

**DIPHENYLAMINE (addendum)**

*First draft prepared by  
A. Protzel  
Environmental Protection Agency  
Washington DC, United States*

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**Explanation**

Diphenylamine was first evaluated by the JMPR in 1969 (Annex 1, reference 12), when an ADI of 0.025 mg/kg bw was established on the basis of a NOAEL of 2.5 mg/kg per day in a two-year study in dogs. Diphenylamine was re-evaluated in 1976 (Annex 1, reference 26), when an ADI of 0–0.02 mg/kg was allocated on the basis of a NOAEL of 1.5 mg/kg per day for Heinz-body formation reported in a six-month study in mice and a safety factor of 100. The 1982 JMPR considered impurities in commercial-grade diphenylamine and concluded that additional data on this aspect were desirable (Annex 1, reference 38); the ADI was made temporary, and the Meeting required additional data on teratogenicity, haematological effects, and mutagenicity. The 1984 JMPR established an ADI of 0–0.02 mg/kg bw for diphenylamine of 99.9% purity on the basis of a NOAEL of 1.5 mg/kg bw per day in mice (Annex 1, reference 342).

**Evaluation for Acceptable Daily Intake****1. Biochemical aspects***(a) Absorption, distribution, and excretion*

Uniformly ring-labelled <sup>14</sup>C-diphenylamine was administered to groups of five male and five female Sprague-Dawley rats orally in corn oil as a single oral dose of 5 mg/kg bw, as a single oral dose of 5 mg/kg bw preceded by 5 mg/kg bw per day of non-radioactive diphenylamine for 14 days, or as a single oral dose of 750 mg/kg bw. Urine, faeces, and cage washes were collected 4, 8, 12,

and 24 h after dosing and at 24-h intervals up to 168 h thereafter. The recovery of radiolabel in urine after 168 h, representing 68–89% of the dose, indicated extensive absorption of the compound. Total recovery of radiolabel 168 h after dosing accounted for 94–105% of the dose. After the single dose of 5 mg/kg bw, the mean percent of radiolabel recovered was 81% in urine, 9.1% in faeces, and 9.2% in cage washes for males, and 72% in urine, 16% in faeces, and 11% in cage washes for females. When this dose was preceded by the 14-day pretreatment, 89% of the radiolabel was recovered in urine, 7.6% in faeces, and 7.7% in cage washes for males, and 68% in urine, 21% in faeces, and 12% in cage washes for females. After the high single dose, 75% of the radiolabel was found in urine, 15% in faeces, and 4% in cage washes for males, and 73% in urine, 8.8% in faeces, and 11% in cage washes for females. The mean percent of the dose in residual carcass plus tissues was 0.41% in males and 0.28% in females at the high dose and 0.14–0.28% of the dose at the other dosages (Wu, 1993).

Uniformly ring-labelled  $^{14}\text{C}$ -diphenylamine was administered in capsules with corn meal to two female Toggenburg goats at a dose of 50 mg/kg bw per day for seven days. The doses were based on feed consumption and were targeted to yield a dose equivalent to the consumption of feed containing diphenylamine at 50 ppm. An additional goat received the vehicle alone. Urine, faeces, and milk were collected twice daily, covering 0–8 h after dosing and 8–24 h after dosing, and cages were washed once daily. The goats were sacrificed 24–26 h after the last dose, and the liver, kidneys, omental and back fat, loin muscle, and leg muscle were analysed for residues and metabolites. Urine was the major route of elimination, the two goats eliminating 85–91% of the daily dose in urine, 3.4–8.6% in faeces, and 0.52–0.78% in milk; the cage washes contained 1–3.8% of the dose. A total of 92–96% of the dose was recovered. The cumulative percent of the dose that was excreted (96.5% for both goats) was very similar to the values for percent of the daily dose excreted, indicating that each dose of the test material was largely excreted within 24 h. The concentrations of residues in milk, expressed as ppm  $^{14}\text{C}$ -diphenylamine equivalents, plateaued on the first day and were 0.77–0.91 ppm for goat 2 and 0.53–0.66 ppm for goat 3 after the 8-h collection period and 0.22–0.43 ppm for both goats after the 16-h periods. The total amounts of radiolabelled residues in tissues were 0.1–0.11 ppm in liver, 0.07–0.12 ppm in kidney, 0.006–0.007 ppm in leg muscle, 0.006–0.008 ppm in loin muscle, 0.021–0.026 ppm in back fat, and 0.02 ppm in omental fat (Kim-Kang, 1994a).

Uniformly ring-labelled  $^{14}\text{C}$ -diphenylamine was administered in capsules with corn meal to 20 laying hens (*Gallus domesticus*, Hyline 6-36) at a concentration equivalent to administration of diphenylamine in the diet at 50 ppm, for seven days. Five additional hens received the vehicle only. Eggs were collected twice a day during treatment; excreta were collected daily. The hens were sacrificed 22–24 h after the last dose, and liver, kidneys, skin (with fat), and thigh and breast muscles were analysed for residues and metabolites. On days 2–7, 84–98% of the daily dose was recovered. Cumulative recovery of radiolabel in the excreta was 91% of the dose. The concentrations of residues in egg yolk, expressed in ppm as  $^{14}\text{C}$ -diphenylamine equivalents, did not plateau during treatment and increased from less than 0.01 ppm on day 1 to 0.31 ppm on day 7; no radiolabel was detected in egg white. The total concentrations of radiolabelled residues in tissues were 0.15 ppm in liver, 0.21 ppm in kidney, < 0.01 ppm in thigh muscle, < 0.01 ppm in breast muscle, and 0.04 ppm in fat and skin (Kim-Kang, 1994b).

#### (b) *Biotransformation*

The biotransformation of diphenylamine was studied in rats, treated as described above (Wu, 1993). Diphenylamine underwent extensive biotransformation, as no more than 2.7% of the dose was found as untransformed diphenylamine in any group. The structures of the metabolites were elucidated by co-chromatography (high-performance liquid or thin-layer chromatography) or mass spectral techniques. The following 12 metabolites were identified at all doses: 4,4'-dihydroxydiphenylamine (unconjugated and as the *O*-sulfate and the *O,O*-disulfate), 4-hydroxydiphenylamine (unconjugated and as the *O*-glucuronide, *N*-glucuronide, *O*-sulfate, and *O,N*-diglucuronide), indophenol (unconjugated and as the *O*-sulfate), 3-hydroxydiphenylamine and 2-

hydroxydiphenylamine. These metabolites plus parent accounted for 82–92% of the dose in excreta and were found mainly as their sulfate and glucuronide conjugates. Some quantitative sex- and dose-related differences in the metabolite patterns were seen. The proposed metabolic pathway for the biotransformation of diphenylamine in rats is shown in Figure 1. Diphenylamine undergoes biotransformation involving hydroxylation at various positions of the phenyl ring, primarily in the *para* position, followed by sulfation and/or glucuronidation and excretion. No cleavage of the diphenylamine structure was observed.

In the study of Kim-Kang (1994a), described above, no metabolites were identified in the urine and faeces of the two lactating goats. Of the total 23% radiolabelled residue found in the liver, 5.9% was identified as diphenylamine, 1.7% as 4-hydroxydiphenylamine, 2.3% as 4,4'-dihydroxydiphenylamine, 2.9% as 4-hydroxydiphenylamine glucuronide, 8.3% as 4-hydroxydiphenylamine sulfate, and 2.1% as indophenol. Of the total 73% radiolabelled residue in kidney, 36% was identified as diphenylamine, 12% as 4-hydroxydiphenylamine glucuronide, 24% as 4-hydroxydiphenylamine sulfate, and 1.3% as indophenol. Of the total 94% radiolabelled residue in milk, 7.4% was identified as diphenylamine, 39% as 4-hydroxydiphenylamine glucuronide, and 47% as 4-hydroxydiphenylamine sulfate. Of the total 40% radiolabelled residue in omental fat, 36% was identified as diphenylamine and 3.6% as 4-hydroxydiphenylamine.

In the study in hens (Kim-Kang, 1994b), no metabolites were identified in urine or faeces. Of the total 85% radioactive residue in egg yolks, 17% was identified as diphenylamine, 4.8% as 4-hydroxydiphenylamine, 0.6% as 4,4'-dihydroxydiphenylamine, 3.2% as 4-hydroxydiphenylamine glucuronide, 57% as 4-hydroxydiphenylamine sulfate, and 1.9% as a polar oligomer conjugate of 4-hydroxydiphenylamine. Of the total 31% radiolabelled residue in liver, 7.9% was identified as diphenylamine, 4.5% as 2-hydroxydiphenylamine, 3% as 4,4'-di-hydroxydiphenylamine, 1.4% as 4-hydroxydiphenylamine glucuronide, 8.6% as 4-hydroxydiphenylamine sulfate, 4.7% as a polar oligomer conjugate of 4-hydroxydiphenylamine, and 1.3% as indophenol. Of the total 39% radiolabelled residue in kidney, 1.1% was identified as diphenylamine, 0.3% as 2-hydroxydiphenylamine, 0.2% as 4-hydroxydiphenylamine sulfate, and 38% as a polar oligomer conjugate of 4-hydroxydiphenylamine. Of the total 58% radiolabelled residue in skin and fat, 35% was identified as diphenylamine and 23% as 4-hydroxydiphenylamine sulfate.

The biotransformation of <sup>14</sup>C-diphenylamine was also studied in Red Delicious apples. Residues of a number of plant metabolites were identified in apple peel and pulp, and untransformed diphenylamine was the major contributor to the total residue 40 weeks after application. The major metabolite was 4-hydroxydiphenylamine, present as the glucose conjugate. Other metabolites identified were 2-hydroxydiphenylamine, 3-hydroxydiphenylamine, and dihydroxydiphenylamine (possibly the 2,4-isomer). These compounds were present free or as conjugates with mono- or oligosaccharides (Kim-Kang, 1993).

## 2. Toxicological studies

### (a) Acute toxicity

The results of studies of the acute toxicity of diphenylamine are summarized in Table 1. After acute oral administration to rats in one study, diphenylamine (purity, 99.9%) was slightly toxic, with an LD<sub>50</sub> of 3000 mg/kg bw in males and 2700 mg/kg bw in females (Spanjers & Til, 1982). In another study, diphenylamine (purity, 99–100.1%) was generally not toxic, the LD<sub>50</sub> being > 15 000 mg/kg bw for animals of each sex (Majnarich, 1991a).

The acute dermal LD<sub>50</sub> after a 24-h exposure to diphenylamine (purity, 99.9–100.1%) was > 2 g/kg bw in New Zealand white rabbits of each sex. No clinical signs were noted (Majnarich, 1991b).

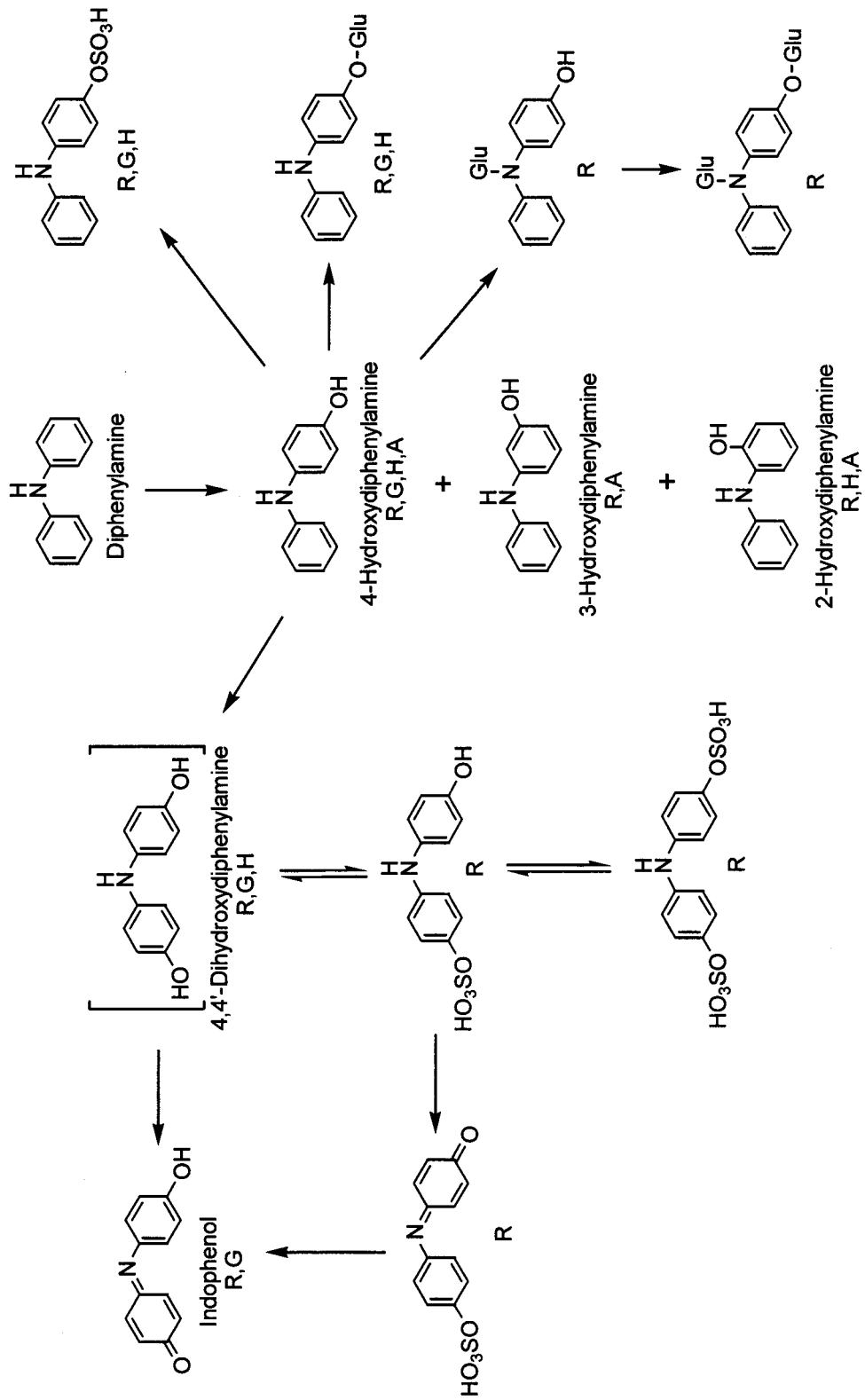


Figure 1. Biotransformation of diphenylamine

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From Wu (1993), Kim-Kang (1993, 1994a,b)  
 R, found in rats; H, found in laying hens; G, found in goats; A, found in apples

**Table 1. Acute toxicity of diphenylamine in rats**

Sex	Route	Purity (%)	LD <sub>50</sub> (mg/kg bw)	Reference
Male Female	Oral	99.9	3000 2700	Spanjers & Til (1982)
Male and female	Oral	99.9–100.1	> 15 000	Majnarich (1991a)
Not reported	Dermal (24 h)	99.9–100.1	> 5000	Majnarich (1991b)

Diphenylamine (purity, 99.9–100.1%) applied to the eyes of one rabbit for seven days without rinsing was corrosive and induced corneal opacity (Kreuzmann, 1991a). The same preparation was not irritating to the skin of rabbits (Kreuzmann, 1991b). Diphenylamine (purity, 99.9%) did not produce dermal sensitization in guinea-pigs (Kiplinger, 1995).

(b) *Short-term studies of toxicity*

*Mice*

Groups of 15 male and 15 female Swiss-derived CD-1 mice received technical-grade diphenylamine in the diet at 0, 10, 520, 260, or 5200 ppm for 90 days, equal to doses of 1.7, 94, 440, and 920 mg/kg bw per day in males and 2.1, 110, 560, and 1100 mg/kg bw per day in females. The animals were observed for clinical signs, deaths, body weight, and food consumption; ophthalmological and haematological examinations were carried out, organs were weighed, and the animals were examined grossly and histopathologically. The hair of animals at the intermediate and high doses had a greenish tint, which may have been due to staining with diphenylamine or a metabolite. Three deaths occurred among controls and among males at the high dose; two of the latter had enlarged spleens, and one also had cystitis, probably related to treatment. There were no treatment-related effects on body weight, food consumption, or ophthalmic parameters. Haematology indicated dose-related decreases in erythrocyte counts and haematocrit in animals at the two higher doses that were statistically significantly different from controls. The values for mean corpuscular haemoglobin, mean corpuscular volume, and mean corpuscular haemoglobin content increased with dose and were statistically significantly different from those of controls in animals at the two higher doses; the mean corpuscular haemoglobin content was also statistically significantly increased in males at 525 ppm. The reticulocyte counts increased with dose and were statistically significantly different from those of controls at the high dose. In males, the absolute and relative weights of the liver and spleen increased with dose and were statistically significantly different from those of controls at the two higher doses; the relative weights of the kidney and heart were statistically significantly different from those of controls in mice at the high dose. In females, the absolute and relative weights of the spleen increased with dose and were statistically significantly different from those of the controls in animals at the two higher doses; the absolute and relative weights of the liver and the relative weights of the kidney were statistically significantly different from those of controls in females at the high dose. Necropsy of females revealed dark, enlarged spleens at the three higher doses, dark livers at the two higher doses, and dark kidneys at the highest dose. In males, necropsy showed dark, enlarged spleens and dark livers at the two higher doses. Histopathological examination of the liver showed increased pigment deposition and slight haematopoiesis in animals of each sex at the two higher doses. The spleen showed haemosiderosis and congestion at the three higher doses, reaching incidences of 14/15 or more at the two higher doses; the severity of spleen haematopoiesis was also increased at the three higher doses. The kidneys showed pigment deposition at the two higher doses. Cystitis was observed in 9/15 males at the high dose and in 2/15 females at 2625 ppm and 8/14 females at 5250 ppm. The cellularity of the bone marrow was increased at the two higher doses. The NOAEL was 10 ppm, equal to 1.7 mg/kg bw per day, on the basis of changes in haematological parameters and findings at necropsy (Botta, 1992).

### *Rats*

Groups of 10 male and 10 female Sprague Dawley rats received technical-grade diphenylamine in the diet at 0, 150, 1500, 7500, or 15 000 ppm for 90 days, equal to doses of 0, 9.6, 96, 550, and 1200 mg/kg bw per day in males and 0, 12, 110, 650, and 1300 mg/kg bw per day in females. The animals were observed for clinical signs, deaths, body weight, food consumption, ophthalmic, urinary, haematological, and clinical chemical end-points, organ weights, and gross and histopathological appearance. Greenish hair was first seen in females at 1500 ppm, later in 60% of males and 100% of females at 7500 ppm, and then in 70% of males and 100% of females at 15 000 ppm. Pale skin was seen in 100% of females at 7500 and 15 000 ppm and in 40% of males at 15 000 ppm. Two males at 15 000 ppm were found dead on day 6 of dosing, apparently due to gastroenteritis; there were no other deaths. The body weights and body-weight gains of animals of each sex at 7500 and 15 000 ppm were consistently and statistically significantly below those of controls; although these values were generally lower than those of controls in animals at 1500 ppm, they were not statistically significantly different. Food consumption was not affected at any dose. The frequency of darkening of the urine increased with dose, starting with one female at 1500 ppm and 100% of rats at 15 000 ppm. Haematological measures indicated decreased erythrocyte counts and haemoglobin values, which were statistically significantly different from those of controls in animals at 7500 and 15 000 ppm at termination. The haematocrits were statistically significantly lower than those of controls in females at the three highest doses. Small, statistically significant increases in alkaline phosphatase activity, albumin content, and albumin:globulin ratio in males and glucose and albumin content and albumin:globulin ratio in females were observed at 7500 and 15 000 ppm. The cholesterol concentration increased with dose in females and was statistically significantly different from that of controls at the three higher doses. In males, the absolute and relative weights of the liver and spleen increased with dose and were statistically significantly raised at 7500 and 15 000 ppm; the relative weights of the kidney and gonad also increased with dose and were also statistically significant at the two higher doses. In females, the absolute and relative weights of the liver increased with dose, and the change in relative weights was statistically significant at doses  $\geq$  1500 ppm. The kidneys were dark in animals of each sex at 7500 and 15 000 ppm, and about 60% of the females at the high dose had dark and/or enlarged livers. The spleens of both males and females at the two higher doses were congested. Histopathological examination revealed an increased incidence of haematopoiesis and pigment in the liver, haematopoiesis, haemosiderosis, and congestion in the spleen, and pigmented kidneys in animals of each sex at 7500 and 15 000 ppm. The spleens of all females at 1500 ppm also showed an increase from minimal to slight haematopoiesis and haemosiderosis. The NOAEL was 150 ppm, equal to 12 mg/kg bw per day, on the basis of increased clinical signs of toxicity, clinical chemical changes, organ weights, and gross and histopathological appearance (Krohmer, 1992a).

### *Rabbits*

Groups of five male and five female New Zealand white rabbits received repeated dermal applications of technical-grade diphenylamine dissolved in distilled water at doses of 100, 500, or 1000 mg/kg bw per day. The material was applied daily for 6 h to an area of clipped skin corresponding to about 10% of the body surface and kept under occlusion for 21 consecutive days, with terminal sacrifice on day 22. Two additional groups of five rabbits of each sex served as vehicle controls. The animals were observed for clinical signs, deaths, ophthalmoscopic parameters, erythema, oedema, desquamation, and other adverse skin reactions, body weight, food consumption, urinary, haematological, and clinical chemical end-points, organ weights, and gross and histopathological appearance, the latter limited to the liver, kidneys, spleen, treated and untreated skin, and gross lesions. There were no deaths or treatment-related effects on clinical signs, body weights, food consumption, or haematological end-points. The only possible treatment-related effects on clinical chemistry were on sodium and potassium concentrations; females at all three doses had depressed sodium values, and those at the intermediate and high doses and males at the high dose had depressed potassium values with respect to controls. Gross necropsy, revealed dark-red foci in the stomachs of rabbits of each sex at the intermediate and high doses, which increased

in frequency with dose: the incidences were 1/5 in males at the intermediate dose, 4/5 in those at the high dose, 1/5 in females at the intermediate dose, and 2/5 in females at the high dose. No dark-red foci were seen in the stomachs of controls or rabbits at the low dose. The NOAEL for systemic toxicity was 100 mg/kg bw per day on the basis of the presence of dark-red foci in the stomachs of males and females. The NOAEL for dermal effects was 1000 mg/kg bw per day, the highest dose tested (Siglin, 1992).

### *Dogs*

Groups of four pure-bred beagle dogs of each sex received technical-grade diphenylamine (purity, > 99%) in gelatin capsules at doses of 0, 10, 25, or 50 mg/kg bw per day for 90 days. They were observed for deaths, clinical signs, body weight, food consumption, ophthalmological, haematological, clinical chemical, and urinary parameters, organ weights, and gross and histopathological appearance. There were no deaths, and no treatment-related changes were seen in any of the above parameters. Statistically significant increases were seen, however, in some clinical chemical parameters including albumin content, the albumin:globulin ratio in males, and bilirubin content in females at the high dose. These effects may have been incidental. The NOAEL was 50 mg/kg bw/day, the highest dose tested (Krohmer, 1992b).

### *(c) Long-term studies of toxicity and carcinogenicity*

#### *Mice*

Groups of 60 CD-1 mice of each sex received diets containing technical-grade diphenylamine (purity, > 99%) at concentrations of 0, 520, 2600, or 5200 ppm for up to 78 weeks, equal to 0, 73, 370, and 760 mg/kg bw per day for males and 0, 90, 460, and 940 mg/kg bw per day for females. Ten mice of each sex per dose were sacrificed at 52 weeks. The animals were observed for clinical signs, deaths, body weight, food consumption, ophthalmological and haematological end-points, organ weights, and gross and histopathological appearance.

An increase in the incidence of greenish staining of the fur, especially around the anogenital area, with dose was seen as the study progressed. By week 26, most of the mice at 5250 ppm were affected, and by the end of the study some mice at 525 ppm group showed staining. The incidence of penile prolapse increased with dose, affecting seven males at 2625 ppm and 17 at 5250 ppm by 78 weeks. The frequency of unkempt appearance also increased with dose, with a higher incidence among males. The mortality rate increased with dose, becoming statistically significantly different from controls for males at 2625 and 5250 ppm by 52 weeks. The deaths were attributed mainly to cystitis among males and amyloidosis in females. The mean body-weight gains were 87, 86, and 91% of control values for males at 5250 ppm and 104, 93, and 93% of control values for females at 5250 ppm at 13, 52, and 78 weeks, respectively. The body-weight gains of males at 5250 ppm and occasionally animals at 2625 ppm were statistically significantly decreased throughout the study (mainly through week 58). The body-weight gain of females at 5250 ppm was significantly decreased during the first three weeks and then occasionally for the remainder of the study. Mean food consumption was statistically significantly decreased during the first week of treatment in males at the high dose and remained increased throughout treatment for males at the two higher doses, with occasional statistically significant differences from controls. The food consumption of females at any dose showed little or no statistically significant difference from that of controls.

At the interim haematological evaluation at 52 weeks, males showed dose-related decreases in haematocrit and erythrocyte counts that reached statistical significance at 2625 and 5250 ppm; and mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin content increased with dose and reached statistical significance in males at these doses. Females showed dose-related decreases in haematocrit that reached statistical significance at  $\geq 525$  ppm; their erythrocyte counts decreased in a dose-related fashion and reached statistical significance at 2625 and 5250 ppm. Mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin content increased with dose, the latter two reaching statistical significance at 2625 and 5250 ppm and the mean corpuscular volume at 5250 ppm. At termination at 78 weeks, males and females showed dose-related decreases in haematocrit and erythrocyte counts that

reached statistical significance at 2625 and 5250 ppm; reticulocyte counts, mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin content increased with dose and reached statistical significance in males at these doses.

At the interim necropsy, darkened spleens were seen in most mice at 2625 ppm and in all those at 5250 ppm. Darkened livers were seen in most mice at 5250 ppm and pale kidneys in many. At terminal necropsy, the livers of some mice at 2625 ppm and most at 5250 ppm were dark. The spleens of most treated mice were dark and often enlarged. Dose-related increases in the absolute and relative weights of the spleen, liver, and heart were seen in animals of each sex at the interim and final sacrifices. At interim sacrifice, the absolute weights of the spleen and liver of males at 2625 and 5250 ppm were statistically significantly different from those of controls, while the increases in the relative liver weights reached statistical significance only at the highest dose. In females, the absolute and relative weights of the spleen were statistically significantly increased only at the highest dose. The absolute and relative weights of the heart were statistically significantly increased in females at 5250 ppm; the increases in males were not statistically different from those in controls. At final sacrifice, the absolute and relative weights of the spleen and liver of males at 2625 and 5250 ppm were statistically significantly increased. In females, the absolute and relative weights of the spleen were significantly increased only at 5250 ppm; the differences in relative liver weight reached statistical significance at 2625 and 5250 ppm. The absolute and relative weights of the heart were statistically significantly increased in animals of each sex at 5250 ppm. Changes in the absolute and relative weights of the pituitary and thyroid were not consistently dose-related and were thus considered not to be of toxicological significance.

Histopathological examination revealed treatment-related effects in the kidney, liver, spleen, bone marrow, urinary bladder, and penis. The incidences of haematopoiesis and pigment deposition in the liver were increased at the intermediate and high doses. Of mice at 2625 ppm that were found dead or moribund or were sacrificed at termination, 19/51 males and 24/51 females showed liver haematopoiesis and 15/51 males and 37/51 females showed liver pigmentation; higher incidences of these effects were seen at the high dose. The incidences of spleen congestion and haemosiderosis were increased in all treated mice. Of the mice at the low dose found dead or moribund or sacrificed at termination, 11/50 males and 8/50 females showed spleen congestion and 8/50 males and 35/50 females showed spleen haemosiderosis; higher incidences of these effects were seen at the higher doses. The frequency of pigment deposition was increased in males at the high dose and in females at the intermediate and high doses. Among females found dead or moribund or sacrificed at termination, pigment was found in 5/51 at the intermediate dose and 7/51 at the high dose. Among males found dead or moribund or sacrificed at termination, 5/51 at the intermediate dose and 7/51 at the high dose showed pigment accumulation; additionally, 5/54 males had pyelonephritis. Although the incidence of spleen haematopoiesis did not increase with dose, the severity scores increased from minimal or slight in controls to mixed scores including moderate or marked at the high dose. The severity of haematopoiesis in the spleen increased from minimal to slight at 525 ppm in contrast to the largely minimal level in controls. Additionally, bone-marrow cellularity changed from predominantly moderate in controls to marked at the higher doses. The incidences of urinary bladder cystitis and dilatation increased with dose, reaching statistical significance at the intermediate and high doses. Among mice at the intermediate dose that were found dead or moribund or were sacrificed at termination, 24/51 males and 13/48 females had cystitis and 18/51 males and 13/48 females had dilatation; higher values were seen at the high dose. The incidence of balanoposthitis apparently increased with dose; however, no statistical analysis was available. In females at 5250 ppm, the incidence of amyloidosis in the thyroid, adrenals, kidneys, stomach, small intestine, ovaries, and uterus was increased. The incidence of tumours was comparable in the treated and control groups. The NOAEL for toxicity was 525 ppm, equal to 73 mg/kg bw per day, on the basis of decreased body-weight gain, decreased survival, and significant haematological and gross and microscopic pathological alterations (Botta, 1994a).

### *Rats*

Groups of 60 male and 60 female Sprague-Dawley rats received diets containing technical-grade diphenylamine (purity, >99%) at concentrations of 0, 200, 750, 3750, or 7500 ppm for males and 0, 150, 500, 2500, or 5000 ppm for females for up to two years, equal to 0, 8.1, 29, 150, and

300 mg/kg bw per day for males and 0, 7.5, 25, 140, and 290 mg/kg bw per day for females. Groups of 10 rats of each sex per group were killed at a one-year interim sacrifice. The animals were observed for clinical signs, deaths, body weight, food consumption, ophthalmological, haematological, clinical chemical, and urinary end-points, organ weights, and gross and histopathological appearance.

The only treatment-related clinical finding was greenish colouration of the fur in the urogenital or ventral cervical area in animals of each sex at the two higher doses. The effect was attributed to the presence of a metabolite in urine or faeces. No treatment-related effects on mortality rates were observed; however, the study was terminated at 102 weeks because of increased mortality rates in controls and animals at the low dose: survival among males was 22% at 0 and 200 ppm and 55% at 7500 ppm. Survival thus seemed to increase with dose, and an analysis of the survival data indicated a statistically significant negative trend for mortality as the dose increased in animals of each sex; the mortality rates at the two higher doses were statistically significantly lower than those for the control group. A dose-related decrease in body weight and body-weight gain was seen throughout most of the study, which reached statistical significance at the two higher doses. The body-weight gains of males at the two higher doses were depressed to 95 and 87% of the control values at 78 weeks and equal to those of controls at 102 weeks; in females, the corresponding values were 78 and 56% of controls at 78 weeks and 80 and 61% at 102 weeks. There was no decrease in food consumption, except during the first week; at other times, food consumption appeared to have increased, possibly due to food wastage. There were no treatment related effects on ophthalmological parameters.

Haematological examination revealed dose-related decreases in erythrocyte counts, haemoglobin, and haematocrit in animals at the two higher doses throughout treatment. The decreases reached statistical significance for erythrocyte count and haemoglobin in males at 3750 and 7500 ppm in week 26 and at termination and for erythrocyte counts, haemoglobin, and haematocrit in females at 2500 and 5000 ppm through most of the treatment and at termination. Although the erythrocyte counts, haemoglobin, and haematocrit were decreased in males at 750 ppm and in females at 500 ppm, the decreases reached statistical significance only sporadically during treatment. The mean corpuscular volume and mean corpuscular haemoglobin content were significantly different from those of controls in males at the three higher doses and in females at the two higher doses.

Dose-related, statistically significant increases in the absolute and relative weights of the spleen were seen in females at the two higher doses at interim sacrifice and at termination. A similar effect on spleen weights was observed in males, except that the changes in rats at 3750 ppm were not statistically significant. The relative weight of the liver was statistically significantly increased in females at 5000 ppm at termination and at 3750 and 5000 ppm at interim sacrifice; no increase in liver weights was observed in males. The increases in spleen and liver weights are consistent with the haematological effects of the compound. Findings of dark and/or enlarged spleens at necropsy in males at  $\geq 750$  ppm and in females at 500 ppm are probably related to the haematological effects. Microscopic examination revealed treatment-related effects in the kidney, liver, spleen, and bone marrow. The incidence of pigment deposition in the kidney increased in a dose-related fashion and reached 44/50 in males and 44/52 in females at the high dose that were found dead or moribund or were sacrificed at termination. The incidences of haematopoiesis and pigment deposition in the liver increased in a dose-related fashion and reached 21/50 and 27/50 in males and 41/52 and 45/52 in females at the high dose that were found dead or moribund or sacrificed at termination. Erythroid hyperplasia was seen at the higher dose but not in controls or at the low dose. The incidence of congestion of the spleen increased in a dose-related fashion and reached incidences of 50/50 in males and 47/52 in females at the high dose that were found dead or moribund or sacrificed at termination. These findings are all related to the observed haematological effects. No treatment-related increase in tumour incidence was observed. The NOAEL for toxicity was 150–200 ppm, equal to 7.5 mg/kg bw per day, on the basis of changes in haematological parameters and in the histopathological appearance of the spleen, kidney, and liver (Botta, 1994b).

### *Dogs*

Four beagles of each sex received diphenylamine (purity, > 99%) by gelatin capsule at a dose of 0, 10, 25, or 100 mg/kg bw per day for 52 weeks and were observed for clinical signs, body

weight, food consumption, ophthalmological, haematological, clinical chemical, and urinary end-points, organ weights, and gross and histopathological appearance. No treatment-related clinical signs were seen at termination. One dog at the intermediate dose and two at the high dose had greenish hair. There were no deaths or treatment-related effects on body weight, food consumption, or ophthalmological parameters. Haematological examination revealed decreased mean erythrocyte counts (by 11% in comparison with controls), haemoglobin (9.3%), and haematocrit (8.7%) in males at the high dose; smaller decreases in these parameters were found in females. The platelet count increased with dose in males at the 13-, 26-, 39-, and 52-week evaluation periods, becoming statistically significant at the intermediate and high doses. There was a dose-related increase in mean total bilirubin concentration, which was statistically significant for animals at the intermediate and high doses throughout the study, in animals at the low dose at week 26, and in females only at week 39. The mean cholesterol concentration appeared to increase with dose at all evaluation periods but was statistically significantly increased only in males at the high dose at week 13 (by 68%) and in females at the high dose at week 39 (by 37%). The blood urea nitrogen concentration was decreased in females at the intermediate (by 16%) and high doses (by 20%) at week 52. The mean absolute and relative weights of the liver and thyroid appeared to increase with dose in males, but only the mean absolute liver weight of males at the high dose was statistically significantly increased. The mean absolute and relative weights of the thyroid decreased with dose in females but did not reach statistical significance at any dose. There were no treatment-related gross or histopathological changes. The NOAEL for toxicity was 10 mg/kg bw per day on the basis of haematological and clinical chemical changes (Botta, 1994c).

(d) *Genotoxicity*

As summarized in Table 2, negative results were obtained for mutation in bacteria (Lawlor, 1992) and for induction of micronuclei in mouse bone marrow *in vivo* (Murli 1992 a,b). A weakly positive response was observed for mutation in mouse lymphoma cells *in vitro* at a dose range that was toxic, and the mutant frequency did not increase with dose (Cifone, 1992). The Meeting concluded that although diphenylamine has some genotoxic potential it is unlikely to be a human genotoxic hazard.

(e) *Reproductive toxicity*

(i) *Multigeneration reproductive toxicity*

In a two-generation study of reproductive toxicity, groups of 28 Sprague-Dawley rats received diphenylamine (purity, 99.8%) in the diet at concentrations of 0, 500, 1500, or 5000 ppm, equal to 0, 40, 120, and 400 mg/kg bw per day for F<sub>0</sub> males and 0, 46, 130, and 450 mg/kg bw per day for F<sub>0</sub> females for 70 days before mating. After weaning, 28 F<sub>1</sub> rats of each sex per group were also given the test diets for up to 70 days before mating and were selected as parents for the F<sub>2</sub> litters. The parent animals were observed for deaths, clinical signs, body weight, food consumption, mating and fertility indices and other measures of reproductive performance, organ weights, and

**Table 2. Results of assays for the genotoxicity of diphenylamine**

End-point	Test object	Concentration	Purity (%)	Results	Reference
<i>In vitro</i> Forward mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538	6.67–333 µg/plate <sup>a</sup> 10–667 µg/plate <sup>b</sup>	99.9	Negative	Lawlor (1992)
Gene mutation	L5178Y <i>tk</i> <sup>+</sup> mouse lymphoma cells	5–80 µg/ml	≥ 93	Weakly positive <sup>b</sup> Negative <sup>a</sup>	Cifone (1992)
<i>In vivo</i> Micronucleus formation	Mouse (ICR strain)	250–1000 mg/kg bw (males) 375–1500 mg/kg bw (females)	99.9	Negative	Murli (1992a,b)

gross and histopathological appearance. The offspring were observed at birth and through lactation for clinical signs, deaths, weights, and sex ratio; all underwent necropsy.

Toxicity was dose-related and was observed in animals of each sex and generations at all doses. In general, females were more affected than males, and F<sub>1</sub> animals were more affected than F<sub>0</sub> animals. In the F<sub>0</sub> generation, the incidences of bluish coats and a bluish fluid in the cages were increased in males and females at 5000 ppm, and similar findings were made in the F<sub>1</sub> generation. Swelling of the mammary glands were seen in females, and palpable masses were noted in lateral or ventral regions, especially in females. These masses appeared to be transient but were not examined grossly or microscopically. Body weight and body-weight gain were significantly decreased at various times in animals at 1500 and 5000 ppm. At 5000 ppm, there was a decrease in body weight compared with controls, of 6–9% for F<sub>0</sub> males, 5–8% for F<sub>0</sub> females, 22–28% for F<sub>1</sub> males, and 11–23% for F<sub>1</sub> females. At 1500 ppm, there was a 5–8% decrease in body weight for F<sub>0</sub> females, 7–9% for F<sub>1</sub> males, and 5% for F<sub>1</sub> females. The relative weights of the kidney, liver, and spleen of F<sub>0</sub> and F<sub>1</sub> males were significantly increased at 5000 ppm, and the relative weights of the liver and spleen of F<sub>0</sub> and F<sub>1</sub> females were significantly increased at 1500 ppm. The relative weights of the liver were significantly increased at 1500 and 5000 ppm in F<sub>0</sub> females but only at 5000 ppm in F<sub>1</sub> females.

Gross observations at necropsy included enlarged spleens in animals of each sex and generation at 1500 and 5000 ppm and in F<sub>1</sub> females at 500 ppm, in addition to blackish-purple spleens in animals of each sex and generation at 500, 1500, and 5000 ppm. The incidences of blackish-purple spleen in F<sub>1</sub> rats at 500 ppm were 9/28 males and 6/28 females; incidences of 27/28 or higher were seen at higher doses. Histopathological examination revealed a brown pigment in the proximal tubules of the kidney in animals of each sex and generation at 5000 ppm; and 3/28 F<sub>1</sub> females at 1500 ppm also had brown pigment. Congestion and haemosiderosis of the spleen were seen in animals of each sex and generation at 500, 1500, and 5000 ppm. Increased spleen erythropoiesis was seen in 4/28 males at 5000 ppm. Hepatocyte hypertrophy was seen in males of both generations at 1500 and 5000 ppm; in females, it was seen at all doses in the F<sub>0</sub> generation but only at 5000 ppm in the F<sub>1</sub> generation. Kupffer cells with brown pigment containing iron were seen in animals of each generation, at 5000 ppm in males and at 1500 and 5000 ppm in females.

Reproductive toxicity was seen in the form of decreased mean pup weight at various times in each generation and reduced litter size at the high dose; no other parameters were affected by treatment. F<sub>1</sub> pups at 5000 ppm had statistically significantly decreased mean body weight (11% less than controls at birth and 14–25% less than controls during lactation). F<sub>2</sub> pups showed statistically significantly decreased mean body weight at 1500 ppm (10–12% less than controls during late lactation) and at 5000 ppm (10–19% less than controls throughout lactation). The mean body weight of pups in the F<sub>2</sub> litters at birth was about 4.8% lower than that of controls, but the difference was not statistically significant. The mean litter size decreased with dose in both generations, by 21% at 5000 ppm in the F<sub>1</sub> generation, and was statistically significantly different from control values. Although the mean litter size in the F<sub>0</sub> generation at 5000 ppm was decreased by 10%, this value was not statistically significant. The decrease in litter sizes with dose correlated with the number of uterine implantation scars observed at necropsy, and the mean uterine implantation scar count decreased with dose in both generations. The mean scar count at 5000 ppm was decreased by 16% and was statistically significant; although the mean scar count in the F<sub>0</sub> generation was decreased by 7.3% at 5000 ppm, the value was not statistically significant.

A NOAEL was not identified for parental systemic toxicity. Although haemosiderin and congestion in the spleen and hepatocyte hypertrophy were seen at all doses, the incidence and intensity of these effects were appreciably smaller at the lower dose. For example, only 4/28 females at the low dose had spleen congestion, rated as minimal, whereas 22/28 females at the intermediate dose had this effect and its intensity was nearly equally divided between minimal and mild. These observations suggest that the lower dose in this study is close to the NOAEL for parental systemic toxicity. The NOAEL for reproductive toxicity was 1500 ppm, equivalent to 120mg/kg bw per day for F<sub>0</sub> animals, on the basis of decreased mean litter size at the high dose. The NOAEL for developmental toxicity was 500 ppm, equivalent to 46 mg/kg bw per day in maternal animals, on the basis of the statistically significantly decreased mean body weights of F<sub>2</sub> pups (Rodwell, 1993).

(ii) *Developmental toxicity**Rats*

In a range-finding study for developmental toxicity, groups of six pregnant Sprague-Dawley Crl:CDTM BR VAF/Plus rats received diphenylamine (purity, 99.9%) in corn oil by gavage at doses of 0, 10, 50, 100, 200, 300, or 400 mg/kg bw per day on days 6–15 of gestation. Maternal toxicity was seen at doses of 100 mg/kg bw per day and higher. Two deaths occurred at 400 mg/kg bw per day, and there were dose-related decreases in food consumption, body weight, and body-weight gain starting at a dose of 100 mg/kg bw per day. Necropsy revealed a purplish-black spleen in one dam at 100 mg/kg bw per day and in all surviving dams at higher doses. The increased incidence of early resorptions was apparent dose-related and was accompanied by decreases in mean fetal weight and gravid uterine weight at doses of 200 and 300 mg/kg bw per day. There were no treatment-related external malformations or developmental variations. On the basis of the results of this study, 0, 10, 50, and 100 mg/kg bw per day were selected for use in the definitive study (Rodwell, 1992a)

In the definitive study, groups of 25 pregnant Sprague-Dawley Crl:CDTM BR VAF/Plus rats received diphenylamine (purity, 99.9%) in corn oil by gavage at doses of 0, 10, 50, or 100 mg/kg bw per day on days 6–15 of gestation. The dams were observed for deaths, clinical signs, body weight, and food consumption. All rats were sacrificed on day 20 of gestation and were necropsied grossly. The spleens were weighed, and the uterus was removed, examined externally, weighed, and opened for internal examination. The fetuses were observed externally, and their viscera and skeleton were examined. There were no deaths or treatment-related effects on clinical signs, body weights, or food consumption. At necropsy, 5/25 dams at the high dose had enlarged, blackish-purple spleens, and the mean weights of the spleens were significantly greater than those of controls. No treatment-related effects were seen on gross necropsy, in the appearance or weight of the uterus, or in the numbers of viable fetuses, early and late resorptions, and corpora lutea, or on external, visceral, or skeletal malformations or variations in the fetuses. The NOAEL for maternal toxicity was 50 mg/kg bw per day on the basis of effects on the spleen in dams at the high dose. The NOAEL for developmental toxicity was 100 mg/kg bw per day, the highest dose tested, in the absence of developmental effects (Rodwell, 1992b).

*Rabbits*

In a study of developmental toxicity, groups of 16–18 pregnant New Zealand white rabbits received diphenylamine (purity, 99.9%) in 1% methyl cellulose by gavage at a dose of 0, 33, 100, or 300 mg/kg bw per day on days 7–19 of gestation. The dams were observed for deaths, clinical signs, body weight, and food consumption. All rats were sacrificed on day 29 of gestation and subjected to gross necropsy; the ovaries and uterus of each animal were removed and examined to determine the number of corpora lutea and the type, distribution, and number of implantation sites. The fetuses were examined externally, and their viscera and skeleton were analysed. No deaths occurred during the study. The mean food consumption of dams at 300 mg/kg bw per day was reduced on days 7–29 of gestation, and a slight decrease in body weight was noted. Green discoloration of the urine was seen in all treated animals. No treatment-related effects were seen at necropsy or on embryonic or fetal growth or development. The NOAEL for maternal toxicity was 100 mg/kg bw per day on the basis of decreased body-weight gain and food consumption early during treatment. The NOAEL for developmental toxicity was 300 mg/kg bw per day, the highest dose tested (Edwards et al., 1983).

(f) *Special studies*(i) *Renal cystic disease*

The issue of tubular cyst formation in diphenylamine-treated rats was reviewed previously (Annex 1, references 13, 27, 39, and 43). Additional observations on the induction of tubular cysts in the kidneys of mice and rats treated with diphenylamine in the diet are summarized below.

Renal histopathology and function were studied in male Sprague-Dawley rats fed pelleted diets containing 1% w/w (i.e. 10 000 ppm) diphenylamine (purity unspecified) for 5–20 months. Control animals received identical diets in which methyl cellulose was incorporated instead of diphenylamine. The animals were observed for renal tubular diameter and renal functional parameters including intratubular hydrostatic pressure, single-nephron glomerular filtration rate, and transit times through the loop of Henle; light and scanning electron microscopy was performed. The luminal diameters of 22 dilated nephrons from diphenylamine-treated rats were 34–110  $\mu\text{m}$ , and those of 10 undilated nephrons were 21–450  $\mu\text{m}$ ; the luminal diameters in six nephrons of two control rats were 26–34  $\mu\text{m}$ . The intraluminal hydrostatic pressure was significantly higher in dilated tubules than in undilated tubules from treated rats; there was no difference in intraluminal hydrostatic pressures among control rats. The single-nephron glomerular filtration rate was also similar in dilated and undilated tubules, consistent with an intrinsic change in glomerular function. The transit times through the loop of Henle were three to four times faster in dilated than in undilated nephrons of treated and control rats. Microscopic examination indicated that dilatation along the proximal and collecting tubules was segmental, not diffuse, some dilated tubules communicated with cysts deep in the renal substance, the dilated collecting tubules occasionally contained debris, tubules adjacent to cysts appeared to be compressed, and diphenylamine-exposed kidneys contained proximal convoluted tubules with segments of apparent narrowing. The authors concluded that cyst formation results from initial obstruction and a subsequent increase in intraluminal pressure arising from sustained glomerular filtration and unaltered water reabsorption (Gardner et al., 1976).

The time-course of development of lesions in the renal collecting tubules was studied in male Sprague-Dawley rats fed pelleted diets containing 1% w/w (i.e. 10 000 ppm) diphenylamine (purity unspecified) for up to 76 weeks. Control animals received standard diet. The animals were observed for renal concentrating ability (urine osmolality) at 2, 4, 5, and 20 weeks of treatment; light and transmission and scanning electron microscopy was performed at 2, 5, 10, 15, 20, 25, 52, and 78 weeks of treatment; and autoradiography with tritiated thymidine was conducted to determine whether hypertrophy was the mechanism of cyst formation. Decreased urine osmolality was seen in treated rats, which was statistically significantly different from control values at 6 and 20 weeks. Structural changes first appeared after five weeks of treatment. Light microscopy of the medullary collecting tubules at five weeks revealed focal areas of apparent thickening along the walls, resulting from layering of cells. By 10 weeks, some tubules were dilated and contained focal areas of cellular necrosis. By 15–20 weeks, many more ducts were dilated and contained cast material. By 24 weeks, frank cysts were visible in the cortex and medulla, and large collecting tubular cysts were seen; some proximal tubules and renal corpuscles were dilated. Over time, cysts were found in every segment of the nephron and collecting tubules. Transmission electron microscopy at five weeks revealed changes in the medullary collecting tubules. The tubular cells appeared to be enriched in mitochondria, and their apical border was studded with long microvilli. At 10 weeks, necrotic cells were seen along the collecting tubules. Scanning electron microscopy showed that the collecting duct cysts were lined with irregular cells which no longer resembled collecting tubule cells. The labelling index for the collecting tubules reached a plateau at weeks 5–15 and had decreased to background levels by week 52. Because the number of nuclei counted on cross-sections of the collecting tubules increased gradually with time, the authors concluded that the increase in labelling index was the result of hyperplasia of the tubular cells and not of a degeneration or regeneration phenomenon. The authors concluded that one of the initial events in the pathogenesis of cystic renal disease is a hyperplastic response of the collecting tubule cells (Evan et al., 1978).

The effect of diphenylamine on the composition of the native renal basement membrane was studied in male C57Bl/6 mice fed pelleted diets containing 2% w/w (i.e. 20 000 ppm) diphenylamine (purity unspecified) for up to 10 months. Control animals received chow without diphenylamine. After 10 months of treatment, the mice were sacrificed and their kidneys processed for light microscopy and immunohistology of bamin, a glycoprotein associated with the matrix of the glomerular basement membrane. Microscopy revealed cysts in the cortical and medullary portions

of the kidney. The cysts were lined with flattened or somewhat cuboidal cells and contained a brownish coagulum that appeared to be constituted of necrotic cells. Brownish discoloration was also seen in the cytoplasm of neighbouring non-cystic cells and in tubular cells of animals sacrificed before the formation of cysts. The authors speculated that the brownish debris preceded the formation of cysts. When immunohistology was performed with antibodies to bamin, the antibodies bound to the glomeruli of control mice but not to those of treated animals. This result was obtained in the three-week experiment. In another series of experiments, male C57Bl/6 mice were injected with EHS tumour cells and five days later received a diet containing diphenylamine (at an unspecified level). After three weeks, the animals were killed, and tumours were removed for analysis of the proteins of the basement membrane. Analysis by sodium dodecylate sulfate-polyacrylamide gel electrophoresis and by immunoblot revealed the presence of a 75–80-kDa protein in controls but little or none in the diphenylamine-treated mice. The authors were unclear how the loss of bamin, a molecule associated with the glomerular but not the tubular basement membrane, would be related to the formation of tubular cysts. They speculated that material from an abnormal glomerular filtrate might damage the tubular epithelial cells and result in the formation of tubular cysts (Rohrbach et al., 1993).

#### (iv) *Renal papillary necrosis*

A series of studies has been reported on the induction of renal papillary necrosis by diphenylamine in hamsters, rats, and Mongolian gerbils. The development of animal models for this lesion is of interest because it is the initial stage in human analgesic-induced nephropathy.

Groups of 10 male Syrian hamsters, 40 Sprague-Dawley rats, and 40 Mongolian gerbils received diphenylamine (purity unspecified) at doses of 0, 400, 600, or 800 mg/kg bw per day in peanut oil by gavage for three days. Animals that became moribund were sacrificed and necropsied; survivors were sacrificed 24 h after the third dose of diphenylamine. All animals were observed for death and were examined for gross and microscopic lesions. Forty percent of hamsters at the low dose and 100% of those at the two higher doses died or were sacrificed *in extremis* during treatment; there were no deaths among the rats or gerbils. At necropsy, brown kidneys and yellow-brown papillae were seen in 50% or more of hamsters at the intermediate and high doses and in none of those at the low dose. The only gross lesion in hamsters at the low dose was splenomegaly (90%), which was not seen at higher doses. Microscopic examination revealed total renal papillary necrosis, i.e. necrosis of all elements of the renal papillae, including collecting tubules, at all doses in 4/10 hamsters at the low, 7/10 at the intermediate, and 4/10 at the high dose. Only two rats at the high dose had renal lesions, which consisted of necrosis of the medullary interstitial cells and vasa recta, limited to the apex, and degeneration of the renal interstitial matrix. As noted by the authors, the limited incidence and extent of the lesions in the Sprague-Dawley rats contrasted with the more extensive effects observed in Wistar rats by Powell et al. (1985). No effects were observed in Mongolian gerbils (Lenz & Carlton, 1990).

A total of 27 male Syrian hamsters were given diphenylamine by gavage (purity unspecified) dissolved in peanut oil at a dose of 600 mg/kg bw. At intervals of 0.5, 1, 2, 4, 8, 16, and 24 h after dosing, three hamsters were perfused *in situ* with fixative and their kidneys were removed for examination by transmission electron microscopy. Starting 1 h after dosing, the basal plasma membrane of the endothelial cells of the ascending vasa recta in the proximal portion of the renal papilla became separated from the basal lamina, forming large subendothelial vacuoles. These alterations persisted during the first 8 h after dosing. By 16 h, the endothelial cell nuclear membranes and the luminal plasma membrane had become convoluted, and by 24 h platelets were seen to adhere to the basal lamina, which had become exposed. By 2 h after dosing, the adjacent interstitial cells started undergoing structural alterations, and by 24 h there was necrosis. Ultrastructural alterations became visible in the thin limb of the loop of Henle by 24 h and in collecting tubule epithelial cells by 14–16 h. The authors speculated that the selective effect on the endothelial cells of the ascending vasa recta were attributable to generation of a toxic metabolite by the endothelial cell or to interference with the metabolism of the endothelial cells by a metabolite of diphenylamine (Lenz et al., 1995).

The toxicity of diphenylamine dissolved in dimethyl sulfoxide to renal papilla was studied in Syrian hamsters, rats, and Mongolian gerbils. Male Syrian hamsters and Sprague-Dawley rats were given diphenylamine (purity unspecified) by gavage at doses of 0, 400, 600, or 800 mg/kg bw per day for up to nine days. Only one of 30 hamsters at 400 mg/kg bw per day showed renal papillary necrosis. This result contrasted with previous results (Lenz & Carlton, 1990) in which oral administration of diphenylamine dissolved in peanut oil produced extensive necrosis in male Syrian hamsters at the same doses; furthermore, pretreatment of the hamsters with dimethyl sulfoxide protected the animals against the renal papillary necrosis induced by administration of diphenylamine. Renal papillary necrosis occurred in 4/30 rats at the high dose; none was seen in Mongolian gerbils. The incidence in rats and gerbils did not differ from that observed when peanut oil was used as the vehicle in a previous study (Lenz & Carlton, 1990). The mechanism by which dimethyl sulfoxide exerts its protective effect in Syrian hamsters was not studied (Lenz & Carlton, 1991).

The effect of diphenylamine on renal glutathione concentrations was studied in groups of eight male Syrian hamsters given diphenylamine (purity unspecified) by gavage at a dose of 0, 200, 400, or 600 mg/kg bw 1 and 4 h after dosing. The concentrations in the cortical area decreased with dose, reaching statistical significance at the low dose at 1 h and at the intermediate dose at 4 h; the concentrations at 1 h were 41% of the control value at 200 mg/kg bw, 31% at 400 mg/kg bw, and 29% at 600 mg/kg bw. The glutathione concentrations in the outer medulla and the renal papilla were not statistically significant decreased.

In order to study the effect of glutathione reduction on toxicity to renal papilla, groups of eight male Syrian hamsters were given buthionine sulfoxime, an inhibitor of glutathione biosynthesis, at 500 mg/kg bw intraperitoneally, diphenylamine in peanut oil at 400 mg/kg bw by gavage, the same doses of buthionine sulfoxime plus diphenylamine, or the peanut oil vehicle alone. The animals were sacrificed 24 h after administration of diphenylamine for gross and microscopic examination of the kidney. No gross or microscopic lesions indicative of papillary necrosis were observed in any group. Because a dose of diphenylamine that is toxic to the papilla did not deplete glutathione in that region and because depletion of glutathione (to 71% of the control value) by buthionine sulfoxime did not enhance the toxicity of diphenylamine to the papilla, the author concluded that this toxic effect of diphenylamine is mediated by mechanisms independent of oxidative injury (Lenz, 1996).

### Comments

After oral administration to rats, goats, or hens,  $^{14}\text{C}$ -diphenylamine was extensively absorbed and rapidly excreted. In rats given a single dose, 45–72% appeared in the urine within 24 h and 68–89% by 168 h after dosing, with less than 0.4% of the dose in the residual carcass and less than 0.05% in any individual tissue. In goats treated with single daily doses for seven days, 85–91% of the daily dose was excreted in urine and 0.5–0.8% in milk; the concentrations of residues in milk plateaued after 24 h. When laying hens were treated with single daily oral doses for seven days, 84–98% of the daily dose was recovered in the excreta; the concentrations in egg yolk reached 0.31 mg/kg on day 7, but no residues were found in egg whites. Diphenylamine underwent extensive biotransformation in rats, goats, and hens, with ring hydroxylation and formation of glucuronide and sulfate conjugates. In addition to untransformed diphenylamine (< 3% of the dose), the following 12 metabolites were identified at all doses: 4,4'-dihydroxydiphenylamine (unconjugated and as the *O*-sulfate and the *O,O*-disulfate), 4-hydroxydiphenylamine (unconjugated and as the *O*-glucuronide, *N*-glucuronide, *O*-sulfate, and *O,N*-diglucuronide), indophenol (unconjugated and as the *O*-sulfate), 3-hydroxydiphenylamine, and 2-hydroxydiphenylamine. These metabolites accounted for about 80–90% of the dose and were excreted largely as their sulfate and glucuronide conjugates. There was no cleavage of the diphenylamine structure. Except for a polar oligomer of 4-hydroxydiphenylamine found only in the eggs and tissues of hens, all of the metabolites reported in hens and goats were detected in rats. Residues of a number of plant

metabolites were identified in apple peel and pulp, but untransformed diphenylamine was the major contributor to the total residue 40 weeks after application. The major metabolite was 4-hydroxydiphenylamine, as the glucose conjugate. Other metabolites identified were 2-hydroxydiphenylamine, 3-hydroxy-diphenylamine, and dihydroxydiphenylamine (possibly the 2,4 isomer). These compounds were either free or conjugated with mono- or oligosaccharides. Although all of the hydroxylated metabolites (aglycones) identified in plants were seen in rats, the conjugating species were generally different.

After acute oral administration to rats, diphenylamine (99.9% pure) was slightly toxic ( $LD_{50}$  about 3000 mg/kg bw) in one study, whereas in another study super-refined diphenylamine (purity, 99.0–100.1%) was essentially nontoxic ( $LD_{50} > 15\ 000$  mg/kg bw).

WHO has not classified diphenylamine for acute toxicity.

In mice given diphenylamine at dietary concentrations of 0, 10, 520, 2600, or 5200 ppm for 90 days, dose-related changes in haematological parameters (decreased erythrocyte counts and packed cell volumes and increased reticulocyte counts) were observed. The mean corpuscular haemoglobin count was significantly increased at dietary levels of 520 ppm and above. Necropsy revealed dark, enlarged spleens with haemosiderosis and congestion at dietary levels of 520 ppm and above. Spleen haematopoiesis was increased in animals of each sex at 520 ppm. The NOAEL was 10 ppm (equal to 1.7 mg/kg bw per day) on the basis of changes in haematological parameters and findings at necropsy in animals at 520 ppm.

In rats that received diphenylamine in the diet at concentrations of 0, 150, 1500, 7500, or 15 000 ppm for 90 days, body weights and body-weight changes, although generally lower than the control values at 1500 ppm, were not significantly different from those of controls at doses below 7500 ppm. The cholesterol concentration increased with dose in females and was significantly different from that of controls at 1500 ppm. In females, the absolute and relative weights of the liver increased with dose, and the relative liver weights were significantly different from those of controls in animals at 1500 ppm and above. Histopathological examination revealed increased haematopoiesis and pigment in the liver, haematopoiesis, haemosiderosis, and congestion in the spleen, and pigmented kidneys in animals of each sex at 7500 and 15 000 ppm. Additionally, the spleens of all females at 1500 ppm showed minimal or slight haematopoiesis and haemosiderosis. The NOAEL was 150 ppm (equal to 12 mg/kg bw per day) on the basis of changes in clinical chemical parameters, increased organ weights, and gross and histological changes in female rats at 1500 ppm.

Groups of rabbits were exposed dermally to diphenylamine in distilled water at doses of 0, 100, 500, or 1000 mg/kg bw per day for 6 h per day. No effects were observed. Gross necropsy revealed dark-red foci in the stomachs of rabbits at the intermediate and high doses, which increased in number with dose. The NOAEL for systemic effects was 100 mg/kg bw per day on the basis of effects on the stomach at 500 mg/kg bw per day in animals of each sex.

Groups of dogs received diphenylamine by gelatin capsule at doses of 0, 10, 25, or 50 mg/kg bw per day for 90 days. There were no treatment-related effects. The NOAEL was 50 mg/kg bw per day, the highest dose tested.

In a study of carcinogenicity, mice received diets containing diphenylamine at concentrations of 0, 520, 2625, or 5200 ppm for 78 weeks. At 520 ppm and above, decreased packed cell volumes were seen in females and spleen congestion and haemosiderosis in animals of each sex. At 2600 ppm and above, clear haematological effects, consistent with regenerative anaemia, were observed. The incidences of tumours were not increased when compared with those in controls. The NOAEL for toxicity was 520 ppm (equal to 73 mg/kg bw per day) on the basis of decreased body-weight gain, reduced survival, and significant alterations in haematological and gross and microscopic pathological parameters at higher levels. Examination of the incidence and severity of some haematological effects at 520 ppm suggested that this dose is close to the NOAEL/LOAEL threshold. There was no evidence of carcinogenicity.

In a combined study of toxicity and carcinogenicity, rats received diets containing diphenylamine at concentrations of 0, 200, 750, 3750, or 7500 ppm (males) and 0, 150, 500, 2500, or 5000 ppm (females). At 500 ppm and above, decreased erythrocyte count, haemoglobin, and packed cell volumes were observed in animals of each sex; these decreases reached statistical significance only sporadically during treatment in males at 750 ppm and in females at 500 ppm. Haematopoiesis was increased in the liver and spleen of males at 750 ppm and above. At 2625 ppm and above, clear

haematological effects consistent with regenerative anaemia were observed. There was no significant increase in the incidence of tumours when compared with that in controls. The NOAEL was 150–200 ppm (equal to 7.5 mg/kg bw per day) based on haematological and histological effects at dietary levels equal to or greater than 500 ppm. This appeared to be close to the threshold dose, since body-weight gain was not depressed and only sporadic haematological changes were observed at 500–750 ppm. There was no evidence of carcinogenicity.

Dogs received diphenylamine by gelatin capsule at doses of 0, 10, 25, or 100 mg/kg bw per day for one year. Platelet counts in males and total bilirubin concentrations in animals of each sex were statistically significantly higher than those of controls. The NOAEL for toxicity was 10 mg/kg bw per day, and the LOAEL was 25 mg/kg bw per day, both based on haematological and clinical chemical changes.

In a two-generation study of reproductive toxicity, rats received diphenylamine in the diet at concentrations of 0, 500, 1500, or 5000 ppm during premating. A NOAEL for parental toxicity was not observed; the LOAEL was 500 ppm (equal to 40 mg/kg bw per day) on the basis of enlarged spleens in F<sub>1</sub> females, increased spleen congestion and haemosiderosis in animals of each sex in all generations, and hepatocyte hypertrophy in F<sub>0</sub> females. The NOAEL for developmental toxicity was 500 ppm (equal to 46 mg/kg bw per day) on the basis of statistically significantly decreased mean body weight in F<sub>2</sub> pups at 1500 ppm and above. The NOAEL for reproductive toxicity was 1500 ppm (equal to 120 mg/kg bw per day). Although haemosiderin, congestion of the spleen, and hepatocyte hypertrophy were observed in parental animals at all doses, they occurred at appreciably lower incidence and intensity at the lower dose than at the higher doses, which suggested that the lower dose was close to the NOAEL/LOAEL threshold for parental toxicity.

In a study of developmental toxicity, rats received diphenylamine by gavage at doses of 0, 10, 50, or 100 mg/kg bw per day on days 6–15 of gestation. The NOAEL for maternal toxicity was 50 mg/kg bw per day on the basis of enlarged, blackish, heavier spleens at 100 mg/kg bw per day. The NOAEL for developmental toxicity was 100 mg/kg bw per day, the highest dose tested.

In a study of developmental toxicity, rabbits received diphenylamine by gavage at doses of 0, 33, 100, or 300 mg/kg bw per day on days 7–19 of gestation. The NOAEL for maternal toxicity was 100 mg/kg bw per day on the basis of decreased body-weight gain and food consumption at 300 mg/kg bw per day. The NOAEL for developmental toxicity was 300 mg/kg bw per day, the highest dose tested.

Diphenylamine has been tested for genotoxicity in three assays. Negative results were obtained for mutation in bacteria and for induction of micronuclei in mouse bone marrow *in vivo*. A weakly positive response was observed only for mutation in mouse lymphoma cells *in vitro* at a dose range in which the toxicity was relatively high and the mutant frequency did not increase with dose. The Meeting concluded that, although diphenylamine has some genotoxic potential, it is unlikely to present a human genotoxic hazard.

An ADI of 0–0.08 mg/kg bw was established on the basis of the NOAEL of 150 ppm, equal to 7.5 mg/kg bw per day, in the two-year study of toxicity and carcinogenicity in rats and a 100-fold safety factor.

An acute RfD was not allocated because diphenylamine is of low acute toxicity. The Meeting concluded that the acute intake of residues is unlikely to present a risk to consumers.

## Toxicological evaluation

### *Levels that cause no toxic effect*

- Mouse: 520 ppm, equal to 73 mg/kg bw per day (78-week study of carcinogenicity)
- Rat: 150–200 ppm, equal to 7.5 mg/kg bw per day (two-year study of toxicity and carcinogenicity)  
500 ppm, equal to 46 mg/kg bw per day (reproductive toxicity in a two-generation study of reproductive toxicity)

Rat (contd)	50 mg/kg bw per day (maternal toxicity in a study of developmental toxicity) 100 mg/kg bw per day (study of developmental toxicity)
Rabbit:	100 mg/kg bw per day (maternal toxicity in a study of developmental toxicity) 300 mg/kg bw per day (highest dose in a study of developmental toxicity)
Dog:	10 mg/kg bw per day (one-year study of toxicity)

*Estimate of acceptable daily intake for humans*

0–0.08 mg/kg bw

*Estimate of acute reference dose*

Not allocated (unnecessary)

*Studies that would provide information useful for continued evaluation of the compound*

Further observations in humans

**List of end-points relevant for setting guidance values for dietary and non-dietary exposure**

*Absorption, distribution, excretion and metabolism in mammals*

Rate and extent of oral absorption	Rapid absorption: at least 68–89% of a dose
Dermal absorption	No data
Distribution	Extensive
Potential for accumulation	Very little
Rate and extent of excretion	At 24 h, 45–72% of a dose found in urine
Metabolism in animals	Extensively metabolized. Parent < 3%. Approximately 80–90% of a dose appeared as 12 metabolites: indophenol and various isomers of mono- and di-hydroxydiphenylamine excreted in urine as sulfate and glucuronide conjugates
Toxicologically significant compounds (animals, plants, and environment)	Parent. Indophenol might undergo electrophilic interactions

*Acute toxicity*

Rat: LD <sub>50</sub> oral	3000 mg/kg bw
Rabbit: LD <sub>50</sub> dermal	> 2000 mg/kg bw
LC <sub>50</sub> inhalation	No data
Skin irritation	None to slight
Eye irritation	Slight to corrosive with corneal opacity
Skin sensitization	Not sensitizing

*Short-term toxicity*

Target/critical effect	Erythrocytes/anaemia
Lowest relevant oral NOAEL	Mouse: 1.7 mg/kg bw per day, 90-day study
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEL	No data

*Genotoxicity*

Unlikely to be a human genotoxic hazard

*Long-term toxicity and carcinogenicity*

Target:critical effect	Erythrocytes/haemolytic anaemia
Lowest relevant NOAEL	Rat: 7.5 mg/kg bw per day
Carcinogenicity	No carcinogenicity

*Reproductive toxicity*

Reproduction target /critical effect	Decreased mean litter size in both generations and decreased mean body weights of F <sub>2</sub> pups at maternally toxic doses
Lowest relevant reproductive NOAEL	Rat: 46 mg/kg bw per day

Developmental target/critical effect	Rat: No developmental toxicity		
Lowest relevant developmental NOAEL	Rat: 100 mg/kg bw per day, highest dose tested		
<i>Neurotoxicity/Delayed neurotoxicity</i>	No data		
<i>Other toxicological studies</i>	No data		
<i>Medical data</i>	No data		
<b>Summary</b>	<b>Value</b>	<b>Study</b>	<b>Safety factor</b>
ADI	0–0.08 mg/kg bw	Long-term toxicity and carcinogenicity, rats	100
Acute reference dose	Not allocated (unnecessary)		

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