XLVI. THE HYDROLYSIS OF GLYCOGEN BY DIASTATIC ENZYMES. Part III. FACTORS INFLUENCING THE END-POINT OF THE HYDROLYSIS.

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In a previous communication [Norris, 1913, 1] the author confirmed the result previously obtained by Philoche [1908] that in the hydrolysis of glycogen by diastatic enzymes, the hydrolysis is as a rule far from complete, and also pointed out that the final degree of hydrolysis, as represented by the maltose production, increases with rising concentrations of enzyme though at a very much slower rate. This is therefore rather a different case from the breaking down of starch, where, although the hydrolysis is not complete, the equilibrium point according to most workers is the same with different concentrations of enzyme, viz. about 80·8 per cent. maltose and 19·2 per cent. dextrin. In this connection however Maquenne and Roux [1906, 1, 2, 3] suggest that the reaction only comes to an end owing to an insufficient activity of the enzyme and state that if the diastase be activated by the addition of traces of acid the hydrolysis proceeds to completion, a result which has been confirmed by Bayliss [1914].

It was also found by Brown [1904] that if the maltose formed were removed by fermentation with yeast the whole of the dextrin was hydrolysed, a result which suggested that the products exerted a retarding influence. As however acid is produced from the yeast this result may be really due to the activation of the enzyme and not to the removal of the products.

Under these circumstances it seemed of interest to examine the causes limiting the hydrolysis of glycogen by the glycogenase of the pancreas and some experiments carried out from this point of view are described in the present communication.

The glycogen used was prepared from oysters by Pflüger's method and

purified by repeated precipitation with alcohol. In those experiments where the influence of salts was under investigation the solutions were dialysed before use for several days against frequent changes of distilled water in presence of toluene.

The enzyme was as a rule prepared from dried pigs' pancreas which had previously been rendered fat free, by extraction with 50 per cent. alcohol and precipitation of the filtered extract with alcohol and ether. The precipitate was again taken up in 50 per cent. alcohol, filtered and reprecipitated, this process being repeated two or three times.

The course of the hydrolysis was followed by estimations from time to time of the reducing power of the solutions by Bertrand's method, the assumption being made that the reduction was due to maltose alone. While this is probably not entirely correct, the method is far more convenient than estimations of the optical rotation. The latter is open to the same objection as the former method and in addition necessitates the employment of very dilute solutions whereby further inaccuracies are in all probability introduced. In many cases the iodine reaction was also followed during the experiment.

Exp. 1. Influence of enzyme concentration on the hydrolysis of glycogen.

A. 105.5 cc. solution containing 1 g. glycogen, 0.1 cc. enzyme and 1 cc. toluene.

B. 105.5 cc. solution containing 1 g. glycogen, 0.5 cc. enzyme and 1 cc. toluene.

C. 105.5 cc. solution containing 1 g. glycogen, 5.0 cc. enzyme and 1 cc. toluene.

The above were incubated at 37° and samples taken from time to time for analysis, the results being shown in the following table.

TABLE I.

Duration of experiment Hours	Percentage hydrolysis		
	A.	B.	C.
1	36.65	52.90	69.8
2	40.45	54.00	77.1
3	42.80	54.30	82.0
4	43.70	54.55	84.8
5	44.60	54.70	87.4
6	45.10	—	89.0
7	45.40	—	90.2
8	45.60	—	91.0
9	45.60	55.10	91.5

In the above experiment it will be seen that while with the highest concentration of enzyme rather over 90 per cent. of the glycogen was completely hydrolysed to maltose, with 0.1 cc. enzyme only about half this quantity of maltose was formed. At the same time all the glycogen had disappeared, having been converted into a dextrin which remained to a large extent unhydrolysed. The intermediate products in the hydrolysis of glycogen have not received a great amount of attention. Tebb [1898] however found that a dextrin was formed which was extremely resistant to further hydrolysis and identified this with the dextrin previously described by Seegen [1879] and termed by him dystropodextrin. This dextrin gives no iodine reaction, in fact with pancreatic glycogenase no erythrodestrins appear to be formed, or if they are produced they are hydrolysed so rapidly that it is impossible to detect them.

It is difficult to believe however that the resistant nature of this dextrin is the cause of the stopping of the hydrolysis. For considering the experiment just described, the rates fell off when the reduction indicated a hydrolysis of rather over 40 per cent. in *A*, of 50 per cent. in *B*, and over 80 per cent. in *C*. In other words the slower the initial rate of hydrolysis, the greater was the quantity of resistant dextrin apparently formed. In the case of *C* the slowing off in the rate could be quite well explained by the exhaustion of the substrate, but in *A* and *B* this explanation cannot hold. Another possible solution is that two enzymes may be concerned. This view in the case of diastase has been advanced by several investigators [Brown and Heron 1879; Kjeldahl 1879; Duclaux 1899; Fraenkel and Hamburg 1906]. Now if the enzyme preparation employed in these experiments consists of a large proportion of glycogenase which only carries the hydrolysis to the dextrin stage and a very small amount of dextrinase the results obtained above are to a certain extent what would be expected. For in this case with the low enzyme concentration there might be enough glycogenase to carry out the first stage of the hydrolysis but insufficient dextrinase to hydrolyse the dextrin thus formed at a corresponding rate. In this way an accumulation of dextrin would take place. With the high concentration of enzyme the rate of the first stage of the hydrolysis would be limited by the concentration of glycogen employed and hence would not increase proportionally with rising enzyme concentrations. The second stage of the hydrolysis however would proceed at a much greater rate and hence the accumulation of dextrin would be less as the concentration of enzyme increased. Now while this is actually the final result found experimentally an examination of Table I shows that

when the slow stage of the hydrolysis is reached, *B* and *C* are proceeding not very much more quickly than *A*. Hence some other factors must be concerned and the two which first suggest themselves are (1) destruction of the enzyme during the course of the experiment, and (2) retarding influence of the products. Before considering these points however it will be as well to describe some further experiments in which the results indicated above were reproduced by other methods.

It is well known that the activity of a glycogenase preparation is almost completely lost on dialysis but can be restored by the addition of certain salts, notably chlorides. A certain minimum concentration of such salts varying with the concentration of enzyme is required to produce the optimum rate of hydrolysis [Cole 1906; Starkenstein 1910, 1; Norris 1913, 2].

Now if a series of dialysed glycogen solutions is made up, each containing the same quantity of dialysed glycogenase but increasing quantities of KCl, not only does the initial rate of hydrolysis increase with rising concentration of salt but the final degree of hydrolysis also rises. These results only obtain of course until the salt concentration reaches the optimum required for the particular strength of enzyme employed.

Exp. 2. Influence of salt content on the total degree of hydrolysis.

A. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene and 0.5 cc. dialysed enzyme.

B. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene, 0.5 cc. N/20 KCl and 0.5 cc. dialysed enzyme.

C. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene and 2.0 cc. N/20 KCl and 0.5 cc. dialysed enzyme.

The solutions were incubated at 37° and the reducing power estimated at frequent intervals.

In this experiment *A* which contained no added salt had still a low activity, the dialysis not having been carried sufficiently far to render the solution entirely salt free. *A* therefore represents a solution with very low salt content.

Results :

TABLE II.

Duration of experiment Minutes	Percentage hydrolysis		
	<i>A.</i>	<i>B.</i>	<i>C.</i>
15	2.07	9.2	13.2
30	3.22	14.6	22.7
60	5.07	21.6	—
75	—	—	30.18
120	10.14	25.68	—
135	—	—	33.71
240	12.89	28.4	—
255	—	—	37.44
360	—	29.28	—
420	16.58	—	38.34
480	—	29.73	39.24

The results are therefore exactly similar to those obtained when different concentrations of enzyme are employed and indicate that a certain amount of the enzyme remains entirely inactive in presence of insufficient salt. This is of some interest as Starkenstein [1910, 2] has shown that even in the absence of any salt, adsorption of diastase by starch still takes place though no chemical change results until salt is added. The salt presumably must have some specific activating effect on the enzyme and its only function cannot be to assist the combination between enzyme and substrate. It also shows that if two enzymes are present they are equally dependent on the presence of salts.

In view of the above experiment it was of interest to see what would be the effect of adding further salt to a solution such as *A* or *B* above when the rate of hydrolysis had become stationary and hence the following experiment was carried out.

Exp. 3. Addition of salt at "stationary point."

A. 50 cc. 2 per cent. glycogen + 0.25 cc. N/20 KCl + 1 cc. toluene + 0.5 cc. enzyme + 48.25 cc. H₂O.

B. 50 cc. 2 per cent. glycogen + 10 cc. N/20 KCl + 1 cc. toluene + 0.5 cc. enzyme + 38.5 cc. H₂O.

C. As *A*.

The glycogen and enzyme solutions were dialysed before use. When the rate of hydrolysis in *A* and therefore in *C* had become extremely slow, further salt was added to *C* so that the concentration of KCl became equal to that in *B*.

The course of the hydrolysis in each case is shown in Table III below.

TABLE III.

Duration of experiment Minutes	Percentage hydrolysis		
	A. Low salt content	B. High salt content	C. Low salt con- tent. Salt added at stationary point
15	3.95	10.14	—
30	7.39	17.40	7.39
240	24.80	33.50	24.90
315	26.50	35.20	26.54
			KCl added here
345	26.80	35.80	28.60
405	27.50	—	31.40
465	27.70	—	32.32
585	28.30	37.80	34.16
705	28.78	38.20	34.63

The results show that the addition of salt to *C* after 315 minutes resulted in an increase in the rate of hydrolysis, *C* increasing 8.1 per cent. during the remainder of the experiment as against the 2.2 per cent. increase shown by *A*. Compared with *B* however, which contained the high salt concentration all the time, *C* still showed a deficit of 3.6 per cent. when the two rates had become equal. This however would be expected as even assuming that all the excess of enzyme not combined with salt were intact when further salt was added, there was at this point already present in *C* a considerable percentage of the hydrolytic products which might retard the freshly activated enzyme. At the same time if the curves shown in Fig. 1 representing this experiment be observed it will be noticed that the rate of action in *C* after the addition of the salt was slightly less than that obtaining in *B* when at the same stage of the hydrolysis, this suggesting that slight injury to the enzyme had occurred.

The case when further enzyme is added at the stationary point is also of interest and will be considered next.

Exp. 4. Addition of enzyme at "stationary point."

A. 50 cc. 2 per cent. dialysed glycogen + 0.25 cc. N/20 KCl + 1 cc. toluene + 0.5 cc. dialysed enzyme.

D. 50 cc. dialysed 2 per cent. glycogen + 10 cc. N/20 KCl + 1 cc. toluene + 2 cc. dialysed enzyme.

E. 50 cc. 2 per cent. dialysed glycogen + 0.25 cc. N/20 KCl + 1 cc. toluene + 2 cc. enzyme.

F. As *A*. Enzyme added at stationary point so that enzyme concentration then became equal to *E*.

G. As *A.* Enzyme and salt added at stationary point so that the mixture was then similar in composition to *D.*

The hydrolyses were carried out in the usual manner till *F* and *G* had become very slow, which occurred after about five hours, when the additions were made, the results being shown in Table IV.

TABLE IV.

Duration of experiment minutes	Percentage hydrolysis				
	<i>A.</i> Low salt, low enzyme content 26.2	<i>D.</i> High salt, high enzyme content 42.1	<i>E.</i> Low salt, high enzyme content 36.0	<i>F.</i> Addition of enzyme 26.2	<i>G.</i> Addition of enzyme + salt 26.2
315	26.5	42.4	36.2	26.54 Enzyme added	— Enzyme and salt added
330	26.65	42.6	36.3	—	32.32
345	26.80	42.8	36.4	29.36	—
390	27.30	—	—	—	37.90
405	27.50	—	—	31.86	38.50
585	28.30	43.15	37.8	35.11	41.60
705	28.78	43.69	38.3	35.60	42.05

Fig. 1.

The first point of interest is that addition of further enzyme to *F* produced an acceleration although the concentration of salt employed was quite insufficient to permit the enzyme initially present to produce its maximum

effect. There is evidently then an equilibrium established between free salt, free enzyme and the salt-enzyme complex which is changed by the addition of further enzyme, more of the complex being formed and the hydrolysis thereby accelerated. The same point is indicated by the two controls *A* and *E*, *E* proceeding at a much greater rate than *A* although having the same low salt content. The results are shown graphically in Fig. 1, together with those of Experiment 3.

The addition of salt and enzyme naturally produces a greater acceleration than the addition of either separately, and this acceleration is in fact greater than the sum of the two components as one would expect, the enzyme in *F* being retarded by an insufficiency of salt. At the same time the rate in *G* after the addition is less than that obtaining in the control *D* when at the same stage of the hydrolysis. Examining the total degree of hydrolysis produced it will be seen that while *G* never quite reached the control *D*, it approached it very nearly and this also applies to *F* and its control *E*, the deficiency being however slightly greater in this case.

All these results are against the view that the stopping of the action is caused by the resistance of the residual dextrin to further hydrolysis, the addition of further enzyme producing initially a fairly rapid rate of hydrolysis which quickly falls off again, thus suggesting an alteration of an equilibrium between enzyme and the hydrolytic products. The only other obvious factor is the possible destruction of the enzyme during the course of the experiment and the degree to which this is concerned was examined in the following experiment.

Exp. 5. Addition of further glycogen at the "stationary point."

A. 100 cc. 1 per cent. glycogen + 1 cc. toluene + 1 cc. enzyme.

B. 100 cc. 2 per cent. glycogen + 1 cc. toluene + 1 cc. enzyme.

C. Initially as *A*. Further glycogen added after 4 hours hydrolysis.

Hydrolysis was allowed to proceed at 37° until the rate in *A* and *C* became slow and then further glycogen was added to *C* so that the total concentration became equal to that originally present in *B*, and the further course of the hydrolysis observed.

The results are shown graphically in Fig. 2, the abscissae representing the duration of the experiment in minutes and the ordinates the amount of hydrolysis which had taken place expressed in percentages of one gram, the latter being calculated as previously explained on the assumption that the reducing power of the solution is due to maltose alone.

The glycogen was added to *C* at 240 minutes and as will be observed was rapidly hydrolysed, showing that a large proportion of the enzyme was still in an active condition although the rate was much lower than that at which the first quantity of glycogen was initially hydrolysed, the relative values for 60 minutes being 9.5 and 17 respectively. As the final degree of hydrolysis observed was practically the same however in *B* and *C* it is extremely improbable that any of the enzyme had been destroyed during the experiment and the difference in the two rates must be attributed to the influence of the products present when the addition of glycogen was made. This view is supported by the next experiment.

Fig. 2.

Exp. 6. Addition of glycogen and enzyme at the "stationary point."

A. 100 cc. 1 per cent. glycogen + 1 cc. enzyme + 1 cc. toluene.

B. 100 cc. 2 per cent. glycogen + 2 cc. enzyme + 1 cc. toluene.

C. Initially as *A*. When the action had slowed down, enzyme and glycogen were added to make the proportion of these similar to those obtaining in *B*.

It was to be expected that the glycogen added to *C* would be hydrolysed with a considerable initial velocity but that this rate would fall off quickly owing to the influence of the products on the freshly added enzyme. For the

same reason it was unlikely that the final degree of hydrolysis would be as great in *C* as in *B*.

An examination of the results shown in Fig. 3 indicates that these hypotheses were verified. Here again the ordinates represent the degree of hydrolysis expressed in the percentages of one gram hydrolysed to maltose.

The glycogen and enzyme were added to *C* after 300 minutes and while the glycogen was readily hydrolysed the rate was actually less than that obtaining initially in *A* which had only half the concentration of enzyme. This result can only be explained by the influence of the products as whatever

Fig. 3.

may have happened to the first quantity of enzyme during the course of the experiment, that added later was in an active condition and yet could not give such a high rate of hydrolysis as a similar quantity of enzyme acting on a solution containing none of these products. Doubtless for the same reason the final degree of hydrolysis in *C* was less than that observed in *B*.

Influence of the hydrolytic products. (a) Maltose.

Exp. 7. A. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 0.5 cc. enzyme.

B. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 0.5 g. maltose + 0.5 cc. enzyme.

The only difference between the two experimental mixtures is that *B* contains initially an amount of maltose corresponding to that found at the conclusion of a hydrolysis with this strength of enzyme. The rates at which hydrolysis proceeded in *A* and *B* were then compared, the results being shown in Table V.

TABLE V.

Duration of experiment	mg. Cu from 20 cc. solution	
	<i>A.</i>	<i>B.</i> Corrected for added maltose
15 mins.	27	28
30 "	46	46
1 hr.	66	65
2 hrs.	80	80
4 "	92	91
8 "	100	99
20 "	108	104
Percentage hydrolysis	49.6	47.6

This quantity of maltose had then very little depressing action on the rate of hydrolysis.

(b) *Influence of mixed products.*

Exps. 8 and 9. 200 cc. of 1 per cent. glycogen solution containing 2 cc. enzyme and toluene were incubated at 37° for 24 hours. The solution was then boiled and the degree of hydrolysis accurately determined.

The following experiments were then carried out.

A. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 1 cc. enzyme.

B. 100 cc. boiled solution containing hydrolytic products + 1 g. glycogen + 1 cc. toluene + 1 cc. enzyme.

Two similar experiments were carried out, the results being shown in Table VI.

A control was carried out in which the boiled solution of products alone was incubated with enzyme but practically no further change took place during the course of the experiment.

In Experiment 9 there was a slightly higher initial proportion of products than in 8. In both cases however there is a well marked retarding influence exerted by the hydrolytic products, the differences observed at the end of the experiment being as follows :

Exp. 8. 5.69 in a hydrolysis of $46.55 = 12.2$ per cent.

Exp. 9. 6.26 in a hydrolysis of $47.10 = 13.2$ per cent.

It is possible that the freshly formed products may have an even greater influence than those examined in the experiments where the solutions had been boiled.

TABLE VI.

Duration of experiment Minutes	Experiment 8		Experiment 9	
	Percentage hydrolysis			
	A.	B.	A ₁ .	B ₁ .
15	21.40	19.0	18.0	15.2
30	29.49	26.6	27.1	21.87
60	33.77	29.55	33.28	27.65
120	38.57	33.37	38.57	32.78
175	—	—	39.03	33.35
240	40.93	34.3	—	—
360	42.78	36.2	—	—
510	43.25	37.15	—	—
675	—	—	43.72	37.9
1375	—	—	47.1	40.84
1380	46.55	40.86	—	—

SUMMARY AND CONCLUSIONS.

1. A solution of glycogen is not completely hydrolysed to maltose by the glycogenase of the pancreas unless very high concentrations of enzyme are employed, although, even with low enzyme concentrations, the whole of the glycogen may disappear.
2. The total degree of hydrolysis produced rises with increasing concentration of enzyme but not proportionally.
3. In dialysed solutions the same result is obtained by increasing the salt content up to a certain optimum concentration.
4. Evidence is brought forward to show that the resistance of the residual dextrin to further hydrolysis is not the main factor in the stopping of the reaction.
5. There is little evidence indicating that destruction of the enzyme takes place to any considerable extent during the course of the experiment.
6. The mixed products formed have a marked retarding influence on the velocity of the reaction and are probably the most important factors in the stopping of the hydrolysis. Maltose alone however has a very small effect.

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