added to the filtrate, followed by ether until faint cloudiness set in. This was let stand overnight at room temperature before placing in the refrigerator for 12 hours. On filtering, 30 mg. of hygroscopic, needle-like crystals were collected, m.p. 173–175°. Melting points between 170 and 177° have been reported by different authors.[22-24] R_f values of the product were identical with hypotaurine isolated from ichthiamin and from the enzymic decarboxylation of cysteinesulfinic acid (see Table I). Oxidation with potassium permanganate gave an equivalent weight of 52.5; calculated for $C_2H_7NO_2S$, 54.5.

Chromatographic Analysis of Alkaline Cleavage Products. 1. **Identification of the Aliphatic Moiety.**—(a) The solution from the barium hydroxide cleavage of icthiamin was spotted on strips of Whatman No. 1 filter paper which had been sprayed previously with a *p*H 12 phosphate buffer[9] and dried. The chromatograms were developed in a phenol solvent buffered at *p*H 12[9] for 15 hours. After being dried thoroughly, the chromatograms were sprayed with or dipped in a 0.1% ninhydrin solution in acetone and dried in an oven for 5 minutes at 90°. A violet spot appeared at R_f 0.49. Synthetic hypotaurine had R_f 0.49. (b) A portion of the alkaline cleavage solution was treated with several drops of bromine water before spotting and developing as above. A violet spot appeared at R_f 0.29. Synthetic hypotaurine treated as above gave a spot at R_f 0.29. Taurine control had R_f 0.29. (c) Several drops of the alkaline cleavage solution were let stand overnight with 1 mg. of sodium bisulfite before spotting and developing. A violet spot appeared at R_f 0.29. Synthetic hypotaurine treated in this way also gave a spot at R_f 0.29. (d) The solution from the alkaline cleavage reaction was let stand overnight with 1 mg. of sodium bisulfite with 0.1% hydroquinone added. On spotting and developing, a single spot appeared at R_f 0.49.

2. **Identification of the Pyrimidine Moiety.**—The solution from the alkaline cleavage was spotted on strips of unbuffered Whatman No. 1 filter paper and developed in four different alcoholic solvent systems (Table II) until the solvent front had travelled 20–24 cm. The dried chromatograms were scanned before an ultraviolet lamp[12] to locate the pyrimidine compounds which appeared as purplish spots. The results are given in Table II.

Chromatographic Analysis of the Aliphatic Fragment from the Bisulfite Cleavage of Icthiamin.—(a) The solution from the bisulfite cleavage reaction was spotted on strips of Whatman No 1 filter paper previously buffered at *p*H 12, and developed in a buffered phenol solvent as before. Treatment with 0.1% ninhydrin in acetone revealed a single spot with R_f 0.29. Taurine control had R_f 0.29.

(b) The solution from the bisulfite cleavage reaction protected by hydroquinone or ethanol was spotted on buffered Whatman No. 1 filter paper and developed in a buffered phenol solvent as before. Ninhydrin treatment showed a heavy spot with R_f 0.49 and a faint spot at R_f 0.29. The hypotaurine control had R_f 0.49.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, FORDHAM UNIVERSITY]

The Action of Fish Tissue on Thiamin. IV.[1] The Synthesis of Icthiamin[2-4]

BY EDWARD E. KUPSTAS AND DOUGLAS J. HENNESSY

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Icthiamin was synthesized by condensing 4-amino-5-bromomethyl-2-methylpyrimidine hydrobromide with 2-phthalimidoethanesulfinic acid, followed by hydrazinolysis of the phthaloyl group. The identity of synthetic phthalylicthiamin and icthiamin dihydrobromide with the corresponding compounds prepared from natural icthiamin was shown by chromatographic, infrared and elementary analyses.

When the structure of icthiamin was proposed as 4-amino-5-(2-aminoethanesulfonyl)-methyl-2-methylpyrimidine,[1c] two routes to its synthesis seemed practical. These were *via* the formation of the corresponding sulfide which could be oxidized to the sulfone, or by the direct formation of the sulfone. The first method had been attempted during the early study of icthiamin by Barnhurst[5] but oxidation of the intermediate 5-(4-amino-2-methylpyrimidyl)-methyl β-phthalimidoethyl sulfide according to the method of Pomerantz and Conner[6] failed to produce phthalylicthiamin.

The formation of sulfones by the reaction of sulfinates with aliphatic halides[7] formed the basis for the successful synthesis.

Zinc 2-phthalimidoethanesulfinate was obtained by reduction of 2-phthalimidoethanesulfonyl chloride with zinc dust in absolute methanol. However, the purification of the zinc or sodium salts of II proved difficult. Reaction of the impure sulfinates with I failed to yield more than traces of phthalylicthiamin (III). The free sulfinic acid II reacts with 4-amino-5-bromomethyl-2-methylpyrimidine hydrobromide (I) in glacial acetic acid in the presence of anhydrous sodium acetate to give phthalylicthiamin hydrobromide in good yield. Hydroquinone was used in the preparations and reactions of the sulfinates to minimize conversion to sulfonates.

Gabriel and Colman prepared the 2-phthalimidoethanesulfinic acid (II) by two different methods.[8] A simpler procedure which gives a pure product in good yield is described in the Experimental section.

During the synthesis, III was found difficult to prepare analytically pure even with repeated recrystallizations. The extremely insoluble free base

(1) Papers I, II and III (a) J. D. Barnhurst and D. J. Hennessy, THIS JOURNAL, **74**, 353 (1952); (b) **74**, 356 (1952); (c) E. E. Kupstas and D. J. Hennessy, **79**, 5217 (1957).

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

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

(4) Presented at the Meeting-in-Miniature, American Chemical Society, New York Section, March 16, 1956, and the Division of Biological Chemistry, American Chemical Society, 130th Meeting, Atlantic City, New Jersey, September, 1956.

(5) J. D. Barnhurst, Thesis, Fordham University, 1951.

(6) A. Pomerantz and R. Conner, THIS JOURNAL, **61**, 3386 (1939).

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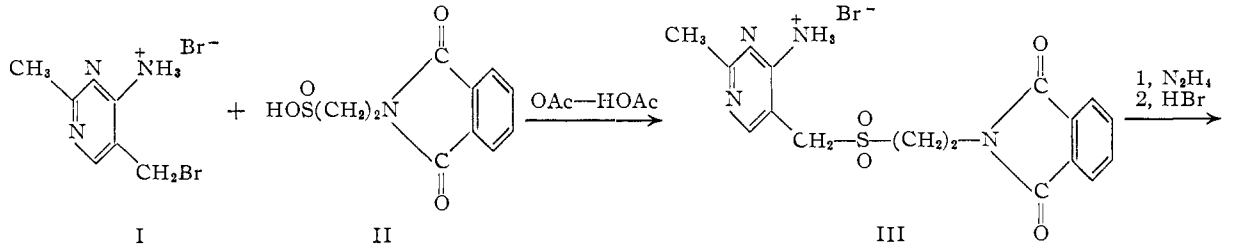

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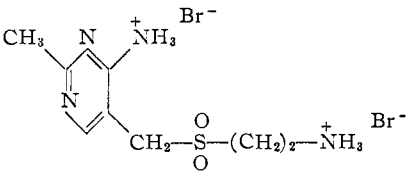

IV

of phthalylicthiamin was formed and freed of the more soluble contaminants by thorough washing. The free base was then readily reconverted to the pure hydrobromide.

Phthalylicthiamin was resistant to the removal of the phthaloyl group by refluxing with concentrated hydrobromic acid or with hydrazine hydrate in ethanol. Elevation of the temperature by substituting isobutyl alcohol for ethanol in the latter procedure resulted in a good yield of icthiamin.

For comparison, phthalylicthiamin was prepared from natural icthiamin by reaction with phthalic anhydride. This was found to be identical with synthetic phthalylicthiamin (III) in every respect, *viz.*, by the melting points of several derivatives as shown in Table I, chromatographic analysis, shown in Table II, and by the infrared spectra.

TABLE I

MELTING POINTS OF PHTHALYLICTHIAMIN AND DERIVATIVES, °C.

Phthalylicthiamin	From clam icth.	Synthetic	Mixed
Phthalylicthiamin	293–294 dec.	293–295 dec.	293–294 dec.
Hydrobromide	286 dec.	288 dec.	286 dec.
Picrate	238–239	237–239	236–238
Picrolonate	253–255 dec.	255–257 dec.	253–256 dec.

Phthalylicthiamin, exposed on a chromatogram to ultraviolet light in the region of 254 mμ, displayed a characteristic pale blue fluorescence. This was conveniently used to determine phthalylicthiamin in reaction product mixtures.

Synthetic icthiamin dihydrobromide was accompanied by small amounts of contaminating material even when recrystallized repeatedly from water–ethanol solvent. Final purification was carried out by conversion to the dipicrate then to the dihydrochloride which analyzed well after recrystallizing twice from water–ethanol. Identity with natural icthiamin was established by superimposability of infrared spectra and similarity of R_f values in chromatography with different solvent systems (Table II).

TABLE II

R_f VALUES OF ICTHIAMIN AND PHTHALYLICTHIAMIN

	Solvent		
	Phenol buffered at *p*H 12	*i*-PrOH:1% acetic acid[a]	EtOH: H_2O[a] (4:1)
Natural phthalylicthiamin·HBr	..	0.35	0.59
Synthetic phthalylicthiamin·HBr	..	.35	.58
Natural icthiamin·2HBr	0.83[b]	..	.41
Synthetic icthiamin·2HBr	.84[b]	..	.42

[a] The spots were located by means of fluorescence excited by ultraviolet light at 254 mμ. ("Mineralight," Will Corporation, New York, N. Y., kindly loaned to us by Dr. L. R. Cerecedo.) [b] The paper had been previously buffered at *p*H 12.[9] The chromatograms were sprayed with a solution of 0.1% ninhydrin in acetone and dried at 90°.

Two typical sulfone infrared absorption bands[10–13] are shown at 7.71 and 8.69 μ and 7.71 and 8.62 μ by phthalylicthiamin (III) and icthiamin (IV), respectively.

We wish to express our appreciation to Sister Marguerite Miriam Casco and Miss Lilia Beauchamp for the infrared analyses. The elementary analyses were done by Mr. Joseph F. Alicino, Metuchen, New Jersey, and by Drs. G. Weiler and F. B. Strauss, Microanalytical Laboratory, Oxford, England.

Experimental

2-Phthalimidoethanesulfinic Acid.—2-Phthalimidoethanesulfonyl chloride[14] (5.46 g., 0.02 mole) in 100 ml. of absolute methanol was reduced at 65° in 5 minutes by an excess of zinc dust (5.5 g.) with 100 mg. of hydroquinone present as an antioxidant. After cooling in an ice-bath, the solid material was collected on a suction filter and washed with several portions of ether. The mixed sulfinate and excess zinc were leached with 80 ml. of boiling water which contained a few milligrams of hydroquinone, and the filtrate was mixed with 10 ml. of cold 6 *N* hydrochloric acid. With cooling, a flaky precipitate formed. This was collected and washed with several small portions of ice-water before drying *in vacuo*. The yield of 2-phthalimidoethanesulfinic acid was 2.88 g. (60%), m.p. 144–146°.[15]

Anal. Calcd. for $C_{10}H_9NO_4S$: C, 50.30; H, 3.79; N, 5.85; S, 13.40; neut. equiv., 239. Found: C, 50.24; H, 3.93; N, 5.45; S, 12.80; neut. equiv., 236.

Phthalylicthiamin Hydrobromide.—2-Phthalimidoethanesulfinic acid (2.8 g., 0.0117 mole), 4-amino-5-bromomethyl-2-methylpyrimidine hydrobromide[16] (3.2 g., 0.0113 mole), anhydrous sodium acetate (0.94 g., 0.0115 mole) and hydroquinone (0.1 g.) were refluxed for two hours in 25 ml. of glacial acetic acid. At the end of this time, 1 ml. of 48%

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(15) Gabriel and Colman report a melting point of 149–149.5°. Successive recrystallizations failed to elevate our melting point.

(16) J. K. Cline, R. P. Williams and J. Finkelstein, THIS JOURNAL **59**, 1052 (1937).

hydrobromic acid was added to ensure complete conversion to the hydrobromide salt before cooling the mixture to give a fine precipitate of phthalylicthiamin hydrobromide. This was collected and recrystallized from glacial acetic acid using Norite to give 3.0 g. of white product. The filtrate from the original reaction mixture was reduced to dryness under vacuum. The residue was dissolved in 10 ml. of warm water, then sodium bicarbonate added until *p*H 7 was reached. The insoluble free base of phthalylicthiamin was collected on a filter. This was stirred into a slurry with a slight excess of concentrated hydrobromic acid and recrystallized from 5 ml. of glacial acetic acid to afford an additional 0.17 g. of phthalylicthiamin hydrobromide. The total yield was 3.17 g. (64%); m.p. 286–288° dec.

Anal. Calcd. for $C_{16}H_{16}N_4O_4S \cdot HBr$: C, 43.55; H, 3.88; N, 12.70; S, 7.26; Br, 18.10. Found: C, 43.30; H, 3.60; N, 12.20; S, 6.94; Br, 17.35.

Phthalylicthiamin.—Phthalylicthiamin hydrobromide was transformed almost quantitatively into the free base by dissolving the hydrobromide in warm water and adding sodium bicarbonate in small portions until *p*H 7 was reached. The insoluble precipitate was collected, suspended in ice-water and filtered. With drying *in vacuo* a fine white powder of phthalylicthiamin was obtained; m.p. 293–295° dec.

Anal. Calcd. for $C_{16}H_{16}N_4O_4S$: C, 52.80; H, 4.40; N, 15.45; S, 8.82. Found: C, 52.10; H, 4.18; N, 15.20; S, 8.40.

Phthalylicthiamin from Natural Icthiamin.—Icthiamin dihydrobromide (82 mg., 0.002 mole) was refluxed in 3 ml. of glacial acetic acid with phthalic anhydride (30 mg., 0.002 mole) and 49 mg. of anhydrous sodium acetate for 2 hours. On cooling a fine precipitate settled out. This was collected and recrystallized from boiling glacial acetic acid using Norite to yield 65.7 mg. (74.5%) of phthalylicthiamin hydrobromide, m.p. 286° dec.

Anal. Calcd. for $C_{16}H_{16}N_4O_4S \cdot HBr$: C, 43.55; H, 3.88; N, 12.70. Found: C, 43.60; H, 3.62; N, 12.70.

The free base was prepared exactly as in the case of synthetic phthalylicthiamin, m.p. 293–294° dec.

Anal. Calcd. for $C_{16}H_{16}N_4O_4S$: C, 52.80; H, 4.40; N, 15.45; S, 8.82. Found: C, 52.50; H, 4.45; N, 15.40; S, 8.63.

Icthiamin Dihydrochloride.—Phthalylicthiamin (2.0 g., 0.0055 mole) was refluxed with 660 mg. of 85% hydrazine hydrate (0.011 mole) in 60 ml. of isobutyl alcohol for 1.5 hours. After cooling, just sufficient bromine was added to destroy the excess hydrazine, as indicated by a permanent faint yellow color. The cold mixture was filtered to remove the precipitated phthalhydrazide and the filtrate was taken to dryness under reduced pressure. The residue was dissolved in 2 ml. of warm water containing a small amount of hydrobromic acid and filtered. To the filtrate, hot ethyl alcohol was added until the cloud point was reached. After cooling slowly and permitting the precipitation to begin, a small portion of ether was added before letting the mixture stand overnight. The fine white needles which were collected weighed 1.32 g. Upon adding ether to the filtrate, 0.21 g. more of icthiamin dihydrobromide was obtained.

The total yield of crude icthiamin dihydrobromide was 1.51 g. (67.5%).

The dipicrate was formed by warming the dihydrobromide in a small quantity of water with a few milliliters of saturated aqueous picric acid solution. Needles of icthiamin dipicrate formed on cooling; m.p. 175–177°. Authentic icthiamin dipicrate melts at 176–178°.[1a]

Anal. Calcd. for $C_8H_{14}N_4O_2S \cdot 2C_6H_3N_3O_7 \cdot H_2O$: C, 33.99; H, 3.13; N, 19.82. Found: C, 34.54; H, 3.53; N, 19.36.

To form the dihydrochloride, the dipicrate was suspended in ether and dry hydrogen chloride passed in with stirring until no yellow solid material was present. The resulting suspension was filtered and washed thoroughly with ether before recrystallizing twice by dissolving in a few drops of hot water and adding hot ethanol, m.p. 235–238° dec. Authentic icthiamin dihydrochloride melts at 237–240° dec.

Anal. Calcd. for $C_8H_{14}N_4O_2S \cdot 2HCl \cdot H_2O$: C, 29.91; H, 5.64; N, 17.44; Cl, 22.08. Found: C, 30.16; H, 5.39; N, 17.78; Cl, 22.00.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, FORDHAM UNIVERSITY]

The Action of Fish Tissue on Thiamin. V.[1] Studies on the Biosynthesis of Icthiamin[2–4]

BY EDWARD E. KUPSTAS AND DOUGLAS J. HENNESSY

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Four possible naturally occurring precursors of the characteristic aliphatic side chain of icthiamin, *viz.*, cysteine, 2-aminoethyl mercaptan, cysteinesulfinic acid, and hypotaurine, were shown to inactivate thiamin in the presence of dialyzed clam extract. Chromatographic analysis of the resulting products and of the products of their reaction with bisulfite showed that only hypotaurine reacted to form icthiamin in significant amounts under these conditions.

The isolation, proof of structure and synthesis of icthiamin, 4-amino-2-methyl-5-(2-aminoethanesulfonyl)-methylpyrimidine, a product of the action of clam tissue on thiamin[1a] affords the basis for the study of the mechanism of the inactivation of this vitamin by the clam. Experimental data reported herein indicate the nature of the compound in clam tissue which displaces the thiazole moiety of thiamin.

It became apparent in the early studies of icthiamin[5,6] that the sulfur-containing moiety had been contributed to its structure by the clam tissue since the thiazole moiety of thiamin could be recovered quantitatively following the inactivation of this vitamin.

The *in vitro* synthesis of icthiamin from 4-amino-5-bromomethyl-2-methylpyrimidine hydrobromide and a hypotaurine derivative, *viz.*, 2-phthalimidoethanesulfinic acid, suggests that icthiamin might be similarly formed by the clam, *i.e.*, *via* an attack by a sulfinic acid on the electron-deficient methylene bridge carbon of thiamin, activated somehow by clam thiaminase.

(1) Papers I, II, III and IV of this series: (a) J. D. Barnhurst and D. J. Hennessy, THIS JOURNAL, **74**, 353 (1952); (b) J. D. Barnhurst and D. J. Hennessy, *ibid.*, **74**, 356 (1952); (c) E. E. Kupstas and D. J. Hennessy, *ibid.*, **79**, 5217 (1957); **79**, 5220 (1957).

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

(4) Presented at the Meeting-in-Miniature, American Chemical Society, New York Section, March 16, 1956, and the Division of Biological Chemistry, American Chemical Society, 130th Meeting, Atlantic City, New Jersey, September, 1956.

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(6) J. D. Barnhurst, Thesis, Fordham University, 1951.