LXXIX. LIVER GLYCOGENASE.

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THE mechanism of the utilisation of liver glycogen has not been so extensively worked out as that of muscle glycogen. The purpose of the present work is to study the action of liver glycogenase by making an active preparation of the enzyme, determining the end-products of its action on glycogen and observing some of the factors influencing this reaction.

Various means of preparing glycogenase have been used. The extracts made by the Buchner (hydraulic press) method are very unstable because they contain proteolytic enzymes which quickly destroy the glycogenase, even when the extracts are kept at a low temperature. Another method of preparation is to grind some of the tissue thoroughly in a mortar, using quartz sand, and then to mix with several volumes of alcohol. The resulting precipitate after standing under alcohol for a few days is collected on a filter-paper and dried *in vacuo*. A weighed portion of the powder can then be taken at any time and ground up into a homogeneous suspension with water. The chief objection to this method is that contact with alcohol appears to alter the potency of the precipitate so that if it is to be used for purposes of comparison care must be taken to see that all the conditions are exactly the same.

In the method of Wiechowski [1910] the tissue is very rapidly macerated to a fine pulp which is spread out on glass plates and these are placed in a rapid current of warmed air until completely dry; the crust is then removed and the scales thoroughly extracted in a suitable extractor with toluene.

More recently Eadie [1927] made an enzyme preparation in which the liver was thoroughly ground, then treated twice with acetone, once with a mixture of equal parts of acetone and ether and finally twice with ether. After drying overnight the powder thus obtained was extracted for several days with 50% glycerol, filtered and dialysed against running water for about 5 hours. The resultant solution was frequently found to be unstable, but it could always be used for 24 hours. When 5 ml. of enzyme solution were allowed to act for 5·25 hours on 5 ml. of 1·5% glycogen solution, the reducing sugar calculated as glucose was about 10% of the amount for total hydrolysis. This amount of reducing sugar represents the hydrolysis of only a small part of the glycogen and indicates that the enzyme preparation is a comparatively inactive one.

The action of glycogenase has been measured by determining from time to time either the amount of glycogen left unchanged or the amount of sugar formed. The former method being somewhat laborious, Salkowski [1906] and Wohlgemuth [1908] elaborated methods in which starch, not glycogen, was used as the substrate, and the hydrolysis was followed quantitatively by the starch-blue reaction with iodine. This procedure is open to criticism because it is based on the unwarranted assumption that starch has the same properties as glycogen (see Haworth [1929]).

Experiments in which the action of pancreatic amylase on glycogen has been followed are also open to criticism, because they are based on the assumption that glycogenase acts exactly like amylase.

Methods.

The enzyme was prepared from the liver of rabbits. The rabbits were killed by severing the carotid artery on one side with a sharp razor and allowing them to bleed. The liver was removed, cut into fine pieces and pressed through cheese cloth in a mortar with twice its weight of acetone. The acetone extraction was repeated once. The acetone extract was filtered, and the residue, which rapidly dried, was put through a sieve. The fine powder thus obtained is a potent preparation of the enzyme.

To study the properties of the enzyme a weighed quantity of the powder was added to a buffered (Clark and Lub) solution of glycogen. The glycogen was prepared from rabbit's liver by the method of Sahyun-Alsberg [1930] and dialysed against running water. The flask containing the mixture was placed in a water-bath at 37° and shaken mechanically. Reducing sugar in samples from the mixture was determined by the Shaffer-Hartmann [1921] method. Glycogen was determined by heating samples from the mixture with an equal quantity of 60 % KOH in a boiling water-bath for 3 hours, precipitating by alcohol, hydrolysing with 2·2 % HCl, and estimating the glucose by the Shaffer-Hartmann method. Thus where simultaneous estimations of substrate and end-product were undertaken the same method for estimating glucose, the Shaffer-Hartmann, was used and possible errors thereby diminished.

Experimental.

A preliminary experiment was carried out to determine the relative amounts of enzyme in the liver of fasted and of fed animals. Accordingly four animals from the same litter were selected and two fed and the others fasted for 2 days; all four were then killed and enzyme preparations made from their livers. That from the fed animals contained a certain amount of glycogen. That the preparations from the fasted animals contained no glycogen was demonstrated by adding some to Clark and Lub buffer solutions and finding no reducing sugar in the mixture over a 5-hour period.

Into each of four flasks were placed 30 ml. of Clark and Lub buffer p_H 7·5 and 1·0 g. of enzyme preparation from each of the four livers. To each flask containing enzyme from the fasted animals was added 0·45 g. of glycogen. The flasks were shaken in a bath at 37° and samples taken and analysed for glycogen at 0, 0·5, 1·5, 3 and 5 hours (see Table I).

Table I.

	% glycogen (as glucose)			
Time (hours)	Fed rabbit	Fed rabbit	Fasted rabbit	Fasted rabbit
0	1·31	1·35	1·46	1·43
0·5	0·93	0·87	0·71	0·73
1·5	0·56	0·53	0·26	0·30
3	0·30	0·29	0·12	0·13
5	0·16	0·12	0·16	0·06

The reaction proceeded faster with the preparations from the fasted animals than with those from the fed animals, but the amount of enzyme in the extracts from the former was greater than that in the latter, and when this allowance is made it will be seen that deprivation of food does not appreciably affect the enzyme content. This is an observation of importance because it renders possible

the preparation of glycogenase free from its substrate glycogen. Hence the enzyme preparations used in the succeeding experiments were derived from the livers of fasted animals.

Influence of p_H.

To each of three flasks containing 30 ml. of Clark and Lub buffer of p_H 6·0, 7·0 and 8·0 with glycogen in solution was added 1·0 g. of enzyme preparation. These were shaken in a bath at 37° and samples taken both for glycogen and sugar at 0, 0·5, 1·5, 3 and 5 hours.

The results are recorded graphically in Fig. 1. This shows that the reaction proceeds more quickly in an alkaline medium.

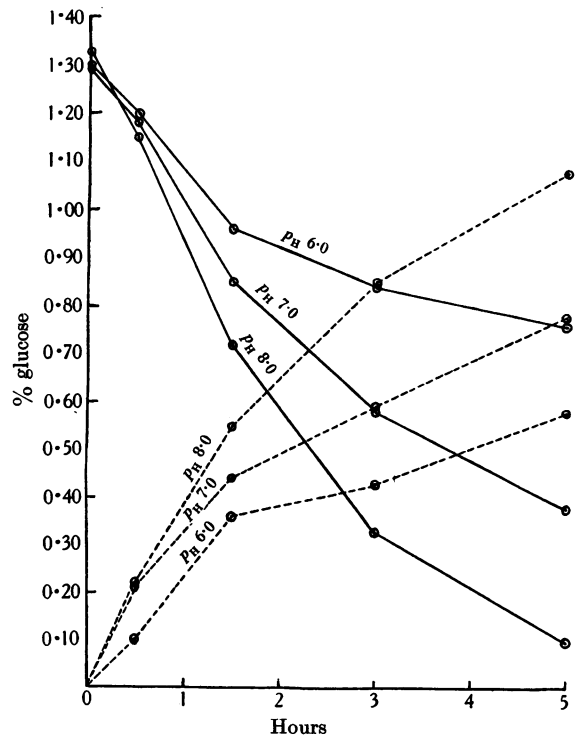

Fig. 1. The relation of glycogenolysis to sugar formation.
—— Glycogen (calculated as glucose). - - - - Reducing sugar (calculated as glucose).

End-product of the reaction.

A further study of the graph disclosed the fact that at any time the contents of the mixture may be expressed in two parts, material estimated as glycogen and reducing sugar, and that the sum of the two parts is approximately equal to the amount of glycogen (expressed as glucose) to start with. This would not be true were maltose present. If the reducing power of glucose with copper reagents be represented by 100, that for maltose may be taken as 55. Hence the presence of maltose would cause a much lower percentage of reducing sugar to be shown at any one time, and the two fractions would not be complementary. Therefore, either no maltose is formed, or, if formed, the rate of its destruction equals that of its formation.

Influence of blood on the reaction.

Eadie [1927] questioned the results of previous workers on the action of liver glycogenase on the grounds that their enzyme preparations contain blood amylase. He performed experiments using a rat liver which had been perfused for 1 hour with warmed Ringer solution and observed an optimum p_H for the enzyme at a lower level than that found by other workers. However, it is known that washing out the liver may either reduce the activity of the enzyme, or destroy it in part, and Davenport [1926] pointed out the fallacy of comparing the activity of unmodified blood serum with blood which has been through an extraction process. In order to investigate this point a quantity of rabbit's blood was subjected to exactly the same procedure as the liver. When the powder thus obtained was allowed to act on a buffered solution of glycogen no reducing sugar was formed over a 5-hour period. Thus the method of extraction which is here employed yields a potent liver glycogenase but destroys blood amylase, and there can be no interference in these experiments by the latter enzyme.

In another experiment 5 ml. of rabbit's blood serum were allowed to act for 5 hours on a 0·2 % buffered solution of maltose; over this period there was no change in the reducing power of the maltose solution, thus indicating no maltase effect.

Effect of the enzyme on maltose.

The preparation of the enzyme free from glycogen makes it possible to obtain a significant result from its action on maltose and lactose. Accordingly to each of four flasks, of which three contained 30 ml. of 0·2 % maltose in Clark and Lub buffered solutions, p_H 6·0, 7·0 and 8·0 respectively, and one contained 30 ml. of 0·2 % lactose solution in Clark and Lub buffer solution p_H 7·0 was added 1·0 g. of enzyme preparation.

The tubes were shaken in a bath at 37° and samples analysed for reducing sugar at 0, 0·5, 1·5, 3 and 5 hours (see Table II).

Table II.

	% reducing sugar (as glucose)			
	Maltose mixtures			Lactose mixture
Time (hours)	p_H 6·0	p_H 7·0	p_H 8·0	p_H 7·0
0	0·110	0·113	0·112	0·144
0·5	0·160	0·147	0·151	0·146
1·5	0·188	0·178	0·184	0·148
3	0·201	0·195	0·197	0·148
5	0·203	0·199	0·201	0·147

The enzyme thus appears to be almost equally active on maltose over a range p_H 6·0–8·0. It is possible however that either there was an excess of enzyme present, thus masking the effect of p_H, or that the optimum p_H lies outside the range investigated.

The enzyme was without effect on lactose at p_H 7·0.

Complete hydrolysis of the mixtures was carried out at the end of the 5-hour period. To 2 ml. of mixture were added 2 ml. of 2·2 % hydrochloric acid and the whole allowed to boil gently under a reflux condenser for half an hour. The solution was then neutralised with sodium bicarbonate and analysed for reducing sugar. The results of this procedure gave values of 0·206, 0·210, 0·203 and 0·206 % respectively, all close to the theoretical. It will thus be seen that almost the whole of the maltose was changed to glucose by the activity of the enzyme.

Influence of sodium chloride.

Four flasks containing glycogen in solution in 30 ml. Clark and Lub buffer p_H 7·4 were prepared and sodium chloride to 0·1, 0·5 and 1·0 % added to three of them. They were shaken in a bath at 37° and samples analysed for sugar at 0, 0·5, 1·5, 3 and 5 hours (see Table III).

Table III.

	% glucose			
Time (hours)	Control	With 0·1 % NaCl	With 0·5 % NaCl	With 1·0 % NaCl
0	0	0	0	0
0·5	0·285	0·250	0·260	0·203
1·5	0·591	0·575	0·580	0·493
3	0·875	0·825	0·845	0·818
5	0·920	0·915	0·920	0·905

These results show that sodium chloride has no appreciable effect on the rate of reaction.

Effect of dialysis.

3 g. of enzyme preparation were dialysed in a collodion sac against distilled water for 48 hours with one change. Three flasks containing glycogen in 30 ml. of Clark and Lub buffered solution p_H 7·4 were taken; to the first was added 1·0 g. of undialysed enzyme preparation, to the second 1·0 g. of dialysed enzyme preparation; and to the third 1·0 g. of dialysed enzyme and sodium chloride to 0·1 %. The flasks were heated in the usual way (see Table IV).

Table IV.

	% glucose		
Time (hours)	Control mixture	Dialysed enzyme mixture	Dialysed enzyme mixture with 0·1 % NaCl
0	0	0	0
0·5	0·280	0·02	0
1·5	0·645	0·09	0·02
3	0·825	0·11	0·05
5	0·930	0·12	0·09

This experiment shows that dialysis destroys the activity of the enzyme and that its activity is not restored by the addition of sodium chloride.

Effect of insulin on glycogenase.

Cammidge and Howard [1924] showed that insulin inhibited the rate of hydrolysis of starch *in vitro* by liver amylase. Visscher [1926], using glycogen as a substrate, reported only slightly less hydrolysis in experiments in presence of insulin. Popper and Wozasek [1933] observed decreased diastase content in fatal insulin hypoglycaemia.

To investigate this problem four animals from the same litter were selected and fasted for 2 days. Two of the animals were each given 12 units of insulin subcutaneously and 2 hours later 12 units intravenously. Both of these were killed in convulsions and the two control animals killed simultaneously. Enzyme preparations were made from the livers, and 1·0 g. extract was added to each of four flasks containing 30 ml. of Clark and Lub buffer p_H 7·4 with glycogen in solution, which were treated in the usual way (see Table V).

Table V.

	% glucose			
			Insulinised animals	
Time (hours)	Control	Control		
0	0	0	0	0
0·5	0·254	0·230	0·200	0·195
1·5	0·650	0·607	0·460	0·483
3	0·750	0·730	0·570	0·580
5	0·780	0·760	0·635	0·640

The small decrease in the activity of the enzyme which followed the lowering of the blood sugar to the convulsive stage is of doubtful significance. It is therefore improbable that insulin, under physiological conditions, has any effect on glycogenase. This is to be expected if insulin does not influence liver glycogenolysis.

Regarding the effect of adrenaline, Langfeldt [1921] claimed that adrenaline not only increased the activity of liver amylase but also altered its optimum hydrogen ion concentration. Later workers could substantiate neither effect, and the present view is that no *in vitro* effect of adrenaline can be demonstrated.

Discussion.

The results obtained show that liver glycogenase converts glycogen quantitatively into glucose, independently of any amylase action on the part of the blood, and that, unlike amylases, glycogenase is not appreciably influenced by variations in the sodium chloride content of the medium. It would also appear that as quickly as maltose is formed it is converted into glucose.

Many attempts have been made to explain how glycogenase and glycogen can exist together in the liver cell. Macleod [1926] advanced the view that glycogenolysis was set up by the local production of a certain amount of acid, and that such a liberation of free acid could be brought about by a curtailment in the arterial blood supply of the hepatic cell, vasoconstriction either from adrenaline or nervous stimulation being suggested as a probable cause. It is thus assumed that at the normal p_H of the liver cell the enzyme is inactive and that with a change in p_H it increases enormously. It has been shown in the present work that the enzyme is by no means inactive at the p_H of the blood, and that its activity is but little altered over any range of p_H likely to be encountered under physiological conditions.

Lesser [Lesser and Kerner, 1920; Lesser, 1920; 1921] advocated the view that the glycogen and amylase were "locked up" in different compartments of the cell. He showed [Lesser and Zipf, 1923] that homologous alcohols in isocapillary concentrations increased sugar formation in the perfused frog's liver by approximately the same amount; that this effect occurred under reversible conditions [Lesser, 1925]; and that the effect was also similar in a perfusion fluid buffered with CO_2 and bicarbonate, p_H 7·4–7·6 [Lesser, 1926]. On the basis of these findings he concluded that a boundary surface phenomenon was involved, and he assumed that two-thirds of the enzyme was adsorbed on the surfaces of the cell and therefore unable to act on the liver glycogen.

On the basis of this view the rôle of adrenaline in glycogenolysis may be considered to be the release of glycogenase by physico-chemical action from intracellular interfaces.

SUMMARY.

1. A method of preparation of glycogenase from rabbit's liver is described.
2. The enzyme converts glycogen into glucose, the rate of reaction being greatest on the alkaline side of neutrality.
3. Maltose is quantitatively changed into glucose by the enzyme.
4. Sodium chloride has no significant effect on the rate of reaction.
5. The enzyme is destroyed by dialysis and its activity is not restored by sodium chloride.
6. No significant decrease in the glycogenase in the liver was observed to follow administration of convulsive doses of insulin.
7. The bearing of these experiments on the problem of glycogenolysis is discussed.

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