# Oral Toxicity of Alkyl Phenylenediamines and Their Activity as Vitamin E Substitutes in Rat Nutrition

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A study was undertaken to determine the chronic toxicity and vitamin E–sparing activity for the rat of two alkyl phenylenediamines with reference to their possible use as antioxidants in animal feeds. These compounds are *N,N'*-bis(1-ethyl-3-methylpentyl)-*p*-phenylenediamine (DOPD-3) and *N,N'*-bis(1-methylheptyl)-*p*-phenylenediamine (DOPD-2). The results demonstrated that both compounds are tolerated at concentrations of at least 0.025%, and possibly 0.1%, in the diet of the pregnant rat without impairment of reproduction. By comparison, *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), which was formerly employed as a feed additive, is toxic at 0.005%. DOPD-3 and DOPD-2 exhibited a somewhat lower activity than DPPD in the prevention and cure of sterility in female rats fed a vitamin E–deficient diet. The presence of 0.1% of the alkyl compounds caused a temporary depression of food intake and growth, probably as a result of decreased palatability, and 0.4% induced a more severe inhibition. 2,6-Di-*tert*-butyl-4-methylphenol (BHT) at comparable levels was found to be inactive as a substitute for vitamin E in rat nutrition.

THE WIDESPREAD use of inedible fats in commercial mixed feeds has created a need for economical antioxidants capable of preventing oxidative rancidity during storage. Peroxidation of dietary unsaturated fatty acids is known to be associated with the occurrence of vitamin E deficiency, and certain nontocopherol antioxidants, notably methylene blue and *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), have been shown to be capable of preventing a deficiency of this vitamin in several animal species.

The discovery that feeding DPPD results in the presence of residues in poultry products (*6*) and that this antioxidant possesses a relatively high order of chronic toxicity for the pregnant rat, as manifested in a prolonged gestation period, hemorrhages, and stillbirths (*1, 2, 5*) were factors in its withdrawal from animal feeds. It has been replaced chiefly by 2,6-di-*tert*-butyl-4-methylphenol (BHT) which is well tolerated by the pregnant rat (*1*).

Previous experiments in this laboratory relative to the replaceability of dietary vitamin E with synthetic antioxidants have shown that 0.005% DPPD is capable of regenerating female rats in which sterility had been induced by feeding a tocopherol-deficient diet (*3*). At concentrations of 0.025 and 0.1%, BHT was found to be totally inactive. In a quest for compounds having a low chronic toxicity and high vitamin E replacement value for the rat, two alkyl phenylenediamines have been evaluated. These are *N,N'*-bis(1-ethyl-3-methylpentyl) - *p* - phenylenediamine (DOPD-3) and *N,N'*-bis(1-methylheptyl)-*p*-phenylenediamine (DOPD-2). Both are dark red liquids with a specific gravity of 0.91 at 60° F.

## Methods

Both compounds were evaluated according to the following criteria: chronic toxicity for the pregnant rat when administered in a stock diet beginning 1 week before mating (short-term toxicity test); chronic toxicity for the female rat when administered in a stock diet from weaning age through the growing period and one reproductive cycle (long-term toxicity test); prevention of sterility in female rats fed a vitamin E–deficient diet from weaning age; and regeneration of sterile female rats fed a vitamin E–deficient diet.

**Short-Term Toxicity Test.** In this test seven groups of 15 to 18, 12-weeks-old female rats of the Sprague-Dawley strain were fed a stock diet (ground Purina Laboratory Chow) containing, respectively, the following supplements: none; 0.025 and 0.1% DOPD-3; 0.025 and 0.1% DOPD-2; 0.025 and 0.1% DPPD. All animals received a vitamin A supplement at the rate of 1000 I.U. per week. The treatments were instituted 1 week prior to mating and continued until after parturition.

**Long-Term Toxicity Test.** This test was conducted using nine groups of 15 to 16, 3-weeks-old weanling females. One group was maintained on the basal stock diet and the others were given the same diet containing the following concentrations of DOPD-3 or DOPD-2: 0.005, 0.025, 0.1, and 0.4%. All animals received 1000 I.U. of supplementary vitamin A weekly. The dietary treatments were imposed continuously until after parturition.

**Prevention of Vitamin E Deficiency.** For estimating the efficacy of these antioxidants in preventing vitamin E deficiency in the rat a purified tocopherol-deficient diet was used. This diet consisted of 64.6% Cerelose, 20% "vitamin-free" casein, 10% molecular-distilled lard, 4% salts, plus vitamins except vitamin E. The details of the diet have been given elsewhere (*2*). Eight groups of 25 weanling female rats were employed and the effect on reproductivity of adding the following supplements to the basal diet was determined: none, vitamin E (30 mg. of α-tocopheryl acetate placed on the diet weekly), 0.005 and 0.025% DOPD-3, 0.005 and 0.025% DOPD-2, 0.025 and 0.1% BHT. These treatments were imposed during a growing period of 7 weeks and one reproductive cycle.

**Remission of Vitamin E Deficiency.** In the regeneration test five groups of 25 weanling females were depleted of vitamin E by feeding the basal purified diet described above. They were mated at about 200 grams body weight and exhibited 100% reproductive failure. Subsequently, they were assigned to a tocopherol-deficient diet containing the supplements indicated, and after a 10-day adjustment period were remated to normal males. In order to reduce the tocopherol content of the basal diet further during the regeneration cycle, the distilled lard was replaced by a methyl linoleate supplement containing no detectable vitamin E (*3*). The supplements administered to five groups during the second cycle were, respectively, as follows: none, vitamin E (30 mg. of α-tocopheryl acetate placed on the diet weekly), 0.005 and 0.025% DOPD-3, 0.025% DOPD-2.

**Histopathology.** Five rats from each group in the long-term toxicity test were sacrificed at the end of the reproductive

**Table I. Results of Short-Term Toxicity Test of DOPD-3, DOPD-2, and DPPD for Pregnant Rat**

| Group No. | Treatment | No. of Females | No. of Litters | No. of Pups Born Total | No. of Pups Born Per litter | % Born Alive | Mortality of Females | Gestation Period, Days[a] |
|---|---|---|---|---|---|---|---|---|
| I | None (stock diet) | 15 | 8 | 94 | 11.7 | 96 | 0 | 23.5 |
| II | 0.025% DOPD-3 | 15 | 15 | 157 | 10.5 | 95 | 0 | 23.2 |
| III | 0.1% DOPD-3 | 15 | 12 | 125 | 10.4 | 97 | 0 | 22.7 |
| IV | 0.025% DOPD-2 | 15 | 10 | 100 | 10.0 | 98 | 0 | 22.3 |
| V | 0.1% DOPD-2 | 18 | 15 | 150 | 10.0 | 87 | 0 | 23.5 |
| VI | 0.025% DPPD | 18 | 14 | 146 | 10.4 | 71 | 0 | 24.1 |
| VII | 0.1% DPPD | 16 | 9 | 61 | 6.8 | 15 | 6 | 24.9 |

[a] Calculated from third day of mating period.

**Table II. Results of Long-Term Toxicity Test of DOPD-3 and DOPD-2 for Reproduction of Female Rat**

| Group No. | Treatment | No. of Females | No. of Litters | No. of Pups Born Total | No. of Pups Born Per litter | % Born Alive | Mortality of Females | Gestation Period, Days[a] |
|---|---|---|---|---|---|---|---|---|
| I | None (stock diet) | 16 | 9 | 86 | 9.6 | 95 | 0 | 22.0 |
| II | 0.005% DOPD-3 | 16 | 7 | 64 | 9.1 | 94 | 0 | 22.7 |
| III | 0.025% DOPD-3 | 15 | 8 | 70 | 8.8 | 93 | 0 | 22.5 |
| IV | 0.1% DOPD-3 | 15 | 7 | 55 | 7.9 | 100 | 0 | 21.9 |
| V | 0.4% DOPD-3 | 15 | Not mated because of depressed growth | | | | | |
| VI | 0.005% DOPD-2 | 15 | 7 | 76 | 10.9 | 100 | 0 | 20.4 |
| VII | 0.025% DOPD-2 | 16 | 15 | 143 | 9.5 | 99 | 0 | 22.3 |
| VIII | 0.1% DOPD-2 | 15 | 7 | 54 | 7.7 | 80 | 0 | 21.7 |
| IX | 0.4% DOPD-2 | 15 | Not mated because of depressed growth | | | | | |

[a] Calculated from third day of mating period.

cycle and samples of the following tissues were taken: skeletal muscle, heart muscle, intestine, spleen, liver, ovary, and pancreas. The tissues were fixed in Bouin's solution, sectioned after mounting in paraffin, and examined histologically. This phase of the experiment was carried out by E. W. Millhouse, Department of Zoology.

### *Results and Discussion*

**Short-Term Toxicity.** A summary of the results of this test is given in Table I. No evidence was obtained to suggest that either DOPD-3 or DOPD-2 at concentrations of 0.025 or 0.1% in the diet exerted any deleterious effect upon reproduction which was reflected in litter size, number of stillbirths, mortality of females, or length of gestation period. The toxic effect of DPPD on reproduction is suggested by the increased proportion of stillbirths and the extended gestation period at the 0.025% level, and is clearly evident at the 0.1% level. At the latter concentration, six of the dams died and no pups survived. Only 12% of the live pups survived in Group VI (0.025% DPPD). That fertility was improved by the alkyl compounds was not substantiated in later tests.

**Long-Term Toxicity.** The results of the growth phase of this experiment are summarized in Figures 1 and 2. It is evident that both antioxidants caused a temporary growth depression at a dietary concentration of 0.1%, and a marked depression at 0.4%. No difference between compounds with respect to their effect on growth is discernible. At the 0.1% level the inhibitory effect appears to have been limited to the initial few days of feeding, indicating that palatability rather than toxicity may have been the primary factor influencing rate of gain. Similarly, the early weight loss at the 0.4% concentration was followed by a period of recovery and weight gain. The possibility remains that the early depression was due partially to a greater intake of the antioxidants per unit body weight in the case of the younger animals; however, the immediate aversion to the higher concentrations indicates that reduced palatability of these diets was a major factor. The incorporation of 0.1% of either DOPD-3 or DOPD-2 imparted a noticeable odor to the diet, and at 0.4% the odor was pronounced. In order to evaluate further the role of palatability in affecting the growth rate, another feeding test was conducted in which 10 trios of weanling rats were fed the basal diet alone and with 0.4% DOPD-3 and 0.4% DOPD-2 on an equal food intake basis. The results are shown in Figure 3. Food intake was severely limited in the antioxidant-fed groups and food utilization was impaired for approximately 2 weeks, following which the performance of all groups was comparable.

The reproductive performance of the various groups is summarized in Table II. The animals which received 0.4% of either antioxidant did not attain breeding weight during the course of the experiment. The only evidence of a deleterious effect of feeding these compounds at the other levels employed lies in the size of the litters produced by the females which received the 0.1% concentration. According to Student's *t* test, litter size was significantly smaller

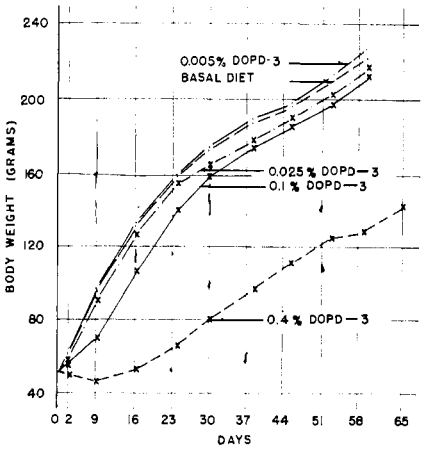


Figure 1. Growth of rats fed stock diet containing various concentrations of DOPD-3

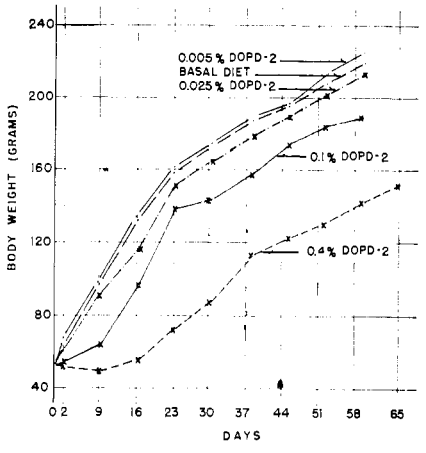


Figure 2. Growth of rats fed stock diet containing various concentrations of DOPD-2

in the 0.1% DOPD-3 and 0.1% DOPD-2 groups than in the control group ($P$ = 0.05 and 0.02, respectively). No indication of an effect upon the length of gestation was observed. With respect to viability of the offspring, 80 to 93% survival was recorded in all groups except Group VIII (0.1% DOPD-2) in which 74% survived. In the 0.1% DOPD-3 group, 93% survived.

The combined results of the short-term and long-term toxicity tests indicate that DOPD-3 and DOPD-2 induce a temporary depression in weight gain in young rats when incorporated into a stock diet at a level of 0.1%. At a level of 0.4% a marked and persistent depression is observed, while at concentrations of 0.005 and 0.025% no significant effect on weight gain is produced. The growth retardation is associated with a decrease in food intake, particularly during the first few days of feeding, and this fact, together with the shape of the growth curve, suggests that a primary cause of growth inhibition is reduced palatability of the diet. To what extent palatability might be a factor in the suitability of these compounds for use as feed additives will depend upon

the outcome of feeding tests with farm animals. The experiments with pregnant rats show clearly that DOPD-3 and DOPD-2 are tolerated at greater dietary concentrations than is DPPD. A level of 0.1% was necessary before any evidence of impairment of reproduction was obtained from feeding the alkyl compounds, and at this level the evidence was inclusive. In contrast, as little as 0.005% DPPD has been found to impair reproduction in the rat (*2*).

**Histopathology.** No evidence of histopathology was discernible in any of the tissue samples taken from animals maintained on the levels of DOPD-3 and DOPD-2 used in the long-term toxicity test (0.005% to 0.4%).

**Prevention of Vitamin E Deficiency.** The effectiveness of DOPD-3, DOPD-2, and BHT in preventing sterility in female rats fed a tocopherol-deficient diet is indicated by the results given in Table III. They show that both alkyl compounds exerted a protective effect against sterility and that 0.025% was more effective than 0.005%. BHT exhibited no protective activity in this test, in keeping with its ineffectiveness in regenerating vitamin E–deficient females (*3*). Matterson and associates (*4*) have reported that BHT is also definitely inferior to DPPD and certain other antioxidants in preventing encephalomalacia in the chick.

At a concentration of 0.005 or 0.025%, DOPD-3 and DOPD-2 appear to be less active in preventing sterility in the rat than 0.005% DPPD (*3*). Unlike the alkyl compounds, DPPD is more effective at 0.005% than at 0.025%, because of its greater toxicity. The protective effect of DOPD-3 and DOPD-2 when used in this diet at 0.025% is roughly comparable to the effect obtained with DPPD at 0.005% in terms of litter efficiency, but is markedly superior in terms of the incidence of stillbirths and postnatal mortality (*3*).

**Remission of Vitamin E Deficiency.** The effect of administering DOPD-3 and DOPD-2 to vitamin E–deficient females is indicated in Table IV. The results show that these antioxidants have a positive, but low-order regenerative effect at the concentrations employed. In similar experiments, 0.005% DPPD was found to have a regenerative efficiency comparable to that of α-tocopherol except for the proportion of stillbirths, whereas BHT was inactive at concentrations of 0.025 and 0.1% (*3*). The data from the present curative experiment confirm those of the preventive test in indicating that DOPD-3 and DOPD-2 have a somewhat lower vitamin E–sparing activity, as well as a lower chronic toxicity, than DPPD for reproduction in the rat.

The foregoing experiments demonstrate that the two alkyl derivatives of phenylenediamine examined do not, within wide limits, cause the appearance of toxicity symptoms in the rat, when the most sensitive criterion found for the diphenyl derivative is applied—i.e., prolongation of the gestation period with consequent reproductive decline. These compounds possess vitamin E–replacement activity with respect to rat fertility, but complete substitution was not obtained at the concentrations employed and their activity in this respect appears to be lower than that of DPPD. Growth depression, which seems to be at least partially attributable to decreased palatability, is obtained at substantially lower dietary concentrations than is the case with DPPD (*2*). The eventual usefulness of these compounds in feedstuffs must be determined by further study of their efficacy in the prevention of other vitamin E deficiency diseases, such as muscular dystrophy; their acceptability in the rations of domestic animals; their effectiveness in the preservation of oxidizable components of mixed feeds; and the analysis of edible animal food products for significant residues.

**Table III. Effectiveness of DOPD-3, DOPD-2, and BHT in Preventing Sterility in Female Rats Fed a Vitamin E–Deficient Diet**

| Group No. | Treatment | No. of Females | No. of Litters | No. of Pups Born: Total | No. of Pups Born: Per litter | % Born Alive |
|---|---|---|---|---|---|---|
| I | None | 25 | 0 | 0 | ... | .. |
| II | Vitamin E | 26 | 16 | 142 | 8.9 | 95 |
| III | 0.005% DOPD-3 | 25 | 2 | 8 | 4.0 | 75 |
| IV | 0.025% DOPD-3 | 25 | 10 | 94 | 9.4 | 97 |
| V | 0.005% DOPD-2 | 26 | 6 | 23 | 3.8 | 83 |
| VI | 0.025% DOPD-2 | 25 | 10 | 73 | 7.3 | 92 |
| VII | 0.025% BHT | 24 | 0 | ... | ... | .. |
| VIII | 0.1% BHT | 25 | 1 | 1 | 1 | 0 |

**Table IV. Effectiveness of DOPD-3 and DOPD-2 in Regenerating Vitamin E–Deficient Female Rats**

| Group No. | Supplement to Basal Diet[a] | No. of Females | No. of Litters | No. of Pups Born: Total | No. of Pups Born: Per litter | % Born Alive |
|---|---|---|---|---|---|---|
| Depletion Cycle | | | | | | |
| I | None | 25 | 0 | 0 | ... | .. |
| II | None | 25 | 0 | 0 | ... | .. |
| III | None | 25 | 0 | 0 | ... | .. |
| IV | None | 25 | 0 | 0 | ... | .. |
| V | None | 25 | 0 | 0 | ... | .. |
| Regeneration Cycle | | | | | | |
| I | None | 25 | 0 | 0 | ... | .. |
| II | Vitamin E (30 mg./week) | 25 | 12 | 83 | 6.9 | 86 |
| III | 0.005% DOPD-3 | 25 | 2 | 17 | 8.5 | 88 |
| IV | 0.025% DOPD-3 | 25 | 4 | 19 | 4.7 | 84 |
| V | 0.025% DOPD-2 | 24 | 6 | 19 | 3.2 | 89 |

[a] Basal diet containing 10% distilled lard as fat source used in depletion cycle. Fat-free diet with methyl linoleate supplement used in regeneration cycle.

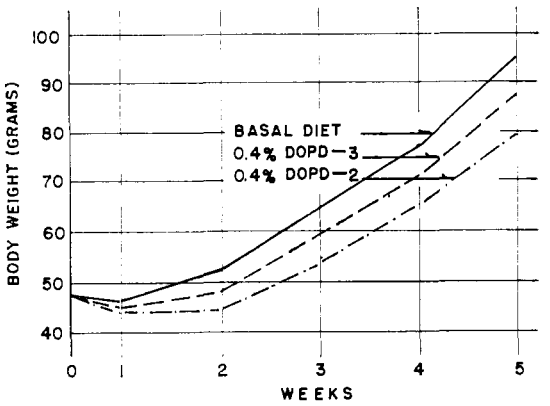


Figure 3. Effect of feeding stock diet containing 0.4% DOPD-3 or 0.4% DOPD-2 on growth of rats when food intake is equated

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