TABLE I.—R_f VALUES AND TIME OBTAINED FOR SEPARATION OF DYES USING THE STANDARD AND ACCELERATED METHODS OF THIN-LAYER CHROMATOGRAPHY

Dyes	R_f Value—Accelerated	R_f Value—Standard
Light green SF yellowish	0.00	0.00
Sudan Black B	0.10	0.15
Indophenol blue	0.25	0.35
Sudan R	0.40	0.45
Oil Red O	0.55	0.65
Dimethylaminoazo benzene	0.65	0.70
Adsorbent—aluminum oxide G		
Solvent benzene-hexane (9:1)		
Development time accelerated—10 min.		
Development time standard—35 min.		

eter, 2.5 mm. thick, and with a 1.2-cm. centered hole to fit the Precision hi-speed chromatography[1] apparatus were used. The plates were coated with aluminum oxide G or silica gel G (with binder) by the spray technique of Bekersky (5) or the technique of Davidek and Prochazka (6). The plates were activated in the usual manner.

[1] Precision Scientific Co., Chicago, Ill.

The samples are spotted at a point 2.5 cm. from the center hole. Ten spots may be applied per plate. The inside of the apparatus was lined with heavy filter paper saturated with the solvent used for developing. The plate is secured to the spindle of the apparatus, and the rotation of the plate set at 500–700 r.p.m. The solvent flow was adjusted to permit a constant flow without overloading and possible washing away of the adsorbent.

Table I shows the R_f values and time obtained for separation of dyes using the standard and accelerated methods of thin-layer chromatography.

Accelerated thin-layer chromatography provides a method of decreasing the time necessary to achieve separation.

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Estimation of Thiamine by Inverse Isotope Dilution

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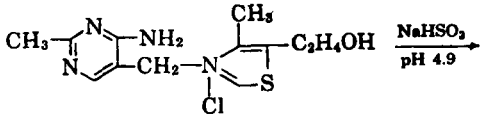
Thiamine hydrochloride was determined quantitatively by inverse isotope dilution in the presence of ascorbic acid, riboflavin, and nicotinic acid. The results obtained, using 100-mg. quantities, indicated an average error of 0.1 per cent and a reproducibility of 0.27 per cent.

THE PURPOSE OF THIS investigation was to study a thiamine assay by inverse isotope dilution analysis. Several advantages are inherent in this technique, including speed and simplicity. However, the most outstanding advantage is that quantitative isolation is not required at any point in the analysis. Thus, thiamine may be determined in the presence of other substances without quantitative extraction.

It was necessary to choose a suitable labeling reagent to react with thiamine to produce a chemically stable derivative. Williams, *et al.* (1), proved the structure of thiamine by a quantitative sulfite cleavage into two moieties. This reaction is

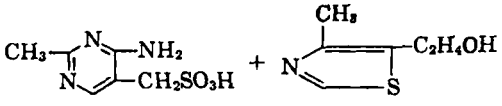


This reaction was used to perform an inverse isotope dilution analysis. Sodium sulfite-S-35 was used as the labeling reagent to form S-35 labeled 2-methyl-6-aminopyrimidyl-5-methanesulfonic acid as the derivative.

The specific activity of the derivative was determined indirectly by carrying out inverse isotope dilution analysis with a known amount of thiamine. The labeled derivative was then mixed with a known amount of carrier. The specific activity of the carrier-diluted derivative was determined by measuring the weight and radioactivity of a portion of the purified material. The weight of the derivative before adding the carrier was calculated by employing the standard inverse isotope dilution formula. The weight of thiamine analyzed was calculated from the weight of the derivative by a gravimetric factor.

EXPERIMENTAL

Optimum Conditions for Cleavage Reaction.—



Thiamine hydrochloride was cleaved under different conditions of temperature, pH, and reaction time. Cleavage at room temperature for 15 hours at a pH of 4.9 was chosen. These cleavage conditions are confirmed by the literature (1).

Physical Properties of Derivative.—The derivative crystallized as fine needle-like crystals insoluble

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in cold water and organic solvents such as pyridine, ethanol, methyl ethyl ketone, and chloroform. The derivative was readily soluble in small amounts of ammonia T.S. and did not precipitate upon dilution with water. The derivative was soluble to the extent of about 1.6% in boiling water after prolonged boiling; it precipitated upon cooling slowly. The compound had no sharp melting point, but began to char at about 410°. These properties match those of the compound separated by Williams, *et al.* (2).

Preparation of Carrier.—Fifty grams of thiamine hydrochloride (Merck, manufacturing grade) was dissolved in 600 ml. of water containing 125 Gm. of anhydrous sodium sulfite (Mallinkrodt, A.R.). Hydrochloric acid, 2.6 *N*, was added dropwise to adjust the pH to 4.95. The mixture was set aside for 2 days; the product was then collected with suction filtration. The product was dissolved in the least amount of ammonia T.S., and glacial acetic acid was then added dropwise until the mixture was distinctly acid to litmus paper. The mixture was set aside for another 2 days to allow for complete crystallization. The supernatant was decanted, and the product was dissolved in 1500 ml. of water by boiling on a hot plate for about 5 minutes. The hot plate was turned off; the solution was allowed to crystallize by cooling slowly to room temperature. The mixture was set aside for 2 days. The supernatant was decanted, and the product was dissolved again in 1500 ml. of boiling water. The product formed after cooling was removed with suction, oven-dried at 120°, and stored in a tightly closed container.

Because the carrier did not have a definite melting point, it was assayed potentiometrically to determine its purity. A weighed portion of the carrier was dissolved in 0.1 *N* sodium hydroxide, and this solution was back titrated with 0.1 *N* hydrochloric acid. The results showed an average of 101.2% carrier for three determinations.

Measurement of Radioactivity.—The radioactivity of all samples was measured with a Tri-Carb liquid scintillation spectrometer, model 314-X (Packard Instrument Co., La Grange, Ill.). The instrument was operated with a 10-90 v. window and the freezer temperature was 1°. The XDC scintillator (3), modified by substituting toluene in place of xylene, was used. The counting vials were 20-ml. screw cap, low potassium glass vials (Packard Instrument Co.).

Sample activities were corrected for variation in counter efficiency by use of a C-14 standard. The energy of the C-14 β particle was considered sufficiently similar to that of the S-35 β particle for this purpose. Sample activities were corrected mathematically for decay. All background measurements were made with a blank containing 15 ml. of scintillator, 1.0 ml. of ammonia T.S., and 1.0 mg. of carrier.

A study of the quenching effect of the carrier was performed; the results indicated that quenching did not occur up to 4.0 mg. of carrier in 1.0 ml. ammonia T.S. per sample.

Determination of the Reproducibility of the Cleavage Reaction.—To determine whether the cleavage reaction was reproducible with the cleavage conditions selected, it was carried out with sodium sulfite-S-35 (New England Nuclear Corp., Boston, Mass.). The cleavage reaction was run with 100 mg. of thiamine hydrochloride, and 500 mg. of carrier was added. One milligram of the carrier-diluted derivative was counted, the specific activity of which was approximately 20,000 d.p.m./mg. The specific activity of the sodium sulfite used was approximately 300,000 d.p.m./mg. A 25-ml. 1 *M* solution with the above specific activity was prepared with sodium sulfite-S-35 and carrier (anhydrous sodium sulfite, Mallinkrodt, A.R.).

The cleavage reaction was run three times with a 2% aqueous solution of thiamine hydrochloride (Roche, U.S.P.). The reaction procedure used was similar to that for the analysis of thiamine hydrochloride in the presence of other vitamins, except that the vitamin mixture was not added, and the carrier-diluted derivative was not recrystallized from ammonia-acetic acid.

For each run, 50.0 mg. of the carrier-diluted derivative was dissolved in 50.0 ml. of ammonia T.S. Six samples of each of these solutions, each containing 1.0 ml., were prepared and counted; the results are shown in Table I. The coefficient of variation for the three runs was 0.20%.

Recrystallization Procedure Necessary to Obtain a Constant Final Specific Activity.—Two methods of recrystallization were employed to purify further the carrier-diluted derivative. The remaining parts of the carrier-diluted derivative from the three runs of the previous experiment were dissolved separately in 5 ml. of ammonia T.S. The pH was adjusted to 4.9 to 5.0 with glacial acetic acid; the mixtures were set aside overnight to complete crystallization. The crystals were collected on a sintered-glass funnel, washed with water, and oven-dried at 120° for 2 hours. A solution containing 50.0 mg. of the purified material in 50.0 ml. of ammonia T.S. was prepared from each run. Six samples from each of these solutions, each sample containing 1.0 ml., were prepared for counting.

The remaining parts of the solids from the ammonia-acetic acid recrystallization were boiled separately on a hot plate with sufficient water to dissolve them. The solutions were allowed to cool slowly overnight. The crystals that formed were

TABLE I.—REPRODUCIBILITY OF THE CLEAVAGE OF THIAMINE HYDROCHLORIDE

Run No.	Av. Sample[a,b] Activity, c.p.m.	Coeff. of Variation, %
1	1746	1.20
2	1752	1.26
3	1752	0.80

[a] Average of six samples, each counted for 4 minutes. [b] Samples were counted 5 months after sodium sulfite-S-35 reagent was prepared.

TABLE II.—DETERMINATION OF THE RECRYSTALLIZATION PROCEDURE NECESSARY TO OBTAIN A CONSTANT SPECIFIC ACTIVITY

Run No.	Noncrystallized,[a,b,c] c.p.m.	Recrystallized[a,c] from Ammonia-Acetic Acid, c.p.m.	Recrystallized[a,c] from Water, c.p.m.
1	1746 ± 21	1708 ± 28	1704 ± 23
2	1752 ± 22	1710 ± 21	1707 ± 23
3	1752 ± 14	1697 ± 25	1723 ± 30

[a] Average of six samples, each counted for 4 minutes. [b] From Table I. [c] The limits of each mean are expressed as the standard deviation.

TABLE III.—INVERSE ISOTOPE DILUTION ANALYSIS OF THIAMINE HYDROCHLORIDE IN PRESENCE OF OTHER VITAMINS

Run No.	Prepn.	Av. Activity,[a] c.p.m.	Coeff. of Variation, %	Recovery,[b] %
1	Standard 1	8072	0.63	...
	Standard 2	8038	0.20	...
	Mixture 1	8072	0.21	100.2
	Mixture 2	8074	0.31	100.2
2	Standard 1	7982	0.66	...
	Standard 2	8067	0.88	...
	Mixture 1	8058	0.45	100.3
	Mixture 2	8006	0.77	99.7

[a] Average of three samples, each counted for 5 minutes. [b] Based on 99.6 mg. thiamine hydrochloride actually present.

collected on a sintered-glass funnel, washed with water, and oven-dried at 120° for 2 hours. Samples were prepared for counting in the manner described above.

The results are shown in Table II. The average activities of the three runs recrystallized from ammonia-acetic acid and water were 1705 and 1711 c.p.m., respectively. The conclusion was that recrystallization from ammonia-acetic acid was sufficient for determination of the final specific activity.

Analysis of Thiamine Hydrochloride in the Presence of Other Vitamins.—The following stock solutions were prepared.

1. A 2% aqueous solution of thiamine hydrochloride (Roche, for ampul use) which was oven-dried at 105° for 2 hours before use.

2. A 5% solution of the carrier in ammonia T.S. oven-dried at 120° for 2 hours before use.

3. An aqueous vitamin mixture containing 25.0 mg. ascorbic acid, 0.50 mg. riboflavin, and 10.0 mg. nicotinic acid in each 2 ml. was prepared by warming a suspension of riboflavin and nicotinic acid in water until a clear solution was obtained, then cooling, and adding the ascorbic acid.

4. A 25-ml. 1 *M* sodium sulfite-S-35 solution was prepared so that the final specific activity of the carrier-diluted derivative would be approximately 15,000 d.p.m./mg., calculated on the basis of using five parts of carrier for each part of thiamine hydrochloride cleaved. These solutions were stored in a refrigerator.

Two runs were carried out on consecutive days under the same conditions. For each run, two standards consisting of 5.0 ml. of the 2% thiamine hydrochloride solution and two mixtures consisting of the above solution plus 2.0 ml. of the vitamin mixture stock solution were used. The assay procedure for the mixtures is described. Into a 30-ml. beaker were pipeted 5.0 ml. of the 2% thiamine hydrochloride solution, 2.0 ml. of the vitamin mixture solution, and 2.0 ml. of the 1 *M* sodium sulfite-S-35 solution. The pH was adjusted to 4.9 to 5.0 by adding 2.6 *N* hydrochloric acid dropwise from a buret. The pH was measured with a pH meter. The beaker was then covered with aluminum foil and set aside overnight. Then, 10.0 ml. of the 5% carrier solution was added. The precipitate already present dissolved in the ammoniacal solution. The pH was readjusted to 4.9 to 5.0 with 2.6 *N* hydrochloric acid. After 3 hours, crystallization was almost complete. The mixture was set aside for 10 hours after which time the supernatant was decanted. The crystals were dissolved in 5.0 ml. of ammonia T.S., and the pH was adjusted to 4.9 to 5.0 with glacial acetic acid. The mixture was set aside to crystallize for 10 hours, then the crystals were collected with a suction flask and a sintered-glass funnel and washed with five 10-ml. portions of distilled water. The funnel and crystals were oven-dried at 120° for 2 hours; the crystals were transferred to a weighing bottle and cooled in a dessicator.

For each mixture and standard, 50.0 mg. of the carrier-diluted derivative was dissolved in 50.0 ml. of ammonia T.S. Three samples of each of these solutions, each containing 1.0 ml., were prepared and counted.

The average activities of both standards for each run were averaged. These averages were used to calculate the initial specific activity of the labeled derivative for each run. The amount of thiamine hydrochloride present in each mixture was calculated. The results are shown in Table III. The average of the four analyses was 99.7 mg. (100.1% recovery), and the standard deviation was 0.27 mg.

SUMMARY

An inverse isotope dilution method for the analysis of thiamine hydrochloride in the presence of other vitamins was studied. The procedure involved cleavage with sodium sulfite-S-35 to form an S-35 labeled derivative. The average error and precision were 0.1 and 0.27%, respectively, using 100-mg. quantities. The method should be applicable to the analysis of considerably smaller quantities of the vitamin contained in complex mixtures.

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