dimethyl-aceto-acetate; mercury-*bis*-aceto-dimethylmethane; aceto-dimethyl-methyl-mercuric chloride; mercuric trimethylacetate.

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NEW METHODS OF SPLITTING PYRIMIDINES. II. THE DECOMPOSITION OF PYRIMIDINES BY MEANS OF FERROUS SALTS

BY MIMOSA HORTENSE PFALTZ[1] AND OSKAR BAUDISCH

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The reactions which we have used in splitting pyrimidines take place under conditions resembling as closely as possible natural conditions in the metabolism of plants and animals. Two methods have been studied intensively, namely, the system, ferrous sulfate plus sodium bicarbonate plus air, and the system, sodium pentacyano-aquo-ferroate,[2] $[Fe^{II}(CN)_5OH_2]Na_3$, plus oxygen or air. In both cases the reactions were carried out at room temperature (primary process), while the complete hydrolysis of the intermediate products (secondary process) was brought about at blood-temperature or at the temperature of the water-bath.

These methods, which were introduced by Baudisch,[3] will be applied to other types of compounds. The use of the system, ferrous sulfate plus sodium bicarbonate plus air, in a very sensitive test for thymine has already been described.[4]

Ferrous salts, which have been used previously only as reducing agents, under suitable conditions exert a strong hydrolyzing and oxidizing action on certain types of compounds. The reactions in the cases which have already been studied are quite similar to the hydrolytic changes brought about by life processes or by light energy. Under mild conditions it is possible to cause a partial hydrolysis of the stable pyrimidine ring with the formation of substances which are completely hydrolyzed by sodium bicarbonate at temperatures between 37° and 80°.

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

When ferrous sulfate is added to an aqueous solution of uracil containing an excess of sodium bicarbonate, a green ferrous carbonate peroxide is precipitated. When the reaction mixture is shaken with air, the ferrous compound is oxidized gradually to ferric hydroxide, while the pyrimidine

[1] This paper is constructed from a dissertation presented by Mimosa Hortense Pfaltz to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy. (O. B.)

[2] Hofmann, *Ann.*, **312**, 1 (1900).

[3] Baudisch, *Ber.*, **54**, 406 (1921).

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

suffers a partial hydrolysis with the formation of an intermediate compound. When the filtered solution, which contains excess sodium bicarbonate, is warmed this intermediate compound is completely hydrolyzed to urea and other products. When thymine is used instead of uracil, we obtain urea, acetol and pyruvic acid.

The amount of bicarbonate in the solution affects the extent of the hydrolysis.

TABLE I

INFLUENCE OF BICARBONATE AND EVAPORATION

In each experiment 2.0 g. of uracil, 30 g. of $FeSO_4.7H_2O$, and 700 cc. of distilled water were used. The reaction mixture, after the addition of solid $NaHCO_3$, was shaken with air in a flask.

Weight of $NaHCO_3$		% Urea formed	
G.	Molecular equivalents	Without evaporation	With evaporation
18	2	0	0
54	6	...	7.43[a]
108	12	traces	12.1

[a] The numerical values for urea do not take into account the slight hydrolysis produced by the distillation with sodium bicarbonate.

The Action of Sodium Pentacyano-aquo-ferroate Plus Oxygen or Air on Pyrimidines

The system, sodium pentacyano-aquo-ferroate[2] ("aquo salt")[5] plus oxygen or air, also brings about a partial hydrolysis of the pyrimidine ring. Four-tenths g. of uracil and 1.00 g. of the complex salt were dissolved in distilled water and a stream of oxygen gas was passed through the solution for 16 hours. Urea could not be detected in the reaction mixture, even after it was warmed on the water-bath. However, after the addition of sodium bicarbonate and evaporation, a quantity of urea was found which corresponded to a split of 6.43%.

The quantity of uracil split can be increased by shaking the dark green solution of uracil and aquo salt with oxygen under a pressure slightly greater than one atmosphere.

TABLE II

INFLUENCE OF "ACTIVATION" AND OXYGEN PRESSURE

In each experiment 0.200 g. of uracil and 0.60 g. of aquo salt were used.

No.	Conditions	Urea formed %
1	Uracil with stream of oxygen for 22 hours	18.3
2	"Activated uracil" with stream of oxygen for 22 hours	15.5
3	"Activated uracil" shaken with oxygen under pressure for 10 hours	53.0

Evidence will be submitted below for the assumption that a loose compound is formed by uracil and aquo salt, the presence of this compound

[5] "Aquo salt" is used for convenience as an abbreviation for sodium pentacyano-aquo-ferroate.

being indicated by a strong green color;[6] when present in this compound, uracil may be said to be activated. In Expt. 3, oxygen displaces the uracil more readily from the compound because of the greater pressure. This oxygen enters the inner sphere of the complex salt and thus becomes activated with the result that it is able to react with activated uracil.

The largest splits are obtained when a freshly prepared solution of aquo salt and uracil is shaken with oxygen under slight pressure. Under these conditions amounts of urea up to 57% of that calculated were found.

It should be emphasized that the presence of ferrous salts is necessary to bring about a decomposition of pyrimidines by the action of molecular oxygen under ordinary conditions.[7] This fact was proved by experiments in which oxygen was passed through aqueous solutions of uracil and thymine for 22 hours. Solid sodium bicarbonate was then added and the solutions were evaporated to dryness. By careful tests with xanthydrol it was impossible to detect any trace of urea.

The Action of Ferrous Sulfate Plus Oxygen and of Ferrous Sulfate Plus Hydrogen Peroxide on Pyrimidines

If oxygen is passed through a solution of uracil or thymine containing ferrous sulfate, only a small split results. The extent of the split is slightly greater if hydrogen peroxide is substituted for oxygen. As stated above, the presence of a ferrous salt is necessary for the decomposition when molecular oxygen is used.

TABLE III

COMPARISON OF ACTIVATION OF OXYGEN BY SIMPLE AND COMPLEX IRON SALTS

In each experiment 0.200 g. of pyrimidine and an equimolecular quantity of the ferrous salt were used. The uracil solutions were shaken 11.5 hours with oxygen under pressure and the thymine solutions 24 hours.

Reagent	With uracil Urea formed %	With thymine Urea formed %
Oxygen	0	0
Aquo salt + oxygen	40.9	13.1
$FeSO_4.7H_2O + H_2O_2$	4.3	3.5
$FeSO_4.7H_2O$ + oxygen	2.9	2.0

Evidence of Compound Formation between Complex Ferrous Ions and Pyrimidines

An explanation of the action of aquo salt as a catalyst in the reactions which have been discussed can best be given by considering the process in the light of Werner's Coördination Theory.

[6] An aqueous solution of aquo salt is light yellow in color, while an aqueous solution of uracil is colorless.

[7] It has been shown by Bass, however, that oxygen is able to split thymine to some extent under the influence of light, even in the absence of ferrous salts. L. W. Bass, THIS JOURNAL, 1924.

It has been shown that sodium pentacyano-aquo-ferroate can form compounds in which aquo (water molecule) has been replaced by whole organic molecules, such as pyridine,[8] nitrosobenzene,[9] *p*-nitrosodimethylaniline,[9] etc.

$$\left[\begin{matrix}\mathrm{Fe^{II}} & \mathrm{OH_2} \\ & (\mathrm{CN})_5\end{matrix}\right]\mathrm{Na_3} \longrightarrow \left[\begin{matrix}\mathrm{Fe^{II}} & \mathrm{C_6H_5NO} \\ & (\mathrm{CN})_5\end{matrix}\right]\mathrm{Na_3}$$

Most of these "penetration compounds"[10] are characterized by their striking colors. For example, in the reaction given above the light yellow color of the aquo salt changes to deep violet. The nitrosobenzene can be displaced from this violet compound by other substances which have a stronger affinity for the iron nucleus, such as sodium nitrite, sodium cyanide and carbon monoxide. In other words, we have to do with a competition for the auxiliary valences of the complex nucleus.

By analogy we are led to the conclusion that the pyrimidines, also, in the reaction with aquo salt primarily become linked to the central iron atom by displacing the water molecule. This conclusion is justified by the striking color changes which take place when a pyrimidine is allowed to react with aquo salt in the absence of air. Uracil and thymine form dark green compounds, while cytosine yields a deep red compound.

As a result of this compound formation the pyrimidine becomes "activated" and is therefore more susceptible to hydrolysis and oxidation. Similarly, oxygen becomes "activated" by other molecules of the complex salt.

$$\left[\begin{matrix}\mathrm{Fe^{II}} & \mathrm{OH_2} \\ & (\mathrm{CN})_5\end{matrix}\right]\mathrm{Na_3} \longrightarrow \left[\begin{matrix}\mathrm{Fe^{II}} & \mathrm{O_2} \\ & (\mathrm{CN})_5\end{matrix}\right]\mathrm{Na_3}$$

While we have definite evidence, in the case which has just been discussed, of the dependence of the reaction upon the auxiliary valences of the iron nucleus, we have no conclusive proof that penetration compounds containing pyrimidines are formed by ferrous carbonate or its peroxide. In this case, however, it was proved indirectly that addition of the pyrimidine to ferrous ion precedes the reaction; hence, we believe that this reaction also is dependent upon the action of auxiliary valences.

If caustic alkali is used in place of the weakly alkaline bicarbonate, the freshly precipitated ferrous hydroxide is able to absorb and activate atmospheric oxygen, but the pyrimidine present is not split under these conditions because the sensitive auxiliary valences of the ferrous ions are already occupied by sodium hydroxide molecules which the pyrimidine is unable to displace. If more alkali is used, even the absorption of oxygen

[8] Manchot and Woringer, *Ber.*, **46**, 3519 (1913).

[9] Ref. 3, p. 414.

[10] Schwarz-Bass, "The Chemistry of the Inorganic Complex Compounds," John Wiley and Sons, New York, **1923**, p. 30.

is prevented,[11] that is, the precipitate of ferrous hydroxide remains white even when oxygen is passed through the reaction mixture.

Additional proof of the assumption that the reaction depends upon the action of auxiliary valences has been brought forward by Bass,[12] who has shown that the catalytic decomposition of pyrimidines by the system, ferrous sulfate plus sodium bicarbonate plus air, may be poisoned by adding small quantities of arsenic trioxide, potassium nitrite, or ammonia to the reaction mixture.

Methods of Retarding the Reaction

The hydrolytic split of pyrimidines by aquo salt plus oxygen can be retarded in two ways. First, substances can be added to the reaction mixture which have a strong affinity for iron and which, therefore, occupy the auxiliary valences of the iron nucleus (poisoning). Second, the pyrimidine ring can be transformed into a more saturated condition, thus retarding or even preventing the formation of a complex compound with the ferrous salt.

In the first case the reaction may be retarded by adding potassium cyanide to the reaction mixture in an amount equivalent to the aquo salt and by allowing the solution to stand for a few minutes before passing in oxygen. When a solution of 0.200 g. of uracil and 0.60 g. of aquo salt was treated with oxygen for 22 hours, a quantity of urea equivalent to a split of 18.3% was obtained. In a duplicate experiment with an equimolecular quantity of potassium cyanide (0.155 g.), only 2.05% of urea was formed. Ammonia may be used in place of potassium cyanide. In an experiment with sodium pentacyano-ammine-ferroate,[2] $[Fe^{II}(CN)_5NH_3]Na_3$, uracil was not attacked, while a duplicate experiment with aquo salt gave a split of 9.7%.

When uracil, in complete absence of air, is shaken with aquo salt, we obtain the deep green solution mentioned previously, which has been shown to contain a complex compound of uracil and aquo salt. If oxygen is passed through this solution, only a small quantity of urea is formed, a fact which indicates that oxygen is not activated by the complex compound.

The second method of retarding the reaction, saturation of the pyrimidine ring, is best illustrated by comparing the extent of the split in uracil and hydro-uracil. In each experiment we used 0.200 g. of pyrimidine with an equimolecular quantity of aquo salt, the solution being shaken with oxygen under pressure for 9 hours. Under these conditions uracil gave 31.9% of urea and hydro-uracil 12.3%. From these results it is seen that saturation of the double bond in uracil decreases the extent of the split.

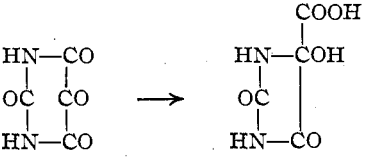
[11] Baudisch and Mayer, *Biochem. Z.*, **107**, 1 (1920).

[12] Bass, unpublished researches.

The Influence of the Methyl Group on the Stability of the Pyrimidine Ring

The influence of the methyl group on the stability of the pyrimidine ring under the conditions which we have described is illustrated by the following results. In experiments using 0.200 g. of pyrimidine, an equimolecular quantity of aquo salt, and oxygen under pressure, the following splits were obtained: uracil, 31.9%; thymine, 14.7%; and 4-methyluracil, 55.6%.

It has been shown by Biltz[13] that in alkaline solution carbon atom 5 of alloxan has a strong affinity for one of the nitrogen atoms of the urea residue and that under suitable conditions alloxanic acid is formed.



This property can undoubtedly be shared by other pyrimidines and explains the fact that thymine is more stable than uracil or 4-methyluracil. In thymine a methyl group is linked to the sensitive carbon atom in Position 5, with the result that any reactions attacking this carbon will be hindered. In 4-methyluracil, on the other hand, the methyl group is attached to a carbon atom which does not have a direct influence on the reaction.

Experimental Part

Apparatus.—For the precipitation of ferrous hydroxide in the absence of air an 800cc. Kjeldahl flask was used which was provided with a ground-glass stopper fused to a glass tube ending in a glass stopcock. The same type of flask was used in experiments which were conducted by shaking the reaction mixtures with oxygen under pressure. In experiments which employed a stream of oxygen, small gas wash bottles of about 300 cc. capacity were used.

Analytical Methods

Urea Determination.—Urea determinations were made in the filtered solutions obtained by dissolving with water the dry residues resulting from the evaporation of the reaction mixtures with sodium bicarbonate. The urea was precipitated according to Fosse's directions for solutions containing from 0.1 g. to 1.0 g. of urea per liter.[14] The proportions are 1 volume of solution, 2 volumes of glacial acetic acid, and 1/20 volume of a 10% solution of xanthydrol in methyl alcohol.

To insure complete precipitation, the solutions were usually allowed to stand overnight. The precipitate was filtered in a Gooch crucible and was washed first with alcohol to remove excess of xanthydrol and then with water until free from acid. After the addition of glacial acetic acid the solutions always turned green when pentacyano salts had been used. This Prussian blue was removed from the precipitates by washing them

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

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

first with water and then with alcohol. The crucibles were dried at 120° and the dixanthylurea was identified by its melting point (257°).

Acetol Test.—Acetol tests were made on the clear distillates obtained from reaction mixtures of thymine. The test depends upon the formation of 3-hydroxy-quinaldine when acetol is treated with *o*-aminobenzaldehyde in alkaline solution.[15] This compound shows a strong blue fluorescence, a phenomenon which makes the test very delicate.

The heated distillate from the reaction mixture was treated with 0.01 g. of *o*-aminobenzaldehyde in alcohol and 5 cc. of sodium hydroxide. Two-thirds of this solution was evaporated by boiling. After cooling, it was acidified with hydrochloric acid and then made alkaline with an excess of solid sodium bicarbonate. A blue fluorescence proved the presence of acetol. If the test obtained was so slight as to be doubtful, some of the solution was poured into a quartz test-tube and illuminated by ultraviolet light from an iron arc. By this means a test was often obtained when the blue fluorescence was not visible in daylight.

Pyruvic Acid Test.—This test was applied to the residues obtained by evaporating thymine reaction mixtures to dryness. It depends upon the formation of indigo when pyruvic acid in alkaline solution is treated with *o*-nitrobenzaldehyde.[16]

After evaporation of the reaction mixture the residue was taken up with water and evaporated again almost to dryness. To this solution were added an aqueous emulsion of *o*-nitrobenzaldehyde and a small quantity of potassium hydroxide solution. The mixture was then shaken with chloroform. A deep blue color in the chloroform layer proved the presence of pyruvic acid in the sample.

Experiments with Ferrous Carbonate Peroxide

The discussion of the experimental work with ferrous carbonate peroxide will be confined to a complete description of one typical experiment.

Two g. of uracil (Expt. 2, Table I) were dissolved with 54 g. of sodium bicarbonate in 700 cc. of boiling water. This solution was boiled for at least one hour in an 800cc. Kjeldahl flask (as described above) to remove all oxygen dissolved in the water. A test-tube containing 30 g. of powdered ferrous sulfate ($FeSO_4.7H_2O$) was lowered into the flask and the contents were boiled for 10 minutes longer. The ground-glass stopper, with the stopcock open, was fitted into the neck. When steam began to come out of the open end of the stopper the flame was removed and the stopcock was closed. After the reaction mixture had been cooled to room temperature the flask was inverted so that the alkaline uracil solution came into contact with the ferrous sulfate. A grayish-white precipitate of ferrous carbonate was formed, which slowly hydrolyzed to ferrous hydroxide.

When all of the ferrous sulfate had dissolved, the stopcock was opened to admit air. The precipitate of ferrous hydroxide immediately darkened and then turned green rapidly as the flask was shaken because of the formation of ferrous carbonate (or hydroxide) peroxide. The contents of the flask were then poured into a 2-liter round-bottom flask fitted with a rubber stopper containing a bent glass tube. This flask was shaken by hand until all the ferrous hydroxide peroxide had become oxidized to ferric hydroxide, a process which required about $1^1/_2$ hours.

When the oxidation was completed the reaction mixture was poured into a 1-liter graduated cylinder to allow the precipitate to settle. The clear supernatant liquid began to turn deep red, the color appearing first at the surface and spreading slowly downward through the liquid. After the liquid had stood overnight, the color varied

[15] Baudisch, *Biochem. Z.*, **89**, 279 (1918). Compare Ref. 4.

[16] Baeyer, *Ber.*, **15**, 2856 (1882). Compare Ref. 4.

from light to dark red, being darkest at the surface. This fact indicated that an absorption of oxygen had taken place, probably by the complex iron salts which exist in the solution.[17]

The volume was then made up to 650 cc. and two portions of 50 cc. each were removed by a pipet for determination of urea. One of these portions was evaporated to dryness before the analysis. (For results see Table I.)

Experiments with Sodium Pentacyano-aquo-ferroate

In this case, also, only one typical experiment will be described in detail.

One g. of uracil was dissolved in about 400 cc. of boiling water and the solution was then cooled to room temperature and diluted to 500 cc. One hundred cc. of this solution was transferred with a pipet to a Kjeldahl flask (as described above) and diluted with 100 cc. of water. Five-tenths g. of aquo salt (containing 0.08 g. of iron) was then added in the solid form and the flask was evacuated with a suction pump until the golden-brown liquid boiled at room temperature. The stopcock was closed and the flask was connected by means of pressure tubing to a gasometer filled with oxygen under a pressure slightly greater than one atmosphere. The stopcock was opened and the flask was shaken with oxygen for $7^1/_2$ hours. At the end of this time the color had changed to dark reddish-brown.

The reaction mixture was poured into a large evaporating dish and after the addition of 2 g. of sodium bicarbonate was evaporated to dryness on a water-bath. During the evaporation the solution became decolorized and ferric hydroxide was formed. The dry residue was dissolved with water, the solution filtered and diluted to 100 cc. A urea determination was made on half of this solution.

The effect of potassium cyanide is discussed on p. 2976. The reactions were carried out by passing oxygen (from a cylinder) through the solutions instead of shaking them with oxygen under pressure.

In Table II the effect of "activated" uracil is shown. Uracil was activated by boiling it for an hour in a Kjeldahl flask (as described above) with about 700 cc. of water and then lowering the required amount of solid aquo salt into the boiling solution by means of a test-tube. After the solution had boiled for 10 minutes longer the ground-glass stopper, with the stopcock open, was fitted into the flask and boiling was continued until steam came out of the open end of the tube. The burner was then removed and the stopcock closed. After cooling to room temperature the contents of the flask and test-tube were mixed by shaking. In Expt. 2 the reaction mixture was allowed to stand for 43 hours after mixing and in Expt. 3, for 24 hours.

Experiments with Ferrous Sulfate Plus Oxygen or Hydrogen Peroxide

The experiments with ferrous sulfate plus oxygen were carried out in the same manner as those with aquo salt and oxygen under pressure. When hydrogen peroxide was used instead of oxygen, it was added to the solution last and the flask was shaken with the stopcock open.

On evaporation with sodium bicarbonate, the iron was precipitated as ferroferric oxide, Fe_3O_4, instead of ferric hydroxide. Before evaporation with sodium bicarbonate the iron was already precipitated to some extent as a red ferric salt; the solution itself, however, remained colorless.

The writers wish to acknowledge the helpful suggestions and criticisms of Professor Treat B. Johnson during the progress of this work.

[17] Chandra, *Dissertation*, Berlin, 1913.

Summary

1. The action of ferrous salts on pyrimidines is described under conditions similar to those found in biological processes.

2. The reagents used were: (a) $FeSO_4.7H_2O$ + $NaHCO_3$ + air; (b) $[Fe(CN)_5OH_2]Na_3$ + O_2 or air; (c) $FeSO_4.7H_2O$ + O_2; (d) $FeSO_4.7H_2O$ + H_2O_2.

3. The cleavage may be considered to take place in two steps: (a) partial hydrolysis and oxidation with the formation of intermediate compounds; (b) complete hydrolysis of the intermediate compounds.

The extent of cleavage is determined by a quantitative estimation of the urea formed. In the case of thymine, acetol and pyruvic acid could be identified as reaction products, a fact which confirms the work of Johnson and Baudisch.[4]

4. It was shown experimentally that the reactions described are brought about by means of auxiliary valences of the ferrous compounds and pyrimidines. The proof was obtained by: (a) occupation (poisoning) of the auxiliary valences of the iron nucleus by sodium hydroxide, potassium cyanide, ammonia or pyrimidines; this poisoning in most cases was indicated by accompanying color reactions; (b) hydrogenation of the pyrimidine ring; (c) stabilization of the pyrimidine ring by a methyl group in Position 5.

5. The relatively greater efficiency of the complex ferrous salt as compared with an ordinary ferrous salt ($FeSO_4.7H_2O$) has been shown.

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THE OXIDATION OF URIC ACID WITH FERROUS SALTS. I

BY MIMOSA HORTENSE PFALTZ

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Introduction.—It was desired to determine the behavior of complex iron salts in the presence of oxygen toward uric acid, the most important member of the purine group. Like the pyrimidines, this compound is of great biological significance, being a product of nuclear metabolism, as well as being further oxidized in the animal body. This oxidation, whatever its nature, takes place under mild conditions (weak alkali and body temperature) and if uric acid could be oxidized in the laboratory under similar conditions, the identification of the products of oxidation might throw some light on the mechanism of uric acid metabolism. With this idea in mind the present investigations were undertaken.

As it has already been shown that pyrimidines can be broken down in the presence of these same complex salts,[1] these investigations were carried out in a manner analogous to those on the pyrimidines.

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