SOME STUDIES ON THE CONTENT OF THIAMINE AND ANTI-THIAMINE FACTOR IN FISHERY PRODUCTS

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ABSTRACT

Data are presented which indicate that most fishery products contain from 50 to 200 micrograms of thiamine per 100 grams of material. Very few species were found to contain the anti-thiamine factor. Experiments are reported on concentration of the anti-thiamine factor from viscera of carp.

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INTRODUCTION

In 1941, when our work was begun, comparatively little was known concerning the factors affecting the chemical assays for thiamine and the anti-thiamine factor in fishery products. It was necessary to investigate various chemical procedures in order to determine their relative importance in permitting true assays. The results presented in this article were made available to interested government agencies and individuals, but no summary has been prepared previously.

The determination of the chemical structure of thiamine and the synthesis of the crystalline compound made it possible to determine the daily requirements of this vitamin for persons of different age groups and physical and physiological activity. These requirements were then defined in terms of an actual weight of thiamine, rather than in the empirical international units of activity which had been adopted at the 1934 conference of the Committee for Biological Standardization, League of Nations.

Later, the Council on Foods and Nutrition, National Research Council, in this country proposed daily allowances of thiamine for various categories of age, sex, and activity. The allowances are, on the whole, rather generous, since an excess amount of thiamine is recommended which will safely permit optimum growth or physiological activity.

The anti-thiamine factor was first discovered as a deficiency disease in foxes which were fed certain raw fish in the diet. A survey of the literature will not be made here, because quite comprehensive reviews are now available to those who are interested in the subject. The anti-thiamine factor is of little consequence in human nutrition since practically no raw fishery products are consumed which contain this factor. For animals, too, it is of only secondary importance because cooking rather easily destroys the factor. Cooked fish can be included in the rations without experiencing any difficulties due to the anti-thiamine factor.

A number of biological and chemical methods have been promulgated for the assay of thiamine. One of the first methods proposed was a curative test with

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pigeons which had been fed a deficient diet until acute symptoms of polyneuritis developed. A similar curative test with rats was standardized and made mandatory for official assays according to the Pharmacopoeia of the United States (XII).

The gain in live weight of dogs or rats initially suffering from polyneuritis, when fed diets containing various levels of thiamine and foods, has been used to estimate potency. One method utilizes the duration of recovery of normal rate of heart beat in rats initially suffering from bradycardia because of thiamine deficiency. Another method is based on the increase in oxygen uptake of avitaminotic pigeon brain in pyruvic acid solution as the result of adding thiamine-containing substances. Biological assay methods with humans to determine requirements have usually involved estimating the urinary excretion of thiamine after feeding known amounts of foods or crystalline thiamine under controlled conditions.

Micro-biological assay methods using yeast and bacteria are being employed quite extensively, particularly the yeast fermentation method. This method is based on the fact that the rate of fermentation by yeast is proportional to the thiamine content of the media under controlled conditions. The quantity of carbon dioxide evolved is the usual measure of activity.

The two chemical methods most widely used are the colorimetric method promulgated by Prebluda and McCollum (1939), and the thiochrome method first developed by Jansen (1936). In the colorimetric method, the thiamine treated with a reagent consisting of diazotized p-aminoacetophenone produces a red dye. The dye is dissolved in xylene, and the concentration is determined by means of a colorimeter or photoelectric photometer. This method has not been found to be sensitive enough for use in assaying foods having a comparatively low concentration of thiamine. The thiochrome method is based on the oxidation of thiamine to thiochrome by alkaline potassium ferricyanide. The thiochrome is extracted with isobutyl alcohol, and the concentration is determined by the amount of fluorescence produced.

EXPERIMENTAL METHODS FOR THIAMINE ASSAYS

For the experimental work reported herein, the thiochrome method was used because it seemed to offer the best possibilities for assaying fishery products containing low concentrations of thiamine. A rat growth method was used as a biological check for some of the assays.

The method finally adopted, after considerable testing of modification in methods, was a variant of the method devised for general use by Hennessy and Cerecedo (1939). A 5 to 15 g. sample, depending on the dry matter content and probable thiamine content, was boiled for 30 minutes in a 250 ml. Erlenmeyer flask with dilute acid. Two percent acetic acid was usually used, although 0.1N sulfuric or hydrochloric acid can be used instead. Sodium acetate (2.5N) was used to adjust the pH to 4.0 to 4.5 after the solution was boiled. One-fourth gram of takadiastase, or other active phosphatase was added and the mixture was incubated overnight at 37° or for 3 hours at 50° C. After incubation, the solution was centrifuged and filtered into a 100 ml. volumetric flask. The residue was stirred with water, heated in a water bath, recentrifuged, and the supernatant liquid was added to the original solution. The solution was made up to volume, and aliquots were used directly in the oxidation procedure, or first given the base exchange treatment.

The necessity for using a phosphatase digestion was recognized when assaying samples of oysters. At first, only a pepsin digestion was used, but the values decreased until no thiamine was found in a sample of oysters from Maryland. A

takadiastase digestion was tried, and 100 micrograms of thiamine were found per 100 g. of raw oysters.

In the base exchange treatment, the modified Hennessy method (1941) was carried out as follows: 80 to 100 mesh Decalso was stirred 4 times with 10-volume portions of 3 percent acetic acid for 10 minutes each. Between the second and third washings, the Decalso was stirred with 5 volumes of 25 percent potassium chloride for 15 minutes. It was finally washed thoroughly with water. The base exchange tubes were about 20 cm. long and 5 mm. in diameter with a capillary tube at the bottom end and a funnel holding about 25 ml. on the top end. A plug of glass wool was placed at the junction of capillary and tube, and the activated Decalso suspended in water was poured into the tube until the settled material was 5 cm. deep.

After the water was drained from the tube, an aliquot of the sample containing up to 10 micrograms of thiamine was added. The liquid was permitted to drain out, and three 20-ml. portions of water were pulled through the Decalso with suction. The suction had to be broken before the water level reached the Decalso column to avoid drawing in air which may have caused channeling. Elution of the thiamine was accomplished by using a solution of 25 percent potassium chloride. The rate of flow should be about 1 ml. per minute. Twenty-five ml. of eluate were collected unless the quantity of adsorbed thiamine was very small. In this case, a smaller quantity was collected to avoid too great a dilution. The standards to be used in reading the unknown were treated in a similar manner. The Decalso columns were generally used only once.

This method of base exchange treatment to remove interfering substances was not usually used in assaying the fishery products. In most cases, the blank readings were small, and there was no evidence of the presence of interfering substances. The base exchange procedure could, however, also be used as a means for concentrating the vitamin, although there is some doubt that the Decalso is able to adsorb all of the vitamin. The error due to incomplete adsorption can be accounted for to a considerable extent by treating the standards with Decalso.

The treatment to prepare the samples for reading of fluorescence was as follows: 13 ml. of isobutyl alcohol were placed in a 30 ml. capacity separatory-centrifuge flask. Five ml. of the water extract, either with or without previous base exchange treatment, were added to the alcohol. Three ml. of oxidizing solution, prepared by dissolving $\frac{1}{2}$ g. of potassium ferricyanide in 50 ml. of 15 percent solution of sodium hydroxide, were added and the mixture was immediately shaken for $1\frac{1}{2}$ minutes, then centrifuged at a low speed for $\frac{3}{4}$ minute. The aqueous layer was drawn off, and $1\frac{1}{2}$ g. of anhydrous sodium sulfate were shaken with the alcohol. The solution was again centrifuged. Ten ml. of the isobutyl alcohol solution were transferred to a cuvette, and an estimation of the fluorescence was made with the aid of a Pfaltz and Bauer fluorometer. A blank was prepared according to the same procedure, except that three ml. of a 15 percent solution of sodium hydroxide were used instead of a like amount of the oxidizing mixture. The value obtained for the blank was subtracted from that of the unknown, and the difference represented the fluorescence due to the thiochrome. The thiochrome content was determined by comparing the fluorescence obtained for the test sample, with that obtained for a similar sample to which a known amount of thiamine had been added.

In conducting the rat growth method of assay, weanling rats with an initial live weight of 40 to 50 g. and not more than 30 days in age were fed a thiamine deficient diet until they ceased to gain in weight. The diet consisted of sucrose, 60; casein, 18; autoclaved peanuts, 10; autoclaved dried brewer's yeast, 5; salt mixture, U.S.P. XII, No. 2, 4; sodium bisulphite treated liver extract, 1;

and cod liver oil, 2 parts by weight. To this was added 100 micrograms of pyridoxine per 100 g. of diet.

When the rats ceased to gain in weight, they were placed in individual cages and allotted into comparable groups. One group was fed the basal deficiency diet. This was the negative control group. Three groups were fed the basal diet containing 15, 25, and 35 micrograms of added crystalline thiamine per 100 g. of diet. These diets were fed ad libitum. The individual increase in weight over a 4-week period was plotted against the thiamine intake in order to establish a standard curve.

Other groups of rats were fed the basal diet ad libitum plus varying daily allowances of the food to be assayed. The food to be assayed was ground thoroughly and quick frozen. The frozen product was weighed out daily, and fed to the individual rats from small glass cups. All rats consuming satisfactory quantities of food as compared with those fed the basal diet plus crystalline thiamine were included as assay animals. The individual increase in weight was plotted against the indicated thiamine intake as derived from the standard curve. The indicated intake of thiamine in micrograms divided by the grams of fish consumed, times 100 equals the thiamine content in micrograms for 100 g. of fishery product.

EXPERIMENTAL DATA FOR THIAMINE ASSAYS

The samples of fresh or frozen fishery products were obtained from local dealers, or were shipped in directly from a firm in the area of production. The samples of canned products were purchased from local retail stores.

Test samples were selected which represented as closely as possible the edible material, either raw or processed, of products available for home consumption. The flesh was finely ground through a food chopper, or, if possible, liquified in a Waring Blendor.

Thiamine in Oysters

The assays with raw shucked oysters (Ostrea virginica) were conducted for two reasons; first, to determine if regional variations occur, and secondly, to determine the effect of different commercial packing methods on the thiamine content. The following data indicate the average value, or range in values found with samples obtained from different areas:

State	Thiamine per 100 grams of raw oysters	State	Thiamine per 100 grams of raw oysters
	Micrograms		Micrograms
Louisiana	110 - 130	Maryland	100 - 103
Georgia	98 - 106	New York	170 - 180
Virginia	100 - 110	Connecticut	170

These data indicate that the oysters in the North contained more thiamine per unit of fresh weight than those from the South. It is well known that the oysters in the North grow at a slower rate, and this may be a factor in determining the concentration of vitamin. The oysters in the South also have a lower dry matter content.

Commercial packers also use different methods of handling the oysters before packaging. Some studies were conducted to determine the effect of different methods, such as shucking dry, shucking into water and holding in water, blowing in

water for varying periods of time, etc., on the thiamine content. The data obtained with oysters from Green Point, Long Island, N. Y. are presented in Table 1.

Table 1 - The Dry Matter and Thiamine Contents of Oysters Handled in Different Ways After Shucking

Sample	Treatment	Dry Matter Content	Thiamine per 100 Grams of Raw Oysters
		Percent	Micrograms
14A	Fresh shucked	18.5	190
14B	Shucked dry, and held dry	18.8	186
14C	Shucked into a perforated can, and held dry	23.3	209
14D	Blown for 3 minutes in fresh water	17.1	170
14E	Blown for 3 minutes in salt water	19.6	191
17F	Fresh shucked, dried on towel	24.0	246
15A	Fresh shucked	18.8	176
15C	Shucked dry, and held dry	21.7	236
15D	Blown for 3 minutes in fresh water	15.1	152
23A	Fresh shucked	19.5	177
23B	Held in water for 2 hours and blown for 10 minutes in fresh water	14.1	166
23C	Held dry for 1 hour and blown for 3 minutes in fresh water	19.0	210

These data indicate that there is a close correlation between the thiamine content and the dry matter content of these oysters. The samples were divided into three groups; namely, those freshly shucked or equivalent (14A, 14E, 15A, and 23A), those held dry or equivalent (14C, 17F, 15C, and 23C), and those held wet (14B, 14D, 15D, and 23B). The chi square test for the goodness of fit showed that the thiamine content was a function of the dry matter content, and there was no appreciable loss due to washing, blowing, or draining soon after the oysters were shucked.

Comparable assays were conducted by the thiochrome and the rat growth methods to determine the desirability of using the thiochrome method for assay of fishery products. Two groups of rats were fed the raw oysters at a rate of 1 and 1½ g. per day per rat. It was necessary to use several lots of oysters in the feeding tests. The average thiamine content obtained by the thiochrome method was about 175 micrograms, and that obtained by the rat growth method was about 160 micrograms per 100 g. of oysters. The difference in mean values is about 9 percent which is small enough to indicate reasonably good agreement.

Thiamine in Other Fishery Products

The data in Table 2 were obtained for the thiamine content of other fishery products. The results are expressed to the nearest 5 micrograms per 100 g. of product, and the range in values are given when more than a single sample was assayed.

It may be noted that most fishery products are a fair source of thiamine. Compared with a recommended allowance of 1.8 mg. of thiamine per day for a moderately active man, a serving portion of fish (equivalent to about 100 g.) would supply less than 1/10 of the daily allowance. These values are for raw seafood; cooked and canned products contain less thiamine. Fishery products are in the same category, however, as lean beef and poultry as a source of this vitamin. An interesting observation is that only a comparatively few species of fish contain the anti-thiamine factor in the flesh.

There were no indications of the presence of substances which interfered with the normal development of fluorescence due to thiamine with most fishery

products. The blank samples showed very little fluorescence, and the base exchange treatment did not effect the values. This was not true with samples of canned Atlantic Coast mackerel. The fluorescence of the blanks prepared from the mackerel extract was four to six times greater than that obtained ordinarily. The base exchange treatment did not reduce this value. There was also a considerable increase in fluorescence in the test samples. The difference between these two values was much greater than could be accounted for by the probable vitamin content. In fact, the canned product was seemingly a better source of thiamine than the raw

Table 2 - Data on the Thiamine Content of Some Fishery Products

Fishery Product	Thiamine per 100 g. of Edible Portion	Fishery Product	Thiamine per 100 g. of Edible Portion
	Micrograms		Micrograms
Fresh or frozen:		Fresh or frozen:	
Anglerfish (Lophius piscatorus)	25	Sea robin (Prionotus, species)	80 - 100
Burbot (Lota maculosa)	450 - 460	Shark, dogfish (Squalus acanthias)	50
Carp (Cyrinus carpio)	X*	Skate (Raja, species)	20 - 30
Cod (Gaddus callarias)	40 - 50	Smelt, lake (Osmerus mordax)	X*
Croaker (Micropogon undulatus)	155	Snapper:	
Haddock (Melanogrammus aeglefinus)	100	Gray (Lutianus griseus)	170
Grouper, black (Garrupa nigrita)	160 - 180	Red (" blackfordii)	170 - 180
Herring, lake (Leucichthys artedi)	100 - 115	Swellfish (Spheroides maculatus)	50
Mackerel:		Crab, blue (Callinectes sapidus)	75
Boston (Scomber scombrus)	170 - 200	Mussels (Mytilus edulis)	X*
King (Scomberomorus regalis)	50 - 60	Cooked:	
Spanish (" maculatus)	160 - 200	Salmon, red, baked	85 - 90
Mullet (Mugil, species)	55	Crab, blue, hard shell	60
Muttonfish (Lutianus analis)	40	Crab, blue, soft shell	85 - 100
Pompano (Trachinotus, species)	400 - 425	Canned:	
Salmon, red (Oncorhynchus nerka)	125 - 135	Crab, blue, white meat	None
		Salmon, pink	25

*X means that the anti-thiamine factor was present.

product. When ½ g. a day of mackerel was fed to rats, the probable thiamine content was calculated to be 230 micrograms per 100 g. for the raw, and 26 micrograms for the canned product. The thiochrome method assayed about 200 micrograms per 100 g. for the raw flesh.

The solution to this problem was found in using a method suggested by Mason and Williams (1942). To a 5 to 25 ml. aliquot of the digested extract which had been adjusted to a pH of 5, were added 25 mg. of anhydrous sodium sulfite. The mixture was heated in a boiling water bath for 15 minutes. This aliquot, and an untreated one were given the base exchange treatment. An aliquot of each eluate was oxidized as usual with the potassium ferricyanide solution. The sample treated with sodium sulfite was used as the blank. The thiamine content thus determined was equal to 28 micrograms per 100 g. of canned mackerel flesh, which was comparable to the value determined by the rat growth method, and in the same range as those reported for other canned fish.

THE ANTI-THIAMINE FACTOR

The presence of a thiamine destroying factor in the flesh, and other organs of some species of fish became known because ranch-raised foxes fed raw fish suffered from a deficiency disease called Chastek's paralysis. The disease could

oftentimes be cured by feeding thiamine-rich concentrates, or by injecting large quantities of pure vitamin. It could be prevented by feeding a diet containing cooked fish, or by feeding raw fish on alternate days. The latter system of feeding permitted enough thiamine from the basal diet to be metabolized to prevent the development of symptoms.

The quantity of anti-thiamine factor present could be determined by adding a known quantity of thiamine to a suspension of a ground portion of the test sample. After incubating for several hours at room temperature, the solution was filtered, and the residue was washed. The combined filtrate and wash water was then assayed for thiamine.

It was found that the quantity of thiamine destroyed was dependent on the length of time the flesh was in contact with the vitamin. The destructive factor was easily destroyed by heat, and no thiamine could be detected in the cooked flesh of the fish containing the anti-thiamine factor.

Studies were begun to extract the active factor from carp, since fairly large specimens were readily available. The anti-thiamine factor apparently is concentrated in the viscera, although Green, et al (1942) reported that the head, skin, and fins contained a considerable concentration of this factor. The viscera of the carp were divided into three parts; namely, the spleen, the intestines, and the remainder of the viscera. These portions were finely chopped, and small samples were suspended in a two percent solution of acetic acid containing thiamine, for 18 hours at room temperature. After that period, a determination was made of the amount of thiamine that was destroyed. The incubation period which was arbitrarily selected was too long for a quantitative estimate of activity per gram of sample. It was definitely indicated, however, that the sample minus the spleen and intestines had less activity than the other two samples. This finding was confirmed by Sealock, et al (1943). They reported that an active preparation could be extracted with 10 percent sodium chloride.

In a preliminary experiment, an active preparation was obtained by extracting 320 g. of viscera with 10 percent sodium chloride, and making up to a final volume of one liter. The liquid was filtered from the solid material through cheesecloth. Ten ml. of this solution destroyed 170 micrograms of thiamine in one hour. This was roughly equivalent to 53 micrograms of thiamine per g. of viscera. The solution lost all activity when dialyzed against tap water. The addition of a small quantity of glacial acetic acid in order to attempt to precipitate the factor reduced the activity of the filtrate so only 12 micrograms of thiamine were destroyed per g. equivalent of viscera. Glacial acetic acid could probably be used to precipitate the active factor.

Later, it was deemed desirable to dialyze the extract against ammonium sulfate to precipitate the active fraction, which could then be reprecipitated with acetone. In the first test, the following molar concentrations were used: 1.39, 1.64, 2.05, and 2.60. The filtrates showed a progressively slight decrease in activity as the concentration of ammonium sulfate increased, except for the 2.60 molar concentration which showed a greater reduction of activity of the filtrate. The precipitates obtained from the 2.05 and 2.60 molar concentrations were dissolved

in water and tested for anti-thiamine activity. Neither exhibited any appreciable activity, but the precipitate from the 2.60 molar solution was definitely more active.

The dialysis was repeated with some of the same extract. The original solution had lost a great deal of its activity. Molar concentrations of 2.0, 2.5, and 3.0 were used. An inactive precipitate and an active filtrate was obtained with dialysis in a 2.0 molar concentration. An active precipitate and an active filtrate was obtained with the 2.5 molar concentration. An active precipitate and inactive filtrate was obtained with the 3.0 molar concentration. The activity of the precipitate was only slightly increased between 2.5 and 3.0 molar concentrations, but there was a great decrease in the activity of the filtrate in the latter concentration. These results were also obtained with a freshly prepared extract from carp viscera.

Two lots of viscera, 592 and 900 g., respectively, were extracted with sodium chloride, and the extracts were dialyzed against various concentrations of ammonium sulfate. The active precipitates obtained in the dialysis were, in some cases, dissolved in water and reprecipitated with acetone.

Table 3 - Data on the Extraction of the Anti-Thiamine Factor from Viscera of Carp

Molar Concentrations of Ammonium Sulfate	Yield of Wet Precipitate (from dialyzed filtrate)	Thiamine Destroyed Per Gram of Precipitate	
		½ hour	1½ hours
	Grams	Micrograms	Micrograms
First extract:			
2.0 to 2.4	1.7	-	143
2.4 to 3.0	2.5	165	-
Above 3.0	1.2	43	100
Fractionation of the precipitate obtained in 2.4 to 3.0 molar extraction:			
2.6		very weak	
2.8		-	69
3.0		-	113
Dialysis of another sample:			
2.0 to 2.4	1.4	68	-
2.4 to 3.0	1.1	135	
		160*	
Above 3.0	4.0	35	
		53*	
		80**	

The active precipitates were obtained by dialysis of extracts obtained with 10 percent sodium chloride solutions against ammonium sulfate solutions of given molarity. * and ** were active precipitates which were dissolved in water and re-precipitated with acetone. * was a wet acetone precipitate and ** was the dried acetone precipitate.

The data in Table 3 indicate that a partial concentration of the active material was effected. Due to the war conditions, it became necessary to begin work on problems of greater national importance so no further studies were carried out with the anti-thiamine factor.

SUMMARY

1. Of the samples tested, the thiamine content of shucked oysters varied from 100 to 190 micrograms per 100 grams.

2. Very little, if any, thiamine was found to be leached from shucked oysters by ordinary methods of handling before packaging.

3. Most raw fishery products contained from 50 to 200 micrograms of thiamine per 100 g. of edible material. This is about equivalent to the thiamine content of lean beef or poultry.

4. Comparatively few species of fish had the anti-thiamine factor in the edible flesh.

5. It was possible to partially concentrate the anti-thiamine factor by extracting carp viscera with a 10 percent solution of sodium chloride, and precipitating by dialyzing against a 2.5 to 3.0 molar concentration of ammonium sulfate.

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