A study of the nutritional role of anti-oxidants in the diet of the rat

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The effectiveness of certain non-tocopherol anti-oxidants in preventing or delaying the appearance of signs of vitamin E deficiency in various species has been demonstrated. These deficiency syndromes include exudative diathesis (Dam, Kruse, Prange & Søndergaard, 1951) and encephalomalacia (Singsen, Bunnell, Kozeff, Matterson & Jungherr, 1955) in the chick, resorption-gestation in the rat (Dam & Granados, 1952), muscular dystrophy in the rat (Moore, Sharman & Ward, 1954), rabbit (Markees, 1954), calf (Blaxter, Brown, Wood & MacDonald, 1953) and lamb (Draper & Johnson, 1956) and infertility in turkeys (Jensen, Carver & McGinnis, 1956). The anti-oxidants that have been investigated most extensively are methylene blue (MB) and NN^1-diphenyl-p-phenylenediamine (DPPD).

Whereas it seems clear that the need for tocopherols in the diet is modified by the administration of certain synthetic anti-oxidants, the question of the nutritional dispensability of vitamin E has not been resolved by the experiments cited. The preventive action of non-tocopherol compounds on vitamin E deficiency might accrue either from a conserving effect on the small quantities of tocopherols present in the diet, gut and tissues or from replacement of tocopherols in their physiological role. In support of a specific metabolic need for tocopherols, Scott, Hill, Norris, Dobson & Nelson (1955) have found that DPPD fails to prevent exudative diathesis in chicks fed on diets essentially devoid of vitamin E, and they imply that its protective effect is due to the conservation of small amounts of tocopherols present in some experimental diets or in the body tissues. However, this syndrome is evidently of complex aetiology, and Stokstad (1957) has found that it is responsive to selenium. Nason & Lehman (1956) reported that D-α-tocopherol specifically enhances the reduction of cytochrome C in vitro and that several other anti-oxidants, including DPPD, do not act in this way. Shull, Alfin-Slater, Deuel & Ershoff (1957) have observed that the protective effect of DPPD and other anti-oxidants against muscular dystrophy in the guinea-pig eventually disappears, and they suggest that these compounds act by conserving tissue tocopherols. The work of Christensen, Dam & Gortner (1956) indicates that the prevention of sterility in rats by MB is dependent upon the presence of small quantities of vitamin E in the diet.

Our study was designed to evaluate further the efficacy of certain anti-oxidants, chiefly DPPD, in preventing and curing resorption-gestation in the female rat. In addition to its possible prophylactic effect, which might accrue from a protective

action on tocopherols present in the tissues, the curative value of DPPD was tested on females depleted of vitamin E by being maintained on diets low in or devoid of tocopherols throughout growth and one reproductive cycle, in the course of which they exhibited reproductive failure.

EXPERIMENTAL AND RESULTS

Series 1. Six groups of ten Sprague–Dawley weanling female rats were fed on a vitamin E-low purified diet containing supplements: group 1, 0·7 mg D-α-tocopheryl acetate/week (given once weekly in triacetin on the diet); group 2, 0·7 mg DL-α-tocopheryl acetate/week (given once weekly in triacetin on the diet); group 3, 0·4 mg DPPD/week (given once weekly in triacetin on the diet); group 4, 0·1 % MB mixed with the diet; group 5, 0·1 % DPPD (reduced in the second and third reproductive cycles to 0·025 and 0·0025 %, respectively) mixed with the diet; group 6, none. The lowest level of DPPD used for group 3 was approximately the molar equivalent of the tocopherol administered to groups 1 and 2, and represented about 0·0006 % of the diet consumed.

The percentage composition of the basal diet was: dextrose (Cerelose) 65·2, Labco 'vitamin-free' casein 20, tocopherol-low (molecular distilled) lard (Distillation Products Industries, Rochester, New York) 10, salts (Spector, 1948) 4, choline chloride (25 % dry mix in starch) 0·4, vitamin premix (in Cerelose) 1. The vitamin premix contained (mg/g): thiamine hydrochloride 2·5, riboflavin 1·6, calcium pantothenate 4, nicotinic acid 10, pyridoxine hydrochloride 0·6, biotin 0·06, folic acid 0·4, menadione 0·1, vitamin B_{12} 0·002. Vitamins A and D were administered twice weekly on the diet to provide 250 and 60 i.u./day, respectively.

The rats were maintained on the experimental diets for 8 weeks after weaning and were then mated to normal males fed on a stock diet. All females were carried through at least two reproductive cycles on the treatments described. Resorption-gestation was detected by weighing the pregnant rats daily. In some instances weanling pups from these females were maintained for 8 weeks on the diet of the dam and were then also mated.

The results are summarized in Table 1, and show clearly that DPPD, as well as MB, has a marked protective effect against vitamin E deficiency in the female rat, as indicated by its ability to sustain reproduction. The reproductive performance of the females receiving the higher levels of DPPD was substantially poorer than that obtained at lower levels, and other evidence of toxicity was encountered, including a prolongation of gestation time by 2–3 days, vaginal haemorrhages, anaemia and a high incidence of stillbirths. Initial failure to promote reproduction with DPPD in this diet was evidently due to its high chronic toxicity for the pregnant, as compared with the growing, rat (Johnson, 1955). The tolerance of the rat for DPPD was examined in further experiments and the results have been published elsewhere (Draper, Goodyear, Barbee & Johnson, 1956). These studies showed that as little as 0·005 % DPPD in the diet produced adverse effects upon reproduction. Similar findings relative to the toxicity of this compound were reported by Ames, Ludwig, Swanson & Harris (1956). Whether the performance of the females receiving the equivalent of 0·0006 %

Table 1. *Reproductive performance of female rats of series 1*

Group no.	Generation no.	Cycle no.	Supplement to basal diet	No. of females	No. of implantations	No. of resorptions	No. of pups born — Total	No. of pups born — Per implantation	Percentage born alive
1	1	1	0·7 mg D-α-tocopheryl acetate/week	10	4	0	30	7·5	100
	1	2	0·7 mg D-α-tocopheryl acetate/week	10	10	1	69	6·9	99
2	1	1	0·7 mg DL-α-tocopheryl acetate/week	10	7	0	64	9·1	100
	1	2	0·7 mg DL-α-tocopheryl acetate/week	10	10	0	92	9·2	97
3	1	1	0·4 mg DPPD/week	10	5	0	35	7·0	100
	1	2		9	9	1	61	6·8	87
	2	1		5	4	0	36	9·0	100
	2	2		5	3	0	18	6·0	100
	3	1		5	2	2	0	0	
4	1	1	0·1% MB	10	9	0	64	7·1	98
	1	2		10	8	1	50	6·2	84
	2	1		5	5	1	25	5·0	84
	2	2		5	3	2	2	0·7	50
5	1	1	0·1% DPPD	10	10	0	21	2·1	15
	1	2	0·025% DPPD	8	7	0	50	7·1	50
	1	3	0·0025% DPPD	8	2	1	5	2·5	100
6	1	1	None	10	7	7	0	0	—
	1	2	None	10	10	7	15	1·5	100

DPPD was depressed by cumulative toxicity is unknown, but it is evident that at this concentration the compound maintained satisfactory reproductivity through two cycles in females that had received a vitamin E-deficient diet from weaning age. Moreover, second-generation females selected from the first litters of their dams and maintained on the same regimen from weaning retained their ability to bear young. The protective action of MB was also shown to extend through two reproductive cycles and to second-generation females.

Series 2. The efficacy of DPPD in the prevention of vitamin E deficiency was studied further on larger numbers of females and an experimental diet containing cod-liver oil instead of distilled lard. Cod-liver oil is well known to accelerate the appearance of vitamin E deficiency in various species, presumably because of its high concentration of unsaturated fatty acids. A level of 4% cod-liver oil was used, with a corresponding adjustment in the level of Cerelose; otherwise the diet was as described above. The dietary treatments were imposed continuously from the weaning age of 3 weeks throughout a 'growing' period of 8 weeks and four successive reproductive cycles. Because the detection of resorptions by body-weight changes was found to be of questionable accuracy, only the numbers of litters and pups were recorded. The results are shown in Table 2.

After 6–8 weeks on the experimental diets, animals in all groups developed a scaliness and fissuring of the tail suggestive of a deficiency of essential fatty acids. Methyl linoleate (60% pure, containing no added anti-oxidant, Nutritional Biochemicals Corporation, Cleveland, Ohio) was administered weekly by mouth at a

Table 2. *Reproductive performance of female rats of series 2*

Group no.	Cycle no.	Supplement to basal diet	No. of females	No. of litters	No. of pups born Total	No. of pups born Per litter	Percentage born alive
1	1*	None	25	0	0	0	—
2	1*	0·025% DPPD	25	4	13	3·2	15
3	1*	0·005% DPPD	25	11	78	7·1	25
4	1*	30 mg D-α-tocopheryl acetate/week	25	9	78	8·7	75
1	2	None	25	0	0	0	—
2	2	0·025% DPPD	21	10	71	7·1	7
3	2	0·005% DPPD	22	16	108	6·7	14
4	2	30 mg D-α-tocopheryl acetate/week	23	17	155	9·1	57
1	3	None	21	0	0	0	—
2	3	0·025% DPPD	14	4	14	3·5	4
3	3	0·005% DPPD	15	7	44	6·3	27
4	3	30 mg D-α-tocopheryl acetate/week	21	4	33	8·2	55
1	4	None	17	0	0	0	—
2	4	0·025% DPPD	11	6	11	1·8	0
3	4	0·005% DPPD	14	7	43	6·1	35
4	4	30 mg D-α-tocopheryl acetate/week	17	6	39	6·5	67

* All females showed dermal signs of essential fatty-acid deficiency at time of mating. Signs disappeared after weekly oral administration of methyl linoleate at a level equivalent to 0·2% of the food intake. The diet used in series 1 experiments (containing distilled lard) was used for cycles 3 and 4.

rate equivalent to 0·2 % of the diet consumed, and the lesions rapidly diminished. The poor reproductive performance of cycle 1 may therefore be presumed to have resulted from a deficiency of essential fatty acids, despite the presence in the diet of 4 % cod-liver oil. The level of methyl linoleate was increased to the equivalent of 0·4 % of the diet during cycle 2. The cod-liver oil used in the basal diet was assayed for vitamin E by the method of Tošić & Moore (1945) and was found to contain 8 mg/100 g. The oil thus contributed a small but significant quantity of vitamin E to the diet (about 3·2 μg/g), and it was therefore decided to revert to the basal diet used in series 1, which contained distilled lard as the fat source. The rats were maintained on the lard diet during cycles 3 and 4.

Although the performance of the rats receiving DPPD was distinctly inferior to that of the animals receiving vitamin E, particularly in the incidence of stillbirths, it was, nevertheless, possible to maintain reproductivity in the DPPD-fed groups through four consecutive cycles on a diet deficient in vitamin E. The record of the animals fed on 0·005 % DPPD was clearly superior to that obtained on the 0·025 % level, a further indication of the compound's toxic effect. To what extent the difference in the efficacy of vitamin E and DPPD may have been due to toxic side-effects of the latter cannot be estimated. Experiments are in progress to determine the effectiveness of other, less toxic, compounds in replacing tocopherols for reproduction in the rat. No clear explanation is available for the mediocre performance of the vitamin E-supplemented groups during the third and fourth cycles, other than the occurrence of a chronic respiratory infection, which affected some members of all groups as age advanced.

Series 3. The experiments of series 1 and 2, which demonstrated that female rats could be maintained on a diet deficient in vitamin E throughout growth and four reproductive cycles without exhausting their reproductivity, indicated either that the dietary requirement for vitamin E is markedly reduced when certain other anti-oxidants are also present or that the requirement for the vitamin in most diets represents a non-specific need for an anti-oxidant to conserve oxidizable components of the diet and tissues. Further evidence in support of the latter explanation would be available if it could be demonstrated that some specific sign of vitamin E deficiency could be cured by the administration of a non-tocopherol anti-oxidant. The experiments of series 3 were designed to determine whether female rats that had exhibited reproductive failure as a result of vitamin E deficiency could be restored to reproductivity by the addition of DPPD to the diet.

This series of experiments was conducted concurrently with those of series 2. Weanling females were fed on the basal diet, containing 4 % cod-liver oil, already described under series 2. All four groups of twenty-five females were maintained on the vitamin E-deficient regimen for 8 weeks after weaning and through four reproductive cycles. As noted for the rats of series 2, signs of essential fatty-acid deficiency appeared at the time of the first mating and methyl linoleate was curative. During the second cycle methyl linoleate was provided orally once weekly at a rate equivalent to 0·4 % of the food consumed. The results are summarized in Table 3.

In the first (depletion) cycle, during which all animals were fed on the basal diet,

only five pups were obtained from a hundred females. In the next (regeneration) cycle, during which DPPD at 0·1 or 0·01 %, or vitamin E was added, 71, 109 and 106 pups, respectively, were obtained. Although nearly all the pups from dams receiving the DPPD-supplemented diets were stillborn, performance in terms of number of pups born was superior at the lower level of DPPD and represented 68 % of the number obtained from females restored with vitamin E.

Table 3. *Reproductive performance of female rats of series 3*

Group no.	Cycle no.	Supplement to basal diet*	No. of females	No. of litters	No. of pups born Total	Per litter	Percentage born alive
1	1	None	25	0	0	0	—
2	1		25	0	0	0	—
3	1		25	1	4	4	0
4	1		25	1	1	1	100
1	2	None	25	0	0	0	—
2	2	0·1 % DPPD	24	15	71	4·7	3
3	2	0·01 % DPPD	25	17	109	6·4	6
4	2	30 mg D-α-tocopheryl acetate/week	25	19	161	8·5	71
1	3	None	21	0	0	0	—
2	3		15	2	4	2	0
3	3		17	1	1	1	0
4	3		23	8	55	6·9	75
1	4	None	17	0	0	0	—
2	4	0·1 % DPPD	13	5	17	3·4	0
3	4	0·01 % DPPD	15	8	44	5·5	32
4	4	30 mg D-α-tocopheryl acetate/week	18	13	105	8·1	67

* Basal diet containing 4 % cod-liver oil as fat source was used during cycles 1 and 2, with methyl-linoleate supplement added in cycle 2. Basal diet containing distilled lard was used for cycles 3 and 4.

The detection of small quantities of tocopherols in the cod-liver oil used in the basal diet raised the possibility that the regenerative effect of DPPD observed in cycle 2 might be attributable to its protective action on the vitamin E contributed by the oil. Accordingly, the females were carried through another depletion cycle on the basal diet described for series 1, in which the fat was provided as distilled lard. This diet was also used during the fourth cycle, in which the regenerative effect of adding DPPD and vitamin E was again determined. The carry-over of the vitamin E supplement administered to group 4 during cycle 2 was evident in cycle 3; otherwise only five pups were obtained from the fifty-three females comprising the other groups.

The regenerative effect of DPPD on the vitamin E-deficient females is again evident from the results of cycle 4. On the 0·01 % level of DPPD, performance was 68 % of that obtained with vitamin E in terms of litter size and 48 % in terms of numbers of live pups. As observed previously, the females receiving 0·1 % DPPD in the diet produced smaller litters and a higher proportion of stillbirths.

Series 4. The experiments of Christensen *et al.* (1956) suggested that the small residual amounts of tocopherols present in the distilled lard used in these experiments

may be sufficient, in the presence of other anti-oxidants, to meet the needs of the rat for reproduction. The level of total tocopherols in the lard is stated by the manufacturer to be less than 5 μg/g, a concentration that would provide approximately 5 μg/rat/day. Although this figure represents a small fraction of the estimated requirement, it was decided to eliminate the fat from the regenerative diet and to supply essential fatty acids as methyl linoleate (60 % pure). The linoleate was given twice weekly by mixing it with a small quantity of the basal diet at a rate calculated to be 1 % of the food consumed. The levels of vitamins A and D were increased fivefold to compensate for less efficient absorption from the fat-free diet. In an attempt to avoid the toxicity associated with feeding DPPD, butylated hydroxytoluene (BHT) was also used in the regeneration cycle. This anti-oxidant has been shown by Ames *et al.* (1956) to be less toxic for the rat, and it has come into common use in animal feeds.

Table 4. *Reproductive performance of female rats of series* 4

Group no.	Cycle no.	Supplement to basal diet*	No. of females	No. of litters	No. of pups born		Percentage born alive
					Total	Per litter	
1	1		25	0	0	—	—
2	1		25	0	0	—	—
3	1	None	25	0	0	—	—
4	1		26	0	0	—	—
5	1		26	0	0	—	—
1	2	None	25	0	0	—	—
2	2	30 mg D-α-tocopheryl acetate/week	25	12	83	6·9	86
3	2	0·005 % DPPD	25	11	83	7·5	63
4	2	0·025 % BHT	26	0	0	—	—
5	2	0·1 % BHT	26	0	0	—	—

* Basal diet contining 10 % distilled lard was used during cycle 1. Fat-free basal diet with methyl-linoleate supplement was used for regeneration during cycle 2.

The outcome of this series is indicated in Table 4. Most noteworthy is the failure of BHT to show any regenerative effect on the deficient females, in contrast to DPPD, which behaved like the tocopherol in terms of the number and size of litters born. The suboptimal performance of the controls was believed to be associated with the general aversion of the rats to the linoleate supplement and their failure to gain normally on the fat-free diet. Nevertheless, the ability of DPPD to rehabilitate vitamin E-deficient females fed on a tocopherol-free diet seems evident. The methyl-linoleate concentrate was assayed for tocopherols according to the procedure of Green, Marcinkiewicz & Watt (1955), and no vitamin E was detectable. The response to DPPD, therefore, was not attributable to its protective effect on tocopherols in the diet.

DISCUSSION

Although the experiments described were confounded to some extent by toxicity of DPPD, the results demonstrate that this compound has both a preventive and a curative effect on the resorption-gestation caused by vitamin E deficiency in the rat.

Markees (1954) has ascribed similar properties to MB. The final experiment indicated that this effect is not necessarily dependent upon the presence of vitamin E in the diet. Hence, unless DPPD participated directly in some physiological function essential to reproduction and normally assumed by vitamin E, it appears necessary to conclude that DPPD in some way activated in the tissues residual amounts of tocopherol that were otherwise inadequate to sustain reproduction.

The long-term feeding experiments on diets very low in tocopherols illustrated that the requirement of the rat for vitamin E is largely represented by a need for a biologically active anti-oxidant, and the apparent failure of BHT to fill this need indicated that a high order of compound specificity may be involved. Partial regeneration of vitamin E-deficient females on a fat-free diet also has been observed after treatment with other phenylenediamines. Experiments are in progress to determine whether the regenerative effect of DPPD persists after more prolonged periods of depletion.

SUMMARY

1. When supplemented with NN^1-diphenyl-p-phenylenediamine (DPPD) or methylene blue (MB), vitamin E-deficient diets containing distilled lard as a fat source sustained reproductivity in sixty female rats when given throughout the growing period and two reproductive cycles.

2. Second-generation females maintained continuously on this diet also retained their ability to produce living young.

3. Females that exhibited reproductive failure when fed on the basal regimen through the growing period and one breeding cycle could be partially restored by administration of DPPD. The regenerative effect of DPPD was also observed when all fat was removed from the diet, whereas in these circumstances butylated hydroxytoluene was inactive. The effectiveness of DPPD was modified by its toxicity, at least at the higher levels employed.

4. The significance of these results in relation to the dietary requirement for vitamin E and its nutritional status is discussed.

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Observations on feeding pigs on a low-fat diet with and without supplementary tocopherol

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The objects of this investigation were twofold, first, to investigate the possible effects of dietary tocopherol on composition of depot and liver fat in pigs, and secondly, to attempt to induce clinically recognizable signs of tocopherol deficiency by the prolonged feeding of a tocopherol-deficient diet.

Bratzler, Loosli, Krukovsky & Maynard (1950) reported that supplementing a fat-deficient, tocopherol-deficient ration with mixed tocopherols led to the formation of depot fat containing more oleic acid than was found in pigs not given the supplement. In these experiments Bratzler *et al.* (1950) fed five weanling male pigs to 75 lb. live weight on the basal ration supplemented, for three of the animals, with 2·87, 55·12 and 110·2 mg mixed tocopherols/kg body-weight/day, giving at slaughter depot fat of iodine value 64·0, 65·8 and 61·0 compared with 57·1 and 55·0 for two unsupplemented (control) pigs. In addition, the work of Hove & Seibold (1955) suggested that the amount of liver fat and the proportions of its component polyethenoid fatty acids may be affected by the absence or presence of dietary tocopherols.

The problem of muscular dystrophy in pigs has recently been reviewed by Blaxter & McGill (1955) who concluded that the aetiology of the many field cases described in

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