

Relationship between vitamin E and encephalomalacia in chicks*

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Encephalomalacia is readily produced in chicks by vitamin E-deficient diets containing polyunsaturated fatty acids of the linoleic acid series or their esters (Dam, Nielsen, Prange & Søndergaard, 1958; Century, Horwitt & Bailey, 1959; Machlin & Gordon, 1960). The vitamin is believed to protect against the development of the disease by acting as an antioxidant *in vivo*, i.e. by preventing the peroxidation of polyunsaturated fatty acids in vital tissues such as the brain. It thus behaves as a biological antioxidant, whereas most synthetic antioxidants inhibit lipid peroxidation *in vitro*, but display little or no protective activity in the chick. There are few biologically active synthetic antioxidants. Among these may be mentioned 6-ethoxy-1,2-dihydroxy-2,2,4-trimethylquinoline or ethoxyquin (Machlin, Gordon & Meisky, 1959) and *N,N'*-diphenyl-*p*-phenylenediamine or DPPD (Bunnell, Matterson, Singsen & Eaton, 1956), only the former being approved in the United States for use in feeding-stuffs.

The factors responsible for outbreaks of encephalomalacia under field conditions are much less known, but are generally believed to be similar to those studied experimentally. Thus, added fish oil has often been implicated, since it has been used extensively in the past as a source of vitamin A in feeds. However, there is a surprising dearth of evidence supporting this supposition. Outbreaks of encephalomalacia have been observed regularly in this country, even after fish oil had been replaced by stabilized, fat-free vitamin A concentrates, and in the absence of any fat supplements. The destruction of vitamin E under unsuitable storage conditions of the feed has also been mentioned as a possible causative factor (Winter, 1958), but we have been unable to find any evidence in the literature or from our own observations in the field that such an occurrence has any bearing on the problem of field encephalomalacia.

The purpose of our study was to clarify some aspects of the relationship between vitamin E and encephalomalacia on the farm under practical conditions. Two main lines of approach to the problem were used in this work, namely (*a*) feeding experiments with commercial mashes taken from farms where outbreaks had occurred, or experiments with mashes variously treated so as to lower their vitamin E content or increase the level of fat oxidation products, and (*b*) assays for vitamin E in mashes and livers of chicks from affected farms and comparison with corresponding values obtained

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in experimentally induced cases of encephalomalacia. In addition, a number of breeding eggs, as well as livers and yolk sacs of newly hatched chicks, were analysed for α -tocopherol. 'Free-radical' damage, previously detected in cerebella of chicks reared on semi-synthetic diets (Budowski & Mokadi, 1961), was determined in some feeding experiments and in chicks from affected farms.

EXPERIMENTAL AND RESULTS

Animals. Chicks used in feeding experiments were 1-day-old White Leghorn cockerels. They were housed in electrically heated batteries with raised wire floors and were given food and water *ad lib*. Up to forty chicks/group were used in the various experiments. Livers of chicks used in feeding experiments or collected from farms were stored in a frozen state until analysed.

Commercial mashes. A composition typical for mashes collected from farms where outbreaks of encephalomalacia had been observed as well as for normal mashes is: grain (sorghum) 64, soya-bean meal 19, fish meal 6, lucerne meal 3, wheat bran 5, vitamin and mineral supplement 3%.

Heated mash. This mash was similar to the commercial type described above, except for the absence of lucerne meal. It was heated in a forced-draught oven at 120° for 16 h, during which time the total content of reducing substances dropped to 5 μ g tocopherol equivalent/g.

Extracted mash. This was a commercial mash, continuously extracted with light petroleum, until the total content of reducing substances was reduced to less than 5 μ g tocopherol equivalent/g.

Oil supplements. When oil was added to the mashes, the protein content was increased by raising the amount of soya-bean meal at the expense of grain, so as to keep the energy:protein ratio approximately constant.

Safflower-seed oil was used as a supplement in some experiments because of its high linoleic acid content. 'Oxidized' oil was prepared by blowing air through samples at a temperature of 180° for various periods of time. In one experiment safflower-seed oil was converted into the mixed fatty acid methyl esters (Hartman, 1956), and the latter were distilled under reduced pressure.

In some feeding experiments, the vitamin E-free semi-synthetic diet used by Ames & Swanson (1958) was given. This diet contains 16% 'stripped' lard as a source of polyunsaturated fatty acids. In other trials, the stripped lard was omitted from the above diet, oxidized safflower-seed oil being used instead. Reference to the semi-synthetic diet will therefore always be accompanied by a description of the fat supplement used.

Analytical techniques. α -Tocopherol was assayed in feeds, oils and some of the livers by two-dimensional paper chromatography (Analytical Methods Committee: Vitamin E Panel, 1959). Total reducing substances were determined in unsaponifiable extracts after passage through Floridin earth XS (British Drug Houses Ltd). Most of the liver samples, as well as egg yolks, and some of the feeds were analysed for α -tocopherol by the simpler column chromatography described by Bieri, Pollard, Prange & Dam (1961).

Oils were analysed for iodine and peroxide values and oxidized fatty acids by official methods (American Oil Chemists' Society, 1961); carbonyl values were determined by the method of Henick, Nenca & Mitchell (1954). The linoleic acid content of safflower-seed oil was determined by the enzymic procedure of MacGee (1959). This method measures total methylene-interrupted polyunsaturated acids, but the only fatty acid of this type known to be present in safflower-seed oil is linoleic acid.

Determination of free-radical damage. The procedure, adapted from that of Blois (1958), measures the reaction between aqueous tissue extracts and the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). This radical may be synthesized by the method of Goldschmidt & Renn (1922); it can be obtained from the Aldrich Chemical Company. Tissues are homogenized in 10% (w/v) trichloroacetic acid (TCA) to yield a 10% (w/v) tissue suspension. After centrifugation, the clear supernatant extract is tested as described below. Into three test-tubes, labelled 'unknown', 'blank' and 'standard', are placed respectively 0.2 ml extract, 0.2 ml 10% (w/v) TCA and 0.2 ml 10% TCA containing 0.1 μ mole cysteine hydrochloride. To each tube are added 3.7 ml of an ethanolic solution of DPPH (prepared by dissolving a few crystals of DPPH in two drops of chloroform and adding enough ethanol to obtain an extinction reading of 0.5–0.8 at 520 m μ and 1 cm light path). Saturated aqueous sodium acetate (0.1 ml) is added and the whole mixed. The extinction of the three solutions is determined after 15 min at 520 m μ , with distilled water for setting the instrument at zero extinction. The difference between the extinction of the blank and that of the unknown or standard is proportional to the concentration of radical-reactive compounds. After the time indicated, the reaction is almost complete with extracts from brain or cerebellum, and it goes to 90% completion with liver extracts, for which a correction factor of 1.1 should be used. SH compounds are determined directly in the TCA extracts (Ellman, 1959). Non-SH compounds reacting with the hydrazyl are then determined by difference.

Feeding experiments with 'suspect' mashes. Mashes were taken from ten affected farms where mortality ranged from 10 to 15% and encephalomalacia had been diagnosed on the basis of the behaviour of the diseased chicks and gross and histological observations of the cerebellum. All these mashes were assayed for α -tocopherol (see p. 351). From three farms where mortalities were among the highest, the suspect mashes were given to groups of forty chicks for a period of 6 weeks. No disease was observed. It is to be noted that none of the mashes contained any added fat.

A similar failure to produce the disease was observed when an extracted commercial mash was given for 6 weeks. A heated mash was similarly found to be ineffective in causing the disease. In these two mashes the tocopherol contents had been reduced to low values, as mentioned previously.

Effect of oxidized safflower-seed oil. Addition of oxidized safflower-seed oil to commercial or semi-synthetic diets resulted in a spectacular incidence of encephalomalacia. Table 1 gives the result of one such experiment. The incidence of encephalomalacia was 100% in all groups, even though the content of total reducing substances was as high as 53 μ g/g. In the experiment reported in Table 1, no chemical characteristics were determined for the oxidized oil. But since this oil had been aerated

for 40 h, the chemical changes involved were presumably similar to those shown in Table 2 for an oil aerated for 42 h.

In a second experiment, the effect of oxidized safflower-seed oil was compared with that of the original oil and of the methyl esters derived from it. Table 2 gives the results obtained, together with some characteristics of the oils and the tocopherol contents of the diets. It will be seen that a high incidence of encephalomalacia was achieved with the oxidized oils, whereas the effect was much less with the methyl esters and absent with the original oil. The number of affected birds seemed to vary

Table 1. *Effect of oxidized safflower-seed oil (OSO)* on incidence of encephalomalacia in chicks*

Diets†	Total reducing substances in diet (μg/g)	Incidence of encephalomalacia‡
Semi-synthetic + 20 % OSO	3.0	10/10
Semi-synthetic + 8 % OSO	1.2	
Commercial + 20 % OSO	53.0	
Extracted + 20 % OSO	21.0	

* Oil aerated at 180° for 40 h.

† These diets had been found previously not to cause encephalomalacia without added oil.

‡ Numerator, no. of chicks exhibiting signs of encephalomalacia; denominator, no. of chicks/treatment.

Table 2. *Effect of oxidation of safflower-seed oil, added to a commercial mash at a level of 20%, on the incidence of encephalomalacia in chicks*

Oil	Time of aeration at 180° (h)	Linoleic acid in oil (%)	Iodine value of oil	Carbonyl value of oil	Oxidized fatty acids in oil (%)	Total reducing substances		Stability of oil*	Incidence of encephalomalacia†
						In oil (μg/g)	In diet (μg/g)		
Safflower-seed A‡	0	77.4	144.5	11	0.5	600	152	30.4	0/15
	42	54.9	130.9	119	3.8	58	43.6	25.2	9/15
	69	49.4	121.5	172	8.6	50	42.0	23.2	10/15
Safflower-seed B‡	33.5	48.0	124.6	123	6.9	41	40.2	—	10/15
Methyl esters of oil B	0	70.5	—	—	—	0	32.0	105	2/12

* Peroxide number (m-equiv./kg) of oil extracted from mash after 30 days' storage at room temperature.

† Numerator, no. of chicks exhibiting signs of encephalomalacia; denominator, no. of chicks/treatment.

‡ Oil A was a refined edible oil of recent production; oil B had been stored for about 1 year.

in direct relation to the amount of oxidation products (carbonyl value, oxidized fatty acids) and the rate of peroxide formation in the oils added to the diets. No relation between incidence and total reducing substances in the diet was apparent. Although α -tocopherol was not determined in these feeds, a reasonable estimate may be obtained from the value of 32 μ g total reducing substances/g found for the diet containing the distilled methyl esters, which are free from such compounds. Since one-quarter of these substances usually is α -tocopherol (see p. 351), the diets listed in Table 2 should have contained at least 8 μ g α -tocopherol/g. Comparison of the diets containing

oxidized oils with those containing methyl esters shows that the latter produced a lower incidence of encephalomalacia in spite of a higher ratio of linoleic acid to α -tocopherol.

Tocopherol levels of 'suspect' and 'normal' feeds. Tocopherol contents of mashes collected from ten affected farms during the years 1958-9 were determined and compared with those of ten normal feeds. None of these mashes contained added fat. Suspect and normal feeds averaged 37.5 and 40 μg total reducing substances/g, and 9.0 and 10.0 μg α -tocopherol/g, respectively. These differences are of no practical significance. Further assays have been carried out since 1961, and the results are given in Table 3, together with α -tocopherol levels in healthy and diseased chicks taken from a number of farms. These feeds generally contained more tocopherol than those of earlier years, probably owing to improved quality of ingredients (especially lucerne meal) and addition of vitamin E concentrates. Again, these feeds contained no added oil, except for three suspect feeds and one normal mash. Both suspect and normal feeds gave a similar range of values.

Liver tocopherol levels of affected and healthy chicks. Although vitamin E is not stored preferentially in any special organ, liver contents are suitable indices of the tocopherol intake and vitamin E status of the chick (Pudelkiewicz, Matterson, Potter, Webster & Singens, 1960; Dicks & Matterson, 1961).

Table 3. Mean α -tocopherol contents of feeds and livers of chicks (4-5 weeks old) collected from diseased and healthy flocks

Farm no.	α -Tocopherol content	
	In feeds ($\mu\text{g/g}$)	In livers ($\mu\text{g/g}$)
	Affected flocks	
1	8.5 (2)	0.5 (3)
1	—	0.9 (2)*
2	16.0 (1)	0.5 (3)
3	—	3.1 (2)
4	—	1.0 (2)
5	16.0 (1)	0.7 (6)
6	24.8 (1)	1.9 (3)
7	—	4.8 (3)†
8	15.0 (3)‡	0.5 (22)
8	—	0.5 (2)
	Weighted mean	1.0 (48)
	Healthy flocks	
9	7.8 (1)	4.0 (4)
10	—	2.7 (4)§
11	15.8 (1)	7.3 (6)
12	15.0 (1)‡	5.0 (16)
	Weighted mean	5.0 (30)

Values in parentheses are the number of samples examined.

* Apparently healthy chicks from affected flocks.

† 3-week-old chicks.

‡ Contained 3% added oil (acidulated soya soapstock).

§ 6-week-old chicks.

Table 3 gives the α -tocopherol levels in livers of diseased and healthy chicks taken from a number of farms, together with assay values for feeds obtained from some of these farms.

It will be seen that affected flocks yield low liver α -tocopherol values ($0.5-3.1 \mu\text{g/g}$) compared with healthy flocks ($2.7-7.4 \mu\text{g/g}$). Only on one farm did diseased chicks give a relatively high value of $4.8 \mu\text{g/g}$, but there the lower age of the chicks may have been the reason, since the liver vitamin E concentration decreases during the first weeks of life. Thus, the affected chicks appeared to suffer from hypovitaminosis E, even though they received mash similar in vitamin E content to feeds given to healthy flocks. Healthy chicks from affected flocks also exhibited low tocopherol values, which indicates that these flocks were deficient in vitamin E.

The low liver tocopherol values found in field cases of encephalomalacia were similar to those found in experimentally induced encephalomalacia. Thus, livers of chicks given the semi-synthetic 'vitamin E free' diet containing 16% stripped lard averaged $0.6 \mu\text{g}$ α -tocopherol/g. Similarly, encephalomalacic chicks from a group receiving a commercial mash supplemented with 20% oxidized safflower-seed oil gave liver values of $0.9 \mu\text{g/g}$.

Table 4. *Concentration of hydrazyl-reactive compounds* in cerebellar extracts of encephalomalacic and healthy chicks*

(Mean values with their standard errors ($\mu\text{equiv./g}$ cerebellum) for four chicks)

Condition of chicks	Age (days)	RH	SH	Non-SH
Chicks on commercial diet + 20% safflower-seed oil				
Encephalomalacic	21-28	6.54 ± 0.29	2.24 ± 0.11	4.39 ± 0.25
Healthy	21-28	7.45 ± 0.17	2.71 ± 0.06	4.75 ± 0.25
Chicks from affected farm				
Encephalomalacic	25	4.40 ± 0.27	1.32 ± 0.19	3.07 ± 0.10
Healthy	25	7.28 ± 0.55	1.78 ± 0.11	5.39 ± 0.69

* RH, total reactive compound; SH, sulphhydryl compound; non-SH, difference RH - SH.

† Significant difference ($P < 0.05$).

‡ Highly significant difference ($P < 0.01$).

'Free-radical' damage. This was determined in encephalomalacic and healthy chicks from a group receiving a commercial mash containing 20% oxidized safflower-seed oil. The results are presented in Table 4. It will be seen that a significant decrease in the concentration of hydrazyl-reactive substances occurred in the affected chicks.

A similar picture was given by chicks from an affected farm (Table 4). Thus, free-radical damage seems to be associated with cerebellar lesions, whether in farm outbreaks or upon feeding with oxidized safflower-seed oil or as a result of a dietary vitamin E deficiency coupled with the presence of linoleic acid, as reported earlier (Budowski & Mokadi, 1961).

Tocopherol values of egg yolks and livers of newly hatched chicks. Since the above results indicate that neither the vitamin E level of the feed nor the presence of excess linoleic acid or deteriorated fat accounted for the outbreaks of encephalomalacia, other explanations must be sought. One possible contributory factor may be found in the low vitamin E reserves supplied by some of the maternal yolks. That this possibility should receive serious consideration is shown by the results obtained with eggs col-

lected from two breeding farms. Five eggs obtained from one farm gave from 243 to 696 μg α -tocopherol/yolk (mean 493 μg /yolk), whereas the values ranged from 618 to 1150 μg /yolk (mean 777 μg /yolk) for nine eggs taken from a second breeding farm. These limited results show that there was not only a considerable variability in the vitamin E content of the eggs within one breeding flock, but also that the mean tocopherol contents of the yolks may be entirely different for different flocks.

A similar variation was noted in liver vitamin E values of newly hatched chicks. Values for nine 1-day-old chicks ranged from 62.0 to 134.8 μg /liver.

A question closely related to the problem of vitamin E reserves is the presence of unresorbed yolk in newly hatched chicks. In nine yolk sacs analysed, the α -tocopherol levels ranged from 45.6 to 401 μg /sac. It is obvious that a considerable part of the α -tocopherol originally present in the maternal yolk may remain in the yolk sac after hatching.

DISCUSSION

The main conclusion to be drawn from these observations on field encephalomalacia is that affected chicks suffered from hypovitaminosis E, although the diets implicated were not especially low in α -tocopherol compared with diets used on 'healthy' farms. The amount of dietary α -tocopherol required to prevent encephalomalacia has been stated to range from 11 to 22 μg /g by Scott, Norris, Heuser & Nelson (1955). Singen, Bunnell, Matterson & Kozeff (1955) arrived at a similar range of 16–24 μg /g, although the diets used were different. Thus, it would appear that most of the values shown in Table 3 are borderline, which might well have enabled other stress factors to become operative. Lowering of the tocopherol content of the mashes by extraction or heating did not result in encephalomalacia in controlled feeding tests. It appears therefore that the real α -tocopherol content of the feed is not of decisive importance under field conditions.

Liver tocopherol values for encephalomalacic chicks, presented in Table 3, are in a much lower range than those reported by Markson, Carnaghan & Parr (1957), who found over 9 μg /g. They also stated that 'the tocopherol status of the encephalomalacic chicks was no lower than that of the normal'. It appears, however, that the figures reported by Markson *et al.* (1957) represent total reducing substances, rather than α -tocopherol. Edwin, Diplock, Bunyan & Green (1960) have shown that such values are much higher than the correct α -tocopherol figures and, further, that differences in values between vitamin E-deficient and normal animals may become obscured. This may explain the discrepancy between our results and the liver tocopherol values published by Markson *et al.* (1957).

Results of feeding tests with added safflower-seed oil showed that, with such diets rich in linoleic acid, the formation of fatty acid oxidation products, or a decrease in the stability of the oil, may be more important for the development of the disease than the real α -tocopherol content of the diets or the ratio of linoleic acid to α -tocopherol (Century *et al.* 1959; Machlin & Gordon, 1960, 1962). Long-chain keto acids have been reported to cause encephalomalacia in chicks (Kokatnur, Okui, Kummerow & Scott, 1960). The possible presence of such compounds is indicated by the elevated carbonyl

values of the oxidized oils. With increasing use of low-grade oil supplements in poultry feeds, the question of oxidation products such as the above should receive careful attention, even though the presence of added fat is not a prerequisite for outbreaks of encephalomalacia.

Chicks suffering from encephalomalacia as a result of oxidized oil in the diet had low liver α -tocopherol values. The same effect was observed with the semi-synthetic diet with added lard or in farm outbreaks. In fact, the experimentally induced disease and field encephalomalacia seem to be characterized by a similar physiological vitamin E deficiency and a similar histopathological picture, although different causative factors may be involved. Determination of free-radical damage further confirmed the similarity of cerebellar damage observed in the field and under experimental conditions.

It is not at present possible to pinpoint the factor causing encephalomalacia in the field. Further work on the role of initial tocopherol reserves of the chicks and the tocopherol status of the mothers may possibly help to clarify the picture.

The question of vitamin E reserves in the baby chick has received some attention in the past. For instance, Singesen *et al.* (1955), in their experiments on encephalomalacia, stressed the importance of using chicks from depleted hens to induce the disease. Considerable variability exists in the tocopherol levels of egg yolks, according to Markson *et al.* (1957), Dju, Quaife & Harris (1950) and Glista (1957). Our results confirm the wide range of yolk vitamin E contents. Initial liver tocopherol values of baby chicks also vary widely. These reserves may become important when the amount of vitamin E supplied by the feed is borderline.

Considerable amounts of the vitamin remain in the unabsorbed yolk after hatching, but it is not known whether or not they contribute to the initial reserves of the chick. The situation is similar to that found for maternal vitamin A, which remains, for the most part, in the unabsorbed yolk at the time of hatching (Parrish, Williams, Hughes & Payne, 1950). According to Almquist (1960), removal of the unabsorbed yolk leads to slower growth during the first few weeks, probably because of a loss of nutrient reserves.

Clearly, the problem of encephalomalacia in the field is complex. Possible factors such as those mentioned above need to be explored.

SUMMARY

1. One-day-old White Leghorn cockerels were fed on commercial mashes collected from farms with a high incidence of encephalomalacia. Feeding tests were also undertaken with commercial mashes modified by heating and extraction or by addition of oxidized oil. Vitamin E assays were carried out on feeds, livers and yolks. Cerebella were tested for 'free-radical' damage.

2. Suspect mashes collected from affected farms, as well as heated or extracted mashes, failed to induce encephalomalacia under controlled conditions, whereas oxidized safflower-seed oil added to commercial mashes at a level of 20% produced a high incidence of the disease.

3. Liver tocopherol values of diseased farm flocks were low (about 1 $\mu\text{g/g}$), even

in apparently healthy animals taken from the same flocks. Healthy flocks gave a higher range of values (about 5 µg/g). Chicks made encephalomalacic by either oxidized safflower-seed oil or a semi-synthetic vitamin E-deficient diet containing stripped lard also had low liver levels of vitamin E (less than 1 µg/g).

4. Free radical damage was observed in the cerebella of diseased chicks, both in the field and under experimental conditions.

5. Evidence is presented to show that preformed oxidation products may be of greater significance in the aetiology of the disease than the ratio of linoleic acid to α -tocopherol.

6. Vitamin E assays on feeds did not reveal any significant differences between 'suspect' and 'normal' mashes.

7. Breeding eggs were found to vary considerably in vitamin E content, both from egg to egg and from flock to flock. Unabsorbed yolks retained a large part of the maternal tocopherol.

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