CLXVII. STUDIES ON PLANT AMYLASES.
I. THE EFFECT OF STARCH CONCENTRATION UPON
THE VELOCITY OF HYDROLYSIS BY THE
AMYLASE OF GERMINATED BARLEY.

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It is clear that, for the determination of enzymic reaction-velocity under readily definable conditions, the states near the initiation of the reaction are decidedly the best.

Usually however this part of the progress has been left unexplored and delineation of it has been based merely upon empirical expressions of doubtful value. In the present paper data have been directly collected within a few minutes of the origination of the reaction on the mixing of enzyme and substrate. From these data "initial slopes" are determined graphically and used as a measure of reaction-velocity. In experiments of this short duration the difference between apparent and true zero-time becomes significant.

THE STARCH-AMYLASE SYSTEM. DESCRIPTION OF AN
"INITIAL SLOPE" METHOD.

Of the various criteria which have been used for following the progress of starch degradation in the presence of amylase, the production of reducing sugar can be most accurately measured. When there is a possibility, as in this reaction, that the final product is the result of a series of transformations, it would be preferable to derive reaction velocity values from data on the disappearance of the original substrate, rather than the accumulation of the final product. In the absence of methods, however, for the accurate determination of the unchanged starch, it is necessary to rely upon determinations of the reducing sugar produced for precise information concerning the progress of the reaction. The objections to this procedure are minimised if the reaction velocity determinations are restricted to the initial period before there is an appreciable falling-off in rate, since it can then be concluded that one particular stage in a possible series of reactions is being measured.

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An analysis of a large number of published experimental data on amylase action has led to the conclusion that many methods which have been used for obtaining reaction velocity values frequently fail to give a reliable measure of the initial reaction velocity, and that the results are accordingly of limited significance. An "initial slope" method has therefore been used in an attempt to obtain more reliable data on the action of certain plant amylases.

The success of such a method depends upon (1) the use of a suitable analytical procedure for the precise determination of small amounts of reducing sugar, and (2) a careful standardisation of the technique for mixing enzyme and substrate, withdrawing samples, and arresting the reaction in these samples.

(a) Sugar determination.

A trial of various existing micro-methods of sugar analysis indicated the desirability of developing an analytical procedure adapted to the specific requirements of the present problem, since existing methods were found to be inadequate either because the range was too limited or the determination of the smaller amounts of sugar was unreliable. Accordingly a method was developed based on that of Hagedorn and Jensen. In this modified procedure, which has been described earlier [Hanes, 1929], the range of the original method was increased about tenfold. Its precision and range have proven satisfactory for the present purpose. It has been found, moreover, that the alkaline ferricyanide reagent used in this method (solution A) is effective for arresting the action of amylase on starch.

The sugar which is formed from starch after prolonged action of the barley amylase preparations used is almost exclusively maltose. It has been assumed, although not experimentally demonstrated, that maltose is also the sugar which appears in the early stages of the reaction. The sugar method was therefore standardised for maltose, and the reducing power is expressed in terms of maltose.

(b) Standardised procedure for following the early part of the reaction.

The reaction is carried out in 75 cc. Erlenmeyer flasks, supported in a water-thermostat. Ordinarily the temperature is maintained at 25°, although it has been advantageous for certain purposes to use lower temperatures.

A total digest volume of 29 cc. has been found suitable for most of the work. This is composed of 25 cc. of substrate preparation, 2 cc. of buffer solution, and 2 cc. of enzyme solution. This volume allows 5 samples to be withdrawn for maltose determination.

The solutions used, and the digests themselves, are kept free from bacterial contamination by saturation with toluene, and the pipettes are guarded with thick plugs of cotton wool, to exclude traces of saliva.

Before starting a digest, the solutions to be used and the reaction flasks are allowed to come to the temperature of the water-bath, and 5 cc. portions of the ferricyanide solution A, for sugar analysis, are pipetted into a series

of numbered boiling-tubes. A digest is prepared by pipetting 25 cc. of substrate and 2 cc. of buffer solution into the reaction flask. Finally the enzyme is run in from a 2 cc. pipette. At the moment of removing the finger to start the delivery of the pipette, a stop-watch is started. This is referred to as "zero time." The delivery of the pipette takes about 8 seconds; when it is nearly empty the last drops are blown out, the tip being held against the wall of the flask. It is then tapped three times against dry portions of the wall. The flask is then removed from the rack and shaken vigorously to wash down the walls and mix the contents. This is usually completed about 20 seconds after zero time.

A clean dry 5 cc. pipette is now filled with digest liquid and allowed to drain back into the flask. It is then refilled, and the level adjusted to the mark. The tip is then freed from adhering drops of liquid and inserted into one of the boiling-tubes, touching the wall about 2 cm. above the level of solution A. At a convenient point on the stop-watch, usually at an even tenth-second, the delivery of the sample is started into the tube. The delivery occupies about 7 seconds, after which the pipette is allowed to drain a further 10 seconds, the tip still in contact with the wall. It is then tapped five times against dry portions of the wall, and removed. The boiling-tube is now inclined in the hand and rotated to wash down the wall. The time of the beginning of the delivery, and the number of the tube are noted. The procedure for subsequent samples is identical. The same pipette is used throughout each digest without washing with water, but before taking each sample it is rinsed out with the digest liquid.

Ordinarily the tubes are left for less than 15 minutes before continuing with the procedure for maltose estimation, although no appreciable differences are found if they are left longer.

When facility with the technique had been acquired, the delivery of the first sample into the "killing" solution could be started as early as 0.6 minute after zero time, all operations being carried out in the standard way. For most purposes, it has been found satisfactory to take the first sample at about 1.0 minute after zero time, and the second and third at about 3.0 and 6.0 minutes respectively. However, it is necessary to bear in mind, when deciding upon the timing of digest samples, that the aim of the procedure is to establish the initial slope of the progress curve. Consequently, if for any reason it is expected that the reaction velocity of a particular digest will fall off relatively earlier, then samples are taken at shorter intervals. An example of this procedure is to be seen in Digest No. 6 (p. 1413) in which the times of successive samples were 0.7, 1.9, 3.1 and 4.3 minutes after zero time. The ideal timing of samples presupposes a knowledge of the particular reaction system under observation.

(c) *The derivation of reaction velocity values from the data.*

It is found on plotting the data obtained by this procedure, that the progress of sugar formation is seldom strictly rectilinear even during the brief

initial periods under observation, although under certain conditions the falling-off in velocity may be slight. Usually a progressive decline in reaction velocity is apparent from the beginning, and it is evident that the earliest measured slope (that is, the slope during the interval between the first and second samples) must be lower than the ideal initial slope. It is accordingly desirable to attempt to obtain by extrapolation a slope value which will approximate more closely to the ideal initial slope.

The extrapolation is carried out by plotting the progress-time data on a large scale graph, and joining the points by a carefully-drawn curve. A black silk thread is stretched across the paper and its position and direction adjusted by sighting along the curve and the thread so that it passes through the earliest experimental point, and a portion of the thread forms an extrapolation of the curve back to zero time. The slope of the thread can then be accurately determined by readings on the squared paper. Three such determinations are usually made and the average value is taken as a measure of the initial reaction velocity. This procedure gives a slope value at a point approximately half-way between zero time and the time of the first sample, in practice, about 20 seconds after zero time.

In explaining the reason for adopting this particular method of extrapolation it is necessary to mention certain complications which arise as a result of the extreme shortening of the time scale which is effected in the present procedure. Two lag effects, which are negligible when the time intervals of sampling are longer, may now become appreciable; these effects are due to (1) the "initial lag" phase after zero time, representing the time for the delivery of the enzyme and its mixing with the substrate and possibly, in addition, for the attainment of equilibrium between enzyme and substrate; and (2) the "killing" lag, representing the time required for arresting the reaction in each digest sample after starting the delivery into the alkaline ferricyanide solution. These lag phenomena have the effect of causing displacements of empirical progress curves in time, the initial lag producing a displacement to the right (when the zero point of the time axis is at the left), the "killing" lag, a displacement to the left. In extrapolating for initial slope values, an analysis has shown that the aim should be to measure the slope at the intersection between the extrapolated progress curve and the horizontal line representing the level of the initial reducing power of the digest components. Consequently, unless the two opposing displacements in time are equal, a "corrected origin" is required for extrapolation. For the present data, the "corrected origin" probably occurs about 10-20 seconds after zero time. The method of extrapolation described above gives a measure of the slope at about 20 seconds after zero time and this has been adopted as a working approximation to the theoretical ideal.

THE EFFECT OF STARCH CONCENTRATION UPON THE REACTION VELOCITY.

The relationship between substrate concentration and reaction velocity in an enzyme-substrate system is of considerable theoretical importance. Briggs and Haldane [1925], in extending the earlier formulations of the Michaelis theory of enzyme action [Michaelis and Menten, 1913], consider the reaction $A \rightarrow B$, unimolecular as regards A , catalysed by an enzyme E . They suppose one molecule of A to combine reversibly with one molecule of E forming an enzyme-substrate compound, which then decomposes irreversibly into free enzyme and B , the product of the reaction. The system considered may be represented:

the specific constants for the different reactions being as indicated, k_1 , k_2 and k_3 .

By applying the law of mass action, an expression is deduced relating reaction velocity and substrate concentration, which would be valid so long as the molar concentration of enzyme is negligibly small compared with that of substrate. This expression is:

$$v = V_{\infty} a / (a + K_s),$$

where a is the molar substrate concentration,

v , the reaction velocity,

V_{∞} , the maximum velocity of the system which would be attained when the substrate concentration is so large that the whole of the enzyme exists in the form AE^1 ,

and K_s is the ratio $(k_2 + k_3)/k_1$.

The relationship between reaction velocity and substrate concentration predicted from this hypothesis is a rectangular hyperbola.

Two attempts have been made to test the applicability of this hypothesis to the action of barley amylase. Sjöberg and Erikson [1924], using as substrate preparations of "amylose" and "amylpectin" prepared according to Gatin-Gruzewska, came to the conclusion that the relationship is of the form predicted by the theory. The experimental evidence offered in support of this conclusion is very limited and far from satisfactory on other grounds. Eadie [1926] in re-investigating the question, using Lintner's soluble starch as substrate, came to the conclusion that the relationship is of an entirely different kind from that predicted by the theory, since he observed that with certain low starch concentrations no reaction occurred. He was therefore of the opinion that the Michaelis theory was not applicable to the action of barley amylase. The reason for the anomalous nature of Eadie's results will be made clear later.

¹ This assumes that no secondary effects of high substrate concentration arise, such as water shortage.

EXPERIMENTAL.

A study of the effects of varying the substrate concentration has been carried out, using the initial slope method as detailed on p. 1407. Several different preparations of amylase from germinated barley have been used.

Method of preparing amylase from barley.

Barley of known variety, locality and season of growth has generally been used. A weighed sample of selected grains, usually 100 or 200 g., is washed thoroughly and allowed to soak in distilled water for about 10 hours. It is then spread in a thin layer on moist filter-paper and set away to germinate in a dark moist chamber. The grains are occasionally stirred and moistened during the germination period. When the plumules, still within the seed coat, have grown to about 1/2 to 3/4 of the length of the seed, the grains are placed in a beaker and killed by stirring with a little toluene, and then ground to a paste in a fine mincer.

This material is then extracted in an ice-chest with dilute acetic acid (usually 0.1 %) for a period varying from 6 to 24 hours, with occasional stirring. The coarser sediment is then removed either by squeezing through fine cloth in a press, or by centrifuging. The turbid liquid is then filtered until almost clear. The enzyme is now precipitated by adding sufficient 95 % ethyl alcohol to make a final concentration of 70 % by volume. The liquid is set away in an ice-chest in a tall vessel for 12–24 hours, during which time the precipitate settles. The bulk of the clear supernatant liquid is then decanted. The remainder is filtered through Whatman's No. 50 paper on a Büchner funnel. The final pinkish residue is allowed to drain on the filter for 15 minutes. The paper bearing the residue is then removed and ground up in a mortar with distilled water. The liquid is poured off and filtered. The successive residues (and papers) are repeatedly extracted to remove the water-soluble fraction. The clear, faintly yellow filtrate is then made up to a definite volume, and toluene is added as a preservative. The preparations obtained in this way have a very low content of reducing sugar. They remain clear for several days but usually on prolonged storage a small flocculent precipitate appears which is inactive. The hydrogen ion concentration is generally in the range p_H 6.4–6.8 and the buffering power is slight. In different preparations the total dry weight has represented from 0.1 to 0.35 % of the dry weight of the original barley.

On occasion, further purification has been attempted by dialysis, reprecipitation, fractional precipitation, and by adsorption on kaolin. Reference to these preparations will be reserved for a later paper.

Exp. 1. Enzyme. Amylase 1, prepared from germinated barley. (See Appendix 1.)

Substrate. Two solutions of soluble starch, 1.25 and 0.145 %, were used in this experiment. They were freshly prepared from a sample of Lintner's soluble starch (Merck) which had been dried to constant weight at room

temperature over sulphuric acid. An amount of starch, sufficient to make 1 litre of the required concentration, is suspended in a small volume of water and then washed into 600 cc. of boiling water. The whole is then brought to the boil, boiled for 8 minutes, cooled and made up to 1 litre. Toluene is added.

Buffer. 2 cc. of acetic acid-sodium acetate buffer, $M/5$ in acetate, in each digest of 29 cc., making the final concentration $M/73$ approximately. The final p_H was 4.71–4.73 in all digests¹. The reaction temperature was 25.0°.

Digests were set up as shown in Table I, and the data obtained from them are given in Table II.

Table I.

Digest No. ...	1	2	3	4	5	6	7	8	9	10	11	12
1.25 % soluble starch cc.	25	15	10	5	3	2	—	—	—	—	—	—
0.145 % soluble starch cc.	—	—	—	—	—	—	10	8	6	3	1	1
Water cc.	—	10	15	20	22	23	15	17	19	22	24	24
Buffer solution cc.	2	2	2	2	2	2	2	2	2	2	2	2
Amylase No. 1 cc.	2	2	2	2	2	2	2	2	2	2	2	2
Final starch concentration	1.078	0.647	0.431	0.216	0.129	0.0863	0.0500	0.0400	0.0300	0.015	0.005	0.005

The first point which becomes evident from this set of data is that there is no evidence of a "critical" starch concentration for the occurrence of the reaction as has been suggested by Eadie [1926]. Even in the digests of lowest

Fig. 1. Progress curves of digests of low starch concentration, from 0.050 % to 0.005 %.

starch concentration (0.005 %) the reducing power increases progressively during the period under observation. This is shown clearly in Fig. 1 in which progress curves of several digests of low substrate concentration have been plotted².

¹ This amylase preparation showed an optimum activity between p_H 4.6 and 4.8. This has been found to be true of nearly all the preparations of barley amylase investigated. The data on the p_H -activity relationships will be discussed in a subsequent paper.

² Since several of the sugar determinations in this experiment fell below the range of the sugar method as originally standardised, the method was re-standardised for maltose down to 0.05 mg. The linear relationship between the amount of maltose and the ferricyanide reduced was found to hold over the entire range investigated.

Table II.

Digest No.	α % starch concentration	t Mins. after zero time	x mg. maltose per 5 cc.	Slope mg./min.	v , initial slope	
					Earliest measured	Extra- polated*
1	1.078	1.0	1.12		0.438	0.445
		3.1	2.04	0.438		
		5.0	2.815	0.408		
		7.0	3.63	0.408		
2	0.647	0.8	1.08		0.433	0.435
		2.6	1.86	0.433		
		4.5	2.665	0.424		
		6.2	3.35	0.403		
3	0.431	0.75	0.575		0.386	0.400
		2.5	1.25	0.386		
		4.3	1.92	0.372		
		5.9	2.485	0.354		
4	0.216	0.7	0.415		0.334	0.345
		2.3	0.95	0.334		
		3.6	1.35	0.308		
		4.9	1.75	0.308		
5	0.129	0.7	0.295		0.300	0.305
		2.0	0.685	0.300		
		3.3	1.035	0.269		
		4.7	1.36	0.232		
6	0.0863	0.7	0.25		0.238	0.260
		1.9	0.535	0.238		
		3.1	0.80	0.221		
		4.3	1.035	0.196		
7	0.0500	0.9	0.23		0.175	0.180
		2.1	0.44	0.175		
		3.3	0.63	0.158		
		4.8	0.79	0.107		
		10.1	1.24	0.085		
8	0.0400	0.7	0.155		0.154	0.165
		1.9	0.34	0.154		
		3.0	0.50	0.145		
		4.1	0.625	0.113		
9	0.0300	0.7	0.15		0.135	0.140
		2.0	0.325	0.135		
		3.4	0.475	0.107		
		6.0	0.655	0.069		
		9.0	0.81	0.052		
10	0.015	0.7	0.12		—	—
		2.0	0.22	0.077		
		6.0	0.39	0.045		
		10.0	0.48	0.028		
		23.0	0.55	0.005		
11	0.005	0.7	0.07		—	—
		2.2	0.13	0.040		
		4.5	0.16	0.013		
		7.7	0.18	0.006		
		10.9	0.20	0.006		
12	0.005	0.7	0.06		—	—
		2.0	0.10	0.031		
		8.0	0.18	0.013		

* The method of extrapolation is described on p. 1409. The values are given to the nearest 5 in the third decimal place. This implies a significance ranging from about 0.5 % in the highest values to about 2 % in the lowest.

Digests 10, 11 and 12 were carried out primarily to determine whether or not hydrolysis occurred and no attempt has been made to measure reaction velocities with such low starch concentrations.

The initial sugar values, calculated from separate determinations of the reducing power of the enzyme and the substrate, were, for digest 10, 0.04 mg.; for digests 11 and 12, 0.025 mg., expressed as maltose per 5 cc. Knowing these values it is possible to calculate the extent of the reaction in these digests. In digest 10 (0.015 %), approximately 59 % of the starch is hydrolysed at 10 minutes; 70 % at 23 minutes. In digests 11 and 12 (0.005 %) 72 % of the starch is hydrolysed at 10 minutes. It is clear that the reaction proceeds to the normal extent in these extremely low substrate concentrations.

These results, which have been confirmed using three different preparations of amylase, are contrary to the findings of Eadie who reported that no hydrolysis took place below a certain critical starch concentration, which varied in his different experiments from about 0.03 % to 0.09 %, as estimated from his graphs.

It is now proposed to consider the information obtainable from the data of digests 1-9 concerning the relationship between substrate concentration and initial reaction velocity. Progress curves of these digests are shown in Fig. 2. The curves have been made to pass through the origin by subtracting

Fig. 2. Progress curves of digests 1 to 9 of substrate concentration varying from 1.078 % in No. 1 to 0.030 % in No. 9 (see Table II).

from each of the observed x values of each digest a quantity equal to the apparent initial sugar value (S_a) for that digest calculated from the earliest measured slope (e.g. for digest 1, $S_a = 1.12 - 1.0 \times \frac{2.04 - 1.12}{2.1} = 0.68$). This arbitrary procedure facilitates the visual comparison of the plotted data, and has been adopted solely for that reason.

The values of initial velocity found by extrapolation, in the manner described on p. 1409, are given in the last column of Table II. It will be seen by comparing the figures in the last two columns that the differences between the earliest measured slopes and the extrapolated values are small. Actually, the conclusions which will be drawn from the data would be almost equally valid if either set of values had been used.

The relationship predicted from the Michaelis theory is

$$v = V_{\infty} a / (a + K_s).$$

The values of the constants V_{∞} and K_s for this expression giving the best fit to the data of digests 1 to 9 have been calculated by the method of least squares. It was assumed that the values of a , the substrate concentration, were without experimental error, as compared with the error in the determinations of v . A set of equations of the form

$$a/v = a/V_{\infty} + K_s/V_{\infty}$$

was constructed from each available pair of a and v values¹. From these, two equations normal in respect to $1/V_{\infty}$ and K_s/V_{∞} respectively were derived in the usual way. By solving these equations, the values of best fit were found to be²: $V_{\infty} = 0.478$, $K_s = 0.077$. The equation

$$v = 0.478 a / (a + 0.077)$$

has accordingly been used to calculate theoretical values for comparison with the observed values of reaction velocity. These are given in Table III.

Table III.

a Starch concentration %	v calculated $v = 0.478 a / (a + 0.077)$	v observed
1.078	0.446	0.445
0.647	0.427	0.435
0.431	0.406	0.400
0.216	0.352	0.345
0.129	0.299	0.305
0.0863	0.252	0.260
0.0500	0.188	0.180
0.0400	0.163	0.165
0.0300	0.134	0.140

It is clear that the relationship is in close agreement with that predicted from the Michaelis theory. The experimental results have been plotted in Fig. 3, together with the curve of the equation $v = 0.478 a / (a + 0.077)$.

Exp. 2. A second experiment, carried out in the same manner as Exp. 1, will be described briefly. The enzyme was amylase 2, prepared from germinated

¹ This method will give those values of $1/V_{\infty}$ and K_s/V_{∞} which give the minimum value for the sum of the squares of the deviations between calculated and observed values of $1/v$ (not of v); but this is good enough for the present purposes.

² Owing to the fact that the molecular weight of starch is unknown, the substrate concentrations have been expressed as percentages. Consequently this K_s value is not comparable with the values obtained for other enzymes.

barley (see Appendix). The substrate in this case was prepared from air-dry soluble starch which contained approximately 4.90 % of water. A 1 % solution was made up following the same procedure as described above. Seven digests of different starch concentration were followed, these being of 29 cc. volume as before, containing 2 cc. of amylase, 2 cc. of $M/5$ acetate buffer (final p_H , 4.70–4.72) and varying proportions of the 1 % starch solution and distilled water. The data obtained from these digests, Nos. 13 to 19, are shown

Fig. 3. The relationship between reaction velocity and substrate concentration. The values of v and a of Exp. 1 have been plotted, together with the rectangular hyperbola of best fit.

Table IV.

Digest No.	a % starch concentration	t Mins. after zero time	x Mg. maltose per 5 cc.	Slope mg./min.	v , initial slope	
					Earliest measured	Extrapolated*
13	0.820	1.0	1.525		0.495	0.505
		3.0	2.515	0.495		
		5.0	3.49	0.487		
		7.0	4.395	0.452		
14	0.459	1.0	0.815	0.465	0.465	0.470
		3.4	1.93	0.459		
		5.6	2.94			
15	0.262	1.0	0.845	0.429	0.429	0.440
		3.4	1.875	0.402		
		5.6	2.76			
16	0.164	1.0	0.91	0.360	0.360	0.370
		3.75	1.905	0.350		
		4.75	2.255	0.326		
		7.25	3.07			
17	0.0983	1.0	0.645	0.286	0.286	0.300
		2.5	1.075	0.253		
		4.0	1.455	0.223		
		6.0	1.90			
18	0.0656	0.82	0.32	0.242	0.242	0.250
		2.08	0.625	0.214		
		3.2	0.865	0.202		
		5.0	1.23			
19	0.0328	0.8	0.255	0.154	0.154	0.165
		2.0	0.44	0.140		
		3.0	0.58	0.103		
		5.0	0.785			

* The method of extrapolation is described on p. 1409.

in Table IV, together with the values of the initial starch concentrations, corrected for the moisture content of the air-dry starch¹.

The values of best fit of K_s and V_∞ calculated for this set of data by the method which was used in Exp. 1, were found to be $V_\infty = 0.554$, $K_s = 0.079$. Values calculated from the equation $v = 0.554 a / (a + 0.079)$, for the different substrate concentrations employed are shown in Table V.

Table V.

a %	v calculated $v = 0.554 a / (a + 0.079)$	v observed
0.820	0.506	0.505
0.459	0.473	0.470
0.262	0.426	0.440
0.164	0.374	0.370
0.0983	0.307	0.300
0.0656	0.251	0.250
0.0328	0.163	0.165

Here again the relationship is in close agreement with that predicted by the theory. Moreover it will be noticed that the value of K_s for this amylase preparation (No. 2) is the same within the limits of the experimental error, as that found for amylase 1.

It is clear from the results of these two experiments that the Michaelis theory offers a quantitative interpretation of the effects of substrate concentration upon the velocity of starch hydrolysis by the amylase of germinated barley. The evidence that the theory is applicable to this enzyme-substrate system seems at least as conclusive as that which exists for other cases, including the invertase-sucrose system.

A discussion of the effects of various factors, including hydrogen ion concentration, upon the magnitude of K_s for this enzyme will be reserved for a later paper. It should be mentioned here that other somewhat less extensive sets of data for other preparations of the enzyme indicate that the K_s value is normally close to the values which have been found for amylases 1 and 2, when the enzyme is acting at the optimum hydrogen ion concentration upon a substrate of soluble starch prepared as described above.

A DISCUSSION OF THE RESULTS OF EADIE.

It seems necessary to discuss further the discrepancy between the results which have now been presented and the results of Eadie [1926].

Eadie came to the conclusion that with low starch concentrations no reaction occurs. His substrate concentration-velocity curves consistently show

¹ In making the correction for the water content of the starch it was unfortunately necessary to rely upon a moisture determination carried out some time after the experiment was done. The values of a and of K_s are therefore subject to an additional error not exceeding 2 %, which is absent from the results of Exp. 1. While the determination of K_s is accordingly slightly less reliable on this account, conclusions regarding the form of the substrate concentration-velocity relationship are of course not affected.

velocities of zero over a short range of low substrate concentration. Several points deducible from the curves shown by Eadie (the primary data are not published) bring the actuality of his results under suspicion. In the first place it is true in general of his data that the more active the enzyme as judged by the reaction velocity with high substrate concentration, the lower is the "critical" starch concentration required for the reaction to take place. Moreover, in the graph showing the relationship between reaction velocity and relative enzyme concentration, the points do not indicate a linear relationship, but strongly suggest that with enzyme concentrations lower than 0.4 on his scale, the reaction velocity would be zero. These points suggest that estimates of low reaction velocities by Eadie's method were unreliable.

An investigation of the technique used by Eadie has shown this to be the case. The method he used was an approach to an initial velocity method. Sugar estimations were made at 1, 5 and 10 minutes. The average rates for the 4 and 9 minute intervals were calculated and these were averaged to obtain the value which was taken as the reaction velocity. The reducing power was determined by the original micro-method of Shaffer and Hartmann [1921]. When this method was used by the writer to follow the progress of sugar production in digests of low starch concentration (0.05 %) using enzymes of low activity, comparable with those used by Eadie, it was found that the progress curves showed an initial flat phase, after which the reducing power began to increase. The duration of the initial flat phase was found to depend upon the amount of enzyme added, the longest observed being about 35 minutes. It was next demonstrated that the reducing power actually increases from the beginning, by adding a small amount of glucose to one of a pair of digests before the enzyme was added. The digest with added glucose showed an increasing reducing power from the beginning; the other showed an initial level phase.

The micro-method of Shaffer and Hartmann was then tested against known small amounts of glucose, added to the standard 5 cc. of the copper reagent. Practically no reduction was apparent until glucose in excess of about 0.1 mg. had been added. Up to 0.3 mg. the method was entirely unreliable, the values being consistently low¹.

This defect in the sugar method used by Eadie makes clear the reason for his anomalous results. When the reaction velocity in a particular digest was so small that the amount of reducing sugar produced in 10 minutes was below the critical value of the method of sugar determination, then a reaction velocity of zero was recorded. This seems to provide the explanation of the various characteristics of Eadie's results which have been mentioned.

There remains no evidence for the view that a certain critical starch

¹ Shortly after this work was done in 1926 similar conclusions as to the unreliability of the Shaffer and Hartmann micro-method with small amounts of glucose were published by Duggan and Scott [1926]. Later a modification was proposed by Somogyi [1926] to correct this defect in the original method.

concentration is necessary for starch hydrolysis by this amylase. Re-investigation of the point with the modified Hagedorn and Jensen method has shown that reducing sugar is produced even when the starch concentration is as low as 0.005 %, and that, moreover, the reaction goes to about 75 % completion, which is the normal extent of the hydrolysis of starch by barley amylase.

THE EFFECT OF AMYLASE CONCENTRATION UPON REACTION VELOCITY.

According to the Michaelis theory, the reaction velocity would be expected to be proportional to enzyme concentration so long as the concentration of enzyme remains small compared with that of the substrate. A number of experiments with different preparations of barley amylase have shown this to be true. A single example will serve to illustrate the experimental establishment of this relationship.

When investigating the effects of some factor other than substrate concentration, it is advantageous to employ a relatively high substrate concentration in order that the falling-off in velocity due to its transformation into products may be minimised. It has been found that the procedure for maltose estimation is somewhat facilitated if the starch concentration is not too high. For most purposes a starch concentration of 0.43 % (25 cc. of 0.5 % starch solution in a 29 cc. digest) has been found satisfactory. It will be seen from Fig. 3 that at this concentration the velocity is approaching its maximum. Occasionally for particular purposes higher starch concentrations have been used.

Exp. 3. Amylase No. 3, prepared from germinated barley, was used. Immediately before the experiment samples of the enzyme were prepared in different dilutions.

Table VI.

Digest No.	<i>e</i> Relative enzyme concentration	<i>t</i> Mins. after zero time	<i>x</i> Mg. maltose per 5 cc.	Slope mg./min.	<i>v</i> , initial velocity		Ratio <i>v/e</i>
					Earliest measured	Extrapolated*	
20	100	0.7	1.40	1.325	1.325	1.35	0.0135
		1.9	2.99	1.28			
		2.9	4.27				
21	75	0.6	1.11	0.993	0.993	1.01	0.0135
		1.9	2.40	0.964			
		3.0	3.46				
22	50	0.7	0.88	0.684	0.684	—	0.0137
		2.9	2.385	0.680			
		4.2	3.27				
23	37.5	0.6	0.64	0.487	0.487	0.495	0.0132
		2.1	1.37	0.480			
		3.5	2.04				
24	25	0.6	0.53	0.352	0.352	—	0.0141
		2.9	1.34	0.350			
		4.1	1.76				
25	12.5	0.75	0.415	0.166	0.166	0.167	0.0134
		3.0	0.79	0.160			
		5.0	1.11				

* The method of extrapolation is described on p. 1409.

Digests were made up of 25 cc. of 0.5 % soluble starch, 2 cc. *M*/5 acetate buffer (final p_H 4.73) and finally 2 cc. of amylase (of different strengths in the different digests). The reaction temperature was 25.0°.

The data obtained from these digests are given in Table VI.

The values of the ratio v/e , shown in the last column of Table VI, are approximately constant. The experimental data have been plotted in Fig. 4 together with the straight line of best fit, whose equation was found by the method of least squares to be $v = 0.001345e + 0.006$. The close distribution of the points about this line is good evidence that the relationship is linear.

Fig. 4. The relationship between reaction velocity and enzyme concentration.

In a similar experiment carried out with an enzyme of considerably lower activity, the relationship was examined over a lower range of reaction velocities. The reaction velocities varied from 0.36 with the highest amylase concentration ($e = 16$) down to 0.022 with the lowest ($e = 1$). Here again there was no doubt that the relationship was linear, although the experimental error in the measurement of very low reaction velocities is noticeably greater¹.

It can be concluded from these experiments that the initial reaction velocity (over the range 1.35 to 0.022) is proportional to the concentration of amylase.

SUMMARY.

An "initial slope" method of measuring the velocity of starch hydrolysis in the presence of amylase is described.

The effect of starch concentration upon reaction velocity has been investigated, using the amylase of germinated barley. The observed relationship is in close agreement with that predicted by the Michaelis theory.

¹ It has been repeatedly observed that the reaction velocity falls off more rapidly in digests containing very low concentrations of amylase than in digests containing greater concentrations of the same enzyme preparations. Since this cannot be due to falling substrate concentration, it suggests that the enzyme at high dilution in the presence of the substrate becomes subject to an inactivation or inhibition. It is impossible at the present time to indicate the possible nature of this effect.

The relationship between initial reaction velocity and enzyme concentration has been found to be linear over a wide range of enzyme concentration.

It is a pleasure to acknowledge my indebtedness to Mr G. E. Briggs and to Dr F. F. Blackman for much helpful criticism and advice.

APPENDIX.

Details of amylase preparations.

Amylase 1. 100 g. Spratt-Archer barley of good malting quality, harvested 2 years earlier near Cambridge. Soaked—10 hours. Germination—4 days at 17–20°. Ground and extracted for 6 hours with 600 cc. of 0.1 % acetic acid at 3°. Precipitated with 70 % (by volume) ethyl alcohol, and allowed to stand 12 hours at 3°. Filtered off precipitate, and made up to 2000 cc. Solid content of preparation, 17 mg. per 100 cc.

Amylase 2. 100 g. Plumage-Archer barley of excellent malting quality, harvested 12 months earlier at Norwich. Soaked—10 hours. Germination—6 days at 20–22°. Extracted for 48 hours with 500 cc. of 0.1 % acetic acid at room temperature. Filtered and precipitated with 50 % alcohol. Filtered off precipitate after 18 hours, and made up to 500 cc. Solid content not determined.

Amylase 3. 500 g. Spratt-Archer barley of fair malting quality, harvested 3 months earlier near Cambridge. Soaked—12 hours. Germination—4 days at 20–22°. Extracted for 24 hours with 1 litre of 0.1 % acetic acid at room temperature. Precipitated with 65 % alcohol. Filtered and made up to 1000 cc. Solid content not determined.

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