XXXII. THE REVERSIBILITY OF THE ACTION OF UREASE OF SOY BEAN.

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SINCE Takeuchi in 1909 discovered that the soy bean contained urease in an easily available and very reactive form, many investigations have been made into the question of the mechanism of the hydrolysis of urea to ammonium carbonate by this enzyme, and several attempts have been made, on the basis of the now widely accepted view that the same enzyme which provokes hydrolysis must also produce synthesis, to show that urease is no exception to this general rule.

Specific reversibility of enzyme action, although shown to take place in the case of lipase by Kastle and Loevenhart [1900], Dietz [1907], Armstrong and Gosney [1914], and in the case of emulsin by Bayliss [1912], Bourquelot and Bridel [1913], is by no means so definitely established in the case of most other enzymes. Thus the synthetic action of invertase and the production of maltose from glucose by maltase, to mention only two cases in which specific reversibility has been claimed, are still matters of controversy. Any new direct evidence of specific synthetic power is therefore of considerable value.

Barendrecht [1920] in the course of a long investigation on the application of his radiation hypothesis to the case of urease, stated that this enzyme was reversible, adducing as his evidence that when soy bean meal is added to the first of two equal quantities of ammonium carbonate solution, less ammonia is given off from the former on treatment with potassium carbonate and aspiration through sulphuric acid. If more soy bean meal is added the shortage of ammonia increases, being roughly proportional to the amount of added meal. The actual difference was small, 2-4 parts per thousand of carbonate used. Urea was not isolated. Matlaar [1920] strongly criticised Barendrecht's conclusion, and using Fosse's xanthhydrol reaction for urea showed that urea was not produced under the conditions described by Barendrecht even to the one-fortieth part of the small extent that the latter had supposed. It was evident that if any direct synthesis of urea by this enzyme took place at all, the position of equilibrium was very near complete hydrolysis.

Fearon [1923] has detected and isolated cyanates in the reaction mixtures during the decomposition of an aqueous solution of urea by soy bean urease,

but he has been unable to find any evidence of the formation of urea from either ammonium carbonate or carbamate, and comes to the conclusion that the normal method of zymolysis of urea is by way of cyanic acid and ammonia, the former being rapidly hydrolysed by the solvent into more ammonia and carbon dioxide. Mack and Villars [1923, 1] have also isolated cyanates under similar conditions, but bring forward evidence [1923, 2] to show that when commercial urease is added to a very strong (10 *N*) solution of ammonium carbonate and carbamate at 55° (the optimum temperature for urease), small quantities of urea are produced and can be separated as the dioxanthyl compound from the reaction mixture. They do not state whether any control experiments with heated inactivated urease were carried out, but find that a control without urease at the same temperature gives a very small precipitate with xanthhydrol.

There is no inherent chemical difficulty in the idea of the formation of urea from ammonium carbonate or carbamate. Lewis and Burrows [1912] showed that even at as low a temperature as 77°, on heating ammonium carbamate or urea in a sealed tube equilibrium was almost reached, after 95 days, at 1 % urea 99 % carbamate-carbonate. This same reaction at a higher temperature is described in a recent patent of the Badische Anilin und Soda Fabrik [1921] in which urea in 25 % yield is obtained by heating ammonium carbamate to 135°–140° under 15 atmospheres pressure.

But at ordinary temperatures and pressures this synthesis has not yet been observed, and the experiments about to be described indicate that the presence of urease is necessary if this synthesis is to take place at a measurable rate at room temperature.

The problem of showing that such a synthesis does take place in these circumstances is really that of separating urea in very minute quantity from a large excess of ammonium carbonate. The two substances are widely different in their chemical and physical properties, and separation may be achieved by taking advantage of the fact that whilst urea is soluble to the extent of 1.1 % in dry, and still more soluble in wet, butyl alcohol, ammonium carbonate is insoluble. Dakin's [1918, 1920] successful use of this partially miscible solvent in the continuous extraction of mono-amino acids from the products of protein hydrolysis led to my adopting it in an endeavour to effect this separation of urea. Since butyl alcohol boils at 118°, at which temperature small quantities of urea are rapidly decomposed, it was necessary to conduct the operation under reduced pressure, and a modification of the apparatus used by Dudley [1919] was employed. The temperature of the butyl alcohol was in this way kept at 60° during the course of the experiment, whilst a rapid stream of the solvent could be bubbled through the reaction mixture. This removed the urea from the reaction mixture almost as soon as it was formed, thus keeping its active concentration low and tending to force the reaction over towards further synthesis. The process of continuous removal from the sphere of reaction of substances synthesised by enzymes seems to

be a common biological phenomenon, and in this sense the method used in these experiments might be said to simulate what occurs in many living processes.

Apparatus used for continuous extraction.

A—reaction bottle.

B—bottle for excess butyl alcohol.

C—extraction flask containing butyl alcohol in bath at 60°.

D—dropping funnel containing urease.

Two preliminary experiments were made to find out whether butyl alcohol would extract urea quantitatively from its mixed solution with ammonium carbonate and carbamate:

(a) 1 g. of urea was dissolved in 100 cc. of a 10 % solution of dry ammonium carbonate, and extracted continuously for twelve hours with butyl alcohol. The residue in the extraction vessel was found to contain insufficient urea in 10 cc. to give a positive reaction with urease, which was easily given by 10 cc. of 0.05 % urea. Urea was shown to be present in the butyl alcohol in large quantities but was not estimated in this experiment. The extraction, then, is fairly complete.

(b) 0.001 g. of urea was dissolved in 100 cc. of a 20 % solution of ammonium carbonate, and extracted continuously for four periods of six hours

each, the separation and estimation of urea being conducted in the manner to be described later in this communication, using xanthhydrol.

					Wt urea extracted
First	six hour period	...	...		·0004 g.
Second	„ „	...	...		·00026 g.
Third	„ „	...	...		·00010 g.
Fourth	„ „	...	...		minute traces only

This gives a total quantity of about 75 % of the original urea extracted by this method from its very dilute solution in 18 hours.

By use of this apparatus, urea in small quantities has been isolated from the reaction mixture of urease + the equilibrium mixture of ammonium carbonate and carbamate which is obtained when strong ammonia solution is saturated with CO_2 gas at room temperatures and the resulting white crystalline precipitate is dissolved in distilled water. No urea whatever was obtained unless both active urease and this mixture were together present in the reaction bottle.

EXPERIMENTAL.

Ammonium carbonate. "Ammonium carbonate" was prepared by saturating portions of 250 cc. of 0·91 ammonia with dry CO_2 under a slight pressure. The precipitated salt was washed on a Buchner with a little cold distilled water, then with alcohol, and allowed to dry for two days in the air. On dissolving 20 g. of the dry solid in 100 cc. of water, some CO_2 was evolved and the resulting solution of p_{H} 8·2–8·4 was found to contain 84 % carbonate and 16 % carbamate, by Lewis and Burrows' [1912] method. During the extraction the p_{H} slowly increased, ending at 8·6–8·8, owing to loss of CO_2 from the solution.

Urease. Urease was prepared by two methods. In the earlier experiments it was obtained by extracting freshly ground soy bean meal with ten times its weight of 0·3 % KH_2PO_4 and allowing to stand for ten minutes. The mixture was then filtered through paper, allowed to stand for at least half-an-hour, and the activity of the urease was tested using a standard buffered solution of urea. It was then filtered into the dropping funnel shown on the left in the diagram, and covered by a layer of butyl alcohol to prevent any slight risk of bacterial contamination. Usually 20 cc. of the enzyme solution was used for a day's (8 hours) extraction, the liquid being added in 1 cc. portions at intervals. This procedure was adopted owing to the fact that butyl alcohol plus ammonium carbonate slowly destroy the enzyme.

Onodera [1915] showed that whilst in small concentrations the monohydric alcohols up to amyl alcohol increased the activity of soy bean urease (this increase being connected with the diminution in surface tension at the interface between the colloidal particles of the urease and the continuous phase), in higher concentrations the action was inhibitory. In a series of preliminary

trials the inhibitory effect of large concentrations of butyl alcohol was confirmed. Some idea of the relative activity of the enzyme before and after the experiment was obtained by noting the times taken by strictly comparable dilutions of the fresh enzyme and of the enzyme from the reaction mixture to change the p_H of a standard buffered solution of urea from 7.0 to 8.0. After a day's extraction the activity gauged in this way was diminished to one-fifth or less. Some of this effect was due to the ammonium carbonate, which on standing with urease for eight hours was found to diminish its activity, as previously observed by Barendrecht and others.

In the later experiments urease was prepared by Van Slyke and Cullen's method [1914] by two precipitations with ten volumes of acetone. The criticism that the urea found might have been present in some protected form in the cruder preparation could not arise in the case of this purified urease. 0.5 g. of the solid, dissolved in 20 cc. of distilled water, and filtered through glass wool was used for each day's (8 hours) extraction.

Methods of detection and analysis of urea. At the end of the period of continuous extraction, the stop-cock leading to the bottle *B* was opened and the butyl alcohol remaining in the extraction flask was distilled into this bottle until the flask was almost dry. The pressure was then released and the contents of the flask washed out with three washings of absolute alcohol into a small evaporating basin. The alcohol was evaporated on a water-bath and the dry residue washed three times with boiling chloroform. This removed most of the colouring matter which had come over in the butyl alcohol and left behind an almost colourless residue which was then extracted three times with 10 cc. of boiling acetone. The filtered acetone solution was evaporated to dryness, and the very small quantity of crystalline residue contained any urea which had been present in the extraction flask at the close of the extraction period. In later experiments it was found that the chloroform always extracted, together with the colouring matter, a trace of urea which could be detected by xanthhydrol precipitation but which was insufficient to weigh.

Two methods were used for the detection and estimation of the urea in the acetone extract.

1. The well-known method, by which the p_H of an indicator solution is changed to the alkaline side when urease of a known p_H and urea solution at the same p_H are added together and warmed to 37° for a short period, was extended to work with very small quantities of urea as follows:

A phosphate buffer mixture ($M/15$ KH_2PO_4 and $M/15$ $KNaHPO_4$) was made of $p_H = 7.0$, and 0.1 cc. of this was added to each of a series of tubes containing 5 cc. of dilute solutions of urea of different, known strengths with 0.5 cc. of 0.01 % phenol red.

Urease was prepared by diluting 5 cc. of the soya bean extract with an equal quantity of water, and adjusting the p_H to 7.0 with a few drops of $N/50$ NaOH. Of this enzyme solution 0.2 cc. was added to each of six tubes containing urea, the p_H immediately noted, and noted again at intervals.

Tube	Amount urea in mgm.	p_H at start	p_H after 15 mins. at 14°	p_H after raising to 37° for 15 mins.	p_H after 90 mins. at 37°
1	1.25	7.0	>8.4	>8.4	>8.4
2	0.25	7.0	7.8	7.9	7.9
3	0.10	7.0	7.35	7.4	7.4
4	0.05	7.0	7.1	7.1	7.15
5	0.025	7.0	7.0	7.1	7.1
6	0.012	7.0	7.0	7.0	7.0
7	1.25	Control with boiled enzyme no change of p_H			

This method is therefore capable of detecting 0.05 mgm. of urea in 5 cc. of liquid, 1 in 100,000, and in quantities somewhat greater than this the change of p_H gives a rough quantitative idea of the amount of urea present. Armstrong has shown how very specific the action of urease is, and this definite change of p_H after the addition of urease is convincing evidence of the presence of urea.

The advantage of this method was that it was possible to follow it by an estimation of the amount of ammonia produced from the synthesised urea by the enzyme, using a micro-Folin apparatus. Potassium carbonate was added to the reaction mixture and the ammonia liberated was aspirated into $N/100$ H_2SO_4 and titrated with $N/100$ CO_2 -free soda in a CO_2 -free atmosphere using methyl red as the indicator. The serious drawback was that half of the solution of urea had to be reserved for a blank determination with heated enzyme. It was always necessary to do this, as the blank gave from one-third to one-sixth of the total amount of ammonia produced by the active enzyme.

2. Xanthhydrol was used in the later experiments for the detection and estimation of urea using Nicloux and Welter's [1921] gravimetric modification of Fosse's well known method.

The acetone solution of urea obtained from the contents of the extraction flask in the usual way was evaporated to dryness and the minute crystalline residue dissolved in 2 cc. of water and filtered. The filter paper was washed with a little water, and to the 3 cc. of urea solution thus obtained were added 5 cc. of glacial acetic acid and 0.5 cc. of a 10 % solution of xanthhydrol in methyl alcohol. The liquid was shaken and allowed to stand at room temperature for ten minutes, when a crystalline precipitate could be observed uniformly throughout the tube. This began to separate, and after two to three hours the liquid was filtered off through a Neubauer micro-crucible and the dioxanthyl urea washed and dried as described by Nicloux and Welter. The weight of urea is exactly one-seventh of the weight of the dioxanthyl urea.

Results. These are tabulated below. About two-thirds of the experiments which have been done are given. Those omitted from considerations of space are duplicates completely confirmatory of the results shown.

The quantities of urea obtained are small, but quite recognisable and capable of estimation. In the later experiments as the technique improved the yields of urea became somewhat greater. Endeavours were made to obtain

Enzyme	Substrate	Time of extraction in hours	Method of testing for urea	Method of estimation	Amount urea found in mgm.
Soya bean extract	(NH ₄) ₂ CO ₃	6	Urease	Urease	>0.1
"	Distilled water only	6	"	"	Nil
"	(NH ₄) ₂ SO ₄ ¹	6	"	"	"
"	(NH ₄) ₂ SO ₄ at <i>p</i> _H =8.6	6	"	"	"
"	Distilled water	16	"	"	"
"	(NH ₄) ₂ CO ₃	18	"	"	0.3*
"	"	18	"	Micro-Folin	0.2
None	"	18	"	Urease	Nil
"	"	20	Xanthhydrol	Xanthhydrol	"
Soya bean extract boiled	"	15	Urease	Urease	"
Soya bean	"	45	"	Micro-Folin	>0.6
Van Slyke urease	"	26	Xanthhydrol	Xanthhydrol	0.37
"	"	16	Urease	Micro-Folin	0.24
None	"	20	Xanthhydrol	Xanthhydrol	Nil
Van Slyke urease ²	"	20	"	"	0.30
"	"	12	"	"	0.35

¹ Of same concentration in NH₄ ions as the ammonium carbonate used in the same series of experiments.

² A particularly active preparation.

* Reaction mixture in bottle kept at 37° during last eight hours of this experiment.

larger quantities of urea by conducting the operation in strong cane sugar solutions, and thus forcing the simple reaction

over towards the side of synthesis by diminishing the amount of available water, but the results were not encouraging:

Enzyme	Substrate	Time of extraction in hours	Method of testing and estimation	Amount of urea found in mgm.
Soya bean extract	(NH ₄) ₂ CO ₃	8	Urease	0.1
"	(NH ₄) ₂ CO ₃ in 25 % cane sugar	14	"	0.1
"	(NH ₄) ₂ CO ₃ in 50 % cane sugar	20	"	Traces only

No further attempts have been made, up to the present, to increase the yield.

The actual change in colour of the indicator, on hydrolysis of half of the urea solution (the other half being kept as control) by means of the same enzyme that had synthesised it, was most striking, particularly with the larger quantities of urea. In the "synthesised" urea solution the rate of colour change was the same as in a solution of urea of the same approximate strength made up for comparison.

In two experiments not given above the synthesised urea was crystallised from a very small quantity of acetone (by evaporation) and compared microscopically with a similar quantity of ordinary urea similarly treated. The resemblance of crystalline form was evident. A small portion of the synthesised urea placed on the outside of a test-tube containing boiling butyl alcohol (118°) did not melt, but when put onto the outside of a test tube in which sulphuric acid was being heated melted below 126°. (Urea melts at 132°.) There was insufficient for a m.p. determination in the ordinary way. The urea

still contained a little pigment. On heating in a small dry tube, ammonia was given off, but as this reaction meant the disappearance of the yield of 18 hours' extraction it was not repeated.

DISCUSSION.

It has been established by these experiments that urea may be extracted from a solution containing *active* urease and ammonium carbonate-carbamate mixture, but not under conditions in which the enzyme is destroyed, or in which either the ammonium carbonate or the active enzyme is absent.

Control experiments as indicated above have shown that the urea does not come from

- (a) ammonium carbonate + carbamate alone;
- (b) the enzyme solution alone;
- (c) the enzyme in presence of another ammonium salt;
- (d) the inactivated enzyme in presence of ammonium carbonate and carbamate.

The control experiments also negative the possibility that the urea found is derived from the oxidation of the butyl alcohol in presence of ammonia to cyanic acid and the transformation of this substance into urea [cf. Fosse and Laude, 1921]. A solution of ammonium sulphate made alkaline with ammonia in presence of active urease was found to give no trace whatever of urea under the same conditions of experiment in which ammonium carbonate gave easily recognisable quantities.

A fair summary of the situation with regard to the various reactions which may theoretically take place during the hydrolysis and synthesis of urea is given by Mack and Villars [1923, 1], and the experiments described above fall most easily into line with the conclusion of these authors that the reaction $\text{urea} \rightleftharpoons \text{ammonium carbamate}$ is the one catalysed by urease and not the reaction $\text{urea} \rightleftharpoons \text{ammonium cyanate}$, or $\text{urea} \rightleftharpoons \text{cyanic acid} + \text{ammonia}$. In the latter case it would be necessary also for the urease to catalyse the reaction $\text{cyanic acid} + \text{ammonia} \rightleftharpoons \text{ammonium carbonate}$ which, as has been known for some time [Walker and Hambley, 1895], is a very slow reaction in alkaline solution even at 69°, and at equilibrium only gives about 4 % of ammonium carbonate. The great rapidity and the completeness with which urea is hydrolysed by urease would therefore seem, in themselves, to put this explanation out of court.

Further experiments are being carried out in an endeavour to throw more light on the mechanism of the action of urease.

SUMMARY.

Urea is produced in very small quantities during the action of urease on a mixture of ammonium carbonate and carbamate in strong solution in water, at room temperatures, under conditions which preclude the formation of urea by any other means than enzymic synthesis. The action of urease is, therefore, reversible.

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