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.

NEW YORK 58, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

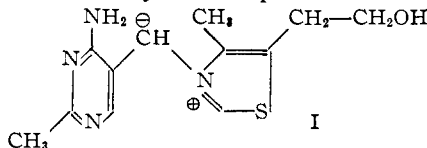
The Thiamin–Pyruvate Reaction

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.

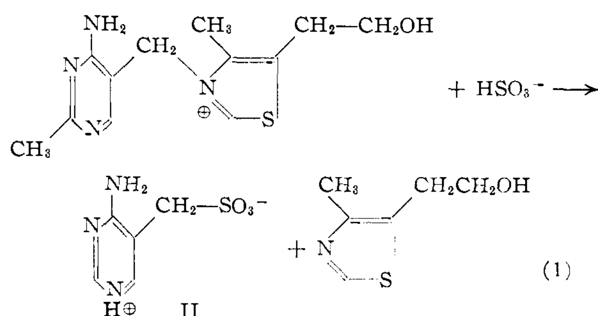
(1) R. Breslow, THIS JOURNAL, **79**, 1762 (1957).

(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).



This conclusion is correct, and has proved an important step in establishing the chemistry of thiamin. The control experiments were in fact adequate to establish the lack of exchange at the methylene group of thiamin, and therefore to eliminate the ylid, I, from consideration. However, the control experiments were not rigid with respect to exchange elsewhere in thiamin, and the statement that "the recovery and crystallization procedure did not wash out deuterium from . . . thiamin . . ." should have been restricted to exchange at the methylene bridge. The control experiments in the sulfonic acid have been repeated and extended and have unambiguously confirmed the original conclusion that there is no exchange at the methylene group of thiamin; the detailed results of these and the earlier experiments were transmitted informally last year to several investigators in the field. The purpose of this article is to present the experimental evidence in detail.

Fundamentally, the best experiments were conducted as follows. The decarboxylation of pyruvate with thiamin was carried out at a *p*H of approximately 9 in D_2O under experimental conditions where the yield of CO_2, based on thiamin, was about 60%. The thiamin was then cleaved by adding $NaDSO_3$ directly to the decarboxylation mixture. The sulfonic acid, II, which crystallized from solution, was recrystallized from D_2O and converted to the sodium salt with $NaDCO_3$ in D_2O. The sodium salt of the sulfonic acid was twice dissolved in water and the water removed by vacuum evaporation; this process removed the exchangeable hydrogen atoms at the amino group of the sodium salt of II. The sodium salt was then analyzed for deuterium; very little was found. The compound had not been in contact with H_2O, but only with D_2O, up to the time the sodium salt of the sulfonic acid was dissolved in cold water. If any deuterium had been introduced into the methylene bridge (*e.g.*, *via* the ylid, I), it could not be lost except during the last step. Such loss is theoretically improbable; it was ruled out experimentally by nuclear magnetic resonance experiments. Measurements of the nuclear magnetic resonance spectrum of the sodium salt of the sulfonic acid in D_2O showed that it is stable in this solution, and does not exchange its hydrogen atoms, from the methylene group or elsewhere, under the experimental conditions of the final washing process. It follows that the methylene group of thiamin does not ionize under the experimental conditions where thiamin catalyzes the decarboxylation of pyruvate, and that the ylid, I, is not an intermediate in the reaction.

Experimental

Decarboxylation.—In a typical experiment, 594 mg. (1.76 meq.) of thiamin chloride hydrochloride, 194 mg. of sodium pyruvate and 3.52 meq. of NaOD solution were dissolved in 9.05 ml. of D_2O. The reaction mixture was frozen and evacuated, and then the reaction flask was heated at 50° for six hours. The reaction flask was cooled, and an amount of glacial acetic acid introduced approximately equal to the NaOD originally added. This introduced approximately 0.4 meq. of hydrogen per cc. of D_2O solution into the D_2O, and reduced the deuterium content of the solvent from 98.5% to about 98.0%. Then 0.345 g. of $NaDSO_3$ was added per ml. of the reaction mixture (the salt was prepared by dissolving $NaHSO_3$ in D_2O, and then evaporating to dryness *in vacuo*) and the mixture allowed to stand for 24 hours at room temperature. The sulfonic acid[5] was isolated by filtration, and 165 mg. of this acid recrystallized from 10 cc. of boiling D_2O; the hot solution was rapidly filtered before cooling. The recrystallized acid was suspended in 2 cc. of D_2O and heated with an equivalent quantity of $NaDCO_3$. The resulting solution was evaporated to dryness *in vacuo*. The resulting salt was dissolved in 2 cc. of H_2O and evaporated on a Rinco rotatory evaporator. The solution was just above 0°, and the evaporation took about an hour. The solution and evaporation was repeated, and the salt then burned for deuterium content.[6] Similar experiments were conducted without added pyruvate. The results are shown in Table I. The sodium salt was identified by showing that its infrared spectrum was the same as that of a sample prepared from authentic sulfonic acid.[5]

TABLE I

D CONTENT OF THE SODIUM SALT OF 5-(2-METHYL-4-AMINOPYRIMIDINE) METHANE SULFONIC ACID

Cond. for decarboxylation	HD/H_2 ratio[a]	% of 1 atom of D incorporated
Pyruvate added	0.0035	1.40
Pyruvate added	.0037	1.48
Pyruvate added[b]	.0035	1.40
Pyruvate added[b]	.0035	1.40
Control (no pyruvate)	.0039	1.56
Control (no pyruvate)	.0037	1.48
Control (no pyruvate)	.0035	1.40
Recrystn. of sulfonic acid and formation of the sodium salt in D_2O	.0005	0.2

[a] After correction for the natural abundance of deuterium. [b] Sample mixed with $K_2Cr_2O_7$ before combustion.

Nuclear Magnetic Resonance.—About 155 mg. of the sodium salt of II was dissolved in D_2O, and the solution evaporated to dryness *in vacuo*. This operation was repeated, and the salt was then dissolved in 0.3 cc. of D_2O, in a 5 mm. glass tube for a determination of the nuclear magnetic resonance spectrum of the solute. The measurements were made in a Varian Associates Model V 4300 B High Resolution N-M-R Spectrometer with the assistance of Dr. A. A. Bothner-By.

The spectrum showed three sharp peaks located at 951, 1103 and 1172 cycles (relative to a toluene standard with 1000 cycles assigned to the aromatic hydrogen atoms and 1197 cycles for the methyl hydrogen atoms). The peaks have been assigned, respectively, to the ring hydrogen atom at position 6, to the methylene group attached to position 5, and to the methyl group at position 2 of the pyrimidine ring; the amino group is deuterated, and so does not appear in the spectrum. The water peak was at a very low level. All the peaks remained unchanged in position and size after 15 days at room temperature, or after an hour at 100°.

Other samples were prepared by dissolving about 155 mg. of the sodium salt of II (without prior treatment with D_2O) in 0.3 cc. of D_2O; the *p*H of such solutions was around 8.3. The three peaks previously located were present in the same ratios, and the water peak was now also present at 1072 cycles. In one experiment, the first measurement was made a few minutes after the salt had been dissolved in the

(6) R. B. Alfin-Slater, S. M. Rock and M. Swislocki, *Anal. Chem.*, **22**, 421 (1956).

D_2O; subsequently the tube was heated at 100°; the positions and ratios of peak heights remained unchanged. In all these experiments, the peaks corresponding to the aromatic hydrogen atoms, to the methylene hydrogen atoms and to the methyl hydrogen atoms were present in approximately the theoretical ratios of 1:2:3, and since the peaks were unchanged with time, the measurements showed that there was little or no exchange with the solvent at the methylene group or elsewhere in the pyrimidine ring, under the experimental conditions employed. However, in most experiments, neither the heights nor the areas under the peaks were in exactly the theoretical ratios; a typical example of peak heights was 1:1.8:3.0, although some others corresponded more nearly to 0.8:2.0:3.0, and one set was exactly 1.0:2.0:3.0. The variations depended to some extent on the precise balancing of the N-M-R Spectrometer. Similar quantitative deviations were obtained with other compounds (*e.g.*, ethanol). Since the ratios of the peaks in the sodium salt of II are strictly independent of time, the possibility of exchange in the salt under the conditions of the experiments is eliminated.

Manometric Measurements.—The decarboxylation was carried out at 50° as in the experiments to measure deuterium exchange. After measured time intervals, the solutions were cooled, and one ml. introduced into a standard Warburg flask; 1 ml. of 2 *N* HCl was introduced into the side arm. When the flask had come to temperature in the thermostat for the Warburg, the solutions were mixed, and the evolution of CO_2 noted. The method was standardized with solutions of K_2CO_3, and all the Warburg flasks calibrated. A solution of 326 mg. of thiamin chloride hydrochloride and 105 mg. of sodium pyruvate in 10 ml. of 0.196 *N* NaOH gave a 40% yield of CO_2 in six hours; a solution of 330 mg. of thiamin chloride hydrochloride and 1.07 g. of sodium pyruvate in 10 ml. of 0.196 *N* NaOH gave 135% yield of CO_2 based on the thiamin present. These manometric experiments were refined and confirmed by Dr. DeLos F. DeTar, who has observed even larger yields of CO_2 based on thiamin introduced.

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CAMBRIDGE 38, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, WEIZMANN INSTITUTE OF SCIENCE]

Poly-L-histidine

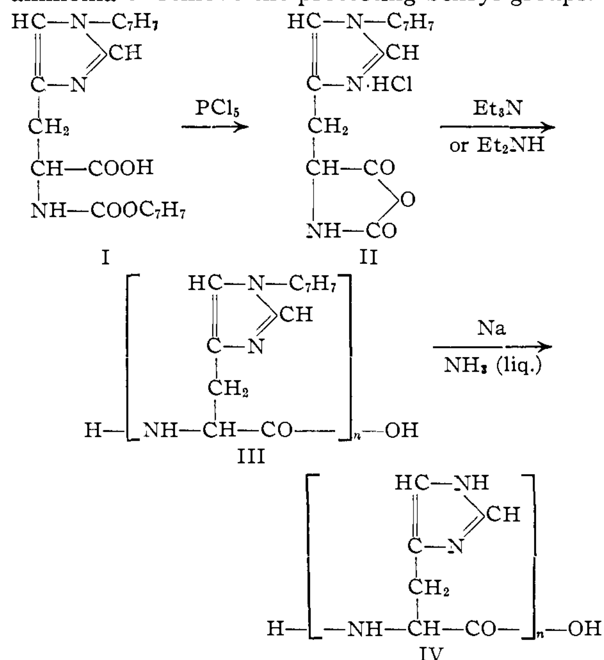
BY ABRAHAM PATCHORNIK, ARIEH BERGER AND EPHRAIM KATCHALSKI

RECEIVED MAY 25, 1957

Poly-L-histidine (IV) was synthesized by the polymerization of 1-benzyl-N-carboxy-L-histidine anhydride (II) in dioxane, and reduction of the resulting poly-1-benzyl-L-histidine (III) with sodium in liquid ammonia. The potentiometric titration of a poly-L-histidine preparation with an average degree of polymerization $n = 15$ could be described by equation 1 assuming an intrinsic dissociation constant, pK_0 6.15, for the imidazolium groups of the polymer. The apparent heat of ionization of the imidazole groups of poly-L-histidine, $\Delta H' = 5.2$ kcal./mole residue, was calculated by means of equation 3. Poly-L-histidine formed insoluble complexes with copper, zinc, magnesium, cobalt, silver and mercury. Poly-1-benzyl-L-histidine formed sparingly soluble salts with many mineral acids.

The imidazole groups of the histidine residues are responsible for most of the buffering capacity of proteins in the physiological *p*H range.[1] They are also capable of combining with various metal ions and appear to constitute the principal sites for metal binding in proteins.[2] Poly-L-histidine was synthesized to provide a model compound for the investigation of the properties of the histidine residue in high molecular weight compounds.

In the synthesis of poly-L-histidine, outlined in the following scheme, the imidazole imino group was reversibly blocked by benzylation. The starting monomer, 1-benzyl-N-carboxy-L-histidine anhydride hydrochloride (II), was prepared from 1-benzyl-N-carbobenzoxy-L-histidine (I)[3] by treatment with phosphorus pentachloride. A solution of the free 1-benzyl-N-carboxyl-L-histidine anhydride in dioxane was obtained by neutralizing II with one mole of triethylamine and removing the insoluble triethylammonium chloride. In this solution polymerization was initiated at room temperature either by diethylamine[4,5] or triethylamine.[6] The poly-1-benzyl-L-histidine (III) which separated out was treated with sodium in liquid ammonia to remove the protecting benzyl groups.[7]



For purification the resulting poly-L-histidine (IV) was dissolved in dilute acid and precipitated with dilute alkali.

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