[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF THE OHIO STATE UNIVERSITY]

SYNTHESIS OF UREA WITH THE ENZYME UREASE

BY EDWARD MACK AND DONALD S. VILLARS

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The reversible action of enzymes has been demonstrated in several instances as by Croft Hill[1] for maltase, by Kastle and Loevenhart[2] for lipase, and by Bayliss[3] for emulsin. But the reversible action of urease in the reaction, $(NH_2)_2CO + 2H_2O = (NH_4)_2CO_3$, has been disputed. Bayliss[4] calls attention to the fact that attempts to synthesize urea from ammonium carbonate and ammonium bicarbonate with urease have been unsuccessful, and Kiesel[5] states that urease does not function reversibly. Barendrecht[6] in a memoir on urease and the theory of enzyme action by radiation, claims to have synthesized urea, starting with ammonium carbonate in the presence of urease, and assuming that the loss of ammonia content which the system suffered was due to the formation of urea. Mataar[7] disputes this result, having conducted experiments of his own and re-performed some of Barendrecht's under as nearly the same conditions as possible. Mataar made use of the Fosse method[8] for the analytical determination of urea.

We have succeeded in showing beyond any doubt that the equilibrium in this reaction can be approached from the side of the ammonium carbonate, and that the presence of urease hastens very decidedly the attainment of equilibrium. Previous failure to detect the effect was due to (1) starting with too dilute a solution of ammonium carbonate, and (2) not waiting a long enough time for the action, which even in the presence of urease is slow.

As long ago as 1912, G. N. Lewis and G. A. Burrows[9] showed that urea is formed in a conc. (10 *N*) solution of ammonium carbonate and carbamate, NH_4OCONH_2, heated in closed tubes. The urea was dissolved out with alcohol and weighed. Equilibrium was reached at 132° after about 2 days, at 110.7° after 5 days, and at 77° after about 95 days. We have let the reaction proceed in this same way at lower temperatures with the addition of the enzyme urease, and have found approximately the same equilibrium concentrations of urea as found by Lewis and Burrows, but

[1] Croft Hill, *J. Chem. Soc.*, **73**, 634 (1908).

[2] Kastle and Loevenhart, THIS JOURNAL, **24**, 491 (1900).

[3] Bayliss, *J. Physiol.*, **46**, 236 (1913).

[4] Bayliss, *Brit. Assoc. Advancement Sci. Repts.*, **85**, 687 (1915).

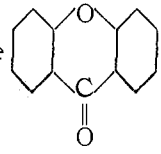
[5] Kiesel, *Z. physiol. Chem.*, **75**, 169 (1911).

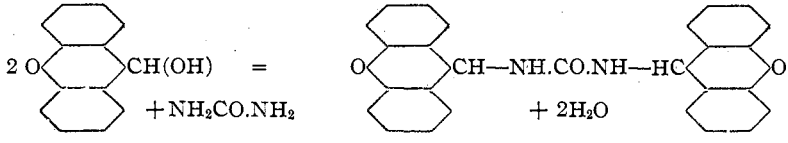
[6] Barendrecht, *Proc. Acad. Sci. Amsterdam.*, **21**, 1126 (1919); *Rec. trav. chim.*, **39**, 2 (1920).

[7] Mataar, *Rec. trav. chim.*, **39**, 495 (1920); **40**, 65 (1921).

[8] Fosse, *Compt. rend.*, **157**, 948 (1913); *Ann. Inst. Pasteur*, **30**, No. XII (1916).

[9] Lewis and Burrows, THIS JOURNAL, **34**, 1515 (1912).

a very much shorter time required for reaching equilibrium, the time depending on the concentration of the enzyme. We have employed Fosse's method[10] in estimating the urea. This method depends on the quantitative precipitation of urea by xanthydrol, a reduction product of xanthone. It is made by reducing xanthone  in alcoholic solution at about 55° with sodium amalgam. The alcoholic solution is then filtered, and the filtrate poured into several times its own volume of water, when white crystalline xanthydrol precipitates. This is washed with water and dried in a desiccator over calcium chloride. When a 10% solution of xanthydrol in absolute methyl alcohol is added to a solution of urea to which a proper amount of glacial acetic acid has been added, 2 molecules of water are eliminated and dixanthyl urea precipitates.



The bulky precipitate, when dried on a filter paper, can be peeled off bodily and weighed by itself on a balance pan, its weight being a little less than 7 times as much as the urea which it contains. This method is exact besides being very sensitive, and has many advantages over the usual methods of determining urea. When the urea concentrations were very small it was necessary to add xanthydrol and let it stand for several hours to insure complete precipitation, and when the precipitate was too small to be weighed directly, it was collected in a capillary tube by centrifugal force and estimated in a manner similar to that described by Olof Arrhenius.[11]

Ammonium carbonate solutions were made by passing carbon dioxide, washed in a 50% sulfuric acid solution, into strong (29%) ammonia solution. The white crystals which formed were allowed to settle, and the supernatant solution was drawn off and analyzed for carbonate and carbamate content by the method of Lewis and Burrows.[9] This consists in precipitating the carbonate with an ammoniacal saturated solution of barium carbonate at 0°, filtering the solid as quickly as possible in an alundum thimble and titrating the precipitate as carbonate with standard hydrochloric acid. The filtrate is heated on a water-bath to transform the carbamate into carbonate and the additional barium carbonate which precipitates is titrated with acid solution as before; this gives the content of carbamate.

[10] We wish to thank M. Fosse, who personally introduced one of us to the technique of his method in the laboratory of the Pasteur Institute in the spring of 1919, and who presented us with a sample of xanthydrol.

[11] O. Arrhenius, THIS JOURNAL, 44, 132 (1922).

Three solutions were prepared having the following normal strengths

	Carbonate	Carbamate	Total
I	3.765	0.451	4.216
II	5.466	4.864	10.330
III	5.133	6.091	11.224

Reaction at 100°.—Sealed tubes, each holding 20 cc. of solution were heated at 100° in a water-bath and opened at intervals for analysis.

TABLE I

REACTION AT 100°

Solution I		Solution II	
Time Hours	Wt. of dixanthyl urea G.	Time Hours	Wt. of dixanthyl urea G.
2.0	0.0000	47.0	0.0298
7.5	0.0000	145.25	0.0562
19.25	0.0000	241.75	0.0000 (tube cracked)
27.0	0.0000	287.75	0.2765
60.5	0.0000	355.50	0.3217
95.5	0.0081	....	....
Calc. wt. if equilibrium had been reached = 0.1471		Calc. wt. if equilibrium had been reached = 0.3604	

In the absence of dixanthyl urea (0.0000) the solution was perfectly clear and had no trace of sediment on the bottom or scum floating on the top.

The equilibrium amounts have been calculated from the data of Lewis and Burrows, by means of the formula,

$$w = n \times c \times \frac{60.052}{2} \times \frac{10}{1000} \times \frac{420.18}{60.052}$$

where w the weight of dixanthyl urea, n is the normality of solution (ammonium carbonate plus carbonate), c is the equilibrium percentage of urea (1.660% for 100°, 0.4314% for 55°, and 0.1825% for 25°), 60.052 is the molecular weight of urea, 10 is cubic centimeters taken for testing, and the last fraction is the ratio of the molecular weights of dixanthyl urea and urea.

In the table just given, the calculated amount is in very fair agreement with the value which we have found after about 15 days, and means that equilibrium had been nearly reached.

Reaction at 55°.—The reaction at 55°, the optimum temperature for urease, was carried out in 2 stoppered flasks, heated in an electrically controlled oven. Solution II was placed in both flasks, and urease, to make 0.1% solution, added to one of the flasks. Samples were withdrawn and tested with xanthydrol.

The results with Solution II show very clearly that the urease increases the velocity of formation of urea. The urease which was used (the soluble white powder which is now procurable from chemical firms) gave no pre-

cipitate whatever with xanthydrol, even after standing with it for 2 days; consequently, it was itself entirely free from urea and did not contribute to the formation of precipitates except in its role as a catalyst.

TABLE II

REACTION AT 55°

Solution II			Solution III		
Time Hours	Ppt. of dixanthyl urea 0% urease	0.1% urease	Time Hours	Ppt. of dixanthyl urea 0% urease	1% urease
0.33	0.0000	0.0000	0.5	0.0000	0.0098
1.5	0.0000	traces	1.0	0.0000	0.0340
4.33	0.0000	traces	2.0	0.0000	0.0382
27.5	0.0000	0.0164	4.0	0.0000	0.0382
52.0	0.0000	0.0188	7.75	0.0000	0.0768
98.33	0.0051	0.0314	23.5	trace	0.0544
...	....	....	47.0	0.0000	0.0513
Calc. wt. if equilibrium had been reached =0.0937			Calc. wt. if equilibrium had been reached =0.1018		

With Solution III and 1.0% urease the velocity of formation of urea is greatly increased. The yield after 1 hour is as large as the yield after 100 hours with 0.1% of urease. The decreasing values for the precipitate at the end were due to evaporation of ammonium carbamate from the flask, whose stopper was blown off by the gas pressure. To prove this, we analyzed the solution remaining at the end for carbonate and carbamate content, and calculated from the data of Lewis and Burrows the amount of precipitate to be expected if urea were present in equilibrium concentration. The number obtained was 0.0488 as compared with 0.0513 actually found *some time before this solution was stoppered to save for analysis.*

Reaction at 25°.—This was carried out in an open flask in a water thermostat.

TABLE III

REACTION AT 25°

Solution II

Time Hours	Ppt. of dixanthyl urea 0% urease	0.1% urease
0.5	0.0000	0.0000
1.5	0.0000	0.0000
4.5	0.0000	0.0000
27.75	0.0000	0.0016
52.25	0.0000	0.0111
98.5	0.0000	0.0048
197.25	0.0000	0.0105
293.25	0.0000	0.0162

Calc. wt. if equilibrium had been reached=0.0396

Summary

1. It has been shown that when concentrated solutions of ammonium carbonate and carbamate are used, the enzyme urease acts reversibly,

that is, it increases the velocity of formation of urea, and hastens the attainment of equilibrium.

2. A 1% solution of enzyme will bring equilibrium in a 10 *N* ammonium carbonate-carbamate solution containing about equal amounts of each in about 10 hours at 55°, the optimum temperature for urease. With 0.1% solution of enzyme, the reaction goes many times more slowly, being about 1/3 completed in 98 hours. Without any enzyme, equilibrium would be attained at 55° only after about 600 days.

COLUMBUS, OHIO

[CONTRIBUTION FROM THE LABORATORIES OF THE OHIO STATE UNIVERSITY]

THE ACTION OF UREASE IN THE DECOMPOSITION OF UREA

BY EDWARD MACK AND DONALD S. VILLARS

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A great deal of experimental work has been done and much speculation indulged in regarding the chemical reactions which occur when urea is transformed into ammonia and carbon dioxide. It seems well established that ammonium cyanate is one of the intermediate products. Thus Burrows and Fawsitt[1] give the reaction as

$$CO(NH_2)_2 \rightleftarrows NH_4^+ + OCN^-;\ NH_4^+ + OCN^- + 2H_2O \rightleftarrows 2NH_4^+ + CO_3^=.$$

Werner,[2] however, advances much more convincing arguments for the view that urea exists largely in the form, H–N = C(NH$_3$)–O, and goes through the reactions,

$$H{-}N{=}C\begin{matrix}NH_3\\|\\O\end{matrix} \longrightarrow (H{-}N{=}\overset{+\,NH_3}{CO} + HO.CN) \longrightarrow NH_4CNO \longrightarrow (NH_4)_2\,CO_3.$$

The transformations of urea into ammonium cyanate, and of ammonium cyanate into urea, are the equivalent of the transformations of the two forms of cyanic acid. Ammonia and the keto form give urea. Ammonia and the enol form give ammonium cyanate.

We are interested here in the course of the reaction when the enzyme urease is mixed with the urea solution.

If the enzyme increases the velocity of formation of ammonium cyanate from urea, it should catalyze the reverse reaction. We have submitted this to test. Enough ammonium chloride and potassium cyanate were mixed with water to make a 0.1 *M* solution of each, and urease was added. The potassium chloride formed by double decomposition has only a slight retarding effect[3] on the urease. The experiment was made at 25° and portions of solution were occasionally withdrawn for determination of the

[1] Burrows and Fawsitt, *J. Chem. Soc.*, **105**, 609 (1914).

[2] Werner, *J. Chem. Soc.*, **113**, 83 (1918).

[3] Armstrong and Horton, *Proc. Roy. Soc.*, **85**, 109 (1912).