CCXXXV. THE STRUCTURE OF UREA WITH REFERENCE TO ITS DEAMINATION AND SYNTHESIS BY UREASE.

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(Received 9 July 1936.)

In theory, the enzymic decomposition of urea may proceed along one of at least three possible paths:

1. Direct hydration [Armstrong & Horton, 1912],

2. Direct hydrolysis to carbamic acid [Yamasaki, 1920; Sumner, 1926],

3. Dissociation into cyanic acid [Werner, 1923, 1; Fearon, 1923, 1926],

Of these possible reactions, the first has always lacked experimental support for the existence of the hypothetical hydrated carbamide, and the work of Sumner and others has shown that the primary detectable effect of the action of urease on urea is the liberation of ammonia and another compound, now recognized to be carbamic acid. The third reaction path is the one that Werner has found to be followed in the hydrolysis and synthesis of urea by purely chemical methods, cyanic acid being an obligatory intermediate in all these changes, and it might be expected that the catalytic decomposition of urea would proceed along the same lines as those of thermal or alkaline hydrolysis.

When urea solutions undergoing zymolysis are treated with an excess of silver nitrate it is possible to obtain precipitates which resemble silver cyanate and differ from silver carbamate in their stability to acids up to pH 5; in higher concentrations of acid these precipitates are hydrolysed with liberation of NH_3 and CO_2 . Furthermore, concentrated solutions of urea undergoing vigorous zymolysis were found to give, under appropriate conditions, various colour reactions characteristic of cyanates. For these reasons, the present author was led to conclude that the primary effect of urease was to catalyse the dissociation of the substrate.

Sumner *et al.* [1931], working with pure preparations of the crystallized enzyme obtained from jack bean extracts, were unable to find any evidence of cyanate formation during the early stages of zymolysis; these results have been

confirmed by the present writer, using the jack bean enzyme, which necessitates the conclusion that the silver precipitates obtained in the earlier experiments were silver carbamate protected in some way by the impurities present in the original soy bean preparations. Whilst Sumner's work has revealed the importance of carbamic acid as the intermediate reactant in the zymolysis of urea, two dependent questions remain unanswered: the form in which urea is attacked by the enzyme, and the significance, if any, of the positive reactions for cyanate obtained in the later stages of hydrolysis on application of the copper-benzidine and other tests.

From the chemical standpoint, the proof that such a stable solute as urea can be deaminated by direct hydrolysis is of much interest; while from the biological standpoint, the acceptance of the highly reactive cyanic acid as a possible metabolite in the physiological history of urea has many bearings on the wider questions of the origin and fate of urea in plants and in animals.

Criticism of the carbamate theory of zymolysis.

1. The detection of carbamic acid by the method employed by Sumner does not preclude the possible existence of *isocyanic* acid as its precursor, since carbamic acid is the first product of the hydrolysis of cyanic acid [Fearon & Dockeray, 1926].

2. Quastel [1932] has observed that potassium cyanate protects urease from the toxic action of brilliant green, whereas ammonium carbamate has little or no effect. This is evidence for combination between cyanic acid (but not carbamic acid) and the enzyme.

3. The carbamide formula for urea affords no clue as to the manner in which deamination can occur. The amino-grouping in urea is much more stable than that of the acid amides or amino-acids, and does not react with formaldehyde or with nitrous acid until it has been unmasked by the addition of a strong acid [Werner, 1923, 1].

Criticism of the cyanate theory of zymolysis.

1. The hydrolysis of metallic cyanates in solution is not accelerated by urease. When potassium cyanate is added in low concentrations to buffered solutions of boiled and unboiled urease kept at temperatures of 10–30° hydrolysis may actually proceed more slowly in presence of the active enzyme.

This difficulty was recognized and evaded in the earlier work by assuming that the cyanate liberated by dissociation of urea was in the labile *iso*-form, $\text{HN}:\text{CO}$, whereas the alkali cyanates are salts of more stable form, $\text{HO}.\text{CN}$. The existence of this isomerism has been shown from a study of the effect of temperature on the polymerization of cyanic acid [Werner & Fearon, 1920], and the recent work of Birckenbach & Kolb [1935] on the Raman spectrum of the cyanate residue has confirmed the occurrence of the two forms of the acid.

2. Cyanate cannot be detected by the very sensitive copper-pyridine test [Werner, 1923, 2], or the copper-benzidine test [Fearon, 1926], in the earlier stages of zymolysis, but only in concentrated solutions of urea which have been allowed to approximate to zymolytic equilibrium. This suggests either that the cyanate is the result of some side-reaction, or else is an artefact produced by the action of the ammonia on the enzyme.

In order to define the ambiguous position of cyanate in the urea-urease system a series of experiments was carried out in presence of reagents capable of immobilizing cyanate as well as carbamate. The conclusions reached are: (1) carbamic acid is formed by the direct deamination of urea, and not by

hydrolysis of a precursor; (2) cyanic acid is formed by dehydration of ammonium carbamate, and can only appear in the later stages of the reaction when the carbamate content has reached a sufficiently high level.

Application of trap methods of analysis to the urea-urease system.

Trap methods of analysis are of two kinds: those designed to stabilize intermediate reactants as solutes, and those designed to remove intermediate reactants as precipitates. Examples of the first type employed were salts of barium and calcium, both of which form soluble cyanates and carbamates. Examples of the second type employed were (1) lead, which forms an insoluble cyanate and carbamate, and (2) the organic bases, aniline, phenylhydrazine and semicarbazide, all of which form sparingly soluble derivatives of cyanic acid but not of carbamic or carbonic acids.

The sources of enzyme employed were: (i) a high-grade sample of jack bean meal, which yielded (ii) the crystallized enzyme according to Sumner's method; (iii) a sample of soy bean meal freed from lipin material by extraction with light petroleum and with warm alcohol, and the commercial preparations of (iv) Dunning and (v) Squibb.

A. Zymolysis of urea in presence of barium and calcium salts.

Exp. 1. 50 ml. solutions of $M/3$ urea were treated with 1 ml. of urease solution prepared by centrifuging 1 % suspensions of (i), (iv) and (v). The mixtures were kept at 10° , and as each became alkaline to phenol red it was neutralized by the

Fig. 1. Zymolysis of urea in presence of calcium acetate. A, "free ammonia". B, "free + carbamate ammonia". C, "Total ammonia".

addition, every 5–10 min., of M calcium acetate in $M/40$ acetic acid. In the mixture containing urease (v) a precipitate of phosphate appeared after each addition of calcium, and was redissolved by further addition of $N/10$ acetic

acid. For analysis, triplicate samples were withdrawn. To the first, 1 ml. of *N* HCl was added, and the mixture kept at 40° to hydrolyze any cyanate or carbamate that might be present. Subsequent nesslerization of this sample gave the "total ammonia" value of the mixture. Each of the two remaining samples was treated with 4 ml. of *N* NaOH. The "free ammonia" in one sample was estimated by nesslerization following centrifugal separation of the precipitated calcium hydroxide. The last sample was incubated at 40° for 2 hours in order to hydrolyze any carbamate that might be present. Under these conditions, as Lewis & Burrows [1912] have shown, cyanate does not liberate ammonia. Subsequent nesslerization of this sample, after removal of precipitated calcium carbonate, gave the "free + carbamate ammonia" value of the mixture.

In all these experiments the value for the "total ammonia" was found to be the same as that for the "free + carbamate ammonia", which indicates that carbamic acid and ammonia are the only identifiable products of the primary attack of urease on urea.

In none of the experiments was any carbonate formation observed during the first hour, although ammonia was being liberated continuously. This indicates that carbon dioxide cannot be a primary product of the zymolysis of urea, as originally suggested by Armstrong & Horton, but must arise from the hydrolysis of the carbamate.

B. Zymolysis of urea in presence of lead salts.

Of the commoner metals available for the fixation of cyanate as an insoluble salt, namely, silver, mercury, lead and copper, only lead was found to be suitable for use with the enzyme. Now, although, as Sumner had pointed out, Pb ions, even in very low concentration, are highly toxic to pure crystallized urease, it is a remarkable fact that the crude jack bean extracts continue to attack urea even when the enzyme has been completely precipitated by lead acetate and is no longer in solution.

Exp. 2. 100 ml. of *M* urea were treated with 5 ml. of a centrifuged 5% aqueous extract of jack bean. The mixture was kept at 10°, and as it became alkaline to phenol red it was neutralized from time to time by the careful addition of 10% lead acetate in *N* acetic acid.

The lead produced a precipitate which carried down the enzyme, but zymolysis continued slowly after adsorption as shown by the colour change of the indicator in the region of the precipitate. After 4 hours the mixture had received 4 ml. of the lead acetate solution. The lead precipitate from half of the mixture was now collected, washed free from ammonia and urea and dried in a desiccator. The remaining half of the mixture was kept for 3 days before collecting the precipitate. The precipitates were tested for cyanate by the copper-pyridine test, and analyzed for cyanate and carbamate by the method of alkaline and acid hydrolysis. In no experiment was there any evidence of cyanate formation.

Precipitate 1. Wt., 0.41 g. Carbamate, 3.9%.

Precipitate 2 consisted entirely of lead carbonate together with jack bean debris.

C. Zymolysis of urea in presence of semicarbazide.

Concurrently with the experiments on the effect of metallic stabilizers, a series was set up to see if an organic compound could be used to trap the hypothetical precursor of the carbamic acid. Aniline, phenylhydrazine and semicarbazide were the bases selected as yielding insoluble ureides with cyanic acid. In the relatively high concentrations used, *M*/10–*M*, aniline and phenylhydra-

zine had much greater inhibiting effects than semicarbazide, which is in agreement with the results obtained by Myrbach [1926] during his study of the action of the aldehyde reagents on yeast saccharase. In lower concentrations, Quastel has shown that semicarbazide has a protective action on urease.

Semicarbazide in acid solution reacts with cyanic acid to form a characteristic crystalline compound, hydrazodicarbonamide, $\text{H}_2\text{N} \cdot \text{CO} \cdot \text{NH} \cdot \text{NH} \cdot \text{CO} \cdot \text{NH}_2$, the solubility of which is about 1:7000, at room temperature, and which melts (with decomposition) at 245° .

Hydrazodicarbonamide occurs in two crystalline forms, according to the conditions of the reaction. When a few drops of fresh 0.1% potassium cyanate are added to about 2 ml. of 5% semicarbazide hydrochloride the dicarbonamide begins to separate out in a few minutes in the form of brilliant tetrahedra. When the semicarbazide reagent is added in small quantities to a warm solution of potassium cyanate, which has been boiled for a few minutes to liberate ammonia, the dicarbonamide appears as a mixture of tetrahedra and ovoid crystals.

On recrystallization from warm water the ovoid crystals revert to the tetrahedral form. Hydrazodicarbonamide is not dissolved in the cold by *N* HCl, *N* NaOH, concentrated NH_3 or the commoner non-polar organic solvents. It dissolves with decomposition in hot, glacial acetic acid.

The semicarbazide reagent was applied to the urea-urease system in two ways. In the first set of experiments, *M* and *M*/3 solutions of urea undergoing zymolysis at 10° were neutralized from time to time by addition of *M* semicarbazide hydrochloride. When the reactions had proceeded for 3–6 hours the mixtures were acidified by addition of excess of the reagent and left overnight. Next day, and for several subsequent days, samples of each mixture were centrifuged and examined for crystal formation, but in no instance was there any sign of hydrazodicarbonamide crystals among the enzyme debris. In the second set of experiments, the urea and semicarbazide hydrochloride were mixed in equimolecular proportions, and the mixture neutralized with *N* NaOH before adding the enzyme. After zymolysis had continued for 24–48 hours at room temperature, the mixtures were acidified with *N* HCl, and placed in a desiccator. Within 24 hours a crystalline precipitate began to appear among the debris. The crystals consisted chiefly of small ovoids closely resembling the ovoid form of the dicarbonamide. Along with these crystals were a few tetrahedra, as well as the polysaccharide granules which are commonly present in suspensions of jack bean extracts. When crystallization appeared to be complete, the residue was collected in a centrifuge-tube, washed with concentrated ammonium hydroxide, *N* NaOH, *N* HCl and finally with several changes of water. The residue was then extracted three times with boiling water, the extracts collected and concentrated. On standing, typical tetrahedral crystals began to separate out, which were collected and identified as hydrazodicarbonamide by their melting-point.

Exp. 3. 2 g. of urea and 2 g. of semicarbazide hydrochloride were neutralized to phenol red by means of *N* NaOH, treated with a suspension containing 75 mg. urease (Dunning) and the total volume made up to 25 ml. After zymolysis for 24 hours the mixture was reduced in a desiccator to about 10 ml. and then slightly acidified with *N* HCl. Crystal formation was observed towards the end of the second day, and after a week the residue was collected, washed with acid and alkali and twice recrystallized from hot water. The yield of hydrazodicarbonamide amounted to 18 mg.

Cyanate formed under these conditions may have at least three possible origins: (i) decomposition of an unidentified impurity accompanying the enzyme preparation, (ii) hydrolysis of part of the semicarbazide itself and (iii) action of the urease, or possibly an associated enzyme, on one of the reactants in the mixture. The third of these possibilities is the most likely, since negative

results as regards hydrazodicarbonamide formation were obtained with the following mixtures:

- (1) Urease digested with semicarbazide in acid and in alkaline solutions.
- (2) Urease incubated with semicarbazide and ammonium hydroxide, chloride or sulphate.
- (3) Urease incubated with semicarbazide and sodium hydrogen carbonate.
- (4) Heat-inactivated urease digested with semicarbazide and urea.
- (5) Heat-inactivated urease digested with semicarbazide and ammonium carbamate-carbonate.

Now although urease does not liberate cyanic acid directly from urea, the possibility remains that it, or some accompanying enzyme, is able to catalyze the dehydration of carbamic acid in solution in a manner comparable with the action of carbonic anhydrase on carbonic acid.

If this hypothesis be true it might be expected that when urease is incubated with concentrated solutions of ammonium carbamate-carbonate urea would be produced from the union of cyanate and ammonia; whereas if urease is incubated with the same substrate in presence of a cyanate stabilizer it should be possible to detect the intermediate reactant.

Mack & Villars [1923], and Kay [1923] were the first to demonstrate the formation of urea by the action of urease on ammonium carbamate-carbonate, thus showing that the outline reaction,

is capable of being carried out in either direction by the enzyme. Now, Werner has obtained a considerable amount of evidence that all the familiar syntheses of urea from ammonium carbonate by purely chemical means necessitate (i) the formation of carbamic acid, and (ii) its dehydration to cyanic acid, which then combines with the ammonia to form urea.

Consequently, it is of interest to determine if the enzymic synthesis of urea retraces the path of deamination in the reverse direction, or if cyanate formation represents an alternative route of synthesis. This aspect of the problem has a bearing on the origin of urea in lower plants, many of which appear to be free from urease [Fosse, 1916; 1928]. The possibility of course remains that cyanate formation is due to the activity of a separate enzyme, quite distinct from urease, and present in soy and jack bean extracts that have not been fractionated by Sumner's method of crystallization.

Before following this line of investigation, which will be made the subject of a later research, it is necessary to determine the optimal conditions for the enzymic synthesis of urea. These are exemplified in the following data obtained from a series of experiments carried out in collaboration with Prof. E. A. Werner, in which yields of urea considerably greater than those of previous investigators have been obtained.

The synthesis of urea by urease.

The enzyme preparations used were: (i) soy bean residues after extraction with fat-solvents, (ii) jack bean residues and (iii) urease Dunning. Other preparations were examined also, including several free from urease, in order to test the

possibility that the urea might arise from sources other than true reversed zymolysis. The substrate was an ammonium carbamate-carbonate mixture, which on analysis represented 4.45 *N* carbamate and 6.4 *N* carbonate. In each experiment, 25 ml. of the substrate were incubated with the enzyme preparation in a closed flask for 8 hours at 55°.

Table I. *Urea formation from ammonium carbamate-carbonate at 55°.*

Enzyme preparation	Urea yield from 25 ml. substrate mg.
None	0.0
Jack bean, heat inactivated, 25 mg.	0.0
Jack bean I, 25 mg.	4.0
Jack bean I, 75 mg.	6.6
Jack bean II, 25 mg.	16.2
Jack bean II, 500 mg.	20.3
Soy bean, 250 mg.	1.7
Soy bean, 250 mg.	1.6
Soy bean, 750 mg.	3.2
Soy bean, lipin-free, 25 mg.	3.7
Soy bean embryo, dried, 250 mg.	1.8
Soy bean embryo, lipin-free, 250 mg.	3.0

The preparation "Jack bean II" is the urease powder marketed by Messrs Dunning.

Table II. *Incubation of preparations free from urease.*

Preparation	Urea yield from 25 ml. substrate mg.
Takadiastase, 250 mg.	0.0
Peptone, Merck, 250 mg.	0.0
Bran, dried, 250 mg.	0.0
Arginine hydrochloride, 33 mg.	(a trace)

Table III. *Incubation of urease preparations with various substrates.*

Preparation	Substrate	Urea yield from 25 ml. substrate mg.
250 mg.		
Soy bean	<i>N</i> NaOH	0.0
Soy bean	<i>N</i> NH ₄ OH	0.0
Soy bean	2.5 <i>N</i> NH ₄ OH	0.0
Soy bean	8 <i>N</i> NH ₄ OH	0.0
Soy bean	10 <i>N</i> H.COO.NH ₄	0.0
Soy bean	Carbamate-carbonate	1.6
Soy bean	Carbamate-carbonate	1.6
Soy bean	<i>N</i> -methylammonium- methyl carbamate	0.0

The urea formed was estimated as follows. After incubation, each mixture was concentrated to 5 ml. on a water-bath to remove excess of ammonia, then diluted with 10 ml. of glacial acetic acid, and treated with 3 ml. of 10 % xanthydrol in methyl alcohol. After remaining for 12–14 hours, the xanthydrol precipitates were removed by filtration, washed with alcohol and ether and dried and weighed, according to the technique of Fosse. The precipitates were identified as dixanthylurea from their micro-crystalline appearance and their melting-point, 260°, as taken in the vapour of diphenylether.

These experiments lead to the conclusion that the formation of urea by the action of urease on concentrated solutions of ammonium carbamate is a true enzymic synthesis and not due to the action of the strong alkali on urea precursors.

The point of attack in the action of urease on urea.

If it be conceded that the point of attack in the zymolysis of urea is the imino-group, and not the amino-group, as commonly assumed, Werner's earlier objections to the direct deamination of urea can be overcome, and, furthermore, the action of urease can be related to those of other enzymes capable of hydrolyzing the C—N linkage. That is to say, the zymolysis of urea is represented as:

the polar groups, —NH₂ and —OH, serving as points of attachment for the enzyme in a manner comparable with that postulated for dipeptidase systems by Bergmann *et al.* [1935]. In general, the imino-linkage appears to be sensitive to enzyme attack when it appears in compounds containing the configuration R—C(OH):N—R, which occurs in many substances capable of exhibiting resonance between enol and keto forms.

Examples of such configurations can be shown to exist at the point of attack in natural substrates such as allantoin, arginine, asparagine, guanine, hippuric acid and the peptides, and the deamination of α-amino-acids may also be explained in terms of oxidative formation and subsequent hydrolysis of an imino-group.

SUMMARY.

The following scheme is proposed as representing the changes occurring in the urea/urease system:

NOTES: *The structure of urea.* Of the five possible structures that may be assigned to urea, it is assumed in the above scheme that the unsymmetrical or *iso*-form is the one attacked by the enzyme. While the symmetrical or true carbamide form is found in ureides and substituted ureas, according to Werner it is too unstable to exist free in solution.

Were it present, the zymolysis of urea might be expected to follow the lines originally suggested by Armstrong & Horton, and result in the simultaneous appearance of ammonia and carbon

dioxide in the solution at the start of the reaction. A cyclic or closed form of urea has been suggested by Werner to account for the fact that in neutral solution the amino group is masked and will not react with aldehydes, but this can also be explained more simply by assuming that urea is in equilibrium with a zwitterionic form, as represented above.

The unexpectedly high value obtained by Wyman [1933] for the dielectric constant of urea solutions supports the suggestion that the solute is present largely in the zwitterionic form.

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