AN INVESTIGATION OF THE THIAMINE AND THIAMINE PHOSPHATE CONTENTS OF CEREALS

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ABSTRACT

Wheat, barley and oats were analyzed for total and free thiamine. The thiochrome method was used for the determination of total thiamine. For free thiamine the same procedure was employed with two modifications. There was a preliminary inactivation of enzymes present in the sample and incubation with Taka-Diastase was omitted. The difference between the total and free thiamine contents is referred to as bound thiamine and wheat, barley and oats were found to contain bound thiamine.

A new method, involving paper chromatographic and ion-exchange chromatographic separation, was developed for the determination of thiamine, thiamine monophosphate and thiamine pyrophosphate, and was found to work satisfactorily with synthetic mixtures and with pure substances added to cereal extracts. When it was used with wheat, oats and barley alone the results showed they contained no thiamine phosphates.

Trypsin and diastase were found to release the bound thiamine in cereals, but not to hydrolyse added

phosphoric acid esters of thiamine to free thiamine and it is concluded that the bound thiamine in cereals is not present as esters of thiamine and phosphoric acid.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

Thiamine, or vitamin B_1, has a widespread distribution. It is entirely absent only from oils and fats, cassava, and refined sugar, otherwise it is present in nearly every food. Among the best sources are brewer's yeast, wheat, rice, rye, corn, nuts, peas and beans. Meat, fish and milk are also quite rich in thiamine. The thiamine content of the organs, particularly liver and kidney, is higher than that of the muscles.

In many countries, especially in the Orient and other less-developed parts of the world, cereals are the main source of energy and the people get most of their thiamine and other B vitamins from these foods. Of the cereals wheat and rice are the most important. Wheat contains about 300-800 γ of thiamine per 100 g. and the thiamine content of rice varies usually between 200-300 γ per 100 g. Thiamine is very unevenly distributed in the kernel of wheat. The content of the inner layer, the endosperm, is the lowest, about 30 γ per 100 g. The aleuron layer and the germ are

much richer sources; by far the richest part is the scutellum, the small layer between the germ and the rest of the kernel. Similarly in rice thiamine is found in the outer layers, and therefore machine-milled rice has comparatively little thiamine. This is one of the reasons for which nutritionists recommend the inclusion of whole cereals in the diet.

Thiamine forms a coenzyme which takes part in many important biochemical reactions. All the known thiamine-containing enzymes consist of a protein part, the apoenzyme, and a coenzyme of lower molecular weight, thiamine pyrophosphate. All these enzymes catalyze either a simple decarboxylation or an oxidative decarboxylation reaction. Both take part in the metabolism of pyruvic acid or, more generally speaking, in the metabolism of α-oxycarboxylic acids. Both thiamine and thiamine pyrophosphate have been identified in animal tissues. Thiamine has been identified in cereals in the free form, but there is still no agreement on whether thiamine pyrophosphate occurs in cereals as well, that is, whether a part of the "bound" thiamine is bound as an ester to pyrophosphoric acid.

That thiamine occurs in cereals in some combined or bound form seems well established. Simple extraction

procedures are not sufficient to bring all the thiamine in cereals into solution in the free form. All standard methods—except certain short ones for wheat and wheat flour—call for an enzyme digestion of the sample, presumably to convert the bound to free thiamine.

It has been demonstrated that thiamine may be made to form a number of phosphates in the laboratory, mono-, di-, tri-, tetraphosphate and so on. The question arises whether any of these occur in cereals.

Recently methods have been proposed for the separation of some of the phosphates from synthetic mixtures of them. The procedures involve paper electrophoresis, paper chromatography or column chromatography. It seemed that an informative investigation would be to test these methods to see if adequate recoveries of the various thiamine phosphates could be obtained from synthetic mixtures and—if they could—to attempt the same with cereals. It was believed that this would show whether the thiamine phosphates occur in cereals and, if so, which ones and in what amounts. This is the investigation this thesis describes.

CHAPTER II

REVIEW OF LITERATURE

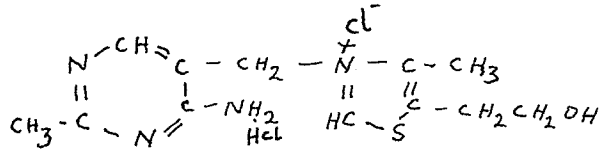
Historical

Since its discovery thiamine (popularly known as vitamin B_1) has engaged the attention of scientists because of its usefulness as an antiberiberi factor and for its overall importance in nutrition. Scientists all over the world attempted to isolate it and determine its chemical structure. Jansen and Donath in 1926 were successful in separating a few milligrams of vitamin B_1. The isolation of crystalline thiamine by Jansen and Donath, by Windaus and his associates, and by Williams and his co-workers is today only of historical interest. Williams (1) gives a good characterization of the efforts in his book: "It is doubtful whether the isolation and identification of any other substance in the history of biochemistry have cost as much labor as have these operations as applied to thiamin. . . The first gram of the pure vitamin must have cost an aggregate of several hundred thousand dollars." Soon its chemical

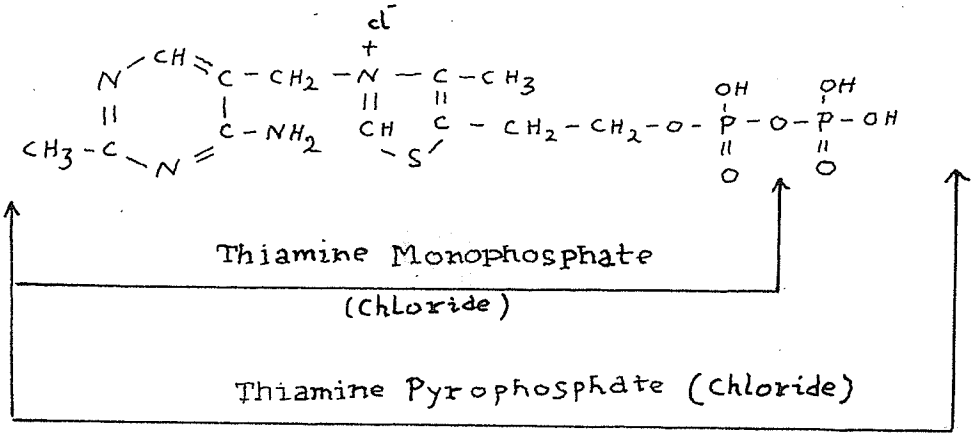
structure was determined and it was synthesized in the laboratory.

Decreased metabolism of thiamine-deficient tissues led to the supposition that it has something to do with cell metabolism but the exact cause was at first not clear. The whole picture was clarified by the brilliant work of Peters and his school in Oxford. In a long series of experiments Peters et al. (2, 3, , 5) and Thompson (4) demonstrated that thiamine plays a decisive role in carbohydrate and, especially, in pyruvic acid, metabolism. Peters (5) and Passmore et al. (6) demonstrated that the rate of oxygen uptake of minced brain from polyneuritic pigeons suspended in pyruvic acid solution could be increased by the addition of thiamine to the system. This was the first instance of a chemical substance catalyzing a reaction in an organ tissue preparation. Further research by Peters et al. (7) and by Westenbrink and Polak (8) led to the concept that the catalysis affecting the oxygen uptake was due not to thiamine itself but to a compound that was synthesized from the free vitamin. The nature of the compound became clear from another series of experiments by Neuberg and his collaborators (9, 10), and by Lohmann and Schuster (11).

The active compound was found to be thiamine pyrophosphate (TPP) which is also called cocarboxylase because it acts as the cofactor in the decarboxylation reaction of pyruvic acid (to acetaldehyde and carbon dioxide). Later it was shown that thiamine pyrophosphate acts as a coenzyme in many other reactions (12) of great biochemical importance and since then more and more work has been done in this field. The chemical formulae of thiamine and thiamine pyrophosphate (TPP) are as follows:



Thiamine Chloride Hydrochloride



Assay of Thiamine

Thiamine can be determined by physical, chemical and biological methods. In the physical method it is estimated by its absorption in the ultraviolet at 265 mμ. This method has been used (13) for measuring the thiamine content of nerve fibres. To distinguish the thiamine from other substances (nuclein components) absorbing at 265 mμ, the absorption may be measured before and after destroying the thiamine by ferricyanide.

The earliest methods that were used for the estimation of thiamine were biological ones. In the beginning when polished rice, an easily obtainable thiamine-free food, was taken as a basal diet, hens and cocks were used as experimental animals. Soon smaller birds, especially pigeons, and mammals (rats) were used as the experimental animals. With the mammals, polished rice could no longer be used as a basal diet because of its deficiencies in other nutrients. Therefore a complex basal diet was essential. The experiments may be prophylactic or curative. Both are apt to give not very accurate results, and both require large amounts of thiamine-rich extracts. These are serious

handicaps and therefore when microbiological and chemical methods became available biolgical methods ceased to be used very much. The advantages of the biological methods are (1) they do not require troublesome extractions and (2) they give specific results.

Schultz et al. (14) were the first to use a fermentation method for the estimation of thiamine. They found that yeast fermentation was enhanced by the presence of free thiamine. They utilized this fact for a quantitative method for its estimation. Used with a Warburg apparatus, the method is suitable for microestimation (15). Thiamine pyrophosphate (cocarboxylase) can be measured by its activity as a coenzyme. Ochoa and Peters (16) were the first to use this method for the quantitative determination of thiamine and cocarboxylase in boiled tissue extracts. Westenbrink (17) developed a micro method which permits the determination of about 0.00005 γ of cocarboxylase and about 0.0005 γ of thiamine separately in a mixture of both compounds.

In 1935 the Swiss investigator Schopfer (18) found that the growth of the mould Phycomyces blakesleeanus

required the presence of thiamine. He used this method for the quantitative estimation of thiamine by measuring its influence on the growth Phycomyces blakesleeanus. The method is very sensitive but its chief disadvantage is that it takes 8 - 10 days before the growth is complete. Later various investigators used other microorganisms— Saccharomyces cerevisiae (19), Lactobacillus fermenti (20)— for the same purpose. The split products of thiamine, the pyrimidine plus the thiazole part, are active also.

Among chemical methods the "Thiochrome method," first reported by Jansen (21) in 1936, is most widely used. It is based on the oxidation of thiamine by potassium ferricyanide in an alkaline medium, extraction by isobutanol of the thiochrome formed, and estimation of the intensity of the violet-blue fluorescence in ultra violet light. Thiamine phosphates can not be assayed by this method, without prior hydrolysis to the free thiamine, because the corresponding thiochrome phosphates are insoluble in isobutanol (22). It could be expected that this method is not only sensitive but also specific for use in biological fluids and extracts. Bouman (23) found, however, that

blighted potatoes (infected with Phytophtera) contain a substance with a blue fluorescence which is soluble in isobutanol. Also urine and other biological fluids contain interfering substances.

When this method was applied to certain cereal extracts and biological fluids high blanks were obtained due to the presence of interfering materials. Various adsorbents (naturally occurring silicates, such as acid clays and fuller's earth) were used to remove the interfering materials but Cerecedo and his coworkers (24, 25, 26) were the first to use a synthetic zeolite (Decalso) for this purpose. They found that thiamine can be adsorbed on Decalso and can be removed later by eluting with neutral salt solutions. They used the method and isolated pure sample of thiamine from rice polishings, brewer's yeast, and wheat germ.

Thiamine in Cereals

Kinnersley and Peters (27) reported that thiamine may be present in more than one form in yeast extracts. They found that about 12% of the activity of their yeast extracts was always associated with the lead precipitate, even upon a reprecipitation. This along with other evidence led them

to suggest that they were probably "dealing with two forms of vitamin B_1, either differently combined or perhaps oxidized and reduced." A similar conclusion was drawn by Guha and Drummond (28) from their studies on wheat embryo extract. This other form of thiamine might have been thiamine pyrophosphate which was later discovered by Lohmann and Schuster (11) to be present in yeast.

Lohmann and Schuster (11) have shown that a large part of the naturally occurring thiamine may be present in the form of its pyrophosphoric acid ester which cannot be estimated as such by the thiochrome method. For this reason some material gave lower values for thiamine than those obtained by biological methods in which TPP is physiologically active. The difficulty was overcome by Hennessy and Cerecedo (29) by enzymically converting cocarboxylase to thiamine during extraction of the sample. An enzyme preparation from beef kidney was used for this purpose. As the preparation of such material is tedious and time consuming, the adaptability of other enzyme products was investigated. The applicability of many enzyme preparations such as Taka-Diastase, Clarase, Polidase, Mylase was studied by various investigators (30, 31) and it was found that Taka-

Diastase and Clarase were quite suitable.

In the usual methods cereals are first treated with such enzyme preparations and then the amount of free thiamine is estimated by the thiochrome method. The applicability of the thiochrome method for the estimation of thiamine in cereals has been studied in detail by various workers (30, 31, 32, 33, 34, 35). A rapid method for the determination of thiamine in wheat and flour has been reported by Hoffer et al. (33) in which the enzymic digestion step was omitted. They extracted the sample with potassium chloride solution (acidified with acetic acid) and determined the thiamine content of the extract by the thiochrome method. They found that by the use of a correction factor the rapid method with unenriched commercial flours gave the same results as obtained by the regular procedure. Without the correction factor it gave lower results. With enriched flour and wheat no correction was required. With bran and shorts the rapid method gave higher thiamine values than the regular procedure, with germ it gave lower values. They explained the low results with germ by assuming that it contained bound thiamine, presumably cocarboxylase, but no explanation was offered for high results with bran and shorts or for the low

results on unenriched flours.

The occurrence of various thiamine phosphates, mono-, di-, and triphosphate in animal tissues, particularly in liver, has been reported by various workers (36,37,38). The presence of thiamine pyrophosphate in blood, animal tissues and yeast has been demonstrated by its cocarboxylase activity and also identified chromatographically. Greiling and Kiesow (36) identified thiamine triphosphate (TTP) and TPP in the liver, kidney and brain of rat by paper chromatography. Recently Rindi and De Giuseppe (37) found TMP, TPP and TTP in rat tissues. Kiessling (38) identified the presence of thiamine, TMP, TPP and TTP in yeast extract by paper chromatography. It has been said earlier that in cereals thiamine occurs both in the free and bound form. But as far as the investigator is aware it has not been proved definitely that the bound thiamine in cereals is thiamine pyrophosphate.

Hennessy and Cerecedo (29) compared the thiamine contents of several samples of whole wheat and wheat germ determined by different methods. They used three methods, namely, bioassay, the thiochrome method without enzymic treatment, and the thiochrome method after enzymic (phosphatase preparation) treatment. They obtained comparable results by all the three methods with two wheat germ samples but with

the third wheat germ sample the first and third methods gave a higher thiamine content than that obtained by the second method, thus indicating the presence of bound thiamine (probably cocarboxylase) in that particular wheat germ sample.

Tauber (39) studied the cocarboxylase content of several plant materials and concluded that with the exception of yeast, plant materials possess very small cocarboxylase activity. The materials he used were cabbage, onions, orange juice, orange seeds, spinach, green pepper, etc. No cereal sample was studied.

Obermeyer, Fulmer and Young (40) reported that wheat flour does not contain any cocarboxylase. They found that all the thiamine of wheat flour can be determined by the thiochrome method without prior treatment with any enzyme preparations. They also observed that wheat flour contained a cocarboxylase-hydrolyzing factor which could hydrolyze any added TPP if the extraction was carried out with cold potassium chloride solution (5 - 25% potassium chloride in 0.005 N sulfuric acid). The factor was found to be heat-labile and could be destroyed by performing the extraction with hot 0.1 N sulfuric acid.

On the other hand Sure (41) found bound thiamine (probably cocarboxylase) in wheat embryo. He studied the efficiency of different commercial enzyme preparations in hydrolyzing the bound thiamine in yeast and wheat embryo at different pH's. He observed that at low hydrogen ion concentrations (pH 2.5 to 2.7), practically no free thiamine was liberated by commercial phosphatase preparations from the cocarboxylase of two brewer's yeasts. However, at the same range of pH, 75 - 88 percent of combined thiamine was hydrolyzed by such phosphatases from commercial wheat embryo. The fact that the cocarboxylase in wheat embryo can be hydrolyzed by phosphatase at much lower hydrogen ion concentrations than the combined thiamine of brewer's yeasts led Sure to suggest that thiamine exists in a differently combined form in wheat germ than in the type of yeasts studied.

Synthesis of Thiamine Phosphates

Of the thiamine phosphates cocarboxylase commanded particular attention on the part of scientists because of its important role as a coenzyme in biochemical reactions. It has been synthesized both by chemical and enzymic methods.

Recently TMP and higher thiamine phosphates have also been synthesized chemically. Weyland and Tauber (42) gave an extensive description for the preparation of thiamine pyrophosphate which substance Weil-Malherbe (43) synthesized by treating thiamine bromide with silver pyrophosphate. Karrer and Viscontini (44), Viscontini et al. (45) and Yusa (46) improved the method of Weyland and Tauber. The improved method is described briefly as follows.

One g. of thiamine chloride hydrochloride and 1 g. of phosphorus pentoxide were added, in several portions, to phosphoric acid mixture at 100 - 105° C. The phosphoric acid mixture was prepared by heating 2.6 g. of orthophosphoric acid at about 320° C till it became semi-solid. By this treatment orthophosphoric acid is converted to a mixture of pyrophosphoric acid ($H_4P_2O_7$) and metaphosphoric acid (HPO_3) (62). The whole mixture was allowed to stand further for 20 minutes at 100 - 105° C with occasional stirring. It was cooled, mixed with 1.6 ml water, and the resulting solution was centrifuged. The supernatant viscous liquid was poured into 40 ml cold ethanol-acetone mixture (1:1 vol.) with vigorous agitation. The white precipitate formed was collected, dissolved in minimal amount of water and precipitated as described above. After the precipitation was repeated thrice, the product was obtained as nearly nonhydroscopic white powder, the yield being 1.26 g. The

mixture (product) thus prepared contained higher phosphates of thiamine including thiamine triphosphoric acid ester (TTP) 7%, thiamine pyrophosphate 20%, thiamine monophosphate 60%, and free inorganic phosphate (below 1%).

Paper Chromatography of Different Thiamine Derivatives.

The first method for separating different thiamine derivatives by paper chromatography was reported by Baldantoni et al. (47) using isobutanol, pyridine and water (1:1:1) as solvent. By using an ascending chromatographic method they were able to separate a pseudo thiamine, thiamine, TMP and TPP. By this method TPP and TTP cannot be separated.

Kiessling (48) reported a procedure by which all the phosphate derivatives of thiamine can be separated. The solvent is 100 ml. isobutyric acid, 60 ml. 1 N ammonia, and 1.6 ml. 0.1 M ethylene diamine tetra-acetic acid. From a mixture of thiamine phosphates prepared according to Viscontini, Bonetti and Karrer (45), nine thiamine compounds can be isolated. From the total and hydrolyzable phosphorus per molecule of each derivative he concluded that those nine spots were due to thiamine, pseudothiamines, thiamine mono-, di-(or pyro-), tri-, tetra-, and pentaphosphates. Pseudothiamines are bromo- or chloro- derivatives of thiamine, generally present as impurities in synthetic thiamine preparation. They contain non-ionizable Cl or Br and were first separated and identified chromatographically by Gaudiano et al.

(63). The spots 8 - 9 have more than five phosphate groups per molecule of thiamine. There is no report in the literature about the mode of linkage of these phosphate groups to the thiamine molecule. Roux et al. (64) assumed that in thiamine triphosphate the third phosphate group is attached to the NH_2 of the pyrimidine nucleus of thiamine molecule. They also proposed that in the higher phosphates of thiamine two separate chains of polyphosphoric acid might be linked each to the NH_2 of the pyrimidine and $-CH_2OH$ group of the thiazole, but no experimental evidence was presented. By using this method he got fairly good separation, the respective R_f values are 0.87, 0.79, 0.77, 0.68, 0.55, 0.46, 0.36, 0.24, and 0.21.

Siliprandi and Siliprandi (49) used a different solvent for the chromatographic separation of thiamine phosphates. The solvent is n-propanol-water-1 M acetate buffer pH 5 (65:20:15). They found that previous washing of the filter paper (Whatman No. 1) with 4 N hydrochloric acid and an alcoholic solution of 8-hydroxyquinoline followed by washing with 50% ethanol resulted in a nearly quantitative separation of thiamine, TMP, TPP, and TTP. The higher phosphates cannot be separated by this technique. The corresponding R_f values of thiamine, TMP, TPP, TTP are 0.64, 0.38, 0.21 and 0.12 respectively.

All the chromatographic methods discussed above are the ascending type.

Paper Electrophoretic Separation of Thiamine Derivatives

Various methods (50-54) for separating different thiamine derivatives by paper electrophoresis have been described in the literature, each involving a different buffer solution of different pH and ionic strength. Rossi-Fanelli et al. (51) used a solution (pH 3.0) consisting of 1000 ml. of 0.1 N sodium chloride and 3.5 ml. glacial acetic acid. They were able to separate thiamine, TMP, TPP and TTP nearly quantitatively. The thiamine derivatives migrated to the negative end of the filter paper at different speed TTP < TPP < TMP < Thiamine.

Siliprandi and Siliprandi (50) used a different solution for electrophoresis. It was composed of 50 ml. 1 M sodium acetate, 50 ml. 1 M acetic acid, 900 ml. distilled water, the final pH of the solution is kept at 5.1 - 5.2. They achieved a good separation of thiamine, TMP, TPP and the higher phosphates. Thiamine migrates towards the cathode, TMP goes more slowly also towards the cathode, TPP goes to the anode, TTP and higher phosphates migrate at greater speeds, respectively, towards the anode.

Other Methods of Separation of Different Thiamine Derivatives

Recently Siliprandi and Siliprandi (49) have studied the problem of separation of thiamine derivatives in greater detail. They studied various methods, namely, chromatographic separation on (a) filter paper, (b) starch column, (c) ion-exchange column and electrophoretic separation by (a) filter paper, (b) starch and cellulose powder column and were able to use all of these methods for the quantitative separation of various thiamine derivatives from a mixture of pure components. In this report they also described a method of desalting a solution containing thiamine derivatives. This desalting step is necessary because the presence of any salt interferes with the separation of different derivatives either by chromatographic or electrophoretic methods. It is described briefly as follows.

The solution containing thiamine derivatives and salt was passed through a small column containing carbon ("Carbo-Activ"), pretreated with cholesteryl stearate (100 mg. per g. carbon), in order to adsorb the thiamine derivatives. Cellulose powder was mixed in the column to obtain a faster flow rate. After washing with distilled water, which removed the inorganic salts still retained in the column,

the thiamine compounds were eluted with 50% ethanol. The treatment of the carbon with cholesteryl stearate was necessary to make the adsorption reversible. The alcohol water eluate was concentrated and used for chromatographic or electrophoretic separation.

In all the cases alcoholic alkaline ferricyanide solution was sprayed on the filter paper, after drying, to detect the position of the different thiamine derivatives. All the thiamine derivatives are oxidized to the corresponding thiochromes which fluoresce in ultraviolet light.

Recently De Giuseppe and Rindi (55) reported a method for the separation of thiamine phosphates by ion-exchange chromatography. They used anion exchange resins in the form of borate and acetate. A mixture of thiamine and its phosphoric acid esters was percolated through two superposed columns A and B containing a strong anion exchange resin in the acetate and borate form respectively. TPP and TTP were retained in column A, while column B held TMP firmly and thiamine very loosely. The successive use of 0.02 M boric acid (H_3BO_3) and 0.1 M sodium chloride in 0.1 M hydrochloric acid solution allowed the elution of thiamine

and TMP, respectively, from column A, while the elution of TPP and TTP was achieved by using 0.02 M sodium acetate in 0.04 M acetic acid and 0.1 M hydrochloric acid respectively.

Those methods, for separation of different thiamine derivatives, described above can not be employed directly with the cereal extracts because they contained many interfering materials (carbohydrates, proteins, salts, etc). So there remains the problem of developing a method which can be used for the quantitative estimation of different derivatives of thiamine in cereals. It might resemble methods already described, but must provide for the removal of interfering substances.

From the above discussion it is clear that the main problem was to (1) determine the free and total thiamine content of the cereal samples, (2) develop a method for determining the various thiamine phosphates in cereal samples both qualitatively and quantitatively and use that method to find which derivatives of thiamine are present in cereals and in what amounts.

CHAPTER III

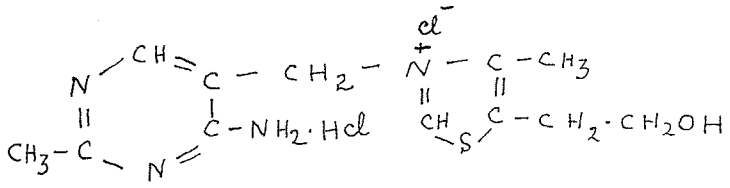
METHODS AND MATERIALS

1. Thiochrome Method for the Determination of the Total Thiamine in Cereals.

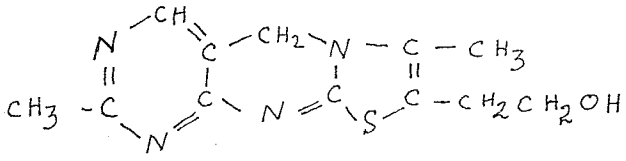
This method was first reported by Jansen (21) and has been modified later by other investigators (22, 29 - 35). Each step of the method has been studied extensively and has been reviewed elsewhere (34, 35). Bechtel and Hollenbeck (35) have recently studied the method elaborately and suggested some modifications. In this investigation the modified method described by them was followed. Since its individual steps enter into the overall experimental procedure of this investigation the method is described in some detail below.

(a) Principle of the method. 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 layer is measured with a sensitive fluorophotometer. The result is compared with that obtained from a solution containing a known amount of thiamine.



Thiamine Chloride Hydrochloride



Thiochrome

(b) Apparatus. (1) Fluorophotometer. Electronic fluorophotometer - Manufactured by Coleman Electronic Co., Inc., Maywood, Ill., U. S. A. The apparatus was equipped with special filters suitable for thiochrome estimation and was used according to the manufacturer's direction. (2) Water bath for a temperature of 100° C. (3) Incubator for a temperature of 37 - 40° C. (4) Decalso adsorption tubes. The reservoir should be approximately 50 mm. long and 25 mm. in diameter, and the adsorption tube 5 to 6 mm. inside diameter and approximately 140 mm. long ending in a capillary tube 10 mm. in length and of such bore that when charged the rate of flow will be not more than 1 ml. per minute (5) Glass-stoppered graduated cylinders of 25 ml. capacity. (6) Reaction vessel. Glass-stoppered centrifuge tubes with conical bottom of 30 ml. capacity.

(c) Reagents. (1) Sodium hydroxide solution, 15%. (2) Potassium ferricyanide solution, 1%. If contained in a stoppered brown bottle and stored in a cool, dark place, this reagent is stable. (3) Alkaline potassium ferricyanide solution. One hundred ml. of sodium hydroxide solution (reagent 1) was mixed with 3 ml. of potassium ferricyanide solution (reagent 2). This reagent was prepared daily just

before use. (4) Sulfuric acid solution, 0.1 N approximately. (5) Sodium acetate solution, 2.5 M. (6) Enzyme preparation. Two percent Taka-Diastase [a] solution in 2.5 M sodium acetate (reagents). This reagent was prepared fresh daily. (7) Isobutanol. This should give a blank reading of 1.5 galvanometer scale divisions or less. It was purified by shaking with activated charcoal (2 g. charcoal per 100 ml. isobutanol) for 15 minutes and was filtered through a fine filter paper (Whatman No. 42). (8) Potassium chloride solution, 25%. (9) Activated Decalso - Synthetic zeolite (sodium aluminium silicate exchanger) - supplied by "The Permutit Company", Birmingham, N. J., U. S. A. (10) Stock thiamine solution. Thiamine chloride hydrochloride [b] was dried over phosphorus pentoxide in a desiccator for at least 24 hours. One hundred mg. of it was dissolved in 25% ethanol and diluted to 1 liter with 25% ethanol. This solution is stable for several months if kept under refrigeration. (11) Standard thiamine solution . Five ml.

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[a] Taka-Diastase. Supplied by "Parke, Davis & Co. Ltd., Wakerville, Ontario, Canada.

[b] Thiamine chloride hydrochloride. Supplied by "Nutritional Biochemicals Corporation", Cleveland, Ohio, U. S. A.

of the stock thiamine solution (warmed to room temperature) was diluted to 100 ml. with 0.1 N sulfuric acid. Four ml. of this intermediate solution was diluted again to 100 ml. with 0.1 N sulfuric acid solution (final concentration 0.2 γ per ml.). This solution was prepared daily. (12) Quinine sulfate solution. One hundred mg. of U. S. P. quinine sulfate was dissolved in 0.1 N sulfuric acid and diluted to 1 liter with the sulfuric acid solution. Three ml. of this solution was diluted to 1 liter with 0.1 N sulfuric acid solution. This solution was stored in a brown bottle.

(d) Sampling of cereals. The whole grain was ground in a Wiley mill to pass a 20 mesh screen. The milled material was then ground finely to a 100-mesh size and mixed thoroughly.

(e) Extraction. About 4 - 5 g. of the sample was weighed and placed in a 250 ml. volumetric flask (pyrex). One hundred ml. of 0.1 N sulfuric acid was added and the mixture was heated in a boiling water bath for fifteen minutes with occasional shaking. The flask was cooled and 10 ml. of enzyme suspension (reagent 6) was added. The pH

was adjusted to 4.5 and the mixture was allowed to incubate for 4 hours at 37° C. Then it was cooled to room temperature and the volume was made up to 250 ml. The contents were mixed thoroughly for 15 minutes and filtered through a fluted filter paper (Whatman No. 1), the first few ml. of the filtrate being discarded. The filter paper had been tested previously and found to adsorb no thiamine.

(f) Purification. This step was necessary to get rid of the interfering material which was present in the cereals and gave a high galvanometer reading for the blank. This was done by passing the extract through a column containing Decalso, a synthetic zeolite composed of sodium aluminium silicate. Decalso acts as a cation exchanger and was used in the H^+ ion form. Thiamine in acidic solution (pH 4-5) is adsorbed by Decalso and can be eluted with 25% potassium chloride solution. The used Decalso was regenerated by repeated washing with 3% hot acetic acid followed by washing with hot water.

A column height of 3-4 cm. and diameter 5-6 mm. was found to be sufficient for the adsorption of 20 γ of thiamine. Before use, the column was washed with boiling water. Both during adsorption and elution the flow rate

was adjusted to about 1 ml. per minute. The flow rate was controlled by putting some glass wool at the bottom of the column and pressing it to the desired degree.

Forty to sixty ml. of the filtrate from the previous section (e) was passed through the Decalso column. The reservoir and the column were washed with three successive 5 ml. portions of hot water. Then the adsorbed thiamine was eluted with 20 ml. of 25% potassium chloride solution and the eluate was collected in a glass-stoppered graduate cylinder. The eluate was made up to 25 ml. with water and shaken well.

(g) Oxidation. Five ml. of the eluate from the previous section was placed in a reaction vessel and 3 ml. of alkaline ferricyanide solution (reagent 3) was added to it and the vessel shaken well. Fifteen ml. of isobutanol was added to the mixture immediately with a quick delivery pipette and the whole was shaken vigorously for 90 seconds. The two layers of water and isobutanol were separated by centrifuging the reaction vessel for 30 seconds at 2000 r.p.m., and the bottom aqueous layer was sucked out with a thin capillary tube, connected to suction. One ml. of 95% ethanol (dehydrating agent) was added to the isobutanol layer and mixed well. About 10 ml. of the mixture was decanted into the cuvette for reading the

fluorescence of the thiochrome. For the blank the same procedure was repeated except 3 ml. of potassium hydroxide solution (reagent 1) was added instead of alkaline potassium ferricyanide solution (reagent 3).

(h) Measurement and calculation. The fluorophotometer was standardized primarily with standard thiamine solution and secondarily with quinine sulfate solution. The apparatus was adjusted in such a way that it gave a fixed suitable galvanometer deflection (70 - 80 scale divisions) with quinine sulfate solution. The concentrations of the standard thiamine and that of the unknown thiamine solution were chosen so that both gave nearly the same galvanometer deflections. The special filters (supplied by the Manufacturers) were used. An example of calculation is shown below.

Microgram (γ) of thiamine chloride hydrochloride per g. of the cereal sample

$$= \frac{R_x - R_{xb}}{R_s - R_{sb}} \times \frac{V}{Z} \times \frac{E}{S} \times \frac{P}{R}$$

where R_x = fluorometer reading with unknown.

R_{xb} = blank fluorometer reading with unknown.

R_s = fluorometer reading with standard thiamine.

R_{sb} = blank fluorometer reading with standard thiamine.

V = volume of extraction (250 ml.) in ml.

Z = volume in ml. of extract passed through Decalso.

E = microgram of thiamine chloride hydrochloride, present in the reaction vessel containing standard thiamine solution.

S = sample weight in g.

P = volume of eluate; and

R = volume of eluate used for oxidation.

A standard curve (Fig. 1) of the galvanometer reading vs. microgram of thiamine content of the final 16 ml. of isobutanol was drawn and in some cases the thiamine content was evaluated from this curve. In all cases, before taking the reading, the fluorometer was adjusted in such a way that the standard quinine sulfate solution gave a galvanometer reading of 70.

The total thiamine contents of different cereals are shown in Tables 4 - 9.

2. Thiochrome Method for the Determination of the Free Thiamine in Cereal.

As mentioned previously in chapter II, p. 12, enzymic digestion of the cereal sample is necessary to bring its bound thiamine into solution. Without enzymic digestion the thiamine content of cereals was found to be smaller in most cases. Obermeyer, Fulmer, and Young (40) 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 sulfuric acid solution for 15 - 30 minutes. Hennessy and Cerecedo (29) have also indicated the presence of bound and free thiamine in one of the wheat germ samples they studied. The amount of free thiamine, extractable by heating the cereal sample with 0.1 N sulfuric acid for 15 minutes is described as the free thiamine content of the cereal. The method used is described briefly as follows.

About 4 - 5 g. of the finely ground cereal sample was placed in a 250 ml. volumetric flask and 100 ml. of hot 0.1 N sulfuric acid solution was added to it. The contents were heated in a boiling water bath for 15 minutes with occasional shaking. The mixture was cooled to room temperature, filtered and the amount of free thiamine in the filtrate was determined (as described in the section 1 of this

chapter) immediately, thus omitting the enzymic digestion. The difference between the total thiamine and the free thiamine content was assumed to be the bound thiamine. The free thiamine contents of the cereals are shown in Tables 4 - 9.

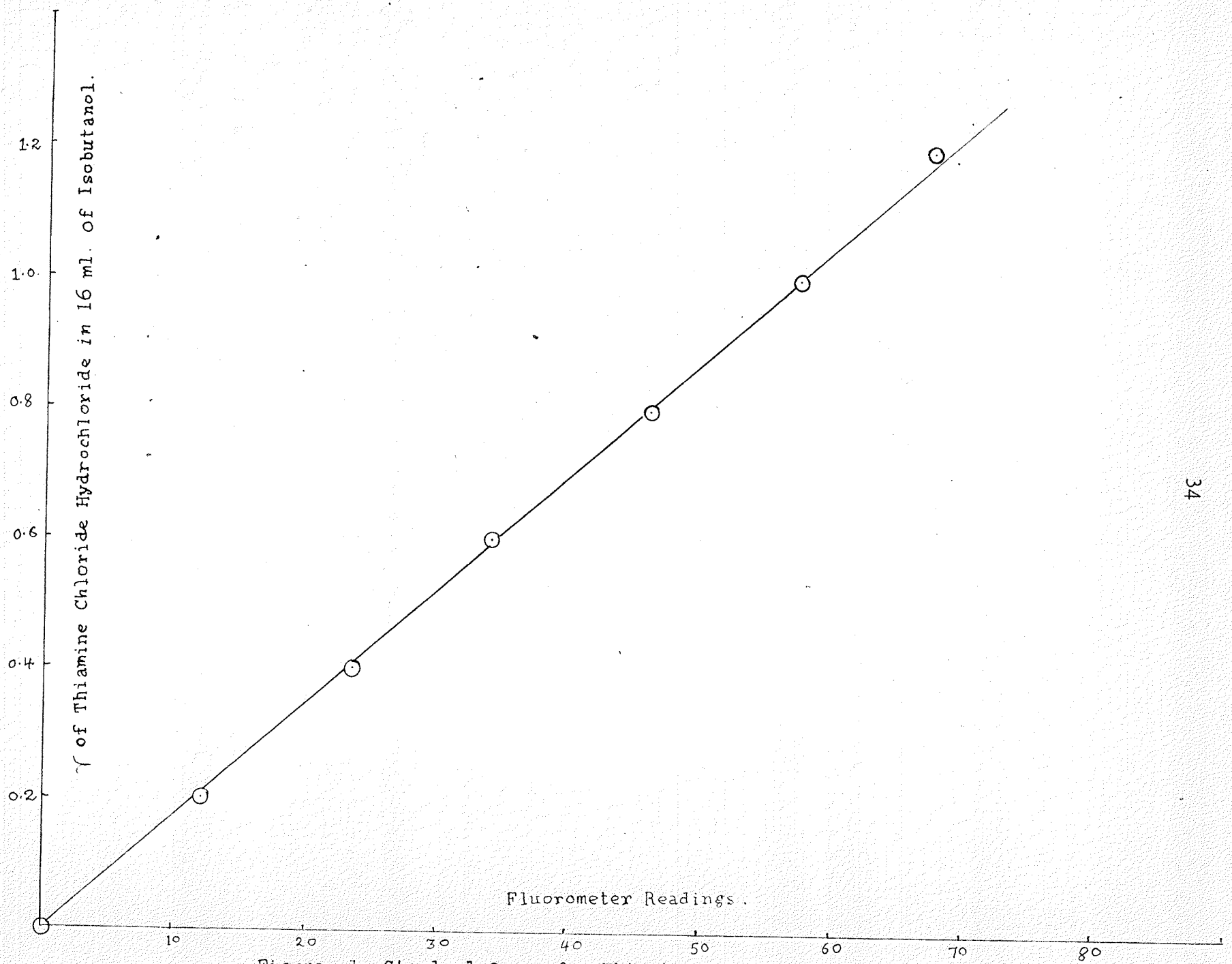

Figure 1. Standard Curve for Thiamine.

CHAPTER IV

DEVELOPMENT OF A METHOD FOR THE DETERMINATION OF DIFFERENT THIAMINE PHOSPHATES IN CEREALS

1. Preliminary Studies

Thiamine and thiamine phosphates are expected to be present in cereals in small amounts, about 4 - 6 γ per g. For chromatographic or electrophoretic separation about 50 - 60 γ of total thiamine derivatives are needed and it should be present in a minimum amount of solution. About 12 - 16 g. of cereal sample, depending on the thiamine content of the particular material, would be necessary for this purpose. When the cereal sample is extracted with 0.1 N sulfuric acid solution, either cold or hot, many other materials — carbohydrates, proteins, other B vitamins etc. — also go into solution and these must be removed and the whole extract (about 300 - 400 ml.) be concentrated to about 0.5 - 1.0 ml. before it can be subjected to paper chromatographic or electrophoretic separation. The usual method used for separating interfering materials is preferential adsorption

and elution. The adsorbents generally used for thiamine are Fuller's earth, charcoal, and Decalso. The suitable eluents should be low-boiling volatile organic liquids preferably neutral or weakly acidic, which can be concentrated easily to a residue-free liquid. Various adsorbents, namely Fuller's earth, Florisil, activated alumina, calcium carbonate, charcoal and Decalso were employed as adsorping agents and eluting capacity of different solutions were studied. They are discussed briefly as follows.

a. Fuller's earth. Fuller's earth and other naturally occurring silicates have been widely used in the earlier days for the adsorption of thiamine and it has been reported (56) that thiamine can be eluted with a mixture of pyridine, acetic acid and water (4:1:1). About 60 γ of thiamine was passed through a column, containing 2 - 3 g. of Fuller's earth, and the column was washed with water. The filtrate was analyzed and found to contain no thiamine, thus indicating the complete adsorption of thiamine. Three solutions, containing different percentage of pyridine and acetic acid, were used for elution and it was found that in no case was the percentage elution over 80%. The following mixtures were tested as eluting agent and the respective elution capacity

are noted side by side.

Pyridine,	acetic acid,	water		(4:1:1)	80%	elution
"	"	"	"	(6:1:1)	69%	"
"	"	"	"	(2:1:1)	73%	"

In each case 50 ml. of eluate was collected, and increasing the volume of eluate had no effect on the results.

(b) Florisil, activated alumina, calcium carbonate. Other materials such as florisil [a], activated alumina, and calcium carbonate were also found to adsorb thiamine, but the adsorbed thiamine could not be eluted completely. Ethanol (50%) and pyridine, acetic acid, water mixture (4:1:1) were tested as eluting agent. In no case was the elution more than 80%.

(c) Charcoal. Activated carbon has been used for a long time for the adsorption of thiamine and this was confirmed in the present investigation. Three samples of charcoal, which were available in the laboratory, namely Norit A [b],

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[a] Florisil. Supplied by "Floridin Company", Warren, Pennsylvania, U.S.A.

[b] Norit A. Supplied by "Fisher Scientific Company Ltd.", Toronto, Canada.

animal charcoal [c], activated carbon [c] were tested and the elution capacity of different eluting agents in each case was studied. About 0.5 g. of charcoal was found to be sufficient to adsorb 60 γ of thiamine chloride hydrochloride. The charcoal powder was mixed with filter paper pulp, which was found to have no thiamine-adsorbing capacity, and was made to a slurry with water. This slurry was used to make the column. The following solutions were tested as eluting agents and the percentage elution in each case is noted in Table I.

It has been reported by Siliprandi and Siliprandi (49) that by the previous treatment of activated carbon with cholesteryl stearate, the adsorption process can be made reversible. This was first proposed by Hagdahl et al. (57). He argued that strongly adsorbable compounds such as hexanol or cholesteryl stearate are adsorbed on the most active sites, thus eliminating the possibility of irreversible adsorption of weakly-adsorbable compounds (in this case thiamine) on the most active sites. The pretreatment of the carbon was effected as follows:

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[c] Animal charcoal and activated carbon. Source unknown.

Table 1

THIAMINE-ELUTING CAPACITY OF DIFFERENT SOLUTIONS FROM DIFFERENT CHARCOAL SAMPLES

Eluting solution	Percentage elution		
	Norit A	Animal charcoal	Activated carbon
Ethanol, 50% (50 ml.)	46	27	$\approx$ 0
Ethanol, 50% (25 ml.) + Methanol, 50% (25 ml.)	49	31	$\approx$ 0
Pyridine, acetic acid, water (4:1:1), 50 ml.	55	30	$<$10

One hundred mg. of cholesteryl stearate [a], dissolved in a 1:1 ethanol-ether mixture, was adsorbed on 1 g. of carbon by shaking the mixture for 48 hours. The ethanol-ether mixture was then diluted with water so that all the cholesteryl stearate was taken up by the carbon.

All the three carbon samples were pretreated with cholesteryl stearate in this way and the improvement in the elution capacity of different eluting agents was studied. The results are given in Table 2.

From these results it is clear that elution of thiamine was not complete and this method was not suitable. Siliprandi and Siliprandi (49) used a carbon sample called "Carbo Activ" which was not available in the market during the time this investigation was carried out.

(d) Decalso. It has been said in Chapter III, p. 28, that Decalso can be used for removing interfering materials from thiamine solution. Thiamine is adsorbed on Decalso and the interfering materials are washed out with water. The

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[a] Cholesteryl stearate. Supplied by "The British Drug Houses Ltd.", Poole, England.

Table 2

THIAMINE-ELUTING CAPACITY OF DIFFERENT SOLUTIONS FROM DIFFERENT PRETREATED CHARCOAL SAMPLES

Eluting solution	Percentage elution		
	Norit A	Animal charcoal	Activated carbon
Ethanol, 50% (50 ml.)	65	35	15
Ethanol, 50% (25 ml.) + Methanol, 50% (25 ml.)	67	37	16
Pyridine-acetic acid-water, (4:1:1), 50 ml.	78	41	23

adsorbed thiamine can be eluted with 5 N hydrochloric acid, but it was undesirable because high concentrations of hydrochloric acid would hydrolyze the thiamine phosphates. Various pyridine-acetic acid-water mixtures and ethanol-water mixtures were tested as eluting agents and it was found that alcohol solution did not elute any thiamine, while maximum elution with pyridine-acetic acid-water mixtures was 80%.

It has been said earlier that thiamine can be eluted completely with 25% potassium chloride solution. This eluate must be freed completely from potassium chloride if it is to be used for chromatography. The investigator was aware of no reagents which could precipitate K^+ ion completely without introducing any foreign metal ion in the solution and so it was a big problem to remove the K^+ ion from the solution. The method of electrolytic desalting has been employed for separating salts from amino acids with success. It was thought that it might solve the problem. The principle of electrolytic desalting is described briefly as follows.

In operation, the solution to be desalted is floated on a mercury surface - the cathode - and is in direct contact with a semipermeable membrane. Electrolyte is

contained above the membrane in the anode cell. The application of direct current causes the inorganic cations to migrate to the mercury, where they are reduced to an amalgam. The anions migrate through the membrane into the anode chamber where they are oxidized at a platinum electrode and swept away by the following electrolyte. The flow of electrolyte also serves to cool the anode. The organic materials have much slower rates of migration, and furthermore their charge tends to be neutralized at the membrane surface. Thus the inorganic ions are effectively removed from solution, while the organic molecules remain. The mercury amalgam is removed by continuous circulation and washing with water. The desalting operation takes 5 minutes to 1 hour, depending on the type and volume of sample solution. Completion of the operation is indicated by a relatively rapid drop in the operating current.

This method was tried with the potassium chloride eluate and after the desalting process was over, the sample solution was found to contain no thiamine. This might have been due to either (1) destruction of thiamine during electrolysis or (2) passing of thiamine through the semi-permeable membrane. Later on it was found that, before the half of the total salt was removed, all the thiamine dis-

appeared from the solution. So this method was unsuitable.

The method of desalting a sample as reported by Siliprandi and Siliprandi has been described earlier in Chapter II, p.20 , and was found to be unsuccessful due to the unavailability of the proper kind of charcoal.

When all these methods failed other cations, easily removable from the solution without introducing any other metal ion, were tested as eluting agents. It is a well-known fact that Ba^{++} ion can be precipitated as barium sulfate with dilute sulfuric acid and it was found to have good eluting power, about 93 - 95% in case of thiamine. The corresponding eluting power in the case of TPP was about 86%. It was thought that barium chloride could be used as an eluting agent and the final result could be corrected by multiplying with suitable correction factors. The corresponding correction factors for thiamine and TPP were 1/0.94 and 1/0.86 respectively. Fifty ml. of 30% barium chloride ($BaCl_2.2\ H_2O$) was used for elution and was found to be sufficient. After elution, barium chloride was precipitated by adding the exact amount of 1 N sulfuric acid required, a little ethanol was added to keep the

solubility of barium sulfate to a minimum. The precipitate was decanted and the precipitate washed. All the supernatant and wash water were collected and concentrated in vacuum at room temperature. It was found that when the solution was concentrated to a very low volume (about 1 ml.) some solid residue appeared. This residue might have been excess barium chloride, or barium sulfate or other impurities which might have been present in the barium chloride reagent. As the amount of barium chloride in the eluate was quite high (about 15 g.) it could contain comparatively large amounts of impurities even when pure barium chloride was used. Another disadvantage of this method was that it was very difficult to precipitate all the barium chloride exactly, and any excess sulfuric acid was undesirable because it, when concentrated, oxidizes all organic matters very rapidly.

Further investigations were carried out and a new method was found, which is described in section 3 of this chapter.

2. Paper Chromatographic and Electrophoretic Separation of Thiamine Derivatives.

The available methods have been discussed in the "Review of Literature." Of the paper chromatographic methods the ones reported by Siliprandi and Siliprandi (49), and by Kiessling (48), were used in this investigation. The former used the solvent n-propanol-water-1 M acetate buffer, pH 5 (65:20:15) and gave a very good separation of thiamine, TMP, and TPP. The higher phosphates seemed not to move at all and so this method is suitable when the higher phosphates are absent. The latter method uses the solvent - 100 ml. iso-butyric acid, 60 ml. N ammonia and 1.6 ml. of 0.1 M ethylene diamine tetracetic acid and gave a good separation of the higher phosphates. It was intended to be used if any higher phosphates of thiamine had been found to be present in the cereal samples. In both the methods Whatman No. 1 filter paper was used. Typical chromatograms obtained by the above two methods are shown in Figures 2 (a) and 2 (b) respectively. Both methods are of ascending type. Figure 2 (a) shows the presence of TMP and TPP in the synthetic mixture of thiamine phosphates, prepared according to the method of Yusa (46), TPP and the higher phosphates are not well

separated from each other. From Fig. 2(b) it is seen that the chromatogram, prepared from the same synthetic mixture, contains eight well-separated spots. The first two top-most spots (near to the solvent front) are due to TMP and TPP respectively. It did not indicate the presence of any thiamine (T) or pseudothiamines. It took about 16 - 20 hours to run the solvents, afterwards the chromatograms were dried in air and sprayed with alcoholic alkaline ferricyanide solution (1 ml. 1% potassium ferricyanide solution, 50 ml. 30% sodium hydroxide and 50 ml. ethanol) and dried in air. When it was viewed in ultraviolet light, all the thiamine derivatives appeared as blue fluorescent spots. The spots were marked with pencil and afterwards photographs were taken using ordinary black and white film (Kodak TX 135-20) in day light.

For the unknown a parallel run with known derivatives of thiamine was made, only the part of the filter paper containing the known compounds was sprayed with ferricyanide reagent and the position of different derivatives was located by comparing with that of the known ones. The respective spots, from the unknown part, were cut into smaller pieces and placed in 25 ml. volumetric flasks

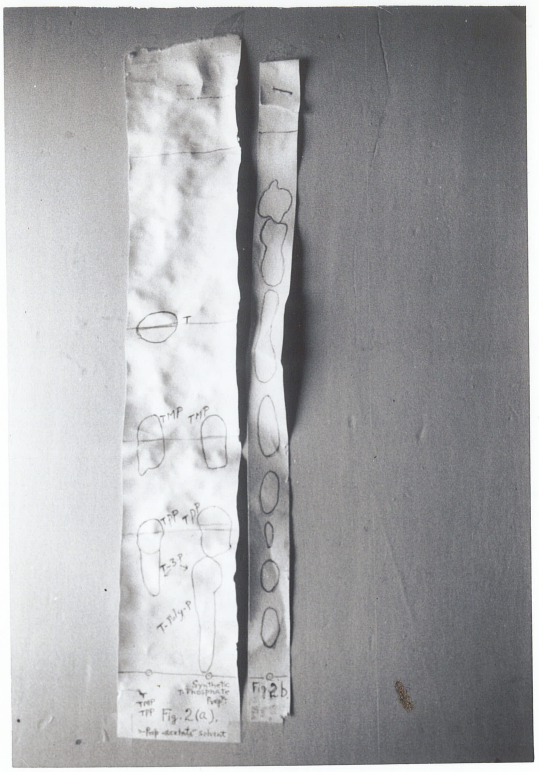

Figure 2. Chromatograms of the Separation of Different Thiamine Derivatives.

(a) As obtained by the method of Siliprandi and Siliprandi using the solvent - n-propanol-water - 1 M acetate buffer, pH 5 (65:20:15).

(b) As obtained by the method of Kiessling using the solvent - 100 ml. isobutyric acid, 60 ml. N ammonia and 1.6 ml. of 0.1 M ethylene diamine tetra-acetic acid.

T = Thiamine; TMP = Thiamine monophosphate; TPP = Thiamine Pyrophosphate; T-3-P = Thiamine triphosphate. T-poly-P refers to Thiamine polyphosphates containing thiamine tetra-, pentaphosphates, etc.

separately and shaken well with 20 ml. of 0.1 N sulfuric acid solution for half an hour. The volume was made up to 25 ml. and mixed well and each solution was analyzed for thiamine after hydrolysis with Taka-Diastase. By comparing with standard known solutions the loss, during all the steps of chromatographic separation and elution, was determined and was found to be negligible. With thiamine, TMP [a] and TPP [a] (20 γ of each) the percentage recoveries (by using the method of Siliprandi and Siliprandi) were found to lie between 99 - 100%. As low as 5 γ of a derivative could be detected by this method.

The mixture of higher phosphates of thiamine was prepared by the method of Yusa (46), as described in Chapter II. All the derivatives were dissolved in 50% ethanol and stored at about -10° C. At this condition all the solutions were found to be stable for nearly two months. The solutions were warmed to room temperature before use.

Of the electrophoretic methods of separation, the one developed by Rossi-Fanelli, Mondovi and Boffi(51) was used. The solution

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[a] Thiamine Monophosphate (TMP) and Thiamine Pyrophosphate (TPP). Supplied by "Nutritional Biochemicals Corporation", Cleveland, Ohio, U. S. A.

used was composed of 1000 ml. of 0.1 N sodium chloride and 3.5 ml. glacial acetic acid, the final pH of the solution was 3.0. It gave good separation of thiamine, TMP and TPP but the higher phosphates were not separated at all. A photograph of the separation of thiamine derivatives is shown in Figure 3. The filter paper (Munktel No. 20) used was about 16 inches in width, a current of 10 milliamperes was passed through the paper for 12 hours. The whole process was done in the cold room. The photograph in Fig. 3 shows two runs side by side, the one on the left is with the synthetic mixture of thiamine phosphates. From the figure it is clear that TPP contained traces of higher phosphates which appear as a long tail just below TPP. It is also seen from the figure that the synthetic phosphate preparation does not contain any free thiamine and the higher phosphates are not separated at all, they appear as a tail at the bottom of TPP. The percentage recoveries of thiamine, TMP and TPP were studied and the loss was found to be quite high. The percentage recoveries for thiamine, TMP and TPP were 92%, 87% and 72% respectively.

Due to better recoveries and better separation of the higher phosphate derivatives, the chromatographic methods are

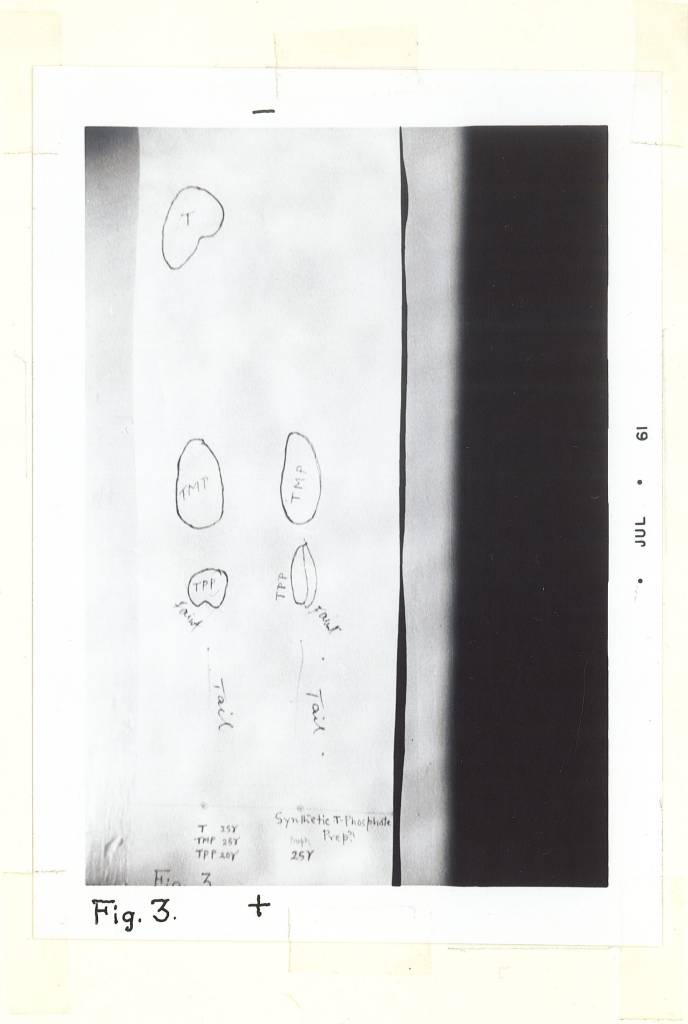

Fig. 3.

Figure 3. Paper Electrophoretic Separation of Different Thiamine Derivatives.

to be preferred to electrophoretic ones.

3. The New Method for the Determination of Different Thiamine Phosphates in Cereals.

It has been mentioned earlier in this chapter, p.35, that after the extraction of the thiamine and its derivatives from cereals it should be purified from other materials before it can be subjected to either paper chromatographic or electrophoretic separation. The purification method with Decalso was not suitable because the thiamine and its derivatives could be eluted only with salt solutions of high concentrations. The method of Siliprandi and Siliprandi for desalting the sample, by the use of adsorbent charcoal treated with cholesteryl stearate solution, did not prove satisfactory. This might have been due to the unavailability of the proper source of charcoal because different charcoal samples gave different results. The method of electrolytic desalting was also found unsuitable because it brought about the disappearance of thiamine within a short time. So a new method of desalting was sought and a long but a fairly satisfactory method was developed. It is described as follows.

(A) Principle. The cereal sample was extracted with a suitable solvent, which will be discussed later in section (B), and the extract was passed through a Decalso column. The column was washed with water, and the thiamine derivatives were eluted with 25% potassium chloride solution. The potassium chloride eluate was dried in a current of dry air and was triturated with 95% ethanol in a glass mortar. The alcohol extract was filtered and dried completely in vacuum. The residue was dissolved in water and the solution was passed through an anion exchange resin which retained only the thiamine pyrophosphate and the higher thiamine phosphates, and allowed K^+ ion, thiamine and thiamine monophosphate to pass through. The filtrate was collected and analyzed for free thiamine and thiamine monophosphate. Thiamine pyrophosphate and the higher phosphates were eluated with 2 N acetic acid and the eluate was concentrated and analyzed by the chromatographic method.

(B) Extraction. During the extraction of the thiamine derivatives from cereals care should be taken so that the phosphate esters of thiamine (if present in the sample) are not hydrolyzed. Thiamine and thiamine monophosphate are quite stable in acidic solution (44, 58, 59)

even at higher temperature (90 -100° C). Thiamine pyrophosphate is stable in weakly acidic solution at room temperature, but slowly hydrolyzes to thiamine monophosphate and inorganic phosphate if kept for long at higher temperature (44, 59). The higher phosphates, namely thiamine triphosphate and thiamine tetraphosphate are stable at room temperature in weakly acidic solution for one or two days but slowly hydrolyze to thiamine monophosphate, thiamine pyrophosphate and inorganic phosphate if kept longer (44, 59). They are very unstable at higher temperature and break down to the same products, as mentioned above. Thiamine and all its derivatives are very unstable in alkaline solution (58, 59). In view of all these factors three different types of extraction procedures were employed. These are discussed below.

(i) Cold sulfuric acid extraction in presence of 10^{-3} M fluoride: Fluoride ion has been reported to be a strong inhibitor (60) of the phosphatases even at concentrations as low as 10^{-6} M. In this investigation it was found that in the presence of 10^{-3} M fluoride (F^-) the phosphatase action of Taka-Diastase was completely inhibited (0.2 g. Taka-Diastase in 100 ml. of 10^{-3} M F^-). A suitable amount of sample of cereal (10 - 15 g.) was stirred with 300 - 400 ml. of 0.1 N sulfuric acid solution.

containing 10^{-3} M sodium fluoride, in the cold for three hours. This kind of extraction inhibited the action of the phosphatases. The mixture was then centrifuged, the supernatant liquid was decanted and the residue was washed twice with 0.1 N sulfuric acid solution, containing 10^{-3} M F^-, and centrifuged again. All the extracts and washings were collected and were used for the next step, a purification process.

(ii) Hot sulfuric acid extraction: This method of extraction is similar to that of Obermeyer's method described on p.32, Chapter III. The chief disadvantages of this method are that the higher phosphates of thiamine are liable to break down at higher temperature and a thick, viscous extract results. This method of extraction should be avoided if possible even though it was hoped that at higher temperatures better recoveries would be obtained.

(iii) Cold perchloric acid extraction: Cold perchloric acid extraction has been employed by Greiling and Kiesow (36) for the extraction of thiamine and its phosphate derivatives from animal tissues. Four ml. of M/3 perchloric acid was used per gram of cereal sample and at this concentration of perchloric acid Taka-Diastase was

found to be completely inhibited. The cereal sample and perchloric acid were triturated for 1 hour in the cold in a glass mortar. The mixture was centrifuged and the supernatant liquid was collected. The residue was washed with M/3 perchloric acid and centrifuged again. All the extract and wash liquid were collected and titrated with molar potassium hydroxide solution in the cold using phenolphthalein as indicator. After the end point was reached, 1 ml. of 0.1 N sulfuric acid was added to the mixture to make the solution acidic. The precipitate of potassium perchlorate was removed by centrifugation in the cold and the precipitate was washed with water, and all the extracts and washings were collected and used for the next step, purification.

(C) Purification: This step is the same as has been described in Chapter III, p.28 . About 8 g. of Decalso was found to be sufficient to adsorb all the thiamine derivatives extracted from 16 g. of cereal sample if the flow rate during adsorption had been kept at 0.5 ml. per minute. A column 5 cm. high and 2 cm. diameter was used. Fifty ml. of 25% potassium chloride solution was found to be sufficient to elute all the thiamine derivatives and the flow rate during

elution was kept at 0.5 ml. per minute. All the steps so far described were carried out in the cold room at about 1° C.

(D) Removal of the bulk of potassium chloride by alcohol extraction: Thiamine is fairly soluble in 95% ethanol, 100 mg. per 100 ml. (58). Though the thiamine phosphates are very slightly soluble in ethanol they could be brought into solution if triturated long enough with a large quantity of ethanol, as they would be present originally in cereals in minute quantities. The potassium chloride extract from the previous step was placed in a glass mortar and dried completely by passing dry air over it. When most of the water had gone, the whole mass was spread evenly on the inner surface of the mortar with the pestle and the passing of dry air was continued. This process of spreading the potassium chloride on the surface of the mortar was repeated until all the sample was dry. This took about four hours. About 25 ml. of ethanol was added to the dry potassium chloride and the whole mass was triturated for 30 minutes. The liquid was decanted and filtered. The residue was washed and filtered with two successive 15 ml. portions of ethanol, each time triturating the whole mass

for 20 minutes. Solubility of potassium chloride in 95% ethanol is quite low, about 0.2 g. per 100 ml. 95% ethanol (61) and in this way most of the potassium chloride was removed. The ethanol extract was dried completely in vacuum at room temperature and the residue was dissolved in a minimum quantity of water containing four or five drops of 0.1 N hydrochloric acid to keep the solution acidic, in which thiamine and its derivatives are more stable.

(E) Anion exchange chromatography of the extract: The small amount of potassium chloride present in the final extract should be removed first, before it could be subjected to chromatographic separation and it was thought that anion exchange treatment might solve the problem. The resin used was Dowex-1 (x 8, acetate form). It is a strongly basic anion exchange resin, and contains quarternary ammonium groups as its functional groups which are attached to a styrene-divinyl-benzene copolymer and is 8 percent cross-linked. After use, the resin was 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 indicated the same pH as that of the distilled water. In acidic solution thiamine is present as cation, the basic

amino group in 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.

The column used was made from an ordinary glass burette cut into smaller lengths. The bottom part of the delivery tube of the burette was connected to a narrow glass tube with a small rubber tubing. The glass tube was bent vertically upwards and it stood erect parallel to the burette. The end of the glass tubing was bent downwards and the height of the delivery end of this tube was kept at the same height as that of the resin bed, thus keeping the column always filled with liquid. A sketch of the column is shown in Figure 4. The whole column was first filled with water and then a little glass wool was placed at the bottom of the tube. A slurry of the resin was poured slowly from the top and the resin was allowed to settle. In this way a compact air-bubble-free column was obtained. Ten to twelve ml. wet volume of the resin was found to be enough to adsorb 0.1 g. potassium chloride and 80 γ mixture of thiamine and thiamine pyrophosphate (1:1) if the flow rate during adsorption process had been kept at 0.3 ml. per minute.

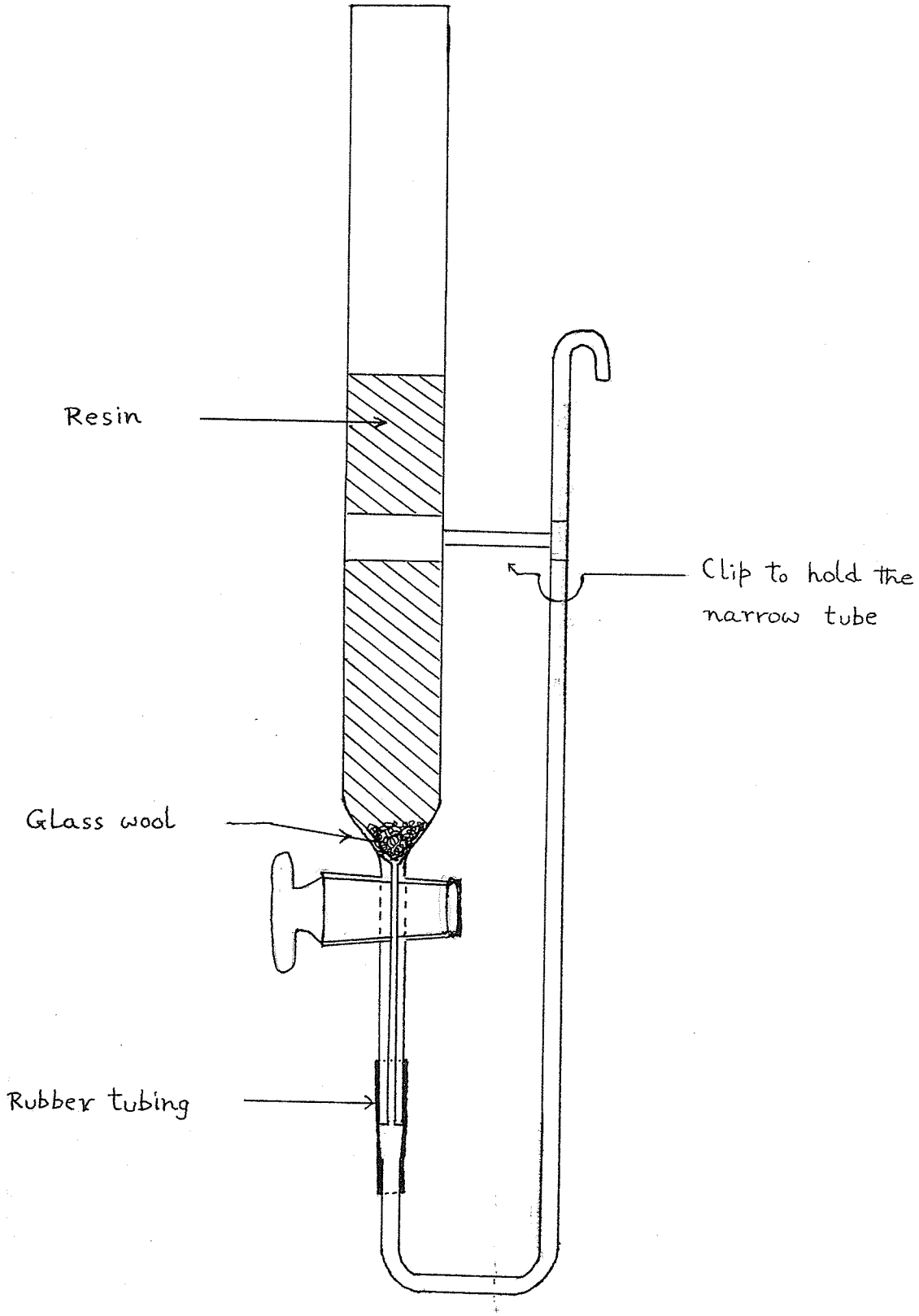

Fig. 4. Column for ion-exchange treatment.

Synthetic thiamine, thiamine monophosphate, and thiamine pyrophosphate were passed separately with 0.1 g. of potassium chloride (total mixture diluted to about 10 ml., pH 5 - 6) through the column and it was found that thiamine and thiamine monophosphate passed through the column and thiamine pyrophosphate was retained. When a mixture of higher thiamine phosphates, as prepared by the method of Yusa (46), was passed through the column with 0.1 g. of potassium chloride, thiamine pyrophosphate and all the higher phosphates (tri-, tetra-, pentaphosphate, etc.) were found to be retained and could be eluted with 60 ml. of 2N acetic acid solution in the cold. The acetic acid eluate was concentrated in vacuum at 20° C and was chromatographed according to the method of Kiessling (as described on p. 46). The higher phosphates were found to be quite stable under the experimental conditions provided the whole experiments were completed within two or three days.

The same method, as described in the last paragraph, was used with the cereal extracts. The extract from step "D" (as described on p. 57) was passed through the column, the resin bed was washed with three bed-volume of water and all the filtrate and washings were collected in a volumetric flask and diluted to the desired volume. This solution was kept for the estimation of thiamine and thiamine monophosphate. Thiamine pyrophosphate and the higher phosphates were eluted with 60 ml. of 2 N acetic acid solution and the eluate was concentrated in vacuum at 20° C and was chromatographed

according to the method of Kiessling (as described on p. 46). The chromatogram was dried in the air, the respective spots containing different phosphate derivatives were cut and eluted separately with 0.1 N sulfuric acid solution. The different eluates containing various thiamine phosphates were analyzed by the thiochrome method. A parallel run was carried out with known thiamine derivatives for locating the spots, which fluoresce in ultra violet light when sprayed with alkaline ferricyanide solution.

(F) Results with known materials: A mixture containing 40 γ of thiamine chloride hydrochloride, 20 γ of thiamine monophosphate and 20 γ of thiamine pyrophosphate was taken and mixed with 400 ml. of a wheat extract (previously freed from thiamine and its derivatives by passing through excess Decalso). Then the whole procedure, starting from step "C" up to the step "E" (pp. 56 - 62) was carried out. The filtrate and washings from the anion exchange column were analyzed for free and total thiamine according to the method described in the Chapter III. The difference between the total thiamine and free thiamine content was accounted to thiamine monophosphate. The acetic acid eluate from the anion exchange column was analyzed for

Table 3

RECOVERY OF THIAMINE, THIAMINE MONO-PHOSPHATE AND THIAMINE PYROPHOSPHATE

	Total amount of the starting material. γ.	Total amount recovered γ	% recovery
Thiamine	40	37.6	94
	40	37.2	93
	40	38.0	95
Thiamine monophosphate	20	18.82	94.1
	20	19.44	97.2
	20	17.64	88.2
Thiamine pyrophosphate	20	18.14	90.5
	20	17.14	85.7
	20	18.28	91.4

thiamine pyrophosphate after enzymic hydrolysis. The experiment was repeated thrice and found to have good recovery and fair reproducibility. The results of these experiments are tabulated in Table 3.

(G) Result with cereals: Cold perchloric acid extraction was employed with the cereals and the same procedure (as described in pp. 55 - 62) was repeated. Two of the cereal samples (Mindum wheat and Garry oats) were analyzed and found to contain no thiamine phosphates. Only the free thiamine was found to come into solution and the value of the free thiamine content was nearly the same as obtained by the Obermeyer method (p. 32). It became doubtful whether they contained any thiamine phosphate and further investigations were carried out to determine the nature of the bound thiamine. This will be discussed in the next chapter (V).

CHAPTER V

NATURE OF THE BOUND THIAMINE IN CEREALS

The fact that the newly developed method did not give any indication of the presence of thiamine phosphates in two of the analyzed sample (Mindum wheat and Garry oats) lead to further investigation on the nature of the bound thiamine. This was done by the following series of experiments which will be discussed one by one to show briefly their underlying principles and their significance.

Treatment 1

Treatment with Diastase [a]: Diastase is an enzyme which hydrolyzes starch to sugars at room temperature quite rapidly at pH 4.2 - 4.6. It was first discovered by Kirchoff in 1814 in wheat extract. This enzyme was treated with thiamine monophosphate and thiamine pyrophosphate in pH range 3 - 6 for 6 hours at 37° C and no free thiamine was

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[a] Diastase (animal source). Supplied by "Nutritional Biochemicals Corporation", Cleveland, Ohio, U. S. A.

found to be present in the reaction mixture thus indicating that it did not hydrolyze either of the substrates. Thus it was concluded that if thiamine phosphates had been present in cereals they would not be hydrolyzed to thiamine on treatment with Diastase preparation.

About 5 g. of ground cereal was placed in a 250 ml. volumetric flask and heated in a boiling water bath for 15 minutes with 100 ml. of hot (90° C) 0.1 N sulfuric acid. This heating destroyed all the enzymes which had been present in the cereal sample and also helped in the extraction of the thiamine. If any thiamine phosphates had been present in the cereal samples they would break down to thiamine monophosphate, not to free thiamine, during this extraction process (see p. 54). The mixture was cooled and the solution was adjusted to pH 4.5 by the addition of 2.5 M sodium acetate solution, and was treated with 0.2 g. of Diastase (dissolved in 5 ml. molar acetate buffer pH 4.5). The mixture was incubated for 6 hours at 37° C and then diluted to 250 ml. and shaken well. It was filtered through a fluted filter paper and the amount of free thiamine in the filtrate was determined by the thiochrome method. Fifty ml. of the filtrate was saved and used in the treatment II. For results of treatment I see Tables 10 - 15.

Treatment II

Treatment with Diastase followed by treatment with Taka-Diastase: Taka-Diastase is a rich source of phosphatases. If there had been any thiamine phosphate present in the cereal sample the total thiamine content obtained by this treatment would be higher than that obtained by treatment I. The increase in the thiamine content would correspond to the amount of thiamine phosphate present in the cereal sample.

A solution of 0.5 g. of Taka-Diastase, dissolved in 2.5 ml. of 1 M acetate buffer pH 4.5, was added to the 50 ml. extract which was saved from treatment I and the mixture was incubated for 3 more hours at 37° C. Then total thiamine in the mixture was determined by the thiochrome method. For results of treatment II see Tables 10 - 15.

Treatment III

Treatment with trypsin [a]: Trypsin is an enzyme which belongs to the class of proteinases, and hydrolyzes large protein molecules to smaller peptides. It acts favorably

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[a] Trypsin. Supplied by "Fisher Scientific Company", Toronto, Ontario.

around pH 6-8, at 37° C. Trypsin was found to have no hydrolyzing action either on thiamine monophosphate or thiamine pyrophosphate at pH 6 for 6 hours.

About 5 g. of cereal sample was placed in a 250 ml. volumetric flask and heated for 15 minutes with hot (90° C) 0.1 N sulfuric acid. The mixture was cooled and adjusted to pH 6 with 2.5 M sodium acetate solution and dilute sodium hydroxide solution. To the mixture 0.2 g. of trypsin was added, it was shaken well and allowed to incubate for 6 hours at 37° C. After incubation the mixture was made up to 250 ml. and shaken well. The extract was filtered through a fluted filter paper and the amount of free thiamine in the filtrate was determined by the thiochrome method. Fifty ml. of the filtrate was saved for use in treatment IV. For results of treatment III see Tables 10 - 15.

Treatment IV

Treatment with trypsin followed by treatment with Taka-Diastase: The significance of this treatment is same as that of treatment II and was done in the same way. For results of treatment IV see Tables 10 - 15.

Treatment V

Treatment with trypsin in the presence of added TPP: About 5 g. of the cereal sample was placed in a 250 ml. volumetric flask and 100 ml. hot (90° C) 0.1 N sulfuric acid and 20 γ of TPP were added to it and the whole mixture was heated in a boiling water bath for 15 minutes. The mixture was cooled and adjusted to pH 6. To it was added 0.2 g. of trypsin and the whole mixture was incubated at 37° C for 6 hours. After incubation the mixture was diluted to 250 ml. and mixed well. It was filtered through a filter paper and the amounts of free and total thiamine were determined by the thiochrome method (as described in Chapter III). The purpose of this treatment was to find out any factor which might be present in cereals that could cause the breakdown of thiamine phosphates. The same conclusion can also be drawn with the higher phosphates of thiamine. It is unlikely that any factor which did not liberate any free thiamine from TPP would do so from the higher phosphates. For results of treatment V see Table 16.

Treatment VI

Treatment of the cereal sample with 0.1 N sulfuric acid solution for 10 hours in the absence of any added enzyme preparation: About 5 g. of cereal was placed in a 250 ml. volumetric flask and was heated in a boiling water

bath with 100 ml. of hot (90° C) 0.1 N sulfuric acid solution for 15 minutes. The mixture was cooled and was incubated for 10 hours at 37° C, with occasional shaking. The mixture was diluted to 250 ml. and mixed well. The extract was filtered through a fluted filter paper and the free thiamine content of the extract was determined by the thiochrome method. The usefulness of this treatment was to find the increase in free thiamine content due to diffusion of free thiamine from the inside of the cells on prolonged contact with the solution. For results of treatment VI see Tables 10 - 15.

CHAPTER VI

RESULTS AND DISCUSSION

Results

Six samples of cereals were studied, two of each, of wheat, barley and oats. These were Mindum wheat, Selkirk wheat, Vantage barley, Montcalm barley, Garry oats and Rodney oats. The results of analysis for their free and total thiamine contents and of various different treatments are presented in tabulated form. Each value corresponds to a separate assay and is the mean of two determinations made on the final solution. That is, from the same solution certain aliquots were taken in duplicate, oxidized to thiochrome and its fluorescence measured. The results for aliquots from a single sample always checked closely, within two per cent.

TABLE 4

FREE AND TOTAL THIAMINE CONTENT OF MINDUM WHEAT. γ/g.

	Total thiamine	Free thiamine	Bound thiamine (by difference)
	4.29	1.94	2.35
	3.86	1.44	2.42
	4.01	1.63	2.38
	3.90	1.45	2.45
	4.28	1.82	2.46
mean	4.07	1.65	2.42

TABLE 5

FREE AND TOTAL THIAMINE CONTENT OF SELKIRK WHEAT. γ/g.

	Total thiamine	Free thiamine	Bound thiamine (by difference)
	2.92	1.25	1.67
	2.97	1.34	1.63
	2.68	1.21	1.47
	2.90	1.22	1.68
	2.99	1.37	1.62
mean	2.89	1.28	1.61

TABLE 6

FREE AND TOTAL THIAMINE CONTENT OF VANTAGE BARLEY. γ/g.

	Total thiamine	Free thiamine	Bound thiamine (by difference)
	4.12	2.42	1.70
	4.21	2.51	1.70
	4.44	2.65	1.79
	4.19	2.46	1.73
	4.33	2.65	1.68
mean	4.26	2.54	1.72

TABLE 7

FREE AND TOTAL THIAMINE CONTENT OF MONTCALM BARLEY. γ/g.

	Total thiamine	Free thiamine	Bound thiamine (by difference)
	3.72	1.89	1.83
	4.33	2.32	2.01
	4.38	2.48	1.90
	3.86	1.92	1.94
	4.05	2.20	1.85
mean	4.07	2.16	1.91

TABLE 8

FREE AND TOTAL THIAMINE CONTENT OF GARRY OATS. γ/g.

	Total thiamine	Free thiamine	Bound thiamine (by difference)
	7.04	4.86	2.14
	6.62	4.38	2.24
	6.31	4.38	1.93
	6.71	4.52	2.19
	6.30	4.27	2.03
mean	6.59	4.48	2.11

TABLE 9

FREE AND TOTAL THIAMINE CONTENT OF RODNEY OATS. γ/g.

	Total thiamine	Free thiamine	Bound thiamine (by difference)
	5.64	3.80	1.84
	5.52	3.47	2.05
	5.43	3.21	2.22
	5.52	3.38	2.14
	5.47	3.28	2.19
mean	5.51	3.43	2.08

Table 10

FREE THIAMINE CONTENT OF MINDUM WHEAT AS OBTAINED BY DIFFERENT TREATMENTS . γ/g.

Type of treatment (see Chapter V)				
I Diastase	II Diastase and Taka-Diastase	III Trypsin	IV Trypsin and Taka-Diastase	VI No enzyme
4.25	4.29	4.47	4.45	2.52
4.40	4.37	4.60	4.61	2.70
3.87	3.92	4.37	4.32	2.50
mean 4.17	4.19	4.48	4.46	2.57

Table 11

FREE THIAMINE CONTENT OF SELKIRK WHEAT AS OBTAINED BY DIFFERENT TREATMENTS. γ/g.

	Type of treatment				
	I Diastase	II Diastase and Taka-Diastase	III Trypsin	IV Trypsin and Taka-Diastase	VI No enzyme
	2.95	2.95	2.70	2.70	2.15
	2.98	3.01	2.98	2.97	2.30
	3.01	2.97	3.12	3.16	2.0
mean	2.98	2.98	2.93	2.94	2.15

Table 12

FREE THIAMINE CONTENT OF VANTAGE BARLEY AS OBTAINED BY DIFFERENT TREATMENTS. γ/g.

Type of treatment				
I Diastase	II Diastase and Taka-Diastase	III Trypsin	IV Trypsin and Taka-Diastase	VI No enzyme
4.20	4.21	4.25	4.24	3.42
4.40	4.40	4.42	4.40	3.50
4.43	4.44	4.19	4.20	3.31
mean 4.34	4.35	4.28	4.28	3.41

Table 13

FREE THIAMINE CONTENT OF MONTCALM BARLEY AS OBTAINED BY DIFFERENT TREATMENTS. γ/g.

	Type of treatment				
	I Diastase	II Diastase and Taka-Diastase	III Trypsin	IV Trypsin and Taka-Diastase	VI No enzyme
	4.10	4.15	3.75	3.77	3.13
	4.25	4.24	4.05	4.05	3.20
	4.05	4.02	3.90	3.95	3.01
mean	4.13	4.14	3.90	3.92	3.11

Table 14

FREE THIAMINE CONTENT OF GARRY OATS AS OBTAINED BY DIFFERENT TREATMENTS. γ/g.

Type of treatment				
I Diastase	II Diastase and Taka-Diastase	III Trypsin	IV Trypsin and Taka-Diastase	VI No enzyme
7.06	7.0	6.98	7.0	5.4
6.66	6.66	6.57	6.45	5.6
6.62	6.71	6.32	6.38	6.0
mean 6.78	6.79	6.62	6.61	5.66

Table 15

FREE THIAMINE CONTENT OF RODNEY OATS AS OBTAINED BY DIFFERENT TREATMENTS. γ/g.

Type of treatment				
I Diastase	II Diastase and Taka-Diastase	III Trypsin	IV Trypsin and Taka-Diastase	VI No enzyme
5.72	5.70	5.51	5.51	4.50
5.41	5.41	5.48	5.48	4.40
5.55	5.50	5.41	5.40	4.37
mean 5.56	5.54	5.46	5.46	4.42

Table 16

THIAMINE CONTENT OF DIFFERENT CEREAL SAMPLES AS OBTAINED BY TREATMENT. V. (Trypsin in the presence of added TPP).

Cereal sample	Free thiamine in the presence of added TPP. γ/g.	Average free thiamine in the presence of added TPP. γ/g.	Percentage recovery of added TPP	Average percentage recovery of added TPP.
Mindum wheat	4.29 4.50	4.39	95.6 96.2	95.9
Selkirk wheat	2.80 3.10	2.95	97.2 93.2	95.2
Vantage barley	4.30 4.40	4.35	97 99	98
Montcalm barley	4.10 3.95	4.02	94.8 97	95.9

Table 16, cont.

Cereal sample	Free thiamine in the presence of added TPP. γ/g.	Average free thiamine in the presence of added TPP. γ/g.	Percentage recovery of added TPP.	Average percentage recovery of added TPP.
Garry oats	6.45 7.0	6.72	97.2 93.3	95.2
Rodney oats	5.8 5.4	5.6	94.8 95.2	95

Thiamine Phosphate Contents of Different Cereal Samples as Obtained by the New Method.

It has been mentioned earlier (p. 64) that when Mindum wheat and Garry oats were analyzed, by the new method, for their thiamine phosphate contents they were found to contain no thiamine phosphates. Other cereal samples, namely, Selkirk wheat, Vantage barley, Montcalm barley and Rodney oats were later analyzed by the same method and it was found that none of them contained any thiamine phosphates. The free thiamine contents in each case were nearly the same as obtained before (Tables 4 - 9).

Discussion

From the results of Tables 4 - 9 it is clear that all the cereal samples studied contain thiamine both in the free and bound form. The fractions of the total thiamine present as bound thiamine in different cereals were found to be as follows:

	% of total
Mindum wheat	59.4
Selkirk wheat	55.7
Vantage barley	40
Montcalm barley	47
Rodney oats	37.7
Garry oats	32

It is also clear from the data of Tables 4 - 15 that some kind of enzymic digestion is necessary to bring the entire bound thiamine into solution, since in every case the results obtained with enzymic digestion (total thiamine) are greater than those obtained without it (free thiamine).

It has been said in chapter IV that two of the cereals (Mindum wheat and Garry oats) when analyzed by the new method did not give any indication of the presence of thiamine phosphates in them. But it was found that the

method worked well with a mixture of thiamine, TMP and TPP and it was concluded that the same method could be employed for the determinaton of thiamine phosphates in cereals.

The problem arises as to the nature of the compound or compounds containing the bound thiamine. It might be one or more of the thiamine phosphates namely, mono-, di-, triphosphates, etc. It might be bound in some other form either with protein or carbohydrate, or both. Thiamine phosphates might also be present either as free phosphates or in combination with protein, carbohydrate or some other materials. Thiamine monophosphate was found to be very stable under the experimental conditions and thiamine pyrophosphate and higher phosphates of thiamine decomposed slightly to thiamine monophosphate and inorganic phosphate if kept for a long time in solution. In this investigation it was also found that trypsin and Diastase did not cause any hydrolysis of thiamine monophosphate or thiamine pyrophosphate. These facts excluded any possibility of the liberation of free thiamine from thiamine phosphates which might be present as such or combined with something else. The results of treatments I and III, with Diastase and trypsin (see Tables 10 - 15), respectively, show that

digestion with either of these enzymes increased the free thiamine content of all the cereals and the free thiamine contents of all the cereals became nearly equal to their total thiamine contents (Tables 4 - 9) in each case. If all of the bound thiamine had been present as phosphoric acid ester there should not have been any increase in the free thiamine contents of the cereals and if a part of the bound thiamine had been present as phosphoric acid ester the free thiamine contents of the cereals after these treatments should not have been equal to the total thiamine contents of the cereal samples. From these results it is concluded that the bound thiamine was not present as thiamine phosphate in any of the cereal samples studied and thiamine phosphates are not present in significant amounts in cereals.

The results of treatments II and IV, digestion with Diastase and trypsin, respectively, followed in each case by digestion with Taka-Diastase, show there is no increase in the free thiamine content of the extracts, over that obtained after treatments I and III, respectively, that is, digestion with Diastase or trypsin alone without further incubation with Taka-Diastase. There is no increase in the

free thiamine content in any case. If thiamine phosphates had been present in the above mentioned extracts the free thiamine should have been increased. These findings also indicate the absence of thiamine phosphates in the cereals studied.

In treatments I - IV, the cereal sample was first heated in a boiling water bath with hot 0.1 N sulfuric acid solution for 15 minutes. This treatment caused the destruction of any phosphatases which might have been present in the cereal. It might have been possible that there was some other factor in the cereal sample or in the reagents and that factor was heat stable and caused the hydrolysis of thiamine phosphates during the extraction process. To exclude this possibility the cereal samples were subjected to treatment V, i.e., treatment with trypsin in the presence of added TPP. If such a factor had been present in the system it should have hydrolyzed the added TPP and the value for free thiamine content should have been increased markedly. The results of Table 16 indicate that there were no increases in the free thiamine contents of the cereals and nearly 95% of the added TPP could be recovered as free thiamine on further treatment with Taka-Diastase. These

facts support the earlier conclusion that no thiamine phosphates were present in the cereals.

Thiamine might have been present inside the cells in combination with proteins or carbohydrates or both. This investigation did not give any idea about the exact nature of the bound thiamine nor did it attempt to do so. More work should be done, along other lines, to get an idea of the exact nature of the binding of the thiamine. Diastase hydrolyzes larger carbohydrate molecules to smaller molecules of sugars and trypsin breaks down larger protein molecules to smaller peptides. Both the enzymes have similar effects--the disintegrating of the cells, and helping the free thiamine to come into solution. In treatment VI the cereal samples, after heating with 0.1 N sulfuric acid, were incubated for 10 hours without any addition of enzymes and the free thiamine contents of the extracts were determined. The results shown in Tables 10 - 15, for treatment VI, indicate that there are considerable increases in the free thiamine contents from those determined previously (Tables 4 - 9). For example the value for Mindum wheat rose from 1.65 to 2.57, for Vantage barley from 2.54 to 3.41 and for Garry oats from 4.48 to

5.66 γ/g. In no case did the new values for free thiamine equal those for total thiamine. This increase might be due either to increased diffusion of the free thiamine from the inside of the cell or slow disintegration of proteins or carbohydrates, and hence of the cells, in acidic solution.

SUMMARY

Free and total thiamine contents of six cereal samples of wheat, barley and oats were determined and it was found that they contain appreciable amounts of thiamine in the bound form.

A new method was developed for the quantitative estimation of thiamine and different thiamine phosphoric acid esters in cereals. The use of this method showed there are no thiamine phosphoric acid esters present in cereals.

Further treatment of cereals with trypsin and Diastase confirmed that thiamine phosphates did not occur in cereals. Further studies are needed to reveal the actual substances of which bound thiamine is a part.

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