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

RECEIVED FEBRUARY 27, 1957

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).

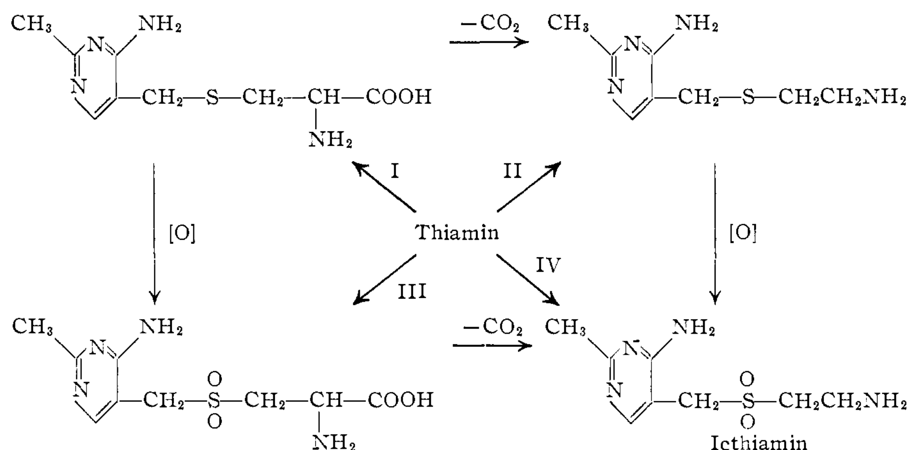
(2) This work was aided by a grant from the Williams-Waterman Fund.

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

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



Alternately, a similar attack on thiamin by the proper sulfhydryl compound, followed by an oxidation to the sulfone to yield icthiamin may be possible enzymatically, even though such a synthesis was unsuccessful *in vitro*.[6]

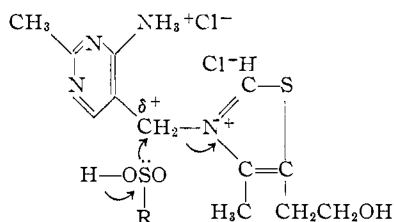


Fig. 1.

The experiments reported herein were undertaken to learn if icthiamin could be formed by exhaustively dialyzed clam tissue extract as a source of thiaminase acting on thiamin in the presence of compounds which could be considered as reasonable precursors of the aliphatic side chain of icthiamin. The dialyzed extract was shown to have no significant destructive effect on thiamin nor did the compounds tested in the presence of heated clam extract have any effect. Hence, inactivation of the thiamin could be attributed to a reaction of the added compound catalyzed by clam thiaminase.

Cysteine (I) appeared to be a possible source of the 2-aminoethanesulfonyl side chain of icthiamin for the following reasons: (a) discounting the carboxylic function, cysteine is composed of the same number of carbon, nitrogen and sulfur atoms as the side chain of icthiamin, (b) cystine, as the source of cysteine, constitutes a fair percentage of many marine fauna proteins[7-9] and (c) cysteine is known to undergo stepwise enzymic oxidation of the sulfhydryl group and decarboxylation,[10] modifications which would be necessary at some stage if icthiamin were to be formed.

Three other compounds were considered. These were 2-aminoethyl mercaptan (II), cysteinesulfinic acid (III) and 2-aminoethanesulfinic acid (hypotaurine) (IV). Cysteinesulfinic acid and 2-aminoethyl mercaptan could by a displacement of the thiazole and subsequent decarboxylation or oxidation, respectively, act as sources of the 2-aminoethanesulfonyl chain of icthiamin. Hypotaurine could form icthiamin directly by displacing the thiazole moiety of thiamin.

An aqueous extract of clam tissue suspension was prepared and dialyzed until its thiamin destroying power was negligible as described in the Experimental section. The dialyzed extract was incubated with thiamin in the presence of each of the compounds: cysteine (I), 2-aminoethyl mercaptan (II),[11] cysteinesulfinic acid (III), prepared according to the method of Schubert,[12] and hypotaurine (IV), obtained by a simultaneous reduction and hydrazinolysis of 2-phthalimidoethanesulfonhydrazide.[1c]

During all the operations, the acidity of the clam suspension and extract was maintained as close to *p*H 3.6 as possible. Tenmatay[5] has shown this to be one of the two *p*H maxima for thiamin inactivation by clam. The destruction of thiamin was followed by a thiochrome assay procedure.[5] The results of a typical run are given in Table I. All four of the compounds tested brought about significant destruction of thiamin in the presence of dialyzed clam tissue extract.

TABLE I

RELATIVE AMOUNTS OF THIAMIN REMAINING AFTER 24 HOURS INCUBATION

Mixture incubated with thiamin[a]	Galvanometer deflection
Standard[b]	50
Dialyzed clam extract, control	48
+ I	3
+ II	10
+ III	3
+ IV	13
Undialyzed clam extract, control	7

[a] One mg. thiamin/10 ml. solution. [b] Solution of thiamin containing 1 mg./10 ml. water.

Following a 24-hour incubation at room temperature, the above samples were treated according to the procedure reported by Barnhurst and Hennessy[1a] for the isolation of icthiamin. The concen-

(7) Y. Okuda, S. Uematsu, K. Sakata and K. Fujikawa, *J. Coll. Agr. Tokyo Imp. Univ.*, **7**, 39 (1919); *C. A.*, **14**, 2827 (1920).

(8) R. J. Block, "Amino Acid Handbook," Charles C. Thomas, Springfield, Ill., 1956, p. 270–271.

(9) J. M. R. Beveridge, *J. Fisheries Research Board Can.*, **7**, 51 (1947).

(10) J. Awapara and W. J. Wingo, *J. Biol. Chem.*, **203**, 189 (1953).

(11) Kindly furnished in the form of the hydrochloride salt by Evans Chemetics, Inc., 250 E. 43rd St., New York 17, New York.

(12) M. P. Schubert, THIS JOURNAL, **55**, 3336 (1933).

trate obtained in this way from each sample was then analyzed by paper chromatography with different solvent systems.

Two additional materials were run as controls. One was pure icthiamin; the other was the concentrate from the sample of undialyzed clam extract containing thiamin treated in the same fashion as the other samples. This yielded icthiamin along with any impurities to be found in the concentrates from the samples containing I, II, III and IV. The results are given in Table II.

TABLE II

CHROMATOGRAPHIC EVIDENCE FOR ICTHIAMIN

Compd. added to dialyzed clam extract and thiamin	Solvents[a,b] A	B	C	D	E
Cysteine (I)	+	−	−	0	−
2-Aminoethyl mercaptan (II)	0	+	0	0	0
Cysteinesulfinic acid (III)	+	−	−	+	0
Hypotaurine (IV)	+	+	+	+	+
Controls					
Icthiamin	+	+	+	+	+
Undialyzed clam extract and thiamin	+	+	+	+	+

[a] Solvents: A, isopropyl alcohol–water–acetic acid (7:2:1); B, phenol–water (4:1); C, phenol buffered at pH 12[13]; D, ethanol–water (4:1); E, ethanol–water (4:1), spotted on paper buffered previously at pH 12.[13] [b] +, positive; 0, doubtful; −, negative.

As shown in the above table, the evidence for icthiamin is very pronounced in the case of hypotaurine, diminishing with cysteine sulfinic acid and cysteine in that order. The spots from the 2-aminoethyl mercaptan reaction had R_f values which were just sufficiently different from those of icthiamin to consider them extremely doubtful rather than negative. Icthiamin, like thiamin,[14] undergoes a distinctive cleavage in the presence of sulfite ions. Upon such a cleavage, icthiamin was first shown to yield a pyrimidinesulfonic acid[1b] and taurine.[5] However, in the presence of an antioxidant, such as hydroquinone, hypotaurine instead of taurine is obtained.[1c] The characteristic R_f values of taurine and hypotaurine suggested using this sulfite cleavage to confirm or disprove the indications of icthiamin in Table II.

Each of the five concentrates carried through the icthiamin isolation steps was divided into two parts. One portion was treated with aqueous sodium bisulfite; the second portion was treated identically except that hydroquinone was added. After 12 hours the solutions were analyzed by paper chromatography, giving the results shown in Table III. Samples of authentic taurine and hypotaurine were used for reference.

From these data it seems clear that in the presence of thiamin and dialyzed clam extract, only hypotaurine of the four compounds tested reacted to form icthiamin. The lower R_f values of the taurine spot from the samples compared to the control may be attributed to the "dragging" in the presence of salts and other polar compounds in the sulfite treated material.

Whether undialyzed clam homogenate could utilize cysteine, cysteinesulfinic acid, or 2-aminoethyl mercaptan to produce icthiamin cannot be said at this time. In dialysis, certain ions or coenzymes may be removed. These could be necessary for the formation of icthiamin from the compounds other than hypotaurine. Hope,[15] for example, has established that pyridoxal phosphate is the coenzyme of a decarboxylase for L-cysteinesulfinic acid.

TABLE III

RESULTS OF BISULFITE CLEAVAGE

Source of product treated with bisulfite	Hydroquinone Present	Absent
Dial. extract–thiamin–cysteine	No spot	No spot
Dial. extract–thiamin–2-aminoethyl mercaptan	No spot	No spot
Dial. extract–thiamin–cysteine sulfinic acid	No spot	No spot
Dial. extract–thiamin–hypotaurine	0.50	0.16
Undial. extract–thiamin	0.51	0.17
Controls		
Taurine	..	0.20
Hypotaurine	..	0.51

The presence of taurine in clam tissue,[16–20] perhaps as an artifact resulting from oxidation of hypotaurine, does seem to us to suggest that hypotaurine is at least in part responsible for the enzymatic conversion of thiamin to icthiamin by clam tissue.

Experimental

Preparation of Dialyzed Clam Extract.—A suspension of clam tissue was prepared by homogenizing 325 g. of freshly opened Quahog clams with 1 liter of cold distilled water in a Waring blendor. The suspension was adjusted to pH 3.6 with 2 N hydrochloric acid and let stand 4 hours in an ice-bath. At the end of this time the suspension was centrifuged for 15 minutes to give a milky centrifugate. This was filtered through glass-wool to remove floating particles. A portion of this undialyzed extract was reserved as a control. The remainder of the extract was dialyzed in 1 in. cellophane tubing for 18–20 hours at 5° against distilled water running at the rate of approximately 1 liter per hour. After dialysis, the pH of the non-dialyzable portion was again adjusted to 3.6.

Inactivation of Thiamin.—In each of seven flasks was placed 2 ml. of solution containing 10 mg. of thiamin. The individual incubation solutions were then made up as

(1) 100 ml. of water (standard)
(2) 100 ml. of undialyzed clam extract (control)
(3) 100 ml. of dialyzed clam extract (control)
(4) 100 ml. of dialyzed clam extract + 30 mg. of cysteine hydrochloride
(5) 100 ml. of dialyzed clam extract +30 mg. of 2-aminoethyl mercaptan hydrochloride
(6) 100 ml. of dialyzed clam extract +30 mg. of cysteinesulfinic acid (a)
(7) 100 ml. of dialyzed clam extract +30 mg. of hypotaurine
(a) Added in the form of a solution after decomposing the barium salt with dilute sulfuric acid.

Samples 8–13 were identical with 2–7 except that the clam extract was heated at 100° for 10 minutes before combining with the other ingredients. No significant destruction of thiamin occurred in these samples.

(13) E. F. McFarren, *Anal. Chem.*, **23**, 168 (1951).

(14) R. R. Williams, R. E. Waterman, J. C. Keresztesy and E. R. Buchman, THIS JOURNAL, **57**, 536 (1935).

(15) D. B. Hope, *Biochem. J.*, **59**, 497 (1955).

(16) M. Henze, *Z. physiol. Chem.*, **43**, 477 (1904–1905).

(17) B. C. P. Jansen, *ibid.*, **85**, 231 (1913).

(18) U. Suzuki, K. Yoshimura and Y. Tanaka, *J. Coll. Agr. Tokyo Imp. Univ.*, **5**, 1 (1912); *Chem. Zentr.*, **84**, *I*, 1043 (1913).

(19) K. Sugino, K. Oodo, T. Sekine, E. Ichikawa and K. Watanabe, *J. Chem. Soc. Japan*, **72**, 252 (1951); *C. A.*, **46**, 658f (1952).

(20) Y. Okuda and K. Sanada, *J. Coll. Agr. Imp. Univ. Tokyo*, **7**, 77 (1919); *C. A.*, **14**, 2806 (1920).

The flasks were left standing 20–24 hours at room temperature before assaying the contents for the thiamin remaining.

Assay of Thiamin.—Two 0.2-ml. samples from each flask were measured into 15-ml. centrifuge tubes. To each tube was added 8 ml. of water. To the first tube was added 5 ml. of 15% aqueous sodium hydroxide and to the second was added 5 ml. of a solution containing 1 ml. of 1% aqueous potassium ferricyanide in 50 ml. of 15% aqueous sodium hydroxide. The samples were centrifuged immediately for one minute and poured into cuvettes. The fluorescence of each sample was then quickly measured in a fluorophotometer.[21] The galvanometer deflection of the second sample minus the deflection of the first indicated the relative amount of thiamin present.

Detection of Icthiamin.—(A) The solution from each flask containing clam extract was adjusted to *p*H 5.5 with 2 *N* sodium hydroxide and heated to 90° on the steam-bath to coagulate the proteinaceous material which was then filtered off. An ion-exchange column for each sample was prepared by adding 2–3 g. of Decalso[18] to a 0.7 × 15 cm. glass tube, having at one end a 0.03 cm. capillary and a 50-ml. reservoir at the other.[22] A tiny piece of gauze was placed at the bottom of the tube to prevent the Decalso from blocking the entrance to the capillary. The column was wetted by drawing through a few milliliters of 3% acetic acid before passing through the filtered incubation mixtures.

The procedure followed from this point was that given by Barnhurst and Hennessy[18] for the isolation of icthiamin, *viz.*, washing the column, eluting the adsorbed material with 15 ml. of saturated sodium chloride solution in 0.1 *N* hydrochloric acid, etc. The precipitations with silicotungstic acid and silver nitrate, as well as the washings, were carried out in 15-ml. centrifuge tubes to facilitate handling.

After decomposition of the silver salts with dilute hydrobromic acid, the solutions were centrifuged and filtered to ensure complete removal of the silver bromide. The filtrates were then reduced to dryness *in vacuo*. The residues obtained were not recrystallized but were dissolved in a few drops of water for chromatographic analysis. For each chromatogram, several microliters of each solution were spotted, as well as a sample containing authentic icthiamin, on a strip of Whatman #1 filter paper. Where buffered paper was used, the strips were previously dipped in *p*H 12 phosphate buffer[13] and dried before spotting. The solvent systems employed were those given in Table II.

At the end of the run, the chromatograms were removed from the solvents and dried. The pyrimidine compounds were located on the chromatograms run in the non-phenolic solvents by scanning the strips in a darkened room before a suitable source of ultraviolet light.[23] The chromatograms run in the phenol solvents were dipped in a trough containing a 0.1% solution of ninhydrin in dry acetone and dried at 90° for 5 minutes. Icthiamin gives a characteristic light brown spot under these conditions.

Sulfite Cleavage Reaction.—The solution from each sample remaining after spotting the above chromatograms was divided into two equal parts of approximately 2 drops each in 3 in. test-tubes. First a small crystal of hydroquinone was added to one part of each sample. Then approximately 5 mg. of sodium bisulfite was added to both parts of each sample, adjusting the *p*H between 5 and 6. The test-tubes were stoppered and let stand at room temperature. After 12 hours, the solutions were spotted on strips of buffered filter paper and hung in a buffered phenol solvent as above. At the end of the run, the chromatograms were dried and treated with ninhydrin as before. Taurine and hypotaurine were located as pinkish violet spots with R_f values of 0.18 ± 0.02 and 0.50 ± 0.02, respectively.

(21) A Pfaltz and Bauer fluorophotometer was used.

(22) Sold by E. Machlett and Son, New York, N. Y.

(23) The source of ultraviolet light was a "Mineralight" lamp with a transmittance peak at 254 mμ. (Will Corporation, New York, N. Y.) kindly loaned to us by Dr. L. R. Cerecedo.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

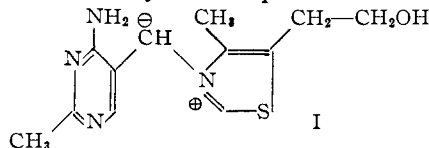
The Thiamin–Pyruvate Reaction

BY K. FRY, L. L. INGRAHAM AND F. H. WESTHEIMER

RECEIVED MAY 29, 1957

The experimental proof is given that the methylene group between the pyrimidine and thiazole rings in thiamin does not ionize during the thiamin-catalyzed decarboxylation of pyruvate.

Breslow[1] has demonstrated recently that the hydrogen atom in the 2-position of thiazolium salts ionizes, and therefore readily exchanges in neutral solution and at room temperature with the hydrogen atoms of water. We have confirmed his remarkable observation. In a prior publication, he had suggested[2] that the hydrogen atoms of the methylene bridge of thiamin could ionize to form the ylid, I. This ylid was postulated to explain



the role of thiamin in the catalyzing of the non-enzymatic decarboxylation of pyruvate,[3] and therefore, by implication, to explain the mechanism of the action of the coenzyme, thiamine pyrophosphate in the decarboxylation of pyruvate catalyzed by the enzyme, carboxylase. Concurrently and independently, Ingraham and Westheimer[4] attempted to test the hypothesis that the ylid, I, is formed in alkaline solution; they carried out the decarboxylation of thiamin in D_2O, and tested for hydrogen–deuterium exchange in the thiamin recovered from the reaction mixture, and particularly in the sulfonic acid, II, obtained from thiamin by cleavage with bisulfite[5] according to eq. 1.

They found about 0.1 atom of deuterium per molecule in the recovered thiamin, but almost none in the sulfonic acid, II. They stated that control experiments demonstrated that deuterium had not been introduced into the thiamin and subsequently lost in the work-up of this compound or of the sulfonic acid, II, and concluded that the ylid, I, is not an intermediate in the thiamin-catalyzed decarboxylation of pyruvate.

(1) R. Breslow, THIS JOURNAL, **79**, 1762 (1957).

(2) R. Breslow, *Chemistry & Industry*, R 28 (1956); *cf.*, K. Wiesner and Z. Valenta, *Experientia*, **12**, 190 (1956).

(3) S. Mizuhara and P. Handler, THIS JOURNAL, **76**, 571 (1954).

(4) L. Ingraham and F. H. Westheimer, *Chemistry & Industry*, 846 (1956).

(5) R. R. Williams, R. E. Waterman, J. C. Keresztesy and E. R. Buchman, THIS JOURNAL, **57**, 536 (1935).