INFLUENCE OF ANTIOXIDANTS AND REDOX SUBSTANCES ON SIGNS OF VITAMIN E DEFICIENCY

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Vitamin E, originally discovered as a substance necessary for reproduction in rats, possesses the properties of an antioxidant, that is, a substance which inhibits autoxidation of fats in contact with molecular oxygen.

Speculation has, therefore, arisen concerning whether or how far the biological effects of vitamin E can be explained on the basis of an antioxidant effect *in vivo* (64).

The view that at least some manifestations of vitamin E deficiency are related to the lack of an antioxidant effect has received support from the observation that certain signs of vitamin E deficiency occur only in the presence of dietary highly unsaturated fatty acids, which easily undergo autoxidation, and that under such conditions lipoperoxides can be detected in the body fat. Further, some antioxidants and redox substances unrelated to vitamin E may afford a partial protection against certain signs of vitamin E deficiency. These problems will be more fully discussed in the following sections.

The principal effect of an antioxidant is the neutralization of a free radical representing the initial stage in the autoxidative chain reaction (Fig. 1), the following stages of which are the formation of a peroxidic free radical, hydroperoxides, keto and hydroxy compounds, and polymerization products. The reaction between the antioxidant and the free radical involves the transformation of the antioxidant into an unstable free radical form which easily undergoes irreversible changes, whereby the antioxidant is destroyed. In the case of vitamin E the free radical is the semiquinone radical demonstrated in 1949 by Michaelis and Wollman (67).

Among the seven known tocopherols (alpha, beta, gamma, delta, epsilon, zeta, and eta) the physiologically most important, alpha, has the least pronounced antioxidant effect *in vitro*; it is, however, the one that accumulates to the largest degree in animal tissue. The tocopherols must be in the free phenolic form in order to act as antioxidants. The esters are inactive until they become hydrolyzed.

In the *in vitro* test for antioxidant activity a certain concentration of the antioxidant is required to obtain maximal effect. Higher concentrations of the antioxidant may result in diminished activity. This also applies to the antioxidant effect of alpha-tocopherol *in vitro*.

Certain substances may act as synergists for an antioxidant; that is, they may increase its effect. Synergists for the antioxidant effect of tocopherol *in vitro* are, for instance, ascorbic acid and phosphoric acid. The mechanism by which the synergism is brought about has not been completely elucidated, but may be related to a stabilization of the antioxidant.

The signs of vitamin E deficiency known at present are very numerous. A

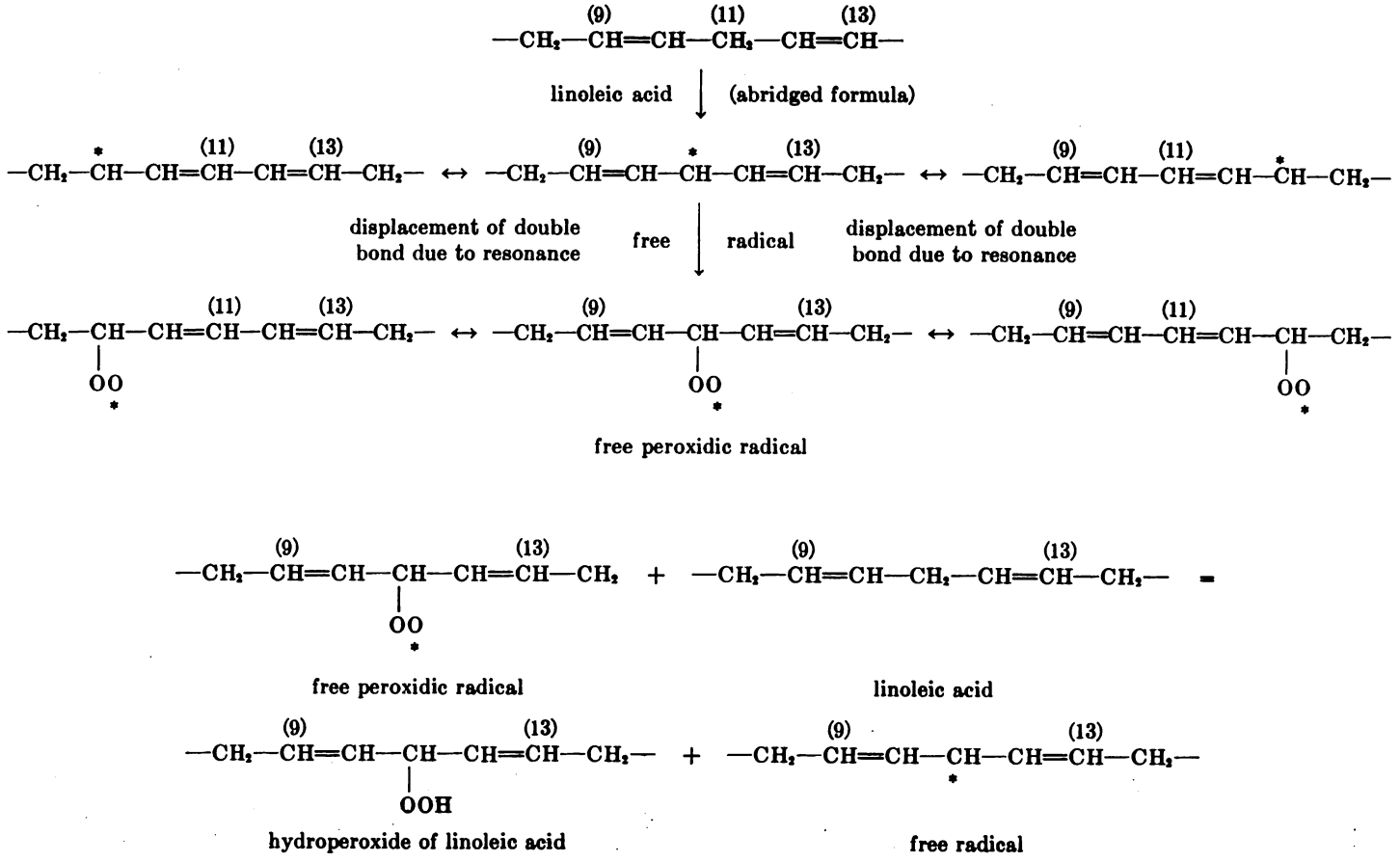

FIG. 1. Some of the initial stages in the autoxidation of linoleic acid

brief description of some of them may be necessary for the understanding of the present problem.

The *exudative diathesis* in chicks was the first instance of a sign of vitamin E deficiency in which peroxidation of fat *in vivo* was demonstrated. This disease (11) affects preferentially the adipose tissue but also muscles and skin. The initial stage is reddening of the tissue due to diffuse hemorrhage. In the second stage plasma exudes from the capillaries of the affected region. The exudate is usually green colored from destruction products of hemoglobin. During the third stage the green color fades and the exudate is absorbed. Sometimes the condition leads to the death of the animal.

The exudative diathesis does not occur when the diet contains sufficient vitamin E or when fat is not present in the diet, but an edematous condition of a different type may occur in chicks reared on fat-free diets; this condition, however, is unrelated to vitamin E deficiency. Both cod liver oil and lard may provoke the exudative diathesis. The effect is connected with the highly unsaturated fatty acid fraction. The saturated acids, oleic acid, and the non-saponifiable fraction are inactive. The total fatty acid fraction from linseed oil may also produce exudative diathesis in chicks. Cod liver oil is the most active among the fats mentioned, and when it is used a yellow-brown color develops in the affected adipose tissue after the exudate is absorbed.

If the highly unsaturated fatty acids are autoxidized to such an extent that the iodine value decreases sufficiently before the fat is ingested, the fat will not give rise to this disorder.

TABLE I

Example of a diet which produces exudative diathesis (and encephalomalacia)

Casein, vitamin test[1]	200	g
Sucrose	547.5	g
Gelatine	80	g
Salt mixture[2]	50	g
Sodium chloride	20	g
Choline chloride	1.5	g
Vitamin mixture no. 4[3]	1	g
Cod liver oil[4]	100	g
Vitamin K substitute[5]	10	mg
	1000	g

[1] From British Chemicals & Biologicals Ltd., 43 Regent Street, Loughborough, England.

[2] McCollum-Simmonds' salt mixture no. 185, supplemented with 13.5 mg KI, 139 mg $CuSO_4$, $5H_2O$, 556 mg $MnSO_3$, $4H_2O$ per 100 g.

[3] Thiamine hydrochloride 3 mg, riboflavin 4 mg, niacin 50 mg, Ca pantothenate 12 mg, pyridoxine hydrochloride 3.5 mg, biotin 0.1 mg, folic acid 2 mg, sucrose 925.4 mg, total 1000 mg.

[4] The cod liver oil must be incorporated in the diet every day and the unfed portion of the ready-made diet kept in the ice box up to 24 hours.

[5] Dicalcium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester (Synkavit®, Roche).

The circumstance that the disease primarily affects adipose tissue is particularly favorable to an examination of the affected tissue for abnormal oxidation products of fatty acids, for instance, lipoperoxides. Dam and Granados (13) undertook such an examination using the iodometric method of King *et al.* (50), slightly modified for the purpose.

It was found that the disorder began approximately at the time when the presence of peroxides could be detected, whereas higher peroxide values were found in the later stages of the disease, *viz.*, when the yellow-brown color of the fat appeared. In chicks receiving vitamin E or no fat the adipose tissue did not contain peroxides. Dam *et al.* (19) found peroxides in the adipose tissue of chicks reared on vitamin E-deficient diets containing 10% lard and no cod liver oil.

These results show that vitamin E may exert an antioxidant effect *in vivo*, and that changes of tissue may occur when this effect is lacking, provided a sufficient amount of easily autoxidizable fat is present. (The fact that dietary unsaturated fatty acids are deposited in the adipose tissue had already been demonstrated in 1901 by Henriques and Hansen (41); that they are deposited in the phospholipids was shown by Sinclair (86).)

The question then arises whether the formation of abnormal oxidation products of fat is the cause of the exudative diathesis or merely a sign of the disappearance of vitamin E from the tissue. Since the disorder has not been observed in the absence of dietary highly unsaturated fatty acids, even when the diet contains a substance such as 2,6-dichlorophenolindophenol which oxidizes free tocopherols, the first alternative seems likely. The explanation may be that some primary autoxidation product of the fatty acids (not necessarily the peroxides, but more likely the free radical) reacts with substances in the wall of the capillaries, and thereby damages the latter.

The diet which produces the exudative diathesis often gives rise to another sign: *encephalomalacia*. This condition was first described by Pappenheimer and Goettsch (77) and extensively studied by them. Clinically there is ataxia, spasm or paralysis. The chicks may die or recover without any change in the diet. Usually the disease begins in the cerebellum. Later the cerebrum may become affected. Localized or diffuse areas of edema, fine hemorrhages and necrosis are usually seen in the cerebellum or the cerebrum. According to Pappenheimer and Goettsch (77), the cerebellar lesions are characterized histologically by edema, with disorganization of the fibrillar and granular elements, degeneration of the Purkinje cells and those of the granular layer, small hemorrhages scattered in both white and grey matter, and hyaline thrombosis of capillaries in and around the necrotic areas. Probably the condition is due to interference with the development of certain structures in the brain. It occurs in the period when the growth of the brain is rapid, but is rare after about two months of age (75).

It is possible to compose a diet which produces encephalomalacia, usually without signs of exudative diathesis, such as the original "diet no. 108" of Pappenheimer and Goettsch (77). A simplified diet which produces encephalomalacia as the main sign is indicated in Table II.

The characteristic features of this diet are: high lard, low carbohydrate and

TABLE II

Simplified diet which produces encephalomalacia as the main sign

Casein, vitamin test[1]	200 g	
Gelatine	80 g	
Salt mixture[2]	40 g	
Sodium chloride	5 g	
Vitamin mixture[3]	1 g	
Choline chloride	1.5 g	
Vitamin K substitute[4]		10 mg
Corn starch	372.5 g	
Lard	300 g	
	1000 g	

[1] From British Chemicals & Biologicals Ltd., 43 Regent Street, Loughborough, England.

[2] McCollum-Simmonds' salt mixture no. 185 supplemented with 13.5 mg KI, 139 mg $CuSO_4$, $5H_2O$, 556 mg $MnSO_4$, $4H_2O$ per 100 g.

[3] Thiamine hydrochloride 3 mg, riboflavin 4 mg, niacin 50 mg, Ca-pantothenate 12 mg, pyridoxine hydrochloride 3.5 mg, biotin 0.1 mg, folic acid 2 mg, sucrose 925.4 mg, total 1000 mg.

[4] Dicalcium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester (Synkavit®, Roche).

absence of vitamin E. Peanut oil deprived of vitamin E by suitable absorption may be used instead of lard.

Although vitamin E prevents encephalomalacia (12, 78), and easily oxidizable fat is necessary for the development of this sign, the search for peroxides in the lipids of brains from chicks with encephalomalacia has, hitherto, been unsuccessful.

Encephalomalacia and exudative diathesis are probably two variations of the same metabolic defect. Certain changes of the diet will favor one or the other of the two disorders. Thus, addition of cholesterol to an encephalomalacia producing diet will depress the tendency to encephalomalacia and favor exudate in fat tissue.

Rats fed diets which produce exudative diathesis and encephalomalacia in chicks fail to develop these conditions. However, vitamin E deficient diets containing a sufficient amount of cod liver oil will produce yellow-brown coloration of the adipose tissue and disappearance of the normal brown color of the incisors in rats.

The yellow-brown fat in the adipose tissue shows a yellow-green fluorescence under the ultraviolet lamp. It has been demonstrated (13) that the development of yellow-brown fat is associated with peroxidation of the fat *in vivo*. Cod liver oil favors the development of the brown fat much more than lard (30). The yellow-brown color of the fat is due to the presence of two brown components, one of which is fat-soluble while the other is insoluble in fat solvents as well as in water. The non-fat-soluble component stains acid-fast with fuchsin and resembles thereby the pigment "ceroid" (34, 52, 53) which occurs in liver, lymph nodes and other tissue of rats reared on diets causing liver damage and con-

taining cod liver oil. There is, in fact, no definite distinction between "ceroid" and the non-fat-soluble component of the brown adipose tissue.

A colorimetric method for the determination of fat-peroxides based on the oxidation of leuco-dichlorophenolindophenol has been developed by Hartmann and Glavind (38).[1] This method can be adopted for use on frozen sections of adipose tissue (26). It is thereby possible to demonstrate the location of the peroxides in the affected adipose tissue. The initial stage is characterized by a homogenous peroxidation of the fat content of some of the cells, but the fat-insoluble component which appears as granules does not necessarily contain peroxides (31).

The pigments in the yellow-brown fat tissue must have originated from the abnormal oxidation of the highly unsaturated fatty acids occurring in the adipose tissue. The fat-soluble component must be assumed to represent the least transformed oxidation products while the non-fat-soluble component consists of higher polymerization products. Cod liver oil autoxidized *in vitro* will also give rise to yellow-green fluorescent fat-soluble and non-fat-soluble reaction products, the latter staining acid-fast with fuchsin. Copolymerization of autoxidized lipids with protein, and catalysis of the autoxidation by hemoglobin has been considered by Tappel (88) as a cause of the pigment formation.

Contrary to the findings in chicks, the hemorrhagic and exudative stages do not occur in rats. The reason could be sought in differences in the fine structure of the capillaries in the two species. Further histochemical studies must decide whether this explanation is correct.

Yellow fat and edema of the adipose tissue have been found in mink reared on fish scraps (39). This adipose tissue also stains acid-fast with fuchsin (62). Vitamin E prevents the development of this effect in mink and linolenic acid provokes it (51). The condition has been studied further by Dalgaard-Mikkelsen *et al.* (10). Robinson and Coey (79) have found brown fat in pigs reared on a vitamin E-deficient diet containing cod liver oil. The rare disease scleredema which affects the adipose tissue in premature infants has been said to respond to vitamin E treatment (25) and thus is believed to be related to the exudative diathesis in chicks.

Martin and Moore (58, 59) have described a *brown discoloration of the uterus* in female rats deprived of vitamin E. Moore and Wang (75) are of the opinion that the brown substance contains oxidized protein. It is likely that the brown pigment of the uterus is closely related to that in the yellow-brown fat tissue. A similar fuchsinophil pigment was found by Escudero *et al.* (24) in the adrenal cortex and by Mason and Emmel (60, 61) in the sex glands and musculature of vitamin E-deficient rats. A fluorescent, acid-fast staining substance has also been found in the nervous system of rats with chronic vitamin E deficiency (23).

Presence of lipoperoxides has been demonstrated in adipose tissue from patients suffering from *peripheral venous disease* (49), and in the arteriosclerotic aorta (27). A relationship of these observations to vitamin E has not been shown experi-

[1] By this method it is sometimes possible to detect traces of peroxide in fat from chicks and rats reared on diets containing 10% cod liver oil and 10 mg % d,l-α-tocopherol acetate (20 and unpublished data from the reviewer's laboratory).

mentally, although some authors have claimed a beneficial effect of vitamin E on certain forms of such disorders (6).

The *depigmentation of rat incisors* was first described by Davies and Moore (22). The fact that fat is necessary for the appearance of this sign was demonstrated by Granados and Dam (32). The normal brown pigment of the incisors is a ferric compound, probably ferric phosphate. The maxillary incisors which are normally more colored than the mandibular are the first to lose their color when the rats are placed on a vitamin E-deficient diet containing suitable fat. Lard, as well as cod liver oil, will produce incisor depigmentation. The effect is associated with the highly unsaturated fraction of the fatty acids. The iron content of the enamel decreases very much and manganese rises somewhat under the influence of the "depigmenting" diet (14). This sign could be in some way related to the lack of an antioxidant effect against autoxidation of fatty acids in the enamel organ.

Hove and Harris (48) have shown that vitamin E increases the normal utilization of a suboptimal level of essential fatty acids in the diet of rats. The explanation is, apparently, that vitamin E protects these metabolites against abnormal oxidation. Thus, when highly unsaturated fatty acids are given in small amounts they disappear in the absence of vitamin E. When given in higher amounts abnormal oxidation products may accumulate.

Moore (70) showed that the feeding of vitamin A to rats (1000 I.U. per week in the form of halibut liver oil on a diet containing 10% lard) results in higher vitamin A levels in the liver when the diet contains vitamin E than when the diet is vitamin E-free. Hickman *et al.* (42) and Miles *et al.* (68) found that the utilization of vitamin A is more efficiently increased by free tocopherol than by its esters. The latter authors also found that increase of the vitamin E content beyond a certain level results in suboptimal utilization of vitamin A. This latter observation agrees with the general experience that there is a certain optimal concentration for the action of an antioxidant. Beyond this concentration its action decreases or may even be reversed. Regarding the sparing effect on vitamin A, Hickman *et al.* (42) found alpha-, beta-, and gamma-tocopherols equally active.

Dam *et al.* (19, 20) found no significant effect of vitamin E on vitamin A storage in the liver of chicks within 5 weeks and of rats within 14 weeks when the diet contained no cod liver oil (vitamin A being given as natural ester), whereas the effect was marked on diets containing cod liver oil. They also found that the vitamin A level in the liver of rats decreased from the 5th to the 14th week when the cod liver oil-containing diet was vitamin E-free. All these observations are in agreement with the assumption of an *in vivo* destruction of vitamin A coupled to the autoxidation of highly unsaturated fatty acids in the absence of antioxidants.

The circumstance that the antioxidant activity of the tocopherols *in vitro* increases from alpha to delta, whereas the biological activity as measured in the fertility test with female rats decreases in the same order is no hindrance for the assumption of an antioxidant effect *in vivo*. Barnes *et al.* (2) and Lundberg *et al.* (55) have shown that the biopotency of the various tocopherols as antioxidants

(measured in the rendered fat of the experimental animals (rats)) is related to the amounts deposited in the fat, alpha being deposited to a larger extent than gamma.

The following signs of vitamin E deficiency occur even when the diet does not contain easily oxidizable fat: 1) reproduction failure in female rats (the classical symptom of vitamin E deficiency); 2) massive liver necrosis in rats (this is greatly accelerated by easily oxidizable fat); 3) hemolysis of erythrocytes in rats; 4) muscular degeneration in chicks. Therefore, these signs do not seem to be due to autoxidation of fat.

Massive liver necrosis (33, 82) and fatal lung hemorrhage (47) occur in vitamin E deficiency under certain dietary conditions, partly in relation to the amount and quality of the protein. Fat with a high content of highly unsaturated fatty acids greatly accelerates the onset of the two pathological effects (15), but liver necrosis has been developed with fat-free diets (66).

When the dietary protein consists of "damaged" casein (82) or is provided by certain types of yeast (37), the incidence of hepatic necrosis is particularly high. Low sulfur amino acids, especially low cystine, is one of the conditions for the development of hepatic necrosis (28, 35, 44, 85). A more detailed review of this subject has been given by Himsworth (43) and McLean and Beveridge (65). However, the dietary conditions are still more complicated, since Schwarz (83, 84) has demonstrated the existence of a "factor 3" in non-necrogenic yeast and in casein which protects against this effect. The factor is not identical with cystine or methionine which, under suitable conditions, will protect against liver necrosis in vitamin E deficiency. Lindan and Work (54) have shown that both reduced and total glutathione as well as ascorbic acid are very much lowered in the necrotic livers of vitamin E-deficient rats. While cystine protects, ascorbic acid does not.

In connection with the role of vitamin E in maintaining the normal state of the liver, its ability to minimize the damage of this organ by carbon tetrachloride should be mentioned (46). It might be that the effect of vitamin E in preventing liver necrosis consists in the protection of cystine, glutathione and some other metabolites which are essential to the normal state of the liver. Accumulation of peroxides in the liver has not been demonstrated, and probably does not occur to any appreciable extent.

György and Rose (36, 80) found that alloxan and dialuric acid produce hemolysis of erythrocytes in vitamin E-deficient rats. From this finding they developed a test for vitamin E deficiency. The effect was believed to be caused by the lack of an antioxidant effect against a free radical representing the initial stage of the autoxidation of dialuric acid. The free radical is supposed to react with a substance in the erythrocytes whereby the latter are damaged. Hemolysis of erythrocytes from vitamin E-deficient rats also occurs in isotonic saline provided oxygen is not excluded (8). Rose and György (81) reported that when administered *in vivo* the effectiveness of the tocopherols in inhibiting dialuric acid hemolysis *in vitro* decreased in the order alpha, beta, gamma, delta, similarly to their activity measured by fetal resorption.

Muscular degeneration in chicks (21) occurs even when the diet does not

contain fat. This condition is greatly ameliorated by increase of dietary cystine. It is possible, therefore, that it is connected with some abnormal metabolism of sulfur amino acids.

Degeneration of the convoluted tubules of the kidney (59) is a sign of vitamin E deficiency which has not yet been studied sufficiently to judge its possible dependence on dietary highly unsaturated fatty acids. Lack of essential fatty acids leads to a similar condition (4, 69).

Studies on the influence of the constant feeding of antioxidants and redox substances on the aforementioned signs of vitamin E deficiency have shown the following results (compare Table III):

The industrially important antioxidant *nordihydroguaiaretic acid* (NDGA) affords some protection against exudative diathesis and encephalomalacia in chicks when fed at a 0.5% level (17, 18). It also increases vitamin A storage in the liver on diets with 10% cod liver oil. At the 0.5% level the substance retards growth. NDGA does not act against muscular degeneration in chicks.

Disulfiram (Antabuse, tetraethylthiuram disulfide) which shows a moderate antioxidant effect in the Swift test, protects fairly well against exudative diathesis in chicks (0.025% of the diet) but not against encephalomalacia (18). It protects vitamin A in the liver of chicks and rats (17, 18), and acts against yellow-brown fat and incisor depigmentation in rats (1), but not against muscular degeneration in chicks.

Ascorbic acid (0.5% of the diet) affords a fairly good degree of protection against encephalomalacia, but much less against exudative diathesis in chicks (15, 16), and no protection of vitamin A in the liver (Dam, Prange and Søndergaard, unpublished). It does not protect against muscular degeneration in chicks.

Cystine (as already mentioned) protects against liver necrosis and lung hemorrhage in rats and against muscular degeneration in chicks. This effect is probably not due to antioxidant properties but rather to a supplementation of a too low level of sulfur amino acids.

Methylene blue (0.126% of the diet) provides fairly good protection against exudative diathesis and encephalomalacia in chicks (18), and protects vitamin A in the liver of chicks and rats to a very high degree (19, 20). It also protects fairly well against yellow-brown fat and peroxidation of the adipose tissue in rats (1), and against hepatic necrosis and lung hemorrhage in rats receiving cod liver oil (16). The effect of methylene blue against liver necrosis in rats has been confirmed by Schwarz (85a). Methylene blue will counteract to a high degree the unfavorable effect on growth of chicks exerted by cod liver oil in vitamin E-deficient diets. As judged from the effect on growth, methylene blue is better tolerated by chicks than by rats, and by rats better than by hamsters.

Whereas methylene blue did not act against muscular degeneration in chicks (21), Blaxter and associates (3) found that it protected against muscular dystrophy in calves induced by the feeding of cod liver oil. The toxic effect of cod liver oil was found to be associated with the polyethenoic acids but did not consist in a lowering of the tocopherol content of the muscles. Methylene blue ameliorated the condition without resulting in higher concentrations of tocopherol

TABLE III

The signs of vitamin E deficiency discussed in the present article and their response to various substances.

Sign	Easily Autoxidizable Fat		Improvement Obtained by															
	Necessary for occurrence of sign	Aggravates or accelerates condition	Nordihydroguaiaretic acid		Disulfiram		Ascorbic acid		Cystine		Methylene blue		Thiodiphenylamine		Several dyes*		Diphenyl-*p*-phenylenediamine	
			a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
Exudative diathesis (chicks)	+			(+)		+		(+)		0		+		+				
Encephalomalacia (chicks)	+			(+)		0		(+)		0		+		+				+
Peroxidation and brown coloration of adipose tissue (chicks and rats)	+			+		+		(+)		0		+		+				
Brown uterus (rats)	?			0		0		0				+		+		+		
Depigmentation of incisors (rats)	+			+		+		0		0		+		+				
Low vitamin A in liver (rats, chicks)	+			(+)		+		0		0		+		+				+
Reproduction failure (female rats)	0										0†	0†						
Massive liver necrosis (rats)	0(?)	+						0		+		+						
												NB						
Lung hemorrhage (rats)		+																
Hemolysis with dialuric acid (rats)	0											(+)						
												NB						
Muscular degeneration (chicks)	0	+	0		0		0		+		0							
Muscular degeneration (calves)		+						0				+						
Degeneration of renal tubules (rats)												+						

a refers to diets without easily oxidizable fat.

b refers to diets containing such fat.

+ means fairly good protection, (+) less marked protection.

NB = disagreement among various investigators.

* New methylene blue, Bindschedler's green, malachite green, methylviolet, rosaniline.

† formerly found positive with less thoroughly depleted rats and with diets containing traces of vitamin E.

in serum, muscle, liver, or perinephric fat. It seems, therefore, that there was a direct protective effect of methylene blue against the toxicity of the polyethenoic acids of cod liver oil. Ascorbic acid and ethyl gallate did not show this effect. The experiments of Blaxter *et al.* (3) show that it may be necessary to distinguish between the effect of methylene blue against a condition as such and against the effect of a noxious substance provoking the condition. Regarding muscular dystrophy caused by lack of vitamin E it is worthwhile to remember that tocopheryl hydroquinone has been assumed to be the specific remedy for this condition (56).

Conflicting statements occur in the literature concerning the influence of methylene blue on reproduction failure in vitamin E-deficient female rats. Thus, Dam and Granados (15) and Markees (57) found that methylene blue counteracts this condition when the substance is added to the diet on which the rats are reared. When methylene blue is given as one single dose at the beginning of pregnancy it has no such effect (57). Moore *et al.* (71) were unable to find any effect of methylene blue on failure of reproduction. Recently, Christensen *et al.* (9) have shown that when the diet is thoroughly freed from traces of vitamin E, which requires that its content of lard is purified by suitable adsorption, methylene blue has no effect on reproduction failure, whereas a marked effect of methylene blue is observed when traces of vitamin E are present in the diet. This must mean that methylene blue retards the depletion by stabilizing traces of vitamin E otherwise insufficient for protection. Such an interpretation is in agreement with the observation of somewhat higher values for tocopherol in adipose tissue of chicks (18) and in blood of rats (57) on vitamin E-deficient, methylene blue-containing diets than found on similar diets without methylene blue. The same interpretation may hold for the effect of methylene blue on incisor depigmentation and dialuric acid hemolysis in rats, items on which there have also been different opinions in the literature (1, 7, 40, 71, 72, 73, 74).

Moore and associates (40, 71, 72, 73, 74) were unable to find any effect of methylene blue on these signs, whereas they found effects on brown coloration of the uterus and against tubular degeneration in the rat. Methylene blue was found in their studies to be effective against brown coloration of the uterus when given in the amount of 0.032% of the diet.

Several *other redox dyes and related substances* such as thiodiphenylamine (18), new methylene blue, Bindschedler's green (which does not contain sulfur), the triphenylmethene dyes—malachite green (apparently toxic to chicks), methyl violet and rosaniline—have effects in rats similar to those of methylene blue (72, 73, 74). Bindschedler's green and toluidine blue also act against encephalomalacia when given in suitable amounts (Dam, unpublished studies). Lately, diphenyl-*p*-phenylenediamine (DPPD) has been found effective against encephalomalacia in chicks (5, 87), and capable of increasing vitamin A in liver and plasma in chicks (63). DPPD was also found effective against encephalomalacia when injected intramuscularly, and the same applied to alpha- and gamma-tocopherols which were nearly equally effective, whereas delta-tocopherol was ineffective. Several antioxidants such as 2,6-di-t-butyl-4-methoxy phenol (BHT),

dibutyl- and diamylhydroquinones, diisobutyl-p-phenylenediamine, and 1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline (Santoquine®) given orally counteracted encephalomalacia. As far as the reviewer knows, no effect of redox dyes or antioxidants has yet been demonstrated against muscular degeneration in chicks.

When interpreting the observed effects of antioxidants and redox dyes on signs of vitamin E deficiency the following points must be taken into account: A. The effects of the substances in question seem to be most pronounced against symptoms occurring in the presence of dietary polyenoic fatty acids. B. 1) As already mentioned, the substances may act by protecting small amounts of vitamin E present in the diet or in the animal, thereby improving the action of otherwise insufficient amounts of the vitamin. 2) The substances may act as antioxidants in cases where vitamin E does the same. 3) In the case of methylene blue and of some related redox dyes, the known ability of these substances to substitute for the cytochrome system may play a part. 4) Such substances may also act in a way similar to that recently reported for vitamin E in the respiratory chain, in which the vitamin is believed to act as a carrier in one path of electron transfer from flavin systems to cytochrome c (76). 5) A combination of some of the possibilities mentioned under B. 1) to 4) may apply.

An obvious requirement for a positive effect following oral ingestion of a redox substance *in vivo* is that the substance be absorbed from the intestine. Furthermore, it must be carried to the proper places within the organism. If the substance is not absorbed, it may at most exert a protective effect as an antioxidant for traces of vitamin E or of other essential metabolites in the food or in the intestine. It has long been known that methylene blue is absorbed from the intestine (89), and its profuse excretion in the bile of chicks after being absorbed is easy to demonstrate. Ingested diphenyl-*p*-diphenylamine is also excreted in the bile of chicks (Dam and Søndergaard, unpublished). Ingested NDGA and Antabuse exert a growth depressing effect on rats and chicks and therefore must also be absorbed.

In order to stabilize vitamin E in the body directly, an absorbable redox substance must be assumed to have a redox potential lower than that of alpha-tocopherol, but it cannot be excluded that a similar substance with a redox potential somewhat higher than that of α-tocopherol may act as antioxidant against the autoxidation of polyenoic fatty acids. The redox potential of alpha-tocopherol is not known with great accuracy. According to Golumbic and Mattill (29) the E of alpha-tocopherol (measured at 75°C in 95% ethanol containing 0.2 N HCl) is between the redox potentials of *p*-toluquinone and *p*-xyloquinone, *i.e.*, between 656 and 597 mV; at pH 7 this probably means between 236 and 177 mV. In the Thunberg tube α-tocopherol in 80% ethanol at room temperature without addition of acid will reduce 2,6-dichlorophenolindophenol (+217 mV) but not tolylene blue (+115 mV), thionine (+62 mV), toluidine blue (+11 mV) or methylene blue (+11 mV).

Possibility B. 1), stabilization of vitamin E, probably applies to the effect of methylene blue observed by some investigators against reproduction failure in female rats, and possibly to incisor depigmentation and dialuric acid hemolysis in rats.

Possibility B. 2), antioxidant effect corresponding to that of vitamin E, seems to be the explanation of the effect of methylene blue, thiodiphenylamine and Antabuse on liver storage of vitamin A, and of the effect of the same substances and NDGA on brown adipose tissue and peroxidation of body fat in chicks and rats, and of the effect of redox dyes on brown uterus in rats. The effect of methylene blue on cod liver oil-induced muscular dystrophy in calves and on cod liver oil-accelerated liver necrosis in rats may be of the same nature.

A combination of the two aforementioned modes of action may apply to the exudative diathesis and encephalomalacia in chicks.

It seems, at present, less easy to apply possibilities B. 3) and 4), roles in the respiratory chain, to the problems under discussion, since there has not been any indication of diminished respiration in connection with the signs of vitamin E deficiency mentioned. On the contrary, increased oxygen uptake has been reported for muscles of vitamin E-deficient animals (45).

Finally, the question may be raised whether practical application may be made of the observed effects of antioxidants and redox substances on signs of vitamin E deficiency. To this may be said that those of the aforementioned substances which are unphysiological are more or less toxic. The color of the dyes is also an obstacle. A substance like NDGA which in some countries is permitted in low concentrations as an additive to certain foods is not active enough to replace vitamin E in artificial diets. The same probably applies to other artificial antioxidants. Consideration has, however, been given to the use of such substances in chicken rations containing fish oils. According to Singsen *et al.* (87), an amount of 0.0125% of DPPD would be sufficient to prevent encephalomalacia under most field conditions. It remains to be seen whether this practice will hold its own in the future.

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