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Synthesis, Isolation and Identification of Cocarboxylase

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From the reaction mixture recently described[1] cocarboxylase may be isolated in pure state by a procedure to be described in the present communication. A series of experiments will be presented which show that the synthetic cocarboxylase as obtained from synthetic thiamin chloride is in every respect identical with the natural cocarboxylase isolated by Lohmann and Schuster[2] from bottom yeast.

Experimental

Synthesis.—One gram of sodium pyrophosphate was placed in a Pyrex test-tube and heated until all of the water of crystallization was removed. Two cc. of orthophosphoric acid (C. P. 85%) was placed in another large Pyrex tube and heated until a slight amount of solid deposit formed on the side of the tube. Then the pyrophosphate was added and the mixture gently heated until solution took place. After cooling 1 g. of thiamin was added. The tube was placed in an oil-bath of 155°, kept there for fifteen minutes and constantly stirred. Then the tube was removed and after cooling the solid mass was dissolved in 15 cc. of cold water. Cold saturated barium hydroxide solution was added until no more precipitate formed and the solution just commenced to turn yellow. The precipitate was centrifuged out and the supernatant fluid was decanted. The precipitate was extracted four times with 80 cc. of cold water. The supernatant fluids (five) were united and 3% sulfuric acid was added to slight blue reaction of congo red paper. The barium sulfate was centrifuged off and discarded. The barium-free solution was concentrated to 30 cc. in vacuum at 25°. It was cooled in ice water and 15 volumes of a mixture of one part of absolute alcohol and two parts of ether were added which precipitated the cocarboxylase in the form of microscopic needles. Sometimes a gummy mass formed which turned into long macroscopic needles on short standing in the cold. The yield was about 1 g. of crude cocarboxylase.

Isolation.—One and one-half grams of crude cocarboxylase was dissolved in 150 cc. of water, neutralized to congo red with ammonium hydroxide and treated with 3 cc. of *N* silver nitrate. This first precipitate was mainly silver chloride and, accordingly was discarded. The clear solution was made neutral to litmus with ammonium hydroxide and 15 cc. of *N* silver nitrate was added. A copious yellow precipitate appeared. It contained most of the active cocarboxylase in the form of the silver salt. This was centrifuged and washed with water, suspended in 150 cc. of water and decomposed with hydrogen sulfide. The precipitate was centrifuged and washed with 20 cc. of water. The liquids were united and the hydrogen sulfide removed by aëration. To the neutral, hydrogen sulfide-free solution 96 cc. of 2.5 *N* hydrochloric acid was added and the mixture was diluted to 300 cc. with water. A moderate excess of a 25% phosphotungstic acid solution was added and the mixture centrifuged. The supernatant liquid was discarded. The precipitate was treated with 150 cc. of acetone which dissolved the phosphotungstic acid whereas the cocarboxylase separated as an oil which tended to crystallize on scratching. The cocarboxylase was centrifuged off and the acetone fraction was discarded. The precipitate was extracted twice more with 50 cc. of acetone. The residue was then extracted with 20 cc. of 0.1 *N* hydrochloric acid and filtered. The insoluble part was treated with 75 cc. of acetone and the acetone discarded. The residue was extracted with 15 cc. of 0.1 *N* hydrochloric acid. To the total (35 cc.) 0.1 *N* hydrochloric acid solution, 350 cc. of acetone was added and the mixture placed in the refrigerator overnight. The supernatant fluid was discarded and the residue dissolved in 15 cc. of 0.1 *N* hydrochloric acid; 150 cc. of acetone was added and the mixture allowed to crystallize in the refrigerator for twenty-four hours. The crystalline precipitate was dissolved in 15 cc. of 0.1 *N* hydrochloric acid and 30 cc. of absolute alcohol and 120 cc. of acetone added. The mixture was allowed to crystallize in the refrigerator for twenty-four hours; yield 180 mg.

Analysis.—The synthetic, practically pure cocarboxylase dried in a vacuum over sulfuric acid had a slightly yellowish color similar to the natural product and melted at 240°. Its elementary composition agrees well with that found for the natural cocarboxylase by Lohmann and Schuster. Calcd.: C, 30.08; H, 4.42; N, 11.71; P, 12.96; Cl, 7.40. Found: C, 30.62; H, 4.20; N, 11.82; P, 12.37; Cl, 7.95.

One more crystallization (50 mg. in 1 cc. of 0.1 *N* hydrochloric acid with 3 cc. of alcohol) gave a cocarboxylase which may be considered to be 100% pure. Found: C, 30.36; H, 4.24; N, 11.30; P, 12.83.

Acid Hydrolysis.—3.206 mg. of synthetic cocarboxylase with 2 cc. of *N* hydrochloric acid was placed in a bath of boiling water for forty minutes. The solution was then evaporated to dryness in vacuum at room temperature, and the ionized phosphate determined: found, 12.978 mg. of ammonium phosphomolybdate (5.89% P) or 47.6% of the total phosphorus. The second phosphate radical is more firmly bound, as also found by Lohmann and Schuster for natural cocarboxylase. Twenty-five micrograms of cocarboxylase in 1 cc. of 0.1 *N* hydrochloric acid boiled for sixty minutes at 100° lost 50% of its coenzyme activity.

Enzymic Hydrolysis.—To 4 g. of minced kidney tissue, 50 micrograms of cocarboxylase in 4 cc. of water was added and incubated at 37°. In thirty minutes 58% of the cocarboxylase was hydrolyzed by the kidney phosphatase, as determined enzymatically, using alkaline washed, cocarboxylase-free dry brewers' yeast.

Thiamin Content and Thiochrome Formation.—It is well known that thiamin may be oxidized to thiochrome

(1) H. Tauber, THIS JOURNAL, **60**, 730 (1938).

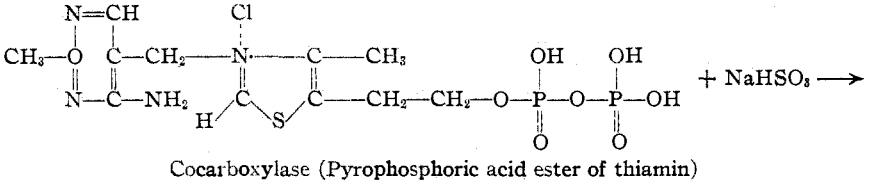
(2) K. Lohmann and P. Schuster, *Biochem. Z.*, **294**, 188 (1937).

by an alkaline potassium ferricyanide solution. Tauber[3] found that the ferrocyanide formed during the reaction may be converted quantitatively to Prussian blue and the color measured colorimetrically. The same is true for cocarboxylase. Its thiamin portion may be converted quantitatively to thiochrome. Cocarboxylase also forms thiochrome on the addition of alkali alone. The thiochrome compound, however, is inactive. It becomes enzymatically active again on acidification.

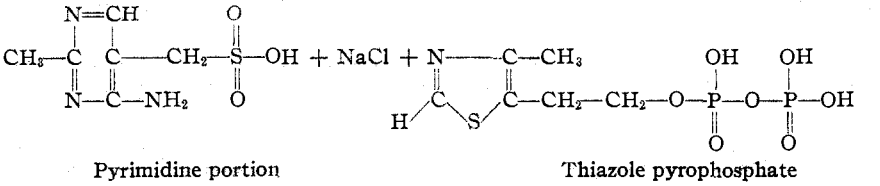
Formaldehyde–Azo Test.—The formaldehyde-azo test[4] gave an intense yellow color with cocarboxylase. Thiamin gave a red color.

Coenzyme Activity.—Synthetic cocarboxylase is as active as natural cocarboxylase (very kindly furnished by Professor Lohmann). Exact comparisons can only be made, however, if the same yeast preparation is employed as the source of carboxylase. The carboxylase content of various yeasts differs considerably.

Sulfite Cleavage.—The synthetic cocarboxylase should split in the same manner as the natural cocarboxylase, *e. g.*, into a phosphorus-free pyrimidine and a thiazolpyrophosphate:



Cocarboxylase (Pyrophosphoric acid ester of thiamin)



Pyrimidine portion Thiazole pyrophosphate

One hundred and twenty-three mg. of cocarboxylase was dissolved in 2 cc. of water and adjusted to *p*H 5 by the addition of 0.27 cc. of *N* sodium hydroxide; 200 mg. of solid sodium bisulfite was added and the mixture allowed to stand for three days at room temperature. In less than one hour crystallization began and after concentration in vacuum at room temperature to 0.5 cc., the crystals were filtered off, washed with 3 drops of water and dried in vacuum to constant weight; yield 44.6 mg., calcd. 52 mg. It was recrystallized by dissolving in hot water, cooling, filtering, washing with several portions of water, and drying.

Anal. Calcd. for the P-free pyrimidine $C_6H_9O_3N_3S$ (m. w. 203.2): C, 35.44; H, 4.46; N, 20.68. Found: C, 35.37; H, 4.45; N, 20.51.

The filtrate from the pyrimidine portion was made slightly alkaline to litmus with ammonium hydroxide; 3 drops of hydrogen peroxide was added and the solution warmed in order to oxidize the sulfite ions to sulfate ions. The solution was then made acid to congo red with 10% nitric acid (5 drops). A slight excess of concd. barium nitrate solution and a slight excess of 10% silver nitrate solution were added. The sulfate and chloride precipitate was discarded and the filtrate was made neutral to congo red with dilute ammonium hydroxide. It was then concentrated to 3 cc. in vacuum at room temperature. An excess of 60% silver nitrate solution was added until no further precipitation occurred. The precipitate was filtered off, washed with 50%, then 90%, then 100% alcohol and finally with ether, and then dried in vacuum; yield 150 mg. silver salt, calcd. 184 mg.

The silver salt was dissolved in 3 cc. of 5% nitric acid, neutralized with ammonium hydroxide and excess 60% silver nitrate was added. The precipitate was filtered, washed with alcohol and ether as above. Analyses indicate the approximate formula: $C_6H_8O_7NSP_2Ag_3(AgOH\cdot AgNO_3)6/10\cdot 3H_2O$ (m. w. 854.75).

Calcd.: C, 8.42; H, 1.72; total N, 2.62; N as nitrate, 0.98; Ag, 54.8; P, 7.26. Found: C, 8.21; H, 1.45; total N, 2.61; nitrate N, 0.87; Ag, 53.0; P, 7.64.

Mild Hydrolysis in *N* Nitric Acid.—3.395 mg. of the cleavage product was heated at 100° for twenty minutes with 1 cc. of *N* nitric acid: found 9.717 mg. of ammonium phosphomolybdate = 4.10% P, hence 53.6% of the total P was hydrolyzed. This indicates that only one of the phosphate radicals is removed readily from the phosphorylated thiazole.

Lohmann and Schuster evidently obtained their cleavage product in a more acid solution since their analysis indicates the formula $C_6H_8O_7NSP_2Ag_3(HNO_3)6/10\cdot 3H_2O$, whereas we obtained a basic double salt.

The nitrate was reduced with Devarda's metal and determined colorimetrically. Nitrogen was determined according to Hayman and Adler.[5]

The analytical work was carried out by D. F. Hayman, W. Reiss and H. Levy, to whom we are indebted. We wish also to express our appreciation to Dr. R. T. Major, for his advice and interest throughout this investigation.

Summary

Synthetic cocarboxylase has been prepared in pure form and identified. Its behavior toward mild acid hydrolysis and its cleavage products as well as its coenzyme activity are identical with those of the natural cocarboxylase, the pyrophosphoric acid ester of thiamin.

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(3) H. Tauber, *Mikrochim. Acta*, **3**, 108 (1938).

(4) H. W. Kinnersley and R. A. Peters, *Biochem. J.*, **28**, 667 (1934).

(5) Hayman and Adler, *Ind. Eng. Chem., Anal. Ed.*, **9**, 197 (1937).