[CONTRIBUTION FROM THE DEPARTMENTS OF VITAL ECONOMICS AND BACTERIOLOGY OF THE UNIVERSITY OF ROCHESTER]

Thiamine Inactivation by the Fresh-Fish or Chastek-Paralysis Factor[1]

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The disease of the fox commonly known as Chastek paralysis represents an unusual type of vitamin deficiency disease in that it results from a destruction of the dietary thiamine before absorption and utilization occur. The exhaustive studies of Green, Carlson and Evans[3] clearly demonstrate that the destruction, and consequently the paralysis, are due to the inclusion of fresh or frozen fish of certain species in the diet of the foxes. Since the addition of excessive amounts of thiamine to a ration containing fish prevents the disease, it was concluded that some component of the fish tissues is responsible for the thiamine loss and further that this loss takes place during the intimate mixing of these tissues with the thiamine-containing portions of the diet occurring in the feed pan and in the digestive tract.

The inability of cooked fish to cause Chastek paralysis[3a] strongly suggested the presence of a heat labile factor whose inactivating influence on thiamine should prove capable of *in vitro* investigation. Consequently an analysis of the biochemical factors involved in the disappearance of the vitamin was initiated. During the course of these experiments, preliminary reports recording the feasibility of the *in vitro* approach to the problem have appeared. Spitzer, Coombes, Elvehjem and Wisnicky[4] not only demonstrated the production of polyneuritis in chicks by the feeding of whole carp and various fractions, but also the destruction of as much as 100% of added thiamine by incubation with raw fish entrails. Woolley[5] has also reported the *in vitro* activity of carp tissue, as well as the heat lability and possible fractionation of the fish principle.

In this communication are included the description of an *in vitro* assay method, the definition of a unit of activity, and an analysis of the distribution and of some of the characteristics of the factor causing the disappearance of thiamine.

(1) A preliminary report of this work was made before the Division of Biological Chemistry of the American Chemical Society at the annual fall meeting at Buffalo, N. Y., Sept. 10, 1942.

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(3) Green, Carlson and Evans, (a) *J. Nutrition*, **21**, 243 (1941); (b) **23**, 165 (1942).

(4) Spitzer, Coombes, Elvehjem and Wisnicky, *Proc. Soc. Exptl. Biol. Med.*, **48**, 376 (1941).

(5) Woolley, *J. Biol. Chem.*, **141**, 997 (1941).

Experimental

Preliminary experiments dealing with diet mixtures[6] containing frozen carp readily demonstrated the destructive action of the fish material. For example, a fox ration when assayed by the fermentation procedure of Schultz, Atkin and Frey[7] was found to contain 1.7 micrograms of the vitamin per gram. A second ration consisting of four parts of the first and one part of frozen whole carp contained only 0.5 microgram per gram. The mixtures in both cases were fairly solid, and had been thoroughly mixed and refrigerated for several days. If one assumed the fish to be completely free of thiamine, dilution of the first mixture with one part of fish should still have yielded a thiamine concentration of 1.4 micrograms instead of the 0.5 actually found.

Further demonstration of the effectiveness of the fish in causing disappearance of the vitamin was accomplished by incubation procedures.[8] The following are typical experiments. In one case 1 g. of carp viscera destroyed 33 micrograms in one hour at 25° out of a total of 100 micrograms of thiamine originally present. Centrifuged extracts equivalent to 1 g. of viscera removed 31 and 67% in one and four hours, respectively. Suspensions of carp head also were active. In one instance 7.5 g. destroyed 3 micrograms or three-fourths of that present in two and one-third hours.

For experiments of this type establishing the *in vitro* destruction, the fermentation procedure served quite adequately in evaluating the thiamine loss. Since a chemical method would permit a greater number of determinations in a given period, the colorimetric diazonium method described by Melnick and Field[9] was adopted for subsequent experiments.

Assay Method and Unit of Activity.—In order to study the distribution and characteristics of the effective principle, it was first necessary to de-

(6) These materials were very kindly supplied by Professor R. G. Green of the University of Minnesota, to whom we are also indebted for his enthusiastic coöperation and interest in this program.

(7) Schultz, Atkin and Frey, *Ind. Eng. Chem., Anal. Ed.*, **14**, 35 (1942).

(8) A very generous supply of thiamine has been furnished by the Medical Department of Merck and Co., Inc., for use in these experiments.

(9) Melnick and Field, *J. Biol. Chem.*, **127**, 515 (1938)

vise a procedure of measuring the activity of different tissues and preparations. The following method has been developed from numerous preliminary investigations, the details of which will not be recorded. In use it has proved relatively simple and rapid.

To 1 to 3 ml. aliquots of fish brei or extract previously adjusted to *p*H 7.4 and contained in a test-tube is added 1 ml. of 0.2 *M* phosphate buffer (*p*H 7.4). If necessary the volume is adjusted to 4 ml. with water and then 1 ml. of thiamine solution containing 2.5 micromoles of the vitamin is added. After incubation for two hours at 37.5°, 5 ml. of 20% trichloroacetic acid is added. The solution is allowed to stand for thirty minutes in order that the precipitated protein may flocculate. After centrifugation 2-ml. aliquots of the supernatant fluid are analyzed for thiamine, comparison being made with a solution identical in every respect except that the trichloroacetic acid is added immediately following the thiamine addition and the incubation is omitted.

One unit has been defined as that amount of activity which under the above conditions will cause the disappearance of 1 micromole (1×10^{-6} mole) of thiamine. In practice a preliminary assay is made with several different levels of the unknown substance. The assay is then repeated using in each tube the amount calculated to contain 1 unit of activity.

The precision which may be obtained with this procedure may be illustrated by the results of one assay. The material used was an extract (made with 2% sodium chloride) of a desiccated preparation of fish viscera. The aliquot in each test-tube contained 50 mg. of material which had dissolved in the salt solution. Ten values with an average of 9.48 ± 0.42 units per gram (range 8.8 to 10.0) were obtained. It is thus evident that an assay of a given extract may be accomplished with an average error not greater than ±5%.

Distribution in Carp Tissues.—Green, Carlson and Evans[3b] have demonstrated that of the whole carp, the meat ("somatic muscle") contains little or none of the principle causing destruction of thiamine. On the other hand the viscera and trimmings (heads, skins, fins and skeletons) were found extremely potent in producing Chastek paralysis. Further investigation of distribution among the separate tissues and organs has been made using the above *in vitro* method of assay. The carp[10] used in these experiments were immobolized by severing the spinal cord. A small incision made possible the collection of blood from the heart and immediately afterward the other tissues were dissected, blotted and weighed. Each tissue was thoroughly minced with 10% sodium chloride in 0.10 *M* phosphate buffer. The *p*H was adjusted to 7.4 and aliquots assayed. Representative values obtained are listed in Table I together with the weight of the fresh tissue. With the materials of lower activity at least 0.5 to 1.0 g. or more was used in each tube.

TABLE I

DISTRIBUTION OF THIAMINE-DESTROYING PRINCIPLE IN CARP TISSUES

Tissue	Weight, g.	Activity units per g.
Spleen	7.8	25.00
	2.5	6.00
	8.2	21.6
Liver and pancreas	95.1	2.5
	53.9	1.90
	34.7	1.48
Gastrointestine	44.4	2.5
	31.3	8.72
	23.7	3.54
Gills	52.1	2.5
	34.0	4.34
	15.2	2.16
Kidney	17.8	1.34
Blood	...	1.23
	...	0.80
Heart	4.1	.33
Testes	392	.25
Brain	2.3	.21
Mucous	21.2	.04
	3.7	.14
Gall bladder and bile	8.4	.11
Eyes	7.8	.06
Ovaries	9.3	.62
Swim bladder	...	0
Muscle	...	0

The rather wide distribution of the principle among the tissues and organs studied suggests that it has an important role in the metabolic processes of the carp. This view is supported by the occurrence of the greatest concentration of activity in four tissues, namely, the spleen, liver and pancreas, gastrointestine and gills. Table I also shows the variance in concentration in the same organs removed from different fish. Whether this is dependent upon seasonal, sexual or other factors we have not attempted to determine.

Preparation of Desiccated Powder.—On numerous occasions when live carp were not readily available, the viscera, accumulated on ice in a local fish market over the course of a day, have been used. No attempt was made to dissect the material except to remove the swim bladders. Since aqueous extracts of even fresh tissues rapidly lose activity

(10) The live carp were very kindly supplied by Mr. W. E Wiedner of Seneca Falls, N. Y.

on standing in the cold (5°) (as much as 36% in ten days), the preparation of a stable dry powder was considered essential. This was accomplished by treatment of the thoroughly minced viscera with 6 volumes of cold acetone. After thirty minutes the insoluble material was removed by filtering with suction and was again extracted for thirty minutes with 4 volumes of cold acetone. The defatted-dehydrated preparation was freed from acetone in a vacuum desiccator over phosphorus pentoxide. With further grinding and separation of the inactive shreds of connective tissue, desiccated powders were obtained which have retained their activity for eight months when stored in the cold.

Each gram of viscera yields on the average 0.15 g. of powder containing 7 to 24 units per g. depending upon the freshness of the original material. This powder has served as a ready source of the thiamine-destroying principle for from it the activity is readily removed by 10% aqueous sodium chloride, 45 to 50% of the total solids dissolving. More dilute sodium chloride solutions may be used for extraction purposes; however, the principle is not as completely removed. As Woolley[5] has reported, it is relatively insoluble in distilled water. The latter solvent is particularly of no value in preparing extracts of the acetone desiccated powders. From this it may be deduced that the active principle, being insoluble in water and soluble in dilute salt solution, is probably a protein substance.

The acetone treatment may likewise be used for preparation of dehydrated-defatted powder from the individual tissues. The liver and pancreas, gills and blood yield upon such treatment 90% of the original activity. From some of the other tissues somewhat smaller yields are obtained, but the preparations prove adequate for further study.

Characteristics of the Thiamine Destruction.—These have been determined utilizing the general principles of the assay procedure with the minor modifications demanded by the nature of the experiment. Aqueous sodium chloride extracts of acetone-desiccated powder have been used as a source of the fish principle in all cases.

Since the activity of extracts was found to vary with the hydrogen-ion concentration of the incubation mixtures, experiments of the type illustrated in Fig. 1 were conducted. Each experiment was made with a different desiccated preparation. The individual values have been corrected for any thiamine destruction which may have occurred due to incubation (without the fish extract) at the particular *p*H value. From the graph it is apparent that the maximum disappearance of thiamine occurs at *p*H 9.1. It is also apparent that the loss of thiamine at this *p*H is approximately four times that at *p*H 7.4, the hydrogen-ion concentration used in the assay method. However, the latter value has been chosen since it represents the most alkaline reaction which may be used without obtaining significant loss of thiamine by the alkalinity of the solution. The use of an additional correction factor in the assay method is thus avoided.

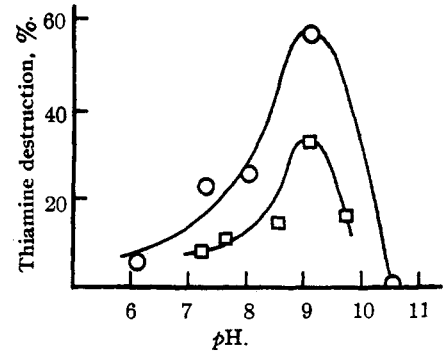

Fig. 1.—Effect of *p*H on thiamine destruction by the fish principle: 5×10^{-4} *M* thiamine, 37.5°; ○, preparation 6-86-A, incubated two hours; □, preparation 6-66-F, incubated one hour.

Not only is the activity of the fish principle dependent upon the *p*H of the incubation mixture, but also upon the temperature at which the mixture is incubated, as is demonstrated in Fig. 2. It is evident that the temperature curve is similar in shape to those usually encountered in enzyme reactions. From the experiment shown Q_{10} values of 2.3 and 2.1 were calculated for the temperature ranges of 30 to 40 and 40 to 50°, respectively.

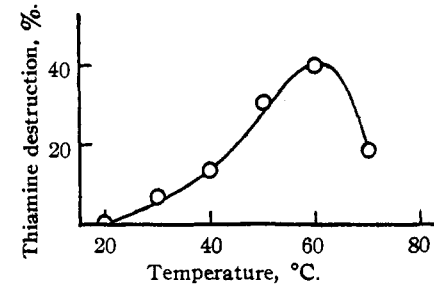

Fig. 2.—Effect of temperature on thiamine destruction by the fish principle: 5×10^{-4} *M* thiamine, *p*H 7.4, incubated two hours.

The marked decrease of activity at temperatures greater than 60° is confirmed by the experiment illustrated in Fig. 3, in which the loss of activity at 100° was determined. With this preparation, which was relatively crude, the activity was almost completely destroyed in thirty minutes. With more dilute or more purified material, complete destruction may be accomplished by heating in a boiling water-bath for five minutes. These findings are in complete agreement with the fact that cooked fish will not cause

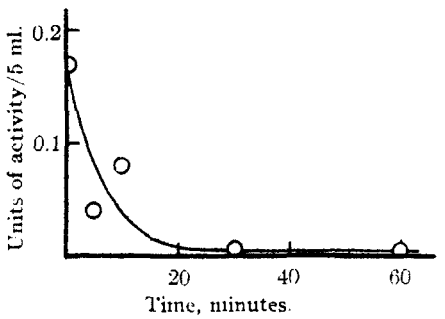

Fig. 3.—Heat inactivation of the fish principle: sodium chloride extract heated in boiling water-bath.

Chastek paralysis in the fox. Further, this heat lability furnishes added evidence that the paralysis-producing factor is of protein nature.

The protein nature of the fish principle is likewise evident in its behavior with the characteristic protein precipitating reagents. It is thrown out of solution by trichloroacetic acid (as in the assay procedure), or by alcohol, acetone, ammonium sulfate, picric acid or metaphosphoric acid.

In the presence of excess quantities of thiamine, the addition of increasing amounts of extract results in an increased destruction of the vitamin approximately proportional to the quantity added as is illustrated in Fig. 4.

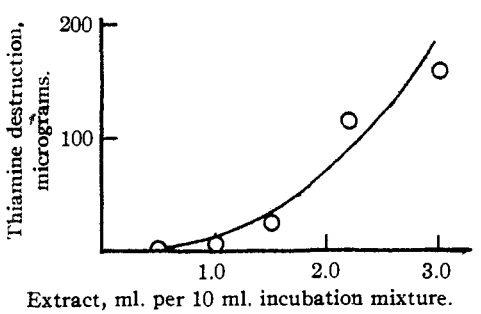

Fig. 4.—Relationship between amount of thiamine destroyed and amount of fish principle present: thiamine, 200 micrograms/10 ml.: incubated four hours at 25°.

Because of the unknown nature of the reaction involved in the loss of thiamine, it is of considerable importance to study the time course of the reaction. Representative experiments are illustrated in Fig. 5. The velocity constants for each experiment (Table II) calculated by means of the first-order equation, exhibit a uniformity quite as satisfactory as that usually obtained with tissue brei or crude extracts. The decrease in rate which occurs in the later stages may probably be explained by the lability of the fish factor under the conditions used. Incubation of the cruder fractions of the principle without thiamine, but otherwise under the same conditions, always results in large losses of activity in relatively few hours. This point will be referred to again in a later paragraph.

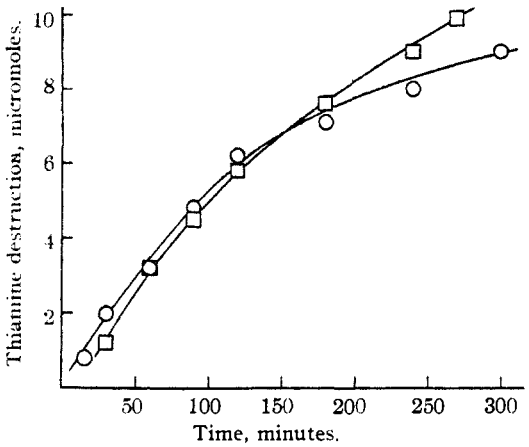

Fig. 5.—Time course of thiamine destruction by fish principle: ○, 75 ml. 5×10^{-4} M thiamine in 0.04 M phosphate buffer containing the sodium chloride soluble portion of 750 mg. desiccated powder, 6-93-A, pH 7.4, 37.5°; □, 50 ml. total volume, containing soluble portion of 500 mg. 6-93-A, other conditions same.

TABLE II

VELOCITY CONSTANTS OF THIAMINE DESTRUCTION

$$k = \frac{2.3}{t} \log \frac{C_0}{C}$$

t, min.	$k \times 10^3$ Expt. 1	Expt. 2
15	2.10	
30	2.78	2.78
60	2.28	2.29
90	2.37	2.21
120	2.38	2.21
180	1.84	2.01
240	1.61	1.86
270		1.86
300		1.46

These characteristics of the thiamine disappearance taken collectively strongly suggest that the loss of thiamine which occurs upon incubation with the fish principle results from enzymatic reaction, a possibility suggested by Spitzer, *et al.*,[4] and Woolley.[5] In fact, such a conclusion might readily have been drawn were it not for the recent disclosure of the biotin–avidin relationship. It has been demonstrated,[11] that biotin in the presence of avidin becomes physiologically nonutilizable through direct combination with the latter rather than through an enzymatic or catalytic action of the avidin. An entirely similar

(11) Eakin, Snell and Williams, *J. Biol. Chem.*, **136**, 801 (1940).

situation may exist between thiamine and the fish principle. On the other hand, if the destruction of thiamine is catalytic, we should predict that one mole of the fish principle would cause the loss of many moles of thiamine in a given unit of time. In other words, a high "turnover number" would be expected. Until the molecular weight of the active principle is determined, the true turnover number cannot be calculated. However, evidence obtained from experiments in this direction may be cited.

In one experiment, 5.0 g. of desiccated powder was successively extracted with 5 portions of 10% sodium chloride in 0.2 *M* phosphate buffer. The supernatant extract obtained by centrifuging was diluted to 50 ml. To 20 ml. were added 10 ml. of 0.1 *M* thiamine, water and sufficient sodium hydroxide to adjust to *p*H 7.4, the final volume being 50 ml. To a second 20-ml. portion was added water and sodium hydroxide to make a total volume of 40 ml. A solution of thiamine (*p*H control) similar to the first in all respects except that the extract was omitted was also prepared and the three solutions were then incubated at 37.5°, 1 ml. of toluene being added to each to prevent bacterial action. For purpose of analysis 5-ml. aliquots were removed from the first and third flasks and 5 ml. of 20% trichloroacetic acid added. From the second flask 4 ml. was removed, 1 ml. of 0.1 *M* thiamine and 5 ml. of trichloroacetic acid being added immediately. Thiamine was then determined in these aliquots in the usual fashion, the aliquots from the second flask serving as standards for those from the first flask. The analyses showed that from the first flask 194 micromoles of thiamine had disappeared in twenty-four hours due to the action of the fish principle.

The 20 ml. of extract in the flask contained 900 mg. of protein as determined by analysis of a separate portion ($N \times 6.25$) and 1090 mg. of organic material as determined in an ashing procedure. The total of 194 micromoles of thiamine lost represents the removal of 1 micromole of thiamine for each 4.6 mg. of protein present. If one assumes for purpose of calculation a protein molecular weight of 40,000, then 1 micromole of protein would equal 40 mg. The molar ratio of thiamine destroyed to fish principle used is then 8.7 to 1. With a protein of lower molecular weight the ratio would be decreased and with higher molecular weight it would be correspondingly increased.

Since the fish principle used in this experiment was obtained by the acetone treatment of whole viscera and subsequent extraction with 10% sodium chloride, it is extremely unlikely that the 900 mg. of protein represents the pure principle. If it is even 10% pure the above ratio becomes 87 to 1 instead of 8.7 to 1.

Since previous indications of the lack of stability of the extracts had been encountered, the possibility was further investigated that the low relative activity of the extracts might be explained on this basis. In one instance an extract entirely similar to that in flask two above was incubated for twenty-four hours at 37.5°. Aliquots removed at the beginning and end of the incubation were assayed by a further two-hour incubation with 2.5 micromoles of thiamine as in the assay procedure. The aliquots at the beginning caused the destruction of 1.34 micromoles of thiamine whereas those removed at twenty-five hours destroyed 0.17 micromole. It is thus apparent that the incubation of the extract in the absence of thiamine for a period of twenty-five hours resulted in a loss of 87.3% of the activity originally present. It is probable that a similar inactivation occurred in the thiamine-containing flask of the previous experiment with the consequent decreased destruction of thiamine.

One other type of evidence further indicates the enzymatic nature of the fish principle. In the case of the biotin–avidin complex, complete recovery of the biotin may be accomplished by heat treatment which alters or destroys the avidin.[12] When similar heat treatment, proteolytic digestion or acid hydrolysis is employed after incubation of thiamine with the fish principle, we have encountered no measurable recovery of the vitamin. Deutsch and Ott[13] have likewise reported that acid hydrolysis of mixtures containing thiamine inactivated by raw smelt yielded no detectable thiamine.

Should further investigation confirm the enzymatic nature of the fish principle, it may then be regarded as a type of thiaminase, since thiamine is the substrate. It may, however, be suggested that a more suitable and specific name may be adopted following elucidation of the biochemical function and activity of the factor. This is even more the case since Bonner and Buchman[14] have already suggested that an enzyme of pea roots capable of synthesizing thiamine from its two component heterocycles is "a thiaminase."

Summary

The Chastek-paralysis or thiamine-destroying activity of fish tissues has been investigated by means of *in vitro* methods, the thiamine loss being measured with a standard colorimetric chemical procedure.

An *in vitro* method of assay has been described and a unit of activity defined as that amount which in two hours will destroy 1 micromole of thiamine in 5 ml. of 0.0005 *M* at *p*H 7.4 and 37.5°.

With the assay procedure it has been shown that the principle is present in the majority of the visceral tissues of the carp, and that the spleen, liver and pancreas, gastrointestine and gills contain the highest concentration.

Preparations of stable dry powders have been

(12) Eakin, Snell and Williams, *J. Biol. Chem.*, **140**, 535 (1941).

(13) Deutsch and Ott, *Proc. Soc. Exptl. Biol. Med.*, **51**, 119 (1942).

(14) Bonner and Buchman, *Proc. Nat. Acad. Sci. U. S.*, **24**, 431 (1938).

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.

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[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.