 2 for female rats fed this amide suggests that female rats excrete a greater percentage of the dose as the N-hydroxy amide than do male rats. In a direct comparison with adult rats of both sexes fed 0.06 per cent of 4-acetylaminoﬂuorene in the diet for 9 or 5 weeks the females excreted, as the N-hydroxy metabolite, approximately twice as much of the ingested amide as did male rats.
dosage is involved. They are probably related in part to the different effects these amides have on the liver of the rat, i.e., 2-acetylaminofluorene is hepatotoxic and gives rise to proliferation of the liver cells, whereas 4-acetylaminobiphenyl has no known effect on the rat liver (16, 20). Further explanation of these data must await analysis at the enzymatic level.

Elution of paper chromatography strips from urines of dogs given 4-acetylaminobiphenyl showed that 7–20 per cent of this amide was excreted as the N-hydroxy metabolite. Minor errors are probably involved in these estimations, because the dog excretes urine less frequently than does the rat and because the collection flasks had to be removed for short times during the daily washing of the dog metabolism cages.

Tests on the carcinogenicity of N-hydroxy-4-acetylaminobiphenyl in the rat.—When sufficient synthetic N-hydroxy-4-acetylaminobiphenyl became available its carcinogenicity in the female rat was determined under several conditions. In adult female rats fed 0.04 per cent of 4-acetylaminobiphenyl or 0.043 per cent of N-hydroxy-4-acetylaminobiphenyl for 16 weeks the N-hydroxy metabolite proved to be as active a mammary carcinogen as the parent amide (Table 3). In addition, the N-hydroxy derivative induced ear duct carcinomas in six of nineteen rats, whereas the parent amide caused only one. Dietary 3-methylcholanthrene at the level of 0.003 per cent had no marked effect on the carcinogenicity of either compound for the mammary gland, but it did appear to reduce the incidence of ear duct carcinomas induced by the N-hydroxy compound. Forestomach papillomas were seen in three rats fed N-hydroxy-4-acetylaminobiphenyl (either with or without methylcholanthrene), and one of these rats also had a squamous-cell carcinoma of the forestomach (Figs. 1 and 2). No forestomach tumors were ob-

<table>
<thead>
<tr>
<th>DIET</th>
<th>NO. RATS</th>
<th>INITIAL WT. (GM.)</th>
<th>WT. GAIN AT 16 WK. (GM.)</th>
<th>NUMBER OF RATS WITH:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mammary carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ear-duct carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Forestomach papillomas</td>
</tr>
<tr>
<td>AABP</td>
<td>16</td>
<td>184</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>AABP+MC</td>
<td>16</td>
<td>181</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>N-HO-AABP</td>
<td>19</td>
<td>186</td>
<td>33</td>
<td>1</td>
</tr>
<tr>
<td>N-HO-AABP+MC</td>
<td>16</td>
<td>182</td>
<td>33</td>
<td>1</td>
</tr>
</tbody>
</table>

* The rats were fed the various diets for 16 weeks, after which they were maintained on the basal grain diet until the surviving rats were killed at 42 weeks.
† The numbers in parentheses indicate the total number of mammary carcinomas in each group.
‡ This rat also had a squamous-cell carcinoma of the forestomach.
served in the rats fed 4-acetylaminobiphenyl, but they have been found frequently in rats fed N-hydroxy-2-acetylaminofluorene (15).

Preliminary data indicate that male rats are also more susceptible to the carcinogenic action of N-hydroxy-4-acetylaminobiphenyl than to that of 4-acetylaminobiphenyl, although the male rats develop tumors only slowly when either compound is administered. In a study still in progress only one of sixteen male rats fed 0.06 per cent of 4-acetylaminobiphenyl in the diet for 81/2 months has developed a tumor (a carcinoma of the ear duct) by 91/2 months. On the other hand, three of sixteen male rats fed an equimolar amount of N-hydroxy-4-acetylaminobiphenyl have developed tumors. These include one rat with both a carcinoma of the ear duct and an adenocarcinoma of the mammary gland and two rats bearing single adenocarcinomas of the mammary gland. In a preliminary test in which N-hydroxy-4-acetylaminobiphenyl and its parent amide were each injected intraperitoneally into adult male rats 3 times weekly for 5 months at a level of 0.01 mmole per 100 gm. of body weight in 1 ml. of 0.9 per cent sodium chloride solution containing 1.75 per cent of gum acacia, one of four rats which received injections of the N-hydroxy derivative developed a carcinoma of the ear duct at 10 months, while no tumors were noted after 11 months in four rats which received the parent amide.

As in the case of N-hydroxy-2-acetylamino-
fluorene and 2-acetylaminofluorene (15), tests in young female rats revealed an increased carcinogenicity of N-hydroxy-4-acetylaminobiphenyl for the mammary gland as compared with 4-acetylaminobiphenyl. The results of the intraperitoneal injection of 4-acetylaminobiphenyl and its N-hydroxy derivative in young female rats are shown in Chart 2. In a second experiment carried out under the same conditions with immature female rats six of seventeen animals given injections of the N-hydroxy metabolite had mammary tumors at 26 weeks, whereas only one of seventeen rats given injections of the parent amide bore a similar tumor. It is clear that under these conditions N-hydroxy-4-acetylaminobiphenyl is more active as a mammary carcinogen than its parent amide.

The mammary tumors induced in the adult and young female rats given 4-acetylaminobiphenyl and its N-hydroxy metabolite were diagnosed histologically as adenocarcinoma (Figs. 3 and 4). These carcinomas are similar to those seen in the rat after the ingestion of compounds such as 2-acetylamino- fluorene and its congeners. The ear-duct tumors induced by N-hydroxy-4-acetyl-
aminobiphenyl were all typical keratinizing squa-
mous-cell carcinomas (Figs. 5 and 6).

Methemoglobinemia was noted in the adult and weanling female rats within 90 minutes after each intraperitoneal injection of N-hydroxy-4-acetylaminobiphenyl, whereas no similar effects were noted in the rats receiving 4-acetylaminobiphenyl. The methemoglobinemia decreased in severity with continued administration of the hydroxy derivative. Various hydroxylamines are known to cause methemoglobinemia (29, pp. 164, 429, and 433).

DISCUSSION

The observations in this paper extend the previous findings on the occurrence and importance of N-hydroxylation in the metabolism and carcinogenicity of 2-acetylaminofluorene and 2-amino-
fluorene (9, 15, 17) to the closely related but less versatile carcinogens 4-acetylaminobiphenyl and 4-aminobiphenyl. The carcinogenicity of the N-hydroxy metabolite of 4-acetylaminobiphenyl indicates that it is a proximate carcinogen in mammary carcinogenesis in the rat by 4-acetylaminobi-
phenyl. The o-hydroxy derivatives, 3-hydroxy-4-
acetylaminobiphenyl and 3-hydroxy-4-aminobi-
phenyl, are the only other metabolites of 4-acetyl-
aminobiphenyl and 4-aminobiphenyl which have been tested for carcinogenic activity. Oral adminis-
tration of these derivatives elicited no tumors in the rat (16), although wax pellets containing 3-
hydroxy-4-aminobiphenyl sulfate induced bladder tumors after implantation in the urinary bladder of the mouse (8).

Recent work has extended knowledge on the occurrence of N-hydroxylation to several other species and to a third carcinogenic aromatic amine. Thus, Troll and Nelson (23) have detected conjugates of N-hydroxy-2-naphthylamine in the urine of dogs and humans given 2-naphthylamine, and Boyland and Manson have isolated the O-sul-
fate of this hydroxylamine from the urine of dogs after administration of 2-naphthylamine. In this paper it has been shown that the dog excretes approximately 10 per cent of an oral dose of 4-acetyl-

7 Bradshaw (6) stated that the experimental period of 13 months in our tests (16) was "inadequate." In these tests the metabolites were fed for 8–10 months at a level equimolar to that employed for the parent amide. By 13 months a few mammary fibroadenomas, probably spontaneous, were seen in the rats given the o-hydroxy derivatives. However, 77 per cent of the rats fed the parent amide, 4-acetylaminobiphenyl, had adenocarcinomas of the mammary gland by 6 months. Even if adenocarcinomas were to develop after 13 months in the rats fed these high levels of the o-hydroxy metabolites, such data could not establish these metabolites as proximate carcinogens.

8 E. Boyland and D. Manson, private communication from Dr. Boyland, Chester Beatty Research Institute, London.
amino-biphenyl as a conjugate of the N-hydroxy derivative. Little or none of this conjugate was detected in dog urine following oral administration of 4-amino-biphenyl. Other studies in this laboratory have demonstrated N-hydroxylation of 2-acetylaminofluorene in the golden hamster; as in the rat (15) oral administration of N-hydroxy-2-acetylaminofluorene to this species induces a high incidence of papillomas and squamous-cell carcinomas of the forestomach, whereas 2-acetylaminofluorene is inactive at this site. Thus, three different carcinogenic aromatic amines and amides are known to be N-hydroxylated in vivo, and N-hydroxylation is known to occur in the rat, mouse (17), hamster, dog, and man. These species are susceptible to the carcinogenic action of aromatic amines. However, N-hydroxylation of 2-acetylaminofluorene was not detected in the guinea pig (17), and so far this species has proved to be totally resistant to the carcinogenic action of aromatic amines and amides (27). These observations and the experimental evidence that the N-hydroxy metabolites of 2-acetylaminofluorene and 4-acetylaminobiphenyl are proximate agents in carcinogenesis by these amines suggest that N-hydroxylation is an important step in carcinogenesis by many aromatic amines and amides.

Further studies are required on the possible interrelationships between N-hydroxylation and o-hydroxylation in the metabolism of aromatic amines. An experiment with a mixture of N-hydroxy-2-acetylaminofluorene and 2-acetylaminofluorene-9-C14 in the rat (13) provides strong evidence that the 1-hydroxy metabolite of 2-acetylaminofluorene is derived from the N-hydroxy metabolite. The other o-hydroxy metabolite, the 3-isomer, is apparently derived both by direct oxidation at the 3-carbon atom of the amide and from the N-hydroxy derivative. The suggestion (17) that N-hydroxy-o-hydroxy metabolites of carcinogenic amines might also be formed has received experimental support from the recent report by Troll and Nelson that dogs given 2-naphthylamine excrete N-hydroxy-1-hydroxy-2-naphthylamine. Hence, in some cases o-hydroxy amines may be derived from N-hydroxy amines; in other cases direct o-hydroxylation may occur alone or before or after N-hydroxylation of the same molecule.

Similarly, further data are needed on the relative roles of N-hydroxylation and of o-hydroxylation in the carcinogenic processes induced by aromatic amines. The N-hydroxy metabolites of 2-acetylaminofluorene and 4-acetylaminobiphenyl are highly carcinogenic when administered either orally or intraperitoneally to the rat, whereas the o-hydroxy derivatives of these amines and of 4-amino-biphenyl have not exhibited carcinogenic activity when fed to the rat (16, 22). Evidence for the carcinogenicity of o-hydroxy amines comes mainly from the induction of bladder tumors in mice after implantation of pellets containing compounds of this type in the urinary bladder. The carcinogenicities of N-hydroxy derivatives under these conditions have not been reported. Thus, from the present data it is possible that o- and N-hydroxy metabolites may each be carcinogenic under certain conditions and that either or both types may undergo further metabolism to other proximate carcinogens which might include compounds such as N-hydroxy-o-hydroxy derivatives.

In view of the interest in the o-hydroxylation hypothesis of carcinogenesis by aromatic amines (7, 25), a brief consideration of its experimental support is pertinent. Most of the evidence has been obtained by Bonser and her group (2, 4, 8) and by Boyland and his associates (1, 5) in studies which employed the implantation in the mouse urinary bladder of pellets of test substances in relatively inert vehicles such as paraffin waxes and cholesterol. Under these conditions certain o-hydroxy amines have induced bladder tumors, whereas the parent amines have shown only borderline activity, although 2-anthramine, which oxidizes very readily, had appreciable activity (8). This ingenious procedure requires only a small quantity of test material which is implanted only once in an easily manageable species. However, this test has some serious disadvantages. Even the most active substances tested have not given tumor incidences over 50 per cent, and pellets of the waxes and cholesterol alone (substances notably variable in composition) have induced bladder tumors, including carcinomas, in up to 9 per cent of the surviving control animals (3). In many of the tests the pellets were prepared by melting the vehicle and test substance together, a procedure which must have led to oxidative changes in such sensitive substances as o-hydroxy amines even before their insertion in the bladder. This problem has been minimized in recent work by the use of compressed pellets (1, 3); however, high concentrations in the pellets (12–20 per cent) of highly reactive compounds might give rise to decomposition and contamination.
Fig. 1.—Squamous-cell carcinoma of the forestomach from a female rat fed N-hydroxy-4-acetylaminobiphenyl in the diet. This section shows extensive infiltration of the submucosa by nests of epithelial cells. X50.

Fig. 2.—Higher magnification of the carcinoma shown in Figure 1. Nests of keratinized squamous epithelial cells are evident. X150.

Fig. 3.—Adenocarcinoma of the breast from a female rat which received repeated intraperitoneal injections of N-hydroxy-4-acetylaminobiphenyl for 3 weeks after weaning. There is extensive proliferation of ductlike elements. X50.

Fig. 4.—Higher magnification of the carcinoma shown in Figure 3. The irregular ductlike structures are separated by strands of connective tissue. X150.

Fig. 5.—Squamous-cell carcinoma of the ear duct from a female rat fed N-hydroxy-4-acetylaminobiphenyl in the diet. The large cysts are distended by infected sebaceous material. X50.

Fig. 6.—Higher magnification of the carcinoma shown in Figure 5. This section shows nests of squamous cells separated by strands of connective tissue. X150.
such as N-hydroxylation have played a role in the bladder implantation technic. Results obtained with the o-hydroxy amines by the other than o-hydroxylation. In any case, it is now important to determine to what degree reactions by other routes has been noted for both o-methyl-compound may be cleaved to the corresponding condensation products with misleading pathological changes are less likely to occur when the com-

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MORRIS et al.--Histopathology and Sulfhydryl during Carcinogenesis

Soluble -SH were causative or merely accompanied by hyperplasia, as is not evident from the data. It is possible that both the pyridine nucleotides and glutathione are involved in reactions which detoxify the carcinogen or react with the estrogen, resulting in decreased glutathione concentration and increased proportion of oxidized TPN. Perhaps some other member of the chain on the way to oxygen--such as flavoprotein or a hemeprotein--has reacted with the carcinogen or with products of cell destruction, and the oxidized flavoprotein or cytochrome c has oxidized the TPN, thus decreasing the TPNH available for keeping the glutathione and the protein -SH reduced. We expect to investigate these alternative possibilities.

The difference between the sexes in the changes of -SH in response to the carcinogen, both during the early inflammatory reaction and the later period of tumor formation, is striking. This effect may be related to the differing action of sex hormones.

It is recognized that analysis of the whole pouch cannot give specific information about the epithelial tissues in which the tumors appear. However, except for the most violent inflammatory reactions, no change in the morphology of the underlying connective tissue and muscle strands has been seen. Therefore, we suggest that the sulfhydryl changes in the total tissue may be correlated with the morphological changes in the epithelial layer. Methods of treating the tissue to separate the epithelial layer from connective layer are being investigated, with the hope that some treatment will be found which will neither change the sulfhydryl values nor inactivate the enzymes.

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condensation products with misleading pathological activities. Although no route of administration is free from objection, it would appear that such changes are less likely to occur when the compounds are introduced into the body at lower concentrations in the vehicle by other procedures such as dietary administration and intraperitoneal injection. In spite of the emphasis on the activity of the o-hydroxy amines, the most active compound noted to date in the bladder implantation tests is 1-methoxy-2-naphthylamine (8). Whereas this compound may be cleaved to the corresponding naphthol in vivo, no evidence in this regard has been presented. Furthermore, an increase in the carcinogenicities of aromatic amines administered by other routes has been noted for both o-methyl- and o-methoxy-substituted amines (19, 24, 25); it is possible that these substitutions favor reactions other than o-hydroxylation. In any case, it is now important to determine to what degree reactions such as N-hydroxylation have played a role in the results obtained with the o-hydroxy amines by the bladder implantation technique.

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The \(N\)-Hydroxylation of 4-Acetylaminobiphenyl by the Rat and Dog and the Strong Carcinogenicity of \(N\)-Hydroxy-4-acetylaminobiphenyl in the Rat

James A. Miller, Carole S. Wyatt, Elizabeth C. Miller, et al.


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