[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

NEW METHODS OF SPLITTING PYRIMIDINES. IV. A STUDY OF THE MECHANISM OF THE DECOMPOSITION OF THYMINE[1]

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Johnson and Baudisch[3] have shown that thymine is partly split into urea, pyruvic acid and acetol by the system ferrous sulfate plus sodium bicarbonate plus air. This reaction can be used as a very sensitive test for thymine, since acetol can be detected according to the method of Baudisch[4] by the formation of 3-hydroxyquinaldine from *o*-aminobenzaldehyde. The other products of the decomposition of thymine, namely, urea and pyruvic acid are detected[3] by means of xanthydrol[5] and *o*-nitrobenzaldehyde,[6] respectively.

The splitting of thymine is of particular chemical and biological interest, since this pyrimidine, as a constituent of the nucleic acids, is present in all plant and animal cells and hence may play an important role in the formation of new cells. The reactions of thymine were therefore given particular attention and were reserved for a more exhaustive study to determine the mechanism of the reaction.

The preceding communication by Pfaltz and Baudisch[7] is concerned in general with the study of the action of ferrous salts on pyrimidines in the presence of oxygen. The mechanism of the split of pyrimidines into urea and other compounds is not explained.

The Action of Hydrogen Peroxide on Thymine

If a solution of thymine containing sodium bicarbonate is treated with concd. hydrogen peroxide and is then hydrolyzed by warming, we obtain, in addition to urea, a large quantity of acetol and only a small quantity of pyruvic acid. If the reaction is carried out under the same conditions except for the fact that a small quantity of a ferrous salt is added to the reaction mixture, the relative quantities of acetol and pyruvic acid are reversed.

From these experimental results we conclude that the primary hydrolytic split of the thymine ring produces a compound which forms acetol and

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[3] Johnson and Baudisch, THIS JOURNAL, **43**, 2670 (1921); *Ber.*, **55**, 18 (1922).

[4] Baudisch, (a) *Biochem. Z.*, **89**, 279 (1918). Other applications of this test are described in papers by Baudisch and Deuel, (b) THIS JOURNAL, **44**, 1581, 1585 (1922).

[5] Fosse, *Ann. chim.*, [9] **6**, 13 (1916).

[6] Baeyer, *Ber.*, **15**, 2856 (1882).

[7] Pfaltz and Baudisch, THIS JOURNAL, **45**, 2972 (1923).

urea when complete hydrolysis occurs on warming with bicarbonate. Pyruvic acid, however, results from the hydrolysis of a compound produced by the oxidation of this primary hydrolytic product. Both the intermediate compound and its oxidation product still contain the urea residue, a fact which is proved definitely by the failure of the xanthydrol test. The end products of the reaction, that is, urea, pyruvic acid and acetol, are formed only when the primary compounds are warmed on the water-bath with sodium bicarbonate.

The Action of Sodium Pentacyano-aquo-ferroate Plus Oxygen on Thymine

The results with hydrogen peroxide are confirmed by those obtained by treating thymine with sodium pentacyano-aquo-ferroate and oxygen. When air or oxygen is passed through an aqueous solution of thymine containing a limited quantity of this complex iron salt and when the reaction mixture is then warmed on the water-bath after the addition of sodium bicarbonate, the products formed are urea and pyruvic acid. Traces of acetol could be detected in only a few experiments. The activation of both thymine and oxygen by aquo salt brings about at once the formation of an oxidation product of the primary hydrolytic product. Hence, only pyruvic acid and urea can be formed by complete hydrolysis.

The Action of Ferrous Sulfate Plus Sodium Bicarbonate Plus Air on Thymine

Acetol and pyruvic acid are obtained in roughly equal quantities by the action of the system ferrous sulfate plus sodium bicarbonate plus air on thymine. This result therefore affords a proof of the fact that ferrous carbonate peroxide possesses the power of splitting the pyrimidine ring by hydrolysis. A partial oxidation of the primary hydrolytic product takes place as a result of the activation of the oxygen of the air.

The Action of Iodine Solution Plus Sodium Bicarbonate on Thymine

It has been shown[8] that many pyrimidines react with iodine solution in the presence of sodium bicarbonate to form intermediate hydrolytic products which are hydrolyzed completely by warming them on the water-bath with sodium bicarbonate. Under these conditions thymine yields urea and acetol, but no trace of pyruvic acid. In this case the primary hydrolytic product is not oxidized by iodine and hence no pyruvic acid can be formed.

The Mechanism of the Decomposition of Pyrimidines

It has been shown that the decomposition of thymine, under the conditions which we have described, leads to the formation of two compounds of great physiological importance, namely, acetol and pyruvic acid. In

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[8] Bass and Baudisch, This Journal, 46, 181 (1924).

order to explain the formation of these compounds it was first assumed that methylglyoxal, $CH_3.CO.CHO$, appeared as a primary product and that it then suffered a Cannizzaro reaction, thus yielding the two compounds which are obtained as final products. This assumption is not tenable because experiments showed that methylglyoxal does not form acetol and pyruvic acid when warmed in aqueous solution with sodium bicarbonate.

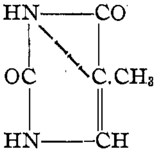
The observation that iodine solution splits thymine into urea and acetol proves that acetol is a direct hydrolytic product and that pyruvic acid is formed by a secondary oxidation. Moreover, experiments showed that pyruvic acid does not result from the oxidation of the acetol formed in the reaction, but that the primary hydrolytic product, which still contains the urea residue, becomes oxidized to a body which, on complete hydrolysis by warming with sodium bicarbonate, decomposes into urea and pyruvic acid. The investigations with ferrous sulfate plus sodium bicarbonate plus air, with hydrogen peroxide (with or without ferrous sulfate), with sodium pentacyano-aquo-ferroate plus air, and with iodine solution, showed that the products of the decomposition, namely, urea, acetol and pyruvic acid, could not be detected after the primary process. In other words, warming with sodium bicarbonate is necessary in every case for complete hydrolysis.

Additional proof of the fact that the primary hydrolytic product, which still contains the urea residue, is oxidized under certain conditions, is obtained from experiments with hydrogen peroxide, either alone or in the presence of ferrous salts, and with aquo salt plus oxygen. The quantity of pyruvic acid formed in these experiments varies with the strength of the oxidizing action; in the case of aquo salt the primary product is practically completely oxidized. It should be noted that the oxidation takes place in neutral solution at room temperature and that a higher temperature and the presence of bicarbonate are necessary for complete hydrolysis of the intermediate product to urea and pyruvic acid.

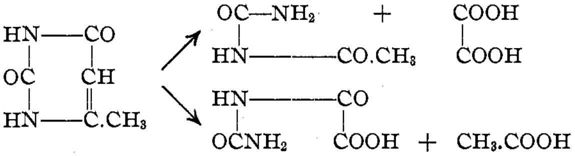
We are, therefore, able to split thymine into urea and acetol, into urea and pyruvic acid, or into urea, acetol and pyruvic acid. The first reaction occurs when oxidizing action is excluded and when only hydrolytic action, introduced by iodine and completed by warming with bicarbonate, is allowed to occur. These results, we believe, give conclusive proof of the fact that in the case of the split produced by the system ferrous sulfate plus sodium bicarbonate plus air or by hydrogen peroxide, the primary process is a partial hydrolytic split of the pyrimidine ring, while the oxidation of the intermediate compound, which still contains the urea residue, follows as a secondary process.

The question now arises as to which atoms are affected by the hydrolysis. From a previous publication[7] it appears that, in analogy with the work of

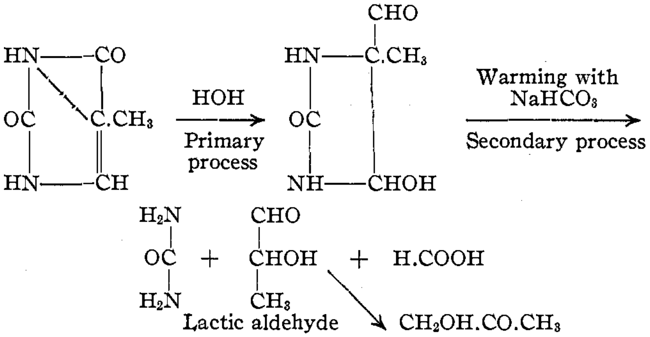
Biltz,[9] carbon atom 5 of the pyrimidine ring has a strong affinity for nitrogen atom 1, a fact which may be indicated in the formula of thymine by a dotted line.



It has been found by other investigators that when a rupture of the pyrimidine ring occurs the double bond between carbon atoms 4 and 5 is broken. For example, Behrend,[10] on oxidizing 4-methyluracil with potassium permanganate, obtained acetyl urea and oxaluric acid, besides small amounts of acetic and oxalic acids.



On the basis of the experimental results which have been given we propose the following mechanism as a possible explanation of the hydrolytic split of thymine under the conditions which we have used.[11]



There is at the present time too little evidence to allow us to formulate the mechanism of the oxidation reaction and of the formation of pyruvic acid from the intermediate oxidized compound. It seems most probable

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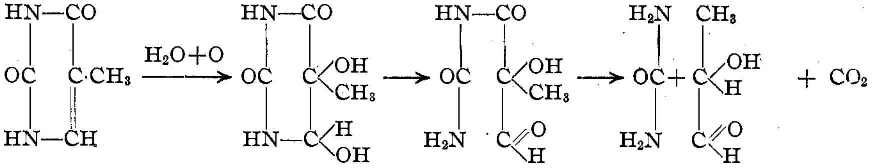
[9] Biltz, Heyn and Bergius, *Ann.*, **413**, 68 (1916). Biltz, *Ber.*, **54**, 1809 (1921).

[10] Behrend, *Ann.*, **309**, 275 (1899); **323**, 178 (1902).

[11] Another mechanism which was considered carefully involves the following steps:



to us that the process depends upon either peroxide formation or dehydrogenation.

The Significance of the Decomposition Products of Thymine for Biochemistry

The formation of lactic aldehyde, which we have assumed in our scheme of the reaction, is of particular chemical and biological interest. Lactic acid appears to play an important role in the metabolism of plants and animals, since it can be detected in many life processes. In particular, its presence has been noted in many cases when a rapid decomposition of cells takes place. For this reason considerable importance should doubtless be attached to lactic aldehyde, although it has not yet been isolated in metabolic processes because of its great lability.

From our investigations it may therefore be concluded that in the decomposition of plant and animal cells lactic acid and lactic aldehyde may be degradation products of thymine as well as of sugars.

Another product of the decomposition of thymine by our methods, pyruvic acid, can form alanine by reacting with ammonia.

Finally, urea has been obtained from nearly all the pyrimidines which we have investigated. It has been observed[12] recently that many fungi contain urea in large quantities but, to our knowledge, the formation of this compound has never been attributed to the degradation of thymine or other pyrimidines.

Experimental Part

Experiments with Hydrogen Peroxide.—1. Ten cc. of concd. hydrogen peroxide (a commercial 30% H_2O_2) was added to a solution of 0.5 g. of thymine in 150 cc. of distilled water and the reaction mixture was shaken for 12 hours in a shaking machine. Five g. of sodium bicarbonate was added and the solution was distilled to a volume of about 50 cc.

The distillate had a strong odor of acetol and with *o*-aminobenzaldehyde gave 3-hydroxyquinaldine, which was identified by its strong blue fluorescence and by its melting point. One-half the residue from the distillation gave a strong urea test with xanthydrol. The remainder gave a very faint indigo test for pyruvic acid when treated with *o*-nitrobenzaldehyde and alkali.

2. Ten cc. of concd. hydrogen peroxide and 0.6 g. of crystallized ferrous sulfate were added to a solution of 0.5 g. of thymine in 150 cc. of distilled water and the reaction mixture was shaken for 12 hours. After the addition of 5 g. of sodium bicarbonate, the solution was distilled to small volume.

Acetol was contained in the distillate in traces. It was necessary to use an iron arc to observe the blue fluorescence in the quinaldine test.[13] Part of the residue from the distillation gave immediately a heavy precipitate of indigo when treated with *o*-nitrobenzaldehyde and alkali, a fact which proved the presence of pyruvic acid. A xanthydrol test on the remainder of the residue proved the presence of a considerable quantity of urea.

3. This experiment is identical with that described in Paragraph 2 except for the fact that 0.05 g. of crystallized ferrous sulfate was used instead of 0.6 g. The distillate

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[12] Iwanoff, *Biochem. Z.*, 136, 1, 9 (1923).

[13] Ref. 4b, p. 1582.

gave a strong test for acetol, just as in Expt. 1. Contrary to Expt. 1, however, the residue from the distillation gave an intense indigo test. The presence of urea was proved by means of xanthydrol.

Experiments with Sodium Pentacyano-aquo-ferroate.—Five-tenths g. of sodium pentacyano-aquo-ferroate was added to a solution of 0.4 g. of thymine in 1 liter of distilled water. A moderate stream of oxygen was then passed through the reaction mixture. After a short time the bright yellow color of the solution changed to deep grass-green. After 2 days the flow of oxygen was stopped. Twenty g. of sodium bicarbonate was added to the clear, deep bluish-green solution and the mixture was then distilled to small volume.

The distillate did not give 3-hydroxyquinaldine when treated with *o*-aminobenzaldehyde, a fact which proves that no acetol was formed.[14] The residue from the distillation gave strong tests for urea and pyruvic acid.

Experiments with Ferrous Sulfate Plus Sodium Bicarbonate.—A solution of 10 g. of crystallized ferrous sulfate in 150 cc. of distilled water was added to a solution of 0.5 g. of thymine and 30 g. of sodium bicarbonate in 500 cc. of distilled water. This reaction mixture was shaken with air in a 1-liter flask with a rubber stopper carrying two glass tubes. The green ferrous carbonate peroxide gradually changed to red ferric hydroxide, the operation requiring about one hour. The precipitate was removed by filtration and the filtrate was distilled to small volume.

The distillate gave a strong acetol test and the residue gave strong tests for urea and pyruvic acid. Acetol and pyruvic acid were always formed in roughly equal quantities.

Experiments with Iodine Solution.—Fifty cc. of an approximately 0.1 *N* solution of iodine[15] was added to a solution of 0.5 g. of thymine and 30 g. of sodium bicarbonate. The reaction mixture, after dilution to 1 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 small volume. The distillate gave a strong acetol test. The residue gave a strong urea test, but no test for pyruvic acid. Although this experiment has been repeated several times, a positive test for pyruvic acid has never been obtained.

Summary

1. The action of the following reagents on thymine has been studied: (a) hydrogen peroxide; (b) hydrogen peroxide plus ferrous sulfate; (c) sodium pentacyano-aquo-ferroate plus oxygen or air; (d) ferrous sulfate plus sodium bicarbonate plus air; (e) iodine solution.

2. The primary products of the reactions still contain the urea residue.

3. The final products, namely, urea, acetol and pyruvic acid, are formed only when the primary products are heated in aqueous solution with sodium bicarbonate.

4. Acetol is formed as a direct hydrolytic product of thymine.

5. Pyruvic acid is formed by the complete hydrolysis of an intermediate oxidized compound.

6. A mechanism has been proposed for the decomposition of thymine under the conditions which have been used.

New Haven, Connecticut

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[14] In some cases it was possible to detect very faint traces of acetol in the distillate.

[15] 13 g. of iodine + 20 g. of KI per liter of solution.