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On the existence and significance of lipid peroxides in vitamin E-deficient animals

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1. A micro-adaptation of the iodimetric method has been used to determine lipid peroxides in the tissues of vitamin E-deficient rats and chicks.

2. No increases in lipid peroxide were found in liver, kidney or adipose tissue of rats with nutritional liver necrosis due to deficiency of vitamin E and selenium. When liver necrosis was induced by giving rats a casein diet and silver acetate solution to drink, the peroxide value of the adipose tissue was not increased.

3. Degeneration of the testes of vitamin E-deficient rats was not accompanied by a rise in the peroxide value of the tissue lipids.

4. There was an increase in cathepsin activity of the kidneys of rats displaying the phenomenon of renal autolysis (*post mortem*), but there was no increase in lipid peroxide content.

5. No rise in lipid peroxide was found in dystrophic chick breast muscle, in cerebellum, brain and adipose tissue of chicks with encephalomalacia nor in the liver of chicks with exudative diathesis.

6. In rat liver, kidney, testis and leg muscle, peroxide values in the range 10–40 μ-equiv./g lipid were found, and these values were not altered either by a substantial change in the degree of unsaturation of the dietary lipid or by the addition of vitamin E to the diet. Dietary addition of *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) or 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin) also failed to affect the peroxide value of liver. The possibility that lipid peroxide is a normal metabolite of these tissues is discussed.

7. Peroxide values of rat adipose tissue were never found to be greater than 40 μ-equiv./g lipid and were readily decreased by the addition of vitamin E to the diet or by a decrease in the unsaturation of the dietary lipid. The peroxide content of this tissue may depend upon the uptake of peroxidized dietary lipid.

8. The conclusion from this study of true lipid peroxides in animal tissues is that the biological role of vitamin E is not connected with lipid peroxidation in vivo, in agreement with our previous studies on the metabolism of the fatty acid substrates of peroxidation and of α-tocopherol and other postulated biological antioxidants.

The hypothesis that vitamin E functions solely as a physiological antioxidant has been critically examined by Green, Diplock, Bunyan, McHale & Muthy (1967). In that study and in two succeeding ones (Diplock, Bunyan, McHale & Green, 1967; Diplock, Green, Bunyan, McHale & Muthy, 1967) we found that the metabolism of [^{14}C]α-tocopherol was unaffected by large amounts of unsaturated lipid in the diet or by the imposition of other stresses on the vitamin E status of the animals and concluded that lipid peroxidation was not causally concerned in the aetiology of vitamin E deficiency disease. Furthermore, in a recent study (Bunyan, Diplock & Green, 1967) we could find no evidence that the peroxidative loss of polyunsaturated fatty acids (PUFA) was a general feature of vitamin E deficiency, although a significant role for vitamin E in the interconversion of highly unsaturated fatty acids was not denied. Although, therefore, most of the evidence for the biological antioxidant hypothesis seems to be circumstantial, there is nevertheless some apparently direct evidence for a

connexion between vitamin E deficiency disease and lipid peroxidation in vivo. Dam & Granados (1945) were the first workers to demonstrate the presence of lipid peroxide in tissues and they showed that greatly increased amounts were present in the adipose tissue of chicks and rats given vitamin E-deficient diets containing cod-liver oil. These findings have often been cited and have been seminal in the development of the biological antioxidant hypothesis. Later, Glavind & Hartmann (1961) found peroxides in vitamin E-deficient chick liver and Emmel & LaCelle (1961) correlated the phenomenon of post-mortem renal autolysis in rats given large amounts of unsaturated fats with a rise in the peroxide value of the adipose tissue.

The finding, albeit under somewhat special dietary conditions, of lipid peroxides in the tissues of vitamin E-deficient animals apparently conflicted with our general conclusion as to the role of lipid peroxidation in the development of vitamin E deficiency disease. Furthermore, re-examination of the papers of Dam & Granados (1945), Glavind & Hartmann (1961) and Emmel & LaCelle (1961) suggested that their interpretations might have to be reconsidered for three reasons. First, it is clear that the presence of peroxidized lipid in the gut must be completely excluded if measurements of tissue peroxides alone are to give unequivocal evidence of peroxidation in vivo; or, alternatively, allowance must be made for a quantitative relationship between the peroxides of the gut contents and the peroxides of the tissues. Although Dam & Granados (1945) and Emmel & LaCelle (1961) took certain precautions against gross peroxidation of dietary lipids, our experience with highly unsaturated oils in vitamin E-deficient diets leads us to doubt that these precautions were sufficient to prevent the ingestion of some peroxidized lipid. O'Brien & Frazer (1966) suggest that when the animal is subjected to prolonged feeding of peroxidized oils, the gut barrier to absorption may be lessened. Secondly, the only investigation, hitherto, of a tissue with a specific lesion due to vitamin E deficiency—the cerebella of chicks suffering from encephalomalacia—has failed to reveal any peroxidized lipid, by the methods used (Dam, 1962). Thirdly, there are objections to the methods for peroxide determination used by the three groups of workers (see p. 491).

In consequence we have re-investigated the question of the existence of lipid peroxides in tissues and their relation to vitamin E deficiency. For this work, we used the micro-iodimetric technique of Heaton & Uri (1958), which offered a substantial increase in sensitivity compared to the standard volumetric method.

EXPERIMENTAL AND RESULTS

Methods

Solvents. Chloroform and methanol were boiled for 1 h under reflux with lithium bromide and cadmium filings, distilled and stored under nitrogen: the purified solvents were used within 5 days. Acetic acid (AR) and ethanol were not redistilled before use. The following solvent mixtures were prepared as required: chloroform–acetic acid solution (1:2, v/v), chloroform–ethanol solution (9:1, v/v), chloroform–methanol solution (2:1, v/v), 'rinsing mixture' (chloroform–methanol–water; 12·7:6·3:0·8, v/v) and 'upper layer solvents' (chloroform–methanol–water; 3:48:47).

In addition, a 'solvents blank' solution was prepared equivalent to the combined solvents present in each final test mixture. The solutions required each day, including the water for the solution of KI, were gassed with N_2 before use.

Killing of animals and dissection of tissues. With the exceptions stated below, rats were killed by exposure to N_2 in a polythene bag. Tissues were dissected under N_2 and weighed in a weighing bottle filled with N_2. In the tests on rat liver described in Tables 6 and 8, however, the rats were killed by breaking their necks, and their livers were removed, sectioned and placed immediately in chloroform–methanol solution under N_2. Chicks were killed by breaking their necks and their tissues were dissected and placed immediately under solvent.

Extraction of tissue lipids. The method was based on the technique described by Folch, Lees & Stanley (1957). The tissue was macerated in 19 volumes of chloroform–methanol solution containing 0·5 mg D-α-tocopherol/g tissue in a top-drive homogenizer, operated at about 1000 rev/min for 1 min. The homogenizer goblet, which was cooled in crushed ice, was flushed with N_2 before adding the tissue and solvents and a stream of N_2 was also passed into the top of the goblet during maceration. The extract was centrifuged at 1000 *g* for 5 min and made up to volume with 'rinsing mixture'. A portion of the solution was then washed by stirring well with 0·2 vol. of NaCl solution (0·73 %) and centrifuged. The upper layer was siphoned off and the surface of the lower layer was then washed three times with small volumes of 'upper layer solvents' (as described by Folch *et al.* 1957). The washed solution was made to a convenient volume with 'rinsing mixture' and a sample was then evaporated to dryness under reduced pressure, the flask being opened under N_2. The dried extract was immediately taken up in chloroform–acetic acid solution and quickly filtered through sintered glass. A portion (6·3 ml) of this solution (usually equivalent to 5–20 mg lipid) was pipetted into the spectrophotometer tubes, adapted as described below. A reagent blank was prepared by submitting 40 ml 'rinsing mixture', containing 1 mg D-α-tocopherol, to the same procedure. Precautions were taken to ensure that the tissue extract was handled throughout in an atmosphere of N_2 with the occasional exception of a rapid filtration through glass-wool when the test solution contained undissolved particles.

Determination of lipid peroxide (micro-method). The test was carried out in an atmosphere of N_2 in spectrophotometer tubes fitted with gas inlets and outlets, as described by Heaton & Uri (1958). Two sets of eight tubes were used. N_2 was passed into the tubes in parallel by means of two glass manifolds. The gas was washed by passing it through five Dreschel bottles containing, in order: (1) alkaline pyrogallol solution (20 g KOH and 2·5 g pyrogallol per l.), (2) and (3) a solution containing sodium dithionite (15 g), NaOH (10 g) and anthraquinone-2-sulphonic acid (0·2 g) per l., (4) water and (5) chloroform–acetic acid solution. All connexions were made with PTFE tubing. N_2 was passed through the tubes for 5 min before adding any liquids, which were then introduced from an all-glass syringe attached to a fine polythene tube passed down the gas outlet to the bottom of the spectrophotometer tube. With N_2 still passing, 6·3 ml of test solution or reagent blank were introduced and then, 10 min later, 0·5 ml of KI solution (10 %, w/v) was added (or 0·5 ml water to give a 'sample blank').

After a further 5 min the stream of N_2 was stopped and the gas taps were closed. Each tube was inverted once to mix the contents and then allowed to stand in the dark at room temperature for 1 h. The optical density of the iodine liberated was measured at 380 nm in a Unicam SP 600 spectrophotometer with the 'solvents blank' set at 0. Because of evaporation in the stream of N_2, the final volume of the contents was determined by the measured addition of water up to a calibration mark, and each optical density was corrected to a standard volume of 6·5 ml. The optical density was then corrected by subtraction of the reagent blank, and of the appropriate 'sample blank'. Each test was done in duplicate or triplicate. Calibration was effected by use of standard iodine solution. One μ-equiv. of iodine ($\equiv$ 1 μ-equiv. of peroxide) gave an optical density of 1·226. Fatty acid ester preparations were dissolved in chloroform–acetic acid solution for testing.

Determination of lipid peroxide (macro-method). Method 2 of British Standards Institution (1958) was used.

Determination of peroxides in dietary lipids. If the diet was expected to have a peroxide value of less than 10 m-equiv./kg lipid, it was extracted by the method described for tissues and tested by the micro-method. Other diets were shaken vigorously with 15 ml light petroleum (boiling range 40°–60°) per g diet for 3 min. The extract was filtered through Whatman paper no. 541 and tested by the macro-method.

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

Determination of degree of renal autolysis. Kidneys were decapsulated, cut into quarters and incubated at 37° in a closed tube for the periods of time described in Expt 7. The degree of autolysis was assessed by determining non-protein-nitrogen (NPN) production during incubation, as described by Emmel (1957). NPN was determined by macerating the kidney in trichloroacetic acid solution (10%, v/v) and measuring the optical density of the extract at 280 nm. A solution of tyrosine was used as standard, results being reported as tyrosine equivalents.

Cathepsin activity. The method described by Bunyan, Green, Diplock & Robinson (1967*a*) was used.

Investigations into the method for determining lipid peroxide in tissues

Extraction of lipid. Some of the methods described in other publications were compared with that described in this paper. Glavind & Hartmann (1961) and Glavind, Søndergaard & Dam (1961) ground tissues with anhydrous sodium sulphate and then extracted the lipid in chloroform in a Waring Blendor. In an earlier paper, Glavind & Hartmann (1956) described the use of a grade of chloroform containing ethanol. When we ground rat liver with anhydrous sodium sulphate and extracted the lipids with a 9:1 (v/v) mixture of chloroform and ethanol (Table 1, method 3) we obtained only about one-half of the lipid obtained by the method of Folch *et al.* (1957) (method 1). The greater amount of apparent lipid obtained by method 2 showed the necessity of washing the chloroform–methanol extract free of non-lipid substances. Glavind &

Hartmann (1961) also described a method for chick liver in which the tissue was ground in a mortar with chloroform. With rat liver only about one-tenth of the lipid was extracted in this way (Table 1, method 4). The chloroform–methanol method was also shown to be superior to grinding liver with anhydrous sodium sulphate and then extracting it for $6\frac{1}{2}$ h in a Soxhlet extractor using a 1:1 (v/v) mixture of diethyl ether and light petroleum (boiling range 40–60°) (method 5).

Table 1. *Comparison of methods of extracting lipids from rat liver*

(Livers of vitamin E-deficient male rats were used)

Method of extraction	Apparent lipid content (%)			
	Test 1	Test 2	Test 3	Test 4
1. Blended in chloroform–methanol	4·3	5·0	3·8	6·1, 6·5
2. Blended in chloroform–methanol but not washed	—	6·7	—	—
3. Blended in chloroform–ethanol*	—	—	—	3·8, 2·9
4. Ground with chloroform–ethanol	0·65, 0·44	—	—	—
5. Soxhlet extraction	3·2	2·9	2·8	—

* Liver first ground with anhydrous sodium sulphate.

Table 2. *Reaction of antioxidants with lipid extracts of rat liver*

(Livers of 4-month-old vitamin E-deficient rats were extracted as described. Each result is the peroxide value of a portion of extract, equivalent to 100 mg liver, after reaction with the antioxidant in chloroform–acetic acid solution for 20 min at room temperature in the dark. A different rat was used in each of the six tests)

Antioxidant added	Antioxidant concentration (mg/g liver)	Lipid peroxide (μ-equiv./g liver)				
		Test 1	Test 2	Test 3	Test 4	Test 5
None	—	0·47	0·62	0·43	0·55	0·26
α-Tocopherol	10	0·62	—	—	—	—
	0·25	—	0·65	0·57	—	—
Propyl gallate and citric acid (equal parts)	10	—	—	—	0·31	—
	0·10	—	—	—	—	0·32

Avoidance of free-radical oxidation of lipid extracts. The difference in tocopherol content between the tissues of deficient animals and those given vitamin E could affect the stability of the lipid during the extraction and washing procedures. In order to overcome this difference and to lessen the chance of any further oxidation, a large amount of α-tocopherol (500 μg/g tissue) was added to the extraction solvent. (α-Tocopherol did not react with the lipid peroxide of tissue extracts (Table 2). α-Tocopherol did not affect the reagent blank or standard solutions of I_2, containing KI (10%, w/v)). In addition, an inert atmosphere was maintained during the procedure, as described. That oxidation of lipid had been prevented was shown by tests in which oxidized maize oil gave the same peroxide value whether tested directly or after being blended and washed as for a tissue extract (see Table 3). Furthermore, the apparent

peroxide values of tissue extracts were not changed when the extracts were allowed to remain 20 min longer in contact with the washing solution or for up to 3 h in chloroform–acetic acid solution.

Effect of sample size. In preliminary tests, a solution of oxidized maize oil in chloroform–methanol solution was reacted with KI (10%, w/v) solution with the intention of avoiding the change from extraction solvent to chloroform–acetic acid solution in tests on tissues. However, very high and variable peroxide values were obtained when a sample of 150 μg oil was tested, but not when 300 μg were used (Table 3). When the chloroform–methanol solution was removed under reduced pressure and the extract taken up in chloroform–acetic acid solution, there was no effect of sample size.

Table 3. *Effect of sample size on apparent peroxide value by the micro-method*

(Tests were carried out on a sample of oxidized maize oil)

Solvent mixture	Sample size (μg)	Peroxide value (μ-equiv./g) Micro-method	Macro-method
Chloroform–methanol	300	260 ± 3 (3)*	230
	150	473 ± 151 (8)	230
Chloroform–acetic acid	300	273, 217	230
	150	260, 247	230
	300	250 ± 47 (5)†	245 (interpolated)
	300	280 ± 23 (4)†	264 ± 2 (3)

* Three or more results are given as the mean and standard deviation and the number of tests is shown in parentheses.

† In these tests, the maize oil had been stored longer and its peroxide value had increased. In addition, those samples tested by the micro-method had been submitted to the full process of maceration and washings used for tissue extracts.

Comparison of macro- and micro-methods. Recovery tests. A sample of maize oil, oxidized to a peroxide value of 230 m-equiv./kg, was used as a standard substance. On average, the results of the micro-method (twenty-four tests) were 116% (SD ± 14%) of the results of the macro-method (six tests).

Paper chromatography of lipid extracts. Milas, Harris & Golubovic (1963) could not find significant amounts of lipid peroxide in the tissues of vitamin E-deficient rats and chicks when extracts were submitted to paper chromatography. However, in our tests, a similar chromatographic procedure confirmed the results of the micro-method already described. Extracts of a vitamin E-deficient rat liver were submitted to descending chromatography on Whatman no. 1 paper, using a 45:5:50 (v/v) mixture of *n*-butanol, ethanol and water. The papers were sprayed with a solution containing glacial acetic acid (3 ml), saturated KI solution (2 ml) and starch solution (5 ml).

Animal experiments

Norwegian hooded rats and Warren cockerels were used.

Expt 1. This experiment was designed to investigate the way in which the lipid peroxide content of rat tissues might vary according to the degree of unsaturation of

the lipid in the vitamin E-deficient diet. It was considered that methyl oleate (OLME) and cod-liver oil methyl esters (CLOME) were sufficiently different in degree of unsaturation for this purpose. These lipids were prepared and purified as described by Green *et al.* (1967). Litters of rats (together with their dams) were given diet A 10 Y 3 (Bunyan, McHale & Green, 1963) until 37 days of age. The young rats were then allocated at random, with equal distribution of sexes and litter-mates, to two groups to receive the basal vitamin E-deficient diet containing either 10% OLME or 10% CLOME. The basal diet had the percentage composition: casein ('low vitamin content'; Genatosan Ltd) 20, salt mixture 2·73, $NaH_2PO_4.2H_2O$ 2·2, vitamin mixture (Bunyan, Green, Diplock & Robinson, 1967*b*) 0·4, sugar 44·2, glucose 20·9 and lipid (OLME or CLOME) 10. The salt mixture supplied (g/kg) $CaCO_3$ 18·2, KCl 3·5, Na_2CO_3 1·2, $MgSO_4.7H_2O$ 4, ferric citrate 0·15, $MnSO_4.4H_2O$ 0·2, $ZnSO_4.7H_2O$ 0·06, KI 0·0003, NaF 0·00025, $(NH_4)_6\ Mo_7O_{24}.4H_2O$ 0·002, $CoSO_4.7H_2O$ 0·01, $Al_2(SO_4)_3.K_2SO_4.24H_2O$ 0·0007, $CuSO_4.5H_2O$ 0·02. In order to avoid destruction in the diet, vitamin A was omitted from the vitamin mixture and given by mouth (300 i.u./rat per week). The diet with OLME was made up weekly and stored at 4° between feeds. The diet with CLOME was made up daily. Rats were killed and examined after 13–39 days on diet.

In the rats given CLOME, PUFA contents of liver, kidney, testis and leg muscle were raised, but CLOME did not increase the amount of lipid peroxide in these tissues. Indeed, when considered on a PUFA basis, the peroxide values seem to be lower than for rats given OLME (Table 4). In the adipose tissue of rats given CLOME there was a substantial accumulation of PUFA and also a great rise in peroxide value even when considered on a PUFA basis. Tests on similar diets have shown that a considerable degree of oxidation of dietary lipid occurs even when CLOME is added fresh each day (e.g. in one test, a value of 54 μ-equiv./g lipid was found). Therefore the increased peroxide value of the lipids of the adipose tissue may have been partly due to the ingestion of peroxidized dietary lipid.

Expts 2 and 3. Expt 2 was part of an investigation (to be published) into the effects of dietary fat and vitamin E deficiency in the metabolism of vitamin A. Young rats, at 14 days of age, were given the diet A 10 Y 3 with vitamin A omitted (i.e. a diet deficient in vitamins A and E) and a dose of 15 i.u. vitamin A/rat per week, by mouth. At 10 weeks of age, all the rats were given 10000 i.u. vitamin A palmitate by mouth and then allocated at random to four groups to receive the basal diet of Expt 1 with the inclusion of, respectively: OLME 10%; OLME 10% with D-α-tocopheryl acetate, 120 ppm; CLOME 10%; CLOME 10% with D-α-tocopheryl acetate, 120 ppm. The diets were made weekly and stored at 4° between feeds, and food residues were discarded daily from the food troughs. The rats were killed 12 weeks later. In Expt 3, the effects of adding vitamin E to the CLOME diet of Expt 1 were investigated, with rats of the same age but given the test diets for a longer period. Young rats were given diet A 10 Y 3 until 30 days of age and then divided into groups to receive the 20% casein diet of Expt 1 with inclusions of either CLOME 10% or CLOME 10% with D-α-tocopheryl acetate, 100 ppm (diets were made weekly and stored at 4°). The rats were killed and examined after 47–58 days on diet.

Table 4. *Expt* 1. *Lipid peroxides in tissues of rats given* 10% *methyl oleate (OLME) or* 10% *cod-liver oil methyl esters (CLOME) in a vitamin E-deficient diet*

(Weanling rats were given a 20% casein diet (see p. 481) with 10% OLME or CLOME. Three or more results are given as the mean and standard deviation and the number of results is shown in parentheses. Fewer than three results are given individually)

	Liver			Kidney	Testis	Leg muscle	Adipose tissue		
Days on diet ...	7	14	29–37	30–37	35	32–39	7*	13–16	28–38
				Lipid peroxide† (μ-equiv./g)					
Dietary lipid									
OLME	1·1±0·7 (4)	0·1, 1·2	1·9	0·73, 0·73	0·16, 0·33	0·44	1·1, 4·1	0·4±0·3 (5)	0·7±0·5 (4)
CLOME	1·0±0·6 (3)	0·5±0·2 (3)	1·2	0·98, 0·99	0·21, 0·34	0·42	2·3, 4·0	13·5±7·3 (5)	27±14 (4)
				PUFA‡ (mg/g)§					
OLME	—	4·4, 6·7	3·8, 7·3	6·5	4·1, 4·5	2·3	—	6·1	3·5, 3·2
CLOME	—	12·1, 15·2	9·5, 14·1	10·0	4·7, 5·6	4·0	—	21·9	52·9, 36·3
				Lipid (%)§					
OLME	—	5·1, 5·4	4·6, 5·0	4·1	2·9, 2·2	4·1	—	81	88, 77
CLOME	—	5·1, 5·5	4·5, 4·7	3·9	2·9, 2·3	3·3	—	—	59, 60

* Very little adipose tissue was found in these young rats.
† Each result was obtained by the analysis of the tissues from one rat or two rats combined.
‡ Polyunsaturated fatty acids.
§ Each result was obtained by the analysis of the combined tissues from four to eight rats, except for liver at 14 days on diet, when pairs of rats were used.

The results of these two experiments are shown in Table 5. As in Expt 1, CLOME increased the peroxide value of adipose tissue only, but to a lesser extent than before, in spite of, or perhaps because of, the longer feeding period. The general PUFA content of adipose tissue was greater in this test, but the difference between rats given OLME and those given CLOME was still marked. The large difference in PUFA of leg muscle seen in Expt 1 was not found in Expt 2. Dietary vitamin E did not alter the peroxide values of leg muscle or liver, but it decreased the value for adipose tissue

Table 5. *Expts 2 and 3. Effects of dietary vitamin E on lipid peroxides in tissues of rats given a vitamin E-deficient diet containing* 10% *methyl oleate (OLME) or* 10% *cod-liver oil methyl esters (CLOME)*

(Expt 2. Rats were given a diet deficient in vitamin E and marginal in vitamin A from 2 to 10 weeks of age. Then they were given 10000 i.u. vitamin A by mouth and given a 20% casein diet with the supplements shown. Expt 3. Rats at 30 days of age were given the 20% casein diet with the supplements shown. Three or more results are given as the mean and standard deviation and the number of results is shown in parentheses)

Dietary lipid	Liver		Leg muscle	Adipose tissue	
	47–58*	84†	84†	58*	84†
	Lipid peroxide (μ-equiv./g)				
OLME, 10%, alone	—	—	0·6, 0·8	—	0·3, 1·2
OLME, 10% + vitamin E‡	—	—	0·4, 0·5	—	0·0, 0·4
CLOME, 10%, alone	2·5, 0·5	—	0·5, 0·3	13·6	9·7, 16·6
CLOME, 10% + vitamin E‡	1·5, 0·7	—	0·7, 0·3	0·6	0·0, 0·6
	PUFA§ (mg/g)				
OLME, 10%, alone	—	10·1 ± 1·8 (3)	2·4, 3·7	—	21·2 ± 0·4 (3)
OLME, 10% + vitamin E‡	—	9·5 ± 1·3 (4)	3·4, 3·7	—	19·6 ± 2·4 (3)
CLOME, 10%, alone	17·1, 19·1	12·4 ± 1·6 (4)	3·9, 3·9	—	59·8 ± 10·4 (5)
CLOME, 10% + vitamin E‡	18·9, 18·5	13·4 ± 1·6 (4)	4·1, 3·8	—	83·9 ± 10·1 (4)
	Lipid (%)				
OLME, 10%, alone	—	4·9 ± 1·1 (3)	3·4, 2·2	—	93 ± 4·6 (3)
OLME, 10% + vitamin E‡	—	5·4 ± 0·5 (4)	2·8, 2·4	—	95 ± 0·7 (3)
CLOME, 10%, alone	5·3, 5·7	5·5 ± 0·8 (4)	3·3, 2·7	—	77·5 ± 0·9 (4)
CLOME, 10% + vitamin E‡	7·4, 5·6	6·0 ± 0·3 (4)	3·0, 2·5	—	92·2 ± 4·0 (4)

* Expt 3, one or two rats used per analysis.
† Expt 2, one or two rats used per analysis.
‡ D-α-tocopheryl acetate, 100 ppm.
§ Polyunsaturated fatty acids.

almost to zero. This experiment leaves open, once again, the question whether vitamin E exerted its effect by decreasing peroxidation in the diet or in the tissues. A distinctive feature of Expts 1–3 was the weight depression induced by giving rats 10% CLOME (Table 6). Vitamin E was found largely to prevent this weight loss.

Expt 4. This experiment was designed to evaluate the role of lipid peroxidation in dietary necrotic liver degeneration, induced by a deficiency of vitamin E and selenium. Rats were given the torula yeast necrogenic diet (Bunyan, Green, Diplock & Robinson, 1967*b*) from 14–30 days of age and then divided into groups to receive the same diet or that diet supplemented with Se, 0·05 ppm or D-α-tocopheryl acetate, 100 ppm. Rats were studied in the necrotic and pre-necrotic phases of the disease, but there was no increase in lipid peroxides of liver, adipose tissue, or kidney (Table 7).

Table 6. *Expts* 1, 2 *and* 3. *Effects of vitamin E and type of dietary lipid on weight changes in rats*

(Results are given as mean weight gains with standard deviations and the number of rats is shown in parentheses)

	Weight gains (g)					
Expt no. ...	1		2	3		
Feeding period (weeks of age)...	5–7	5–9	10–22	4–6	4–8	4–11
Dietary lipid:						
Methyl oleate (OLME)	13·4 ± 4·4 (9)*	26·7 ± 6·4 (9)†	115 ± 20 (5)	—	—	—
OLME + vitamin E	—	—	117 ± 41 (6)	—	—	—
Cod-liver oil methyl esters (CLOME)	4·3 ± 3·1 (9)	15·6 ± 5·3 (9)	84 ± 29 (6)	9·0 ± 4·8 (5)	20·2 ± 1·5 (5)	32·2 ± 7·0 (5)
CLOME + vitamin E	—	—	127 ± 45 (6)	12·0 ± 2·8 (4)	37·3 ± 12·3 (4)‡	56·8 ± 20·1 (4)‡

* Significantly higher than CLOME group, $P < 0.001$.
† Significantly higher than CLOME group, $P < 0.01$.
‡ Significantly higher than CLOME group, $P < 0.05$.

Expt 5. In this experiment liver necrosis was induced by treatment with silver acetate. Young rats were given diet A 10 Y 3 (Bunyan, McHale & Green, 1963) until 32 days of age and then divided into two groups. Both groups received a diet whose percentage composition was: casein ('low vitamin content'; Genatosan Ltd) 8·3, salt mixture (Diplock, Green *et al.* 1967) 5·33, vitamin mixture (Diplock, Green *et al.* 1967) 0·4, glucose 81, CLOME 2·5 and the methyl esters of maize oil (MOME) 2·5. MOME was prepared and purified as described by Green *et al.* (1967). Vitamin A was given by mouth (300 i.u./rat per week). One group was given water to drink and the other group received a solution of silver acetate (0·15 %). Lipid peroxides were

Table 7. *Expts* 4 *and* 5. *Lipid peroxides in tissues of rats developing liver necrosis due to deficiency of selenium and vitamin E or treatment with silver acetate*

(Expt 4. Liver necrosis was induced by giving rats a torula yeast diet (see above). Expt 5. Liver necrosis (95 % incidence, 5–15 days) was induced by giving rats a casein diet (see p. 481) and Ag acetate (0·15 %) in their drinking water. Three or more results are given as the mean and standard deviation and the number of analyses is shown in parentheses)

Expt no.	No. of days on diet	No. of rats per analysis	Tissue	Lipid peroxides (μ-equiv./g)		
				Rats developing liver necrosis	Normal rats	
4	9–48	2	Liver*	1·35 ± 1·07 (15)†	1·25 ± 0·67 (13)‡	1·0 ± 0·42 (8)§
	13–20	3	Adipose tissue	1·7 ± 1·2 (4)†	—	1·9 ± 1·0 (4)§
5	10–13	2	Kidney	0·62 ± 0·10 (5)†	0·34, 0·34‡	0·6 ± 0·2 (3)§
	6–9	3	Adipose tissue	2·17 ± 2·2 (4)‖	1·07 ± 0·5 (4)¶	—

* Thirteen rats given the torula yeast diet had necrotic livers.
† Rats given the torula yeast diet.
‡ Rats given Se, 0·05 ppm, as sodium selenite.
§ Rats given D-α-tocopheryl acetate, 100 ppm.
‖ Rats given silver acetate solution to drink.
¶ Rats given water to drink.

studied in the adipose tissue of these animals because it was thought that, in view of the results of Expts 1–4, any possible pro-oxidant effect of silver acetate would be shown in the adipose tissue, if anywhere. However, no real difference was found (Table 7).

Expt 6. This was an experiment on the role of lipid peroxides in the degeneration of rat testes due to vitamin E deficiency. Young male rats were given either diet A 10 Y 3 or this diet supplemented with D-α-tocopheryl acetate, 100 ppm (A 10 Y 3 + E) from birth until 4–6 months of age, when they were killed and their testes were removed, weighed and tested. Testicular degeneration had clearly occurred, to judge by the organ weights, but the lipid peroxide contents of both normal and deficient tissues were very low and there was no difference between them (Table 8). This finding contrasts with the elevated levels of β-glucuronidase activity (total and free) found in similar degenerate testes (Bunyan, Green *et al.* 1967*b*). Total PUFA of the deficient testes was lower than normal, in general agreement with the work of Bieri & Andrews (1964) and Witting & Horwitt (1966).

Expt 7. In order to produce the phenomenon of renal autolysis (Moore, Sharman & Symonds, 1958; Emmel & LaCelle, 1961), rats were given diet A 10 Y 3 until

31 days of age and then a diet with the percentage composition: casein ('low vitamin content'; Genatosan Ltd) 25, dried brewer's yeast (Marmite Ltd) 10, salt mixture (as in Expt 1) 2·73, $NaH_2PO_4.2H_2O$ 0·97, vitamin mixture (Bunyan, Green *et al.* 1967*b*) 0·4, sugar 41·3 and cod-liver oil 20. For comparison, some rats were given the same diet with the addition of D-α-tocopheryl acetate, 120 ppm, and others received a diet with 3 % lard in place of 20 % cod-liver oil. The results of these tests are shown in Table 9.

Table 8. *Expt* 6. *Lipid peroxides in the testes of vitamin E-deficient rats*

(Rats were reared on diet A 10 Y 3 or diet A 10 Y 3 + E (see pp. 481, 485) until 4–6 months of age. Three or more results are given as the mean and standard deviation and the number of results is shown in parentheses)

	Testis wt (g)	Lipid peroxide (μ-equiv./g)	PUFA (mg/g)
Deficient rats	0·73 ± 0·31 (6)*	0·18 ± 0·12 (3)	4·3, 4·1
Normal rats	1·22 ± 0·18 (4)	0·27 ± 0·22 (3)	6·1, 6·2

* Including one high value of 1·36 g for a vitamin E-depleted rat with non-degenerate testes.

The rats were about 4 months old when tested. Evidence of post-mortem renal autolysis was provided by the values for NPN production in the kidneys that had been sectioned and kept at 37° for periods of 6–24 h. At the longer times of incubation, an odour of putrefaction was observed from the kidneys of rats given 3 % lard or 20 % cod-liver oil. These kidneys were also partly liquified, with the probable loss of some NPN from the remaining tissue. NPN production was greater in the kidneys of rats given cod-liver oil than in those given lard. Dietary vitamin E appeared to decrease the degree of autolysis of the kidneys of rats given cod-liver oil. Cathepsin activity (total and free) was elevated in the kidneys of rats given cod-liver oil, in confirmation of an earlier observation by T. Moore (1966, private communication). After 24 h incubation, cathepsin activity decreased in all three types of kidney with a concomitant decrease in latency.

Lipid peroxides were measured only in the kidneys of rats given cod-liver oil with and without vitamin E. No real differences were found between the two groups, all values being of the order of 1 μ-equiv./g. Nor was there any clear indication of a change in peroxide value after incubation. The differing dietary lipids had only small effects on the PUFA content of kidney.

Expts 8 *and* 9. In these experiments, we attempted to decrease lipid peroxidation in rat tissues by prolonged feeding of vitamin E and/or other antioxidants. In Expt 8, rats were given either the vitamin E-deficient diet A 10 Y 3 or diet A 10 Y 3 + E from 14 days of age for 8 months. No difference was found in the lipid peroxide of liver but there were less peroxides in the kidneys of the rats given vitamin E (Table 10). In Expt 9, rats were given diet A 10 Y 3 + E until 3 months old. They were then divided into groups to receive either daily doses of 50 mg D-α-tocopheryl acetate by mouth or dietary supplements of DPPD, 0·1 %, or ethoxyquin, 0·1 %. Prolonged dosage with these substances did not decrease peroxides in liver compared to control rats given diet A 10 Y 3 throughout (Table 10).

Table 9. *Expt 7. Post-mortem autolysis and lipid peroxides in vitamin E-deficient rat kidneys* 0, 6, 18 *or* 24 *h after death*

(Rats were reared from 1 to 4 months old on the basal diet (25 % casein and 10 % brewer's yeast, see p. 486) with added lipids as described below. Zero-time analyses were carried out immediately after death and the others were done on kidneys that had been decapsulated, quartered and left for the time stated in a closed tube at 37°)

	Degree of autolysis (as mg tyrosine/g)			Cathepsin activity* (units/g)		Lipid peroxide (μ-equiv./g)				PUFA (mg/g)	Lipid (%)
Dietary lipid	6 h†	18 h†	24 h‡	0 h‡	24 h‡	0 h†	0 h‡	18 h†	24 h‡	0 h†	0 h†
Lard (3 %)	2·4	7·7	2·8	131 (47)	35 (94)	—	—	—	—	7·8	4·8
Cod-liver oil (20 %)	9·4	16·8	12·5	227 (81)	98 (71)	0·87	0·72	1·00	1·55	8·4	5·1
Cod-liver oil (20 %) + vitamin E, 120 ppm	4·1	10·0	7·0	138 (46)	64 (75)	1·11	1·00	1·32	0·87	7·2	4·2

* Figures in parentheses indicate 'free activity' (enzyme activity of homogenate in sucrose solution expressed as a percentage of the activity with added Triton X-100).

† Each result was obtained by the analysis of the kidneys of one rat.

‡ Two female rats from each group were used for these assays: one kidney of each rat was used for the zero-time analysis and the others for the analysis after 24 h.

Table 10. *Expts* 8 *and* 9. *Lipid peroxides in tissues of rats given vitamin E,* DPPD *or ethoxyquin, for various periods of time*

(Rats were given diet A10 Y3 from 14 days old and then the treatments described)

Expt no.	Treatment	No. of rats per analysis	Tissue	Lipid peroxide (μ-equiv./g) Treated rats	Lipid peroxide (μ-equiv./g) Control rats (given diet A10Y3)
8	Dietary vitamin E*, 8 months	2	Liver	1·06	0·96
		2	Kidney	0·08	0·36
9	Dietary vitamin E*, 3 months, then:				
	8 daily doses of 50 mg vitamin E	2	Liver	0·79	0·84
	23 daily doses of 50 mg vitamin E	2	Liver	1·65	0·66
	DPPD†, 0·1 % of diet, 9 days	2	Liver	0·22	0·75
	DPPD, 0·1 % of diet, 21 days	2	Liver	0·82	0·41
	DPPD, 0·1 % of diet, 37 days	2	Liver	0·46	0·42
	Ethoxyquin,‡ 0·1 % of diet, 14 days	2	Liver	0·30	0·49
	Ethoxyquin, 0·1 % of diet, 15 days	1	Liver	1·17	1·16

* D-α-tocopheryl acetate, 100 ppm, added to diet A10Y3 (i.e. diet A10Y3+E).
† *N,N'*-diphenyl-*p*-phenylenediamine.
‡ 6-Ethoxy-1,2-dihydro-2,2,4-trimethylquinoline.

Expts 10, 11 *and* 12. In these tests we examined the effects of vitamin E deficiency diseases on the lipid peroxide content of chick tissues.

In Expt 10, chicks were given the α-protein diet (diet 4, described by Bunyan, Green *et al.* 1967*a*) with the addition of DL-methionine, 0·5 % for 34 days. Muscular dystrophy was induced in some of the chicks by reducing their methionine supplement to 0·25 %, and chicks from both groups were killed and tested between 38 and 54 days of age. The peroxide contents of normal and degenerate breast muscle were found to be the same (Table 11).

Exudative diathesis (Expt 11) and encephalomalacia (Expt 12) were induced in chicks by giving them the diets described by Bunyan, Green *et al.* (1967*a*). Liver was studied in Expt 11, because Se has been shown to cause a significant depression in the peroxidizability in vitro of liver homogenates, as measured by the formation of malondialdehyde (Bunyan, Diplock, Edwin & Green, 1962). However, in contrast to this finding, the lipid peroxide content of the liver in vivo was not affected by dietary Se, even though all the chicks given the unsupplemented diet had exudative diathesis. No increases in peroxides were found in the cerebellum, brain or adipose tissue of chicks suffering from encephalomalacia; but there was some evidence of a slight decrease in PUFA of cerebellum (Table 11).

DISCUSSION

Much of the evidence for lipid peroxidation in vivo as a causal factor in vitamin E deficiency disease is indirect. It derives from a great body of early work on the effects of unsaturated (and often oxidized) fats in accelerating the course of certain vitamin E

Table 11. *Expts* 10, 11 *and* 12. *Lipid peroxides in the tissues of chicks with vitamin E-deficiency diseases*

(Expt 10. Muscular dystrophy (100% incidence) was produced by giving chicks a soya-bean diet (see p. 486); others were protected by dietary methionine, 0·25%. They were all 38–54 days old when tested. Expt 11. Chicks were given a torula yeast diet to produce exudative diathesis (see p. 488). Some chicks were protected by dietary Se, 0·15 ppm, as sodium selenite. They were all 20–26 days old when tested. Expt 12. Casein–lard diet (see p. 488) was used to induce encephalomalacia. Fifteen deficient chicks were tested: four were prostrate, eight had severe ataxia and three had mild ataxia. Some chicks were protected by dietary D-α-tocopheryl acetate, 50 ppm. They were all 21–26 days old when tested)

	Expt 10*	Expt 11†	Expt 12‡								
	Lipid peroxide (μ-equiv./g)§					PUFA (mg/g)			Lipid (%)		
	Breast muscle	Liver	Cerebellum	Brain	Adipose tissue	Cerebellum	Brain	Adipose tissue	Cerebellum	Brain	Adipose tissue
Diseased chicks	0·24±0·11 (5)	0·55±0·36 (9)	0·82±0·47 (3)	1·9, 0·4	0·4, 0·0	5·5	7·4, 7·5	66, 65	5·0	6·7, 6·9	90, 88
Normal chicks	0·20±0·17 (6)	0·57±0·33 (9)	1·08±0·72 (3)	1·7, 0·4	0·5, 0·0	6·9	7·7, 7·3	64, 59	6·0	7·1, 7·9	95, 92

* One chick per analysis.

† Two chicks per analysis. All the deficient chicks had exudative diathesis.

‡ Two chicks per analysis.

§ Three or more results are given as the mean and standard deviation and the number of analyses is shown in parentheses.

deficiency diseases, the formation of acid-fast pigment in deficient animals given large amounts of dietary unsaturated fat, and the ability of certain substances with 'anti-oxidant' properties to prevent or delay the onset of the deficiency disease. We have already reviewed a number of these factors (Green *et al.* 1967).

In recent years many workers have attempted to provide more direct evidence for peroxidation in vitamin E-deficient tissues by using the thiobarbituric acid reaction to determine malondialdehyde, which is believed to be formed during the oxidation of unsaturated fatty acids. However, malondialdehyde is known to be metabolized by tissues (Caputto, McCay & Carpenter, 1961), and, if precautions are taken to exclude the possibility of peroxidation in vitro after removal of the tissues, zero time measurements detect only traces of malondialdehyde and these cannot be quantitatively correlated with the development of vitamin E deficiency diseases (Bunyan *et al.* 1962; Bunyan, Green & Diplock, 1963). Most work has been concentrated on determining malondialdehyde in tissue homogenates after a preliminary incubation in vitro. Under these conditions the production of malondialdehyde is quantitatively related to the formation of peroxide during the incubation (Bieri & Anderson, 1960) and many workers regard the extent of peroxide formation in vitro as a quantitative indication of the existence of 'lipid peroxidation' in the original tissue and of value in establishing a role for vitamin E as an inhibitor of biological peroxidation (Bieri & Anderson, 1960; Zalkin & Tappel, 1960; Zalkin, Tappel & Jordan, 1960; Pritchard & Singh, 1960; Zalkin, Tappell, Caldwell, Shibko, Desai & Holliday, 1962; Bird & Szabo, 1964; Recknagel & Ghoshal, 1966). However, it is doubtful whether the in vitro test carried out on tissues ruptured in air can provide useful information about the situation in vivo. As Barber (1963) has shown, the in vitro test simply reveals the effective balance of pro- and anti-oxidant components of the incubated homogenate. In some tissues, such as brain, liver and kidney, antioxidant systems that operate in vivo may be broken down by homogenization and dilution. Furthermore, in liver necrosis, which is of critical importance in the vitamin E problem, even the formation of lipid peroxides in vitro is unrelated to the function of α-tocopherol and Se in preventing the disease (Corwin, 1962; Bunyan, Green & Diplock, 1963).

Previously we have attempted to obtain direct evidence of a connexion between vitamin E deficiency and lipid peroxidation in vivo by studies of some of the reactants directly involved—the postulated antioxidants (Green *et al.* 1967; Diplock, Bunyan *et al.* 1967) and the fatty acid substrates (Bunyan, Diplock & Green, 1967). The results were uniformly negative. We have therefore studied another component of the postulated peroxidation mechanism, true lipid peroxides in vivo.

There are many difficulties in determining peroxides in tissues. The main problems are, first, to eliminate any artefactual peroxide formation, which may commence as soon as the tissue is dissected, and, secondly, to measure accurately the small quantities of peroxide involved. The micro-method of Heaton & Uri (1958) was used because it followed the accepted procedure of iodimetry, with avoidance of the oxygen-induction effect, and yet it was somewhat more sensitive than the standard titrimetric procedures. Fairly satisfactory agreement with the macro-method was found and quantitative recoveries of peroxides added to rat liver were obtained. Taking 0·010 as the least

difference in optical density to be detected with confidence and 0·04 g as the largest sample of lipid that was soluble in the chloroform–acetic acid solution (6·3 ml), the lower limit of detectable peroxide value was 0·2 μ-equiv./g lipid or about 0·01 μ-equiv./g liver. According to Dam & Granados (1945) the iodimetric method that they used (King, Roschen & Irwin, 1933) has a lower limit of accuracy of 0·5 μ-equiv. peroxide/g lipid, although peroxide values below 1 μ-equiv./g would seem to be difficult to determine by that method. Furthermore, the reaction of peroxide with KI was carried out in air, which can lead to erroneously high values (Lea, 1952); we found that failure to maintain an atmosphere of nitrogen, when using the macro-method, raised the apparent peroxide value of an oil from 72 to 204 m-equiv./kg.

In preparing the lipid extract, vitamin E was added to the solvent mixture to limit further oxidation during handling. Emmel & LaCelle (1961) took similar precautions, but used a mixture of propyl gallate and citric acid. On the other hand, Glavind & Hartmann (1961) were concerned about losses of small amounts of peroxides and they added an excess of hydrogen peroxide while extracting, to saturate the peroxide-destroying systems. The amount of lipid peroxide destroyed in their tests was about 0·1 μ-equiv./g liver, which approximates to a 5 % loss in our recovery tests with oxidized maize oil and is within the error of determination. Even so, a loss of this amount would not detract from the value of the results when used to compare deficient and normal tissues. Milas *et al.* (1963, in discussion) found considerable destruction of peroxide by preparations of intestine, but none by preparations of liver, muscle or brain.

Milas *et al.* (1963) found no peroxide in six tissues, including adipose tissue, of vitamin E-deficient rats given a diet with 15 % cottonseed oil and 3 % methyl arachidonate, and found only traces of peroxide, no more than in the controls, in the tissues of chicks with encephalomalacia. These authors used methods differing from ours in several respects. They used 10, rather than 19, volumes of chloroform–methanol solvent per g of tissue, added no antioxidant, and did not wash the extract free of non-lipid substances. However, in our experience, these procedures did not appreciably decrease the peroxide values found when the extracts were tested by the micro-iodimetric method. Furthermore, we were able to detect the lipid peroxides in these extracts by using one of the chromatographic systems and sprays described by Milas *et al.* (1963). The negative findings by these authors may possibly have been due to loading too little extract on to the paper.

Lea (1952) has dealt fully with the defects in indophenol and thiocyanate methods similar to those used by Glavind & Hartmann (1961), Emmel & LaCelle (1961) and Milas *et al.* (1963). The results of both methods are too high when carried out in air and too low in nitrogen. Of the two, the thiocyanate method is the more sensitive and reproducible but the necessary correcting factors vary for different lipids, thus limiting its use for tissue extracts of variable lipid composition.

Among more recent developments in this field there is the highly sensitive iodimetric method of Oette, Petersen & McAuley (1963) that uses 0·0001 N-sodium thiosulphate solution and an amperometric end-point, and Hamm, Hammond, Parvanah & Snyder (1965) have recently revived interest in the diphenylcarbazide reaction of Stamm (1925).

The most significant finding of our investigation would seem to be the lack of change in the peroxide value of tissues that are primarily affected by vitamin E deficiency. Thus, in rats, lipid peroxides were not increased in necrotic liver, degenerate testis or in the kidney that autolysed in vitro. Similarly, in chicks, dystrophic breast muscle and degenerate cerebellum and brain showed no rise in peroxide value compared with normal controls. Furthermore, there was no rise in kidney and adipose tissue of rats with liver necrosis nor in the adipose tissue of chicks with encephalomalacia. Although a specific pathological lesion had not been found in the liver of chicks with exudative diathesis, this organ has a raised titre of cathepsin activity (Bunyan, Green *et al.* 1967*a*) and, when homogenized and incubated in vitro, it produces more malondialdehyde than control livers from chicks given Se or vitamin E (Bunyan *et al.* 1962). These abnormalities evidently cannot be explained by the existence of a raised peroxide value in vivo.

In the present study, raised cathepsin activity has also been found in the kidneys of rats given 20% dietary cod-liver oil for long periods, but, as already stated, this was not associated with a rise in lipid peroxides in the same tissue, either in vivo or after incubation in vitro. Emmel & LaCelle (1961) did not investigate kidney peroxides, but found great, although very variable, increases in the peroxide value of adipose tissue; a maximum mean value of 690 (range 0–2300) m-equiv./kg was reported after the rats had received the diet for 7 weeks. These authors postulated a causal relationship between adipose tissue peroxides and the renal autolytic phenomenon. However, our tests suggest that there is a special relationship between the dietary lipids and those of the adipose tissue that does not hold true for other tissues. Thus, adipose tissue responds most readily to changes in the degree of unsaturation and peroxide value of the dietary lipid. Furthermore, when vitamin E is given with the unsaturated dietary lipid, the peroxide value of adipose tissue lipid is greatly decreased, but other tissues are not affected (see Expts 2 and 3). The results of Expts 8 and 9 show that the lipid peroxides of liver are not decreased even when rats are given prolonged high doses of vitamin E, DPPD or ethoxyquin.

In summary, therefore, it would appear that, with the exception of adipose tissue, all the rat and chick tissues studied have a peroxide value in the range 10–40 μ-equiv./g lipid, whatever the dietary lipid and whether vitamin E is given or not. The peroxide value of adipose tissue from rats given diets containing OLME, with or without vitamin E, or CLOME with vitamin E, and from chicks given 20% lard, were much lower, being in the range 0·3–3·0 μ-equiv./g lipid. It was only in rats given CLOME without vitamin E for relatively long periods that the peroxide values in adipose tissue were elevated; even so, the values were within the range found for all other tissues studied, deficient or not. These results agree with the finding of El-Khatib, Chenau, Carpenter, Trucco & Caputto (1964) that there was no difference in tissue peroxides between vitamin E-deficient and -supplemented rats and rabbits. However, their estimates of lipid peroxides, obtained by testing butanol extracts by the thiocyanate method and chloroform extracts by an unspecified iodine method, were much lower than ours. We have never found, in adipose tissue, peroxide values approaching those found by Dam & Granados (1945) and Emmel & LaCelle (1961), who quote maximum figures of 190 and 2300 μ-equiv./g respectively, nor did we

observe the great variation, including numerous zero values, that they did. Some of these differences may be explicable in terms of the methods used, as we have suggested above. Christensen, Dam, Prange & Søndergaard (1958) also found high peroxide values in adipose tissue of vitamin E-deficient rats given casein diets containing 20% cod-liver oil for long periods, but recorded zero values for rats given 20% lard, in agreement with the earlier work of Aaes-Jorgensen (1949). Christensen *et al.* (1958) found zero peroxide values in adipose tissue of rats given diets containing 40% torula yeast and supplemented with Se, although it was clear from their experiments, in agreement with our present findings, that Se had no antioxidant effect, for it did not decrease the peroxide value of adipose tissue from rats given the casein diets with cod-liver oil; in fact the peroxide value increased considerably. Thus, the elevation of peroxide values in adipose tissue that is often cited (Tappel, 1962; Dam, 1962) in discussing the antioxidant function of vitamin E would seem to be irrelevant to the real problem of vitamin E deficiency, the manifestations of which are readily produced in many species with diets containing only small amounts of unsaturated lipid. Rather, such elevated values as may be found, and our experiments showed that the true values may be much lower than usually cited, must be regarded as related to the special effects of feeding excessively large amounts of highly unsaturated fatty acids. This conclusion is the same as that already arrived at from our other studies on tocopherol metabolism.

What, then, is the origin, in most tissues, of this apparently stationary level of peroxides, and why is it unaffected by vitamin E or by substantial amounts of dietary unsaturated lipid? There seems little reason to doubt that the methods used do measure, specifically, true lipid peroxides. The fact that the method reveals nearly zero values with certain types of test material, such as the adipose tissue of rats given vitamin E (Expt 2) and pure preparations of methyl esters of unsaturated fatty acids (unpublished), shows that artefacts were not formed at the stage of reaction with KI. The artefactual formation of peroxide during extraction of the tissue appears unlikely because oxidized lipid added to tissue preparations could be recovered virtually quantitatively; if peroxidation were to occur at this stage, co-oxidation, with a greater than theoretical recovery of peroxide, would be expected. It remains possible, however, that the peroxides found are artefactual in the sense that they are formed after death and before extraction. If, on the other hand, the tissue peroxides are truly present in vivo and arise by the continual peroxidation of lipids and if this reaction is inhibited by tocopherol, then the only way in which the peroxide level could remain constant is if tocopherol also decreased the rate of decomposition of the formed peroxides. However, such a situation would seem to be inadmissible; we have shown (Table 2) that α-tocopherol does not affect the decomposition of peroxides in vitro at room temperature, whilst Privett & Quackenbush (1954) demonstrated that, at elevated temperatures, α-tocopherol accelerated peroxide decomposition. Glavind *et al.* (1961) studied the metabolism of injected triglyceride peroxide in the chick and found that it was unaffected by the vitamin E status of the animals; they concluded that the tocopherols are probably not involved in peroxide metabolism. It may be noted that DPPD and ethoxyquin are also without effect in vivo (Expt 9).

It would seem difficult to account for all these observations on the basis of current theories of peroxidation mechanisms. Witting & Horwitt (1964) pursuing the idea that such mechanisms are fully operative in vivo and are, indeed, the sole cause of vitamin E deficiency disease, have calculated the 'peroxidizability' of tissue lipids from their PUFA composition. They have stated that peroxidation in vivo is simply a function of this peroxidizability and the vitamin E content of the tissue. Using their method, it is possible to calculate that the 'peroxidizability' of many of the tissues of our rats given CLOME had substantially increased. It follows from our results that there is no relationship between the so-called 'peroxidizability' of the tissue or the vitamin E status of the animal and the existence of lipid peroxides in vivo.

A satisfactory solution of the dilemma is not immediately apparent. Hove & Harris (1946) suggested that peroxidation in vivo of polyunsaturated fatty acids might serve a metabolic function and that linoleic acid peroxides might act as terminal hydrogen acceptors for some chain of enzymic oxidation, such as the β-oxidation of fats. Schwarz (1962) has also considered the possibility that lipid peroxides might themselves be normal metabolites, possibly involved in oxygen transfer systems. It is perhaps not altogether unlikely that they are formed in tissues by a mechanism different from that of autoxidation in vitro. It seems to be certain, however, that their presence is unrelated to the biological role of vitamin E.

Against this background, the flexibility of the peroxide level in adipose tissue may be regarded, not as typical of the whole body situation, but as exceptional. Possibly the behaviour of adipose tissue is related to the ingress of peroxidized fatty acids from the diet. This we have shown to be difficult to control even by stringent precautions (Expt 1 and other, unpublished, observations).

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