Petroleum ether added to a mixture of a hard fat, acid, water and castor bean lipase accelerates hydrolysis to a great extent. The hydrolysis of oils is similarly somewhat hastened.

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RESEARCHES ON PYRIMIDINES. XCII. NEW METHODS OF IDENTIFYING THE PYRIMIDINE, THYMINE.

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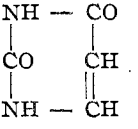
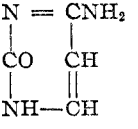
Of the three pyrimidines which have so far been found as products of hydrolysis of nucleic acids only two, namely, uracil (I) and cytosine (II), can be identified by means of characteristic color reactions. Wheeler and Johnson[1] developed a delicate test for these two pyrimidines which



(I).



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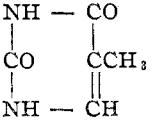


(III).

is applied by first oxidizing them in aqueous solution with bromine and then treating the resulting solution with an excess of barium hydroxide. A characteristic purple color develops under such conditions, due to the formation of the barium salt of dialuric acid, which permits of the identification of these two pyrimidines when present in very small quantities. Thymine (III) or any pyrimidine of this type containing alkyl groups in Positions 4 or 5 of the pyrimidine ring fails to respond to this characteristic test.

On account of the important rôle which thymine plays in the chemical processes operating during the growth and destruction of the living cell, it is very important to have available also a characteristic color reaction for the detection of this pyrimidine. A color test which would permit the identification of thymine in the presence of uracil, cytosine and sugar would be of the greatest value in any investigation dealing with the constitution of nucleic acids. The basic properties of cytosine permit its separation from uracil and thymine, but up to the present time we have had no method of identifying small quatities of thymine in the presence of uracil and sugars.

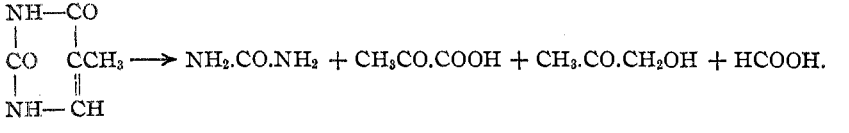
We are able to announce in this paper the discovery of color reactions which are as characteristic for thymine as the dialuric acid reaction is for the identification of the pyrimidine uracil. We find that the thymine molecule is completely destroyed at ordinary temperature, when subjected

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

in aqueous solution to the oxidizing and reducing action of the system, ferrous sulfate plus sodium hydrogen carbonate plus air,[2] with cleavage of the pyrimidine ring and formation of pyruvic acid, acetol, urea and formic acid. The urea is easily identified by the properties and melting point of its characteristic grouping with xanthydrol,[3] while the formic acid is recognized by its reducing action on silver salts.

For the detection of the two remaining products of oxidation namely, pyruvic acid and acetol, we have made use of reactions already described in the chemical literature, and it is on two specific reactions that we base our color tests, which we now bring forward as an indirect proof of the presence of thymine. The identification of pyruvic acid as a product of oxidation of thymine is shown conclusively by its behavior towards *o*-nitro-benzaldehyde in alkaline solution when, as is well known, they interact to form the dye indigo.[4] The color reaction of pyruvic acid with sodium nitroprusside cannot be utilized in our work as a direct test for this reagent on account of the presence of urea, which we find interacts also with the nitroprusside giving a similar highly colored solution. For the detection of acetol we utilize the very susceptible and specific reaction first developed by Baudisch,[5] who has shown that this ketone alcohol interacts smoothly in alkaline solution with *o*-amino-benzaldehyde with formation of 3-oxyquinaldine. Both of these reactions are extremely sensitive and applicable in very great dilution.

These reactions, which are applicable for the detection of these two aliphatic combinations resulting by oxidation of thymine, serve, therefore, for the indirect identification of this pyrimidine. The discussion of the mechanism of the change whereby thymine undergoes destructive oxidation is reserved for a future paper. At present we may represent this transformation as follows.



The discovery that the pyrimidine ring in such combinations as uracil, cytosine and thymine may be destroyed by oxidation at such low temperature in the presence of iron salts, and the identification of pyruvic acid and acetol as normal products of oxidation of thymine are new facts of the greatest biochemical interest in that they reveal possible fundamental chemical changes which the pyrimidine ring actually undergoes in the normal metabolic transformations of the cell.

[2] Baudisch, *Ber.*, **54**, 406 (1921).
[3] R. Fosse, *Compt. rend.*, **145**, 813 (1907); *Ann. inst. Pasteur*, **30**, 525,673 (1916).
[4] Baeyer, *Ber.*, **15**, 2856 (1882).
[5] Baudisch, *Biochem. Z.*, **89**, 279 (1918).

Experimental Part.

While it has been shown that the oxidation of uracil, cytosine and thymine leads to products of physiological interest, a description of these experiments and the discussion of the theory of these oxidations will be deferred for publication in a later paper. At this time we shall confine ourselves to the practical method of applying our new color reactions for the detection of thymine in the presence of other pyrimidines.

A description of a single experiment will suffice to reveal the interesting behavior of thymine when subjected to the oxidizing action of the system, ferrous sulfate plus sodium hydrogen carbonate plus air. Two and six-tenths g. of recrystallized thymine was dissolved in about 100 cc. of hot water and this solution then poured into one of 200 g. of sodium hydrogen carbonate dissolved in 2 liters of water. A 5-liter flask serves best for this operation. To this solution of thymine and bicarbonate is then added 100 g. of crystallized ferrous sulfate dissolved in 500 cc. of water, and the resulting mixture then agitated violently. Colorless iron hydrogen carbonate precipitates at once and will remain in this condition if protected from the air. On agitation of the solution, however, with exposure to the air this carbonate precipitate gradually assumes a green color, due to the absorption of oxygen from the air, with formation of an unstable peroxide. One proceeds to agitate the mixture thoroughly when the oxidation of the thymine takes place immediately and the greenish-gray color of the carbonate precipitate disappears slowly and the carbonate finally assumes a brownish color and the appearance of iron hydroxide. This oxidation and change in the carbonate take about $^3/_4$ hour. The mixture is now poured into a filtering jar and allowed to stand overnight when a clear colorless solution is obtained and the iron hydroxide deposits as a dense layer on the bottom of the jar. This clear solution is not characterized by any special odor and is colorless.

The final change in the process of oxidation is brought about by transferring the supernatant liquid to a large evaporating dish and heating the solution on a steam-bath. Hydrolysis takes place under these conditions and the solution acquires an odor characteristic of an alkaline sugar solution when heated. After heating long enough to bring about the above change, 100 to 200 cc. of the clear solution is evaporated to dryness; the residue dissolved again in distilled water and the evaporation repeated. Pyruvic acid is not volatile with steam while acetol vaporizes easily. The dry residue is then dissolved in a small volume of water and the indigo test for pyruvic acid applied as follows: to 5 to 10 cc. of the cold solution are added a few drops of *o*-nitro-benzaldehyde, and the mixture is shaken with chloroform after the addition of 1 to 2 cc. of conc. potassium hydroxide solution. On shaking, the indigo forms immediately and dissolves in the chloroform giving a characteristic blue solution. The formation of indigo

is hastened by warming the solution to be tested and agitating this with the nitro-benzaldehyde until an emulsion is formed and then shaking with chloroform and alkali. Under these conditions a deep blue chloroform solution is obtained and the reaction is so delicate that this characteristic blue color can be obtained easily with solutions representing as low as 2 to 5 mg. of thymine.

In order to apply successfully the test for acetol the remainder of the original thymine solution is subjected to distillation by boiling over a free flame. The distillate at first possesses no reducing properties and is odorless, but as the distillation is continued the characteristic sugar smell develops and finally the distillate acquires the property of reducing Fehling's solution and also ammoniacal silver solution. In order to test for acetol the total distillate is combined with a few drops of *o*-amino-benzaldehyde, potassium hydroxide solution is added until the mixture is distinctly alkaline, and the solution is finally boiled for a few minutes in a beaker. After cooling, the solution is acidified directly with dil. hydrochloric acid, and then made it alkaline again with sodium hydrogen carbonate solution when a characteristic blue fluorescence develops as a result of the formation of 3-oxyquinaldine. For a more exact determination of the acetol the sodium hydrogen carbonate solution is extracted with ether, the ether solution carefully dried and finally distilled when nearly colorless needles are obtained, which have the correct melting point and possess the characteristic properties of 3-oxyquinaldine. An alcoholic solution of the quinaldine develops after dilution with water a deep blue fluorescence, and on adding an alcoholic solution of iron chloride the characteristic deep red complex iron salt of 3-oxyquinaldine is obtained. This red salt is destroyed immediately by addition of acid.

The formation of 3-oxyquinaldine from acetol and *o*-amino-benzaldehyde is a reaction which was first discovered by Baudisch[5] and is one which is specific for this ketone alcohol. The formation of the quinaldine in the distillate of the oxidized thymine proves the formation of acetol as a product of oxidation of this pyrimidine, which is a discovery of the greatest biochemical interest.

Uracil behaves in an entirely different manner from thymine towards the system ferrous sulfate plus sodium hydrogen carbonate plus air. Both pyrimidines are oxidized apparently in a similar manner, but uracil cannot lead to the production of pyruvic acid on account of the absence of a methyl group in Position 5 of the pyrimidine ring. Consequently according to our procedure uracil and thymine can be tested for in the presence of each other, which has not been possible hitherto.[6] In fact, we have been able to demonstrate that it is possible to detect thymine easily in the presence of uracil and cytosine by application of our method. Mixtures of 0.5 g.

[6] See *J. Biol. Chem.*, **4**, 407 (1908).

each of uracil and cytosine and also of uracil and cytosine with small quantities of thymine (0.15 g.) were oxidized according to the procedure described above, and the test for thymine applied. In no case were we able to obtain the characteristic blue color reactions except in those mixtures into which thymine had been introduced.

In this paper we have recorded only the qualitative characteristics of our colorimetric test for the pyrimidine, *thymine*. When the pyrimidine has been prepared in quantity and, therefore, sufficient material is available for research this oxidation reaction will be investigated more carefully and from a quantitative point of view.

Our work to date shows that the system, ferrous sulfate plus sodium hydrogen carbonate plus air, is characterized by its specific oxidizing and reducing action, which may be utilized for bringing about fundamental changes in organic combinations of biochemical interest. The writers are now engaged in the study of several of these transformations and will extend the investigation to new fields of research in which it is anticipated that the ferrous hydroxide-peroxide reagent will reveal still further new transformations of biochemical interest.[7]

Summary.

1. It has been shown that ferrous hydroxide-peroxide is a sufficiently strong oxididizing agent to destroy the pyrimidine ring at a very low temperature.

2. Uracil, thymine and cytosine are completely destroyed by interaction with this reagent leading to the formation of products which are easily hydrolized to urea and aliphatic combinations.

3. In the case of thymine it has been definitely established that pyruvic acid and acetol are normal products of oxidation. These compounds can be identified easily by characteristic color reactions, which serve indirectly as reliable tests for thymine.

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[7] Our methods of testing for uracil, cytosine and thymine will find immediate practical application in the study of constitution of nucleic acids, and in the research on *tubercle bacillus* now in progress in this laboratory (T. B. Johnson).