precipitate was purified by redissolving in concentrated ammonia and subsequent reprecipitation with glacial acetic acid. An analytical sample was prepared by vacuum sublimation.

5-Imidazolone-4-carboxamide (VIII).—Two grams of aminomalonamide (IV) and 25 ml. of ethyl orthoformate were heated for two hours at 145°. The excess ortho ester was removed under reduced pressure and the bluish-green residue was recrystallized several times from aqueous alcohol, using Darco as decolorizing agent. The grayish crystals possessed no sharp melting point but decomposed between 270 and 275°; yield 1.7 g. (78%).

Anal. Calcd. for $C_4H_6O_2N_2$: C, 37.79; H, 3.97; N, 33.07. Found: C, 37.91; H, 3.79; N, 33.09.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF FORDHAM UNIVERSITY]

The Action of Fish Tissue on Thiamin. I. The Isolation of Icthiamin[1,2,3]

BY JAMES D. BARNHURST[4] AND DOUGLAS J. HENNESSY

Thiamin is inactivated by a constituent of clam tissue. The principal pyrimidine derivative formed thereby is icthiamin of empirical formula $C_8H_{14}N_4O_3S$. A method of isolation of icthiamin as its dihydrobromide is presented, utilizing ion exchange, precipitation with silico-tungstic acid and precipitation with Ag^+. An assay method is presented for the estimation of icthiamin during the isolation.

It has been known for a number of years that the raw tissues of several species of aquatic fauna are able to inactivate thiamin. For example, Green, Carlson and Evans[5,6] have reported the occurrence of a thiamin avitaminosis in foxes fed on a diet containing 10% or more of raw carp. Woolley[7] and Sealock, *et al.*,[8] have described the thiamin-inactivating activity of raw carp *in vitro*.

More than a dozen other pertinent papers have appeared in the literature in most of which the problem of the nature of the inactivation has been attacked by studying the behavior of the active principle responsible for the inactivation.

To our knowledge, only two reports have appeared in which the problem has been attacked through a study of the structures of the products into which thiamin is converted by such inactivation. The first of these reports is that of Krampitz and Woolley[9] who isolated from carp-inactivated thiamin, 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole. The second report is that of Hennessy and Warner[10] who isolated in low yield from clam-inactivated thiamin, the crystalline dihydrochloride of a base of undetermined structure which they named "icthiamin" and to which they assigned the empirical formula $C_8H_{16}N_4O_3S\cdot 2HCl$. These latter workers showed also that one of the products of inactivation of thiamin by carp tissue extract and by smelt tissue extract is similar to icthiamin in its reaction with sodium bisulfite and with the thiazole moiety of thiamin in the presence of live yeast.

Our report describes a procedure for the routine isolation of icthiamin in quantities large enough to allow a determination of its structure.

Icthiamin is formed by allowing thiamin to react with a finely ground aqueous suspension of clam tissue. The icthiamin thus formed is then isolated by a method patterned after the procedure of Cerecedo and Hennessy[11] for the isolation of thiamin from rice polishings. The bulk of the clam tissue is removed by causing it to coagulate by *p*H adjustment and heat. The resulting solution is then passed through an ion-exchange column which takes up the icthiamin. Elution of icthiamin from the column is followed by precipitation with silicotungstic acid. After decomposition of the silicotungstate precipitate with barium hydroxide, icthiamin is further purified by fractionation with silver ion, with which it forms a sparingly soluble salt. The silver salt is then decomposed with hydrobromic acid and icthiamin is finally isolated as its dihydrobromide. The details of the isolation procedure are described in the experimental part.

In order to determine the efficiency of each step of the isolation, an assay for icthiamin was employed, the basis for which is the following[12]: (1) Icthiamin and 2-methyl-4-amino-5-hydroxymethylpyrimidine, when subjected to the conditions of the thiochrome method for the assay of thiamin, yield no fluorescent products.

(2) Icthiamin and 2-methyl-4-amino-5-hydroxymethylpyrimidine, with an equivalent or excess of the thiazole moiety of thiamin, added to live yeast in the presence of a suitable nutrient, yield thiamin quantitatively equivalent to the amount of pyrimidine derivatives used, as determined by thiochrome assay.

(3) If icthiamin is treated in aqueous solution with sodium bisulfite at *p*H 5, it yields a sulfited product which cannot be converted to thiamin by the treatment with the thiazole moiety of thiamin and yeast.

(4) 2-Methyl-4-amino-5-hydroxymethylpyrimi-

(1) This work was aided by a grant from the Williams-Waterman Fund.

(2) Presented before the division of Biological Chemistry, American Chemical Society, 117th Meeting, Philadelphia, Penna., April, 1950.

(3) This paper is based on a portion of a thesis submitted by J. D. Barnhurst to the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(4) Wallace and Tiernan Co., Inc., Belleville, N. J.

(5) R. G. Green, W. E. Carlson and C. A. Evans, *J. Nutrition*, **21**, 243 (1941).

(6) R. G. Green, W. E. Carlson and C. A. Evans, *ibid.*, **23**, 165 (1942).

(7) D. W. Woolley, *J. Biol. Chem.*, **141**, 997 (1941).

(8) R. R. Sealock, A. H. Livermore and C. A. Evans, THIS JOURNAL, **65**, 935 (1943).

(9) L. O. Krampitz and D. W. Woolley, *J. Biol. Chem.*, **152**, 9 (1944).

(10) D. J. Hennessy and S. Warner, Abstracts, 109th Meeting, American Chemical Society, Atlantic City, N. J., April, 1946.

(11) L. R. Cerecedo and D. J. Hennessy, THIS JOURNAL, **59**, 1617 (1937).

(12) In order to simplify the interpretation of the results of the assay, the assumption is made that the only thiamin-regenerable pyrimidine derivatives formed in significant amounts by the action of clam tissue on thiamin are icthiamin which is sulfite sensitive and 2-methyl-4-amino-5-hydroxymethylpyridmidine which is sulfite insensitive.

dine yields thiamin after the above thiazole–yeast treatment whether or not it is first sulfited.

Thus by treating a non-sulfited aliquot of an isolation step with the thiazole moiety of thiamin and live yeast, a quantity of thiamin is formed which can be assayed by the thiochrome method and which is a measure of both the icthiamin and the 2-methyl-4-amino-5-hydroxymethylpyrimidine present. By repeating the above treatment on a sulfited aliquot of the same isolation step, a quantity of thiamin is formed which can be assayed by the thiochrome method and which is a measure of only the 2-methyl-4-amino-5-hydroxymethylpyrimidine present. The difference between the amounts of thiamin formed from the sulfited and the non-sulfited aliquots of an isolation step is therefore the thiamin due to the icthiamin in the aliquot. Thus the amount of icthiamin in any isolation step may be measured, and the efficiency of that step determined. The details of the assay method are described in the experimental part.

TABLE I

ASSAY OF ICTHIAMIN DURING ITS ISOLATION[a]

Isolation step	Icthiamin dihydrobromide recovered grams	% [b]
Tissue filtrate	2.53[c]	100
Filtrate from ion-exchange column	0.14	6
Eluate	2.28	90
Mother liquor of silicotungstate	0.00	0
Decomposed silicotungstate	1.70	67
Mother liquor of silver salt	0.01	0
Decomposed silver salt	1.32	52
Crystalline icthiamin dihydrobromide	0.94[d]	41
	0.10	

[a] In this isolation run, 2.83 g. of thiamin was treated with clam tissue. [b] The percentages were calculated on the assumption that all the icthiamin formed is present in the tissue filtrate. [c] This represents a conversion of 74% of the thiamin to icthiamin. [d] The upper figure is the result of an assay of the first crop of icthiamin dihydrobromide: the lower figure is the result of an assay of the recrystallized residue obtained from the mother liquor of the first crop of crystals.

The results of assays of icthiamin in each step of a typical isolation run are summarized in Table I. The table shows that in the inactivation of thiamin by clam tissue, 74% of the thiamin is converted into icthiamin and that 41% of the icthiamin formed is isolated as the crystalline icthiamin dihydrobromide.

TABLE II

ELEMENTARY ANALYSES OF ICTHIAMIN DERIVATIVES

	C	H	N	S	Halogen
Icthiamin dihydrobromide, m.p. 245–248° dec.					
Calcd. for $C_8H_{14}N_4O_3S\cdot 2HBr$	23.54	3.95	13.73	7.86	39.16
Found	23.55	4.21	13.74	7.93	39.41
Icthiamin dihydrochloride, m.p. 237–240° dec.					
Calcd. for $C_8H_{14}N_4O_3S\cdot 2HCl$	30.10	5.05	17.55	10.04	22.22
Found	30.00	5.55	17.58	10.17	21.84
Icthiamin dipicrate, m.p. 176–178°.					
Calcd. for $C_8H_{14}N_4O_3S\cdot 2C_6H_3N_3O_7$	34.10	2.86	19.88	4.55	
Found	34.10	2.83	19.91	4.53	

Microanalyses were performed by Dr. F. A. Buehler, formerly of this Laboratory, and Dr. J. F. Alicino, P. O. Box 267, Metuchen, N. Y.

Melting points and elementary analyses of the dihydrobromide, dihydrochloride and dipicrate derivatives of icthiamin are shown in Table II. The elementary analyses suggest a formula $C_8H_{14}N_4O_3S$ for the free base.

Experimental

(I) **Isolation of Icthiamin (a) Inactivation of Thiamin.**—A 25% aqueous suspension of 4.7 kg. of shelled Quahog clams[13] is prepared by blending the clams with water in a Waring blender for two minutes. The suspension in an enamelware pot is then adjusted to *p*H 3 with 2 *N* hydrochloric acid. Thiamin, 2.83 g. dissolved in a small amount of water, is added to the suspension which is then well mixed and allowed to stand for 40 hours at room temperature. At the end of this time, an assay for thiamin by the thiochrome method shows a complete inactivation of the vitamin. Tenmatay[14] has shown two *p*H maxima, at 3.6 and 9.0, for the inactivation of thiamin by clam tissue. He has also shown that the sulfite sensitive inactivation product is formed in higher yield at the lower pH_a. The lower *p*H suppresses microbial growth so that inactivation at room temperature with its rate faster than at ice-box temperatures may be employed.

(b) **Removal of the Clam Tissue.**—The suspension now containing icthiamin is adjusted to *p*H 5.5 with 2 *N* sodium hydroxide. Upon heating the suspension to 85° by introduction of a current of steam, the clam tissue coagulates in a form which permits its facile removal by filtration of the hot suspension through a 50-cm. fluted filter paper. In this manner, approximately 18 liters of milky opalescent tissue filtrate are obtained.

(c) **Ion-exchange.**—After cooling to room temperature, the tissue filtrate containing icthiamin is passed through a cylindrical bed of 40–60 mesh Decalso,[15] 5 cm. in diameter and 120 cm. deep, at a rate of 15 ml. per minute.[16]

(d) **Elution of Icthiamin from the Ion-exchanger.**—After the tissue filtrate has passed through, the ion-exchanger containing icthiamin is washed thoroughly with water. The icthiamin is then eluted with 0.1 *N* hydrochloric acid, saturated with sodium chloride. The eluant is passed through the ion-exchanger at a rate of 4 ml. per minute. Approximately 7 liters of eluate are collected.

(e) **Precipitation of the Silicotungstate of Icthiamin.**—The eluate containing icthiamin is first treated with concentrated ammonium hydroxide to raise the solution to *p*H 7.5 at which point the aluminum in the eluate precipitates as its hydroxide. The aluminum is present in the eluate as a result of a partial decomposition of the ion-exchanger by the acidic eluant. The aluminum hydroxide is removed and the icthiamin in the resulting clear solution is precipitated by adding with stirring a 10% solution of silicotungstic acid in 1% sulfuric acid until no more precipitate forms on the addition of a few ml. of the precipitant. The precipitate is collected and washed with a 0.1% solution of silicotungstic acid until the washings give no test for chloride ion.

(f) **Decomposition of the Silicotungstate.**—The precipitate containing icthiamin silicotungstate is then transferred to a Waring blendor and blended for several minutes with 300 ml. of cold water to give a fine suspension of the precipitate. A cold slurry of finely recrystallized barium hydroxide is then added to the suspension in large enough excess to raise the suspension to approximately *p*H 11 after it has blended for several minutes with the barium hydroxide. The pasty mass resulting from this treatment contains insoluble barium silicotungstate, excess barium hydroxide and icthiamin in solution. The mass is centrifuged for 15 minutes and the resulting supernatant liquid is filtered through a thin layer of filter-aid to ensure complete removal of barium silicotungstate. The centrifugates are resuspended in small quantities of water, treated with

(13) The clams were purchased from the West-English Fish Co., and Teddy's, The House of Sea Food, Inc., Fulton Market, N. Y. 7, N. Y.

(14) A. L. Tenmatay, Thesis, Fordham University, 1950.

(15) Decalso is an ion-exchanger of the aluminosilicate type sold by the Permutit Company, 330 W. 42nd St., N. Y. 18, N. Y.

(16) Decalso is prepared for use by stirring it thoroughly three times with ten volumes of water and three times with ten volumes of 1% acetic acid.

additional barium hydroxide, blended for a few minutes and then recentrifuged. The supernatant liquid from the second centrifuging is filtered through filter-aid and combined with the first supernatant liquid. To these combined liquids, there is added without delay, 1 *N* sulfuric acid until *p*H 5 is reached. The barium sulfate formed here is removed, leaving a clear straw-colored solution containing icthiamin sulfate.

(g) **Precipitation of the Silver Salt of Icthiamin.**—To the solution containing icthiamin sulfate is added 10 g. of silver nitrate in 50 ml. of water, whereupon a slight precipitate forms. This is removed and concentrated ammonium hydroxide is added to the clear solution drop by drop with continual swirling. This elevation of the *p*H of the solution (to approximately *p*H 8) causes icthiamin to precipitate as its silver salt. The addition of the base is continued until no precipitate forms on the addition of a few more drops. An excess of ammonia must be avoided because this causes the salt to redissolve. The salt is collected on a buchner funnel, washed with 4 × 5 ml. of distilled water adjusted to *p*H 8 with ammonium hydroxide and partly dried by suction for ten minutes.

(h) **Decomposition of the Silver Salt.**—The moist cake of precipitate containing icthiamin silver salt is transferred to a 50-ml. centrifuge tube, made into a paste with a few ml. of water, and decomposed into silver bromide and soluble icthiamin dihydrobromide by adding with stirring 20 ml. of 5% aqueous hydrobromic acid.[17] After additional stirring to ensure complete decomposition of the organic silver salt, the silver bromide is removed by centrifuging, and the supernatant liquid evaporated to dryness *in vacuo* at room temperature in the presence of sodium hydroxide pellets and phosphorus pentoxide in separate compartments of a vacuum desiccator.

(i) **Crystallization of Icthiamin Dihydrobromide.**—The yellow-brown residue containing icthiamin dihydrobromide is then triturated with 20 ml. of absolute ethanol. The trituration removes from the residue most of the color, and the small amount of gummy material present and facilitates the collection of the crude icthiamin dihydrobromide on a small funnel. The material on the funnel is washed with 5 ml. of absolute ethanol and then recrystallized by dissolving it in twice its weight of hot water, adding absolute ethanol until the cloud-point is reached and allowing the solution to cool slowly. In this manner 950 mg. of icthiamin dihydrobromide is obtained as white needles. An additional 100 mg. of product may be obtained by evaporating to dryness the mother liquor of the first crop of crystals and recrystallizing the residue.

Icthiamin dipicrate may be obtained by dissolving icthiamin dihydrobromide or dihydrochloride in a small amount of water, adding a saturated aqueous solution of picric acid and warming the solution. On cooling, icthiamin dipicrate crystallizes as yellow needles.

II. Assay of Icthiamin (a) General Remarks.—50-ml. centrifuge tubes marked at the 10- and 25-ml. levels are used as reaction vessels. The yeast is a low-thiamin material.[18] The yeast nutrient and the sulfiting procedure followed are those described for the standard fermentation method for the assay of thiamin.[19]

(b) **Regeneration of Thiamin.**—Six reaction vessels are required for the assay of the icthiamin in an isolation step. Vessel A is a blank and contains no thiamin other than that native to the yeast used in the assay.[20]

To vessel B is added 1 ml. of a solution containing 10 μg. of thiamin.

To vessel C is added 1 ml. of a sulfited solution which before sulfiting contained 10 μg. of thiamin.

To vessel D is added 1 ml. of a solution containing 5 μg. of 2-methyl-4-amino-5-hydroxymethylpyrimidine and 1 ml. of a solution containing 5 μg. of 4-methyl-5β-hydroxyethylthiazole.[21,22]

To vessel E is added 1 ml. of a solution containing 5 μg. of 4-methyl-5β-hydroxyethylthiazole and an aliquot of an isolation step[23,24] estimated to contain a concentration of icthiamin and 2-methyl-4-amino-5-hydroxy-methylpyrimidine which will result in the synthesis of 5 to 10 μg. of thiamin.

To vessel F is added 1 ml. of a solution containing 5 μg. of 4-methyl-5β-hydroxyethylthiazole and a sulfited aliquot of the isolation step of the same dilution as the aliquot of vessel E.

To each vessel is then added 2 ml. of yeast nutrient and 2 ml. of a 5% aqueous suspension of yeast. The vessels are then diluted to the 10 ml. mark with water, mixed thoroughly and allowed to stand overnight at room temperature.

(c) **Assay of the Regenerated Thiamin.**—To each vessel is then added 1 ml. of glacial acetic acid after which they are heated in a boiling water-bath for 30 minutes. When the vessels have cooled they are each treated with 5 ml. of 4 *M* sodium acetate, diluted to the 25-ml. mark with water and thoroughly mixed. The vessels are centrifuged to settle the yeast and each one treated as follows:

Two 10-ml. samples, 1 and 2, are measured into 20-ml. centrifuge tubes. To sample 1 is added 5 ml. of 15% aqueous sodium hydroxide and to sample 2 is added 5 ml. of a solution containing 1 ml. of 1% aqueous potassium ferricyanide in 50 ml. of 15% aqueous sodium hydroxide. The two samples are then immediately centrifuged for one minute, and approximately 12 ml. of each supernatant liquid transferred to a cuvette and measured for fluorescence in a fluorophotometer.[25] The galvanometer reading of sample 2 less that of sample 1 is the vessel galvanometer deflection due to fluorescence of the thiochrome formed from the thiamin in the samples.

(d) **Determination of Icthiamin.**—The weight in grams of icthiamin, as its dihydrobromide, is calculated by use of the following equation in which the letters refer to the galvanometer deflections of the corresponding reaction vessels of the thiamin assay, part (c) above:

$$\text{grams of icthiamin dihydrobromide} = \frac{E - F}{B - C} \times 1.2 \times 10^{-5} \times \text{dilution factors}^{26}$$

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(17) Icthiamin may be isolated as its dihydrochloride by substituting hydrochloric acid at this step.

(18) The yeast was kindly supplied by Dr. C. N. Frey, Standard Brands, Inc., New York, N. Y.

(19) The Association of Vitamin Chemists, Inc., ed., "Methods of Vitamin Assay," Interscience Publishers, Inc., New York, N. Y., 1947, pp. 86, 88.

(20) The galvanometer deflection corresponding to vessel A is not used in the assay calculations; it serves as a check that the yeast is not extraordinarily rich in native thiamin.

(21) The galvanometer deflection corresponding to vessel D is not used in the assay calculations; it serves as a check of the ability of the yeast to synthesize thiamin from its pyrimidine and thiazole halves. The deflection of this vessel should approximate that of vessel B.

(22) The 4-methyl-5β-hydroxyethylthiazole was generously furnished by Merck & Co., Inc., Rahway, N. J.

(23) In order that the aliquots contain no components which will interfere with the assay, the following purifications are performed: (1) The mother liquor of the silicotungstate precipitate is treated with excess barium hydroxide to remove any excess silicotungstic acid as insoluble barium silicotungstate. Excess barium hydroxide is removed with dilute sulfuric acid.

(24) The assay of the eluate must be performed within a day after the eluate is collected, otherwise ambiguous results are obtained.

(25) A Pfaltz and Bauer fluorophotometer was used.

(26) The factor 1.2 is the ratio of the molecular weight of icthiamin dihydrobromide to that of thiamin chloride hydrochloride.