XLIV. ON LIVER AMYLASE.

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IN view of certain peculiarities in the relation of the velocity of hydrolysis of starch by the amylase of germinated barley to its concentration [Eadie, 1926], it was decided to investigate the relations which hold in the case of liver amylase and glycogen, in the hope of throwing more light on the physiology of glycogen in the liver and the regulation of carbohydrate metabolism. Early in this work the disconcerting fact was disclosed that the optimum hydrogen ion concentration varied with the preparation used, and was now at p_H 7 and now at p_H 6. Earlier workers have uniformly stated it to be about p_H 7 (see for example, Holmbergh [1923], who gives references to the earlier papers), although careful search of the literature shows that this has not always been justified by their results. During the course of these experiments a paper by Davenport [1926] was published in which this peculiarity was noticed when liver amylase acted on starch.

Since the liver preparations used contained more or less blood, it seemed possible that there were really two or more amylases present. The amylase of the blood [Euler, 1922] is probably derived for the most part from the pancreas, and pancreatic amylase has an optimum about p_H 7. This suggested that the more alkaline optimum in the case of the liver preparations was due to amylase derived from the blood present in the liver, and that the liver amylase itself was responsible for the more acid optimum.

METHODS.

The extent of hydrolysis was estimated by determining the amount of sugar produced, using the method of Shaffer and Hartmann. Reducing power was calculated as glucose. The objections raised by Visscher [1926] to the use of this method in the presence of phosphate buffers apparently do not apply when the concentration of phosphate is as low as in these experiments [Davenport, 1926].

The liver was prepared by a method similar to that described by Holmbergh [1923]; it was thoroughly ground, then treated twice with acetone, once with a mixture of equal parts of acetone and ether, and finally twice with ether. The quantities and times recommended by Holmbergh were not strictly followed. After drying overnight the powder thus obtained was extracted for several days with 50 % glycerol, filtered and dialysed against running water for about five hours. The resultant solution was frequently found to be unstable, but it could always be used for 24 hours.

Clark and Lubs' phosphate buffers were used and checked electrometrically.

Liver from various sources was tried, but the most active preparations came from the rat rather than from the pig or sheep.

The glycogen used was prepared by Pflüger's method, for the most part from rabbit liver, but in a few experiments that from mussels was used. No difference was found between the glycogens from these two sources.

During hydrolysis the reacting solutions were kept in a thermostat at 37°.

EFFECT OF PERFUSION ON RAT LIVER¹.

A rat liver was treated by the method just outlined after washing off the blood but without perfusion, and its activity tested in the following way.

Into each of five flasks were placed 5 cc. of 1.5 % glycogen solution, 0.5 cc. of 2.5 % sodium chloride solution, 5 cc. buffer (of varying p_H) and 15 cc. water. These were warmed to 37°, and 5 cc. of the enzyme solution were added to each. Samples removed immediately after mixing showed the absence of sugar. Samples were taken in 2, 4 and 24 hours. The results for the 4-hour samples are shown in Fig. 1 (continuous line), and those for the other two periods are in substantial agreement with these. An almost identical curve was obtained with sheep's liver. It will be noted that there are two maxima, one about p_H 6 and another about p_H 6.8 or 6.9.

Fig. 1. Effect of perfusion.

— Unperfused liver.

--- Perfused liver. The figures on the ordinate refer to the unperfused liver.

For the actual values for the perfused liver see text.

Another rat was then taken and perfused for about an hour with warmed Ringer's solution. After this time the liver showed no traces of blood. Treated in an exactly similar way, it gave the results shown below.

p_H : 5.95, 6.35, 6.72, 7.14, 7.41.

Glucose % after 5½ hours: 0.0525, 0.0510, 0.0480, 0.0465, 0.0440.

These results are plotted in Fig. 1 (dotted line) on the same scale as the other curve, but to prevent the curves overlapping, another base line is used.

¹ I am indebted to Mrs Holmes for her kindness in perfusing these livers.

The figures in the margin should each be increased by 0.0025 to apply to this curve.

Here the maximum between 6.7 and 7.1 has disappeared. Evidently the perfusion has washed out the blood and with it an amylase having its optimum about the neutral point.

Since the rat liver is rather small, I endeavoured to find a method for freeing from blood livers from the slaughter-house. For this purpose a quantity of pig's liver was finely ground, stirred up with water, centrifuged and again treated with water, the process being repeated four times.

After being treated in the usual way this preparation was allowed to act on glycogen under the same conditions as before. After 5 hours' hydrolysis the following amounts of sugar were found:

p_H : 5.95, 6.35, 6.72, 7.14, 7.41.

Glucose %: 0.0016, 0.0028, 0.0032, 0.0040, 0.0020.

This was clearly unsuccessful.

Another plan was then tried based on that used by Thunberg for washing muscle. The ground liver was placed in a tall glass cylinder covered with gauze through which a glass tube passed leading to the bottom of the cylinder. Tap water was run through the glass tube until the water coming away was clear. The residue was treated in the usual way and the extract allowed to act as before. In $4\frac{1}{2}$ hours the amounts of sugar produced were:

p_H : 5.95, 6.35, 6.72, 7.14, 7.41.

Glucose %: 0.0056, 0.0050, 0.0062, 0.0056, 0.0053.

There has been a shift of the optimum towards the acid side here but not so completely as in the case of the perfused rat liver. Obviously some blood amylase is still present although in relatively less amount.

Since the rat liver was so much more active and so much easier to wash free from blood, it was decided to continue with it. Lesser [1920] considers that washing of the liver takes out some of the liver amylase, and I find it associated with considerable loss of activity. Since the pig's liver was not very active to begin with there could be very little activity left after sufficient washing to free it from blood.

It should be noted here that this change in optimum p_H is not of the kind described by Michaelis and Pechstein [1914] among others in the case of ptyalin, where it varies with the salt present in the mixture, for in all cases here the same amounts of phosphate and sodium chloride were used. Moreover, the presence of two optima in certain experiments with unwashed liver could not be explained in this way.

INFLUENCE OF SUBSTRATE CONCENTRATION.

Fig. 2 shows the amounts of sugar formed in an hour under the following conditions. Six flasks were prepared containing 0.5 cc. of 2.5 % sodium chloride solution, 5 cc. phosphate buffer of p_H 5.95, varying quantities of

1.5 % glycogen solution and water to make a total volume of 25.5 cc. After being warmed to 37°, 5 cc. of enzyme solution were added to each.

Fig. 2. Effect of substrate concentration.

Fig. 3. Showing initial stages of hydrolysis.

That the rate of reduction is practically linear over this range is shown by Fig. 3, which refers to a solution of a similar composition containing 0.45 % of glycogen. During the time covered by the curve 0.06 % of glucose was produced, *i.e.* 10 to 15 % of the amount for total hydrolysis. This is about the extent of hydrolysis in the experiment illustrated in Fig. 2. Any deviation from the linear relation is likely to produce results which are too low in the case of the lower concentrations of glycogen and (relatively) too high in the case of the higher concentrations.

The smooth curve in Fig. 2 is based on the theory proposed by Michaelis and Menten [1913], who deduce from it the equation:

$$v = k \cdot \frac{S}{S + K_s}$$

Here v is the velocity, S the substrate concentration, K_s the "affinity constant" or Michaelis constant, and k is another constant. The curve in Fig. 2 is plotted from this equation, putting $K_s = 0.095$ and $k = 0.00021$. This value for the affinity constant is of the same order of magnitude as those obtained by Michaelis in the paper quoted.

It will be seen that the agreement of the values found with those predicted from theory is within the limits of experimental error. Closer examination reveals that the first two experimental points lie below the curve and the three following above it, which might indicate a constant trend. I think that this is due to the fact that the velocity (as mentioned above) falls off somewhat with time, and affects the points corresponding to the smaller concentrations first. I have tried several other types of curve, but no other seems to fit the results nearly so well.

These results therefore differ markedly from those obtained with starch and the amylase of germinated barley, and resemble those obtained with such a system as sucrose and invertase.

An attempt was made to do this experiment with a preparation made from unwashed liver. The curve obtained resembled this one somewhat, but, as might be expected, was rather more complex and did not fit exactly any well-known type of curve.

EFFECT OF SALT CONCENTRATION.

Since it is probable that most previous workers have used mixtures of blood and liver amylase to investigate this point, it was thought worth while to repeat the experiment.

Flasks were set up each containing 5 cc. of 1.5 % glycogen solution, varying quantities of 0.30 % sodium chloride solution, and water to make 20 cc. 5 cc. of enzyme solution were added to each in the manner described above. The concentration of glycogen thus was 0.25 %. The sugar formed in one hour was estimated and the results are given in Fig. 4.

Fig. 4. Effect of salt concentration.

It will be seen that this amylase resembles closely the other amylases of animal origin in being extremely sensitive to the presence of sodium chloride. The optimum is apparently about 0.1 %.

EFFECT OF ADRENALINE.

These experiments were done in the spring of 1926. Since that time two other contributions to the subject have been made. It will be recalled that Langfeldt [1921] claimed that adrenaline not only increased the activity of the liver amylase, but also altered its optimum hydrogen ion concentration.

Working with liver amylase and glycogen I was not able to find any alteration in the optimum p_H in the presence of adrenaline. There was, however, increased activity, but this disappeared when the concentration of sodium chloride in the controls and in the solutions containing adrenaline was made equal. Commercial preparations of adrenaline contain considerable amounts of sodium chloride.

Visscher [1926] using glycogen as a substrate found no alteration of the optimum p_H , but his enzyme was evidently badly contaminated with blood amylase, and he did not discover the chloride effect. Later, Davenport [1926] reported that with starch as a substrate adrenaline had no effect, apart from the sodium chloride contained in its preparations. Davenport used several enzyme preparations, some of which contained blood amylase in large quantity while others did not. I have found a similar variation in the blood amylase content in preparations made by Holmbergh's method; the method used by Davenport evidently gives similar results.

It appears therefore that Langfeldt's claims are not substantiated.

DISCUSSION.

In view of the failure of earlier workers to recognise that their preparations of liver amylase might be badly contaminated by that from the blood, a good deal of their work requires revision.

One of the most detailed studies of the enzyme is that by Holmbergh [1923]. He states that the optimum p_H in the presence of sodium chloride and phosphate buffer is 6.9. This indicates that his preparation contained so much blood amylase that its activity overshadowed that of the liver; consequently his estimates of the quantity of amylase in the liver are probably much too high. Similar suspicion also attaches to other estimates for in no case is there proof that liver amylase alone is being estimated. Indeed, to estimate the amount of liver amylase is a matter of some difficulty; no method of washing out the blood seems satisfactory, because, as Lesser and Kerner [1920] have shown, thorough perfusion also washes out some of the true liver amylase.

My experiments have also some bearing on the question of glycogenolysis. For the problem of how amylase and glycogen can exist together in the liver cell, several solutions have been offered. One of these is that, to quote Macleod [1922], "glycogenolysis in the liver cell could be set up by the local production of a certain amount of acid. Such a liberation of free acid could be brought about by a curtailment in the arterial blood supply of the hepatic cell..." and vasoconstriction either from adrenaline or nervous stimulation is suggested as a probable cause. The argument includes the remark that "diastatic enzymes are particularly susceptible to the reaction (c_H) of their environment, a very slight degree of acidity favouring and a trace of alkalinity markedly depressing their activities." It is apparently assumed that at the normal p_H of the liver cell the enzyme is inactive, and that its activity enormously

increases with the degree of acidity. It seems unlikely that a physiological process such as vasoconstriction would lead to such a change as from p_H 7.4 to 6. Yet we see that the enzyme is by no means inactive at the former p_H , and in changing to the latter its activity increases by less than 25 % (cf. Fig. 1). A change from p_H 7.4 to 6.7, which is probably much closer to changes occurring in the living cell, increases the activity by about 10 %.

Since the rate of hydrolysis is very nearly constant over a considerable period at the beginning, the figures from which these calculations are made give a pretty close measure of the initial rates, and may be used in place of them without any error which would affect this argument in the slightest.

The liver amylase itself is either not an active enzyme compared with that of the blood, or is present in very small concentrations, for the presence of blood in the organ from which the preparation is made is able to mask it almost completely. It is very much weaker than that of the pancreas. These facts, in connection with the flatness of the activity- p_H curve, render the explanation quoted above much less probable than it appeared to be in the absence of more detailed knowledge of the enzyme.

Lesser [Lesser and Kerner, 1920; Lesser, 1920, 1921] has advocated another view, viz. that the glycogen and amylase are, as it were, "locked up" in different compartments of the cell. This explanation unfortunately adds little to our knowledge because it suggests no mechanism whereby the two are kept separate. Lesser finds a loss of activity on perfusion which he attributes to the washing-out of liver amylase only, for he maintains, on the basis of experiments by himself and by Fröhlich and Pollak, that the amylase of the blood can have no action on the liver glycogen. He reports [1921] an experiment in which he perfused livers with solutions of varying p_H and measured the hourly output of glucose. The figures for the first hour's perfusion show maximal amounts at p_H 7.44 and 5.66. The figures for the second, third and fourth hours show maxima at 6.45. (There was no solution of p_H intermediate between 5.66 and 6.45.) These values refer of course to the perfusion fluid and are not to be taken as indicating very accurately the conditions under which the actual hydrolysis occurs, although they must influence it to a great extent and one which becomes greater as time goes on.

These facts would be simply explained if we admit that the blood amylase is active during the first hour, but is washed out by perfusion much more rapidly than the liver amylase. If this be admitted, I suggest that it is quite possible that blood amylase may act on glycogen under physiological conditions.

SUMMARY.

1. The optimum c_H for liver amylase is shown to be at least as high as that indicated by p_H 6, and the previously accepted value is attributed to the amylase of the blood.
2. The effect of substrate concentration on the activity has been determined and shown to be in accordance with the theory of Michaelis.

3. The effect of sodium chloride is shown to be similar to that found with other amylases of animal origin, and the effect of adrenaline solutions is found to be due solely to the sodium chloride contained in them.

4. The bearing of these experiments on the problem of glycogenolysis in the liver is discussed.

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