LINOLEIC ACID INTAKE AND VITAMIN E REQUIREMENT

Dit proefschrift met stellingen van

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De Rector Magnificus van de Landbouwhogeschool,

H.A. Leniger

Wageningen, 7 mei 1973

F. C. JAGER

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
PROF. DR. IR. H. A. LENIGER,
HOOGLERAAR IN DE TECHNOLOGIE,
IN HET OPENBAAR TE VERDEDIGEN OP WOENSDAG 6 JUNI 1973
DES NAMIDDAGS TE VIER UUR IN DE AULA VAN DE
LANDBOUWHOGESCHOOL TE WAGENINGEN

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STELLINGEN

1. De verhouding tussen de hoeveelheden vitamine E en meervoudig onverzadigde vetzuren (E/PUFA ratio) geeft geen informatie over de vitamine E status van een dieet. (Dit proefschrift.)
2. De conclusie van Herting et al. dat een verhoogd vetgebruik de behoefte aan vitamine E vergroot is niet gerechtvaardigd. D.C. Herting, M.I. Ludwig en E.E. Drury, J. Nutr. 99, 481 (1969).
3. Het veelvuldig gebruik van de term PUFA (polyunsaturated fatty acids), als het uitsluitend linolzuur betreft, suggereert ten onrechte een uniforme biologische werking van alle meervoudig onverzadigde vetzuren.
4. Het is wenselijk naast minimale hoeveelheden voedingsstoffen, ook maxima aan te bevelen voor verschillende bevolkingsgroepen.
5. Endogeen insuline kan atherogeen werken. R.W. Stout, Br. Med. J. 3, 685 (1970). R.W. Stout en J. Vallance-Owen. The Lancet I, 1078 (1969). G.L. Duff en G.C. McMillan, J. Exp. Med. 89, 611 (1949).
6. Harman meent dat door toevoeging van stoffen aan de voeding die de reacties van vrije radicalen afremmen, de gemiddelde levensduur van de mens met tenminste zeven jaar verlengd zou kunnen worden. Deze opvatting vindt onvoldoende steun in de uitkomsten van zijn experimentele werk. D.H. Harman, Am. J. Clin. Nutr. 25, 839 (1972). D.H. Harman, J. Gerontol. 23, 476 (1968).
7. De huidige technologische ontwikkeling van de voedingsindustrie zal problemen voor de veevoeding oproepen.
8. Het effect van prostaglandine E_1 op de baarmoeder berust niet op een directe stimulering van het gladde spierweefsel, maar op een sensibilisering van het orgaan voor oxytocine. J. Favier en W.J. Rietveld, Am. J. Obstet. Gynecol. 115, 33 (1973).
9. Een effectief faunabeheer onder leiding van biologen is gewenst; in dit kader dient de verlening van jachtrechten aan particulieren te verdwijnen.

Proefschrift van F.C. Jager
Wageningen, 6 juni 1973

PROMOTIE IN DE AULA DER LANDBOUWHOGESCHOOL

GENERAAL FOULKESWEG 1A

RECEPTIE NA AFLOOP VAN DE PROMOTIE

IN HET KLEIN-AUDITORIUM VAN HET AULAGEBOUW

Aan alle medewerkers van Unilever Research Vlaardingen die hebben bijgedragen aan het tot stand komen van dit proefschrift.

C O N T E N T S

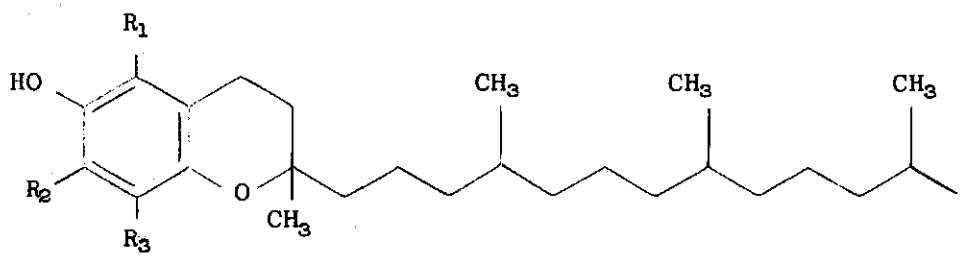
I. INTRODUCTION

Edible fats contain small amounts of nonglyceride components, one of them being vitamin E. This vitamin occurs mainly in fats of vegetable origin; the richest source is wheat germ oil. Animal fats, in general, have a low vitamin E content.

Vitamin E was discovered by Evans and Bishop (1922), when they concluded that the resorption gestation occurring in their feeding experiments with rats was due to a deficiency of a fat-soluble factor in the food. Since this discovery was related to reproduction, vitamin E was long regarded as a special "fertility vitamin". However, rats later proved to be an exceptional case: most animals would have died from myopathy, anemia or encephalomalacia, long before reproduction could have occurred. The resorption gestation occurs because the embryo dies after 12-14 days due to affection of the vascular tissue. Although the females remain fertile, male rats may become sterile after prolonged feeding on a vitamin E deficient diet, because of testis degeneration.

A review of the early history of vitamin E research has been given by Evans (1962).

The name vitamin E refers to a group of tocopherols (Fig. 1), which differ in structure, biopotency and antioxidant capacity. The latter is supposed to be the natural function of vitamin E in oils, especially in relation to polyunsaturated fatty acids (PUFA). Vitamin E most commonly occurs as D-α- or as D-γ-tocopherol. The other tocopherols occur so rarely, or have such low biopotencies, that they are not of practical value. Synthetic vitamin E is usually D-α- or DL-α tocopheryl acetate. The biopotencies of the acetate esters are about 9% less than those of the corresponding free tocopherols (Brubacher and Weiser, 1967).



R_1	R_2	R_3	Name	Biopotency (I.U./mg)
CH_3	CH_3	CH_3	α-tocopherol	1.5
CH_3	H	CH_3	β-tocopherol	0.4
H	CH_3	CH_3	γ-tocopherol	0.2
H	H	CH_3	δ-tocopherol	0.02

Fig. 1 Structure and biopotency of naturally occurring tocopherols. One mg DL-α-tocopherol acetate has a biopotency of 1 international unit (I.U.) by definition. The biopotencies mentioned are those of the natural D-forms.

The vitamin E requirement may be influenced by different dietary factors, the effect being dependent on the species studied and the tissue affected. Such factors are: synthetic antioxidants (encephalomalacia, embryonic degeneration, in vitro haemolysis), selenium (exudative diathesis, liver necrosis, myopathy), the amino acids, cystine and menthionine (myopathy) and polyunsaturated fatty acids (encephalomalacia, in vitro haemolysis, myopathy) (see also Scott, 1970). Most factors have a beneficial effect, but polyunsaturated fatty acids may increase the need for vitamin E. The term PUFA comprises both the highly unsaturated hexaenes and pentaenes from fish oils as well as linoleic acid. The PUFA contained in edible fats and margarines relates almost exclusively to linoleic acid.

In recent years there has been a growing interest in the consumption of vegetable oils with a high content of linoleic acid, such as sunflower, safflower or maize oil. Much confusion

has existed around the question whether or not the linoleic acid rich vegetable oils naturally contain sufficient vitamin E to balance their PUFA content. Harris (1962) calculated that in natural oils a positive correlation exists between the content of total tocopherols and the content of linoleic acid plus linolenic acid. From data obtained from human studies by Horwit (1960) and from a literature review of animal studies, Harris and Embree (1963) were led to suggest a vitamin E requirement of 0.6 mg D-α-tocopherol per gram of linoleic acid ingested. This E/PUFA ratio of 0.6 means that most vegetable oils which are rich in linoleic acid do not contain sufficient vitamin E.

Some experimental work designed to obtain quantitative information on the relation between linoleic acid intake and vitamin E requirement was done by Weber et al. (1964) on rats and by Søndergaard and Dam (1966) on chickens. The former found that linoleic acid had a profound effect on the vitamin E requirement, whereas no such effect was noted by the latter.

In view of these and other confusing results, we carried out experiments with rats and ducklings in order to quantify the effect of linoleic acid intake on vitamin E requirement. In this work, vitamin E was not given in single doses, but incorporated into the food, in order to ensure good absorption (Pudelkiewicz and Nakiya, 1969). Care was taken that the diets contained sufficient essential fatty acids (EFA), as much confusion has arisen from the fact that many researchers did not realize they were working with EFA-deficient diets.

II. DETERMINATION OF VITAMIN E REQUIREMENT

The principle of the determination of vitamin E requirement is simple. Animals are for some time given diets containing increasing amounts of vitamin E. The amount of vitamin E that is found to be just sufficient to prevent deficiency symptoms may be regarded as the actual requirement for this vitamin. The

difficulty is to find an animal species that develops a suitable deficiency symptom within a short time and to express the degree of disease quantitatively. Moreover, it is important that no other deficiency symptoms occur at the same time. The in vitro haemolysis in rats (Jager, 1968) and the myopathy in ducklings (Jager et al. 1969, Jager and Vles 1970, Jager and Verbeek-Raad 1970) were found to fulfil these requirements. In addition to these methods the use of some other deficiency criteria will be discussed.

A. IN VITRO HAEMOLYSIS

Rose and György (1950a) discovered that the erythrocytes of rats kept for some time on vitamin E deficient diets, haemolyzed in vitro, after the addition of dialuric acid. Vitamin E is supposed to act as an antioxidant and, therefore, oxidizing agents such as hydrogen peroxide might have the same haemolytic action on red cells (in vitro) of vitamin E deficient animals as dialuric acid. This was indeed found to be true (Rose and György, 1952). It was found later (Christensen et al. 1956) that in vitro haemolysis of vitamin E deficient erythrocytes may also occur spontaneously. This phenomenon of spontaneous in vitro haemolysis was used by Jager (1968) to determine the vitamine E requirement of rats. It proved to offer a better sensitivity and reproducibility than the dialuric acid method (Christensen et al. 1956; Jager 1968; Draper and Saari Csallany 1969). Horwitt et al. (1956) showed that the hydrogen peroxide haemolysis may give widely varying results, dependent on the way in which the test is carried out.

Some insight into the cause of in vitro haemolysis was given by Tsen and Collier (1960), who demonstrated that it is associated with lipid peroxidation and that addition of α-tocopherol to the erythrocyte suspension decreased or completely prevented haemolysis.

Jacob and Lux (1968) studied the mechanism by which hydrogen peroxide causes in vitro haemolysis of vitamin E deficient red cells of rats. They demonstrated that the locus of attack by hydrogen peroxide was the red cell membrane, in which especially one phospholipid, i.e. phosphatidyl ethanolamine was destroyed prior to the onset of haemolysis. Phosphatidyl ethanolamine is presumably so vuluerable to oxidative destruction, because it contains the largest proportion of unsaturated fatty acids in red cell membranes (Ways and Hanahan, 1964). An initial sequence of fatty acid destruction in phosphatidyl ethanolamine may lead to membrane disorganization, which results in the observed haemolysis.

The relation between in vitro haemolysis and fatty acids in the red cell membrane was clearly demonstrated by Bieri and Poukka (1970). They found in (EFA deficient) rats a linear relationship between the peroxidizable index - calculated after Holman (1954) and Witting and Horwitt (1964a) - of the erythrocyte polyunsaturated fatty acids and the α-tocopherol content of the red cells required to prevent 10% in vitro haemolysis by dialuric acid.

The spontaneous haemolysis of washed red cells incubated at 37^{o}C may be attributed to small amounts of hydrogen peroxide generated continuously during aerobic incubation. The hydrogen peroxide may be eliminated by glutathione peroxidase (Mills, 1957; Cohen and Hochstein, 1963) and under certain conditions by catalase (Theorell and Ehrenberg, 1952; Nitowski and Tildon, 1956; Gross et al., 1967). The glutathione peroxidase pathway, however, requires the presence of glucose to maintain adequate levels of reduced glutathione (Wittels and Hochstein, 1966), as otherwise the initiated reaction chain of lipid free radicals may be broken by a lipid soluble antioxidant as is vitamin E (Witting, 1965). There is no proof so far that dietary selenium has any effect of practical importance upon the results of the in vitro haemolysis test (Christensen et al., 1958; Gitler et al., 1958; Krishnamurty and Bieri, 1961), nor is this the case with

cystine or methionine or the dietary protein level (Gitler et al., 1958; Scott, 1970; Jager, unpublished results).

Leonard and Losowski (1967) studied the relationship between the plasma vitamin E level and the results of the hydrogen peroxide haemolysis test in human subjects. They found that a negative correlation exists between the plasma tocopherol level and the percentage in vitro haemolysis. This found confirmation in the work of Silber et al. (1969) and Poukka and Bieri (1970), who showed that the tocopherol content of the erythrocyte membrane depends upon the tocopherol concentration of the blood plasma and that a complete exchange takes place between plasma and red cells within a few hours.

Besides tocopherol, the red cells take up fatty acids from the surrounding medium and incorporate them in the phospholipids of the erythrocyte membrane (Oliveira and Vaughan, 1964; Jacob and Lux, 1968). The fatty acid pattern of the red cell membrane is a reflexion of the fatty acid composition of the blood plasma (Waku and Lands, 1968).

Jacob and Lux (1968) observed that the in vivo survival time of ^{59}Fe-labelled red cells was identical for vitamin E deficient and vitamin E supplemented rats. This initially somewhat unexpected observation means that in vivo the destruction of vitamin E deficient red cells is not increased. The destruction is most probably prohibited by a reparative mechanism in the erythrocyte membrane; damaged fatty acids in the phosphoethanolamine are exchanged for intact fatty acids from the plasma. When the ^{51}Cr-method was used it was found that in the case of vitamin E deficiency the red cells survival time was decreased (Horwitt et al., 1963; Marvin, 1963). Binder and Spiro (1967) showed that this was due to a labelling artefact.

Erythrocytes of vitamin E deficient rats have been found to be no more sensitive to haemolysis by hypotonic solutions than were those of normal controls, when examined by the usual

saline osmotic resistance test. At the same time, however, the in vitro haemolysis test may be strongly positive (Christensen et al. 1956; Walker and Kummerow, 1964). Porter et al. (1962) found that in vitamin E deficient monkeys, despite a severe degree of anemia, the erythrocytes of the vitamin E deficient animals did not differ significantly from the controls in the osmotic resistance test. Remarkable was the finding that when in two monkeys the red cell osmotic fragility test was repeated, some days after vitamin E administration, an increased fragility could be demonstrated.

1. In rats

(a) *Dose-response effect* (Jager, 1968)

(i) Experimental design. In order to determine the dose-response effect of vitamin E intake on the percentage spontaneous haemolysis in vitro, newly weaned male albino Wistar rats (SPF) were kept for 16 months on vitamin E deficient diets (see Table I) to which increasing doses of vitamin E (DL-α-tocopheryl acetate) had been added. The diet contained 35 cal% lard.

Table I

Composition (weight%) of vitamin E deficient diets containing 35 cal% fat for rats and ducklings

Components	Rats	Ducklings
Casein	29.3	36.0
Sucrose	48.5	
Maize starch		34.6
Fat	17.8	14.4
Salt mixture	4.0	5.8
Cellulose powder		8.9
Vitamin mixture*	0.4	0.3

*See Jager and Houtsmuller (1970) and Jager and Vles (1970)

Male rats were choosen as test animals because, according to Ward (1963), they are more susceptible to vitamin E deficiency than the females. The 48 rats were divided into 6 groups of 8 animals. The successive groups received 3, 8, 13, 18, 23 and 43 mg DL-α-tocopheryl acetate per kg food, respectively. Food and drinking-water were given ad libitum. Regular determinations of the food intake of all animals revealed that the intake of all dietary components, other than vitamin E, was the same for all groups. Determinations of vitamin E requirement in rats by means of an in vitro heamolysis test had been done before by Rose and György (1950b), Friedman et al. (1958) and Ward (1963). In all these cases, vitamin E was administered in single doses, daily or weekly per os. According to Ward (1963), however, under these circumstances the result of the haemolysis test is dependent on the time elapsing between the vitamin E administration and the moment at which the test is carried out. For this reason in the experiment presented here the vitamin E was taken up in the food in graded levels, to ensure an almost constant plasma tocopherol level for each group.

(ii) The spontaneous haemolysis test. This test was performed with the following reagents; stock solution (1); buffer, pH 7.4; 136 g KH_2PO_4 + 30 g solid NaOH was made up to 1000 ml with distilled water. Stock solution (2): physiological saline: 170 g NaCl, was made up to 1000 ml with distilled water. The test solution was made by: 50 ml solution (1) + 75 ml solution (2), made up to 2000 ml with aq. dest., and saturated with oxygen before use by shaking with air; the pH was adjusted at 7.4, if necessary. 0.1 ml blood was taken from the rats' tails, immediately suspended in 7.5 ml test solution and centrifuged for 10 min at 500 x g at 15°C. The supernatant was removed by suction and the red cells were resuspended in 7.5 ml test solution. After homogenization, 1.0 ml suspension was pipetted into: (A) 4.0 ml test solution (determination 1), (B) 4.0 ml test solution (determination 2), (C) 4.0 ml aq. dest. (total

haemolysis). The tubes (A), (B) and (C) were incubated for 4 h at 38°C, homogenized and centrifuged for 10 min at 500 x g at 15°C. The degree of haemolysis in the supernatant was determined colorimetrically at 543 nm, setting the test solution at 100% transmission. The percentage haemolysis was calculated from: extinction of the determination x 100, divided by the extinction from the total haemolysis test. The animals were not previously fasted, as is usual in a blood test, because it was found that after they had been fasted for 24 h the degree of haemolysis had decreased (Jager, unpublished results). In some cases, besides the spontaneous method, dialuric acid haemolysis was performed (Moore et al., 1957).

(iii) Results. The relationship between the dietary vitamin E content and the percentage in vitro haemolysis is a somewhat sigmoid-shaped curve (Fig. 2).

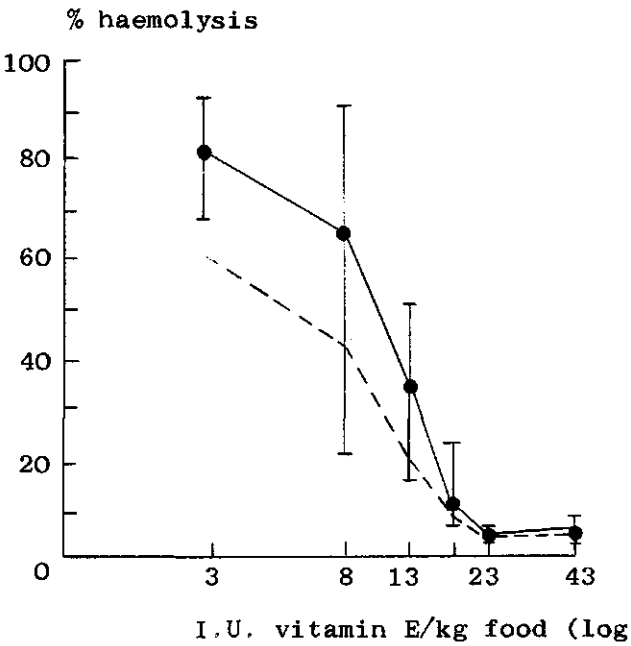


Fig. 2 (Jager, 1968). Relationship between vitamin E content of food and in vitro haemolysis.
Drawn line: spontaneous haemolysis.
Dotted line: dialuric acid haemolysis.

A linear relationship appeared to exist from 8 to 18 I.U. vitamin E (DL-α-tocopheryl acetate) per kg food. Within one dosing group, considerable differences in percentages of haemolysis could be observed. When the individual percentages of haemolysis within one dosing group were compared over a certain period, there were no clear indications that invariably the same animals had a high or low percentage of haemolysis. There was a certain individual level, but its significance in comparison with the total of the variations within a dosing group was slight. Vitamin E requirement, as determined by spontaneous haemolysis in vitro, was somewhat arbitrarily defined as the amount needed to prevent 10% haemolysis. This amount was determined from the steeply-descending part of the dose-response curve, where necessary, by linear extrapolation. In view of the fact that with higher doses of vitamin E 4-6% haemolysis is still observed, the 10% mentioned has to be considered as an initial haemolysis. The percentages of haemolysis by dialuric acid were somewhat less than those found by the spontaneous method, as is indicated in Fig. 2. The spontaneous haemolysis test was found to have a better reproducibility.

The influence of the incubation time is illustrated in Fig. 3. Since the dose-response relationship did not change appreciably after 4 h, this incubation time was maintained in further experiments.

The value of the spontaneous in vitro haemolysis test will be highly dependent on the extent to which vitamin E requirement, as calculated from this test, is found to remain constant throughout the lifetime of the rat. The results of such an investigation are given in Fig. 4. The initially high level can be explained by the fact that during the first 14 days the animals received no vitamin E. Apparently it took some weeks before a steady level was reached after the administration of vitamin E. After the 262nd day, the level again increased and, until the 408th day, all measuring points were higher than those over the entire preceding period.

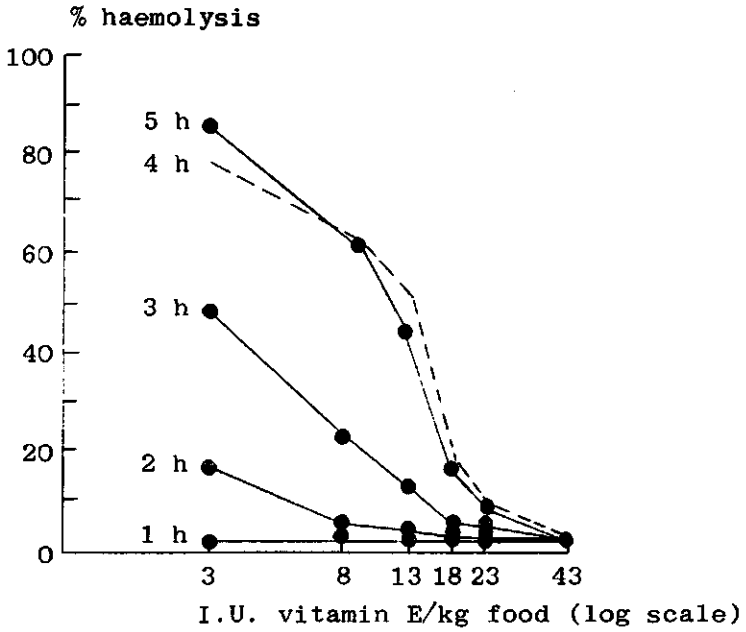


Fig. 3 (Jager, 1968). Influence of the incubation time (at 38°C) on the dose-response relationship between in vitro haemolysis and dietary vitamin E content.

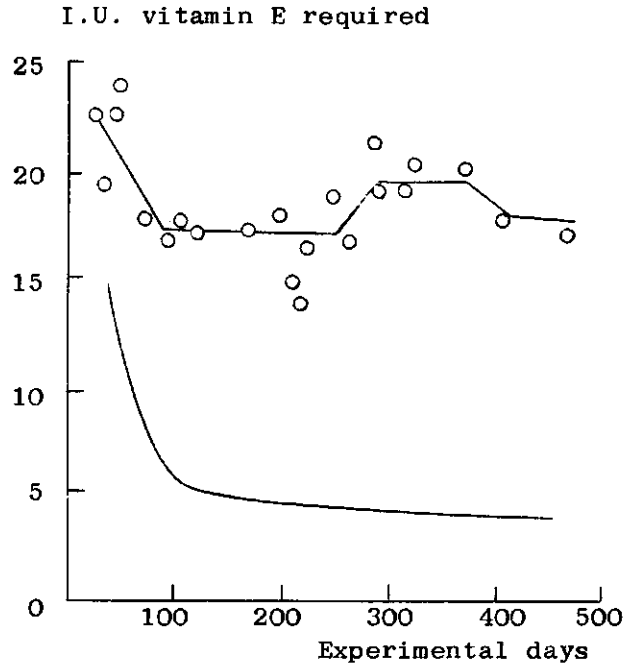


Fig. 4 (Jager, 1968). Influence of the duration of the experiment on the amount of vitamin E required to prevent 10% haemolysis in vitro.
Measured points: found amounts (per kg food).
Drawn line (x 10): calculated amounts per kg body weight per day.

From the 263rd until the 375th day, a different batch of lard was used; however, analysis of this lard with respect to fatty acid composition and tocopherol content gave no indication whatsoever to explain this increase. Nevertheless it can be stated that over a period of 15 months no major changes occurred in the vitamin E requirement calculated per kg food (mean 19.0 I.U./kg). When calculated per kg body-weight, however, there was a continuous decrease in the requirement for vitamin E, for the food intake remained fairly constant, whereas the mean body-weight increased from 200 g on the 35th day to 660 g on the 480th day. From these results we may conclude that vitamin E requirement is related to food intake (or perhaps, better, calorie intake) and not to body-weight.

(iv) Calculation of human requirement for vitamin E. A mean vitamin E requirement of 19 I.U./kg food for the rat, corresponds with 4.0 I.U./1000 cal in the diet applied in this experiment. Extrapolation to man with a consumption of 2500 cal/day gives a daily requirement of 10.0 I.U. vitamin E. One mg DL-α-tocopheryl acetate (= 1.0 I.U. vitamin E, by definition) is equivalent to 0.65 mg D-α-tocopherol (Fig. 1), and therefore man would need 6.5 mg D-α-tocopherol per day. Harris and Embree (1963) calculated that the average diet in the USA supplied 14.9 mg D-α-tocopherol per day. Bunnell et al. (1965), however, calculated from their analysis of different foods that the daily intake of D-α-tocopherol in the USA varied between 2.6-15.4 mg with average of 7.4 mg/day.

These calculations were based on an analysis of raw materials. For processed foods (cooked, fried, etc.), Losowsky and Kelleher (1970) and Smith et al. (1971) found that in the United Kingdom in about 80% of the cases investigated the food supplied less than 5.0 mg D-α-tocopherol per day.

Dayton et al. (1965) performed the hydrogen haemolysis test on a group of seven men each of whom received 2.4 mg D-α-tocopherol per day from a diet containing 40 cal% saturated fat.

The degree of haemolysis was 12% and therefore their daily intake must be considered insufficient.

Considering all these facts together, we must conclude that, based on the in vitro haemolysis test, a rather high percentage of human beings are, in fact, border-line cases of vitamin E deficiency.

(b) Relation to other criteria. Significance of the haemolysis-test (Jager, 1972a). A positive haemolysis test is generally considered to indicate a state of vitamin E deficiency. However, it was not clear so far to what extent the results of an in vitro test may be indicative of an actual pathological condition (Weber and Weiser, 1967. Anonymus, 1969). Therefore, in male rats, long-term, dose-response effects of vitamin E in relation to different possible deficiency symptoms were studied in order to correlate these with the degree of spontaneous in vitro haemolysis.

(i) Experimental design. 48 newly-weaned male albino Wistar rats (SPF), divided into 6 groups of 8 animals, were kept for 22 months on vitamin E deficient diets, as indicated in Table I. Lard was used as a source of fat. Vitamin E was added to the diet as D-α-tocopheryl acetate. The 6 groups received 0.5, 1.5, 2.5, 4.5, 8.5 and 16.5 mg D-α-tocopheryl acetate per kg food respectively.

After 22 months the surviving animals were killed by decapitation, dissected and observed for gross pathology. The musculi gastronemii, liver and testicles were removed and examined microscopically. In the cross-section of the muscles, the affected fibres were counted with a maximum count of 10 per section. Testis degeneration was also evaluated in a cross-section, using a score ranging from 0-4. Score 0 was given if less than 3 tubuli were affected (excluding the outer border, because on the outside of the testis some degenerated tubuli were frequently found, independent of the vitamin E dose). Scores 1, 2, 3 and 4 indicate that up to 25, 50, 75 and 100% respectively of the

tubuli were degenerated. The scores of both testicles were added (total score 0-8).

(ii) Results and discussion. Although about 25% of the animals died before the end of the experimental period, no relation between the vitamin E content of the diet and mortality was observed.

Only the group receiving the lowest dose of vitamin E (0.5 mg D-α-tocopheryl acetate per kg food) showed a decreased weight gain, this becoming significant after about one year.

Liver necrosis was not observed; this is, however, only to be expected if the diet not only has a very low vitamin E-content, but is also deficient in selenium (Schwarz and Foltz, 1957; Bonetti and Stirpe, 1962).

The leg muscles showed myopathy. The amount of hyaline-degenerated fibres showed the best response to the dose of vitamin E and was therefore regarded as a typical result of vitamin E deficiency. The relation between the three most important deficiency symptoms, that is, myopathy, testis degeneration and *in vitro* haemolysis is given in Table II (Vles and Jager, 1970).

Table II

Relation between myopathy, testis degeneration and spontaneous *in vitro* haemolysis in vitamin E deficient rats

	D-α-tocopheryl acetate (mg/kg food)					
	0.5	1.5	2.5	4.5	8.5	16.5
Number of animals studied	7	8	6	6	6	4
Mean number of hyaline degenerated fibres per leg muscle cross-section	10.0	7.8	5.6	2.4	0.8	0.4
Testis degeneration (score 0-8)	7.6	0.9	0.7	0.7	0	0.5
Spontaneous *in vitro* haemolysis (%)	90	87	80	41	19	3

No signs of disability in locomotion were observed. Obviously, an amount of 0.5 mg D-α-tocopheryl acetate per kg food was already enough to protect the animals in this respect.

According to Table II, even 1.5 mg D-α-tocopheryl acetate per kg food was sufficient to prevent testis degeneration. Some degeneration was, it is true, found in the groups receiving 1.5 mg vitamin E or more, but this is likely to be the result of old age (Berg, 1967).

Over a period of 22 months, the vitamin E requirement to prevent more than 10% haemolysis was practically constant (12 mg D-α-tocopheryl acetate per kg food). This was in good agreement with the results mentioned previously (Jager, 1968). In Fig. 5, the individual relation between the number of hyaline-degenerated fibres per muscle cross-section and the percentage spontaneous haemolysis in vitro is expressed. An haemolysis of less than 50% is accompanied by hardly any myopathy.

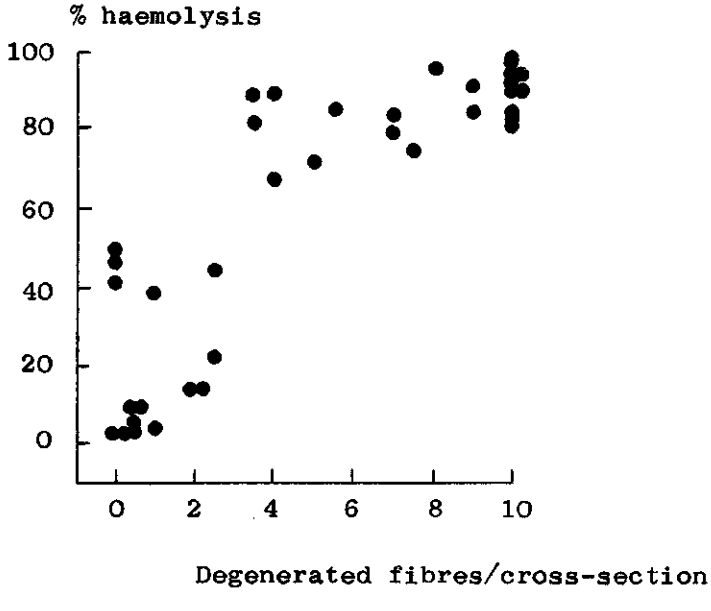


Fig. 5 Relation between number of hyaline-degenerated fibres per muscle cross-section and degree of in vitro haemolysis in rats.

Severe myopathy may be expected if the haemolysis is more than 70%. We might conclude, therefore, in contrast with the opinion of Alfin Slater et al. (1969a), that the in vitro haemolysis test is a valid indicator of vitamin E nutriture. A high percentage of haemolysis indicates that the vitamin E status is so low that it may predispose to myopathy (Jager, 1969a).

2. In other species

In the case of vitamin E deficiency, in vitro haemolysis, either by the addition of dialuric acid or hydrogen peroxide or spontaneously, is to be expected in all mammalian erythrocytes. In man in vitro haemolysis is often found in new-born or premature infants; in the latter case it is mostly associated with some degree of anemia (György et al., 1952; Gordon and Nitowsky, 1956; Hanna et al., 1965; Oski and Barness, 1967). Obviously only little tocopherol passes the placenta as the plasma tocopherol level of the new-borns is mostly very low, whereas the mother has normal values. After administration of vitamin E to the children, the haemolysis disappears and the haemoglobin levels become normal within two weeks (Oski and Barness, 1967). In vitro haemolysis in adult men, under experimental vitamin E deficiency, has been reported by Horwitt et al. (1956). In patients with gastro-intestinal diseases, associated with severe steatorrheo, a positive haemolysis test is often found, frequently concomitant with creatinuria (Binder et al. 1965). A true nutritional vitamin E deficiency was found in some Jordanian children by Majaj et al. (1963). The disease, however, was complicated by a protein malnourishment. Also Whitaker et al. (1967) cured anemia in protein-malnourished children by the additional administration of vitamin E.

Vitamin E deficiency, as expressed in a positive haemolysis test, has been reported in various animals: monkeys (Fitch et al., 1964), pigs (Hill, 1963), rabbits (Draper, 1959, Ringler and Abrams, 1971) and dogs (Haynes and Rousseau, 1970). In mammals,

a positive haemolysis test may occur for a long time, without any concomitant changes in histological structures, indicative for vitamin E deficiency. The in vitro haemolysis test is therefore regarded as the most sensitive criterion for vitamin E deficiency in mammals. In contrast to this, the erythrocytes of birds seem to be very resistant to in vitro haemolysis. Christensen et al. (1956) noticed that chickens on diets that produced severe encephalomalacia, showed almost no in vitro haemolysis, whether spontaneous or induced by adding dialuric acid. The same phenomenon was observed by Jager and Vles (1970) in ducklings, where despite serious myopathy of leg muscles and gizzard, the spontaneous in vitro haemolysis remained very low. These findings are probably related to the difference in erythrocyte structure between birds and mammals. The red cells from the first still contain a nucleus and those from the latter do not.

Although in vitro haemolysis has been found to occur in a wide variation of different animals, it has only been used in rats for the actual determination of vitamin E requirement (Rose and György, 1950b; Friedman et al., 1958; Ward, 1963; Jager, 1968).

B. MYOPATHY

Myopathy of the skeletal muscles is the most frequently occurring manifestation of vitamin E deficiency in mammals and birds. This general occurrence would make it suitable for the determination of vitamin E requirement. However, this is only possible when myopathy is a rather sensitive criterium for all the animal species studied and when the myopathy is not complicated by other vitamin E deficiency symptoms at the same time.

Myopathy may be studied by direct microscopical observation of the diseased muscle cells, but also by recording secondary effects as the increased excretion of creatine in the urine (creatinuria) (Gerber et al., 1952) or the elevated level

of different serum enzymes, which are supposed to leak from the diseased muscle cell into the bloodstream (Jager et al., 1969). Gerber et al. (1952) concluded that creatinuria in rats was not caused by a release of creatine by the diseased muscle cell, but that the uptake of creatine by the muscle was diminished in the vitamin E deficient animal, so that, in consequence, the size of the free creatine pool increases and creatinuria results.

Although the skeletal muscles are in general the most sensitive to vitamin E deficiency, myopathy may also be found in heart muscle (Bowman and Dyer, 1960) and in the smooth muscles of stomach (Jungherr and Pappenheimer, 1937) and intestine (Jager, 1972b).

The study of myopathy in vitamin E deficient animals is somewhat complicated by the fact that the disease may be prevented in part or completely by the addition of cystine or menthionine to the diet (Dam et al., 1952; Machlin and Shalkop, 1956; Scott and Calvert, 1962; Witting and Horwitt, 1964b). Another complication is formed by selenium, which was originally discovered as the factor giving protection against dietary liver necrosis in rats (Schwarz and Foltz, 1957). It was also found that only trace amounts in the diet are needed to prevent almost completely myopathy in vitamin E deficient chicks (Dam and Sóndergaard, 1957; Scott, 1962) ducklings (Jager, 1972b) and rats (Moore and Sharman, 1964). It was, however, found ineffective in rabbits (Hove et al., 1958; Proctor et al., 1961).

Jager (1972b) found that for the protection of ducklings against myopathy the activity of 0.1 mg Se is equivalent to at least 20 mg D-α-tocopheryl acetate. This effect of Se could not be explained by a raised blood tocopherol level as Desai and Scott (1965) concluded from their experiments on chickens. Already 0.1 mg Se/kg food protected ducklings fully against myopathy, despite a serum tocopherol level of only 1.2 μg/ml. In ducklings not receiving Se, a serum tocopherol level of 5.3 μg/ml was needed.

A survey of selenium treatment against myopathy in a wide range of different animals has been given by Wolf et al. (1963).

The function of vitamin E in the prevention of myopathy is still not quite clear, Zalkin et al. (1962) found a release of lysozomal enzymes in vitamin E deficient rabbit muscle and advanced the theory that for lack of antioxidant the primary damage would consist of free radical damage to lipoprotein membranes of the cell and its subcellular organelles. Macrophages and phagocytic leucocytes, which are particularly rich in lysozomes, enter the regions of lipid peroxidation and could undergo rupture due to peroxidation damage. This process would set free lysozomal enzymes, leading to cell necrosis. Morphologic studies on rats (Howes et al., 1964), chickens (Cheville, 1966) and rabbits (van Vleet et al., 1968) indicated that the primary injury involves mitochondria. Van Vleet et al. (1968) draw attention to their observation that although lysozomes were numerous in degenerated muscle cells, they were not observed in skeletal muscle fibres during the early stages of degeneration; they therefore concluded that lysozomes served a secondary role in muscle cell lysis. Jager (1972a) found that vitamin E deficient rat muscle fibres at an early stage of degeneration frequently showed an increased activity of succinic dehydrogenase (SDH), just as was noted by Cheville (1966) for chicken muscle. But the most conspicuous feature in these rat muscle fibres was the very strong activity of lactic dehydrogenase (LDH). It was found (Jager, 1972a) that as the state of degeneration increased, the SDH-activity decreased and finally disappeared, whereas LDH activity increased and remained highly active until complete myolysis of the fibre set in. This is demonstrated in Fig. 6: strong LDH activity is especially found in the swollen fibres, a stage just preceding complete myolysis. These phenomena indicate that apparently an increasing part of the required cell energy is taken up by glycolysis when the mitochondria fail to supply sufficient energy by normal oxidation.

(a)

(b)

Fig. 6 (a) Cryostat section (140x) of muscle of vitamin E deficient rat showing hypertrophic and atrophic fibres and an increase of interstitial cells. Staining: haematoxylin-eosin.

(b) Alternate section of Fig. 5. Swollen fibres show strong LDH-activity.

When the glycolysis, as a last source of energy, also fails, myolysis starts. Therefore, muscle degeneration obviously finds its cause in a failure of cellular energy production.

With respect to the mitochondria, indications have been found that in some way vitamin E is involved in oxidative phosphorylation (Corwin, 1965; Naito et al., 1966; Carabello et al., 1971). Another phenomenon that might be related to mitochondrial disfunction has been found by Molenaar et al. (1968, 1970), who observed in electron microscopy of intestinal epithelial cells of man and ducklings a decreased membrane contrast in state of vitamin E deficiency. This was most distinctly observed in the outer mitochondrial membrane. This contrast-loss in membranes was explained as a critical loss of double bonds and therefore probably represented a visualization of lipid peroxidation. In agreement with this explanation, Vos et al. (1972), who found an analogous loss of contrast in liver tissue of vitamin E deficient ducklings, demonstrated that on analysing the fatty acids of these liver mitochondria the largest decrease in arachidonic acid was found in the outer membranes.

These observations strongly suggest that vitamin E may function in some way as an antioxidant in vivo. This view on vitamin E has found support by many researchers (Tappel, 1962, 1965, 1970; Horwitt, 1965; Witting, 1965). Bunyan et al. (1967), however, were not able to detect an increase in lipid peroxides in different parenchematous tissues of vitamin E deficient rats. Such an increase may, however, in some instances be found in adipose tissue of vitamin E deficient animals (Glavind et al., 1971; Green, 1972). Several observations have been reported (Diplock et al., 1968; Green, 1972) that do not quite fit in with an in vivo antioxidant function of vitamin E and it is quite obviously not the only function of this vitamin (Gilbert and Birky, 1971). The antioxidant concept still remains the most widely accepted view on vitamin E function.

1. Dose-response effects in ducklings (Jager and Verbeek-Raad, 1970)

(a) Introduction. Ducklings are suitable animals for vitamin E research: when placed, after hatching, on a vitamin E deficient diet, the animals develop, within a few weeks, a severe myopathy. The disease is characterized by a hyaline degeneration of the striated muscles; symptoms of encephalomalacia or exudative diathesis are not observed (Pappenheimer and Goettsch, 1934; Pappenheimer, 1940).

The suitability of the duckling for determination of vitamin E requirement was investigated by studying the dose-response effects of vitamin E intake versus different criteria. It was found by Jager and Vles (1970) that the myopathy itself could be best expressed by awarding a score after microscopical examination of a muscle cross-section. The myopathy score of each section ranged from 0-4, indicating that less than 3 fibres (0), up to 25% (1), 50% (2), 75% (3) and 100% (4) of the muscle fibre was affected. The myopathy score of an individual animal was obtained by adding together the myopathy scores of all the muscles examined. This myopathy score was found to give better results than the often used incidence of myopathy (percentage of animals affected) or even than the precise determination of the percentage of affected fibres per muscle cross-section.

Concomitant with myopathy, several other changes are to be expected in the case of vitamin E deficiency that might be suitable criteria for determining the vitamin E requirement. An increased activity of the following serum enzymes might be observed; aspartate amino-transferase (GOT) (Walter and Jensen, 1964; Anonymus, 1966), lactic dehydrogenase (LDH) (Giese, Erzoy and Hill, 1965; Anonymus, 1966) and creatine phosphokinase (CPK) (Bird and Carabello, 1966). Besides leucocytosis (Dinning, 1952), a change in serum protein composition should be expected (Goldstein and Scott, 1956).

(b) Experimental design. Six groups of 10 male Peking ducklings were kept from the day of hatching on a lard diet, as indicated in Table I. Vitamin E was added to the diet in increasing doses as D-α-tocopheryl acetate. After 4 weeks the survivors were killed by decapitation, but just before that, blood was obtained by heart puncture for differential leucocyte counts and serum determinations of proteins, enzyme activities and total tocopherol. At autopsy, the bodies were observed for gross pathology and parts of the heart and gizzard removed for microscopical examination. The myopathy score was determined by taking 3 pairs of muscles from the legs, viz., M. gastrocnemious pars interna, M. sartorius and M. semimembranosus. Since 6 cross-sections were examined the maximum score per animal was 24.

(c) Results. The dose-response effects of vitamin E according to different criteria are summarized in Table III. The myopathy of the skeletal muscles manifested itself at autopsy by longitudinal, white striations and a waxy appearance. Concomitant with the severest cases of myopathy of the legs, occasionally haemorraghe and a greenish exudate were found. Microscopically the disease was characterized by the typical Zenker's degeneration (Fig. 7) as described by Pappenheimer and Goettsch (1934).

Even at high doses of vitamin E, some affected fibres may occasionally be found. Vitamin E requirement was therefore defined as the amount needed to prevent a myopathy score of more than one. A linear relationship was found between the logarithm of the vitamin E dose and the myopathy score (Fig. 8). It was estimated that 16 mg D-α-tocopheryl acetate per kg food were required to prevent myopathy of the skeletal muscles.

The lesions in the gizzard were recognizable macroscopically as circumscribed white areas (Fig. 9). The histological picture showed necrocalcinosis and proliferation of muscle nuclei.

Table III

Dose-response effects of vitamin E in ducklings after 4 weeks (mean values)

Criterion (* = in survivors) \ Vitamin E (mg/kg diet)	1.7	2.5	3.8	5.0	9.5	15.5
1 Myopathy score (0-24) (n=10)	10.0	9.2	6.2	4.8	3.7	1.1
2 Myopathy score*	7.2	5.5	5.8	4.8	4.0	1.1
3 Gizzard myopathy (number of animals) (n=10)	4	4	3	0	0	0
4 Heart lesions (number of animals) (n=10)	4	1	0	0	0	0
5 Mortality (number of animals) (n=10)	4	5	1	0	1	1
6 Weight gain (g)*	820	840	860	980	1000	910
7 C.P.K. (Wroblewski units)*	1900	1900	2400	2900	1800	800
8 G.O.T. (Karmen units)*	149	138	121	55	49	45
9 L.D.H. (Wroblewski units)*	2090	2120	1670	1400	1200	700
10 Albumin (mg/ml)*	13.9	17.1	20.2	22.0	22.7	25.8
11 Tocopherol (µg/ml)*	0.6	1.7	2.6	4.2	4.7	7.6
12 Differential leucocyte count:*						
Heterophilic granulocytes (%)	53	38	38	28	41	33
Heterophils with rods (%)	20	12	7	6	7	4

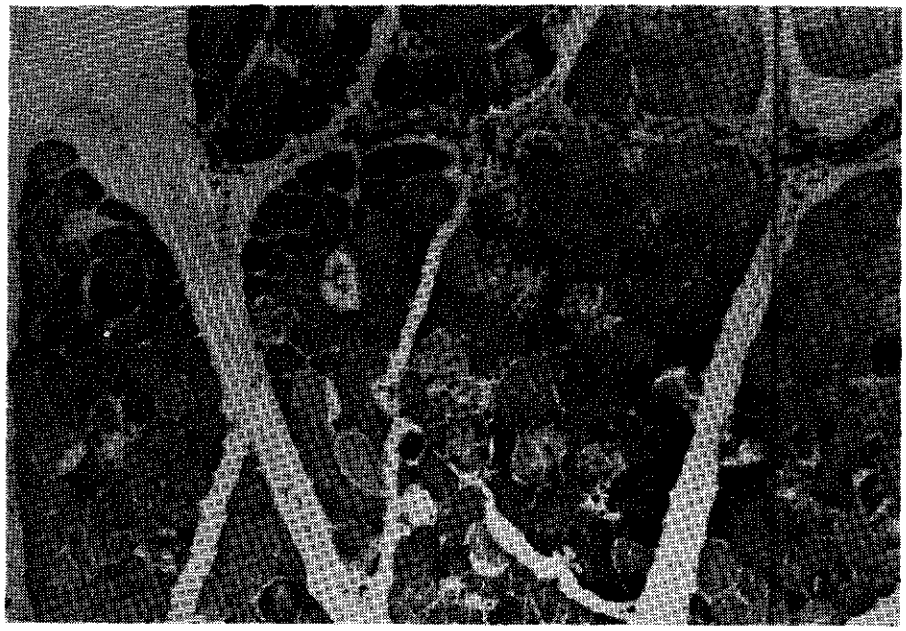


Fig. 7 Cross-section (140x) of leg muscle of vitamin E deficient duckling, showing hyaline necrosis of scattered muscle cells, associated with infiltration of macrophages and cellular reaction in perimysium.
Staining: Masson trichrome.

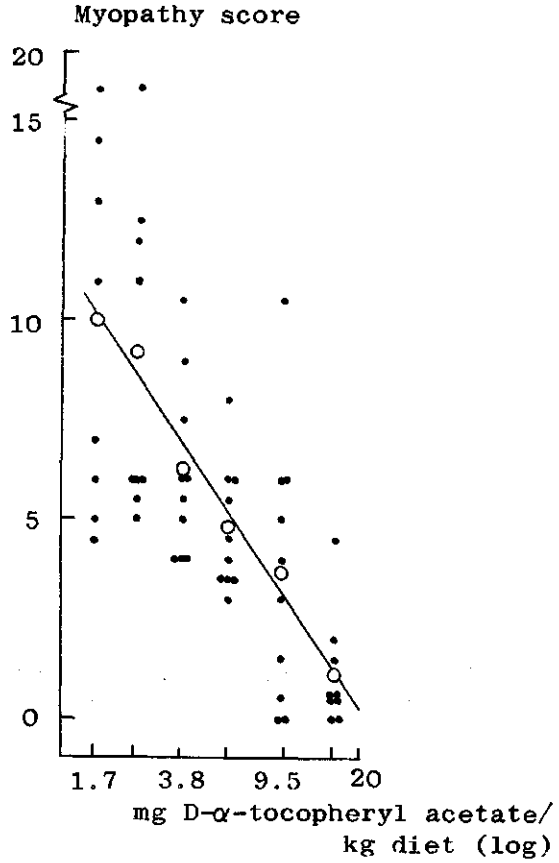


Fig. 8 Effect of vitamin E on myopathy in ducklings, 4 weeks after hatching. O = mean value.

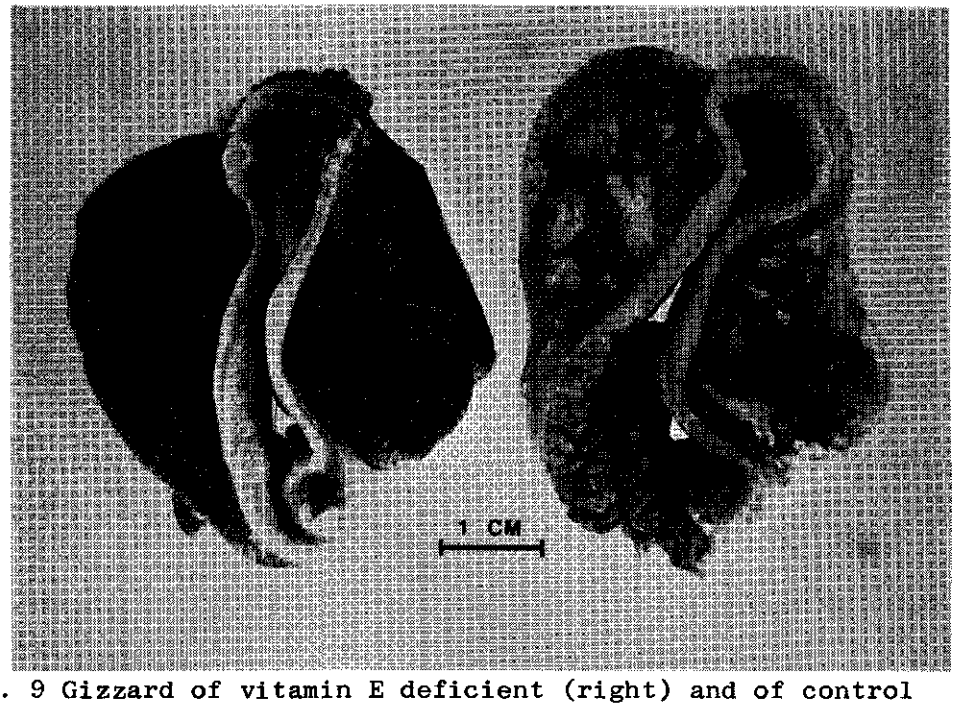


Fig. 9 Gizzard of vitamin E deficient (right) and of control (left) ducklings. Severe calcinosis is shown by the white areas in the cross-section of the left gizzard.

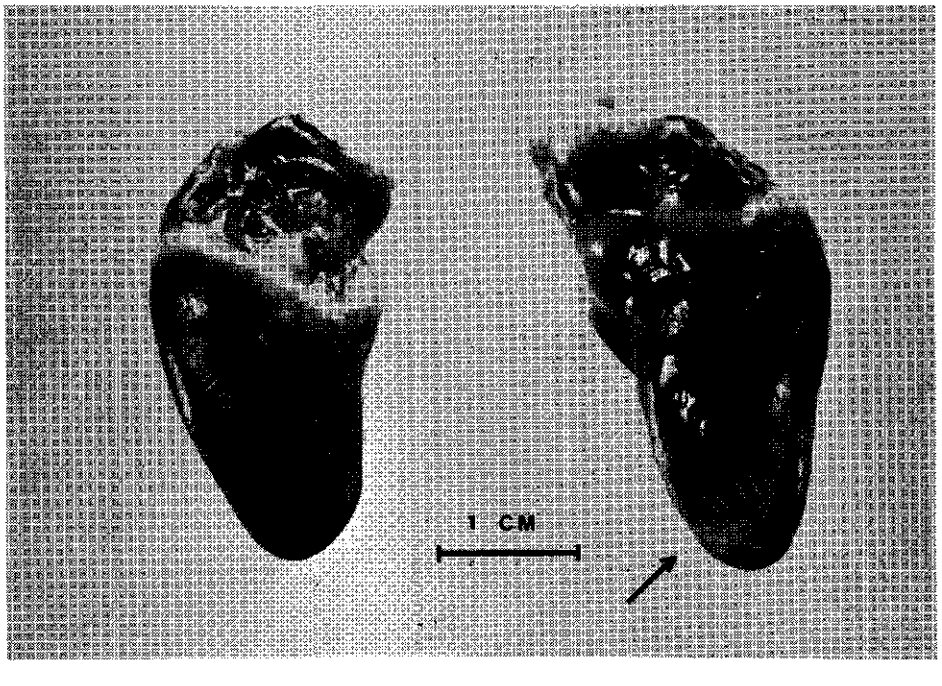


Fig. 10 Heart of vitamin E deficient (right) and of control (left) ducklings. Site of calcinosis is indicated by arrow.

This picture was different from that observed by Scott et al. (1967) in the gizzard of turkey poults, where, besides proliferation of muscle nuclei, edema with infiltration of heterophilic leucocytes and fibrosis were the main features; calcinosis was not found. Obviously as little as 6 mg D-α-tocopheryl acetate per kg food was sufficient to protect the smooth muscles of the gizzard from myopathy, while even less (4 mg) was needed to protect the heart musculature. The heart lesions were also characterized by *necrocalcinos* (Figs. 10 and 11). In addition, proliferation of muscle nuclei, edema and haemorraghe were observed.

Besides in heart and gizzard of vitamin E deficient ducklings, necrocalcinosis may also occur in the smooth musculature of the intestine, as was found by Jager (1972b) in a later experiment on ducklings (see also Table VI). The disease is illustrated in Figs. 12 and 13.

Mortality occurred for the most part during the 3rd experimental week. Practically all animals with a low body weight (⟨ 800 g) showed gizzard myopathy or severe myopathy of the legs (score ⟩ 6). Jager and Vles (1970) found a significant correlation between the mean food consumption and weight increase per vitamin E dosing group. The decreased weight-gain in the groups with the lower vitamin E doses seems to be a secondary effect, caused by a reduced food intake as a result of myopathy of the gizzard. Further, an impaired mobility and reduced food and water intake was observed, due to weakness of the skeletal muscles. The disease seems to reach rather suddenly a critical point, as during the first two weeks no differences in weight gain or food intake were observed between the groups. Within each group the increase in weight varied greatly. An amount of 6 mg D-α-tocopheryl-acetate/kg food appeared to be sufficient for a normal growth response.

An increased CPK activity is considered typical of muscular diseases.

Fig. 11 Heart-muscular tissue (140x) of vitamin E deficient duckling. Areas of necrocalcinosis are characterized by dark, coarse granular appearance and pycnotic nuclei. Staining: haemotoxylin-eosin.

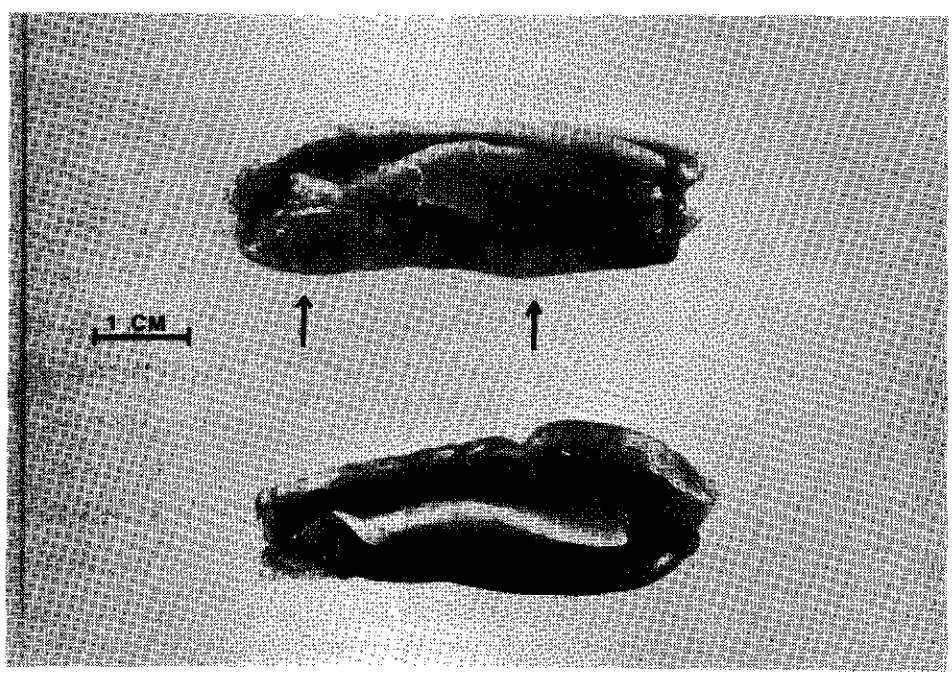


Fig. 12 Loop of duodenum around pancreas of vitamin E deficient (upper) and control (lower) duckling. Calcinosis, indicated by arrows, manifests itself by white cross striations.

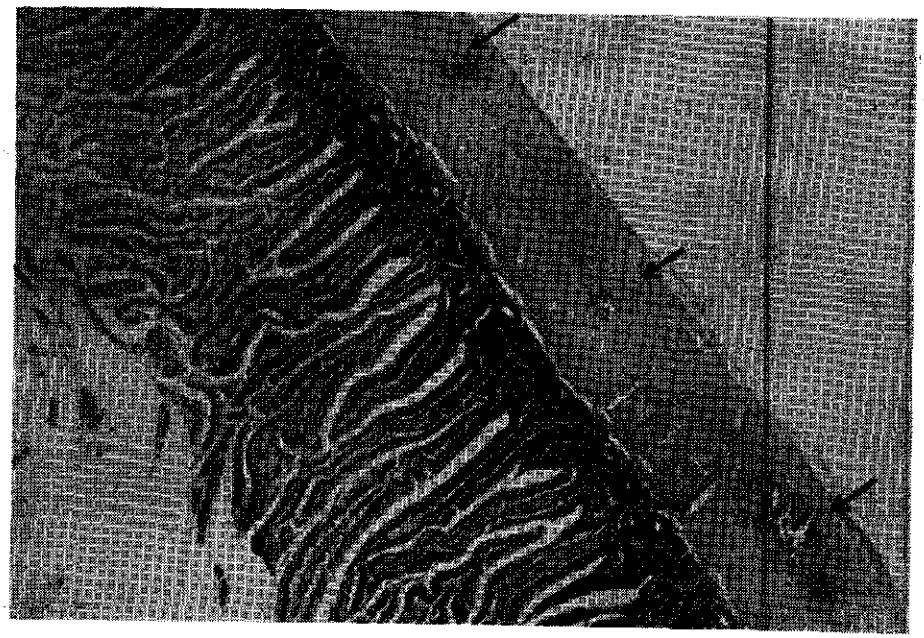


Fig. 13 Duodenum (55x) of vitamin E deficient duckling. Sites of necrocalcinosis are indicated by arrows. Staining: haemotoxylin-eosin.

The general concept is that the serum enzyme is of muscular origin and enters into the bloodstream as a result of an increased permeability of the muscle cell membrane (Bird and Carabello, 1966). On the basis of this concept, an increase in CPK activity in the blood could be expected, the ultimate activity being the result of an equilibrium between release and breakdown of the enzyme. A positive correlation was indeed found between the myopathy score and the CPK activity.

However, at the lowest vitamin E doses, where high myopathy scores prevailed, the CPK level - in contrast to GOT and LDH - was low. This is probably explained by the earlier onset of myopathy at the lowest vitamin E doses and the faster disappearance of CPK from the blood than the GOT and LDH (as known to occur after myocardial damage).

A significant negative relationship was found to exist between the logarithm of the vitamin E dose and the GOT activity, suggesting an S-shaped curve, although there was no

proof of a significant deviation from a linear relationship because of the great variations within the groups.

The LDH activity showed a linear relationship to the log dose vitamin E. The serum level of this enzyme seems to offer a sensitive criterion for establishing vitamin E deficiency (myopathy), in addition to the advantage of a linear relationship to determine vitamin E requirement.

The serum protein concentrations appeared to be linearly related to the log dose vitamin E (Fig. 14). The albumin concentrations decreased, whereas those of $\alpha_1 + \alpha_2$-globulin and γ-globulin increased at low vitamin E doses. These results suggest that vitamin E is in some way, involved in protein metabolism, although for the lowest vitamin E doses, the low serum albumin content might be partly caused by a reduced food intake.

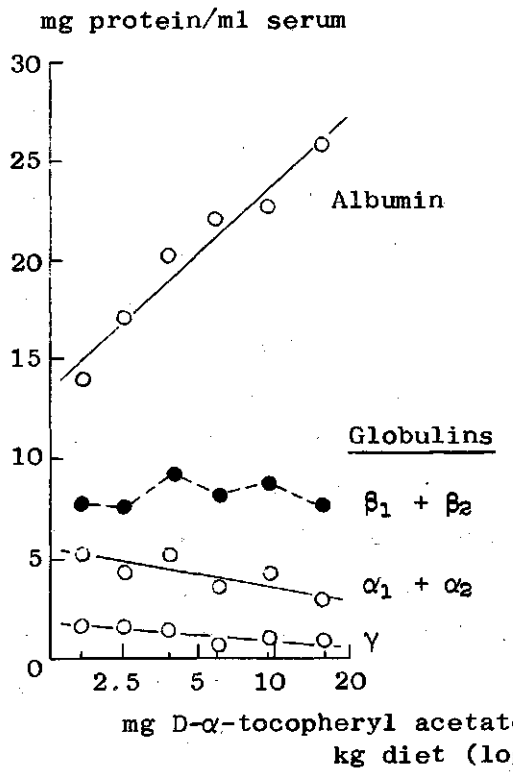


Fig. 14 Effect of vitamin E on serum-protein concentration in ducklings (n = 5-10).

A linear relationship was found between the log dose vitamin E and the serum tocopherol level, but the tocopherol concentrations varied greatly within one group, e.g. for the group receiving 5.9 mg vitamin E per kg food from 0.4 to 10.0 µg/ml serum.

Statistical analysis revealed that there was no significant influence of vitamin E on total white blood cell count (number/µl) or on the percentages of heterophils with granular eosinophilic bodies, eosinophils, basophils and monocytes, but from 1.7 to 5.9 mg vitamin E there was a significant increase of lymfocyte percentage and a decrease of the percentage of total heterophils and of heterophils with rodshaped eosinophilic bodies. Severe granulocytosis, as found by Dinning (1952) in rabbits and by Nafstad (1965) in pigs, was only occasionally observed in the vitamin E deficient ducklings. Obviously the differential white blood cell count does not offer a criterion sensitive enough for the determination of vitamin E requirement in ducklings.

(d) Conclusions. Myopathy of the skeletal muscles proves to be the most sensitive and reliable criterion for vitamin E deficiency studies in ducklings. As in fact - as far as known - myopathy in any form is the only disease occurring in vitamin E deficient ducklings, the importance of the criteria investigated in the serum depends upon their relation to the myopathy. The correlation coefficients between the myopathy score and the criteria investigated were as follows:

LDH	+0.71
log GOT	+0.70
log CPK	+0.55
albumin (mg/ml)	-0.52
tocopherol (µg/ml)	-0.49
heterophilic granulocytes	+0.53

LDH and GOT showed good correlation with the myopathy score. It must, however, be realized that perhaps better correlations would be obtained if the skeletal muscles were the only source of these enzymes, as it may be expected that at the lowest doses of vitamin E the gizzard and heart muscles also contribute to the enzyme levels in the blood. The correlation with CPK was low, as might be expected for reasons explained previously.

Although both serum albumin concentration and the myopathy score showed an linear relationship to the log dose vitamin E, the correlation between both was poor. The explanation is that the changes in serum protein concentrations must be considered a vitamin E deficiency phenomenon, independent of myopathy and are probably caused by some disfunction of the liver.

The serum tocopherol level appeared to give only a rough indication of the state of muscular disease. The correlation coefficients between the serum tocopherol level and the above criteria, determined in the same way as for the myopathy score, were either much lower or entirely absent.

Although in this experiment the correlation coefficient between the myopathy score and the percentages of heterophilic granulocytes was only poor (+0.53), in another experiment on vitamin E deficient ducklings by Jager and Vles (1970) a much better correlation of +0.67 was found. This suggests that the granulocytosis is the direct result of the muscle disease.

The ultimate conclusion from this experiment must be that, in ducklings, the histologically assessed score of myopathy of the skeletal muscles offers the best criterion for determining vitamin E requirement. It was ultimately found (Jager, 1972b) that when, concomitant with degeneration of the skeletal muscles, severe myopathy of gizzard, heart and intestine occurs, the determination of serum LDH activity does not offer a suitable criterion for the determination of vitamin E requirement. The differential serum protein determination appeared to offer a far less accurate and sensitive criterion that the myopathy score.

Myopathy has been used only incidentally as a criterion for the determination of vitamin E requirement. Most work on myopathy in relation to dietary vitamin E has been done on chicken. Vitamin E requirement of chickens in relation to dietary selenium was determined by Scott (1962), using the incidence of myopathy as a criterium in combination with a score for the severity of the disease. This score was assessed by gross observation of the skeletal muscles at autopsy. The same method in vitamin E deficient chickens was used by Jenkins et al. (1962) and by Søndergaard and Dam (1970).

Another method is to determine the time needed until the onset of creatinuria, as has been done by Witting and Horwitt (1964a).

2. Dose-response effects in rats (Jager, 1972a)

The pathology of vitamin E deficient rats is mainly characterized by damage of the testicles and the skeletal muscles. The muscular disease in young rats has been described by Olcott (1938) and Pappenheimer (1939), while Evans et al. (1938) described the degeneration of cross-striated musculature in vitamin E deficient old rats. Vitamin E deficiency diseases in young rats may be observed by taking the offspring from vitamin E deficient (or nearly deficient) mother animals. When the experiment starts with young from mothers or normal diets, it may take several months before any histological changes due to vitamin E deficiency are observed.

Long-term, dose-response effects of vitamin E in relation to myopathy, as found by Jager (1972a), are given in Table IV. Besides myopathy of the skeletal muscles, degeneration of the testis was found (Table II). As appears from Table IV, not all phenomena mentioned showed clear dose-response effects. Some phenomena occur, almost to the same extent, at all doses of vitamin E. This is most probably due to the old age of the rats. Dystrophic changes, resembling those occurring in vitamin E deficiency may then be observed (Berg, 1967).

Table IV

Effect of vitamin E on morphology of both the musculi gastrocnemii in rats, after 22 months
Number of affected fibres observed per cross-section
(maximum count = 10)

D-α-tocopheryl acetate (mg/kg)	0.5	1.5	2.5	4.5	8.5	16.5
n	7	8	7	6	6	4
Fibre with one or more centralle located sarcolemmic nuclei	10.0	10.0	10.0	10.0	10.0	10.0
Swollen fibre	10.0	5.4	8.0	8.8	3.2	4.5
Swollen fibre with one or more centrally located sarcolemmic nuclei	9.0	4.5	5.6	3.4	1.1	1.5
Hyaline-degenerated fibre, infiltrated with histiocytes	10.0	7.8	5.6	2.4	0.8	0.4
Degenerated fibre with centrally located myoblasts	10.0	1.3	2.6	1.7	0.0	0.0
Fibre with centrally degenerated cylinder	0.6	0.3	0.3	0.0	0.0	0.0
Interfibrilar fat cells	10.0	8.4	6.3	9.6	5.1	4.2
Atrophied fibre, associated with increase of interstitial cells or with centrally located nuclei in the fibre	6.4	2.5	3.7	1.0	1.0	1.8

The amount of the hyaline degenerated fibres (Table IV) showed the best response to the dose of vitamin E and this degeneration can therefore be regarded as a typical result of vitamin E deficiency. Besides the different types of diseased muscle fibres mentioned in Table IV, an increase in interstitial cells was observed at decreasing doses of vitamin E.

The results obtained indicate quite clearly that myopathy in rats is not a sensitive enough criterium to serve the determination of vitamin E requirement.

3. Effects in other species

Except in ducklings and rats, nutritional myopathy, caused by vitamin E deficiency has been found in many different species: monkeys (Dinning and Day, 1957), cows (Vawter and Records, 1947), horses (Jones and Reed, 1948), sheep (Metzger and Hagan, 1927), pigs (Adamstone et al., 1949; Forbes and Draper, 1958), goats (Madsen et al., 1933), dogs (Anderson et al., 1939), minks (Mason and Hartsough, 1951), guinea pigs and rabbits (Goettsch and Pappenheimer, 1931), hamsters (Houchin and Mattill, 1942), chickens (Dam et al., 1952), turkeys (Jungherr and Pappenheimer, 1937) and guppy fish (Cumings, 1942).

Although severe myopathy may occur in monkeys, the most important symptom of vitamin E deficiency in these animals is formed by anemia (Dinning and Day, 1957). In chickens the myopathy is complicated by the occurrence of encephalomalacia and exudative diathesis at the same time (Cheville, 1966; Scott, 1970).

Pure nutritional myopathy caused by vitamin E deficiency has not been reported to occur in man. But tocopherol deficiency, manifested by creatinuria and lesions in the skeletal muscles, was found in patients who suffered from severe steatorrhea of long duration (Binder et al., 1965).

C. ENCEPHALOMALACIA AND DOSE-RESPONSE EFFECTS IN CHICKENS

Of all other symptoms of vitamin E deficiency (encephalomalacia, exudative diathesis, liver necrosis, anemia, resorption gestation, testis degeneration and diminished fertility) only the incidence of encephalomalacia in chickens has actually been used for the determination of vitamin E requirement (Dam and Søndergaard, 1964; and Søndergaard and Dam, 1966). The dose-response showed a sigmoid shaped surve (Fig. 15) that pointed to a vitamin E requirement (for a diet containing 50 cal% lard) of the same order as found by Jager (1968, 1972b) in rats (in vitro haemolysis test) and in ducklings (myopathy score).

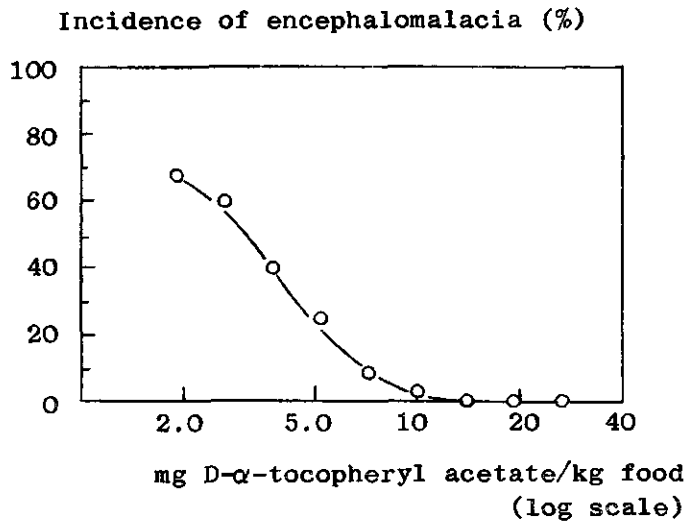


Fig. 15 (Søndergaard and Dam, 1966). Effect of dietary vitamin E on incidence of encephalomalacia in chickens (n=10).

III. EFFECT OF HIGH LINOLEIC ACID INTAKE ON VITAMIN E REQUIREMENT

Even in the early days of vitamin E research it was known that the intake of polyunsaturated fatty acids (PUFA) may increase vitamin E requirement. The addition of cod liver oil to vitamin E deficient diets was a known procedure to speed up the appearance of vitamin E deficiency symptoms. Although the effect of highly unsaturated PUFA and also linolenic acid on vitamin E requirement may be interesting from a theoretical point of view, PUFA in edible fats and oils consist almost exclusively of linoleic acid. For this reason the experiments mentioned here will relate only to this fatty acid.

Weber et al. (1964) calculated from their experiments on rats, using in vitro haemolysis as a criterion, that 0.5 mg DL-α-tocopheryl acetate per extra gram of linoleic acid would be needed. In contrast with these results, Sóndergaard and Dam (1966), in their experiments on chickens in which the incidence of encephalomalacia was used as a criterion, did not find any effect of linoleic acid intake on vitamin E requirement.

Weber et al. (1964) used single oral doses of vitamin E. This procedure may give too high values for the requirement, as the absorption of vitamin E is expected to be better, when incorporated in small quantities in the food (Pudelkiewicz and Nakiya, 1969). Moreover, the coconut oil-diet which they used as a control has to be considered EFA deficient.

A. EXPERIMENTS ON RATS (in vitro haemolysis) (Jager, 1969b,c)

1. Experimental design

96 newly weaned male albino Wistar rats (SPF) were kept for 4 weeks on a vitamin E deficient lard diet (Table I), in order to allow the animals to use up their vitamin E reserves. In a second period of 3 weeks, the animals were divided in 4 groups

each of 24 animals, that received increasing doses of vitamin E (D-α-tocopheryl acetate) in their diets as indicated in Fig. 16. In the third period of 9 weeks, half the animals of each group of the second period received tocopherol-free maize oil (Jager, 1969c) instead of lard in their diets.

Spontaneous in vitro haemolysis (Jager, 1968) was used as a criterium to indicate vitamin E requirement.

The fatty acid composition of the lard and maize oil is given in Table V.

2. Results

The mean results of weekly determinations during the experimental period are presented in Fig. 16. The difference in vitamin E requirement between the lard and maize oil group appeared to be very significant.

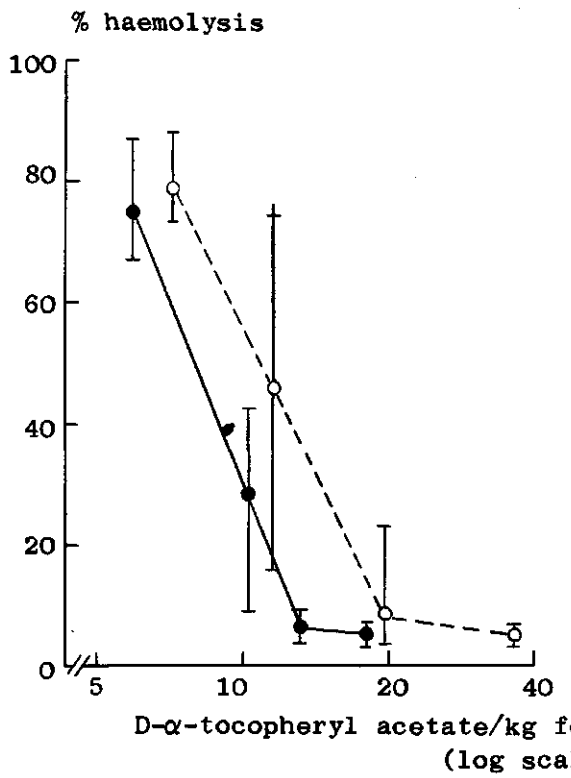


Fig. 16 (Jager, 1969b). Effect of type of diet on dose-response curves of vitamin E (n=12).
● lard
○ maize oil.

Table V

Fatty acid composition (%) of lard and maize oil

Fatty acid	Lard	Maize oil
C 14:0	2.0	-
C 16:0	29.5	13.0
C 17:0	0.5	-
C 18:0	18.0	1.7
C 18:1, ω9	36.0	27.0
C 18:2, ω6	10.5	56.7
C 18:3, ω3	-	1.1
C 20:0	1.5	-
C 20:1	0.5	-
C 20:2	0.5	-
Iodine value	58	125

For both diets a linear relationship was found between log vitamin E content of the diet and the haemolysis percentage. The regression coefficients of the two dose-response curves, however, differed significantly and therefore the lines in Fig. 16 do not run parallel.

According to the haemolysis test, the vitamin E requirement for the lard diet (4.0 cal% linoleic acid) was 12.4 mg D-α-tocopheryl acetate per kg food and that for the maize oil diet (20.2 cal% linoleic acid) was 19.0 mg; this is 2.4 and 3.7 mg D-α-tocopherol per 1000 kcal of food.

3. Conclusion

Table V shows that the great difference in PUFA content between lard and maize oil almost exclusively concerns linoleic

acid. Calculated per kg food, the difference in linoleic acid content between the lard and the maize oil diet is 81.2 g, and the difference in need of vitamin E is 6.6 mg D-α-tocopheryl acetate. This difference is (Brubacher and Weiser, 1967) equivalent to 136/149 x 6.6 = 6.0 mg D-α-tocopherol. On the assumption that the need of vitamin E increases linearly with increasing linoleic acid content, this need is 6.0/81.2 = 0.074 mg D-α-tocopherol per extra gram of linoleic acid. We may therefore conclude that the intake of vegetable oils with a high linoleic acid content, such as sunflower, maize and safflower oil, may cause an increased requirement for vitamin E, but that this increase is amply covered by the natural vitamin E content of these oils, as 74 mg D-α-tocopherol per kg linoleic acid would itself be sufficient to cover this extra need.

B. MYOPATHY IN DUCKLINGS (Jager, 1972b,c)

1. Experimental design

Newly-hatched male Peking ducklings were given diets as indicated in Table I, the dietary fat being lard or stripped maize oil (tocopherol-free). The division into groups (n=8) with respect to the dietary vitamin E content is given in Table VI. After 4 weeks, the surviving animals were killed by decapitation. All animals were examined for gross pathology and different organs were removed for microscopic examination. The myopathy of the leg muscles was expressed in a score (0-24) as described before (II B. 1). Just before killing the survivors at the end of the experiment, blood was obtained by heart puncture for determination of the serum tocopherol level.

Table VI

Number of affected ducklings per group (n=8), after 4 weeks on diets containing lard (L) or tocopherol-free maize oil (M)

Criterion	Fat	D-α-tocopheryl acetate (mg/kg)					
		2.5	4.0	6.5	11.0	18.0	34.0
Mortality	L	8	6	1	0	1	0
	M	4	5	7	2	1	1
Fatty liver	L	3	5	2	2	0	0
	M	0	2	1	0	0	0
Necro-calcinosis:							
Heart	L	1	3	1	0	0	0
	M	4	5	1	3	0	0
Intestine	L	4	7	6	2	0	0
	M	4	3	4	4	3	0
Gizzard	L	8	8	1	2	0	0
	M	8	8	8	7	2	0

2. Results

The result of the assessment of the score of myopathy of the leg muscles is shown in Fig. 17. After square-root transformation, a linear relationship was found between dietary vitamin E content and myopathy score for both the lard and the (stripped) maize oil diet. The lines in Fig. 17 were found to run parallel. Twice as much vitamin E was needed for the maize oil diet (21.8 cal% linoleic acid) as for the lard diet (3.0 cal% linoleic acid); 27.0 and 13.5 mg D-α-tocopheryl acetate per kg diet respectively, or 6.4 and 3.2 mg D-α-tocopherol per 1000 cal of food.

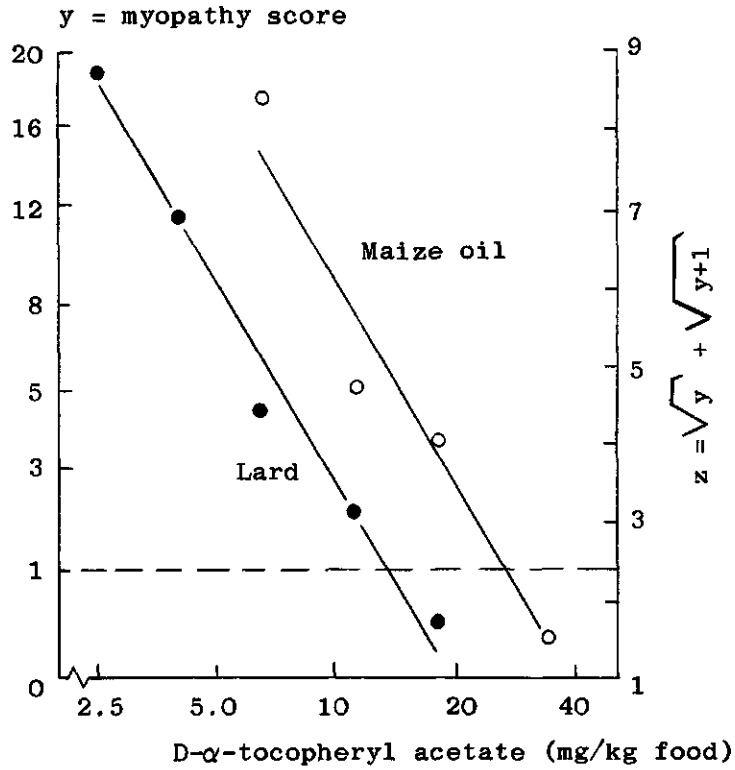


Fig. 17 Effect of dietary fats with low (lard) and high (maize oil) linoleic acid content on the need for vitamin E to prevent myopathy (score 1) of the skeletal muscles in ducklings.

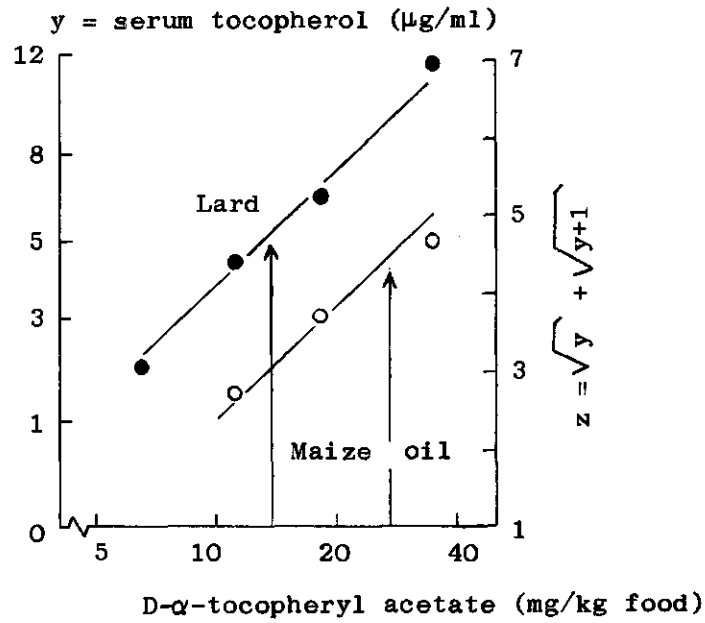


Fig. 18 Effect of dietary fats with low (lard) and high (maize oil) linoleic acid content on the serum-tocopherol level of ducklings. Arrows indicate amount of vitamin E needed to prevent myopathy (score 1).

The effects of linoleic acid intake on criteria other than the skeletal muscles, are indicated in Table VI. Although the values found were not so suitable for an exact calculation of vitamin E requirement as the myopathy score of the leg muscles, the general trend is the same. An exception is the occurrence of fatty liver, which showed a higher frequency in the lard groups than in the maize oil groups.

Fig. 18 illustrates the effect of a high linoleic acid intake on the serum tocopherol level. As for the myopathy score, a linear relationship was found with the vitamin E content of the diet, for both the lard and maize oil groups. The lines in Fig. 18 appeared to run parallel. The serum tocopherol levels of the maize oil groups were 43% of that of the lard groups. This means that the increased vitamin E requirement caused by the maize oil can be completely explained by the drop in blood-tocopherol level.

Obviously, the lard diet needed a somewhat higher serum-tocopherol level to avoid skeletal-muscle myopathy than the maize oil diet (5.3 and 4.6 μg/ml serum respectively). This difference might have been due to a difference in selenium content of the fats, although no selenium could be detected in any of the dietary fats used (thus, the concentration must have been below 0.1 mg per kg fat).

3. Conclusions

The important conclusion from this experiment is that high linoleic acid intake does not induce an increased need for vitamin E in the tissues, as the required blood-tocopherol level of the maize oil diet was not higher than that of the lard diet. The increased need for dietary vitamin E on the maize oil diet is obviously caused by destruction of vitamin E in the gastro-intestinal tract and perhaps by a decreased absorption, which results in a drop in the blood-tocopherol level.

For both the lard and maize oil diet, ducklings needed more vitamin E than rats, which is indeed to be expected for such rapidly growing animals as far as the basic requirement is concerned. Furthermore, at high dietary linoleic acid concentrations their feeding habits favour oxidation of the food components much more than is the case with rats.

The dietary fats are to be considered the natural sources of vitamin E. From this point of view it could be calculated that in order to cover the need for vitamin E, the lard should contain at least 86 mg D-α-tocopherol per kg fat. However, lard contains by nature seldom more than about 10 mg/kg fat. The maize oil should contain twice as much i.e. 172 mg D-α-tocopherol per kg fat, which is indeed amply covered by the natural vitamin E content of this oil. This conclusion was experimentally confirmed; ducklings on a lard diet all died within 3 weeks because of severe myopathy, when no extra vitamin E was added to the diet. On the other hand, ducklings on a diet containing natural maize oil did not need extra vitamin E. After 4 weeks on the (natural) maize oil diet all animals were in good condition and no signs of myopathy were observed.

Obviously, a mainly saturated animal fat such as lard must be considered inadequate in its vitamin E content, whereas a mainly unsaturated vegetable oil like maize oil is more than adequate.

The same conclusion can be drawn from an experiment by Dayton et al. (1965) who found that a group of men on a diet containing 40 cal% saturated fat showed a positive haemolysis test. This test, however, remained negative on an identical diet containing unsaturated vegetable fat.

C. ENCEPHALOMALACIA IN CHICKENS (Søndergaard and Dam, 1966)

From the day of hatching, chickens were given a vitamin E-free ration for one week, followed by a diet containing 50 cal% fat for 6 weeks. The fat was one of a series of mixtures of lard and (tocopherol-free) sunflower oil, which provided 3.3 cal% linoleic acid for the diet lowest, and 9.7 cal% for the diet highest, in linoleic acid. Each group at one dietary linoleic acid level was subdivided into 8 groups (n=10) receiving increasing doses of D-α-tocopheryl acetate in order to determine vitamin E requirement by means of the incidence of encephalomalacia (Fig. 15).

Increasing the level of dietary linoleic acid from 3.3 to 9.6 cal%, proved to have no effect upon the requirement for vitamin E, which remained 2.3 mg D-α-tocopherol per 1000 cal of food. Obviously, within this range of linoleic acid intake, no relationship with vitamin E requirement exists. An increase in the latter might, however, be seen at linoleic acid intakes above 9.7 cal%. This is, at least, strongly suggested by the results of Vogtmann and Prabucki (1971), who found that the liver vitamin E level of chickens after 3 weeks on a sunflower oil diet (22.5 cal% linoleic acid) was 67% of that of chickens on a lard diet (3.8 cal% linoleic acid). The dietary fats were tocopherol-free and vitamin E was added to both diets in equal doses of 4.0 mg DL-α-tocopheryl acetate/1000 cal of food.

D. SERUM TOCOPHEROL LEVEL AND IN VITRO HAEMOLYSIS IN MAN (Horwitt, 1960)

Information on vitamin E requirement of man is scarce. No direct facts with regard to vitamin E requirement of man are available. Horwitt (1960), in experiments on man (n=6), found that when a daily ingestion of 30 g lard was replaced by 30 g (tocopherol-free) maize oil, this caused a slight drop in the serum-tocopherol level (about 5%), which again fell by about 10% when the maize oil intake was increased to 60 g per day. From

these facts it could be calculated that at linoleic acid intakes of about 3, 9 and 15 cal% of the diet the corresponding serum-tocopherol levels were: 10, 9.5 and 8.0 μg/ml respectively, at an constant vitamin E intake of 15 mg D-α-tocopheryl acetate per day (+ 2 mg free tocopherol, contained in the food). At these serum-tocopherol levels, the in vitro haemolysis test remained negative.

IV. DOSE-RESPONSE EFFECTS OF LINOLEIC ACID INTAKE

A. VITAMIN E REQUIREMENT (Jager and Houtsmuller, 1970)

For 3 weeks, 192 newly weaned male rats were given a vitamin E deficient lard diet (Table I) in order to deplete them from their vitamin E reserves. Thereafter the animals were divided into 6 groups of 32 each that received for 5 weeks lard diets to which increasing doses of D-α-tocopheryl acetate were added; 2.5, 4.0, 6.5, 10.0, 15.5 and 23.5 mg/kg food respectively. In this period a steady state between vitamin E intake and the blood tocopherol level was reached.

Finally each group of 32 animals was split into 4 subgroups of 8 animals that received, for 5 weeks, increasing doses of linoleic acid in their diets: 0.5, 2.0, 7.0 and 25.6 cal% respectively. For this reason the lard in the diet was replaced by 4 different mixtures of coconut oil and (tocopherol-free) safflowerseed oil. In fact, the group receiving only 0.5% cal% linoleic acid, was on an essential fatty acid deficient diet (Holman, 1960), but an EFA deficient status was not attained during this period of 5 weeks, as the preceding periods on lard diets had supplied enough linoleic acid to build up sufficient reserves. During this last period, vitamin E requirement was determined weekly by the spontaneous haemolysis test in vitro (Jager, 1968).

The mean results over the experimental period are given in Table VII, together with those of other previously mentioned experiments.

Table VII

Relation between linoleic acid intake and vitamin E requirement or status

Dietary linoleic acid (cal%)	Vitamin E requirement		
	mg D-α-tocopherol/ 1000 kcal of food	Percentage of basic need	
0.5	2.5	100*	Rat; haemolysis test (Jager and Houtsmuller 1970)
2.0	2.5	100	
7.0	2.5	100	
25.6	3.5	140	
4.0	2.4	100	Rat; haemolysis test (Jager, 1969c)
20.2	3.7	154	
3.0	3.2	100	Duckling; myopathy score (Jager, 1972)
21.8	6.4	200	
3.3	2.3	100	Chickens; incidence of encephalomalacia (Søndergaard and Dam, 1966)
5.7	2.3	100	
8.1	2.3	100	
9.7	2.3	100	
	Tocopherol concentration in liver** (μg/g)		Chickens (Vogtmann and Prabucki, 1971)
3.8	52.2 ± 3.2		
22.8	35.3 ± 4.4		
	Serum-tocopherol level** (μg/ml)		Man (Horwitt, 1960)
3	10.0		
9	9.5		
15	8.0		

*Animals were not yet EFA-deficient because of a preceding period on a diet containing 4.0 cal% linoleic acid

**At equal vitamin E intake (see text)

They indicate, that up to an intake of about 10 cal%, linoleic acid has no effect on vitamin E requirement or status. Between 10 and 20 cal% dietary linoleic acid, the vitamin E requirement may increase up to 200%.

It is obvious that there exists a basic need for vitamin E, unaffected by linoleic acid intake. The increased requirement for vitamin E above 10 cal% linoleic acid intake is apparently caused by an increased destruction (and/or decreased absorption) of tocopherol in the intestinal tract, as shown by a decreased tocopherol level in liver and blood. Weber et al. (1964) made the remarkable observation that linoleic acid intake in rats had no effect on different tissue tocopherol levels, when vitamin E was administered intraperitoneally in contrast to peroral administration. Green et al. (1967) also showed that linoleic acid has no effect on tissue tocopherol levels, when interaction of this fatty acid with tocopherols in the gut was avoided, by giving them at separate times. When tocopherol and fatty acids were given together and interaction in the gut could take place, only about half as much tocopherol was recovered from rats given linoleate as from those given oleate. This was attributed to loss of tocopherol in the gut by increased peroxidation due to the presence of linoleate.

B. VITAMIN E REQUIREMENT AND DIETARY E/PUFA RATIO

It has been suggested by many researchers that PUFA intake should be balanced by a concomitant intake of vitamin E. Harris and Embree (1963) were led to calculate, that the dietary E/PUFA ratio should be at least 0.6. That is, the intake of each gram of PUFA would need a concomitant intake of at least 0.6 mg D-α-tocopherol, as otherwise symptoms of vitamin E deficiency could be expected. For this reason the results of the (spontaneous in vitro) haemolysis test in rats in relation to the effect of linoleic acid intake on vitamin E requirement (Jager, 1969b,c, Jager and Houtsmuller, 1970) have been compiled in

Fig. 19. They indicate quite clearly that as no fixed critical dietary E/PUFA ratio, exists any attempts to calculate such a ratio for the evaluation of the vitamin E adequacy of a certain diet, is pointless.

C. VITAMIN E REQUIREMENT AND EFA DEFICIENCY (E/PUFA ratio in membranes)

Literature data concerning the relation between linoleic acid intake and vitamin E requirement often seem contradictory. We find, on the one hand, experiments in which the addition of even small amounts of linoleic acid to the diet caused an increase in vitamin E requirement (Dam et al., 1958; Hutcheson et al., 1963; Calvert et al., 1964; Weber et al., 1964; Bieri and Poukka, 1970), and on the other, experiments in which linoleic acid had no such effect (Søndergaard and Dam, 1966; Jager and Houtsmuller, 1970). This discrepancy is due to the fact that in the first series of experiments, all diets used were EFA deficient. In this case the addition of linoleic acid to the diet causes an increase in the PUFA content (mainly arachidonic acid) of the bio-membranes, which is reflected by an increase in the need for vitamin E. This is, for instance, clearly demonstrated by the experiments of Bieri and Poukka (1970), who used the in vitro haemolysis test to determine vitamin E requirement. They found in their EFA-deficient rats a linear, positive relationship between the peroxidizable index of the erythrocytes and the required plasma α-tocopherol level needed to prevent haemolysis (Fig. 20).

The peroxidizable index (P.I.) is used to indicate the sensitivity to oxidative destruction of the erythrocyte lipids. The index represents an estimate of the relative peroxidizability of the PUFA and is obtained by multiplying the percentages of the various fatty acids by the following factors: 1, 2, 4, 6 and 8 for fatty acids containing: 2, 3, 4, 5 and 6 double bonds respectively (Holman, 1954; Witting and Horwitt, 1964a).

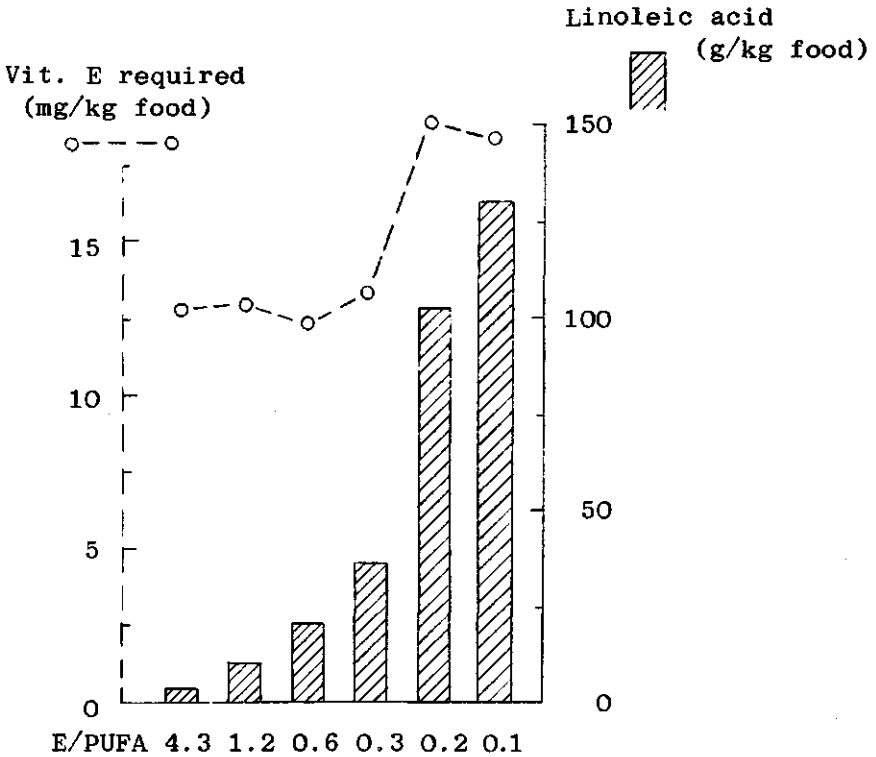


Fig. 19 Effect of intake of linoleic acid on vitamin E requirement of rats. E/PUFA = intake of D-α-tocopherol (mg) divided by intake of linoleic acid (g).

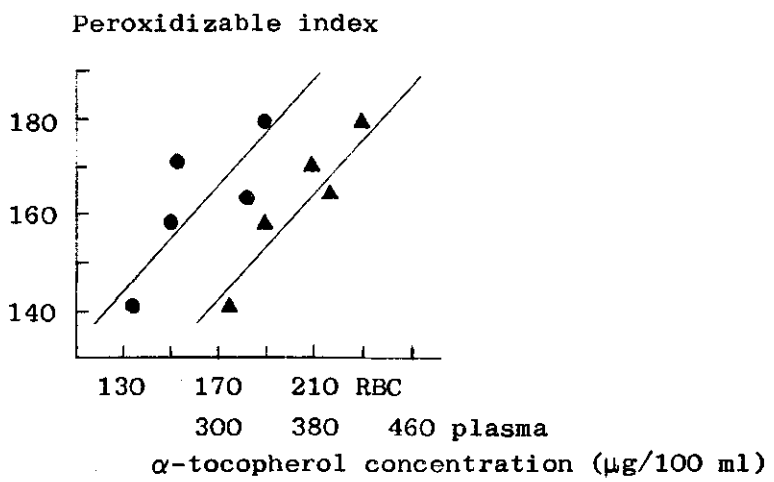


Fig. 20 (Bieri and Poukka, 1970). Relationship between the peroxidizable index of the erythrocyte polyunsaturated fatty acids and the α-tocopherol content of the red cells (●) or plasma (▲) required to prevent 10% haemolysis in EFA-deficient rats.

A case comparable to that in Fig. 20 was mentioned by Witting (1972), who found that in EFA and vitamin E deficient rats, the time needed to produce creatinuria decreased as the percentage ω6-fatty acids in the muscle fatty acids was increased by the addition of linoleic acid to the diet.

These examples show that although the E/PUFA ratio is not critical in diets, it is very probably critical in the biomembranes of erythrocytes and muscle. In other words, a certain amount of PUFA incorporated in biomembranes needs a circumscribed amount of α-tocopherol for protection against (oxidative) destruction. This view fits in quite well with a hypothesis by Diplock et al. (1971) and Lucy (1972) put forward on the basis of studies with molecular models. Lucy (1972) suggested that poly-unsaturated lipids can be packed in an orderly manner in a bilayer membrane by association with either vitamin E or cholesterol molecules. A special relation is assumed to exist between arachidonic acid and α-tocopherol. The position of two methyl groups of the alkyl-side-chain of the α-tocopherol molecule is thought here to be opposite ("space filling") to the system of double bonds of arachidonic acid. In the light of this special relation between α-tocopherol (which would fit in better than the other tocopherols) and arachidonic acid, it is easy to explain the increased need for vitamin E of EFA-deficient animals given additional dietary linoleic acid, as a proportional increase in arachidonic acid in the biomembranes results from linoleic acid intake in EFA-deficient animals (Holman, 1960). In non-EFA-deficient animals, however, the increase in dietary linoleic acid has no effect upon the vitamin E requirement, because the biomembranes are already "saturated" with arachidonic acid, and there is at least no important increase in PUFA. As shown in Table VIII (Jager, 1972c), the erythrocyte lipids increase their linoleic acid content, mainly at the expense of oleic acid (Jager and Houtsmuller, 1970). The increased need for vitamin E at an intake of 25.6 cal% linoleic acid is obviously not due to a

change in the fatty acid composition of the erythrocyte membrane, but is the result of a lowered blood tocopherol level, as explained previously. The P.I.s are lower than those found by Bieri and Poukka (1970), but this must be due to differences in the strains of rats used.

In order to get enough essential fatty acids, male rats would need 1-2 cal% linoleic acid in their diets (Holman, 1960). For chickens these figures are 3.6 and 1.8 cal% for males and females respectively (Menge, 1970).

Table VIII

Dietary linoleic acid content, fatty-acid composition of erythrocyte lipids and vitamin E requirement (haemolysis test) of rats

Linoleic acid		Erythrocyte lipids (%)				Vitamin E** requirement
Weight %	Cal%	C 18:1 ω9	C 18:2 ω6	C 20:4 ω6	P.I.*	
0.27***	0.5	13.7	5.2	18.8	128	12.9
0.98	2.0	12.2	7.9	19.5	130	12.8
3.6	7.0	9.9	9.4	20.9	135	12.7
13.0	25.6	9.0	13.3	20.7	145	18.5

*P.I. = peroxidizable index of erythrocyte lipids

**D-α-tocopheryl acetate (mg/kg food)

***This diet was in fact deficient in essential fatty acids, but no important decrease in erythrocyte arachidonic acid was found because of a preceding period of 8 weeks on a diet containing 4 cal% linoleic acid

V. VITAMIN E ADEQUACY OF FATS AND OILS

For the calculation of the vitamin E adequacy of a fat, it should be noted that in a diet containing a normal amount of fat (40 cal%) the fat component must contain sufficient tocopherol to cover the total dietary requirement for vitamin E.

Table VII shows that dietary linoleic acid up to an intake of about 10 cal% does not affect the basic requirement for vitamin E. This corresponds with a linoleic acid content of 100/40 x 10 = 25% in the dietary fat.

As a general conclusion from Table VII, we may state that 3 mg D-α-tocopherol per 1000 kcal of food will be sufficient to meet the basic need for vitamin E, which need may increase to 200% at linoleic acid intakes above 10 cal%. Calculated on this basis we may say that a dietary fat (9.4 kcal/g) at an intake of 40 cal%, needs 100/40 x 3 x 9.4 ≈ 70 mg D-α-tocopherol/kg fat, in order to contain enough vitamin E, if the fat contains more than 25% linoleic acid, the need may increase to 140 mg/kg fat.

More vitamin E per kg fat would be needed at a lower dietary fat level. For a diet containing 30 cal% fat, the basis need would be 93 and 186 mg D-α-tocopherol/kg fat, for fats containing less or more than 25% linoleic acid respectively. For the sake of convenience we might state that at a normal range of fat intake (30-40 cal%), 100 mg D-α-tocopherol/kg fat will be sufficient, an amount that will increase to 200 mg, if the dietary fat contains more than 25 cal% linoleic acid.

The vitamin E "adequacy" of different fats and oils are compiled in Fig. 21, based on a dietary fat intake of 35 cal%. The vitamin E content of the (refined) oils is based on data obtained from Weber et al. (1962), Herting and Drury (1963), Swern (1969) and Slover (1971). The total of different tocopherols has been expressed as equivalents of mg D-α-tocopherol,

according to the bio-potencies given by Brubacher and Weiser (1967) (see Fig. 1).

The most striking fact to appear from Fig. 21 is that obviously all highly saturated fats are deficient in vitamin E, whereas most vegetable oils with a high linoleic acid content must be considered natural sources of vitamin E. This was not only found from the experiment of Jager (1972b,c), but was also supported by the work of Dayton et al. 1965) and Alfin-Slater et al. (1969a,b), who found linoleic rich vegetable oils to be adequate in vitamin E.

An exact evaluation of soybean oil meets with some difficulty because of its relatively high content of linolenic acid. Quantitative experimental data, with respect to the effect of dietary linolenic acid on vitamin E requirement in non-EFA-deficient diets, are not available.

It was noted in several cases (in EFA- and vitamin E-deficient animals) that whereas dietary linoleic acid aggravated symptoms of vitamin E deficiency, dietary linolenic acid did not; these symptoms were encephalomalacia in chicks (Dam et al., 1958), myopathy in chicks (Hudcheson et al., 1963) or testicular degeneration in rats (Witting et al., 1967; Witting, 1970).

In a border-line case of dietary EFA deficiency, however, it was noted that ample dietary linolenic acid increased the onset of creatinuria in rats (Witting and Horwitt, 1964a; Witting, 1970). This was associated with an increase of ω3-fatty acids in the muscle phospholipids.

Therefore, although dietary linolenic acid might affect the fatty acid composition of biomembranes and consequently the requirement for vitamin E, this effect will be relatively unimportant in circumstances where the diet supplies ample linoleic acid. This may be concluded from feeding experiments on rats by Walker (1972), when the P.I. of different organ lipids is calculated from their fatty acid compositions.

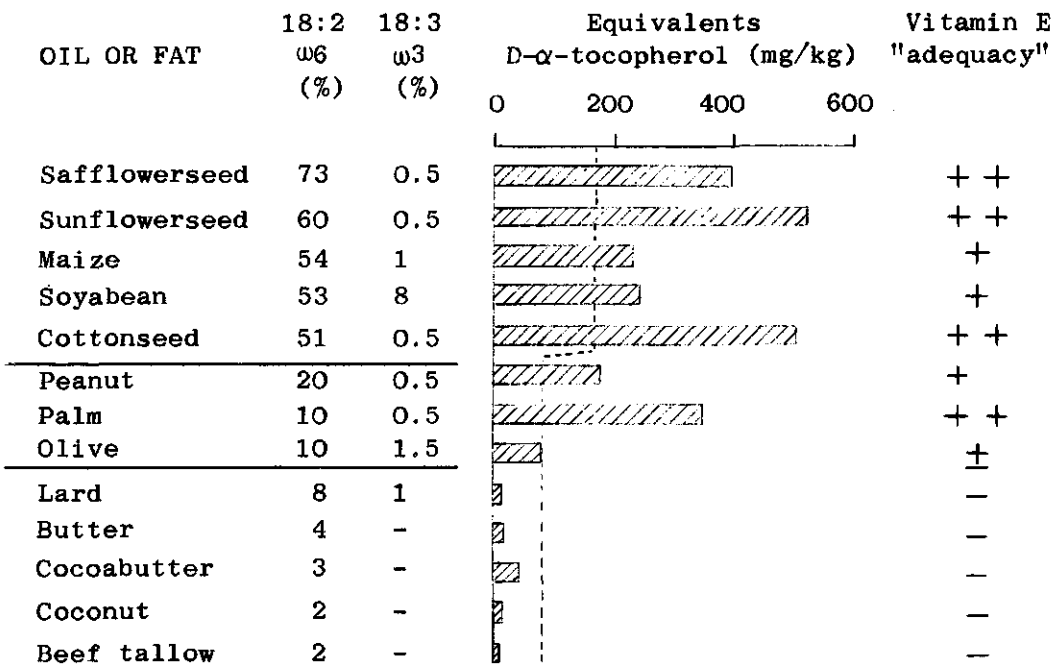


Fig. 21 Linoleic and linolenic acid contents and vitamin E adequacy of several oils and fats. Dotted line: amount of vitamin E required.

For this reason linolenic acid must be judged on its peroxidizability, i.e. on the extent to which an increased destruction of vitamin E in the intestinal tract (as is supposed to occur at high linoleic acid intake) may be expected. The P.I. of soybean oil is not higher than that of safflowerseed oil and therefore soybean oil must be regarded as adequate in vitamin E.

VI. CONCLUSIONS

In the study of the effect of linoleic acid intake on vitamin E requirement, much confusion has arisen from the fact that many researchers did not realize that they were working with EFA-deficient animals. Under the conditions of EFA deficiency, the addition of only small amounts of dietary linoleic acid results in an increased PUFA content (especially arachidonic acid) of membrane structural lipids, which is obviously associated with a proportionally increased need for vitamin E (Fig. 22; phase AB).

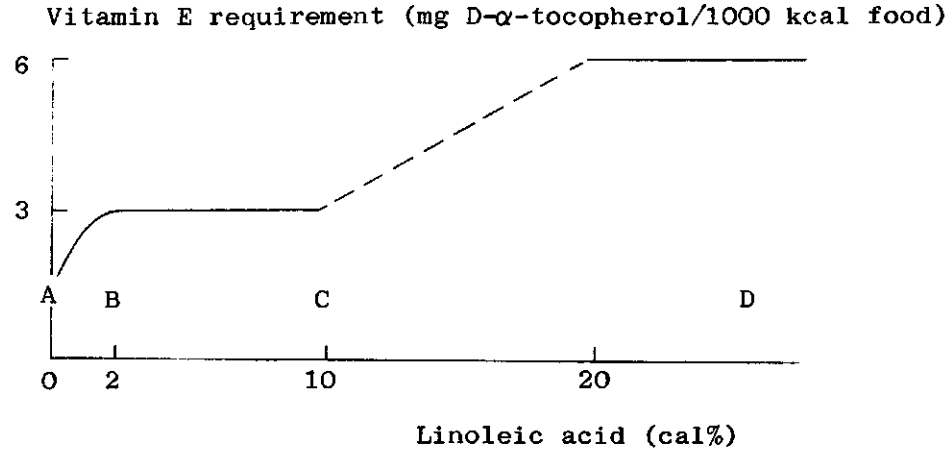


Fig. 22 Relationship between dietary content of linoleic acid and vitamin E requirement.

In normal animals, when biomembrane systems are "saturated" with essential fatty acids, dietary linoleic acid has no effect on the vitamin E requirement (Fig. 22; phase BC) except when very high intakes of linoleic acid are involved (Fig. 22; phase CD), which appears to be associated with a lowered blood tocopherol level, probably because of an increased destruction of tocopherol in the intestinal tract.

A critical dietary E/PUFA ratio, which might otherwise be used for evaluating the vitamin E adequacy of a given diet, does not exist.

Most saturated fats are inadequate in vitamin E; linoleic acid rich vegetable oils may be considered the natural sources of vitamin E.

At a normal range of fat intake (30-40 cal%), 100 mg D-α-tocopherol/kg fat is sufficient to meet the need for vitamin E. but if the dietary fat contains more than 25% linoleic acid this value increases to a maximum of 200 mg/kg fat.

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SUMMARY

In experiments with rats and Peking ducklings it has been investigated to what extent the linoleic acid content of the diet is of influence on the requirement of vitamin E. This requirement was determined by adding D-α-tocopheryl acetate in increasing doses to vitamin E-free diets and to determine how much vitamin E was necessary to prevent deficiency symptoms, such as *in vitro* haemolysis in the rat and myopathy in the duckling. For these symptoms methods were developed with with the deficiency grade could be expressed by a number.

The erythrocytes of rats with a vitamin E deficiency haemolyse spontaneously *in vitro*. Use was made of this phenomenon to determine the requirement of vitamin E. For this purpose, newly weaned male rats were placed on diets with increasing doses of vitamin E. After 2 weeks the haemolysis test was positive. The test was repeated weekly. Good results were obtained when a 0.01% erythrocyte suspension was incubated for 4 hours in a solution of physiological saline buffered with phosphate, at pH 7.4 and 38°C. It was found that there was a negatively linear relation between the logarithm of the vitamin E content of the food and the percentage *in vitro* haemolysis. By linear extrapolation it could be calculated how much vitamin E was necessary to prevent haemolysis. This is *in vitro* haemolysis, which cannot in itself be regarded as a deficiency symptom, was found, after prolonged vitamin E deficiency in rats, to show a clear relation with the degree of myopathy of the leg muscles.

The experiment with Peking ducklings was started with one-day-old chickens which were kept for 4 weeks on diets with increasing doses of vitamin E. In contrast with rats, in these ducklings the erythrocytes showed *in vitro* only a very slight spontaneous haemolysis. On the other hand, strong myopathy occurred. The strongest was the hyaline-degeneration of the

skeletal muscles. Moreover, necrocalcinosis was found in the smooth muscles of stomach and intestines and in the heart muscle. In the duckling, besides the degree of myopathy in the various organs, certain other criteria were investigated for their suitability for determining the requirement of vitamin E: weight increase, differential counting of leucocytes, activity of the serum enzymes creatine-phosphokinase, aspartate-aminotransferase and lactic acid dehydrogenase, differential determination of the serum proteins and the serum tocopherol level. All these criteria showed a clear connection with the vitamin E content of the food. The histologically determined degree of myopathy of the skeletal muscles was found to be the most suitable for the determination of the requirement of vitamin E. The degree of myopathy of a duckling was expressed as a score (0-24) which was determined by a microscopic assessment of the cross-sections of three pairs of leg muscles (score 0-4 per muscle). A linear relation was found between the myopathy score and the logaritm of the vitamin E content of the food. The amount of vitamin E needed to prevent myopathy could be calculated by linear extrapolation.

Myopathy in ducklings was found to occur only if the food contained little vitamin E at the same time as very little selenium. As little as 0.1 mg Se (as Na_2SeO_3) per kg food was sufficient to prevent myopathy completely. This amount has an anti-myopathic effect that is equal to at least 20 mg D-α-tocopheryl acetate.

All diets, of the rats as well as of the ducklings, contained 35 cal% fat. The linoleic acid content of the diet was varied by the use of fats with a low (lard and coconut fat) or with a high linoleic acid content (maize oil and safflower oil). Lard and coconut fat contain very little vitamin E; in the case of maize oil and safflower oil, the tocopherols were removed. Increasing doses of linoleic acid were obtained by the use of mixtures of coconut fat and safflower oil. Study of the effect

of an increasing linoleic acid content of the food led to the following conclusions:

- Up to 10 cal% the linoleic acid content of the food has no influence on the requirement of vitamin E. This requirement is roughly 3.0 mg D-α-tocopherol per 1000 Kcal food.

- At a linoleic acid uptake of more than 10 cal% the requirement of vitamin E can increase to 6 mg D-α-tocopherol per 1000 Kcal food. This is probably the result of an increased breakdown of vitamin E in the gastro-intestinal tract.

- From the ratio between the content of vitamin E and linoleic acid it is not possible to deduce whether or not a diet contains sufficient vitamin E.

- Many investigators have observed an increase in the requirement of vitamin E after only very small doses of linoleic acid. However, they were working with diets which contained insufficient essential fatty acids (EFA). With these diets (0-2 cal% linoleic acid) linoleic acid uptake causes a proportional increase in the requirement of vitamin E, which no longer increases once the EFA-deficiency has been corrected. It seems probable that in biomembranes a critical ratio exists between vitamin E and polyunsaturated fatty acids.

- At a fat consumption of 30-40 cal% a dietary fat will need to contain 100 mg D-α-tocopherol (or an equivalent amount of other tocopherols) per kg fat to meet the requirement of vitamin E. This requirement can rise to 200 mg, if the fat contains more than 25% linoleic acid.

- In general, vegetable oils with a high linoleic acid content contain ample vitamin E. Strongly saturated fats often contain too little vitamin E.

SAMENVATTING

Bij ratten en Peking-eenden werd onderzocht in hoeverre het linolzuurgehalte van het voedsel invloed heeft op de behoefte aan vitamine E. Deze behoefte werd vastgesteld door aan vitamine E-vrije diëten opklimmende doseringen D-α-tocoferylacetaat toe te voegen en te bepalen hoeveel vitamine E benodigd was om deficiëntiesymptomen zoals in vitro-hemolyse bij de rat en myopatie bij de eend te voorkomen. Voor deze symptomen werden methoden ontwikkeld, waarmee de deficiëntiegraad in een getal kon worden uitgedrukt.

De erytrocyten van ratten met een vitamine E deficiëntie hemolyseren spontaan in vitro. Van dit verschijnsel werd gebruik gemaakt om de behoefte aan vitamine E te bepalen. Voor dit doel werden pas gespeende mannelijke ratten op diëten met opklimmende doseringen vitamine E geplaatst. Na 2 weken was de hemolysetest positief. De test werd wekelijks herhaald. Goede resultaten werden verkregen, wanneer een 1‰ erytrocytensuspensie gedurende 4 uur werd geïncubeerd in een met fosfaat gebufferde oplossing van fysiologisch zout, bij pH 7,4 en 38°C. Er bleek een negatief lineair verband te bestaan tussen de logaritme van het vitamine E-gehalte van het voedsel en het percentage in vitro-hemolyse. Door lineaire extrapolatie kon worden berekend hoeveel vitamine E benodigd was om hemolyse te voorkomen. Deze in vitro-hemolyse, die op zichzelf niet als een deficiëntiesymptoom kan worden beschouwd, bleek na langdurige vitamine E-deficiëntie bij ratten een duidelijk verband te vertonen met de graad van myopatie van de pootspieren.

Het experiment met Peking-eenden werd gestart met ééndagskuikens, die gedurende vier weken op diëten met opklimmende doseringen vitamine E werden gehouden. In tegenstelling tot ratten, vertoonden bij deze eenden, de erytrocyten in vitro slechts een geringe spontane hemolyse. Er trad daarentegen een sterke myopatie op. Het sterkst was de hyaline-degeneratie van de skeletspieren. Daarnaast werd necrocalcinose gevonden in de

gladde spieren van maag en darm en in de hartspier. Bij de eend werden naast de graad van myopatie in de verschillende organen diverse andere criteria onderzocht op hun geschiktheid om de behoefte aan vitamine E te bepalen: gewichtstoename; differentiële telling van leukocyten; activiteit van de serumenzymen creatinefosfokinase, aspartaat-aminotransferase en melkzuur-dehydrogenase; differentiële bepaling van de serum-eiwitten en het serumtocoferol niveau. Al deze criteria vertoonden een duidelijk verband met het vitamine E gehalte van het voedsel. De histologisch bepaalde graad van myopatie van de skeletspieren bleek het meest geschikt om de behoefte aan vitamine E te bepalen. De graad van myopatie van een eend werd uitgedrukt in een score (0-24), die werd bepaald door een microscopische beoordeling van de dwarsdoorsneden van drie paar pootspieren (score 0-4 per spier). Er werd een rechtlijnig verband gevonden tussen de myopatie-score en de logaritme van het vitamine E gehalte van het voedsel. De hoeveelheid vitamine E, benodigd om myopatie te voorkomen kon door lineaire extrapolatie worden berekend.

Myopatie bij eenden bleek alleen voor te komen, indien het voedsel naast weinig vitamine E, tevens zeer weinig selenium bevatte. Reeds 0,1 mg Se (als Na_2SeO_3) per kg voedsel kon myopatie geheel voorkomen. Deze hoeveelheid heeft een anti-myopatie-effect, dat gelijk is aan tenminste 20 mg D-α-tocoferylacetaat.

Alle diëten, zowel van ratten als eenden, bevatten 35 cal% vet. Het linolzuurgehalte van het dieet werd gevarieerd door het gebruik van vetten met een laag (reuzel en cocosvet) dan wel hoog linolzuurgehalte (mais- en saffloerolie). Reuzel en cocosvet bevatten zeer weinig vitamine E, bij de mais- en saffloerolie werden de tocoferolen uit de oliën verwijderd. Opklimmende doseringen linolzuur werden verkregen door mengsels van cocos- en saffloerolie te gebruiken. Bestudering van het effect van een toenemend linolzuurgehalte van het voedsel, leidde tot de volgende conclusies:

- Tot 10 cal% heeft het linolzuurgehalte van het voedsel geen invloed op de behoefte aan vitamine E. Deze behoefte bedraagt globaal 3,0 mg D-α-tocoferol per 1000 Kcal voedsel.

- Bij een linolzuuropname van meer dan 10 cal% kan de behoefte aan vitamine E toenemen tot 6 mg D-α-tocoferol per 1000 Kcal voedsel. Dit is waarschijnlijk het gevolg van een verhoogde afbraak van vitamine E in het maagdarmkanaal.

- Uit de verhouding tussen het gehalte aan vitamine E en linolzuur valt niet af te leiden of een diëet al dan niet voldoende vitamine E bevat.

- Vele onderzoekers namen reeds na geringe doseringen linolzuur een toename van de behoefte aan vitamine E waar. Zij werkten echter met diëten, die onvoldoende essentiële vetzuren (EFA) bevatten. Bij deze diëten (0-2 cal% linolzuur) veroorzaakt linolzuuropname een evenredige toename in de behoefte aan vitamine E, die niet meer toeneemt, zodra de EFA-deficiëntie is opgeheven. Het lijkt waarschijnlijk, dat er in biomembranen een kritische verhouding bestaat tussen vitamine E en meervoudig onverzadigde vetzuren.

- Bij een vetconsumptie van 30-40 cal% zal een voedingsvet 100 mg D-α-tocoferol (of een equivalente hoeveelheid andere tocoferolen) per kg vet dienen te bevatten om de behoefte aan vitamine E te dekken. Deze behoefte kan tot 200 mg stijgen, indien het vet meer dan 25% linolzuur bevat.

- Plantaardige oliën met een hoog linolzuurgehalte bevatten als regel ruim voldoende vitamine E. Sterk verzadigde vetten bevatten veelal te weinig vitamine E.

Persoonlijke gegevens

De schrijver van dit proefschrift werd geboren op 22 augustus 1930 te Vledder (Dr.). De middelbare school (H.B.S.-B) bezocht hij in Steenwijk. Na het afleggen van het doctoraal examen biologie (bijvak biochemie) aan de Rijksuniversiteit te Groningen, was hij van 1958-1963 als leraar biologie werkzaam bij het middelbaar onderwijs. Sedert 1963 is hij als wetenschappelijk medewerker verbonden aan de Groep Biologie van het Unilever Research Laboratorium te Vlaardingen.

In de laatste jaren werd een belangrijk deel van zijn werk te Vlaardingen gevormd door een experimenteel onderzoek bij ratten en eenden naar het verband tussen de opneming van linolzuur en de behoefte aan vitamine E. De resultaten van dit onderzoek werden gepubliceerd in een aantal artikelen die als basis van dit proefschrift dienden.

Zijn huidige werkzaamheden liggen op het terrein van de biologische electronenmicroscopie. De belangstelling is in hoofdzaak gericht op het effect dat de voeding, met name het type vet, kan hebben op de ultrastructuur van verschillende organen. Als voorbereiding op deze werkzaamheden bracht hij een jaar door op het laboratorium voor medische electronenmicroscopie (Prof. Daems) te Leiden.