[CONTRIBUTION FROM THE CHARLES F. KETTERING FOUNDATION]

Preparation of the Hydrazone and Azine of Pyridoxal-5-Phosphate

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In connection with some biochemical experiments, pure hydrazine derivatives of pyridoxal-5-phosphate were desired. Pyridoxal-5-phosphate hydrazone was prepared from pyridoxal-5-phosphate in the presence of a ten-fold excess of hydrazine and obtained in pure crystalline form by controlled acidification. It was also prepared from the corresponding azine in the presence of excess hydrazine. Pyridoxal-5-phosphate azine was also prepared from pyridoxal-5-phosphate and hydrazine. Both compounds were purified by recrystallization and examined for homogeneity by colorimetric analysis of their hydrazine content and by paper chromatography. The ultraviolet absorption spectra were examined in 0.1*N* hydrochloric acid and 0.1*M* potassium phosphate *p*H 7. Observations indicative of the instability of the azine in these solvents are presented.

The involvement of pyridoxal-5-phosphate as the coenzyme in many enzymatic reactions of amino acids is well established. These have recently been reviewed by Meister.[1] The compound is also found as the prosthetic group of crystalline muscle phosphorylase.[2] In connection with some biochemical experiments, hydrazine derivatives of pyridoxal-5-phosphate were desired.

This paper desdribes the preparation and some properties of pyridoxal-5-phosphate hydrazone and of the corresponding azine. The hydrazone was prepared by addition of the aldehyde to a ten-fold excess of hydrazine hydrate. The azine was prepared by adding hydrazine hydrate to a solution of pyridoxal phosphate. Both compounds can be purified by recrystallization induced by controlled acidification of the potassium salts.

EXPERIMENTAL

Pyridoxal-5-phosphate hydrazone (2-methyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric acid hydrazone). Pyridoxal phosphate[3] (0.330 g.) was dissolved in 20 ml. of distilled water at 55–60°. The resulting solution was added dropwise with stirring to a 10-fold excess of hydrazine hydrate (0.714 g. of an 85% solution) in 15 ml. of water at 55–60°. The mixture was maintained at the 55–60° temperature for 10 min. and then filtered. Filter and beaker were washed with a total of 5 ml. of water which was added to the main filtrate. Hydrochloric acid (0.1*N*) was added dropwise, reducing the *p*H to 5.7, at which point feathery crystals began to appear. As preliminary work had demonstrated that a somewhat discolored and impure product is obtained at low *p*H values, acidification was stopped at *p*H 5.7 and the solution allowed to stand for 2 hr.

The fine near-white needles with a barely perceptible yellow tinge were collected by suction filtration, washed twice with distilled water, three times with 95% ethanol, and six times with ether. The product was dried over phosphorus pentoxide at atmospheric pressure and stored in air. The yield was 0.161 g. (47.6%). Examination of the melting characteristics revealed preliminary darkening at 227° followed by decomposition at 236–237°. Analysis of the products agreed with theoretical values calculated for the monohydrate.

Anal. Calcd. for $C_8H_{12}O_5N_2P \cdot H_2O$: C, 34.45; H, 5.06; N, 15.00; P, 11.10. Found: C, 34.51; H, 5.25; N, 15.02; P, 11.10. The expected water of hydration was lost upon heating to constant weight at 120°.

A second batch of crystals was obtained from the filtrate, which had a *p*H value of 6.1 after removal of the first crystals, by the dropwise addition of 0.1*N* hydrochloric acid to *p*H 5.6. After standing overnight the crystals were collected and washed as described above. The yield was 0.066 g. (19.5%). These crystals appeared to be identical with those obtained in the first crystallization and had the same melting characteristics. The combined yield was thus increased to 67.3%.

Recrystallization of similarly prepared material has been effected by suspending the material in water and dissolving with the dropwise addition of 0.1*N* potassium hydroxide, filtering, and lowering the *p*H to 5.7 by the dropwise addition of 0.1*N* hydrochloric acid. The recovered material (60%) gave the same carbon-hydrogen analysis as the original preparation and exhibited the same melting characteristics.

Careful control of *p*H is recommended. When uncontrolled acidification is used, a discolored and impure product is obtained. When, for example, in an otherwise identical procedure, the *p*H during the crystallization was allowed to drop to 3.0, dark pink-brown needles were obtained which darkened at 217° and decomposed at 230–231°. This material can be recrystallized as described above to produce the pure product. The final yield, however, is reduced to 36%.

Pyridoxal-5-phosphate azine (2-methyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric acid azine). Hydrazine hydrate (0.136 g. of an 85% solution) was added with stirring to a solution of pyridoxal phosphate (0.50 g.) in 17 ml. of 0.87 *N* potassium hydroxide. The solution was stirred for 10 min. in a water bath at 45°, cooled to room temperature, and the *p*H was lowered to approximately 3 by the dropwise addition of 1*N* hydrochloric acid. The yellow precipitate was collected by suction filtration, washed twice with distilled water, twice with 95% ethanol, and three times with ether. Air was then drawn through the filter for 30 min.

Although the precipitate initially is quite sticky and filtration and washings are slow, it becomes flaky when the ether is removed and is readily separable from the filter paper as a bright yellow powder. The yield was 0.478 g. (87.8%).

The product was further purified by dissolving it in 0.1 *N* potassium hydroxide. The resulting solution was filtered and the *p*H lowered to 3 by the dropwise addition of 0.1 *N* hydrochloric acid. The resulting precipitate was filtered and washed as described above. The yield was 0.436 g. corresponding to an overall yield of 80% and a 91.3% recovery in the purification. The product was dried over phosphorus pentoxide at atmospheric pressure and stored in air. In the melting point apparatus this material darkened about 195°

(1) Alton Meister, "Biochemistry of the Amino Acids," Academic Press, Inc., New York, N. Y., 1957, pp. 202–213.

(2) Carl F. Cori and Barbara Illingworth, *Proc. Natl. Acad. Sci.* **43**, 547 (1957).

(3) The commercial material obtained from the Nutritional Biochemical Corp., Cleveland, Ohio, was used as received.

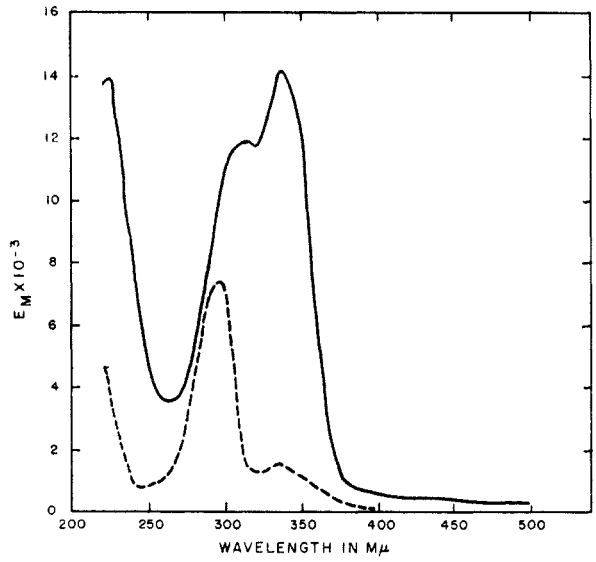

Fig. 1. Absorption spectra in 0.1*N* hydrochloric acid. Spectra determined on 2.6 × 10^{-5}*M* solutions. Solid line, pyridoxal phosphate hydrazone; dashed line, pyridoxal phosphate. E_M denotes molecular extinction coefficient (molar absorbance, ϵ, or molar absorbancy index, α_m)

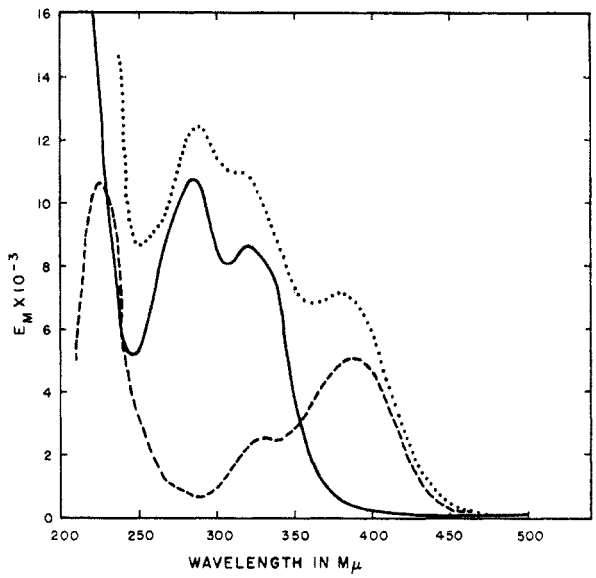

Fig. 2. Absorption spectra of 0.1*N* potassium phosphate *p*H 7.0. Dotted line, pyridoxal phosphate azine—ninety minutes after dissolving in buffer; solid line, pyridoxal phosphate hydrazone; dashed line, pyridoxal phosphate. EM denotes molecular extinction coefficient

and became progressively darker to 280°. Analysis of the material agreed with the theoretical values calculated for the trihydrate.

Anal. Calcd. for $C_{16}H_{20}N_4P_2O_{10}\cdot 3H_2O$: C, 35.22; H, 4.79; N, 10.28; P, 11.44. Found: C, 35.11; H, 5.03; N, 10.26; P, 11.44. The expected water of hydration was lost upon heating to constant weight at 120°.

Conversion of pyridoxal-5-phosphate azine into pyridoxal-5-phosphate hydrazone. Pyridoxal phosphate azine (0.237 g.) was suspended in 5 ml. of water and mixed with a solution containing 0.248 g. of 85% hydrazine hydrate in 5 ml. of water. The mixture was stirred to dissolve the azine and heated on a water bath at 85° for 30 min. with frequent stirring. The hot solution was then filtered and the beaker and filter were washed with a total of 5 ml. of water. Hydrochloric acid (0.1 *N*) was added dropwise with stirring to reduce the *p*H to 5.9 and the solution was allowed to stand for 1 hr. The crystals were collected by suction filtration, washed twice with water, three times with ethanol, six times with ether, and dried in air. The yield was 0.159 g. (65.3%). The product was further purified by recrystallization as described previously, dried over phosphorus pentoxide, and stored in air. The crystals darkened at 224° and decomposed at 235–236°. Analysis agreed with theoretical values calculated for the monohydrate.

Anal. Calcd. for $C_8H_{12}O_5N_3P\cdot H_2O$: C, 34.45; H, 5.06; N, 15.00; P, 11.10. Found: C, 34.45; H, 4.95; N, 14.90; P, 11.26.

RESULTS AND DISCUSSION

Colorimetric determination of hydrazine content. In the presence of hydrochloric acid, *p*-dimethylaminobenzaldehyde reacts with hydrazine forming a quinoid structure possessing a yellow-orange color.[4] The color was observed to develop rapidly when 1 ml. of the reagent prepared as described by Pesez and Petit[4] is added to 5 ml. of solution containing the hydrazine. To insure completeness of the reaction, the mixture was allowed to stand 20 min. before examination. A standard curve was prepared from optical density values at 455 mμ obtained with known concentrations of hydrazine sulfate using a Beckman Model B spectrophotometer with 1 cm. cuvettes. The curve is linear with increasing hydrazine concentrations up to 0.44 μg. hydrazine per ml. of final reaction mixture. These values are within the range described by Audrieth and Ogg.[5] Various dilutions of standard solutions of the two pyridoxal phosphate derivatives were subjected to the same procedure. The hydrazine content of each determined from observed optical density values agreed well with calculated values of the hydrazine content. The results are presented in Table I.

TABLE I

Determination of Hydrazine Content[a]

Azine		Hydrazone	
Calcd.	Found	Calcd.	Found
0.274	0.260	0.330	0.340
0.220	0.210	0.275	0.270
0.164	0.164	0.221	0.218
0.107	0.105	0.166	0.166
0.054	0.058	0.110	0.119

[a] Expressed as μg. hydrazine per ml.

Ultraviolet absorption spectra. The ultraviolet absorption spectra of pyridoxal-5-phosphate and the corresponding hydrazone and azine were examined in 0.1 *N* hydrochloric acid and 0.1 *M* potassium phosphate at *p*H 7. The spectra of the preparation of pyridoxal phosphate, which was used as the

(4) M. Pesez and A. Petit, *Bull. Soc. Chim. France*, 1947, 122–123 (2 g. *p*-dimethylaminobenzaldehyde, 10 ml. conc. hydrochloric acid, 100 ml. 95% ethanol).

(5) L. F. Audrieth and Betty A. Ogg, "The Chemistry of Hydrazine," John Wiley and Sons, Inc., N. Y., 1951, p. 164.

starting material, are in good agreement both qualitatively and quantitatively with those obtained by Peterson and Sober[6] in these solvents.

Spectra obtained in 0.1 *N* hydrochloric acid are presented in Fig. 1. With the hydrazone, maxima are observed at 314 mμ (ϵ = 11,920) and 337 mμ (ϵ = 14,160). The 337 mμ maximum should be useful for spectrophotometric determinations, as the absorption of pyridoxal phosphate and pyridoxamine phosphate is minimal in this region.[6] The spectrum obtained with the azine in 0.1 *N* hydrochloric acid was essentially coincident with that resulting from the addition of the spectra of pyridoxal-5-phosphate and the hydrazone. Thus hydrolysis of the azine is essentially complete at low concentrations in acidic medium.

Spectra obtained in 0.1 *M* potassium phosphate at *p*H 7 are presented in Fig. 2. Upon formation of the hydrazone the maximum at 388 mμ which is specific for the –CHO group of pyridoxal phosphate[6] disappears as expected and maxima are observed at 284 mμ (ϵ = 10,800) and 320 mμ (ϵ = 8,630). Extinction values at the maxima were reproducible during the time required to examine the spectrum and exhibited no significant change after standing 14 hours in the buffer. The spectrum is indicative of the homogeneity of the hydrazone.

In addition to maxima observed at 288 and 316 mμ, the azine has a maximum at 379 mμ in the buffer. Similar azine structures show a maximum in this general region, e.g. 2,2′-dihydroxybenzalazine has a maximum at 355 mμ.[7] Immediately following solution of the azine in phosphate buffer *p*H 7 an initial decrease in absorption at both the 288 and 379 mμ maxima was observed. Reproducible values were obtained after approximately one hour and decreased less than 2% in 12 hours. The azine spectrum in Fig. 2 was obtained after 90 minutes. The spectrum of a mixture of equimolecular quantities of pyridoxal phosphate and its hydrazone in 0.1 *M* potassium phosphate buffer *p*H 7 was essentially coincident with that of the azine. This was interpreted as evidence for the existence of an equilibrium among azine, hydrazone, and pyridoxal phosphate in the solutions of the azine itself. Further support for this conjecture was obtained when additions of the separate spectra of the hydrazone and pyridoxal phosphate produced a curve which was not coincident with that of the azine. In the latter case the maximum at 379 mμ was about 25% lower than that for the azine itself. Available information does not permit a more complete explanation of this observation.

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(6) E. A. Peterson and H. A. Sober, *J. Amer. Chem. Soc.* **76**, 169 (1954).

(7) E. R. Blout and R. M. Gofstein, *J. Amer. Chem. Soc.* **67**, 13 (1945).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF FAIRFIELD UNIVERSITY, THE DEPARTMENT OF CHEMISTRY AND CHILDREN'S HOSPITAL, UNIVERSITY OF BUFFALO, AND ROSWELL PARK MEMORIAL INSTITUTE]

A 2-Trifluoromethyl Analog of Thiamin[1]

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Two synthetic routes for thiamin, adapted from classical methods, were found satisfactory for the preparation of 3-[(4-amino-2-trifluoromethyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride hydrochloride [(VII) "trifluorothiamin"]. Solvolysis of intermediates occurred under certain conditions. "Trifluorothiamin" is biologically active as a thiamin antagonist in microorganisms and in mice where paralysis and opisthotonus occur. Growth of a transplanted carcinoma and a leukemia was suppressed in mice on a thiamin-deficient diet.

Although many analogs of thiamin have been prepared,[3a-d] the synthesis of a trifluoromethyl analog seemed pertinent because the presence of such a group could have a significant electronic effect on the molecule, but should not have a significant steric effect. In a preceding paper,[4] the syntheses of 4-amino-2-trifluoromethyl-5-hydroxymethylpyrimidine (I) and 4-amino-5-aminomethyl-2-trifluoromethylpyrimidine (IV) from trifluoroacetamidine were described. Their biological activity[5] contributed to our further interest in 2-trifluoromethylpyrimidines.

This paper is concerned with the use of compounds I and IV as starting materials for the preparation of 3-[(4-amino-2-trifluoromethyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazo-

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(4) J. A. Barone, E. Peters, and H. Tieckelmann, *J. Org. Chem.*, **24**, 198 (1959).

(5) Unpublished results.