The Role of Free Fatty Acids on Antioxidant Effectiveness in Unsaturated Oils[1]

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STUDIES of the comparative effectiveness of various antioxidants in marine oils revealed that EMQ[2] was considerably more effective than were NDGA or PG in samples of crude menhaden, herring, anchovy, and other fish oils. On the other hand, EMQ and NDGA were equally effective in refined cod-liver oil; and in lard NDGA was a more powerful antioxidant than was EMQ (Table I, A). An investigation of the various possible factors causing these differences revealed that the amount of free fatty acid in the oils played an important role.

Further studies were then undertaken to determine the effect of free fatty acid on the stability of other substrates and on the effectiveness of other antioxidants. The results obtained are described in this paper.

Experimental

Materials and Methods. The U. S. Fish and Wildlife Service furnished samples of crude fish oils and the highly purified fractions of sardine oil methyl esters and alcohols. Refined cod-liver oil was obtained through the courtesy of W. M. Cox Jr., Mead Johnson and Company. Purified fatty esters, acids, and glycerides were Hormel Institute products. The unsaturated fraction from menhaden oil was supplied by W. A. Lundberg, Hormel Institute. The lard used was an especially prepared commercial sample free from antioxidants obtained through the helpfulness of Ruth Okey. The refined vegetable oils were purchased in the local markets. The antioxidants used were those described previously (1). Myristic and palmitic acids were recrystallized from alcohol.

The effectiveness of the antioxidants was estimated from the length of the induction period as determined by the weighing method recently described (2). Samples of oil with or without antioxidant were incubated in constant-temperature draft ovens. Once daily they were removed, allowed to cool, weighed, and replaced. Sample size was 1 g. in a 30-ml. beaker or 200 mg. in a 10-ml. beaker. The oleate, lard, and vegetable oils were held at 60°; the fish oils and trilinolein, at 50°; and the esters, at 40°C. No qualitative differences in relative antioxidant effectiveness have been traceable to temperatures within this range.

At the beginning of this work the additives were added to the beaker (as an aliquot of a solution in a volatile solvent, usually absolute alcohol), and the solvent was carefully removed in a stream of nitrogen prior to the addition of the oil. A later, preferred technique was to add the aliquot of solution to the weighed sample of oil. The solvent volatilized from the uncovered beakers in a few hours at 50 or 60° in the draft ovens. Some of the data presented in this paper were obtained by the first procedure, and some by the second one.

The end of the induction period was estimated to the nearest half-day as judged from the increase of weight characteristic of the beginning of rapid oxidation and was arbitrarily chosen to be the time when the weight had increased by 0.5%. All of the highly unsaturated substrates had induction periods of only a few hours at the temperatures employed; hence the data cannot be used to compare the relative stabilities of the unprotected oils or esters. Although all of the substrates were held at 0°F. in containers, the free space of which was flushed with nitrogen after each use, nevertheless their stabilities changed during storage. Accordingly the comparisons within a single run are of more significance than those between runs.

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[2] Abbreviations used are as follows: EMQ, 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline (Santoquin); NDGA, nordihydroguairetic acid; PG, propyl gallate; HQ, hydroquinone; DPPD, diphenyl-p-phenylenediamine; BHA, 2(or 3)-tertiarybutyl-4-methoxyphenol; BHT, 2,6-ditertiarybutyl-4 methyl phenol; α-T, d-α-tocopherol; γ-T, d-γ-tocopherol.

TABLE I

Relative Effectiveness of EMQ, NDGA, and PG in Fish Oils, Lard, and Purified Fatty Esters and Acids

Substrate	Temperature	Concentration of antioxidant	Induction period—days			
			None	EMQ	NDGA	PG
A. Fish Oils and Lard	°C.	%				
Crude menhaden oil	50	0.05	0.5	10.0	7.5	3.5
Crude herring oil	50	0.05	1.0	5.0	1.5	3.0
Crude anchovy oil	50	0.02	0.5	5.0	2.0	1.5
Refined cod liver oil	50	0.05	1.0	5.0	5.0	6.0
Lard	60	0.025	1.5	11.5	25.0	19.0
B. Esters						
Methyl oleate	60	0.01	5.0	23.5	54.0	24.0
Methyl linoleate	40	0.02	0.5	13.0	35.0	36.0
Trilinolein	50	0.05	0.5	8.5	21.0	12.0
Methyl linolenate (95%)	40	0.02	0.5	5.0	7.0	4.0
Methyl arachidonate	40	0.10	0.5	11.0	17.0	8.0
Methyl esters of sardine oil fatty acids[a]	40	0.05	0.5	3.0	3.5	1.0
Methyl esters of menhaden oil fatty acids[b]	40	0.05	0.5	4.0	6.0	1.5
C. Fatty Acids						
Oleic acid	60	0.01	2.0	14.0	6.0	5.0
	60	0.025	2.0	31.0	16.0	10.0
	60	0.05	2.0	68.0	30.0	25.0
Linoleic acid	40	0.05	0.5	6.5	1.5	1.0
Linolenic acid	40	0.05	0.5	8.0	1.5	1.0
Menhaden oil fatty acids	40	0.05	1.0	9.0	2.5	2.5
Cod liver oil fatty acids	40	0.05	0.5	34.5	1.0	1.0
Cottonseed oil fatty acids	40	0.05	5.5	36.0	6.5	6.5

[a] Fraction I.V. 360. [b] Fraction I.V. 350–360.

Results

One obvious hypothesis was that the number and type of double bonds in the substrates affected relative antioxidant activity. However the data obtained with purified methyl esters of oleic, linoleic, linolenate, and arachidonate (1, 2, 3, and 4 unsaturated bonds, respectively) showed that NDGA was superior to EMQ in all of these (Table I, B). NDGA also was superior to EMQ in very highly unsaturated fractions of esters of menhaden and sardine oils (iodine values 350 to 360). Trilinolein was also protected more by NDGA than by EMQ. These combined observations indicated that neither the amount of unsaturation nor the type of ester linkage could account for the superiority of EMQ in crude fish oils.

In contrast, the free fatty acids corresponding to the esters were protected much more effectively by EMQ than by NDGA (Table I, C). This pattern also was demonstrated with fatty acids prepared from menhaden, cod-liver, and cottonseed oils.

Experiments were then carried out with mixtures of purified esters and acids. The data given in Table II illustrate the effect of added linoleic acid on the antioxidant activity of EMQ and NDGA on trilinolein. They show that the added free fatty acid exerted an effect at unexpectedly low concentrations and that it affected adversely the antioxidant effect of NDGA relatively more than it did that of EMQ. This observation offered a relatively simple explanation for the results obtained with the crude fish oils (2–4% free fatty acid) and suggested that if the free fatty acids were removed, the resultant neutral oil would be protected better by NDGA than by EMQ. Accordingly free fatty acids were washed out of a solution of crude menhaden oil in petroleum ether with a solution of dilute sodium carbonate and were recovered by acidification of the aqueous portion and extraction with petroleum ether. The neutral oil and free fatty acid fractions were washed separately, freed of solvents by vacuum distillation, and used for the assays in Table III. Similar results were obtained when menhaden oil fatty acids prepared by careful saponification were added. The data support the free fatty acid hypothesis.

TABLE II

Effect of Added Linoleic Acid on the Antioxidant Activity of EMQ and NDGA in Trilinolein (50°C.)

Added linoleic acid (%)	Induction period—days	
	0.05% EMQ	0.05% NDGA
None	7.0	16.5
0.5	6.5	5.0
1.0	4.5	3.0
2.0	4.0	1.5
4.0	3.0	1.0

Next it was important to determine whether the effect of free fatty acid depended upon the nature of the acid or acid mix added. A comparison of various fatty acids at hand indicated that the effect was not unique with linoleic acid but could be demonstrated with several saturated and mono- and dibasic fatty acids (Table IV). The results with adipic and sebacic acid suggest that only one of the two carboxyl groups was effective. Citric and phosphoric acids were exceptions, as was to be expected from their known activity as synergists (3). Oleic acid was used for most of the subsequent experiments on the nature of the free fatty acid reaction since it was more readily soluble than were the saturated fatty acids and was more stable toward oxidation than were the more highly unsaturated ones (Table I, C).

TABLE III

Effect of Added Menhaden Oil Fatty Acids on Antioxidant Activity of EMQ, NDGA, and PG in Fatty Acid-Free Menhaden Oil and Trilinolein (50°)

Substrate	Fatty acids added	Amount of antioxidant added	Induction period—days		
			EMQ[a]	NDGA	PG
	%	%			
Menhaden oil	None	0.1	5.5	6.0	3
Menhaden oil	2.5	0.1	8.0	3.5	3
Trilinolein	None	0.05	11.0	26.5	19
Trilinolein	2.0	0.05	14.0	7.5	10

[a] These experiments were run by the first technique described under "Methods." It is believed that loss of EMQ during removal of solvent accounts for the apparent lower effectiveness in the absence of added fatty acids.

The data in Table V indicate that the addition of oleic acid to trilinolein decreases the antioxidant activity of all of the antioxidants used, but to varying degrees. Amounts as low as 0.1% of oleic acid modified the antioxidant action of α- and γ-tocopherols (Table VI). Inasmuch as the tocopherols are considered to be the natural antioxidants in vegetable oils, these observations suggested that oleic and other fatty acids would reduce the induction period of such oils. Results obtained with refined products are shown in Table VII. The induction period of lard, in contrast, was not measurably affected.

TABLE IV

Effect of Type of Fatty Acid in Modifying Antioxidant Activity of NDGA (μ mole) in Trilinolein, and in Menhaden Oil[a] (50°)

Acid	Induction period—days		
	Trilinolein	Menhaden oil	
	5 μ mole acid	1.5 μ mole acid	5 μ mole acid
None	10	14	7
Oleic	2	9	5
Myristic	2	9	5
Palmitic	2	10	5
Adipic	4[b]	9	5
Sebacic	4[b]	8	5
Citric	30	25	20
Phosphoric	....	....	>31

[a] Freed of fatty acids, 200-mg. sample. The runs with menhaden oil were made with two different preparations.
[b] 2.5 μ mole.

TABLE V

Comparison of the Effect of Varying Amounts of Oleic Acid on Antioxidant (0.05% level) Effectiveness in Trilinolein (50°)

Antioxidant	Induction period—days			
	Concentration of oleic acid			
	None	0.5%	1.0%	2.5%
NDGA	29	8.5	5.0	1.5
HQ	22	5.5	1.5	1.5
DPPD	18	....	3.0	2.5
PG	14	....	7.0	5.5
BHA	8	....	5.0	3.0
EMQ	8	....	3.0	3.0
α-T	8	....	....	1.0
γ-T	8	....	....	1.5

TABLE VI

Effect of Oleic Acid on Induction Period of Trilinolein Containing Added Tocopherols (0.1%) (50°)

Antioxidant	Induction period—days				
	Oleic acid added				
	None	0.05%	0.1%	0.2%	0.5%
α-Tocopherol	10.5	9	7.5	7	4
γ-Tocopherol	8.5	....	6.0	....	4

TABLE VII

Effect of Added Oleic Acid on Stability of Vegetable Oils and Lard (60°)

Oil	Induction period—days				
	Concentration of added oleic acid				
	None	0.1%	0.2%	0.5%	1.0%
Cottonseed	11.0	9	8.0	8.0	5.0
Peanut	11.0	7	3.0	....	3.0
Olive	8.0	5	6.0	3.0	3.0
Corn	8.0	6	5.0	4.0	2.0
Soy	6.0	6	5.0	4.0	2.0
Lard	2.5	....	2.5	2.5	2.5

Discussion

These experiments demonstrate that antioxidant action can be modified by the presence of free fatty acids in triglyceride systems. The following tentative hypothesis is proposed: triglycerides are relatively non-polar. In comparison, the antioxidants by virtue of their free hydroxyl or imino groups have polar character, as do the free fatty acids. It therefore may be that free fatty acids exist in the oil in close association with the antioxidant and thus modify its effectiveness. Sufficient kinetic data are not yet available to interpret the results in terms of the proposals of Hammond *et al.* (4) or of others that are investigating antioxidant behavior.

This hypothesis would require that free fatty acids added to triglycerides or other esters in the absence of antioxidants should have little or no effect. Thus the induction period of lard, which is presumably almost free of natural antioxidants, was not appreciably changed by the addition of oleic acid (Table VII). However the methods used in this investigation are inadequate for an accurate measure of short induction periods. Some comparisons of weight gain with and without added fatty acid appear in Table VIII. The results support the hypothesis that only minor changes in the induction period occur in the absence of antioxidants. A preliminary comparison of oxygen uptakes at 35° of trilinolein with trilinolein containing 1% oleic acid suggested that the induction period was not appreciably affected but that the subsequent absorption of oxygen was accelerated in the presence of free acid. Since it is known that the build-up of peroxides proceeds to a much greater degree during the autoxidation of methyl esters than it does during the autoxidation of the free acids (5, and others), it is reasonable to believe that the free acid could be associated and/or react with peroxides as well as with antioxidants and thus might accelerate the rate of their decomposition. The effect of free fatty acids on the oxidation of antioxidant-free triglycerides merits further study.

One obvious conclusion to be drawn from these combined observations and particularly from the results shown in Table VI is that the removal of free fatty acids from crude vegetable oils by the refining process may be expected to yield oils of increased stability. It is not known whether the benefits of refining could be detected separately from the losses in stability because of the removal of other stabilizing influences (gossypol, phospholipides, etc.) during the process.

It would appear that the effects of free fatty acid on antioxidant activity might have been observed previously. A search of text books, reviews, and the more accessible technical literature however failed to uncover pertinent statements. Ogden (6) claimed that high free fatty acid in tallows cuts down the carotene stability in dried alfalfa to which it is added, but this possibility is not supported by the observations of Bickoff *et al.* (7).

Conclusions

a) In purified, unsaturated esters and triglycerides NDGA is a superior antioxidant to EMQ at all levels of unsaturation.

b) In long-chain unsaturated fatty acids EMQ is a superior antioxidant to NDGA.

c) The addition of free fatty acids to triglycerides, even in amounts as small as 0.05%, modifies the comparative effectiveness of antioxidants. The effect on the antioxidant activity of NDGA is more marked than is that on the activity of EMQ.

d) The effect of free fatty acid on relative antioxidant activity is demonstrable with long- and short-chain, saturated and unsaturated fatty acids.

e) Comparisons are given of the effect of the addition of free fatty acids on various antioxidants in various substrates.

f) The effectiveness of tocopherols in triglycerides is decreased by the addition of free fatty acid. Similarly the induction period of several vegetable oils is reduced when free fatty acids are added.

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TABLE VIII

Increase in Weight of Unsaturated Substrates (200-mg. samples) With and Without Addition of Fatty Acid

Substrate	Acid	Temperature	Time	Weight increase				
				acid added				
				None	0.5%	1.0%	2.0%	4.0%
			hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Trilinolein	Linoleic	50°	18	3.0	3.3	4.9	3.9	4.4
Lard	Oleic	60°	69	1.0	1.2	1.0	1.5	....
			114	2.7	3.1	2.9	3.2	