STUDIES ON THE DETERMINATION OF THIAMINE AND RIBOFLAVIN IN FOODS

by

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TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS 1

PART I .... 2 - 35

INTRODUCTION .... 3 - 6

EXPERIMENTAL ..... 7 - 34

1. Method of extraction from foods 7

2. Enzymatic hydrolysis 9

3. Adjustment of the pH of the extracting medium 11

4. Use of external indicators in the adjustment of the pH 12

5. Details of the extraction technique... 13

6. Effect of filtering extracts 14

7. Purification of extracts by selective adsorption 14

8. Effect of prior extraction with isobutanol 15

9. Use of hydrogen peroxide as a clarifying agent..... 17

10. Effect of omitting methyl alcohol during oxidation. 19

11. Optimum amount of potassium ferricyanide..... 21

12. Optimum amount of sodium hydroxide.... 21

13. Amount of isobutanol required for extraction..... 23

14. Use of oxidizing agents other than potassium ferricyanide.. 24

15. The effect of potassium cyanide and potassium iodide 24

Page

16. Destruction of thiochrome in ultraviolet light 25

17. Instrument used for measurement of fluorescence 26

18. Outline of modified method 27

19. Preparation of a calibration curve... 30

20. Comparison of results by various methods..32

SUMMARY 36

PART II ..... 37 - 50

INTRODUCTION ..... 38 - 39

EXPERIMENTAL ..... 40 - 49

1. Use of benzyl alcohol in the purification of riboflavin extracts... 40

2. Use of stannous chloride as a reducing agent 40

3. Use of zinc acetate in the purification of extracts 41

4. Effect of using an excess of zinc acetate 42

5. Use of pure benzyl alcohol as a blank 43

6. Use of benzyl alcohol saturated with sodium chloride..... 44

7. Methods of extraction 45

8. Modified method for the determination of riboflavin 46

9. Application of the modified method... 49

SUMMARY 50

CONCLUSION 51

BIBLIOGRAPHY ..... 52 - 61

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PART I

Studies on the Determination of Thiamine (Vitamin B_1) in Foods.

PART I. INTRODUCTION

The vital importance of thiamine as a dietary constituent makes it imperative that rapid and accurate methods should be available for its determination. However until comparatively recently, the time-consuming and expensive biological assay has given the most satisfactory results.

The curing of the head retraction of pigeons, fed on thiamine deficient diets, was used as a criterion of the thiamine content of foodstuffs by Coward and associates (14). More recently the use of chicks has been proposed by Jukes and Heitman (33). The rat curative method has also proven useful and modifications have been introduced by Kline, Tolle and Nelson (37). Birch and Harris (3) were the first to suggest the rat-bradycardia procedure and subsequent work by Baker and Wright (1) has established its reliability. The growth response of rats has formed the basis of another satisfactory method and according to Munsell (45) has a sensitivity of one microgram of thiamine.

The short life cycle of micro-organisms favours the development of a rapid method for thiamine. Schultz, Atkin and Frey (58 (59) found that thiamine stimulated the rate of alcoholic fermentation and they have developed a procedure which gives values in close agreement with other biological assays. Schopfer and coworkers (56) employed the growth response of Phycomyces blakesleeanus but later (57) reported that substances, which are not

destroyed by autoclaving, also affect the growth rate of this organism.

A wide variety of chemical methods has also been proposed for the quantitative determination of thiamine. A number of colorimetric procedures consist of coupling the vitamin with certain diazotized aromatic amines and subsequent measurement of the coloration produced. Jansen and Donath (30) obtained a Pauly reaction when thiamine was treated with diazotized sulphanilic acid. Willstaedt (68) and later Willstaedt and Barany (69) employed 2,4-dichloroaniline in an alkaline medium to give a yellow-red pigment soluble in ether. The use of p-aminoacetophenone in alkaline solution was suggested by Prebluda and McCollum (48) (49). A purple-red complex was formed which was insoluble in water but soluble in certain organic solvents. A quantitative method based on this reaction was developed by Melnick and Field (41) (42) (43) who used xylene as the solvent. Yang and Platt (70) and Emmett, Peacock and Brown (19) have since suggested minor modifications. Bismuth potassium iodide was found by Naiman (46) to give an orange-red precipitate with thiamine but the reaction was not specific.

The ability of solutions containing the antineuritic vitamin to reduce ferric ferricyanide with the formation of a blue colour, was first described by Jendrassik (31). Levine (39) later showed that the reaction was given by ortho and polyphenols. Barger and coworkers (2) oxidized solutions of the vitamin with alkaline ferricyanide and isolating the crystalline oxidation product. This substance was found to give an intense blue fluorescence when ex-

posed to ultra-violet light; it is now known as thiochrome and is formed from thiamine alone. The measurement of the intensity of this fluorescence after extraction into isobutanol, has formed the basis of a very sensitive and highly specific method for the estimation of vitamin B_1.

In 1936 Jansen (29) profiting by the work of Peters (47) and Kinnersley and coworkers (36), was able to apply the reaction to the quantitative determination of thiamine. Since that time various alternative techniques and special modifications for particular materials have been suggested. Karrer and Kubli (34) substituted visual comparison for the photoelectric instrument used by Jansen. Pyke (50) (51) (52) introduced peptic digestion and by a visual titration method was able to obtain satisfactory results on foodstuffs. Frankonite was used by Westenbrink et al (65) (66) to adsorb the vitamin and thus eliminate interfering substances but Hennessy and Cerecedo (27) (9) found "Decalso", a synthetic zeolite, to be superior for this purpose. Conner and Straub (12) have recently suggested improvements in the apparatus used in the extraction of the thiamine. Tauber (62) proposed a colorimetric method in which the vitamin was converted to thiochrome by alkaline ferricyanide and the resulting ferrocyanide measured as Prussian blue. However such a modification greatly reduces the sensitivity of the procedure.

The literature contains numerous modifications of the thiochrome method for the estimation of vitamin B_1 in urine and these differ mainly in the procedure employed for purification and concentration. Westenbrink and Goudsmit (64) used frankonite K.L. for this purpose while Widenbauer et al (67) preferred kaolin. A "Decalso"

column was employed by Ferrebee and Carden (21) and Jowett (32) used the same material but merely shook the solution with a small quantity of the powdered zeolite. Wang and Harris (63) preferred to use only a preliminary washing with isobutanol and then compared the solutions by a visual method.

It was evident on examining the literature that the thiochrome method was the most promising for the analysis of foodstuffs with a low thiamine content. The investigations recorded in this thesis are concerned with an attempt to modify the method so as to avoid the use of adsorption procedures to eliminate interfering substances. A modified thiochrome method is proposed which is based on a reinvestigation of the quantitative application of the thiochrome reaction.

EXPERIMENTAL

1. Method of extraction from foods.

Hennessy and Cerecedo (27) recommended the refluxing of a finely divided sample for 3 minutes with 5 to 20 parts of 2% acetic acid. After cooling and centrifuging, the residue was reextracted and the filtrates combined. In a later paper Hennessy (26) suggested the use of 0.1 N sulphuric acid but stated that the ratio of sample to volume of extracting liquid, should not be less than 15 to 1. Melnick and Field (42) preferred a simple aqueous extraction or the use of 80% methyl alcohol, which was claimed to be more specific for the vitamin. Conner and Straub (12) have recently employed 0.04 N sulphuric acid, with a liquid to solid ratio of 10-1. All these methods however, require constant stirring. Pyke (52) and Booth (4) have used a peptic digestion for 12 hours at 37°C. with satisfactory results. This would seem to be a logical procedure since thiamine is known to be intimately associated with protein. A number of these extraction methods have been investigated with the results shown in Table I.

TABLE 1 - Comparison of Various Methods of Extracting Thiamine from Different Materials (Results expressed in International Units (micrograms ÷ 3) per 100 gm.)

Method of extraction*	Dry yeast**			Wheat germ***	Special breads Dried***	
	Number of determinations	Range	Average		A	B
I. Incubating at 37°C. for 12 hr.						
(a) Pepsin-HCl at pH 2.0	6	730-737	733	980	92	112
(b) HCl at pH 2.0	2	725-730	727	991	-	-
(c) Papain at pH 4.5	4	675-680	677	698	-	-
II. Refluxing for 1 hr.						
(a) HCl at pH 2.5	2	722	722	976	88	95
(b) CH_3COOH at pH 2.5	4	570-604	590	1023	66	90
III. Autoclaving at 120°C. for 40 min.						
H_2SO_4 at pH 3.0	2	360-397	379	446	-	-

* Followed, in all cases, by takadiastase treatment at pH 4.5.

** Standard Brands Incorporated; Type 700-H; Guaranteed 600-900 I.U. per 100 gm.

*** Average of two determinations agreeing closely in each case. Bread A contained 5% wheat germ and Bread B was made from flour produced by a special milling process.

Under the conditions of these experiments peptic digestion or incubation with hydrochloric acid alone, under identical conditions, gave virtually the same extraction of thiamine. Papain digestion as recommended by Emmett et al (19) gave definitely low values. Less consistent results were obtained by refluxing with acids and, under the conditions employed, autoclaving is unsatisfactory. In the earlier work, methyl alcohol (42) was employed to reduce the organic matter content of the extracts. After digestion was complete, the solution was made 50 or 80% with methyl alcohol and then centrifuged. The alcohol was then removed by vacuum distillation, the residue again centrifuged, the supernatant liquid removed and made up to volume. This procedure satisfactorily removed a considerable portion of the organic matter but the advantage gained was more than offset by the increased pigmentation of the final extract. de Caro and Butturini (8) recently employed saturated ammonium sulphate solution in the extraction of thiamin from tissues. However when their procedure was applied to foodstuffs it proved entirely unsatisfactory. In view of these experiments, digestion overnight with a 0.2% solution of pepsin at pH 2 was adopted as a standard procedure.

2. Enzymatic hydrolysis.

In most foodstuffs, a portion of the thiamine occurs as the phosphoric acid ester, cocarboxylase. The oxidation product

of the ester, which is formed on treatment with alkaline ferricyanide, is not soluble in isobutanol and thus remains in the aqueous layer (55). An enzymatic hydrolysis is therefore required to convert the ester into free thiamine. A wide variety of enzyme preparations has been employed to effect this conversion, including takadiastase, mylase, clarase, diastase -Merck, kidney phosphatase and yeast preparations (26). Takadiastase however, has been most widely used and proved quite satisfactory in this investigation. Booth (4) suggested that since wheat contains little or no cocarboxylase, the enzyme treatment might be omitted with such products, but it was found that a clearer extract was obtained when the takadiastase was included and thus it was used in all analyses. The effect of omitting the enzyme in the determination of thiamine in a sample of yeast is shown in Table II.

TABLE II. - Effect of the omission of takadiastase.

Treatment		Results *(I.U. per 100 gm.)
Pepsin only	A	591
	B	651
Pepsin + takadiastase	A	753
	B	760

* International Units.

3. Adjustment of the pH of the extracting medium.

Pyke (52) considered that the addition of sufficient 0.1% pepsin in 0.33% hydrochloric acid, to bring the total volume of the sample and solution up to 97.4 ml., would also give a pH optimal for peptic digestion. Similarly the addition of 2.6 ml. of N sodium hydroxide, after incubation overnight, was assumed to change the solution to pH 4.5 for takadiastase hydrolysis. A series of experiments have shown that such was not the case. The amount of hydrochloric acid and sodium hydroxide required, varies with the particular material being assayed, as indicated in Table III. In each case 50 ml. of 0.1% pepsin in 0.33% hydrochloric acid was added to the sample.

TABLE III. - Showing variations in pH when Pyke's method was employed with various materials.

Material	pH 1 hour after addition of pepsin solution only.	ml. of N sodium hydroxide or N hydrochloric acid to adjust to pH 2.	ml. N sodium hydroxide required to adjust to pH 4.5
Wheat germ	3.5	1.5 acid	1.5
Grass	3.3	2.0 acid	3.7
Bread A	1.0	1.4 alkali	1.0
Bread B	1.2	1.1 alkali	1.0

These results indicate that the buffer capacity of the particular substance undergoing analysis is a major factor in the fluctuations of the pH. Conner and Straub (12) used a sodium acetate-acetic acid buffer because they believed that the addition of N sodium hydroxide might form "local areas of high alkalinity" and thus result in the destruction of the vitamin in those areas. In this investigation there was no evidence of any such destruction and the solutions were adjusted to optimum conditions by the addition of N hydrochloric acid or N sodium hydroxide.

4. Use of external indicators in the adjustment of the pH.

In the early course of this study a Hellige pH meter was used in all pH measurements. However this procedure involved the transference of a portion of the solution into a small beaker, after each addition of hydrochloric acid or sodium hydroxide, and a subsequent possibility of a loss of thiamine through inadequate washing. If the beaker and electrodes were rinsed thoroughly, it invariably resulted in a large volume of solution and this prevented an adequate washing of the residue after centrifuging. Thus a number of external indicators were used in order to test their value in making the necessary adjustment. Thymol blue for pH 2 and bromcresol green for pH 4.5 were found the most satisfactory and the accuracy of the procedure is illustrated in Table IV. The pH was adjusted as close as possible to pH 2 and 4.5 by means of the indicators and then checked with the pH meter.

TABLE IV. - Adjustment of the pH by means of External Indicators.

Material	Actual pH by glass electrode	
	Peptic digestion pH 2	Takadiastase hydrolysis pH 4.5
Wheat germ	2.0	4.4
Yeast	1.8	4.5
Milk	1.8	4.7
Liver	1.9	4.7
Cabbage	2.0	4.4
Carrot	1.8	4.6

In no instance was the variation greater than 0.2 and this is of no significance. Thus the use of external indicators was adopted in all subsequent work.

b. Details of the extraction technique.

Pyke (52) recommended that sufficient pepsin solution be added to a 20 gm. sample to give a total volume of 97.4 ml. After incubation overnight, 2.6 ml. of sodium hydroxide were added to give a total volume of 100 ml. He then assumed that, on completion of the digestion, the thiamin was evenly distributed throughout the solution and the solid residue. As Booth (4) has noted this assumption would only be correct if the digestion were complete. Since this is not the case, the practice of centrifuging the extract and washing the residue twice with distilled water, was adopted. The washings were then combined in a volumetric flask and made up to volume.

6. Effect of filtering extracts.

Occasionally an extract did not separate clearly on centrifuging and it was necessary to resort to filtration. This was particularly true in regard to extracts from bread. In order to check the possible adsorption of the vitamin on the filter paper, an extract was divided into 2 portions, one part filtered and the other centrifuged. The readings after oxidation were 18.6°, indicating that there had been no loss due to adsorption. There was also no evidence of the necessity of giving the filter papers a prior extraction with isobutanol as suggested by Wang and Harris (63).

7. Purification of extracts by selective adsorption.

Many substances have been used as adsorbents in concentrating and purifying crude thiamine extracts. Most European workers employed frankonite or similar clays but Cerecedo and Hennessy (9) introduced a synthetic zeolite, "Decalso", which they claimed was more selective. In this case the thiamine was removed by a hot, 25% potassium chloride solution. Melnick and Field (42) preferred the use of a potassium chloride solution acidified with sulphuric acid to pH 2 for the elution procedure. These workers (42) found however, that the presence of salts, non-aqueous solvents and large concentrations of other organic material, prevented quantitative adsorption of the vitamin. Only 65% recoveries were obtained when the thiamine solutions contained

sodium chloride in amounts comparable to those found in an equal volume of urine. Jowett (32) reported recoveries which averaged only 68% but were comparatively constant. Recently however Conner and Straub (12) have employed a 5 cm. column of activated, 60 to 80 mesh "Decalso", resting on a plug of spun glass in a specially designed adsorption tube. They have reported recoveries of added thiamine, by this method, of 89.5 to 100%. In this investigation the adsorption column proved quite unsatisfactory. In addition to the disadvantages already mentioned, it was found that the "Decalso" concentrated the iron in grass extracts and thus interfered with the subsequent oxidation. Ritsert (64) and Wang and Harris (63) both claimed that any advantages accruing from adsorption procedures were more than offset by the disadvantages and thus preferred direct methods. The latter used a prior extraction with isobutanol and this modification seemed to be worth investigating.

8. Effect of prior extraction with isobutanol.

Wang and Harris (63) suggested that 2 drops of 10% hydrochloric acid should be added before giving the solution a prior extraction. However when this treatment was applied to extracts of grass and other pigmented materials, the colour still appeared in the final isobutanol layer. It was apparent that some of the chromogens were extracted from strongly alkaline solution only, thus

an experiment was carried out to check the effect of extracting with isobutanol after the alkali had been added. This practice resulted in very clear solutions but also indicated a considerable loss of thiamine as shown in Table V.

TABLE V - Effect of extracting after addition of alkali
(Results I.U. per 100 gm.)

Material	No prior extraction	Alkali added and one prior extraction
Flour A	202	159
B	200	156

Since this was definitely not satisfactory, the results obtained after a prior extraction at pH 5, were compared with those obtained after the addition of 2 drops of 10% hydrochloric acid. The values are given in Table VI.

TABLE VI - Effect of prior extraction at pH 5
(Results I.U. per 100 gm.)

Material	pH 5	After addition of 2 drops of 10% hydrochloric acid
Bread A	54.6	75.6
" B	74.0	95.0

It was evident that even at pH 5 there was a definite loss of the vitamin due to this treatment and since a higher pH was required for successful purification, this modification was of no use.

9. Use of hydrogen peroxide as a clarifying agent

It was found at this point in the investigation, that the use of an unoxidized sample as a blank, was not satisfactory. If sufficient pigment were present to be detected visually, it was noticeably less after oxidation and an error was thus introduced. As Hills (25) has pointed out, fluorescence due to substances other than pigments might also be either increased or reduced during the oxidative process. Ritsert (54) attempted to overcome this difficulty by using as a blank, that amount of isobutanol extract, freed of thiamin by adsorption, which contained the same quantity of non-thiochrome fluorescence as was present in the test solution. This involved the personal factor, in that it depended on the ability of the observer to judge the amount of greenish fluorescence present in the test sample in addition to the blue-violet of the thiochrome. Jowett (32), aware of the effect of oxidation on interfering substances, took an arbitrary value for his blank, halfway between the "total blank" and the "reagent blank". It appeared, therefore, that some type of controlled oxidation, by which the treatment given to the test sample and the blank could be duplicated more closely, would be a definite advantage. Hydrogen peroxide seemed to be a logical choice since Kinnersley, O'Brien and Peters (36) stated that it did not oxidize thiamine to thiochrome. However, in alkaline solution, it rapidly oxidized to a colourless state, anthoxanthins and non-thiochrome fluorescing substances contained in the blank and test solutions. A series of ex-

periments were therefore performed to check the recovery of thiamine after the addition of hydrogen peroxide. 1 ml. of a 30% solution of the reagent was added in each case and the results are indicated in Table VII.

TABLE VII - Effect of hydrogen peroxide on the recovery of added thiamine.

Thiamine added (γ per ml.)	Thiamine recovered (γ per ml.)	% recovery
.124	.121	97.6
.124	.123	99.3
.124	.118	95.2
.124	.128	103.0
.144	.137	95.2
.144	.144	100.3

These values give an average recovery of 98.4% of the added vitamin. Since this value is well within the experimental error of the procedure, it is evident that there is no appreciable destruction due to hydrogen peroxide. When this treatment was applied to a sample of whole wheat flour, the error of using an unoxidized sample as a blank, became quite apparent. The values obtained are given in Table VIII.

TABLE VIII - Effect of hydrogen peroxide.
(Results I.U. per 100 gm).

Material	No hydrogen peroxide	1 ml. 30% hydrogen peroxide
Whole wheat flour 1	307	288
" 2	303	279

In cases where the extract was more highly pigmented, the error would be considerably larger. In view of these results, the hydrogen peroxide treatment was included in all further experiments.

10. Effect of omitting methyl alcohol during oxidation.

It soon became evident that, with materials such as grass and bran, the hydrogen peroxide did not completely remove the interference due to pigments. However, when methyl alcohol was omitted from the oxidation mixture, the final extract was found to be perfectly clear. Pyke (52) used 2 ml. of methyl alcohol because he believed that it stabilized the oxidative process and made the amount of potassium ferricyanide less critical. However a comparison of the curves given in Fig. I and II indicated that such was not the case. The data for Fig. I was obtained with an extract from wheat germ, using a 2 ml. aliquot, 1 ml. of methyl alcohol, 1 ml. of 30% sodium hydroxide and 1 ml. of 30% hydrogen peroxide. The experiments which gave the results indicated in Fig. II were conducted with an extract from bran and the reagents were the same as those above except that the methyl alcohol was

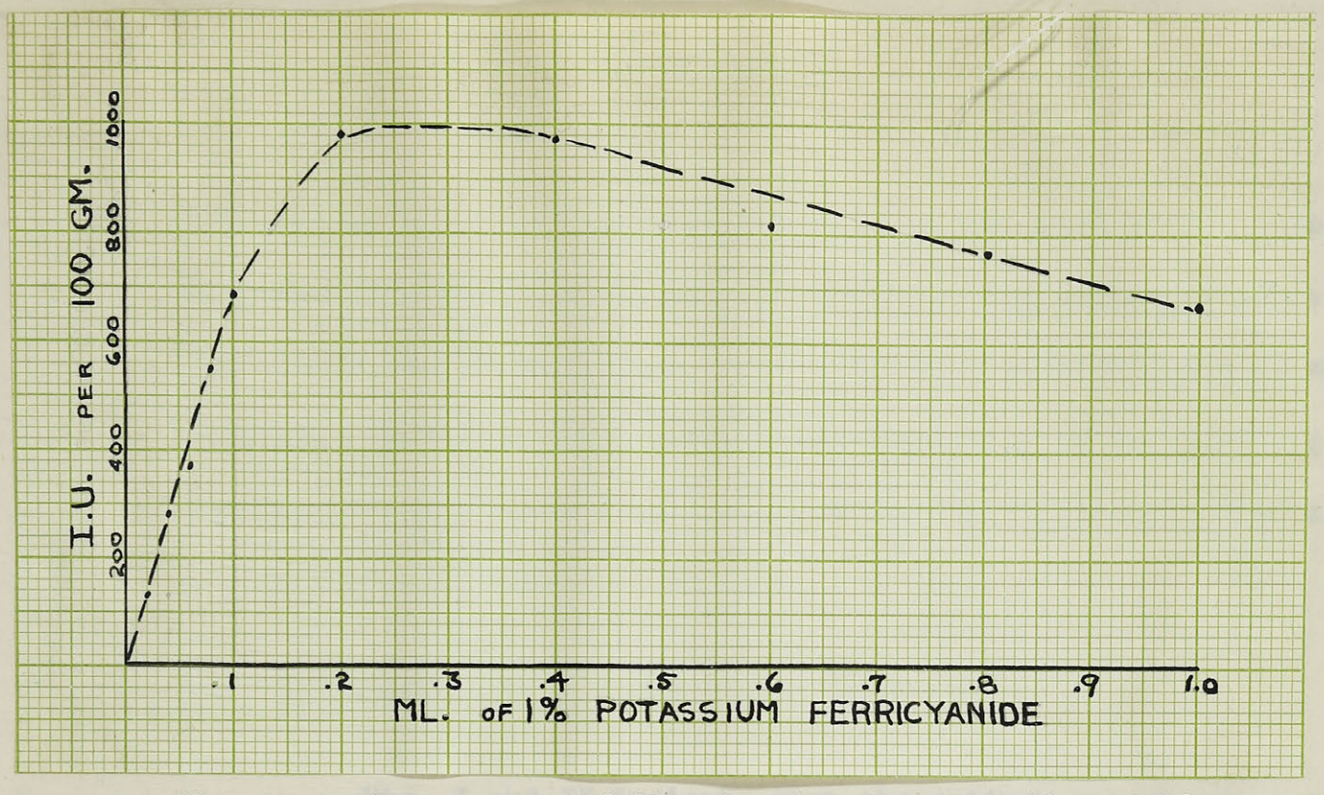

Fig. I Optimum amount of 1% potassium ferricyanide solution using 1 ml. of 95% methyl alcohol.

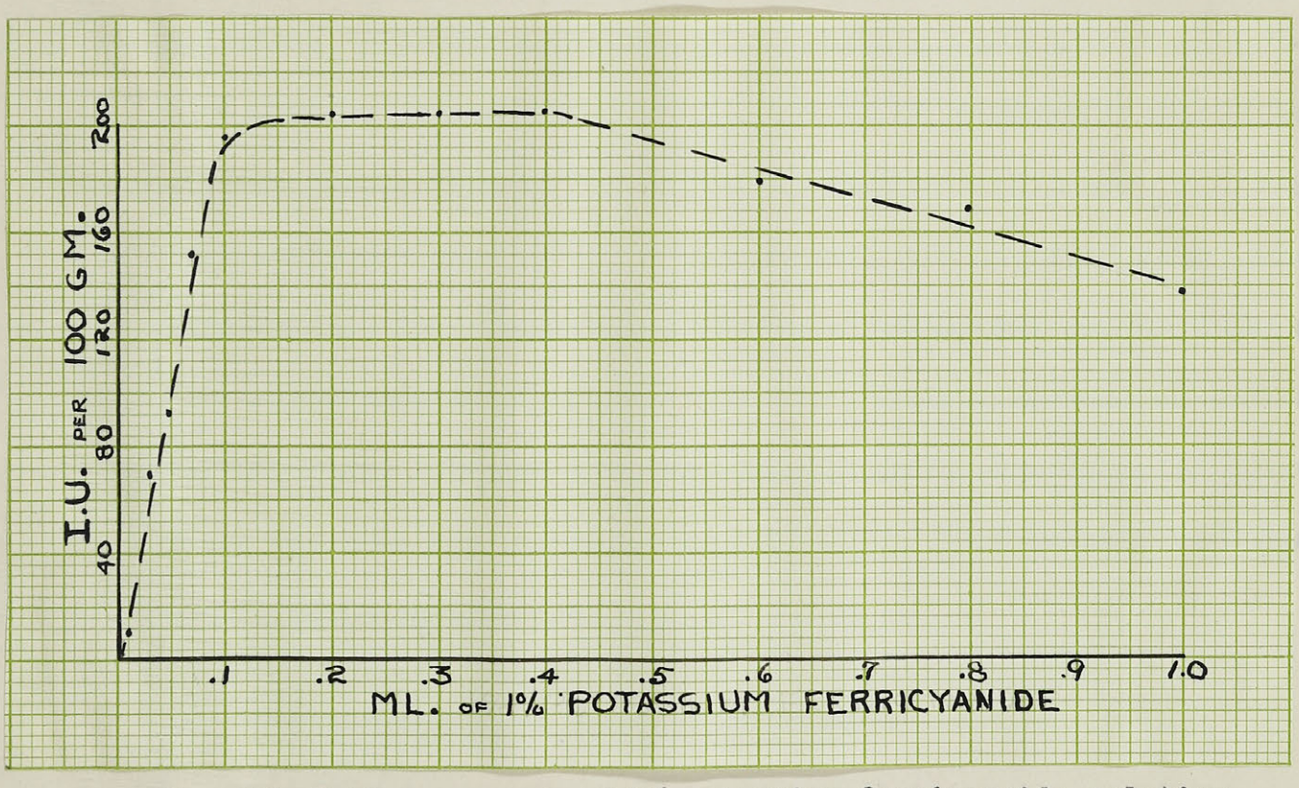

Fig. II Optimum amount of 1% potassium ferricyanide solution with methyl alcohol omitted.

replaced by 1 ml. of distilled water. It was evident that the addition of methyl alcohol did not stabilize the oxidation since the amount of potassium ferricyanide was less critical when it was omitted. Since the alcohol had the disadvantage of facilitating the transfer of pigments into the isobutanol, it was not used in any further experiments.

11. Optimum amount of potassium ferricyanide.

Fig. I and II indicate that the optimum amount of potassium ferricyanide lies between .1 and .4 ml. of 1% aqueous solution. However as Hills (25) pointed out the amount of oxidizing agent depends on the total amount of oxidizable substances present rather than on the amount of vitamin B_1 and thus will vary with different materials. To meet this difficulty, three aliquots were taken. One, which was to act as a blank, was not oxidized and the others were treated with slightly different amounts of potassium ferricyanide. If the readings did not agree closely a different range was used and the oxidation repeated. This modification has proven quite satisfactory.

12. Optimum amount of sodium hydroxide.

Pyke (52) recommended the use of 1 ml. of 30% sodium hydroxide in a total volume of 7 ml. It was found however that this strength of solution did not give the maximum fluorescence. A series of readings were taken, employing varying concentrations of the alkali

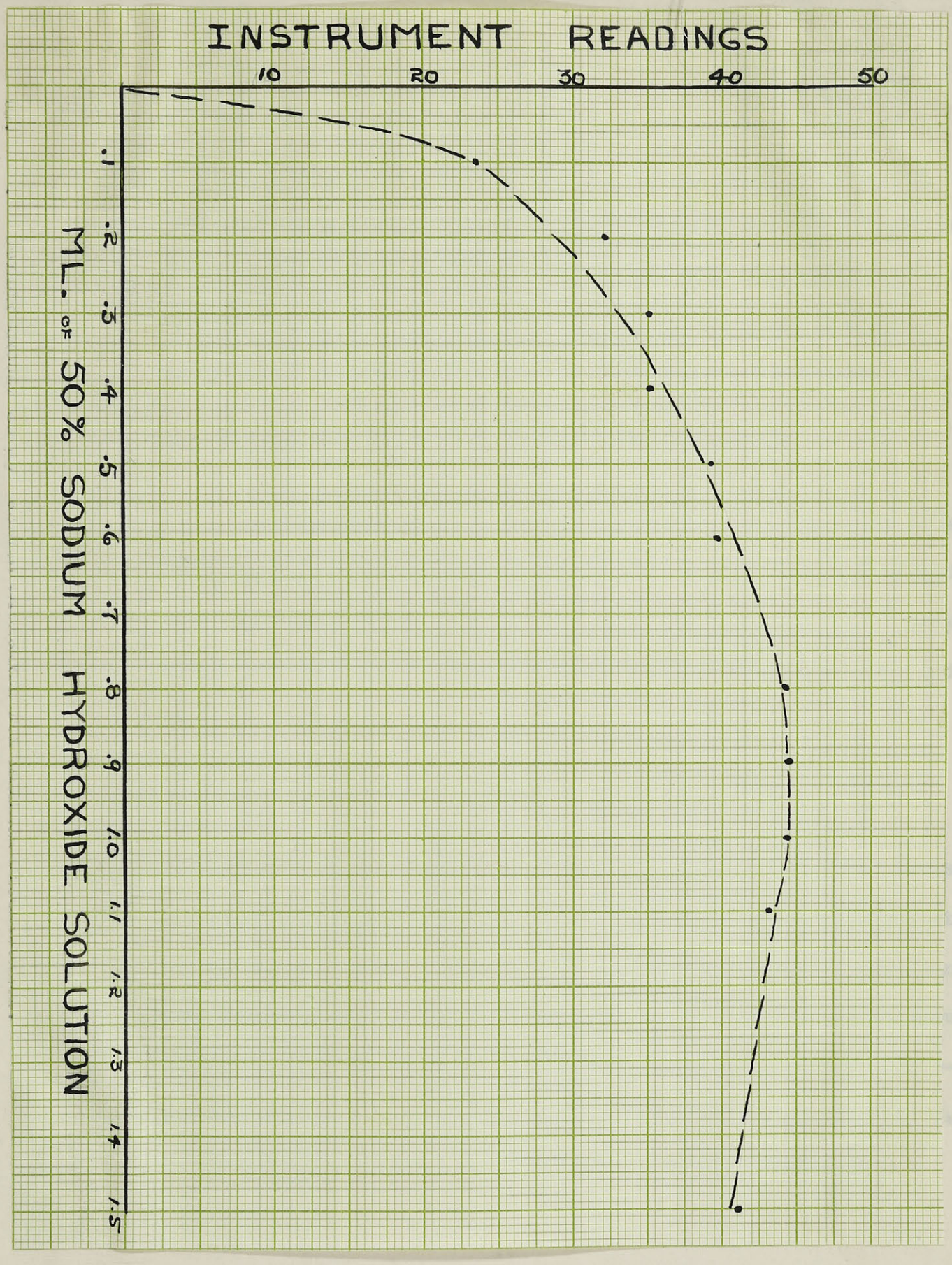

Fig. III. Optimum amount of 50% sodium hydroxide for maximum fluorescence.

and the results obtained are shown in Fig. III. It is evident from this data that the optimum amount lies within the range of .75 to 1.1 ml. of 50% sodium hydroxide in 4.2 ml. of solution. Since a 50% solution of sodium hydroxide is quite viscous and difficult to pipette, 1 ml. of 40% aqueous solution was found most convenient. Conner and Straub (12) have recently confirmed these results by stating that .45 gm. of sodium hydroxide in a total volume of 5 ml. gives the optimum conditions for the oxidation.

13. Amount of isobutanol required for extraction.

The literature reveals that varying amounts of isobutanol have been employed to extract the thiochrome from aqueous solution. Pyke (52) used 13 ml. of alcohol to 7 ml. of the vitamin solution. Hills (25) and Wang and Harris (63) have followed this ratio fairly closely. Conner and Straub (12) increased the volume to 20 ml. but state that there is no increase in the percentage extraction. Callegari (6) has shown however, that satisfactory results were obtained when the volumes of the extracting liquid and the aqueous solution were equal while Mukherji (44) found that it was advantageous to reduce the volume to 3 ml. In this investigation it was found that moderate variations in the ratio did not influence the amount of the thiochrome extracted. Since the smaller quantity of isobutanol increased the sensitivity considerably, a ratio of 5 ml. of isobutanol to 5 ml. of aqueous solution was adopted.

14. Use of oxidizing agents other than potassium ferricyanide.

Thiamine is known to be quite unstable in strongly alkaline solution but such a solution is required for oxidation by potassium ferricyanide and thus there is a possibility that some destruction may occur during the oxidative process. A number of oxidizing agents have been tested in an effort to find a substance which would convert the thiamine to thiochrome in neutral or acid solution. Negative results were obtained however with potassium molybdicyanide, cupric sulphate, ferric lactate, ferric chloride, ferric citrate, manganese dioxide, potassium dichromate and potassium permanganate. However 1 ml. of a solution of 5 gm. of cupric sulphate and 5 gm. of tartaric acid in 100 ml. of water produced a definite fluorescence when added to thiamine solutions. Since this reagent also required an alkaline medium and the fluorescence produced was much less than that obtained with potassium ferricyanide, no advantage would be gained by its use.

15. The effect of potassium cyanide and potassium iodide.

de Caro and Butturini (8) recently introduced the use of 1 ml. of 2% potassium iodide solution to remove the glutathione which they claimed interfered with the oxidation. In addition they recommended the use of 2 drops of 20% potassium cyanide solution to stabilize the thiochrome. These recommendations were tested with the results tabulated in Table IX.

TABLE IX. - Effect of potassium iodide and potassium cyanide.
(Results in I.U. per 100 gm.)

Material	Author's method	de Caro and Butturini's method.
Liver	123 121	61.5 121
Whole Wheat flour	121 119	121 123
Feed	484 467	472 474
Flour	27.8 29.7	29.8 30.8

The low value of 61.5 I.U. was obtained by adding 2 drops of potassium cyanide to 1 ml. of an extract while in the remaining experiments the solution was diluted to 4 ml. before this addition. This indicates that a high concentration of potassium cyanide may actually destroy the vitamin. These results show no evidence that potassium cyanide has a stabilizing effect nor do they indicate that glutathione interferes.

16. Destruction of thiochrome by ultra-violet radiations.

Prebluda and McCollum (49) have criticized the thiochrome method because of the known instability of the oxidation product in the presence of ultra-violet radiations. To check this point a thiochrome solution was placed in the instrument and readings

recorded every two minutes with the results indicated in Table X.

TABLE X - Rate of destruction of thiochrome in ultra-violet light.

Time in minutes	Reading	Conc.γ per ml.	Loss γ per ml.
0	49.6	.345	0
2	44.8	.305	.040
4	43.5	.282	.023
6	38.1	.230	.052
8	33.3	.182	.048
10	28.0	.129	.053

These values indicate a loss of .0215. γ per minute of exposure or a destruction of approximately 6% of the original. Since the time required for a reading is not more than 5 seconds, the error due to this factor, would be negligible.

17. Instrument used for measurement of fluorescence.

A wide variety of instruments have been employed in the estimation of thiamine. Pyke (50) originally used the Cohen fluorimeter (10) as Jansen (29) suggested but later introduced a visual titration method similar to that of Wang and Harris (63). However Booth (4) makes the statement that the photoelectric cell and filter in lieu of visual matching have given much closer agreement between replicates and greater overall accuracy. This claim was confirmed by the results obtained on duplicate samples as tabulated in Table XI.

TABLE XI. - Comparison of visual and photoelectric matching.

Visual matching	Photoelectric fluorimetry.
204	202
196	202

In this investigation, the fluorimeter described by Froman and McFarlane (22), was used throughout. The instrument is of the compensating two-photocell type and thus is not affected by fluctuations in line voltage.

18. Outline of modified method.

The procedure described below is based upon the results of the foregoing experiments:

Grind solids as fine as possible to facilitate quantitative extraction of the vitamin. Transfer a 5 to 20 gm. sample, the weight depending on its thiamine content, e.g., 5 gm. of yeast or of wheat germ and 10 to 20 gm. of bread or flour, to a 250 ml. centrifuge bottle and add a 0.2% solution of pepsin in 0.33% hydrochloric acid (50 ml. to the 5 to 10 gm. sample and 75 ml. to the 20 gm. sample). Let stand for one hour, then adjust to pH 2.0 with N hydrochloric acid or N sodium hydroxide, using thymol blue as an external indicator, and incubate overnight at 37°C. Adjust to pH 4.5 with bromcresol green as an external indicator, add 0.1 gm. of takadiastase and incubate at 37°C. for a further three to four hours. Centrifuge at maximum speed, transfer the supernatant to

a 100 ml. volumetric flask and wash the residue twice with distilled water. Combine the extract and washings and dilute to volume with distilled water. At this point it may be advantageous, with some extracts, to further clarify a portion of the solution by filtering through a filter paper before applying the thiochrome test, which is carried out as follows:-

Pipette a 1 ml. aliquot of the extract into each of three 10 ml. graduated, glass-stoppered cylinders. The optimum amount of potassium ferricyanide in the test solutions varies according to the amount of organic matter present and, under the conditions prescribed here for 1 cc. of the extract, falls within a range of 0.1 to 0.4 ml. of a 1% solution. If 2 or 3 ml. of the extract is taken for the test, the optimum range of ferricyanide concentrations must be redetermined. To cylinders No. 1 and No. 2 add 0.2 and 0.3 ml., respectively, of a 1% aqueous solution of potassium ferricyanide. Dilute the contents of the three cylinders to 3 ml. with distilled water. Cylinder No. 3 contains no ferricyanide and acts as a blank. Add 1 ml. of 40% sodium hydroxide to each cylinder, mix thoroughly and let stand for two minutes. Add 1 ml. of 30% hydrogen peroxide, again mix thoroughly, and allow to stand for about five minutes. Add 5 ml. of isobutanol, stopper, and shake vigorously for one minute. Remove the stoppers and centrifuge at minimum speed (zero step on Q.V. circular rheostat), using the 250 ml. trunnion cups, containing the customary rubber pads. In addition, hold the cylinder in position with a No. 11 stopper bored so as to fit over the neck of the cylinder. Record the volume of the isobutanol layer.

Carry out the fluorescence measurements as follows:- Transfer a 4 ml. aliquot of each supernatant to three fluorimeter tubes (22) and add 1 ml. of 95% methanol to remove a slight turbidity due to moisture. Special care should be taken to ensure complete mixing, and the outside of the test tube should be perfectly clean. The writer has modified the procedure previously described (22) for making the readings. At least 15 min. before making a reading, switch on the ultra-violet lamp, with the galvanometer connected in the circuit. During that time a test-tube, painted inside with black enamel, is in the test-tube block and the polaroids are set at zero reading.

About five minutes before making a reading remove the blackened test tube and open the polaroids. Place tube No. 3 (which has not been treated with potassium ferricyanide) in the instrument, with the polaroids at zero and the resistance adjusted to give no deflection of the galvanometer. Replace the blank with one of the test solutions and rotate the polaroids until the galvanometer again reads zero. Record the polaroid reading and ensure that the voltage has not changed appreciably in the interim; this is done by replacing the test solution with the blank solution and observing that there is no deflection of the galvanometer at zero reading on the polaroids. If the results of the two tests do not agree closely, repeat the test making a small change in the volumes of 1% potassium ferricyanide solution.

19. Preparation of a calibration curve.

There are certain difficulties involved in the preparation of a calibration curve of thiochrome using aqueous solutions of thiamine, which are not encountered with extracts. For example the amount of ferricyanide required is very much less and more critical for thiamine in pure solutions than in extracts containing other oxidizable substances. A series of experiments indicated that under the conditions employed, 0.2 ml. of 0.1% aqueous potassium ferricyanide gave the maximum fluorescence. An attempt was made to obtain data for a calibration curve under conditions similar to those in an actual test by adding organic matter in the form of a 1% dextrin solution. However it was soon evident that the dextrin did not give the same effect as an actual extract and thus was of no use. It was also observed that with pure solutions the time which was allowed to elapse between the addition of alkaline ferricyanide and the hydrogen peroxide was important. The results of a number of experiments to investigate this point are indicated in Table XII.

TABLE XII - Effect of standing on intensity of fluorescence.

Elapsed time	Reading obtained
30 seconds	36.5
1 minute	40.5
2 minutes	42.5
3 minutes	42.0
4 minutes	40.0
5 minutes	40.0
10 minutes	37.5

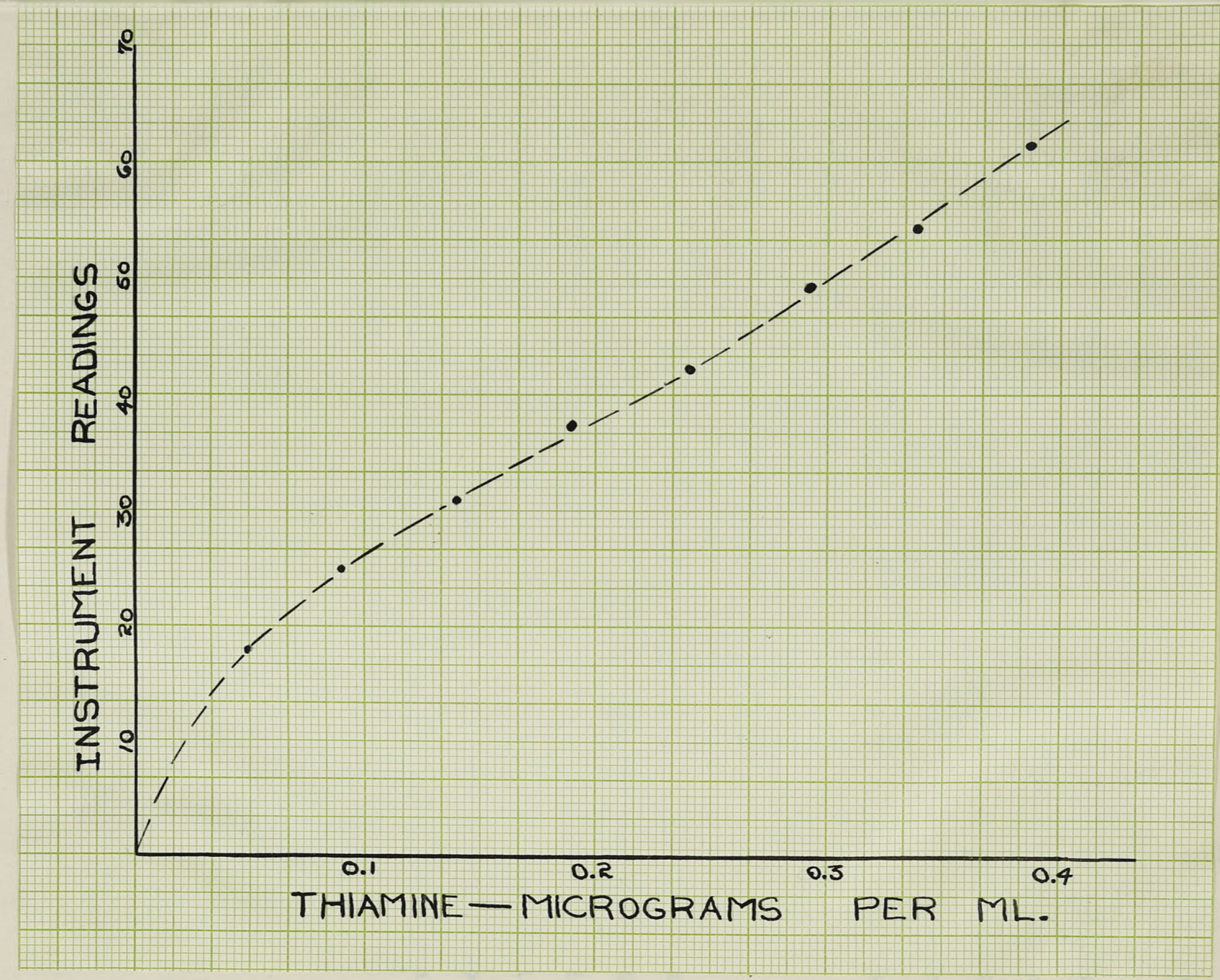

Fig. IV. Calibration curve for thiamine.

It was evident that, with pure solutions, the oxidation should be allowed to continue for two minutes before the addition of hydrogen peroxide. The calibration data is plotted in Fig. IV. The shape of the curve is characteristic of the instrument and parallels closely the curve which would be obtained with quinine sulphate. The thiamine content of unknown solutions can be calculated by reference to the calibration curve or from the equation (22) $C = K^1 \sin^2 \Theta$ where K^1 is a constant for the instrument and fluorescent substance and which by calculation from the data plotted in Fig. IV, was found to be 0.523 ± 0.021.

20. Comparison of results by various methods.

It was important to compare the results obtained by the modified method with those obtained by other modifications of the thiochrome method and by biological assay. Samples were analyzed in four different laboratories, two of which used a "Decalso" adsorption technique, a third employed a visual titration method and a fourth bio-assay. The results of such a study are shown in Table XIII.

TABLE XIII - A comparison of the thiamine content of two special breads as determined in different laboratories by different methods.

(Results expressed in International Units per 100 gm.)

Methods of analysis	Bread A	Bread B
Biological assay	99	113 and 130
Thiochrome method:		
(1) Decalso adsorption		
Laboratory A - photoelectric fluorimetry	58	78
Laboratory B - photoelectric fluorimetry	53	91
(2) Modified Pyke's Method		
Laboratory C - visual titration	86	132
Author's - photoelectric fluorimetry	92	112

It is evident that the modified method gives results of the same order as those obtained by the visual titration method and by bio-assay. However values given by the "Decalso" technique are considerably lower. A further confirmation of the reliability of the writer's method was obtained by analysis of samples secured from the National Research Council in a recent collaborative study, the results of which are tabulated in Table XIV. The figures for rat-growth, photoelectric and fermentation methods are average values which varied widely between different laboratories. The visual titration procedure was conducted in a single laboratory. The results in column No. 5 compare favourably with the other average values.

TABLE XIV - Research Council Collaborative Study Data.
(Results expressed as micrograms per gm.)

Sample	Rat-Growth Method	Thiochrome visual titration	Thiochrome photo-electric fluorimetry	Fermentation	Author's modified method
1. Whole wheat flour	5.01	5.10	5.07	5.19	5.07
2. Feed	13.7	14.07	13.77	14.73	14.22
3. Patent flour	.87	2.04	.90	.87	.89
4. White Bread plus Thiamin	5.22	5.01	4.65	4.71	5.06
5. Dried Hi-B-1 Yeast	654.0	777.0	681.0	693.	762.
6. Hi-B-1 Bread	5.22	5.31	4.62	4.62	5.04

The procedure gives equally satisfactory results when applied to beef liver, cabbage, carrots and whole milk. Values of 105, 37, 36 and 15 I.U. of thiamine per 100 gm., respectively, were obtained; these appear reasonable when compared with average values obtained by bio-assay as recorded in the literature. The liver, cabbage and carrots were put through a meat-chopper and 20 gm. of the finely minced material used for the test. In the case of milk, 25 ml. was employed.

SUMMARY

Various modifications of the thiochrome method for the determination of thiamine have been investigated and improvements suggested.

Hydrogen peroxide was found to be an efficient reagent for the clarification of crude thiamine extracts. There was no oxidation of thiamine to thiochrome or destruction of pre-formed thiochrome by the treatment but interfering substances were rapidly oxidized to a colourless state. Methyl alcohol was shown to be unnecessary for the oxidation of thiamine by alkaline ferricyanide and its elimination resulted in clearer isobutanol extracts. The optimum amounts of potassium ferricyanide and sodium hydroxide have also been determined.

The method as outlined makes it possible to apply photoelectric fluorimetry to the determination of thiamine without the use of an adsorption technique.

PART II

Studies on the Determination of Riboflavin (vitamin B_2) in Foods.

PART II INTRODUCTION

During the early stages of the study of riboflavin, biological methods, based on the Bourquin and Sherman (6) rat-growth procedure, have proven quite valuable. More recently however, Sneil and Strong (60) have introduced a rapid and sensitive microbiological method which involves the response of Lactobacillus casei to the vitamin. The riboflavin is determined by measuring the turbidity or acidity of the culture.

Since the discovery by Kuhn and Wagner-Jauregg (38) that riboflavin solutions gave off a brilliant yellow-green fluorescence when activated by light of certain wave-lengths, numerous methods have been proposed for the determination of the vitamin based on the intensity of this fluorescence. Early procedures involved the extraction of the riboflavin by an organic solvent such as acetone or methyl alcohol, followed by purification by adsorption on frankonite or a similar product. Emmerie (16) employed lead sulphide for this purpose and eluted with a mixture of water, pyridine and acetic acid. He followed this treatment by purification with potassium permanganate and hydrogen peroxide as recommended by van Eekelen and Emmerie (15). Sullivan and Norris (61) introduced sodium hydrosulphite as a reducing agent to destroy the fluorescence of the riboflavin and determined the vitamin content by the difference between a reduced and unreduced sample. Stannous chloride was included in the procedure by Hodson and Norris (28) because they claimed that in the presence of this reagent, riboflavin could be

reoxidized by shaking with air but interfering substances were not. Recently Ferrebee (20) and Conner and Straub (12) have adsorbed the vitamin on a granular floridin earth, "Supersorb", and after elution with pyridine and acetic acid, purified the extracts by controlled oxidation before measuring the intensity of the fluorescence.

Riboflavin, when exposed to high illumination in alkaline solution is converted into lumiflavin, a compound which is soluble in chloroform. Reindel and Fleischmann (53) have used this reaction as the basis of a method in which the intensity of the blue fluorescence of lumiflavin is considered proportional to the vitamin B_2 content. However Lunde and coworkers (40) have stated that values obtained by this method are always low due to destruction of the vitamin during the process. Several colorimetric methods (16) (40) have been proposed but since they are much less sensitive than the fluorimetric procedures they are not applicable to the analysis of foodstuffs.

A close study of these various methods revealed that the adsorption process was a highly critical procedure and often resulted in loss of the vitamin. Purification by controlled oxidation (15) appeared to be satisfactory when the extracts were comparatively clear but when caramelization had occurred (61) or the material was highly pigmented, the interference was not entirely removed. Thus it was evident that, in order to improve the method, a more satisfactory procedure for the removal of interfering substances must be devised.

EXPERIMENTAL

1. Use of benzyl alcohol in the purification of riboflavin extracts.

In 1937 Greene and Black (23) reported that vitamin B_2 could be extracted from aqueous solutions by shaking with benzyl alcohol. The following year Emmerie (17) stated that only the free flavins were extracted by this method and that the phosphorylated compound remained in the aqueous layer. He found that a single extraction, with an equal volume of benzyl alcohol, removed 76-79% of the free riboflavin and in 1939 (18) employed the reagent in a method for the determination of vitamin B_2 in urine. It appeared, therefore, that benzyl alcohol might be of use in removing riboflavin from crude extracts of foodstuffs and rough determinations indicated that such an extraction was worth investigating. However, in order to check its feasibility, it was necessary to find a reducing agent which would react with riboflavin in benzyl alcohol in order that a proper blank could be obtained.

2. Use of stannous chloride as a reducing agent.

Hydroxylamine, hydrazine hydrate, titanous chloride, p-hydroxyphenyl glycine, sodium hydrosulphite, cuprous salts and ferrous salts were tested without success. However it was found that stannous chloride reduced the riboflavin to a non-fluorescent state when it was added to a benzyl alcohol solution

of the vitamin. This reduction only occurred in the presence of ultra-violet light, a phenomenon which confirmed the report of Haring and Walton (24) that the autoxidation of stannous chloride was a photochemical chain reaction. It was found necessary to twice recrystallize the stannous chloride from hot 95% ethyl alcohol in order to remove impurities, otherwise a cloudy precipitate appeared which interfered with the fluorimetric readings. 1 ml. of a 3% solution was capable of reducing the riboflavin in a 3 ml. aliquot of all extracts. If a stronger solution was employed there was danger of cloudiness in the final benzyl alcohol extract. 95% ethyl alcohol was first employed as a solvent for the stannous chloride but benzyl alcohol later proved more satisfactory.

3. Use of zinc acetate in the purification of extracts.

The emulsion, which formed on shaking benzyl alcohol with pure riboflavin aqueous solutions, broke easily on standing but when extracts of foodstuffs were employed considerable difficulty was encountered. Materials such as flour and liver formed heavy emulsions which did not separate even after centrifuging at high speed. It was quite evident that some method of purification had to be employed and a number of protein precipitants were tested. Trichloracetic acid, mercuric chloride, mercuric acetate, barium chloride and lead acetate were of no use since a precipitate developed on the addition of stannous chloride. However when 1 ml. of 16% zinc acetate solution in a total volume

of 5 ml. was shaken with an equal volume of benzyl alcohol, no precipitate appeared on the addition of the reducing agent to the final extract. It was later found that with an occasional sample a slight cloudiness was present but this was due to the crude riboflavin extract rather than to the presence of zinc acetate and could be avoided by decreasing the aliquot to 1 ml.

4. Effect of using an excess of zinc acetate.

When 1 ml. of 16% zinc acetate solution was employed the benzyl alcohol layer still had the greater density and thus remained on the bottom after centrifuging. Under these circumstances the zinc precipitate remained in the benzyl alcohol layer after the aqueous solution had been siphoned off and it was thus necessary to pass it through a dry filter before taking a reading in the fluorimeter. It was possible to avoid this inconvenience by using 2 ml. of a saturated solution of zinc acetate. In this case the density of the aqueous layer was increased to such an extent that the benzyl alcohol remained on the top and the precipitate accumulated in the lower aqueous portion. By this treatment it was possible to eliminate the filtration and the clear benzyl alcohol layer could be pipetted directly into the fluorimeter tube without the removal of the aqueous solution. It was necessary, however, to check the effect of the zinc acetate on the extraction of the vitamin. This was accomplished by using a 2 ml. aliquot, making the mixture up to 5 ml. with varying amounts

of saturated zinc acetate solution and distilled water, and extracting with 5 ml. of benzyl alcohol. The results are tabulated in Table XV.

TABLE XV. - Effect of zinc acetate on the extraction of riboflavin by benzyl alcohol.

Sample no.	Ml. of sat. zinc acetate sol.	Readings obtained
1	0.0	36.3
2	0.1	35.2
3	0.3	36.4
4	0.5	36.9
5	0.7	35.5
6	1.0	36.3
7	2.0	36.3
8	3.0	36.6

Sample No. 2 did not have sufficient zinc acetate and the flocculent precipitate which formed, interfered with the filtration. The density of the aqueous layer in No. 5 was very close to that of benzyl alcohol and consequently separation was extremely slow. These factors may have interfered with the extraction and may be responsible for the slightly lower results. In samples 5 to 7 the aqueous layer was heavier than the benzyl alcohol. These results indicate that the presence of zinc acetate has no appreciable effect

on the extraction of riboflavin from aqueous solutions. However when zinc acetate was added in sufficient amount to give the aqueous layer a greater density than that of benzyl alcohol, it unfortunately caused a precipitate with stannous chloride. Thus if this method was employed it was necessary to use pure benzyl alcohol as a blank in making the readings.

5. Use of pure benzyl alcohol as a blank.

It was quite apparent that a serious error would be introduced if pure benzyl alcohol did not give the same reading in the fluorimeter as a benzyl alcohol extract in which the fluorescence of the riboflavin had been destroyed by reduction. A number of experiments were therefore carried out to check this point. A 3 ml. aliquot of an aqueous extract of some food material was pipetted into a 15 ml. centrifuge tube. 1 ml. of a 16% zinc acetate solution, 1 ml. of distilled water and 7 ml. of benzyl alcohol were added. The tubes were then shaken vigorously and centrifuged at 3200 r.p.m. for 10 minutes. The aqueous layer was siphoned off and the lower benzyl alcohol solution passed through a dry filter paper. 5 ml. of the clear benzyl alcohol was transferred to a fluorimeter tube and 0.5 ml. of a 3% solution of purified stannous chloride in benzyl alcohol, were added. The reagents were mixed, the tube placed in the fluorimeter and allowed to stand in the path of the ultra-violet light until the galvanometer remained constant. The reduction was usually complete in 3 minutes. The reading obtained was compared with that given by benzyl alcohol which had been put through the above procedure except that 3 ml. of distilled water replaced the original extract and pure benzyl alcohol was used in place of the stannous chloride solution. The results obtained are tabulated in Table XVI.

TABLE XVI. - Deviations of readings obtained with riboflavin solutions reduced by stannous chloride, from those given by pure benzyl alcohol.

Material	Galvanometer deviations in cm. from reading of pure benzyl alcohol.
Pure riboflavin	0.00
Whey powder	0.11
Yeast	0.10
Whole milk	0.00
Chicken liver	0.01
Calf liver	0.00
Flour	0.00
Skim milk powder	0.08
Egg white	0.00
Grass clippings	0.00
Bran	0.00

These results indicated that in some cases there are substances other than riboflavin which are extracted by benzyl alcohol and which give a yellow fluorescence in ultra-violet light. A deviation of 0.1 cm. involves an error of approximately 5% at a reading of 30°. Thus if more accurate results are required, 1 ml. of 16% zinc acetate must be employed as outlined on the previous page, in order that a solution may be reduced by stannous chloride to serve as a blank.

6. Use of benzyl alcohol saturated with sodium chloride.

Greene and Black (23) and Emmerie (17) have stated that the addition of sodium chloride to benzyl alcohol increased the solubility of the riboflavin. This was found to be true in a

range from 0.5 γ to 2.5 γ per ml. Outside this range, for some unexplained reason, the solubility decreased and gave results almost identical with those obtained with benzyl alcohol alone. Since the advantage gained by the addition of sodium chloride was only very slight it was not included in the final method.

7. Methods of extraction.

Acid extraction (13) or extraction by an acid-acetone solution (61) have been the chief methods proposed for the preparation of a crude extract from foodstuffs. In this investigation however the acetone procedure could not be used due to the miscibility of the benzyl alcohol and acetone. In addition some enzymatic treatment was necessary to free the vitamin, present in a phosphorylated state since the phosphoric acid ester is not extracted by benzyl alcohol. It appeared logical to use the peptic digestion as outlined for the determination of thiamine so that it would thus be possible to obtain values for both vitamin B_1 and B_2 on the same extract. Since riboflavin is sensitive to light, the incubation at 37° C. was carried out in a dark cabinet and the crude extracts, after being made up to volume, were placed in a refrigerator. However, it was not found necessary to protect the solutions from light during the actual test. In order to investigate the possibility of any destruction, a pure riboflavin solution was put through the entire procedure and 98.5% of the original vitamin was recovered.

8. Modified method for the determination of riboflavin.

Prepare an extract as outlined for the determination of thiamine (page 27). A rough test must then be conducted for each particular material by the method indicated on page 44 to determine the feasibility of using benzyl alcohol as a blank. If the reduced sample and the pure benzyl alcohol give the same reading the following procedure may be employed. Pipette a 3 ml. aliquot of the extract, 2 ml. of saturated zinc acetate solution and 7 ml. of benzyl alcohol into a 15 ml. graduated centrifuge tube and shake vigorously. Centrifuge at 3200 r.p.m. for 10 minutes or until the benzyl alcohol layer is quite clear. Record the volume of the supernatant liquid. Transfer 5 ml. of the benzyl alcohol to a fluorimeter tube and add 0.5 ml. of 95% ethyl alcohol to remove any trace of cloudiness due to moisture. Prepare a blank in a similar manner by replacing the 3 ml. aliquot with 3 ml. of distilled water. However if the rough test indicates that the readings are not comparable, the determination must be conducted as follows:- Pipette a 3 ml. aliquot of the extract, 1 ml. of 16% stannous chloride solution, 1 ml. of distilled water and 7 ml. of benzyl alcohol into each of two 15 ml. graduated centrifuge tubes and shake vigorously. Centrifuge at 3200 r.p.m. for 10 minutes and then siphon off the upper aqueous layer. Pass the benzyl alcohol layer through a dry filter paper and transfer 5 ml. of the clear solution to a fluorimeter tube. To one tube add 0.5 ml. of 3% stannous chloride in benzyl alcohol and 0.5 ml. of 95% ethyl alcohol. To the other add 0.5 ml. of benzyl alcohol and 0.5 ml.

Fig. V. Calibration curve for riboflavin.

of 95% ethyl alcohol. Carefully mix the reagents and place the tube containing the stannous chloride in the instrument (22) and allow it to remain in the path of the ultra-violet light until the galvanometer remains constant. Adjust to zero and replace the blank with the test solution and rotate the polaroids until the galvanometer again reads zero. The riboflavin content of the solution is calculated from a calibration curve prepared from pure riboflavin solutions and employing 2 ml. of saturated zinc acetate solution. Such a curve is shown in Fig. V. The equation $C = K^1 \sin^2 \theta$, cannot be used in this case because K^1 was found to be a variable quantity.

9. Application of the modified method.

Values obtained for the riboflavin content of various materials are tabulated in Table XVII.

TABLE XVII - Riboflavin content of various materials analyzed by the modified method.

Material	Riboflavin content (γ per gm.)
Calf liver	32.0
Bran	2.7
Whole wheat flour	1.7
Buttermilk powder	31.3
Dried yeast	18.7
Skim milk powder	26.4
Chicken liver	18.6
Egg yolk	5.2
Egg white	3.1
Grass clippings	1.8
Whole milk	1.0
Whey powder	28.1

These values agree closely with the average figures reported in the literature for similar products. A comparison of results obtained by this method with those given by other procedures is in progress.

SUMMARY

A method has been described for the estimation of riboflavin in foodstuffs. Benzyl alcohol was used as an immiscible solvent for the extraction of the vitamin from aqueous solutions and by such a procedure interfering substances were largely removed. A 3% solution of stannous chloride in benzyl alcohol was employed to reduce the riboflavin, a reaction which took place only in the presence of ultra-violet radiations. Zinc acetate was used as a protein precipitant with satisfactory results.

CONCLUSION

The chemical methods for the determination of thiamine and riboflavin have been carefully investigated and modified methods are proposed which can be applied to the same extract from a given material. Peptic digestion followed by takadiastase hydrolysis was found to be the most convenient and effective method for the extraction of both thiamine and riboflavin from foodstuffs.

The fluorimeter of Froman and McFarlane has performed satisfactorily in making the fluorimetric readings.

The values obtained for thiamine and riboflavin agree closely with the average values given in the literature.

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