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THE PRIMARY AROMATIC AMINES: THEIR BIOLOGICAL PROPERTIES AND STRUCTURE-ACTIVITY RELATIONSHIPS

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INTRODUCTION

Primary aromatic amines are compounds of considerable industrial and commercial importance. They are used as intermediates in the synthesis of numerous organic compounds including the azo dyes and as antioxidants in consumer goods including most rubber products. Also, they are produced environmentally when plant and other organic material are burned (1). Some aromatic amines have been shown to have potent toxicological properties. They are one of the classes of substances about which there is much concern from both an occupational and environmental point of view, particularly with regard to their carcinogenic activity. The continuously rising incidence of bladder cancer among workers in some industries, despite the removal of known carcinogens, highlights the importance of this problem (2).

This review is limited to primary aromatic amines specifically excluding the acetamides, such as phenacetin and N,2-fluorenylacetamide, since these substances have been subject to numerous reviews. Aliphatic amines and polyamines, many of which are of considerable biological importance, also are not covered. Unfortunately, many aromatic amines of industrial importance have not been studied toxicologically, or the toxicity data are not in the open literature. With the current realization that many of these substances have serious toxic potential, this situation will hopefully be rectified.

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Structure-activity relationships among the primary aromatic amines are of much interest from both a theoretical and a practical point of view. Nevertheless, these relationships have not been intensively investigated as have the polynuclear hydrocarbons. The present state of knowledge of these relationships, which concern carcinogenic activity and methemoglobin formation, are reviewed here. Since their mechanism of action undoubtedly involves metabolic activation and probably also detoxication, the metabolism of these substances in humans and animals is discussed.

Further detailed information on the toxicology of these substances may be obtained from discussion in textbooks on chemical carcinogenesis (3–6). Additional data concerning their metabolism in human and animals may be obtained from texts on detoxication (7, 8). Several volumes of the IARC also contain additional data and an overall evaluation of the carcinogenic hazards of some of these amines (9–11).

The aromatic amines are, of course, bases that are nonpolar and poorly water-soluble in this form, but are readily converted to highly water-soluble hydrochloride salts. As the free base the amines are usually quite unstable to light, heat, and oxygen, oxidizing to quinoneimines and quinones, which condense to unidentified purple-colored polymerization products. Rather unusually, 2-naphthylamine forms a red photooxidation product which has been identified as 2-amino-1,4-naphthoquinone-N,[4]2-naphthylamine (12).

THE NAPHTHYLAMINES

2-Naphthylamine

Suspicion that aromatic amines are a potential bladder carcinogen was first voiced by Rehn in 1895 (13), when he observed that several of his patients with bladder cancer worked in the same dyestuffs factory. For some time these tumors were ascribed to aniline largely because aniline was the most common chemical in these factories. There was no reason to suspect 2-naphthylamine (β-naphthylamine) (2-NA) until 1938, when Hueper (14) induced very similar bladder tumors by the administration of this substance to dogs. Subsequent epidemiological investigations have amply confirmed the fact that 2-NA is a bladder carcinogen in humans (15–21). Chlornaphazine (a chemotherapeutic agent formerly used for polycythemia), which is metabolized to 2-NA, caused bladder cancer in many patients treated with 100 g or more (22). Hueper's results in dogs have been duplicated a number of times (23–25). After 1937, the manufacture of 2-NA was continued under reduced exposure conditions until it was finally abandoned in 1953.

2-NA has also been tested in other species. Contrary to widespread opinion, 2-NA is also a bladder carcinogen in rats, although, the maximum

incidence is 10% or less, whereas in the dog the incidences approach 100% if given in sufficient dose for a sufficient period (24, 26, 27) (R. M. Hicks, personal communication). Rabbits, guinea pigs, and hamsters also develop bladder cancer when administered 2-NA, although the incidence never exceeds about 50% (27–30). Conzelman et al (31), in a most difficult experiment involving the daily oral administration of capsules of 2-NA, demonstrated that the rhesus monkey was susceptible to the bladder carcinogenic action of this substance. Mice, on the other hand, appear to be quite resistant to the bladder carcinogenic action (32) but develop liver tumors after oral and subcutaneous administration (27, 33).

One of the most critical questions bearing on the degree of environmental and occupational hazard from 2-NA is the matter of the quantitative sensitivity of man and the various animal species. While it is apparent from the literature that the early occupational exposures to 2-NA were severe, it is hard to judge whether the amounts ingested were in the microgram, milligram, or gram range. Even more important, there are no data bearing on minimum hazardous exposures. The best estimate may be based on a determination of the amount of free amine present in urine of early 1-naphthylamine workers. These values were on the order of 10–40 mg excreted per day (15). Data from our laboratory with both 1-NA and 2-NA indicate that about 5% of the total amine derived substances present in urine is free amine (unpublished). Therefore, multiplying the above figure by 20 gives a value for the dose absorbed on the order of 10 mg/kg per day, indicating that man may be about equally as sensitive as the dog. The rat, hamster, and rhesus monkey appear to be an order of magnitude more resistant. Conzelman et al (34) in a dosage-response experiment with dogs, observed a clear dosage-response relationship in the range of 6.25 to 50 mg/kg. From his data, he calculated a value of $n = 4$ and $k - 1.9 \times 10^6$ for Druckrey's equation (35, 36) ($dt^n = k$), expressing dosage-response relationships in carcinogenesis. Using these values, he calculated that dogs would get bladder tumors from daily doses of 0.1 mg/kg in 5.5 years and 0.01 mg/kg in 10 years. Experiments to test his hypothesis were planned but never carried out because of the time and expense involved.

It is worthwhile to point out that in all exposures to 2-NA, a marked degree of individual variability was observed. For instance, in the Conzelman study (31) one monkey developed bladder tumors at a dose of 6.25 mg/kg in 6 months while another survived doses that were increased from 100 mg/kg to 200 mg/kg to finally 400 mg/kg over a 5-year period without effect. A strong impression of similar variability has been also observed from occupational studies (15–20). Since the etiology of bladder cancer is widely held to involve activation of a metabolite, it seems possible that genetic differences in metabolism may be operative.

METABOLISM The metabolism of 2-naphthylamine has been extensively studied primarily in an attempt to elucidate the biochemical mechanism of carcinogenic action of the compound on the bladder. Initially, it was shown that the major metabolite of 2-NA in the dog was 2-amino-1-naphthyl sulfate by Wiley (37). This observation was followed up by the extensive work of two pioneers in the field of chemical carcinogenesis, E. Boyland and G. M. Bonser. Although the above metabolite was subsequently shown to represent 80–90% of the radioactivity excreted in the urine following an oral dose of 2-NA-14C, Boyland eventually succeeded in demonstrating the presence of the 14 urinary metabolites in the rabbit, a species that also develops bladder cancer from 2-NA (7). This forcefully demonstrated that questions concerning detoxification may appear simple at first, but prove not to be so upon deeper exploration. Unfortunately, there is no reason to assume that the biological activity of a compound is associated with the major metabolite; it can as easily be due to a very minor one.

Following the discovery of N-hydroxylation and the demonstration of its importance in carcinogenic activation, attention focused on the possibility of N-hydroxylated metabolites of 2-NA in bladder cancer induction. The occurrence of free N-hydroxy-2-naphthylamine in the urine of dogs given large doses of 2-NA was demonstrated by Troll & Nelson (38) and by Boyland & Manson (39). This observation was confirmed by two different procedures: TLC and electrophoresis (40). It should be pointed out that this metabolite is a hydroxylamine which is quite distinct in chemical and biological properties from N-hydroxy-2-acetylaminofluorene discovered by the Millers (41, 42), which is a hydroxamic acid. Hydroxylamines are basic substances, very nonpolar, and unstable, while hydroxamic acids are quite stable and, of course, acidic in nature. The formation of N-hydroxy-2-naphthylamine in the liver was demonstrated by several investigators by in vitro incubation with liver microsomal preparations (40, 43–45).

A considerable body of evidence has accumulated indicating that N-hydroxy-2-naphthylamine is the active urinary carcinogen of 2-NA (46–49). Subsequent in vitro experiments demonstrated that the N-hydroxy compound was glucuronidated in the liver and that the glucuronic acid conjugate formed was N-(β-1-glucosiduronyl)-N-hydroxy-2-naphthylamine (49). Presence of the same conjugate in the urine of dogs given 2-NA led to the conclusion that this conjugate was the carrier form responsible for the transport of the activated metabolite, N-hydroxy-2-naphthylamine, from its site of formation in the liver to its site of action in the bladder (J. M. Poupko, unpublished observations). Its resistance to hydrolysis at blood and tissue pH explain the failure of 2-NA administered orally or inhaled to produce tumors in other tissues of the body besides the bladder.

Mutagenicity tests conducted on 2-NA and its isomers support the above concept of the role of N-hydroxylation in the induction of bladder cancer. When *Neurospora crassa* was used, N-hydroxy-2-naphthylamine was mutagenic without activation while 2-NA was not. Only following metabolic activation was 2-NA itself mutagenic (51). Similar results were obtained with *Escherichia coli* (52) and *Salmonella typhimurium* (the Ames test) (53).

The influence of phenobarbital and methylcholanthrene stimulation on the ability of the dog and rabbit liver to N-hydroxylate 2-NA was investigated by Uehleke & Brill (54) and by Brill & Radomski (40). It was found that phenobarbital administered i.p. for 2 weeks induced a 10-fold stimulation of the N-hydroxylating ability of the liver, while methylcholanthrene had no stimulatory effect whatever. Phenobarbital appeared to reduce the induction time of the appearance of bladder tumors when given simultaneously with 2-NA (55) but the results were not convincing.

2-NA is a weak inducer of methemoglobinemia in dogs. A dose of 70 mg/kg only induced an average of 1.2 g/100 ml of methemoglobin in the blood of dogs (46). The mechanism of methemoglobin formation is believed to involve the same N-hydroxy metabolite. This is discussed subsequently in the section on aniline.

1-Naphthylamine

1-Naphthylamine (α-naphthylamine) (1-NA) has been produced commercially for approximately 60 years. Production in the United States is reported to have risen to 2.6 million kg in 1948. Since then, primarily because of suspicions concerning its possible carcinogenicity, production and sales have fallen considerably. Only one company in the United States is still producing 1-naphthylamine, although it continues to be manufactured in considerable quantities in Germany, England, and Japan. 1-Naphthylamine is an intermediate in the synthesis of a large number of compounds, including dyes, herbicides, and antioxidants. It is reported to have been used as an intermediate in the production of 150 different dyes. Rubber antioxidants manufactured from 1-naphthylamine include aldo-1-naphthylamine condensate and N-phenyl-1-naphthylamine. 1-Naphthylamine is present in coal and cigarette smoke (56).

The removal of 2-naphthylamine as a contaminant from 1-naphthylamine is difficult and expensive. Consequently, early commercial production samples of 1-naphthylamine contained 4–10% of 2-naphthylamine. The modern commercial product, however, contains on the average only 0.1% (56). Reports of individual cases of tumors of the urinary bladder attributed to occupational exposure to 1-naphthylamine have appeared in the literature since 1929 (56). Case, however, in his investigation of occupational

exposure to aromatic amines, found 19 cases of bladder cancer among 1-naphthylamine workers who supposedly had no other exposure to known carcinogenic substances (15). Since it is usually difficult to be certain that a worker in a chemical industry is exposed only to one compound and not to others, these results may be regarded skeptically. Even if these results are accurate, since commercial grades of 1-naphthylamine contain considerable quantities of 2-naphthylamine, these data cannot be taken as evidence that 1-naphthylamine itself is a bladder carcinogen.

Early tests of the carcinogenicity of 1-naphthylamine in animals must also be regarded as inconclusive because of the contamination of the 1-naphthylamine used with varying amounts of the 2-isomer. Bonser gave two dogs oral doses of 0.5 g three times a week for 9 years. At the end of this period one of the two dogs had a bladder papilloma (24). On the other hand, Gehrmann et al (23) gave five dogs 300 or 330 mg doses five times a week for 4.5 years, and did not observe the occurrence of bladder tumors. As a result of the inconclusiveness of these experiments, two experiments were initiated approximately 10 years ago to study the carcinogenicity of highly purified 1-naphthylamine in the dog. The first experiment conducted in this country has just been terminated with the sacrifice of all dogs after 9 years of the administration of a dose of 15 mg/kg in corn oil solution on each of 5 days of the week. Six purebred beagle dogs were utilized, three males and three females. When the bladders were removed and examined they were found not only to be free of any evidence of tumors, but also to be quite normal in gross appearance. Microscopic examination confirmed this observation; there were no tumors and no histological changes. Nor was there significant evidence of a tumorgenic effect of 1-naphthylamine on any other tissues or organs (J. L. Radomski, unpublished observations). The second experiment, being conducted in England although the animals have not yet been sacrificed, appears to be producing the same result (I. Purchase, personal communication). These results, coupled with experience of the DuPont Corporation who have monitored their α-naphthylamine workers for the past 15 years without detecting a single case of bladder cancer (J. A. Zapp, personal communication), appear to demonstrate that 1-naphthylamine is not a bladder carcinogen.

1-Naphthylamine has been tested in the mouse and hamster. A dose of 0.01% of the hydrochloride in the drinking water for a period of 84 weeks was administered to Swiss mice. No significant neoplastic changes were observed. 1-Naphthylamine was also tested in newborn Swiss mice in two separate experiments of 10 months and 12 months duration. No significant increase in tumor incidence was observed (57). 1-Naphthylamine administered in the diet at concentration of 0.1% for life and 1% for 70 weeks to Syrian golden hamsters failed to show a carcinogenic effect (29, 30).

METABOLISM STUDIES The metabolism of 1-naphthylamine has not been extensively investigated. Basically 1-naphthylamine is metabolized by hydroxylation in the 2-position, the 4-position, and the nitrogen atom (N-hydroxylation). The amine may also be conjugated with acetic acid, sulfuric acid, and glucuronic acid. The major metabolites and the percentage of radioactive label found in the urine is shown in Table 1. Experiments have also indicated, using radio C-14 label 1-naphthylamine, that there are an additional six metabolites that remain unidentified (J. L. Radomski, unpublished observation). These may be metabolites which have been ring oxidized and are conjugated on more than one functional group. There has been no evidence of metabolites resulting from the oxidation in five, six, seven, or eight position. The hydroxylamine metabolite of 1-naphthylamine is also conjugated with glucuronic acid. As a matter of fact, in in vitro experiments this conjugate appears to form more readily than the analogous conjugate of N-hydroxy-2-naphthylamine (49). In addition, N-hydroxy-1-naphthylamine by intraperitoneal administration is more carcinogenic to rats than N-hydroxy-2-naphthylamine (58). By subcutaneous injection in newborn mice, however, the two compounds appear to be equally carcinogenic (59). N-hydroxy-1-naphthylamine is an active mutagen in *Salmonella typhimurium* according to the Ames test (53).

These observations do not correlate with the lack of carcinogenicity of 1-NA.

Other Naphthylamines

N-PHENYL-2-NAPHTHYLAMINE This compound is used primarily in this country as an antioxidant in the rubber industry. The commercial product is contaminated with 20–30 mg/kg of 2-naphthylamine. It is of such commercial importance in this country that it has been estimated that 15,000 workers are potentially exposed (60).

Table 1 Summary of the quantitative analysis of urinary metabolites of 1-naphthylamine-1C-14

	1-Naphthylamine			
Metabolite	Dog no. 1 (%)	Dog no. 2 (%)	Dog no. 3 (%)	Average (%)
1-Naphthylamine	0.8	1.4	5.0	2.4
1-Amino-2-naphthyl sulfate	27.5	29.8	22.4	26.6
1-Amino-4-naphthyl sulfate	61.0	60.1	57.2	59.4
1-Amino-2-naphthyl glucuronide	2.1	2.7	3.3	2.7
1-Amino-4-naphthyl glucuronide	5.9	4.5	6.4	5.6
Unidentified	2.7	1.5	5.7	3.3

In an extensive epidemiological study of the incidence of bladder cancer in rubber workers, Veys (61) found an increased cancer incidence in all workers exposed to materials known to contain 2-naphthylamine including N-phenyl-2-naphthylamine. However, when the study was narrowed to N-phenyl-2-naphthylamine itself, no increase was observed.

When tested in dogs at the high dose of 540 mg, five days a week for 4.5 years, no bladder tumors or other tumors were observed (23). However, when evaluated for carcinogenicity by oral and subcutaneous administration as part of the Innes experiment, a possibly significant increase in tumor incidences (primarily hepatomas) was observed (62).

At issue is not only the presence of small amounts of 2-naphthylamine as a contaminant in the N-phenyl product, but also the possibility of metabolic removal of the phenyl group. Experiments in dogs showed very small amounts (up to 10 μg) in the urine of dogs given 5 mg/kg (63). No N-hydroxy metabolites were detected in the urine. In addition, only 2.8% of the administered radioactivity appeared in the urine following administration of 1,4,5,8-14C-N-phenyl-2-naphthylamine.

At present the available epidemiological, toxicological, and metabolic data do not support an indictment of this substance as an occupational carcinogen.

Another derivative of 2-naphthylamine, 3-methyl-2-naphthylamine, was found to be a potent carcinogen in rats inducing tumors of the gastrointestinal tract, breast, cecum, ileum, and skin when tested by oral administration (64, 65).

THE BIPHENYLAMINES

4-Aminobiphenyl

4-Aminobiphenyl was manufactured and used as a rubber antioxidant in this country for 20 years. There is no direct evidence that it was used commercially elsewhere in the world, but there is a strong suspicion that it was. Fortunately, in 1955, a convincing epidemiological investigation establishing its role as a potent occupational bladder carcinogen terminated further commercial use (66). In 1971, a follow-up study covered a total of 315 exposed workers of which 53 had bladder tumors (67). Finally, a total of 503 exposed workers were identified, 35% of which had histologically proven bladder cancer (68, 69). Unfortunately, since there is no way to quantitate the degree of exposure these men received, there is no way to evaluate the carcinogenic potency in the human in order to compare it to the animal model.

4-Aminobiphenyl was first observed to be a bladder carcinogen in test animals by Walpole et al in 1954 (70), one year prior to the cessation of its

manufacture in this country. Only two dogs were tested, both of which developed tumors in 33 months of 6 times a week oral administration. However, this observation was soon confirmed by Deichmann et al in 1958 in four additional dogs all of which developed tumors in 21–34 months of daily (5 times a week) dosing (71). In an experiment designed to evaluate the possible synergistic action of aromatic amines, no synergistic or additive effect was observed. However, the experiment clearly demonstrated that 4-aminobiphenyl was the most potent aromatic amine carcinogen, for it alone of the compounds tested produced tumors in all six dogs at the dose of 1 mg/kg 5 times a week. 2-Naphthylamine and benzidine were negative at this dose administered for 3 years (72). The possibility was also tested that a single massive dose of this amine might result in the induction of cancer. However, no tumors were found after a 5-year observation period (73).

Rabbits also develop bladder tumors from orally administered 4-aminobiphenyl (74). In mice, the primary carcinogenic effect observed from oral administration was a significant increase in the incidence of hepatomas (75).

4-Aminobiphenyl has not been tested by chronic oral administration to rats. Subcutaneous administration daily, however, resulted in the induction of intestinal tumors (76).

METABOLISM Considering that 4-aminobiphenyl is the most potent bladder carcinogen known, at least in the dog, there are surprisingly little data in the literature on its metabolism in humans and experimental animals. Early English workers, Bradshaw & Clayson (77), identified by paper chromatography 4-amino-3-diphenylsulfate, the sulfuric acid conjugate of the *o*-hydroxylated amine, in the urine of dogs fed 200 mg/kg. This observation was confirmed when it was demonstrated that this metabolite constituted 70–80% of the radioactivity present in the urine following the administration of 4-aminobiphenyl-(^{3}H). Other metabolites identified in these experiments were 4-aminobiphenyl-N-glucuronide (5–10%). Approximately 10–15% of the free amine was found in urine (78). These experiments also showed the presence of the N-oxidized compounds, 4-nitrosobiphenyl and N-hydroxy-4-aminobiphenyl, in the urine of dogs given the amine. In vitro incubation studies with fortified liver microsomes demonstrated its formation in the liver (49). However, it was hard to imagine how this unstable, insoluble metabolite could make its way from its site of formation in the liver to its site of action in the bladder. This enigma was resolved by the demonstration of the presence in the urine of a water-soluble glucuronic acid conjugate which on acid and β-glucuronidase catalyzed hydrolysis liberated N-hydroxy-4-aminobiphenyl (79, 80). This conjugate is

apparently the carrier form of the active carcinogen from the liver to the bladder.

The conjugate was prepared synthetically by two different procedures, one a direct condensation of the N-hydroxy compound with glucuronic acid (80) and the other a condensation between N-hydroxy-4-aminobiphenyl and methyl (tri-*o*-acety-d-D-glucopyranosyl bromide) (81). The latter synthesis is considered unequivocal for the preparation of an N-C conjugate. The conjugate was also obtained by the incubation of N-hydroxy-4-aminobiphenyl with uridine-5'-diphosphoglucuronic acid and dog liver microsomes (49). Chromatographic and infrared analysis of the two synthetic conjugates, the urinary conjugate, and the conjugate obtained by incubation showed them all to be identical chemical substances.

This evidence establishes the identity of the glucuronic acid conjugate as sodium (N,4-biphenyl-N-hydroxy-β-glucuroniosylamine). The compound could also be named as N-(β-1-glucosiduronyl)-N-hydroxy-4-aminobiphenyl. It is a new type of conjugate lacking the stability of an ether or an ester glucuronide. It is readily converted by oxidation or disproportionation to a nitrone. While it is generally assumed to be only a carrier it may be the active metabolite, reacting with macromolecules of the bladder mucosa. It is mutagenic for *Salmonella typhimurium* in the Ames test (80).

Unlike 2-NA or 1-naphthylamine, 4-aminobiphenyl is an acutely toxic substance, largely by virtue of its potent ability to induce methemoglobin formation in animals and man (46). A dose of 10 mg/kg in corn oil may be acutely fatal in the dog. The mechanism of methemoglobin induction is believed to also involve the N-hydroxy metabolite as is discussed subsequently in the section on aniline.

Benzidine (4,4'-diaminobiphenyl)

In the United States, the production of benzidine amounts to many millions of pounds a year. More than 250 dyes are derived from benzidine. These dyestuffs are manufactured by coupling tetrazotized benzidine with phenols and amines. There has been concern recently that such dyes may have unreacted benzidine residues as contaminants (82). In addition the azo group has been shown to be readily reduced in the gut (83, 84). Therefore these dyes may undergo hydrolytic fission with the liberation of free benzidine. Recently, the National Institute of Occupational Safety and Health has recommended that the three most widely used benzidine-derived dyes, Direct Black 38, Direct Blue 6, and Direct Brown 95, be handled as human carcinogens (85). Analysis of the urine of workers handling these dyes indicated the presence of higher than expected concentrations of benzidine. Similar results were seen in test animals given these dyes indicating that the dyes are being metabolized to benzidine (86). Benzidine is also used in the rubber industry and in the manufacture of plastics. It is used in clinical

laboratories for the detection of occult blood and in other colormetric determinations (82).

Although a suspicion was expressed by Hueper in 1942 (87) that benzidine was responsible for the induction of bladder cancer in workers, it was not until 1954 that this was convincingly documented (15, 82, 88). The general feeling expressed in these epidemiological studies is that benzidine has a considerably lower attack rate or potency than 4-aminobiphenyl and 2-naphthylamine. There is recent work suggesting that only exposed benzidine workers with low serum properdin, an important immunoprotein, develop bladder cancer, an observation that may explain the high individual variability observed with this and other carcinogenic amines. It is possible that it may also induce tumors of other sites in humans, although the evidence is not convincing (82).

By oral administration to the rat and hamster, benzidine induced liver tumors exclusively: cholangiomas, liver cell carcinomas, and hepatomas. Rats fed 0.017% benzidine in a diet for 424 days developed cholangiomas and liver cell tumors (90). Hamsters fed benzidine and benzidine dihydrochloride in the diet of the concentration of 0.1% developed cholangiomas, hepatomas, and hepatocellular carcinomas (29, 30). When tested in dogs, three of seven dogs given 200 to 300 mg/day, 6 days/week for 5 years developed bladder carcinomas in 7, 8, and 10 years. The other four dogs sacrificed after 9 years were negative (91). This is clearly a lower order of response to that observed from 2-naphthylamine and 4-aminobiphenyl. That benzidine is a weaker carcinogen in the dog was confirmed subsequently (72). These observations coincide with the impression gained from the observation of occupational exposures to benzidine in the human. Hepatomas were also induced in mice and rats given repeated injections of benzidine subcutaneously (92).

METABOLISM The metabolism of benzidine has been studied in the mouse, the rat, the guinea pig, the rabbit, and the dog (77, 93–96). The principal metabolite was the mono *o*-hydroxylated derivative excreted either free or conjugated with sulfuric acid (94). Monoacetylated benzidine and benzidine conjugated on one of the amine groups with sulfuric acid and glucuronic acid have also been observed (93, 95). As expected, no acetylated derivatives of benzidine were observed after administration to the dog. Diacetylated benzidine was found when especially high concentrations were given to guinea pigs, rabbits, and rats (96).

Other Biphenyl Derivatives

Other derivatives of biphenyl have been evaluated for their carcinogenic activity mostly for the purpose of evaluating structure-activity relationships. It has been reported that 2-aminobiphenyl is noncarcinogenic to rats

and that rats do not excrete an N-hydroxylated metabolite in urine (97). 3:2'-Dimethyl-4-aminobiphenyl, however, has been found to induce bladder tumors in hamsters (100 mg/kg for 158 days) (98) as well as a few colon adenocarcinomas in rats (99).

Diacetylbenzidine induces glomerular nephritis in rats and mice fed the compound. A low incidence of ear duct tumors was also observed (100). Liver tumors were induced after s.c. injection while ear duct, mammary, and skin tumors were observed following i.p. administration (101).

An isomer of benzidine, 2,4'-diaminobiphenyl, had no toxic effect in rats from a lifetime dose of 2 mg per day (102). Tested in 2 dogs for 7 years, it did not induce bladder cancer (5 mg/kg, 6 times a week). The dogs had nodular hyperplasia of the spleen at autopsy (102).

3,3'-Dichlorobenzidine is a commercially important compound used as an intermediate in the production of many dyes and pigments. It is also used as a curing agent for polyurethane plastics. It is clearly carcinogenic when orally administered to rats, hamsters, and dogs. Rats developed bladder, mammary, and ear duct tumors (103–105). Hamsters developed bladder tumors and liver tumors (29, 30). Most interesting were the results of a 7.1 year test in six dogs involving oral administration of a dose of 100 mg, 5 times a week. All dogs had an elevated SGPT activity for the first three years of the test, some much longer. All dogs, except one that was tumor free, had bladder papillary carcinomas. Four of these five dogs also had liver cancer. This compound is clearly a potent bladder and liver carcinogen (106).

Its methoxy analogue, 3,3'-dimethoxybenzidine given by gavage, also induced tumors at various sites including the bladder (only 2 out of 60 rats), the intestines, skin, and Zymbol gland (107–109). Hamsters fed 0.1 and 1.0% in the diet did not develop a significant number of bladder tumors, but at the 1% feeding level, did develop a 37% incidence of forestomach papillomas (29, 30).

BENZENE DERIVATIVES

Aniline

Aniline is one of the oldest and most widely used industrial compounds. It is produced in virtually every industrial country in the world. It is used primarily as an intermediate in the synthesis of other organic compounds such as rubber processing chemicals, antioxidants, dyestuffs, photographic chemicals, pharmaceuticals, and agricultural chemicals (110). For a compound of such widespread industrial importance, it is difficult to understand why there has been no adequate chronic toxicity or carcinogenicity test conducted throughout the 150 years of its utilization. Rehn, who first

observed the induction of bladder cancer in industrial workers with aromatic amines, believed these tumors to be due to aniline and called them *aniline bladder cancers.* Later these tumors were attributed to 2-naphthylamine and benzidine. While these tumors can certainly be attributed to these other amines, there is at the same time no convincing evidence that aniline was not involved.

In spite of the suspicions that it may be a bladder carcinogen, aniline has not been adequately tested in dogs, the prime test species for the evaluation of bladder carcinogens. Only one experiment on only three dogs is in the literature. No tumors were observed after 4 years of daily administration (23). Aniline as the hydrochloride was given in the drinking water to rats in an amount calculated to provide a dose of 22 mg/day. The experiment lasted for 750 days. One half of the rats survived for more than 425 days. No tumors of the bladder, spleen, liver, or kidneys were observed (111). Aniline, either as the free base dissolved in lard or olive oil or the hydrochloride dissolved in water, was not found to be carcinogenic by subcutaneous injections in mice. The experiments ranged from 12–15 months (112, 133). It is clear that this compound needs further epidemiological and experimental investigation, especially in view of its continued enormous usage.

Aniline is generally regarded as an acutely toxic compound, although there is little in the literature to justify its reputation. Other than the induction, primarily by acute exposures, of methemoglobinemia (ferrihemoglobinemia) no other acute or chronic toxic effects have been reported. Undoubtedly its widespread usage in industry and its volatility have contributed to its reputation. Even in the induction of methemoglobinemia (a freely reversible condition), judging from experiments in the dog, 4-aminobiphenyl is 10 to 20 times more potent and, from experiments in the cat, *p*-dinitrobenzene is 50 to 80 times more potent (114).

Sensitivity to the induction of methemoglobin by aromatic amines varies markedly. The cat is the most sensitive, man is about 60% as sensitive as the cat, the dog about 30% as sensitive, the rat 5%, and the rabbit and monkey seem to be completely insensitive (114). Experiments in man indicate that an oral dose of at least 25 mg was necessary to induce detectable methemoglobinemia (115). Because of the cat's exquisite sensitivity, most studies of the mechanism of methemoglobin induction have been conducted with this species. The preponderance of evidence supports the concept that N-hydroxylation is primarily involved in methemoglobin formation from the aromatic amines (116). Although many organic species are capable of initiating the oxidation of hemoglobin to methemoglobin including aminophenols, quinoneimines, and quinones, the aryhydroxylamines are the most potent (115). However, more than one of these metabolites of aniline may be responsible for the production of methemoglobin (117).

The aryhydroxylamines participate in a cyclic reaction which catalyzes this oxidation. As many as 50 mmol of methemoglobin may be produced by 1 mmol of hydroxylamine. The reactions involved appear to be as follows: Phenylhydroxylamine in the presence of oxygen reacts with hemoglobin yielding methemoglobin and nitrosobenzene. Nitrosobenzene is reduced by the diaphorase NADP-methemoglobin reductase in the presence of NADP back to phenylhydroxylamine which in turn can oxidize another molecule of hemoglobin. Apparently a small amount of nitrosobenzene is reduced all the way to its amine, which eventually terminates the reaction. NADP methemoglobin reductase is the enzyme responsible for the physiological reduction of methemoglobin to hemoglobin. However, the nitroso metabolite seems to have a greater affinity for this enzyme than methemoglobin, a factor that inhibits the reconversion of methemoglobin back to hemoglobin as long as the nitroso compound is present (116). The nitroso compound also appears to be inactivated by glutathione present in red cells, a second factor that tends to break up the cycle. Nitro aromatics, which are also potent inducers of methemoglobin, enter this same cycle after being reduced by nitro reductase to a nitroso metabolite (116).

Some individuals have a hereditary absence of NADP-methemoglobin reductase. The trait is due to an autosomal recessive allele and the disease is manifested in homozygotes of both sexes. The disease normally presents as cyanosis after birth. Such individuals are abnormally sensitive to nitrite (118). They are usually presumed to be more sensitive to aniline and other aromatic amines, nitro compounds, and acetamides that induce methemoglobinemia (118), but the evidence for this is not conclusive. While the lack of NADP-methemoglobin reductase retards the reconversion of methemoglobin back to hemoglobin it also may prevent the reduction of the nitroso compound back to the hydroxylamine, a factor that may mitigate the effect.

Aniline is also metabolized to *p*-aminophenol which is a less active inducer of methemoglobin than phenylhydroxylamine (116). *p*-Aminophenol also requires oxygen to oxidize hemoglobin to methemoglobin. Formation of the *p*-quinoneimine is observed which may go back to *p*-aminophenol in a manner analogous to the hydroxylamine-nitroso cycle. However, only a few equivalents of methemoglobin are produced by one equivalent of *p*-aminophenol (116).

METABOLISM In experimental animals and man, aniline basically is acetylated, *o*-hydroxylated, *p*-hydroxylated, and N-hydroxylated (119–121). The amine group may also be conjugated with glucuronic acid in the formation of an N-glucuronide or with sulfuric acid in the formation of a sulfamate (122). The ring hydroxyl groups may be either glucuronidated or sulfated. The relative amounts of the various metabolites formed vary considerably from species to species, except that in all species the major

metabolite excreted in the urine is *p*-aminophenol (119). Since the dog does not acetylate, acetyl derivatives are not found in this species. Phenylhydroxylamine, the N-hydroxy metabolite of aniline, is formed in the liver and is present in the blood where it is partially oxidized to nitrosobenzene (122). It has never been detected in the urine of animals given aniline, however (116). This is critically important, if N-hydroxylated derivatives of aromatic amines are indeed responsible for the induction of bladder cancer, and would explain the failure to observe this disease in man or experimental animals exposed to aniline. It appears that the phenylhydroxylamine produced may be entirely consumed in reactions with hemoglobin.

Other Benzene Derivatives

A large number of aniline derivatives are manufactured and used commercially in this country. However, toxicological data are available on but a few of them.

ortho-Toluidine is widely used primarily as a starting material for the production of dyes, pigments, and antioxidants. It is available primarily as the dihydrochloride. Mice given *o*-toluidine in the diet developed vascular tumors while rats developed subcutaneous fibromas and fibrosarcomas, an unusual carcinogenic response to oral administration (123). The compound is clearly carcinogenic to rodents. Intramuscular and subcutaneous administration to rabbits and guinea pigs induced papillomas of the bladder (28, 124). Bladder tumors were also observed in a more recent feeding experiment in rats (123). *p*-Toluidine similarly tested did not induce bladder tumors in rats but did induce hepatomas in mice. No significant incidence of tumors was observed in either rats or mice receiving *m*-toluidine (123). All three toluidines were tested as bladder carcinogens in a 6-year experiments in dogs, at a dose of 100 mg 5 times a week. No tumors were induced by any of the three isomers (126). Thus, the issue of bladder carcinogenicity remains in doubt, tumors being induced in rats but not in dogs. Perhaps the dose given to dogs was not adequate.

The metabolism of *o*-toluidine has been studied. It appears to be excreted in the urine of rats and mice primarily as the sulfate conjugate of the *p*-hydroxylated compound and N-acetyl-4-amino-*m*-cresol. The N-glucuronide was also an important metabolite of the amine. Glucuronides of these hydroxyl derivatives were also observed in small quantities (127).

There is epidemiological evidence that *o*-toluidine may be a bladder carcinogen in humans. A number of reports of bladder tumors in workers handling this substance are in the literature (128–130). None of these in themselves are convincing, mostly because of possible exposures to β-naphthylamine. Taken together, however, especially in view of the recent work in animals, it seems possible that this substance is indeed a bladder carcinogen.

An interesting derivative of *o*-toluidine, *p*-chloro-*o*-toluidine, is an industrial compound used as an intermediate in the production of pigments, dyes, and insecticides. Although the only epidemiological study on the workers exposed to this substance was negative, it involved only a small group of men (128). On the contrary, there is a report of hematuria occurring in workers exposed to this substance (131). This suggests cancer-inducing potential. It was observed that it induced a high incidence of hemangiosarcomas of various organs in mice fed the compound for life (132). Unfortunately no chronic toxicity tests have been conducted in rats and dogs.

Both 2,4- and 2,5-xylidine had been reported to cause liver tumors and possibly also fibrosarcomas in rats (123). 2,4-Toluenediamine, an intermediate in the production of urethane foam, induced liver cancer when fed to rats (133).

AROMATIC AMINES USED AS HAIR DYES Considerable attention has been focused recently on several substances used in dying human hair and animal fur because of several epidemiological studies indicating that hairdressers who use these substances occupationally may have a heightened incidence of bladder cancer. These substances are usually diaminobenzene derivatives which are themselves colorless, but are readily oxidized to colored quinoneimines, quinones, and various polymerized reaction products of these substances. When used as hair dyes, these amines are usually mixed with hydrogen peroxide immediately before use, producing the above oxidation products that then react with sulfhyryl groups present in hair with the formation of a permanent bond. They include 2,4-diaminoanisole, 4-amino-2-nitrophenol, 1,2-diamino-4-nitrobenzene, 1,4-diamino-2-nitrobenzene, meta-phenylenediamine, and para-phenylenediamine. All of these substances have been tested by repeated local applications to experimental animals (134). Most of these substances have not been tested for carcinogenicity by long-term or life-time feeding studies.

2,4-Diaminoanisole has been tested by long-term skin application studies in mice. No toxicity or increase in incidence of tumors either at the site of application or elsewhere in the body was observed. The amines were mixed with 6% hydrogen peroxide before application (135).

PHENYLENEDIAMINES In addition to being used as hair dyes, the phenylenediamines are widely used as synthetic intermediates. Application of either phenylenediamines to the skin of mice (after mixing with 6% hydrogen peroxide) for 18 months did not induce an elevated incidence of tumors (135). Subcutaneous injection in rats of the hydrochloride was also essentially negative (136). para-Phenylenediamine was also tested (inadequately) by daily oral administration to rats for 8 months (136). No tumors

were observed. That these substances may be absorbed through the skin was demonstrated in dogs following the application of an aqueous gel (137).

4-Amino-2-nitrophenol, 1,2-diamino-4-nitrobenzene, and 1,4-diamino-2-nitrobenzene have been similarly tested by skin application to mice and/or rats for experiments lasting approximately 1.5 years without producing significant toxic or carcinogenic effects (134).

It is surprising that these substances to which exposure of the human population is very wide have been so poorly tested toxicologically. Apparently it has been the policy of government regulatory agencies to limit requirements for testing to those immediately relevant to the intended use of the compound, namely application to the skin and hair. Several of these substances are now in the process of being evaluated in the NCI Carcinogenesis Bioassay Program. Because of the instability and the formation of a multitude of compounds of unknown properties, they represent a nightmarish toxicological picture insofar as their adequate safety evaluations are concerned. Yet the diamines and their oxidation products are highly reactive substances which could be expected to react with tissue nucleophiles with the production of biological effects.

BRIDGED BENZENE AMINES

Following the recognition of the hazardous degree of carcinogenicity of 2-naphthylamine, 4-aminobiphenyl, and benzidine, their function in industry as antioxidants was in part replaced by compounds in which a carbon, nitrogen, oxygen, or sulfur atom was inserted between the two benzene rings.

4,4'-Methylenedianiline

4,4'-Methylenedianiline (MDA) has been produced commercially in this country and throughout the world for many years. It is used primarily as an intermediate in the synthesis of some extremely important plastics and synthetic fibers. This compound was considered responsible for an outbreak of toxic hepatitis in industrial workers (138). Between 1966 and 1972, 12 cases of chemical hepatitis were identified among workers in a large manufacturing firm. Absorption was believed to be principally through the skin. The victims experienced jaundice and dark urine. Biochemical evidence of liver damage including increased serum bilirubin, SGOT and SGPT (139). Previously accidental contamination of flour with MDA had resulted in an outbreak of jaundice of varying degrees in 84 persons in Epping, England. This was documented by liver biopsies and clinical studies (140, 141).

Single small oral doses of MDA induced liver and kidney injury in rats, rabbits, dogs, and cats. Cats also developed anemia, methemoglobinemia,

Heinz body formation, and irreversible blindness (142, 143). Repeated smaller oral doses also resulted in liver cirrhosis. Oral administration to rats for 121 days produced cirrhosis in the liver of all animals. Two hepatomas were found when the animals were observed for a period up to 3 years. Other suggestive evidence of hepatocarcinogenicity was obtained by subcutaneous administration over a period of 700 days. In this case, 4 out of 50 Wistar rats revealed hepatomas (144). These results are equivocal, however, and the carcinogenicity of the compound has not been clearly demonstrated in animals or man.

In a chronic toxicity test designed to determine primarily the tendency of MDA to produce bladder cancer, a dose of 70 mg given 3 times a week was administered to nine dogs for as long as 7 years. All dogs showed clinical evidence of chemical hepatitis and/or cirrhosis. Four of the nine dogs survived for the 7-year period. None of the dogs developed bladder tumors although there was evidence of epithelial hyperplasia and Von Brunn's nests (considered to be preneoplastic changes) (145). It appears that this compound is a potent hepatic toxicant. Whether or not it is a bladder carcinogen or a hepatocarcinogen is not yet certain. It must be pointed out that because of the liver toxicity the amount administered to the dogs was limited to approximately 3 mg/kg on a 5 day a week basis, and it has required 5 mg/kg administered 5 days a week for 2-naphthylamine to induce bladder cancer in dogs.

4,4'-Methylene-bis-(2-chloroaniline)

4,4'-Methylene-bis-(2-chloroaniline), also known as MOCA, is also a carbon bridge compound but with two chlorine atoms ortho to the amine groups. It is of great commercial importance. It is widely used as a curing agent in isocyanate containing polymers and other plastics. No evidence of an occupational hazard from bladder cancer due to exposure to this compound has so far been reported, although only one modest study has been conducted (146). However, in a recently published experiment in which six male dogs were give MOCA for as long as 9 years at a dose of 100 mg, 5 days a week for most of the experiment, this compound was clearly demonstrated to be a bladder carcinogen. All five of the dogs surviving more than 8 years had transitional cell carcinomas of the urinary bladder (147). It has also been demonstrated to be carcinogenic in the mouse and rat after oral administration, (105, 148) and to produce tumors distant from the site of injection by subcutaneous administration. Primary lung tumors in both mice and rats were the most striking observation. Liver tumors were also observed (149).

It would be surprising if more careful epidemiological investigations in extensive occupationally exposed individuals did not turn up an elevated incidence of bladder carcinoma.

4,4'-Methylene-bis-(2-Methylaniline)

4,4'-Methylene bis-(2-methylaniline) is a compound that has been of minor industrial importance. At present, it is not produced commercially in this country, although it is produced in Japan. This compound is clearly a liver carcinogen also producing lung and skin but no bladder tumors in rats (146). In a recently published experiment the compound was administered orally 5 days a week in a daily dose of 100 mg to six beagle dogs for 7 years. This dose was toxic to the dogs and none survived beyond 7 years. Liver degeneration was observed in all of the dogs. All three dogs that survived for more than 5.2 years had hepatocellular carcinomas; two of them had primary lung tumors. No tumors of the urinary bladder were observed (150).

2,2'-bis (4-Aminophenyl) Propane

2,2'-bis (4-Aminophenyl) propane (known as Bis Aniline A), another carbon bridged compound, was given in a dose of 300 mg 2 or 3 times a week for 6 years. One of the six dogs had 12 bladder papillary carcinomas. The other dogs had only evidences of chronic bladder irritation, such as petechia and hyperplasia (151).

4,4'-Imidocarbonyl-bis (N,N'-Dimethyl) Aniline

This dye, known as auramine, is used in the coloring of paper and cardboard. It has been used in some countries as a food dye. It has been shown to be a liver carcinogen in mice and rats (152). A single epidemiological study has implicated it as an occupational bladder carcinogen in the death of six workers of bladder cancer in England and Wales (16). However, when tested for seven years in dogs (estimated approximate dose, 4 mg/kg given 5 days a week) no bladder tumors were observed (153). The possibility of exposure to other compounds in the above cases of bladder cancer must be considered.

Other Bridged Benzene Compounds

4-Aminobiphenylamine, a nitrogen bridged compound, was tested in six dogs at a dose of 50 mg, 3 times a week. No tumors were produced after six years. However, preneoplastic changes such as hyperplasia and desquamation of the epithelial surface were observed (151). In addition, because of liver toxicity the dose was only about 2 mg/kg calculated on a 5 day a week basis.

4,4'-Diaminobiphenylether, a compound of some industrial importance in the United States and Japan, has been tested in rats and mice by both oral and subcutaneous administration (154). It produced tumors at various sites but the experiments are difficult to evaluate because of the variety of tumors observed and the fact that controls were not used.

A chlorinated derivative, 3,3'-dichloro-4'-diaminobiphenylether, induced ear canal tumors in rats (155).

A sulfur bridged aniline derivative, 4,4'-thiodianiline, induced breast tumors when administered orally to rats (156).

STRUCTURE-ACTIVITY RELATIONSHIPS

Although the primary aromatic amines present a variety of fascinating contrasts in biological activities and behavior with differences in chemical structure, there has been very little progress in the understanding of these relationships on either a biochemical or molecular basis. Unfortunately, research dealing with these relationships has been negligible compared to the effort that has been devoted to the study of structure-activity relationships among the polynuclear hydrocarbons. An understanding of structure-activity relationships among organic compounds is perhaps the ultimate goal of pharmacological and toxicological research. It holds promise of a future in which all biological effects of organic substances will be explainable on a molecular basis and predictable from a consideration of the electronic properties of the compound. Unfortunately progress in this direction has been discouragingly slow, for it is an extremely difficult undertaking for many reasons. First, one must have a good idea of the specific chemical reaction that the compound or activated metabolite undergoes in order to initiate the biological effect. This is a formidable undertaking in itself. Then one must understand the metabolic steps which the compound undergoes from the time it enters the body to the final reactive metabolite. One must also understand the detoxication and disposition of the compounds for these may well play an important role. Then one must have an accurate measure of the potency of the compound with regard to the particular biological effect being examined. This would normally seem a relatively easy undertaking except that accurate measurements on the same species of test animal do not usually exist. Finally, one must have a meaningful way of calculating the electronic properties of the organic compound in order to give significant molecular parameters. This in itself is an area undergoing constant development but satisfactory results have not been achieved with complex compounds.

The usual approach to the study of structure-activity has been to attempt to correlate some one molecular property of a series of carcinogens with their carcinogenic potency as derived from literature sources. This approach is based on the assumption that there is one electronic property of this series of molecules that determines whether or not, or to what extent the effect is produced. In fact, there probably are different reasons why a particular member of a series of compounds is inactive or of lower potency

than another. For instance, one compound may not be active because it does not react with the target of tissue, while another compound may be inactive because of particularly efficient detoxication involving an entirely different organic reaction. It therefore seems advisable to limit the series of compounds to those that have been studied in the same species of animal and under the same conditions biologically and to those that exert their effect by a common mechanism of action. Table 2 represents an attempt to quantitate three different biological effects, all observed by continuous oral administration of a series of primary amines for a period of 5 to 10 years. Unfortunately, the compounds were not given in the same dosage and some were given dissolved in vegetable oil while others were given dry. However, these factors most likely have not influenced that result materially. Adjustments were made in quantitating the potency for the difference in dosage administered. Of this series of compounds it can be seen that only two of them are potent methemoglobin inducers, 4-aminobiphenyl and aniline. 2-Naphthylamine is weak and the others are, as far as it is known, inactive. There seems to be a clear-cut difference between those compounds having conjugating benzene rings which do not markedly affect the liver and those having a carbon or nitrogen bridge between the two benzene rings which induce severe liver toxicity.

Table 2 Effects of the chronic administration of primary amines on the blood, liver, and bladder of dogs[a]

	Methemoglobin formation	Liver toxicity and/or carcinogenesis	Bladder carcinogenesis
4-Aminobiphenyl	+++	–	+++
2-Naphthylamine	+	–	++
1-Naphthylamine	–	–	–
Benzidine	–	–	+
Aniline	++	–	–
Dichlorobenzidine	–	+	++
Methylenedianiline	–	+++	±[b]
4, 4′-Methylene-bis-(2-chloroaniline)	–	+	++
4, 4′-Methylene-bis-(2-methylaniline)	–	+++	–
Bis aniline A	–	±	+
4-Aminobiphenylamine	–	++	±[b]

[a] Modified from data from the following sources (23, 47, 48, 71, 72, 91, 106, 126, 145, 147, 150, and 151).
[b] ± indicates only preneoplastic changes, no tumors.

Considerable evidence supports the theory that N-hydroxylation is the primary mechanism for the induction of bladder and liver cancer by aromatic amines (46–49). In general, acetamides (e.g. N,2-fluorenylacetamide) induce liver cancer while the amines are primarily bladder carcinogens. The acetamide is N-hydroxylated in the liver to form a hydroxamic acid which then undergoes either transacetylation or esterification yielding a high reactive species which attacks critical nucleophiles of liver cells. The hydroxamic acid, alternatively, is conjugated with glucuronic acid. This conjugate is stable and does not hydrolyze to release N-hydroxy compound in the bladder. On the other hand primary amines in the dog are N-hydroxylated to yield hydroxylamines that are not reactive at usual tissue pH with nucleophiles and instead are conjugated with glucuronic acid. This conjugate *is* hydrolyzed at urinary pH liberating the hydroxylamine which does react at acidic pH with tissue nucleophiles inducing bladder cancer. In the rat, primary amines induce liver cancer presumably because they are acetylated, forming hydroxamic acids. The mechanism of reaction of the hydroxylamine appears to proceed with generation of a nitrenium ion in acidic media. Alternatively a free radical may be the ultimate reactive species. Whichever of these mechanisms is the one responsible for the binding of the carcinogen to tissue macromolecules it is likely that the relative stabilizing effect of the resonance and inductive properties of the aromatic system and its substituents determines the order of potency within a series of primary aromatic amine carcinogens.

In their discussion of structure-activity relationship among carcinogens, Arcos & Argus (5) recall Druckrey's enunciation of the "para principle" stating that in order to be carcinogenic an aromatic amine must have a long uninterrupted conjugated system with the amino group attached to one of the terminal (para) carbons. Specifically those aromatic amines which have a resonance form in which the carbon atom *p*- to the amino group is conjugated with at least one more aromatic ring are generally carcinogens. Those amines in which the conjugation through the *p*-carbon is interrupted by a single carbon bridge and those in which the *p*-carbon is not connected to another conjugate ring are generally not bladder carcinogens. The one exception to this rule, methylene-bis-2-chloroaniline, apparently involves substituent effects because the homologous methylene-bis-2-methylaniline is not a bladder carcinogen.

The importance of resonance in conveying carcinogenic activity on an aromatic amine may be thought of in terms of its stabilizing effect on the ultimate reacting species. Whether it be ion or radical this intermediate must possess an unstable electronic distribution. The more this electronic imbalance can be delocalized through resonance the more stable and therefore the more readily formed will be the intermediate. This effect is observed

with respect to ease of acid catalyzed carbonium ion formation which increases in the order: alkyl alcohol, benzyl alcohol, diphenylmethyl alcohol, triphenylmethyl alcohol. The resonance effect of free radical stability is readily observed in the case of N,N-diphenyl picrylhydrazyl, a stable free radical (157).

With respect to those compounds, i.e. aniline and 1-naphthylamine, in which the carbon *p*- to the amino group is not part of a conjugate linkage to another aromatic system, a recent observation indicates that in acid urine, the N-hydroxy metabolite of 1-naphthylamine rapidly rearranges to 1,4-aminonaphthol which is oxidized to 1,4-naphthoquinone. 2-Naphthylamine and 4-aminobiphenyl do not undergo this type of 1,4-Bamberger rearrangement and the N-hydroxy metabolite liberated from the glucuronic acid conjugate has greater stability, presumably enabling it to penetrate and react with macromolecules of the bladder mucosa.

Substituents on the aryl moiety of aromatic amines can influence the carcinogenic activity in two ways. If the substituent is one that is rapidly metabolized itself or facilitates detoxication the compound may be diverted to a noncarcinogenic metabolite. If not, it may influence the carcinogenic potency of the parent amine by an inductive effect transmitted through the conjugated aromatic system. The effect of such substituents will depend both on the direction and intensity of their inherent inductive effect and on the effectiveness with which the inductive effect is transmitted to the amino group. The inductive effect of substituents would influence the stability of the ultimate reactive species in opposite directions depending on whether that intermediate is a nitrenium ion or a free radical. A nitrenium ion with a positive charge on its nitrogen should be most stabilized by substituents which can release electrons through the aromatic rings to the nitrogen thereby helping to delocalize its charge. On the other hand electron withdrawing substituents should help to delocalize the unpaired electron(s) of a free radical into the π electron cloud of the aromatic system (157). The observation that an electron releasing substituent such an $-NH_2$ (benzidine) decreases the potency of 4-aminobiphenyl whereas electron withdrawing substituents such as Cl (dichlorobenzidine) increase the potency for bladder cancer induction suggest that the active intermediate may be a free radical. A detailed study of the effect of varying substituents on the carcinogenic potency of, for example, 4-aminobiphenyl should shed considerable light on not only these structure-activity relationships but also on the mechanism of activation of the aromatic amine carcinogens.

Another factor must be taken into account regarding structure-activity relationships if progress is to be made in this area. Chemical carcinogenesis of the liver and of the bladder is coming to be recognized as being a two or more staged process. These different stages undoubtedly involved differ-

ent mechanisms and different organic reactions. They are going to have to be separated in structure-activity considerations. This has become clear in so far as bladder cancer is concerned by recent data emphasizing the possibility that promotion is a more critical process than initiation in determining whether an individual animal or man develops cancer or whether one chemical is carcinogenic or noncarcinogenic. Surely this is an inviting field for research. It is hoped that this review will stimulate interest in this fascinating problem.

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