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Comparison of the activities of the acetates of d-, d,l- and l-α-tocopherols against encephalomalacia in chicks*)

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With 2 figures and 4 tables

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Prevention of encephalomalacia in chicks under standardized conditions provides a relatively simple means for comparison of the biological activities of various forms of vitamin E.

Encephalomalacia occurs in young chicks when the diet is deficient in vitamin E and contains a certain amount of a polyunsaturated fatty acid belonging to the linoleic acid series, e. g., linoleic acid or arachidonic acid (DAM et al., 1958; DAM and SØNDERGAARD, 1962). If, further, a trace of selenium dioxide is added to the diet, development of exudative diathesis is prevented and encephalomalacia will appear as the only gross sign of vitamin E deficiency. The diagnosis can be made by the clinical signs: ataxia, spasm and paralysis, and the gross autopsy of the cerebellum and cerebrum. Commercial lard is usually so low in vitamin E that it can be used as source of linoleic acid in encephalomalacia producing diets without any special treatment for removal of vitamin E.

Experimental

Day-old chicks were kept in brooders with wire screen bottom and given the vitamin E-free starter ration indicated in table 1 for 6 days, whereafter they were divided into groups of 10 and given the encephalomalacia producing diets No. 1, 2, 3, and 4, the composition of which are also shown in table 1.

The amount of lard in the diet and the duration of feeding suitable for the trials were chosen after running a preliminary experiment with diets containing 5, 10, 20, and 30% of lard, resp.

Fig. 1 shows the results of the preliminary experiment. It is seen that the incidences of encephalomalacia obtained after 4 and 5 weeks increase with increasing content of lard in the diet. The highest incidence, 10 out of 10 chicks (10:10), being reached with 30% lard. Although a somewhat higher content of linoleic acid than that corresponding to 30% lard might be desirable, it is not suitable to use diets with more than 30% lard since, otherwise, the consistency of the diet will be too greasy (the greasiness will be even more pronounced if

*) A preliminary report on part of this study was presented at the 6th International Congress of Nutrition, Edinburgh, 9–15 August, 1963.

sucrose is used instead of corn starch). Except in the trial with 30% lard, the incidences of encephalomalacia were higher after 5 weeks than after 4 weeks of feeding. Therefore, and since it is not practical to keep the chicks in the cages for a longer period of time, a feeding period of 35–36 days was chosen for the final experiments in which the activities of the tocopherols were to be compared.

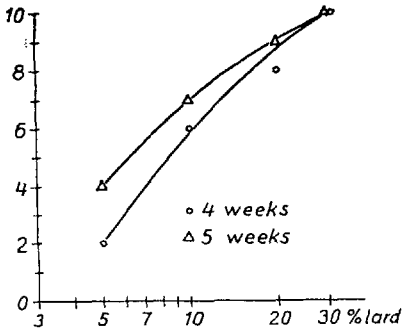

Fig. 1. Relation between dietary levels of lard and incidence of encephalomalacia. Horizontal: Dietary levels of lard. Vertical: Incidences of encephalomalacia (number of chicks with encephalomalacia in groups of 10 chicks). ○ = after 4 weeks of experimental feeding. △ = after 5 weeks of experimental feeding.

Table 1. Diets

	"Starter ration"	Experimental diets No. 1	No. 2	No. 3	No. 4
Casein, crude[1]	20 g				
Casein, Vitamin Test[2]		30 g	30 g	30 g	30 g
Fleischmann Yeast 50 B[3]	20 g				
Gelatin	3 g	3 g	3 g	3 g	3 g
Salt mixture[4]	5.17 g	5.17 g	5.17 g	5.17 g	5.17 g
Vitamin B mixture[4]	0.1 g	0.1 g	0.1 g	0.1 g	0.1 g
Choline chloride	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
Corn starch	46.53 g	56.53 g	51.53 g	41.53 g	31.53 g
Lard[5]	5 g	5 g	10 g	20 g	30 g
	100.00 g	100.00 g	100.00 g	100.00 g	100.00 g
Vitamin K substitute[6]	1 mg	1 mg	1 mg	1 mg	1 mg
Selenium dioxide		0.14 μg	0.14 μg	0.14 μg	0.14 μg

1. "Dairinex", from A/S Dansk Mejeri Industri & Export Kompagni, Stege, Denmark.
2. From Genatosan Ltd, Loughborough, England.
3. From Standard Brands Inc., New York, U.S.A.
4. DAM and SØNDERGAARD (1953).
5. Obtained from Andelssvineslagteriet, Hillerød, Denmark.
6. Synkavit, Roche (di-calcium salt of 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester).

Vitamins A and D_3 were given in the form of 0.1 ml of an aqueous solution (DAM et al., 1957) twice a week, corresponding to 250 i. u. vitamin A and 20 i. u. vitamin D_3 per day.

Two experiments were made with d-, d, l- and l-α-tocopherol acetates, and one with d- and d, l-α-tocopherol acetates.

The levels of tocopherol acetates (mg%) incorporated into diet 4 are indicated in table 2. Within each experiment the levels of tocopherols tested were given to groups of 10 chicks.

Results and Discussion

The incidences of encephalomalacia in the various groups (after 35–36 days) are shown in table 2.

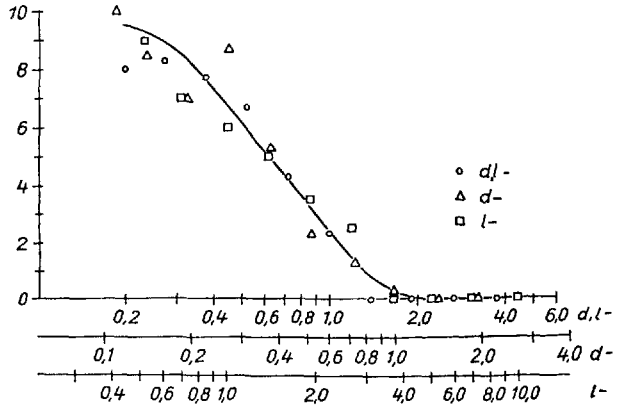

Fig. 2. Relation between dietary levels of d,l-, d-, and l-α-tocopherol acetates and incidences of encephalomalacia. Horizontal: Dietary levels of α-tocopherol acetates. Vertical: Incidences of encephalomalacia (number of chicks with encephalomalacia in groups of 10 chicks). ○ = d,l-α-tocopherol acetate. △ = d-α-tocopherol acetate. □ = l-α-tocopherol acetate.

In fig. 2 the average incidences of encephalomalacia are plotted graphically against the logarithm of the levels of the tocopherol acetates. The abscissae for each form of tocopherol acetate (d-, d, l- and l-) are different and so adjusted that the points for all forms fall as near as possible on a smooth curve.

Table 2. Summary of the experiments indicating the dietary levels of the acetates of the three tocopherols (d-, d, l-, and l-), and the corresponding incidences of encephalomalacia (number of chicks with encephalomalacia in groups of 10 chicks) within 35–36 days

mg% α-tocopherol acetate	Incidence of encephalomalacia									Average incidence of encephalomalacia in all three experiments		
	Experiment 1			Experiment 2			Experiment 3					
	d-	d,l-	l-	d-	d,l-	l-	d-	d,l-		d-	d,l-	l-
0.100	10									10		
0.139	8			9						8.5		
0.193	5	8		8			8			7	8	
0.269	9	8		9	9		8			8.7	8.5	
0.373	4	7		8	8		4	8		5.3	7.7	
0.519	1	6	9	3	7		3	7		2.3	6.7	9
0.721	0	5	6	3	6	8	1	2		1.3	4.3	7
1.000	0	1	6	1	4	6	0	2		0.3	2.3	6
1.390	0	0	5		0	5		0		0	0	5
1.930	0	0	2		0	5		0		0	0	3.5
2.690		0	1			4					0	2.5
3.730		0	0			0					0	0
5.190			0									0
7.210			0									0
10.000			0									0

The levels representing the incidence 50% are as follows: 0.37 mg% for d-α-tocopherol acetate, 0,62 mg% for d, l-α-tocopherol acetate, and 1.4 mg% for l-α-tocopherol acetate. This means that if the potency of d, l-α-tocopherol acetate is chosen as 100, the potency of the d-form will be 168, and that of the l-form 44. The accuracy of the determinations is not very great. It could be increased by using more chicks in the groups and shorter intervals between the doses. However, the amount of l-α-tocopherol available was not sufficient for more than two experiments of the dimensions indicated. The tocopherol levels giving an incidence of 50% are believed to be more accurately determined than the levels giving the incidence 0.

Under the circumstances of the present experiment, the incidence 0 seems to be reached with 1.19 mg% of the d-form, 2.00 mg% of the d,l-form and 4.52 mg% of the l-form, all as the acetates, or, by stoichiometric calculation, with 1.08 mg%, 1.82 mg% and 4.12 mg%, respectively, of the free tocopherols.

Table 3. Comparative biopotencies of α-tocopherol acetates

Test method	α-tocopherol acetate			Reference
	d, l-	d-	l-	
Resorption gestation bioassay (rats)	100	136		Harris and Ludwig (1949)
	100	167	35	Ames et al. (1963)
Hemolysis test (rats)	100	147		Friedman et al. (1958)
	100	142–151	62.5	Weiser et al. (1963)
	100	174–213	51	Brüggemann et al. (1963)
Muscular dystrophy (chicks)	100	146	36	Scott and Desai (1964)
Encephalomalacia (chicks)	100	168	44	Present study

A direct comparison of the activities of the free tocopherols was not made.

A comparison between the relative biopotencies found in the present study and those found by other investigators using different methods of assay appears from table 3.

Provided that the activities of the free d-α-tocopherol and its acetate have the same activity on a molar basis, the following considerations can be made.

When the fatty acid composition of the diet and the small amount of d-α-tocopherol occurring in the lard are known, the data obtained in our study can be used for determining the amount of d-α-tocopherol (in mg) necessary for balancing 1 g of linoleic acid under the conditions of the experiment.

Table 4 shows the results of gas-liquid-chromatographic analyses of the methyl esters of the fatty acids from the lard and from the oil extracted from the corn starch.

The extraction of corn starch (210 g) was carried out in Soxhlet with methanol (30 h) and with ethyl ether (6h). The evaporated extract was taken up in benzene, dried, and weighed. The amount of oil obtained in this way was 0.47 g per 100 g corn starch. The oil contained 97% fatty acids.

From the data presented it can be calculated that the diet contained 1.57% linoleic acid, viz. 1.50% from lard and 0.07% from corn starch.

Table 4. Gas chromatographic determination of individual fatty acid methyl esters as per cent of total fatty acid methyl esters from lard and corn starch fat

	Lard (column of Reoplex)	Corn starch fat (Column of diethylene glycol succinate)
C 14:0	1.63	0.10
C 14:1		trace
C 16:0	33.69	34.76
C 16:1	3.30	trace
C 17:0 (?)	0.51	trace
C 18:0	14.56	1.09
C 18:1	41.05	8.29
C 18:2	5.26	51.37
C 18:3		2.33
C 18:4 or C 20:1		0.17
C 20:4		0.22
C 20:5		1.02
C 22:6		0.64

The amounts of d-α-tocopherol contained in lard and corn starch oil were determined by the method described by BIERI et al. (1961). The lard was found to contain 5.5 microgram per g and the corn starch oil 15 microgram per g. The total content of d-α-tocopherol originating from lard and corn starch oil was 0.17 mg + 0.0022 mg per 100 g diet. (The tocopherol content found in corn starch oil was apallingly low in comparison with that reported for corn oil, e.g. by HERTING and DRURY (1963), but under all circumstances the amount of tocopherol originating from corn starch is negligible in the present connection. Even if it had been 10 times as high it would only have contributed 0.02 mg per 100 g diet).

The amount of tocopherol necessary for balancing 1.57 g linoleic acid was 1.08 mg + 0.17 mg = 1.25 mg, corresponding to 0.8 mg d-α-tocopherol per g linoleic acid.

It is not known whether the same ratio applies in cases where the levels of linoleic acid in the diet are materially different from that used in the present study.

It is of some interest to compare the ratio 0.8 found in the present study with the ratio 0.83 between mg d-α-tocopherol and g linoleic acid, and the ratio 0.63 between mg d-α-tocopherol and g total polyunsaturated fatty acids in the average diet of the population of the U.S.A., as estimated by HARRIS and EMBREE (1963) (14.9 mg d-α-tocopherol, 18.0 g linoleic acid and 5.5 g other polyunsaturated fatty acids). These ratios are considered by the just mentioned authors to represent border lines below which the intake of vitamin E is insufficient.

Dietary vegetable fats containing linoleic acid and only trace amounts of linolenic acid, and no other antioxidant than vitamin E, would therefore seem to be balanced with vitamin E from the standpoint of human requirements if, in repeated trials, they do not produce encephalomalacia in the test described, when given in amounts furnishing about 1.5–1.6% of linoleic acid in the diet.

The same conclusion cannot be drawn if linolenic acid is present in considerable amount, since linolenic acid does not produce encephalomalacia in chicks

(DAM et al., 1958), and the possibility exists that considerable amounts of linolenic acid may tend to minimize or delay the response in the encephalomalacia test.

Other investigators have determined the amount of vitamin E required for balancing 1 g of linoleic acid when other criteria are used. Thus WEBER et al. (1964) used the hemolysis test in experiments with rats. Under these circumstances they found that addition of linoleic acid (in the form of methyl linoleate) to the diet of rats increases the requirement of vitamin E by 0.5 mg d, l-tocopherol acetate per g linoleic acid. Using the ratio between the biopotencies of d- and d, l-α-tocopherol acetates determined by the hemolysis test in rats (WEISER et al., 1963), it can be calculated that 0.5 mg d. l-α-tocopherol acetate corresponds to 0.34 mg d-α-tocopherol acetate or 0.31 mg d-α-tocopherol per g linoleic acid.

It is not surprising that tests using different criteria and different species do not result in the same figure for the border line ratio between d-α-tocopherol and linoleic acid. In this connection it is worth to remember that encephalomalacia is specifically dependent upon dietary polyunsaturated fatty acids of the linoleic acid family, whereas increased tendency to hemolysis probably is furthered by increasing degree of unsaturation of polyunsaturated fatty acids in general.

Acknowledgements

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Summary

The activities of the acetates of d-, d, l- and l-α-tocopherols against encephalomalacia in chicks reared on a diet with 30% lard as the main source of linoleic acid and 31.53% corn starch as source of carbohydrate were found to be 168 for the d-form, 100 for the d, l-form, and 44 for the l-form (the activity of d, l-α-tocopherol acetate arbitrarily being set at 100). The accuracy of the determinations is not very great. It could be increased by using more chicks for the doses giving an incidence of encephalomalacia of about 50%, and smaller intervals between the doses.

Full protection against encephalomalacia was obtained with 1.19 mg% of the d-form, 2.00 mg% of the d,l-form, and 4.52 mg% of the l-form, all as the acetates.

Assuming that the free tocopherols have the same activity as their acetates on a molar basis, full protection would be reached with 1.08 mg% of the d-form, 1.82 mg% of the d, l-form, and 4.12 mg% of the l-form, all as the free tocopherols.

Analysis of the lard and corn starch used in the experiments showed that the diet with 30% lard contained 1.57% linoleic acid, viz. 1.50% from lard and 0.07% from corn starch. Further, the diet contained 0.17 mg% d-α-tocopherol originating from lard. The content of d-α-tocopherol originating from corn starch was negligible.

From the above mentioned data it can be calculated that a total amount of 1.25 mg d-α-tocopherol was required for compensation of the encephalomalacia producing effect of 1.57 g of linoleic acid under the conditions of the experiment.

Zusammenfassung

Die Schutzwirkungen der Acetate von d-, d, l- und l-α-tocopherol gegen Encephalomalacie in jungen Küken wurden untersucht.

Die Encephalomalacie hervorrufende Nahrung enthielt 30% Schweineschmalz als Hauptquelle von Linolsäure, und 31.35% Maisstärke als Kohlenhydrat-Quelle.

Wenn die Aktivität des d, l-α-tocopherol-acetats willkürlich auf 100 gesetzt wird, wurde die Aktivität des d-α-tocopherol-acetats auf 168, und die des l-α-tocopherol-acetats auf 44 gefunden. Die Genauigkeit der Bestimmungen ist nicht sehr groß. Größere Genauigkeit würde mehrere Küken und kleinere Differenzen zwischen den gewählten Dosen erfordern.

Volle Schutzwirkung gegen Encephalomalacie wurde mit 1.19 mg% des d-α-tocopherol-acetats, 2.00 mg des d, l-α-tocopherol-acetats und 4.52 mg% des l-α-tocopherol-acetats erreicht.

Unter der Voraussetzung, daß die freien Tocopherole dieselbe Schutzwirkung pro Mol. haben wie die entsprechenden Acetate, läßt es sich berechnen, daß volle Schutzwirkung mit 1.08 mg% der d-Form, 1.82 mg% der d, l-Form und 4.12 mg% der l-Form des freien α-tocopherols erreicht wird.

Analysen des verwendeten Schweineschmalzes und der verwendeten Maisstärke zeigten, daß die 30% Schweineschmalz enthaltende Nahrung 1.57% Linolsäure enthielt, nämlich 1.50% von Schweineschmalz und 0.07% von Maisstärke herrührend. Die Nahrung enthielt 0.17 mg% d-α-tocopherol vom Schweineschmalz herrührend, während die von der Maisstärke herrührende Menge von d-α-tocopherol vernachlässigt werden konnte.

Es läßt sich somit berechnen, daß voller Schutz gegen die Encephalomalacie-hervorrufende Wirkung von 1.57 g Linolsäure unter den vorliegenden Versuchsbedingungen eine Gesamtmenge von 1.25 mg d-α-tocopherol erfordert hat.

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