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OVERVIEW

DIPHENYLAMINE

Toxicity

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OVERVIEW

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I. INTRODUCTION

Diphenylamine (DPA) is a pesticide used primarily for the control of apple "scald," an important problem in the apple industry both from the growers' view (economics) and from the consumers' view (esthetics). Recognizing the potential large-scale exposure to the general population, the U.S. Department of Agriculture conducted studies on the toxicity of DPA in the 1950's and sponsored additional studies with Albany Medical College in the late 1960's and early 1970's. The critical toxic endpoints associated with ingestion of DPA were the formation of Heinz bodies and cystic renal tubules.

The following overview deals with the results of chronic studies in mice, rats, and dogs, to establish a NOEL and to define the toxic effects associated with the LOEL and higher doses. Special studies on the critical endpoints indicated above will be covered in the overview both with respect to methodology (evaluation of any effect in toxicology is dependent on the method, its sensitivity, limitations, reliability, etc.) and to the results obtained with these methods. In the array of studies reviewed, 16 are published animal studies and one deals with human exposure to a structurally related chemical (Accession No. 254669).

II. CHRONIC STUDIES

A. Mice: A two-part chronic toxicity study was conducted by Abraham and Goldberg (1969), Abraham et al. (1969), and Ford et al. (1972) [Reference to this study is made in Abraham and Goldberg (1969) and the Booth abstract (1963)] in an effort to

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establish an unequivocal NOEL for DPA in mice. In the first part, equal numbers of male and female CD-1 mice (Charles River) were fed diets containing either 0, 50, 100, or 250 ppm DPA (100-200 mice/sex/dose). Ten animals per group were sacrificed after 6, 12, and 18 months on study. An additional 20 mice per group were sacrificed at 80 weeks. The remaining animals were fed the diets containing DPA for 92 weeks, after which these animals were transferred to the control diet for 5 weeks.

The authors reported that DPA administration caused a dose-related increase in the incidence of Heinz bodies at all three doses. Furthermore, there was an increase in liver and spleen weights of females and liver weights of males fed DPA at 250 ppm. Organ weights were not taken for animals dosed at 50 or 100 ppm. All other hematological findings (including methemoglobin formation), gross pathology, and histopathology were unaffected by DPA administration at all three dose levels. There was no increase in the incidence of tumors at any dose level. However, this study did not follow Core guidelines for a chronic/oncogenicity study with respect to the numbers of tissues examined at termination.

In the second part of the study, animals of the same source and strain were given diets containing 0, 5, 10, 50, 100, 250, or 1000 ppm of DPA. Five mice/sex/dose for the four higher doses were sacrificed on days 2, 4, 5, 7, 9, 11, and 14 for determinations of erythrocyte glutathione reductase and glucose-6-phosphate dehydrogenase (G6PD) activities. Heinz bodies were counted in blood samples from four to six animals/sex/dose for all groups daily for the first 20 days on study, thereafter at day 30, and monthly for the next 5 months.

The authors reported that a compound-related decrease in G6PD activity was seen in mice fed 250 and 1000 ppm DPA and that this decrease corresponded to the initial appearance of Heinz bodies. Heinz bodies first appeared on days 5, 8, 12, and 14 for mice fed

DPA at 1000, 250, 100, and 50 ppm, respectively. An increase in Heinz body formation was not seen in the 5- and 10-ppm groups. The authors demonstrated by oral administration of DPA to mice that a NOEL of 10 ppm could be assessed from the incidence of Heinz bodies; however, based on other effects the NOEL is 100 ppm.

B. Rats: Booth (1963) summarized the unpublished work of F. De Eds in which groups of 20 rats per sex were fed diets containing DPA at 0, 10, 100, 1000, 5000, or 10,000 ppm for 2 years. Body weight gain was reduced in the 5000- and 10,000-ppm dose groups for both sexes. At 10,000 ppm, hemoglobin and red cell counts were reduced. The latter effect was reversible on cessation of dosing. Survival was not affected at any dose. Histopathologic findings were limited to the kidney where cystic renal tubules, accompanied by chronic interstitial nephritis, were present in both sexes at 1000, 5000, and 10,000 ppm. The NOEL in rats is 100 ppm based on the cystic changes in the kidney at 1000 ppm. A core classification cannot be designated for this study because this is a second-hand summary of the data and does not present all aspects of the study.

In a chronic rat study reported by Thomas et al. (1957), six female rats/dose were fed diets containing DPA at 0, 250, 1000, 5000, 10,000, and 15,000 ppm for 226 days. Body weights were reduced in the 5000-, 10,000-, and 15,000-ppm groups, but food consumption was unchanged. At termination, necropsy revealed enlarged kidneys with poorly defined nodularity and spongy cut surfaces at 5000 ppm and above. Microscopic examination of liver, kidney, spleen, adrenal, and heart of the animals fed 5000 ppm or more DPA revealed cystic kidneys and pigment deposition in the liver and kidney as the only microscopic abnormalities. The pigment tested positive with Prussian blue; therefore, it is assumed to be derived from the blood. The severity of cystic tubule formation increased with dose. The

NOEL for this chronic study was 1000 ppm; however, hematological parameters were not investigated. This study would be classified as core supplemental by present standards.

In another chronic study, Thomas et al. (1967) fed 20 rats/sex/dose 0, 10, 100, 1000, 5000, or 10,000 ppm DPA in a semisynthetic diet for 2 years. Body weight was significantly reduced ($p < 0.01$) in male rats at 5000 and 10,000 ppm and in female rats at 1000, 5000, and 10,000 ppm. Food consumption was significantly reduced ($p < 0.01$) in both sexes at 5000 and 10,000 ppm. The only other adverse effects reported were cystic dilation of the renal tubules with interstitial proteinaceous fluid, often with blood-derived, iron-positive pigment. The lesions were present at doses of 1000 ppm and above. The tissues of animals fed DPA at 100 ppm could not be distinguished histologically from those of the control. The NOEL for this chronic rat study was 100 ppm based on cystic dilation of the renal tubules; the LOEL was 1000 ppm. In a separate chronic study, the authors indicated that recovery to normal hemoglobin values (hemoglobin had been elevated prior to recovery for this study) and normal body weights occurred. In an associated reproduction study, the average litter size was decreased with increasing concentration of the DPA. These studies would be classified as core supplemental by present standards.

C. Dogs: Booth (1963) summarized the unpublished work of F. De Eds in which groups of two dogs per sex were fed diets containing DPA at 0, 100, 1000, or 10,000 ppm for 2 years. Growth was decreased at 10,000 ppm in both sexes. Hemoglobin and red cell counts were reduced and red cell fragility was increased at 10,000 ppm. At termination, no histopathologic alterations were described but liver weights were increased in the 10,000-ppm group due to increased fat content. There was some hepatic intracellular bilirubin and the spleen, kidney, and bone marrow showed hemosiderosis correlating with red cell destruction.

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This study would be considered invalid by today's standards because of the small number of animals used and because the dogs suffered from distemper during part of the study.

III. RENAL EFFECTS

Crocker et al. (1971) reviewed cystic kidney disease and malformations of the renal tubules in man and animals. They also reviewed evidence that DPA (technical grade) could produce abnormalities in the kidneys of rats that are similar to those seen in humans. In studies where DPA was fed to pregnant rats for the last week of gestation (2 mL alcohol solution of 1 percent DPA per day) the kidneys of the dams were normal while the tubules of the progeny were dilated. On microdissection of the newborn rats, cystic lesions were found in the proximal area of the nephron rather than in the collecting system, which is the site of DPA-induced cysts in adult rats. Other reports indicated that these effects were due to an unidentified impurity in the technical DPA.

To further elucidate the effects cited above, Crocker et al. (1972) obtained samples of DPA from J. T. Baker (DBA-B) and Eastman Organic Chemicals (DBA-K). Both samples had three trace impurities (designated E, F, and G) revealed by thin-layer chromatography (TLC); the largest amounts were in the DBA-K sample, which had aged 2 years. DPA was fed to pregnant rats for the last 7 days of gestation at 1.5 or 2.5 percent. Kidneys of mothers fed DPA from either source were comparable to the controls. In the progeny the most severe lesions resulted from the 2.5 percent DPA-K (aged sample) or with contaminant E from the TLC separation (50 µg/day). When purified DPA from DPA-K was fed, no cystic changes resulted. No relationship was drawn between contaminant E level and the effect of aged DPA-K nor were the impurities, probably antioxidation products, identified.

The effects of DPA on the structure and function of the rat kidney, as well as susceptibility to pyelonephritis, was reported by Kime et al. (1962). A diet containing 2.5 percent DPA was fed to weanling and adult Sprague-Dawley rats for up to 12 months. At necropsy, tissues were weighed and samples of the right kidney, spleen, liver, and heart were taken for examination by light and electron microscopy. Renal function was evaluated by creatinine clearance, total creatinine chromogen, and urinary osmolality. Urinary sediment, protein, and glucose were also determined.

Animals fed 2.5 percent DPA showed tubular dilation and cyst formation regardless of their age at the start of the study. Incidence and severity of both effects were increased with the duration of DPA administration. Both effects were prominent at the critical medullary junction. Electron microscopic examination revealed proliferation of the brush borders of the proximal tubular epithelial cells and cytoplasmic swelling of the convoluted tubules. No histopathologic findings were found in the other organs examined. Renal function studies indicated a decreased clearance that was proportional to the severity of cystic involvement, a decrease in urinary osmolality, and an increase in blood urea nitrogen. Finally, the polycystic kidneys were found to have a markedly increased susceptibility to pyelonephritis.

In a study by Safouh et al. (1970), DPA was administered in the diet to Sprague-Dawley rats for 1 to 12 months using the following dose groups: 40 rats received 15,000 ppm, 60 rats received 25,000 ppm, 10 rats received 15,000 ppm DPA supplemented with 2500 ppm sulfur-containing amino acids (equal parts of D-L-methionine and L-cysteine), 10 rats received 25,000 ppm DPA supplemented with 5000 ppm sulfur-containing amino acids, and 20 rats served as controls. Rats were killed every 2 months for histopathologic examination of the kidneys and microdissection of the nephrons.

At both the 15,000- and 25,000-ppm doses, cystic tubules occurred as early as 2 months and the effect was clearly dose related. The lesions were most prominent in the medullary region of the kidney and consisted of focal cystic changes in the collecting tubules. In the rats fed sulfur-containing amino acids in combination with DPA, protection was anticipated. This did not occur and, in fact, the rats fed 25,000 ppm DPA and 5000 ppm sulfur-containing amino acids developed the most severe cystic disease. The urinary concentrating capacity was decreased significantly in rats fed 25,000 ppm DPA at 5 weeks and at 7 months. The severity of the effects increased with time.

IV. BLOOD ALTERATIONS

A. Methemoglobin Formation: Since DPA is structurally related to other cyclic amines that are capable of inducing the formation of methemoglobin, it was important that methemoglobin formation be examined in a comprehensive evaluation of DPA toxicity. Calabrese (1983) reviewed the mechanism and interspecie susceptibilities of methemoglobin formation using acetanilide and acetophenetidine as methemoglobin-inducing agents. The interspecies' order of susceptibility, from most susceptible to least susceptible, appeared to be cat, man, dog, rat, rabbit, and monkey, although there was indication that this susceptibility ranking was subject to considerable variation. Despite the structural relationship of DPA to other cyclic amines found capable of forming methemoglobin, no methemoglobin was formed in the chronic studies reported above.

B. Heinz Body Formation: The characterization, detection, and possible physiological effect of Heinz body formation were reviewed by Jain (1969; 1973) and Jain and Keeton (1975). Unfixed smears of feline blood stained with 0.5 percent methylene blue were satisfactory for examining Heinz bodies, but the stains

were temporary and could not be stored. Methylene blue and potassium oxalate also stained the Heinz bodies and was permanent, but destroyed the leucocytes. Jain described a new method using the copper-sulphate/Wright-Leishman technique. Heinz body counts using this method compared favorably with the methylene blue method. The preparations were permanent, the leucocytes were not adversely affected, and the distinctive features of the leucocyte were preserved for detailed cytological examination. Although bright-field microscopy was adequate for satisfactory viewing of these slides, phase contrast microscopy was found to provide a superior method of visualization. Smears of intact and partially lysed blood elements were also studied using supravital stains. Intact samples were found to have large Heinz bodies with unusual features.

Using scanning electron microscopy, Heinz bodies were recognized as large, rounded, elevated areas within the cell membrane, but projecting outward to varying degrees. Sometimes the projection was pinched off by the erythrocyte membrane and sometimes appeared on a thin stalk. Hemolysed smears showed small to large irregular masses of dense material in many erythrocyte ghosts. Sometimes the granules coalesced to form a single large Heinz body.

Jain studied the formation of Heinz bodies in cats, their rate of disappearance, the role of the spleen in their removal from circulation, and the effect of transfusion on splenectomized or intact cats with regard to Heinz body removal. The role of steroid-induced stress was also studied with regard to Heinz body formation. In cats treated with aniline, disappearance of Heinz bodies was rapid, with a 50 percent reduction within 5 to 14 days of dosing. Less than 5 percent of the erythrocytes contained Heinz bodies by 30 days. Splenectomy had little effect on reducing Heinz body count. The number of transfused intact

or splenectomized cats used were too few to arrive at a conclusion. The administration of steroids had no effect on Heinz body formation.

Jain suggested that Heinz body formation reduces the plasticity of the erythrocyte, resulting in difficult capillary passage. It was proposed that Heinz body formation could result in anemia, but this does not generally occur in cats unless greater than 50 percent of the erythrocytes are affected.

V. MISCELLANEOUS STUDIES

Volodchenko (1975) reviewed the acute toxicity of DPA and two related compounds, hydroxydiphenylamine (HODPA) and dinitroparahydroxydiphenylamine (DNHODPA). In humans, HODPA was reported to produce headache, rapid fatigue, nausea, and pain in the epigastric region. Hematologic studies indicated that exposure to these compounds resulted in reduced hemoglobin, reticulocytosis, and methemoglobinemia. In acute oral rat studies, the LD_{50} values for DPA and HODPA were equal, 3.2 and 3.3 g/kg, respectively. DNHODPA had a similar LD_{50} of 2.61 g/kg following intra-abdominal injection, indicating that DNHODPA was probably less toxic. At high doses (50 percent of the LD_{50}) DPA and HODPA produced methemoglobinemia and Heinz body formation. DNHODPA did not produce methemoglobinemia. The author indicated that in the oral studies, DPA and HODPA produced unspecified changes in the nervous system, which was suggested to result from hypoxia due to the changes in hemoglobin. However, this report was poorly written, leaving out important methodology and numerical data. Therefore, these findings are considered to provide only supplementary information.

In a publication by Gosselin et al. (1976), acute human poisoning following exposure to aniline or nitrobenzene was reviewed. Both compounds are structurally related to DPA but no data on DPA were

reported. Both aniline and nitrobenzene produced symptoms of weakness, headache, slate blue cyanosis of the skin and mucous membranes, shock, and coma. In addition, vomiting was seen with nitrobenzene. Cyanosis produced by both aniline and nitrobenzene was due to methemoglobin formation and, under severe conditions, Heinz body formation. The lethal dose for aniline was 6 g but larger quantities have been tolerated. The corresponding NOEL was 0.07 to 0.21 mg/kg.

VI. SUMMARY

The chronic toxicity of DPA was studied in mice. Effects were limited to increased liver weights in both sexes and increased spleen weights in the females at 250 ppm, without corresponding histopathological alterations, and reversible Heinz body formation in both sexes at 50 ppm and above. There were no indications of an oncogenic effect.

Chronic exposure of rats to DPA produced body weight suppression in females at 1000 ppm and in both sexes at 5000 ppm and higher levels. In one study, food consumption was also suppressed at 5000 and 10,000 ppm, but in other studies food consumption was not affected by chronic exposure of up to 10,000 ppm DPA. One investigator found reduced hemoglobin and red cell counts at 1000 ppm while no effects on formed elements or hemoglobin were observed in two other studies. Reversible increases in hemoglobin were reported in one study and no effects in another. Heinz bodies were found in the erythrocytes of rats fed DPA at 100, 250, or 1000 ppm, while in several other studies, no Heinz bodies were reported at comparable doses. In all chronic studies cystic kidneys were observed. Some authors found associated proteinaceous material or blood-derived, iron-positive pigment in the tubules. The cystic tubules were formed at 1000 ppm and the severity increased with dose. There were no indications of an oncogenic effect.

In dogs fed diets containing DPA at 1000 ppm for 2 years, growth was decreased, hemoglobin and red cell counts were reduced, red cell fragility was increased, liver weights were increased, ether-extractable lipids in the liver were increased, and the spleen, kidney, and bone marrow all showed hemosiderosis. Cystic kidneys were not seen at any dose, and the above findings were not observed at 100 ppm.

Generally, the rodent studies indicate that a NOEL for chronic dietary administration of DPA based on adverse effects (cystic tubule formation) is 100 ppm. However, the true NOEL, considering Heinz body formation, is 10 ppm. These parameters cannot be determined in dogs because the only dog study reviewed was invalid based on the small number of dogs used at each dose. Despite the weakness of the dog study, where it is apparent that it is the least sensitive species, it appears that a provisional acceptable daily intake could be safely established using a large safety factor, for example 1000 times.

Because rats and mice developed cystic kidneys when exposed to DPA, extensive study has been focused on this finding. Crocker et al. found the collecting tubules to be cystic when 2.5 percent DPA was fed to adult rats. When administered to pregnant females at lower levels (2 mL of 1 percent solution/day), cystic lesions in the newborn progeny were found in the proximal area of the nephron. The cystic kidney effect was shown to be caused by an unidentified impurity in DPA rather than DPA itself. Renal function studies indicated decreased clearance proportional to cystic involvement together with decrease in urinary osmolality and an increase in blood urea nitrogen at a dose of 2500 ppm. Studies on rats also demonstrated that DPA increased rat susceptibility to pyelonephritis. Safouh showed that development of cystic tubules is clearly dose related, with lesions seen at doses of 15,000 and 25,000 ppm occurring as early as 2 months after initiation of dosing. Rats fed up to 5000 ppm sulfur-containing amino acids in combination with DPA did not show the anticipated protection and in fact exhibited the most severe lesions.

Since some of the studies also showed changes in blood hemoglobin content or the formation of Heinz bodies, special attention was paid to these findings. A staining method using the copper-sulphate/ Wright-Leishman stain was described by Jain as a preferred method for visualizing Heinz bodies. The effects of transfusion, splenectomy, and steroid stress were evaluated with regard to Heinz body formation or removal. Following Heinz body formation in aniline-dosed rats, disappearance of affected cells is rapid, a 50 percent reduction occurring within 5 to 14 days of dosing with less than 5 percent of the erythrocytes containing Heinz bodies remaining after 30 days. Splenectomy had little effect on the removal of Heinz bodies. Steroids had no effect on Heinz body formation. Larger numbers of animals were needed to reach a definitive conclusion on the effects of transfusion or splenectomy. Although bright-field microscopy was adequate for viewing feline blood for Heinz bodies, provided the staining was adequate, phase contrast microscopy was found to provide a superior method for visualization. Scanning electron microscopy provided some advantage with regard to defining morphology but no advantage existed for quantification.

From reports of human exposure to structurally related materials such as aniline or nitrobenzene, the symptoms of DPA intoxication can be anticipated to be weakness, headache, cyanosis, shock, and coma. Supportive therapy should take into consideration the reduced oxygen-carrying capacity of the blood, decreased urinary clearance, changes in urinary osmolality, susceptibility to pyelonephritis, and possible consequences of cystic tubule alteration.

BIBLIOGRAPHY

Abraham, R. and Goldberg, L. (1969) Determination of the toxicity of diphenylamine in mice. Report from Albany Medical College, Albany, New York (unpublished).

Abraham, R., Ford, W., Rockwood, W., Goldberg, L., and McChesney, E. W. (1976) Long-term toxicity of diphenylamine in mice. Report from Albany Medical College, Albany, New York (unpublished).

Booth, A. N. (1963) Summary of toxicological data--chronic toxicity on diphenylamine. Food Cosmet. Toxicol. 1:331-333.

Calabrese, E. J. (1983) Methemoglobin formation, in: Principles of Animal Extrapolation, 1st Ed. John Wiley Publishing Co., New York, pp. 307-320.

Crocker, J. F. S., Brown, D. M., and Vernier, R. L. (1971) Developmental defects of the kidney--a review of renal development and experimental studies of maldevelopment. Pediatr. Clin. North Am. 18:355-376.

Crocker, J. F. S., Brown, D. M., Borch, R. F., and Vernier, R. L. (1972) Renal cystic disease induced in newborn rats by diphenylamine derivatives. Ame. J. Pathol. 66:343-349.

Ford, W., Abraham, R., Rockwood, W., and Goldberg, L. (1972) Observations consequent upon a long-term study of diphenylamine in mice. Toxicol. Appl. Pharmacol. 22:302 (Abstract No. 73).

Gosselin, R. E., Hodge, H. C., Smith, R. P., and Gleason, M. N. (1976) Acute poisoning by aniline, in: Clinical Toxicology of Commercial Products, 4th Ed. The Williams and Wilkins Co., Baltimore, MD, pp. 29-35.

Jain, N. C. (1969) A staining technique to demonstrate erythrocyte refractile bodies in cat blood. Br. Vet. J. 125:437-441.

Jain, N. C. (1973) Demonstration of Heinz bodies in erythrocytes of the cat. Bull. Am. Soc. Vet. Clin. Pathol. 2:13-23.

Jain, N. C. and Keeton, K. S. (1975) Scanning electron microscopy of Heinz bodies in feline erythrocytes. Am. J. Vet. Res. 36:1691-1695.

Kime, S. W., Jr., McNamara, J. J., Luse, S., Farmer, S., Silbert, C., and Bricker, N. S. (1962) Experimental polycystic renal disease in rats: electron microscopy, function, and susceptibility to pyelonephritis. J. Lab. Clin. Med. 6:64-78.

Safouh, M., Crocker, J. F. S., and Vernier, R. L. (1970) Experimental cystic disease of kidney-sequential, functional, and morphological studies. Lab. Invest. 23:392-400.

Thomas, J. O., Cox, A. J., Jr., and DeEds, F. (1957) Kidney cysts produced by diphenylamine. Stanford Med. Bull. 15:90-93.

Thomas, J. O., Ribelin, W E., Wilson, R. H., Keppler, D. C., and DeEds, F. (1967) Chronic toxicity of diphenylamine in albino rats. Toxicol. Appl. Pharmacol. 10:362-374.

Volodchenko, V. A. (1975) Toxicological characteristics of diphenylamine and certain of its derivatives depending on the chemical structure. (Russian) Gig. Sanit. 10:114-116. [English translation by Kaspin, A. (1984)]