

THE THIAMIN CONTENT OF VARIOUS FOODS

by

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INTRODUCTION

Thiamin or vitamin B₁ is one of the chief deficiencies of the American dietary. Evidence of the growing recognition of the importance of this vitamin is indicated by the recent development of "Enriched Flour." Scientists are pointing out that thiamin may be an important factor in maintaining the morale of the people in the present emergency. In addition to thiamin, efforts are being made to restore to cereal products such amounts of vitamins and minerals as may be necessary to restore the food to its level before refining. At present, "Enriched Flour" contains added thiamin, nicotinic acid and iron. Riboflavin, and calcium and phosphorus may soon be required in addition.

Since it is doubtful whether it will be possible to introduce sufficient amounts of thiamin into the diet of the American people by fortification of flour alone, one may expect that the practicability of fortifying other foods, such as sugar, will soon be considered.

Though the fortification of foods such as flour and sugar with crystalline vitamins offers a convenient means of improving the dietary, this is not the only alternative. A large number of food products are in themselves rich sources, not only of thiamin, but many other vitamins and minerals. Some

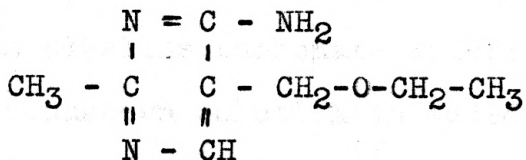
of these are sufficiently familiar to the average housewife to offer no particular problem in their preparation, or in their incorporation into easily made dishes. Others, however, are less familiar and their successful incorporation into the American dietary may require considerable experimentation. Wheat germ, for example, is rich in thiamin, riboflavin, vitamin E and other vitamins and minerals. It should prove highly desirable if methods could be developed for successfully including rather high percentages of this material in cooked cereals, meat loaf and other dishes to make a palatable product. This accomplished, it should be important to know whether significant losses of vitamins are brought about in the preparation of the food.

In the present study, worked out in collaboration with the Food Economics and Nutrition Department, wheat germ was added to cream of wheat, and to meat loaf, in an attempt to increase the thiamin content of the food. Biological assays were carried out in the Food Economics and Nutrition Department and chemical determinations by the Chemistry Department. In addition, the losses incurred in cooking were determined chemically.

REVIEW OF LITERATURE

Fermentation Method

Schultz, Atkin and Frey (1937) discovered that crystalline vitamin B₁ and the synthetic product are powerful accelerators of fermentation. This led to the development of a rapid method of vitamin assay, based on the measurement of carbon dioxide evolved from a reaction mixture containing yeast, sugar, a buffer salt mixture and distilled water. The unknown was added to this mixture. The rate of fermentation was measured by recording gasometer readings of gas evolved at convenient intervals. It was found that 2-methyl-5-ethoxymethyl-6-aminopyrimidine also stimulated fermentation, but rat tests served to show that with the exception of autoclaved yeast there was no natural source of it. The gas method of vitamin assay is a rapid and reliable one for research and industry.



2-methyl-5-ethoxymethyl-6-aminopyrimidine

Colorimetric Methods

In recent years it has been shown that there are chemical reactions of thiamin which lend themselves to the colorimetric and to the fluorometric determinations of the vitamin. Naiman (1937) reported that with thiamin-containing substances bismuth potassium iodide gives an orange-red precipitate which can be filtered, dried and weighed. The weight of the precipitate was found to be proportional to the amount of vitamin used. This is a test for the thiazole nucleus.

Raybin (1938) reported that one milligram of thiamin dissolved in a few cubic centimeters of saturated borax gives an orange color increasing in intensity when a drop of alcoholic 2,6-dibromoquinonechloroimide is added.

The principal colorimetric tests are the azo tests, which are modifications of the Ehrlich-Pauly reaction. Kinnersley and Peters (1934) developed one of the first tests with some semblance of usefulness. Diazotized sulfanilic acid is added to an alkaline carbonate solution and, after a short interval, the unknown solution is added to the diazo solution. The color which is yellow at first soon turns pink and is stable for many days. Kinnersley and Peters (1938) modified the original test by using 30 percent ethyl alcohol

instead of water; by extracting the dye with butyl alcohol and further extracting this extract with hydrochloric acid. In addition, samples containing cocarboxylase were treated with phosphatase. Since highly purified solutions must be used, some of the vitamin is lost in purification. Acetone and reducing agents interfere with the test, as do also some metals such as copper, mercury and silver.

Prebluda and McCollum (1936) found that when a solution of thiamin is treated with diazotized *p*-aminoacetanilide or diazotized methyl-*p*-aminoacetophenone a characteristic purple-red compound is produced which is stable and highly insoluble in water. It is soluble in acetone, amyl alcohol and other solvents and when dissolved therein can be compared in a colorimeter with known amounts of thiamin similarly treated. High salt concentrations and metallic ions influence the effectiveness of the reagent.

Melnick and Field (1939) confirmed the test described by Prebluda and McCollum (1939) and reported that the purple-red compound could be quantitatively extracted with xylene and that the extract lent itself to colorimetric evaluation. These workers used permutit as a means of isolating thiamin from interfering substances. The method is specific for free thiamin and not for the phosphorylated form. Melnick and Field have more recently described a method for hydrolyzing the thiamin pyrophosphate to free thiamin by incubation with active yeast powder containing a phosphatase.

Emmet, Peacock and Brown (1940) modified somewhat the Melnick-Field chemical method for determining thiamin quantitatively by substituting the Lovibond tintometer for the colorimeter, and by replacing the complicated permutit adsorption and elution steps with a simpler procedure using superfiltrol. Riboflavin, nicotinic acid, pyridoxine and pantothenic acid do not interfere even when present in comparatively large amounts. Ascorbic acid in excess gives low values, but this can be overcome by adsorption with superfiltrol. In general with simple and complex mixtures, the method gives quantitative results which are in conformity with data obtained by bioassay methods.

Willstaedt (as cited by Roth, 1938), demonstrated the formation of a yellow pigment, which could be extracted with ether from the reaction of thiamin and 2,4-dichlorobenzene-diammoniumchloride. The pigment was adapted to chromatographic adsorption. Willstaedt and Bárány (1938) described a similar method which depends upon the formation of an ether-soluble azo dye by the reaction of diazotized 2,4-dichloroaniline with vitamin B₁ in an alkaline solution and the detection of the color in a Pulfrich photometer.

Thiochrome Assay

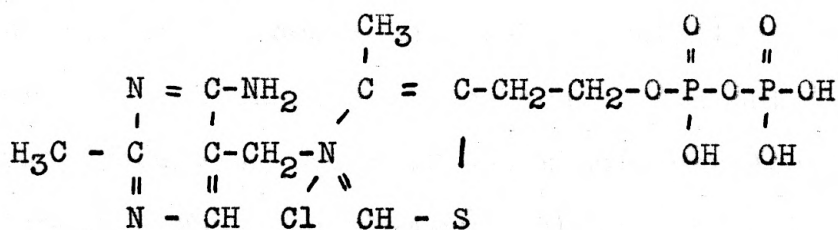
Jendrassik (1923) found that solutions containing

Jansen (1936) studied the quantitative transformation of thiamin to thiochrome in alkaline solution by quantitative oxidation with potassium ferricyanide, extraction with isobutanol and estimation of intensity of fluorescence by comparison in a Cohen fluorometer. Certain substances interfered with this reaction, since complete recovery of thiamin added to urine could not be obtained. The method also involved the use of special apparatus and the results varied as much as twenty percent from the bradycardia method of rat assay. It is the most exact chemical method to date.

Karrer and Kubli (1937) modified the procedure with a standard of comparison prepared by the oxidation of thiamin to the fluorescent thiochrome. By this method results varying only 20 percent from those given by the electrocardiographic method of rat assay were obtained.

Westenbrink and Goudsmit (1938) developed a method for determining the thiamin content of body tissues and fluids, particularly urine. They recognized the difficulties in thiamin determination of urine caused by disturbing substances, foreign materials, atmospheric oxidation and fluorescent colorations originally present. These difficulties were largely overcome by working with dilute solutions, by adsorption on franconite and elution prior to oxidation in an atmosphere of nitrogen, and by allowing for the fluorescence of untreated eluates.

Pyke (1937) made a rather extensive study of the thiochrome method as applied to the determination of thiamin in a wide range of foodstuffs and biological materials. Westenbrink and Goudsmit (1938) reported that there was a large discrepancy between the results of biological assay of thiamin and the original thiochrome method of Jansen when both methods were applied to animal tissues. Their belief was that Pyke had estimated only a small part of the total amount of vitamin B₁ actually present in the animal tissues which he investigated, as the thiamin is present in two forms in animal tissue. Westenbrink and Jansen (1938) described the determination of the two forms of vitamin B₁, thiamin and cocarboxylase or thiamin pyrophosphoric acid, separately by means of the thiochrome method. The thiochrome formed from thiamin is extracted quantitatively from the aqueous mixture by isobutanol, while the thiochrome pyrophosphoric acid from the cocarboxylase remains quantitatively in the aqueous layer.



Cocarboxylase

Westenbrink and Goudsmit applied this method to various animal tissues. They used pepsin to digest the tissue

proteins previous to the adsorption of the vitamin on franco-nite. This helped to reduce the "blank" fluorescence of the material before oxidation with potassium ferricyanide.

Hennesy and Cerecedo (1939) reported success in the elimination of non-specific fluorescence by employing synthetic base exchanging zeolites for adsorbing the vitamin. By the use of a more sensitive fluorometer they determined 0.1 microgram of thiamin, and by employing an enzyme preparation obtained from the kidney they succeeded in assaying the cocarboxylase content of various foods and biological materials obtaining results agreeing with biological tests.

Biological Assay

Since it has been the purpose of this paper to study the applicability of chemical methods to the analysis of food materials, the bioassay technique will not be elaborated to a great extent. Sister Rose Genevieve, a graduate student in the Department of Food Economics and Nutrition, will present a paper on the bioassay, giving the values obtained on studies with rats receiving cream of wheat and meat loaf, both with and without a wheat supplement. The increase in thiamin content due to the inclusion of wheat germ will thus have been determined.

Bioassays have been carried out with rats and pigeons.

Several methods for rat assay have been described by Coward (1938). Response in rats may be measured by their increase in weight, by the cure of convulsions, or by the cure of bradycardia. In the former there is usually a steady increase in weight during the first ten to fourteen days of feeding on the thiamin-free diet, during which time the rats are weighed twice a week. In the test period, three weeks in duration, daily doses of the test substance are given. Yeast concentrates should give a prompt and complete recovery of polyneuritis, or convulsions, with a remission in 3 to 15 days, depending upon the size of dose. Paralysis will recur again if no more vitamin is administered and it may be alleviated by additional doses. The paralysis is characterized by lameness of the hind and forelimbs, incoordination, spastic gait and cartwheel and rolling movements. A curve of response should be used for working out the results.

In the bradycardia method 40 gram rats are given a thiamin-free diet and after three weeks electrocardiograms are taken. Four groups with at least five rats for a group are used as in the above method. A single dose of the standard is given each rat with the diet as before. Electrocardiograms are taken again 24 hours after dosing. If the rate is not increased, a larger dose is given. There should be a rise to normal in 24 hours and a gradual slackening.

In the cure of convulsions method used by the Food Economics and Nutrition Department young rats were chosen, weighing from 50 to 60 grams. The young rats were kept with the mother rat during the preparation period. The rats were kept separately throughout the test period in ordinary wire cages. The rats were placed several inches above the floor of the cage to prevent access to their faeces, as the faeces contain thiamin synthesized in the rat body. If the rats obtained this material, the experiment would be ruined. The rats placed on the thiamin-free diet were weighed weekly until they ceased gaining or were losing weight. At that time the rats were weighed daily until they showed signs of polyneuritis, characterized by convulsions and paralysis. The rats were then injected with a standardization dose of two International Units, or six micrograms, of thiamin, causing the rats to gain 5 to 8 grams which should last about ten days. After the rats again developed polyneuritis, an amount of food to be tested was given so that the rats would gain approximately the same amount as on the standardization dose.

All of these methods but the "increase in weight" method are specific. The cure of convulsions in rats takes longer, but if there is a large supply of rats in preparation the test can be completed in a short time. The cure of bradycardia takes three weeks of preparation and one dose of the

test substance to each rat. The length of time to complete a cure is the same as the cure of convulsions. Also the cost of the electrocardiograph is prohibitive for many laboratories. The "increase in weight" method requires two weeks for preparation and then daily or one-half weekly doses for two weeks at least. It requires a longer time for results than for other methods. This method requires more labor for giving doses and is not specific for thiamin, as there are other factors to take into consideration. This method is more accurate than the other methods but is not so specific.

Pigeons develop retracted neck without thiamin. For pigeons 0.03 gram of the fuller's earth adsorption product of an extract of rice polishing, or three units of thiamin, is given, and this should give a fifty percent cure of the pigeons with retracted neck. The amount is weighed on a watch glass, mixed with distilled water and placed in the pigeon's neck.

A simultaneous test of the standard of reference should be made whenever a determination of thiamin is made, the object being to find a dose of the substance examined to give an equal response. The desired response is about half-way between no response and "complete response".

EXPERIMENTAL PROCEDURE

As has been shown there have been many methods, chemical and biological, worked out for the determination of vitamin B₁. The biological methods have unavoidable limitations to which the chemical methods are not subjected. These have been discussed elsewhere. The chemical methods are based on the measurement of the concentration of substances produced from the vitamin in standardized procedures.

Since colorimetric methods have been shown to be undesirable, and since a sensitive fluorophotometer of the Pfaltz and Bauer type was available, it was thought best to use the fluorophotometer. The thiochrome method seemed to have the best possibilities for determining the foods to be used in this study. Modifications of the method of Hennessy and Cerecedo (1939), an adaptation of the method of Jansen (1936), and that of Merck and Company (1940) were used, as well as the modifications later described by Hennessy (1941). A few modifications in these methods were also made.

Much of the time was spent in working out the technique, so that the determinations would be highly sensitive. In the early part of the experimentation period, much time

was lost in learning the use of the fluorophotometer. However, this was done so that as many of the sources of error as possible could be overcome, and so that uniform results could be obtained.

The Fluorophotometer

The fluorophotometer manufactured by Pfaltz and Bauer was used for the determination of thiamin. When used for fluorometric means it operates upon the following principle, as explained by Loewenstein (1940-41):

The exciting beam from a mercury vapor lamp passes through the solution in the cuvette causing it to emit a bluish fluorescence with thiamin. This strikes a photocell which transforms the light into electric energy. This photoelectric current is registered on a galvanometer. Since the intensity of fluorescence depends upon the intensity of the exciting light and upon the concentration of the fluorescent material, this concentration can be determined by using a fixed exciting intensity. The galvanometer deflection is generally in linear relationship to the concentration of the fluorescent material within the limits used.

The exciting light source is an 85 watt mercury capillary arc enclosed in a protective glass bulb. Since the fluorophotometer works on the principle of a constant light intensity, the standardization of the instrument must be made after the mercury arc reaches its maximum in respect to light intensity. This intensity is reached approximately 10 to 15 minutes after the lamp is lighted. As the age of the lamp

progresses, its original light intensity gradually diminishes so that a daily standardization of the fluorophotometer in terms of fluorescence of a quinine sulfate standard should be made.

The "zero reading" on the galvanometer must be checked before all readings. The galvanometer should be kept in the off position when readings are not being made.

Loewenstein (1940-41) further described the fluorophotometer:

For thiamin determination an ultra-violet transmitting filter with a peak of about 3700 Å is used, which filters out all visible light. Between the glass cuvette and the round photocell, two spectral filters with a peak at approximately 4600 Å are placed, which permit a high transmission of the fluorescent light. The yellow filter cuts off all the ultra-violet light and bluish-green filter all light of wave-lengths above 4700 Å. With these filters, the results obtained are in accordance with Lambert-Beer's law. The fluorophotometer may be calibrated by means of a standard solution of various concentrations of quinine sulfate or pure thiamin hydrochloride.

Standardization of the Fluorophotometer

Following is the method used for the standardization of the fluorophotometer.

A cuvette was filled with the "quinine sulfate working standard" (made by dissolving 0.0108 gram of quinine sulfate in a liter of 0.1 N H₂SO₄ and diluting this, one part to three of 0.1 N H₂SO₄). The cuvette was placed in the

cuvette chamber and the lid closed. With the switch in the F1 position, the deflection on the galvanometer was adjusted to some arbitrarily selected position (17 gave good results) by opening or closing the iris diaphragm (to about 30) in front of the light housing. This value had to be checked each day, always seeing that the galvanometer rested at zero between the readings. The instrument was standardized in terms of units deflection per microgram of thiamin hydrochloride. The technique of oxidation, extraction of thiochrome and reading of the instrument was identical with that employed in the actual determination.

Into each of three 60-cc. separatory funnels 4 cc. of distilled water was introduced along with 1 cc. of thiamin hydrochloride working standard, containing 10 micrograms of thiamin hydrochloride per cc. The latter was measured accurately with a one cc. pipette. The solutions were then mixed. The reacting vessels were designated as No. 1, No. 2, and No. 3. Three cc. of mixed reagent (15 percent NaOH, containing 0.1 cc. of 1 percent potassium ferricyanide in each three cc. of mixed reagent) were added to the first two vessels and mixed. Twenty cubic centimeters of isobutyl alcohol was added and the solution shaken for 1.5 minutes, using a stop watch. To No. 3 were added 20 cc. isobutanol and 3 cc. of 15 percent NaOH after which it was immediately shaken vigorously. No. 3 vessel was the blank and compen-

sated for fluorescence present in the reagents. The routine of adding the reagents and timing of their addition must not be varied. Timing at this oxidative stage was critical; the entire process had to be carried through to completion without delay in any of its stages. All the reagents were added to one vessel and the shaking completed before starting another.

The alcoholic and aqueous phases were allowed to separate. In carrying out a series of oxidations this occurred while the samples in other vessels were being oxidized. The lower aqueous layer was drawn off and the alcoholic layer filtered through a funnel containing one-half inch of anhydrous sodium sulfate crystals in the filter paper. This took out the water and clarified the alcohol layer containing the thiochrome.

The cuvette containing the extracted thiochrome in isobutanol was introduced into the cuvette chamber and the lid closed. The switch was thrown onto the F1 position and the deflection read on the galvanometer. The value recorded was the one assumed at its first momentary position of rest, since thiochrome is destroyed by ultra-violet light.

The galvanometer values for each of the three solutions were obtained one directly following another under identical conditions.

Table 1 gives calculations which are typical of values obtained following the technique described above.

Table 1. Standardization calculations.

Reaction vessel No. 1	Reaction vessel No. 2	Reaction vessel No. 3
10.0 microgr. vit. B ₁	10.0 microgr. vit. B ₁	Blank
21.10	21.15	8.35
8.35 (blank)	8.35	
$\frac{12.75}{10} =$	$\frac{12.80}{10} =$	
1.28 units per microgram	1.28 units per microgram	

In this case the deflection due to one microgram of thiamin hydrochloride was 1.28 units on the galvanometer scale. This entire standardization procedure was performed each day along with the other tests.

In the oxidation procedure, instead of carrying out the tests in 25-cc. glass-stoppered graduated cylinders as suggested by Hennessy and Cerecedo (1939) or in centrifuge tubes as suggested by Merck and Company, 60-cc. separatory funnels were used, and operations carried out in them. Pipettes were used to measure the quantities of the various reagents added. This eliminated the use of several different vessels to carry out the tests. It was found in these cases that centrifuging was not necessary, as, in carrying out several tests at a time, the isobutanol and water layers had time to separate without centrifuging. Usually three or four samples with two determinations each were carried out

at one time. Using the separatory funnels, the water layer was drawn off and the isobutanol layer run through a funnel containing anhydrous sodium sulfate. Twenty cc. of isobutanol was used instead of 13 cc. as suggested in several of the procedures. This was done so that the cuvette of the fluorophotometer might be filled for the readings and thus variations avoided.

Steps in the Assay Procedure

The assay procedure may well be considered a four-step operation involving first, the extraction of the vitamin from the sample; second, purification and concentration of the extracted vitamin through a base exchange step; third, the oxidation of thiamin hydrochloride to thiochrome by potassium ferricyanide in an alkaline medium, extracting the thiochrome formed by isobutanol; and lastly, the quantitative determination of the thiochrome so formed by measuring the intensity of the violet-blue fluorescence in ultra-violet light by means of the fluorophotometer.

Extraction Procedure

The foods which were tested in this series of determinations were wheat germ, cream of wheat with and without wheat

germ added to it, and meat loaf with and without added wheat germ. In addition some of these foods were analyzed for their thiamin content both cook and uncooked.

Different methods of extracting or digesting the test materials were tried in an effort to determine which was the best. In the early tests the materials were refluxed in a water or steam bath for two thirty-minute periods with 2 percent acetic acid. In cases of gelation where good separation did not occur the 2 percent acetic acid solution was made 30 percent with alcohol (Hennessy and Cerecedo, 1939). Autoclaving at 120° C. (15 pounds pressure for twenty minutes) has also been used by some workers. Later it was pointed out by Hennessy (1941) that refluxing and heating in a boiling water bath with 0.1 N sulfuric acid has been found to be sufficient if the ratio of extracting liquid per gram is from 15 to 50 cc. per gram.

From 2 to 5 grams of the sample were accurately weighed and suspended in 60 to 70 cc. of 0.1 N sulfuric acid, or so that the pH was 3. It was desirable to use enough of the sample to yield approximately 20 to 40 micrograms of vitamin B₁ in the final volume (100 cc.) of extract. The mixture had to remain acid during the digestion period, as thiamin hydrochloride was readily destroyed in alkaline solution. The mixture was digested with a reflux condenser in a boiling water bath for one hour.

As the thiochrome assay was performed there were three alternate procedures: direct assay (Method I), assay following base exchange (Method II), and assay following enzymatic hydrolysis with or without base exchange (Method III). Method I was used where the blank was comparatively small and the recovery of vitamin, added in equal quantity to that present was quantitative. There should be left in the aqueous layer, following extraction, not more fluorescence (observed visually) than in the same layer of a sample containing an equal quantity of pure vitamin B₁. If direct assay was not practical Method II was used. When obvious residual fluorescence was noted in the aqueous layer, Method III was employed. In these experiments Method II was used at first, but it proved to be unsatisfactory, so that Method III with base exchange was used finally.

Method I. Direct Assay

Three 60-cc. separatory funnels were used for each determination. No. 1 contained the sample (diluted so that 5 cc. contained approximately 1 to 5 micrograms of thiamin hydrochloride); No. 2 contained the same amount of sample plus 10 micrograms of vitamin B₁; and No. 3 was the blank, containing the same amount of sample and all the reagents but potassium ferricyanide, and it gave the fluorescence due to the reagents used. The difference in galvanometer

readings of Nos. 1 and 2 equaled the deflection due to 10 micrograms of thiamin hydrochloride. In this way one was assured that the sample was neither under- nor over-oxidized.

If the approximate thiamin hydrochloride content was not known, a preliminary determination was carried out. Then an aqueous dilution (pH 3 to 4) of the original solution or material was prepared so that 5 cc. contained from 1 to 2 micrograms of thiamin hydrochloride.

For the direct determination the sample and reagents were added, by pipette, in the order given in Table 2, completing one before starting another. The aliquot and the standard vitamin B₁ were added to the reacting vessels before adding the reagents. After adding the reagents they were shaken 1.5 minutes, and the procedure was continued as directed in the "Standardization of the Fluorophotometer" from "The alcoholic and aqueous phases.....etc."

Table 2. Method I. Direct assay procedure and calculations.

Reaction vessel No. 1	Reaction vessel No. 2	Reaction vessel No. 3
5 cc. aliquot	5 cc. aliquot	5 cc. aliquot
3 cc. mixed reagent	10 microgr. vit. B ₁	20 cc. isobutanol
20 cc. isobutanol	3 cc. mixed reagent	3 cc. 15 per- cent NaOH
	20 cc. isobutanol	

Calculations

12.15
 $\frac{8.35}{3.80}$ (blank)
 3.80 units for
 sample

25.00
 $\frac{12.15}{12.85} \div 10 =$
 1.28 units per
 microgram

8.35

$\frac{3.80}{1.28} = 2.97$ or 3.0 micrograms vitamin B₁ found in 5 cc of the aliquot. This volume multiplied by the dilution factor will yield the vitamin B₁ content of the material.

Method II. Base Exchange Procedure

In the base exchange procedure a portion of the sample was run through a column of especially prepared zeolite, decalso or other adsorbent, to adsorb the vitamin and thus get rid of some of the substances which interfered with the thiochrome test.

Zeolite was prepared for use by boiling it with 2 percent acetic acid while stirring, allowing it to settle, and decanting the supernatant liquid. It was washed in the same way with 25 percent potassium chloride solution, followed by four or five washings with distilled water to remove all the potassium chloride. The zeolite was dried at about 100° C. by spreading the material out to hasten the drying process. The "prepared zeolite" was stored in suitable containers until ready for use. It was customary and desirable to check the percentage recovery of thiamin hydrochloride on each new prepared stock of zeolite.

A sufficient number of base exchange tubes was available so that several samples could be run at once. The tubes were made by welding a 15 mm. hard glass test tube onto 12 mm. hard glass tubing. A small roll of fiber glass was placed in the bottom of the stem and approximately 65 mm. of the prepared zeolite added. It was tapped gently to "set" the zeolite, and then washed with boiling distilled water. The deliver was approximately 1 cc. per minute.

An aliquot of the original dilution or extract was measured accurately into a 60-cc. Erlenmeyer flask, to yield approximately 10 to 20 micrograms of vitamin B₁. In some cases 40 to 45 cc. were used. Usually two or three portions of the 100 cc. extract were passed through columns according to these directions in order to give checks. The solution

was heated to 80° C. and passed in small portions through the hot zeolite bed. The flask was rinsed with boiling distilled water and passed through the zeolite bed when all the original solution had disappeared. The bed was washed with boiling distilled water, using at least three 5 cc. portions, allowing each to drain thoroughly before adding more.

The thiamin hydrochloride was eluted with small portions of boiling 25 percent potassium chloride solution in 0.1 N hydrochloric acid, and 25 cc. of eluate was collected in a graduated cylinder and mixed thoroughly. Two determinations were made, using three 4 cc. portions for each. This gave checks for the oxidation which was carried out as described under the direct determination procedure.

Calculations for determining the thiamin content per gram are given in Table 3.

Table 3. Calculations for the base exchange procedure.

Reaction vessel No. 1		Reaction vessel No. 2		Reaction vessel No. 3
12.15		24.90		8.31
<u>8.31</u> (blank)		<u>12.15</u>		
3.84		<u>12.75</u> ÷ 10 =		
		1.28 units per		
		microgram		
<u>3.84</u>	x	<u>25</u>	x	<u>100</u> = 93.75 micrograms
1.28		4		20
				in original
				sample
galvanometer	dilution	25-cc. aliquot		
deflection	factor for	through base		
ratio	eluate	exchange		
<u>93.75 micrograms</u>	=	18.55 micrograms of vitamin B ₁		
5.0 grams orig.		per gram of original		
sample		sample		

Method III. Enzymatic Hydrolysis

The occurrence of thiamin in some sources as a phosphoric ester made necessary an enzymatic hydrolysis which followed immediately after the extraction. It has been shown that dried yeast may contain as much as 75 percent of its thiamin in the esterified form, while in rice polish and wheat germ, the major portion exists as the free vitamin (Melnick and Field, 1939). Takadiastase, requiring an incubation period of at least two hours at 45° to 50° C., has

been used by various workers, and was used in the last of the determinations made in this study. Maintenance by a buffer of the solution at the pH of the optimum activity of the phosphatase was necessary during the incubation period. The takadiastase broke down the starch molecules as well as converted the thiamin esters into the free vitamin.

After the material had been extracted with 0.1 N sulfuric acid as described above, it was cooled, and the pH adjusted to 4.0 to 4.5 by adding 0.1 volume of 1.2 M sodium acetate solution to the extraction mixture. The enzymatic hydrolysis was brought about in 2 to 5 hours at 45° C. by using 0.5 percent takadiastase at pH 4.0 to 4.5. This step was not necessary, possibly, for all foodstuffs, but it was used for all of the later determinations described in this paper. The first tests were made without takadiastase. After the hydrolysis, the total volume was made up to 100 cc. and centrifuged, after which the supernatant liquid was decanted from the solid materials left. This was then carried through the base exchange procedure and the oxidation steps, as described previously.

Preparation of Samples

Cream of wheat supplemented with wheat germ was prepared in the Department of Food Economics and Nutrition as follows: cream of wheat, 70 grams; wheat germ, 50 grams;

and water, 696 grams; giving a total uncooked weight of 816 grams and a finished product of 733 grams. Moisture determinations were made on it as well as other samples, in order that they might be compared on a moisture-free basis.

The plain meat loaf was prepared as follows: ground beef, 125 grams; ground pork, 62.5 grams; egg, 25 grams; and tomato juice, 30 grams. The supplemented meat loaf contained, in addition, 25 grams of wheat germ. The meat loaves were cooked at an oven temperature of 350° C. for 50 minutes. Samples of both the cream of wheat and the meat loaves were used in the biological assay. In the latter the samples were analyzed both cooked and uncooked to determine the losses in cooking. Uncooked wheat germ was analyzed also.

RESULTS

Several analyses were run on the different foods. At first the same zeolite was used over and over. However, this proved unsatisfactory, due to incomplete recovery of the vitamin. It was found that thiamin left from one extraction was carried over to the next sample passed through the column. Thus new zeolite was used each time. To determine the effect of smaller division of sample particles on the extraction, the wheat germ and cream of wheat were

ground in a Wiley mill. Finally, due to the large amount of fluorescence in the aqueous layer after the oxidation of thiamin, the enzyme, takadiastase, was used for hydrolysis of the cocarboxylase. The final series of tests made use of takadiastase.

In the early tests where the zeolite was used several times uncooked cream of wheat was found to have from 2.0 to 3.8 micrograms of vitamin B₁ per gram. However, in the cases in which fresh zeolite was used for the adsorption columns following hydrolysis with takadiastase the blank reading was larger than the reading with the sample in every case, so it was concluded that the cream of wheat which was used contained no thiamin hydrochloride.

Wheat germ gave the values which are shown in Table 4.

Table 4. The thiamin content of wheat germ using different methods of determination.

Sample	Original moisture content-7.41 percent	Moisture-free basis	Percent extraction
Wheat germ	15.00	16.15	37.25
Wheat germ, ground	23.06	24.84	57.27
Wheat germ and takadiastase	40.26	43.73	100.00

With the addition of wheat germ the uncooked cream of wheat, on a moisture-free basis had 10.23 micrograms of

vitamin B₁ per gram as compared to 12.32 micrograms after grinding and 17.47 micrograms, using takadiastase. The cooked cream of wheat supplemented with wheat germ showed 5.46 micrograms per gram on a moisture-free basis and 8.13 micrograms per gram using takadiastase.

From these figures it can be seen that the finer the particles of wheat germ used in the extraction procedure, the greater was the extraction of thiamin hydrochloride. No values were obtained for cream of wheat cooked by itself. Enzymatic hydrolysis proved to be very necessary to obtain a greater percentage yield of thiamin.

The values for the cream of wheat supplemented with wheat germ indicated a 46.6 percent loss in cooking when analyzed without the takadiastase hydrolysis and a 53.5 percent loss in cooking when analyzed using takadiastase. However, the increased extraction gave a higher value for the latter, in spite of the seemingly higher loss in cooking.

Only one figure, that for cream of wheat supplemented with wheat germ, was available from the bioassay which is being carried out in the Department of Food Economics and Nutrition. The value was 1.71 micrograms of thiamin per gram of cream of wheat with wheat germ. Using takadiastase 1.56 microgram per gram was obtained with the chemical assay, showing an 8.7 percent difference in the two methods. Other figures from the bioassay were not available.

The values obtained from the meat loaf samples were not as satisfactory as those from the wheat germ and cream of wheat samples. That is, the readings of the isobutanol layer containing the thiochrome did not check with each other very well on the same sample of meat loaf. In several cases the uncooked meat seemed to have some substances present which interfered with the thiochrome determination.

On the moisture-free basis the uncooked meat loaf without wheat germ contained 25.03 micrograms of vitamin B₁ per gram as compared with 11.9 micrograms per gram in the cooked meat loaf, showing a 52.38 percent loss in cooking. The meat loaf with the wheat germ, on the same basis, showed a gain of 8.54 percent in thiamin content on cooking when analyzed without takadiastase, but a loss of 28.4 percent with the enzyme. The latter value is undoubtedly more nearly correct.

The analysis showed a loss of 9.75 percent by adding the wheat germ to the uncooked meat loaf, but a gain of 35.6 percent in the cooked product.

There are a number of places where errors might have been introduced into the results. It has been shown that the size of the particles influenced the extraction of the vitamin, as did also the hydrolysis by an enzyme.

In the early determinations the volume of the liquid at the end of the extraction procedure was measured after centrifuging the extracted material rather than before as was

done in the last series of determinations. In this way there is a possibility that some of the vitamin remained in the particles after extraction and that this was not accounted for.

There was not a complete recovery of the vitamin from the zeolite columns as was indicated by a recovery check. That is, a definite amount of thiamin hydrochloride was run through the column and the oxidations carried out as with the regular samples. Recoveries of 80 percent, 86.8 percent and 91.7 percent were obtained from zeolite prepared at different times. Each new batch of zeolite prepared was tested in this way. In the calculation of the results these were taken into consideration and the corrected values were presented in the data.

The routine of adding the reagents was carried out the same in each case, but there was a possibility that the timing varied a little from time to time. This would mean that the sample was over- or under-oxidized, causing some variation in the results. Care was taken, however, to avoid this as much as possible.

The fluorophotometer was possibly the biggest source of error, as at times it seemed to vary in the readings. As the age of the mercury lamp progressed its original light intensity gradually diminished. For this reason a daily standardization in terms of fluorescence of quinine sulfate was made. Each time the fluorophotometer was allowed to reach its maximum of light intensity which required 10 to 15

minutes after the lighting of the lamp. A change was often noted while making readings in any one day, but since the three readings for each determination were made, one following the other, in the same order each time, there was not a great possibility of error.

SUMMARY

The thiamin contents of cream of wheat, meat loaf, cream of wheat with added wheat germ, and meat loaf with added wheat germ, cooked and uncooked, have been determined by the thiochrome method.

Various modifications in existing methods have proved advantageous. Best results were obtained by the use of an enzyme, takadiastase, for the liberation of free thiamin from thiamin pyrophosphate. In the event the enzyme is not to be used samples should be ground very finely before digestion.

In the cases reported, cooking resulted in losses of from 28 percent to 53 percent. The addition of wheat germ to cream of wheat and to meat loaf gives a palatable product with an increased thiamin content.

Table 5. Thiamin content of cream of wheat, wheat germ, and meat loaf, alone, and in combinations, cooked and uncooked.

Sample	Fresh uncooked ¹				Fresh cooked				Moisture-free uncooked ³				Moisture-free cooked				Losses in cooking percent	
	Thiamin		Moisture		Thiamin		Moisture		Thiamin		Moisture		Thiamin		Moisture			
	per gram	No. of	percent	per gram	No. of	percent	per gram	No. of	Date	per gram	No. of	Date	per gram	No. of	Date			
	Micro-grams	Units	Detns.	Micro-grams	Units	Detns.	Micro-grams	Units	Detns.	Micro-grams	Units	Detns.	Micro-grams	Units	Detns.			
Cream of wheat, ground ²	-	-	3	7.58			0			-	-	3	2/21					0
Cream of wheat ²	3.79	1.2	5	7.58			0			4.10	1.3	5	1/21					0
Cream of wheat	2.10	0.7	2	7.58			0			2.27	0.8	2	2/21					0
Cream of wheat and takadiastase	-	-	4				0					4	4/25					0
Wheat germ	15.00	5.0	3	7.18			0			16.15	5.4	3	1/20					0
Wheat germ, ground	23.06	7.7	4	7.18			0			24.84	8.3	4	2/21					0
Wheat germ, with takadiastase	40.26	13.4	3	7.18			0			43.37	14.5	3	4/25					0
Cream of wheat and wheat germ	9.48	3.2	3	7.41	0.818	0.3	4	85.04		10.23	3.4	3	2/21	5.46	1.8	4	1/14	46.63
Cream of wheat and wheat germ, ground	11.41	3.8	4	7.41			0			12.32	4.1	4	2/21					
Cream of wheat & wheat germ with takadiastase	16.17	5.4	3	7.41	1.56	0.5	3	80.93		17.47	5.8	3	4/25	8.13	2.7	3	4/25	53.47
Meat loaf with takadiastase	10.83	3.6	4	56.31	5.90	2.0	3	50.50		25.03	8.3	4	4/25	11.92	4.0	3	4/25	52.38
Meat loaf and wheat germ	7.43	2.5	4	64.52	8.73	2.9	4	61.52		20.94	7.0	4	3/24	22.73	7.6	4	3/12	8.54% gain
Meat loaf and wheat germ with takadiastase	11.08	3.7	3	50.96	8.09	2.7	4	50.07		22.59	7.5	3	4/25	16.17	5.4	4	4/25	28.42

¹ For convenience in the preparation of the table the heading "Fresh uncooked" refers to the sample in its original moist basis even though that moisture was low.

² Passed through a column which had been used before. Slight recovery of thiamin from the zeolite. Blank larger than sample in many cases.

³ Samples were placed in a drying oven at 100° C. for three days to drive out all the moisture present.

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