LI. ON THE UREASE OF THE SOY-BEAN AND ITS "CO-ENZYME."

By NAOSUKE ONODERA.

From the Physiological Department of University College, London.

(Received November 15th, 1915.)

In 1897 Bertrand introduced the term "co-enzyme." Since that time, co-enzymes have been discovered for various enzymes. The mode of action of co-enzymes is not yet quite clear, but the constitution of some co-enzymes is definitely determined. For instance, hydrogen ion for pepsin, hydroxyl ion for trypsin, electronegative ions such as chlorine and bromine ions for pancreatic amylase (Henri) and bile-salts for the lipase of liver (Magnus); [for literature see Bayliss, 1914, p. 123].

The constitution of other co-enzymes is not yet known and it is likely that these co-enzymes consist of a complicated system of several components. Harden and Young's [1906, 1908, 1910] co-enzyme of the zymase of yeast juice consists apparently of unknown components. Recently Neuberg and Schwenk [1915] found that salts of 2-ketonic acids act on yeast juice or dried yeast like the co-enzyme, but some important factor is wanting since the effect of salts of 2-ketonic acids is not as strong as that of the natural co-enzyme.

The "co-enzyme" of urease belongs also to the group of those with unknown constitution. Indeed, its nature, as will be seen, may throw some doubt on its claim to the title.

EXPERIMENTAL.

Methods employed.

The method employed for the estimation of ammonia which was produced by urease action was the same as that described in the previous paper [Onodera, 1915]. Only a few words may be added. Precipitated urease prepared according to van Slyke was used as the source of enzyme. The contents of each digestion bottle were always 50 cc. and that always included

5 cc. of 1.5 % urea solution. This amount of urea corresponds to 25 cc. of N/10 NH_3 . The digestion bottles were left in a water bath at room temperature.

1. *Urease loses its activity by dialysis.*

Dialysis was always carried out at room temperature by means of parchment paper, the urease solution being covered with toluene. Distilled water was used as a dialysate and it was either changed at intervals or continuously by means of a syphon. Dialysis was stopped when the chloride reaction of the dialysate was negative. The loss of activity of the enzyme by dialysis is due either to change of enzyme sustained during the course of dialysis or to the passing of enzyme particles through the parchment paper or to the loss of co-enzyme.

Loss of activity of enzyme owing to preservation is excluded since the control undialysed urease preserved under toluene was always very active (see below).

Urease is capable of passing to some extent through thin parchment paper, but not through the parchment paper employed in these experiments. Moreover an experiment is recorded in section 4 showing that this did not take place. It appears, therefore, that some essential constituent was removed from the urease by dialysis. This constituent may, for brevity, be called "co-enzyme."

In the following sections, several experiments are described proving that assumption.

2. *A small amount of fresh urease is able to revive the lost activity of the dialysed urease.*

TABLE I.

1.5 % urease dialysed for 5 days in cc.	0.5 % fresh urease added in cc.	N/10 NH_3 developed in 16.5 hours in cc.
5	—	0.08
5	0.5	4.62
5	1.0	8.53
—	1.5	4.03
—	1.0	1.23
—	0.5	0.15
Control urease not dialysed, 3 times diluted		
5	—	23.19

As is seen in this table, 1.5 % urease lost its activity by 5 days' dialysis, and 5 cc. of this dialysed urease developed only 0.08 cc. of $N/10 \text{ NH}_3$, while 5 cc. of the control urease, even three times diluted, hydrolysed almost all the urea present in nearly the same time. The addition of 0.5 and 1.0 cc. of 0.5 % fresh urease revived the lost action markedly, while the sum of the ammonia produced by the dialysed and the fresh urease each separately was much less.

The question arises whether this reviving action of the fresh urease is not an illusory phenomenon and whether it is not a simple additional effect of fresh urease on the urease which might be diluted by dialysis. The curve of urease concentration and ammonia development given in the previous paper [Onodera, 1915, p. 570] shows that the amount of ammonia produced by the quantity $(a + b)$ cc. of urease is greater than the sum of ammonia which is produced by a cc. and b cc. of urease respectively. From this curve we obtained the following data:

0.5 % urease in cc.	$N/10 \text{ NH}_3$ developed in cc.
0.5	1.0
1.0	2.0
1.5	4.6
2.0	9.4
2.5	12.8
3.0	15.2
3.5	17.2
4.0	18.8

Making use of these data, if we suppose that a certain quantity of the dialysed urease can produce 1.0 cc. $N/10 \text{ NH}_3$, then that quantity corresponds exactly with 0.5 cc. of 0.5 % urease. Now suppose that we add to that dialysed urease 1 cc. and 1.5 cc. of 0.5 % fresh urease respectively. By the simple addition of the corresponding quantities of ammonia we get 3 cc. and 5.6 cc. of $N/10 \text{ NH}_3$ respectively. But in fact 1.5 and 2.0 cc. of 0.5 % fresh urease produced 4.6 and 9.4 cc. respectively.

The increase of the amount of ammonia produced by the further addition of urease, as is seen in the previous data, is not so great as it is in Table I.

In the former case, the increase of yield of ammonia does not exceed twice the arithmetic sum of the separate amounts of ammonia. In the latter case, the increase reaches to ten or twenty fold. According to these considerations, the presence of co-enzyme in the fresh urease and the loss of co-enzyme by dialysis was definitely settled.

3. *Is this co-enzyme an inorganic salt and is it soluble in ether?*

A certain volume of 1 % urease was dried, incinerated and was dissolved in a quantity of distilled water equal to its original volume. 5 cc. of 1.5 % urease, which was dialysed for 5 days, developed 0.13 cc. of N/10 NH_3 in 16.5 hours. The same quantity of the dialysed urease with 5 cc. of the solution of the ash developed only 0.22 cc. of N/10 NH_3 during the same time.

Rosenheim's co-enzyme or activator of pancreatic lipase could be extracted from the ash only by means of hydrochloric acid [Pekelharing, 1912]. I tried this procedure also with urease. Urease was incinerated with hydrochloric acid and the ash was dissolved in water. 5 cc. of the same dialysed urease developed 0.33 cc. of N/10 NH_3 in 20 hours while an equal quantity of the dialysed urease with the solution of the ash produced only 0.36 cc. of N/10 NH_3 .

20 cc. of 1 % urease were shaken with 50 cc. of ether and the ether separated. After evaporation of the ether the residue was suspended in 20 cc. of distilled water. 15 cc. of this solution mixed with 5 cc. of the dialysed urease developed 0.33 cc. of N/10 NH_3 in 20 hours, while the dialysed urease alone produced 0.36 cc. of N/10 NH_3 during the same time. I tried to ascertain whether the co-enzyme was in the original solution. The urease solution after ether extraction was freed from ether by drawing an air current through it, the aeration being continued nearly half an hour until the smell of ether disappeared. With this solution the following experiment was made.

TABLE II.

Dialysed urease in cc.	1 % urease after extraction with ether	N/10 NH_3 developed in 17.7 hours in cc.
5	—	0.67
—	5	1.84
5	5	2.62

It will be seen that the urease action was inhibited very strongly and no activator was found in it. We can judge from this fact that ether destroyed the co-enzyme and that it could not be restored by the driving away of ether.

The dialysed urease employed in this series of experiments was, of course, reactivated when fresh urease was added.

We can conclude that the co-enzyme is not an inorganic salt and does not pass into ether but is destroyed by ether.

4. *Can the co-enzyme be found in the dialysate?*

For the purpose of solving this question, the following experiment was carried out. 0.5 % urease was subjected to dialysis for 4 days, the amount of dialysate being 5 times as great as the urease. The dialysate was not changed. After dialysis the dialysed urease solution was found in the same quantity as at starting. During dialysis both the urease solution and the dialysate were covered by toluene. The data obtained with these materials are given in Table III.

TABLE III.

Urease in cc.		N/10 NH ₃ developed in 16.3 hours in cc.
0.5 % urease not dialysed	5.0	15.70
0.5 % urease dialysed	5.0	0.11
Dialysate	25.0	— 0.06
0.5 % urease dialysed	5.0	0.22
Dialysate	25.0	

By this experiment it was proved that the urease inactivated by dialysis could not be reactivated by the addition of dialysate. It was moreover shown that no urease particles passed through the parchment paper. This dialysed urease solution recovered its activity considerably when co-enzyme was added, as the following table shows.

TABLE IV.

Dialysed solution in cc.				0.5 % fresh urease in cc.	N/10 NH ₃ developed in 16.5 hours in cc.
0.5 % urease not dialysed, as control	...	...	2.5	—	3.36
0.5 % urease dialysed	...	...	2.5	—	0.08
Dialysate	...	...	12.5	1.0	7.70
0.5 % urease dialysed	...	...	2.5		
Dialysate	...	...	12.5	1.0	0.60
—				1.0	

Co-enzyme was lost by dialysis but it could not be detected in the dialysate. What change then might have occurred to the urease during dialysis?

(a) Is co-enzyme dialysable but destroyed immediately after passing through parchment paper into the dialysate?

(b) Is co-enzyme not dialysable but changed into an inactive state through the diminution of salts owing to dialysis? It is possible that the precipitation of proteins may have been the principal cause. In fact, notable precipitation occurred in the urease solution during dialysis, while the control urease solution remained unchanged.

(c) Is co-enzyme adsorbed by the dialysing parchment paper?

No definite ground could be found for answering the first question with confidence. It is however very unlikely.

To answer the second question, the following experiments were carried out. After dialysis, the dialysed urease solution was filtered and the precipitate was suspended in distilled water, the volume of the suspension being made equal to that of the filtered urease. (To avoid confusion I use in the following description the abbreviations (d.f.) for the filtrate and (d.p.) for the suspension of the precipitate from the dialysed urease.) With d.f. and d.p. experiments were carried out.

TABLE V.

1.5 % urease dialysed for 5 days in cc.	0.5 % fresh urease in cc.	N/10 NH ₃ developed in 17 hours in cc.
d.f. 5	—	0.95
d.p. 5	—	0.88
d.f. 5 }	—	3.07
d.p. 5 }		
d.f. 5	2.0	16.05
d.p. 5	2.0	8.70
—	2.0	2.00
1.5 % urease not dialysed, as control 5	—	22.66

TABLE VI.

0.5 % urease dialysed in cc.	0.5 % fresh urease in cc.	N/10 NH ₃ developed in 17 hours in cc.
d.f. 5	—	0.01
d.p. 5	—	0.23
d.f. 5	2.0	7.52
d.p. 5	2.0	5.62
—	2.0	1.70

In Table V we see that d.f. and d.p. were not able to activate each other. It indicates that co-enzyme was neither in the solution nor in the precipitate of the dialysed urease.

If we compare the activities of d.f. and d.p. alone, we do not find any distinct difference in Table V but in Table VI d.p. was a little stronger than d.f. When d.f. and d.p. had been activated by addition of fresh urease they showed a distinct difference. The activated d.f. developed markedly more ammonia than the activated d.p. The increased development of ammonia in the activated d.p. gives us no proof that the increase was chiefly to be ascribed to the activating effect of co-enzyme added (fresh urease).

From the data of Tables V and VI we may conclude that inactivated enzyme was present in the filtrate d.f. The precipitate d.p. contained a

smaller quantity of enzyme proper than the filtrate, but still, although very small, some quantity of co-enzyme. This co-enzyme might possibly be supposed to be fixed by the enzyme. For this reason, the precipitate, in spite of its activity, could not activate the filtrate. From these facts I distinguish two kinds of co-enzyme of urease. One is the fixed co-enzyme and the other is the free co-enzyme.

In the following experiments it is clearly seen that the free co-enzyme has less resistance to various modes of treatment (such as dialysis, influence of heat, acid and alkali) than the fixed one. This fact agreed also with an experiment on the germination of soy-bean (see later).

Some reactions of the precipitate were studied. 1.5 % urease was subjected to dialysis for 4 days. The precipitate was collected and was made up with distilled water to a suspension equal in volume to the original solution. The dialysis was not quite sufficient, consequently the dialysed urease still showed some activity. The suspension was like milk and quite neutral. The addition of two drops of saturated MgSO_4 solution, 0.2 cc. of N/10 NaOH, or four drops of N/10 H_2SO_4 to 2 cc. of the suspension cleared away the milky state. This reaction was interesting, remembering that urease solution always becomes turbid when it is mixed even with a very weak acid. CaCl_2 had no effect. The suspension showed a distinct biuret-reaction. An experiment on digestion of the precipitate was made. 5 cc. of 1 % trypsin were added to 20 cc. of the suspension. The mixture was subjected to digestion at 37° for 2 days and was then dialysed for 2 days; the biuret-reaction was negative in this dialysed mixture.

An experiment was then made with the mixture.

10 cc. of the digested and then dialysed suspension developed 4.75 cc. of N/10 NH_3 in 18 hours.

5 cc. of 1 % trypsin developed 0.27 cc. of N/10 NH_3 .

10 cc. of the suspension alone developed 7.42 cc. of N/10 NH_3 in 21 hours.

From this we learn that the urease was not destroyed by trypsin digestion. Van Slyke also found that urease resisted trypsin digestion.

I tried also to restore the co-enzyme from the precipitate, supposing this precipitation to be reversible. The precipitate was dissolved in a small quantity of NaOH, H_2SO_4 , or MgSO_4 as described above and was added to dialysed urease, but these experiments all failed to restore the co-enzyme.

It seems very likely that the disappearance of co-enzyme was due to precipitation during dialysis and that it remained in the dialysed solution in an inactive state, but I could not succeed in proving it.

As to the third question, the adsorption of co-enzyme by parchment paper was definitely excluded. The following experiment shows this.

5 cc. of dialysed urease developed 0.08 cc. of N/10 NH_3 in 16.4 hours.

5 cc. of dialysed urease with parchment paper cut in pieces developed 0.06 cc. of N/10 NH_3 in 16.4 hours.

5 cc. of dialysed urease + 1.0 cc. of 0.5 % fresh urease developed 8.53 cc. of N/10 NH_3 in 16.4 hours.

5. *Can co-enzyme be filtered through a Berkefeld porous filter?*

Rosenheim's co-enzyme of pancreatic lipase could easily be separated by filtration through Pukall's porous clay filter [Umeda, 1915]. The same experiment was therefore tried with urease.

After filtration, a white precipitate remained on the filter. The precipitate was collected and treated with a quantity of distilled water equal to that of the filtrate so as to make a suspension.

Investigations of the activity of the filtrate as well as of the suspension and of their contents of co-enzyme were carried out. The results obtained were as follows:

TABLE VII.

0.5 % urease dialysed in cc.	Urease treated with clay filter in cc.		N/10 NH_3 developed in 17.25 hours in cc.
	Filtrate	Residue	
5	—	—	0.13
5	2	—	2.48
5	—	2	3.92
—	2	—	0.68
—	—	2	0.80

TABLE VIII.

0.5 % urease dialysed in cc.	Urease treated with clay filter in cc.		N/10 NH_3 developed in 18.7 hours in cc.
	Filtrate	Residue	
5	—	—	0.08
5	1	—	3.82
5	—	1	1.27
—	1	—	0.62
—	—	1	0.09
—	5	5	22.07

As is seen in Tables VII and VIII, the activities of the filtrate and the residue were not constant; in the case of Table VIII the activity of the residue was very small. This difference was caused probably by the mode of preparing the urease solution and the conditions at filtration, but it was shown that

no urease was lost by filtration through a porous filter. As Table VIII shows, the mixture of filtrate and residue in corresponding proportions had a very strong activity.

Further, the filtrate and the residue both contained co-enzyme. Their activity went quite parallel to their contents of co-enzyme. It was therefore clear that in the case of urease the enzyme proper and the co-enzyme both passed through the porous filter.

6. *Investigation of the distribution of urease in soy-bean or in its germ.*

It is known that some enzymes are distributed only in certain parts of plants, cells, etc. The distribution of the enzyme itself and of its co-enzyme might be different, so that the following experiment was made.

I separated the shell of the soy-bean from the bean substance or kernel and made a 1 % water extract from each of these two parts, with which experiments were made.

TABLE IX.

1.5 % urease dialysed in cc.	1 % extract of bean-shells in cc.	1 % extract of bean-kernel in cc.	N/10 NH ₃ developed in 16.4 hours in cc.
5	—	—	0.45
5	2	—	0.50
5	—	2	1.73
—	2	—	-0.01
—	—	2	0.22

We learn from these data that the shell contained neither urease nor co-enzyme while the bean substance contained both.

I investigated then whether germination of the soy-bean was able to change the distribution of enzyme and co-enzyme.

Soy-beans were washed with distilled water and were arranged in rows on sheets of filter paper and were then covered again by several sheets of filter paper. They were all kept in glass vessels and moistened with distilled water. After keeping them in darkness for three days, the commencement of the growth of the germs was noticed. Here the germs, shell and proper substance were separated from each other. 1 % water extracts were prepared from each of the three parts and the following experiment made:

TABLE X.

5 cc. of 1 % germ-extract	developed	17.34 cc. of N/10 NH ₃ in 18.2 hrs.
„ kernel-extract	„	9.42 „ „ „
„ shell-extract	„	0.18 „ „ „

The weights of dry substance of the germs and the bean substance proper were 10.47 and 38.79 % respectively. Consequently, a 1 % watery extract of them nearly corresponded to 0.1 and 0.4 % respectively referred to the dried substances.

When we look at Table X with this knowledge, we notice immediately that the content of active urease in soy-bean germs must be very high. It is higher than in the precipitated urease itself. In contrast to this fact the absence of activating power on dialysed urease interested me very much (see Table XI).

TABLE XI.

1.5 % urease dialysed in cc.	1 % germ- extract in cc.	1 % kernel- extract in cc.	1 % shell- extract in cc.	N/10 NH ₃ developed in cc.
5	—	—	—	0.13 in 16.5 hours
5	2	—	—	3.05 "
5	—	2	—	0.83 "
5	—	—	2	0 "
—	2	—	—	3.29 "
—	—	2	—	0.67 "
—	—	—	2	-0.02 "
5	—	—	—	0.04 in 17.7 hours
5	2	—	—	2.65 "
—	2	—	—	2.63 "

The process of germination of soy-bean is to be compared with dialysis, because in both processes the exchange of substances takes place between the urease or the bean-kernels and the surrounding water through a vegetable membrane. As a result, the co-enzyme of the germs and kernels was lost through the germination.

7. *Upon which constituent of urease, the diffusible or non-diffusible one, does acid or alkali act?*

An investigation was made to see which factor is influenced by these reagents. N/1000 HCl was allowed to act on urease for ten minutes, and was then neutralised by NaOH. Co-enzyme (fresh urease) and urease without co-enzyme (urease heated for an hour to 80° C., see following experiments) were added to the above-mentioned mixture and the activity measured with the result shown in Table XII.

It is to be noticed that the data concerning the inhibitory effects of various substances were not always uniform. Therefore in such cases I will describe the results of parallel experiments as obtained.

From this table one can easily see that N/1000 HCl only destroyed the diffusible part, and the remaining urease was put in action again by the

TABLE XII.

H ₂ O in cc.	0.5 % urease in cc.	N/200 HCl in cc.	Neutralised by addition of N/200 NaOH in cc.	H ₂ O in cc.	0.5 % urease in cc.	1.5 % urea in cc.	N/10 NH ₃ developed in 20 hours in cc.
45	5	—	—	12.5	—	5	22.10
35	5	10	10	2.5	—	5	0.10
35	5	10	10	2.5	—	5	0.05
35	5	10	10	1.5	1	5	9.53
35	5	10	10	1.5	1	5	9.98
35	5	10	10	0.5	2*	5	—0.02
40	—	10	10	0.5	2*	5	—0.03
40	—	10	10	1.5	1	5	0.20

* Heated to 80° C. for an hour.

addition of co-enzyme but not by the addition of the urease heated to 80° C. In a similar manner the effect of alkali was investigated. N/50 NaOH was allowed to act on urease for an hour.

TABLE XIII.

H ₂ O in cc.	0.5 % urease in cc.	N/10 NaOH in cc.	Neutralised by addition of N/10 H ₂ SO ₄ in cc.	H ₂ O in cc.	0.5 % urease in cc.	1.5 % urea in cc.	N/10 NH ₃ development in 27.7 hours in cc.
45	5	—	—	11	—	5	22.52
35	5	10	10	1	—	5	7.17
35	5	10	10	1	—	5	6.53
35	5	10	10	—	1	5	17.35
35	5	10	10	—	1	5	17.00
40	—	10	10	—	1	5	0.74

From this table we may safely conclude that NaOH destroyed the greater part of the co-enzyme and this co-enzyme lost could be restored by the addition of fresh urease. I regret the data were not decisive, but those of Table XV supply this deficiency.

If acid and alkali act on co-enzyme only, we may assume that these substances will not be able to affect the dialysed urease, which is free from co-enzyme.

Experiments concerning this point are shown in Tables XIV and XV.

TABLE XIV.

H ₂ O in cc.	0.5 % urease dialysed in cc.	N/200 H ₂ SO ₄ in cc.	Neutralised by addition of N/200 NaOH in cc.	H ₂ O in cc.	0.5 % urease in cc.	1.5 % urea in cc.	N/10 NH ₃ developed in 18.7 hours in cc.
45	5	—	—	11	—	5	0.16
35	5	10	10	1	—	5	0.00
35	5	10	10	1	—	5	0.01
35	5	10	10	—	1	5	2.87
35	5	10	10	—	1	5	2.91
40	—	10	10	—	1	5	0.29
45	5	—	—	10	1	5	3.00

TABLE XV.

H ₂ O in cc.	0.5 % urease dialysed in cc.	N/10 NaOH in cc.	Neutralised by addition of N/10 HCl in cc.	H ₂ O in cc.	0.5 % urease in cc.	1.5 % urea in cc.	N/10 NH ₃ developed in 27.5 hours in cc.
35	5	10	10	1	—	5	0.03
35	5	10	10	1	—	5	0.01
35	5	10	10	—	1	5	3.41
40	—	10	10	—	1	5	0.71

From these tables we see that the previous hypothesis was confirmed. Dialysed urease itself was not inhibited by acid at all and could develop as much ammonia as the urease without the acid influence when fresh urease (co-enzyme) was added to them both. Acid and alkali therefore only destroy the co-enzyme but not the urease itself.

8. *What is the influence of heat on urease?*

Van Slyke and Cullen [1914] found that the optimum temperature for urease action was 55°; heating of urease to 60° for 30 minutes was ineffective, heating of urease to 70° for 30 minutes deprived it of 25 % of its activity, but heating to 80° for 30 minutes destroyed it completely. A first experiment was made with urease which was heated to 60° for half an hour.

TABLE XVI.

0.5 % urease heated to 60° for an hour in cc.	0.5 % fresh urease in cc.	N/10 NH ₃ developed in 20.3 hours in cc.
5	—	22.31
5	1	22.28
—	1	0.44

TABLE XVII.

1.5 % urease dialysed for 5 days, in cc.	0.5 % fresh urea in cc.		N/10 NH ₃ developed in 17.7 hours in cc.
	Not heated	Heated to 60° for half an hour	
5	—	—	0.04
5	2	—	12.32
5	—	2	10.76
—	2	—	1.54
—	—	2	1.57

In Table XVI we note that the urease was not injured at all by heating to 60° for an hour. The heated urease solution contained obviously a sufficient amount of urease proper and co-enzyme to hydrolyse the amount of urea present. Accordingly, the addition of a small amount of urease proper and co-enzyme did not show any appreciable effect.

But heating to 60° even for half an hour was not without effect on the urease system. As Table XVII clearly shows, some amount of the free co-enzyme was lost, and in regard to the capacity of reactivating dialysed urease the heated urease was inferior to the unheated.

Experiments were carried out with the urease which was heated to a higher temperature as follows:

TABLE XVIII.

0.5 % urease heated to 80° for an hour in cc.	0.5 % fresh urease in cc.	N/10 NH ₃ developed in 20.3 hours in cc.
5	—	0.06
5	1	11.61
—	1	0.42

TABLE XIX.

0.5 % urease heated up to boiling point in cc.	0.5 % urease in cc.	N/10 NH ₃ developed in 17.25 hours in cc.
5	—	—0.02
5	1	10.93
—	1	0.43

It is clearly shown in these tables that heating to 80° for an hour or heating just up to boiling point totally destroyed the co-enzyme in the urease solution both fixed and free. But the addition of co-enzyme (fresh urease) enormously revived the action of the urease.

By these experiments the statements of van Slyke and Cullen as to the effect of heat on urease were confirmed. Further the heating of the urease solution above 80° totally destroyed the co-enzyme but not the urease proper. But the free co-enzyme was somewhat labile, it was already somewhat injured by heating to 60° for half an hour.

As a natural consequence it occurred to me that dialysed urease should not be destroyed by heating since it has no co-enzyme. An experiment to test this was made:

TABLE XX.

0.5 % urease dialysed for 5 days, in cc.	0.5 % urease in cc.	N/10 NH ₃ developed in 18.8 hours in cc.
5	—	0.26
5	1	4.57
—	1	0.06
Heated to 80° for an hour		
5	—	0.10
5	1	2.59
Heated up to boiling point		
5	—	0.02
5	1	1.68

The conclusion was shown to be correct. Only the yield of ammonia produced by the dialysed urease was smaller if it had been heated. This difference may be ascribed to the total destruction of the co-enzyme which remained fixed. The heating up to boiling point, however, will very likely destroy a certain amount of urease proper.

9. *To what change is the diminution of the activity of urease during preservation to be ascribed?*

Since it has been shown that co-enzyme is in every respect very labile, it is probable that the diminution of the activity of urease during preservation is due to change of co-enzyme.

An experiment was made with urease which was kept with toluene in an incubator at 36°·5 for 15 days. The urease solution was 0·5 % and 5 cc. of it developed 22·6 cc. of N/10 NH₃ in 20·3 hours at the start.

TABLE XXI.

Old urease in cc.	0·5 % fresh urease in cc.	N/10 NH ₃ developed in 18·7 hours in cc.
5	—	5·17
5	0·5	10·87
—	0·5	0·11

It is very likely that the co-enzyme was subjected to some change during the preservation, but the data are not sufficiently convincing. Whether the urease proper remained quite intact is also uncertain.

10. *Does blood serum contain co-enzyme beside "auxo-urease"?*

Margarete Falk [1914], and Jacoby and Umeda [1915] studied "auxo-urease" in blood sera which considerably accelerated the urease action. These statements interested me and suggested the attempt to find the co-enzyme of urease in blood.

TABLE XXII.

0·5 % urease dialysed in cc.	Serum of ox in cc.	0·5 % urease heated to 80° for an hour in cc.	0·5 % urease in cc.	N/10 NH ₃ developed in 17 hours in cc.
—	—	—	2	1·70
—	1	—	—	—0·02
—	1	—	2	17·17
5	—	—	—	0·26
5	1	—	—	1·21
—	—	5	—	0
—	1	5	—	0·05
—	—	5	1	10·08

As is shown in this table ox serum contains no urease, but a small quantity of the serum accelerates the urease action to an enormous amount; no true co-enzyme was however found in the serum. The urease which was heated at 80° for an hour lost its activity totally, but the addition of the serum could not revive the action at all, while the addition of a small quantity of the fresh urease (co-enzyme) restored the lost activity considerably. The dialysed urease employed was not quite free from the fixed co-enzyme, consequently the addition of the serum caused appreciable increase of activity. This effect was undoubtedly due to an accelerator in the serum, the "auxo-urease."

DISCUSSION.

Since urease loses its activity by dialysis and recovers it by the addition of something contained in fresh solutions, we are justified in calling this something a "co-enzyme," in view of the present ignorance as to what a co-enzyme really is. The facts that it is destroyed by heat and by the action of acid, alkali, etc. suggest a protein nature, but its apparent passage through parchment paper negatives this suggestion, unless it be assumed that it requires for its activity the presence of some diffusible substance. But, again, the fact that addition of the dialysate is ineffective is remarkable and, on the hypothesis suggested, could only be accounted for by supposing that the protein or colloidal activator undergoes some irreversible change. What is distinctly proved is that what is called "urease" can be brought into an inactive state which can be restored to activity by the addition of a trace of some substance or substances which are contained in fresh extracts of soy-bean.

It is clear that further work is necessary to decide which constituents are to be called the enzyme proper and which the "co-enzyme." The possibility cannot be excluded that the residue in the dialyser might contain a substance capable of increasing the activity of fresh urease, although this does not seem probable.

CONCLUSIONS.

(1) The urease of soy-bean loses its activity on dialysis. The lost activity is restored by the addition of a small amount of fresh urease. This indicates that the fresh urease contains co-enzyme. The co-enzyme could not be separated, accordingly its nature is not yet known.

(2) It is very likely that the co-enzyme is a system consisting of two groups of components, one of which is dialysable and the other undialysable. The dialysable component undergoes some irreversible change during dialysis.

(3) The co-enzyme consists of two parts, fixed and free.

(4) Heating and dialysis destroy the free co-enzyme first, then the fixed co-enzyme. The last portion of the fixed co-enzyme is found in the precipitate produced by dialysis, resisting the influence of heating and dialysis tenaciously.

(5) The inhibitory effects of heat, acid and alkali are exerted upon the co-enzyme, but not upon the urease proper.

(6) In germination, urease accumulates in the germs of the soy-beans in large proportion, but free co-enzyme is absent.

(7) Although ox serum has an accelerating power, it contains no substance which can be compared with the co-enzyme.

In conclusion I have pleasure in expressing my cordial thanks to Prof. Bayliss for his advice.

REFERENCES.

- Bayliss (1914). *The nature of enzyme action*. London.
Falk, Margarete (1914). *Biochem. Zeitsch.*, **59**, 298.
Harden and Young (1906). *Proc. Roy. Soc. B*, **78**, 369.
— — (1908). *Proc. Roy. Soc. B*, **80**, 299.
— — (1910). *Proc. Roy. Soc. B*, **82**, 321.
Jacoby and Umeda (1915). *Biochem. Zeitsch.*, **68**, 23.
Neuberg and Schwenk (1915). *Biochem. Zeitsch.*, **71**, 135.
Onodera (1915). *Biochem. J.*, **9**, 544.
Pekelharing (1912). *Zeitsch. physiol. Chem.*, **81**, 355.
van Slyke and Cullen (1914). *J. Biol. Chem.*, **19**, 141.
Umeda (1915). *Biochem. J.*, **9**, 38.