[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

RESEARCHES ON PYRIMIDINES. CV. A NEW TEST FOR THYMINE AND 5-METHYLCYTOSINE IN THE PRESENCE OF URACIL AND CYTOSINE[1]

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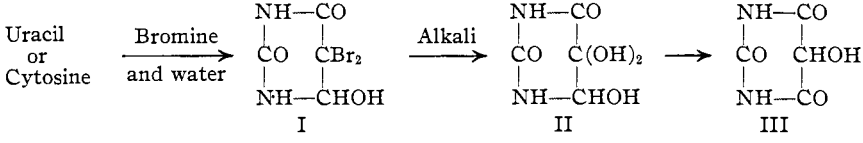
RECEIVED NOVEMBER 7, 1928 PUBLISHED APRIL 5, 1929

Introduction

In 1907 Wheeler and Johnson[3] showed that when uracil or cytosine is treated first with bromine water, and then with an alkali, the following reactions take place



The dialuric acid formed, III, is characterized by its property of interacting with barium hydroxide to give a purple, insoluble barium salt.

Since 1907 this reaction has been used widely by biochemical workers as a practical test for these two pyrimidines. It was found that thymine, IV, would not give this purple barium salt, a fact which could be predicted from inspection of the structural formula of the pyrimidine. Johnson[4] later described a method for separating thymine from uracil by means of nitric acid, but this method of analysis was not proposed by him as a test for thymine in the presence of uracil. We have available, therefore, delicate tests for uracil and cytosine, and as thymine is also a normal constituent of many nucleic acids, it was very important to develop a correspondingly delicate test for this pyrimidine which would be applicable in the presence of either uracil or cytosine. The object of the research discussed in this paper was to develop, if possible, from known reactions a practical test for thymine which would meet these conditions.

The first attempt to perfect a specific test for thymine was made by Johnson and Baudisch,[5] who made a preliminary study of the oxidation products of thymine. It was found that the thymine molecule, in the presence of the system *ferrous sulfate* + *sodium bicarbonate* + air yields after acid hydrolysis urea, pyruvic acid, acetol and formic acid. Urea

[1] Constructed from a dissertation presented by Henry Harvey Harkins, in June, 1927, to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy.

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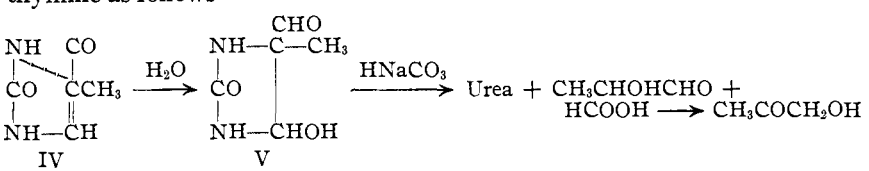
[3] Wheeler and Johnson, *J. Biol. Chem.*, **3**, 183 (1907).

[4] Johnson, *Am. Chem. J.*, **40**, 19 (1908).

[5] Johnson and Baudisch, THIS JOURNAL, **43**, 2670 (1921).

is identified by means of its xanthydrol derivative;[6] the pyruvic acid is converted to the dyestuff indigo by interaction with *o*-nitrobenzaldehyde, the acetol is identified as 3-oxyquinaldine formed by warming the acetol in alkaline solution with *o*-aminobenzaldehyde,[7] while formic acid is identified by its reducing action on silver nitrate in ammoniacal solution. It was found that pure thymine, IV, in quantities of milligrams only (2 to 5 mg.) may be detected by this procedure. No postulations were made regarding the mechanism of this interesting degradation of the thymine molecule. Deuel and Baudisch[8] later reported a modification of this test for the detection of thymine in the presence of carbohydrates.

This research was continued later by Baudisch and Bass,[9] who investigated the action of other oxidizing reagents on thymine, namely, (1) hydrogen peroxide, (2) hydrogen peroxide + ferrous sulfate, (3) sodium penta-cyano-aquo-ferroate + air, (4) ferrous sulfate + sodium bicarbonate + air and (5) iodine + sodium bicarbonate. They found that the five oxidizing agents except iodine were productive of urea, acetol and pyruvic acid when the products of oxidation are heated in aqueous solution with sodium bicarbonate. Acetol is formed as a direct hydrolytic product of thymine, while pyruvic acid is formed by the hydrolysis of an intermediate oxidation product of unknown constitution. Baudisch and Bass were apparently only interested in the mechanism of these various changes, and interpreted the course of the hydrolytic split of thymine as follows



With the first three oxidizing reagents the time required to carry out the complete operation is from twelve to forty-eight hours. The following quotation from the paper by Baudisch and Bass[9] reveals their method of oxidizing thymine with iodine "50 cc. of an approximately 0.1 *N* solution of iodine was added to a solution of 0.5 gram of thymine and 30 grams of sodium bicarbonate. The reaction mixture after dilution to one liter was allowed to stand overnight. The excess of iodine was then removed by blowing a rapid stream of air through the solution until it was decolorized (about four hours). The colorless solution was then distilled to a small volume. The distillate gave a strong acetol test. The residue gave a strong test for urea but no test for pyruvic acid. Al-

[6] Fosse, *Compt. rend.*, **145**, 813 (1907).

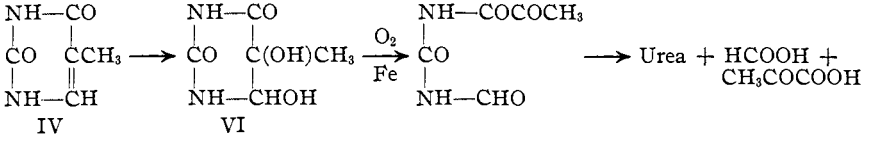
[7] Baudisch, *Biochem. Z.*, **89**, 279 (1918).

[8] Deuel and Baudisch, THIS JOURNAL, **44**, 1581 (1922).

[9] Baudisch and Bass, *ibid.*, **46**, 181, 184 (1924).

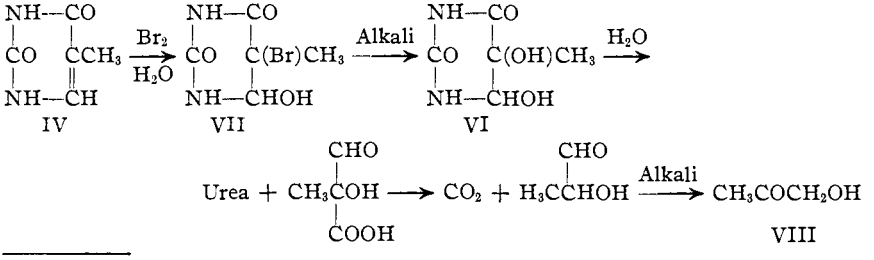
though this experiment has been repeated several times, a positive test for pyruvic acid has never been obtained." This quotation will serve to illustrate the technique of these tests as compared with that of the simplified test described in detail in the Experimental Part of this paper. Furthermore, the mechanism of change brought about by the action of iodine on thymine is undoubtedly identical in principle with our new procedure, but Baudisch and Bass were not conscious of this at the time of their work.

The correct formulation of the mechanism of the oxidation of thymine was worked out by Baudisch and Davidson.[10] They rejected the previous explanation postulated by Baudisch and Bass[9] and concluded that the mechanism of reaction leading to the formation of pyruvic acid is to be explained as follows



In other words, they demonstrated that the different end-products resulting from degradation of thymine by oxidation and hydrolysis can result from the breakdown of an intermediate pyrimidine-glycol represented by Formula VI. But here again these workers emphasized only the mechanism of reaction in their work and did not point out any improved applications that could be made of their technique for the testing for thymine in the presence of other compounds. With these data at hand we have now been able to establish the basis of a very sensitive test for thymine applicable in the presence of uracil and cytosine.

Basis of the Thymine Test.—Thymine IV is converted quantitatively into 4-hydroxy-5-bromohydrothymine, VII, by the action of bromine water.[11] Baudisch and Davidson prepared this pyrimidine according to Jones' directions and showed that it undergoes hydrolysis easily with formation of carbon dioxide, acetol and urea. From these two observations we can now formulate reactions which lead to an important and intensively delicate test for thymine. The changes are expressed as follows



[10] Baudisch and Davidson, *J. Biol. Chem.*, **64**, 233 (1925).

[11] Jones, *Z. physiol. Chem.*, **29**, 20 (1900).

To carry out this simple test one treats thymine in aqueous solution with an excess of bromine and then removes the excess of halogen by boiling the solution. Barium hydroxide is then added in excess and the solution distilled, when acetol is carried over into the distillate. The acetol, VIII, is then tested for according to the method of Baudisch.[7] The usefulness of this test is apparent if one bears in mind the fact that when uracil and cytosine are treated in a similar manner they both yield almost quantitatively the purple barium salt of dialuric acid. Therefore, we now have available a procedure whereby these two pyrimidines can be detected easily in the same solution containing thymine, and can be removed from solution so as not to interfere with the acetol test for thymine. The reactions are easy to perform and the tests for the three pyrimidines in the same solution can be applied in a few minutes.

Application of the Acetol Test with 5-Methylcytosine.—On account of the structural relationship existing between thymine and 5-methylcytosine and between uracil and cytosine, respectively, and since uracil and cytosine are both converted by action of bromine water and alkali into dialuric acid, it was logical to suppose that 5-methylcytosine would give the same end-products as thymine, IV. Experimentally this has been found to be the case and the detection of the four pyrimidines, uracil, thymine, cytosine and 5-methylcytosine, thus becomes a simple matter. The technique of this analytical procedure is discussed in detail in the Experimental Part of this paper. The investigation is being continued.

Experimental Part

Synthesis of Thymine.—Thymine was prepared by hydrolysis of 2-ethylmercapto-5-methyl-6-oxypyrimidine.[12] This mercapto-pyrimidine can be obtained in much better yields than according to the procedure described in the previous paper.

Sodium Ethylformylpropionate.—For the preparation of this salt the necessary reagents were used in the following amounts: 204 g. of ethyl propionate, 222 g. of ethyl formate, 1000 cc. of anhydrous ether and 46 g. of sodium. The two esters were mixed and then added slowly through a dropping funnel to ether in which was suspended the sodium. In order to accelerate the reaction the sodium was granulated by melting under the toluene and thoroughly shaking while cooling. The toluene was then decanted, the sodium washed with anhydrous ether and finally covered with dry ether in a large, round-bottomed flask connected to a reflux condenser. The ether solution of mixed esters was then dropped onto the sodium during a period of two days and the mixture allowed to stand for a third day to complete the reaction. Water was then added cautiously and the sodium salt of ethylformylpropionate dissolved. The water layer was separated and used in the condensations described below. Several preparations of this salt were made and in each condensation reaction the water layer was diluted to 1000 cc. and then divided into aliquot parts as required. Seven pyrimidine condensation reactions were then made using salt units corresponding to known weights of ethyl propionate (see Table I).

It may be concluded from the results of these experiments that the addition of

[12] Wheeler and Johnson, *Am. Chem. J.*, **31**, 591 (1904).

alkali is not necessary in this condensation, and also that 102 g. of ethyl propionate is productive of sufficient condensation product—*ethyl sodium formylpropionate*—to interact with only 23 g. of pseudo-ethylthiourea hydrobromide (0.125 mole).

TABLE I

CONDENSATIONS WITH PSEUDO-ETHYLTHIOUREA

No.	Pseudo-ethylthiourea hydrobromide, g.	Ethyl propionate in form of sodium-formylpropionate, g.	Yield of mercapto-pyrimidine, g.	Sodium hydroxide, g.
1	185 (1 mole)	102	31	None
2	185 (1 mole)	102	26	40
3	92.5 (0.5 mole)	102	30	None
4	92.5 (0.5 mole)	102	20	20
5	46.0 (0.25 mole)	102	31.5	None
6	46.0 (0.25 mole)	102	36.00	None
7	23.0 (0.125 mole)	102	19.00	None

Theoretical yield for (Expt. 7) 31.00 g.

Preparation of 5-Methylcytosine.—This pyrimidine was synthesized from 2-ethylmercapto-5-methyl-6-oxypyrimidine according to the method described by Wheeler and Johnson.[3] This process involves treatment of the 2-ethylmercaptopyrimidine with phosphorus pentachloride to obtain 2-ethyl-mercapto-5-methyl-6-chloropyrimidine and then heating this chloro compound under pressure with ammonia. The resulting mercapto-aminopyrimidine is then converted into 5-methylcytosine by hydrolysis with hydrochloric acid. Ninety-three grams of the 2-ethylmercaptopyrimidine were productive of 82 g. of the chloro compound boiling at 145–147° at 15–18 mm. By heating this chloride at 125–120° in alcohol saturated at 0° with ammonia nearly a quantitative yield of the corresponding aminopyrimidine was obtained. After digesting this mercaptopyrimidine for six hours with hydrochloric acid the evolution of mercaptan had ceased and on evaporating the solution the hydrochloride of 5-methylcytosine was obtained in excellent yield. Practically no thymine, IV, is produced in this hydrolysis. The free base was separated according to the directions of Wheeler and Johnson.

Application of the Acetol Test with Uracil, Thymine, Cytosine and 5-Methylcytosine: Thymine.—To 0.0100 g. of pure thymine dissolved in 50 cc. of water, bromine is added until a red color is permanent. The excess of bromine is then removed by boiling and to the solution is added 1.0 g. of barium hydroxide crystals. The mixture is then refluxed for fifteen minutes and then distilled. The distillate is made strongly alkaline with sodium hydroxide and tested for acetol as follows.

"3.0 grams of crystalline *o*-nitrobenzaldehyde is mixed with 50 grams of crystalline ferrous sulfate and to this is added 75 cc. of concentrated aqueous ammonia solution. This ammoniacal solution is then heated on the steam-bath for one hour. It is then distilled with steam and several cubic centimeters of the distillate added to the alkaline solution containing acetol described above. This mixture is then evaporated by heating over a Bunsen flame to a volume of 25 cc. After cooling, hydrochloric acid is added until distinctly acid, and the solution then made alkaline by addition of sodium bicarbonate and the solution filtered. The solution thus obtained shows a deep blue fluorescence even in diffused daylight."[7]

In order to test the delicacy of this reaction successively smaller quantities of thymine were used and the solution containing acetol and *o*-aminobenzaldehyde was evaporated to a small volume, in some cases to a volume of 1 cc. It was observed that a blue fluorescence could be obtained with 0.001 g. of thymine. Although the

characteristic fluorescence produced with this small quantity of pyrimidine is not observable in ordinary light, it can easily be seen in the light of a mercury arc lamp.

Baudisch[7] does not mention in his paper how to preserve the *o*-aminobenzaldehyde reagent. It was found that the aqueous solution containing *o*-nitrobenzaldehyde, ferrous sulfate and ammonia, heated as described above, could be allowed to stand in the laboratory for two weeks without destruction of the aminobenzaldehyde, *i. e.*, the steam distillate from such a mixture gave the fluorescence characteristic of 3-oxyquinaldine at the end of this time.

Uracil and Cytosine.—From inspection of the formulas of uracil and cytosine it can be seen that neither of these pyrimidines can give rise to acetol when treated with bromine and alkali. Uracil prepared by the hydrolysis of 2-methylmercapto-6-oxypyrimidine, when tested for acetol was found, however, to give a distinct, blue fluorescence, which persisted even when the uracil thus obtained was recrystallized several times from water. The same quantity of synthetic cytosine made by the pseudothiourea method[3] was also found to give a distinct fluorescence. Apparently these pyrimidines contained a small trace of thymine and 5-methylcytosine, respectively, as impurity. The presence of these two impurities can be traced back to the original aliphatic ester used in the first condensation of the synthesis, namely, ethyl acetate. It is necessary that this reagent be absolutely free from ethyl propionate in order to obtain uracil or cytosine that will not respond to the acetol test.

In order to prove that uracil, when free from thymine, will not give the acetol test, this pyrimidine was synthesized from urea and malic acid according to the method of Davidson and Baudisch.[13] The uracil so obtained cannot contain thymine. When 0.5 g. of this product was tested for acetol no fluorescence whatever could be detected thereby proving that uracil and cytosine cannot produce the blue fluorescence characteristic of thymine.

5-Methylcytosine.—5-Methylcytosine hydrochloride prepared as described above responds to the acetol test strongly. In order to make certain, however, that the test in this case is due to 5-methylcytosine and not to thymine, the methylcytosine was precipitated repeatedly as its phosphotungstate and the recovered methylcytosine then tested for acetol as before. A strong fluorescence was obtained, showing that 5-methylcytosine undergoes the same reaction with bromine and alkali as thymine. This is to be expected since uracil and cytosine yield the same product—dialuric acid—when treated with bromine and alkali.

Summary

1. An improved chemical or color test for detecting thymine, based on the experimental work of Johnson, Baudisch and co-workers, has been described.

2. The test is dependent on the formation of acetol by action of bromine and alkali on thymine.

3. The test applies equally well to 5-methylcytosine.

4. Both of these pyrimidines can be detected in quantities as small as one milligram.

5. The acetol test for thymine and 5-methylcytosine is applicable in the presence of uracil and cytosine.

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[13] Davidson and Baudisch, THIS JOURNAL, **48**, 2379 (1926).