Vitamin E and stress

2. The metabolism of D-α-tocopherol and the effects of stress in vitamin E deficiency in the chick

By A. T. DIPLOCK, J. BUNYAN, D. McHALE and J. GREEN

Walton Oaks Experimental Station, Vitamins Ltd, Tadworth, Surrey

(*Received* 6 *June* 1966—*Accepted* 19 *September* 1966)

1. The metabolism of small amounts of [5-Me-^{14}C]D-α-tocopherol and [5-Me-^{3}H]D-α-tocopherol has been studied in the vitamin E-deficient chick. Small doses of the labelled tocopherol were given to chicks, which were then subjected to stress by giving them diets formulated to produce encephalomalacia, exudative diathesis or muscular dystrophy.

2. Tocopherol concentrations in the cerebella and brains of chicks with incipient encephalomacia were the same as those in normal chicks in which the dietary fat stress was absent.

3. α-Tocopherol delayed the onset of encephalomalacia by a mean value of 3·5 days when its concentration in the cerebellum was about 2×10^{-7}g/g of lipid. This concentration is considerably below the usual effective concentrations of antioxidants in vitro.

4. Selenium deficiency, under conditions leading to a high incidence of exudative diathesis, was not associated with lowered tocopherol levels and did not result in detectable destruction of tocopherol.

5. Nor was there any destruction of tocopherol or significant effect on its metabolism in the processes leading to muscular dystrophy: on the contrary, the affected muscles of dystrophic chicks, which had received a diet deficient in sulphur amino acids, contained significantly more tocopherol than muscles from control birds.

6. These results do not support the hypothesis that lipid peroxidation is a causative process in the aetiology of vitamin E-deficiency diseases in the chick. The relationships between unsaturated lipid, Se, sulphur amino acids and tocopherol in the chick require further exploration.

When the vitamin E-deficient rat is subjected to the dietary stress of high levels of polyunsaturated fatty acids, there is little effect on the metabolism of tracer doses of [^{14}C]D-α-tocopherol in any organ except (to a small degree and after a long time) in adipose tissue. It was concluded (Green, Diplock, Bunyan, McHale & Muthy, 1967) that lipid peroxidation is probably not significantly concerned in the aetiology of vitamin E deficiency disease in the rat and that the antioxidant theory was not adequate to explain the role of vitamin E. In this paper, results of a study of [^{14}C]α-tocopherol metabolism in the chick are described.

Vitamin E deficiency leads to three well-defined diseases in the young chick. Encephalomalacia is produced, usually between 3 and 5 weeks of age, by adding polyunsaturated fatty acids (especially linoleic acid) to the diet, which in most published experiments has been based on casein. The disease is apparently due to fragility of the vascular system of the cerebellum and is generally regarded as being caused by peroxidation *in situ* in the cerebellum (Dam, 1944*a*, *b*; Century, Witting, Harvey & Horwitt, 1963). The specific effect of linoleic acid is considered to be related to its preferential incorporation into brain tissue. The disease can be prevented by extremely small amounts of α-tocopherol and also by synthetic antioxidants (in larger quantities, usually), but it is only delayed, not prevented, by selenium.

With diets containing smaller amounts of fat but deficient in Se as well as vitamin E, chicks of about 3 weeks of age develop exudative diathesis, which is characterized by the appearance of copious green exudates in the subcutaneous spaces (Dam & Glavind, 1940; Dam, 1942). The disease is influenced by a variety of other dietary factors, being sensitive to mineral balance (Bieri, Briggs & Pollard, 1958; Gitler, 1958) and traces of toxic substances. It is especially related to the presence of small amounts of fatty acids of the linolenic series in the diet. It is not clear why linolenic acid seems specially involved as a stress for this condition (Dam, Nielsen, Prange & Søndergaard, 1958*a*), nor has it been clearly established whether the production of the disease requires the presence of some minimal amount of the dietary unsaturated lipid. If the latter, the amount must be small, for exudates can be produced with diets containing yeast almost free of dienoic fatty acids and with no polyenoic fatty acids (Dam, Nielsen, Prange & Søndergaard, 1958*b*). Exudates are not readily produced on non-fat diets containing casein as the source of protein, but this may be because the condition is alleviated by minute amounts of Se and many types of casein contain sufficient quantities of this trace element.

Exudative diathesis is often said to be caused by an increase in capillary permeability, which, in turn, is considered to be produced by 'lipid peroxidation'. However, Dam *et al.* (1958*b*) were unable to detect peroxides in the tissues of birds suffering from the disease.

On certain casein-based diets and in the absence of severe fat stress, chicks develop another deficiency disease, muscular dystrophy. The condition develops during the first 4–5 weeks of life and was first described by Dam Prange & Søndergaard (1952). The disease seems to be precipitated primarily by a deficiency of sulphur amino acids and can be prevented by dietary cystine (Dam *et al.* 1952) or methionine (Machlin & Shalkop, 1956). Nesheim, Calvert & Scott (1960) showed that the disease could be produced by increasing dietary arginine, and it now seems clear that dystrophy occurs when sulphur amino acids are limiting for growth (Jenkins, Hill, Hutcheson & Branion, 1962). The dystrophy is readily preventable by tocopherol, even when sulphur amino acids are limiting. The disease can occur in the total absence of dietary fat and can then be partially prevented by fairly high levels of Se (0·1–0·5 ppm: exudates are prevented with as little as 0·03 ppm). The presence of about 0·5 % linoleic acid (but apparently, not linolenic acid) in the diet has a pronounced stress effect on the dystrophy and, in contrast to what happens under conditions leading to exudative diathesis, the presence of the linoleic acid renders even high concentrations of dietary Se ineffective (Jenkins *et al.* 1962). Tappel (1962) has suggested that muscular dystrophy is primarily produced by lipid peroxidation, leading to a breakdown of the lysosomal membranes and eventually to death of the muscle cells.

If any of the three diseases in the chick is in fact associated with tissue peroxidation, it should be possible to show this by an effect on the metabolism of isotopically labelled α-tocopherol in the period immediately before the onset of disease.

EXPERIMENTAL AND RESULTS

Chicks. One-day-old male chicks (either Rhode Island Red × Light Sussex strain or Warren hybrids) were obtained from a commercial breeder. They were reared in electrically heated wire-floored cages and provided with food and water *ad lib.*

Diets. The diets for experiments on encephalomalacia had the percentage composition: casein ('low vitamin' content; Genatosan Ltd) 18, gelatin 10, L-arginine 0·4, DL-methionine 0·4, glucose 59·85, either methyl oleate (OLME) or maize-oil methyl esters (MOME) 4, salt mixture (Bunyan, Diplock, Edwin & Green, 1962) 6·75, vitamin mixture (Bunyan *et al.* 1962) 0·2, choline dihydrogen tartrate 0·4, chlortetracycline 0·2 ppm, vitamin A at 25 i.u./g and vitamin D_3 at 3·3 i.u./g (the vitamins as gelatin-coated beadlets). The exudate-producing diet had the percentage composition: torula yeast 60, lard 4, DL-methionine 0·6, L-arginine 0·2, glycine 0·5, glucose 27·75, and had the same additions of salt and vitamin mixtures, choline, chlortetracycline, vitamins A and D as for the encephalomalacia-producing diet. The dystrophy-producing basal diets 1, 3 and 4 were those described by Bunyan, Green, Diplock & Robinson (1967).

Methyl esters. These were obtained from oleic acid and maize oil by methods described previously (Green *et al.* 1967).

Tocopherol. D-α-tocopherol was prepared from α-tocopheryl acid succinate, and [5-Me-^{14}C]D-α-tocopherol and [5-Me-^{3}H]D-α-tocopherol were prepared as previously described (Green *et al.* 1967).

Dosing. Doses of radioactive tocopherol were freshly prepared as emulsions in 0·9 % (w/v) NaCl (with Tween 80) and given orally, intraperitoneally or intravenously (via the median vein).

Analyses. Radioactive α-tocopherol and its metabolites in carcasses and muscle were determined as described by Green *et al.* (1967). Brains and cerebella contained too much lipid for the general method to be satisfactory; these tissues were therefore saponified, in the presence of carrier non-radioactive tocopherol, by the method of Mervyn & Morton (1959) and sterols were removed from the non-saponifiable extract with digitonin (Diplock, Green & Bunyan, 1963). The remainder of the analytical procedure was then the same as for other tissues.

Encephalomalacia

Expt 1. Since encephalomalacia has been considered to be caused by peroxidation *in situ* in the brain or cerebellum the concentration of tocopherol in these organs was measured in the normal and pre-encephalomalacic chick. The concentration of tocopherol in the cerebellum is extremely difficult to measure by the usual methods of chemical analysis, but valuable information can be obtained by the use of isotopically labelled tocopherol. The first experiment was a preliminary one to determine the smallest amount of labelled tocopherol that could be measured in the cerebellum and a suitable dose that could be given to the chick without greatly affecting the course of the disease.

Thirteen 1-day-old cockerels (Rhode Island Red × Light Sussex) were given the

diet containing OLME. On the 7th and 8th days of age each chick was given an oral dose of 77·2 μg (4070 dps (disintegrations/sec)) [^{14}C]α-tocopherol. On the 9th day, the chicks were divided into two groups. Group 1 continued to receive the diet with OLME and group 2 received the diet with MOME. Three chicks from each group were killed on the 17th day, and their carcasses, specimens of breast muscle and brains were analysed for [^{14}C]α-tocopherol. Tissues were stored at $-20°$ before analysis. The remaining chicks in each group continued to receive their diets and the appearance of encephalomalacia was recorded. All three birds in group 1 remained healthy up to the 30th day. The four birds in group 2 developed encephalomalacia on days 19, 20, 20 and 22, respectively.

Table 1. *Expt* 1. *Metabolism of* [^{14}C]α-*tocopherol in chicks receiving an encephalomalacia-producing diet* (*with* 4% *maize oil methyl esters* (*MOME*)) *compared to chicks receiving a control diet* (*with* 4% *methyl oleate* (*OLME*))

(Each bird received orally 154·4 μg (8140 dps) [5-Me-^{14}C]α-tocopherol. For details of dosing, see above. Each result was found on a pooled sample from three birds in each group)

Dietary lipid	Mean chick wt at death (g)	[^{14}C]α-tocopherol Mean carcass (total dps)	Brain (dps/g)	Muscle (dps/g)
OLME	188	1014	0·9	6·7
MOME	172	1108	1·4	5·3

Table 1 gives the results. There was no difference between the groups in the concentration of tocopherol in the carcass or breast muscle. The counts in the brain were extremely small and no significance can be attached to them. The experiment therefore suggested that if significant counts in brain or cerebellum were to be obtained a larger dose of radioactive tocopherol would have to be given. It was realized that a larger dose would delay the onset of disease by several days (cf. Horwitt, Harvey, Century & Witting, 1961), but a compromise appeared necessary.

Expt 2. This experiment was essentially a repetition of Expt 1, but ten times as much tocopherol was given and, to increase sensitivity still further, [^{3}H]tocopherol of about ten times the specific activity of the [^{14}C]tocopherol was used (Green *et al.* (1967) have shown that [^{3}H] in the 5-methyl group of α-tocopherol does not undergo exchange reactions in vivo). It was desirable to allow the labelled tocopherol to remain in the pre-malacic chicks for as long as possible, for presumably it is in the period before the onset of clinical disease that lipid peroxidation might be expected to have its effect. On the other hand, it was realized that, since the disease must occur only when there is a threshold amount of tocopherol in the brain, it would not be possible to leave the birds right up to the time when the signs of disease appeared, for this would merely repeat the conditions of Expt 1 in which we were unable to detect significant amounts of labelled tocopherol in the brain.

Twenty-eight 1-day-old Warren cockerels were given the diet containing OLME for 8 days. They were then weighed and allocated in pairs to one of two groups, so that a pair in group 1 was as close in weight as possible to a pair in group 2. Four pairs in

each group were then given a single intraperitoneal dose of 1 mg (677 500 dps) [^{3}H]α-tocopherol per bird; the four pairs dosed were chosen to be as close in weight to each other as possible. The remainder of each group was each given a similar dose of unlabelled α-tocopherol. Group 1 continued to receive the diet with OLME, and group 2 were transferred to the diet containing MOME. At 17 days of age the chicks given the radioactive tocopherol were killed and their cerebella and the remainder of the brains and carcasses (after removal of the intestinal tract) were analysed for [^{3}H]tocopherol and its metabolites. Petechial haemorrhages of the cerebellum were observed at this time *post mortem* in two birds from group 2. The remainder of each group continued to receive their diets until encephalomalacia was observed in group 2.

Table 2. *Expt 2. Metabolism of* [^{3}*H*]α*-tocopherol in chicks receiving an encephalomalacia-producing diet (with* 4% *maize-oil methyl esters (MOME)) compared to chicks receiving a control diet (with* 4% *methyl oleate (OLME))*

(Each chick received intraperitoneally 1 mg (677 500 dps) of [5-Me-^{3}H]D-α-tocopherol on day 8. For details of dosing see above. Chicks were killed for analysis on day 17 and analysed in pairs. Results are given as means with standard deviations)

	Dietary lipid	
	OLME	MOME
No. of pairs of chicks analysed	4	3
Wt when dosed (g)	62 ± 6	61 ± 4·8
Wt gain (days 8–17) (g)	48 ± 8·9	60 ± 14·8
Encephalomalacia in chicks not killed on day 17* (6 birds per group)	None	33 (2), 34 (2), 37 (2)
Carcass		
Tocopherol (dps/chick)	84 800 ± 13 700	98 400 ± 17 950
Metabolites (dps/chick)	47 700 ± 42 500	44 600 ± 16 140
Cerebellum		
Weight (g)	0·23 ± 0·02	0·25 ± 0·02
[^{14}C]tocopherol		
dps/g	50 ± 35	73 ± 57
ng/g	39 ± 26	54 ± 42
Remainder of brain		
Weight (g)	1·27 ± 0·04	1·50 ± 0·29
[^{14}C]tocopherol		
dps/g	78·6 ± 24	59·0 ± 16
ng/g	58 ± 18	43 ± 12

* Figures indicate age of chick when disease developed, with numbers in parentheses.

The results (Table 2) show that there was no significant difference between the two groups in the metabolism of tocopherol. This is so whether the results from the carcass or from the brain and cerebellum are considered. The variance in the results for the cerebellum was high, possibly because of bird-to-bird variation, but also related to analytical difficulties with the small amounts of tissue. The concentration of radioactive tocopherol was the same in the cerebellum as in the remainder of the brain. This concentration was only one-thousandth of the average carcass concentration, illustrating the relative slowness with which brain tissue takes up exogenous lipids.

In Expt 2, encephalomalacia developed later than in Expt 1. Since this difference

could have been partly due to the comparatively large dose of labelled tocopherol given in Expt 2, we carried out a separate experiment to measure the delaying effect of a 1 mg dose of α-tocopherol. We found, that compared to controls, this dose delayed the mean time of onset of the disease by 3·5 days; details are not given in the tables, but are available.

Exudative diathesis

Expt 3. Twenty-eight 1-day-old Warren cockerels were given the basal exudate-producing diet until they were 8 days old. They were then weighed and allocated equally to two groups, so that each pair in group 1 was close in weight to a corresponding pair in group 2. Four pairs of chicks in each group, selected to be as close in weight as possible, were then given a single intraperitoneal dose of 250 μg (135000 dps) [^{3}H]α-tocopherol. The remainder of the birds were given a similar dose of unlabelled α-tocopherol. After 24 h the diet of the birds in group 2 was supplemented with 0·15 ppm Se, as sodium selenite. On the 16th day the chicks that had received the radioactive tocopherol were killed and their carcasses, with alimentary tract and contents removed, were analysed in pairs for radioactive tocopherol and its metabolites. The remainder of the birds continued on test for another 6 days.

Table 3. *Expt* 3. *Metabolism of* [^{3}*H*]α*-tocopherol in chicks receiving an exudate-producing diet compared with that in chicks receiving the same diet supplemented with selenium* (0·15 *ppm*)

(Each chick received intraperitoneally 250 μg (135000 dps) [5-Me-^{3}H]D-α-tocopherol on day 8. For details see above. Results are given as means with standard deviations)

Diet	No. of chicks analysed	Chick wt when dosed (g)	Wt gain (days 8–16) (g)	Carcass	
				Tocopherol (dps/chick)	Metabolites (dps/chick)
Basal	8	64·2 ± 4·1	59·1 ± 14·6	18200 ± 5076	5275 ± 1676
Basal + Se	8	64·2 ± 3·3	56·5 ± 13·7	21500 ± 5656	8550 ± 1676

Table 3 gives the results. Five out of six of the surviving birds in group 1 developed exudative diathesis during the 6 days after the birds used for analysis were killed. In addition, three of the eight birds of group 1 taken for analysis on the 16th day showed slight exudates *post mortem*. No exudates occurred in group 2. The analytical results show that the metabolism of tocopherol was unaffected by the biological processes leading to exudative diathesis.

Muscular dystrophy

Muscular dystrophy in the vitamin-E deficient chick can be produced at about 35 days of age by rearing birds on a diet deficient in sulphur amino acids or by withdrawing methionine at about 30 days from a diet adequate in this amino acid (Desai, Calvert & Scott, 1964). Nesheim *et al.* (1960) produced dystrophy by adding arginine to the diet from the 1st day, thus producing a relative deficiency of sulphur amino acids. It occurred to us that the addition of arginine, if delayed until about 30 days,

might precipitate dystrophy within a few days, as does methionine withdrawal. These two procedures were of special interest to us for two reasons. First, they would provide a means of producing a vitamin E deficiency disease by a dietary stress other than unsaturated fat. Secondly, it would be possible to introduce the stress immediately after giving the tracer dose of radioactive tocopherol in the expectation that the tocopherol would be subjected to the metabolic consequences of the stress condition during virtually the whole of the critically important period before the onset of disease.

Expt 4. This was a pilot experiment in which dystrophy was produced in three ways. The experiment was divided into several sections, carried out simultaneously.

Section 1: twenty-three 1-day-old cockerels (Rhode Island Red × Light Sussex) were divided into four groups, which were treated as follows:

Group 1 received the basal dystrophy-producing diet 1.

Group 2 received diet 1 with the addition of 0·44 % DL-methionine.

Group 3 received diet 1 with the addition of 0·44 % DL-methionine for 29 days and, thereafter, this diet with 0·44 % methionine and supplemented with 0·33 % L-arginine.

Group 4 received the same diet as group 2 for 29 days and, thereafter, diet 1 (i.e. the methionine was withdrawn).

On the 29th day, two chicks from each group were given, by intravenous injection, 48·4 μg (6437 dps) [^{14}C]α-tocopherol and two other chicks from each group were given, similarly, a dose of unlabelled α-tocopherol. All the chicks were killed on the 35th day of test and examined for dystrophy. The breast muscles and carcasses of the chicks given the radioactive dose were analysed for [^{14}C]α-tocopherol.

Section 2: six 1-day-old cockerels (as above) were divided into two groups. Group 1 received the dystrophy-producing diet 3 for 35 days; group 2 received this diet for 29 days and, thereafter until the 35th day, the diet supplemented with 0·4 % DL-methionine. On the 29th day, two chicks from each group were given 48·4 μg of the [^{14}C]α-tocopherol intravenously. These chicks were killed on the 35th day and their breast muscles and carcasses analysed.

Section 3: nine 1-day-old cockerels (as above) were divided into three groups and treated as follows:

Group 1 received the dystrophy-producing diet 4 for 35 days.

Group 2 received diet 4 with 0·6 % DL-methionine for 35 days.

Group 3 received the diet of group 2 for 29 days and thereafter diet 4.

Two chicks in each group were given 48·4 μg of the [^{14}C]α-tocopherol intravenously on the 29th day, and all the birds were killed on the 35th day. Analyses were as for the other birds.

The incidence of disease is recorded in Table 4. Dystrophy was produced with all three basal diets, 1, 3 and 4, and prevented by dietary methionine. When arginine was introduced into the methionine-supplemented diet at 29 days of age (section 1, group 3), mild dystrophy was produced only in one chick during the next 6 days (cf. Nesheim *et al.* (1960), who found a high incidence of dystrophy when they gave

Table 4. *Expt 4. Metabolism of [^{14}C]α-tocopherol in chicks given diets stressed in various ways so as to produce muscular dystrophy*

(Each chick received 48·8 μg (6437 dps) [5-Me-^{14}C]D-α-tocopherol by intravenous injection on the 29th day of age, and then received the stressed diets for another 6 days. Each analysis relates to one pair of birds. For details of dosing and diet changes, see p. 109).

Section	Group	Diet and change of diet*	No. of birds in group	No. with dystrophy at 35 days and rating†	Mean chick wt		[^{14}C]tocopherol			
							Carcass		Breast muscle	
					29 days (g)	35 days (g)	dps/chick	μg/chick	dps/g	μg/g
1	1	1	6	1^{+}, 2^{++}	279	389	3087	23	2·4	0·018
	2	1+methionine	5	0	320	432	3079	23	1·1	0·008
	3	1+methionine (+arginine at 29 days)	6	1^{+}	347	500	3703	28	1·6	0·012
	4	1+methionine → 1	6	1^{++}, 5^{+++}	342	430	3404	26	3·7	0·028
2	1	3	4	1^{+}, 1^{++}, 2^{+++}	165	208	3477	26	7·7	0·058
	2	3+methionine	2	0	168	262	3815	29	5·5	0·041
3	1	4	4	2^{+}, 2^{+++}	146	190	3336	25	7·2	0·054
	2	4+methionine	2	0	229	312	2824	21	3·6	0·027
	3	4+methionine → 4	3	1^{++}, 2^{+++}	293	327	3259	25	6·4	0·048

* Diet change at 29 days. † Slight = +; moderate = ++; severe = +++.

Table 5. *Expt 5. Metabolism of [^{14}C]α-tocopherol in chicks in which dystrophy was produced by withdrawal of methionine from the diet*

(Each chick received 48·83 μg (5650 dps) [5-Me-^{14}C]D-α-tocopherol by intravenous injection on the 27th day of age, and then received the stressed diets for another 7 days. Each analysis relates to one pair of birds. For details of dosing and diets, see p. 111. Results are given as means with standard deviations)

Group	Dietary methionine from days 27 to 34 (as % of requirement)	No. in group	No. with dystrophy at 35 days and rating*	Chick wt (27 days) (g)	Wt gain (days 27–34) (g)	Carcass			Breast muscle		
						[^{14}C]tocopherol		[^{14}C]metabolites	[^{14}C]tocopherol		[^{14}C]metabolites
						dps/chick	μg/chick	(dps/chick)	dps/10 g	μg/g	(dps/10 g)
1	100	6	0	248±12	133±15·5	2138±370	18·5±3·2	1270±252	16·5±6·7	0·015±0·005	3·5±1·0
2	72	6	2^{+}, 3^{++}, 1^{+++}	249±14	124±18·9	2066±66	17·9±0·6	1770±248	36·6±3·3‡	0·032±0·003‡	4·5±1·3
3	47	4	4†	248±6	34·7±11·9†	2429±358	21·0±3·1	1310±144	31·2±7·1§	0·027±0·006§	5·2±1·8

* Slight = +; moderate = ++; severe = +++. † Group 3 significantly lower than group 1 or 2, $P < 0·001$. ‡ Group 2 significantly higher than group 1, $P < 0·01$. § Group 3 significantly higher than group 1, $P < 0·05$.

arginine from the 1st day of age). Methionine withdrawal produced severe dystrophy (section 1, group 4 and section 3, group 3), as found by Desai *et al.* (1964).

Table 4 also gives the analytical results. The values for chick carcasses show no evidence of destruction of tocopherol during the onset of dystrophy; in sections 1 and 3, values for dystrophic chicks were not higher than values for controls; in section 2 there was a small decrease. Groups 2 and 4 of section 1 and groups 2 and 3 of section 3 are especially important, for an advantage of producing dystrophy rapidly by the methionine-withdrawal method is that the final weight of the dystrophic birds is hardly depressed; whereas if continuous methionine deficiency is used to produce the dystrophy, there is a marked depression of growth which complicates to some extent the interpretation of the results. In all three sections of the experiment, however, the results for breast muscle show an important trend in that the onset of dystrophy is characterized, not by a decrease, but by an increase (up to twofold) of tocopherol concentration in the muscle. Compare, for example, groups 1 and 2 of section 1, groups 1 and 2 of section 2, and groups 1 and 2 of section 3, where the dystrophy was prevented by methionine supplementation; or compare groups 2 and 3 of section 1, where the dystrophy was precipitated (although only mildly) with arginine; or groups 2 and 4 of section 1 and groups 2 and 3 of section 3, where the disease was precipitated by methionine withdrawal.

Expt 5. This experiment was a more detailed study of muscular dystrophy induced by methionine withdrawal, which in the previous experiment had produced the most severe muscle lesions. Twenty-eight 1-day-old Warren cockerels were given the dystrophy-producing diet 4 supplemented with 0·5% DL-methionine for 27 days. They were then weighed and examined through the skin for signs of dystrophy. All the birds seemed healthy. Pairs of chicks were selected to give a total weight of 490–501 g per pair, and eight such pairs were randomized between three groups, which were then given the diets as follows: group 1 continued to receive diet 4 supplemented with 0·5% DL-methionine, which contributed 100% of the chick's estimated methionine requirement; group 2 received the same diet in which the added methionine was reduced from 0·50% to 0·25% (the modified diet contained only 72% of the estimated requirement); group 3 received diet 4 without added methionine (this diet contributed only 47% of the chick's estimated need). Before being given these diets, each chick was given intravenously 48·83 μg (5650 dps) [^{14}C]α-tocopherol. The chicks received the three diets for 7 days, after which they were killed and then examined for dystrophy. The carcass, without the intestinal tract and contents, and a sample of breast muscle from each pair were analysed as before.

Table 5 gives the results. Moderate to slight dystrophy of the breast muscle was found in all the birds of group 2, but only slight dystrophy occurred in group 3. The birds in group 2 gained in weight almost as well as those in group 1, but there was a severe growth retardation in group 3. There was no significant change in the metabolism of tocopherol in the carcasses of the affected birds, and the amounts of tocopherol and its metabolites recovered from all three groups were similar. In agreement with the results of the previous experiments, however, there was a significant difference in the tocopherol content of the breast muscle, the affected birds having twice

as much as the controls. In groups 1 and 2, the increase was most marked and was obviously unaffected by any consideration of differences in weight gain over the experimental period.

DISCUSSION

We have discussed previously (Green *et al.* 1967) the use of tracer amounts of radioactive tocopherol to detect the possible occurrence of lipid peroxidation in the rat. Using the same methods, we have studied the three vitamin E deficiency diseases in the chick and our results suggest that they are not caused by lipid peroxidation.

Dietary unsaturated fatty acids are definitely required to produce encephalomalacia. Thus Machlin, Marco & Gordon (1962), who found significant changes in the unsaturated fatty acid pattern of the cerebella of chicks given linoleic acid, considered that encephalomalacia is 'a result of increased concentrations of such fatty acids in the brain concomitant with a depletion of antioxidant from this tissue'. That there must be such a depletion is, as we have said, a necessary corollary of the antioxidant theory of vitamin E function. However, we have been unable to find an increased rate of destruction of tocopherol in either cerebellum or brain of chicks given sufficient polyunsaturated fatty acid esters to produce the disease. Budowski & Mokadi (1961) and Glavind & Søndergaard (1964) found a decrease in the concentration of certain antioxidant substances in the brains and cerebella of chicks with encephalomalacia. However, the latter authors found, and their findings agree in this respect with ours, that there was no change in the concentration of fat-soluble antioxidants, but only of 'water-soluble antioxidants' such as ascorbic acid and thiols. Glavind & Søndergaard (1964) comment on the unexpected nature of this result, for these 'water-soluble antioxidants' have no effect on encephalomalacia. It is possible that the changes recorded by Budowski & Mokadi (1961) and Glavind & Søndergaard (1964) were a late manifestation of degeneration of the brain tissue; whereas, in the experiments described here, we have compared tissues in their pre-pathological state when, however, the causative biochemical changes should be operative.

It was found that a single dose of 1 mg of D-α-tocopherol delayed the onset of encephalomalacia by several days. It can safely be concluded that the resulting concentration of tocopherol in the cerebellum must be effective in overcoming for this length of time the metabolic stress imposed by the dietary polyunsaturated fatty acids, whatever the nature of this stress is. It is of interest, therefore, to consider the concentration of [^{3}H]α-tocopherol we found in the cerebellum (Expt 2), which was about 2×10^{-5} % on a lipid basis, assuming about 50 % lipid in the tissue. Contrast this with antioxidant concentrations known to be effective in vitro. Thus, α-tocopherol is generally found to be of maximum effectiveness at about 0·01–0·05 %. Lea & Ward (1959) found some prolongation of the induction period of peroxidizing methyl linoleate when tocopherols were used at 3×10^{-3} %. Machlin, Gordon & Meisky (1959) found that a concentration of about 0·02 % (on a lipid basis) of α-tocopherol was necessary to reduce the peroxidation of liver homogenates in vitro by 50 %. It seems that the effective cerebellar concentration of tocopherol is at least two orders of magnitude below known effective antioxidant concentrations in vitro.

Exudative diathesis can be regarded as a stress condition that is produced in the vitamin E-deficient chick by a deficiency of Se. We found (Expt 3) that the metabolism of α-tocopherol was unaffected by Se deficiency or, presumably, by the biochemical events leading to the disease. It must be concluded that Se does not spare tocopherol in the molecular sense. If the two substances act synergistically in the chick (Scott, 1962), it seems more likely that they function in alternate biochemical pathways.

The muscular dystrophy experiments yielded the significant finding that the tocopherol concentration of dystrophic muscle was about twice as high as in muscle from control birds, although no change was found in the rest of the carcass. This difference could not be accounted for by a change in the gross weight of the affected tissue. It would appear to indicate a diminished power of the dystrophic tissue to utilize tocopherol for its normal purposes. The results of Expts 4 and 5 suggested indeed that the highest tocopherol concentrations were associated with the greatest severity of the dystrophy. Whatever the reason for the difference, it seems irreconcilable with the requirements of the lipid peroxidation hypothesis. This confirms the recent view of Desai *et al.* (1964) that peroxidation is not a primary process in muscular dystrophy, but that this disease is primarily concerned with sulphur amino acid metabolism, which is mediated in some way by tocopherol and, in some circumstances, by Se. It is of interest that Blaxter, Brown & MacDonald (1953) obtained results similar to ours when they studied tocopherol levels in liver, muscle, adipose tissue and plasma of the dystrophic calf. They found no significant differences in any tissue except degenerate white muscle, which contained more tocopherol than normal muscle. They commented that, even within the muscle, higher tocopherol concentrations were associated with the severity of the dystrophy.

Dam (1944*a*), in his classical study of chick deficiency diseases, suggests that the effect of polyunsaturated fatty acids in these conditions may not be associated with the destruction in vivo of vitamin E, but rather with abnormal tissue reactions in its absence. The nature of the interrelationships between unsaturated lipid, tocopherol, Se and sulphur amino acids in the chick are still far from being understood; it would seem, from our results, that further elucidation of the problem will come from focusing attention on aspects of metabolism other than lipid peroxidation.

REFERENCES

Bieri, J. G., Briggs, G. M. & Pollard, C. J. (1958). *J. Nutr.* **64**, 113.
Blaxter, K. L., Brown, F. & Macdonald, A. M. (1953). *Br. J. Nutr.* **7**, 287.
Budowski, P. & Mokadi, S. (1961). *Biochim. biophys. Acta* **52**, 609.
Bunyan, J., Diplock, A. T., Edwin, E. E. & Green, J. (1962). *Br. J. Nutr.* **16**, 519.
Bunyan, J., Green, J., Diplock, A. T. & Robinson, D. (1967). *Br. J. Nutr.* **21**, 127.
Century, B., Witting, L. A., Harvey, C. C. & Horwitt, M. K. (1963). *Am. J. clin. Nutr.* **13**, 362.
Dam, H. (1942). *Proc. Soc. exp. Biol. Med.* **52**, 285.
Dam, H. (1944*a*). *J. Nutr.* **27**, 193.
Dam, H. (1944*b*). *J. Nutr.* **28**, 297.
Dam, H. & Glavind, J. (1940). *Naturwissenschaften* **28**, 207.
Dam, H., Nielsen, G. K., Prange, I. & Søndergaard, E. (1958*a*). *Nature, Lond.* **182**, 802.
Dam, H., Nielsen, G. K., Prange, I. & Søndergaard, E. (1958*b*). *Experientia* **14**, 291.
Dam, H., Prange, I. & Søndergaard, E. (1952). *Acta path. microbiol. scand.* **31**, 172.
Desai, I. D., Calvert, C. C. & Scott, M. L. (1964). *Archs Biochem. Biophys.* **108**, 60.
Diplock, A. T., Green, J. & Bunyan, J. (1963). *Br. J. Nutr.* **17**, 199.

Gitler, C. S. (1958). Studies of vitamin E deficiency with torula yeast diets in the rat and the chick. PhD Thesis, University of Wisconsin, Madison, USA.
Glavind, J. & Søndergaard, E. (1964). *Acta chem. scand.* **18**, 2173.
Green, J., Diplock, A. T., Bunyan, J., McHale, D. & Muthy, I. (1967). *Br. J. Nutr.* **21**, 69.
Horwitt, M. K., Harvey, C. C., Century, B. & Witting, L. A. (1961). *J. Am. diet. Ass.* **38**, 231.
Jenkins, K. J., Hill, D. C., Hutcheson, L. M. & Branion, H. D. (1962). *Poult. Sci.* **41**, 61.
Lea, C. H. & Ward, R. J. (1959). *J. Sci. Fd Agric.* **10**, 537.
Machlin, L. J., Gordon, R. S. & Meisky, K. H. (1959). *J. Nutr.* **67**, 333.
Machlin, L. J., Marco, G. J. & Gordon, R. S. (1962). *J. Am. Oil Chem. Soc.* **39**, 229.
Machlin, L. J. & Shalkop. W. T. (1956). *J. Nutr.* **60**, 87.
Mervyn, L. & Morton, R. A. (1959). *Biochem. J.* **72**, 106.
Nesheim, M. C., Calvert, C. C. & Scott, M. L. (1960). *Proc. Soc. exp. Biol. Med.* **104**, 783.
Scott, M. L. (1962). *Vitams Horm.* **20**, 621.
Tappel, A. L. (1962). *Vitams Horm.* **20**, 493.

Printed in Great Britain