SUCROSE INVERSION BY BAKERS' YEAST AS A FUNCTION OF TEMPERATURE

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The velocity of many physiological processes increases exponentially with temperature according to the Arrhenius equation

$$k = ze^{-\mu/RT}$$

where k is the rate of the reaction, z is a constant, e is 2.718, R is the gas constant, T is the absolute temperature, and μ represents the energy of activation in calories per mol of the pacemaker reaction in the catenary series of events controlling the rate (Crozier and Hoagland, 1934). Since most biological reactions are catalyzed by enzymes, the change in rate is determined by the enzyme, just as in chemical systems the temperature characteristic is indicative of the specific catalyst employed in the reaction. For certain isolated enzyme systems the data on rate of reaction as a function of temperature fit the Arrhenius equation. For yeast invertase, using different experimental conditions and measuring rate of inversion in a variety of ways, μ was found to be independent of temperature and has the value of 11,000–11,500 (Sizer, 1937, 1938). Crozier reported (1924) a constant μ = 16,700 obtained by analyzing Quastel's data on succinic dehydrogenase of *E. coli*. Craig (1936) isolated a fat oxidation system from *Lupinus albus* and obtained a μ = 11,700, when the system was saturated with oil, but the situation was more complicated if oil were not added. Certain other enzyme systems yield constant temperature characteristics at least over narrow ranges of temperature (Barnes, 1937).

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The ultimate interpretation of temperature characteristics for physiological processes will depend on the isolation and study of the chemical reactions involved, especially the slowest in the series and those capable of becoming the pacemaker under different environmental conditions. One approach to the problem is to compare, as a function of temperature, the kinetics of a chemical process carried out inside the living cell with the same reaction catalyzed by an enzyme from that cell. The same temperature characteristic might be expected in the two cases, unless more than one enzyme capable of activating the substrate were present in the cell. In the latter case the temperature analysis might be complex and influenced by unknown factors which determine which one of the several enzymes will play the dominant rôle. A comparison between intracellular behavior of an enzyme and its characteristics after separation from the cell and purification would also be obtained from such a study.

In this study the mechanism of sucrose inversion *in vivo* was measured and compared with enzyme hydrolysis. The kinetics of inversion catalyzed by yeast invertase as a function of temperature was compared with that for inversion using the yeast cells.

EXPERIMENTAL

The hydrolysis of sucrose was followed by measuring chemically the amount of reducing sugars present in the digest at successive time intervals. This method has an advantage over the polarimetric technique of following the reaction, since in the former mutarotation of the products of hydrolysis is not a complicating factor. Sumner's dinitrosalicylic acid method (1925, 1935) of measuring colorimetrically the amount of reducing sugars was used, since it had proved to be an excellent method of determining the activity of yeast invertase (Sizer, 1938).

Stock solutions of sugar and yeast were kept in the refrigerator. The sugar solution contained 6 per cent sucrose made up in M/15 KH_2PO_4, and the yeast solution was prepared by making up a 2.5 per cent suspension of compressed yeast in M/15 KH_2PO_4. In the first two series of experiments Fleischmann's bakers' yeast cake was used, while in the last series a pure strain of bakers' yeast, *Saccharomyces cerevisiae* (G.M. No. 21062 supplied by the Fleischmann laboratory), was employed. This strain is essentially the same as the yeast in yeast cake and was used by Stier and Stannard (1937) in their quantitative study of carbohydrate metabolism. Both stock solutions were adjusted to the temperature of the water bath (temperature controlled to ± 0.05°C.) and then 2 cc. 2.5 per cent yeast suspension were added to 10 cc. 6 per cent sucrose. The yeast suspension was rendered homogeneous by thoroughly shaking before using. At successive time

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intervals, 1 cc. samples of the digest (stirred before removing sample) were added to 3 cc. dinitrosalicylic acid reagent in 25 cc. Folin-Wu sugar tubes. The tubes were kept in a boiling water bath for 5 minutes, then placed under the tap for 3 minutes, and finally diluted to 25 cc. The amount of reducing sugars present was determined by comparing these solutions in a colorimeter with a standard solution treated in a fashion similar to that used on the samples of the digest. A standard containing 5 mg. glucose/cc. was used in all the work with bakers' yeast but a 4 mg. standard was used for work with the pure strain of yeast. Results were expressed in terms of milligrams of reducing sugar per cubic centimeter of digest.

Sucrose Hydrolysis by Bakers' Yeast Cake

Sugar inversion catalyzed by Fleischmann's yeast cake was studied as a function of digestion time and of temperature. When the concentration of reducing sugars is plotted as a function of the duration of digestion, the points fall along a smooth curve (Fig. 1, upper curve). This curve is identical in appearance with that obtained using yeast invertase as the catalyst (Sizer, 1938). Rate of digestion can be calculated from the curve by determining the reciprocal of the time required to produce a given amount of invert sugar. Rate expressed as milligrams of invert sugar per minute was calculated from the time required to produce 5 mg. invert sugar as indicated by the curve drawn through the plotted points. Rate can be calculated equally as well from other phases of the reaction, although, during the later stages of inversion, interfering factors such as enzyme destruction and inhibition by the accumulated glucose and fructose may complicate the situation.

When log rate is plotted against $1/T$ (Fig. 2, upper curve), the data are best fitted by drawing through the points two straight lines which intersect at a critical temperature of 17°C. The large number of experimental points obtained indicate that the data can be fitted only by drawing through them two straight lines. The slope of the line drawn through the points for from 0 to 17°C. corresponds to a μ = 10,700. This agrees closely with the value of 11,000 which Sizer (1938) reported over the same temperature range using a similar technique with yeast invertase. The data of Euler and Laurin (1920) indicate a μ = 10,700 between 0 and 20°C. for yeast invertase, although they agree with other workers that μ decreases with rise in temperature. This agreement between the μ values for yeast and yeast

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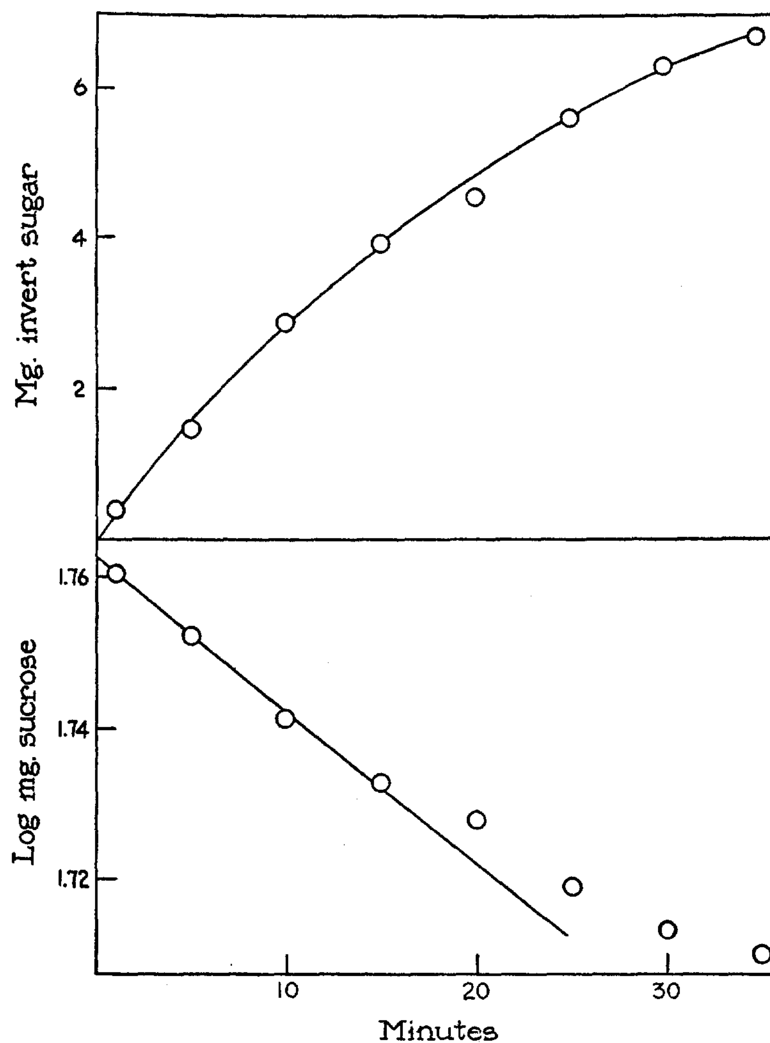

FIG. 1. Inversion of 5 per cent sucrose catalyzed by 0.417 per cent bakers' yeast at 2.1°C.

Upper Curve.—Milligrams invert sugar liberated during the hydrolysis plotted against elapsed time in minutes.

Lower Curve.—Log sucrose concentration (log (58 − milligrams invert sugar)) plotted against the duration of digestion in minutes. Monomolecular velocity constant = 0.00200.

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invertase is evidence that the enzyme behaves identically *in vivo* and *in vitro*, at least with respect to temperature activation. Above 17°C. the straight line drawn through the plotted points has a slope corresponding to a μ = 8,300. Above 40°C. the points fall off from the curve indicating temperature inactivation of the enzyme system.

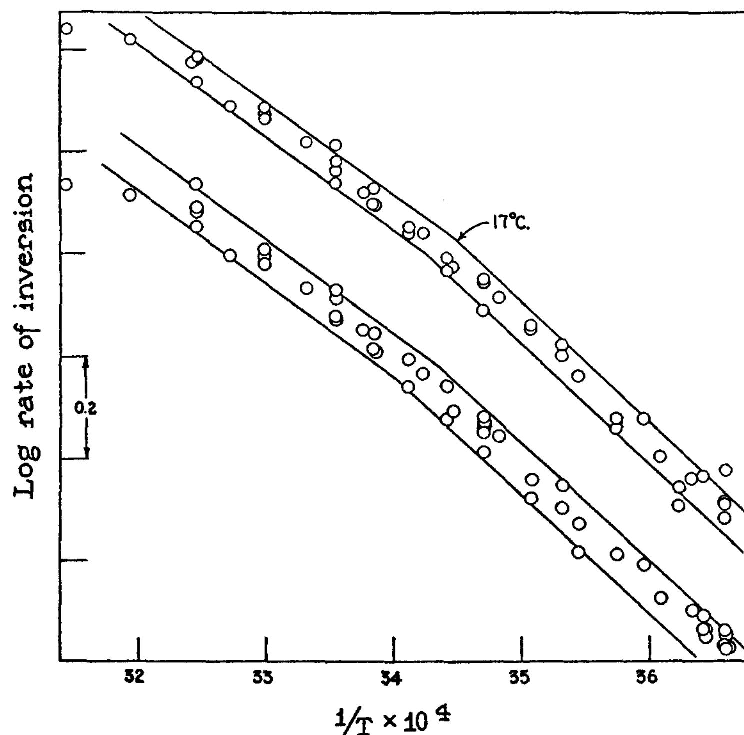

FIG. 2. Log rate of inversion of 5 per cent sucrose by 0.417 per cent bakers' yeast plotted against the reciprocal of absolute temperature. Below 17°C. μ = 10,700, above 17°C. μ = 8,300. Inactivation occurs above 40°C.

Upper Curve.—Rate expressed as milligrams invert sugar/min.

Lower Curve.—Rate expressed as monomolecular velocity constants.

The new μ value of 8,300 suggests that in the living yeast there exists another enzyme (*e.g.* yeast maltase) capable of hydrolyzing sucrose, and which plays the dominant rôle in inversion above 17°C. Yeast invertase, on the other hand, is the important catalyst for sugar inversion below that temperature.

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Other methods are available for calculating rate of inversion. For example, the monomolecular velocity constants may be computed from a monomolecular plot of the data and used as a measure of rate. When log sucrose concentration is plotted against elapsed time (Fig. 1, lower curve), the first few points fit roughly along a straight line; *i.e.*, the reaction follows the monomolecular equation. After about 4 mg. reducing sugar have been liberated the points fall off from the straight line. This phenomenon is precisely that obtained with yeast invertase (Sizer, 1938). Monomolecular velocity constants were calculated from the slopes of the straight lines drawn through the plotted points for the various temperatures. The graph of log k *vs.* $1/T$ (Fig. 2, lower curve) is practically identical with that obtained when log rate, expressed as milligrams invert sugar/minute, is related to $1/T$. It is obvious that results obtained on the kinetics of inversion by living yeast as a function of temperature are independent of the method of analyzing the experimental data. It should be emphasized, however, that both methods employed deal only with data for the first part of the hydrolysis. It seems likely that information obtained for later stages of hydrolysis could not be interpreted in terms of the Arrhenius equation due to interfering factors which complicate matters. A similar situation was postulated for yeast invertase.

Sucrose Inversion by Yeast Killed with Toluene

Are the kinetics of inversion as a function of temperature related in any way to vital processes in the yeast, or determined by the morphology of the living cell? A study of inversion catalyzed by dead cells was made to answer this question. Formaldehyde and *o*-cresol were unsatisfactory lethal agents for they not only destroyed the cells, but also almost completely inhibited the enzymic hydrolysis of sucrose. Toluene proved satisfactory for it killed the cells, yet did not inhibit their inversion powers. As would be expected, toluene also does not impede the action of yeast invertase. Although the solution saturated with toluene was lethal, the cells were not cytolyzed, but remained intact, during the 2 weeks that the preparation was used. The sucrases were present and active inside the intact dead cells rather than in the surrounding medium (see p. 704).

The inversion curve obtained with the dead cells was very similar

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to that procured with the living ones. Rate was calculated from the time required to liberate 5 mg. invert sugar, and an Arrhenius plot was made of the data (Fig. 3, upper curve). Two straight lines intersecting at 17°C. best fit the plotted points. The lower and steeper

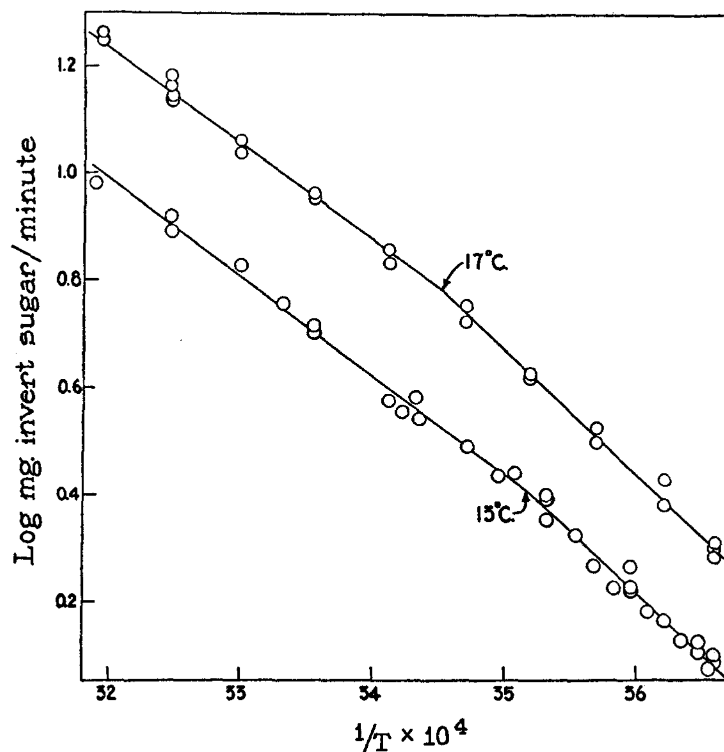

FIG. 3. Log 10 times the rate of inversion of 5 per cent sucrose by 0.417 per cent yeast plotted against $1/T$. Below 13–17°C. μ = 10,700, above this temperature μ = 8,300. Inactivation occurs above 40°C.

Upper Curve.—Inversion catalyzed by bakers' yeast killed with toluene. The critical temperature is 17°C.

Lower Curve.—Inversion catalyzed by a pure strain of *Saccharomyces cerevisiae*, G.M. No. 21062. The critical temperature is 13°C.

line has a slope corresponding to a μ = 10,700, while for the upper one μ = 8,300. Heat inactivation sets in above 40°C. A comparison of these results on dead yeast with those obtained on living yeast reveals an excellent agreement. It seems evident that the inversion mecha-

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nism of the cell is not dependent on vital processes, nor is it determined by that part of the cell structure which changes when death sets in.

Inversion by a Pure Strain of Yeast

The common yeast cake may contain more than one strain of yeast as well as certain impurities such as starch which might complicate the inversion picture. In view of this, it was considered advisable to repeat the work on inversion as a function of temperature using a pure strain of *Saccharomyces cerevisiae*. A pure strain of Fleischmann's bakers' yeast, G. M. No. 21062, proved satisfactory for this study.

The course of inversion as a function of the duration of digestion is very similar to that for bakers' yeast cake. Since inversion did not proceed quite as rapidly, however, the rate was calculated from the time required to produce 4 mg. invert sugar instead of 5 mg. As before, the time required to produce a given amount of the hexoses was interpolated from the graph. Duplicate determinations were made at each temperature and the plotted points in the Arrhenius graph (Fig. 3, lower curve) represent the average of two inversion rates. The analysis of the data for the pure strain yields μ's of 10,700 below 13°C., and 8,300 above that temperature. Enzyme inactivation occurs above 40°C. These results are almost identical with those obtained with bakers' yeast cake, and with yeast killed with toluene. It becomes apparent that the kinetics of inversion as a function of temperature are the same using yeast from different sources and under widely different experimental conditions. It is interesting to point out that the critical temperature for the pure strain is 13°C. as compared with 17°C. for bakers' yeast.

Factors Which Might Complicate Inversion

1. Change in Number of Living Cells.—An increase or decrease in the number of cells in the stock solution or even a change in their metabolism might alter the kinetics of inversion, especially since the culture was used over a period of several weeks. Yeast cells suspended in M/15 KH_2PO_4 are so called "resting cells" and do not multiply. Microscopic examination reveals a very small but constant number of budding cells. Neither do they die over the period that they are used, since there is no increase in the number of cells (roughly 5 per cent) which take up the dye when the differential methylene blue

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staining technique (Nelson, Palmer, and Wilkes, 1932) was used. This was true for both the ordinary yeast and the pure strain, and agrees with the results of Stier and Stannard (1937). Another indication that no significant change has occurred in the culture is the fact that the rate of inversion at a given temperature does not vary with the age of the yeast suspension. During the course of inversion the sugar solution does not appear to be toxic, since there is no increase in the number of dead cells over a period of several hours. In the case of the yeast treated with toluene about 98 per cent of the cells stained blue indicating that they were dead.

2. Change in pH of the Digest.—As a result of the normal respiration of the yeast, acids such as carbonic are formed which might inhibit hydrolysis by bringing the sucrases further from the pH for optimum activity. (4.7 is the optimum pH for yeast invertase.) The increase in acidity of the digest was followed continuously with the glass electrode using a Beckman pH meter. In one experiment the pH decreased from 4.22 to 4.04 in 45 minutes at 24°C. In a second the change was from 4.23 to 3.90 in 83 minutes. A similar increase in acidity was recorded with the quinhydrone electrode in a third test. An average of the three experiments indicated a decrease in pH of about 0.02 unit in the 5 minutes required to liberate 5 mg. invert sugar. This slight increase in acidity would not be expected to alter the rate of inversion appreciably.

3. Fermentation.—As soon as the sucrose is hydrolyzed a part of the invert sugar may be utilized in the systems of respiration and fermentation. This would seriously complicate the measurement of inversion, since it depends upon the determination of the reducing sugars produced. Fermentation was studied using the sensitive micro-method for alcohol determination recommended to the author by Dr. Allison and used successfully on yeast by Stier and Stannard (1937). An average of three different experiments indicated that 0.05 mg. alcohol was produced at 35°C. in the 5 minutes required to liberate 4 mg. invert sugar. On the assumption that fermentation may be represented by the equation

$$C_6H_{12}O_6 \rightarrow 2CO_2 + 2C_2H_5OH$$

(Harrow and Sherwin, 1935) this 0.05 mg. represents 0.10 mg. hexose which has been fermented. Since, during the liberation of 4 mg.

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reducing sugars, only 0.10 mg. is fermented, it seems evident that during the early part of inversion fermentation does not play a serious complicating rôle.

Another indication that fermentation and respiration are not seriously confusing the inversion picture is the fact that, in the toluene-killed cells, the kinetics of inversion as a function of temperature are identical with those for living cells. Yet in the dead cells the processes of fermentation and respiration have been interrupted.

Is Inversion Extra- Or Intracellular?

Workers in general agree that yeast invertase is active inside the cell or attached to its surface and is not present in the solution (Nelson, 1933). Since in this work there appear to be two enzymes involved, it seemed advisable to determine whether they were both intracellular. An old suspension of bakers' yeast was filtered through a Seitz filter, and the hydrolytic activity of the cell-free filtrate was tested. The activity was very slight and amounted to only 2.6 per cent of that of the unfiltered material. A similar experiment was performed with the toluene-treated yeast where the filtrate had 5.7 per cent of the activity of the original suspension. In both cases the enzyme activity is associated with intact cells and cannot be readily separated from them.

DISCUSSION

Warburg and certain others believe that information concerning cellular processes which can be obtained from studying enzyme systems is definitely limited. They suggest that the intracellular behavior of an enzyme may be quite different from its properties after separation from the cell and subsequent purification. There is no evidence for the validity of this statement with respect to yeast invertase. The kinetics of inversion *in vitro* and *in vivo* are the same (Sizer, 1938); Nelson and associates (1932) found that inversion followed the same equation in the two cases. The identity *in vivo* and *in vitro* of the relationships between rate of inversion and (H^+) or sucrose concentration led Nelson and associates (1933) and Wilkes and Palmer (1933) to the belief that in the living cell invertase was present in or near the cell membrane. Increase in inversion rate as a function of temperature below 13–17°C. is also identical in the yeast

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cell and yeast invertase solution. This lends support to the hypothesis that the change in rate of physiological processes with temperature is determined by the energy of activation of the enzyme-substrate complex of the pacemaker link in the series of reactions controlling the rate.

Above 13–17°C., however, there is a sharp contrast between the behavior of the yeast cell and yeast invertase with respect to inversion as a function of temperature. For yeast invertase the temperature characteristic is 11,000, while for bakers' yeast above 13–17°C. it is 8,300. This difference in μ values suggests that in yeast there is present a second sucrase different from the familiar yeast invertase with respect to the fact that it has a lower energy of activation.

SUMMARY

Inversion of sucrose by bakers' yeast follows the same course as inversion catalyzed by yeast invertase. Rate of inversion increases exponentially with temperature; the temperature characteristic in the Arrhenius equation is 10,700 below 13–17°C., and 8,300 above that temperature. Temperature inactivation occurs above 40°C. The effects of temperature upon rate of inversion were the same using Fleischmann's yeast cake, the same yeast killed with toluene, and a pure strain (G. M. No. 21062) of bakers' yeast. The last differed from the other two only in the fact that its critical temperature was 13°C. as compared with 17°C. for the others.

The catalytic inversion is associated with enzyme activity inside the cell, not in the medium, and is independent of any vital processes inside the cell such as respiration and fermentation. Since invertase activity is the same inside the cell as it is after extraction, it appears possible to relate the temperature characteristics for physiological processes to the catalytic chemical systems which determine their rate.

At least two enzymes are capable of inverting sucrose in the yeast cell. The familiar yeast invertase (μ = 10,700) is active below 13–17°C. while a second enzyme (μ = 8,300) plays the dominant rôle above that temperature.

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