

THE DETERMINATION OF THIAMINE
AND ITS PHOSPHATE ESTERS
IN CEREAL GRAINS



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ABSTRACT

The method recently developed by Rindi and De Giuseppe for the determination of thiamine and its mono-, di-, and triphosphates in animal tissues utilizes cholesteryl stearate treated charcoal for purification of the extract and Dowex -1 and Amberlite IRC -50 resins for separation of thiamine and its various phosphate esters. This method was tested on rabbit liver by addition of known quantities of these substances (except triphosphate) to the tissue extract and determining the amount recovered. The method was not strictly quantitative as shown by recoveries of over one hundred per cent.

The method was then applied to different samples of wheat, oats and barley and was found not to be quantitative because of recoveries of less than one hundred per cent. Nevertheless, the method does indicate trace amounts of these esters in the samples studied. The purification steps of this method and the paper chromatographic method of Siliprandi and Siliprandi were used together but no conclusions could be drawn due to excessive tailing resulting presumably from insufficient purification.

The "Revised Thiochrome Procedure" for the determination of free and total thiamine in cereal products developed by Bechtel and Hollenbeck was used on the above samples, and the results indicated a greater amount of total thiamine than was revealed by the method of Rindi and De Giuseppe.

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⁺ Method of Bechtel and Hollenbeck (1).

[†] Method of Rindi and de Giuseppe (7).

INTRODUCTION

Vitamins are those dietary essentials that are frequently referred to as "accessory food factors". Vitamin B₁ or thiamine occurs in cereals both in the free and in a combined form (1). Tauber (2) has shown that only a very small fraction of the total thiamine is present as co-carboxylase in vegetables and fruits. Yeast, however, may have as much as 75% of its thiamine as co-carboxylase (3). Pyke (4) claimed that proteolytic digestion was required for the complete liberation of thiamine from natural products, but it has been observed (6) that milk is the only material which must be treated with papain or pepsin. The combined thiamine in milk was not in the form of co-carboxylase but a complex which was not dialyzable and had to be hydrolyzed with a proteolytic enzyme (5). Others have used proteolytic digestion and claimed that it was necessary for the complete extraction of thiamine in cereal products and meats, but none of these workers presented any evidence showing that the proteolytic digestion actually was essential (6).

Thiamine forms a co-enzyme which takes part in many biochemical reactions. This co-enzyme, cocarboxylase, described usually as thiamine diphosphate or thiamine pyrophosphate, combines with an apoenzyme (a specific protein) and metal ion to form the carboxylase system. This enzyme catalyzes simple decarboxylation or oxidative decarboxylation reactions and also takes part in the metabolism of α -oxycarboxylic acids (e.g.

pyruvic acid).

It has been shown that thiamine can be made to form a number of phosphates in the laboratory, mono-, di-, tri-, tetraphosphate and so on. The most common of the thiamine compounds, however, is the diphosphate or cocarboxylase mentioned previously. The question arises whether the combined form of thiamine in cereal grains includes thiamine phosphate esters.

Recently, Rindi and de Giuseppe (7) developed a method for the quantitative assay of thiamine and its mono-, di- and triphosphates in animal tissues. They investigated rat tissue and found that thiamine diphosphate was the most abundant thiamine phosphoric ester, but small amounts of thiamine mono- and triphosphate were also detected. It seemed desirable to apply this method to cereal grains to determine whether thiamine phosphates also occur in them. Prior to this, however, the reliability of the method should be tested by determining the recovery of known amounts of added thiamine and thiamine esters, first from animal tissue and then from cereal grains. It is desirable to compare the results obtained by this method with those of another accepted method. This is the investigation this thesis describes.

REVIEW OF LITERATURE

Historical

There is still considerable work being done on methods of analysis for thiamine as shown by the papers which continue to appear. Around 1940 the activity in this field was at a peak. The main factors which prompted this widespread interest were the concerted efforts to isolate the vitamin during the early part of the century, the necessity of evaluating foods for their vitamin content and the desire to set up analytical methods for checking the vitamin content of enriched foods and vitamin preparations.

There are an infinite number and variety of methods for the analysis for thiamine. Prior to the isolation of crystalline thiamine, the ones most commonly used were animal assays but the time and labor involved in these have resulted in their gradual replacement by other procedures, particularly chemical ones. At present the thiochrome technique is one of the few assays that is being used as much as, or more than, when it was developed.

Table I shows the chemical methods used up to the present time (18, 19). The following abbreviations are used throughout this thesis:

- T - Thiamine
- TMP - Thiamine monophosphate
- TDP - Thiamine diphosphate
- TTP - Thiamine triphosphate

TABLE I

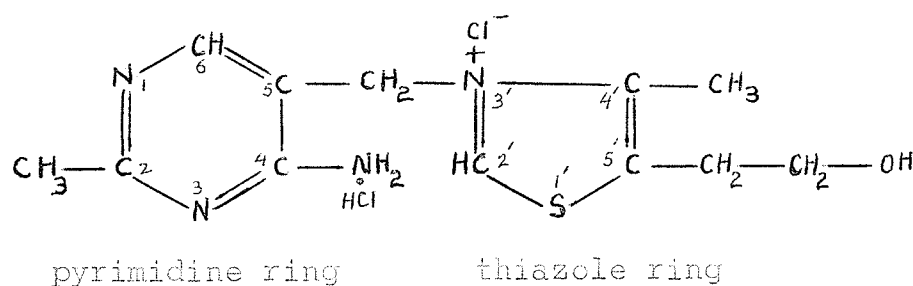
CHEMICAL METHODS USED FOR THE DETERMINATION OF THIAMINE

Method	Sensitivity	Remarks
1. Thiochrome	as little as 0.1 μg . (in isobutanol extract)	used extensively.
2. Colorimetric (diazotized p-amino- acetophenone)	20-100 μg . in aliquots used for color development	limited use due to interfering substances.
3. Precipitation a) Ammonium Reineckate b) Silicotungstic acid	2-5 mg. in 5 ml. 5 mg. of T required	used in pharmaceutical field. pure T solutions needed.
4. Titration a) Sodium hydroxide b) Perchloric acid	600 mg. of T required	used to verify the structure. many interfering substances.
5. Spectrophotometric (UV absorption)	6 μg ./ml. of T required	great aid in determining the structure of T.
6. Paper chromatographic	0.1-100 μg . detectable	useful for separating T and its phosphate esters.
7. Paper electrophoresis	50 μg . -2 mg. are required	useful for separating T and its phosphate esters.
8. Polarographic (30)		requires solutions of known composition.
9. Infra Red (31)		requires solutions of known composition.
10. Fluorescent micro- spectrography (32)		used for localizing T in tissue cells.

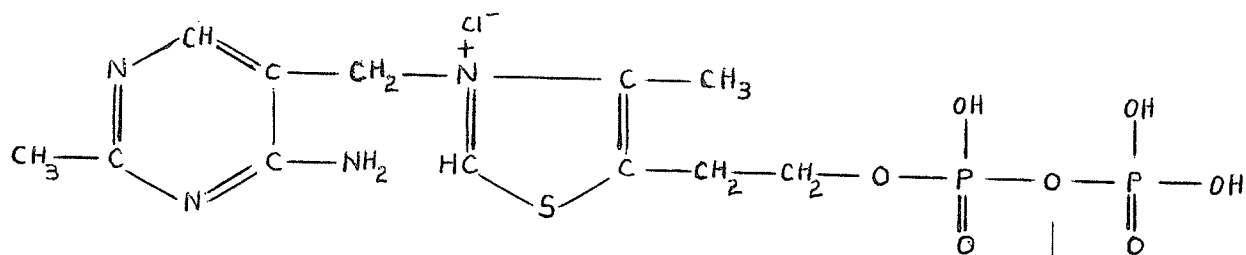
Structure of Thiamine, Thiamine Mono- and Diphosphate Esters

Thiamine or vitamin B₁ is composed of substituted pyrimidine and thiazole rings joined together by a methylene group. The chemical name is 4'-methyl-5'-β-hydroxy-ethyl-N- $\{[2\text{-methyl-4-amino-pyrimidyl-(5)]-methyl}\}$ -thiazolium-chloride-hydrochloride. Thiamine is the only natural substance to contain thiazole and it is the only natural pyrimidine to have an alkyl group in position 2.

The biological action of vitamin B₁ is due mainly to the action of its pyrophosphoric acid ester. Vitamin B₁ apparently acts as an acceptor of phosphoric acid (e.g. from Adenosine triphosphate) to form the co-enzyme cocarboxylase (thiamine diphosphate) (8).



Thiamine Chloride Hydrochloride



Thiamine Monophosphate (Chloride)

Cocarboxylase or Thiamine Pyrophosphate (Chloride)

The Nature of Thiamine in Cereals

It has been demonstrated that thiamine can combine with phosphoric acid to form phosphate esters. It is known that thiamine is bound to a protein in milk (5), and that it could possibly combine with carbohydrate. Also the thiamine phosphates themselves may be bound to protein or carbohydrate or some other substance. Some authors claim that cereal germ (e.g., wheat germ) contains some thiamine in the phosphorylated form and consequently should be treated with an enzyme (9). Analytical methods indicate that most of the thiamine in cereals is in the free form. Many authors doubt the existence of thiamine phosphates in cereals. However, most of these decisions have been arrived at by deduction rather than straightforward determinations of thiamine phosphates (e.g. Sure's (10) work on the cocarboxylase in wheat embryo and brewer's yeast). Recently Sen and Robinson (11) developed a method which showed that there were no thiamine phosphates in cereals. This was supported by hydrolysis with a variety of enzymic treatments. Non-phosphatase enzymes (diastase and trypsin) gave a total thiamine content that was almost the same as enzymes known to contain phosphatases (e.g. Taka-diastase). From this it is seen that the combined thiamine in cereal grain is either not in the form of the phosphate esters, or, if phosphate esters are present, they must occur in trace amounts difficult to detect. Other authors have used proteolytic digestion and claimed that it was necessary for the complete extraction of thiamine in cereal products (12).

DISCUSSION OF THE VARIOUS STEPS IN CHROMATOGRAPHIC ANALYSIS

This discussion is based on the thiochrome procedure (in contrast to the colorimetric method) after separation by ion exchange resin. All chemical procedures have certain preliminary steps in common. The vitamin must be extracted, the extract purified, different combined forms separated and hydrolyzed to thiamine and finally converted to thiochrome, a fluorescent compound.

(a) Extraction

The purpose of this step is to obtain the vitamin both as the free and combined form in solution. It has been suggested that the combined forms may include the phosphate esters (9). Thiamine and thiamine monophosphate are quite stable in acid solution (13) even at a high temperature (90-100°C). Thiamine pyrophosphate is stable in weak acid solution at room temperature, but slowly hydrolyzes to thiamine monophosphate if kept for long at higher temperature (14). The higher phosphates, namely thiamine triphosphate and thiamine tetraphosphate, are stable at room temperature in weak acid solution for one or two days, but slowly hydrolyze to thiamine monophosphate, thiamine pyrophosphate and inorganic phosphate if kept longer (14). They are very unstable at higher temperatures and break down to the products mentioned above. Thiamine and all its derivatives are very unstable in alkaline solution (13). Thus an acid extracting

medium is desirable. However, Obermeyer, Fulmer and Young (15) found that wheat contained a thiamine pyrophosphate (cocarboxylase) hydrolyzing factor which is thermolabile and could be destroyed by performing the extraction with hot (90°C) 0.1 N sulphuric acid for 15-30 minutes. Thus hot acid extractions of various sorts have been used such as H_2SO_4 , HCl and even acid KCl solution. Heat is not the only inhibitor of any phosphatases occurring in grain for Sumner and Myrback (16) found fluoride ion to be a strong inhibitor and this has been added to sulphuric acid to enable cold extractions to be done. Cold perchloric acid extractions have also been successful, for Taka-Diastase was found to be completely inhibited at the concentration used (M/3) (17). Rindi and de Giuseppe (7) tried both 0.5 N perchloric acid and 5% trichloroacetic acid for extraction of the vitamin from rat tissues, but chose the latter because they found less protein in the deproteinized extract. (Both were tried with cereal grains by myself and each was found to be equally effective). Although at higher temperatures better recoveries would be obtained, the possibility of breaking down the higher phosphates is always present.

(b) Purification

Not only thiamine and its combined forms (including the phosphate esters) are extracted from cereals, but proteins, carbohydrates, other B vitamins, salts and nucleotides are extracted simultaneously. This presents a serious problem because

of the low concentration of thiamine and its compounds in comparison to these other substances, and also because some interfere with the determination by the thiochrome method. They do this because some fluoresce in ultra violet light (giving high blank readings) and/or others form oxidation products which fluoresce in ultra violet light (giving high sample readings). Furthermore, the presence of any salt interferes with the separation of the different derivatives either by chromatographic or electrophoretic methods. A purification procedure is therefore necessary to eliminate as much as possible of the material that will interfere with the final estimation of the vitamin. A secondary effect of purification is that it may be used to concentrate the vitamin.

A wide range of substances have been used with corresponding eluting agents. Some (18) of these are shown in Table II. Of these substances, adsorbent charcoal and Decalso are the ones used most widely.

Siliprandi and Siliprandi (19) used "Carbo Activ" to purify thiamine extracts from inorganic salts. After washing the column with distilled water, which removes the inorganic salts still retained on the column, the thiamine compounds were eluted with 50% ethanol. They noticed that when thiamine esters are adsorbed on pure carbon from water solutions it is possible to elute them completely with 50% ethanol. When, however, these compounds are adsorbed from saline solutions (as in the case of

TABLE II

SOME MATERIALS USED FOR PURIFICATION

Materials	Eluting Agents (where applicable)	References
Adsorbent charcoal	10% propanol (normal)	(7)
Decalso	25% KCl	(1)
Fuller's earth	pyridine:HAc:water	(33)
Florisil	pyridine:HAc:water	(17)
Amberlite IRC-50 (sodium form)		(34)
Extraction with iso- butanol		(35)
Extraction with benzyl alcohol		(36)
Electrolytic desalting		(17)
Several clays (e.g., frankonite, superfiltrol etc.)		(18)

cereal extracts) T, TMP, TDP, but not TTP can be eluted. Hagdahl et al (20) first proposed to decrease the adsorption capacity of the carbon by treating it with strongly adsorbable compounds such as hexanol or cholesteryl stearate, thus eliminating the irreversible adsorption on the most active sites. It was found that cholesteryl stearate treated charcoal gave optimum adsorption.

A base exchange method using the adsorbent zeolite was introduced by Cerecedo et al (21). Zeolite is a class of hydrated silicates of aluminum and sodium or calcium or both. The term was originally applied to the naturally occurring minerals which were used extensively for water softening. Artificial zeolites are made in a variety of forms ranging from gelatinous to porous sand. The latter is made by the Permutit Co., New York City, under the trade name Decalso. Decalso is effective for purification only when employed as a column. It acts as a cation exchanger and is used in the H^+ ion form. In acidic solution thiamine is present as cation, the basic amino group of the thiamine molecule carries the positive charge, while the thiamine phosphates carry both positive and negative charges, the latter being carried by the phosphate ion.

(c) Separation

Both paper chromatography and electrophoresis have been used successfully on pure mixtures but the standard purification methods do not give clear separation by these techniques.

Recently Sen and Robinson (11) developed a long but fairly satisfactory method of desalting cereal extracts, which enabled them to determine thiamine pyrophosphates and the higher phosphates by paper chromatography. Thus the disadvantage of paper chromatography and electrophoresis is that they require pure samples in high concentration.

Thus the attention has been turned to column chromatography and two resins, Dowex 1-X8 and Amberlite IRC-50, have been used. Dowex 1-X8 is a strongly basic, anion exchange resin. The anion exchangers are of polystyrene, strong base (i.e. quaternary ammonium) type in the form of spherical beads (200-400 mesh). The degree of cross linking (i.e. % divinyl benzene used to link the linear vinyl benzene molecules into a 3-D structure) specified is usually 8-10% and such material may be regarded as standard. Difference in per cent cross linking changes the property of the resin. The success of the adsorption step in using ion exchange resins depends on the equilibrium between solution and exchanger (which is determined principally by the ionic strength of the medium) and the rate of exchange (which depends on flow rate and mesh size and the charge on the ions in question). Both equilibrium constant and rate of adsorption depend on charge which is influenced by pH; hence, this is the most important factor in determining how successful the adsorption step will be. Dowex 1-X8 contains quaternary ammonium groups as its functional groups which are attached to a styrene-divinyl-benzene co-polymer. After use the resin is regenerated by passing excess 3 M sodium acetate solution through

it followed by washing with 3 N Acetic acid and water until the wash water indicates the same pH as that of distilled water. The advantage of using a strongly anionic instead of a weakly cationic resin on the extract lies in the fact that with the former it is possible to fix and then to separate sharply the phosphoric esters of thiamine which have acid functions (22).

Amberlite is the trademark of Rohm and Haas Co., Philadelphia, for insoluble cross-linked polyelectrolytes used as ion exchange resins. Amberlite IRC-50 resin is a carboxylic acid-type cation exchanger which is weakly acidic. In the hydrogen form, it allows TTP to pass readily, retards TDP and fixes TMP and thiamine (22).

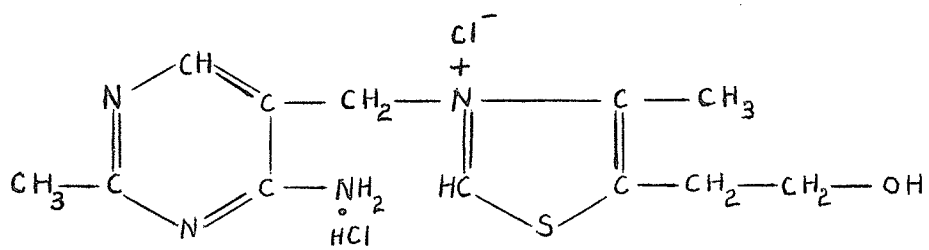
(d) Hydrolysis

Thiamine must be in the free form to be estimated chemically. This is so because on oxidation cocarboxylase (TDP) is converted to a thiochrome derivative which cannot be extracted by isobutanol and so would not be measured. Phosphate esters are, therefore, hydrolyzed enzymatically to thiamine before oxidation. Kinnersley and Peters (23) were apparently the first to find Takadiastase suitable for the hydrolysis of cocarboxylase. Takadiastase is the trade name for the preparation resulting from the action of spores of the fungus Aspergillus oryzae (a fungus of the Roji-yeast used in making rice wine) on wheat bran (18). Today the commercially available preparations such as Takadiastase, Clarase, Polidase, Mylase and Diastase or

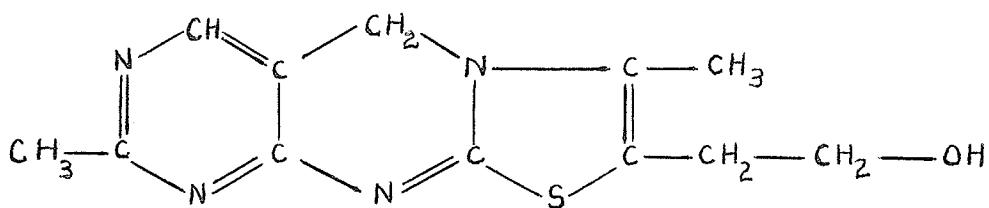
Maltin, are widely used in thiamine analysis. Takadiastase, Mylase and acid phosphatase were tried with wheat and the results were quite similar. These enzymes preparations are of added value since they hydrolyze starches, thus decreasing the amount of insoluble material and making filtration easier (24). Some authors claim that proteolytic digestion is required for the complete liberation of thiamine from natural products (4), but this has to be investigated more thoroughly for cereal grains.

(e) Oxidation

Thiamine, when treated with alkaline ferricyanide solution, is oxidized to thiochrome which fluoresces in ultraviolet light. The thiochrome is very unstable in aqueous solution and the brilliancy of fluorescence is also less in aqueous than in alcoholic solution. For this reason, after oxidation, the thiochrome is immediately extracted with isobutanol and the fluorescence of the isobutanol is measured with a sensitive fluorophotometer. Most investigators agree that approximately 67% of the thiamine is converted to thiochrome over the concentration range 0.2-2 μ g. of thiamine (25). This yield, however, is very constant under standardized conditions.



Thiamine Chloride Hydrochloride



Thiochrome

METHODS AND MATERIALS

1. A New Chromatographic Method for the Determination of Thiamine and its Mono-, Di- and Triphosphates in Animal Tissues as Applied to Cereal Grains.

This method for animal tissues was first reported by Rindi and de Giuseppe (7) on the basis of previous work (22) where they reported the analytical conditions for the separation and determination of thiamine and its phosphoric esters in pure solutions by using a strong anionic resin. Since most of the work was done on this method, it will be described in detail.

(a) Principle of the Method

In the case of liver, the tissue was extracted and deproteinized by homogenization in trichloroacetic acid. Cereals were ground to a 100-mesh size prior to extraction. The extract, brought to pH 6.7-6.8, was adsorbed on a charcoal column and eluted with 10% (v/v) propanol. The vacuum concentrated eluate was applied to a column of Dowex-1 (X8 acetate form), which retained TDP and TTP and allowed thiamine and TMP to flow through. The thiamine and TMP content of the percolated fluid and the combined water washings were determined. TDP was eluted from the Dowex column with 0.02 N sodium acetate in 0.04 N acetic acid, and TTP by M acetate buffer, pH 4.5. TDP and TTP were determined in the eluates.

(b) Apparatus

(i) MSE homogenizer. (ii) Centrifuge--Servall centrifuge. (iii) Columns. Charcoal columns (11 mm. x 111 mm.) with a bulb at the top. Dowex and Amberlite columns (8 mm. x 80-90 mm.). All the chromatographic columns were made from Pyrex glass tubes, fitted with a sintered glass disk and fused to a ground-glass stopcock. (iv) pH meter--Photovolt Model 110, Photovolt Corp., N. Y., U. S. A. (v) Rinco rotating evaporator--Rinco Instrumental Co., INC., Greenville, Ill. used in conjunction with (vi) Vacuum pump. (vii) Incubator for a temperature of 37-40°C. (viii) Reaction vessel. Glass-stoppered centrifuge tubes of 30 ml. capacity with conical bottom. (ix) Mechanical shaker. (x) Mechanical stirrers. (xi) Fluorophotometer. Electronic Photofluorometer--Coleman Electronic Co., Inc., Maywood, Ill., U. S. A.

(c) Reagents

(i) Adsorbent charcoal--This was prepared as described by Siliprandi and Siliprandi (19) by suspending 10 g. of an activated charcoal (3 SL Acticarbono produced by C.E.C.A. 24, Rue Murillo, Paris-VIII) in a solution containing 1 g. of cholesteryl stearate (British Drug Houses Ltd.) in 300 ml. of ethyl ether plus 300 ml. of ethanol, and continuously stirring for 48 hours at room temperature. After addition of a large excess of water, the charcoal was filtered on a Buchner funnel and thoroughly washed with water. It was dried in the air and stored in an amber colored bottle.

(ii) 5% (v/v). Trichloroacetic acid. (iii) 40% Sodium hydroxide. (iv) 10% (v/v) 1-Propanol (normal). (v) Dowex-1 resin (X8, acetate form). This was prepared from a batch of Dowex -1 (X8, 200-400 mesh) (British Drug Houses Ltd.) as indicated by de Giuseppe and Rindi (22) and was stored in 0.5 M acetic acid. Before use it was thoroughly washed with water. De Giuseppe and Rindi included the series of washes suggested by W. E. Cohn (26) calculated to remove organic material from the polystyrene matrix. This was done by the following wash sequence, water, acetone-water, acetone, petroleum-ether, acetone, acetone-water, water. Conversion to the chloride form was accomplished after this sequence by washing with strong HCl. (This also removes acid-soluble impurities). After a water wash to remove excess HCl, the resin was converted to the acetate form with sodium acetate (since acetic acid, because of its low ionization is very inefficient at replacing chloride). This was done until the silver nitrate test on the washings showed the absence of chloride. Before storage a final water wash to remove excess anion was performed. (vi) Amberlite CG-50 Type 2. This synthetic weakly acidic cation exchange resin (carboxylic acid type--hydrogen form) was used instead of Amberlite IRC-50 because it is an improved form of the latter mentioned which is not manufactured any more. Amberlite IRC-50 had indicated an efficiency of 92-95% in the adsorption of thiamine

in the sodium form of the resin (27). Two favorable properties of this resin are that adsorbed ions can be effectively eluted by moderate concentrations and volumes of acid, and it can be easily buffered, thereby affecting its adsorptivity for weakly basic compounds. The resin was buffered at pH 4.5, according to the method of Vanatta and Harris (27) using 10% NaOH, 15% NaAc and HAc. (vii) Cellulose powder. Whatman, ash-free, cellulose powder was washed with 50% (v/v) ethanol, filtered off in a Buchner funnel, thoroughly rinsed with water and dried. This powder was mixed with the charcoal to obtain a faster flow rate. (viii) 2 M acetate buffer. (ix) 0.02 M sodium acetate in 0.04 M acetic acid. (x) M acetate buffer pH 4.5. (xi) 1 N HCl. (xii) 0.1 N HCl. (xiii) Phosphatase solution. This was prepared just before use by suspending 1 g. of Takadiastase (Parke, Davis and Co., Ltd.) in 10 ml. of 2 M acetate buffer, pH 4.5. (xiv) Solid Potassium Chloride. (xv) Standard Thiamine solution. (0.2 μ g./ml.). (xvi) 1% Potassium ferricyanide solution. (xvii) 15% sodium hydroxide. (xviii) 0.1 N H_2SO_4 . (xix) Isobutanol. This was redistilled and the fluorescence observed on the fluorimeter was negligible. (xx) Quinine sulphate solution. (0.3 μ g./ml. in 0.1 N H_2SO_4). (xxi) Vitamin B₁ Monophosphoric ester. (xxii) Cocarboxylase.

Preparation of charcoal columns: A portion (1-2 ml.) of a 10% cellulose suspension in water was placed on the sintered glass disk of the column. A homogenous mixture of 300 mg. charcoal and 1.5 g. cellulose powder was prepared and

suspended in water. (This was capable of adsorbing about 35-45 μg . of thiamine). The suspension was placed on the settled cellulose layer by means of a wide-mouthed pipette, whose tip was kept below the water surface. The charcoal was allowed to sediment completely, by opening the column stopcock, without eliminating all the water. Finally 1 ml. of cellulose suspension in water was placed on the charcoal column after thorough cleaning of the walls by means of a glass rod wadded with cotton.

(d) Procedure

Sampling of cereals: The whole grain was ground in a Wiley mill to pass a 100 mesh screen and was mixed thoroughly.

Extraction: About 20-40 g. (depending on thiamine content) were weighed and extracted twice with 5% (v/v) trichloroacetic acid in the cold room (0°C). The first extraction was done overnight and the second one for 3-4 hours. Two extractions of about three hours each gave almost identical results. The sample was placed in a stoppered Erlenmeyer flask and stirred quite vigorously with a magnetic stirrer during extraction. The two extracts were centrifuged and the supernatant fluids were combined and made up to a specific volume (250 mls. or 500 mls. depending on thiamine content) with 5% trichloroacetic acid.

Purification: An aliquot was taken and was neutralized (pH 6.7-6.8) with 40% sodium hydroxide. After centri-

fuging again, the neutralized aliquot was passed through the charcoal column. The flow rate during all the analytical steps (adsorption, washing, elution) was adjusted to 10-15 drops/min. The column was washed with 20-25 ml. of water. The thiamine compounds were eluted with 60-70 ml. of 10% (v/v) propanol and collected in a ground glass round-bottomed liter flask.

Concentration of eluate: The propanol eluate was concentrated to about 5 ml. in a rotating evaporator under vacuum, on a water bath at 25-30°C., the pressure being reduced gradually to avoid bumping. The flask was then accurately washed and the volume brought to 15 ml. with water.

Chromatography on Dowex and Amberlite resins: After addition of 0.8 ml. of 0.1 N HCl, the concentrated eluate was passed through a layer (8 mm. x 25 mm.) of Dowex -1 (X8, acetate form, 200-400 mesh) placed in a chromatographic glass column (8 mm. x 80-90 mm.). The resin was washed with water, and both eluate and washing were collected in the same 25 ml. volumetric flask, up to the mark, with thorough mixing. Then 0.5 ml. of 2M acetate buffer was added to 12.5 ml. of this solution in an Erlenmeyer flask (fraction I), and 0.5 ml. of phosphatase solution was added to the remaining 12.5 ml. (fraction II). About 20 ml. of 0.02 M sodium acetate in 0.04 M acetic acid was put on the column. Phosphatase solution (1 ml.) was added to the eluate (fraction III), which contained only TDP, in a 25 ml. volumetric flask, the volume

being adjusted to the mark with water if necessary. Finally, TTP was eluted from the resin with about 19 ml. of M acetate buffer, pH 4.5. The eluate (fraction IV) was treated as indicated for TDP. After thorough mixing, all four fractions were incubated overnight at 37°C. Fraction IV, diluted with 20 ml. of water, was then passed through a bed (8 mm. x 40 mm.) of buffered (pH 4.5) Amberlite CG-50 Type 2 resin in a glass column (8 mm. x 80 mm.). The resin was washed with 15 ml. of water and eluted with 20 ml. of N HCl; the eluate was collected in a 25 ml. volumetric flask and the volume was adjusted to the mark (fraction IVA).

Determination of Thiamine: Five ml. of the fractions were placed in duplicate in two centrifuge tubes provided with ground-glass stoppers. Five ml. of thiamine solution (containing 0.2 $\mu\text{g.}/\text{ml.}$) were treated in the same way and served as the standard. Solid potassium chloride (1.25 g.) was placed in each tube, which was then shaken until the solid dissolved.

Both oxidation with ferricyanide and extraction with isobutanol (15 ml.) were accomplished according to association of Vitamin Chemists (37). For the fluorimetric measurements, the instrument was set with a quinine sulphate solution (0.3 $\mu\text{g.}/\text{ml.}$) in 0.1 N H_2SO_4 . The fluorimetric readings were corrected for the blanks without oxidation.

The percentage of recovery of added thiamine and its

phosphates was measured by the method just described. A mixture of 20 μ g. thiamine chloride, 20 μ g. thiamine monophosphate, and 20 μ g. thiamine diphosphate was added to 100 ml. of extract from the different samples which had previously been passed through excess charcoal. The recoveries are shown in Tables III, V, XIII, XVI and XX.

2. Paper chromatography of thiamine and its phosphoric esters.

The method developed by Siliprandi and Siliprandi (19) was employed. Whatman No. 1 papers were used and they were washed successively with 4 N HCl, a saturated solution of 8-hydroxyquinoline in 50% ethanol and finally with 50% ethanol. All the papers were run by ascending chromatography over a 30-hour period. Prior to spotting the papers, purification of the extract was carried out with cholesteryl stearate treated charcoal as described in the previous method. This was followed by concentration of the eluate to about 1 ml. with the Rinco evaporator. After drying in the fume chamber at room temperature, the papers were sprayed with a mixture of 2 parts of 96% ethanol, 1 part of 10% NaOH and 0.05 parts of 2.5% $K_3Fe(CN)_6$. The solvent used was N-propanol-water-1 M acetate buffer pH5 (70:20:10). Thiamine and its esters are in this way transformed into corresponding thiochromes and appear as fluorescent greenish-blue spots in ultraviolet light.

3. Thiochrome Method for the Determination of Thiamine in Cereals.

The revised method of Bechtel and Hollenbeck (1) was used to determine the total and free thiamine of the various cereal grains. The thiochrome method was first reported by Jansen (28) and has been modified later by other investigators. The revised method is both shorter and simpler than the older methods. Sulphuric acid extraction followed by sodium acetate buffer replaces acetic acid and sodium hydroxide of the old method and thus gives greater convenience. An improved rapid method for activating Decalso is included. The 50-80 mesh product is used to give ease of flowing. A 100 g. portion of Decalso is washed with a 3% solution of hot acetic acid in a Buchner funnel. The acid is drained off by vacuum after being in contact with the Decalso for 10-15 minutes. This is followed by a wash with approximately 250 ml. of hot 25% potassium chloride solution which remains in contact with the Decalso 10-15 minutes before draining by vacuum. After another acid wash, the Decalso is washed with several portions of hot water until the extract is free from chloride as shown by a silver nitrate test. The activated Decalso is dried in an oven below 100°C and stored in a stoppered bottle. About 0.5 g. of the activated Decalso is used, and this gives a column height of 3-4 cm., in a 5-6 mm. diameter column. This is enough for the adsorption of 20 µg. of thiamine. Older procedures require a column

height of 8-10 cm., but the smaller amount of Decalso used in this revised procedure permits one to discard the used adsorbent without having to purify or reactivate it.

Principle of the method: Two weighed samples such that the final portion (5 ml.) to be oxidized would contain approximately 1 μ g. of thiamine were placed in Erlenmeyer flasks and 50 ml. of 0.1 N H_2SO_4 added to each. They were heated in a boiling-water bath for 10 minutes. After cooling to below $40^\circ C.$, 5 ml. of enzyme suspension (2% solution of Takadiastase in 2.5 M sodium acetate) were added to flask A and 5 ml. of 2.5 M sodium acetate added to flask B. Flask A was incubated at $37^\circ-40^\circ C.$ for at least four hours. Instead of filtering, the alternative process of centrifuging was carried out (13,000 r.p.m.). The solutions were then made up to volume and an aliquot was passed through the Decalso column. The column was washed with hot water and thiamine was eluted with boiling 25% KCl solution into a 25 ml. volumetric flask. The volume was made up to the mark and 5 ml. samples were taken for oxidation. Oxidation with ferricyanide and extraction in isobutanol were carried out in the same manner as described on page 22.

RESULTS

Eleven samples of cereals were studied, after the method was tried with rabbit liver. At least two samples of each of Selkirk, Ramsey and Pembina wheats, Carry and Rodney oats, Vantage and Montcalm barleys, and wheat germ, shorts, middlings and flour were used. Recoveries of thiamine, thiamine mono- and diphosphates were determined for wheat, oats and barley extract previously freed of all thiamine. The sum of thiamine from thiamine and the esters of each sample is compared with a separate determination of total thiamine. The per cent recovery is calculated as a fraction of the sum of the determined value. The final column of the tables which show the T, TMP, TDP and TTP content of the various samples is headed "% of total T". This percentage is based on the thiamine equivalent of thiamine and its phosphate esters.

All the samples used were 1962 grain except for barley which was 1955 grain.

TABLE III

RECOVERY OF THIAMINE, THIAMINE MONOPHOSPHATE,
AND THIAMINE DIPHOSPHATE FROM RABBIT LIVER

TRIAL 1

	Total amount of starting material $\mu\text{g.}$	Total amount recovered $\mu\text{g.}$	% recovery
Thiamine	20	23.1	115.5
Thiamine monophosphate	20	25	125
Thiamine diphosphate	20	27.1	135.5

TRIAL 2

	Total amount of starting material $\mu\text{g.}$	Total amount recovered $\mu\text{g.}$	% recovery
Thiamine	20	23.5	117.5
Thiamine monophosphate	20	22.15	111.4
Thiamine diphosphate	20	23.81	119.1

TABLE IV

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENTS OF RABBIT LIVER

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T.
Thiamine	0.28	0.28	10.4
Thiamine Monophosphate	1.45	1.15	43. *
Thiamine diphosphate	1.51	0.987	36.9
Thiamine triphosphate	0.49	<u>0.27</u>	10.1
Thiamine due to T, TMP, TDP, TTP (i.e. sum)		2.687 $\mu\text{g./g.}$	
Thiamine due to everything (determination)		2.29 $\mu\text{g./g.}$	
% recovery		=	117%

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T.
Thiamine	0.30	0.30	11.15
Thiamine monophosphate	1.40	1.105	41.
Thiamine diphosphate	1.63	1.06	39.4
Thiamine triphosphate	0.41	<u>0.226</u>	8.4
T due to T, TMP, TDP, TTP (i.e. sum)		2.691 $\mu\text{g./g.}$	
T due to everything (determination)		2.38 $\mu\text{g./g.}$	
% recovery		=	113%

* TO READ - 43% of the total thiamine found in rabbit liver comes from TMP.

TABLE V

RECOVERY OF THIAMINE, THIAMINE MONOPHOSPHATE,
AND THIAMINE DIPHOSPHATE FROM SELKIRK WHEAT

TRIAL 1

	Total amount of starting material $\mu\text{g.}$	Total amount recovered $\mu\text{g.}$	% recovery
Thiamine	20	15	75
Thiamine monophosphate	20	18.1	90.5
Thiamine diphosphate	20	12.3	61.5

TRIAL 2

	Total amount of starting material $\mu\text{g.}$	Total amount recovered $\mu\text{g.}$	% recovery
Thiamine	20	15.5	77.6
Thiamine monophosphate	20	17.45	87.3
Thiamine diphosphate	20	13.16	65.9

TABLE VI

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENT OF SELKIRK WHEAT

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	2.1	2.1	95.5
Thiamine monophosphate	0.065	0.05	2.26
Thiamine diphosphate	0.18	0.12	5.4
Thiamine triphosphate	0.08	0.049	2.25
T due to T, TMP, TDP, TTP (i.e.sum)		2.319 $\mu\text{g./g.}$	
T due to everything (determination)		2.22 $\mu\text{g./g.}$	
% recovery = 104.2%			

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	2.5	2.5	98.8
Thiamine monophosphate	0	0	0
Thiamine diphosphate	0.03	0.02	0.77
Thiamine triphosphate	0.02	0.011	0.43
T due to T, TMP, TDP, TTP (i.e.sum)		2.531 $\mu\text{g./g.}$	
T due to everything (determination)		2.36 $\mu\text{g./g.}$	
% recovery = 107.1%			

TABLE VII

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENTS OF RAMSEY WHEAT

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% Total T
Thiamine	3.5	3.5	95.5
Thiamine monophosphate	0.078	0.061	1.67
Thiamine diphosphate	0.129	0.084	2.29
Thiamine triphosphate	0.038	0.021	0.57
T due to T, TMP, TDP, TTP (i.e. sum)		3.666 $\mu\text{g./g.}$	
T due to everything (determination)		3.78 $\mu\text{g./g.}$	
		% recovery = 97.2%	

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% Total T
Thiamine	3.2	3.2	95.6
Thiamine monophosphate	0.06	0.047	1.4
Thiamine diphosphate	0.140	0.091	2.72
Thiamine triphosphate	0.024	0.013	0.39
T due to T, TMP, TDP, TTP (i.e. sum)		3.351 $\mu\text{g./g.}$	
T due to everything (determination)		3.60 $\mu\text{g./g.}$	
		% recovery = 93%	

TABLE VIII

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENT OF PEMBINA WHEAT

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	3.9	3.9	98
Thiamine monophosphate	0	0	0
Thiamine diphosphate	0.075	0.049	1.23
Thiamine triphosphate	0.062	<u>0.034</u>	0.85
T due to T, TMP, TDP, TTP (i.e. sum)		3.983 $\mu\text{g./g.}$	
T due to everything (determination)		3.6 $\mu\text{g./g.}$	
		% recovery = 111.5 %	

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	3.5	3.5	97
Thiamine monophosphate	0.064	0.05	1.38
Thiamine diphosphate	0.0585	0.038	0.05
Thiamine triphosphate	0.0383	<u>0.021</u>	0.58
T due to T, TMP, TDP, TTP (i.e. sum)		3.609 $\mu\text{g./g.}$	
T due to everything (determination)		3.55 $\mu\text{g./g.}$	
		% recovery = 102%	

TABLE IX

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENTS OF WHEAT GERM

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	13.7	13.7	97.5
Thiamine monophosphate	0	0	0
Thiamine diphosphate	0.44	0.286	2.06
Thiamine triphosphate	0.11	0.061	0.43
T due to T, TMP, TDP, TTP (i.e. sum)		14.047 $\mu\text{g./g.}$	
T due to everything (determination)		15.5 $\mu\text{g./g.}$	
		% recovery = 90.6%	

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	13.8	13.8	98
Thiamine monophosphate	0	0	0
Thiamine diphosphate	0.36	0.234	1.63
Thiamine triphosphate	0.085	0.047	0.35
T due to T, TMP, TDP, TTP (i.e. sum)		14.081 $\mu\text{g./g.}$	
T due to everything (determination)		16.1 $\mu\text{g./g.}$	
		% recovery = 87.4%	

TABLE X

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENTS OF WHEAT SHORTS

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	12	12	92.5
Thiamine monophosphate	0.9	0.71	5.47
Thiamine diphosphate	0.35	0.23	1.77
Thiamine triphosphate	0.05	<u>0.027</u>	0.20
T due to T, TMP, TDP, TTP (i.e. sum)		12.967 $\mu\text{g./g.}$	
T due to everything (determination)		13.75 $\mu\text{g./g.}$	
		% recovery = 94.5%	

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	12.13	12.13	91.5
Thiamine monophosphate	1.1	0.87	6.55
Thiamine diphosphate	0.34	0.22	1.66
Thiamine triphosphate	0.08	<u>0.044</u>	0.30
T due to T, TMP, TDP, TTP (i.e. sum)		13.264 $\mu\text{g./g.}$	
T due to everything (determination)		13.61 $\mu\text{g./g.}$	
		% recovery = 97%	

TABLE XI

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENTS OF MIDLINGS

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	$\frac{\%}{\text{of total T}}$
Thiamine	4.6	4.6	96.1
Thiamine monophosphate	0	0	0
Thiamine diphosphate	0.21	0.136	2.84
Thiamine triphosphate	0.08	0.044	0.92
T due to T, TMP, TDP, TTP (i.e. sum)		4.780 $\mu\text{g./g.}$	
T due to everything (determination)		4.6 $\mu\text{g./g.}$	
		% recovery = 104%	

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	$\frac{\%}{\text{of total T}}$
Thiamine	4.4	4.4	95.9
Thiamine monophosphate	0	0	0
Thiamine diphosphate	0.23	0.15	3.27
Thiamine triphosphate	0.09	0.049	1.1
T due to T, TMP, TDP, TTP (i.e. sum)		4.599 $\mu\text{g./g.}$	
T due to everything (determination)		5.68 $\mu\text{g./g.}$	
		% recovery = 81%	

TABLE XII

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENTS OF FLOUR

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	0.38	0.38	96.2
Thiamine monophosphate	0	0	0
Thiamine diphosphate	0.02	0.013	3.3
Thiamine triphosphate	0.004	0.002	0.56
T due to T, TMP, TDP, TTP (i.e. sum)		0.395 $\mu\text{g./g.}$	
T due to everything (determination)		0.42 $\mu\text{g./g.}$	
		% recovery = 94%	

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	0.36	0.36	99.4
Thiamine monophosphate	0	0	0
Thiamine diphosphate	0.003	0.002	0.52
Thiamine triphosphate	0.001	0.0005	0.014
T due to T, TMP, TDP, TTP (i.e. sum)		0.363 $\mu\text{g./g.}$	
T due to everything (determination)		0.39 $\mu\text{g./g.}$	
		% recovery = 93%	

TABLE XIII

RECOVERY OF THIAMINE, THIAMINE MONOPHOSPHATE
AND THIAMINE DIPHOSPHATE FROM GARRY OATS

TRIAL 1

	Total amount of starting material $\mu\text{g.}$	Total amount recovered $\mu\text{g.}$	% recovery
Thiamine	20	20.8	108
Thiamine monophosphate	20	9.45	47.2
Thiamine diphosphate	20	11.3	56.5

TRIAL 2

	Total amount of starting material $\mu\text{g.}$	Total amount recovered $\mu\text{g.}$	% recovery
Thiamine	20	21.2	112
Thiamine monophosphate	20	10.9	54.5
Thiamine diphosphate	20	13.2	66

TABLE XIV

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENTS OF GARRY OATS

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	2.6	2.6	82
Thiamine monophosphate	0.62	0.49	15.45
Thiamine diphosphate	0.079	0.052	1.64
Thiamine triphosphate	0.046	0.025	0.79
T due to T, TMP, TDP, TTP (i.e. sum)		3.167 $\mu\text{g./g.}$	
T due to everything (determination)		3.2 $\mu\text{g./g.}$	
		% recovery = 99.1%	

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	2.68	2.68	81.5
Thiamine monophosphate	0.67	0.53	16.1
Thiamine diphosphate	0.098	0.064	1.94
Thiamine triphosphate	0.035	0.019	0.58
T due to T, TMP, TDP, TTP (i.e. sum)		3.293 $\mu\text{g./g.}$	
T due to everything (determination)		3.1 $\mu\text{g./g.}$	
		% recovery = 106%	

TABLE XV

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENTS OF RODNEY OATS

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	2.44	2.44	85.5
Thiamine monophosphate	0.39	0.308	10.8
Thiamine diphosphate	0.13	0.085	2.9
Thiamine triphosphate	0.056	0.031	1.1
T due to T, TMP, TDP, TTP (i.e. sum)		2.864 $\mu\text{g./g.}$	
T due to everything (determination)		2.72 $\mu\text{g./g.}$	
		% recovery = 105%	

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	2.42	2.42	84.5
Thiamine monophosphate	0.45	0.36	12.5
Thiamine diphosphate	0.125	0.081	2.82
Thiamine triphosphate	0.027	0.015	0.52
T due to T, TMP, TDP, TTP (i.e. sum)		2.876 $\mu\text{g./g.}$	
T due to everything (determination)		2.77 $\mu\text{g./g.}$	
		% recovery = 103.5%	

TABLE XVI

RECOVERY OF THIAMINE, THIAMINE MONOPHOSPHATE
AND THIAMINE DIPHOSPHATE FROM VANTAGE BARLEY

TRIAL 1

	Total amount of starting material $\mu\text{g.}$	Total amount recovered $\mu\text{g.}$	% recovery
Thiamine	20	21.4	107
Thiamine monophosphate	20	12	60
Thiamine diphosphate	20	13.3	66.5

TRIAL 2

	Total amount of starting material $\mu\text{g.}$	Total amount recovered $\mu\text{g.}$	% recovery
Thiamine	20	20.8	104
Thiamine monophosphate	20	13.4	67
Thiamine diphosphate	20	13.6	68

TABLE XVII

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENTS OF VANTAGE BARLEY

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	1.79	1.79	86.3
Thiamine monophosphate	0.285	0.225	10.81
Thiamine diphosphate	0.068	0.044	2.12
Thiamine Triphosphate	0.036	0.02	0.96
T due to T, TMP, TDP, TTP (i.e. sum)		2.079 $\mu\text{g./g.}$	
T due to everything (determination)		2.0 $\mu\text{g./g.}$	
		% recovery = 103.9%	

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	1.84	1.84	85.5
Thiamine monophosphate	0.33	0.26	12.08
Thiamine diphosphate	0.06	0.039	1.79
Thiamine triphosphate	0.029	0.016	0.75
T due to T, TMP, TDP, TTP (i.e. sum)		2.155 $\mu\text{g./g.}$	
T due to everything (determination)		2.04 $\mu\text{g./g.}$	
		% recovery = 105.2%	



TABLE XVIII

THIAMINE, THIAMINE MONOPHOSPHATE, THIAMINE DIPHOSPHATE
AND THIAMINE TRIPHOSPHATE CONTENTS OF MONTICAIN BARLEY

TRIAL 1

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	2.55	2.55	91.4
Thiamine monophosphate	0.274	0.216	7.9
Thiamine diphosphate	0.029	0.019	0.72
Thiamine triphosphate	0.0109	0.006	0.22
T due to T, TMP, TDP, TTP (i.e. sum)		2.791 $\mu\text{g./g.}$	
T due to everything (determination)		2.6 $\mu\text{g./g.}$	
		% recovery = 107%	

TRIAL 2

	$\mu\text{g./g.}$	Thiamine equivalent $\mu\text{g./g.}$	% of total T
Thiamine	2.54	2.54	88.4
Thiamine monophosphate	0.38	0.30	10.4
Thiamine diphosphate	0.029	0.019	0.66
Thiamine triphosphate	0.018	0.010	0.35
T due to T, TMP, TDP, TTP (i.e. sum)		2.869 $\mu\text{g./g.}$	
T due to everything (determination)		2.7 $\mu\text{g./g.}$	
		% recovery = 106.4%	

TABLE XIX

AVERAGE RESULTS FOR ALL SAMPLES. $\mu\text{g./g.}$

	T Content	TMP Content	TDP Content	TTP Content	Total T content (i.e.determination)	Literature value for total T content
Rabbit liver	0.29	1.43	1.57	0.45	2.34	
Selkirk Wheat	2.3	0.033	0.11	0.05	2.29	2.89 (11)
Ramsey Wheat	3.35	0.069	0.135	0.031	3.69	} 2.34 - 5.94 (29)
Pembina Wheat	3.7	0.032	0.067	0.05	3.58	
Wheat Germ	13.75	0	0.4	0.098	15.8	21.3 (29)
Wheat Shorts	12.07	1.0	0.345	0.065	13.68	15.15, (29)
Wheat Middlings	4.5	0	0.22	0.085	5.14	
Wheat Flour	0.37	0	0.012	0.003	0.405	0.81 (29)
Garry Oats	2.64	0.65	0.089	0.041	3.15	6.59 (11)
Rodney Oats	2.43	0.42	0.128	0.042	2.75	5.51 (11)} 4.8 - 10.8 (29)
Vantage Barley	1.82	0.308	0.064	0.034	2.02	4.26 (11)
Montcalm Barley	2.55	0.327	0.029	0.015	2.65	4.07 (11)} 5.4 - 7.5 (29)

TABLE XX

AVERAGE RECOVERY OF ADDED THIAMINE
THIAMINE MONO- AND DIPHOSPHATES. %.

Item	Thiamine	TMP	TDP	Total thiamine
Rabbit liver	116.5	118.2	127.3	119.9
Selkirk wheat	76.3	88.9	63.7	77.4
Carry oats	110	50.9	61.25	76
Vantage Barley	105.5	63.5	67.25	80

TABLE XXI

FREE AND TOTAL THIAMINE IN CEREAL GRAINS⁺ $\mu\text{g.}/\text{g.}$

Item	TRIAL 1		TRIAL 2	
	Free Thiamine	Total Thiamine	Free Thiamine	Total Thiamine
WHEAT				
Selkirk	2.03	3.10	1.73	2.68
Ramsey	2.86	3.91	2.71	3.98
Pembina	3.15	4.08	2.98	4.25
Wheat germ	14.03	17.21	12.36	16.85
Wheat shorts	12.81	14.10	11.32	12.98
Wheat Middlings	4.02	6.11	3.81	5.12
Wheat flour	0.40	0.51	0.38	0.42
OATS				
Garry oats	3.46	5.89	2.69	5.81
Rodney oats	2.48	4.31	2.12	4.11
BARLEY				
Vantage barley	1.97	4.58	1.76	4.03
Montcalm barley	2.72	3.85	2.12	3.36

⁺ According to the Modified Method of Bechtel and Hollenbeck (1).

TABLE XXII

AVERAGE RESULTS FOR FREE AND
COMBINED THIAMINE IN CEREAL GRAINS⁺

Item	Free Thiamine µg./g.	Total thiamine µg./g.	Combined Thiamine µg./g. (by difference)	Free Thiamine %	Total Thiamine %
Selkirk wheat	1.88	2.89	1.01	65.1	34.9
Ramsey wheat	2.79	3.95	1.16	69.6	30.4
Pembina wheat	3.07	4.17	1.10	73.5	26.5
Wheat germ	13.20	16.53	3.33	79.8	20.2
Wheat shorts	12.07	13.54	1.47	89.0	11.0
Wheat middlings	3.92	5.62	1.70	69.7	30.3
Wheat flour	0.39	0.47	0.08	83.0	17.0
Carry oats	3.08	5.85	2.77	52.7	47.3
Rodney oats	2.30	4.21	1.91	54.7	45.3
Vantage barley	1.87	4.31	2.44	43.5	56.5
Montcalm barley	2.42	3.61	1.19	67.1	32.9

⁺ According to the Modified Method of Bechtel and Mollenbeck (1)

TABLE XXIII
 AVERAGE RESULTS FOR FREE AND
 COMBINED THIAMINE IN CEREAL GRAINS⁺

Item	Free Thiamine µg./g.	Total Thiamine µg./g.	Combined Thiamine µg./g. (by difference)	Free Thiamine %	Total Thiamine %
Selkirk wheat	2.30	2.43	0.13	94.6	5.4
Ramsey wheat	3.35	3.51	0.16	95.5	4.5
Pembina wheat	3.70	3.80	0.10	97.4	2.6
Wheat germ	13.75	14.06	0.31	97.8	2.2
Wheat shorts	12.07	13.12	1.05	92.0	8.0
Wheat middlings	4.50	4.69	0.19	96.0	4.0
Wheat flour	0.37	0.38	0.01	97.4	2.6
Garry oats	2.64	3.23	0.59	81.8	18.2
Rodney oats	2.43	2.87	0.44	84.7	15.3
Vantage barley	1.82	2.11	0.29	86.3	13.7
Montcalm barley	2.55	2.83	0.28	90.0	10.0

⁺ According to the method of Rindi and deGiuseppe (7).

DISCUSSION

Rindi and De Giuseppe (7) did not report doing any work with known quantities of esters carried through their method. This, it was thought, should have been done as proof of the reliability of the procedure and, therefore, conclusions on its accuracy and reliability are based on the recoveries of known amounts of synthetic thiamine, thiamine monophosphate and thiamine diphosphate. At least two trials were performed with each sample. The results of the two trials were quite close, thus the method gives good checks.

The results with rabbit liver (Table IV) show that thiamine is present in the free form and as its mono-, di- and triphosphates, just as was found for rat liver by Rindi and De Giuseppe.

	Rabbit liver $\mu\text{g./g.}$	Rat liver $\mu\text{g./g.}$
T	0.29	0.24
TMP	1.43	0.66
TDP	1.57	6.81
TTP	0.45	0.92

Nevertheless, when known quantities of esters were added to the liver extract, somewhat greater amounts than were added were recovered (Tables III and XX). This indicates that the results reported for thiamine and its esters in rabbit liver should be correspondingly lower than those given in Table IV.

Recovery of the known quantities of T, TMP and TDP from Selkirk wheat shows that TMP was recovered to the greatest extent and TDP to the least with thiamine in between (Tables V and XX). However, average recoveries were well below one hundred per cent. This could be due to several reasons, these being incomplete adsorption on the charcoal column and/or incomplete elution from the column. Both reasons are invalid because preliminary work done on the charcoal column shows the total amount of thiamine found in the liquid percolated through the charcoal and on further elution with 10% propanol was negligible. Another reason for this low value could be absorption of T and TMP on the Dowex column. Preliminary experiments with known amounts of T and TMP also showed this absorption to be negligible (approximately 1%). The low values for TDP might be due to incomplete adsorption and/or incomplete elution from the Dowex column. Here again preliminary experiments with a known amount of TDP passed through the Dowex column showed this to be negligible. It seems, then, that interfering substances due to incomplete purification on the charcoal column can somehow inhibit complete oxidation of thiamine to thiochrome.

The recoveries of added T, TMP and TDP from Garry oats and Vantage barley (Tables XIII, XVI and XX) are similar to those for Selkirk wheat except for recoveries of over one hundred per cent thiamine. This suggests hydrolysis of TMP and TDP occurs during the process.

Table XIX is a summary of average results obtained on all samples studied. Detailed results for each sample are given separately in the corresponding tables. (e.g. Table VI for Selkirk wheat). Considering wheat as a whole (Table XIX) free thiamine occurs in the largest amount, with the values becoming less from TDP, to TMP, to TTP. For wheat germ, middlings and flour, the same trend occurs except that no TMP was found in these three samples. Wheat shorts shows presence of TMP and the value decreases from thiamine to the higher phosphates. Both oats and barley show the decrease in content from thiamine to the higher thiamine phosphates. It is possible that higher phosphates than thiamine triphosphate occur in cereals, but only in very minute amounts. The foregoing observations are shown on a percentage basis in the Table XXIV. Samples with similar contents are represented as a group

Since recoveries from cereals by this method are not strictly quantitative, it must be regarded as mainly of qualitative value only when applied to material of this sort.

TABLE XXIV

T, TMP, TDP, TTP IN THE GRAIN SAMPLES
(AS % OF TOTAL THIAMINE)

	T	TMP	TDP	TTP
Wheat	95.5-98.8	0- 2.26	0.77-5.4	0.39 -2.25
Wheat germ, middlings flour	95.9-99.4	0	0.52-3.30	0.014-1.10
Wheat shorts	91.5-92.5	5.47- 6.55	1.66-1.77	0.20 -0.30
Oats	81.5-85.5	10.8 -16.1	1.64-2.90	0.52 -1.10
Barley	85.5-91.4	7.9 -12.08	0.66-2.12	0.22 -0.96

Using the cholesteryl stearate treated charcoal process of this method for the purification step, paper chromatographic separation according to Siliprandi and Siliprandi (19) was tried. About 25 μ g. of each of T, TMP, TDP, and TTP is needed to produce good chromatograms. Condensing these substances in cereal grains also condenses the impurities and excessive tailing results. Several passes through charcoal to effect sufficient purification and usage of thin layer chromatography instead of paper chromatography will speed up the process and might give a quantitative separation.

The Revised Thiochrome procedure for the determination of thiamine in cereal products by Bechtel and Hollenbeck (1) was applied to all the grain samples studied, to determine the free and total thiamine contents. At least two trials were carried out with each sample, and the results are shown in Table XXI. The average results for free and combined thiamine of the cereal grains as determined by this method are shown in Table XXII and a similar table (Table XXIII) by the New Chromatographic Method of Rindi and de Giuseppe was drawn up for comparison purposes.

The outstanding difference between the results in these two Tables is the higher total thiamine values obtained by the Bechtel and Hollenbeck method. Since the free thiamine is quite similar in both methods, the combined thiamine (determined by difference) is much higher as determined by the above method. The reason for this must lie in the different extraction proce-

dures. In the above method, extraction was with 0.1 N sulphuric acid in a boiling water bath for approximately ten minutes. In the method of Rindi and de Giuseppe extraction was with 5% (v/v) trichloroacetic acid in the cold room (approximately 0°C) from two hours to overnight. Apparently the former method freed all the combined thiamine whereas the latter method freed the thiamine and thiamine phosphates only. Suppose thiamine is linked with protein or carbohydrate or both, and also to phosphates which may occur as free phosphates (mono-, di-, tri-, etc.) or may be combined with protein or carbohydrates or both. Then the results shown in Tables XXII and XXIII suggest that one method frees all of the combined thiamine whereas the other method frees the combined thiamine phosphates only. Thus the percentage of free and combined thiamine varies considerably by the two methods. For instance by the method of Bechtel and Hollenbeck, Selkirk wheat contains 65.1% free thiamine and 34.9% combined thiamine; whereas by the method of Rindi and de Giuseppe, 94.6% of the thiamine obtained was due to free thiamine whereas 5.4% was due to combined thiamine. Thus the percentages obtained by the two methods must be considered together with the actual amounts obtained.

Thus there are trace amounts of thiamine phosphates in cereal grains but the bulk of the combined thiamine does not occur as thiamine phosphates.

SUMMARY

1. Thiamine, thiamine mono-, di-, and triphosphates of several grain samples were determined according to the New chromatographic method of Rindi and deGiuseppe. It was found that the method was qualitative but not quantitative due to unfavorable recoveries of known amounts of esters. However, trace amounts of the phosphate esters were indicated.

2. Purification of the extract by this method was not sufficient to give a good separation by paper chromatography.

3. Total thiamine as determined by the revised thiochrome procedure of Bechtel and Hollenbeck differed from that obtained by the other method. However, the free thiamine was almost the same. The combined thiamine (determined by difference) was consequently different being much greater by the Bechtel and Hollenbeck method. This was attributed to the bulk of the combined thiamine occurring not as thiamine phosphates.

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