CXXVI. THE EFFECT OF SUBSTRATE CONCENTRATION ON THE HYDROLYSIS OF STARCH BY THE AMYLASE OF GERMINATED BARLEY.

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It has been frequently assumed in the theory of enzyme action that the enzyme forms a combination with the substrate which breaks up to yield the products of the reaction and the free enzyme again. Further assumptions, such as that the velocities of combination of enzyme and substrate, and of the decomposition of their compound, follow the law of mass action, permit the deduction of mathematical expressions which connect the velocity of the reaction as a whole, at least in the initial stages, with the concentration of enzyme and substrate. Such expressions have been derived by Van Slyke and Cullen [1914], Michaelis and Menten [1913], and Briggs and Haldane [1925], and have been used by many authors. They have been particularly successful in the case of invertase.

An attempt to apply this theory to the case of barley amylase and starch has been made by Sjöberg and Ericson [1924]. Their data, however, seemed too scanty to afford a convincing proof that this theory was applicable, and indeed seemed to indicate that the relation which held between the initial velocity and the substrate concentration was other than that predicted by this theory. Their experiments have therefore been amplified.

METHODS.

For substrate Lintner's soluble starch was used. Sjöberg and Ericson have shown that the difference between the rates of action of the enzyme on amylopectin and amylose is very slight.

The enzyme was prepared by allowing barley to germinate for 4 days; it was then ground thoroughly, extracted with water for 48 hours and filtered. Before use the extract was usually dialysed for several days against either distilled water or the buffer solution to be used. The extract before dialysis contained, of course, a large amount of reducing substance.

For buffer, a solution of sodium acetate and acetic acid was used, 0.2 *M* in acetate, and of p_H 5.1 unless otherwise noted.

The reacting solutions were brought to 15° before mixing and the reaction was allowed to take place in a vessel immersed in a water-bath at that temperature.

Reducing power was estimated in 5 cc. samples by the micro-method of Shaffer and Hartmann. The results are calculated as glucose.

The velocity was determined in the first 10 minutes in order to confine the estimation to the linear portion of the velocity curve. Estimations were usually made 1, 5 and 10 minutes after adding the enzyme and the average of the velocities in 4 and 9 minutes calculated. This gives double weight to the first period and was done in order to minimise any error from a falling-off of velocity in the second period. As a rule, the velocities in the first and second periods were identical, but in some cases experimental error was evident, in others the reaction was apparently going more slowly as it proceeded.

The production of reducing sugar from starch is probably the result of a series of reactions; but, since during the experimental period the rate is linear, it is evident that it is always the same reaction that is being measured. We are, therefore, not confused by the possibility of several reactions being reflected in varying degrees in the results.

RESULTS.

The relation between velocity and substrate concentration.

Several curves were obtained, and a typical one is given in Fig. 1. Inspection of the curve shows at once that it differs very definitely from that predicted by Michaelis or Willstätter. These authors prefer to plot the velocity against the logarithm of the substrate concentration, and obtain a curve which is formally identical with that of the dissociation-residue curve of a weak acid. If the velocity be plotted thus it will be seen that two approximately straight lines are produced. Of these one represents the rising part of the curve, the other the flat portion where the velocity is zero.

Fig. 1. With dialysed enzyme showing initial lag

Experiments in which undialysed enzyme was used gave similar results, as will be seen from Fig. 2.

In considering the significance of these results two possibilities present themselves. First, that the Michaelis type of curve would be found to apply

to systems of pure enzyme and substrate and is not found here because of the presence of impurities or on account of other experimental conditions. With regard to the last it will be shown that the curve is unaffected in shape by variations of p_H and temperature; nor will the absence of the proper salt concentration in the mixture explain the divergence. The criticism based on the presence of impurities cannot, of course, be disposed of until a pure enzyme preparation has been obtained, but various considerations render it improbable. The fact that experiments with undialysed enzyme show no essential difference in the shape of the curve points in this direction. Moreover, the considerable degree of purification of invertase achieved by Willstätter and by Euler has produced no essential alteration in the curve originally made by Michaelis with a relatively impure preparation.

Fig. 2. With undialysed enzyme.

On the other hand, if this curve is really significant of the relation of velocity to substrate concentration, and I think that this must be assumed in the absence of proof to the contrary, we must conclude that the combination of enzyme and substrate follows in this case a rule other than the simple law of mass action from which the formulae referred to above have been derived; *i.e.* it is probably a heterogeneous equilibrium. This may mean some type of adsorption. While the curve has certain similarities to the adsorption curve, it differs decidedly near the zero point. If we take Freundlich's adsorption equation:

$$y = a.C^n \quad \dots\dots(1)$$

where C is the concentration of adsorbed substance, y is the amount adsorbed, and a and n are constants, we may put C equal to the concentration of substrate (the concentration of the enzyme is constant), and y proportional to the rate, since the rate is presumably proportional to the amount of substrate combined, either "physically" or "chemically," with the enzyme. It is easy to show that this equation does not hold.

It is quite possible to obtain an empirical equation which will fit the results very exactly. Such an equation is

$$v = a + b \log C \quad \dots\dots(2)$$

where v is the velocity, C is the concentration of substrate, and a and b are constants.

Fig. 3. The curve is plotted from equation (2): $\times$ experimental points.

This is illustrated in Fig. 3. The data for this have been chosen from another experiment in which more points were obtained for the rising part of the curve, although none for concentrations of starch so small as to give zero velocity (cf. Fig. 1). The curve is plotted from the equation

$$1000v = 20.4 \log 10x - 10.1.$$

To avoid decimals $1000v$ and $10x$ are used. Negative values of v are assumed to be without significance for the purpose of plotting the curve.

It may be noted in passing that a similar equation was used by Quastel and Whetham [1925] for the rate of reduction of methylene blue by *B. coli* in the presence of varying amounts of hydrogen donator.

I am unable to suggest any theoretical basis for this equation, but it forms a convenient method of comparing curves obtained under slightly different conditions.

The most interesting part of the curve is at the beginning. Until the concentration of starch rises above 0.03 %, the amount of hydrolysis in 10 minutes is nothing. In one or two experiments which were continued for 30 minutes no hydrolysis had occurred in this time. This limiting concentration appeared to vary only slightly with the enzyme preparation used; in some cases slight hydrolysis was seen at this concentration, but never with concentrations of starch less than this. Above this value, or sometimes a slightly greater one, there is a rapid increase in the rate of hydrolysis with increasing concentration of substrate, and the curve gradually falls off in a logarithmic manner.

The effect of neutral salts.

Because the activity of the enzyme is considerably decreased by dialysis, and in view of the well-known effect of sodium chloride on animal diastases, it was thought worth while to investigate this effect, although other workers have shown that plant diastase differs from animal in this respect.

The results are as follows:

Salt used	Concentration (normality)	Rate of hydrolysis
None	0.0	0.006
NaCl	0.064	0.007
"	0.32	0.006
"	1.62	0.009
KCl	0.038	0.006
K ₂ SO ₄	0.154	0.007

The starch concentration was 0.385 %.

It will be seen that salts in large concentration have a slight effect. This, however, is insufficient to account for the loss of activity on dialysis.

The effect of enzyme concentration.

Two experiments were made using different enzyme preparations. The results of one of these are given in Fig. 4. It will be seen that the velocity is, within the limits of experimental error, directly proportional to the enzyme concentration. The concentration of starch was the same in all these experiments, viz. 0.385 %.

The other experiment which was made under the same conditions gave quite similar results with a thirty-fold variation in the relative concentration of enzyme.

Fig. 4. The effect of enzyme concentration.

From these it is concluded that alteration in the amount of enzyme would have no effect on the general shape of the curve. This conclusion is further substantiated by the fact that, although several enzyme preparations of varying activity were used to determine the activity-substrate-concentration curve, it was always of the same shape.

Fig 5. Showing the effect of p_H .*The effect of hydrogen ion concentration.*

Curves were made for p_H 4.3 and 6.1. From Fig. 5 it will be seen that the effect is chiefly on the slope of the curve and only very slightly on the point where the zero value is reached. This is shown by the equations for these curves. The a of the equation affects the position of the point representing zero velocity, while the b (the coefficient of the $\log 100 C$ term) affects the slope.

At p_H 4.3 the equation is

$$1000v = 9.9 \log 100C - 3.9 \quad \text{.....(3)}$$

and at p_H 6.1

$$1000v = 7.7 \log 100C - 3.8 \quad \text{.....(4).}$$

The difference between 3.9 and 3.8 is possibly due to experimental error; at any rate it is not large enough to make a definite statement on this point possible.

The effect of temperature.

A curve was made at p_H 4.3 and a temperature of 5° which may be compared with the one made at p_H 4.3 and 15° and given in the preceding section.

The equation for the curve at 5° is:

$$1000v = 4.2 \log 100C - 3.5 \quad \text{.....(5).}$$

From this and the corresponding Fig. 6 it can be seen that again the greatest effect is on the slope of the curve, but here there is a definite shift to the right.

It should be mentioned that the curves in this and the preceding section were made with all precautions to ensure that the activity of the enzyme was the same throughout. The enzyme preparation used had stood some months. At first its activity decreased rapidly from day to day, but by this time it had reached a practically stationary value. This is in agreement with the results of Sjöberg and Ericson. The curves were made on successive days and repetition of certain points on the curve showed that the enzyme had not changed.

Fig. 6. Showing effect of temperature

The equations were deduced from the experimental data by the method of least squares.

It is interesting to calculate the temperature coefficients for varying starch concentrations. This may be done conveniently from equations (3) and (5). It is obvious from the figure that the use of the actual experimental data would lead to a similar result.

Starch concentration %	Q_{10}
0.0681	∞
0.0682	1000
0.0698	100
0.0716	50
0.0931	10
0.133	5
0.291	4
2.78	3

These figures show the great dependence of the temperature coefficient on the substrate concentration. In this case at least the temperature coefficient is meaningless without further details.

Glycogen as substrate.

An experiment was done using glycogen as substrate:

Glycogen concentration %	Rate
0.029	0.000
0.058	0.000
0.144	0.002
0.202	0.003
0.276	0.004
0.288	0.004
0.394	0.008
0.473	0.010
0.590	0.013
0.720	0.013
1.01	0.016
1.38	0.020

It will be seen that the affinity of the enzyme for glycogen is much less than for starch; the curve commences to rise only when a higher concentration of glycogen is used, and rises much less steeply. It is still rising, however, at the highest concentration used, while the curve for starch is practically horizontal in that region.

DISCUSSION.

The bearing of these experiments on the velocity equation for the hydrolysis of starch may be pointed out. A great many equations have been proposed: they have been summarised by Kuhn in Oppenheimer's *Handbuch*.

The ordinary mono-molecular equation

$$dx/dt = k(a - x) \quad \dots\dots(6)$$

implies that the initial rate ($x = 0$) is directly proportional to a , i.e. the initial concentration of substrate. Thus if, keeping everything else constant, one varies the substrate concentration, the relation found should be expressed by the formula

$$v = k.c \quad \dots\dots(7),$$

where k is a constant, and v and c have the same significance as before. A comparison with equation (2) shows that this does not hold. It is therefore obvious that since (7) does not hold, (6) cannot represent the true course of the reaction, nor can any other equation which does not reduce, when $x = 0$, to (2) or an equivalent expression. None of the equations quoted by Kuhn apparently does so.

It is quite realised, however, that equation (2) is entirely empirical, and is not necessarily the only one which fits the facts, nor the one which may ultimately be found best to do so. On the other hand, any equation which seeks to embody the time-relations for the hydrolysis of starch must necessarily reduce under these conditions to this form or to an equivalent one.

SUMMARY.

1. The relation of velocity of hydrolysis of starch by the diastase of germinated barley to the concentration of starch has been described and shown to differ from the relations previously described for other enzymes.
2. The effects on this of changes of salt concentration, of hydrogen ion concentration and of temperature are described.
3. A comparison is made with glycogen as substrate.
4. The temperature coefficient of the reaction is shown to vary from three to infinity according to the concentration of starch used.

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