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Free Radical Theory of Aging: Effect of the Amount and Degree of Unsaturation of Dietary Fat on Mortality Rate[1,2]

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FREE radicals formed more-or-less at random throughout a biological system from both enzymatic and non-enzymatic sources would be expected to produce a multiplicity of deleterious changes (Harman, 1956, 1962, 1969). From this point of view, increasing the ingestion of dietary components prone to participate in free radical reactions should increase the rate of biological degradation. Fat is such a dietary component.

The tendency of triglycerides, the major component of dietary fat, to react with molecular oxygen (Holman, 1954) increases with the degree of unsaturation of the triglyceride fatty acid moieties. Hence, increasing the degree of unsaturation of dietary lipids, which tends to raise the unsaturation of structural lipids as well as those in transport (Carroll, 1965; Century, Witting, Harvey, & Horwitt, 1963; Marco, Macklin, Emery, & Gordon, 1961; Witting, Harvey, Century, & Horwitt, 1961), should increase the rate of initiation of more-or-less random free radical reactions throughout an organism. Increasing the amount of fat ingested should also increase lipid peroxidation as the quantity of lipid in transport in the blood and tissues is enhanced.

The above potential adverse effects of dietary fat can be decreased by increasing the dietary intake of Vitamin E, the natural lipid antioxidant, and/or other antioxidants (Harris & Embree, 1963; Witting, 1965; Witting & Horwitt, 1964). However, even though the level of *in vivo* lipid peroxidation should be kept below that associated with overt Vitamin E deficiency, the small increases in free radical reactions expected to be produced throughout an organism by increasing the amount and/or degree of unsaturation of dietary fat might be sufficient to produce over a period of time recognizable differences in biological degradation. To evaluate the foregoing possibility, using the mortality rate as a measure of biological degradation, groups of mice and rats were fed diets throughout life containing 5, 10, or 20% by weight of either lard, olive oil, corn oil, safflower oil, or distilled triglycerides of menhaden oil as the sole source of lipid; the diets contained 20 mg. of α-tocopherol acetate per 100 gm. of finished diet, an amount estimated to be in excess of that needed to prevent Vitamin E deficiency in animals receiving the most readily peroxidizable diet (20% menhaden oil).

METHODS

Groups of C3H/HeJ female (Jackson Laboratory, Bar Harbor, Me.) and Swiss male mice (Charles River CD-1 pathogen-free mice; Charles River Breeding Laboratories, Brookline, Mass.) were obtained shortly after weaning and caged 10 per cage (stainless steel, 12 × 8

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× 5 in.); the animals were allowed to live out their life-span with their original cage mates, i.e., cages were not consolidated as animals died. Prior to being placed on special diets, the mice were fed commercial mouse pellets (Rockland; Tekland, Inc., Monmouth, Ill.). The animals were maintained throughout life in an air-conditioned room at 76-78 F. at a humidity of 50-60%; light was from two large windows on the south side of the room as well as from overhead fluorescent lamps (these were turned off at night except for one small night light). The ventilating system provided 6 air changes (fresh air) per hour, through baffles to prevent drafts. Cages were changed 2-3 times per week. The bedding was sterilized shredded corn husks (San-i-Cel, Paxton Processing Company, Paxton, Ill.).

When the C3H mice were 8 weeks old, and the Swiss mice 5 weeks old, the animals were divided into groups of 50-60 (i.e., 5 or 6 cages) and placed on a semi-synthetic diet, fed daily, containing 5, 10, or 20% by weight of either lard, olive oil, corn oil, safflower oil, or distilled triglycerides of menhaden oil; the lard (antioxidant-free), olive oil, corn oil, and safflower oil were all edible grade, marketed for human consumption. The approximate percentages of the major fatty acid constituents of these five lipids (Goddard & Goodall, 1959) are given in Table 1. Lard, olive oil, corn oil, and safflower oil differ from one another primarily in the relative amounts of 16:0, 18:0, 18:1, and 18:2 fatty acids. In contrast, the unsaturated moieties of menhaden oil are mainly those with 5 and 6 double bends, i.e., 20:5, 22:6 and a lesser amount of 22:5.

Table 1. Percentages of Major Fatty Acids in the Fats Used in the Present Experiment.

	Fatty Acid Composition of Dietary Fats									
Fat	Sat.[a]	16:1	18:1	20:1	16:2	18:2	18:3	18:4	20:4	Iodine No.
Lard	40.0		48.0			11.0	0.6			64.0
Olive oil	15.0		75.0			10.0				90.2
Corn oil	12.4	1.2	30.0	1.7		54.1	0.6			126.7
Safflower oil	8.2		15.4			76.4				146.0
Menhaden oil	42.3	7.9	13.4	0.9	0.8	1.1	0.9	1.9	1.2	*ca.* 180

[a]Sat. = saturated fatty acids; these are 12:0 and above mainly 16:0 and 18:0 although menhaden oil contains 8.0% 14:0.

Menhaden oil also contains relatively large amounts of ester fatty acids with 5 and 6 double bonds (20:5 — 10.2%; 22.5 — 1.6%; 22:6 — 12.8%) as well as small amounts of other components, such as branched chain fatty acids which are not listed.

Table 2. Values of the Ratios of the Weight of Vitamin E in the Experimental Diets to the Estimated Minimum Amount Required to Prevent Vitamin E Deficiency.

	Weight of Vitamin E in Diet/Minimum Amount Needed		
	% by Weight of Lipid in Diet		
Fat	5	10	20
Lard	45.7	23.3	12.0
Olive oil	57.2	29.1	15.0
Corn oil	12.6	6.6	3.6
Safflower oil	9.4	5.1	2.9
Menhaden oil	5.2	2.6	1.3

Twenty mg. of DL-α-tocopherol acetate were added per 100 gm. of diet; this amount was estimated to be more than sufficient to prevent development of Vitamin E deficiency in the animals receiving the most readily peroxidized diet (20% menhaden oil). This procedure caused the ratio of the weight of Vitamin E in the diet to that estimated to be required to prevent Vitamin E deficiency to vary from diet-to-diet. The approximate value of this ratio was calculated for the 15 diets, taking into account the percentage of polyunsaturates (in terms of the estimate peroxidizability (EP)—see below) and the amount of Vitamin E in the oils (Herting & Drury, 1963), on the basis of two assumptions: (*a*) 0.6 mg of α-tocopherol per gram of polyunsaturates in safflower oil is just sufficient to prevent Vitamin E deficiency (Harris & Embree, 1963) and (*b*) that the relative Vitamin E requirements of different oils are proportional to the estimated peroxidizability of the oils (Witting, 1965; Witting & Horwitt, 1964); the ratios are given in Table 2.

The estimated peroxidizabilities of the five oils used in the calculations for Table 2 were determined from the equation:

$$EP = 0.025(C{=}C)_1 + 1.0(C{=}C)_2 + 2.0(C{=}C)_3 + 4.0(C{=}C)_4 + 6.0(C{=}C)_5 + 8.0(C{=}C)_6,$$

where $(C{=}C)_1$, $(C{=}C)_2$, etc. are the percentages of monoenoic, dienoic, etc. fatty acids in the oil; the foregoing numerical values (i.e., 0.025, 1.0 etc.) are based on reported *in vitro* maximal rates of oxidation (Holman, 1954). The estimated peroxidizability of the lipids in the present study, calculated on the basis of the approximate percentages of the major fatty acid constituents—given in Table 1 above are: lard, 15; olive oil, 12; corn oil, 56; safflower oil, 77; distilled triglycerides of menhaden oil, 131.

Judging from appearance, activity and mortality rates none of the animals in this experiment became overtly Vitamin E deficient. In agreement with this, at least for the 5% diets, are the results of an experiment (Derrick &

Wishner, 1967) with groups of male rats—from the same breeding stock as used in the present study (see below)—receiving diets containing either 5% stripped corn oil or 5% cod liver oil with and without 20 mg. DL-α-tocopherol acetate per 100 gm. of diet.

The composition of the 5% by weight lipid diet has been published (Harman, 1968); 10 and 20% by weight lipid diets were prepared at the expense of the glucose monohydrate. The percentage of the calories derived from fat in the 5, 10, and 20% by weight diets were 11, 21, and 36%, respectively; the latter figure is somewhat less than the fat calories present in the average human diet in the United States (40-50%) (US Dept. of Agriculture, 1961).

The diets were prepared at intervals of 1 to 2 weeks and stored in closed glass jars prior to use; the safflower and menhaden oil diets were kept in a deep freezer while the other diets were stored in a cold room at refrigerator temperature. Samples of the diets were analyzed several times for peroxides by the iodiometric method (AOCS Official Method Cd 8-53); none were found.

The attempt was made to feed the animals of each strain isocalorically; food consumption was determined about once a month for a period of 1 week—the average amount of food (in calories) eaten per animal per day in the dietary group eating the least during this period of a week was fed to all the groups during the ensuing month. In spite of the foregoing the body weights tended to increase with the level of dietary fat. Judging from the way the animals ate, the diets were all about equally palatable.

Fresh food was given to the mice each day; food dishes designed to minimize spillage were employed (LC-306 food cup, Geo. Watmann Mfg. Co., Baltimore).

The mice, ear-coded, were weighed and counted each month, and the presence of any gross tumors noted.

A similar experiment was conducted at the same time using 400 male rats (Charles River CD male rats; Charles River Breeding Laboratories, Brookline, Mass.); 28 rats in most groups —4 per cage (11½ × 18¼ × 6½ inches, stainless steel). The rats were maintained in a room by themselves; the environmental conditions, etc., were the same as with the mice.

Whenever possible, dead animals were autopsied and tissues—heart, skeletal muscle, liver, lung, kidney, spleen, testes, and intestine as well as tumor, if present—taken for microscopic examination.

Table 3. C3H Female Mice: Effect of Dietary Fat on Mean Life-Span and Body Weight.

Fat	% by Weight of Fat in Diet					
	5		10		20	
	MLS[a]	W[b]	MLS	W	MLS	W
Lard	14.7±4.8(58)[c]	26.7	14.4±4.4(55)	28.2	14.5±4.5(59)	30.0
Olive oil	15.7±5.0(56)	27.0	14.8±3.6(55)	28.0	13.9±3.9(58)	31.4
Corn oil	14.8±4.3(54)	28.1	14.4±3.9(58)	28.9	12.9±3.6(57)	29.9
Safflower oil	14.4±4.7(54)	27.8	13.8±3.9(59)	28.2	13.4±3.9(54)	28.2
Menhaden oil	13.6±4.2(58)	26.4	13.7±3.3(54)	26.5	13.3±3.7(57)	25.9

[a]Mean life-span in months±standard deviation.
[b]Average body weight for the period 3 mo. to end of life.
[c]Number of animals in original group.

Table 4. Swiss Male Mice: Effect of Dietary Fat on Mean Life-Span and Body Weight.

Fat	% by Weight Fat in Diet					
	5		10		20	
	MLS[a]	W[b]	MLS	W	MLS	W
Lard	15.6±7.6(59)[c]	38.7	17.0±7.0(54)	41.3	17.1±7.2(61)	41.8
Olive oil	15.9±7.2(59)	40.0	15.4±7.2(56)	40.7	18.1±6.9(54)	42.5
Corn oil	17.2±7.5(58)	41.0	17.2±7.3(53)	41.9	16.7±6.5(58)	44.3
Safflower oil	18.6±8.9(52)	40.0	17.6±7.2(59)	40.6	17.5±7.1(55)	42.7
Menhaden oil	16.3±7.6(54)	40.9	15.9±7.2(57)	40.2	17.6±7.9(55)	41.5

[a]Mean life-span in months±standard deviation.
[b]Average body weights for the period 3 mo. to the end of life.
[c]Number of animals in original group.

Results

The mean life-spans in months and average body weights for the period 3 mo. to the end of life of the mice and rat groups are given in

Table 5. Charles River Male Rats: Effect of Dietary Fat on Mean Life-Span and Body Weight.

Fat	% by Weight Fat in Diet					
	5		10		20	
	MLS[a]	W[b]	MLS	W	MLS	W
Lard	29.9±6.1 (28)[c]	519.6	28.0±5.5 (28)	539.9	29.8±5.1 (24)	562.3
Olive oil	27.8±7.9 (28)	533.0	30.7±5.0 (29)	523.6	27.8±5.4 (24)	601.9
Corn oil	30.2±7.0 (28)	516.6	29.8±5.8 (28)	544.0	26.6±4.4 (23)	653.2
Safflower oil	30.2±6.2 (28)	510.3	29.6±6.3 (28)	537.2	24.8±6.6 (24)	615.5
Menhaden oil	28.7±6.3 (28)	519.4	29.7±7.1 (28)	556.9	29.5±5.6 (24)	595.3

[a]Mean life-span in months±standard deviation.
[b]Average body weight for the period 3 mo. to the end of life.
[c]Number of animals in original group.

Tables 3 (C3H female mice; this table also includes the mean life-spans for mice dying with and without a gross tumor), 4 (Swiss male mice), and 5 (Charles River strain male rats). It is to be noted that the mean life-spans of the animals receiving the commercial-like diets, i.e., the 5% olive oil or lard diets, are as high, or higher than those reported for C3H female mice (Russell, 1966), Swiss male mice (Evans, 1948; Haman, 1961), or Sprague-Dawley male rats (Jones & Kimeldorf, 1963). The foregoing attests both to the nutritional adequacy of the semi-synthetic diets and to the generally good level of animal care. There were no significant differences in maximum life-span between the dietary groups.

Statistical analysis (analysis of variance) gave the following results:

C3H mice: (*a*) life-span decreased significantly ($p<0.05$) with increase in degree of dietary fat unsaturation or amount of dietary fat; (*b*) body weight was not significantly influenced by the degree of unsaturation or the amount of dietary fat; and (*c*) no correlation between body weight and life-span.

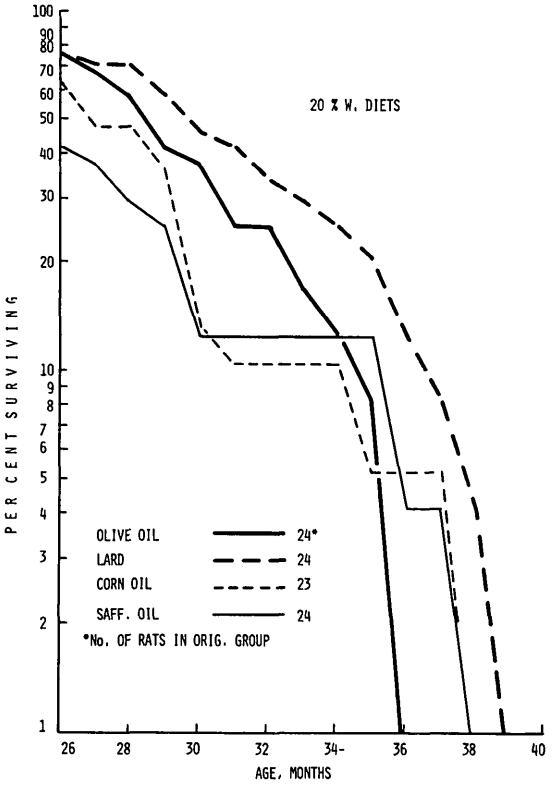

Fig. 1. Charles River Male Rats: Effect of dietary fat unsaturation on mortality rate.

Swiss male mice: (*a*) the life-span was not dependent on the amount or degree of unsaturation of dietary fat; (*b*) body weight increased with the level of dietary fat; and (*c*) no correlation between body weight and life-span.

Charles River strain male rats: (*a*) the life-span was not influenced significantly by the amount or degree of unsaturation of the dietary fat—this result was not changed by omitting the menhaden oil data from the analysis; (*b*) body weight increased with the level of dietary fat; and (*c*) life-span decreased with increasing weight.

Although there were no significant differences in mortality rates between the Swiss mice or Charles River strain male rat dietary groups, mortality rate trends in the lard, olive oil, corn oil, and safflower oil diet groups were present similar to those observed for C3H mice: (*a*) the mortality rates were generally somewhat higher for animals receiving the 20% fat diets than for those on the 5 and 10% diets, possibly relating in part to the greater body weight of the 20% groups; the differences were most evident for the corn and safflower oil groups; (*b*) at the 20% dietary fat level, animals on saturated diets tended to live longer on the average than those on polyunsaturated diets; this is most clearly shown by the rat curves (Fig. 1).

The C3H female mouse gross tumor data (mammary carcinomas on microscopic section) are given in Table 6; 320 (37.8%) of the 847 mice died with a gross tumor. Analysis of the

Table 6. C3H Female Mice: Effect of Amount and Type of Dietary Fat on Accumulative Number of Mice Dying with a Gross Tumor.

% by Weight Fat in Diet	Age (Mo.)	Dietary Fat: Lard	Olive Oil	Corn Oil	Safflower Oil	Menhaden Oil
5	8	0(58)[a]	0 (56)	0 (54)	2 (54)	0(58)
	10	0	0	1	2	0
	12	2	0	2	3	1
	14	3	2	4	6	3
	16	6	5	12	10	8
	E[b]	16(27.6)[c]	11 (19.6)	19 (35.2)	20 (37.0)	12 (20.7)
10	8	0(55)	1 (55)	0 (58)	0 (59)	0 (54)
	10	0	1	0	1	2
	12	1	3	1	3	4
	14	3	6	7	10	6
	16	15	13	16	18	12
	E	20(36.4)	23 (41.8)	26 (44.8)	27 (45.8)	18 (33.3)
20	8	0(59)	0 (58)	0 (57)	0 (54)	0 (58)
	10	0	0	1	2	0
	12	6	1	7	3	4
	14	7	7	14	11	11
	16	17	11	18	18	18
	E	28(47.5)	26 (44.8)	24 (42.1)	26 (48.1)	24 (41.4)

[a]Number of mice in original group.
[b]E—age when last mouse died.
[c]Percentage of the original group that died with a gross tumor.

tumor data (analysis of variance) showed that the total number of tumors in each group increased significantly ($p<0.05$) as the amount of fat in the diets was increased. Inspection of Table 6, ignoring the menhaden oil data (see below), shows that mice receiving the corn and safflower oil diets tended to die with gross tumors at earlier ages than mice on the more saturated diets; at 14 mo. of age the difference in the number of mice dying with gross tumors in the combined lard and olive oil groups (14) is significantly less ($p<0.05$) than the corresponding value (25) for the combined corn oil and safflower oil groups. In all groups the mean life-span (Table 3) of the mice dying with a gross tumor paralleled, but was higher, than that for the group as a whole.

None of the Swiss mice died with gross tumors.

Fifty-two rats, 13.0% of the original 400, had gross tumors (these appeared to be fibromas) at death. The total number of tumor-bearing rats in the 5, 10, and 20% groups, combined because of the small numbers in each group, were: lard—5(6.3% of original group); olive oil—10(12.3%); corn oil—11(13.9%); safflower oil—12(15.0%); menhaden oil—14(17.5%). Although the rat tumor incidence appears to increase with increasing unsaturation of dietary fat, the differences are not statistically significant.

On the basis of the estimated peroxidizability of the dietary fat, the mortality rates and gross tumor incidences of the menhaden oil groups were anomalous in comparison with data for the other dietary fat groups: Thus, for C3H mice the mortality rates were as expected but the tumor incidences were similar to those of mice receiving olive oil diets while the mortality rates of the Swiss mice and of the rats were like those of animals on lard or olive oil diets. These differences may be due in part to the less ready enzymatic hydrolysis of the 20:5 and 22:6 fatty acids (Botting, Vandenburg, & Reiser, 1967) present in the menhaden oil, so that the lipid actually absorbed from the intestinal tract may have been more saturated than that ingested, and to a preferential utilization of highly unsaturated fatty acids for energy production (Dupont & Mathias, 1968).

The autopsy data are summarized below; cannibalism and the variable time interval between death and autopsy—up to about 24 hours—limited the number of autopsies as well as the accuracy of pathological diagnosis.

C3H female mice: 93 of the 862 mice, 10.8%, were autopsied. Mammary carcinoma was present in 47 cases (50.5%). Small scattered areas of calcification were present in the hearts of 13 mice (14.0%); possibly more prevalent in the polyunsaturated oil groups. One mouse had leukemia (20% safflower oil group).

Swiss male mice: 119 of the 853 mice, 14.0%, were autopsied. Amyloidosis of the liver was present in 10 mice (8.4%); 4 cases in the olive oil groups and none in those receiving menhaden oil. Nine mice (7.6%) had lung carcinomas.

Charles River strain male rats: 133 of the 400 rats, 33.3%, were autopsied. Myocardial fibrosis was found in 19 rats (14.3%), amyloidosis in 3 rats (2.3%), and leukemia in two (1.5%). Although a number of tumors have been found in the male Sprague-Dawley rat (Ross & Bras, 1965; Thompson, Huseby, Fox, Davis, & Hunt, 1961) all those found in this study appeared to be fibromas.

Discussion

Increasing the amount and/or degree of unsaturation of dietary fat decreased the mean life-span, without influencing the maximum life-span, of female C3H mice even though the base diet contained an amount of Vitamin E sufficient to keep the level of *in vivo* peroxidation below that associated with overt Vitamin E deficiency for the most readily peroxidizable diet. The mortality rates of Swiss male mice and Charles River male rats on the same diets showed similar trends.

A part of the increased mortality rate of the C3H mice associated with increases in the amount and/or unsaturation of the dietary fat appeared to be due to an increased rate of mammary tumor induction. The foregoing suggests that lipid-free radicals, like those derived from ionizing radiation (Moore, 1962), increase the carcinogenicity of the mammary tumor agent.

The effect of lipid peroxidation on over-all mortality rate was rather small. Decreasing the ratio(R), amount of Vitamin E in the diet/amount of dietary Vitamin E estimated to be needed to prevent Vitamin E deficiency, from about 50 to around 3—by varying the amounts of lard, olive oil, corn oil, or safflower oil—was associated with progressive decreases in the

mean life-span of female C3H mice to a maximum difference of approximately 10%. In a somewhat similar study (Morin, 1967), utilizing male LAF_1/J and C3H/HeJ mice (60 mice/group) given diets containing 15% by weight of either hydrogenated coconut oil or safflower oil (R was calculated to be greater than 40 for the coconut oil diet and about 1.5 for the safflower oil diet), no significant effect of lipid unsaturation was observed; however, the C3H/HeJ mice receiving the safflower oil diet had a mean life-span (15.8 $\pm$ 3.8—standard deviation—months) 8.7% less than the corresponding value (17.3 $\pm$ 3.9 mo.) for the coconut oil group. Thus, marked increases in the amount and/or degree of unsaturation of dietary fat produce at most decreases of around 10% in mean life-spans when the amount of dietary Vitamin E exceeds the minimum quantity estimated to prevent overt signs of Vitamin E deficiency.

The present and published data on the effect of dietary fat on life-span (Duel, 1957; French, Ingram, Uram, Barron, & Swift, 1953; Silberberg & Silberberg, 1954) and cancer (Silverstone & Tannenbaum, 1950; Tannenbaum & Silverstone, 1953) in mice and rats are compatible with the possibility that lipid peroxidation contributes to the degradation of biological systems but is not the major factor determining the mortality rate when the diet contains lipid antioxidants in amounts normally employed to prevent signs of Vitamin E deficiency.

Summary

Free radical reactions have been implicated in the degradation of biological systems. On this basis increasing the amount and/or degree of unsaturation of dietary fat might be expected to increase the rate of biological degradation. To evaluate this possibility semi-synthetic diets containing 5, 10, or 20% by weight of either lard, olive oil, corn oil, safflower oil, or distilled triglycerides of menhaden oil were fed to groups of C3H female mice, Swiss male mice, and Charles River strain male rats throughout life, starting shortly after weaning. Twenty mg of α-tocopherol acetate were added to the base diet, an amount estimated to be sufficient to prevent Vitamin E deficiency in the animals on the most readily peroxidized diet—20% by weight menhaden oil.

The mean life-span of C3H female mice was decreased significantly, in part due to an increased incidence of mammary carcinoma, by increasing the amount and/or degree of unsaturation of the dietary fat; similar trends were produced in the rats and Swiss mice, but these effects were not statistically significant.

The present data are compatible with the possibility that lipid peroxidation contributes to biological degradation but is not the major factor determining the mortality rate when the diet contains amounts of antioxidants in the range ordinarily employed to prevent signs of Vitamin E deficiency.

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