[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Formation of Fumaramic Acid from Asparagine in Phosphate Buffer

BY EUGENE A. TALLEY, THOMAS J. FITZPATRICK[2] AND WILLIAM L. PORTER

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The compound isolated from the reactions of asparagine in phosphate buffer and previously reported to be 4-carboxy-2-azetidinone, has been shown to be fumaramic acid. Authentic fumaramic acid was synthesized from fumaryl dichloride and compared with the compound from asparagine by chemical means, by infrared absorption and by X-ray powder diagrams.

In a publication[3] describing results of investigations of analytical methods for use with amides from potatoes, the authors reported the isolation of a compound from the reaction mixture resulting from heating asparagine in *p*H 6.7 buffer (Na_2HPO_4, KH_2PO_4) at 100° for 24 hours. This compound, from the available analytical data, apparently corresponded to a structure described by the name 4-carboxy-2-azetidinone. Carbon, hydrogen and nitrogen analyses, molecular weight, neutralization equivalent, ninhydrin activity and reaction with the Rydon–Smith reagent[4] all were consistent with this structure.

At the suggestion of Dr. Ernest Sondheimer,[5] we re-examined the compound with the possibility in mind of its being fumaramic acid, the mono-amide of fumaric acid, first synthesized by Griess[6] by treatment of asparagine with methyl iodide and potassium hydroxide. This compound has the same molecular weight and empirical formula as the isolated compound. The possibility of fumaramic acid had not been considered previously by the authors since its production would require the deamination of the asparagine under extremely mild conditions leaving the amide group intact.

This re-examination has led to explanations of certain difficulties encountered in the earlier work such as the failure to esterify by the usual procedure involving hydrochloric acid. It has also led to the discovery of an error in interpretation, namely, the stability of the compound toward hydrolysis by 6 *N* HCl. Hydrolysis had been checked originally by chromatographing the hydrolysate on paper in several solvents. The compound moved, in these solvents, almost identically with the unhydrolyzed material, was acidic, negative to ninhydrin and positive to the Rydon–Smith reagent. However, when restudied, the product was found to be fumaric acid which was also determined to give a positive Rydon–Smith reaction, an unexpected and previously unknown fact.

Hydrolysis with hot sodium hydroxide produced, after desalting by ion-exchange, mainly fumaric acid but also malic, succinic and tartaric acids (identified by paper chromatography and derivatives), as would be expected from the literature.[7]

An authentic sample of fumaramic acid was synthesized from fumaryl dichloride by preparation of monomethyl fumarate according to the method of Lutz.[8] This compound was treated with concentrated ammonium hydroxide to form the ammonium salt of fumaramic acid. After desalting by ion-exchange, the fumaramic acid was recrystallized from water. Determination of the melting point on the microscope hot-stage gave a sublimation range of 191–193° as reported for the so-called 4-carboxy-2-azetidinone. However, determination in a capillary tube in an oil-bath showed both compounds to melt at 216.5–217.5°, the melting point given in the literature for fumaramic acid. Molecular weight, melting point, ultimate analyses, R_f data by paper chromatography as well as infrared absorption and X-ray powder diagram data have led to the conclusion that the compound previously isolated was fumaramic acid.

Treatment of fumaric acid with concentrated ammonium hydroxide for three weeks at room temperature does not produce any detectable amount of aspartic acid while fumaramic acid under the same conditions goes quantitatively to asparagine. When fumaric acid is treated with ammonium hydroxide, the ammonium salt is formed and under these mild conditions the addition to the double bond is difficult. However, in the same treatment of fumaramic acid, the carboxamide group does promote the substitution of the double bond. These observations fit the experimental data and are consistent with data in the literature[9] concerning activation of double bonds by carboxamide groups and esters. Aspartic acid in phosphate buffer under the conditions employed with asparagine produces no fumaric acid. The removal of the amino group of asparagine is an example of a base-catalyzed elimination reaction in which the carboxamide function of the asparagine has activated the adjacent methylene group. Preliminary studies indicate that time, temperature and basicity have an influence on the relative ratio of the products (fumaramic acid and aspartic acid). Increase in *p*H to 7.50 produced approximately 25% fumaramic acid based upon the asparagine as compared to about 5% at *p*H 6.5.

(1) Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. Article not copyrighted.

(2) Candidate for a Ph.D. degree at the University of Massachusetts, Amherst.

(3) E. A. Talley, T. J. Fitzpatrick and W. L. Porter, THIS JOURNAL, **78**, 5836 (1956).

(4) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).

(5) Ernest Sondheimer, New York State College of Forestry, private communication.

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(7) J. M. Weiss and C. R. Downs, THIS JOURNAL, **44**, 1118 (1922).

(8) R. E. Lutz, *ibid.*, **52**, 3423 (1930).

(9) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953.

Experimental

Fumaramic Acid from Asparagine.—Method described in original paper[1]:

Anal. Calcd. for $C_4H_6O_3N$: C, 41.74; H, 4.38; N, 12.18; mol. wt., 115.09. Found: C, 41.45; H, 4.24; N, 12.04; mol. wt. (freezing point depression using glacial acetic acid), 103.2; neut. equiv., 109.3; m.p. (capillary tube 216.5–217.5° (cor.).

Fumaric Acid Monomethyl Ester.—The method of Anschütz and Baeumges[10] as modified by Lutz[8] was employed. A solution of dry methanol (10.7 g.) in 145 ml. of dry benzene was added dropwise to 51.0 g. of fumaryl dichloride with constant stirring. The temperature was kept below 30° and hydrogen chloride was evolved. After standing overnight, under dry conditions, the benzene was boiled off at atmospheric pressure. The product was fractionally distilled through a 12-inch column filled with small porcelain saddles at a pressure of about 18 mm. Four fractions were taken with boiling ranges 62–71°, 71–82°, 82–84° and 84–85°. At 84–85° a product solidified in the column (dimethyl fumarate). The fractions boiling from 71–84° were combined and stirred vigorously with cold water until the emulsion, first formed, solidified and crystallized. After cooling in ice, the suspension was filtered and the crystals washed with ice-water. The yield, after drying at 50° *in vacuo*, was 8.2 g. or 18.9% of crystals melting at 144.0–144.5°.

(10) R. Anschütz and P. Baeumges, *Ann.*, **461**, 188 (1928).

Fumaramic Acid from Fumaric Acid Monomethyl Ester.—The fumaric acid monomethyl ester (8.2 g.) was treated with 25 ml. of concentrated ammonium hydroxide at room temperature. Solution occurred immediately. After shaking for several minutes, a product began to crystallize. The suspension of crystals was allowed to stand two hours and then evaporated to dryness in a rotary evaporator. The dry product was taken up in water and redried to remove the last traces of free ammonia. The product was dissolved in water and passed through a column of Dowex 50[11] in the H^+ ion cycle to desalt. After washing with three bed-volumes of water, the effluent and washings were combined, evaporated to a small volume in the rotary still at less than 40° and allowed to crystallize. After recrystallization from water and drying at 50° *in vacuo*, the yield of fumaramic acid was 6.3 g. or 16.4% based upon the fumaryl dichloride. X-Ray powder diagrams and infrared absorption in KBr disks showed the two samples of fumaramic acid to be identical.

Anal. Calcd. for $C_4H_5O_3N$: C, 41.74; H, 4.38; N, 12.18; mol. wt., 115.09. Found: C, 41.85; H, 4.60; N, 11.96; mol. wt. (freezing point depression in glacial acetic acid), 102.2; neut. equiv., 110.4; m.p. (capillary tube), 216.5–217.5°; hydrogenation (g./mole H_2), 118.2.

(11) Mention of commercial products does not imply endorsement by the Department of Agriculture over others of similar nature not mentioned.

PHILADELPHIA 18, PENNA.

[CONTRIBUTION FROM THE MALLINCKRODT LABORATORIES OF HARVARD UNIVERSITY]

The Role of Thiamin in Carboxylase

BY D. F. DETAR[1] AND F. H. WESTHEIMER

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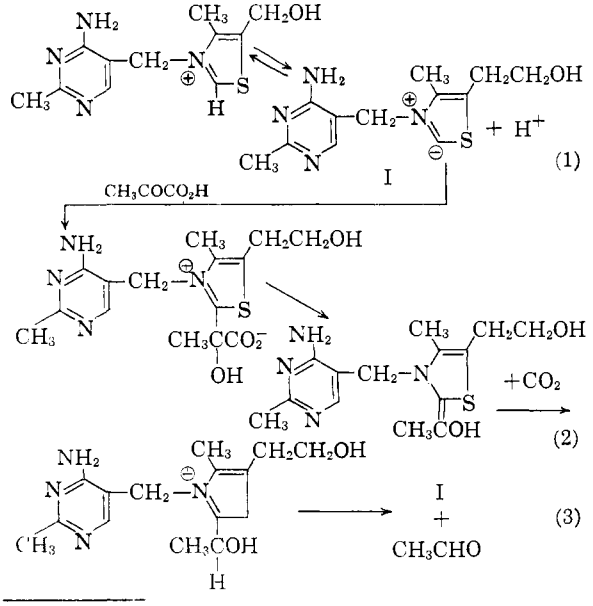
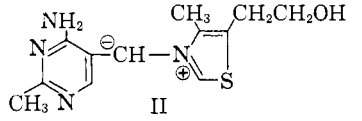
The decarboxylation of pyruvate with yeast carboxylase has been carried out in tritiated water. The coenzyme, thiamin pyrophosphate, was released from the enzyme. Carrier was added, and the coenzyme was cleaved to pyrimidinesulfonic acid. The compound so obtained contained no tritium. This finding for the enzymatic reaction accords with the facts for the known model system which catalyzes the decarboxylation of pyruvate, and with the probable mechanism for the enzymatic and "model" systems.

A mechanism for the action of thiamin has recently been advanced by Breslow.[2] This mechanism involves the ionization of a proton from the 2-position of the thiazolium ring, to form an ylid; this ylid is postulated as the essential intermediate in the decarboxylation of pyruvic acid.



The ionization shown in equation 1 has been demonstrated by deuterium exchange, and the mechanism will account for the reactions observed in model systems.[3] Earlier the ylid II was postulated[4] as an intermediate in the process, but this possibility has been disproved for the model systems.[5]



Although it seems reasonable to assume that the model system and the enzymatic one operate by very similar mechanisms, this point remains for experimental demonstration. The present research is concerned with a study of the mechanism of the enzymatic decarboxylation of pyruvic acid with the

(1) National Science Foundation Senior Post-doctoral Fellow. Professor of Chemistry, University of South Carolina, Columbia, S. C.

(2) R. Breslow, THIS JOURNAL, **80**, 3719 (1958).

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

(4) R. Breslow, *Chemistry & Industry*, R28 (1958); a related species has been suggested by K. Wiesner and Z. Valenta, *Experientia*, **12**, 190 (1956).

(5) L. Ingraham and F. H. Westheimer, *Chemistry & Industry*, 846 (1956); K. Fry, L. Ingraham and F. H. Westheimer, THIS JOURNAL, **79**, 5225 (1957).