Lipid Oxidation Products and Chick Nutritional Encephalopathy

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ABSTRACT

Safflower oil and its distilled methyl esters were thermally oxidized and fed to young chicks in a vitamin E deficient diet. At a dietary level of 10%, the oxidized lipids caused more severe nutritional encephalopathy (NE) than the unoxidized methyl esters, indicating that factors other than dietary linoleic acid and vitamin E affect the development of NE. A polar lipid extract from oxidized methyl esters accelerated the induction of NE, as did the synthetic methyl esters of keto-octadecenoic and keto-octadecadienoic acids. Dicumarol exerted a protective action against NE. The possibility is discussed that conjugated keto-polyenoic fatty acids, provided by oxidized oils or formed endogenously in vitamin E deficiency, may play a role in causing NE.

INTRODUCTION

Nutritional encephalopathy (NE), better known as encephalomalacia, is induced in young chicks by diets deficient in α-tocopherol and containing polyunsaturated fatty acids (1). The disease is characterized by degenerative changes mainly in the cerebellum, accompanied by ataxia, prostration, and death. Ultrastructural changes in the cerebella of chicks affected with NE have been described by various authors (for a brief review, see ref. 2). The dietary lipid causing NE is linoleic acid or its esters, whereas derivatives of linolenic acid are inactive (3-5).

Autoxidized polyunsaturated oils have occasionally been used to induce NE in chicks (4,5), and we found thermally oxidized safflower oil to be very effective for that purpose (2,6,7). The α-tocopherol level of such oil is greatly reduced while the linoleic acid content is still high. Since the treatment of the oil results in the accumulation of oxidation products, the question arises whether or not some of these products may play an active role in causing NE. The evidence presented in this report points in this direction.

MATERIALS AND METHODS

Animals and Feeds

Day-old crossbred New Hampshire X White Leghorn male chicks were housed in thermostatically heated battery brooders with raised wire floors and had free access to water and feed.

The compositions of the two vitamin E deficient diets are presented in Table I. The diets contained 4 or 10% lipids, and the composition was adjusted so as to ensure a constant ratio of metabolizable energy to protein. The linoleic acid in these diets was provided by distilled safflower methyl esters or by thermally oxidized safflower oil or methyl esters, prepared as described below. The diets contained 0.005%, 2,6-di-*tert.*-butyl-4-methylphenol (BHT), a level of antioxidant which ensured the stability of the dietary linoleic acid and α-tocopherol for over a week at room temperature. During the experiments, the diets were kept at -18 C and dispensed daily.

Dietary Lipids

Refined edible safflower oil was purchased

TABLE I

Composition of Diets

Ingredient	Percent	
Lipid[a]	4.00	10.00
Extracted soybean meal	52.00	56.00
Cellulose	1.00	3.00
D,L-methionine	0.14	0.15
Mineral mix[b]	4.00	4.00
Vitamin mix[c]	0.50	0.50
BHT[d]	0.005	0.005
Glucose monohydrate	38.36	26.35

[a]Safflower oil or safflower methyl esters.

[b]Supplying per kg feed: dicalcium phosphate 28 g; limestone 7 g; NaCl 3.5 g; $MnSO_4 \cdot H_2O$ 370 mg; $ZnCO_3$ 145 mg; ferric citrate 165 mg; $CuSO_4 \cdot 5H_2O$ 11.8 mg; KI 2.35 mg; and $CoCl_2 \cdot 6H_2O$ 1.21 mg.

[c]Supplying per kg feed: vitamin A 3000 U; vitamin D_3 200 U; menadione sodium bisulfite 1 mg; thiamine 3.6 mg; riboflavin 7.2 mg; Ca pantothenate 20 mg; niacin 55 mg; pyridoxine 6 mg; biotin 0.2 mg; folic acid 2.4 mg; vitamin B_{12} 0.02 mg-choline chloride 1.3 g. These amounts were premixed with 3.6 g glucose monohydrate.

[d]2,6-di-*tert.* butyl-4-methylphenol.

[1]Mention of firm names or trade products does not imply endorsement or recommendation by the Department of Agriculture over other firms or similar products not mentioned.

TABLE II

Effect of Thermally Oxidized Safflower Oil (SO-24)[a] and Freshly Distilled Safflower Methyl Esters (ME-0) on Nutritional Encephalopathy[b]

Dietary lipids	Analysis of lipids		Incidence per 20 chicks	
	α-Tocopherol	Linoleic acid	Ataxia	Mortality
	μg/g	%		
			At 3 weeks	
SO-24 4%	10-20	60-65	7	5
ME-SO 4%	5-12	70-76	15	9
			At 2 weeks	
SO-24 10%			12	11
ME-0 10%			8	1

[a]Safflower oil aerated at 145 C for 24 hr.

[b]The lipids were fed from hatching at the levels indicated.

from Shemen Ltd., Haifa and from Teth-Beth Ltd., Petah-Tikva. Different batches contained from 71 to 76% linoleic acid and ca. 350 μg α-tocopherol/g. Methyl esters were prepared from the oil by a modification of the transmethylation procedure of Hartman (8), followed by vacuum distillation.

Thermal oxidation of safflower oil and distilled methyl esters was done by heating batches of 1-2 kg to 145 C ± 2 C under a stream of air (0.5 l/min). The length of the thermal treatment was 24 hr for the oil and 3 hr for the esters. The following abbreviations were used: SO-24 for the oxidized oil; ME-3 for the oxidized methyl esters; and ME-0 for the fresh methyl esters.

A crude extract of polar lipids was prepared from thermally oxidized methyl esters by repeated partition between hexane and 90% (v/v) ethanol, using six separatory funnels arranged in countercurrent fashion. The final ethanolic extract was concentrated under reduced pressure and extracted with ethyl ether. The yield of polar lipids averaged 6%.

Methyl esters of conjugated keto-octadecenoic acid and keto-octadecadienoic acid were prepared from methyl oleate and linoleate, respectively, as described elsewhere (9). The oleate-derived product contained 96% conjugated keto esters consisting of an isomeric mixture of methyl 8-, 9-, 10-, and 11-oxo-octadecenoate. The linoleate-derived product contained 91% keto-dienes consisting mainly of methyl 13-oxo-9,11- and 9-oxo-10,12-octadecadienoate.

For testing in chicks, lipid fractions or synthetic products were dissolved in safflower methyl esters in the amounts indicated.

Quantitative Expression of NE

Chicks were inspected twice daily and the times at which the first signs of ataxia were observed and when death occurred, were recorded. Inspection of the cerebella always confirmed that the affected chicks were stricken with NE. Results were expressed as number of chicks affected per total number of chicks per treatment at the age indicated. Alternatively, curves representing the cumulative incidence of ataxia or death have been plotted.

Analytical Determinations

α-Tocopherol in the dietary lipids was determined by saponification, fractionation of the unsaponifiables by thin layer chromatography on Silica Gel G with hexane/ethyl ether (8:2), and colorimetric reaction of the α-tocopherol fraction with ferric chloride and bathophenanthroline (10).

The fatty acid composition of the lipids was determined after transmethylation of the samples with 3% (w/v) H_2SO_4 in methanol at reflux temperature for 1 hr and extraction of the methyl esters with hexane. The esters were submitted to isothermal gas liquid chromatography at 180 C on Gas Chrom W coated with 15% DEGS. All materials were obtained from Packard Ltd., Jerusalem. Methyl esters prepared for feeding experiments were injected directly into the chromatograph. Glyceryl triheptadecanoate and methyl heptadecanoate were added as internal standards to the oil and methyl ester samples, respectively, for calculation of the true linoleic acid content of the oxidized samples.

RESULTS

In a first trial, two encephalopathogenic diets were compared: thermally oxidized safflower oil, SO-24, and freshly distilled

TABLE III

Effect of Fresh and Thermally Oxidized Safflower Methyl Esters on Nutritional Encephalopathy

Dietary lipids[b]	Analysis of lipids		Vitamin E[a] added to feed	Incidence per 20 chicks at 19 days	
	α-Tocopherol	Linoleic acid		Ataxia	Mortality
	μg/g	%	μg/g		
ME-0	5.0	75.9	0	14	6
			1	13	4
ME-3	0.35	68.5	0	18	14
			1	19	14

[a]D,L-α-Tocopheryl acetate.

[b]ME-0, freshly distilled safflower methyl esters; ME-3, methyl esters aerated at 145 C for 3 hr. The lipids were fed as 10% of the diet from the 8th day, after the chicks received 4% ME-0 during the first week.

safflower methyl esters, ME-0. The oxidized oil had less linoleic acid but no less α-tocopherol than the fresh esters (Table II). When these lipids were fed as 4% of the diet, ME-0 caused a greater incidence of ataxia and mortality than did SO-24. However, at the 10% level, the oxidized oil was more active than the fresh methyl esters.

A similar comparison was made between fresh and oxidized methyl esters, ME-0 and ME-3. Table III shows that ME-3, with less linoleic acid, nevertheless produced a more severe incidence of NE, compared to ME-0. This greater activity of ME-3 was not due to the lower α-tocopherol content of the oxidized vs. the fresh esters, since the difference in tocopherol content between the two diets was no more than 0.5 μg tocopherol/g diet, whereas even the addition of 1 μg DL-α-tocopheryl acetate/g diet had virtually no effect on NE (Table III).

Extracts of polar lipids were prepared from ME-3 by repeated partition between hexane and 90% ethanol. The polar lipids were added to encephalopathogenic diets and their influence on NE was studied. The results of one such experiment, illustrated in Figure 1, show that the polar lipids increased the incidence of NE.

Two synthetic fatty acid oxidation products were tested in the same chick model: a keto monoene prepared from methyl oleate and a keto diene obtained from methyl linoleate. Both compounds accelerated the induction of NE (Figs. 2 and 3).

Fibrin clots have previously been observed in cerebellar capillaries of chicks affected with NE (2). The present chick model was used to study the effect of dicumarol on the incidence of NE. Table IV shows that the anticoagulant exerted a protective effect which increased in direct relation to its concentration in the diet. In this experiment, the diet contained 4% ME-0, but similar results were obtained with diets containing 10% oxidized safflower oil.

DISCUSSION

Several authors (4,5) have reported that the incidence of NE in vitamin E deficient chicks is directly related to the amount of linoleic acid consumed by the chicks. This is seen to be the case in the first experiment in which SO-24 and ME-0 were fed at the 4% level (Table II). However, the reversal of activities of the two lipids at the 10% level does not agree with this concept. One possible explanation for the apparently contradictory results is that SO-24 contains oxidation products which are encephalopathogenic and which, at the higher dietary level, are absorbed in sufficient amounts to overcome the opposite reaction expected from the lower linoleic acid content of SO-24 vs. ME-0.

The above explanation receives support from the observation that the oxidized esters, in spite of their lower linoleic acid content, caused a more severe incidence of NE than the fresh esters (Table III), while more direct evidence for the involvement of lipid oxidation products is provided by results obtained with polar lipids extracted from oxidized methyl esters (Fig. 1).

The effects of the two synthetic ketoenoic fatty acid esters (Figs. 2 and 3) are of interest for the following reasons: 12-oxo-9-*cis*-octadecenoic acid was previously reported to increase the severity of NE when given orally with stripped corn oil (11), but not after injection (12). Subsequently, this compound was shown to possess strong prooxidant activity in vitro (13) and eventually its activity on NE was ascribed, according to a communication from the same laboratory (14), to destruction of residual tocopherol in the diet during the

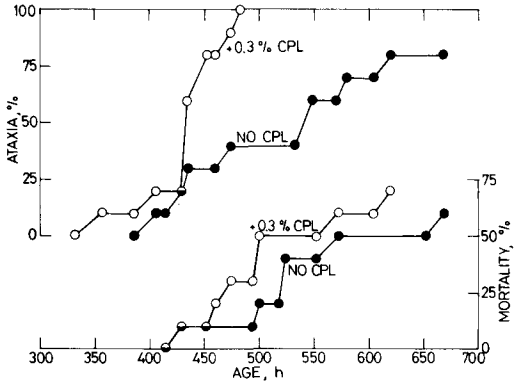

FIG. 1. Effect of crude polar lipids (CPL) on cumulative incidence of encephalopathy induced by 4% safflower methyl esters. The polar lipids were obtained from thermally oxidized safflower methyl esters and were fed with 3.7% fresh safflower methyl esters from the 8th day. During the first week, the chicks received 4% fresh safflower methyl esters. The control chicks received 4% fresh esters throughout the entire period. There were 20 chicks per treatment.

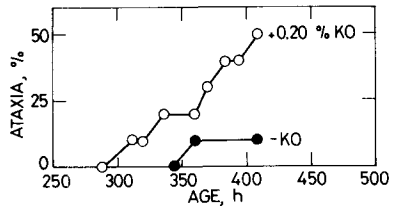

FIG. 2. Effect of conjugated methyl keto-octadecenoate (KO) on cumulative incidence of ataxia induced by 4% safflower oil methyl esters. KO was fed with 3.8% fresh safflower methyl esters from the 8th day. During the first week, the chicks received 4% safflower methyl esters. The control chicks received 4% safflower methyl esters throughout the entire period. There were 10 chicks per treatment.

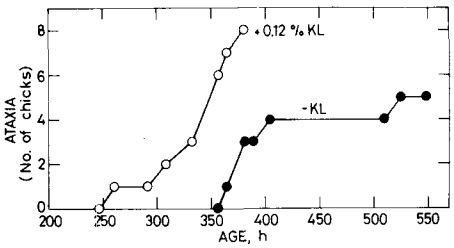

FIG. 3. Effect of conjugated methyl keto-octadecadienoate (KL) on cumulative incidence of ataxia induced by 4% safflower methyl esters. The safflower methyl esters were fed from the first day and KL was added from the 8th day. There were eight chicks per treatment.

tests. The same keto oleate has recently been reinvestigated by Fukuzawa and Sato (15) who reported that it had definite anti-vitamin E activity in the rat and that it specifically combined with bovine serum albumin to form a strongly fluorescent compound (16). These authors postulated that the keto oleate undergoes isomerization to the conjugated 12-oxo-10-*trans*-ene and that the conjugated enone then condenses with albumin amino groups to form fluorescent Schiff base compounds (16). That keto oleate is absorbed by rats when administered by stomach tube was mentioned in a recent review by Perkins (17).

Keto oleate is of little nutritional or pathological significance, because it is unlikely to be found in autoxidized polyunsaturated oils, nor would it be expected to form in vivo. On the other hand, allylic ketoenes have been found in autoxidized methyl oleate (18), and conjugated keto-dienes have been found in autoxidized methyl linoleate (19). Thus, the results of this study with synthetic conjugated ketoenes indicate that this type of compound could be one of the active species contributing to the NE-accelerating effect of oxidized oils rich in linoleic acid.

Moreover, conjugated keto-dienes could be expected to form in vivo, even in the absence of any exogenous supply. In vitamin E deficiency, for instance, polyunsaturated membrane lipids are believed to undergo peroxidation (20,21), and the hydroperoxides formed could yield conjugated keto compounds by several enzymic and nonenzymic reactions, such as those discussed by Gardner (22) for linoleic acid hydroperoxide. In fact, an increase in monocarbonyls has been reported in adipose tissue of vitamin E deficient rats (23). Lipid hydroperoxides are also readily reduced to the corresponding allylic hydroxy compounds by the ubiquitous glutathione peroxidase (EC 1.11.1.9) (24,25); this reaction raises the intriguing question of the possible biological activity on NE of these compounds, or their desaturation to active keto-dienes.

The mechanism by which the synthetic ketoenes enhance the severity of NE is not known. One possibility is suggested by the reaction of 12-oxo-9-octadecenoic acid with albumin, referred to above (16). A similar reaction of conjugated enones or dienones with amino lipids or proteins of cell membranes must result in impaired membrane function, a result usually ascribed to peroxidation of membrane lipids in vitamin E deficiency. The additional possibility that such a condensation reaction might lead to fluorescent products deserves to be investigated, although the fluorophore formed in this case would differ from the 1-amino-3-imino group derived from

TABLE IV

Effect of Dietary Dicumarol on Nutritional Encephalopathy[a]

Dicumarol concentration	Incidence per 20 chicks at 3 weeks	
	Ataxia	Mortality
μg/g		
0	12	8
200	8	7
400	1	1

[a]Chicks received 4% freshly distilled safflower methyl esters from hatching.

malondialdehyde and postulated to form in vitamin E deficiency (26).

The results on the protective effect of dicumarol (Table IV) indicate that the blood coagulation system plays a role in the etiology of NE, in agreement with the histological observation on the presence of fibrin clots in the cerebral vessels (2). Whether or not the process is triggered by thrombocyte aggregation remains to be clarified, but the inability of linolenic acid to induce NE (3-5) would indicate that the cyclo-oxygenase system is involved. For instance, linolenic acid and especially its long chain metabolites are strong inhibitors of prostaglandin formation from arachidonic acid (27), and all *cis*-5,8,11,14,17-eicosapentaenoic acid has recently been reported to be a precursor of a powerful anti-aggregating substance (28). Also, among brain tissues of the rat and guinea pig, the cerebellum has the greatest capacity for PGE_2 formation (29).

The possibility that conjugated keto-enoic fatty acids play a role in the etiology of NE and other syndromes of vitamin E deficiency deserves further study. From a nutritional point of view, attention should be given to the formation and concentrations of conjugated keto-dienes in artificially and commercially heated polyunsaturated oils.

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