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SOME CHARACTERISTICS OF INVERTASE ACTION

BY J. M. NELSON AND GROVER BLOOMFIELD

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Brown[1] observed that the rate of hydrolysis of sucrose in the presence of invertase reaches a maximum and remains constant above a certain substrate concentration, but did not actually determine what this concentration was.

Nelson and Vosburgh[2] discussed a number of curves obtained from their own data and those of Michaelis and Menten[3] relating the velocity of hydrolysis with the sucrose concentration and showed that the maximum rate of inversion was obtained in each case at about 5 g. of substrate per 100 cc. of solution. At higher concentrations there was practically no change in velocity, while with less sugar the rate was smaller.

The fact that two distinct invertase preparations used at different temperatures (Nelson and Vosburgh worked at 37°, Michaelis and Menten at 25°), although causing different actual rates of hydrolysis, gave a maximum velocity of inversion at about the same sucrose concentrations, is very striking for it indicates that there is some sort of phenomenon which is independent of the enzyme preparation and the temperature.

The Relation between the Sucrose Concentration and the Rate of Hydrolysis at Various Temperatures and Hydrogen-Ion Concentrations

The fact that the temperature does not seem to affect the sucrose concentration at which the hydrolysis of the substrate reaches a maximum, but does affect the actual rate of the reaction appeared so striking that it was deemed desirable to obtain further data on this point. It was hoped that more light would thus be thrown upon the nature of the hydrolytic process. Hence, the influence of the temperature upon the relation between the sucrose concentration and the rate of the hydrolysis and, in addition to this, the influence of the hydrogen-ion concentration were studied.

Experimental.—Curves were obtained in such ranges of temperature and hydrogen-ion concentration as to make the results significant for the subsequent part of this investigation.

Since changes of the hydrogen-ion concentration and temperature affect the velocity of hydrolysis, the curves obtained by plotting the latter against the sucrose concentration would, naturally, not be comparable. In order to make a comparison possible (and this was the

[1] Brown, *J. Chem. Soc.*, **81**, 373 (1902).

[2] Nelson and Vosburgh, THIS JOURNAL, **39**, 790 (1919).

[3] Michaelis and Menten, *Biochem. Z.*, **49**, 333 (1913).

object of the following experiments), the velocities all had to be reduced to a single scale. This was done by giving the value of unity to the maximum velocity for each curve, so that the lower velocities in each case would become fractions of the maximum.

The relative velocities of hydrolysis were obtained by Michaelis and Menten's method[3] of comparing the initial rates of inversion. If, at the beginning of the reaction, the amounts of sucrose hydrolyzed are plotted against the corresponding times, practically straight lines are obtained. The slopes of these initial straight-line sections were assumed to give the relative velocities.

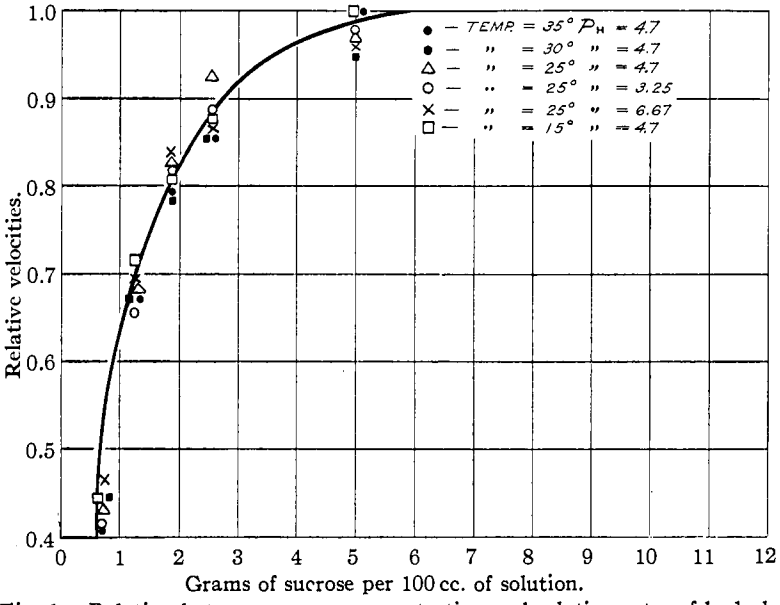

Fig. 1.—Relation between sucrose concentration and relative rates of hydrolysis at different temperatures and hydrogen-ion concentrations.

This method of comparing the rates of hydrolysis at the beginning of the reaction rather than at some later stage, as was done by Nelson and Vosburgh, has the advantage that in the former case practically no invert sugar is present, while in the latter sufficient invert sugar may have formed to introduce a complicating factor due to its retarding influence on the hydrolysis.

Time-change of rotation curves were plotted and the initial velocities compared for each set of experiments. The greatest velocities were always taken as unity while the others were given in fractions thereof. In this way all data were reduced to one scale. The relative velocities thus obtained are given in Table I and plotted in Fig. 1.

TABLE I

RELATION OF VELOCITY OF HYDROLYSIS TO THE SUCROSE CONCENTRATION FOR VARIOUS CONDITIONS OF HYDROGEN-ION CONCENTRATION AND TEMPERATURE

Sucrose per 100 cc. G.	Temp. P_H	Relative velocities 15° 4.7	25° 3.25	25° 4.7	25° 6.67	30° 4.7	35° 4.7
12		1.000	1.000	1.000	1.000	...	...
10		1.000	1.000	1.000	1.000	1.000	1.000
5		1.000	1.000	0.085	0.977	0.972	1.000
2.5		0.889	0.894	.924	.878	.872	0.870
1.875		.813	.818	.821	.823	.778	.792
1.25		.707	.654	.680	.693	.677	.677
0.625		.448	.425	.445	.459	.450	.411

The points of the different sets of experiments, as is seen in Fig. 1, show no consistent shifting due to changes of temperature and hydrogen-ion concentration, and fall on a practically smooth curve. This shows that the temperature and hydrogen-ion concentration do not affect the relation between the sucrose concentration and the relative rates of the hydrolysis; or, stated differently, that the effect of the temperature and hydrogen-ion concentration on the velocity of the reaction is independent

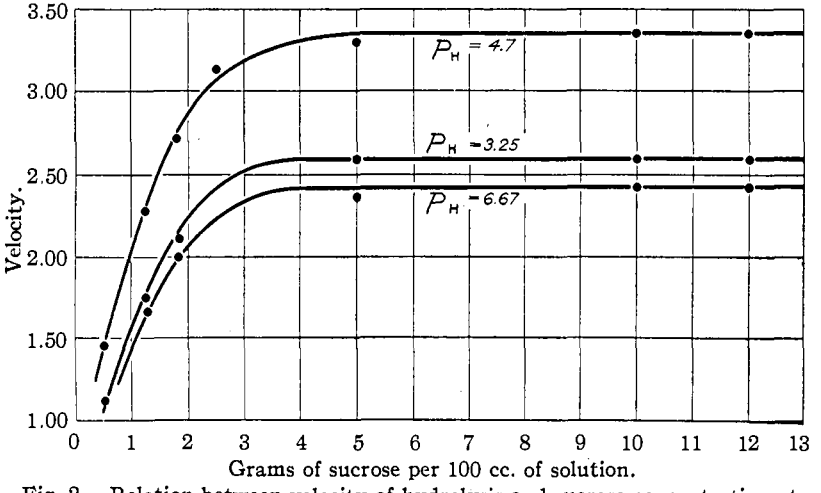

Fig. 2.—Relation between velocity of hydrolysis and sucrose concentration at different hydrogen-ion concentrations.

of the sucrose concentration. Furthermore, the results show that the sucrose concentration at which the hydrolysis reaches its maximum velocity is independent of the temperature and the hydrogen-ion concentration.

When the actual velocities in the above experiments, at the three hydrogen-ion concentrations at 25°, were plotted instead of the relative velocities, the curves shown in Fig. 2 were obtained.

The fact that these curves could be made to coincide (within the limits of the experimental error) by changing the ordinate scale means that the ratio of the velocities for any arbitrarily chosen set of sucrose concentrations (say 2.5, 5 and 10 g. per 100 cc. of solution) is the same for each curve, or what amounts to the same thing, for each hydrogen-ion concentration.[4] Conversely, if the ratio of the velocities for any arbitrarily chosen set of sucrose concentrations is the same for each hydrogen-ion concentration curve, then these curves can be made to coincide (as was the case in Fig. 1) by a proper transformation of the velocity scale.

Michaelis and Rothstein[5] performed a series of experiments to test Michaelis and Davidsohn's idea[6] that invertase in the more alkaline region of its activity behaved as though it were an acid in which the unionized portion was responsible for the catalytic properties of the enzyme. Michaelis and Rothstein's experiments, however, furnished data which serve as a confirmation of the results obtained in the present investigation, namely, that the hydrogen-ion concentration has no effect on the shape and position of the curve obtained by plotting the rate of hydrolysis against the sucrose concentration.

They determined the dependency of the velocity of hydrolysis on the hydrogen-ion concentration, using a different amount of sucrose for each set of experiments. The data gave a set of curves similar in shape to the right-hand branches of the curves in Fig. 3, each curve, however, representing a different sucrose concentration. They claimed that when the ordinates were properly reduced the curves all coincided. This means that the ratio of the velocities for the various sucrose concentrations is the same for any one hydrogen-ion concentration than it is for any other since, as the authors stated, the curves are superimposable. But, as was just mentioned, this fact means that the curves obtained by plotting the rate of hydrolysis against the sucrose concentration can be made to coincide, no matter what the hydrogen-ion concentration is. Thus, the data of Michaelis and Rothstein can be used as a confirmation of the results obtained in this investigation that the hydrogen-ion concentration has no effect on the shape and position of the curve obtained by plotting the velocity of the hydrolysis against the sucrose concentration, and hence on the sucrose concentration at which the reaction reaches a maximum velocity.

In view of the above results that the sucrose concentration at which the hydrolysis reaches a maximum velocity is independent of the temperature

[4] Since this article appeared in dissertation form, 1922, Kuhn [*Z. physiol. Chem.*, **125**, 45 (1923)] has reached the same conclusion, that the relation between the concentration of sucrose and velocity of hydrolysis is independent of the hydrogen-ion concentration.

[5] Michaelis and Rothstein, *Biochem. Z.*, **110**, 217 (1920).

[6] Michaelis and Davidsohn, *ibid.*, **35**, 387 (1911).

and hydrogen-ion concentration, it follows that the influence of these two factors must be ascribed to that part of the process which appears to be more intimately related to the actual catalytic effect of the enzyme.

The Influence of the Temperature and Hydrogen-Ion Concentration on the Catalytic Effect of the Enzyme

This influence was studied as follows. Curves relating the hydrogen-ion concentration to the rate of hydrolysis were obtained at three temperatures in order to study (1) the effect of the hydrogen-ion concentration on the velocity of the reaction, to determine the shape of the curves and whether it was affected by changes of temperature; (2) the temperature coefficient of the reaction at different hydrogen-ion concentrations.

A Criterion for the Rate of Hydrolysis.—Nelson and Vosburgh[2] have definitely established that the hydrolysis of sucrose in the presence of invertase does not follow the unimolecular law. The constant unimolecular coefficients obtained by Hudson[7] must have been due to special conditions holding for his particular experiments.

Nelson and Hitchcock[8] derived an expression for the rate of hydrolysis which is given by $n=\frac{1}{t}\left(\log\frac{100}{100-p}+0.002642\ p-0.0_5886\ p^2-0.0_61034\ p^3\right)$; n, which remains constant throughout any one hydrolysis (if the latter is not deviated from its normal course by the inactivation of the enzyme, or for some other reason), is a measure of the velocity of the reaction, and p is the percentage of sucrose hydrolyzed in time t.

This expression, as the authors have pointed out, is purely empirical. It was intended to be used in those hydrolyses only in which the sucrose concentration was 10 g. per 100 cc. of solution, and was shown to hold in the temperature interval 15° to 35° and between P_H 4.5 and 6.5.

Experimental.—The experiments were performed with the initial sucrose concentration described above and 5.56 cc. of invertase per 100 cc. of solution throughout. The hydrogen-ion concentrations were maintained constant with 0.01 M citrate buffers, except in a few cases where, as indicated, borate buffers were used. The progress of the hydrolyses was followed by the polariscopic method which Vosburgh[9] has shown to be applicable. The invertase preparation numbered 7 in this Laboratory was used for all experiments.

These temperatures were chosen because much of the previous work had been done in this range and a means was thus provided for fruitful comparison. Besides, practical considerations made working at lower and higher temperatures inadvisable. Below 25° the rate of hydrolysis was so slow that the time of the experiments became unduly long unless

[7] Hudson, THIS JOURNAL, **30**, 1160, 1564 (1908).

[8] Nelson and Hitchcock, *ibid.*, **43**, 2632 (1921).

[9] Vosburgh, *ibid.*, **43**, 219 (1921).

the amount of invertase was increased. Above 35° the range of constant n-values was unduly reduced due to the inactivation of the enzyme in the more acid region of its activity.

The results of the experiments are arranged in Table II so as to bring out the relation between the velocity of the reaction (of which n is a measure) and the hydrogen-ion concentration of the solution.

TABLE II

RELATION BETWEEN THE VELOCITY OF THE REACTION AND SÖRENSEN (P_H) VALUES

25°		30°		35°	
P_H	$n \times 10^5$	P_H	$n \times 10^5$	P_H	$n \times 10^5$
8.43[a]	10	8.41[a]	18	8.46[a]	26
7.70	113	7.67	170	7.70	243
7.43	193	7.45	269	7.32	428
7.33	213	7.21	364	7.28	452
6.92	375	6.75	603	7.31	471
6.67	474	6.52	686	6.85	753
6.48	520	5.80	825	6.62	845
6.26	572	5.29	861	6.56	889
5.67	654	4.88	871	6.16	987
5.32	678	4.68	871	5.70	1069
4.88	682	4.27	855	5.30	1081
4.59	681	3.66	789	4.94	1098
4.27	671	3.24	737	4.69	1094
4.06	660	2.93	693	4.47	1092
4.02	654	2.38	606	3.65	966
3.76	641	..	...	3.25	885
3.68	631	..	...	2.93	805
3.25	587	..	...	2.39	663
2.85	553	..	...	..	..
2.76	540	..	...	..	..
2.37	492	..	...	..	..
2.18	455	..	...	..	..

[a] Borate buffer used.

The values of n, which are a measure of the velocity of the hydrolysis, are calculated according to the equation of Nelson and Hitchcock. When they are plotted against the corresponding Sörensen (P_H) values, smooth curves can be drawn through the points thus obtained. These (Fig. 3) show the familiar phenomenon of an optimum zone of the enzyme's activity.

Discussion of the Curves.—Fig. 3 shows that the curves extend from the extreme alkaline limit of the enzyme's activity to about P_H 2.0–2.5. Beyond the latter points the acid inactivation of the invertase was so great that no reliable measure could be obtained of its activity. The straight line is drawn through the three curves to divide the region of constant values of n on the right from that on the left where n falls off during the reaction. The constancy of n is thus established (but see above remarks

as to the borate buffer) from P_H approximately 3.0 to approximately 8.5, which is a considerable extension of the limits for which Nelson and Hitchcock originally applied it (P_H 4.5 to 6.5). It is thus established that invertase acts uniformly between above limits.

The straight line in Fig. 3 slopes toward the right with reference to the P_H-axis. This means that at lower temperatures a greater acidity is necessary (P_H 2.75 at 25°) to bring about first inactivation of the invertase with the resultant falling off of n than at higher temperatures where a smaller hydrogen-ion concentration suffices to bring about this effect

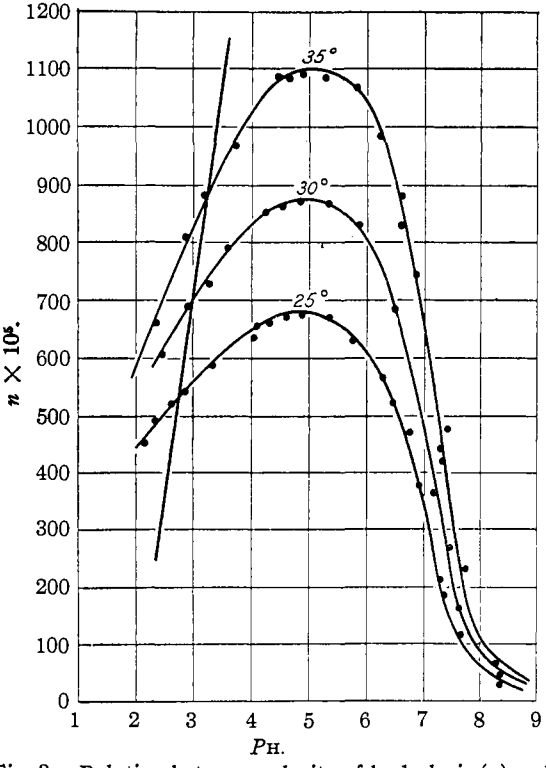

Fig. 3.—Relation between velocity of hydrolysis (n) and P_H at 25°, 30° and 35°.

(P_H 3.0 at 30° and 3.3 at 35°). This result is supported by data of Sörensen[10] and Michaelis and Menten. The former, at 52.1°, indicate that invertase is first progressively inactivated at P_H approximately 3.90, while the latter, at 22.3°, show this to be the case at P_H approximately 2.5.

The data are collected in Table III and plotted in Fig. 4 in order to bring out the effect more clearly.

[10] Sörensen, *Biochem. Z.*, **21**, 131 (1909).

TABLE III

RELATION BETWEEN THE TEMPERATURE AND THE HYDROGEN-ION CONCENTRATION AT WHICH THE INACTIVATION OF INVERTASE DURING THE HYDROLYSIS FIRST BECOMES NOTICEABLE

Temp., °C.	22.3	25.0	30.0	35.0	52.1
Critical P_H	2.5	2.75	3.00	3.3	3.9

The curves of Fig. 3 further show that the optimum activity at all three temperatures lies between P_H 4.5 and 5.0. The corresponding values obtained by Sörensen were P_H 4.0–5.0; by Michaelis and Davidsohn, P_H 3.5–5.2; and by Euler and Emberg, P_H 4.2–5.2. It is seen that the present method of using n as a criterion of velocity gives the optimum zone as lying within a narrower range than that determined by these workers.

Michaelis and Davidsohn[6] were the first to study the shape of the curve obtained by plotting the rate of hydrolysis against the hydrogen-ion concentration from a quantitative standpoint. They showed the velocity of hydrolysis to be inversely proportional to the time required for the inversion of any given fraction of the substrate and expressed this by the following relation: Velocity of Hydrolysis $= f(a, x)$, where a is the initial concentration of the substrate and x the fraction inverted in time t. Bayliss[11] had previously pointed out that this method was least open to objection, so long as no accurate expression for the course of the hydrolysis was available.

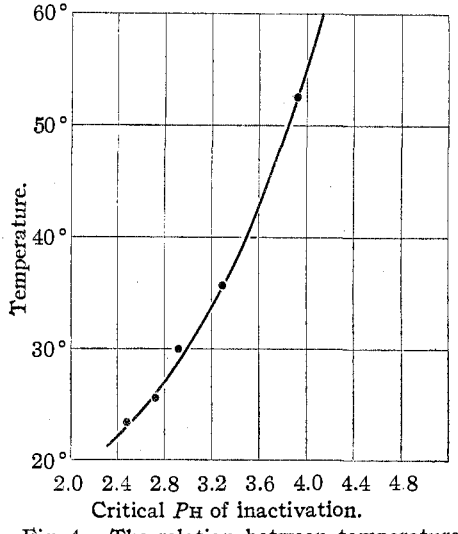

Fig. 4.—The relation between temperature and the critical hydrogen-ion concentration of inactivation of invertase.

Michaelis and Davidsohn's method of determining the velocity of inversion required the plotting of what they termed a standard curve and the comparison with this of the data obtained from all other hydrolyses. It was in order to avoid the necessity of this comparison method that Nelson and Hitchcock derived n as measure of the velocity of the reaction.

If, in the case of any reversible reaction $HA \rightleftarrows H^+ + A^-$, the undissociated fraction of the compound is plotted against the logarithm of the hydrogen-ion concentration, a curve called by Michaelis[12] a "Dissozia-

[11] Bayliss, *Proc. Roy. Soc., London*, **84B**, 90 (1911).

[12] Michaelis, *Biochem. Z.*, **33**, 182 (1911).

tionsrest" curve, and by Clark[13] a "dissociation residue" curve, is obtained. Michaelis[14] has pointed out that this curve is described by the relation

$$\rho = \frac{[H^+]}{[H^+] + k} \quad (1)$$

where ρ is the fraction of the compound not dissociated and k is the dissociation constant; k can be determined because, as is seen, it is equal to the hydrogen-ion concentration corresponding to $\rho = 1/2$.

Now, Michaelis and Davidsohn found that the more alkaline branch of the curve they obtained by plotting the rate of hydrolysis against the hydrogen-ion concentration resembled very closely in shape a dissociation-residue curve. This led Michaelis and Rothstein[5] to consider that the invertase-sucrose compound which Michaelis and Menten claimed to be present, behaved as if it were an acid. They also proposed that it was the undissociated part of the compound which was responsible for the hydrolysis. The reason for this view was that the rate of hydrolysis was greater in the region of the higher hydrogen-ion concentration than in that of the lower, so that if the compound was an acid its dissociation would be most repressed in the former region.

By transforming the n-scale in Fig. 3 to the ρ-scale and comparing the resulting curves with the theoretical dissociation-residue curves given by Equation 1, it was possible to determine how closely the experimental results agreed with the theory of Michaelis and Rothstein.

If, from the experimental curves in Fig. 3, one determines the values of k by graphically finding the hydrogen-ion concentration corresponding to one-half the maximum values of n (n, the rate of hydrolysis being proportional to ρ) and substitutes these in Equation 1, one finds the theoretical values of ρ corresponding to these dissociation constants and any chosen values of the hydrogen-ion concentration. These theoretical values of ρ can then be compared with the corresponding values on the experimental curves which are obtained by changing the n-scale in Fig. 3 in such a way that the maximum value of n is in each case reduced to 1, while all other n-values are diminished in proportion.

The values of k obtained from Fig. 3, as well as a value taken from a curve of Michaelis and Davidsohn, are as follows: for temperatures of 22.3°, 25.0°, 30.0°, 35.0°, the values ($k \times 10^8$) 20 (Michaelis and Davidsohn), 10, 7.94, and 6.76, respectively.

The constants for transforming the n-scale to the ρ-scale are the reciprocals of 682, 871 and 1098 at 25°, 30° and 35°, respectively, since these numbers give the maximum values of n at these temperatures.

In Table IV the theoretical values of ρ obtained by the above method are

[13] Clark, "The Determination of Hydrogen Ions," Williams and Wilkins Co., 1920.

[14] Michaelis, "Die Wasserstoffionenkonzentration," Berlin, 1914, p. 22.

arranged beside those obtained from the experimental curves by reducing the n-scale. The original values of n are also indicated.

TABLE IV

COMPARISON OF THE THEORETICAL VALUES OF ρ WITH THOSE OBTAINED BY REDUCING THE SCALE OF THE EXPERIMENTAL CURVES

	25°			30°			35°		
P_H	$n \times 10^5$	ρ Expt.	ρ Theor.	$n \times 10^5$	ρ Expt.	ρ Theor.	$n \times 10^5$	ρ Expt.	ρ Theor.
5.00	680	0.996	0.990	869	0.998	0.992	1097	0.999	0.994
5.50	665	.975	.969	852	.978	.976	1080	.983	.979
6.00	618	.906	.909	803	.922	.926	1027	.935	.937
6.50	516	.757	.760	695	.798	.799	900	.819	.824
7.00	341	.500	.500	484	.556	.557	655	.596	.597
7.50	160	.235	.240	242	.278	.285	342	.311	.319
8.00	50	.073	.091	79	.091	.112	115	.104	.129
8.50	10	.015	.031	16	.018	.038	24	.022	.045

The theoretical values of ρ at 25° in Table IV are plotted in Fig. 5 and a broken-line curve is drawn through them to make possible a graphical comparison of the dissociation-residue curve thus obtained with the re-

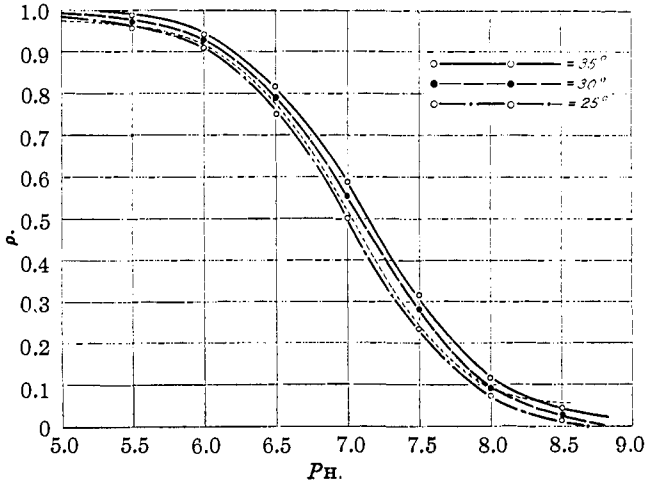

Fig. 5.—Relation of P_H and ρ. The curves of Fig. 4 reduced to a common scale.

duced experimental curve. The coincidence is quite close, except near the two ends of the curves, a discrepancy being especially noticeable in the more alkaline region.

The reduced experimental curves at 30° and 35° are also drawn in Fig. 5 but, as the coincidence of these with the theoretical curves is as good as was the case at 25°, the latter are omitted.

Since the three experimental curves are all similar in shape to a dissociation-residue curve, they must also be similar in shape to one another.

Hence, one may conclude that the shape of the curves showing the relationship between the velocity of hydrolysis (n) and the hydrogen-ion concentration (as given by the Sörensen value) is not affected by a change in the temperature in the range investigated.

Discussion of the Temperature Coefficient.—All recent workers[15] on the temperature coefficient of the hydrolysis of sucrose in the presence of invertase have discussed the same, not in terms of the coefficients themselves, but of a function of these, first derived by Arrhenius[16] who, in a study of the acid hydrolysis of sucrose, showed that an experimental equation could be derived which quite accurately described the influence of the temperature.

The expression of Arrhenius[17] is given by $2A = \frac{RT_1T_2}{T_2-T_1}\log_e\frac{k_2}{k_1}$, where k_2 and k_1 are the unimolecular velocity coefficients (at T_2 and T_1, respectively) which in general are constant for the acid hydrolysis of sucrose, but not for the reaction in the presence of invertase.

The expression is used in this discussion on account of the physical significance that has more recently been attached to it by Marcelin,[18] Lewis[19] and others. Lewis uses E in place of $2A$ and terms it the "critical increment." The values of $2A$ for the hydrolysis of sucrose in the presence of invertase obtained by various workers are collected in Table V.

The values of $2A$ from the data of Tammann, Kjeldahl and O'Sullivan and Tompson were calculated by Euler and af Ugglas. The data are plotted in Fig. 6. The results, as a whole, indicate a decrease of $2A$ with the temperature. Vosburgh, after plotting the data, concluded that a straight-line relation existed between $2A$ and the temperature and obtained the following expression relating the two, $2A = 12{,}300 - 117t$, which is graphically shown by the straight line in Fig. 6.

A similar equation has also been proposed by Euler and Laurin,[15c] $2A = 11{,}400\,(1 - 0.009t)$.

The data of Table V were obtained from experiments performed in or near the zone of the optimum activity of invertase. In no case has a systematic investigation been made of the effect of the hydrogen-ion concentration, throughout the region of the enzyme's action, on the temperature coefficient of the hydrolysis.

Now, it is known that in and near the optimum zone, the activity of

[15] (a) Euler and af Ugglas, *Z. physiol. Chem.*, **65**, 124 (1910). (b) Euler and Laurin, *Arkiv Kemi, Mineral. Geol.*, **7**, No. 24 (1919). (c) Euler and Laurin, *Z. physiol. Chem.*, **110**, 55 (1920).

[16] Arrhenius, *Z. physik. Chem.*, **4**, 226 (1889).

[17] Arrhenius actually used A and not a multiple thereof; $2A$ is here considered owing to its identity with E.

[18] Marcelin, *Compt. rend.*, **158**, 116 (1914); *Ann. Physik*, **3**, 120 (1915).

[19] Lewis, *J. Chem. Soc.*, **109**, 796 (1916).

TABLE V

THE RELATION BETWEEN 2 A AND THE TEMPERATURE

Author	Temperature interval	Mean temperature	2 A
Euler and Laurin[15c]	0.8–10.4°	5.6°	11,400
Euler and af Ugglas[15a]	0 –20	10	11,000
Euler and Laurin[15c]	0.8–18.9	9.9	10,900
Euler and Laurin[15b]	10 –20	15	10,500
Euler and Laurin[15c]	10.4–18.9	14.7	10,200
Vosburgh[20]	15 –20	17.5	10,110
Vosburgh[20]	20 –25	22.5	9,850
Euler and af Ugglas[15a]	20 –30	25	8,040–9,340
Tammann[21]	20 –30	25	7,000
Vosburgh[20]	25 –30	27.5	8,925
Vosburgh[20]	30 –35	32.5	8,690
Euler and Laurin[15c]	20 –45.3	32.7	9,200
Kjeldahl[22]	30 –40	35	8,000
Euler and Laurin[15b]	20 –52.2	36.1	8,800
Euler and Laurin[15c]	20 –52.2	36.1	8,400
O'Sullivan and Tompson[23]	45 –50	47.5	7,000
Euler and Laurin[15c]	45.3–52.2	48.8	5,800

invertase is little affected by changes in the hydrogen-ion concentration. But if the activities at two temperatures are practically unaffected by changes of the hydrogen-ion concentration, then naturally the temperature

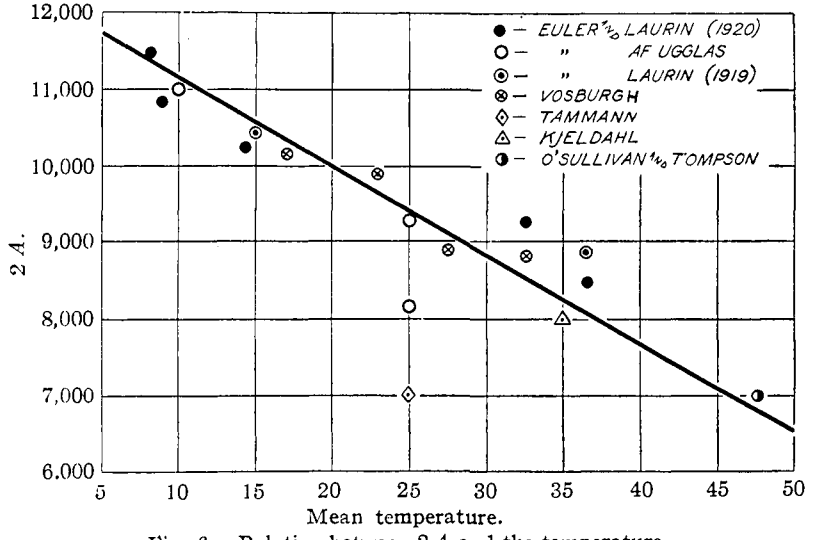

Fig. 6.—Relation between 2 A and the temperature.

[20] Unpublished results from some measurements made in this Laboratory by W. C. Vosburgh.

[21] Tammann, *Z. physiol. Chem.*, **16**, 271 (1892).

[22] Kjeldahl, *Medd. Carlsberg Lab.*, 335 (1881).

[23] O'Sullivan and Tompson, *J. Chem. Soc.*, **57**, 834 (1890).

coefficients, which are the ratios of the activities, will also be but little affected by changes of the hydrogen-ion concentration; and since 2 A is a function of the coefficients, it can also be but little changed.

On the other hand, it was thought possible that conditions as to the temperature coefficients might be different in the regions on both sides of the optimum zone where changes in the hydrogen-ion concentration have a considerable effect on the velocity of the hydrolysis.

For this reason, the values of n in Table IV (which correspond to the values of k in the expression of Arrhenius) were compared at intervals of hydrogen-ion concentration corresponding to P_H 0.5 throughout the region of the enzyme's activity, and the temperature coefficients and values of 2 A calculated from these.

TABLE VI

THE TEMPERATURE COEFFICIENTS ON THE MORE ALKALINE SIDE OF THE OPTIMUM

P_H	$\frac{n_{30°}}{n_{25°}}$	$\frac{n_{35°}}{n_{30°}}$	$\frac{E}{25–30°}$	$\frac{E}{30–35°}$
5.00	1.277	1.263	8,853	8,706
5.5	1.281	1.268	8,945	8,861
6.0	1.299	1.278	9,441	9,181
6.5	1.347	1.295	10,740	9,640
7.0	1.419	1.383	12,630	11,290
7.5	1.512	1.414	14,900	12,900
8.0	1.580	1.455	16,510	14,020
8.5	1.600	1.500	16,960	15,130

It is seen that the coefficients increase with decreasing acidity and decrease with the temperature. The variation of 2 A with the hydrogen-ion concentration is shown graphically in Fig. 7. The variation is greatest in the region between the optimum zone of the enzyme's activity and the zone of its smallest activity. In these zones it is quite small.

Now, when one examines the curves in Fig. 5 which were obtained by reducing the experimental curves of Fig. 3 to a common scale, the reason for this dependency of the temperature coefficient on the hydrogen-ion concentration becomes evident. If the curves of Fig. 3 had all occupied the same position with regard to the P_H-axis, they would have been reduced to a single curve in Fig. 5 and in this case the temperature coefficient of the hydrolysis would have been the same at every hydrogen-ion concentration. As it is, however, the curves show a shift along the P_H-axis with increase in temperature, which is brought out clearly in Fig. 5, and it is this shift which accounts for the increase in the temperature coefficients.

Thus, raising the temperature of the hydrolysis seems to have two independent effects, one being an increase in the rate of the reaction and the other a shifting of the curve along the P_H-axis.

It is interesting to note that while in the acid hydrolysis of sucrose the

temperature coefficient is independent of the temperature, as is indicated by some calculations of Arrhenius from data of Urech[24] and Spohr, and of the hydrogen-ion concentration,[25,26] the coefficient changes with both of these in the hydrolysis of sucrose in the presence of invertase.

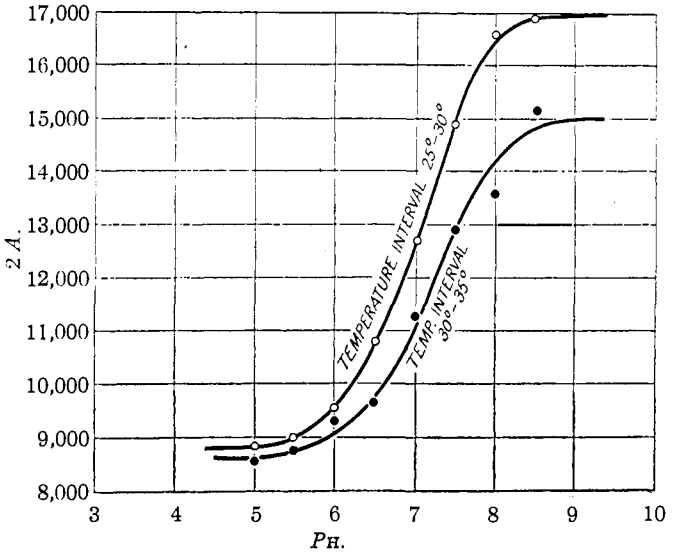

Fig. 7.—Dependency of 2 A upon the hydrogen-ion concentration.

The temperature coefficients on the more acid side of the optimum zone are given in Table VII.

TABLE VII

THE TEMPERATURE COEFFICIENTS ON THE MORE ACID SIDE OF THE OPTIMUM

P_H	$\frac{n_{30°}}{n_{25°}}$	$\frac{n_{35°}}{n_{30°}}$	$\frac{E}{25–30°}$	$\frac{E}{30–35°}$
5.0	1.277	1.263	8,853	8,706
4.5	1.276	1.256	8,792	8,507
4.0	1.262	1.247	8,392	8,233
3.5	1.249	1.212	8,011	7,166
3.0	1.237	1.171	7,663	5,895
2.5	1.238	1.104	7,696	3,685

As is seen here, just as in the more alkaline region, the coefficients fall off with increasing acidity. This might on first thought be attributed to the faster acid inactivation of the enzyme at the higher temperature than at the lower. A careful study of Fig. 3, however, shows that this cannot be the only cause, for the decrease in the coefficients is noticeable not only in the region of decreasing n, but also where the latter are con-

[24] Urech, *Ber.*, **16**, 765 (1883); **17**, 2175 (1884).

[25] Spohr, *Z. physik. Chem.*, **2**, 195 (1888).

[26] Wilhelmy, *Pogg. Ann.*, **81**, 413 (1850).

stant and no inactivation occurs. This means that the temperature coefficients fall off where there is no indication whatever of the enzyme being inactivated.

The phenomenon could be accounted for, just as has been done in the case of the more alkaline branch of the curve, by the assumption that an increase of the temperature not only raises the P_H activity curve, but also has the effect of shifting it toward the right. This would cause the coefficients to be smaller where the activities are small (the most acid part of the curves) than where they are large (near the optimum).

The Critical Increment.—The interest in Arrhenius' equation has been greatly augmented in recent years by a number of new contributions to the theory of chemical reaction. These theories postulate that only those molecules react which are in an active or "critical state." The fact that they lead to Arrhenius' original equation (in a slightly modified form) may be considered as strong evidence in favor of their validity.

The subject is of special interest in this work owing to the discrepancy in the temperature coefficients in the hydrolysis of cane sugar by acids and by invertase. It is to be hoped that thus in time one or more of these theories may furnish a basis for gaining a closer insight into the nature of the enzyme's action. It is, therefore, deemed advisable at least to indicate them.

Marcelin[18] discusses reaction velocity on a thermodynamic basis and also on some considerations of the theory of probability, and postulates that only those molecules react which reach a "critical" condition, that is, acquire a definite amount of energy in excess of the system's average energy per molecule. Lewis[19] has termed this energy difference between the mean state and the critical the "critical increment." Marcelin obtains a constant, E, which is expressed in the same form as Arrhenius' A, but has twice the latter's magnitude. E has been shown to be practically identical with the critical increment per gram molecule.

Rice[27] has extended the results of Marcelin's analysis on the basis of statistical mechanics and by some limiting assumptions has been able to define more closely the critical condition.

Perrin,[28] Lewis[19] and Tolman[29] considered radiation as the source from which the energy of activation necessary for chemical action was obtained.

Lewis has shown that for homogeneous reactions the temperature coefficient and E, the critical increment, are modified by the catalyzing agent. A negative catalyst increases E, a positive catalyst decreases E; and the better (more positive) a catalyst is, the smaller will E be. In support of this idea, Lewis quotes experiments of Halban,[30] Bredig and

[27] Rice, *Brit. Assoc. Rept.*, **1915**, 397.
[28] Perrin, *Ann. Physik*, **11**, 5 (1919).
[29] Tolman, THIS JOURNAL, **42**, 2506 (1920).
[30] Halban, *Z. physik. Chem.*, **67**, 139 (1909).

Lichty,[31] de Bruyn and Sluiter,[32] and others. In the light of these facts and on the assumption that the hydrolysis of sucrose in the presence of invertase is a homogeneous reaction (a view taken by Michaelis[33]) the observations in this investigation on the temperature coefficient of the reaction would mean: (1) that invertase at room temperature is a better (more positive) catalyst than hydrogen ion, since in the presence of the former, E, the energy necessary to change an inactive gram molecule to an active one, is equal to approximately 7700–16,000 calories (depending upon the acidity of the medium) while in the latter, E is equal to 26,000 calories; this means that in the presence of invertase less energy is required to convert inactive molecules of the reacting substances to active molecules than is the case in the catalysis by hydrogen ions; (2) that invertase is a better catalyst in the most acid zone of its activity (P_H = approx. 2.5) where E is smallest, than in the more alkaline regions where E increases as the acidity diminishes.

It must be pointed out, however, that the change of the temperature coefficient and 2 A with the hydrogen-ion concentration can equally well be accounted for by the theory of Michaelis and Davidsohn, that the curves relating to the hydrogen-ion concentration and the velocity of hydrolysis are dissociation-residue curves of an acid. As previously pointed out, the dependency of the temperature coefficients and 2 A upon the hydrogen-ion concentration was shown in a shift of the curves (Fig. 5) along the P_H-axis. According to the theory of Michaelis and Davidsohn these are acid dissociation-residue curves and the shift is due to a change in k, the dissociation constant of the acid, with the temperature. That changes in the value of k should have this effect follows from the relationship pointed out in Equation 1.

Experimental Details

Preparation of Materials.—The invertase preparation designated as No. 7 by Nelson and Hitchcock was used in all experiments. It was made by previous workers of this Laboratory by the method of Nelson and Born[34] and had been kept for several years saturated with toluene in an ice box. Its activity did not change during the present work, as is shown by Expts. 40 and 49, which were performed about six months apart.

The best commercial sugar was used. Its solution in distilled water was stirred with charcoal, filtered and recrystallized by shaking according to the procedure of Bates and Jackson.[35] Its rotation agreed within 0.13% with that calculated from the formulas of Landolt and Schönrock.[36]

[31] Bredig and Lichty, *Z. Elektrochem.*, **12**, 459 (1906); *J. phys. Chem.*, **11**, 225 (1907).

[32] de Bruyn and Sluiter, *Proc. akad. Wetenschappen Amsterdam*, **6**, 733 (1904).

[33] Michaelis, *Biochem. Z.*, **115**, 269 (1921).

[34] Nelson and Born, THIS JOURNAL, **36**, 393 (1914).

[35] Bates and Jackson, *Bur. Standards Sci. Paper*, **268**, 75 (1916).

[36] Landolt and Schönrock in (a) Browne's, "A Handbook of Sugar Analysis," **1912**, pp. 177–178.

Expt. 40 November 28, 1921				Expt. 49 May 17, 1922			
Time Minutes	Observed rotation Degrees	Per cent inverted	$n \times 10^5$	Time Minutes	Observed rotation Degrees	Per cent. inverted	$n \times 10^5$
0	13.05	...	...	0	13.07	...	...
33	7.96	30.22	682	333	8.00	30.10	680
45	6.31	40.05	682	45	6.35	39.90	680
59	4.56	50.44	682	59	4.59	50.35	679
75	2.83	60.84	682	75	2.85	60.67	680
		Mean	682			Mean	680

C. P. Chemicals were used throughout. Buffers and solutions during hydrolysis were kept in Nonsol bottles. All volumetric apparatus was calibrated.

Apparatus.—The temperature was kept constant in a water thermostat. The fluctuation was $\pm 0.01°$ at 25° and $\pm 0.02°$ at 30° and 35°.

The solutions were placed in 200-mm. polariscope tubes and the rotations read with a Schmidt and Haensch polarimeter reading accurately to 0.01°. Uniform length of the tubes was assured by reading the same 10% sucrose solution through all of them. The temperature of the tubes was kept constant at 25° ± 0.05° by the thermostat described by Nelson and Beegle.[37]

The light from a mercury-vapor arc was passed through two Wratten filters, No. 77, one of which had been re-cemented with a green film in place of the yellow. Monochromatic rays of wave length 546.1 $\mu\mu$ were thus obtained. The filters were prepared by Dr. C. E. K. Mees of the Eastman Kodak Company, to whom thanks are due.

Control of the Hydrogen-Ion Concentration.—The hydrogen-ion concentration was kept constant by means of buffer mixtures. The citrate buffers were prepared according to Sörensen,[10] the borate buffers after Clark.[13] They were always used in such amounts that their concentration in the solutions undergoing hydrolysis was 0.01 *M*. This concentration was so small that the salt effect could be considered practically negligible, as Fales and Nelson[38] have pointed out.

Measurement of the Hydrogen-Ion Concentration.—The hydrogen-ion concentration was measured by the electrometric null method. Saturated potassium chloride-calomel electrodes and a saturated potassium chloride salt bridge, as recommended by Mudge and Fales,[39] were used on account of the advantages indicated by these authors.

The relationship between e.m.f. and P_H at the different temperatures was obtained from data of Fales and Mudge as to the e.m.f. given by the combination, calomel—sat. KCl—sat. KCl salt bridge—0.1 *M* HCl, and of A. A. Noyes[40] as to the degree of ionization of 0.1 *N* hydrochloric acid at different temperatures. Some calculations from the latter paper were obtained from Dr. J. C. Morrell to whom thanks are due.

The relationships obtained are given in Table VIII.

TABLE VIII

DEGREE OF IONIZATION AND POTENTIAL IN COMBINATION WITH THE CALOMEL-SATURATED POTASSIUM CHLORIDE CELL OF 0.1 *M* HYDROCHLORIC ACID

Temp., °C	25	30	35
Degree of ionization	0.9245	0.9251	0.9218
Potential, volt	.3100	.3070	.3043

[37] Nelson and Beegle, THIS JOURNAL, **41**, 559 (1919).

[38] Fales and Nelson, *ibid.*, **37**, 2770(1915).

[39] Mudge and Fales, *ibid.*, **42**, 2446 (1920).

[40] Noyes, "The Electrical Conductivity of Aqueous Solutions," Carnegie Inst. **Pub. 63**, 141–339 (1907).

With these values and the Nernst equation the following relationships between e.m.f. and P_H were finally obtained: at 25°, e.m.f. = 0.2488 + 0.05911 P_H; at 30°, e.m.f. = 0.2448 + 0.06010 P_H; at 35°, e.m.f. = 0.2411 + 0.06110 P_H.

Procedure.—The procedure followed was in general that recommended by Vosburgh.[41] The solutions containing buffer and sucrose were made up to volume and such a portion of the latter was taken that when an even number of cubic centimeters of invertase was pipetted in, the required concentration of each component was obtained. The composition of the solutions undergoing hydrolysis was as follows: sucrose 10 g. per 100 cc.; buffer 0.01 *M;* invertase 5.56 cc. per 100 cc. of solution.

The solutions were shaken while being mixed. The pipets delivered in from 6 to 10 seconds, except the one used for the invertase which delivered in 14 seconds.[42] The mean delivery time of the pipet was taken as the time of observation. 25-Cc. samples of the reaction mixture were added to 5 cc. of 0.1 *M* sodium carbonate solution, which stopped the reaction. Readings were made between 15 minutes and two hours after this, as recommended by Hudson.[43] For the initial rotation, solutions of like composition to the above were made up, but the invertase was inactivated by the sodium carbonate before the sucrose was added. In taking the rotation of the solutions, the polariscope tube was rotated after each reading to avoid errors due to possible strains in the cover glasses.[44] Four readings were thus taken. The zero point was determined by readings through distilled water.

In calculating the percentage inverted, the total change in rotation was always taken as 16.84° (except where other values are indicated). For justification of this procedure, see Nelson and Hitchcock.[8]

Owing to the extreme constancy in the n values during the course of an hydrolysis, it was considered sufficient to make four observations only during an experiment. These were in most cases taken at the middle part of the reaction, due to the fact pointed out by Nelson and Hitchcock that the experimental errors are the largest in the early part of the inversion. Since, in addition, taking observations toward the latter part of the reaction involves a considerable loss of time, especially if the hydrolysis is slow, it was decided to take readings at the middle part whenever this was possible.

Readings were taken in the experiments, where the acidity of the medium was so great as to cause the activity of the invertase to decrease during the reaction, the effect showing in a falling off of n, during the early part of the reaction, before the inactivation of the enzyme could advance very far. It was thought this would give the most accurate measure

[41] Vosburgh, THIS JOURNAL, **43**, 1693 (1921).

[42] Simons, *Dissertation*, Columbia University, **1921**.

[43] Hudson, THIS JOURNAL, **30**, 1546 (1908).

[44] **Ref. 35a, p. 156.**

of the activity of the invertase. In the experiments where borate buffer was used, readings had to be made in the early part of the hydrolysis for another reason. Here some unaccounted-for effect was noticeable after several hours. In every case the first part of the inversion progressed at a rate to be expected considering the hydrogen-ion concentration of the medium, and n remained constant; but eventually the velocity of the reaction took a sudden jump, the n values increased correspondingly and the hydrogen-ion concentration of the solution fell off.

The authors desire to expresss their thanks to the Harriman Research Laboratory, whose financial assistance made this work possible.

Summary

1. It has been shown that the sucrose concentration at which the hydrolysis in the presence of invertase attains a maximum velocity is independent of the temperature and the hydrogen-ion concentration and that the effect of these latter upon the rate of the reaction is independent of the sucrose concentration.

2. The limits of the hydrogen-ion concentration within which the hydrolysis with invertase follows a normal course (using n as a criterion) have been extended from $10^{-2.75}$–$10^{-3.3}$ (depending upon the temperature) in the acid region to $10^{-8.4}$ in the more alkaline region.

3. The hydrogen-ion concentration at which invertase first shows inactivation (the critical hydrogen-ion concentration) has been determined at 25°, 30° and 35° and has been shown to decrease regularly between these temperatures.

4. The zone of the optimum action of invertase at 25°, 30° and 35° has been found to lie between the hydrogen-ion concentrations $10^{-4.5}$ and $10^{-5.0}$, a narrower region than had heretofore been determined.

5. The relation between the activity (n) and the hydrogen-ion concentration approximately satisfied the equation for the dissociation-residue curve, as claimed by Michaelis and Davidsohn. The temperature did not affect this relation.

6. It was found that the temperature coefficient of the hydrolysis of sucrose in the presence of invertase was a function of the hydrogen-ion concentration and increased with decreasing acidity, and that hence the hydrolysis was inherently different from that by acid where the temperature coefficient is independent of the hydrogen-ion concentration.

7. The hydrolysis of cane sugar in the presence of invertase involves at least two distinct stages. One of these, which is characterized by the sucrose concentration at which the hydrolysis attains a maximum velocity, is independent of the temperature and hydrogen-ion concentration, while the other changes with each of these.

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