XII. UREASE. PART I. THE CHEMICAL CHANGES INVOLVED IN THE ZYMOLYSIS OF UREA.

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UREA in aqueous solution is readily converted into ammonia and carbon dioxide by a definite class of ferments. The chemistry of this change attracted attention as far back as the end of the eighteenth century, when Fourcroy and Vauquelin (1798) proved that when urine undergoes alkaline fermentation on exposure to the air the ammonia formed is produced from the urea originally present in the urine.

Pasteur [1861] showed that a living organism was responsible for the alkaline fermentation of urine. The organism was isolated three years later by van Tieghem [1864], and was given the name *Micrococcus ureae*. Musculus [1874, 1876] obtained an enzyme from putrid urine, which he found capable of decomposing urea in aqueous solutions. Lea [1885] cultivated the *Micrococcus ureae* in quantity, and confirmed the results of Musculus.

Over 30 different micro-organisms capable of decomposing urea were recognised and described by Miquel [1890], who first suggested the name *urease* for the enzyme common to all these ureaclastic organisms. Since the discovery by Takeuchi [1909] of soy-bean urease, a plentiful supply of the enzyme has been assured, and much attention has been devoted to the study of the decomposition of urea by urease during the intervening years. Results of great practical value in connection with the estimation of urea have been obtained, but the actual chemical mechanism of the change does not seem to have been investigated very closely since Dumas [1830] suggested the equation

to represent the direct hydrolysis of urea in aqueous solution.

The zymolysis of urea has very generally been taken without question to be a simple direct hydration of the urea molecule, either free in solution or combined with the enzyme.

Armstrong and Horton [1912] considered the possibility of urea being in solution in the hypothetical hydrated form of "carbamide," $\text{C}(\text{OH})_2(\text{NH}_2)_2$, and producing on hydrolysis ortho-carbonic acid and ammonia.

Yamasaki [1920], from the reaction rates and temperature coefficients of the zymolysis, maintains that the decomposition of urea takes place in two definite stages, with the formation of ammonium carbamate as the inter-

mediate product. However, neither of the suggestions affects the general assumption that urea is broken down in solution by a direct hydration process.

If this be so, it seems rather remarkable that the attempts to obtain urea by a reverse synthesis from ammonium carbonate and carbamate, have been so unconvincing, more especially when it is considered that urea is a much more stable compound than either ammonium carbonate or carbamate.

Barendrecht [1919, 1921] has put forward a "radiation" theory of enzyme action. He asserts that when the urease "radiation" strikes a molecule of urea it is absorbed, and as a result the urea is hydrolysed. No explanation is offered as to how this hydrolysis takes place. In support of the "radiation" theory, Barendrecht claims to have observed a reverse zymolysis in solutions where the urease is "decaying," and to have obtained a synthesis of urea from ammonium carbonate solutions and urease "decaying" through the combined influences of alkalinity, time, and temperature.

The validity of Barendrecht's claim is questioned by Mattaar [1920], who asserts that small quantities of urea when added to solutions of ammonium carbonate are destroyed by urease under conditions comparable with Barendrecht's alleged synthesis.

When it is realised that such a synthesis of urea is based on an entirely erroneous conception of the relationship between urea and ammonium carbonate, it will be seen that its occurrence is open to doubt.

The decomposition of urea in aqueous solution.

It was first proved by Fawsitt [1902] that the hydrolysis of urea by acids, alkalies, or water alone at the temperature of 100° does not proceed along the lines of the familiar equation suggested by Dumas in 1830. An intermediate cyanate stage was demonstrated, and Fawsitt concluded that urea in solution is first converted by an intramolecular change into ammonium cyanate, and that the ammonium cyanate is hydrolysed to ammonium carbonate. The influence of acids, alkalies, and alcohol on the change were also investigated [Burrows and Fawsitt, 1914].

E. A. Werner, in the course of his extensive work on the constitution of urea, has discussed these experiments in detail [1918], and has shown that the so-called "hydrolysis" of urea under all conditions consists of two stages: (a) the simple dissociation of the urea molecule into ammonia and free cyanic acid, and (b) the hydrolysis of the cyanic acid by the solvent [1913, 1918, 1920].

It has, of course, long been known that cyanic acid is formed when aqueous solutions of urea are heated above 70°, and advantage of this fact has been taken by Walker and Hambly [1895] to prepare pure silver cyanate, but the significance of this formation of cyanic acid has only recently been realised.

Up to the present, the Werner formula for urea has not been considered in connection with the enzyme decomposition of urea. If urease acts in the manner generally ascribed to catalysts and brings about zymolysis by accelerating greatly a normal chemical transformation, it seems probable that the mechanism of the decomposition of urea by urease is comparable with the decomposition of urea by acids, and by alkalies.

If this be so, a careful chemical examination of the system during zymolysis should reveal the presence of the products of dissociation.

THE UREA/UREASE SYSTEM.

In studying an enzyme system attention must be devoted to the following aspects: (1) the enzyme preparation, (2) the application of the enzyme, (3) the substrate, (4) the methods of analysis.

(1) *The enzyme.* A very satisfactory preparation of urease is obtained by extracting the fresh finely ground meal of soy-bean with ether in a Soxhlet extractor for six to twelve hours. By this means most of the fatty material is removed and a very active uniform dispersion is obtained by shaking up with water or with weak alcohol [Folin, 1919]. A better preparation may be got by extracting with absolute alcohol immediately after the ether treatment, but for the present work, attention was devoted to obtaining a standard preparation of uniform zymolytic properties rather than an enzyme preparation of approximate purity.

(2) *The application of the enzyme.* In a qualitative study of enzyme-action it is convenient to have some means of separating the enzyme from the main bulk of the substrate solution so that the subsequent analysis will not be complicated by the presence of enzyme material and debris. This was accomplished by enclosing the enzyme in a collodion tube in one group of the experiments, so that although the substrate had free access to the enzyme the latter did not escape into the bulk of the solution.

Several types of collodion tubes were examined. The most satisfactory ones were made by the Hatschek formula [1920]. The tubes were tested by dialysing urease solutions against distilled water and solutions of ammonium carbonate. Tubes that allowed sufficient enzyme material to escape in 24 hours at room temperature to give a positive biuret reaction on testing the surrounding fluid were rejected.

These collodion tubes are very permeable towards urea and ammonium salts.

In employing collodion separators in enzyme research it is necessary to consider the possible inactivation of the enzyme.

Urease suspensions of the strength used in the experiments were not found to be seriously affected by being kept in the tubes for periods up to two days. After that time, inactivation was apparent in the weaker suspensions, although this seemed to be not so much due to the loss of a dialysable component, or

“co-enzyme,” as claimed by Onodera [1915], but rather to be an instance of membrane adsorption of the enzyme.

To obtain a large surface of enzyme suspension exposed to the substrate, a test-tube of smaller diameter was inserted into each collodion tube so that the urease occupied the space between the two tubes. Inverted specimen jars of capacity 100 to 1000 cc. with narrow necks were used to contain the substrate. The collodion tubes were of such size that they almost filled the necks of the jars.

The hydrogen ion concentration of the system was kept within the working range of the enzyme (*i.e.* p_H 6.8–7.2) by means of centinormal nitric acid and the use of a Cole-Onslow comparator.

(3) *The substrate.* The urea used was purified by two crystallisations from water, and was free from all cyanate, carbonate, carbamate, and ammonia reactions. Solutions of urea in water are readily attacked by the micro-organisms of the ordinary laboratory air, and, consequently, the substrate solutions were made up with sterile water and kept in clean stoppered bottles.

(4) *Methods of analysis.* The analysis of the products of urea decomposition in simple solutions is not a difficult matter, but when the solutions are liable to contain materials extracted from the enzyme special precautions have to be taken. Ammonia was detected by the usual Nessler method, and was estimated by aspiration and Nesslerisation or titration in a comparator.

The presence of traces of ammonia in the urease was of no consequence in most of the experiments. When necessary, it can either be estimated and allowed for, or removed by a few days extraction of the enzyme by absolute alcohol at 30°. Folin [1919] has advocated the use of “permutite” but the common fuller’s earth seems to be effective also.

It may be noted, in passing, that solutions of urease will develop ammonia when kept for some hours in contact with strong alkalies at room temperatures.

Carbon dioxide was detected and estimated by aspiration into standard barium hydroxide solutions.

Cyanic acid was detected and isolated as the silver salt, by which means it has been found possible to separate it from carbonates and carbamates.

This separation is effected by adjusting the hydrogen ion concentration of the solution to p_H 5.0 by means of $N/50$ HNO_3 , and a comparator, using methyl-red as indicator. Under these conditions, all the silver carbonate (and carbamate, if any) is dissolved, and silver cyanate remains.

The presence of the cyanate radicle in the silver precipitate may be demonstrated by three colour tests.

For detecting small traces of cyanate the following method was devised. The silver precipitate, separated from carbonate and carbamate, is collected on a small filter paper and washed thoroughly with distilled water until the washings no longer give any ammonia reaction with Nessler’s reagent. The precipitate on the paper is treated with a few drops of warm $N/2$ HCl ; this liberates and hydrolyses any cyanic acid present, and on washing the

precipitate once more, the filtrate gives a positive Nessler reaction. This is an exceedingly delicate test for cyanates, provided proper precautions are taken to see that the reagents are ammonia-free. The cyanate radicle may also be demonstrated by treating the precipitate with a few drops of 5 % hydroxylamine sulphate, and after a couple of minutes adding a little dilute ferric chloride; a purple colour is produced. This test is due to Hantzsch and Sauer [1897]. The colour is due to the liberation of cyanic acid and the combination of this with hydroxylamine to form a hydroxy-urea, the ferric salt of which is dark purple in solution. It has been developed by the present author into a method for estimating cyanates colorimetrically. Certain precautions must be taken in applying the test to traces of cyanates. The presence of much ammonia interferes with the test by obscuring the characteristic black-purple shade. The hydroxylamine must remain in contact with the free cyanic acid for sufficient time to interact to form the hydroxy-urea. In the presence of excess of the hydroxylamine the coloration is not permanent as the ferric salt is soon reduced to an almost colourless ferrous salt. The colour can be restored by careful addition of more ferric chloride or by adding a drop of hydrogen peroxide solution (which must be free from acetanilide, or any other preservative which gives a colour with ferric salts). Once the solution has been freed from uncombined hydroxylamine the colour is permanent.

Cyanates formed in solution may also be identified by the fact that the free cyanic acid combines with urea to form biuret.

Cyanates in quantity can be most rapidly shown (as, for example, the sublimate formed by heating dry urea in a test-tube) by the intense blue colour given with solutions of cobalt acetate or nitrate.

The delicacy of these tests under general conditions is as follows. Nessler method, 1 in 400,000; hydroxylamine reaction, 1 in 2000; cobalt method 1 in 90; but the delicacy can be greatly increased by using the centrifuge to collect the silver precipitates and by careful washing with ice-cold water.

Biuret was identified and estimated colorimetrically by the copper method, as described by Werner [1913], under conditions when the absence of foreign protein material derived from the enzyme was assured.

These analytical methods are grouped here for convenience, as they will be referred to in subsequent papers.

*Experimental. Series I. Detection of cyanates in the
urea/urease system.*

Fresh solutions of pure urea in distilled water saturated with CO_2 were prepared of concentrations 1, 5, 10, 20 %, and submitted to the action of freshly extracted urease (1 g. in each collodion tube). The solutions were kept at low temperature, $1^\circ\text{--}5^\circ$, to retard hydrolysis of the cyanic acid. At hourly intervals samples were drawn off from the outer containing jars, treated with $N/10 \text{ AgNO}_3$, the solution set to $p_{\text{H}} 5$ by $N/50 \text{ HNO}_3$, and filtered through a close filter paper.

After two hours all the solutions gave positive reactions for cyanic acid by the Nessler method, this continued for several hours in the solutions, but entirely disappeared from the 1 % solution on keeping the flasks at room temperature overnight. All the solutions next day gave a definite biuret reaction, most marked in the strongest solution of urea. By means of a control it was found that this did not arise from the escape of protein material from the dialysing tubes, but was due to the interaction between cyanic acid and the substrate.

Cyanic acid was also shown in solutions of urea undergoing zymolysis in ordinary flasks in direct contact with the enzyme, by treating samples drawn off from time to time with excess of colloidal ferric hydroxide, which precipitates nearly all the ammonia from the solution and brings down the enzyme with the precipitate. On centrifuging the samples, cyanates were found to be present in the clear supernatant layer by applying the hydroxylamine test, and by pipetting off the clear solution and applying the silver method.

The conclusion from a large number of experiments of this type is that cyanic acid is produced during the decomposition of urea by urease. The yield of cyanic acid as silver cyanate in these experiments was always small; up to 0.1 g. from a litre of 5 % urea solution being obtained.

Experimental. Series II. Estimation of the cyanic acid formed during the zymolysis of urea.

Method A. Substrate, 5 % urea. Enzyme, 5 % extracted urease suspension which had been kept for three days at 40°, until the coarser particles had subsided. Samples of 20 cc. of substrate and 10 cc. of enzyme suspension were mixed at 40°, set to p_H 6.5, and incubated at 40°.

At hourly intervals, duplicate pairs of flasks were withdrawn and each treated with 1 cc. of $N/1$ $AgNO_3$. One flask was then titrated to p_H 5 with 0.6 N HNO_3 , using methyl-red paper¹ and a standard flask for comparison.

The acid used gave a measure of the relative degree of zymolysis, and also dissolved any silver carbonate or carbamate that might be present, leaving silver cyanate, silver chloride and precipitated enzyme.

The contents of the other flask were then treated with the same amount of 0.6 N HNO_3 , to bring it to p_H 5 using no indicator. Both samples were then filtered and the precipitates washed three times with a fine jet of cold distilled water. The silver in the filtrate was determined by titration with thiocyanate of such strength that 1 cc. corresponded to 0.015 g. of potassium cyanate. The difference between these values and the value of the silver originally added represented the silver left on the filter paper. By titration, the amount of silver nitrate combining with 10 cc. of the uniform enzyme suspension was determined under comparable conditions, and the difference

¹ Methyl-red paper was used in preference to the solution, so as to have a colourless liquid for the subsequent titration.

between the amount of silver required to precipitate the enzyme together with associated inorganic salts, and the amount of silver left on the filter paper gave the silver fixed as silver cyanate. The presence of cyanic acid in the silver residue was confirmed by the tests described.

The enzyme suspension employed was sufficiently uniform to give an almost constant silver value in a series of six controls.

The chief error was found to lie in the washing of the precipitate on the filter paper. If it is washed for some time with distilled water at room temperature the silver gets ultimately washed away from its union with the protein, but by adopting a uniform technique this error was rendered negligible.

Table I.

Time in hours	Percentage urea decomposed	Percentage cyanic acid present
0	0	0
1	11.16	Not estimated
2	24.84	0.007
3	30.78	0.009
4	32.28	0.011
5	35.98	0.010
7	45.00	0.010

Temperature, 40°.

Enzyme, 10 cc. uniform suspension of urease decanted from 5 % suspension.

Substrate, 20 cc. 5 % urea.

Initial p_H 5.5.

Here it will be seen that the concentration of cyanic acid rises to a maximum and remains remarkably constant. The concentration will obviously depend on the difference between the rate of formation and the rate of removal by hydrolysis of the cyanic acid. When this difference in the rates is constant the concentration of cyanic acid will be constant. Since cyanic acid is very readily hydrolysed in weak aqueous solution at room temperature, it might be expected that the lower the temperature of the urea solution undergoing zymolysis the higher would be the concentration of cyanic acid, since it would accumulate in the solution. A series of experiments showed that this is not so. The amount of cyanic acid in solution is a function of the rate at which the urea is being decomposed, and this decomposition has a higher temperature coefficient than has the decomposition of the cyanic acid by hydrolysis.

To reduce as far as possible the errors introduced into the estimation by the presence of excess of urease material a method was devised whereby the cyanic acid could be estimated in solutions free, or almost free, from enzyme.

Method B. Estimation of cyanic acid. The urease was enclosed in a collodion sac and the cyanic acid was estimated in samples drawn from the surrounding solution. The proportions were 10 cc. of 10 % urease solution in the sac to every 100 cc. of 2 % urea solution in the containing vessel. At definite intervals 20 cc. samples were drawn off in duplicate, set to p_H 5.0 using 0.7 *N* HNO_3 , and methyl-red paper. One sample was boiled for half a minute to destroy any cyanic acid present, then each sample was treated with an excess of

$N/10$ $AgNO_3$, filtered, and the silver estimated in the filtrate. The difference in the silver value gave the amount of cyanate present. The substrate and the enzyme in these experiments were in the ratio of 100 cc. 2 % urea to 10 cc. 2 % urease.

Table II.

Temperature of experiment	Maximum percentage of cyanic acid
2-3°	0.0016 after 45 hours
5°	0.0043 " 30 "
7.5°	0.0034 " 20 "
10°	0.0301 " 24 "

At temperatures above 10° the collodion sacs became very fragile. In general, the lower the temperature the longer the cyanic acid took to attain its maximum value. In the experiments carried out at a temperature just above freezing point, the cyanic acid attained a concentration of 1.6 mg. per 100 cc. substrate when 5.01 % of the total urea had been decomposed. On leaving the solution at 5° for another period of 15 hours the concentration of the cyanic acid had fallen to 0.3 mg. per 100 cc. owing to its slow hydrolysis in the alkaline liquid.

Significance of the presence of cyanic acid in the urea/urease system.

Cyanic acid may represent the intermediate stage in the zymolysis of urea, or may be the result of some comparatively unimportant side reaction. Since the acid is not formed from sterile solutions of urea at the temperature of the experiments, it must be produced by the action of the enzyme on the urea (urease alone, of course, contains no cyanates nor gives rise to them in simple solution). From what is known of the part played by this substance in the normal decomposition of urea, it is probable that it is the true intermediate compound in the enzyme decomposition of urea.

Up to the present, Yamasaki [1918, 1920] appears to be the only worker who claims to have detected ammonium carbamate in the urea/urease system. Unfortunately, his results can hardly be considered conclusive since the method he adopts for estimating carbamate is equally applicable to cyanate. Yamasaki, working on the basis of the old "carbamide" formula for urea, does not appear to have considered cyanic acid at all in connection with the change, which he ascribes to a successive hydrolysis of the urea by simple catalytic action in the substrate—an explanation that affords no light on the mechanism of the process.

By the methods described in the present paper, cyanic acid can be detected and isolated as the silver salt in the presence or absence of carbamates, and, while there is no experimental evidence that ammonium carbamate is not formed during the enzyme decomposition of urea, there is evidence that cyanate does occur and that the decomposition of urea by urease proceeds along the same chemical lines as the normal decomposition of urea in solution by acids, alkalies, or the action of heat.

The change may be represented as follows:

Urease is not directly concerned in the "hydrolysis" of urea. The function of the enzyme is to bring about dissociation of the urea into ammonia and cyanic acid. The hydrolysis of the cyanic acid follows as a secondary change in the presence of water. This has been supported by observations on the action of urease on solutions of urea in alcohol, where the cyanic acid could not undergo hydrolysis.

It will be shown that this conception of urease as a *dissociating* enzyme and not a *hydrolysing* enzyme provides a new light for the investigation of enzyme mechanism, and accounts for, amongst other things, the specific action of the ferment.

CONCLUSIONS.

1. Cyanic acid has been isolated as the silver salt from solutions of urea undergoing decomposition by urease.
2. Cyanic acid attains to a maximum concentration in the urea/urease system, and is being continually produced as fast as it is removed by the hydrolytic action of the solvent.
3. Cyanic acid and ammonia are the intermediate products in the enzyme decomposition of urea.
4. The enzyme decomposition of urea proceeds along the same lines as the normal decomposition of urea in solution by acids, alkalies, or heat.
5. Urease is a *dissociating* enzyme; decomposing the neutral urea molecule into an alkaline component, ammonia, and an acid component, cyanic acid, which is rapidly hydrolysed by the solvent into ammonia and carbon dioxide.

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