[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

RESEARCHES ON PYRIMIDINES. CIV. ISOURACIL AND ITS DERIVATIVES. A PRELIMINARY STUDY OF THE METHODS OF SYNTHESIS

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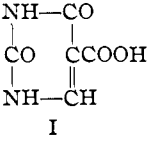
The Constitution of Orotic Acid

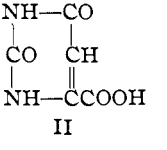
Various chemical aspects of the vitamin problem have stimulated an interest in the study of the chemistry of isouracil and its derivatives. Our interest in this type of pyrimidine compounds as possible vitamine constructions has been stimulated as a result of the continuation of some pyrimidine research which was inaugurated as early as 1907.[2] This research, which was of a preliminary nature, had to do with the question of determining whether orotic acid, $C_5H_4O_4N_2 \cdot H_2O$, is to be represented structurally as a pyrimidine compound.

Biscaro and Belloni[3] reported the isolation of this organic acid from milk whey in 1905 and described several of its derivatives. Sufficient experimental evidence was produced by these investigators to show that the acid is probably a heterocyclic compound having an ureide structure but insufficient data were obtained to enable them to draw a positive conclusion regarding its constitution.

The properties of orotic acid suggested the possibility of its being a pyrimidine having the constitution of one of the uracil-carboxylic acids represented by Formulas I and II, respectively. Wheeler, Johnson and Johns[2] prepared the compound represented by Formula I, and later Wheeler[4] made a careful study of the isomeric pyrimidine II. Neither of these two pyrimidines possessed the specific properties which characterized the orotic acid described by Biscaro and Belloni. The non-identity of the



I



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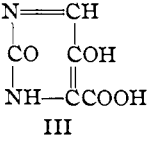


III

two pyrimidines I and II with orotic acid being established, there now remained to be considered the third pyrimidine possibility, namely, isouracil-carboxylic acid represented by Formula III. The further study of this

[1] Constructed from a dissertation presented by William T. Caldwell, in June, 1923, to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy. (T. B. J.)

[2] Wheeler, Johnson and Johns, *Am. Chem. J.*, **37**, 392 (1907).

[3] Biscaro and Belloni, *Estratto dell'Annuario della Soc. Chimica di Milano*, II, fasc. 1 (1905); *Chem. Zent.*, I, 63, 64 (1905); II, fasc. 2 (1905).

[4] Wheeler, *Am. Chem. J.*, **38**, 358 (1907).

interesting problem has now introduced us to many new features of pyrimidine chemistry which promise to become of immediate biochemical interest. It is not improbable that the easiest approach to isouracil, NCONHCH=C(OH)CH, which is the only 2-oxypyrimidine isomeric with uracil, NHCONHCH=CHCO, will be by decarboxylation of isouracilcarboxylic acid, III. Tafel and Houseman[5] described what they believed to be two isomeric modifications of this pyrimidine but state that, in consequence of the fact that all attempts to rearrange one form into the other were unsuccessful, the formulas assigned were given with reservation. No method has yet been developed for the synthesis of an isouracil of known structure. This paper contains a preliminary account of new work in this field.

It is of interest to note at this time that Biscaro and Belloni also made a preliminary study of the physiological action of orotic acid. Injection of an aqueous solution of the potassium salt into a frog produced a violent excitation followed by a complete paralysis of the central nervous system. The same experiment repeated on guinea pigs with corresponding body doses produced less violent results, indicating, therefore, that orotic acid is apparently tolerated in some degree by warm-blooded animals. A remarkable property reported as characteristic of orotic acid is its specific affinity for potassium. The acid is said to be able to liberate hydrochloric and nitric acids in aqueous solutions of their respective potassium salts. The Italian investigators postulated that orotic acid is possibly a natural factor influencing the relative proportions of sodium and potassium found in the different fluids and in muscular tissues of the animal body. To date no exhaustive physiological examination of any pyrimidine-carboxylic acids has been carried on. In the light of the known physiological activity of the different carboxyl derivatives of pyridine and quinoline, for example, it seems not improbable that some of the carboxyl derivatives of the reduced forms of pyrimidine may prove interesting pharmacologically.

Our present research has been confined to the study of two different methods proposed for synthesizing carboxyl derivatives of isouracil. Neither of the two procedures studied has led thus far to practical results. In method one we started with ethyl diethoxy-acetoacetate[6] IV, and prepared the mercapto-pyrimidine V. This proved to be a compound characterized by very desirable properties and was easily converted into the pyrimidine VI by interaction with phosphorus pentachloride. By reduction of this halogen compound we were also successful in synthesizing 2-ethylmercapto-4-ethoxymethyl-5-ethoxypyrimidine VII, which on hydrolysis apparently yields smoothly 2-oxy-4-ethoxymethyl-5-ethoxypyrimi-

[5] Tafel, *Ber.*, **34**, 258 (1901); Tafel and Houseman, *Ber.*, **40**, 8743 (1907).

[6] See Johnson and Hadley, THIS JOURNAL, **38**, 1850 (1916).

dine VIII. The method of operating, involving as it does so many steps, does not yield this pyrimidine, however, in sufficient quantity to proceed through the last two steps of the synthesis represented by Formulas IX and III.

An interesting pyrimidine obtained in our research was the alcohol represented by either of the two formulas, X and XI. This proved to be very resistant to the action of hydrochloric acid, a result quite in accord with the behavior of other alcohols of this type in the uracil series.[7]

IV $C_2H_5OCH_2COCH(OC_2H_5)COOC_2H_5$

V $\overline{NHC(SC_2H_5)=NC(CH_2OC_2H_5)=C(OC_2H_5)CO}$

VI $\overline{NC(SC_2H_5)=NC(CH_2OC_2H_5)=C(OC_2H_5)CCl}$

VII $\overline{NC(SC_2H_5)=NC(CH_2OC_2H_5)=C(OC_2H_5)CH}$

VIII $\overline{NCONHC(CH_2OC_2H_5)=C(OC_2H_5)CH}$

IX $\overline{NCONHC(CH_2OH)=COHCH}$ X $\overline{NHCONHC(CH_2OH)=C(OH)CO}$

XI $\overline{NHCONHCH(CH_2OH)COCO}$

In method two the starting point was the compound ethyl oxalo-ethoxy-acetate, XII. This was found to interact with pseudo-ethylthiourea with formation of the pyrimidine XIII, but less favorably than the ketone ester IV under similar conditions. This observation is quite in accord with that of Wheeler,[8] who failed to obtain mercapto-pyrimidines by condensing pseudothioureas with ethyl oxalo-acetate. On the other hand, Johnson[9] found that ethyl oxalopropionate condenses smoothly with pseudothioureas. The pyrimidine XIII is converted by hydrolysis in hydrochloric acid solution into its corresponding diethoxy derivative, XIV, the mono-ethoxy compound, XV, and finally to isobarbituric acid XVIII with loss of carbon dioxide. It has been found also that the mercapto-pyrimidine XIII interacts normally step by step (XVI ⟶ XVII ⟶ III) up to the last stage of the synthesis, but not until we have found more favorable experimental conditions for the pseudothiourea condensation will it be practicable to apply this method of synthesis for the production of isouracil-carboxylic acid, III, in quantities needed for our researches.

XII $C_2H_5OOCCOCH(OC_2H_5)COOC_2H_5$

XIII $\overline{NHC(SC_2H_5)=NC(COOC_2H_5)=C(OC_2H_5)CO}$

XIV $\overline{NHCONHC(COOC_2H_5)=C(OC_2H_5)CO}$

XV $\overline{NHCONHC(COOH)=C(OC_2H_5)CO}$

[7] Johnson and Chernoff, THIS JOURNAL, **35**, 585 (1913); see Kircher, *Ann.*, **385**, 293 (1911).

[8] Wheeler, *Am. Chem. J.*, **38**, 358 (1907).

[9] Johnson, *J. Biol. Chem.*, **3**, 299 (1907).

XVI $\overline{NC(SC_2H_5)=NC(COOC_2H_5)=C(OC_2H_5)CCl}$

XVII $\overline{NC(SC_2H_5)=NC(COOC_2H_5)=C(OC_2H_5)CH}$ XVIII $\overline{NHCONHCH=C(OH)CO}$

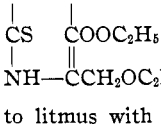
III $\overline{NCONHC(COOH)=COHCH}$

Biscaro and Belloni state that orotic acid fuses with decomposition at 260°. As to whether this is a sharp and definite decomposition point we have no knowledge, but it is an interesting fact that the acid III, obtained by us according to the second outlined method of synthesis, melted with decomposition at the same temperature, 259–260°.

A complete record of our experimental work is given in the final section of this paper. This investigation is being continued by other co-workers in this Laboratory.

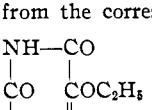
Experimental Part

2-Thio-4-ethoxymethyl-5-ethoxy-6-oxypyrimidine.—This pyrimidine was prepared by condensing thiourea with ethyl α,γ-diethoxyacetoacetate as follows: 7.6 g. of thiourea and 21 g. of ethyl α,γ-diethoxyacetoacetate were added to a solution of sodium alcoholate made by dissolving 2.3 g. of metallic sodium in 75 cc. of absolute alcohol. The resulting mixture was then refluxed in an oil-bath for eight hours, during which time the liquid changed in color from orange to dark red. On cooling, needle-shaped crystals separated from the solution. The alcohol then was distilled off under diminished pressure and the residue dissolved in 100 cc. of water and made acid to litmus with hydrochloric acid. Crystals of the pyrimidine separated and were purified by recrystallization from water after decolorizing with animal charcoal. The thiopyrimidine is practically insoluble in cold water and requires about 100 parts of boiling water for solution. It crystallizes in colorless needles, giving strong tests for nitrogen and sulfur, and melts at 178°.



Anal. Calcd. for $C_9H_{14}O_3N_2S$: N, 12.17. Found: N, 12.28, 12.29.

2-Oxy-4-ethoxymethyl-5-ethoxy-6-oxypyrimidine.—This pyrimidine was obtained from the corresponding thio-compound by the action of chloro-acetic acid. 2.53 g. of the above thiopyrimidine was treated with 3.16 g. of chloro-acetic acid in 60 cc. of distilled water and the mixture refluxed for five hours. On cooling, the 2-oxypyrimidine separated in large crystals and melted to a colorless liquid at 168°. The yield was 1.93 g., or 82% of the theoretical. The sulfur from the thiopyrimidine remained in the mother liquid combined in the form of thioglycolic acid, as shown by the characteristic and beautiful color test with ferric chloride and ammonia.



Anal. Calcd. for $C_9H_{14}O_4N_2$: N, 13.08. Found: N, 13.13.

2-Oxy-4-hydroxy-5-hydroxy-6-oxypyrimidine, IX.—0.92 g. of the above pyrimidine was placed in a pressure tube with 10 cc. of concentrated hydrochloric acid, sealed and heated for two hours at 120–140°. After cooling, the tube was opened and the gas which escaped burned with a green flame; the odor of ethyl chloride was noticeable. Before heating, the pyrimidine dissolved in the hydrochloric acid, forming a clear solution; during the reaction the liquid became bright yellow and a yellow precipitate separated which was practically insoluble in water. This was digested with distilled water, filtered off and dried at 100°. The yield was 0.63 g., or 93% of the theoretical. The substance burned up completely when ignited on platinum foil. It did not melt when heated to 320°.

Anal. Calcd. for $C_5H_6O_4N_2$: N, 17.72. Found: N, 17.61.

2-Ethylmercapto-4-ethoxymethyl-5-ethoxy-6-oxypyrimidine, V.—This compound was prepared by adding 20 g. of pseudo-ethylthiourea hydrobromide to a solution of 20 g. of ethyl α,γ-diethoxyacetoacetate in water containing 10 g. of sodium hydroxide. The mixture was then heated on the water-bath, upon which it became darker in color and ethyl mercaptan was evolved. After heating for eight hours the solution was cooled and acidified, giving a dark colored and rather oily mixture which was set aside to crystallize. This process was slow in starting but on long standing there separated dark colored, but very well formed, stocky crystals. These were purified by recrystallization from alcohol and decolorization with animal charcoal, when they were obtained in colorless crystals. The yield of the purified product was 4 g. The pyrimidine was practically insoluble in water but soluble in alcohol and acetone and gave good tests for nitrogen and sulfur. It melted at 123°.

Anal. Calcd. for $C_{11}H_{18}O_3N_2S$: N, 10.85. Found: N, 10.99, 10.91.

This compound was also obtained in the following way without first isolating the ketone ester; in general this latter method was preferable: 8.7 g. of metallic sodium in small pieces was added directly to 100 g. of ethyl ethoxyacetate in a dry flask connected with a reflux condenser. After cooling during the first stage of the reaction, the flask was suspended in a water-bath and heat applied until all of the sodium had dissolved. Then 70 g. of pseudo-ethylthiourea hydrobromide in absolute alcohol was added to the crude condensation product, the mixture warmed on the water-bath and then set aside to stand at room temperature. The formation of crystals in the dark-colored, viscous liquid was very slow in starting; alcohol and acetone were added in order to thin the material and the whole was then allowed to stand. During this time the crop of crystals gradually increased; after purification they were obtained in white, crystalline form, melting sharply at 123°. The yield was 14 g.

2-Ethylmercapto-4-ethoxymethyl-5-ethoxy-6-chloropyrimidine, VI.—9.53 g. of finely powdered phosphorus pentachloride was added to 11.7 g. of 2-ethylmercapto-4-ethoxymethyl-5-ethoxy-6-oxypyrimidine contained in a dry flask attached to a reflux condenser. After thorough mixing, no reaction was apparent after standing at room temperature for half an hour. The mixture was then moistened with 2 cc. of phosphorus oxychloride, forming a thick paste with a light yellow color but causing no further reaction. After half an hour the color was still unchanged, so the mixture was heated in a water-bath until the temperature rose to 80°, when the reaction started. With brisk evolution of hydrogen chloride an orange-red liquid was formed, but no odor of mercaptan was noticed. This liquid rapidly became darker in color; after fifteen minutes the water was raised to the boiling point and the heating continued for two hours. After the evolution of hydrogen chloride had ceased, the phosphorus oxychloride was distilled off on the water-bath under diminished pressure. The residue was treated with crushed ice, then warmed gently, neutralized with successive portions of ether and the solution placed over calcium chloride to dry.

The ethereal extract was then heated to remove ether and the residue of oil distilled under diminished pressure. It boiled at 165–166° under a pressure of 9–10 mm., distilling as a light yellow oil which did not solidify on cooling to 0°. The substance had an odor very suggestive of naphthalene. The yield was 7 g.

This pyrimidine also was prepared by allowing the reaction to take place in chloroform solution: 6.52 g. of the pyrimidine was dissolved in 50 cc. of chloroform and treated with 5.25 g. of phosphorus pentachloride dissolved in 66.7 cc. of chloroform. After refluxing for an hour the liquid changed from yellow to dark green in color. Five cc. more of the chloroform solution of phosphorus pentachloride was added in order to give a slight excess of this reagent and the heating continued for several hours, during

which time the liquid became brown. The chloroform then was removed and the residue worked up from this point as in the previous case, the pyrimidine being obtained as an oil, insoluble in water.

Anal. Calcd. for $C_{11}H_{17}O_2N_2SCl$: Cl, 12.81. Found: Cl, 12.90.

2-Ethylmercapto-4-ethoxymethyl-5-ethoxypyrimidine, VII.—The 6-chloropyrimidine described above was dissolved in 115 cc. of alcohol and 75 cc. of water. This solution then was boiled under a reflux condenser with 35 g. of 87% zinc dust that was added in portions in the course of two hours. This liquid then was filtered from the residual zinc while hot, washing well with fresh alcohol. The solvent was removed by warming under diminished pressure until but a small quantity of water remained, containing a heavy red oil. This was extracted with ether and dried over calcium chloride.

The ether was distilled from the ethereal solution and from the residue there separated small crystals, which were first washed with alcohol to free them from adhering oil, finally with ether and petroleum ether and further purified by recrystallization from alcohol. The pyrimidine separated in the form of plates and gave good tests for both nitrogen and sulfur but not for chlorine and melted sharply at 167° to a clear oil.

Anal. Calcd. for $C_{11}H_{18}O_2N_2S$: N, 11.57. Found: N, 11.58. *Mol. wt.* (boiling point method using benzene as solvent). Calcd.: 242. Found: 244.

On hydrolysis of a small amount of the above pyrimidine with hydrochloric acid, ethylmercaptan was evolved and we obtained crystals of a substance which darkened very considerably at 281–284° with vigorous effervescence and decomposition. As little material was available this was not investigated but it was very probably 2-oxy-ethoxymethyl-5-ethoxypyrimidine.

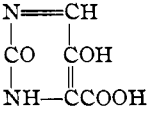
2-Ethylmercapto-4-ethylcarboxylate-5-ethoxy-6-oxypyrimidine, XIII.—Thirty-seven g. of freshly distilled ethyl oxalate and 33 g. of ethyl ethoxyacetate were dissolved in 100 cc. of anhydrous ether and placed in a dry flask attached to a reflux condenser. Then 5.75 g. of metallic sodium in wire form was added. After completion of the condensation with disappearance of the sodium, 41 g. of pseudo-ethylthiourea hydrobromide was added. The liquid emulsified and became yellow in color. It was warmed on the water-bath in order to distil off most of the ether, during which time the color became deeper; upon adding a solution of 14 g. of potassium hydroxide in alcohol to this, solid material began to collect in the flask and the liquid became darker in color. The mixture was then warmed on the water-bath for half an hour and in an oil-bath at 110–120° for two hours, during which time mercaptan was constantly evolved and the color became a deep brown. On cooling, a thick, almost solid, dark-colored mass remained. This was treated as follows: fifteen grams of glacial acetic acid was added in portions, the mixture warmed with 50 cc. of water and then cooled in ice. The crystals and semi-solid material which separated were filtered off and triturated with cold water, when we succeeded in obtaining a colorless product in the form of needles. This was purified by recrystallization from boiling water containing animal charcoal and dried in a vacuum desiccator. The substance gave strong tests for both nitrogen and sulfur and melted at 82–83°. The yield of this pyrimidine was small, but the condensation reaction leading to its formation is promising of further development and it will be studied exhaustively, as this combination would make possible many interesting syntheses if it were available in quantity. Its investigation will be continued.

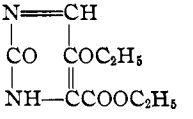
Anal. Calcd. for $C_{11}H_{16}O_4N_2S$: N, 10.29. Found: N, 10.03.

That this pyrimidine behaves normally and is a stable construction was evidenced by its reactivity with phosphorus oxychloride: 0.5 g. was treated with 2 cc. of the oxychloride, whereupon the compound dissolved immediately. Hydrogen chloride

was evolved and a clear solution was obtained after heating for half an hour on the water-bath. The excess of oxychloride then was removed by warming on the water-bath under diminished pressure. After adding ice and gently warming the mixture it was made neutral to litmus with dilute potassium hydroxide. On extracting with ether and evaporating the solvent, a red oil was obtained which contained both chlorine and sulfur and was undoubtedly mercapto-4-ethylcarboxylate-5-ethoxy-6-chloropyrimidine. As no micro-chemical apparatus was available it was impossible to obtain any analytical determinations confirming this constitution.

In order to determine whether this chloropyrimidine is easily reducible, all the material available was dissolved in 20 cc. of absolute alcohol and reduction applied by boiling with zinc dust under a reflux condenser for three hours after adding 10 cc. of water. The dark red color soon disappeared, the supernatant liquid becoming light yellow, almost colorless. After filtering off the zinc, the filtrate was evaporated and extracted with ether. On evaporating the ether a light yellow oil was obtained which finally solidified in the form of hexagonal plates melting at 55–56°. This compound was free from chlorine and its low melting point indicated that we were dealing with the new pyrimidine, 2-ethylmercapto-4-ethylcarboxylate-5-ethoxypyrimidine. It gave a strong test for sulfur. When this compound was heated at 120° with 10 cc. of hydrochloric acid, ethylmercaptan was evolved, showing that the original mercapto grouping had not been destroyed by the previous treatment with phosphorus oxychloride and zinc. The liquid in the bomb tube was yellow in color. On concentrating the acid solution and cooling, a beautiful crystalline substance separated in the form of prisms which melted at 259°. Whether we are dealing here with 2-oxy-4-carboxyl-5-hydroxypyrimidine (orotic acid?) or its corresponding diethyl derivative remains to be determined by further work.





2-Oxy-4-ethylcarboxylate-5-ethoxy-6-oxypyrimidine, XIV.—Thirty-six g. of ethyl oxalethoxyacetoacetate was mixed with a solution of 30 g. of pseudo-ethylthiourea hydrobromide in water. To this was added 15 g. of sodium hydroxide dissolved in water. After standing overnight the mixture was heated on the boiling water-bath, cooled and acidified with hydrochloric acid, whereupon a large amount of mercaptan was evolved from the dark-colored mixture. As it was difficult to stimulate crystallization of any of the products of the condensation, the material was set aside and allowed to stand. From the crystals that ultimately separated this pyrimidine was isolated and purified by several recrystallizations from water, in which it was only moderately soluble. After decolorizing with animal charcoal, it was obtained in the form of colorless needles. It gave no test for sulfur and melted at 230°.

Anal. Calcd. for $C_9H_{12}O_5N_2$: C, 47.36; H, 5.3; N, 12.28. Found: C, 47.68; H, 5.77; N, 12.07.

2-Oxy-4-carboxylic Acid 5-Ethoxy-6-oxypyrimidine, XV.—This compound was obtained along with the pyrimidine just described, from which it was separated by fractional crystallization from alcohol and water. It is practically insoluble in cold water, from which it separates in hexagonal crystals melting sharply at 260° with decomposition. Some of the same pyrimidine was obtained in one experiment in which thiourea and oxalethoxyacetoacetic ester were allowed to react in alcohol containing sodium alcoholate. In this case the reaction product stood for a long time after acidification with hydrochloric acid before the pyrimidine was isolated.

Anal. Calcd. for $C_7H_8O_5N_2$: C, 42.0; H, 4.0; N, 14.0. Found: C, 42.18, 42.4; H, 4.51; N, 13.27, 13.18.

2,6-Dioxy-5-hydroxypyrimidine (Isobarbituric Acid), XVIII.—One g. of 2-oxy-4-carboxylic acid 5-ethoxy-6-oxypyrimidine was sealed in a tube with 10 cc. of concentrated hydrochloric acid and heated for two hours at 110°. Under these conditions no reaction took place. It was therefore heated again for ten hours at 160–165°. The reaction product was found to be insoluble in water and also difficultly soluble in alcohol. On recrystallization from alcohol it separated in the form of microscopic crystals arranged in clusters. These decomposed without melting at 350–355°. The compound was identified as isobarbituric acid. In other words, the carboxyl group is removed by intensive hydrolysis, the pyrimidine behaving in an entirely different manner than uracil-4-carboxylic acid,[2] which resists hydrolysis when heated with 20% sulfuric acid at 200°.

Anal. Calcd. for $C_5H_4O_5N_2$: N, 16.27; for $C_4H_4O_3N_2$: N, 21.87. Found: N, 21.71.

Summary

1. Isouracil-carboxylic acid can exist theoretically in several isomeric modifications and is isomeric with *orotic acid*, a substance stated to have been isolated from milk by Biscaro and Belloni.

2. Experimental evidence has been obtained indicating that isouracil-carboxylic acid may be identical with orotic acid.

3. Two different methods of synthesis have been applied for preparing isouracil-carboxylic acid. Only one thus far gives promise of leading successfully to this pyrimidine.

4. Several new derivatives of isouracil have been prepared.

NEW HAVEN, CONNECTICUT

[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, UNIVERSITY OF MISSOURI]

THE SEPARATION OF THE SOLUBLE PROTEINS OF RABBIT MUSCLE

BY WALTER S. RITCHIE AND ALBERT G. HOGAN

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The earlier work on the separation of muscle proteins has been adequately reviewed,[1] so we will omit a detailed report of those investigations. Howe[2] has recently described a very ingenious procedure which deserves further study, in order to define more precisely the limits of its usefulness. There are several other recent papers[3] that have some bearing on the problem of extracting and separating muscle proteins, though they do not con-

[1] G. Mann, "Chemistry of the Proteids," Macmillan and Co., London, **1906.**

[2] Howe, *J. Biol. Chem.*, **61,** 493 (1924).

[3] Lloyd, *Proc. Roy. Soc. London*, **89B,** 277 (1916); Collip, *J. Biol. Chem.*, **50,** xlv (1922); Granstrom, *Biochem. Z.*, **134,** 589 (1922); Weber, *ibid.*, **158,** 443 (1925); **184,** 407 (1927); Salter, *Proc. Soc. Exptl. Biol. Med.*, **24,** 116 (1926); Wohlisch and Schriever, *Z. Biol.*, **83,** 265 (1925).