STUDIES OF THE INTERMEDIATE PRODUCTS FORMED DURING THE HYDROLYSIS OF UREA BY UREASE

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Cyanic Acid Is Not Formed by the Action of Urease on Urea

In 1923, Fearon\textsuperscript{1} stated that he had been able to detect traces of cyanic acid in solutions in which urea was being decomposed by soy bean urease. He proposed that the reaction concerned is not a true hydrolysis, but rather a cleavage of the urea molecule into cyanic acid and ammonia and that the cyanic acid is at once spontaneously hydrolyzed to ammonia and carbon dioxide.\textsuperscript{2}

Sumner,\textsuperscript{3} in 1926, objected to Fearon's theory not only because he could not detect cyanic acid after allowing exceptionally pure preparations of urease to react with urea, but also because he contended that cyanic acid, if formed, would not be hydrolyzed in an alkaline or neutral medium, but would accumulate so that the end-product of urease action would be ammonium cyanate and not ammonium carbonate. Sumner is not the only author whose opinion and results are contrary to those of Fearon. Armstrong and Horton,\textsuperscript{4} in 1912, failed to detect cyanic acid among the decomposition products of urea acted on by urease and Iwanoff,\textsuperscript{5} in 1924, could find no cyanic acid when urease was allowed to act upon urea dissolved in 80 per cent alcohol. Kay\textsuperscript{6} has ob-

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\textsuperscript{1} Fearon, W. R., Biochem. J., 17, 84, 800 (1923).
\textsuperscript{2} Werner, E. A., The chemistry of urea, New York (1923).
\textsuperscript{3} Sumner, J. B., J. Biol. Chem., 68, 101 (1926).
\textsuperscript{5} Iwanoff, N. N., Biochem. Z., 150, 108 (1924).
\textsuperscript{6} Kay, H. D., Biochem. J., 17, 277 (1923).
Urea Hydrolysis by Urease

jected to Fearon's theory because of the great rapidity and completeness with which urea is hydrolyzed by urease.

In 1926, Fearon stated in another paper\(^7\) that he fully realized the validity of the objections of Kay and of Sumner, but that he had been able to demonstrate the formation of traces of cyanate by the use of a new color test. The test could not be used directly because the soy bean meal added as a source of urease interfered. Fearon allowed the meal to react with urea for 10 hours, after which the solution was treated with silver nitrate. The precipitate was decomposed by adding a sufficient amount of hydrochloric acid and the color test was then applied to the solution.

Fearon worked under the disadvantage of having to employ an impure urease preparation. Using crystalline urease\(^8\) we have had no difficulty in applying Fearon's test in slightly modified form directly to solutions of urea that are undergoing hydrolysis by urease and in showing that within the limit of delicacy of the test no cyanic acid is ever present.

The original test described by Fearon states that one should add 2 to 6 drops of a 6 per cent alcoholic benzidine solution to 5 cc. of water, followed by 2 to 6 drops of cupric acetate and that, after mixing, the solution suspected of containing the cyanate should be added. In the presence of cyanate a purplish precipitate appears. We have found it necessary to use much less copper acetate because if too much is used there is formed in the presence of ammonium chloride a blue, green, or gray precipitate which spoils the test. In our experiments large amounts of ammonium chloride were present.

Our procedure consisted in adding to 2 gm. of urea 5 cc. of water, 3 drops of 0.04 per cent methyl red, and 1 cc. of phosphate-free crystalline urease of from 50 to 150 units activity. After being mixed, 0.1 N hydrochloric acid was run in, drop by drop, with constant rotation to neutralize the ammonia, care being taken never to make the solution acid to methyl red. We have usually added about 30 cc. of acid. To stop the action of the urease we have next added 1 drop of cupric acetate, after which 0.1 N hydrochloric acid has been added cautiously to bring the solution to pH 5. Cyanate has then been tested for by adding 1 drop of

\(^7\) Fearon, W. R., *J. Biol. Chem.*, 70, 785 (1926).

freshly prepared 6 per cent alcoholic benzidine to 3 cc. of the solution. In all cases the tests, as indicated by lack of a purplish precipitate, have been negative.

As a check of the delicacy of the test runs have been made similar to the above except that known amounts of pure potassium cyanate have been added at the start. It was found that the purple precipitate was obtained if the final solution contained as much as 0.13 mg. of potassium cyanate per cc. Pure potassium cyanate solutions gave positive tests with as little as 0.025 mg. per cc.

We have employed the silver nitrate test for cyanate in a similar manner, but here have neutralized the ammonia with 0.2 N acetic acid and have removed the urease by means of aluminum hydroxide before adding the silver nitrate. In all cases the tests were negative as shown by lack of a precipitate. When potassium cyanate was added at the beginning of the experiment a precipitate of silver cyanate was formed if as much as 0.15 mg. of cyanate was present in the final solution.

Ammonium Carbamate Is Produced by the Action of Urease on Urea

Whereas the hydrolysis of urea by water, acids, and alkalis is best explained by the intermediate formation of ammonium cyanate,9,10 the intermediate product of the hydrolysis of urea by urease has been considered by the majority of observers in this field to be ammonium carbamate. As early as 1885 Fenton11 suggested that ammonium carbamate is formed by the hydrolysis of urea under the influence of ferments. Armstrong and Horton4 stated that ammonium carbamate is the only intermediate compound which should be considered and that urease, by determining the resolution of urea in an abnormal direction, serves a most important purpose.

As far as a search of the literature shows, Yamasaki12 has been the only investigator who has made quantitative analyses to show that carbamate is actually formed from urea by urease. He used the method of Fenton11 for the determination of urea and carbamates in this study.
mate. He added carbon dioxide to solutions of urea that were being hydrolyzed by soy bean urease and found this to have a retarding effect upon the spontaneous decomposition of the carbamate. He found that the concentration of the carbamate reached a maximum and that its rate of decomposition was measurable. Yamasaki calls his methods semi-quantitative, nevertheless his findings have been fully confirmed by us.

We felt that the experimental work showing ammonium carbamate to be the intermediate product should be repeated, using crystalline urease and, if possible, employing a method more dependable than that used by Yamasaki.

Method for Determination of Carbamic Acid

The first step necessary was to find some reliable way of analyzing for carbamic acid. At the start we used in a qualitative manner the procedure outlined by Lewis and Borrows. Here, carbonate is removed by adding ice-cold barium hydroxide and filtering in the cold. If the clear filtrate is warmed gently, or allowed to stand out of contact with the air, the carbamate decomposes and gives a precipitate of barium carbonate. We intended to adapt this procedure for quantitative use but did not because we were successful in finding a better one. Our method depends upon the fact that when a solution of ammonium carbamate is Nesslerized only the nitrogen of the ammonium radical reacts. The carbamic acid is stabilized by the Nessler reagent and does not decompose for as long as 1 hour.

To carry out the analysis one pipettes 1 cc. of the carbamate solution directly into a 100 cc. volumetric flask which already contains 3 drops of saturated sodium hydroxide mixed with about 70 cc. of water. The alkali serves to stabilize the carbamate. The solution to be analyzed should contain approximately 1 mg. of ammonia nitrogen. The standard should contain the right amount of ammonium sulfate and the same amount of sodium hydroxide, urea, and enzyme as the unknown. The enzyme should be inactivated by potassium-mericuric iodide before being added to

the standard. Both flasks are treated with 10 cc. of the Nessler solution of Folin and Wu, diluted, mixed, and read in the colorimeter. This reading gives the value for ammonium nitrogen. To obtain the value for the total of ammonium and carbamate nitrogen one pipettes 1 cc. of the carbamate solution into a 100 cc. volumetric flask which contains 1 cc. of N hydrochloric acid. The carbamate is instantly decomposed to ammonium chloride and carbon dioxide. The solution is Nesslerized and compared with a standard containing the same amount of acid and an appropriate amount of ammonium sulfate, urea, etc. In experiments where the carbamate is increasing or decreasing rapidly the two analyses must be made by two persons at the same time, or else the result of one analysis must be interpolated to the time of the other. Subtraction of the value of the analysis to which alkali was added from the result of that to which acid was added gives the carbamic acid nitrogen in mg. per cc.

The method has been shown to give accurate results with pure ammonium carbamate. Here the salt is dumped from a weighing bottle into dilute sodium hydroxide and the solution mixed and analyzed. The method is not accurate for determining carbamate when the carbamate amounts to as little as 10 per cent of the total of ammonium and carbamate nitrogen, since the determination is by difference.

Our pure ammonium carbamate was prepared by passing ammonia gas, dried over sodium hydroxide sticks, and carbon dioxide, dried by bubbling through strong sulfuric acid, into a bottle. The solid carbamate was loosened from the bottom of the bottle by pounding with a glass rod. Samples of the salt dissolved in barium hydroxide without giving a precipitate of carbonate.

**Proof That Carbamate Is the Intermediate Product**

With the procedure described above it has been shown that when urease acts on urea in alkaline solution ammonium carbamate is formed in large quantities and that after the urease has been inactivated the carbamate decomposes to carbonate at a measurable rate.

*Experiment 1*—100 cc. of 1 per cent urea chilled by crushed ice were treated with 3 cc. of crystalline urease of about 50 units per cc. The solution was mixed at once. After 5 minutes the
urease was inactivated with 1 drop of 17 per cent potassio-mercuric iodide. Samples were taken at intervals and analyzed for carbamate by our procedure. The percentages of ammonium carbamate, of the total of ammonium carbonate and carbamate are plotted in Fig. 1.

Experiment 2—In Fig. 2 are shown results of an experiment
similar to the above except that the urease was not poisoned with potassio-mercuric iodide.

Experiment 3—Fig. 3 shows the spontaneous hydrolysis of ammonium carbamate after addition of 261 mg. of the pure salt to 100 cc. of water at 0°.

Experiment 4—We wished to see if any carbamate could be detected when urea is decomposed by urease at 0° in the presence of phosphate buffer or acetate buffer, but no trace of carbamate could be detected either by our method or by the more sensitive method of Lewis and Borrows. Hence it appears that under these conditions carbamate is not formed, or, what is more probable, that carbamate is decomposed as fast as it is formed.

Experiment 5—We were interested to see if the spontaneous decomposition of carbamate could be prevented by the presence of an alkaline buffer. A buffer solution just alkaline to phenolphthalein was made from pure sodium bicarbonate. This was chilled to 0° and into it were dumped 191 mg. of ammonium carbamate. Fig. 4 shows that the rate of hydrolysis is hastened by the buffer, since after 16 minutes nearly all of the carbamate has become decomposed.

Experiment 6—Yamasaki states that carbonic acid somewhat prevents the spontaneous hydrolysis of carbamate. We tested
this by dumping 220 mg. of ammonium carbamate into 100 cc. of water at 0° through which a rapid stream of carbon dioxide was passing. After 6 minutes the reaction of the solution became acid to phenol red. Analyses made 7 and 24 minutes after the addition of the carbamate gave respectively 87 and 84 per cent ammonium carbamate, showing that carbonic acid delays the decomposition of carbamic acid.

Fig. 4. Decrease in percentage of carbamate in Na₂CO₃-NaHCO₃

Fig. 5. Decrease in percentage of carbamate in presence of CO₂
Experiment 7—Here 100 cc. of 0.2 per cent urea were chilled to 0° and saturated with carbonic acid. 1 cc. of urease was added at the rate of 1 drop per minute. After 15 minutes the urease was inactivated by 1 drop of potassio-mercurec iodide. Analyses were made at intervals. As can be seen from Fig. 5 the rate of hydrolysis of carbamate is decreased in this experiment.

The possibility exists that the ammonium carbamate formed by the action of urease on urea is a secondary product produced from carbon dioxide and ammonia, although it is difficult to understand what primary substance there could be that would split into carbon dioxide and ammonia. In Experiment 8 it is shown that when ammonia is treated in the cold with carbonic acid the primary product is largely ammonium carbamate.

Experiment 8 Ice water was saturated with a stream of carbon dioxide. Ammonium hydroxide was added in small quantities at a time until the total nitrogen of the solution amounted to 1 mg. per cc. The solution, which at this time was acid to phenol red, contained 82 per cent of its nitrogen in the form of ammonium carbamate.

SUMMARY

We have shown that cyanate is not formed during the hydrolysis of urea by urease.

We have confirmed the work of Yamasaki and have shown that when urea is acted on by urease in alkaline solution ammonium carbamate is formed in large amount and that the carbamate spontaneously decomposes at a measurable rate. We have confirmed Yamasaki's finding that carbonic acid delays the decomposition of carbamate. No carbamate is formed by urease in the presence of buffers. There is no reason to doubt that ammonium carbamate is an intermediate product of the action of urease on urea, but it is not necessarily the first intermediate product.
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