obtained from active fish tissues by acetone treatment.

The solubility of the factor in dilute salt solutions, the rapid destruction by heat, and the precipitability by common protein precipitants indicate the fish principle to be of protein nature.

The destruction of thiamine is maximal at *p*H 9.1 and 60°, is proportional to the amount of principle used and is characterized by first-order velocity constants. These facts together with the evidence of the catalytic nature of the reaction strongly suggest that the principle effecting the thiamine loss is an enzyme.

ROCHESTER, N. Y. RECEIVED APRIL 6, 1943

[CONTRIBUTION NO. 37 FROM THE LABORATORIES OF DISTILLATION PRODUCTS, INC.]

α-Tocopherol, a Natural Antioxidant in a Fish Liver Oil

BY CHARLES D. ROBESON AND JAMES G. BAXTER[1]

This paper describes the isolation of a natural antioxidant from shark liver oil (Mangona, from Brazil; 3800 units of vitamin A per g.) and identifies it as natural α-tocopherol (hereafter the term, "tocopherol" means "natural tocopherol"). Evidence is further presented to show that α-tocopherol is the major antioxidant in the oil. This finding is of interest because it indicates that the tocopherols may serve as natural antioxidants in fish liver as well as vegetable oils. Conflicting evidence has previously been reported on the occurrence of vitamin E in cod liver oil.[2,3]

Mangona shark liver oil was used in the investigation because the livers were available and could be processed in this Laboratory. A tocopherol concentrate was also prepared from soupfin shark liver oil (*Eugaleus Galeus*, 94,400 units of vitamin A per g.), but since the oil was obtained commercially, it could not be proved that the tocopherol had not been added during processing, *e. g.*, by using some vegetable oil to extract the livers.

Isolation of Antioxidant.—The isolation method employed was based on the hypothesis that the antioxidants in the liver oil would, like the tocopherols in vegetable oils, be concentrated by molecular distillation in the more volatile fraction. Therefore, the oil extracted from the livers was distilled and the fraction distilling below 225° at 0.003 mm. pressure was collected. The antioxidant in this distillate was detected by measuring the activity of the fraction in protecting vitamin A against atmospheric oxidation. The test method was as follows.

A solution of vitamin A in a substantially antioxidant-free oil (5500 units per g.) was prepared by dissolving crystalline vitamin A in distilled sardine oil.[4] To aliquots of this (3 cc.) was added 5% of the undistilled shark liver oil, 5% of the distillate, and 5% of the distillation residue. These samples together with a blank sample containing no added fish liver oil were exposed to air, in thin layers, at 55° for two and five hours. At the end of each heating period, the vitamin A potency was determined by the antimony trichloride method. The vitamin A recoveries are given in Table I.

TABLE I

ANTIOXIDANT ACTIVITY OF MANGONA SHARK LIVER OIL FRACTIONS

Sample	% of original vitamin A potency after exposure to air at 55° for 2 Hrs.	5 Hrs.
1. Solution of crystalline vitamin A in distilled sardine oil (5500 units/g.)	31	10
2. No. 1 + 5% Mangona shark liver oil (M.S.L.O.)	58	25
3. No. 1 + 5% M.S.L.O. distillate	95	91
4. No. 1 + 5% M.S.L.O. residue	41	20

It was concluded from the data that the shark liver oil contained an antioxidant (or antioxidants) which was concentrated in the distillate since the residue had little antioxidant activity. Subsequent isolation work was, therefore, confined to the distillate which contained glycerides, vitamin A esters, and sterols as impurities. The method for separating the antioxidant from the impurities was based on the hypothesis that it was a phenolic compound like the tocopherols.

The distillate (II, Experimental Part) was esterified with succinic anhydride. The half suc-

(1) Presented before the Division of Biological Chemistry of the American Chemical Society, Buffalo meeting, September, 1942.

(2) Sure, *J. Biol. Chem.*, **74**, 45 (1927).

(3) Nelson, Ohrbeck, Jones and Taylor, *Am. J. Physiol.*, **85**, 476 (1928).

(4) Distilled sardine oil was prepared by stripping sardine oil in a molecular still and discarding the first 15% containing natural antioxidants. The next 50% of the distillate was the diluent used.

cinates were separated from vitamin A esters and glycerides by neutralization in absolute alcohol, dilution with water to 83% ethyl alcohol, and extraction of the non-hydroxylic constituents with petroleum ether. After acidifying the alcohol layer, the half succinates (IV) were recovered, saponified, and the sterols were separated by crystallization from ethyl formate solution at low temperatures. A reddish oil (VI) was recovered from the filtrate and distributed between petroleum ether and 83% aqueous ethyl alcohol. Free vitamin A plus some of the antioxidant passed into the alcohol layer. The petroleum ether fraction (VII) was then fractionated by adsorption on Floridin earth.[5] After elution an antioxidant concentrate (VIII) was obtained as a reddish-yellow oil, 0.048% of which had the same antioxidant activity as 5% of the distillate (Table II).

TABLE II

ACTIVITY OF ANTIOXIDANT CONCENTRATE FROM MANGONA SHARK LIVER OIL

Sample	% of original vitamin A potency after exposure to air at 55° for hours					
	2	4	7	9	10	11
1. Solution of crystalline vitamin A in distilled sardine oil residue (5500 units/g.)	31	10				
2. (a) No. 1 + 13% Mangona shark liver oil distillate (II)	89	93	82	82		60
(b) No. 1 + 5% II	95	91	75	72	63	
(c) No. 1 + 1.75% II	94	87	69	62		
3. (a) No. 1 + 0.125% antioxidant concentrate (VIII)	96	93	85	84		69
(b) No. 1 + 0.048% VIII	95	89	77	74	64	
(c) No. 1 + 0.017% VIII	89	81	63	55		

Concentrate (VIII) had these properties characteristic of the tocopherols: (1) It gave a red color when heated with nitric acid in alcohol solution. (2) By the tocopherol assay method of Emmerie and Engel,[6] it gave a red color corresponding to 78.5% tocopherol. (3) It had an ultraviolet absorption band at 292 mμ similar to that of α-tocopherol (Fig. 1).

A crystalline palmitate of the antioxidant was prepared from VIII. The crystals (laths, m. p. 40.5–41.5°) appeared to be identical with those of natural α-tocopherol palmitate (laths, m. p. 42–43°).[7] The carbon and hydrogen analysis supported this conclusion. The absorption spectra of the two esters and their extinction coefficients were nearly the same (Fig. 2). The vitamin E activity of the palmitate was determined by Dr. P. L. Harris and M. H. Joffe of this Laboratory.

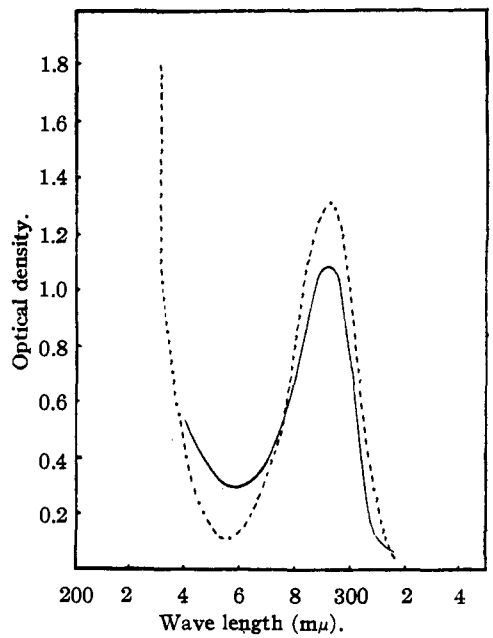

Fig. 1.—Comparison of absorption spectra of: —— antioxidant concentrate (VIII), ($E\,^{1\%}_{1\,cm.}$ 292 mμ = 62.5), and - - - - - natural α-tocopherol, ($E\,^{1\%}_{1\,cm.}$ 292 mμ = 73).

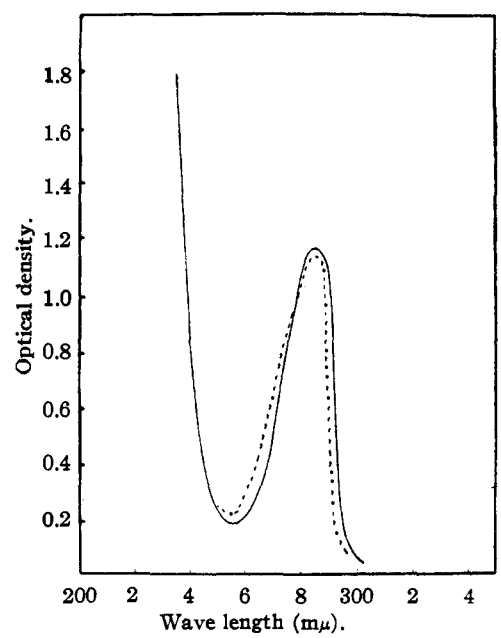

Fig. 2.—Comparison of absorption spectra of: —— palmitic ester of antioxidant, ($E\,^{1\%}_{1\,cm.}$ 286 mμ = 28.1), and - - - - - - natural α-tocopherol palmitate, ($E\,^{1\%}_{1\,cm.}$ 286 mμ = 26.8).

(5) Floridin earth (Floridin Company, Warren, Pa.) is a special grade of fuller's earth recommended by Emmerie and Engel [*Rec. trav. chim.*, **58**, 283 (1939)] for the removal of carotenoids.

(6) Emmerie and Engel, *Rec. trav. chim.*, **57**, 1351 (1938).

(7) Baxter, Robeson, Taylor and Lehman, THIS JOURNAL, **65**, 918 (1943).

The ester was found to be equivalently as active as natural α-tocopherol.[8] It was concluded that the antioxidant isolated from Mangona shark liver oil was α-tocopherol.

Evidence was obtained indicating that α-tocopherol is the major antioxidant in this oil. To prove this, the antioxidant activity of three concentrations of the liver oil distillate (II, 1.75–13%) was compared with three concentrations of the α-tocopherol concentrate prepared from it (VIII, 0.017–0.125%) (Table II).

It appears from the data that 5% of II had substantially the same antioxidant activity as 0.048% of VIII. Thus, if 5% of II contained the same amount of α-tocopherol as 0.048% of VIII, it would seem justifiable to conclude that α-tocopherol is the principal antioxidant in the oil. The percentage of α-tocopherol in II could not be determined directly because the large amount of vitamin A present interfered. However, VI, obtained from it and containing only a small amount of vitamin A, could be assayed by the Emmerie–Engel procedure and the percentage of α-tocopherol in II calculated. From 159 g. of II was obtained 3.35 g. of VI containing 39.4% α-tocopherol, corresponding to 0.83% α-tocopherol in II. Therefore, 5% of distillate II contained the same amount of α-tocopherol as 0.053% of VIII (78.5% α-tocopherol), which agrees well with the value 0.048%.

We, therefore, conclude that α-tocopherol is the major natural antioxidant in Mangona shark liver oil. It was calculated that the fish liver oil contained about 0.01% α-tocopherol, which is about one-tenth as much as is present in unrefined cottonseed oil.

From soupfin shark liver oil by the same procedure a concentrate was obtained containing 76.7% tocopherol by Emmerie–Engel assay. The ultraviolet spectrum of this preparation had $E^{1\%}_{1\,cm.}$ 292 mμ = 77, a value greater than that of pure α-tocopherol. This indicated the presence of extraneous absorption. The concentrate was not further investigated for reasons previously stated. The percentage of tocopherol in the original fish liver oil was calculated to be approximately 0.04%.

Experimental

Isolation of Antioxidant from Mangona Shark Liver Oil

Extraction of Oil from Livers.—The livers (34.9 kg.) were ground in a meat grinder. The slurry was mixed with 1.5 volumes of water containing sodium hydroxide (698 g.). The mixture was blown with steam for fifteen minutes and the oil layer was allowed to separate. After washing with hot water and centrifuging, the liver oil (I, 15.55 kg.) was obtained.

Distillation.—The fish liver oil (I) was distilled in a molecular still and the more volatile fraction distilling from 145–240° at 0.003 mm. pressure was collected (II, 159 g.). In collecting this fraction, the temperature was raised in 10° intervals and two cycles were taken at each temperature.

Esterification.—The distillate (II) in pyridine (400 cc.) containing succinic anhydride (57 g.) was heated for two hours at 70°. The solution was then poured into dilute hydrochloric acid, extracted with ether, and the extract successively washed with acid and water. After drying, the solvent was distilled, leaving a red viscous oil (III, 173 g.).

Extraction.—The esterified distillate (III) in absolute ethyl alcohol (1200 cc.) was neutralized with 2 *N* aqueous sodium hydroxide. The solution was adjusted to 83% alcohol with water and extracted with four 450-cc. portions of petroleum ether. The alcohol layer was acidified with 5% hydrochloric acid and extracted with ether. The extract was washed with water, dried, and the solvent was distilled. The impure antioxidant half succinate was a viscous red oil (IV, 73 g.) which solidified at room temperature.

Saponification.—The acid succinate (IV) was saponified by a procedure previously described.[7] The ester in absolute ethyl alcohol (700 cc.) was refluxed in an atmosphere of nitrogen for ten minutes to remove dissolved air, and aqueous potassium hydroxide (70 cc. concentration, 50 g. per 100 cc.) was added. After refluxing for thirty minutes, the saponification mixture was diluted with water and extracted with ether in the usual way. After distillation of ether under reduced pressure (r. p.), a tan-colored solid (V, 15.5 g.) was obtained.

Removal of Sterols.—The concentrate (V) in ethyl formate (150 cc.) was cooled to 5° for fifteen hours and filtered to remove sterols (11.7 g.). The solvent in the filtrate was distilled (r. p.), leaving a dark red oil (VI, 3.35 g.) containing 39.4% tocopherol by Emmerie–Engel assay.

Distribution.—Fraction VI, in petroleum ether (100 cc., b. p. 30–65°), was extracted four times with 100-cc. portions of 83% aqueous ethyl alcohol which had been saturated with petroleum ether. Solvent was removed from the petroleum ether fraction by distillation (r. p.), giving a viscous oil (VII, 1.2 g.) containing 59% tocopherol.

Adsorption.—Fraction VII was adsorbed from petroleum ether (15 cc.) on a column (9″ × 12 mm.) of Floridin earth. After development with petroleum ether (80 cc.) two bands were observed. The upper half of the column was bluish-green, the lower half was colorless and contained tocopherol which was eluted with ether containing 5% methyl alcohol. The eluate was freed of solvents by distillation (r. p.). The residue, a reddish-yellow oil (VIII, 0.59 g.), contained 78.5% α-tocopherol by Emmerie–Engel assay and had $E^{1\%}_{1\,cm.}$ 292 mμ = 62.5.

Antioxidant Palmitate.—A portion of VIII (0.28 g.) in ethylene chloride (2 cc.) and pyridine (1 cc.) was treated with palmitoyl chloride (0.9 cc.), in ethylene chloride

(8) Harris and Jolfe, THIS JOURNAL, 65, 925 (1943).

(2 cc.), at room temperature. After standing at room temperature for twenty hours, the reaction mixture was poured into ether and the ether extract was washed successively with dilute hydrochloric acid, 0.5 *N* aqueous potassium hydroxide and water. The ether was removed by distillation leaving a white solid containing free palmitic acid. Palmitic acid was removed by adsorbing the crude ester from petroleum ether (15 cc.) on a column of Doucil[9] (9″ × 12 m.m.). The column was washed with petroleum ether (75 cc.) and filtrate and washings were evaporated leaving a residue (0.302 g.) which was crystallized from ethyl alcohol (6 cc.) at 5°. The antioxidant palmitate formed lath-like, white crystals (0.18 g.), m. p. 40.5–41.5°, which were identified as α-tocopherol palmitate.

(9) Doucil (American Doucil Company, 121 South 3d Street, Philadelphia, Pa.) is a sodium aluminum silicate.

Anal. Calcd. for $C_{45}H_{80}O_3$: C, 80.76; H, 12.06. Found: C, 80.81; H, 11.91.

Summary

A natural antioxidant has been isolated from Mangona shark liver oil and identified as natural α-tocopherol. Evidence was obtained indicating that α-tocopherol is the major antioxidant present in this fish liver oil.

This finding is of interest since it indicates that the tocopherols may act as natural antioxidants in fish liver oils as well as in vegetable oils.

ROCHESTER, NEW YORK RECEIVED FEBRUARY 8, 1943

[CONTRIBUTION FROM THE FRICK CHEMICAL LABORATORY, PRINCETON UNIVERSITY]

Reactions of Organometallic Compounds with Alkyl Halides. I. The Action of Sodium Ethyl on (−)2-Bromoöctane

BY NORMAN G. BRINK,[1] JOHN F. LANE AND EVERETT S. WALLIS

Previous studies[2] in this Laboratory have shown that the Wurtz reaction of an optically active alkyl bromide with sodium leads to a completely optically inactive product. Thus, when (+)2-bromobutane is treated with sodium at room temperature, the resulting 3,4-dimethylhexane shows no detectable optical activity. This result would be consistent with a formulation[3] of the Wurtz synthesis involving initial formation of free radicals

$$Na + RX \longrightarrow R\cdot + NaX \qquad (1)$$

followed by their combination to give the expected coupling product

$$2R\cdot \longrightarrow R\text{–}R \qquad (2)$$

or by their disproportionation to alkane and alkene by-products

$$2R\cdot \longrightarrow RH + \text{olefin.} \qquad (3)$$

Secondary alkyl radicals would be expected to show extreme optical instability, since they possess numerous planar resonance states comparable in energy to the normal state. In this they resemble the corresponding carbonium ions more closely than the corresponding carbanions, the planar resonance states of which are considerably higher in energy than the normal state.[4]

(1) Sayre Fellow in Applied Chemistry, 1942–1943.

(2) Wallis and Adams, THIS JOURNAL, **55**, 3838 (1933).

(3) *Cf.* Hückel, Kraemer and Thiele, *J. prakt. Chem.*, **143**, 207 (1935); Bachmann and Clark, THIS JOURNAL, **49**, 2089 (1927); Richards, *Trans. Faraday Soc.*, **36**, 956 (1940).

(4) Baughan, Evans and Polanyi, *Trans. Faraday Soc.*, **37**, 377 (1941).

More recently,[5] sodium alkyls have been shown to act on alkyl halides in a manner typical of the salts of very weak acids, either to effect substitution of the carbanion for halogen

$$(Na^+)R_1^- + R_2X \longrightarrow (Na^+)X^- + R_1 - R_2 \qquad (4)$$

or by their strongly basic action to remove the elements of hydrogen halide with the formation of an olefin

$$(Na^+)R_1^- + R\text{—}CH_2\text{—}CH_2X \longrightarrow R_1H + R\text{—}CH{=}CH_2 + (Na^+)X^- \qquad (5)$$

In addition, when X is bromine or iodine, metal-halogen interchange[5b]

$$NaR_1 + R_2X \longrightarrow NaR_2 + R_1X \qquad (6)$$

can occur. It has been further suggested[5b] that the initial stage of the Wurtz synthesis may involve direct formation of a sodium alkyl

$$2Na + RX \longrightarrow NaR + NaX \qquad (7)$$

and that subsequent stages of the reaction involve the action of the sodium alkyl on additional alkyl halide according to equations (4) and (5). If this be granted, the observed products of the Wurtz synthesis may be accounted for without recourse to the concept of free radicals as critical reaction intermediates.

In order to account for the complete optical inactivity observed in the formation of 3,4-dimethylhexane from (+)2-bromobutane and sodium, however, such an interpretation must be ampli-

(5) (a) Whitmore and Zook, THIS JOURNAL, **64**, 1783 (1942); (b) Morton, Davidson and Hakan, *ibid.*, **64**, 2242 (1942).