The role of carotenoids and vitamin A in encephalomalacia

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1. The relationship of vitamin A and carotenoids with encephalomalacia was examined in chick-raising farms and under laboratory conditions. 2. In encephalomalacic chicks on the farms a significant decrease in liver carotenoid content was demonstrated. In the diseased chicks the carotenoid content was below 0·30 mg/100 g liver, as against the normal values of 0·40–1·0 mg/100 g. 3. Under experimental conditions incidence of encephalomalacia could be decreased by oral treatment with the synthetic carotenoid ethyl β-apo-8′-carotenoate. This implies that deficiency in carotenoid is not merely a concomitant sign of encephalomalacia, but has also a causative role, as sufficient carotenoid reserves confer a certain protection against the disease. 4. The pathogenetic role of carotenoid metabolism in encephalomalacia was supported by certain observations. Factors inhibiting the accumulation of ethyl β-apo-8′-carotenoate in the liver predispose chicks to the disease. In chicks 4–5 weeks old in addition to those with vitamin A reserves higher than 800 i.u./g liver, the accumulation of carotenoids in the liver was about 10% of that measured in chicks more than 7 weeks old, or in those with vitamin A reserves not higher than from 100 to 200 i.u./g. In fact, encephalomalacia occurs mainly among chicks 3–5 weeks old. Also, reserves of vitamin A exceeding 800 i.u./g liver were found to predispose chickens to encephalomalacia.

On the basis of the studies by Pappenheimer & Goettsch (1931) in earlier years, encephalomalacia was considered a result of vitamin E deficiency. These authors fed chicks on a diet known to cause vitamin E deficiency in rats. The experimental birds developed nervous signs that could be cured by added vitamin E.

Recent reports, however, attribute encephalomalacia not so much to an inadequate vitamin E content of the diet as to oxidation of lipids in the diet (Mokadi & Budowski, 1963). Singsen, Bunnell, Mattersen, Kozeff & Jungherr (1955) and Markson, Carnaghan & Parr (1957) found that in encephalomalacic chicks the hepatic and serum levels of vitamin E were not lower than in normal birds. They concluded that the disease is due to rancid fat ingredients of the diet rather than to an absolute decrease in its vitamin E content. Dam, Nielsen, Prange & Søndergaard (1958), Machlin & Gordon (1960) and Machlin (1961) showed that, of all dietary fatty acids, linoleic acid is most liable to induce encephalomalacia. Tsuchiyama, Nishida & Kummerow (1962) succeeded in producing encephalomalacia even with parenteral linoleic acid hydroperoxide.

In fact, encephalomalacia can be prevented by inhibiting the oxidation of fatty acids whether in vivo or in vitro. The protective effect of vitamin E also results from its antioxidant action, but reducing compounds other than tocopherol (*N*,*N*′-di-phenyl-p-phenylene-diamine, 6-ethoxy-1,2-dihydro-2,2,4-trimethyl-quinoline) can prevent and cure encephalomalacia (Singsen *et al.* 1955; Machlin, Gordon & Meisky, 1959; Prohászka, 1963).

In this paper we report observations made on encephalomalacia, partly in chick-raising farms and partly under laboratory conditions.

EXPERIMENTAL AND RESULTS

Findings with chicks raised on farms

In the chick-raising farms, where occasional outbreaks of encephalomalacia had occurred, the percentage composition (g/100 g) of the diet was maize grit 53, barley grit 3, oat grit 2, soya-bean meal 20, wheat germ 5, fishmeal 4, meat meal 4, dried brewer's yeast 1, dried baker's yeast 1, wheat bran 2, ground limestone 2, premix 3. The composition (g/100 kg) of the premix was: Foszkal (Phylaxia, Budapest) (P_2O_5 45%) 600, iodinated salt 400, manganese sulphate 20, oxytetracycline 3, thiamine 0·2, cyanocobalamin 0·002, nicotinic acid 1·5, vitamin A 800000 i.u., vitamin D_3 100000 i.u. In some flocks reared on this mash the incidence of encephalomalacia reached 5–20% by the 8th week of life. The same diet did not produce encephalomalacia under laboratory conditions, incidence being low in smaller groups of birds. In chick farms where several thousands of birds are raised intensively, a 5–20% incidence is practically a mass incidence, whereas in the laboratory where no more than twenty to fifty birds are kept in cages a similar percentage occurrence of the disease involves but one or two birds or, in less fortunate instances, none at all. As in a small group of chicks given a mildly toxic diet the probability of encephalomalacia is lower, the experimental animals were given another diet known to produce a 60–80% incidence. For this purpose, 10% of cottonseed oil, treated with lauryl peroxide as suggested by Machlin (1961) was included in the mash. In some experimental groups for which the concentration of carotenoids was purposely kept low, the maize grit was replaced by barley grit.

The vitamin A contents of the livers were assayed by the Carr–Price reaction, as adapted by Kyrning (1956). Determination of vitamin E was carried out by the method of Feldheim (1960). Tissue was saponified with KOH and extracted with light petroleum. Vitamin E was separated by chromatography in benzene on a Floridin XS column and determined by the Emmerie–Engel reaction. Carotenoids were determined by the procedure of the Association of Official Agricultural Chemists (1958), that is, by extraction with acetone, measuring the carotenoid concentration in a Pulfrich photometer with S 47 filter and expressing it in terms of β-carotene. The peroxide content of the dietary fats was estimated by titration with 0·01 N-$Na_2S_2O_3$ solution (Prohászka & Erdész, 1961).

In chick flocks fed on freshly prepared mash with a peroxide number lower than 5, no encephalomalacia occurred. A notable incidence was, however, recorded in farms where mash had been used after storage for 1–2 months. In the course of storage the feeds became rancid and their peroxide value exceeded 15, generally ranging from 15 to 30. Rancidity occurred when there was an average of 3·0–3·5% fat and total absence of antioxidant. It may be noted that, under practical conditions, feeding with the same rancid mash resulted in dissimilar incidences of encephalomalacia in different flocks, being in some only 5–8% and in others 15–20%. This prompted us to examine a possible relationship of hepatic vitamin A concentration with the frequency of encephalomalacia.

In a chick-raising farm a group of 2000 1-day-old New Hampshire birds was reared

on a rancid mash with 3·4% fat content and a peroxide value of 20 (group A). Another group (group B), also consisting of 2000 day-old New Hampshire chicks, was fed on the same mash, but received 10000 i.u./bird of aqueous vitamin A solution in the drinking water on one occasion when 10 days old. A third group (group C) consisting of 6000 chicks, was fed on freshly prepared mash of the same composition but with a low peroxide value.

Table 1. *Vitamin A, vitamin E and carotenoid values in the livers of healthy chicks and chicks with encephalomalacia given rancid or non-rancid diets with or without vitamin A*

Type of mash	Group	Peroxide value of dietary fats	Mortality from encephalomalacia (%)	Content in liver (mean values with their standard errors) No. of assays	Vitamin A (i.u./g)	Vitamin E (mg/100 g)	Carotenoid (mg/100 g)
Rancid	A	20	4·1	10*	72·5 ± 30·5	0·65 ± 0·25	0·12 ± 0·04
Rancid	B	20	21·5	10*	956·2 ± 82·3	0·70 ± 0·30	0·10 ± 0·04
Non-rancid	C	2	0	12†	105·0 ± 35·2	0·62 ± 0·28	0·65 ± 0·20

* Encephalomalacic chicks. † Healthy chicks.

Results are summarized in Table 1. In group A the frequency of encephalomalacia was much lower than in group B. When the disease showed an increased incidence, in both groups 0·1% of antioxidant (6-ethoxy-1,2-dihydro-2,2,4-trimethyl-quinoline) was added to the mash for 3–4 days. No further incidence occurred after the antioxidant had been used for 2–3 days. As soon as the disease stopped appearing, the original antioxidant-free mash was given again. Subsequent incidence of encephalomalacia was higher in group B than in group A. In group C there was no encephalomalacia.

Results of assays for vitamins A and E and carotenoids in the livers of diseased chicks from groups A and B and those of healthy chicks from group C are shown in Table 1. The assays were carried out at 3–4 weeks of age. The vitamin A contents of the livers were roughly similar in groups A and C, but much higher in group B, which is explained by the treatment of the latter group with vitamin A solution. The vitamin E contents of the livers were roughly similar in all three groups. The carotenoid contents were high in group C, but remarkably low in groups A and B. In fact, among the diseased chicks a decrease in liver carotenoid content was more regularly encountered than a decrease in vitamin E content. Results for groups A and B differed only in the vitamin A contents of the livers, implying that high hepatic concentrations of this vitamin may predispose chicks to encephalomalacia. This hypothesis was confirmed experimentally.

Laboratory examinations

Two groups of fifteen 2-week-old New Hampshire chicks were fed on a mash containing 10% cottonseed oil treated with lauryl peroxide. Until 2 weeks of age the chicks received normal mash without added oil. One group was treated with the aqueous vitamin A preparation, 10000 i.u./chick, when 10 days old. In this group ten cases of encephalomalacia occurred up to 5 weeks of age, whereas in the other group there were only two. Results are presented in Table 2. They verified the supposition that a high vitamin A content of the liver predisposes chickens to encephalomalacia.

Effect of carotenoids on the development of encephalomalacia. As shown in Table 1, liver contents of carotenoid were much lower in affected flocks than in healthy ones. The influence on encephalomalacia of a synthetic carotenoid (ethyl β-apo-8′-carotenoate, a crystalline preparation kindly supplied by Messrs Hoffman-La Roche, Basle, Switzerland) was examined under experimental conditions.

Table 2. *Effect of vitamin A content of the liver on experimental encephalomalacia in chicks*

Group	Incidence of encephalomalacia*	Content in liver (mean values with their standard errors) No. of assays	Vitamin A (i.u./g)	Vitamin E (mg/100 g)	Carotenoid (mg/100 g)
Control	2/15	10†	58 ± 12	0·74 ± 0·20	0·14 ± 0·05
Treated with aqueous vitamin A solution	10/15	10‡	805 ± 95	0·85 ± 0·18	0·12 ± 0·04

* Numerator, no. of chicks exhibiting signs of encephalomalacia; denominator, no. of chicks/treatment.
† Livers from two chicks with encephalomalacia and from eight not exhibiting nervous signs.
‡ Livers from encephalomalacic chicks.

Table 3. *Effect of ethyl β-apo-8′-carotenoate on experimental encephalomalacia in chicks given a diet with* 10% *rancid cottonseed oil of a peroxide value* 33

Group	Incidence of encephalomalacia*	Content in liver (Mean values with their standard errors) No. of assays	Vitamin A (i.u./g)	Vitamin E (mg/100 g)	Carotenoid (mg/100 g)
Control	15/20	5†	900 ± 120	0·92 ± 0·18	0·06 ± 0·03
Treated with ethyl β-apo-8′-carotenoate (5 mg/bird)	5/20	5†	843 ± 132	0·74 ± 0·15	0·18 ± 0·06

* Numerator, no. of chicks exhibiting signs of encephalomalacia; denominator, no. of chicks/treatment.
† Livers from encephalomalacic chicks.

Two experimental groups of twenty New Hampshire chicks were formed. Both groups received normal mash until 2 weeks old, and then modified mash in which the maize grit was replaced by barley grit and 10% cottonseed oil was added. Maize grit was omitted to avoid interference of its carotenoid content with the effect of the synthetic carotenoids. Both groups were treated orally with 10000 i.u. aqueous vitamin A solution per bird when 10 days old. In one group each chick received a total of 5 mg ethyl β-apo-8′-carotenoate daily for 7 days, from 3 weeks of age on. The other group served as untreated controls.

Encephalomalacia was developed between the 3rd and 4th weeks of life by fifteen of the twenty chicks in the control but only by five of the twenty in the carotenoid-treated group. Results are summarized in Table 3.

In a subsequent experiment the preparation Carophyll 10 (Hoffman-La Roche) was supplied as carotenoid source, mixed in the diet at a 0·05% concentration. Carophyll 10

contains 10% ethyl β-apo-8′-carotenoate. The preparation reduced the incidence of encephalomalacia to much the same degree as ethyl β-apo-8′-carotenoate itself.

Table 3 shows that there was no notable difference between the contents of vitamins A and E in livers from the two groups. Although in chicks treated with carotenoid both the liver and serum concentration of carotenoids had been higher, in none did they attain the level (0·65 mg/100 g) demonstrated in chicks fed on normal mash containing 53% maize (Table 1, group C). Hence the incidence of the disease was apparently reduced by treatment with carotenoids, although the carotenoid concentration remained low in both serum and liver of the chicks.

Factors inhibiting the hepatic accumulation of ethyl β-apo-8′-carotenoate. It remained to be explained why the liver carotenoids remained low in spite of the large amount of added carotenoid. It was found that the ability to accumulate ethyl β-apo-8′-carotenoate in the liver depended partly on the ages of chicks and partly on the vitamin A contents of their livers. The age-dependence of the accumulation of synthetic carotenoid was examined as described below.

New Hampshire chicks more than 3 weeks old were used, as up to that age the liver carotenoid derived from the maternal yolk decreased to a level that did not interfere with the measurement of carotenoid absorbed from the feed. From 1 day of age onwards, the chicks were fed on a low-carotenoid diet, replacing with barley grit the maize grit in the mash described above.

Table 4. *Accumulation of carotenoid by chicks of different ages given* 100 *mg Carophyll* 10 (*containing* 10 *mg active substance*) *per chick*

Group	Content in liver (mean values with their standard errors)				Percentage of administered carotenoid absorbed as calculated from liver carotenoid content
	No. of assays	Vitamin A (i.u./g)	Carotenoid mg/100 g	Carotenoid mg/liver	
3-week-old	5	60±24	0·43±0·11	14±4	0·14
5-week-old	5	107±13	1·50±0·60	65±39	0·65
7-week-old	5	92±39	2·20±0·74	212±90	2·12

* Highly significant difference ($P < 0·01$).

Three groups of chicks, 3, 5 and 7 weeks old, received Carophyll 10 in pills daily for 3 days, totalling 100 mg (10 mg active substance). Subsequently the chicks were bled and the carotenoid contents of their livers were determined. Results are given in Table 4. Controls fed on a low-carotenoid diet and not given Carophyll 10 had at the corresponding intervals carotenoid values lower than 0·15 mg/100 g liver. Vitamin A levels were 70–120 i.u./g liver in three age groups.

Table 4 shows that accumulation of dietary carotenoid in the liver was low in 3-week-old and higher in 5-week-old chicks, reaching at 7 weeks of age a level of 1·5–3·0% of that administered. This implies the occurrence in chicks from 4 to 5 weeks

old of alterations in metabolic activity that enhance the accumulation of ethyl β-apo-8′-carotenoate.

High vitamin A contents of the livers depress the accumulation of synthetic carotenoid. This phenomenon was examined in chicks more than 7 weeks old, because at this age the ability to accumulate carotenoids is greater. From 2 weeks of age, the chicks were fed on the low-carotenoid mash used in the previous experiment. When 7 weeks old, the birds were treated orally with aqueous vitamin A solution on one occasion. Several groups were formed, to represent gradually rising liver concentrations of vitamin A. When 8 weeks old, all the chicks were treated with Carophyll 10 daily for 3 days, totalling 100 mg (10 mg crystalline substance) per animal. Subsequently the chicks were bled and the carotenoid contents of their livers and serums were determined. Results are given in Table 5.

Table 5. *Effect of liver reserve of vitamin A on the accumulation of carotenoid in liver and serum of 8-week-old chicks given 100 mg Carophyll 10 (containing 10 mg active substance) per chick*

Added aqueous vitamin A solution (i.u./bird)	Content in liver (mean values with their standard errors)					Content in serum	Percentage of ingested carotenoid absorbed as calculated from liver carotenoid content
	No. of assays	Vitamin A (i.u./g)	Carotenoid mg/100 g	Carotenoid mg/liver		Carotenoid (mg/100 ml)	
20000	6	1120±192	0·29±0·08	54±13	NS	0·17±0·08	0·54
10000	6	406±82	0·77±0·59	135±120		0·38±0·18	1·35
2000	6	118±24	2·25±0·34	446±90	*	0·82±0·24	4·46

NS, not significant. * Highly significant difference ($P < 0·01$).

Table 5 shows that vitamin A concentrations of about 1000 i.u./g liver markedly inhibited the accumulation of carotenoid. A slight increase in the liver carotenoid content was observed with 400 i.u./g liver, and with 100 i.u./g liver of vitamin A the level of accumulated carotenoid reached 4–5 %.

It was found that a high vitamin A content in the liver did not depress the accumulation of carotenoid (mainly lutein) derived from the maize ration. Apparently, the inhibitory effect of vitamin A varies with the carotenoid.

Feeding experiments on laying birds suggested the conclusion that a high vitamin A content in the liver depresses the accumulation but not the absorption of synthetic carotenoids. Twenty New Hampshire layers were fed on a low-carotenoid mash for 4 weeks. During the feeding experiment the carotenoid content of the eggs decreased from 2–3 mg/100 g yolk to 0·5–1·0 mg/100 g yolk. At the end of the 4th week the layers were treated orally on one occasion with aqueous vitamin A solution, 60000 i.u./bird. In this way the vitamin A level could be increased to 1500–2000 i.u./g liver. From the 4th week on, the layers were divided into two groups, ten of them receiving 10 g added Carophyll 10 per 100 kg in the diet, which thus contained 1 mg synthetic carotenoid/100 g. The other group served as control, being kept on the low-carotenoid

mash. In a third group the birds were not given aqueous vitamin A but 10 g Carophyll 10/100 kg were added to their diet. The experiment lasted for 8 weeks. Afterwards the eggs and livers of the layers were assayed for carotenoid content. The values are given in Table 6. The high vitamin A content of the liver apparently did not inhibit the absorption of carotenoid, which was demonstrable at high concentrations in the yolks, whereas in the livers it hardly exceeded the level found in the controls.

Table 6. *Effect of high liver reserves of vitamin A on ethyl β-apo-8′-carotenoate content of egg yolk, liver and serum of laying hens (mean values with their standard errors)*

	Liver (from four assays)			Egg yolk (from fifteen assays)	
Carotenoid in the diet	Vitamin A content (i.u./g)	Carotenoid content (mg/100 g)	Serum carotenoid (from four assays) (mg/100 ml)	Vitamin A (i.u./g)	Carotenoid (mg/100 g)
Carophyll 10 0·01 %	1820 ± 140	0·17 ± 0·04	0·04 ± 0·01	16·2 ± 1·6	3·16 ± 1·19
Control	1810 ± 165	0·15 ± 0·04	0·03 ± 0·01	17·2 ± 2·1	0·34 ± 0·20
Carophyll 10 0·01 %	275 ± 46	1·20 ± 0·35	0·72 ± 0·18	18·1 ± 1·9	3·45 ± 1·50

DISCUSSION

The studies reported here suggest a parallelism between susceptibility to encephalomalacia and the biochemical mechanism regulating the accumulation of ethyl β-apo-8′-carotenoate in the liver. The factors inhibiting the accumulation of ethyl β-apo-8′-carotenoate simultaneously predispose the chicks to encephalomalacia. One of these factors is the age of the birds. Encephalomalacia occurs exclusively in chicks from 3 to 6 weeks old. There is experimental evidence that at that critical age the liver's capacity to accumulate ethyl β-apo-8′-carotenoate is much less than in birds more than 7 weeks old.

Another factor is the high (approx. 1000 i.u./g) vitamin A content of the liver, which inhibits the accumulation of ethyl β-apo-8′-carotenoate, thus predisposing chicks to encephalomalacia.

The role of carotenoids in encephalomalacia is also indicated by the observation that in affected chick flocks the hepatic carotenoid level is much lower than in normal ones. As demonstrated by Ratcliff, Day & Hill (1961) and Anjaneyalu, Kurnick & Reid (1963), the reduction of carotenoid level in the organism results from oxidation processes which can be prevented by antioxidants. In healthy flocks fed on non-rancid maize diet, the average liver carotenoid content was about 0·50–1·0 mg/100 g, made up of 60–70% lutein and 30–40% carotene and kryptoxanthin. Observations have shown that in encephalomalacic chicks the liver carotenoid level varied between 0·10 and 0·20 mg/100 g and in none exceeded 0·40 mg/100 g.

The protective effect of carotenoids is also supported by the finding that feeding with ethyl β-apo-8′-carotenoate resulted in a notably decreased incidence of encephalomalacia. In these experiments only small amounts of carotenoid were accumulated in the liver owing partly to the high vitamin A content of the liver and partly to the young age of the chicks. Nevertheless, the small amount of synthetic carotenoid actually utilized brought about a notable reduction of incidence. Although experi-

mental evidence has been lacking, there is reason to suppose that the natural carotenoids derived from maize and lucerne meal (lutein, kryptoxanthin, zeaxanthin and carotene) have a similar protective action. It is also possible, however, that the chemical and biochemical differences between the synthetic ethyl β-apo-8′-carotenoate and natural carotenoids may show up in their protective action.

Chicks are susceptible to encephalomalacia usually up to 6–7 weeks of age. Older chicks only infrequently develop the disease. Apparently, at 5–6 weeks of age physiological changes take place that render chicks resistant to factors inducing encephalomalacia. This physiological change is also indicated by the fact that on feeding with ethyl β-apo-8′-carotenoate carotenoid accumulation in the liver was more efficient in 7-week-old chicks than in those 3–5 weeks of age.

As to the mechanism of action in encephalomalacia of vitamin A and ethyl β-apo-8′-carotenoate we can merely advance hypotheses. The predisposing effect of vitamin A may be explained by its antagonism to vitamin E, as demonstrated by Brubacher, Schärer, Studer & Wiss (1965) using a radioisotope technique. Our results failed to confirm this finding as no notable change was demonstrated in the hepatic concentration of vitamin E with varying vitamin A levels of the liver. Probably the method applied by us for vitamin E assay may not have been sensitive enough as it measures tocopherol together with other tocopherol-like substances.

In this context the supposition seems to be more feasible that a high vitamin A level of the liver may, by an as yet unknown mechanism, inhibit the accumulation of ethyl β-apo-8′-carotenoate in the liver and serum. With high hepatic vitamin A the binding of ethyl β-apo-8′-carotenoate by proteins is limited and so is its accumulation, whence it cannot play an active part in the organism's biochemical reactions nor in its protective mechanism.

Thus, the protective effect of ethyl β-apo-8′-carotenoate remains to be investigated. Further studies on the metabolism of this compound and of the other carotenoids will probably permit a closer appraisal of the biochemical processes responsible for resistance to encephalomalacia.

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