

## On the postulated peroxidation of unsaturated lipids in the tissues of vitamin E-deficient rats

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1. The micro-iodimetric method has been used to study some factors affecting the concentration of lipid peroxides in the adipose tissue of vitamin E-deficient rats.

2. Cod-liver oil methyl esters (CLOME) or maize oil methyl esters (MOME) with peroxide values ranging from 3 to 330  $\mu$ -equiv./g were given by mouth to vitamin E-deficient rats deprived of food before and after the dose. Lipid peroxides did not accumulate in the adipose tissue of these rats.

3. Experiments with dietary CLOME and MOME of varying peroxide values (2–230  $\mu$ -equiv./g) showed that exogenous lipid peroxide accumulates in the adipose tissue when the rats received these lipids at 10% in the diet for 4 weeks, but not if the dietary concentration was only 4% or if the diet with 10% lipid was given for 5 days only.

4. Rats were given dietary CLOME for 4 weeks. Their adipose tissue was then found to contain about 50  $\mu$ -equiv. lipid peroxide/g. They were divided into three groups. One group was given a fat-free diet and, after 10 days, the adipose tissue concentration of lipid peroxide had decreased to about 10  $\mu$ -equiv./g. The other groups were given the fat-free basal diet supplemented with vitamin E or DPPD (*N,N'*-diphenyl-*p*-phenylenediamine). Neither supplement significantly affected the rate of disappearance of the peroxides from the adipose tissue.

5. It was shown that neither  $\alpha$ -tocopherol nor DPPD reacted with the lipid peroxides of CLOME or MOME *in vitro*, at room temperature or even after 65 h at 37°.

6. It was concluded that unsaturated lipids do not become peroxidized after incorporation into the adipose tissue of vitamin E-deficient rats. Lipid peroxides taken up from the diet into the adipose tissue are not of fleeting existence, having a half-life of about 6 days. Dietary vitamin E probably prevents the accumulation of exogenous lipid peroxides in the adipose tissue by reinforcing the barrier to their absorption in the gut.

7. These studies provide further evidence that current concepts of lipid peroxidation in vitamin E-deficient animals are incorrect. In fact, vitamin E-deficient animals have low concentrations of peroxide in their adipose tissue, unless they have received large amounts of unsaturated lipid for long periods, and the role of vitamin E in controlling this concentration is not due to any effect on peroxidation *in vivo*.

Dam & Granados (1945) first demonstrated the presence of lipid peroxide in the adipose tissue of chicks and rats given vitamin E-deficient diets containing cod-liver oil. Confirmatory evidence was provided by Aaes-Jorgensen (1949) and by Christensen, Dam, Prange & Søndergaard (1958). These findings have often been cited as evidence for the biological antioxidant hypothesis of the action of vitamin E (e.g. Tappel, 1962). However, in a recent investigation (Bunyan, Murrell, Green & Diplock, 1967) we found rat liver, kidney, testis and leg muscle to contain a fairly constant concentration of lipid peroxide regardless of the degree of unsaturation of the dietary lipid and whether or not the rats were given vitamin E. Only in the adipose tissue was the peroxide concentration altered by these dietary variables. Vitamin E deficiency in chicks did not raise the peroxide content of breast muscle, liver or brain; the adipose tissue of chicks with encephalomalacia also had a low peroxide value. Aaes-Jorgensen (1949) and Christensen *et al.* (1958) also reported zero peroxide values for the adipose tissue of

animals given more than enough lard to produce vitamin E-deficiency disease. The high peroxide values often quoted seem, therefore, to be unrelated to vitamin E deficiency as such, but rather to some special effect of dietary cod-liver oil and other unsaturated lipids on the adipose tissue (cf. Emmel & LaCelle, 1961). The experiments carried out by Bunyan *et al.* (1967) left open the question of the incorporation into adipose tissue of exogenous lipid peroxide from the diet or gut. It was decided, therefore, to continue the study of the occurrence of lipid peroxidation *in vivo* with experiments in which more stringent precautions were taken to prevent the intake of exogenous peroxides. If lipid peroxides were then found in the adipose tissue of such rats, this would be evidence for the occurrence of lipid peroxidation *in vivo*. Conversely, if it could be shown that polyunsaturated fatty acids (PUFA) accumulated in these rats without a rise in peroxides, then the evidence would be against peroxidation *in vivo*.

Two general methods were available for controlling the ingress of peroxidized fatty acids. One was the use of the antioxidant IONOX 330 (2,4,6-tri-(3',5'-di-*tert.*-butyl-4'-hydroxybenzyl) mesitylene). This substance has been reported to be excreted quantitatively by rats (Wright, Crowne & Hathway, 1965); its antioxidant activity should not, therefore, extend to the gut wall or beyond but it should prevent oxidation within the gut contents. The other method was to give the lipids orally to rats deprived of food for some hours before and after the dose. Furthermore, the uptake of exogenous lipid peroxide could be studied by giving the rats lipids that were already partially peroxidized to various extents, and some such lipids could be stabilized by the addition of IONOX 330.

## EXPERIMENTAL AND RESULTS

### *Methods*

*Determination of lipid peroxides in diets, tissues and gut contents.* The methods described by Bunyan *et al.* (1967) were used for diets and tissues. Gut contents were treated as for tissues.

*Determination of total polyunsaturated fatty acids (PUFA).* The lipoxidase method of MacGee (1959) was used. Total lipid was determined in the extracts by evaporating samples under reduced pressure.

*Preparation of fatty acid methyl esters.* Methyl oleate (OLME), cod-liver oil methyl esters (CLOME) and maize oil methyl esters (MOME) were prepared free of vitamin E as described by Green, Diplock, Bunyan, McHale & Muthy (1967). When necessary, these lipids were peroxidized by heating at 90°, with a stream of air passing through them.

### *Animal experiments*

*Animals and diets.* The vitamin E-deficient diet A 10 Y 3 (Bunyan, McHale & Green, 1963) was given to Norwegian hooded rats and their dams from birth. At 4–6 weeks of age the young rats were divided at random, with litter-mate control and equal distribution of the sexes, into groups to receive the 20% casein diet described by Green *et al.* (1967) with the addition of the lipids described below. Additions to the diet, which contained 11 i.u. vitamin A/g, were made by replacing sugar. Diets containing lipids were made every 10 days and stored at 4° (except for group 6 of Expt 3, where

the diet was made each day as required). Food was discarded from the rats' feeding troughs after 24 h, except in Expt 3 (Table 4) and as described in Table 3.

*Dosing.* Doses of methyl esters or vitamin E (dissolved in OLME) were given orally into the stomach by means of an olive-tipped needle.

Table 1. *Expt 1. Lipid peroxides in the tissues of rats given various lipids by mouth*

(Rats were given a vitamin E-deficient diet until 38 days of age. Then, for about the next 4 weeks, they were given three oral doses per week of the lipids described below. The rats were deprived of food on Monday, Wednesday and Friday of each week when they received the lipids and on the other days they were given the fat-free 20% casein diet (see above for details of the diet and the dosing arrangements))

Group	Lipids given*	Peroxide value of lipid ( $\mu$ -equiv./g)	Lipid intake (ml/rat per week)	Adipose tissue			Liver Lipid peroxide§ ( $\mu$ equiv./g)
				Lipid peroxide ( $\mu$ -equiv./g)	PUFA† (mg/g)	Lipid† (%)	
1	OLME	5	1.75	0.3	7.3	74	0.4
2		5	2.9	0.1	9.5	—	—
3		5	5.3	1.3, 1.0	—	63	—
4	CLOME	3	1.75	0.4	8.3	75	0.4
5		3	2.9	0.4	12.4	—	—
6		29	1.75	0.8	8.1	75	0.4
7		75	1.75	2.1	3.0	77	1.0
8		110	2.9	0.0	8.1	—	—
9		200	2.9	0.7	8.7	—	—
10	CLOME + IONOX 330	3	1.75	0.3	9.1	72	0.6
11		75	1.75	0.0	5.7	73	1.1
12		75	2.9	0.0	11.4	—	—
13		200	2.9	0.8	5.4	—	—
14	MOME	15	1.75	0.1	30.6	88	0.3
15		15	5.3	0.7, 0.7	11.0	75	—
16		330	2.9	0.1	50.6	—	—
17	MOME + IONOX 330	15	1.75	0.0	36.3	78	0.7
18		330	2.9	0.2	55.1	—	—

\* OLME = methyl oleate; CLOME = cod-liver oil methyl esters; MOME = maize oil methyl esters; IONOX 330 = 2,4,6-tri(3',5'-di-*tert.*-butyl-4'-hydroxybenzyl) mesitylene, 0.01% of the lipid.

† Each analysis was carried out on the combined tissues from four rats.

‡ Polyunsaturated fatty acids.

§ Each analysis was carried out on the combined livers of one male and one female rat.

*Expt 1.* In this experiment, two precautions were taken to avoid peroxidation of lipids in the vitamin E-deficient diet and the gut before absorption. The first was to give rats the lipids by mouth, starving them for 5 h before and 17 h afterwards. The second was to add IONOX 330 to some of the lipids, and, in addition, some of them were partially peroxidized so that the absorption of exogenous lipid peroxide could be detected. Each week, the rats were starved three times for periods extending from 11.00 h one day to 09.00 h the next day, the periods of starvation being alternated with three feeding periods extending from 09.00 h one day to 11.00 h the next. On the 7th day, food was given *ad lib.* Doses of the appropriate lipids were given at 16.00 h on each

day of starvation, total amounts of 6.8–22 ml being given during the 4-week period. The results are shown in Table 1.

Bunyan *et al.* (1967) showed that when vitamin E-deficient rats were given a diet containing 10% CLOME for several weeks their adipose tissue contained high concentrations of lipid peroxide (e.g. 10–40  $\mu$ -equiv./g). Such values were not found in this experiment when CLOME was given by oral dosage, even though the CLOME had peroxide values of up to 200  $\mu$ -equiv./g. The adipose tissue values were, in fact, similar to those for rats given either OLME or CLOME of low peroxide value. However, to judge by the PUFA concentrations found, only small amounts of the fatty acids of the CLOME accumulated in the adipose tissue under these conditions. CLOME of peroxide value 76  $\mu$ -equiv./g even seemed to decrease the tissue PUFA, but this effect was not seen with the more oxidized CLOME. The administration of MOME, however, produced results of great interest, for the tissue PUFA was raised four- to five-fold, yet the peroxide concentration of the adipose tissue was unaffected. This was so even when the MOME had been oxidized to 330  $\mu$ -equiv. peroxide/g. These results therefore clearly indicate that polyunsaturated fatty acids do not necessarily become peroxidized when incorporated into the tissues of the vitamin E-deficient animal; rather they indicate the existence of an efficient barrier to the absorption of exogenous peroxide. In view of the uniformly low peroxide values of adipose tissue, the precaution of adding IONOX 330 to some of the lipids proved unnecessary. The lipid peroxide content of liver was unaffected by the lipids given, in agreement with our previous findings (Bunyan *et al.* 1967). Allowing for a lipid content of about 5%, these liver values are in the range 7–23  $\mu$ -equiv./g lipid, in contrast to the range of adipose tissue values of 0–2.7  $\mu$ -equiv./g lipid.

*Expt 2.* The low peroxide values found in Expt 1, in contrast to the high values found when the lipids were added to the vitamin E-deficient diet, could have been caused by several factors; for instance, the periodic starvation procedure, the route of administration of the lipid, the amount of lipid given, and, possibly, its peroxide value. This last factor was investigated first by finding the extent to which the OLME or CLOME that had been used in previous studies (Bunyan *et al.* 1967) became oxidized in a vitamin E-deficient diet and in the gut during digestion. Three-month-old vitamin E-deficient male rats or weanling rats that had received diet A 10 Y 3 were given the 20% casein diet with added lipids, as described in Table 2. After various feeding periods, the gut contents and adipose tissue of these rats were analysed for lipid peroxides (Table 2). The peroxide values of these and similar diets used previously are given in Table 3. Diets with 10% CLOME became peroxidized even when stored at 4° and much more so when they were placed in the rats' feeding troughs (at 20°). The addition of IONOX 330 inhibited this peroxidation for a total of 10 days at 4° and 2 days at 20°, but not when the diet was kept for 6 days at 20°. IONOX 330 also controlled the peroxide value of peroxidized CLOME when added to the diet (Table 3). D- $\alpha$ -Tocopheryl acetate had a slight inhibitory effect on the oxidation of dietary CLOME; a small amount of the antioxidant  $\alpha$ -tocopherol may have been formed by hydrolysis. The rate of peroxidation of dietary OLME was much less than that of CLOME, as would be expected. When cod-liver oil was mixed into a vitamin

Table 2. *Expt 2. Lipid peroxides in the gut contents and adipose tissue of rats given a vitamin E-deficient diet containing cod-liver oil methyl esters (CLOME)*

Addition to 20% casein diet*	Age of vitamin E-deficient rats used (months)	Feeding period (days)	Time of storage of diets		Estimated peroxide value of diet† ( $\mu$ -equiv./g lipid)	Gut contents		Adipose tissue. Lipid peroxides‡ ( $\mu$ -equiv./g)
			At 4° (days)	At 20° (days)		Lipid peroxides† ( $\mu$ -equiv./g lipid)	Lipid† (%)	
10% CLOME	3	4	7	1	600	200	6.0	—
10% CLOME + IONOX 330§	—	—	—	—	9	29	4.9	—
10% CLOME	1	36	0	1	50	—	—	26
			1	1	75	—	—	24
			5	1	400	39	4.8	—
			7	1	600	77	2.5	—
10% CLOME + IONOX 330§	1	36	0	1	9	—	—	16
			1	1	9	—	—	18
			5	1	9	24	4.7	—
			7	1	9	28	3.1	—

\* See p. 98 for details.

† Approximate estimates of the peroxide value of the diet at that time based on the results given in Table 3.

‡ Each analysis was carried out on the combined tissues of three rats.

§ 2,4,6-Tri-(3',5'-di-*tert*-butyl-4'-hydroxybenzyl) mesitylene, 0.1% of the lipid.

E-deficient diet similar to that used by Dam & Granados (1945), it became peroxidized to only a moderate extent (20–24  $\mu$ -equiv./g lipid) and D- $\alpha$ -tocopheryl acetate was not inhibitory.

Table 3. *Lipid peroxidation in vitamin E-deficient diets containing cod-liver oil (CLO), cod-liver oil methyl esters (CLOME) or methyl oleate (OLME)*

(Diets were tested after various periods of storage in the refrigerator and in the animal rooms)

Addition to basal diet*	Peroxide value of added lipid ( $\mu$ -equiv./g)	Time of storage of diets*		Lipid peroxides in diet ( $\mu$ -equiv./g lipid)
		At 4° (days)	At 20° (days)	
10% OLME	0.4	7	0	2.7
	0.4	6	1	27
10% CLOME	15	1	0	75
	15	4	0	180
	10	7	0	350
	10	0	1	54
	5	3	1	100, 180
	4	6	1	500
10% CLOME + vitamin E†	5	3	1	78
10% CLOME + IONOX 330‡	5	7	0	9
	5	10	0	9
	0.7	0	1	9
	5	3	1	3
	0.7	10	2	8
	4	6	6	105
10% oxidized CLOME	50	0	1	89
10% oxidized CLOME + IONOX 330‡	50	0	1	36
20% CLO	6	0	1	20
	6	7	1	24
20% CLO + vitamin E§	6	0	1	20
		7	1	24

\* The 20% casein diet (see p. 98) except for the tests with CLO for which the diet contained 25% casein and 10% dried brewer's yeast, replacing sugar.

† D- $\alpha$ -Tocopheryl acetate, 250 ppm.

‡ 2,4,6-Tri-(3',5'-di-*tert.*-butyl-4'-hydroxybenzyl) mesitylene, 0.1% of the lipid.

§ D- $\alpha$ -Tocopheryl acetate, 100 ppm.

The adipose tissue peroxide values for rats given dietary CLOME (Table 2) are typical of those found before (Bunyan *et al.* 1967). These values were decreased by IONOX 330; since this antioxidant is believed to be unabsorbed, this suggests that the values found were at least partly due to lipid peroxides formed before absorption of the lipid. However, IONOX 330 did not decrease the values to zero, although it kept the dietary peroxide at a low level. This suggests either that some peroxidation occurs in the gut or that even a low, continuous level of peroxide intake can result in the accumulation of peroxides in the adipose tissue. The peroxide values found for the gut contents are difficult to interpret. Peroxides may possibly be both formed and destroyed in the gut. Furthermore, whether the lipid peroxides are absorbed or not, the apparent peroxide value of the gut contents at any time will depend upon the

relative rates of absorption of oxidized and unoxidized lipid. These apparent values were higher than the dietary peroxide value when this was kept low by IONOX 330, but were much lower when the CLOME had been oxidized to high values, in the absence of IONOX 330. It was clear from these results that the peroxidation of lipids before absorption could not be completely obviated solely by the use of IONOX 330. Other precautions would be required, if peroxidation *in vivo* were to be differentiated from peroxidation in the gut contents. However, the degree of oxidation of the lipid before absorption does not seem to be the only factor influencing the accumulation of lipid peroxides in the adipose tissue.

Table 4. *Expt 3. Lipid peroxides in the adipose tissue of rats given various lipids in a vitamin E-deficient diet*

(Rats were given a vitamin E-deficient diet until 38 days old and then divided into groups. All the groups, except groups 6 and 10, were then given the diets described for the times stated. Rats of group 6 were deprived of food on Sundays, Tuesdays and Thursdays. On Mondays, Wednesdays and Fridays they were caged individually and given 5 g each of the diet described (made fresh). When this had been eaten, the rats were given the fat-free basal diet for the rest of the day. They also received this diet on Saturdays. Rats of group 10 were given the fat-free basal diet for the first 3 days of each week and then the diet with 17% CLOME (see below) for the next 4 days. On the 2nd day of each week, these rats were each given 7 mg D- $\alpha$ -tocopheryl acetate by mouth)

Addition to basal 20% casein diet*	Peroxide value of added lipid ( $\mu$ -equiv./g)	No. of days on test	Estimated lipid intake (ml/rat per week)	Adipose tissue	
				Lipid peroxide† ( $\mu$ -equiv./g)	PUFA‡ (mg/g)
1. 10% MOME	10	28	8	21, 23	179
2. 10% CLOME	7	28	8	17, 15	72
3. 10% CLOME (5 days only)	22	5	8	0.1	—
4. 5% MOME + 5% CLOME	2	28	8	19, 16	270
5. 5% MOME + 5% CLOME + IONOX 330	2	28	8	48	250
6. 20% CLOME + IONOX 330 (3 days per week, see above)	200	28	3.3	0.7, 0.5	21
7. 4% CLOME + IONOX 330	200	28	3.2	1.7, 2.2	35
8. 10% CLOME + IONOX 330	200	28	8	62, 19	78
9. 10% CLOME + vitamin E§	230	28	8	0.3, 0.8	72
10. 17% CLOME (4 days per week, see above)	230	28	6	2.0	17

Vitamin E given by mouth,  
see above

\* See p. 98 for details of this fat-free, vitamin E-free diet. MOME = maize oil methyl esters; CLOME = cod-liver oil methyl esters; IONOX 330 = 2,4,6-tri-(3',5'-di-*tert.*-butyl-4-hydroxy-benzyl) mesitylene, 0.1% of the lipid.

† Each value is the result of an analysis carried out on the combined tissues of two rats.

‡ Polyunsaturated fatty acids. Each analysis was carried out on one rat.

§ D- $\alpha$ -Tocopherol, 100 ppm.

*Expt 3.* In this experiment, we investigated some of the other factors that may have been responsible for the differences between the results of giving rats the lipids by oral dosage (*Expt 1*) and those for rats given CLOME in the diet (Bunyan *et al.* 1967). Weanling vitamin E-deficient rats were given the 20% casein diet (Green *et al.* 1967)

with the lipid supplements described in Table 4, for about 4 weeks. As described in Expt 1, MOME, when given by mouth, greatly raised the PUFA content of adipose tissue without raising its peroxide value. However, when this lipid was included in the diet it induced adipose tissue peroxide concentrations as high as those found for 10% CLOME (cf. groups 1 and 2). A mixture of these two lipids (group 4) had a similar effect and IONOX 330 allowed the peroxide concentration of the adipose tissue to rise above the general level found in Expt 1, even though it prevented oxidation of the lipid in the diet. Another group of rats (group 3) was given 10% CLOME in the diet for 5 days only. The diet was also left exposed to the air in the animal room so as to enhance its peroxidation. Even so the adipose tissue of these rats was almost free of lipid peroxide. It follows that the peroxide value of the adipose tissue is not linearly related to the length of the feeding period or to the amount of PUFA ingested. It may be that a critical feeding period must be exceeded for the absorption of exogenous peroxide to occur. The effect that the periodic starvation procedure, used in Expt 1, may have had on the results was studied with groups 6, 7 and 8. Group 6 rats were given, each week, 3 g (3.3 ml) CLOME, oxidized and then stabilized with IONOX 330, and they were also deprived of food for 3 days per week. In this way they were given the same treatment as group 13 of Expt 1, except that the lipid was given with the food. The peroxide value of the adipose tissue of these rats remained low. When a similar amount of the same peroxidized CLOME was given in the diet, but without periodic starvation, the peroxide value of the adipose tissue was again low (group 7). When, however, the weekly intake of this lipid was raised from 3.2 to 8 ml/rat (group 8), high peroxide concentrations were found in the adipose tissue.

Thus it would seem that lipid peroxides only occur in the adipose tissue when the unsaturated lipids are given in the diet and that both the length of time of feeding and the dietary inclusion rate are important in deciding the results. As described before, these high peroxide concentrations in adipose tissue are, in any event, not causally related to the production of vitamin E-deficiency diseases: 3-month-old male rats from our vitamin E-deficient colony were found to have lipid peroxide concentrations of 0 in adipose tissue, 6  $\mu$ -equiv./g lipid in liver and 10  $\mu$ -equiv./g lipid in brain.

Previous work (Bunyan *et al.* 1967) showed that the high peroxide values of the adipose tissue of rats given 10% dietary CLOME were decreased almost to zero by the addition of  $\alpha$ -tocopheryl acetate to the diet. This finding is difficult to explain, for, as now seems evident, the lipid peroxides of that tissue are solely of dietary origin and yet  $\alpha$ -tocopheryl acetate lacks the antioxidant properties of the free tocopherol (see Table 3). Even the production of  $\alpha$ -tocopherol by hydrolysis in the gut would not offer an explanation in antioxidant terms, because by then the gut contents would already include some lipid peroxide formed during storage of the diet (see Table 3). The effect of  $\alpha$ -tocopherol or its acetate, could, however, be exerted on the absorption or retention of lipid peroxides. In this experiment, an attempt was made to distinguish between the effects of vitamin E in the diet and gut contents and possible effects on lipid peroxides after absorption. Group 9 rats were given 10% CLOME and  $\alpha$ -tocopheryl acetate in the diet. Their adipose tissue contained very little peroxide. Group 10 rats received vitamin E separately from the dietary lipid, but their adipose



tissue value was also low. However, this experimental scheme may not have been adequate to ensure complete separation of the CLOME and tocopherol, since the latter would have been re-excreted into the gut during the days following the dose (Green *et al.* 1967). Furthermore, with this procedure of giving the rats 17% CLOME for 4 days only per week, very little PUFA was taken up.

*Expt 4.* This experiment was designed to study more directly the possible effects of vitamin E on lipid peroxides after absorption in the adipose tissue. Weanling vitamin E-deficient rats were given 10% CLOME in the diet for 4 weeks, after which the peroxide content of their adipose tissue was found to be high. The rats were then

Table 5. *Expt 4. Effects of vitamin E and DPPD (N,N'-diphenyl-p-phenylenediamine) on the metabolism of lipid peroxides in adipose tissue*

(Rats were given a vitamin E-deficient diet until 38 days old and then, for 28 days, the 20% casein diet (see p. 98) with 10% CLOME added. Then the CLOME was withdrawn from the diet and the rats were divided into three groups. One group was given the fat-free basal diet, while the other two received that diet supplemented with, respectively, vitamin E and DPPD)

Addition to basal 20% casein diet	No. of days after removing CLOME from diet							PUFA† of adipose tissue* (mg/g)	
	0	2	3	4	8	9	10	0	4
	Lipid peroxide of adipose tissue* ( $\mu$ -equiv./g)								
None	70, 36	31	44	—	13	—	14	57, 81	43
Vitamin E‡	—	12	—	36	11	7	—	—	53
DPPD	—	—	4	24	—	13	5	—	76

\* Each value is the result of an analysis of the combined tissues of two rats.

† Polyunsaturated fatty acids.

‡ D- $\alpha$ -Tocopheryl acetate, 100 ppm.

divided into three groups and given the fat-free 20% casein basal diet. One group also received dietary D- $\alpha$ -tocopheryl acetate and another group was given the synthetic antioxidant DPPD (*N,N'*-diphenyl-*p*-phenylenediamine) for comparison. Rats were taken in pairs from the three groups during the next 10 days and their adipose tissue was analysed. The results of these tests (Table 5) clearly showed that the lipid peroxides of this tissue are not of 'fleeting existence' as is often supposed (Ghoshal & Recknagel, 1965; Kokatnur, Bergan & Draper, 1966). Ten days after removing the source of the lipid peroxides the tissue concentration in the control rats was still many times the usual resting level. The results for rats given vitamin E and DPPD were lower than controls after 2 or 3 days, but the later results did not confirm this trend. In view of the variability encountered, it could not be shown that vitamin E or DPPD accelerated the rate of decay of lipid peroxides in the adipose tissue. Any effect would seem to be small. The ability of dietary vitamin E to prevent the accumulation of lipid peroxides in adipose tissue cannot, then, be explained by an effect on the peroxides after they have been taken up by the tissue.

*Reaction of antioxidants with lipid peroxides*

*Expt 5.* The absence of reaction between  $\alpha$ -tocopherol and lipid peroxides in vivo complements our previous finding (Bunyan *et al.* 1967) that  $\alpha$ -tocopherol does not react with the lipid peroxides of tissue extracts at room temperature. In this experiment, we studied the effects of reacting  $\alpha$ -tocopherol and DPPD with oxidized MOME for 3 h at room temperature. The micro- and macro-methods of analysis were used. No effect was found (Table 6). In addition,  $\alpha$ -tocopherol and DPPD were incubated at 37° for 65 h with CLOME that had been oxidized to about the same peroxide value as found in the adipose tissue at the start of Expt 4. The peroxide value of the CLOME decreased, under these conditions, by about 10 %, but neither  $\alpha$ -tocopherol nor DPPD accelerated the rate (Table 6). It should be noted that, when measuring the optical density of iodine solutions that also contain DPPD, an additional 'blank' measurement must be made to allow for the decrease in the optical density of the DPPD solution due to reaction with KI. If this correction is not made, it will appear that DPPD has destroyed the lipid peroxide.

Table 6. *Expt 5. Reaction of antioxidants with the lipid peroxides of oxidized maize oil methyl esters (MOME) and oxidized cod-liver oil methyl esters (CLOME)*

(Oxidized MOME was tested by both micro- and macro-methods as described below. Oxidized CLOME was tested by the macro-method at zero time and after 65 h at 37° in an atmosphere of N<sub>2</sub>)

Antioxidant added	Oxidized MOME		Oxidized CLOME, macro-method*	
	Micro-method	Macro-method	0 h	After 65 h at 37°
	None	64 78	63	52.1 ± 0.2 (2)
D- $\alpha$ -Tocopherol†	56	—	52.1 ± 1.2 (2)	47.6 ± 0.9 (3)
DPPD‡	91	63	50.3 ± 0.2 (2)	44.3 ± 0.5 (3)

\* Results are given as means with standard deviations and the number of replicate analyses is given in parentheses.

† 5 mg/g oxidized MOME and 100  $\mu$ g/g oxidized CLOME.

‡ *N,N'*-Diphenyl-*p*-phenylenediamine, 18 mg/g oxidized MOME and 100  $\mu$ g/g oxidized CLOME.

#### DISCUSSION

Cod-liver oil and maize oil have often been added to vitamin E-deficient diets to accelerate the onset of vitamin E-deficiency disease. However, most of the accepted signs of deficiency can be induced in rats and chicks by using lard and, under these conditions, lipid peroxides do not accumulate in the adipose tissue (Aaes-Jorgensen, 1949; Christensen *et al.* 1958; Bunyan *et al.* 1967). Even when the peroxide concentration of the adipose tissue has been elevated (e.g. above 10  $\mu$ -equiv./g) by the use of cod-liver oil, the already higher concentrations in other tissues are unaffected (Bunyan *et al.* 1967). Furthermore, dietary vitamin E decreases the concentration of lipid peroxides in adipose tissue only. The finding of lipid peroxides in the adipose tissue of rats given highly unsaturated dietary lipid would seem therefore to be un-

related to the main physiological role of vitamin E. However, such findings have led to the establishment of a *prima facie* correlation between vitamin E and lipid peroxides that is suggestive of a true biological action of vitamin E. Although our previous studies on the metabolism of radioactive  $\alpha$ -tocopherol (Green *et al.* 1967) led us to the view that lipid peroxidation is not a significant process *in vivo*, it seemed of great importance to investigate this apparent correlation in more detail.

The work described here has in fact provided additional evidence that lipids do not become peroxidized in the adipose tissue of the vitamin E-deficient rat. This conclusion rests partly upon the results obtained by giving rats the peroxide-free lipids by mouth. The results for MOME are of special importance because of the high tissue PUFA concentrations induced by it. The conclusion is further supported by the low peroxide values found in the adipose tissue of rats given only small amounts of CLOME in the diet (Table 4, groups 6 and 7). We consider that all the results we have obtained and the evidence of other workers can be accounted for by the existence of a partially effective barrier to the absorption of exogenous lipid peroxide, which is influenced by vitamin E. The barrier proved effective in preventing the absorption of peroxide when rats were given oral doses of peroxidized CLOME and MOME (Expt 1), when rats were given the lower dietary intake (4% in the diet) of peroxidized CLOME (Expt 3) and also when the higher dietary concentration (10%) of CLOME was given for a short time only (Expt 3). The prolonged feeding of MOME or CLOME, whether peroxidized or not, at 10% in the diet, however, overcame the barrier to absorption. The presence of other food in the gut at the same time as the lipid may also enhance the absorption of peroxides, since none were absorbed after the largest oral doses of MOME (Expt 1), although the weekly intake approached that of rats given 10% dietary CLOME.

The unabsorbed antioxidant IONOX 330 proved of less use in these experiments than expected. It was unnecessary as an adjunct to the experiments with oral doses; and, although an effective antioxidant in the diet, it seemed to be less so in the gut, allowing peroxides to form and become incorporated into the adipose tissue (see Table 4, group 5).

The way in which vitamin E controls the level of lipid peroxides in the adipose tissue of rats given dietary unsaturated lipids for long periods is not entirely clear. It does not destroy preformed peroxide *in vivo* or *in vitro* (at 20° or 37°) and it cannot act by preventing peroxidation in the adipose tissue itself if, as our results indicate, no peroxidation occurs there.  $\alpha$ -Tocopherol, formed by hydrolysis of dietary  $\alpha$ -tocopheryl acetate, can act as an antioxidant in the gut, but this does not explain how vitamin E prevents the uptake of exogenous lipid peroxide (Table 4, group 9). The profound effect of vitamin E on the adipose tissue peroxide concentration must, then, be due to its ability to reinforce the barrier to the absorption of exogenous lipid peroxides. O'Brien & Frazer (1966) commented that the mucosa of the small intestine may be a major site of inactivation of lipid peroxides and may suffer damage in the process. Vitamin E may promote the regeneration of damaged intestinal mucosa in a similar fashion to its effects on the regeneration of rat liver after partial hepatectomy (Marcos, Fodor, Kovacs & Katonai, 1966).

The biological antioxidant hypothesis of vitamin E function and its dependence on the concept of 'lipid peroxidation' in vivo has existed almost since the discovery of the vitamin. As we have previously discussed (Green *et al.* 1967), the theory rests on two main pillars of evidence: the association between unsaturated fat and vitamin E deficiency disease and, secondly, the vitamin E-like activity of certain synthetic substances with antioxidant properties. It cannot be stressed too strongly that current concepts of the role of vitamin E are indissolubly linked with the concept that, in its absence, the whole organism is subject to widespread, proliferating and uncontrolled peroxidation. Thus Tappel (1965) states that 'lipid peroxidation is widespread throughout the vitamin E-deficient animal' leading to a 'wide array of metabolic derangement'; Witting (1965) considers that 'in all cases the primary deficiency response is lipid peroxidation'; Kummerow (1964) states that 'if the tissues contain polyunsaturated fatty acids and a fat-soluble antioxidant is not available, peroxidation must inevitably occur'. The failure of investigators to detect lipid peroxides in the tissues pathologically affected in vitamin E deficiency diseases (e.g. Dam, 1962) has been assumed to be due to the rapid disappearance of these metabolites once formed (Ghoshal & Recknagel, 1965; Kokatnur *et al.* 1966). Indeed, critical examination of the foundations of the antioxidant theory demonstrates that none of the essential prerequisites for its direct proof exist; and, as our results described here and previously (Green *et al.* 1967; Diplock, Bunyan, McHale & Green, 1967; Bunyan *et al.* 1967) show, all attempts to substantiate the existence of peroxidation in vivo have failed. The biological antioxidant theory must, in the absence of such direct proof, be regarded as circumstantial in origin and the relation between polyunsaturated fatty acid metabolism and vitamin E may be due to other causes (Green *et al.* 1967). The problem of the vitamin E-like activity of certain synthetic antioxidants and also of substances such as quinones of the ubiquinone series and their related chromanols (Folkers, Smith & Moore, 1965) remains. However, the common molecular features that endow these varied groups of substances with their observed antioxidant properties in vitro, under normal conditions of oxygen tension (i.e. hydrogen donation or electron acceptance within certain redox potential limits), are probably those that confer upon them their common biological activity, modified by their different degrees of absorption and retention. Several recent studies by workers who are moving away from the biological antioxidant concept suggest specific ways in which substances like  $\alpha$ -tocopherol, with the required redox properties, may function; for instance, Guha & Roels (1965) consider that vitamin E may affect the permeability of membranes by ways 'more complex than mere peroxidation'; Brown, Button & Smith (1967) suggest that vitamin E affects the synthesis of mucopolysaccharides, particularly in the formation of collagen; and Carpenter (1967) found that vitamin E increased the synthesis of microsomal oxidative demethylating enzymes.

To return once again to the problem of lipid peroxides in vivo, we would like to draw attention to the formation of ceroid and lipofuscin pigments in the tissues of animals after prolonged vitamin E deficiency. From almost the earliest studies on vitamin E, the occurrence of these pigments has been attributed to the peroxidation and polymerization of lipids in association with proteins in vivo (Martin & Moore,

1939; Filer, Rumery & Mason, 1946; Mason, Dam & Granados, 1946, and many others). Tappel (1965) regards the formation of these pigments as the clearest evidence for lipid peroxidation in the vitamin E-deficient animal. However, our results now suggest that these pigments may be formed not as a result of the peroxidation of lipids *in vivo*, but rather by the polymerization of exogenous lipid peroxides absorbed from the gut contents. This is why, we believe, and as Filer *et al.* (1946) pointed out, 'little or no pigment occurs in rats and hamsters fed E-deficient diets low in fat', although it is clear that the tissues of such animals must contain 'peroxidizable' lipid; and, secondly, this is why linoleic acid only produces pigment if given 'in sufficient amounts for a long enough period of time' (Filer *et al.* 1946). In summary, therefore, the conditions required to produce pigment are identical with those found in our experiments to permit the absorption of exogenous peroxides.

A further problem is the origin of the lipid peroxides, at approximately stationary concentrations, in tissues other than adipose tissue (Bunyan *et al.* 1967). If these are the normal type of fatty acid hydroperoxide and they are formed by the normal processes of free radical chain reaction, there would seem to be no reason why vitamin E or DPPD should not prevent their formation; indeed this would be required if tocopherol acted as an antioxidant in these tissues. Since they are unaffected by vitamin E or DPPD (Bunyan *et al.* 1967), they must either be formed in compartments separate from the vitamin E or they are produced by a mechanism dissimilar from the normal *in vitro* process of autoxidation and one which is not controlled by tocopherol. In this connexion, it is interesting to note that Slater (1962) criticised the whole concept of biological peroxidation by questioning whether the postulated processes could be effective at the extremely low oxygen tensions existing in tissues. The significance of these tissue peroxides, however they are formed, is unknown.

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*Note added in proof*

Further evidence against the long-held belief that ceroid production is caused by lipid peroxidation is provided by the recent work of A. J. Patek, F. E. Kendall, N. M. deFritsch & R. L. Hirsch (1967) (*Archs Pathol.* **94**, 295). They found that the formation of ceroid in the livers of rats given linoleic acid and a 4 % casein diet for 16 weeks is unaffected by large amounts of  $\alpha$ -tocopherol (2.4 mg/day). No ceroid was found in the livers when the rats were given linoleic acid and a 30 % casein diet, even in the absence of vitamin E.