Dietary Fat Composition and Tocopherol Requirement: III. Quantitative Studies on the Relationship Between Dietary Linoleate and Vitamin E

ROSLYN B. ALFIN-SLATER, YAICHIRO SHIMMA[1], HERBERT HANSEN, PENELOPE WELLS and LILLA AFTERGOOD, School of Public Health, University of California, Los Angeles, and DANIEL MELNICK, Best Foods Research Laboratories, CPC International, Union, New Jersey

ABSTRACT

The present study has involved biologically titrating linoleate vs. vitamin E using the male rat as the indicator. In the first of the titration studies, the dietary tocopherol level was held constant, while in the second study the linoleate intake was held constant. The investigation was conducted with male rats since these have a much higher linoleate requirement than females. By first depleting such animals of their stores of essential fatty acids by feeding a fat-free diet from weaning, a sensitive test organism was provided. These animals have an immediate need for linoleate during the repletion periods. If an imbalance between linoleate and vitamin E content existed in any of the dietary regimens, such an imbalance would have been more likely noted in test animals actively metabolizing the ingested linoleate. Based upon various nutritional and biochemical indices, the amount of tocopherol ordinarily included in the basic diets fed to our rats, 0.01% as dl-alpha-tocopheryl acetate, was adequate even when the diet provided up to 5% linoleate; an amount corresponding to ca. 12% of the total calories and providing a ratio of linoleate to the tocopherol of ca. 500:1. In the reverse biological titration with all test diets now providing the constant level of 5% linoleate, ratios of linoleate to vitamin E were satisfactory even in a ratio of as much as 2500:1 (or 0.4 mg gram of vitamin E per polyunsaturated fatty acid). The control animals continued on the fat-free diet indicated that there is a need for added tocopherols even in the absence of linoleate according to a number of biochemical indices. Based upon a number of accepted bioanalytical approaches, the minimum requirement for linoleate by the fat-depleted male rat was found to be between 100-200 mg/day or ca. 1-2% of the caloric intake. Although the fatty acid composition of tissue lipid fractions is markedly affected by the amount of linoleate in the diet, dietary tocopherol supplements have little effect on these values.

INTRODUCTION

Several groups of investigators have suggested that there is an increased requirement for dietary tocopherols, and especially for vitamin E, as the unsaturation of the fat in the diet is increased (1-3). It is true that polyunsaturated fatty acids (PUFA) are far more readily oxidized *in vitro* than are the more saturated fatty acids, but whether such related oxidation also occurs in vivo to yield metabolites which may be potentially harmful is still open to debate. Although Tappel (4) has presented evidence for the occurrence in vivo of damaging lipid peroxidation products in the vitamin E-deficient animal, and Pritchard and Singh (5) have found decreased levels of PUFA in heart, liver, adrenals and plasma of vitamin E-deficient rats which they attributed to lipid peroxidation, Bunyan et al. (6), using more refined techniques, were *not* able to show decreases in the concentration of the PUFA in rat liver, kidney, heart, spleen, brain, adrenal and adipose tissue during experimental periods of vitamin E deficiency lasting 13 months.

Recently there has been considerable emphasis directed toward increasing the polyunsaturated fatty acid content of the diet at the expense of the more saturated fat in order to delay or possibly prevent certain aspects of cardiovascular disease (7). A direct relationship between the vitamin E intake and the polyunsaturated fatty acid intake has been postulated by Horwitt (8) who reported that elevated dietary linoleate levels increase the tocopherol requirement. This observation was supported by studies on exudative diathesis in chicks where increased lipid peroxides were found in tissue when the dietary level of PUFA was elevated without a concomitant increase in tocopherol (9,10). Hayes and coworkers (11), reporting on his studies with dogs, concluded that although the requirement for tocopherol was directly related to PUFA consumption, this was associated with the metabolism of the fat and not with

TABLE I

Composition of Diet

Dietary constituents[a]	Per cent
Sucrose	67.53
Casein	22.23
Celluflour	4.00
Salt mixture	4.00
Choline chloride	0.24
Vitamin mixture[b]	2.00

[a]When linoleate was included in the diet, it was added at the expense of sucrose. Tocopherol was added as dl-α-tocopheryl acetate powder (1 gm = 250 IU) in amounts depending on the conditions of the experiment.

[b]The vitamin mixture had the following composition: Vitamin Test Casein 61.35 g; *p*-aminobenzoic acid 2.42 g; inositol 2.0 g; (dl-α-tocopherol acetate powder [1 gm = 250 IU]) 1.3865 g; ascorbic acid 0.8 g; thiamine 0.288 g; Ca-pantothenate 0.24 g; niacin 0.24 g; vitamin B_{12} 0.24 g; riboflavin 0.11 g; pyridoxine 0.108 g; crystalets (500,000 U.S.P./g vitamin A, 50,000 U.S.P./g vitamin D) 0.052 g; folic acid 0.046 g; menadione 0.022 g; biotin 0.016 g; 2 g mix supplies; 1.77 g casein.

[1]Present address: Freshwater Fish Research Laboratories, Tokyo, Japan.

TABLE II

Growth of Male Rats Previously Fed a Fat-Free Diet and Thereafter Fed Diets Varying in Linoleate (L) or in dl-α-Tocopheryl Acetate (T)

A. Experiment I. 0.01% T in Diet; Varying Concentrations of L

Group	L, %	No. rats	Weight, g		
			At start	At end	Gain after 4 weeks
I	(FF;OL)	10	237 ±22[a]	230 ±31	-7
II	.25	9	221 ±30	280 ±48	59
III	.50	10	225 ±31	278 ±34	53
IV	1.0	8	242 ±20	298 ±23	56
V	4.0	10	230 ±37	301 ±40	71
VI	5.0	10	232 ±24	210 ±25	78

B. Experiment II. 5% L in Diet; Varying Concentrations of T

Group	T, %	No. rats	Weight, g		
			At start	At end	Gain after 4 weeks
1	0	10	255 ±17	320 ±31	65
2	.002	10	258 ±28	326 ±24	68
3	.004	10	252 ±20	299 ±10	47
4	.006	10	254 ±35	310 ±15	56
5	.008	10	243 ±29	313 ±31	70
6	.010	10	247 ±15	317 ±46	70

[a]SD.

the antioxidant role of the vitamin.

On the other hand, Jager and Houtsmuller (12) reported that, using erythrocyte hemolysis as an index, there was no direct relationship between linoleic acid intake and vitamin E requirement, although the group with the highest linoleic acid intake had the most hemolysis. In human studies, Ahrens et al. (13) found no evidence of creatinuria, myopathy, encephalopathy or other vitamin E deficiency symptoms in patients fed highly unsaturated menhaden oil (low in tocopherol) or corn oil at 40% of calories for 5.5 months. Similarly in studies with rats, Alfin-Slater et al. (14,15) found no evidence of vitamin E deficiency or of an increased vitamin E requirement when vegetable seed oils rich in polyunsaturates were fed at a level of 30% of the diet without a supplementary source of vitamin E. These studies included evaluations of growth, reproduction, lactation, testicular development and performance, and various aspects of lipid metabolism as well as tests for lipid peroxidation products in the tissues. The unreliability of the in vitro peroxidation test as a measure of vitamin E nutriture was also demonstrated. However, since the vegetable seed oils were rich in natural tocopherols, it seemed of interest and importance to attempt to quantitate the linoleate-tocopherol relationship with respect to the amount of tocopherol needed to afford antioxidant protection to linoleate in vivo.

In this report two experiments were designed to further explore the interdependency between PUFA and vitamin E. In the first experiment increasing linoleate supplements were given to groups of rats fed a diet considered adequate with respect to vitamin E content with this latter nutrient remaining constant as a dietary supplement. In the second experiment linoleate was kept constant, and the level of dl-α-tocopheryl acetate in the diet was varied. Various nutritional and biochemical indices were examined to determine the nutritional adequacy of the various diets. These indices included growth and weight gain, analyses for tissue peroxides, cholesterol and lipid determinations in plasma and liver, fatty acid analyses of lipids of plasma, red blood cells and liver, and in vitro hemolysis and fragility tests on red blood cells.

EXPERIMENTAL PROCEDURES

Only male rats from our colony (the former USC strain) were used. In the first experiment animals were maintained from weaning on a fat-free diet containing 0.01% dl-α-tocopheryl acetate (T) for 4 months and were subsequently given supplements of methyl linoleate (L) as follows: 0, 50, 100, 200, 800 and 1000 mg per animal per day for 5 weeks. In the second experiment animals were maintained on a fat-free diet with supplements of dl-α-tocopheryl acetate ranging from 0-0.01% until symptoms of EFA deficiency appeared (14 weeks) and thereafter were given a diet varying in the dl-α-tocopheryl acetate content and containing 5% linoleate for 4 weeks. By using male animals which have a much higher linoleate requirement than do females (16) and by first depleting their stores of essential fatty acids, a very sensitive test organism is provided; such animals are actively metabolizing the ingested linoleate. At the end of the experimental periods the animals were killed.

The composition of the fat-free diet is listed in Table I, and the experimental design is given in Table II. The diet was adequate in all respects except for essential fatty acids as indicated. The linoleate supplements of 50, 100 and 200 mg in Experiment I were administered orally; the higher levels were included in the diet since it was not feasible to give this large amount of material by oral intubation. The basal fat-free diets were prepared weekly and stored in the refrigerator prior to the daily feedings. The diets containing 4% and 5% linoleate were prepared daily. It had been observed previously that the male rats weighing ca. 230 g, and growing rapidly, exhibited a weight gain of ca. 75 g in a 4 week period and consumed on the average 20 g of the diet per day; hence the diets containing 4% and 5% methyl linoleate provided ca. 800 mg and 1000 mg, respectively, of linoleate per rat per day. In the tables all linoleate supplements are expressed as per cent of diets. The dl-α-tocopheryl acetate level was kept constant at 0.01% of the diet, and therefore the daily intake of the dl-α-tocopheryl acetate amounted to ca. 2.0 mg per rat per day.

In Experiment II the linoleate was included at 5% in all the diets as shown under Experiment IIB in Table III; the control groups (IIA) received no linoleate supplement. The dl-α-tocopheryl acetate varied from 0-.010%.

At the end of the experimental periods the animals were killed by removal of blood from the heart under Nembutal anesthesia. Livers were quickly excised and trimmed of fat. Plasma, red cells and liver were treated with lipid solvents

TABLE III

dl-α-Tocopheryl Acetate (T) and Linoleate (L) Content of Diets and Erythrocyte Hemolysis

Group designation	Diet						Red blood cells		
	T, %	L, %	T/L, mg/g	E/PUFA,[a] mg/g	L/T	PUFA/E	Hemolysis, %	Fragility, % NaCl	T, μg/ml
Experiment I (8-10 animals per group)									
I	.010	0	---	---	---	---	2.4[b]	.39[b]	---
II	.010	0.25	40.0	38.0	25/1	26/0	0.3	.38	---
III	.010	0.50	20.0	19.0	50/1	52/1	0.8	.38	---
IV	.010	1.0	10.0	9.5	100/1	104/1	3.9	.35	---
V	.010	4.0	2.5	2.4	400/1	416/1	5.3	.33	---
VI	.010	5.0	2.0	1.9	500/1	520/1	4.2	.36	---
Experiment IIA (6 animals per group)									
1A	0	0	---		---		18.4 ± 2.1[c]	---	---
2A	.002	0	---		---		14.9 ± 4.7	---	---
3A	.004	0	---		---		15.2 ± 2.4	---	---
4A	.006	0	---		---		10.7 ± 3.6	---	---
5A	.008	0	---		---		13.4 ± 1.8	---	---
6A	.010	0	---		---		3.3 ± 1.0	---	2.58
Experiment IIB (10 animals per group)									
1B	0	5.0	---		---		90.2 ± 5.5	---	0.95
2B	.002	5.0	0.4	0.37	2500/1	2700/1	5.5 ± 2.3	---	---
3B	.004	5.0	0.8	0.75	1250/1	1350/1	5.1 ± 1.8	---	1.20
4B	.006	5.0	1.2	1.11	833/1	900/1	4.8 ± 1.0	---	---
5B	.008	5.0	1.6	1.50	625/1	675/1	5.5 ± 0.8	---	1.20
6B	.010	5.0	2.0	1.87	540/1	540/1	3.4 ± 0.8	---	2.95

[a]Vitamin E (α-tocopherol)/polyunsaturated fatty acid (linoleic acid).

[b]These are average values of duplicate tests done on two pooled samples (representing 4 or 5 animals each) of erythrocytes.

[c]SD; values obtained on individual rats.

for subsequent cholesterol, lipid and fatty acid analyses. In Experiment I red cells were tested for their susceptibility to hemolysis by hydrogen peroxide using the method of Rose and Gyorgy (17), for fragility using varying concentrations of sodium chloride (18), and, in Experiment II, for hemolysis by the method of Draper and Csallany (19). Tocopherol analyses were performed by a modification of the method of Quaife et al. (20). Cholesterol analyses were carried out on lipid extracts of plasma and liver using a modification of the Sperry-Schoenheimer method as reported by Nieft and Duel (21). In Experiment I fatty acid analyses were done on extracts of total lipids of plasma, red cells and liver. In Experiment II lipids were separated into their triglycerides, phospholipids and cholesterol ester components, and each was analyzed separately. In experiment I fatty acid determinations were done using a Barber-Coleman Model 20 gas chromatograph (15.4% diethylene glycol succinate polyester on 80-100 mesh chromosorb WAW), and in Experiment II, a Varian Aerograph model 204C (15% diethylene glycol succinate polyester, 80-100 mesh chromosorb WAW) was used. Chromatographic peaks were identified either by comparing retention times with those of standards or from a graph representing the relationship between log retention time and number of carbon atoms.

RESULTS AND DISCUSSION

The gain in weight over the experimental period is shown in Table II. To some degree the growth of the animals over the 4 week period reflected the amount of linoleate which they ingested. In Experiment I, as was to be expected, the essential fatty acid-deficient animals receiving no linoleate supplementation lost weight during the experimental period. There appeared to be a plateau in growth response of the rats receiving from 0.25-1.0% linoleate per day. At the higher levels of linoleate intake, further gains in weight were noted. In experiment II the weight gain of the EFA-deficient rats receiving 5% linoleate was the same at all dl-α-tocopheryl acetate levels, except possibly at the 004% T (although this was shown statistically to be a nonsignificant difference), and confirmed the weight gain seen in the rats fed the 5% linoleate and 0.01% dl-α-tocopheryl acetate in Experiment I.

The results on erythrocyte hemolysis and erythrocyte fragility in saline are shown in Table III. Also included are the amounts of dl-α-tocopheryl acetate and linoleate in the diet and the ratios of these nutrients to each other. In Experiment I the hemolysis figures are all low and quite similar, indicating that the erythrocytes were not subject to hemolysis under the conditions of test, and that no increased susceptibility to hemolysis was produced by the increased linoleate, and therefore by the increased ratio of linoleate to dl-α-tocopheryl acetate from 25:1 to 500:1 in the diet. The average cell fragility values are also in the range reported to be normal for human blood (0.39-0.33% NaCl). In Experiment II, in the animals on the 5% linoleate diet, increasing the ratio of linoleate to dl-α-tocopheryl acetate from 500:1 to 2500:1 again resulted in no increased susceptibility of erythrocytes to hemolysis due to peroxidation in vivo, indicating that this ratio of L/T (corresponding to a vitamin E/PUFA ratio of 0.37 mg/g) provided adequate protection against tissue lipid oxidation and therefore presumably adequate vitamin E nutriture. This figure is lower than the estimation of 0.6 mg/g for the E/PUFA ratio proposed by Harris and Embree (3), and may indeed be even lower as a result of further experimentation. Chen and associates (22) reported that α-tocopherol had to be added back to tocopherol-stripped corn oil in the ratio of only 0.36 mg/g for E/PUFA, whereas this was 1.12 mg/g in the case of the tocopherol-stripped lard to prevent liver peroxidation in the male rat. It is also interesting to note that Dayton et al. (23), using hydrogen peroxide hemolysis in vitro as a criterion, found that in most of the patients on diets containing 4.8 cal% PUFA, a E/PUFA ratio of 0.20 mg/g provided a satisfactory vitamin E status. Since animals on the fat-free diet would be expected to have little linoleic and arachidonic acids in red cell lipids, it is interesting to note that in these animals a larger amount of dietary dl-α-tocopheryl acetate (0.010%) is required to prevent erythrocyte hemolysis than when a 5% linoleate is present in the diet (0.002%%). Although the possibility exists that the absorption of the fat-soluble vitamin E might be impaired on the fat-free diet, the ratio of erythrocyte tocopherol (Table III) to plasma tocopherol (Table IV), when T is included in the diet at 0.01%, is the same in the fat-free and linoleate-supplemented animals—0.34 and 0.32, respectively. In the studies of Bieri and Poukka (24)

TABLE IV

Cholesterol and Tocopherol Content of Plasma and Cholesterol and Total Lipid Content of Liver of Male Rats Fed Diets Varying in Linoleate (L) and dl-α-Tocopheryl Acetate (T)

A. Experiment I. (.01%T) (8-10 Animals Per Group), Varying Linoleate Levels

		Plasma		Liver	
Group no.	L, %	Tocopherol, μg/ml	Cholesterol total, mg/100 ml	Cholesterol total, mg/g	Total lipids, mg/g
I	0	---[a]	48.3 ± 1.6[b]	3.06 ± 0.17[c]	65.7 ± 2.3[d]
II	0.25	---	62.1 ± 4.2[b]	2.39 ± 0.13[c]	61.0 ± 1.6
III	0.5	---	59.9 ± 2.6	2.54 ± 0.18	65.3 ± 2.2
IV	1	---	54.8 ± 2.3	2.07 ± 0.15	55.4 ± 2.5[d]
V	4.0	---	62.3 ± 1.5	2.06 ± 0.06	51.1 ± 1.5
VI	5.0	---	64.7 ± 4.1	2.37 ± 0.23	50.8 ± 2.6

B. Experiment II

		Plasma		Liver	
Group no.	T, %	Tocopherol, μg/ml	Cholesterol total, mg/100 ml	Cholesterol total, mg/g	Total lipids, mg/g
Experiment IIA. Fat-free diet (6 animals per group), varying T levels					
1A	0	<1.0	61.2 ± 6.5[g]	3.34 ± .78[g]	64.4 ± 4.3[h]
2A	.002	8.2 ± 1.9	62.9 ± 6.2	2.66 ± .11	57.4 ± 6.4
3A	.004	7.7 ± 1.1	62.8 ± 9.7	2.81 ± .13	59.4 ± 3.9
4A	.006	8.0 ± 1.8	56.7 ± 2.8	2.60 ± .50	61.7 ± 11.6
5A	.008	6.3 ± 1.1	60.4 ± 6.4[e]	2.66 ± .61	58.4 ± 5.0
6A	.010	7.6 ± 3.8	46.6 ± 3.7[e,f]	2.64 ± .11	55.6 ± 4.9
Experiment IIB. 5% L diet (10 animals per group), varying T levels					
1B	0	2.4 ± 0.9	56.0 ± 5.3	1.93 ± .13[g]	34.1 ± 3.1[h]
2B	.002	8.6 ± 3.3	65.9 ± 6.3	1.88 ± .17	35.7 ± 4.1
3B	.004	5.8 ± 1.7	62.6 ± 5.5	1.74 ± .19	33.9 ± 3.2
4B	.006	6.6 ± 3.0	57.4 ± 6.4	1.79 ± .14	34.8 ± 2.4
5B	.008	7.8 ± 1.3	57.8 ± 9.4	1.86 ± .31	34.8 ± 6.6
6B	.010	9.2 ± 1.8	67.5 ± 4.9[f]	2.06 ± .28	38.7 ± 8.0

[a]Tocopherol concentration not measured.

[b,c,d,e,f,g,h]Matched superscripts indicate $p<.005$

the ratio of erythrocyte tocopherol to plasma tocopherol was 0.45 over a range of tocopherol intake varying from 0.12-0.41 mg/day. In these experiments 0.27 mg α-tocopherol was required by rats fed 5% stripped lard to reduce erythrocyte hemolysis to less than 10%. Herting and coworkers (25) reported that vitamin E was as effectively absorbed and utilized by animals on both low fat and high fat diets. They also reported that erythrocytes from animals fed the low fat diets were less susceptible to hemolysis than those fed the high fat diets. However in Herting's study animals fed fat-free diets were not examined.

In Experiment I, at a dl-α-tocopheryl acetate level of 0.01%, plasma cholesterol values (Table IV) are significantly ($p<.001$) increased in male rats fed fat-free diets to which linoleate is added, as compared with the animals receiving no linoleate supplement (Experiment I, compare groups II and V with I; in Experiment II, compare group 6B with 6A). However no significant differences are apparent in the total plasma cholesterol content as a result of dl-α-tocopheryl acetate administration in the animals fed the various linoleate supplements in Experiment I or fed the 5% linoleate in Experiment II. In Experiment II plasma cholesterol levels of the fat-free animal were the same as those in the linoleate-fed group except at the 0.010% T level, in spite of the fact that the plasma tocopherol levels were the same in this latter case.

Liver cholesterol and liver lipids were high in animals fed the fat-free diets and lower in the animal fed the linoleate-supplemented diets regardless of the tocopherol content of the diet. In Experiment I a dietary supplement of 0.25% linoleate was necessary to reduce the elevated cholesterol levels, whereas a 1% supplement was necessary to reduce liver total lipids. This linoleate-cholesterol relationship in rats is well documented (26). Rats fed fat-free diets have lower plasma and higher liver cholesterol values. The addition of adequate amounts of linoleate to the diet results in a decreased liver cholesterol and increased plasma cholesterol levels.

The fatty acid composition of total lipids or lipid fractions of plasma (triglycerides [TG], phospholipids [PL] and cholesterol esters [CE]) is shown in Table V and Va; of red cells in Table VI; and of liver in Table VII and VIIa.

Plasma fatty acids change rather quickly in response to dietary fatty acids and can be used as a reflection of the composition of dietary fat in short range experiments (27). The fatty acids in plasma of rats fed the EFA-deficient diet (Experiment I(Group I)) are low in linoleic (18:2) and arachidonic (20:4) acids but are high in oleic (18:1), palmitic 16:0) and palmitoleic (16:1) acids. In addition the plasma of the EFA-deficient animal has a high concentration of eicosatrienoic acid (20:3). This eicosatrienoic acid occurs in large quantities in lipids of EFA-deficient tissues (28). It is biosynthesized from oleic acid in the fat-deficient rat and has been shown to be 5,8,11-eicosatrienoic acid (29).

In Experiment IIA, it can be seen that the eicosatrienoic acid component of the lipids is present in phospholipids and cholesterol esters and not in the triglycerides. Less eicosatrienoic acid, less oleic and more palmitoleic acid appear in the plasma lipids of the rats fed the higher levels of tocopherol in the diet. In Experiment I linoleic acid supplements result in increases in linoleic and arachidonic acids and decreases in oleic, palmitic and palmitoleic acids. Stearic acid remains essentially constant. The amount of eicosatrienoic acid is decreased markedly by as little as 50 mg of linoleate (0.25% L) and disappears completely after 4 weeks on a diet containing 4% linoleate (the 800 mg linoleate per rat per day). Holman (28) has proposed the use of the triene-tetraene ratio as a measure of essential fatty acid deficiency; a value over 0.4 indicates an

TABLE V

Major Fatty Acid Composition of Lipids in Plasma of Male Rats Fed Diets Varying in Linoleate (L)

A. Experiment I. 0.01% T

Group no.	L, %	Fatty acid, % 16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:6	20:3/20:4
I	0	18.2	13.3	9.8	35.6	3.7	16.0	1.2	---	15
II	0.25	18.5	14.0	9.7	30.7	5.7	5.6	4.9	---	1.1
III	0.5	18.0	12.4	10.9	30.3	7.8	4.5	13.2	---	0.34
IV	1.0	20.4	13.3	9.7	30.1	6.6	3.8	15.1	---	0.25
V	4.0	17.4	8.7	9.9	25.1	15.2	0	23.0	---	0
VI	5.0	16.7	7.3	9.4	17.4	28.0	0	19.8	---	0

B. Experiment II.

Group no.	T, %		Fatty acid, % 16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:6	20:3/20:4
Experiment IIA. fat-free diet											
1A	0	TG[a]	24.6	13.3	2.3	48.8	4.1	0	0	0	---
		PL	22.0	7.9	11.0	23.5	5.8	7.4	3.1	3.9	2.4
		CE	11.8	17.6	2.1	24.9	5.7	23.4	5.7	0	4.1
6A	.01	TG	25.5	18.3	2.4	39.6	5.8	0	0	0	---
		PL	20.6	8.4	15.5	19.8	6.7	7.1	3.0	5.6	2.4
		CE	18.5	21.1	3.1	21.2	4.4	15.6	4.2	---	3.7
Experiment IIB. 5% L diet											
1B	0	TG	20.6	7.3	3.5	32.2	25.1	0	3.7	0	---
		PL	23.8	6.0	19.3	15.2	12.8	0.9	10.2	5.4	.08
		CE	11.6	5.1	1.9	8.6	14.3	1.9	44.2	0	.04
2B	.002	TG	19.2	7.8	2.5	30.9	31.3	0	2.9	0	---
		PL	23.6	5.0	18.5	14.3	12.1	0	11.9	5.1	---
		CE	11.6	8.6	3.2	8.3	15.2	1.0	41.0	0	.02
3B	.004	TG	15.3	6.2	2.4	26.0	39.0	0	3.9	0	---
		PL	21.7	5.4	16.9	15.9	15.7	0	8.7	4.9	---
		CE	13.3	5.2	2.4	8.9	16.2	0.8	40.3	0	.02
4B	.006	TG	17.2	7.0	2.4	26.4	35.1	0	4.0	0	---
		PL	21.6	6.6	16.1	16.2	13.9	0	9.6	4.2	---
		CE	13.3	6.5	3.2	10.1	17.1	1.4	37.7	0	.04
5B	.008	TG	18.5	8.4	2.7	29.4	32.1	0	3.1	0	---
		PL	24.9	8.0	16.3	17.4	9.1	0	8.4	4.3	---
		CE	15.4	6.1	4.9	11.0	14.9	0	35.9	0	---
6B	.01	TG	19.8	6.0	3.6	29.0	26.8	0	4.9	0	---
		PL	20.6	6.1	18.8	15.2	14.5	0	13.5	0	---
		CE	12.3	6.4	3.4	9.1	17.4	0	43.6	0	---

[a]TG = triglyceride, PL = phospholipid, CE = cholesterol esters.

TABLE VI

Major Fatty Acid Composition of Lipids of Erythrocytes of Male Rats Fed Diets Varying in Linoleate and dl-α-Tocopheryl Acetate Content

A. Experiment I. 0.01% T

Group no.	L, %	Fatty acids, % 16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:3/20:4
I	0	22.5	3.5	16.4	21.8	1.4	24.4	4.6	5.3
II	0.25	24.2	2.8	18.8	19.0	2.9	12.8	14.3	0.89
III	0.50	27.2	2.2	19.2	16.9	2.9	8.4	18.2	0.46
IV1	1.0	27.2	3.7	18.2	16.3	3.7	7.2	19.0	0.38
V	4	27.4	2.3	14.1	13.1	7.8	5.5	25.5	0.22
VI	5	26.1	2.2	18.4	11.6	7.7	4.3	23.7	0.18

B. Experiment IIB. 5% L diet

Group no.	T, %	Fatty acids, % 16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:3/20:4
1B	.0	29.1	4.5	14.7	16.9	6.6	3.6	8.8	0.41
2B	.002	21.1	2.2	14.1	18.4	9.2	4.9	14.6	0.34
3B	.004	27.2	3.6	15.2	18.2	7.1	3.8	13.2	0.29
4B	.006	22.8	4.1	14.4	15.7	7.6	4.4	16.1	0.27
5B	.008	23.7	3.5	16.7	16.8	7.5	3.8	15.1	0.25
6B	.010	23.6	3.6	15.8	14.7	6.9	3.4	13.0	0.26

TABLE VII

A. Major Fatty Acid Composition of Liver Lipids of Male Rats Fed Diets Varying in Linoleate (L)

Experiment I. 0.01% T

Group no.	L, %	Fatty acids, %							
		16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:3/20:4
I	0	21.8	15.4	9.5	39.1	2.2	9.7	1.6	6.1
II	0.25	19.7	14.0	13.3	34.6	4.3	5.8	7.5	0.77
III	0.50	20.9	13.4	13.4	33.5	4.8	3.5	9.9	0.35
IV	1.0	23.9	13.7	13.4	33.2	5.3	0	9.8	---
V	4.0	21.7	9.3	15.9	24.8	11.8	0	16.3	---
VI	5.0	24.5	9.4	14.4	20.7	13.1	0	17.8	---

B. Major Fatty Acid Composition of Liver Lipids of Male Rats Fed Diets Varying in Linoleate (L) and dl-α-Tocopheryl Acetate (T)

Experiment IIA. Fat-free diet

Group no.	T%		Fatty acids, %								
			16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:3/20:4	22:6
1A	0	TG	30.1 ± 1.2	14.5 ± 2.6	1.3 ± 0.9	50.5 ± 3.9	1.9 ± 1.1	---	---	---	---
		PL	17.3 ± 2.8	6.7 ± 1.5	17.0 ± 2.0	24.3 ± 2.7	3.6 ± 1.2	18.8 ± 2.9	6.7 ± 2.2	2.8	---
		CE	15.9 ± 2.6	17.8 ± 2.9	5.2 ± 1.9	42.5 ± 3.8	5.1 ± 2.3	3.7 ± 1.9	1.2 ± 1.0	3.1	---
6A	.010	TG	30.4 ± 2.7	15.4 ± 0.6	2.1 ± 2.0	47.1 ± 3.2	2.4 ± 1.4	---	---	---	---
		PL	17.5 ± 2.3	7.2 ± 1.9	18.1 ± 3.9	23.0 ± 3.7	3.7 ± 1.3	17.7 ± 1.6	8.3 ± 2.3	2.1	---
		CE	19.1 ± 2.1	18.0 ± 6.0	4.9 ± 1.5	33.1 ± 7.0	7.1 ± 3.0	5.5 ± 3.6	2.3 ± 1.4	2.4	---
Experiment IIB. 5% L											
1B	0	TG	39.5 ± 1.3	9.0 ± 1.4	7.6 ± 1.0	32.1 ± 1.9	8.7 ± 2.0	---	Trace	---	
		PL	19.2 ± 2.0	3.3 ± 0.9	26.0 ± 1.6	12.8 ± 1.9	11.1 ± 1.3	Trace	18.6 ± 1.7	---	4.7 ± 0.8
		CE	28.0 ± 3.2	16.6 ± 1.1	14.6 ± 1.9	23.1 ± 2.4	7.5 ± 1.5	---	4.3 ± 2.0	---	---
2B	.002	TG	38.0 ± 3.0	9.8 ± 2.0	5.1 ± 1.8	34.1 ± 2.0	9.4 ± 2.4	---	1.0 ± 0.7	---	---
		PL	17.1 ± 1.2	2.9 ± 0.9	20.9 ± 3.5	12.0 ± 1.7	10.2 ± 1.4	Trace	24.1 ± 4.6	---	6.8 ± 1.0
		CE	23.7 ± 2.9	14.4 ± 2.5	12.5 ± 1.6	23.2 ± 2.4	9.6 ± 1.2	---	8.3 ± 1.4	---	---
3B	.004	TG	42.4 ± 3.8	9.2 ± 3.0	4.3 ± 0.9	31.8 ± 2.9	9.9 ± 4.1	---	2.7 ± 1.3	---	---
		PL	13.9 ± 1.0	3.5 ± 1.0	20.8 ± 1.5	11.9 ± 1.5	11.5 ± 0.8	Trace	29.4 ± 1.7	---	6.3 ± 1.0
		CE	29.3 ± 3.5	10.4 ± 2.8	7.6 ± 2.3	25.8 ± 1.6	11.8 ± 4.0	---	7.7 ± 2.3	---	---
4B	.006	TG	40.2 ± 2.8	7.8 ± 1.6	4.8 ± 1.7	35.4 ± 4.4	9.1 ± 1.0	---	1.4 ± 1.9	---	---
		PL	16.2 ± 2.9	4.1 ± 1.8	22.1 ± 2.6	11.9 ± 2.0	11.6 ± 1.2	Trace	22.7 ± 4.5	---	5.2 ± 1.2
		CE	27.1 ± 3.8	11.6 ± 3.5	11.6 ± 2.0	23.7 ± 2.3	10.5 ± 1.9	---	10.9 ± 1.9	---	---
5B	.008	TG	39.2 ± 1.6	9.3 ± 3.2	5.9 ± 2.7	33.3 ± 4.4	10.1 ± 1.8	---	1.7 ± 2.0	---	---
		PL	16.0 ± 2.4	3.1 ± 0.5	23.5 ± 2.5	12.0 ± 1.6	12.0 ± 0.4	Trace	22.7 ± 4.0	---	5.7 ± 1.6
		CE	22.6 ± 3.0	11.7 ± 2.4	10.3 ± 1.8	23.1 ± 1.9	13.9 ± 3.0	---	13.0 ± 1.9	---	---
6B	.010	TG	32.2 ± 4.0	8.2 ± 3.3	9.0 ± 2.7	30.6 ± 3.6	12.6 ± 2.4	---	6.0 ± 4.1	---	---
		PL	24.5 ± 1.6	3.0 ± 1.4	21.4 ± 1.6	10.5 ± 1.2	10.5 ± 1.2	Trace	30.3 ± 4.1	---	6.1 ± 1.0
		CE	23.3 ± 5.6	12.8 ± 0.9	9.4 ± 2.8	26.3 ± 5.1	9.7 ± 5.9	---	11.8 ± 1.7	---	---

EFA-deficient state; under 0.4, EFA adequacy. Using this criterion it can be seen from the plasma fatty acids that the animals in Group II of Experiment I receiving the lowest EFA supplement (0.25%) are still EFA-deficient after 4 weeks of linoleate supplementation of 50 mg per rat per day. Linoleate at 0.5% alleviates the EFA deficiency.

In Experiment II linoleate in the diet increases both the linoleate and arachidonate components of the plasma triglycerides at the expense of primarily oleic and palmitoleic acids. The tocopherol content of the diet has little effect on the fatty acid composition of the triglyceride fraction. In the phospholipid fraction similar effects are observed; the linoleate-containing diet increases the amount of linoleate and arachidonate, and decreases the oleic and eicosatrienoic acid levels. Again the tocopherol content of the diet has little effect on the fatty acid composition. Differences in lipid patterns are apparent between triglycerides and phospholipids; triglycerides are low in stearic acids, high in oleic and linoleic acids; phospholipids are high in stearic acid and high in the more polyunsaturated acids, C22:4 and C22:6. The characteristic fatty acid and the one occurring in most abundant quantities in cholesterol esters is arachidonic acid. Here again there is no significant effect of the amount of tocopherol in the diet.

Red cell lipids are predominantly phospholipids; these lipids change more slowly than do plasma lipids. It has been proposed that equilibrium between dietary fatty acids and erythrocyte fatty acids in the non-EFA-deficient organism is not complete until after 4-6 weeks (27). In the deficient animal which has immediate requirements for EFA, this equilibrium is probably achieved in a much shorter period of time. An examination of the fatty acid composition of erythrocytes reveals a pattern similar to plasma in certain respects, and different in others (Table VI). The erythrocyte lipids of the fat-free animals are low in linoleic (18:2) and arachidonic (20:4) acids, high in oleic (18:1), palmitic (16:0) and eicosatrienoic (20:3) acids. Similar results were reported by Walker and Kummerow (30) studying the fatty acid composition of erythrocyte lipids of rats fed diets low in essential fatty acids. But whereas the plasma under the present conditions of test has considerable palmitoleic (16:1) and less of stearic acids (18:0), the erythrocytes have little palmitoleic (16:1) and have considerable stearic acid content. Essential fatty acid supplementation results in decreases in oleic and eicosatrienoic acids, and increases in arachidonic and linoleic acids. Palmitoleic, palmitic and stearic acids remain relatively unchanged. An examination of the triene-tetraene ratios reveals that in this test a value of below 0.4 is not achieved until the animal received a linoleate supplement of 200 gm/day or 1% L in the dietary intake (Group IV).

In Experiment II the arachidonic acid content of the erythrocyte lipids of the rats receiving no tocopherol in their diet is lower than those receiving even the lowest

TABLE VIII

Fatty Acid Composition of Brain Phospholipids of Male Rats Fed Diets Containing Varying Levels of Linoleate (L) and dl-α-Tocopheryl Acetate (T)

T, %	Weight of organ, g	TBA value[a]	Fatty acid, %[b] 16:0	16:1	18:0	18:1	20:3	20:4	Higher polyunsaturates[c]	20:3/20:4
Fat free diet										
0	1.45 ± 0.6	.426	21.9 ± 1.6[d,e,f,g]	0.7 ± 0.2[i]	17.1 ± 1.1	20.8 ± 1.0	4.0 ± 0.2	7.0 ± 1.1	13.6	0.57
.002	1.47 ± .05	.439	16.1 ± 0.7	1.0 ± 0.5	17.6 ± 0.2	19.8 ± 1.3	4.8 ± 0.7	7.2 ± 0.6	19.0	0.67
.004	1.47 ± .08	.290	13.8 ± 1.8[d]	6.1 ± 1.0[i]	19.2 ± 5.3	18.4 ± 3.8	4.1 ± 0.3	6.5 ± 0.9	17.0	0.63
.006	1.48 ± .04	.438	13.3 ± 0.5[e]	6.5 ± 2.2	19.0 ± 1.4	18.8 ± 1.1	4.3 ± 0.3	6.7 ± 0.9	19.5	0.64
.008	1.42 ± .07	.456	11.4 ± 0.9[f]	4.6 ± 1.4	20.2 ± 0.9	17.2 ± 3.1	4.6 ± 0.5	7.6 ± 1.4	21.4	0.60
.010	1.42 ± .10	.169	14.1 ± 1.5[g]	6.9 ± 2.6	20.0 ± 4.9	22.4 ± 3.4	3.9 ± 0.9	7.6 ± 1.1	17.9	0.51
5% Linoleate diet										
0	1.48 ± .14	.287	18.0 ± 2.3[h]	1.2 ± 0.7[j]	17.6 ± 1.5	19.0 ± 1.9	2.0 ± 0.6	8.4 ± 1.0	18.0	0.24
.002	1.51 ± .07	.201	21.6 ± 4.2	1.2 ± 0.5	17.8 ± 3.0	18.0 ± 2.6	1.8 ± 0.3	8.8 ± 0.4	18.1	0.20
.004	1.46 ± .06	.243	15.6 ± 2.3	2.6 ± 0.4	19.6 ± 1.9	19.3 ± 2.4	2.0 ± 0.4	9.0 ± 1.8	19.8	0.22
.006	1.51 ± .17	.260	13.1 ± 2.5	5.3 ± 1.1[j]	20.4 ± 0.5	15.9 ± 3.5	2.2 ± 0.3	10.2 ± 0.8	21.8	0.22
.008	1.53 ± .09	.220	15.1 ± 0.8	6.0 ± 0.3	18.6 ± 0.7	16.6 ± 1.1	1.7 ± 0.2	9.4 ± 0.4	20.9	0.18
.010	1.52 ± .09	.101	10.7 ± 1.2[h]	5.9 ± 1.3	19.9 ± 1.5	17.5 ± 2.4	1.8 ± 0.3	9.5 ± 1.1	20.7	0.19

[a]Absorbance at 530 mμ.

[b]Only traces of 18:2 were present.

[c]22:3, 22:4, 24:2, 22:6.

[d,e,f,g,h,i]Matched superscripts indicate significant differences between values at $p < .005$.

tocopherol supplement of .002% (8.8 vs. 14.6%). Additional tocopherol supplements do not result in further changes. These data do not agree with the report of Jager and Houtsmuller (12) that the composition of erythrocyte lipids is not influenced by vitamin E content of the diet and only moderately by the linoleic acid content. However the diets these workers used contained 17.8% fat with 0.27 wt% of linoleic acid as the lowest linoleic acid value. Bieri and Poukka (24) also reported that feeding different levels of α-tocopherol with either hydrogenated coconut oil alone or hydrogenated coconut oil mixtures with either stripped lard or stripped corn oil, to provide linoleate at 0.23% and 1.1% in the diet, had no effect on the red cell fatty acid patterns. The level of linoleate (5%) in our diets, however, was considerably higher than that used in this experiment done by these other investigators.

The liver total lipid fatty acid pattern (Table VII) is qualitatively similar to the plasma lipid fatty acid pattern (Table V). In Experiment I the EFA-deficient animals are low in linoleic and arachidonic acids, high in oleic, palmitic, palmitoleic acids, and relatively high in eicosatrienoic acid although much less of this acid is present here than occurs in plasma and red blood cells lipids. Linoleate supplementation causes an immediate increase in arachidonic acid, followed by an increase in linoleic acid. Palmitic acid remains relatively constant. Stearic acid increases with the lowest linoleate supplement and remains at a constant level regardless of the increasing linoleate supplements. Palmitoleic and oleic acids decrease and the eicosatrienoic acid disappears in animals given the supplement of 200 mg linoleate per day. A value below 0.4 for the triene-tetraene, indicating recovery from essential fatty deficiency, is achieved by the linoleate supplement of 100 mg per rat per day (0.5% in the dietary intake).

Doubling the linoleate content of the diet to 200 mg per rat per day (1%) resulted in no change in the fatty acid composition of the lipids other than complete disappearance of the eicosatrienoic acid. Increasing the linoleate to 4% in the diet doubled both the linoleate and arachidonate content of the lipids at the expense of 16:1 and 18:1. Increasing the linoleate further to 5% resulted in only minor changes.

In Experiment II in animals fed the fat-free diet there were no changes in fatty acid composition in triglyceride, phospholipids or cholesterol esters attributable to the presence of increasing levels of tocopherol in the diet. When 5% linoleate was included in the diet, the fatty acid composition of the triglyceride fraction changed in the direction of increased stearic, linoleic and arachidonic acids, and decreased palmitoleic and oleic acids; no changes attributable to the tocopherol content of the diet were observed. However, in the case of the phospholipids, increasing the tocopherol content of the diet leads to a significant increase in arachidonic acid. In the case of the cholesterol esters, striking increase in arachidonic acid occurs at an intake of 0.006% T in the diet.

In subjecting various tissues of the animals fed the diets indicated in Experiment II to analysis for peroxides by the thiobarbituric acid method (31), the tissues examined, i.e., testes, kidney, and liver, had very low TBA values (measured by absorbance at 540 mμ) ranging from 0.03-0.22 with no correlation evident with regard to tocopherol level with or without the added linoleate. However the values for brain (reported in Table VIII along with the fatty acid composition of brain phospholipids) were considerably higher. It can be seen that dl-α-tocopheryl acetate at 0.01% was necessary to reduce TBA values in brain lipids, especially in those animals fed the fat-free diet. When the fatty acid composition of the brain phospholipids were examined, only traces of linoleate were present in all groups of animals; decreases in 16:0 and increases in 16:1 were apparent in the fat-free group when the tocopherol content was increased from 0-0.004%. In the brain lipids of animals fed linoleate, dl-α-tocopheryl acetate at 0.01% effected a decrease in 16:0, but as little as 0.006% was effective in bringing about a significant increase in palmitoleate (16:1).

REFERENCES

1. Filer, L.J., Jr., R.E. Rumery and K.E. Mason, "Biological Oxidants," Edited by C.G. Mackenzie, Josiah Macy Jr. Foundation, New York, 1946, p. 67.
2. Horwitt, M.K., C.C. Harvey, G.D. Duncan and W.C. Wilson, Amer. J. Clin. Nutr. 4:408 (1956).
3. Harris, P.L., and N.D. Embree, Ibid. 13:385 (1963).
4. Tappel, A.L., Fed. Proc. 24:73 (1965).
5. Pritchard, E.T., and H. Singh, Biochem. Biophys. Res. Commun. 2:184 (1960).
6. Bunyan, J., A.T. Diplock and J. Green, Brit. J. Nutr. 21:217 (1967).
7. "Diet and Heart Disease," American Heart Association, 1968.

8. Horwitt, M.K. Fed. Proc. 18:520 (1959).
9. Century, B., and M.K. Horwitt, J. Nutr. 72:357 (1960).
10. Witting, L.A., and M.K. Horwitt, Ibid. 82:19 (1964).
11. Hayes, K.C., S.W. Nielsen and J.E. Rousseau, Jr., Ibid. 99:196 (1969).
12. Jager, F.C., and U.M.T. Houtsmuller, Nutr. Metab. 12:3 (1970).
13. Ahrens, E.H., Jr., J. Hirsch, M.L. Peterson, W.J. Insull, W. Stoffel, J.W. Farquhar, T. Miller and H.J. Thomasson, Lancet 1:115 (1959).
14. Alfin-Slater, R.B., H. Hansen, R.S. Morris and D. Melnick, JAOCS 16: 563 (1969).
15. Alfin-Slater, R.B., R.S. Morris, L. Aftergood and D. Melnick, Ibid. 16:657 (1969).
16. Greenbert, S.M., C.E. Calbert, E.E. Savage and H.J. Deuel, Jr., J. Nutr. 41:473 (1950).
17. Rose, C.S., and P. Gyorgy, Amer. J. Physiol. 168:414 (1952).
18. Wintrobe, M.M., "Clinical Hematology," Fourth Edition, Lea and Febiger, Philadelphia, 1956, p. 159.
19. Draper, H.H., and A.S. Csallany, J. Nutr. 98:390 (1969).
20. Quaife, M.L., N.S. Scrimshaw and O.H. Lowry, J. Biol. Chem. 180:1229 (1949).
21. Nieft, M.L., and H.J. Duel, Jr., J. Biol. Chem. 177:143 (1949).
22. Chen, L.H., S. Liano and L.V. Packett, Nutr. Rep. Intern. 4:9 (1971).
23. Dayton, S., S. Hashimoto, D. Rosenblum and M.L. Pearce, J. Lab. Clin. Med. 65:739 (1965).
24. Bieri, J.G., and R.K.H. Poukka, J. Nutr. 100:447 (1970).
25. Herting, D.C., M.J. Lundwig and E.E. Drury, Ibid. 99:481 (1969).
26. Alfin-Slater, R.B., JAOCS 34:574 (1957).
27. Farquhar, J.W., and E.H. Ahrens, Jr., J. Clin. Invest. 42:675 (1963).
28. Holman, R.T., J. Nutr. 70:405 (1960).
29. Fulco, A.J., and J.F. Mead, J. Biol. Chem. 234:1411 (1959).
30. Walker, B.L., and F.A. Kummerow, J. Nutr. 82:329 (1964).
31. Zalkin, H., and A.F. Tappel, Arch. Biochem. Biophys. 88:113 (1960).

[Received October 27, 1971]