Dietary Fat Composition and Tocopherol Requirement: IV. Safety of Polyunsaturated Fats[1]

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ABSTRACT

In a long-term multigeneration study, conducted in our laboratories for 32-years, with occasional longevity and histopathological evaluations included, rats of our own inbred strain (originally of Wistar derivation) were fed semisynthetic diets comprising whole wheat, skim milk powder, and fat in the form of margarine products. The total source of tocopherols was the dietary fat itself. Saturated fatty acid content (S) remained relatively constant at about 20% of the fat and total tocopherol level also remained constant at about 0.12% of the fat. Polyunsaturated fatty acid (P) content, however, progressively increased almost fourfold, from 7.5% to 28.5% and alpha-tocopherol levels decreased to one-half level, from 0.033% to 0.016% of the fat. Hence, the ratio of polyunsaturated fatty acids to alpha-tocopherol content changed markedly from 227:1 to 1780:1, with other factors (relative to fat composition) held constant during the 32-year period of feedings and observations. Fat level in the diet increased over the years from 9.2% to 16.0% or from about 21% to about 33% of the caloric intake. Thus, quality and quantity of the fat in the diet progressively changed, and the impact of these changes was evaluated by comparing biological performances of the successive generations. Growth and reproduction and lactation performances were noted to be regularly satisfactory and comparable from generation to generation throughout the experimental period. Longevity studies conducted on arbitrarily selected generations also provided data showing no deleterious effects associated with a dietary change. Histopathological examinations of tissue revealed minimal myocarditis and no malignant tumors which could be attributed to a dietary factor. No vitamin E deficiencies were observed. Even the in vitro peroxide hemolysis values for the red blood cells of the animals, fed the diets containing the higher levels of polyunsaturated fatty acids, were low, indicating that the dietary fats provided sufficient absorbable tocopherol to protect the potentially oxidizable unsaturated fatty acids in the erythrocyte membrane. Biochemical data reflected responses to aging and not to any specific diet fed. It is concluded that a diet providing as much as 33% of the calories as a fat, the latter containing up to 28.5% polyunsaturated fatty acids, substantially of the essential fatty acid type, with a P/S ratio of up to 1.6:1 and a polyunsaturated fatty acid to alpha-tocopherol ratio as high as 1780:1 produces no undesirable effects in the rat.

INTRODUCTION

In 1957, a third report from our laboratory describing the satisfactory nutrition status of our rats, maintained for 46 generations on a modified Sherman diet containing skimmed milk powder and partially hydrogenated vegetable margarine fat, was published (1). This report included data on physiological and biochemical indices, i.e. growth, reproduction, longevity, and cholesterol levels in liver and plasma; all values were within normal ranges and were comparable to data presented earlier (2).

In recent years much research has appeared on the role of fat in the diet, especially concerning the etiology of the disease atherosclerosis. The value of fats containing increased amounts of polyunsaturated fatty acids in reducing hypercholesterolemia and therefore possibly in reducing the incidence and severity of coronary artery disease has been documented in many reports (3-9). However, there are still unresolved questions. Of considerable importance is to determine what constitutes an optimal amount of polyunsaturated fatty acids in the diet, what is the optimal ratio of polyunsaturated to saturated fatty acids in the diet; and whether the introduction of additional quantities of polyunsaturated fat products increases the requirement for antioxidants, specifically the tocopherols (10-13) or is in any way detrimental to the organism. Two recent papers (14,15) have reviewed the published evidence and concluded there are no hazards involved in the proposed and attainable dietary modifications for the control of the serum cholesterol levels. Recent papers from this laboratory have dealt specifically with dietary fat composition and tocopherol requirements; they have indicated that sufficient antioxidant protection is afforded naturally in unsaturated fat products which are commercially available (16,17).

During the years, the type of margarine fat used in our continuing multigeneration and longevity studies has been modified to reflect the changes which have taken place in product development and manufacture; the principal change in recent years has been an increase in the polyunsaturated fatty acid content. In this presentation are reported the results of feeding these newer fats to rats as evaluated by weight gain, reproduction and lactation performances, longevity, biochemical analyses, and histopathological examinations. In addition, level of fat in the diet also was increased to a level providing about 33% of the calories as the test fat. The total source of tocopherols was the dietary fat itself.

MATERIALS AND METHODS

The animals used in our experiments are an inbred strain, originally of Wistar derivation, then designated as USC rats and now referred to, for purposes of convenience,

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TABLE I

Composition of Diets

Component	Group		
	MM (%)	MM and MC (%)	MMM and MCC (%)
Ground whole wheat	66.5	64.5	59.7
Skim milk powder	23.3	23.3	23.3
Sodium chloride	1.0	1.0	1.0
Margarine fat[a]	9.2	11.2	16.0
% of Calories as fat	21	25	33

[a]But fed as margarine, with an average of 1200 USP units of vitamin D included per pound of product.

as A-S rats. The compositions of the several diets fed are shown in Table I. Descriptions of the various margarine fats used in the diets are summarized in Table II; the feeding schedules also are shown. For conducting tests on the fats by the more reliable modern methods of analyses, it was necessary to prepare duplicates of the fats fed during the earlier periods of this long-term investigation.

Generations MM 1-26 were given a diet containing 9.2% margarine fat, discounting the moisture contributed by the margarine. Starting with generation MM 27, the amount of margarine fat was increased to 11.2%. The margarine oil in the margarine fed to the rats until the 44th generation was a commercial vegetable oil consisting of equal parts of soybean oil and cottonseed oil, each hydrogenated directly to margarine constants. Fatty acid composition is shown in Table II. This product also contained 35.3% *trans* acids and some polyene isomers, i.e. the difference between total polyunsaturated fatty acids and the essential fatty acid (EFA) values. In generation 44, margarine with a revised formula for greater plasticity and with an increase of EFA to 6.5% was incorporated into the diet. This product also provided about 35% *trans* acids and polyene isomers also were present to a signifiant degree.

Further dietary changes occurred with generation MM 61, when the EFA of the margarine fat was increased to 27%. Just before that time, another multigeneration experiment, series MC, had been initiated using another margarine product with liquid corn oil as the major ingredient (24). These liquid oil products contained only 20% *trans* acids and no isomers of the polyunsaturated fatty acids (24). After attaining the 75th generation of MM and the 15th generation of MC, these series were terminated and 2 other series, MMM and MCC, were initiated in 1961. In these latter multigeneration experiments, the dietary concentration of the respective hydrogenated margarines was increased to 16.0%, equivalent to 33% of the caloric intake, to more closely reflect the recommended fat intake for human ingestion proposed by the American Heart Association and the Council on Food and Nutrition (3,4). The MMM and MCC series are similar in EFA content (27.5 vs. 28.0%); however, the MMM series contain margarine fats made with either liquid non-hydrogenated cottonseed oil or liquid non-hydrogenated soybean oil as the major ingredient, whereas the margarine fat in the MCC series is made with liquid non-hydrogenated corn oil as the major ingredient.

The tocopherol content of these fat products, and also the ratio of the polyunsaturated fatty acids (P) to tocopherol (T), content also is shown in Table II. It can be seen that the concentration of polyunsaturated fatty, acids in the diets fed, increased to a greater extent than did the tocopherol content particularly in the α-tocopherol; and therefore, the ratio of P/T also increased to a greater extent than did the ratio of P/T total.

Table III shows the growth of male and female rats of representative generations throughout the study; it can be seen that the gain in weight is rather reproducible with inconsistencies due to seasonal variations and the unfortunate presence at times of a chronic respiratory infection. However, the weights (Table III) at 10 weeks are rather consistent although the animals fed the MC and MMM diets are slightly heavier than the others due to the higher food intake. However, tibiae lengths measured on male rats, which is perhaps a better index of actual growth rather than of stored adipose tissue, are very similar in all generations.

Reproductive performance of representative generations of our early studies and more recent experiments is shown in Table IV; reproduction and lactation continue to be very satisfactory with variations due to seasonal changes.

Results of longevity studies done on randomly selected generations are shown in Table V. Although our colony suffers from a chronic respiratory infection which occasionally interferes with longevity, survival for the most part is good. At 75% mortality, animals were killed, and various biochemical and/or histopathological analyses were performed. Biochemical data, i.e. total cholesterol determinations in plasma and liver, moisture, and protein analyses, are reported in Table VI. Although comparisons of data are complicated by the fact that the results are on animals of different ages, plasma cholesterol levels seem to increase with age and not with the specific diet fed. This agrees with earlier work by Keys, et al. (25). Carcass moisture content is remarkably constant and per cent protein in carcass is similar in all groups. Total liver cholesterol values are also comparable in all groups and are very similar to results obtained in our colony when rats are fed laboratory chow diets.

In vitro hemolysis tests using hydrogen peroxide (26) were done on erythrocytes of male rats of generations MM 70, MC 10, MMM 17, and MCC 17. The P/T of the margarine fat fed to rats of group MM 70 was 794:1; of MC 10, 1781:1; of MMM 17, 1375:1; and of MCC 17, 1781:1. The ratio of P/T was 229:1, 190:1, 222:1, and 190:1 respectively. Hemolysis values ranged from 0 to less than 10% in all cases indicating that there was sufficient absorbable tocopherol to protect the potentially oxidizable unsaturated fatty acids in the erythrocyte membrane against oxidation. This is not surprising in view of the fact that previously we have found low hemolysis values (5.5%) with a P/T ratio of 2500:1 in the diet (27). Others have recently reported (28) that, with the ingestion by rats of oxidized fats to the point of toxicity, there was no effect on the susceptibility of erythrocytes to hemolysis which might indicate a need for additional vitamin E; indeed, the vitamin did not appear to have any effect on the toxic reactions of oxidized fats.

Periodic histological examinations of tissues of animals in this multigeneration experiment have not revealed the presence of abnormalities attributable to any dietary component. When the multigeneration experiment was initiated, the tissues—brain, heart, lung, spleen, stomach, testes-ovaries, small intestine, large intestine, liver, pancreas, adrenal, kidney—on 17 animals were examined, 9 of which had died previous to the sacrifice at the end of the 2-year experimental period. The 9, which died after 40, 57, 60, 69, 71, 78, 80, 82, 93 weeks respectively, due primarily to

TABLE II

Margarine Fats, Fed to Rats as Margarine, During Long-Term Multigeneration and Longevity Studies

Margarine products fed					Fatty acid composition (per cent of triglycerides)					Tocopherol content (T)%[d]					Ratio of total polyunsaturated fatty acids (P) to tocopherols	
Year	Group fed: Identity	Group fed: Generation	Margarine fat in diet %	Description of margarine fat[a]	Total polyunsaturated fatty acids as linoleic (P) [b]	EFA[c]	Saturated fatty acids (S)[b]	P/S	EFA/S	Procedure	Alpha (Tα)	Gamma	Delta	Total (Tt)	P/Tα	P/Tt
1940	MM	1-26	9.2	Hydrog. directly to margarine constants 50:50, SBO:CSO[e]	7.5	3.0	22.0	0.3	0.1	Color	---	---	---	0.12	---	63:1
	MM	27- 43	11.2							GLC	0.033	0.063	0.015	0.111	227:1	68:1
Sept. 1954	MM	44-60	11.2	Blend of under- and overhydrog. fats - 60:40, SBO:CSO	12.0	6.5	20.5	0.6	0.3	Color	---	---	---	0.12	---	100:1
										GLC	0.029	0.067	0.016	0.112	414:1	107:1
Sept. 1961	MM	61-75	11.2	Blend of non-hydrog. CSO and hydrog. fat - 52:48, liq. CSO:hydrog. SBO	27.0	27.0	23.0	1.2	1.2	Color	---	---	---	0.11	---	246:1
	MMM	1-14	16.0							GLC	0.034	0.062	0.015	0.111	794:1	243:1
Sept. 1968 to present	MMM	15-25 (and continuing)	16.0	Blend of non-hydrog. SBO and hydrog. fats - 41:40:19, liq. SBO: hydrog. SBO:hydrog. CSO	27.5 (incl. 3.0% as linolenic)	27.5	18.0	1.5	1.5	Color	---	---	---	0.12	---	229:1
										GLC	0.020	0.075	0.018	0.113	1380:1	243:1
Dec. 1960 to present	MC	1-15	11.2	Blend of non-hydrog. CO and hydrog. fats - 48:43:9 liq. CO:hydrog. SBO:hydrog. CSO	28.5	28.0	18.2	1.6	1.5	Color	---	---	---	0.12	---	237:1
	MCC	1-25 (and continuing)	16.0							GLC	0.016	0.076	0.012	0.104	1780:1	274:1

[a]Code: SBO = soybean oil; CSO = cottonseed oil; CO = corn oil; hydrog. = hydrogenated; liq. = liquid non-hydrogenated.

[b]Obtained by gas liquid chromatography (18).

[c]Essential fatty acids (EFA), obtained by the lipoxidase method (19,20) with biological confirmation obtained in testing selected samples (21).

[d]Color = colorimetric method (22); GLC = gas liquid chromatography of the tocopherol acetates by a proeedure related to that reported by others (23); 5 ml of pyridine acetic anhyoride (2:1 v/v) were allowed to react overnight with the separated unsaponifiables from 4 g of oil. A hydrogen flame detector was used for measuring the effluent from 4 ft. x 3/16 in., 2% SE-30 on80/100Diatoport S. column operated at 230 C and a helium flow of 70 cc/min.

[e]Under, and over-hydrogenated fats are fats hydrogenated either too little or too much to attain "margarine constants" and therefore not individually suitable for making margarine but may be blended to achieve margarine constants.

TABLE III

Growth of Rats of Representative Generations

Group	Generation	Weight at 90 days (g.)[a]		Tibia length (mm)
		Males	Females	Males
MM	40	290	200	37.2
MM	60	270	160	37.4
MM	70	259	202	37.5
MM	75	310	212	---
MC	10	252	188	37.5
MC	15	269	202	---
MMM	5	283	219	---
MMM	10	299	212	37.8
MMM	15	247	188	---
MMM	20	314	209	37.7
MMM	25	278	209	---
MCC	5	268	193	---
MCC	10	258	185	37.5
MCC	15	260	183	---
MCC	20	274	192	37.6
MCC	25	232	174	---

[a]Groups of males contained 12 rats each and groups of females contained 24 rats each.

TABLE IV

Reproduction Performance of Representative Generations

Group	Generation	Body weight at breeding (Females) (g.)	Successful pregnancies %	No. of rats per litter	Average weight of weanlings (21 days) (g.)	No. of weanlings[a]
MM	40	198	65	8.2	35.5	72
MM	65	216	85	12.0	35.6	84
MM	70	215	80	8.9	35.2	74
MM	74	218	100	10.9	37.4	123
MC	5	199	90	9.0	36.6	63
MC	10	199	80	9.6	36.0	101
MC	14	202	100	9.7	37.7	123
MMM	5	217	75	8.6	41.8	72
MMM	10	223	95	8.1	38.3	100
MMM	15	187	85	8.6	34.1	93
MMM	20	224	75	10.1	37.1	92
MMM	25	214	90	11.1	33.7	119
MCC	5	194	95	9.2	40.5	126
MCC	10	190	100	9.2	40.5	126
MCC	15	190	70	7.8	38.9	81
MCC	20	195	95	10.7	37.0	128
MCC	25	185	95	10.2	34.1	124

[a] At 3 days of age litters were cut, where possible, to 7 pups per litter.

TABLE V

Longevity Studies of Representative Generations of Rats

Group generation	No. of animals at start		% Survival			
			75%[a] weeks	50% weeks	25% weeks	
MM 34	Males	12	55	80	106	
	Females	17	74	101	112	
MM 75	Males	12	50	81	84	
	Females	24	64	80	100	
MC 15	Males	12	70	90	92	
	Females	24	57	78	92	
MMM 2	Males	12	71	83	96	
	Females	33	74	93	99	(39% survival)[b]
MMM 5	Males	12	36	78	87	
	Females	24	23	59	89	
MMM 10	Males	12	43	65	90	(33% survival)[b]
	Females	24	38	83	89	(46% survival)[b]
MMM 17	Males	12	77	83	98	
	Females	24	74	93	102	
MCC 17	Males	12	99	105	111	
	Females	24	83	95	104	

[a]Week at which % survival was reached.

[b]Animals killed for biochemical analysis of tissues.

TABLE VI
Biochemical Analysis on Male Rats of Representative Generations

Group designation	Age weeks	Total cholesterol[a] Plasma mg/100 ml	Total cholesterol[a] Liver mg/g	Lipid (total)[b] Liver mg/g	Carcass Moisture %	Carcass Protein[c] %
MM 46 (12)[d]	15	51.5 ± 2.0[e]	2.03 ± .04[c]	40.8 ± 0.9[e]	61.8	15.1
MM 70 (12)	15	80.8	—	—	62.1	18.0
MM 75 (3)	85	105.7 ± 21.9	1.62 ± .56	—	64.2	14.9
MC 10 (12)	15	85.0	—	—	63.9	18.7
MC 15 (5)	94	90.5 ± 13.3	1.60 ± .13	—	57.4	14.4
MMM 10 (12)	22	72.2 ± 3.6	1.72 ± .18	41.1 ± 7.0	—	—
MMM 20 (12)	15	51.9 ± 2.8	1.80 ± .10	41.8 ± 4.7	63.9	15.6
MCC 10 (12)	22	77.4 ± 5.6	1.68 ± .13	43.7 ± 5.2	—	—
MCC 20 (12)	15	55.3 ± 1.8	1.74 ± .03	41.5 ± 1.2	62.3	15.0
MMM 17 (4)	98	93.4 ± 12.4	2.00 ± .16	32.5 ± 4.5	—	—
MCC 17 (4)	111	109.3 ± 38.6	1.79 ± .23	32.2 ± 7.3	—	—

[a]Nieft, M.L., and H.J. Deuel, Jr. J. Biol. Chem. 177:143 (1949).
[b]Determined gravimetrically.
[c]Kjeldahl determination on technicon autoanalyzer.
[d]Numbers in parentheses indicate number of animals on which determinations were done.
[e]Includes the standard deviation.

TABLE VII
Gross Pathology Among MMM Rats, Generations 2, 5, and 10

Group	No. rats	Age at sacrifice (wks)	No. of rats with visible lesions[a] Liver	Kidney	Ovaries testes	Pituitary	Adrenals
Males							
MMM 2 and 5	8	104	3	2	2	1	0
MMM 10	8	52	0	0	0	0	0
MMM 10	8	88	1	0	0	0	0
Females							
MMM 2 and 5	12	104	3	3	3	3	0
MMM 10	18	52	2	0	1	1	0
MMM 10	11	88	3	0	2	5	1

[a]Includes hemorrhagic spots, mottling, fluid accumulation etc.

respiratory diseases, e.g., chronic bronchitis, bronchiectasis, suppurative bronchitis, and pneumonitis of various type, also exhibited other pathology, e.g. some metastatic calcification and tumor formation. Of the 8 survivors who were killed after 104 weeks, 2 were without pathological findings; 4 animals had lung involvement, primarily peribronchitis of pneumonitis; 2 had minimal intersititial nephritis; and in one case there was a focal myocarditis (1 lesion). The over-all conclusion of the pathologist at that time was that the animals exhibited some diseased tissue but that this was probably attributable to old age rather than to diet.

Histopathological examination of tissues of rats of subsequent generations yielded similar unspectacular results. Survivors of the longevity experiment involving animals of generation MM 75 and MC 15 which were killed after 100 weeks on their respective diets revealed lung congestion and an occasional mammary tumor but no significant liver pathology or internal tumors.

Gross and histopathology of tissues of rats of MMM 2, 5, and 10 are shown in Tables VII and VIII. Again, lesions found in these animals are essentially functional; they are observable microscopically, are of unknown significance, and seem to be related to the aging process. As a result of the continuing negative results of histopathological examinations throughout the course of this investigation, the number of tissues selected for examination was gradually reduced.

In view of reports linking fats with high concentrations of polyunsaturated fatty acids with a high incidence of fibrosis (29), the hearts of animals of MMM 17 and MCC 17 were subjected to histopathologic examination. No abnor-

TABLE VIII
Histopathological Examination of Tissues of MMM 2 and 5[a] at 104 Weeks

Tissue pathology	Males (8)[b] No. of animals with lesion	Females (12) No. of animals with lesion
Liver		
Carcinoma	0	0
Parenchymatous nodules	1	0
Cholangioma	0	1
Focal atrophy	2	1
Hemorrhage of necrosis	0	2
Pituitary		
Hyperplasia	0	0
Adenoma	1	4
Adrenal		
Channelization	1	0
Adenoma	1	2
Thyroid (adenoma)	0	0
Mammary Gland (tumor)	—	0
Uterus (tumors)		
Benign	—	1
Adeno-carcinoma	—	1
Ovary (cysts)	—	2
Testes (atrophy)	2	—
Kidney		
Pyelonephritis	2	2
Tubular dilatation	2	0
Stomach		
Gland dilatation	1	4

[a]Histology of MMM 10 at 52 and 88 weeks revealed essentially normal tissues in all cases.
[b]Numbers in parentheses are numbers of animals examined.

malities were found in the hearts of the females of either MMM 17 or MCC 17. In the males, in 2 of the 4 animals in MMM 17 and in 3 of the 4 animals in MCC 17, there were no abnormalities; male rats of MMM 17 had slight chronic interstitial myocarditis, and 1 male rats of MMM 17 and 1 male rat of MCC 17 exhibited questionable myocarditis. Since this slight degree of myocarditis had also been seen in the early stages of this study, when a much more saturated margarine product had been used in the diet (7.5% P in the MM diet vs. 28% in the MMM and MCC diets), this incidence of myocarditis cannot be attributed to the polyunsaturated fatty acid component of the diet.

It is concluded that a diet providing as much as 33% of the calories as fat, the latter containing up to 28.5% polyunsaturated fatty acids, principally of the essential fatty acid type, with a P/S ratio of up to 1.6:1 and a polyunsaturated fatty acid to alpha-tocopherol ratio as high as 1780:1 produced no undesirable effects in the rat.

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