ON THE CHEMISTRY OF THE PYRIMIDINES.

VI. NEW COLOR TESTS FOR URACIL AND CYTOSINE.

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(Received for publication, March 26, 1924.)

The study of the action of ferrous bicarbonate plus air on the pyrimidines has been continued and the chemical change in the pyrimidine ring examined in more detail.

If an aqueous solution of cytosine is treated with the system ferrous sulfate plus sodium bicarbonate plus air, hydrolysis of the amino group occurs with the formation of uracil and the liberation of ammonia. Since uracil is formed from cytosine it might be assumed that in the further reaction of the system ferrous bicarbonate plus air, identical results should be expected, whether cytosine or uracil were used as the starting material. That is, however, not the case because the ammonia released from cytosine influences the reaction. The subsequent course of this influence will be described later in this paper.

Before the mechanism of the chemical changes in the pyrimidines, uracil and cytosine, was cleared up, it seemed as if uracil and cytosine behaved entirely differently, since different but characteristic color reactions were obtained in each case.

In our previous experiments with uracil and cytosine, we had studied the behavior of these compounds towards sodium-penta-cyano-aquo ferroate (aquo salt) and had found that the two pyrimidines form penetration compounds of different colors.

With the salt mentioned, uracil forms a deep green, and cytosine, a red compound. This is due to the fact that in uracil the ethylene group with its partial valences is linked to the central iron atom. Ethylene itself forms with the aquo salt a deep green compound. In cytosine not the ethylene group but the nitrogen of the amino group determines the character of the compound, just as in indole. Due to the nitrogen of the pyrrole ring of indole, a red penetration compound is formed and not a green one. This experimental result must be emphasized here, since it apparently shows, very directly, the affinity of pyrimidines for ferrous salts—a fact which is of great importance for further study with pyrimidines. Another result of our previous investigations on uracil and cytosine is the fact that after treatment with ferrous bicarbonate plus air, the ring of the pyrimidines is still intact, but after warming on the water bath, hydrolysis takes place with the formation of urea. Nothing could be said at that time regarding the fate of the remaining three carbons of the ring, so the experiments were continued with a view to obtaining a deeper insight into the chemical changes involved in the disruption of the pyrimidine ring.

The present paper describes the results of a new observation which should be emphasized at this point before passing on to a more detailed description. Uracil and cytosine form on treatment with ferrous bicarbonate and air and subsequent autooxidation of the colorless intermediate products, the same brilliant lemon-yellow pigment possessing special chemical properties. The formation of this pigment from the colorless intermediate compounds by such mild treatment is not only quite remarkable from biological, but also from a physical standpoint, since by this change the absorption of light is extended far into the visible spectrum.²

It should also be strongly emphasized that the pyrimidines, uracil and cytosine, which are stable in a weakly alkaline solution, become, on treatment with ferrous bicarbonate and air, autooxidizable systems which form a number of biochemically important compounds on successive absorption of oxygen.

² It is worth mentioning that fresh urine, the color of which resembles the aforementioned yellow pigment, also gives all the characteristic reactions, so that one may suspect that the new compound may be contained in urine.
The system ferrous sulfate plus sodium bicarbonate plus air does not, as one would expect, oxidize the pyrimidines, but causes the addition of 1 molecule of water to the ethylene group.

\[
\begin{align*}
\text{HN-CO} & \quad \text{HN-CO} \\
\text{OC} & \quad \text{OC} \\
\text{HN-CH} & \quad \text{HN-C} \\
\text{C} & \quad \text{C}
\end{align*}
\]

\[
+ \text{H}_2\text{O} = \quad \text{OC} & \quad \text{C} \\
\text{HN-CH} & \quad \text{HN-C}
\]

The same compound is formed from uracil and cytosine, namely dihydro-isobarbituric acid, which, up to the present time, could not be isolated, but its formation is indicated by several characteristic chemical reactions.

After treatment of an aqueous cytosine or uracil solution with ferrous bicarbonate and air and filtration of the red ferric hydroxide, a colorless, strongly autooxidizable solution is obtained. This solution is then gradually converted by the further absorption of oxygen from the air into the deep red complex ferrous salt of isobarbituric acid. On further autoxidation the deep red solution changes color from the surface downward into brilliant yellow. In alkaline solution at 37°C. this yellow pigment is weakly autooxidizable itself—a fact which is illustrated by the gradual disappearance of the yellow color from the surface.

If one removes the iron of the deep red complex ferrous salt obtained from cytosine after treatment with ferrous bicarbonate and air and subsequent autoxidation, by the addition of nitrosophenyl-hydroxylamine-ammonium (Cupferron)\textsuperscript{3} in a weakly acid solution in the cold, a brilliant yellow solution of the new compound is also obtained which is free from iron.

The same yellow compound is formed from a synthetized isobarbituric acid if the aqueous solution of the acid, together with small amounts of ferrous sulfate and bicarbonate, is allowed to stand in an open dish for 2 or 3 days. After addition of m-phosphoric acid and evaporation to dryness in the water bath, a brilliant yellow residue remains, which shows all the characteristic properties of the yellow compound obtained from uracil or cyto-

sine. The question now arises as to the chemical constitution of the new yellow compound. Although it has not been possible to analyze the yellow pigment because of the small quantities of pyrimidine which were at our disposal, it seems probable that its formation occurs in the following way.

\[
\begin{align*}
\text{Uracil} &\rightarrow \text{Yellow pigment} \rightarrow \text{Dihydro-isobarbituric acid.} \\
\text{Isobarbituric acid.}
\end{align*}
\]

According to this scheme it will be seen that we are dealing with the formation of a bimolecular pinacone-like compound, with the linking of two carbon atoms, 5 of the pyrimidine ring, a property which is characteristic of the atomic group.

*This compound resembles tetra methyl-dehydro-hydurilic-acid

\[
\begin{align*}
N(CH_3)-CO &\quad OC-N(CH_3) \\
OC &\quad C-C \\
N(CH_3)-CO &\quad OC-N(CH_3)
\end{align*}
\]

which was first prepared by Biltz and Hamburger (Biltz, H., and Hamburger, T., *Ber. chem. Ges.*, 1916, xlix, 655).
This type of reaction, as we shall see later, plays an important rôle in biochemical oxidation-reduction processes and in biochemical processes in general. As concerns the experimental part of the conversion of cytosine or uracil by means of ferrous bicarbonate and air, it was very apparent that the yellow pigment was formed much more quickly from cytosine than from uracil. This was found to be due to the fact that the ammonia produced in the cytosine experiment acts as a catalyst and hastens the autoxidation and, therefore, the formation of the yellow pigment. This pigment, which is characterized by a great stability toward both acids and alkalies, has special properties which will now be described in more detail.

The chemical reactions of the yellow pigment are quite different according to whether one deals with weakly acid or weakly basic solution. In basic solution, even at a very high dilution, the following characteristic reactions were observed. Ammoniacal silver nitrate solution is slowly reduced in the cold and is reduced very quickly on heating. The reducing power is very marked towards phosphomolybdic acid with the formation of a deep blue color. Especially sensitive is the reaction with diazobenzenesulfonic acid, which gives an intense, but temporary, brilliant red color. When the solution is cooled with ice before adding the diazo compound, the red color can be maintained for a considerable time.

The reaction with methylene blue is also very characteristic. Methylene blue solutions are decolorized quickly but an intense blue color appears at the surface due to the absorption of oxygen from the air. This reaction characterizes the reduction and oxidation power of the yellow pigment in alkaline solutions. Methylene blue is reduced to the leuco compound because of the loosely linked hydrogen atoms, while on the surface of the liquid a peroxide is formed by the absorption of oxygen which afterwards decomposes with the formation of hydrogen peroxide. Such oxidizing and reducing systems are particularly important in biochemical
processes, and groups which bring about such reactions appear in very different aliphatic and aromatic compounds in nature. In acid solution, the yellow pigment is very susceptible to reducing agents. For instance, zinc dust easily decolorizes it. On warming the colorless solution in an open bath, the yellow color returns on autoxidation.

These new experimental results in the study of the pyrimidines, uracil and cytosine, give, I believe, quite a new insight into the part which they play in biochemical processes—a field in which we have heretofore been completely in the dark.

Significance of the Formation of a Yellow Pigment from Cytosine and Uracil for Biochemistry.

Among the decomposition products of uric acid we find a number of pyrimidine combinations which have aroused the attention of biochemists since the early days of Liebig and Wöhler. The simple pyrimidines, uracil, cytosine, and thymine, however, have not been given sufficient attention by the biologist or the biochemist, and, therefore, we know practically nothing of the metabolism of these compounds in the plant or animal organism.

Prior to the investigations of Kossel and of Levene and Jacobs, it was still an unsolved question whether the pyrimidines appear as such in the nucleic acids or whether they are decomposition products of the purines. As a result of the investigations of Levene and Jacobs\(^4\) we now know that there are simple combinations of pyrimidines with sugar and phosphoric acid, so called nucleotides, which may be considered as the real components of nucleic acid. Still unsolved remained the biochemistry of the pyrimidine ring itself and nothing was known about the chemical properties of uracil, cytosine, and thymine as regards their relation to other compounds occurring in the plant or animal kingdom. In the following description will be given an outline of such relationships which are based on experimental observations, with instances such as indole, chrysarobin, quercetin, and adrenalin.

In the pyrimidine ring we have a very reactive group, namely the ethylene group, which we find also in indole and somewhat

masked in anthracene. On addition of 1 water molecule a new atomic configuration is obtained

\[
\begin{array}{c}
\text{CH} \\
+ \text{H}_2\text{O} \rightarrow \\
\text{CH}
\end{array}
\]

of a very reactive type and of special biochemical significance. Three reactions are particularly characteristic of this group as we shall now see. First, in alkaline solution this atomic configuration absorbs oxygen from the air with the formation of an intermediate peroxide which itself is unstable and decomposes with the production of hydrogen peroxide.

Second, 2 hydrogen atoms of the group in the presence of OH ions are very reactive and readily reduce to the leuco compounds such substances as methylene blue or indigo carmine. Third, one of the carbon atoms of the group possesses the power of linking other carbon atoms which results, of course, in the formation of bimolecular compounds according to the following scheme.

Such a bimolecular compound is obtained, for instance, with adrenalin and a solution of this substance gives all the previously described color reactions which are characteristic for an aqueous solution of isobarbituric acid or of the yellow compound ob-
tained from cytosine and uracil. If we now compare indole and uracil

\[
\begin{align*}
&\text{CH}_2\text{NH} \\
&\text{H} \\
&\text{O} \\
&\text{H} \\
&\text{N}--\text{CH} \\
&\text{CH} \\
&\text{NH} \\
&\text{OC} \\
&\text{H} \\
&\text{N}--\text{CO}
\end{align*}
\]

we will find that because of the ethylene groups in the ring many chemical properties of the two substances are alike. For instance, on treatment of indole with ferrous bicarbonate and air, a colorless solution is first obtained which gives the same characteristic reactions observed after treatment of uracil or cytosine with the same reagent, as for instance in its behavior towards such reagents as ammoniacal silver nitrate, phosphomolybdic acid, diazobenzenesulfonic acid, and the like.

The colorless solution obtained after shaking indole, with the system ferrous sulfate plus sodium bicarbonate and air is strongly autoxidizable, and due to the absorption of oxygen from the air becomes greenish yellow and finally indigo is formed.

A similar behavior is found among anthracene compounds which are widely distributed in nature. For instance, chrysarobin, a well known remedy for skin diseases, which forms on reduction dihydroanthranol

\[
\begin{align*}
&\text{OH} \\
&\text{OH} \\
&\text{OH} \\
&\text{CH}_3 \\
&\text{H} \\
&\text{H} \\
&\text{H} \\
&\text{H} \\
&\text{O} \\
&\text{C} \\
&\text{C} \\
&\text{OH} \\
&\text{OH} \\
&\text{O}_2(\text{Air}) \\
&\text{H}_2
\end{align*}
\]

1,8-Dihydroxymethyl dihydroanthranol. Chrysarobin.

and as a by-product anthrapinacone.

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It is quite interesting to compare all the steps involved in the transformations of anthracene to anthraquinone and of uracil to alloxan. All the compounds listed in the following table have been isolated and are described in the literature except dihydroisobarbituric acid which is first mentioned in this paper.

\[
\begin{align*}
&\text{Anthracene.} \\
&\text{Dihydroanthracene.} \\
&\text{Dihydroanthranol.} \\
&\text{Anthranol.} \\
&\text{Oxanthrol.} \\
\end{align*}
\]

\[
\begin{align*}
&\text{HN—CO} \\
&\text{OC—CH} \\
&\text{HN—CH} \\
&\text{HN—CO} \\
&\text{OC—CH}_2 \\
&\text{HN—CH}_2 \\
&\text{HN—CO} \\
&\text{OC—C—OH} \\
&\text{HN—C—H} \\
&\text{HN—CO} \\
&\text{OC—C—OH} \\
&\text{HN—C—OH}
\end{align*}
\]
Quite similar to the behavior of the anthracene derivatives concerning the attachment of 1 water molecule to a double carbon linkage, is the behavior of the group

\[
\begin{align*}
\text{Anthraquinone.} & \quad \text{HN--CO} \\
\text{OC} & \quad \text{CO} \\
\text{HN--CO} & \quad \text{Alloxan.}
\end{align*}
\]

which occurs in the anthocyanins. The formation of a cyanidine pseudo base by the addition of a water molecule to the carbon atom and finally oxidation to a flavone compound

\[
\begin{align*}
\text{O} & \quad + H_2O \rightarrow \\
\text{C} & \quad + O \\
\text{OH} & \quad \text{O}
\end{align*}
\]

resembles very much the aforementioned synthesis of dihydroisobarbituric acid from uracil. Also, in this case we have to deal with a system which is stable in acid solution and which becomes very reactive and autoxidizable in weak alkaline solution. It must be emphasized that in the natural pigments mentioned as well as in the pyrimidines, we have to do mostly with the glucosides of compounds which, according to our knowledge of certain anthocyanines, have a stronger reactivity than the compounds themselves.

If we summarize our observations we can assume that the pyrimidines, uracil and cytosine, or their decomposition products dihydrobarbituric acid, isobarbituric acid, and the brilliant yellow compound, possess the functions of respiration pigments. This explanation at the same time gives a deeper insight into the necessity of iron which, as is well known, can be found in every cell nucleus.
EXPERIMENTAL WORK.

Experiment 1.—On adding a solution of 50 gm. of ferrous sulfate in 100 cc. of distilled water to a solution of 50 gm. of sodium bicarbonate and 0.1 gm. of uracil in 1 liter of water, a white precipitate of ferrous bicarbonate was formed which gradually turned red on shaking with air. After filtration of the ferric hydroxide a clear, colorless solution remained which, on being allowed to evaporate in vacuum to about 150 cc., yielded a liquid which was clear and colorless (Solution S) but which immediately turned reddish upon contact with air. The reddish solution gave the following characteristic reactions.

With ammoniacal silver nitrate, reduction took place even in the cold; with phosphomolybdic acid an indigo blue color was formed. With diazo-benzenesulfonic acid a brilliant red color, quickly fading to yellow, was produced. On acidifying with m-phosphoric acid and evaporating to dryness in the water bath, there remained a brilliant lemon-yellow residue. But on acidifying with glacial acetic acid a residue remained which had an eosin-like color.

All these reactions are given only with a uracil solution that had been treated with ferrous bicarbonate and not with the uracil solution itself.

Another striking color reaction is the gradual change from colorless into red, then brilliant yellow, and again colorless, which took place if one allowed Solution S to stand in the incubator. The color change always begins at the surface, which indicated that the chemical process was due to an autoxidation process.

Experiment 2.—This experiment was an exact repetition of the first one, with the exception only that the mixture was not shaken by hand but stirred with a very good stirrer for several hours. After filtration and evaporation in vacuum to dryness the white residue became eosin red on contact with the air. While in the first experiment the reddish color was due to the formation of a complex ferrous salt of isobarbituric acid, here we had to do with an organic compound of red color which, however, gave all the same reactions described above.

Experiment 3.—In this experiment, cytosine was taken instead of uracil. The conditions were exactly the same as in Experiment 1 and the shaking was done by hand for 1 hour. Also, in this case the remaining solution after vacuum distillation was colorless, but changed to a deeper red than in the case of the uracil experiment. All the above mentioned characteristic reactions were observed, with the single exception that in the phosphomolybdic acid reaction, the color was greenish blue instead of indigo blue. This is due to the fact that in the cytosine experiment the yellow pigment was more rapidly formed than in the case of uracil and the mixture of blue and yellow gave the more green appearance.

Experiment 4.—The working conditions were changed as follows: In a 2 liter Pyrex round bottom flask 1½ liters of distilled water were boiled for 1 hour and then 0.1 gm. of uracil and 10 gm. of NaHCO₃ were added. After this, a test-tube was filled with 10 gm. of powdered ferrous sulfate
and suspended in the neck of the bottle. After further boiling for about 15 minutes, the bottle was closed air-tight, quickly cooled by tap water to room temperature, and then, by turning the flask, the ferrous sulfate mixed with the sodium bicarbonate was emptied out of the test-tube into the solution. The precipitated snow-white ferrous bicarbonate takes up oxygen rapidly and after vigorous shaking with air, all the iron had changed in about 20 minutes into the red ferric hydroxide. After filtration and evaporation to dryness in vacuum, a colorless residue remains, which, after solution in water, becomes reddish. This solution gave all the aforementioned reactions.

Experiment 5.—This experiment was similar to Experiment 4, except that cytosine was used. In this case the white ferrous bicarbonate did not change so quickly into the red ferric hydroxide because of the ammonia split off from the cytosine by hydrolysis. A dark blue-green precipitate was formed, which changed after further shaking with air into the red ferric salt. The residue from the vacuum distillation was again colorless but still could be changed with air into a reddish or eosin red color. By dissolving in water, the color changes only to yellow. This liquid also gave all the characteristic reactions.

Sensitiveness of the New Color Reactions for Uracil and Cytosine.6

A solution of 0.01 gm. of uracil or cytosine in 1 liter of water was boiled for half an hour and then 5 gm. of sodium bicarbonate were added.

The ferrous sulfate was put in a test-tube and not allowed to be mixed with the boiling liquid. After 10 minutes boiling the flask was closed air-tight and suddenly cooled by tap water. After mixing the ferrous sulfate with the liquid, white ferrous bicarbonate was precipitated and it took only about 20 minutes to change it into red ferric hydroxide. After filtration and evaporation in vacuo, there resulted a colorless residue which was dissolved in 30 cc. of water. The solution changed gradually to a yellowish red color. For the different reactions only 1 cc. of this liquid was taken for each one. The reactions were as follows:

With ammoniacal silver nitrate solution on warming—intensive reduction. With diazobenzene sulfonic acid—a brilliant red color which quickly faded. With phosphomolybdic acid—a deep blue color.

This experiment proves the very great sensitiveness of the new color reactions for uracil and cytosine.

Experiment 6. Experiment 5 was repeated, but the precipitated ferrous bicarbonate was allowed to stand in the absence of air for about 3 hours before it was shaken with air. The aged ferrous hydroxide absorbed the oxygen much more slowly and after 1 hour of vigorous shaking, the iron precipitate was only greenish brown and not red. After filtration and evaporation in vacuum, the residue did not give any of the characteristic reactions.

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J. Biol. Chem. 1924, 60:155-166.

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