[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

Researches on Pyrimidines. CXL. Pyrimidines Derived from Carbethoxymalonic Aldehyde

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Ethyl γ,γ' - diethoxyacetoacetate, $(C_2H_5O)_2CHCOCH_2COOC_2H_5$, was used by Johnson and Cretcher[2] as the basis of their synthesis of uracil-4-aldehyde. An investigation of an isomeric ketone ester which was expected to serve for the synthesis of uracil-5-aldehyde has now been undertaken. This ketone ester, represented by formula (A), should theoretically be formed by application of a Claisen condensation between ethyl β,β'-diethoxypropionate and ethyl formate. The product obtained, however, reacted in accordance with the constitution of the sodium salt of carbethoxymalonic aldehyde (B), instead of the salt of the expected acetal derivative (A).

$(C_2H_5O)_2CHCH(CHO)COOC_2H_5$ (A)

$OHCCH(CHO)COOC_2H_5$ (B)

The salt obtained, I, gave with aqueous copper sulfate an immediate precipitate of a crystalline copper salt conforming to formula II. Moreover, when an aqueous solution of the salt I was treated with ethyl pseudothiourea hydrobromide, an addition product of the composition V was formed at once. Furthermore, the salt I reacted with phenylhydrazine in acetic acid solution to give 1-phenyl-4-carbethoxypyrazole,[3] III.

$OHCC(COOC_2H_5)=CHONa$

I

$[OHCC(COOC_2H_5)=CHO-]_2Cu$

II

$C_6H_5NN=CHC(COOC_2H_5)=CH$

III

$OHCC(COOC_2H_5)=CHOK$

IV

$NH=C(SC_2H_5)NH_2OHCC(COOC_2H_5)=CHOH$

V

Information about the composition of the salt I was necessarily secured by a study of its derivatives, since the salt itself could not be obtained in a sufficiently pure condition for analysis, nor could the free carbethoxymalonic aldehyde be isolated. A similar behavior of nitromalonic aldehyde was observed by Hill and Torrey.[4]

The addition product of carbethoxymalonic aldehyde and pseudoethyl thiourea, V, was stable enough to permit recrystallization and analysis. The molecular composition indicated by the analyses was supported by the formation, upon saponifying the substance with alcoholic potash, of a crystalline potassium salt having the formula IV. Moreover, the compound V gave with phenyl hydrazine in acetic acid the same pyrazole as the sodium salt I. Additive compounds similar in type to the product V were obtained by Behrend and Ernert[5] from the interaction of certain ureas with acetoacetic ester.

The addition product V could be dehydrated by acetic anhydride to form the pyrimidine VI, which was transformed by the usual methods into the pyrimidines VII, VIII and IX, respectively. The compounds VIII and IX are of interest in the study of fundamental pyrimidine types, because they represent simple substitution products of 2-oxypyrimidine.[6]

$N=C(SC_2H_5)N=CHC(COOC_2H_5)=CH$

VI

$N=C(SC_2H_5)N=CHC(COOH)=CH$

VII

$NHCON=CHC(COOH)=CH$

VIII

$NHCON=CHC(COOC_2H_5)=CH$

IX

$NH_2CON=CHC(COOC_2H_5)=CHOH$

X

Other derivatives of carbethoxymalonic aldehyde were prepared by condensing the sodium salt I with urea and thiourea to form the ureide X and the thioureide XI. These compounds are analogous to Hale and Brill's[7] derivatives of nitromalonic aldehyde. The ureide X could be closed to form a ring only with great difficulty, since most dehydrating agents were ineffective or caused the splitting off of urea. By the use of alcoholic sodium ethylate, however, a small quantity of the cyclic ester IX was obtained from X.

(1) Research Fellow in Organic Chemistry of the Chemical Foundation, Inc., 1932-1933.

(2) Johnson and Cretcher, THIS JOURNAL, **37**, 2145 (1915).

(3) Wislicenus and Bindemann, *Ann.*, **316**, 36 (1901).

(4) Hill and Torrey, *Am. Chem. J.*, **22**, 90 (1899).

(5) Behrend and Ernert, *Ann.*, **233**, 11 (1886).

(6) Johnson and Joyce, THIS JOURNAL, **37**, 2163 (1915).

(7) Hale and Brill, *ibid.*, **34**, 83, 295 (1912).

The thioureide XI was dehydrated by heat to give a cyclic compound which differed in properties from Hale's metathiazine,[7] and which appeared to be a thiopyrimidine as expressed by formula XII. This substance was soluble in alkali and gave with chloroacetic acid a product in which the acetic acid radical was attached to the sulfur, XIII. This latter structure was shown by the ready elimination of thioglycolic acid upon hydrolysis.

$NH_2CSN{=}CHC(COOC_2H_5){=}CHOH$

XI

$NHCSN{=}CHC(COOC_2H_5){=}CH$

XII

$N{=}C(SCH_2COOH)N{=}CHC(COOC_2H_5){=}CH$

XIII

Experimental

Ethyl β,β′-Diethoxypropionate.[8]—The following modification of Sugasawa's method[9] was used. Fifty grams of the powdered sodium salt of ethyl formylacetate[10] was added in small portions, during stirring and cooling, to 370 g. of absolute ethanol containing 60 g. of dry hydrogen chloride. The mixture was stirred for a total of three hours, neutralized with dry sodium bicarbonate, and filtered from the inorganic salts, which were washed thoroughly with absolute ethanol. The filtrate and washings were concentrated under reduced pressure at 60°, and the residual oil distilled. Twenty-four grams of colorless acetal was obtained, which was analyzed after one redistillation; b. p. 65° at 2 mm.; n_D^{25} 1.4101.

Anal. Calcd. for $C_9H_{18}O_4$: C, 56.81; H, 9.54. Found: C, 56.82; H, 9.35.

Sodium Salt of Carbethoxymalonic Aldehyde, I.—When a mixture of 0.1 mole of ethyl β,β′-diethoxypropionate and 0.12 mole of neutral, freshly distilled ethyl formate was dropped slowly into 90 cc. of dry ether containing 0.12 mole of powdered sodium, hydrogen was evolved and the sodium dissolved, forming a red solution. After standing for at least twenty-four hours, the ether was evaporated in the cold. The residual gum, when treated with a very small quantity of water, yielded a precipitate of a salt which was shown by its reactions to consist chiefly of the sodium salt of carbethoxymalonic aldehyde, I.

This was insoluble in ether, slightly soluble in alcohol, moderately soluble in water, but could not be made to crystallize from any solvent nor could it be purified completely. An aqueous solution of the salt, when treated with phenylhydrazine and glacial acetic acid, deposited pale yellow needles of 1-phenyl-4-carbethoxypyrazole,[11] which melted at 99–100°[12] after recrystallization from aqueous acetic acid. The substance was identified by Knorr's pyrazole reaction,[13] by saponification to give 1-phenyl-4-carboxypyrazole,[14] and by analysis.

Anal. Calcd. for $C_{12}H_{12}O_2N_2$: N, 12.97. Found: N, 12.89.

Attempts to obtain the free carbethoxymalonic aldehyde by mild acid hydrolysis of the sodium salt suspended in ether gave a mixture of unidentified oils and crystals of ethyl trimesate, m. p. 133°. This was identified by a mixed melting point with an authentic specimen. In the pyrimidine condensation described below the solid salt was ordinarily not isolated, but was used in the form of the red gum or its aqueous solution.

Copper and Potassium Salts of Carbethoxymalonic Aldehyde, II and IV.—The copper salt II was precipitated from an aqueous solution of the sodium salt I by interaction with copper sulfate, and the green precipitate obtained was washed with dilute sulfuric acid. One gram of the copper salt was dissolved in 530 cc. of boiling water, and on cooling 0.8 g. separated in the form of narrow blue-green plates, which melted with decomposition at about 205°.

Anal. Hydrate. Calcd. for $C_{12}H_{14}O_8Cu + 2H_2O$: H_2O, 9.34. Found: H_2O, 9.40. Anhydrous form (dried at 76°, 20 mm.). Calcd. for $C_{12}H_{14}O_8Cu$: C, 41.18; H, 4.04; Cu, 18.18. Found: C, 41.20; H, 4.00; Cu, 17.96.

The potassium salt IV was obtained by saponification of the ethyl pseudothiourea addition product V with potassium hydroxide in boiling absolute ethanol solution. The salt crystallized from absolute ethanol (1 g. soluble in 50 cc. of boiling ethanol) in the form of colorless plates, m. p. 264° with decomposition.

Anal. Calcd. for $C_6H_7O_4K$: C, 39.51; H, 3.87; K, 21.46. Found: C, 39.89; H, 4.03; K, 21.48.

Addition Product of Carbethoxymalonic Aldehyde and Ethyl Pseudothiourea, V.—The salt of carbethoxymalonic aldehyde prepared from 0.1 mole of ethyl β,β′-diethoxyacetate was dissolved in 100 cc. of cold water and treated with 0.12 mole of ethyl pseudothiourea hydrobromide. Stirring caused the immediate precipitation of 11.8 g. of a molecular addition product of carbethoxymalonic aldehyde and ethyl pseudothiourea V (46% yield). The product, melting at 143°, with effervescence, was crystallized from absolute ethanol. Eleven grams dissolved in 200 cc. of boiling absolute ethanol, and upon cooling the solution, 5.5 g. separated in the form of colorless transparent blocks, melting at 143.5–144.5° with effervescence. Concentration of the filtrate caused some decomposition.

Anal. Calcd. for $C_9H_{16}O_4N_2S$: C, 43.50; H, 6.50; N, 11.29; S, 12.92. Found: C, 43.72; H, 6.58; N, 11.14; S, 12.66.

The mother liquor from the reaction product yielded upon further standing 0.2 g. of 2-ethylmercapto-5-carbethoxypyrimidine, VI.

The addition product V was soluble in dilute acid or alkali with decomposition. When dissolved in warm acetic acid and treated with phenylhydrazine, a pyrazole was formed which was identical with the 1-phenyl-4-carbethoxypyrazole obtained from the salt I. Hydrolysis

(8) We are indebted to Miss Anne E. Litzinger for an experimental study of the preparation of this substance.

(9) Sugasawa, *J. Pharm. Soc. Japan.*, **545**, 551 (1927); *Chem. Zentr.*, II, 1814 (1927).

(10) Wislicenus, *Ber.*, **20**, 2931 (1887).

(11) Wislicenus and Bindemann, Ref. 4, reported the melting point of 1-phenyl-4-carbethoxypyrazole as 96–97°.

(12) All melting points given in this paper are corrected.

(13) Knorr, *Ann.*, **238**, 200 (1887).

(14) Claisen, *ibid.*, **295**, 319 (1897).

of the product V with aqueous alkali, followed by treatment with phenylhydrazine in acetic acid solution, gave 1-phenyl-4-carboxypyrazole, which corresponded in properties and analysis to Claisen's compound.[14]

Anal. Calcd. for $C_{10}H_8O_2N_2$: N, 14.90. Found: N, 14.84.

The addition product V underwent saponification with alcoholic potassium hydroxide with the formation of the potassium salt IV. Dehydration of the addition product yielded the pyrimidine VI. Attempts to obtain a similar addition product from methyl pseudothiourea sulfate and the salt I were unsuccessful.

2-Ethylmercapto-5-carbethoxypyrimidine, VI.—A solution of 5.6 g. of the addition product V in 15 cc. of acetic anhydride was heated for four hours at 100°. The acetic anhydride was evaporated by a stream of air, and the residue was iced and diluted with water. The precipitate obtained was purified by dissolving in a warm mixture of 30 cc. of alcohol and 10 cc. of water. This solution, on slow cooling, deposited 4.1 g. of colorless rhombic plates, m. p. 47–48°. The pyrimidine VI is easily soluble in most organic solvents and concentrated acid. It is soluble in water and alkali.

Anal. Calcd. for $C_9H_{12}O_2N_2S$: N, 13.21; S, 15.13. Found: N, 13.03, 13.00; S, 15.05.

2-Ethylmercapto-5-carboxypyrimidine, VII.—This acid was formed by careful hydrolysis of the ester VI. A solution of 2.4 g. of the ester VI dissolved in 8 cc. of ethanol was treated with 8 cc. of a 10% alcoholic potassium hydroxide solution, and the mixture was heated to boiling. It was then cooled, the solvent evaporated, and the residue in aqueous solution was acidified with cold dilute hydrochloric acid. Two grams of the mercapto acid was obtained, which was recrystallized from water. One gram dissolved in 225 cc. of boiling water, and 0.7 g. separated on cooling as long, colorless needles, m. p. 182–183°.

Anal. Calcd. for $C_7H_8O_2N_2S$: N, 15.21; S, 17.43. Found: N, 15.13; S, 17.00.

2-Keto-5-carboxypyrimidine, VIII.—This acid was prepared by hydrolysis of the mercapto ester VI or the acid VII. A solution of 1 g. of the mercapto ester VI in 10 cc. of concentrated hydrochloric acid was heated on the steam-bath under reflux for four hours, and then evaporated to dryness. The residue consisted of an unstable hydrochloride of the pyrimidine, which gave 0.45 g. of the acid on hydrolysis. The product was recrystallized in 10 cc. of boiling water, from which it separated on cooling as colorless prisms (0.3 g.). The substance did not melt, but gradually turned brown above 220°.

Anal. (Hydrate). Calcd. for $C_5H_4O_3N_2 + H_2O$: H_2O, 11.39. Found: H_2O, 11.25. Anhydrous form, dried at 100°. Calcd. for $C_5H_4O_3N_2$: C, 42.86; H, 2.88; N, 20.02. Found: C, 42.87; H, 3.02; N, 19.96.

The pyrimidine VIII is easily soluble in either acid or alkaline solution.

2-Keto-5-carbethoxypyrimidine, IX.—This pyrimidine was obtained by esterification of the acid VIII or in small yield by closure of the acylic ureide X with sodium ethylate. The former method, carried out with absolute ethanol and hydrogen chloride, gave a mixture of the ester IX, melting at 163–164° when pure, with a substance melting at 186–187°, which has not been fully studied. The ester IX crystallized from water in the form of colorless plates containing a mole of water.

Anal. (Hydrate). Calcd. for $C_7H_8O_3N_2 + H_2O$: H_2O, 9.68. Found: H_2O, 9.61. (Anhydrous form). Calcd. for $C_7H_8O_3N_2$: C, 49.97; H, 4.80; N, 16.67. Found: C, 49.95; H, 4.93; N, 16.77.

Ethyl β-Urea-α-formylpropionate, X.—The ether solution resulting from the condensation of 16.4 g. of ethyl β,β′-diethoxypropionate with ethyl formate and sodium was aerated to remove the ether, and then treated with a solution of 6.2 g. of urea in 60 cc. of glacial acetic acid. The mixture was warmed and shaken until solution was complete, and then allowed to stand for twenty-four hours. After evaporation of the solvent *in vacuo*, the residue was diluted with 200 cc. of water. A precipitate of 5.5 g. of ureide X was obtained (34% yield) which was recrystallized from 50 cc. of boiling ethanol. The product separated on cooling in the form of dense clusters of short needles, 4.3 g., m. p. 175° with effervescence. The same substance was obtained by using acetic anhydride instead of acetic acid.

Anal. Calcd. for $C_7H_{10}O_4N_2$: C, 45.14; H, 5.42; N, 15.06. Found: C, 45.24; H, 5.39; N, 15.00.

This ureide was neutral to litmus, soluble in alkali and acid, and did not reduce Fehling's solution nor ammoniacal silver nitrate. The ureide could be transformed into the pyrimidine IX in 16% yield by mild hydrolysis with one equivalent of sodium ethylate. Other agents for closing the ring, such as heat, acid, acetic anhydride and zinc chloride, either caused decomposition, or were ineffective.

Ethyl β-Thiourea-α-formylpropionate, XI.—The sodium salt obtained from the condensation of 20 g. of ethyl β,β′-diethoxypropionate with ethyl formate was dissolved in 150 cc. of water, 9.6 g. of thiourea (1.2 equiv.) was added, and the mixture was treated with concentrated hydrochloric acid during stirring and cooling until no further precipitate or oil was produced. The stirring was continued for five hours, and the mixture was placed in the ice-box for sixteen hours. The powdery product consisted of 7.3 g. (34% yield) of the thioureide, which was crystallized from absolute ethanol. One gram was soluble in about 15 cc. of boiling, and in 50 cc. of cold absolute ethanol.

Anal. Calcd. for $C_7H_{10}O_3N_2S$: N, 13.86; S, 15.87. Found: N, 13.72; S, 15.83.

Dehydration of the thioureide XI to give the thiopyrimidine XII could be partially accomplished by heating with alcoholic sodium ethylate, or concentrated hydrochloric acid, but most successfully by heating *in vacuo*.

2-Thio-5-carbethoxypyrimidine, XII.—When 1.7 g. of the thioureide XI was heated at 150–160° under 3 mm. pressure for one hour, water was evolved, and 0.9 g. of the thiopyrimidine XII sublimed and condensed on the cooled portions of the flask. The substance took the form of bright yellow needles, m. p. 214–216° with decomposition. The residue was a tar.

Anal. Calcd. for $C_7H_8O_2N_2S$: C, 45.60; H, 4.38; N, 15.21; S, 17.43. Found: C, 45.57; H, 4.59; N, 15.14; S, 17.69.

The thiopyrimidine is slightly soluble in boiling water (1 g. in 300 cc.) in boiling ethanol (1 g. in 90 cc.) and soluble in alkali and acids. It sublimes readily below its melting point.

2-Mercaptoacetic Acid 5-Carbethoxypyrimidine, XIII.—A solution of 1 g. of thiopyrimidine XII and 1 g. of chloroacetic acid in 125 cc. of hot water was boiled for half an hour. On cooling, 0.95 g. of the mercaptopyrimidine XIII separated as long needles which melted at 175–176.5° after one recrystallization from water. One gram of the substance was soluble in about 100 cc. of boiling and in 500 cc. of cold water.

Anal. Calcd. for $C_9H_{10}O_4N_2S$: N, 11.52; S, 13.25. Found: N, 11.42; S, 12.85.

The pyrimidine dissolved in acid with the evolution of thioglycolic acid. The residue left after acid hydrolysis has not yet been identified.

Summary

1. Salt derivatives of carbethoxymalonic aldehyde were obtained by application of a Claisen condensation with ethyl β,β'-diethoxypropionate and ethyl formate. Crystalline copper and potassium salts were isolated in addition to an amorphous sodium salt.

2. The interaction of the sodium salt of carbethoxymalonic aldehyde with (a) urea, (b) thiourea, and (c) pseudoethylthiourea hydrobromide resulted in the formation of (a) a monoureide, (b) a monothioureide and (c) a molecular addition product of pseudoethylthiourea and carbethoxymalonic aldehyde. Each of these substances, when dehydrated, formed the corresponding (a) 2-keto, (b) 2-thio and (c) 2-mercaptopyrimidines.

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The Dissociation Constant of Nitrogen-Nitrogenase in Azotobacter

BY HANS LINEWEAVER, DEAN BURK AND W. EDWARDS DEMING

This paper presents the results of an investigation concerning the nature of the dissociation of the complex formed between nitrogen gas and the enzyme nitrogenase in nitrogen fixation by Azotobacter at ordinary temperatures and pressures.

Methods for the evaluation of dissociation constants of enzymic and other reactions have been extended recently by Lineweaver and Burk.[1] The results to be presented here show that the mechanism of nitrogen fixation by Azotobacter may be represented by

$$N_2 + E \underset{v_2}{\overset{v_1}{\rightleftharpoons}} N_2E \xrightarrow{v} E + P \qquad (1)$$

$$v = k'(N_2)(E) = k(N_2E) \qquad (2)$$

$$K_{N_2} = (E)(N_2)/(N_2E) \qquad (3)$$

where one molecule of N_2 combines reversibly with one molecule, or independently reacting group, E (nitrogenase) in the Azotobacter cells. v and k are the velocity and velocity constant of the irreversible decomposition of N_2E (nitrogen-nitrogenase), v_1 and v_2 are the velocities of reversible formation and dissociation of N_2E, K_{N_2} is the respective dissociation constant at equilibrium, and P is the reaction product. (N_2E) is a rectangularly hyperbolic function of (N_2).

This type of mechanism leads to the equation

$$1/v = K_{N_2}/V_{max.}(N_2) + 1/V_{max.} \qquad (4)$$

where $V_{max.}$ is a numerical constant representing the maximum velocity at high nitrogen pressures where the enzyme exists completely in the form N_2E ($V_{max.} = k(E_{total})$). A plot of the reciprocal of the velocity against the reciprocal of the nitrogen pressure ($1/v$ against $1/(N_2)$) yields a straight line whose slope $K_{N_2}/V_{max.}$ and ordinate intercept $1/V_{max.}$ evaluate K_{N_2}, as illustrated in Fig. 1. K_{N_2} is established as a thermodynamic dissociation constant if it does not vary as a function of k, or $V_{max.}$, that is, if v is negligible compared to v_2 (*cf.* also Haldane,[2] p. 40). In this study, several factors influencing k have been varied, *viz.*, P_H, humic acid concentration, oxygen pressure and temperature.[3] Several other factors have also been varied, *viz.*, concentrations of calcium, strontium and oxalate, and P_H, which were previously shown[3,4] to be specific in fixation as distinguished from Azotobacter growth. These might alter $V_{max.}$ by altering the concentration

(1) H. Lineweaver and D. Burk, "The Determination of Enzyme Dissociation Constants," *in press.*

(2) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930.

(3) D. Burk and R. T. Milner, *Ind. Eng. Chem., Anal. Ed.*, **4**, 3 (1932).

(4) D. Burk, H. Lineweaver and C. K. Horner, *J. Bact.*, in press; *cf.* also D. Burk, "Azotase and Nitrogenase in Azotobacter," a review chapter in "Ergebnisse der Enzymforschung," by F. F. Nord and R. Weidenhagen, Vol. III, in press, Leipzig, 1934.