Dietary Fat Composition and Tocopherol Requirement: I. Lack of Correlation Between Nutritional Indices and Results of in Vitro Peroxide Hemolysis Tests

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Abstract

In general, the native tocopherols in polyunsaturated vegetable oils such as cottonseed oil, corn oil and their lightly hydrogenated products include sufficient vitamin E for growth, reproduction, lactation and normal lipid metabolism in the rat. The administration of vitamin E to animals fed diets deficient in essential fatty acids (e.g., a hydrogenated coconut oil or a fat-free diet) does not stimulate growth or reproductive performance per se, although testes development in the male rats is improved and some improvement in lipid metabolism is also noted. Hemolysis of the erythrocytes in vitro by hydrogen peroxide is increased in animals on diets rich (30%) in polyunsaturated vegetable oils or on diets providing no essential fatty acids at all. However, the conditions of the in vitro hemolysis test are not related to those in vivo and the in vitro test is not a measure of erythrocyte fragility. In addition, the in vitro hemolysis test does not necessarily reflect plasma tocopherol levels nor an abnormal nutritional state as a result of subsistence on high linoleate, low tocopherol intake, but rather measures the susceptibility to oxidation of a labile biological substrate and indicates the effective balance between potentially oxidizable lipids (polyunsaturates) in the stroma of the red blood cell and the antioxidant present (tocopherol or vitamin E). The labile lipid substrate may be either of exogenous origin (diet) or may be formed endogenously through tissue synthesis (as a result of an essential fatty acid deficiency). It is concluded that the in vitro hemolysis test may not be a valid indicator of vitamin E nutriture unless it is used in conjunction with other nutritional tests.

Introduction

The role of vitamin E as an in vivo lipid antioxidant has been proposed as a result of many investigations. After Dam and Granados (1) found lipoperoxides in tissues of vitamin E-deficient animals and subsequently observed peroxides in brain tissue of chicks fed cod liver oil, Dam (2) concluded that these peroxides were formed as a result of the disappearance of vitamin E from tissues containing polyenoic fatty acids. Harris and Embree (3) and Horwitt (4) have postulated a direct relationship between vitamin E requirement and polyunsaturated fatty acid intake; elevated dietary linoleate levels increased the tocopherol requirement. Investigators studying exudative diathesis and encephalomalacia in chicks have supported these observations in reports relating these diseases to tissue lipid peroxidation resulting from elevated levels of dietary linoleate unaccompanied by increased tocopherol (5–8). However, Ahrens et al. (9) 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 5½ months.

Recent recommended changes in dietary fats involve the substitution of polyunsaturated for the more saturated fats in the diet. Most naturally occurring polyunsaturated fats contain one or more of the tocopherols varying in amount and varying in antioxidant potency (10,11). However, the possibility has been suggested that an increased tocopherol intake may be required.

The present study was undertaken to determine the effects of certain dietary fats with widely different polyunsaturated fatty acid-tocopherol ratios on the vitamin E status of rats and to ascertain whether there was sufficient vitamin E in the tocopherols of these fats to satisfy the requirement. Various nutritional and biochemical indices, i.e., growth, reproduction, lactation and tissue cholesterol levels were used to establish the adequacy of the test diets. With the results of these tests as background, the value of the in vitro hemolysis test has been assessed as an indicator of vitamin E nutriture.

Experimental Procedures

Methods

Experiment A. Weanling male and female rats from our colony (the former USC strain) were fed the following oils at 30% of the diet: nonhydrogenated winterized cottonseed oil (CSO), nonhydrogenated corn oil (CO), lightly hydrogenated cottonseed oil (Chipper Oil), hydrogenated soybean oil (SBO Shortening) and hydrogenated coconut oil (HCNO). Animals fed fat-free diets (FF) were included for comparison. No tocopherol was added to the diet of these groups. To other groups of animals fed the corn oil, coconut oil and fat-free diets dl-alpha tocopherol acetate (T) was added to supply additional vitamin E, i.e., an increase of 0.05% of the oil (or 0.015% of the diet).

The composition of the basal diets is shown in Table I. The fatty acid composition determined by both spectrophotometric (12) and gas liquid chromatographic analysis (13) and the tocopherol contents of the test oils estimated by colorimetric (14) and gas liquid chromatographic procedures (15) are shown in Table II. Animals were fed the respective diets for 10 weeks at which time they were bred. Where breeding experiments were successful, second and third generations were also given the experimental diets for 10 week periods and their growth recorded. After these feeding periods, susceptibility of red blood cells to hemolysis in vitro by hydrogen

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TABLE I

Composition of Basal Diet[a]

Constituent	Percentage
Casein	20.00
Fat or oil	30.00
Salt mixture[b]	5.00
Celluflour	5.00
Vitamin mixture[c]	0.44
Sucrose	39.56

[a] Diets were made every seven days and stored in the refrigerator until consumed.
[b] Wesson modification of Osborne-Mendel Salt Mixture; Science 75:339 (1932).
[c] The vitamin mixture was tocopherol-free and contained the following vitamins: p-amino benzoic acid 36.3%; inositol 30.4%; ascorbic acid 12.2%; thiamine 4.4%; niacin 3.5%; vitamin B_{12} triturate 3.64%; calcium pantothenate 3.64%; riboflavin 1.7%; pyridoxine 1.7%; vitamin A and D (crystallets) 0.76%; folic acid 0.61%; menadione 0.30%; biotin 0.15%. When tocopherol was added to certain diets it was added as dl α-tocopheryl acetate at a level of 0.015% of the diet (or 0.05% of the oil).

peroxide was determined using the method of Rose and Gyorgy (16) as modified by Horwitt et al. (17). Determinations of tocopherol in the plasma in selected groups were carried out using the method of Quaife and Harris (18). Cholesterol determinations were made on lipid extracts of plasma and liver using a modification of the Sperry-Schoenheimer method (19). Red blood cell lipid extracts of pooled samples were analyzed for fatty acid composition using a Barber-Colman Gas Chromatograph (Model 20, 9.5 ft × 1/4 in. column, packed with 15.0% by weight of diethylene glycol succinate polyester on 80–100 mesh acid-washed Chromosorb W). 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 carbon number.

Experiment B. In a separate but similar experiment other groups of weanling male and female animals were fed cottonseed oil and corn oil unsupplemented with vitamin E, and fat-free diets with and without tocopherol supplements in order to study further the effect of these diets on erythrocyte integrity in vitro.

Results and Discussion

The gain in weight of male and female rats fed the experimental diets over a 10 week period is presented in Table III. Weight gains seem to be dependent on linoleate content of the diet rather than on the tocopherol content since lower weight gains are observed in the groups fed the hydrogenated fats deficient in linoleate and the fat-free diets (with or without supplemental tocopherol) as compared with the groups fed the corn or cottonseed oils. It would seem that all the fat diets contained adequate vitamin E to support growth since the tocopherol additions to the diets of animals fed the various fats had little effect on weight gain. In the fat-free group, the addition of tocopherol resulted in a slight improvement of growth.

The reproductive performance of the animals on the various diets is shown in Table IV. Since the ability of an animal to reproduce is an indication of both essential fatty acid and vitamin E adequacy, diets deficient in both or in either one of the nutrients would interfere with normal reproduction. As expected, rats fed the fat-free diet failed to reproduce. Reproduction was also poor for the animals on the more saturated fat diets low in linoleate (the hydrogenated SBO shortening and the hydrogenated coconut oil diets) although there is some indication of a possible EFA sparing action by hydrogenated coconut oil resulting in a better reproductive performance in the animals fed the hydrogenated coconut oil compared with the rats fed a diet devoid of fat. In some instances, with animals fed the same diets through three generations, reproductive performance in Generation 2 was not as good as in Generation 1. However, in Generation 3, an improved reproductive performance was observed. Reproductive performance for the animals on the liquid nonhydrogenated vegetable oils diets (cottonseed oil and corn oil) remained good through three generations in spite of the fact that these diets contained the highest ratios of polyunsaturates to natural tocopherols.

Supplementation with tocopherol (vitamin E) did not improve the reproductive performance of the animals fed the diets deficient in essential fatty acids (the fat-free or hydrogenated coconut oil diet). Lactation performance (survival of the young) was comparable among all groups exhibiting satisfactory reproductive performance.

Histological examination of testes of male rats

TABLE II

Fatty Acid Composition and Tocopherol Content of Oil Samples

Oil	Iodine no. (Wijs)	Fatty acid composition, per cent of total fatty acid[a]					Tocopherol content, per cent[b]					Ratio of polyunsaturated fatty acid to tocopherols	
		Method	18:3	18:2	18:1	Sat.	Method	α	γ	δ	Total (Tt)	P/Tα	P/Tt
Liquid cottonseed oil (CSO)	115.4	Spec.	0.00	56.3	20.9	22.8	Color.	....	....	....	0.10	....	563:1
		GLC	0.0	56.2	19.3	24.5	GLC	0.054	0.042	0.010	0.106	1041:1	530:1
Lightly hydrogenated cottonseed oil (Chipper oil)	84.3	Spec.	0.00	24.2[c]	48.2	27.6	Color.	....	....	....	0.09	....	269:1
		GLC	0.0	25.1	43.9	28.0	GLC	0.055	0.041	0.009	0.105	456:1	239:1
Hydrogenated soybean oil (SBO shortening)	67.1	Spec.	0.00	1.2	75.7	23.1	Color.	....	....	....	0.13	....	9:1
		GLC	0.5	2.0	74.3	23.2	GLC	0.012	0.083	0.034	0.129	208:1	19:1
Liquid corn oil (CO)	126.4	Spec.	0.9	57.5	28.3	13.3	Color.	....	....	....	0.11	....	531:1
		GLC	0.8	58.1	27.4	13.7	GLC	0.013	0.075	....	0.088	4531:1	669:1
Hydrogenated coconut oil (HCNO)	1.0	Spec.	0.00	0.0	1.0	99.0	Color.	....	....	....	0.008	....	0
		GLC	0.0	0.0	1.4	98.6	GLC	0.00[d]	0.00[d]	0.00[d]	0.00[d]	0	0

[a] Spec. refers to the spectrophotometric method (12); GLC to gas liquid partition chromatography (13), wherein the methyl esters were prepared by refluxing the oil with sodium methoxide and methanol. Wherever GLC values do not add up to 100%, small quantities of unidentified components are present.
[b] Color refers to the colorimetric method (14) while GLC refers to gas liquid chromatography of the tocopherol acetates by a procedure related to that reported (15), using 5 ml of pyridine-acetic anhydride (2:1 v/v) to react overnight with the separated unsaponifiables from 4 g of oil. A hydrogen flame detector is used for measuring the effluent from a 4 ft × 3/16 in. 2% SE-30 on 80/100 Diatoport S column operated at 230 C and a helium flow of 70 cc/min.
[c] Of which about 2% are conjugated dienes.
[d] Less than 0.004%.

TABLE III

Gain in Weight of Male and Female Rats Fed Fat and Fat-Free Diets With and Without Tocopherol Supplements Over a 10 Week Period

Diet	Expt. No.	Males Generation 1 g	Males Generation 2 g	Males Generation 3 g	Expt. No.	Females Generation 1 g	Females Generation 2 g	Females Generation 3 g
CSO	A	245 ± 6[a] (15)[b]	243 ± 7(6)	240 ± 5(6)	A	169 ± 3(20)	155 ± 4(12)	146 ± 4(10)
	B	235 ± 19(10)			B	171 ± 26(10)		
Chipper oil	A	238 ± 6(15)	221 ± 10(7)	224 ± 6(6)	A	176 ± 3(20)	154 ± 6(12)	150 ± 5(9)
SBO shortening	A	179 ± 6(15)			A	131 ± 8(20)		
CO	A	229 ± 10(14)	252 ± 13(6)	229 ± 8(6)	A	163 ± 3(20)	169 ± 10(12)	143 ± 7(12)
	B	230 ± 27(10)			B	168 ± 8(10)		
CO + T	A	241 ± 6(15)	227 ± 11(6)	210 ± 10(6)	A	162 ± 4(20)	152 ± 3(12)	137 ± 5(12)
HCNO	A	160 ± 5(12)	153 ± 8(5)	....	A	112 ± 3(20)	99 ± 5(12)	....
HCNO + T	A	144 ± 4(12)	....	....	A	116 ± 2(20)	....	....
FF	A	160 ± 6(12)	....	....	A	124 ± 8(20)	....	....
	B	147 ± 22(9)	....	....	B	102 ± 18(10)	....	....
FF + T	A	172 ± 9(12)	....	....	A	129 ± 2(20)	....	....
	B	165 ± 31(10)	....	....	B	127 ± 11(10)	....	....

[a] Standard error of the mean.
[b] Figures shown in parentheses represent the number of animals.

showed large testes with large tubules containing active, maturing, spermatogonial cells undergoing spermatogenesis with sperm in the lumina in those animals receiving the corn oil, cottonseed oil and lightly hydrogenated cottonseed oil diets. No evidence of vitamin E deficiency was observed. The rats on the fat-free diet had small testes with degenerative seminiferous tubules severely depleted of sperm indicating an essential fatty acid, and also vitamin E, deficiency. The addition of tocopherol to the diets of the rats fed the fat-free ration seemed to improve the condition of the testes resulting in slightly larger organs and less degeneration. The animals fed the hydrogenated coconut oil diets had testes which were qualitatively histologically similar but not as severely damaged as those observed in the fat-free groups.

Horwitt et al. (20) have reported that feeding a polyunsaturated oil with its natural tocopherols destroyed by oxidation lowers the plasma tocopherol level, and this, in turn, contributes to an increase in the susceptibility of erythrocytes to in vitro hemolysis. Extending the hypothesis further, since a vitamin E deficiency is characterized by a high value for in vitro hemolysis (21), animals with red blood cells that are easily hemolyzed by in vitro procedures may be vitamin E deficient. This hypothesis was investigated in the present study.

The results of erythrocyte hemolysis determinations and plasma tocopherol analyses performed on some of the groups of animals of Experiment A are shown in Table V. Red blood cell hemolysis is low in the animals fed the CSO diet but is significantly higher in some of the animals fed the CO diet and in the animals fed the HCNO and the FF diets. The addition of tocopherol to these latter diets results in an increase in plasma tocopherol levels and in a decrease in the erythrocyte hemolysis values. Horwitt et al. (22) have reported that the degree of hemolysis of erythrocytes by the in vitro test is inversely proportional to the plasma tocopherol levels. The results obtained in these experiments in general support this theory. However, this procedure merely demonstrates the use of a biological test system to measure the presence of an antioxidant (tocopherol) in protecting a labile substrate.

Since both corn and cottonseed oils contained approximately the same amount of polyunsaturated fatty acids (linoleic) (approximately 55%) and total tocopherol (0.10%) and therefore the same P/Tt ratio (approximately 550:1), the difference in erythrocyte hemolysis of the rats fed these two oils was unexpected. It must be pointed out that the tests on these two liquid vegetable oils were conducted a year apart and the in vitro hemolysis was done with different solutions of the reagents at the two periods of test. It is also pertinent that the composition of the tocopherols in these two oils differs substantially (see Table II). Cottonseed oil has more than four times as much α-tocopherol as does corn oil. Herting and Drury (10) have suggested that among the common edible polyunsaturated oils (cottonseed, corn, safflower and soybean) only cottonseed oil (α-tocopherol content of 338–434 μg/g as compared with an α-tocopherol content of 147–236 μg/g in corn oil) supplied sufficient α-tocopherol to balance the effect of its polyunsaturated fatty acids.

In an attempt to achieve a better understanding of the meaning of the in vitro test of erythrocyte hemolysis, further feeding studies were undertaken. In Experiment B groups of male and female rats were again fed corn and cottonseed oils at a level of 30% with no other source of vitamin E and a fat-free diet with and without the tocopherol supplement for a 10 week period. Erythrocyte hemolysis tests were again performed. In addition, to test the possibility of the in vitro erythrocyte

TABLE IV

Effect of the Various Diets on Reproduction and Lactation

Diet	Successful pregnancies Generation 1 %	Successful pregnancies Generation 2 %	Successful pregnancies Generation 3 %	Mortality of young (3–21 days) Generation 1 %	Mortality of young (3–21 days) Generation 2 %	Mortality of young (3–21 days) Generation 3 %
CSO	95(19/20)[a]	73(8/11)	92(11/12)	18	2	7
Chipper oil	55(11/20)	33(4/12)	67(8/12)	12	0	4
SBO shortening	50(10/20)	....	....	38	....	..
CO	84(16/19)	73(8/11)	92(11/12)	6	25	4
CO + T	95(19/20)	83(10/12)	100(12/12)	20	24	2
HCNO	73(14/19)	0(0/9)	....	31	....	..
HCNO + T	60(12/20)	....	....	100	....	..
Fat-free	0(0/20)	....	....	....	....	..
Fat-free + T	0(0/20)	....	....	....	....	..

[a] Number of females casting litters per number of females bred.

TABLE V

Erythrocyte Hemolysis and Plasma Tocopherol Levels (Experiment A)

Diet		Erythrocyte hemolysis %	Tocopherol in plasma (Pooled samples) mg/100 ml
CSO	M	3(8)[a]	0.47
	F	12(8)	0.24
CO	M	80(8)	0.36
	F	{55(8); 3(8)}	0.36
CO + T	M	3(8)	0.43
	F	9(7)	......
HCNO	M	99(8)	0.28
	F	96(8)	0.32
HCNO + T	M	4(8)	0.37
	F	6(8)	0.52
FF	M	86(8)	......
	F	94(7)	0.11
FF + T	M	4(8)	0.55
	F	1(6)	0.57

[a] Numbers in parentheses are numbers of animals on which tests were performed.

hemolysis test reflecting erythrocyte weakness in vivo, erythrocyte fragility was determined by examining the hemolysis of red blood cells in salines of varying concentrations. In this test (23), venous blood is added to tubes containing concentrations of sodium chloride varying from 0.72% to 0.21%. The samples are refrigerated for 2–3 hr after which they are centrifuged. Complete hemolysis is indicated by the absence of a residue at the bottom of the tube or by the absence of any cloudiness in shaking. The results are shown in Table VI.

In this series of tests, complete erythrocyte hemolysis was obtained following hydrogen peroxide treatment of the red blood cells of animals fed the fat-free, unsupplemented diet and also in the case of those fed the corn and cottonseed oil diets, whereas little or no hemolysis was observed in the groups of animals fed the fat-free diet supplemented with tocopherol. These results, obtained on blood samples available and tested at the same time, show no difference in susceptibility to hemolysis when the peroxidation test is conducted in erythrocytes from animals fed either the cottonseed or corn oil diets. Insofar as vitamin E nutriture is concerned, no differences between these two oils were noted in the growth, reproduction and lactation studies reported earlier. Apparently there is sufficient vitamin E occurring naturally in both corn and cottonseed oils to satisfy vitamin E requirements in vivo and only a borderline quantity when the criterion is the nonspecific in vitro hemolysis test. An analogous situation was noted in another of our studies (to be published) where the results of the in vitro test of erythrocyte hemolysis failed to correlate with biological responses based upon growth, reproduction, lactation and tissue lipid peroxidation.

No correlation was apparent between the erythrocyte hemolysis and erythrocyte fragility tests. Differences in hemolysis measured by the hydrogen peroxide test were not accompanied by differences in erythrocyte fragility as measured by hemolysis in salines of low concentration. In fact, all values for this latter test were practically the same and were within, or very close to, the values reported for normal blood where beginning hemolysis ranges from 0.45% to 0.39% NaCl and complete hemolysis from 0.33% to 0.30%.

Hemolysis of blood cells is a normal process. Approximately 1 % of the red blood cells are destroyed

TABLE VI

Erythrocyte Hemolysis and Erythrocyte Fragility as a Function of Diet (Experiment B)[a]

Diet		Erythrocyte hemolysis (%)	Erythrocyte fragility (% NaCl)	
			Start	Complete
CSO	M	100	0.41	0.28
	F	100	0.44	0.30
CO	M	100	0.44	0.28
	F	100	0.46	0.30
FF	M	100	0.49	0.33
	F	100	0.48	0.34
FF + T	M	7	0.47	0.32
	F	0	0.48	0.34

[a] Ten animals per group.

and replaced daily. It has been theorized that the presence of fatty materials in increased quantities (after a meal) causes normal lysis of the erythrocyte (24). It was also found that the erythrocytes of normal men became more susceptible to hypotonic hemolysis after exposure to lipemic serum (25) but that the normal bone marrow could replace this extra loss of red blood cells and therefore it is possible that the degree of erythrocyte hemolysis observed depends to some extent on when the blood sample is drawn and tested.

It has also been suggested (26) that the high degree of erythrocyte hemolysis observed in animals fed a fat-free diet unsupplemented with tocopherol is a result of stored peroxidizable lipid in these animals. Holman (27) has reported that in essential fatty acid deficiency the concentration of arachidonic acid ($C_{20:4}$) decreases whereas that of an eicosatrienoic acid ($C_{20:3}$) increases in several tissues including the erythrocytes. This eicosatrienoic acid with double bonds at positions 5, 8 and 11 is endogenously synthesized from oleic acid by the essential fatty acid-deficient rat (24). In Table VII the per cent of C_{18} and C_{20} fatty acids contained in the erythrocyte lipids of animals fed the various diets are reported. In the animals fed the saturated coconut oil and the fat-free diet with or without tocopherol supplements the linoleic ($C_{18:2}$) and arachidonic ($C_{20:4}$) acid concentrations are quite low, but the level of eicosatrienoic acid ($C_{20:3}$), absent in the animals fed diets with adequate linoleate, is quite high. This triene is potentially as easily oxidized as is either linoleic or arachidonic acid and undoubtedly accounts for the high degree of erythrocyte hemolysis observed in these tocopherol-unsupplemented groups.

Therefore, polyunsaturation in red blood cells and tissues may occur not only from the ingestion of polyunsaturates in high concentrations but also may develop from oleic acid in the absence of essential fatty acids in the diet (28). In the absence of an

TABLE VII

C_{18} and C_{20} Fatty Acids of Erythrocyte Total Lipids[a] in Male Rats

Diet	Expt. No.	Fatty Acid 18:0 %	18:1 %	18:2 %	20:3 %	20:4 %
CSO	A	17.9	10.8	17.3	......	30.3
	B	19.0	9.2	16.4	......	22.1
CO	A	17.7	12.3	14.0	......	16.3
	B	20.7	10.9	14.0	......	21.9
CO + T	A	14.0	16.0	10.2	......	17.9
HCNO	A	14.2	15.8	4.3	18.2	7.2
HCNO + T	A	14.0	15.4	3.1	20.4	6.4
FF	A	12.6	23.1	6.2	20.3	1.4
	B	14.4	21.8	1.6	19.4	6.2
FF + T	A	12.2	23.2	1.6	22.2	2.1
	B	13.9	20.7	1.8	21.9	7.3

[a] Pooled samples of 8–10 animals per group.

TABLE VIII

Plasma Cholesterol and Liver Cholesterol and Total Lipid Levels in Rats Fed Fat and Fat-Free Diets With and Without Supplemental Vitamin E

	Expt. No.	Plasma cholesterol (mg/100 ml)		Liver cholesterol (mg/g)		Liver lipid (mg/g)	
		Males	Females	Males	Females	Males	Females
CSO	A[a]	49.6 ± 4.3[b]	64.7 ± 3.5	2.74 ± 0.09	2.06 ± 0.06	62.7 ± 3.7	47.7 ± 2.0
	B[a]	61.3 ± 10.6	66.6 ± 8.3	2.59 ± 0.14	1.97 ± 0.07	64.5 ± 4.1	47.1 ± 3.5
Chipper oil	A	57.9 ± 3.0	67.7 ± 5.5	2.13 ± 0.08	2.01 ± 0.08	54.8 ± 1.4	46.7 ± 1.9
SBO shortening	A	40.6 ± 2.0	45.2 ± 1.9	2.19 ± 0.08	2.12 ± 0.05	46.9 ± 1.4	39.7 ± 0.9
CO	A	50.6 ± 2.5	60.8 ± 3.7	2.88 ± 0.18	2.10 ± 0.09	60.3 ± 4.1	50.8 ± 2.2
	B	66.1 ± 11.0	69.1 ± 8.6	2.77 ± 0.13	2.01 ± 0.06	60.1 ± 3.3	44.2 ± 1.3
CO + T	A	67.8 ± 3.5	52.1 ± 4.3	2.72 ± 0.13	2.00 ± 0.05	64.3 ± 1.9	49.5 ± 3.0
HCNO	A	45.0 ± 2.5	54.1 ± 5.3	2.18 ± 0.12	2.18 ± 0.08	62.0 ± 7.1	64.0 ± 4.3
HCNO + T	A	45.1 ± 1.7	60.0 ± 2.3	2.21 ± 0.08	1.88 ± 0.06	64.4 ± 5.7	49.7 ± 2.2
FF	A	30.5 ± 3.0	50.2 ± 3.3	3.90 ± 0.20	2.38 ± 0.23	75.7 ± 5.9	40.5 ± 1.9
	B	32.4 ± 2.6	43.4 ± 3.0	3.88 ± 0.04	2.29 ± 0.04	72.3 ± 1.2	50.5 ± 1.1
FF + T	A	35.6 ± 4.1	44.5 ± 2.0	3.35 ± 0.17	2.19 ± 0.11	69.5 ± 3.2	42.7 ± 2.7
	B	37.6 ± 3.5	45.7 ± 3.8	3.31 ± 0.22	2.58 ± 0.07	59.4 ± 1.9	57.4 ± 1.9

[a] Experiment A, 8–16 animals per group; Experiment B, 10 animals per group.
[b] Standard error of the mean.

antioxidant in significant amount in the test system, high values for the in vitro erythrocyte hemolysis test result not only where there is ingestion of polyunsaturated vegetable oils in large amounts but also where there is saturated fat or no fat in the diet. If an antioxidant e.g., tocopherols or vitamin E, is present in significant amount, the red blood cell is protected against in vitro hemolysis induced by hydrogen peroxide. This requirement for in vitro protection against hydrogen peroxide may differ from that required in vivo and hence the in vitro hemolysis test may not be a valid indicator of vitamin E nutriture unless it is used in conjunction with other nutritional tests.

In long term feeding studies with humans where polyunsaturates have replaced the more saturated fats in the diet, several investigators (29,30) have not found any evidence for the need of additional vitamin E other than that provided by the polyunsaturated vegetable oils. Furthermore, despite high levels of polyunsaturation in the tissue lipids no signs of hemolysis in vivo or anemia have been noted. In another of our studies involving multigeneration experiments and long-term observations covering almost the entire life span of the rat fed a polyunsaturated diet of practical value, no extra supplements of vitamin E were required above that native to the vegetable oil component.

The results of our studies are in agreement with those reported by Bunyan et al. (31,32) who found that unsaturated lipids do not become peroxidized after incorporation into the adipose tissue of vitamin E deficient rats. They also reject the in vivo antioxidant theory of vitamin E activity proposed by others (4,26,33–36) since the existence of peroxidation in vivo in the vitamin E deficient animal has not been substantiated (31,32). In further support for our studies, Barber (37) has shown that the in vitro hemolysis test simply reveals the effective balance of pro- and antioxidant components in a system which is not related to conditions in vivo.

The final series of studies conducted in the present investigation are concerned with plasma and liver cholesterol levels and with liver lipid levels. In general, plasma cholesterol values are lower in rats fed the saturated and fat-free diets (Table VIII). Although liver cholesterol levels are lower in male rats fed the saturated fats than in the males fed diets containing linoleic acid (38), the liver cholesterol values in the male animals fed the fat-free diets are considerably higher than those in other groups. Hepatic synthesis of cholesterol is depressed in rats fed fat-free diets and diets inadequate in linoleic acid (38) and it has been postulated that in fat-deficient animals, transport of cholesterol from the liver is also inhibited so that hepatic cholesterol levels are increased (39). This elevation in cholesterol in the liver may also reflect impaired bile acid synthesis. Administration of extra tocopherol (vitamin E) has little effect on plasma or liver cholesterol levels or on liver lipid levels when the linoleate in the diet is provided by a polyunsaturated vegetable oil containing natural tocopherols. In some instances, however, tocopherol administration does result in differences in cholesterol and lipid values in animals fed diets deficient in linoleate. Changes in plasma and liver cholesterol levels and liver lipid levels are apparent in the female rats fed the hydrogenated coconut oil; tocopherol administration results in decreased liver cholesterol from 2.18 to 1.88 mg/g ($p<0.01$) and liver lipid (from 64.0 to 49.7 mg/g, $p<.001$) values. In the animals fed the fat-free diets, tocopherol administration results in decreased liver cholesterol levels in male rats (3.90 to 3.35 mg/g in Experiment A and 3.88 to 3.31 mg/g in Experiment B, $p<0.01$). These changes are in the direction of improvements in lipid metabolism.

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