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# Induction of Hepatomas by 4-Aminobiphenyl and Three of Its Hydroxylated Derivatives Administered to Newborn Mice [1,2]

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SUMMARY—Groups of approximately 25 male and 25 female Swiss mice received injections of 200 μg 4-aminobiphenyl, 4-amino-3-hydroxybiphenyl, 4-hydroxylaminobiphenyl, or 4-amino-4′-hydroxybiphenyl on each of the first 3 days of life. The materials were injected as solutions/suspensions in 3% aqueous gelatin. A large control group was treated with the vehicle only and a further control group left untreated. As a positive control, 20 μg of 7,12-dimethylbenz[a]anthracene (DMBA) was injected into 49 newborn mice on each of the first 3 days of life. In males, a marked and significant increase in the incidence of hepatomas above the control level was seen in response to 4-aminobiphenyl itself and to each of its three derivatives. In females, a slight but probably significant increase was noted in response to three of the test compounds, but not to 4-amino-3-hydroxybiphenyl. In neither sex was there an increase in the incidence of neoplasms at other sites. DMBA-treated mice of both sexes developed, as expected, pulmonary tumors (59%) and lymphomas (14%). In addition, a high incidence of hepatomas was recorded in the males, but none in the females. The results with regard to the four test substances are of interest because tests for carcinogenicity in other systems have given negative results and because the liver is the sole target organ. Further investigation of the difference in response of the two sexes to neonatally injected carcinogens is overdue. The results suggest that no evaluation of carcinogenicity may be complete unless it includes tests in neonates.—J Nat Cancer Inst 41: 403–410, 1968.

4-AMINOBIPHENYL (*para*-xenylamine) is a powerful carcinogen that produces tumors in a

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variety of species. In man (*1*), dogs (*2*), and rabbits (*3*), exposure to 4-aminobiphenyl induces neoplasms of the urinary bladder, whereas in rats (*4*) it gives rise to intestinal and liver tumors. The fact that feeding this carcinogen produces neoplasms of the bladder implies that carcinogenesis is mediated via an active metabolite, but despite extensive study of the metabolism of 4-aminobiphenyl (*5*, *6*), the routes leading to the formation of the proximate carcinogen or carcinogens remain obscure (*7*).

In this paper, the carcinogenic activity of 4-aminobiphenyl and three of its hydroxylated derivatives has been studied in newborn mice. The derivatives 4-amino-3-hydroxybiphenyl, 4-hydroxylaminobiphenyl, and 4-amino-4′-hydroxybiphenyl are known to be formed *in vivo* and they are normally excreted in the urine as conjugates of acetic, glucuronic, or sulfuric acids (*7*).

## MATERIALS AND METHODS

### Chemical Substances

*4-Aminobiphenyl* was a commercial product obtained from Koch-Light Laboratories, Colnbrook, Bucks, England. It was distilled under reduced pressure before use.

*4-Hydroxylaminobiphenyl* was prepared by the reduction of 4-nitrobiphenyl with aluminum at 20° C. The material was recrystallized from benzene before use.

*4-Amino-3-hydroxybiphenyl* and *4-amino-4′-hydroxybiphenyl* were prepared by the reduction of the corresponding nitro compounds with hydrazine hydrate in the presence of palladium on charcoal. The aminophenols were recrystallized as the hydrochlorides. From these, the parent compounds were then liberated by treatment with alkali and recrystallized from ethanol.

The four amino compounds all gave single discrete spots when examined by thin-layer chromatography (*8*).

7,12-Dimethylbenz[*a*]anthracene (DMBA) was obtained from the Koch-Light Laboratories.

All test compounds were dissolved, or suspended by ultrasonication, in 3% aqueous gelatin before injection.

### Mice

Four hundred and sixty newborn Swiss (Porton) mice were used. The animals were obtained from a cesarean-derived strain maintained under barrier conditions (*see below*). Within 24 hours of birth the mice were randomized among 7 experimental groups. Except for one control group, they received injections of one of the compounds listed. After the first injection the animals were returned to a mother, each of whom was given 10 neonates.

### Administration of Test Substances and Conduct of Experiment

The mice were given subcutaneous injections in the interscapular region on each of the first 3 days of life as follows:

| | |
|---|---|
| Group I: | Three injections of 200 μg 4-aminobiphenyl in 0.02 ml aqueous gelatin. |
| Group II: | Three injections of 200 μg 4-amino-3-hydroxybiphenyl in 0.02 ml aqueous gelatin. |
| Group III: | Three injections of 200 μg 4-hydroxylaminobiphenyl in 0.02 ml aqueous gelatin. |
| Group IV: | Three injections of 200 μg 4-amino-4′-hydroxybiphenyl in 0.02 ml aqueous gelatin. |
| Group V: | Three injections of 0.02 ml aqueous gelatin only. |
| Group VI: | No injections. |
| Group VII: | Three injections of 20 μg DMBA in 0.02 ml aqueous gelatin. |

Groups I–IV thus constitute the principal test groups and V–VII are control groups. The last of these, Group VII, was included to demonstrate that Swiss (Porton) mice are sensitive to known carcinogens such as DMBA.

The mice were weaned at 4 weeks and the sexes segregated. They were housed in plastic cages, 10 in each, fed an autoclaved cubed diet (Small Animal Diet, Spillers Ltd., London), and given water *ad libitum*. Barrier conditions were maintained and all bedding was sterilized. During the experiment, the mice were inspected daily, and thoroughly examined at weekly intervals. Sick animals were killed promptly and the survivors were killed between 48 and 52 weeks after birth. Full postmortem examinations were carried out on all mice. The liver, kidneys, and urinary bladder were removed routinely, together with other

organs that showed any abnormalities and fixed in Bouin's solution. Paraffin sections were prepared at 5 $\mu$ stained with hematoxylin and eosin and, where necessary, with hematoxylin and van Gieson, Masson's trichrome, Gordon and Sweet's silver impregnation method for reticulin, and periodic acid-Schiff.

## RESULTS

The number of mice alive at weaning and at the termination of the experiment (at 48–52 weeks) is shown in table 1. Survival was better in Groups I–VI than in Group VII, and the overall survival of mice in all groups until 48–52 weeks was 87.6%.

The distribution of hepatoma-bearing mice in the 7 experimental groups is summarized in text-figure 1. The baseline incidence of liver tumors in Group VI was low and the neoplasms that did occur were entirely in males. In both sexes, treatment with aqueous gelatin only (Group V) was associated with a slightly raised incidence of hepatomas—an observation of doubtful significance. The proportion of mice with hepatomas was considerably increased in the group treated with DMBA (Group VII), but comparable or even larger proportions were seen in the groups treated either with 4 aminobiphenyl or with one of the three hydroxylated derivatives (Groups I–IV). The parent substance, 4-aminobiphenyl, was the most potent

TABLE 1.—Survival of mice in Groups I–VII

| Experimental group | Number of mice given injections at <24 hrs | Number of mice alive at weaning | Number of mice alive at 48–52 weeks | Survivors at 48–52 weeks as % of those alive at weaning |
|---|---|---|---|---|
| Group I: 4-Aminobiphenyl | 52 | 51 (♂ 24, ♀ 27) | 43 (♂ 20, ♀ 23) | ♂ 85. 4, ♀ 92. 0 |
| Group II: 4-Amino-3-hydroxybiphenyl | 55 | 55 (♂ 23, ♀ 32) | 48 (♂ 19, ♀ 29) | ♂ 82. 6, ♀ 90. 6 |
| Group III: 4-Hydroxylamino-biphenyl | 56 | 55 (♂ 20, ♀ 35) | 52 (♂ 19, ♀ 33) | ♂ 95. 0, ♀ 94. 3 |
| Group IV: 4-Amino-4′-hydroxybiphenyl | 50 | 49 (♂ 22, ♀ 27) | 44 (♂ 18, ♀ 26) | ♂ 81. 8, ♀ 96. 3 |
| Group V: Aqueous gelatin | 100 | 98 (♂ 48, ♀ 50) | 87 (♂ 41, ♀ 46) | ♂ 85. 4, ♀ 92. 0 |
| Group VI: Untreated | 98 | 96 (♂ 45, ♀ 51) | 90 (♂ 42, ♀ 48) | ♂ 93. 3, ♀ 94. 1 |
| Group VII: 7,12-Dimethyl-benz[*a*]anthracene | 49 | 49 (♂ 26, ♀ 23) | 39 (♂ 19, ♀ 20) | ♂ 73. 1, ♀ 87. 0 |

hepatic carcinogen, followed by 4-hydroxylaminobiphenyl. In most affected animals in Groups I–IV and in Group VII, multiple liver tumors were present; in Groups V and VI, no more than two hepatomas were observed in any one animal. If the total number of female mice with hepatomas in Groups I–IV is compared with that in Groups V and VI, the difference is not significant at the 5% level ($\chi^2 = 3.65$; $P$ just $>0.05$). However, no hepatomas were seen in females in Group II; the excessive incidence of hepatoma-bearing females in Groups I, III, and IV can therefore be regarded as probably being due to treatment, especially as 5 of 9 had more than two tumors each.

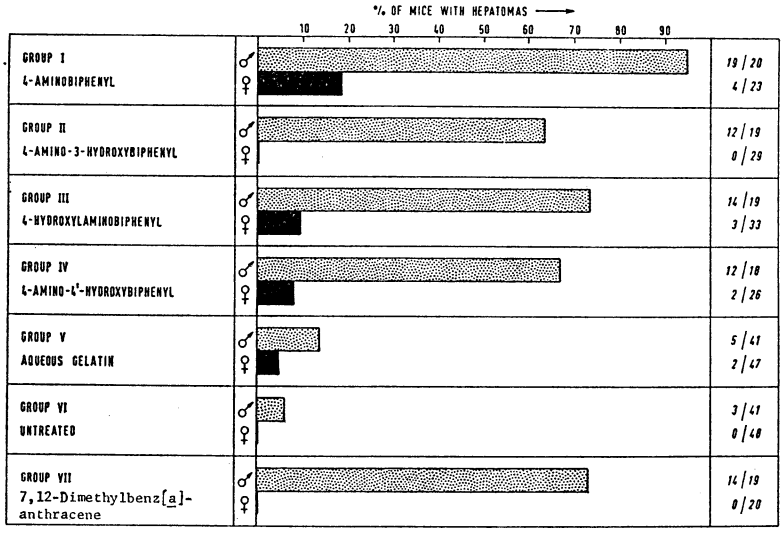
In view of the high proportion of animals that survived for the full duration of the experiment, the times at which hepatomas began to appear in the various experimental groups could not be assessed. However, 7 of the 50 mice that died before 48 weeks were found to have hepatomas at necropsies carried out between 31 and 47 weeks after the beginning of the experiment.

The occurrence of tumors other than hepatomas is shown in table 2. Few additional neoplasms were seen in mice from Groups I–VI, and the rarity of pulmonary adenomas and the absence of bladder tumors are noteworthy. By contrast, animals given injections of DMBA had a predictably high incidence of pulmonary adenomas and a less-pronounced increase in lymphatic neoplasms.

## Histopathologic Findings

*Liver.*—The macroscopic and microscopic appearances of the hepatomas were similar in all experimental groups. The lesions were usually multiple and showed no predilection for any particular zone of the liver. They varied in size, ranging from small nodules to large, protruding masses measuring up to 2.5–3.0 cm in diameter. The predominant cellular pattern was of well-differentiated cords, interspersed with large, blood-filled spaces. Hyaline cytoplasmic inclusions were seen in several tumors from all experimental groups. No bile-duct elements were identified. The tumors often showed patchy necrosis or fatty degeneration, and such changes were not necessarily accompanied by parallel abnormalities in the surrounding non-neoplastic parenchyma. Foci of hemorrhage and necrosis were sometimes prominent and some tumors showed extensive infarction. The surrounding parenchyma was often compressed but no extension of tumors into or beyond the hepatic capsule was evident.

Livers from mice that did not develop hepatomas were either normal or showed nonspecific paren-




TEXT-FIGURE 1.—Incidence of hepatomas in mice of Groups I–VII killed at termination of experiment (48–52 weeks).

TABLE 2.—Incidence of tumors other than hepatomas

| Experimental group | Tumors | | | | |
|---|---|---|---|---|---|
| | Pulmonary adenoma | Thymic lymphoma | Lympho-sarcoma of spleen | Generalized malignant lymphoma | Other tumors |
| Group I: 4-Aminobiphenyl | 1 | 1 | 1 | — | — |
| Group II: 4-Amino-3-hydroxybiphenyl | 2 | — | — | — | — |
| Group III: 4-Hydroxylaminobiphenyl | — | — | — | 1 | — |
| Group IV: 4-Amino-4′-hydroxybiphenyl | 1 | — | — | 1 | — |
| Group V: Aqueous gelatin | 1 | 1 | — | — | — |
| Group VI: Untreated | 2 | — | 1 | — | — |
| Group VII: 7,12-Dimethylbenz[*a*]anthracene | 29 | 3 | — | 4 | 1 granulosa-cell tumor of ovary |

chymal abnormalities, such as margination of cytoplasm, hyaline, hydropic or fatty changes, and necrosis. Such changes were characteristically patchy and were not localized consistently to any structure in or around the hepatic lobule. No lesions regarded as preneoplastic were observed.

*Other tissues.*—Degenerative changes were seen in the renal tubules of several animals. Most bladders examined were normal: Squamous metaplasia was observed in three mice and epithelial atypia in one. The vesical epithelium in this mouse showed some variation in size and shape of the component cells and their normal regular polarity was somewhat disrupted. Nuclear structures were, however, largely normal, mitotic figures were rarely seen, and there was no evidence of proliferation into the lumen of the bladder or downward through the basement membrane—the latter structure was intact.

## DISCUSSION

It is commonly held that aromatic amines are converted *in vivo* into "active" metabolites through a process which involves hydroxylation by enzymes localized in the microsomes of liver cells (*9–11*). With 4-aminobiphenyl, hydroxylation has been shown to occur on the nitrogen atom (*12*) and on the 3 or 4′ positions of the ring structure (*6*), but the metabolic routes leading to the initiation of carcinogenesis by 4-aminobiphenyl are unknown. Furthermore, information on the carcinogenic activity of derivatives of 4-aminobiphenyl is scanty and conflicting. The implantation of pellets containing 4-amino-3-hydroxybiphenyl into the bladders of mice led to the induction of vesical tumors, but pellets containing 4-hydroxylaminobiphenyl or 4-amino-4′-hydroxybiphenyl were inactive in this respect (*13–15*). By contrast, 4-amino-3-hydroxybiphenyl was inactive when incorporated into the diet of rats (*16*), but 4-acetamido-*N*-hydroxybiphenyl, a conjugate of 4-hydroxylaminobiphenyl, produced a high incidence of mammary carcinomas when administered by the same route (*17*).

In an attempt to clarify this situation, the carcinogenic effects of 4-aminobiphenyl and three of its hydroxylated derivatives were examined after their administration to newborn mice. Activity of the microsomal hydroxylating enzymes is low or absent during the first few days of life (*18–20*) and the ability to synthesize glucuronides and other conjugates is also poorly developed (*21, 22*). It was therefore thought that the newborn mouse would provide a suitable model to test whether any of the

hydroxylated derivatives of 4-aminobiphenyl was more likely to be the proximate carcinogen than the parent compound, since further metabolism of such compounds, either by hydroxylation or by conjugation, should be minimal. Another advantage is that the various substances could be expected to remain unchanged in the test animals for relatively long periods, compared with adult mice, thus permitting longer exposure to any putative carcinogen.

The present results clearly fail to implicate any one of the hydroxylated derivatives of 4-aminobiphenyl as the proximate carcinogen. With the production of hepatomas as the parameter for carcinogenic activity, it is obvious that the parent amino compound is the most active of the substances tested, and that the hitherto unconsidered metabolite, 4-amino-4′-hydroxybiphenyl, the hydroxylamino compound, and the *ortho*-aminophenol all produce high yields of hepatomas.

The activity of the hydroxylated derivatives cannot at present be explained; one possibility is that the metabolic inactivity of the newborn mouse is only relative, or is even selective, and metabolism of these compounds does occur during the first few days of life. There is no relevant information on this point, but it is known that neonatal mice can hydroxylate urethan and that *N*-hydroxyurethan can be reduced to urethan and also converted to a metabolite, thought to be glucuronide (*23*, *24*), although such reactions occur at only 12–20% of that observed in adults. *N*-hydroxylation of *p*-chloraniline occurs at approximately the same rate in hepatic microsomes from either young or adult rats, rabbits, or cats (*25*), but no information is available on the ability of young mice to carry out this type of oxidation. *Ortho*-aminophenol uridine diphosphoglucuronyl transferase activity is demonstrable in the newborn mouse, but only at 20% of the adult level (*26*).

In common with other reports on the production of hepatomas in mice that have received the carcinogen during the neonatal period (*27–29*), it was found that males are strikingly more susceptible than females. The relative response of the sexes seen when adult mice are treated with an aromatic amine varies with the compound administered. Repeated monthly injections of 2-amino-5-azotoluene in mice, begun when they were approximately 2 months old, gave rise to a higher incidence of hepatomas in females than in males (*30*). In some of the strains of mice studied, the amine was virtually inactive in males. Administration of the same compound to newborn mice, however, gave rise to hepatomas in males, but not in females (*31*). Armstrong and Bonser (*32*) failed to find any sex difference in the susceptibility of adults of 5 different strains of mice in the action of 2-acetylaminofluorene in producing hepatomas. However, Leathem (*33*) found that when the same compound was mixed with a semipurified diet and fed to adult Swiss mice, males were more susceptible to hepatoma induction than females. 4-Aminobiphenyl, itself, produces higher yields of hepatomas in adult female mice of the C57 × IF strain (*34*), though it was without effect on hepatoma incidence in AB × IF mice of either sex (*35*). This suggests that genetic factors unrelated to sex also determine susceptibility to liver tumor induction. If metabolism of amino compounds is indeed a prerequisite for carcinogenic activity, then such results suggest a marked sex difference in metabolic pathways. Such a difference has been described in adult rats (*36*), but has not previously been found in mice (*37*, *38*).

Castro and Gillette (*39*) have recently shown that the kinetic constants for the *N*-demethylation of ethylmorphine are markedly different in adult male and female mice but they stress that this is not significant at the substrate concentration observed in *in vivo*. The sex difference observed in the metabolism of ethylmorphine by rats has now been partially explained by Davies (*40*), who found that the rate of metabolism was related to the reduction of the drug-cytochrome P-450 complex. Ethylmorphine, however, combines with cytochrome P-450 to give a type 1 spectrum (*41*), whereas the compounds used in the present study all give type 2 spectra (*42*).

Perhaps the most important aspect of the findings reported here is that introduction of compounds into newborn animals has revealed evidence of carcinogenicity not demonstrable by other tests (*43–45*). A corollary of this finding is that critical evaluation of theories of metabolic pathways of carcinogens should be regarded as incomplete unless supposedly negative compounds have been studied in neonatal animals.

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