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Investigations on Malt Amylase

I. On the Viscosimetrical Determination of α-Amylase

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Staudinger and co-workers [1] established, for polymeric homologous substances in collodial solution, the following relation between viscosity, concentration and molecular weight:

$$\eta_{sp} = K_m c_{gm} M$$

where

η_{sp} = the specific viscosity,
K_m = the viscosity-molecular weight constant,
c_{gm} = the concentration in basic moles per litre, and
M = the molecular weight.

This relation applies, among other substances, to dilute solutions of starch and partially degraded starch [2] up to a maximum concentration depending on the molecular weight.

Myrbäck and Sillén [3, 4] showed that α-amylase would break all linkages between the basic molecules of starch at the same rate, except certain linkages at the ends of the molecules and near the points of ramification. Since there are comparatively few linkages of the latter kind, we will disregard them in this work.

The enzymic decomposition of starch by α-amylase will hence follow the equation established by the present author [5]:

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$$A^{t^\circ}_{\alpha/s} = c^2 \cdot \frac{d\frac{1}{\eta_{sp}}}{dt}$$

where

$A^{t^\circ}_{\alpha/s}$ = enzymic activity in units per gram of solution at t° C,
t° = temperature,
α/s = abbreviation for α-amylase and starch,
c_s = concentration of starch in grams per gram of solution, and
t = time in seconds.

PREVIOUS INVESTIGATIONS

According to Davison [6], the activity of an enzyme is to be defined as a function of the time necessary to effect a certain change in a substrate, rather than a function of the change effected in a certain time. He expressed the enzyme concentration as the reciprocal of the time in hours required for the reduction of the viscosity of a Lintner [7] starch solution, the original viscosity of which was about 2 ½ times that of water, by 20 %. Davison attempted to obtain the required initial viscosity by choosing a suitable concentration for the starch solution. The effect of the concentration may not, however, be neglected when the results of series of experiments with different starch solutions are compared.

This was also the opinion of Thompson, Johnson and Hussey [8], who used a 7 % solution of the s a m e batch of Baker's soluble starch in all their experiments, which made the initial viscosities almost identical in all experiments. The enzyme concentration proved to be inversely proportional to the time necessary to reduce the viscosity by 20 %.

Broeze [9] employed the specific viscosity as an expression for the internal state of a sol. He refered to Einstein's [10] formula, which was calculated for a suspension of particles:

$$\eta = \eta_0 (1 + \varkappa\varphi)$$

where

η = viscosity of the »solution»,
η_0 = viscosity of the »solvent»,
$\varkappa$ = a constant depending on the shape of the particles, and
φ = ratio between the total volume of the dispersed substance and the volume of the »solution».

Broeze used a solution of Merck's soluble starch of constant concentration (2 %). In relative enzyme determinations he compared the reaction curves obtained by plotting the specific viscosity at the time *t*, expressed in per cent of the specific viscosity at the beginning of the experiment, against the time *t*. Broeze claimed that the times required for equal decomposition are comparable, although he did not read the absolute enzyme concentration from the reaction curves.

To obtain the time for which a certain viscosity measurement was valid, Broeze added half the outflow time of the liquid to the time at which the measurement was commenced. This is mathematically correct, provided that the fluidity is a linear function of the time.

Instead of solutions of Lintner starch, enzymically degraded starch etc., Józsa and Gore [11] used potato-starch solutions, with which they obtained fairly reproducible viscosities. However, the concentration employed was so high (4.211 %; diluted in the experiments with 1/10 enzyme solution), that Staudinger's formula, $\eta_{sp} = K_m c_{gm} M$, was no longer valid in the beginning of the experiment, since the volume of the hydrated starch must be low in relation to the volume of the free solvent.

In amylase determination, Józsa and Gore calculated a function **D** of the viscosity of a starch solution submitted to enzymic degradation. For mixtures of undegraded and completely degraded starch, they empirically determined a curve showing the relation between **D** and the percentage of starch decomposed.

Józsa and Gore used this standard curve to calculate, from viscosity measurements, the percentage of starch decomposed per hour. They defined the activity (the »liquefying power», **LP**) of the enzyme preparation as the number of grams of starch decomposed by 1 g enzyme preparation per hour at 21° C.

To give this definition real significance, we must suppose that the enzyme preparation will decompose as many 1,4 linkages per hour as occur in the above quantity of starch (or half that number, if we prefer to regard maltose as the elementary component, or perhaps some other fraction). When employing their standard curve, Józsa and Gore tacitly assumed that the viscosity of a starch solution changes as though every starch molecule were completely degraded before the next one is attacked.

This supposition is unpermissable, as has been shown experimentally by Fletcher and Westwood [12]. These authors, however, found a proportionality within a certain range, which allows the application of the method, provided that almost equal and adequate quantities of amylase are used in the experiments. Fletcher and Westwood also pointed out that the viscosity of Józsa and Gore's starch solution varies appreciably with the time of stirring.

Willaman, Clark and Hager[13] attempted to improve Józsa and Gore's method by using a 2 % starch solution and introducing a formula for the calculation of the enzymic activity. According to Józsa and Gore, the function **D** (»decline in outflow time») is

$$\mathbf{D} = 100 \cdot \frac{\tau_0 - \tau_t}{\tau_0 - \tau_\infty}$$

where τ_t is the outflow time of the solution measured in a viscosimeter at the time t after the start of the enzymic degradation etc. Under the above experimental conditions, the percentage **L** of starch decomposed per hour is, according to Willaman, Clark and Hager,

$$\mathbf{L} = \frac{\mathbf{m}}{\frac{\mathbf{a}}{\mathbf{D}} - 1}$$

where **m** and **a** are constants. In the calculation of **LP** (grams of starch decomposed per gram of enzyme preparation) it is said to be necessary to increase **L** to the empirically found constant 1.6.

An examination of Willaman, Clark and Hager's expression **L** suggests that the expression for the enzymic activity

$$A^{t^\circ}_{\alpha/s} = \mathbf{c}_s^2 \cdot \frac{d\frac{1}{\eta_{sp}}}{dt}$$

may be transformed in the following manner:

$$A^{t^\circ}_{\alpha/s} = \mathbf{c}_s^2 \cdot \frac{\frac{1}{\eta} - \frac{1}{\eta_0}}{t} = \mathbf{c}_s^2 \cdot \frac{\eta_0 - \eta_t}{\eta_0 \cdot \eta_t \cdot t} = \mathbf{c}_s^2 \cdot \frac{\eta_0 - \eta_t}{\eta_0 \left[\eta_0 - (\eta_0 - \eta_t) \right] t} =$$

$$= \mathbf{c}_s^2 \cdot \frac{1}{\eta_0 \left(\frac{\eta_0}{\eta_0 - \eta_t} - 1\right) t} = \mathbf{c}_s^2 \cdot \frac{1}{\eta_0 \left(\frac{\eta_0 \cdot 100}{(\eta_0 - \eta_\infty)\, \mathbf{D}} - 1\right) t}$$

if **D** is rewritten as $\mathbf{D} = 100 \cdot \frac{\eta_0 - \eta_t}{\eta_0 - \eta_\infty}$, where η denotes the specific viscosity.

The expression thus obtained can easily be identified with Willaman, Clark and Hager's term for **L**, whereby

$$\mathbf{m} = \frac{\mathbf{c}_s^2}{\eta_0 \cdot t} \text{ and } \mathbf{a} = \frac{100 \cdot \eta_0}{\eta_0 - \eta_\infty}$$

Johnston and Jozsa [14, 15] finally corrected Józsa and Gore's method by graphical extrapolation of a series of measurements to the time of the start of the experiment. This constituted a clear advance in comparison with the method of Willaman, Clark and Hager, which required several empirical constants for the calculations.

Blom and Bak [16] took up, and applied in practice, the idea of Davison [6] and Broeze [9] that the quantity of enzyme is directly proportional to the time required for a certain degree of degradation of the substrate. These authors prepared a homogenous potato-starch solution by stirring a starch suspension at room temperature under the addition of caustic soda [17], after which they adjusted the pH with acetic or phosphoric acid. They eliminated the errors originating from the circumstances that starch solutions of the same concentration may have different initial viscosities and that the mixing of the enzyme and starch solutions requires a certain time, by timing the enzymic activity, not, as hitherto, from the moment of the addition of the enzyme to the starch solution, but from a time at which degradation has proceeded to an arbitrary, but welldefined, stage. As the termination of the enzymic activity, they take the time at which the starch solution has reached another stage of degradation.

These two degradation stages were taken as having been attained when the viscosity of the mixture was twice and equal to the viscosity of a 45 % cane-sugar solution respectively. These times were interpolated from outflow times obtained at different intervals after the beginning of the experiment.

The expression for the enzymic activity

$$A_{\alpha/s}^{t^\circ} = \mathbf{c}_s^2 \cdot \frac{d\dfrac{1}{\eta_{sp}}}{dt}$$

may be written:

$$A_{\alpha/s}^{\circ} = \mathbf{c}^2 \cdot \frac{\dfrac{\eta_w}{\eta_{t_2} - \eta_w} - \dfrac{\eta_w}{\eta_{t_1} - \eta_w}}{t_2 - t_1}$$

where η_t denotes the viscosity of the mixture at the time t and η_w that of water. If $\mathbf{c}_s$, η_{t_2} and η_{t_1} are fixed and another unit chosen, this expression may,

according to Blom and Bak, be written as the enzymic activity per gram of enzyme preparation

$$= \frac{1000}{a(t_2 - t_1)}$$

where a is the weight of enzyme preparation in grams, which, although this is not expressly stated by the author, when in 5 ml of aqueous solution, is added to 50 ml of a 3 % starch solution, will reduce the viscosity in the above-mentioned measure during the time $t_2—t_1$ at the temperature + 20° C.

EXPERIMENTAL

Enzyme solution

Malt meal was stirred with 3 parts of water and after one hour the mixture was centrifuged. β-amylase in the liquid was inactivated by heating according to Ohlsson [18]. After filtration, the amylase was purified according to Weidenhagen [19] by precipitation with tannin, washing with acetone, dissolution in water and filtration.

Starch solution

An approximately calculated quantity of starch was weighed accurately into a round-bottomed flask of 250 ml with a wide neck. 200 ml of water was added. The contents were stirred with a motor-driven propeller. After the starch had been completely suspended, 20 ml of 2 N sodium hydroxide was added at room temperature. The mixture became viscous, but the stirring was made effective by short-circuiting some series resistances of the motor. After 15 minutes, a quantity of 4 N acetic acid (standardized against the sodium hydroxide) equivalent to 25 ml of the sodium hydroxide was added, followed by enough water to give the starch solution the required concentration. The final weight of the mixture was about 250 g. After stirring for another 15 minutes, the solution was poured into a 300 ml conical flask which was placed in a thermostat at 30°.

A layer of starch paste adhing to the interior of the round-bottomed flask indicated that stirring had not been effective enough to make the solution homogenous. On such occasions the solution was rejected, since its concentration was unknown.

Viscosimeter

An Oswald viscosimeter was used in the experiments. If

η = viscosity in CGS units,
τ = time of flow,
D = specific gravity, and
k_1 and k_2 = apparatus constants,

the viscosity is expressed by the equation:

$$\eta = D\tau\,(k_1 - \frac{k_2}{\tau^2})$$

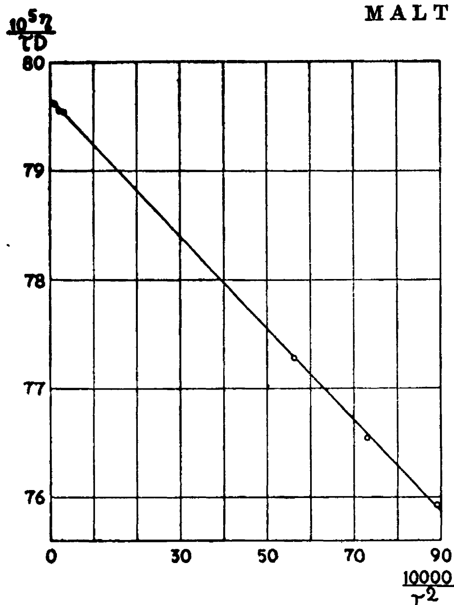

Fig. 1. Calibration of the viscosimeter.

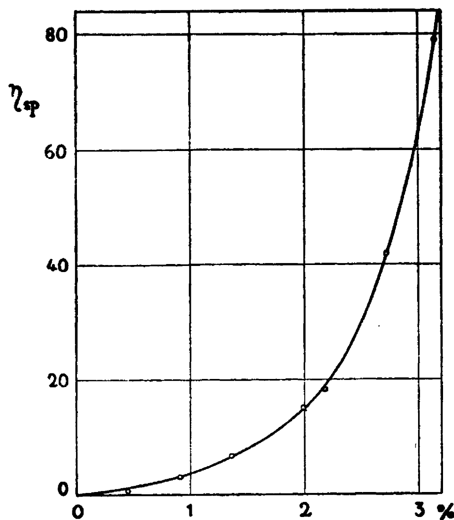

Fig. 2. Specific viscosities of the potato-starch solutions.

The apparatus constants were determined empirically by measurements of the times of flow for water and known cane-sugar solutions. The specific gravity of water has been determined by Thiesen, Scheel and Diesselhorst[20], and the specific gravities of sugar solutions have been calculated from the determinations of Plato[21]. Bingham and Jackson[22] determined the viscosities of water and of cane-sugar solutions at, among other temperatures, 30°. From these values the viscosities of the sugar solutions used to determine the apparatus constants were calculated. In these calculations the following interpolation formula was employed:

$$\frac{1}{3 + \log \eta} = 0.72654 - 0.012035\ (C-30) + 0.00002743\ (C-30)^2 + 0.000000202\ (C-30)^3$$

where C is the percentage concentration of the sugar solution.

The viscosimeter was cleaned with bichromate-sulphuric acid, rinsed with water and finally with alcohol. It was then placed in a thermostat at 30.00 $\pm$ 0.01° C, and dried by suction for about 10 minutes. 3 ml fluid was then introduced. The values obtained in the calibration of the viscosimeter are shown in fig. 1, and the apparatus constants are $k_1 = 0.0007966$ and $k_2 = 0.00421$.

Viscosity of starch solutions

The method employed for measuring the viscosity of starch solutions correspond to the method of determining enzymic activity described below. The results of viscosity measurements on some of my potato-starch solutions of different concentrations are shown in fig. 2.

Measurement of enzymic activity

3 ml enzyme solution was transferred to a 50 ml conical flask which was placed in a thermostat at 30°. The water reached the neck of the flask. A motor-driven stirring propeller was inserted into the flask, the neck of which was packed with cotton. The temperature became constant after few minutes, whereupon the starch solution was added with a 30 ml pipette. A stopwatch and the stirring apparatus were started. The pipette, containing residues of the starch solution, was weighed. The pipette was also weighed both dry and filled with the starch solution. In the latter case, wighing is rendered easier by allowing the bulk of the starch solution to flow into a tared flask. These weighings made possible an exact calculation of the quantity of starch solution introduced and thus of the final concentration. With a pipette, brought to the correct temperature by standing in the thermostat in a glass tube with a fused bottom, 3 ml of the mixture was transferred to the viscosimeter in the termostat. The outflow time for the mixture was determined an adequate number of times, the moment at which the outflow began being read on the stopwatch and noted down.

Calculation of the results

Calculations of series of specific viscosities are best made in the following manner (the index w indicates that the value is for water):

$$\eta = \tau\, D\, (k_1 - \frac{k_2}{\tau^2}) = \tau\, Dk_\tau$$

$$\eta_w = \tau_w D_w (k_1 - \frac{k_2}{\tau_w^2}) = \tau_w D_w k_w$$

$$\frac{1}{\eta_{sp}} = \frac{\eta_w}{D \cdot k_\tau} \cdot \frac{1}{\tau - \frac{D_w k_w}{D \cdot k_\tau} \tau_w} = \frac{\eta_w}{D \cdot k_\tau} \cdot \frac{1}{\tau - \tau_0}$$

The value τ_0 is tabulated against τ; example: Table 1. Furthermore, $\frac{\eta_w}{Dk_\tau}$ is tabulated against τ; example: Table 2.

To obtain the time for which a measurement is valid, we add half the outflow time to the time of starting[9].

$\frac{1}{\eta_{sp}}$ is plotted graphically against time. The points are joined by a straight

Table 1. τ *and* τ_0.

τ	τ_0
	10.0
40	
	10.1
20	
	10.2
15.1	
	10.3
12.6	
	10.4
11.2	
	10.5

Table 2. τ *and* $\frac{\eta_w}{Dk_\tau}$.

2.5 % stock starch solution.

τ	$\frac{\eta_w}{Dk_\tau}$
	9.93
84	
	9.94
57	
	9.95
45	
	9.96
38	
	9.97
34	
	9.98
30.5	
	9.99
28.0	
	10.00
26.0	
	10.01
24.7	

line, the slope of which, $\Delta \frac{1}{\eta_{sp}} / \Delta t$, is determined. The activity of the enzyme *in the mixture with starch* is

$$A^{t^\circ}_{\alpha/s} = \mathbf{c}_s^2 \cdot \frac{\Delta \frac{1}{\eta_{sp}}}{\Delta t}$$

If **a** g enzyme solution (preparation) has been mixed with **b** g starch solution, the activity of the original enzyme solution (preparation) will be

$$A^{t^\circ}_{\alpha/s} = \frac{\mathbf{a} + \mathbf{b}}{\mathbf{a}} \mathbf{c}_s^2 \frac{\Delta \frac{1}{\eta_{sp}}}{\Delta t}$$

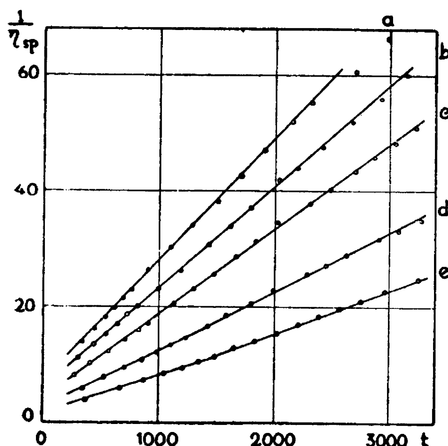

Fig. 3. Enzymic degradation of starch solutions of various concentrations with the same α-amylase solution.

a) $c_s = 0.01995$.
b) $c_s = 0.02179$.
c) $c_s = 0.02402$.
d) $c_s = 0.02719$.
e) $c_s = 0.03173$.

EXPERIMENTS

The results of specific gravity determinations on starch solutions will be found in table 3.

Table 3. Specific gravity of starch solutions with acetate buffer.

Starch %	D_4^{30}
0.000	1.0029
1.995	1.0113
2.179	1.0118
2.402	1.0130
2.722	1.0142
3.173	1.0159

Table 4. Enzymic activity at different substrate concentrations.

c_s	$\frac{1}{\eta_{sp}}$	$A^{30°}_{\alpha/s} \cdot 10^8$
0.01995	0.14—0.66	90.3
0.02179	0.11—0.60	90.8
0.02402	0.08—0.51	91.1
0.02719	0.06—0.15	80
	0.15—0.25	86
0.03173	0.05—0.12	75
	0.13—0.21	85

An enzyme solution, which had been kept in a refrigerator, was measured against several starch solutions of different concentrations. The values obtained in the different series are given in fig. 3 and table 4.

Potato starch was treated with 1.5 N hydrochloric acid at room temperature for 10 hours, washed and dried. The activity of an enzyme solution was measured in comparative experiments with solutions of this starch and of potato starch. The results are given in tables 5 and 6.

Table 5. 3.00 g enzyme solution + 29.63 g of a 2.495 % stock potato-starch solution.

$t + \frac{\tau}{2}$	τ	$\frac{1}{\eta_{sp}}$
344	201.2	0.0520
578	173.2	0.0609
801	156.8	0.0677
991	145.4	0.0734
1178	135.1	0.0795
1398	125.4	0.0862
1574	118.6	0.0916

$d\frac{1}{\eta_{sp}}/dt = 0.0310 \cdot 10^{-3}$. $c_s = 0.02214$.

$A^{30°}_{a/s} = 16.5 \cdot 10^{-8}$.

Table 6. 3.00 g enzyme solution + 29.96 g of a 2.322 % stock solution of potato-starch treated with hydrochloric acid.

$t + \frac{\tau}{2}$	τ	$\frac{1}{\eta_{sp}}$
224	48.7	0.258
300	48.3	0.260
607	46.8	0.271
781	45.9	0.278
990	45.2	0.283
1372	43.7	0.296
1612	42.8	0.304

$d\frac{1}{\eta_{sp}}/dt = 0.0335 \cdot 10^{-3}$. $c_s = 0.02211$.

$A^{30°}_{a/s} = 16.4 \cdot 10^{-8}$.

To ascertain to what extent the method of preparing a starch solution affects the enzymic decomposition of the latter, the activity of an enzyme solution was measured with several starch solutions prepared in different ways. The results are recorded in tables 7—9.

Table 7. 3.00 g enzyme solution + 29.60 g of a 1.998 % stock starch solution prepared by the method described in the text.

$t + \frac{\tau}{2}$	τ	$\frac{1}{\eta_{sp}}$
303	119.3	0.0908
473	112.8	0.0966
698	106.2	0.1031
995	99.0	0.1107
1373	91.0	0.1228
1764	84.6	0.1330
2005	80.9	0.1402
2304	78.1	0.1459

$d\frac{1}{\eta_{sp}}/dt = 0.0282 \cdot 10^{-3}$. $c_s = 0.01814$.

$A^{30°}_{a/s} = 10.0 \cdot 10^{-8}$.

Table 8. 3.00 g enzyme solution + 29.65 g of a 1.998 % stock starch solution prepared by the method described in the text, but subsequently heated to 95° for 15 minutes.

$t + \frac{\tau}{2}$	τ	$\frac{1}{\eta_{sp}}$
260	105.5	0.1040
469	100.3	0.1100
614	97.2	0.1139
858	92.1	0.1209
1151	88.2	0.1270
1476	82.5	0.1370
1773	79.3	0.1432
2069	75.2	0.1522

$d\frac{1}{\eta_{sp}}/dt = 0.0260 \cdot 10^{-3}$. $c_s = 0.01814$.

$A^{30°}_{a/s} = 9.3 \cdot 10^{-8}$.

Table 9. 3.00 g enzyme solution + 29.59 g of a 2.000 % stock starch solution prepared from an aquous suspension of starch by boiling and violent stirring for 15 minutes. Acetate buffer was added on cooling.

$t + \frac{\tau}{2}$	τ	$\frac{1}{\eta_{sp}}$
617	198.8	0.0526
850	183.9	0.0571
1216	154.5	0.0687
1569	137.7	0.0778
1979	122.6	0.0882
2403	111.1	0.0981
2892	98.9	0.1119

$$d \frac{1}{\eta_{sp}} / dt = 0.0260 \cdot 10^{-3}. \ c_s = 0.01816 \cdot A^{30^\circ}_{a/s} = 9.4 \cdot 10^{-8}.$$

DISCUSSION OF THE EXPERIMENTAL RESULTS

Table 4 and fig. 3 show that, when 3.5 and 3 % stock starch solutions are used, deviations from Staudinger's[1] formula are so serious in the beginning of the experiment, that the points of the graph no longer lie on a straight line. These concentrations are hence too high. However 2.65, 2.40 and 2.20 % stock starch solutions give very satisfactory results. A comparison with fig. 2 shows that, at these low concentrations, the deviations from Staudinger's formula begin to assume moderate proportions for undegraded starch. The deviations may be expected to decrease rapidly as the starch is degraded. The measurements cannot, in point of fact, begin before the starch has been appreciably degraded.

Fig. 3 also shows that starch with a high initial degree of degradation cannot be a suitable substrate for the viscosimetric determination of α-amylase, since $d\frac{1}{\eta_{sp}}/dt$ is independent of the time t only before degradation has proceeded to a certain stage. Lintner's [7] starch is therefore not a suitable substrate in this case.

When very low enzyme concentrations are to be determined, it is important to degrade the starch, before the beginning of the viscosity measurement, sufficiently to permit the application of Staudinger's formula with respect to the concentration of the starch solution. We gather from tables 5 and 6 that the same result is obtained in enzyme determinations with starch mildly degraded by hydrochloric acid, and with potato starch previously untreated. In the preparation of a starch solution by working the material with an alkali,

the starch is also subjected to degradation. There was good reason to expect the same result in these cases, since the degradation of starch by α-amylase and that by acids and alkalis must be supposed to take place similarly, *i. e.* all 1,4 linkages are exposed to the same probability of rupture (there are, however, certain exceptions [3, 4]). For the determination of low enzyme concentrations, it is therefore advantageous to use starch that has been very mildly degraded with hydrochloric acid.

It appears from tables 7—9 that a starch solution, made up by dissolving starch in alkali and adjusting the acidity with acetic acid, when afterwards heated to 100° for some time, will be digested at the same rate as a solution made by dissolving starch in boiling water with subsequent addition of the buffer solution.

Howevers, a starch solution that has not been heated, will have its viscosity diminished more rapidly by the influence of amylase than a heated solution, just as if its concentration were less, *i. e.* as if all starch particles had not been completly dissolved. Thus, a starch solution for viscosimetric amylase determination, which has been made up by dissolving starch in alkali and adjusting the pH with acid, should be heated to almost 100° C for some minutes before immersed in the thermostat.

SUMMARY

A relation — recently deduced theoretically by the present author — connecting the enzymic activity, the substrate concentration, the specific viscosity and the time — has been applied in the examination of the reliability of the viscosimetrical methods used earlier in calculating the activity of α-amylase solutions and preparations.

It has been demonstrated experimentally that when using the formula the degradation of starch solutions of various concentrations with the same α-amylase solution results in the same values of enzymic activity.

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