# Nutritive Value of Marine Oils. II. Effects of *in Vivo* Antioxidants in Feeding Menhaden Oil to Swine[1, 2, 3]

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## Abstract

Five pair-groups of swine were fed diets composed of crude feed materials to which 10% of clay-bleached, light cold-pressed menhaden oil was added. The oil was allowed to oxidize under controlled conditions and aliquots of fresh and oxidized oil were removed at peroxide values (PV) of 2.6, 15.5, and 61—each of which was fed to one group of animals. Two additional animal groups received diets containing the highly oxidized oil (PV=61) plus either alpha-tocopherol acetate or ethoxyquin. All oils were stored frozen and were mixed with the diets daily. Lard was fed at a similar 10% level to a control group of pigs. Both feed intakes and weight gains were progressively reduced as the diet oil was more highly oxidized. As oxidation of the oil increased, intensity of "yellow fat" increased and hemoglobin and hematocrit levels were progressively decreased. Both alpha-tocopherol acetate and ethoxyquin acted *in vivo* to improve feed intake, rate of gain, and blood condition, and eliminated the incidence of steatitis.


## Introduction

CERTAIN paradoxical effects have been associated with the inclusion of unsaturated lipids and particularly those of marine origin, in diets for various animal species and man. On the positive side, such lipids are obviously useful sources of the essential fatty acids known to be dietary requirements of rats (6) and of chickens (5,20) not only for maintenance of dermal integrity but also for proper utilization of saturated fat (24). Their contribution to dietary supplies of vitamins A and D has long been recognized (18). It has been further demonstrated that a positive relationship exists between unsaturation in dietary fatty acids and depression of levels of serum cholesterol (33). Holman has pointed out (14), however, that this may not be a simple binary relationship. In addition, there is the basic consideration that all fats, regardless of degree of saturation, contribute significantly to the dietary energy supply. That fish oils may serve in this capacity has been demonstrated experimentally with swine (24).

On the other hand, considerable evidence has been accumulated suggesting that undesirable changes may take place in unsaturated dietary lipids, which lead to profound effects in animals ingesting them. Among resultant symptoms have been listed retarded growth and diarrhea in rats (10,22), depressed growth in chicks (21), and in pigs (4); seborrhea in rats and rabbits (22) and deposition of a yellowish-brown pigment ("steatitis") in depot fat of rats (13), swine (9), cats (7), and mink (16). In most if not all of the above-mentioned instances, data suggest that the dietary lipids involved had been abused in some way prior to feeding. Two types of damage were most frequent: oxidative deterioration and thermal polymerization. Many examples of each of these situations have been documented, but perhaps the data of Andrews et al. (3) for the former and Crampton and associates (8) for the latter are typical. Andrews' studies showed growth depression in rats to be proportional to extent of oxidation of diet fats from peroxide numbers of 100–1200, while Crampton's work indicated a similar proportionality to exist for length of heat treatment of oils held under carbon dioxide (27).

Since most oils in their processing are somewhere subjected to conditions conducive to oxidation, the study reported herein was initiated with the twofold purpose of: 1) examining animal response to dietary lipids oxidized to various known levels, and 2) investigating the effects of added antioxidants on such changes. Menhaden oil, which was specially prepared in a very fresh condition, was chosen for the study. The antioxidants tested were alpha-tocopherol acetate and ethoxyquin. Both of these are known to be effective biological antioxidants, and they gave the opportunity of comparing a natural and a synthetic material. Swine were used as test animals because of their known susceptibility to changes in dietary fat (11).

### Methods and Materials

Six pair of Berkshire swine averaging 76 lb in weight and 10 weeks in age were penned individually and fed a basal diet composed of crude natural ingredients with modifications as listed in Table I.

The choice of levels of antioxidants was made after reference to the literature, where successful applications of from 2.4–40 mg alpha-tocopherol per 100 g diet are reported (32,28,19), and 12.5 mg ethoxyquin per 100 g diet appeared to be generally accepted. Residual antioxidant activity in the crude diet ingredients was probably present, but at unknown levels.

Controlled oxidation of portions of the menhaden oil for use in the diets of lots 3–6 was accomplished at room temperature in a closed glass system, to retain volatiles, using a slight positive oxygen pressure. It was assumed, following the work of Privett, et al. (14), that this temperature was satisfactory for for-

TABLE I

Diet Treatments

| Lot No. | Diet description[a] |
|---|---|
| 1 | Lard diet (lard PV 0.0, TBA number 1)[b] |
| 2 | Menhaden oil diet (fresh oil P.V. 2.6, TBA number 35.3) |
| 3 | Menhaden oil diet (medium oxidized oil PV 15.5, TBA number 48.8) |
| 4 | Menhaden oil diet (highly oxidized oil PV 61.0, TBA number 162) |
| 5 | Lot 4 diet + 4.375 mg alpha-tocopherol acetate/100 g diet |
| 6 | Lot 4 diet + 12.5 mg ethoxyquin/100 g diet |

[a] The basal diet composition was ground barley 60%, lard or menhaden oil 10%, soybean oil meal 20%, meat meal 9%, ground limestone 0.5%, and iodized salt 0.5%. Supplementary vitamins A, D, riboflavin, pantothenic acid and $B_{12}$ and lysine and methionine were provided to meet NRC recommendations (23).

[b] PV = peroxide value; TBA number = Thiobarbituric acid number = mg Malonaldehyde/Kg lipid.

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TABLE II
Growth and Feed Data

| Lot No. | Treatment | Avg daily gain, lb | Avg. daily feed intake, lb | Avg efficiency lb feed/lb gain |
|---|---|---|---|---|
| 1 | Lard diet | 1.66 | 5.83 | 3.51 |
| 2 | Fresh menhaden oil diet | 1.27 | 4.32 | 3.40 |
| 3 | Med. oxid. menhaden oil diet | 1.19 | 3.89 | 3.27 |
| 4 | High oxid. menhaden oil diet | 0.82 | 3.33 | 4.07 |
| 5 | High oxid. menhaden oil diet + alpha tocopherol acetate | 1.45 | 5.02 | 3.45 |
| 6 | High oxid. menhaden oil diet + ethoxyquin | 1.42 | 4.90 | 3.45 |

mation of stable peroxides. In order to maintain diet lipids at the desired state of oxidation, aliquots of lard or menhaden oil were weighed into No. 1 cans, vacuum sealed and quick-frozen at −18F, after which they were stored at 0F. These portions were then mixed with appropriate amounts of dry ration ingredients daily, immediately before feeding. The antioxidants were added to the oil-fed lots 5 and 6 just prior to mixing with the dry feed. Any feed residues remaining were removed and the feed container cleaned prior to addition of new feed.

Feeding of the test diets was continued over a 100-day period, during which time the animals were weighed weekly. At the start and finish of the test, blood samples were drawn from each animal and subjected to determinations for hemoglobin using the Spencer hemoglobinometer and hematocrit using a microhematocrit centrifuge. Plasma cholesterol levels were determined by the method of Zlatkis, et al. (35). At the conclusion of the test the animals were slaughtered, their carcasses examined for evidence of steatitis and other abnormalities, and samples of various body fats were obtained. Peroxide values and iodine numbers (1) and thiobarbituric acid numbers (30) were used as criteria of lipid quality.

## Results

Gross evidence of the general state of health of the animals is provided through data relating to gains in body weight, feed intake, and efficiency of conversion of feed to animal weight gains. Such data are summarized in Table II.

Statistical analysis indicates that the lard-fed animals made faster gains than those fed menhaden oil, irrespective of condition of oil or presence of antioxidant under conditions of this experiment. As oils of higher oxidation levels were fed, feed intakes and weight gains were apparently reduced, such reduction becoming statistically significant ($P<0.01$) in the comparison of "medium" and "high" oxidation oils (Lots 3 and 4). Addition of the antioxidants to the highly oxidized oil resulted in significantly improved animal performance over that on the medium and highly oxidized, but unprotected, oil diets, equalling that made on the fresh oil diet.

Since the composition of the blood is widely used as a criterion of nutritional status, examination of data in Table III yields further information on the response of the animals to the various dietary lipid treatments.

It is considered normal for growing pigs to show increases in blood hemoglobin levels and hematocrits with increasing age, due in part at least to the low iron reserves of the milk-fed weanlings. Evaluation of effects of the diets in this experiment on blood characteristics is perhaps best made therefore by comparisons of initial and final values. On this basis, there would appear to be a lowering effect of menhaden oil on both hemoglobin and hematocrit levels when compared with the lard diet with aggravation of the effect when oils of higher oxidation levels were used. Differences between final and initial hemoglobin values during the trial were as follows for lots 1–6, respectively: +2.5, +1.3, +1.2, −1.2, +3.9, and +2.6 g %. Similar changes in hematocrit values were: +10.9, +4.7, +2.4, −2.5, +9.0, and +5.0% in the same order. Addition of the antioxidants to the highly-oxidized oil resulted in hemoglobin and hematocrit values equal or superior to those noted on the fresh oil. The significance of the blood cholesterol changes is somewhat masked by the variation in these values at the start of the trial. It is evident however that while feeding lard resulted in an increased blood cholesterol figure, feeding menhaden oil generally resulted in decreased values. The only exception to this trend was where the highly oxidized oil was fed, unprotected (Lot 4). Use of the *in vivo* antioxidants reversed this oxidation effect.

A lowering of ether-extractable materials in both epinephric and subcutaneous adipose tissue occurred when unprotected fish oil was fed; however there was not a direct correlation with the degree of oxidation of the oil. Inclusion of the two antioxidants in the diets resulted in considerably increased deposition of fat (or ether-extractable materials) to approximately the levels reached in the lard-fed control animals (Lot 1). In addition to the quantitative differences noted, qualitative changes in depot fat were observable. That in animals fed unprotected fish oil was markedly yellow in color—the depth of color being roughly proportional to extent of oxidation of the oil. The fat of the animals fed antioxidant-treated oil was white.

The weights of livers, hearts, spleens, and both kidneys were recorded at slaughter and are presented in Table IV. Although there was an apparent trend towards hypertrophy of some of the organs from animals fed fish oil, differences in the weights of hearts, spleens, and kidneys among the various treatment groups lacked statistical significance. On the other hand, livers from the group fed highly oxidized oil were significantly heavier than those from the groups fed lard, fresh oil, or highly oxidized oil protected with alpha-tocopherol acetate or ethoxyquin. Significantly heavier livers were also shown for the animals

TABLE III
Blood Data

| Lot No. | Treatment | Hemoglobin, g % | | Hematocrit, % | | Plasma cholesterol, mg % | |
|---|---|---|---|---|---|---|---|
| | | Start | Finish | Start | Finish | Start | Finish |
| 1 | Lard diet | 11.3 | 13.8 | 35.3 | 46.2 | 114 | 126 |
| 2 | Fresh menhaden oil diet | 10.6 | 11.9 | 34.6 | 39.3 | 164 | 132 |
| 3 | Medium oxidized menhaden oil diet | 9.8 | 11.0 | 31.6 | 34.0 | 156 | 137 |
| 4 | High oxidized menhaden oil diet | 10.6 | 9.4 | 33.2 | 30.7 | 129 | 138 |
| 5 | High oxidized menhaden oil diet + alpha-tocopherol acetate | 11.3 | 15.2 | 36.0 | 45.0 | 128 | 100 |
| 6 | High oxidized menhaden oil diet + ethoxyquin | 12.0 | 14.6 | 37.3 | 42.3 | 153 | 101 |

fed medium oxidized oil in comparison with those from animals fed highly oxidized oil protected with alpha-tocopherol acetate.

## Discussion

An interesting feature of the results of this experiment is the degree of protection afforded the animals by addition of antioxidants to the menhaden oil portion of the diet. There seems little doubt that this antioxidant activity took place *in vivo*, rather than in the feed mix prior to consumption. The antioxidants were added immediately before the daily rations were mixed and fed; however it is possible that delay in consumption of all the ration by the animals might have resulted in contact of antioxidant and diet lipid *in vitro* for several hours. More important evidence for *in vivo* activity, however, is the fact that alpha-tocopherol acetate was used, and this would not become biologically active until the acetate moiety was split off, in the digestive tract (2). It should be borne in mind that the oil used in the antioxidant-treated diets was already in an advanced state of oxidation as evidenced by its peroxide value of 61 and TBA no. of 162. These levels are, incidentally, considerably greater than those encountered in most fish oil.

Improvement of feed intakes in the cases of the antioxidant-treated diets was an interesting phenomenon. One would expect *in vitro* antioxidant activity, by depressing oxidative changes in diet fats prior to consumption, to exert a positive effect on feed intake. It is more difficult to reconcile such an effect with *in vivo* activity taking place after considerable preliminary oxidation. One may speculate that such increased feed intakes are rather a reflection of general health and well-being of the animals concerned than an indication of "palatability" per se.

The blood changes, although not extreme, were generally illustrative of a tendency toward anemia as oils of higher oxidation states were fed. Reports exist (31) of occurrence of severe anemia in mink fed raw hake *(Merluccius productus)*, which is, however, a fish of rather low oil content, and of a milder anemia in Eskimos who habitually consume large amounts of dried or raw, frozen fish (29); but in neither of these instances was oxidation level of the dietary lipid implicated. It is significant that deterioration of the blood picture in this experiment was prevented by the use of the two antioxidants. The cholesterol data appear of limited interest, except for the observation that fish oils in the diet generally caused some reduction during the course of the trial. The only exception to this was in the case of the highly oxidized oil, where it may be argued that the points of unsaturation were largely modified. It must be remembered that these feeding treatments were not superimposed on feeding of a hypercholesteremic diet as has been

TABLE IV
Organ Weights

| Lot No. | Treatment | Liver, g | Heart, g | Spleen, g | Both kidneys, g |
|---|---|---|---|---|---|
| 1 | Lard diet | 1721.0 | 290.0 | 158.0 | 348.5 |
| 2 | Fresh menhaden oil diet | 1920.0 | 376.0 | 179.5 | 449.0 |
| 3 | Med. oxid. menhaden oil diet | 2016.5 | 361.0 | 198.0 | 459.0 |
| 4 | High oxid. menhaden oil diet | 2263.5 | 324.5 | 177.5 | 407.0 |
| 5 | High oxid. menhaden oil diet + alpha-tocopherol acetate | 1632.0 | 308.5 | 151.0 | 314.5 |
| 6 | High oxid. menhaden oil diet + ethoxyquin | 1916.5 | 329.0 | 140.5 | 405.5 |

TABLE V
Diet and Body Fat Characteristics

| Lot No. | Iodine numbers | | | TBA numbers[a] | | Ether extractable material, % | |
|---|---|---|---|---|---|---|---|
| | Diet fat | Back fat | Kidney fat | Inner | Outer | Back fat | Kidney fat[b] |
| 1 | 61 | 68.2 | 58.8 | 1.7 | 1.0 | 86.9 | 93.5 |
| 2 | 184 | 91.6 | 90.3 | 40.8 | 10.0 | 44.1 | 75.2 |
| 3 | 183 | 84.8 | 87.1 | 36.2 | 11.0 | 56.1 | 64.1 |
| 4 | 181 | 82.1 | 74.1 | 38.5 | 18.2 | 54.0 | 66.7 |
| 5 | 181 | 95.9 | 79.5 | 3.8 | 16.2 | 92.2 | 89.2 |
| 6 | 181 | 95.7 | 86.2 | 12.5 | 19.1 | 81.3 | 89.0 |

[a] TBA numbers taken on inner and outer back fat.
[b] The designations "Back fat" and "Kidney fat" refer to adipose tissue from these regions.

the case in other studies (26,34); therefore the opportunity for plasma cholesterol reduction was slight.

Differences in depot fat were perhaps most marked by differences in extent of fattening of animals in the various treatment groups, which is a measure of the overall rate of growth. Iodine numbers of body depot fat tended to reflect iodine numbers of dietary lipids; however it is evident from the data in Table V that considerable depot fat must have been synthesized from carbohydrate or some source other than the fish oil. Differences in iodine numbers were not large, but this observation is supported by evidence in the literature that a relatively advanced state of deterioration must be reached before changes in iodine numbers are detectable (17). Generally speaking, iodine numbers decreased with increased oxidation of the diet fat, and this was particularly noticeable in the case of the kidney fat. Antioxidant activity was in the direction of promoting more unsaturated animal depot fat, presumably by protecting double bond sites in the dietary lipids during digestion. It may be of interest that inner backfat appeared to be protected more effectively than outer backfat, as evidenced by TBA values, through the addition of the antioxidants. Garton (12) has previously indicated greater unsaturation in outer than in inner backfat of a pig fed whale oil.

The biological significance of the increased liver weights where menhaden oil was fed, and the heightening of this effect as the oil became more highly oxidized is not explained by the data available. The extent of the liver increases is accentuated when one considers that total body size was simultaneously decreasing (cf. Table II). *In vivo* activity of the alpha-tocopherol apparently reversed the trend toward heavier livers with oxidized dietary lipid.

One may conclude from these data that certain of the adverse effects of oxidized fish oils in diets of swine may be related to the level of oxidation existing in the oils during their metabolism by the animals. Such effects may largely be prevented by inclusion in the diet of such *in vivo* antioxidants as alpha-tocopherol acetate or ethoxyquin at appropriate levels. The two antioxidants apparently performed equally well at the levels used.

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# Dietary Antioxidants in Young Swine[1,2]

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## Abstract

Young swine obtained by hysterectomy were fed purified diets low in vitamin E and supplemented with d-α-tocopheryl acetate and ethoxyquin (Santoquin [R]).

It was demonstrated that with very low levels of polyunsaturated fatty acids (PUFA) in the diet, both tocopherol and Santoquin protected the tissues of the pig from increased thiobarbituric acid (TBA) values and from increased hemolysis usually associated with low vitamin E status. When the dietary PUFA were increased to levels over 5%, the supplements of tocopherol and Santoquin protected against increased TBA values of tissue homogenates, but not against increased hemolysis of erythrocytes, even when blood serum showed substantial amounts of tocopherol.

Some of the interrelationships of dietary PUFA and α-tocopherol were demonstrated. It was shown that for each 1% of peroxidized corn oil added to the diet above 4%, roughly 100 mg of d-α-tocopheryl acetate was necessary to protect the pigs from erythrocyte hemolysis.

The failure to reach a "zero" TBA value in vitamin E-deficient swine tissue homogenates substantiated the theory of *in vivo* lipid autoxidation, and the increased TBA values of incubated tissue homogenates demonstrated *in vitro* lipid autoxidation in tissues not protected by a biological antioxidant.

## Introduction

DIETARY ANTIOXIDANTS have been used in several species of animals for several years to replace vitamin E. The results have varied, depending upon the antioxidant used, the species of animal, and the symptom studied. Several investigators (1,2,3,4) reported varying success in substituting methylene blue and NN'-diphenyl-p-phenylenediamine (DPPD) in the diet of the rat in place of tocopherol, and Draper et al. (5) were able to carry rats through two generations using DPPD in place of vitamin E. The antioxidant DPPD has been shown also to prevent muscular dystrophy in lambs (6). Shull et al. (7) found DPPD and Monsanto's Santoquin to be partially effective in preventing muscular dystrophy in guinea pigs. Studies with the vitamin E-deficient chick have shown a protective action of DPPD and Santoquin against encephalomalacia (8,9), and a protective action with Santoquin against exudative diathesis and muscular dystrophy. Selenium has been shown also to protect the vitamin E-deficient chick from exudative diathesis (10,11).

In the past 10 yr, several workers have described the *in vitro* hemolysis of erythrocytes from vitamin E-deficient animals of several species (12,13,14,15,16). The degree of hemolysis of erythrocytes of animals has been considered an indication of (and actual assay for) their vitamin E status (17). Those animals whose erythrocytes showed a high degree of *in vitro* hemolysis, either by $H_2O_2$ or by dialuric acid, were considered to be vitamin E-deficient.

More recently, it has been found that certain symptoms of vitamin E deficiency are either more readily apparent or appear only under the stress of adding significant amounts of PUFA to the diet (18,19,20,21, 22). Studies involving dietary PUFA have shown that tissue extracts from vitamin E-deficient animals show large increases in TBA reactant material (malonaldehyde) in *in vitro* incubation, in contrast to tissues from normal controls, which show very little or no increase in TBA value (23,24). This criterion has been assumed to indicate a high level of lipid autoxidation in vitamin E-deficient tissues, and that vitamin E prevents this autoxidation *in vivo* and *in vitro*. It has been shown in this laboratory that TBA values measure malonaldehyde release from the autoxidation of PUFA with three or more double bonds, but not from linoleic acid (25).

## Experimental

The experiments to be described here are the results of several years' work on the relationship of vitamin E and other antioxidants, mainly ethoxyquin (or Santoquin) in the nutrition and blood characteristics of young swine.

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