Summary

The oxidation of α-naphthol and *p*-phenylenediamine in the presence of activated charcoal appears to involve several simultaneous and successive processes. The measurement of the indophenol production is not an adequate test of the charcoal activity.

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[CONTRIBUTION FROM THE RESEARCH LABORATORY OF MERCK & CO., INC.]

Cocarboxylase and Related Esters

BY JOHN WEIJLARD

In previous publications concerning synthetic cocarboxylase, a rather indefinite acid mixture prepared by dehydrating orthophosphoric acid has been employed for the phosphorylation.[1,2,3] A different method was employed by H. Weil-Malherbe,[4] who prepared cocarboxylase by interaction of bromo-thiamine with silver pyrophosphate, a reaction that does not fall within the scope of this paper.

The obvious method to prepare cocarboxylase would be the interaction of pure pyrophosphoric acid and thiamine hydrochloride (I). It was found, however, that pyrophosphoric acid itself did not form any thiamine ester whatever, but with anhydrous sodium pyrophosphate as catalyst produced thiamine orthophosphoric acid ester (IV) in fair yields instead of the expected pyrophosphoric acid ester, as shown by the fact that mild hydrolysis produced no phosphoric acid.[2] Similarly, 4-methyl-5-hydroxyethylthiazole (II) formed 4-methyl-5-hydroxyethyl thiazole orthophosphoric acid ester (VI) with pyrophosphoric acid and pyrophosphates. Concentrated sulfuric acid with anhydrous sodium pyrophosphate produced thiamine sulfuric acid ester (III) from thiamine hydrochloride in good yields, but no thiamine pyrophosphoric acid ester could be detected in the reaction mixture.

Phosphorus pentoxide was hydrated to give metaphosphoric acid,[5] and with the acid thus produced it was actually possible to esterify thiamine hydrochloride to thiamine pyrophosphoric acid ester chloride (cocarboxylase) (V). Yields similar to those previously reported[3] of thiamine pyrophosphoric acid ester were obtained when thiamine hydrochloride was esterified with a mixture of phosphorus pentoxide dissolved in pyrophosphoric acid with sodium pyrophosphate as catalyst. Such a solution should be a mixture of meta and pyro acids, with perhaps some unchanged phosphorus pentoxide, similar to the mixture obtained upon dehydrating orthophosphoric acid.[6,7] The mixture from dehydrated orthophosphoric acid gave with 4-methyl-5-hydroxyethylthiazole the compound, 4-methyl-5-hydroxyethylthiazole pyrophosphoric acid ester (VII), isolated as the silver salt,[2,8] which varies in composition according to varying *p*H and salt concentration.

2-Methyl-4-amino-5-bromomethylpyrimidine hydrobromide (VIII) was condensed with 4-methyl-5-hydroxyethylthiazole pyrophosphoric acid ester (VII) on the one hand, and with 5,β-chloroethyl-4-methylthiazole (IX) in the presence of silver pyrophosphate on the other to form cocarboxylase. Cocarboxylase was obtained in these reactions as ascertained by biological tests, but the yields were no higher than those obtained by the more simple direct phosphorylation of thiamine hydrochloride.

Experimental

Thiamine Orthophosphoric Acid Ester.—Five grams of pyrophosphoric acid was heated to faint fuming to remove any excess water, 2 g. of anhydrous sodium pyrophosphate was dissolved in the hot acid, 2 g. of thiamine hydrochloride was added and the mixture held at 150–155° for twenty minutes while stirring continuously. The cooled mass was dissolved in 100 cc. of water, and a slight excess of concentrated barium hydroxide solution was added. The precipitate was removed by centrifugation and discarded, the excess barium removed by adding a slight excess of 10% sulfuric acid and centrifuging. The solution was then concentrated to 60 cc. *in vacuo*. Sufficient one normal silver nitrate solution was added to precipitate the chloride, and the precipitate was removed by centrifuging and was discarded. The solution was neutralized with ammonia and an excess of 1 *N* silver nitrate solution added. The

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(3) John Weijlard, *ibid.*, **63**, 1160 (1941).
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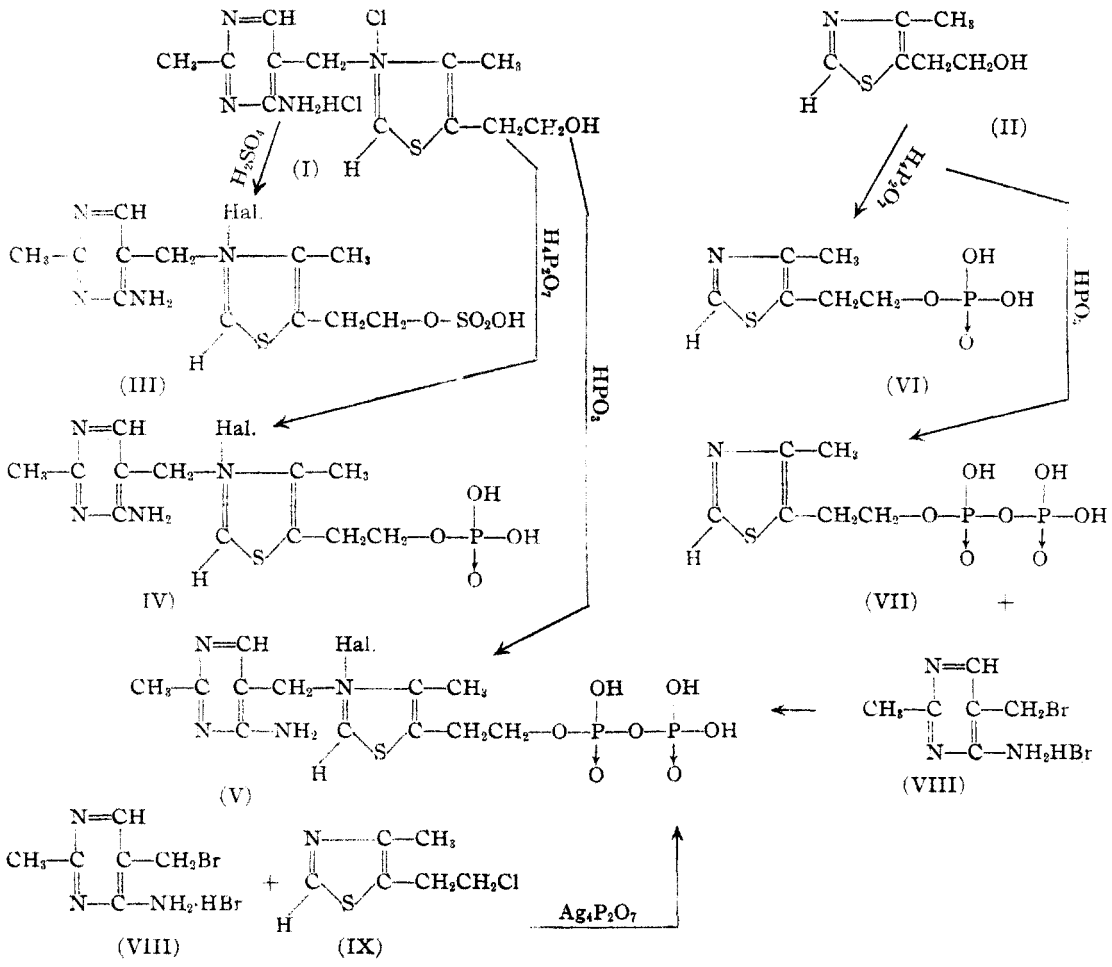

mixture was centrifuged, the silver salt washed with water and suspended in 100 cc. of water and decomposed with hydrogen sulfide. The sulfide was filtered off and the solution aerated to remove excess hydrogen sulfide. Sufficient hydrochloric acid was added to make the solution eight-tenths normal. An excess of 25% phosphotungstic acid was added, the mixture was centrifuged and the solution discarded. The solid was treated with acetone to cause disintegration of the solid and precipitation of the ester. The mixture was chilled and the supernatant liquid decanted and discarded. The acetone insoluble material was dissolved in 50 cc. of 0.1 *N* hydrochloric acid and 10 volumes of acetone added. The solution was chilled at 0° overnight and filtered. The crude ester was recrystallized twice by dissolving in 0.1 *N* hydrochloric acid to form a 5% aqueous solution, adding three volumes of alcohol and seven volumes of acetone and cooling in ice; yield, 0.40 g. m. p. 200–202°. Hydrolysis with normal acid for one-half hour at 100° produced no phosphoric acid, hence no pyrophosphoric acid ester was present.

Anal. Calcd. for $C_{12}H_{18}O_4N_4ClSP\cdot 2H_2O$: C, 34.50; H, 5.31; N, 13.42; P, 7.44. Found: C, 34.59; H, 5.53; N, 13.22; P, 7.81.

Thiamine Sulfuric Acid Ester.—One gram of anhydrous sodium pyrophosphate was added to 2.5 cc. of concentrated sulfuric acid and heated to 150°. One gram of thiamine hydrochloride was added and the mixture heated at 150° for seven minutes. The mass was dissolved in 40 cc. of water and worked up essentially as above. The ester was finally recrystallized from 12 cc. of 0.1 *N* hydrochloric acid and 60 cc. of alcohol; yield 0.54 g., m. p. 258–259° with decomposition. No phosphorus was present. Its aqueous solution gave no precipitate with barium chloride, but on prolonged boiling with dilute hydrochloric acid the ester hydrolyzed slowly and the sulfate could be precipitated.

Anal. Calcd. for $C_{12}H_{17}O_4N_4ClS_2\cdot H_2O$: C, 36.10; H, 4.80; N, 14.05; S, 16.08. Found: C, 35.73; H, 4.93; N, 14.16; S, 16.26.

4-Methyl-5-hydroxyethylthiazole Orthophosphoric Acid Ester.—Four grams of pyrophosphoric acid was heated to faint fuming. One and seventy-five hundredths grams of 4-methyl-5-hydroxyethylthiazole was added and the mixture stirred and held at 150–160° for one hour. The cooled mass was dissolved in 50 cc. of water and the phosphoric acids removed by adding a slight excess of barium hydroxide solution and centrifuging. The excess barium was removed by adding a slight excess of sulfuric acid and centrifuging. The solution was made neutral with ammonia and an excess of normal silver nitrate solution was added. The precipitate was collected by centrifugation, washed with water several times, then suspended in 50 cc. of water and decomposed with hydrogen sulfide. The

silver sulfide was removed by filtration and the solution concentrated to dryness *in vacuo* at 30°. The residue, a thick oil, was dissolved in absolute alcohol and precipitated with ether. The flocculent white precipitate which resulted was collected on a filter and dried *in vacuo;* yield, 0.45 g., m. p. 162°.

Anal. Calcd. for $C_6H_{10}O_4NSP \cdot H_2O$: C, 29.85; H, 5.01; N, 5.80; P, 12.86. Found: C, 30.00; H, 4.87; N, 5.67; P, 12.98.

4-Methyl-5-hydroxyethylthiazole Pyrophosphoric Acid Ester Isolated as the Silver Salt.[2,8]—Four cc. of 85% orthophosphoric acid was heated until heavy fumes were produced and the hot acid appeared milky (290–310°). Two grams of anhydrous sodium pyrophosphate was dissolved in the hot acid and the mixture cooled down to 150°. One gram of 4-methyl-5-hydroxyethylthiazole was added and the mixture was stirred for one-half hour at 150–155°. The mixture was cooled and dissolved in 40 cc. water and a slight excess of barium hydroxide solution was added. The precipitate was centrifuged off and washed with water. The filtrate and washings were made slightly acid to congo red with 10% nitric acid, then concentrated *in vacuo* at 35° to 30 cc. The solution was made neutral to congo with ammonia, but was still acid to litmus. A few drops of normal silver nitrate solution was added and a small amount of silver chloride filtered off. Fifteen cc. of 50% silver nitrate solution was added and the mixture concentrated at room temperature *in vacuo* to one-half volume. The silver salt crystallized out slowly in the form of needles. The mother liquor was decanted off after three days of standing and the crystals washed first with 50% alcohol, then 90% alcohol, finally 100% alcohol followed by an ether washing; yield 0.40 g.

Anal. Calcd. for $C_6H_8O_7NSP_2Ag_3 \cdot 3/10AgNO_3 \cdot 6/10HNO_3 \cdot 3H_2O$ (mol. wt., 766.6): C, 9.39; H, 1.92; N total, 3.47; N nitrate, 1.64; P, 8.09; Ag, 46.4. Found: C, 9.48; H, 1.89; N total, 3.82; N nitrate, 1.75; P, 8.00; Ag, 48.2. Hydrolysis under mild conditions (normal nitric acid for one-half hour at 100°): found 4.45% P which is 55.6% of the total phosphorus.

Thiamine Pyrophosphoric Acid Ester from Phosphorus Pentoxide Hydrated to Give Metaphosphoric Acid.—To 5 grams of phosphorus pentoxide, 0.65 g. of water was added in a closed vessel, and the mixture held at 150° until the mass was nearly transparent. Two grams of thiamine hydrochloride was added and the mixture held at 150° for one-half hour with stirring. The mass was cooled and dissolved in 100 cc. of water, then worked up as outlined in a previous paper[3]; m. p. 238–240°.

Anal. Calcd. for $C_{12}H_{19}O_7N_4ClSP_2 \cdot H_2O$: C, 30.08; H, 4.42; N, 11.71; P, 12.96. Found: C, 30.01; H, 4.35; N, 11.43; P, 13.43.

Thiamine Pyrophosphoric Acid Ester from Pyrophosphoric Acid with Added Phosphorus Pentoxide.—Five grams of pyrophosphoric acid was mixed with 1.5 g. of phosphorus pentoxide and heated until clear. A mixture of 0.5 g. of anhydrous sodium metaphosphate and 0.5 g. of anhydrous sodium pyrophosphate was added followed by 2 g. of thiamine hydrochloride. The mixture was held at 150–155° for fifteen minutes and worked up according to standard procedure[3]; yield 0.23 g. of cocarboxylase, which is the approximate yield obtained using the indefinite mixture resulting from dehydrating orthophosphoric acid.[2,3]

Anal. Calcd. for $C_{12}H_{19}O_7N_4ClSP_2 \cdot 3/4H_2O$: C, 30.41; H, 4.36; N, 11.80; P, 13.08; H_2O, 2.84. Found: C, 30.45; H, 4.39; N, 11.40; P, 12.80; H_2O, 2.75.

Condensation of 4-Methyl-5-hydroxyethylthiazole Pyrophosphoric Acid Ester and 2-Methyl-4-amino-5-bromomethylpyrimidine Hydrobromide.—A suspension of 0.33 g. of silver salt of 4-methyl-5-hydroxyethylthiazole pyrophosphoric acid ester in water was decomposed with hydrogen sulfide, the sulfide was filtered off, the filtrate concentrated *in vacuo* at room temperature to dryness and the residue dried *in vacuo;* yield, 140 mg. of 4-methyl-5-hydroxyethyl thiazole pyrophosphoric acid ester, a glass that did not readily crystallize. This ester was mixed with 150 mg. of 2-methyl-4-amino-5-bromomethylpyrimidine hydrobromide. Three cc. of liquid petrolatum was added and the mixture held at 110° for five minutes with continuous stirring. The mixture was cooled and the oil washed out with ether. The residue was dissolved in 10 cc. of water and the bromide ion removed by the addition of a slight excess of normal silver nitrate solution and centrifugation. The excess silver was precipitated with hydrochloric acid and the precipitate removed by centrifugation. The clear solution (acid to congo) was mixed with 10 volumes of acetone and the mixture chilled in the ice box overnight. The mother liquor was decanted and the residue crystallized by dissolving in 10 cc. of 0.1 *N* hydrochloric acid, adding 30 cc. of alcohol and 70 cc. of acetone and cooling in ice for two days. After decantation, the crystals were washed with alcohol and ether; yield, 110 mg. The cocarboxylase activity indicated a yield of about 10% cocarboxylase.

Condensation of 5,β-Chloroethyl-4-methylthiazole with 2-Methyl-4-amino-5-bromo-methylpyrimidine Hydrobromide in Presence of Silver Pyrophosphate.—One-half gram of 5,β-chloroethyl-4-methylthiazole (distilling at 82.8–83.8° at 2 mm.), 2 g. of anhydrous silver pyrophosphate and 0.9 g. of 2-methyl-4-amino-5-bromomethylpyrimidine hydrobromide were mixed rapidly with 5 cc. of liquid petrolatum and held at 110° for ten minutes with stirring. The cooled mixture was washed with ether to remove the oil, then disintegrated by shaking with 30 cc. of water and the solution made acid to congo with hydrochloric acid. The solid was centrifuged off and the clear solution mixed with 10 volumes of acetone, then chilled overnight. The crude material was recrystallized as outlined above; yield 0.15 g. The cocarboxylase activity indicated about 10% cocarboxylase.

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Errata.—The formulas for analyses in the previous article[3] are given as $C_{12}H_{21}O_8N_4ClSP_2 \cdot {}^1/_2\,H_2O$. They should read: $C_{12}H_{19}O_7N_4ClSP_2 \cdot {}^1/_2\,H_2O$.

Summary

It has been shown that pure pyrophosphoric acid produces the orthophosphoric acid esters of thiamine as well as of 4-methyl-5-hydroxyethyl thiazole, and that metaphosphoric acid or phosphorus pentoxide calculated to give metaphosphoric acid is necessary to form the pyrophosphoric acid ester of thiamine (cocarboxylase). Thiamine sulfuric acid ester has been prepared as well as the pyrophosphoric acid ester of 4-methyl-5-hydroxyethyl thiazole. The latter ester was condensed with the pyrimidine portion of cocarboxylase to give a product with cocarboxylase activity. A similar condensation was carried out with the pyrimidine portion and chlorothiazole in the presence of silver pyrophosphate, the isolated reaction product showing cocarboxylase activity.

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[CONTRIBUTION FROM THE DIVISIONS OF ANIMAL HUSBANDRY AND CHEMISTRY, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA AT DAVIS]

Absorption of Oxygen by Glutathione in Alkaline Solutions. I. Kinetics of the Reaction at *p*H 9 to 11

BY M. B. YOUNG AND H. A. YOUNG

It has been shown[1] that, in the presence of a copper salt as catalyst and at a *p*H below approximately 8.5, a solution of glutathione is oxidized by oxygen from the mercaptan to the disulfide, that the rate of the reaction increases with increase in *p*H, and that the factor determining the rate is the concentration of the dissociated ion.

It has also been shown[2] that as the *p*H is increased beyond 8.5, the reaction becomes more complex both with regard to the products formed and to the interpretation of the reaction rates. From approximately *p*H 9 to 11, the volume of oxygen absorbed per mole of glutathione is slightly greater than that calculated to change the mercaptan to the disulfide. The absorption, however, comes to a definite and abrupt end and there is no indication of a further reaction involving the formation of an oxidation product higher than the disulfide. Colorimetric tests with titanium sulfate have shown this excess absorption to be caused by the production of small amounts of hydrogen peroxide, which appears to be quite stable, particularly at *p*H 9, even in the presence of the copper catalyst. As the *p*H increases beyond 11, the reaction involving the disulfide formation

$$2GS^- + {}^1/_2O_2 + H_2O = GSSG + 2OH^- \quad (a)$$

is followed by another reaction involving oxygen and the disulfide produced n (a) and indicated by a continued slow increase in oxygen absorption. This follow reaction increases in rate as hydroxide ion increases while the rate of reaction (a) remains essentially constant until finally all discontinuity in the resulting curve is lost. The volume of oxygen absorbed indicates that the principal product formed is the salt of the sulfinic acid of glutathione and that the follow reaction is

$$GSSG + {}^3/_2O_2 + 2OH^- = 2GSO_2^- + H_2O \quad (b)$$

Again the oxygen absorbed is a few per cent. high, possibly caused by the formation of more hydrogen peroxide or some sulfonic acid.

The reaction has been studied over the *p*H range 9 to 13.3 with glutathione concentration 0.00110 to 0.00880 molar, copper sulfate concentration 0 to 25 $\times$ 10^{-6} molar and oxygen pressure 0.2 to 1.0 atm. The results of three exemplary experiments are shown in Fig. 1 in which approximately equal amounts of glutathione, 5 cc. of 0.00220 molar solution in the presence of copper sulfate of approximately 5 $\times$ 10^{-6} mole per liter, were oxidized by oxygen at 1 atmosphere at hydroxide ion concentrations of 10^{-5}, 0.0431, and 0.171 mole per liter, respectively. The lines A and B indicate the calculated volumes of oxygen to change the mercaptan to the disulfide and to the sulfinic acid, respectively. There is a distinct break in curve 2 near A.

The present publication is concerned with an attempt to explain the kinetics of the simplest of the reactions, *i. e.*, the oxidation of the mercaptan to the disulfide as it occurs at *p*H 9 to 11.

Experimental

The experiments were carried out in the Warburg apparatus at 37°. Four cc. of glutathione

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