ENZYMES FROM THE STANDPOINT OF THE CHEMISTRY OF INVERTASE

JOHN M. NELSON

Department of Chemistry, Columbia University, New York, New York

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The name invertase, or sucrase, is applied to any enzyme which catalyzes the inversion of cane sugar. Although the chief source of invertase is ordinary brewer's or baker's yeasts, it occurs widely spread in nature, being present in the tissues and fluids of many plants and animals. In most instances, however, it occurs only in small amounts, and often it is difficult or impossible to separate it even in a crude form from these tissues and fluids. For this reason, most studies of this enzyme have been carried on with invertase preparations obtained from yeast, from which it can be obtained in relatively large yields with a minimum amount of labor. The reason why invertase has been studied probably more than any other enzyme is because of its stability judged from the constancy of its activity, and because its substrate, sucrose, is a relatively simple substance as substrates go and has only one linkage which is affected by the enzyme.

Several methods have been employed in obtaining the enzyme from yeast. Some of the earlier investigators (4, 91) first dried the filtered yeast at about 100°C. so as to render the accompanying proteins more insoluble, and then extracted the dried material with water. Emil Fischer (30, 31) dried the yeast at about room temperature and extracted with water containing an antiseptic such as toluene. Others (49, 64) ground the pressed yeast with sand and then extracted with water saturated with chloroform.

Permitting the yeast to autolyze and then working up the liquid autolysate is the method mostly used at present. Among the first to employ this method were O'Sullivan and Tompson (88) in their excellent study of the enzyme. They allowed the pressed

yeast to stand, at room temperature, until the yeast cells died and partially liquefied. Hudson (41) improved greatly upon the procedure of O'Sullivan and Tompson by adding cell poisons, especially toluene (although he also tried others like ethyl acetate and chloroform), to the yeast. In this way he was able to cut down the time required for the autolysis to a few days.

In preparing the crude invertase from the autolysate, Hudson first removed a considerable quantity of the accompanying soluble protein and gums by precipitating with lead acetate and removing the excess lead by means of potassium oxalate and subsequent dialysis. The crude enzyme was then precipitated from the resulting solution by the addition of an equal volume of alcohol, and was finally redissolved in water. Euler (16, 18) and his coworkers, however, found that the lead treatment is not essential, and that just as active preparations can be obtained by precipitating the original filtered autolysate with alcohol, extracting the precipitated protein and enzyme material with water, or dilute alcohol, and repeating the alcohol precipitation.

By assuming invertase to be a true chemical compound whose activity is directly proportional to its concentration, it becomes possible to measure the amount of the enzyme contained in a given weight of material, or in other words, the degree of purity of the enzyme preparation. O'Sullivan and Tompson determined the number of minutes required by 50 milligrams of a preparation to hydrolyze to zero rotation at 15.5°C., 25 cc. of solution containing 16 per cent of sucrose and 0.5 cc. of acid sodium phosphate. According to this measure or time-value, ordinary brewer's yeast runs from 150 to 400 minutes per 50 milligrams of yeast cells. By means of the alcohol treatment of yeast autolysates, followed by subsequent treatment with kaolin to remove more of the extraneous proteins, Euler and Svanberg (19) succeeded in obtaining an invertase preparation which had a time-value of only 3.6 minutes. At the time, this was probably the most active or highly purified invertase preparation ever obtained.

Since then, Willstätter and coworkers (109, 118) have succeeded in obtaining preparations having time-values as low as 0.1

minute, or 36 times more active than that prepared by Euler and Svanberg in 1919. The German chemists modified the Hudson method of rapid autolysis by what they termed fractional autolysis. The latter consists mainly in interrupting the autolysis two or three hours after the toluene or chloroform has been added to the yeast, filtering, discarding the filtrate, which usually contains very little of the enzyme, and finally again adding 10 per cent of toluene and two volumes of water to the residue and permitting the autolysis to proceed to completion.

Still another modification of Hudson's method used by the Munich workers is what is known as neutral autolysis. In the ordinary Hudson method for autolysis, the autolysate tends to become quite acid, the pH ranging from 4.0 up to 5. Willstätter and his associates reasoned that this high acidity might disturb the proteolytic enzymes involved in the liberation of the soluble invertase from the yeast protoplasm. Therefore, in some of their work, they kept the autolysis mixture neutral by means of either dilute ammonia or diammonium phosphate. They also state (110) that straight acid autolysis is apt to yield invertase preparations from which subsequently it is difficult to remove accompanying material that responds to the Millon test for proteins. Neutral autolysates, therefore, yield preparations which show only a faint Millon reaction. On the other hand, these investigators claim that acid autolysis has the advantage over the neutral of yielding preparations entirely free from tryptophan. Their best preparations obtained by means of the neutral method contained about 3 per cent of this amino acid. Preparations fully as active as those reported by the German workers, and made in the writer's laboratory either by the acid or neutral autolysis of yeast, all gave positive tests for the presence of tryptophan.

Probably the most outstanding contribution to the chemistry of invertase made by Willstätter and his students is the use of adsorption in the purification of the enzyme. The fact that enzymes can be adsorbed to other substances is not new. As far back as 1861 Brücke (7) and others adsorbed invertase from its aqueous solutions to colloidal matter like calcium phosphate, cholesterin and lecithin. About 1907 Michaelis (64, 67), in trying

to determine whether invertase was acid or basic in character, noticed that the enzyme was adsorbed to basic substances like ferric hydroxide and alumina, but not to more acid substances such as kaolin. This led him to use kaolin for removing extraneous protein matter present in the invertase solution, much in the same manner as that subsequently employed by Euler and Svanberg, as mentioned above. Willstätter and Racke were the first, however, to take up the subject of the adsorption of invertase in a systematic and thorough manner. They studied not only the influence of the age of the autolysate, but also the concentration of the enzyme, etc., on the extent of the adsorption of invertase to alumina. They also made an elaborate study of the form or state of the alumina best suited for the purpose (50, 51) and of the conditions as well as the reagents most suitable for the subsequent elution of the invertase from the adsorbent. In the case of kaolin (117) they found, contrary to the claims of Michaelis, that invertase can be adsorbed to this material provided that the solution is sufficiently acid and dilute.

The procedure usually followed by the Munich workers is first to subject the autolysate to a pretreatment, such as dialysis, or removal of some of the extraneous matter with lead, as done by Hudson, or more often precipitation with alcohol and dialysis. After the pretreatment, the solution is acidified by dilute acetic acid, the invertase is adsorbed to kaolin and eluted by means of dilute ammonia, sodium carbonate, or phosphate, and the resulting solution is dialyzed. The pH of the dialyzed solution is next adjusted to between 5 and 6, the invertase is adsorbed to alumina and eluted by means of disodium phosphate or arsenate, and the resulting solution is dialyzed. The alumina treatment is often repeated three to four times, when a solution is obtained containing very active material, judged by its low time-value. The kaolin adsorption usually removes most of the yeast gum which always occurs together with the invertase in its cruder preparations, while the alumina tends to remove nitrogenous matter. The accompanying outline illustrates the procedure followed by the Munich workers in the case of one of their most active preparations.

Fresh Autolysate (100)
Chloroform neutral autolysate (room temperature) no fractionation
Time-value of autolysate = 90 to 140 minutes

↓

Alcohol precipitation and extraction of the precipitate with $M/50$ acetic acid
Time-value 15 to 30 minutes

↓

Kaolin adsorption (1 unit in 500 cc. of $M/50$ acetic acid). Concentrating the solution after elution and removing some of the protein precipitated by means of sulfuric acid.

Time-value = 1 minute

↓

Repeated alumina adsorptions (2 to 4 times) with fractionation[1] from neutral or very weakly acid solutions ($M/1000$ to $M/5000$ acetic acid) and in varying dilutions (1 unit in 50 to 500 cc.).

After elution and dialysis
Time-value = 0.2 to 0.16 minute

The final preparation of invertase gave no test for gum, a negative Millon test, and a slight ninhydrin reaction, and contained 5 to 10 per cent of tryptophane. In some cases, especially from yeast whose invertase content had been enriched by feeding it sugar, the tryptophan content amounted to 2 to 3 per cent.

Schneider, in his chapter on carbohydrases in Oppenheimer and Pincussen's *Methodik der Fermente*, page 849, states that Willstätter and his coworkers were unable to obtain by adsorption methods, invertase preparations having a time-value better than 0.2 to 1.5 minutes. They (122) succeeded, however, in some instances, although not always, by adding diammonium phosphate to invertase solutions (time-value 0.2 minute) and then an equivalent amount of lead acetate, in carrying down by the lead phosphate formed material present in the solution to such an extent as to reduce the time-value of the enzyme material still remaining in the solution to 0.1 minute.

According to Lutz, working in the writer's laboratory (unpublished work), preparations having time-values of about 0.2 minute and obtained by the Willstätter adsorption procedure, are often mixtures of at least two invertase complexes differing in their respective time-values or degree of activity. He finds that a part of these highly active preparations obtained by the adsorption procedure is soluble in saturated ammonium sulfate, but not

[1] Fractional adsorption—using either less adsorbent than required for complete adsorption, or incomplete elution.

all. The soluble part, after removal of the ammonium sulfate, has a time value of 0.1 minute, while the insoluble part is less active. Judging from Lutz's experience, it might be that the lead phosphate, in the Willstätter and Schneider experiments mentioned above, carried down the invertase complex insoluble in ammonium sulfate leaving the soluble part with the time-value of 0.1 minute remaining in the solution.

The compositions of the most active preparations obtained by Euler and Willstätter and their respective groups of coworkers varied quite widely, depending upon the procedure followed in preparing them. The ash contents ranged from 0.45 per cent (119) to 5.7 per cent (23). Willstätter, Grasser, and Kuhn (116) have been able to lower the phosphorus content to 0.006 per cent, indicating that it is not an essential component of the enzyme. Euler and Josephson found 1.4 per cent sulfur in the preparation whose ash content was 5.7 per cent, but some of their other highly active preparations contained less. Most of the very active preparations prepared in the Munich laboratory ran quite high in nitrogen, containing from 5 to 12 per cent (119).

Euler and Josephson (22) are inclined to look upon invertase as a complex compound, consisting of a soluble protein intimately associated with an active group. Although the latter constitutes the seat of the catalytic properties of the enzyme, still the protein part plays a significant rôle in the way it influences the specificity as well as the activity of the enzyme. Some of the evidence which they offer as support for their view is as follows: proportionality between the nitrogen content and the activity (21); the presence of tryptophan and sulfur in their preparations (22); the acid and base dissociation constants, K_a and K_b, suggesting an isoelectric point like that of most proteins (pH = 4.5 to 5); molecular weight of about 20,000 (by diffusion) (27); and the inactivation of invertase by proteolytic enzymes like trypsin (22).

Willstätter (107) regards the above evidence put forth by Euler and Josephson as not convincing. In his laboratory they claim to have prepared invertases, not only free from tryptophan but likewise practically free from phosphorus, on the presence of both of which Euler and Josephson place so much weight. Nei-

ther have Willstätter and his students found any parallelism between nitrogen content and activity, in fact, they claim to have obtained invertase preparations free from protein and others free from yeast gum. Their concept (121, 122) of yeast invertase is that it consists of a catalytically active group, of still unknown constitution, adsorbed or at least loosely bound to a colloidal carrier. The carrier need not always be the same substance, but several of the substances, such as proteins, peptides, gums, etc., which usually accompany the enzyme in its preparations may play this part. The colloidal carrier (116) does not determine how the enzyme acts catalytically and is therefore different from Euler's concept of the enzyme, but the destruction of the carrier leaves the otherwise active group inactive. From this point of view, Willstätter and his students interpret Euler and Josephson's inactivation of invertase by trypsin as due to the latter breaking down a protein carrier.

One of the classical methods for obtaining naturally occurring substances in the pure state is crystallization. This method also has been applied to enzymes, and the first crystallized enzyme was obtained by Sumner (97) when he succeeded in isolating from jack-bean meal a crystalline globulin having 730 times more activity than the purest preparation of urease obtained up to that time.

Since then Northrop (85) has crystallized pepsin in large quantities from solutions of commercial pepsin preparations, and lately, together with Kunitz (87), has also isolated trypsin in the crystalline state. Recently Caldwell, Booher, and Sherman (9) reported the crystallization of pancreatic amylase. So far, however, yeast invertase has not been obtained in the crystalline form.

The question naturally arises as to whether the crystalline enzyme preparations are the enzymes in the pure form. If they are, then, of course, these enzymes at least are proteins. Willstätter (109) who claims, as has been mentioned above, that enzymes are made up of a catalytically active group, usually associated with a protein as a carrier, points out that all the crystalline enzymes obtained so far were obtained by using methods for crystallizing proteins, and hence one would expect the crystals

to be protein. Both Northrop and Sumner admit the possibility of active groups being attached to their crystalline protein preparations, or of crystalline proteins having an active prosthetic group, like the porphyrin group in hemoglobin. But Northrop emphasizes the point that if the enzyme consists of a protein and an active group, then these two are held together chemically and not by adsorption as claimed by Willstätter (108). That crystalline pepsin is a single chemical individual and not a mixture, Northrop has shown by the fact that repeated crystallization has no influence on the optical activity of the product or on the composition or the degree of activity. He has also examined the solubility of the crystalline enzyme in a series of different salt solutions and in every case the material behaved as a single compound. As still further evidence for the protein nature of pepsin, Northrop partly denatured the protein and found the extent of denaturation and decrease in activity paralleled each other. He also found that after allowing the denatured protein to revert to its native state, according to the method of Anson and Mirsky (2), the activity returned and the recovered enzyme was indistinguishable from the original crystalline pepsin.

Waldschmidt-Leitz and Steigerwaldt (102) claim to have hydrolyzed crystalline urease by means of trypsin, until the solution no longer gave a precipitate with sulfosalicylic acid, without impairing the enzyme's activity. Sumner (99), on the other hand, finds that trypsin only attacks the protein in crystalline urease after it has been denatured, and the enzyme is no longer active.

Another point indicating enzymes to be proteins is the claim made by several investigators that enzymes possess antigenic properties. Lüers and Albrecht (62) state that they have obtained a serum from rabbits, previously injected with malt amylase preparations, which exerted considerable retarding effect on the activity of this enzyme. Recently Sumner and Kirk (98) have succeeded in preparing a serum which strongly inhibited the action of urease. Matsuoka (63) claims similar results in the case of yeast invertase.

The various methods for isolating invertase from yeast, the

methods for securing highly active preparations, and the determination of the compositions of these active preparations, thus far described, may be looked upon as a more or less direct approach to the unravelling of the chemical nature of the enzyme. In contrast to this direct method of attack, a sort of indirect one based on the kinetics of the hydrolysis reaction has also received considerable attention. Conditions such as the pH of the solution in which the enzyme is acting, the concentrations of sucrose and invert sugar, and the presence of other substances, all have a bearing on the magnitude of the activity of the enzyme.

Sörensen (95) was among the first to examine, in a quantitative way, the relation between the activity of invertase and the pH of the solution. Unfortunately his experiments were carried on at such a high temperature (52.5°C.) that the results obtained may possibly have been influenced by some destruction of the enzyme. Since then Michaelis and Davidsohn (69) have repeated Sörensen's experiments, but at room temperature. The relationship which they found to exist between activity and pH is illustrated graphically in figure 1. The curve shows that the enzyme manifests a maximum activity at about pH = 4.5 and that the velocity of hydrolysis decreases both on the more acid and more alkaline sides of this optimum pH. The portion of the curve on the more alkaline side, pH = 4.5 to 8, resembles the shape of the ionization curve of a weak acid, having as its ordinates the fraction of the acid remaining unionized or $(1 - \alpha)$, and as abscissae the pH of the solution. This, as Michaelis and Davidsohn point out, suggests that within this pH range invertase behaves as though it were a weak acid ionizing as

$$\text{Invertase acid} \rightleftarrows \text{invertase anion and } H^+$$

and that only the unionized invertase is capable of catalyzing the hydrolysis of sucrose. Similarly, the portion of the curve corresponding to the more acid range, pH = 2 to 4.5, resembles the ionization curve of a weak base and has led Michaelis and Davidsohn to claim that invertase is amphoteric.

This amphoteric behavior is very much in line with the claim that invertase is a protein with an isoelectric point in the neigh-

borhood of pH = 4.5, the isoelectric point common to many proteins. Too much weight, however, must not be attached to the shape of the more acid branch of the curve in figure 1, because invertase is very easily inactivated when the acidity of the hydrolyzing sucrose solution is greater than pH = 4.0. This makes the experimental error in this region considerable (75), and it

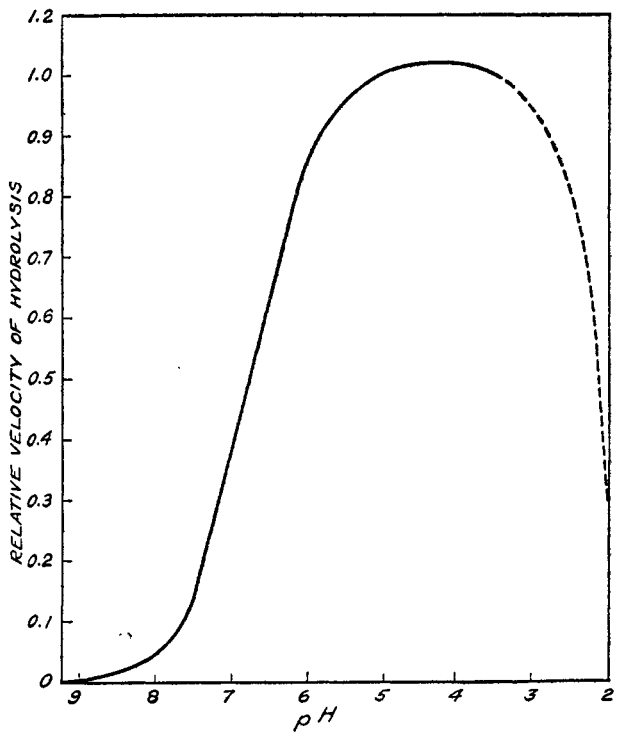

FIG. 1. RELATIONSHIP BETWEEN THE pH AND ACTIVITY OF A YEAST INVERTASE PREPARATION

Michaelis and Davidsohn: Biochem. Z. **35**, 405 (1911)

will be observed that Michaelis and Davidsohn represented the acid branch of the curve by a dotted line.

There was a great deal of speculation by the earlier investigators as to the way in which invertase acted on the sucrose molecule. In 1902 Adrian Brown (8) hydrolyzed, using a constant amount of enzyme in equal volumes, a series of sucrose solutions, ranging in concentration from 5 to 40 per cent, and

found that the amount of sugar inverted per unit time was practically constant, irrespective of the sucrose concentration. This constancy in the quantity of sucrose hydrolyzed led him to formulate the hypothesis that the invertase combines with sucrose according to the simple mass-law principle, i.e., molecule for molecule, to form an invertase–sucrose compound, and that the velocity of hydrolysis is proportional to the concentration of the compound.

Since then, Michaelis and Menten (68) and others (73) have found that in the case of sucrose solutions below 5 per cent the velocity increases gradually as the sugar concentration is increased, and reaches a maximum value when the concentration is about 5 per cent. Looking at this gradual increase in the rate of hydrolysis from the standpoint of Brown's hypothesis, it appears that the equilibrium point of the reaction

$$\text{Sucrose} + \text{invertase} \rightleftarrows \text{sucrose–invertase compound} \qquad (1)$$

is such that below 5 per cent sucrose part of the invertase is present in the solution in the free condition. It is, of course, impossible to determine directly the relative amounts of the enzyme present in the solution as combined and free enzyme. Therefore, instead of trying to test experimentally the mass-law expression,

$$\frac{\varphi}{\phi} = \frac{S}{S + K_s} \qquad (2)$$

in which φ and ϕ represent respectively the combined and total invertase concentrations,[2] S the concentration of free sucrose, and K_s the equilibrium constant, Michaelis and Menten had the clever idea of using relative initial velocities as the measure of the first term in equation 2.

$$\text{Relative initial velocity} = \frac{S}{S + K_s} \qquad (3)$$

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[2] The term $\frac{\varphi}{\phi}$ in equation 2 represents the fraction of the enzyme combined with sucrose. Since the velocity of hydrolysis is proportional to the concentration of the combined enzyme, it follows that the maximum velocity occurs when $\frac{\varphi}{\phi} = 1$. Therefore, putting the velocities corresponding to a series of dilute solutions, all

Figure 2 contains a comparison of the Michaelis and Menten velocity measurements (○) with the theoretical mass-law curve (full-line curve), derived by substituting different values for S in the second member of equation 2 and using the proper value for the equilibrium constant K_s. The agreement between experiment and theory is quite satisfactory.

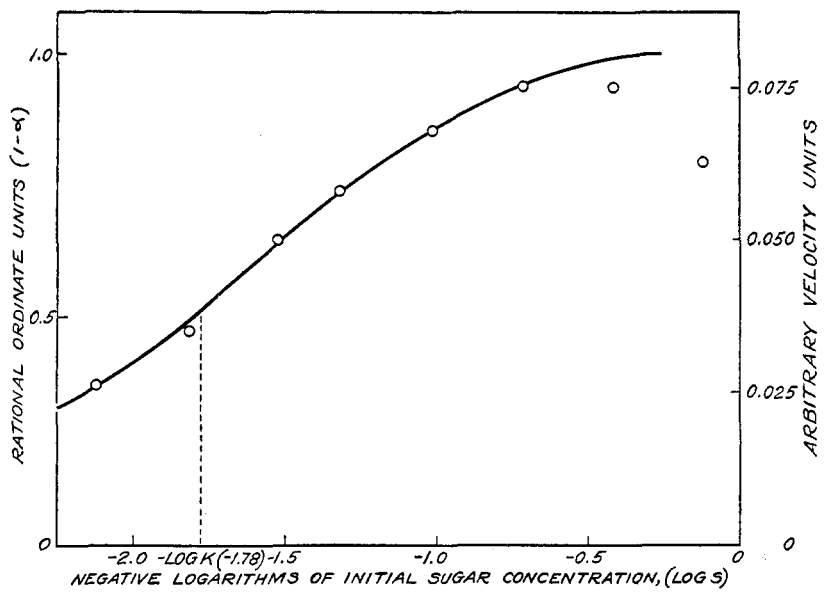

FIG. 2. COMPARISON OF THE MASS-LAW CURVE WITH THE CHANGE IN ACTIVITY OF YEAST INVERTASE AS THE CONCENTRATION OF SUCROSE IS VARIED

Michaelis and Menten: Biochem. Z. **49**, 333 (1913)

Two other points arising from Michaelis and Menten's work also should be mentioned. It is evident that when the first term

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containing the same concentration of enzyme but varying concentrations of sucrose, on a relative basis with the maximum velocity equal to 1, then these relative velocity values can be substituted for the term $\frac{\varphi}{\phi}$ in equation 2. Initial velocities were used because at the beginning of the reaction, practically no sucrose has been hydrolyzed. This, coupled with the assumption that the concentration of invertase is negligible compared to that of sucrose, makes it possible to use the original concentration of sucrose to represent S, the concentration of uncombined sucrose.

in equation 3 is equal to 1/2, then S equals K_s. This means that the concentration of sucrose corresponding to half the maximum velocity of hydrolysis is numerically equal to the dissociation constant of the sucrose–invertase compound. In other words, here is a method for determining the affinity of the enzyme for sucrose. The second point relates to retardation. The presence of any foreign material in the hydrolyzing sucrose solution is apt to affect the activity of invertase. Even the invert sugar formed during the course of the hydrolysis slows up the velocity and hence makes the kinetics of this type of inversion different from that brought about by mineral acids. Michaelis and Menten also examined the relationship between the extent of retardation caused by fructose and glucose and the concentration of the retardant and reached the conclusion that these sugars retard by also combining with the invertase, thereby leaving less of the latter free to unite with the sucrose.

All retardants, however, do not affect the enzyme's activity in the way glucose and fructose do. Thus Michaelis and Pechstein (70) claim that substances such as ethyl alcohol and α-methylglucoside affect the rate at which the sucrose–invertase compound breaks down into invert sugar and free enzyme. In other words, the action of invertase can be retarded in either of two ways,—the retardant may compete with sucrose for the enzyme, as for example glucose and fructose do, or it may affect the medium in such a way that the sucrose–invertase compound breaks down more slowly, as alcohol and α-methylglucoside do. Haldane (37) has suggested the descriptive names, "competitive" and "non-competitive," for these two types.

Michaelis' publications on the kinetics of invertase action served as a great stimulus for further researches along these lines. Euler (24), commenting on Michaelis' work, states: "Michaelis, as is well known, was the first to determine the affinity constant of an enzyme for its substrate as well as for its cleavage products, and on the basis of these measurements he has developed a theory for the kinetics of enzyme action which, together with certain modifications, constitutes one of the fundamental principles upon which scientific enzyme studies rest."

Among the first to apply the Michaelis-Menten method for determining the affinity of an invertase preparation for sucrose were Euler and Laurin (20). The interesting outcome of their measurements was the fact that their enzyme preparation, from a Swedish brewery yeast, gave a higher value, $K_s = 0.025$, instead of 0.0167 as reported by Michaelis and Menten. Shortly afterwards, Kuhn (52), working in Willstätter's laboratory, measured the dissociation constants, K_s, for a series of invertase preparations. Some of these preparations were obtained from the same yeast, but differed from each other in the degree of purification to which they had been subjected. Other preparations were prepared from different strains of yeast. Some of Kuhn's values

TABLE 1

Values of K_s for a series of invertase preparations

ENZYME PREPARATION	DEGREE OF PURITY TIME-VALUES	K_s
	minutes	
Distillery yeast, Race 12	Very crude	0.016
American brewery yeast	6.3	0.020
Lion Brewery yeast	180.0	0.029
Highly purified preparation from Lion Brewery yeast	0.85	0.029
Preparation from another Lion Brewery yeast	0.20	0.040

are given in table 1, and it will be noted that purification appears to have no effect on the value of the constant. On the other hand, the values for K_s for preparations from different yeasts varied among themselves quite widely.

The fact that the degree of purification seems to be without influence on the value of the dissociation constant, Kuhn points out, constitutes further support for Willstätter's claim that the invertase particle consists of a colloidal carrier to which a catalytically active group is attached, and that the colloidal condition of the carrier, its composition, etc., have no influence on the catalytic properties of the active group. He also points out that for analytical purposes, such as the quantitative estimation of invertase units, etc., which depend on velocity measurements,

the value of the dissociation constant is an important factor, because the size of the constant determines the concentration of the sucrose–invertase compound upon which the velocity of hydrolysis depends. For this reason Willstätter and Kuhn (114) recommend that the value of the dissociation constant of the sucrose–invertase compound be included in the description of invertase preparations.

The Michaelis method for determining the dissociation constant, K_s, or its reciprocal, the affinity constant, has been widely used by the European chemists, especially Euler and Willstätter and their respective collaborators, as a guide in their studies of specificity and the classification of enzymes. The experiences of the present writer and his coworkers, however, have made them hesitate in adopting the method in such a whole-hearted way. In the first place, Michaelis and Rothstein (71) found that the two relationships, the pH-activity curve of Michaelis and Davidson (figure 1) and the ps-activity[3] curve of Michaelis and Menten (figure 2) can not be reconciled. Expressing (69) the two relationships, by letting ψ, ϕ, ϕ^- and φ represent total, unionized, ionized, and combined invertase, respectively, and S the concentration of uncombined sucrose, then

$$\frac{\phi^- \cdot H^+}{\phi} = K_H, \quad \frac{\phi \cdot S}{\varphi} = K_s, \quad \text{and } \psi = \phi + \phi^- + \varphi$$

and

$$\text{Relative initial velocity} = \frac{\varphi}{\psi} = \frac{1}{1 + \frac{K_s}{S} + \frac{K_s K_H}{SH}} \tag{4}$$

According to equation 4, if the Michaelis-Davidsohn pH-activity curve were determined at several different concentrations of sucrose, a family of curves should result, distributed along the pH axis in figure 1. Michaelis and Rothstein found this not to

[3] In the case of the curve in figure 2, the negative logarithm of the sucrose concentration is plotted against velocity, just as in the case of the pH-activity curve in figure 1 the negative logarithm of the hydrogen-ion concentration is plotted against the velocity. And just as the latter is often termed the pH-activity curve, so the former is often called the ps-activity curve.

be the case, but instead the same curve resulted, irrespective of the sucrose concentration employed. This obviously means that the pH-activity curve is independent of the sucrose concentration. Similarly, looking at Michaelis and Rothstein's results from the standpoint of the pH-activity curve, the latter turns out to be independent of the hydrogen-ion concentration. On the strength of Michaelis and Rothstein's work, Kuhn (52) suggests that the pH of the hydrolyzing sucrose solution has nothing to do with the reaction involved in the union of the sucrose and invertase, but that it influences the rate at which the sucrose–invertase compound breaks down into invert sugar and enzyme.

Contrary to the findings of Michaelis and Rothstein, A. H. Palmer in this laboratory (unpublished observations) finds that the ps-activity curve (figure 2) is influenced by pH, especially when different buffers are employed to regulate the pH of the hydrolyzing sucrose solutions. For example, the curve resulting when sodium citrate is the buffer has a different slope from the curve resulting, at the same pH, when sodium phosphate is used. Larson (59, 79), using citrates and phosphates and a series of sucrose solutions of different pH values, was unable, in many instances, to find any close agreement between the experimental and theoretical ps-activity curves.

If the reader will look at the ps-activity curves in figure 2, he will notice that the velocity of hydrolysis commences to decrease when the concentration of sucrose exceeds 5 per cent ($\log S = -0.84$). Other curves of the same kind in the Michaelis and Menten paper (68) show even greater deviation from the theoretical (full-line curve in figure 2) than the one shown in figure 2. Michaelis (66) suggests that this discrepancy between the experimental data and the theory may be due to the mass-law no longer holding rigidly in the more concentrated sugar solutions. It must be borne in mind, however, that water is also a reactant in the hydrolysis of sucrose, and the Brown-Michaelis theory does not take it into account. Curve 1 in figure 3 shows the change in the velocity of hydrolysis of sucrose by yeast invertase as the concentration of sucrose is increased up to 70 per cent. Its shape suggests that between 5 and 70 per cent sucrose the velocity is

some simple function of the sugar concentration. But as Ingersoll (43, 44) has pointed out, the concentration of water also drops off linearly with the increase in sucrose concentration, and when the velocity is plotted against the concentration of water, curve 1 in figure 4 results. Since these two curves are so similar in shape, Ingersoll was unable to decide whether it is the concentration of sucrose or that of water which determines the magnitude of the velocity of hydrolysis. Nelson and Schubert (81), however, found it possible to vary the concentrations of water and sucrose independently of each other by adding given quantities of alcohol to the hydrolyzing sucrose solutions. Their results are shown graphically by means of curves 2 and 3 in figures 3 and 4. As has already been stated, the curves marked 1 in figures 3 and 4 represent the velocities corresponding to different concentrations of sucrose and water respectively, while curves 2 and 3 represent the velocities when part of the sucrose has been replaced by alcohol. It will be noticed that when the velocities are plotted against water concentration, then the curves 2 and 3 in figure 4 lie very close to curve 1 for sucrose alone; on the other hand, when the velocities are plotted against the sucrose concentration, curves 2 and 3 in figure 3, then they are spread farther apart. Since the alcohol curves, 2 and 3 in figure 4, fall so close to curve 1, for sucrose alone, it becomes evident that the velocities are nearly the same, irrespective of the particular amount of alcohol and sucrose which the solution contains, provided that the concentration of water is the same. In other words, the concentration of water is a primary factor in determining the magnitude of the velocity of hydrolysis when the concentration of sucrose is large, and the concentration of sucrose is only of minor significance.

Since the concentration of water exerts such a dominating influence on the velocity in the case of solutions containing more than 5 per cent of sucrose, it would not be surprising to find this influence to extend to solutions containing less than 5 per cent of sucrose, but gradually diminishing as the sugar solutions became more and more dilute. If there is an influence due to the water concentration in the region below 5 per cent of sucrose, then obviously the Brown hypothesis does not describe the reaction be-

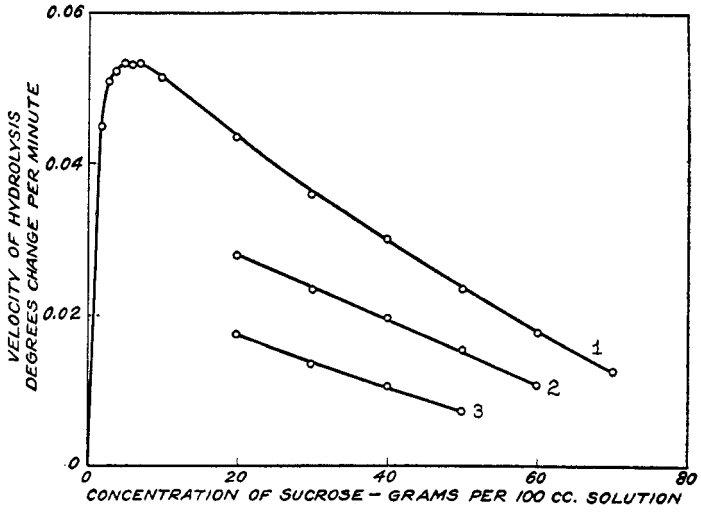

FIG. 3. CHANGE IN THE RATE OF HYDROLYSIS WITH CHANGE IN CONCENTRATION OF SUCROSE, IN THE ABSENCE AND IN THE PRESENCE OF 10 AND 20 PER CENT ETHYL ALCOHOL

Nelson and Schubert: J. Am. Chem. Soc. **50**, 2188 (1928)

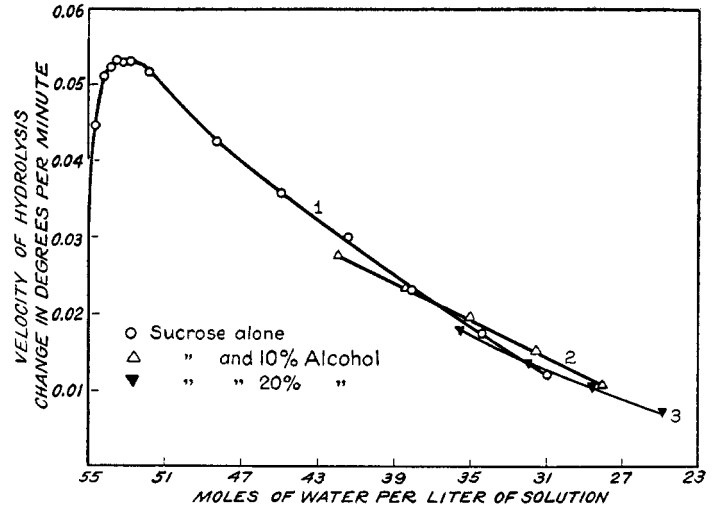

FIG. 4. THE SAME DATA AS REPRESENTED IN FIGURE 3, EXCEPT THAT THE RATE OF HYDROLYSIS HAS BEEN PLOTTED AGAINST WATER CONCENTRATION

Nelson and Schubert: J. Am. Chem. Soc. **50**, 2188 (1928)

tween the enzyme and sucrose, and the Michaelis-Menten dissociation constant loses its significance.

It is quite generally recognized that invertase, up to the present at least, must be regarded as colloidal, and hence strictly speaking, its reaction with sucrose must be heterogeneous rather than homogeneous, as assumed in the Brown-Michaelis theory. Bayliss (5), in his *Nature of Enzyme Action*, not only favored the view that the reaction of an enzyme like invertase belongs to the heterogeneous class, but he also argued that water is an important reactant in the hydrolysis of the substrate, and hence the rôle which it plays should also be taken into account in the formulation of any hypothesis as to the chemical mechanism of the reaction. "We may suppose that in the hydrolysis, for example, the substrate is brought into intimate relation with water on the surface of the particles of the enzyme. . . . . It must be understood that adsorption is merely a preliminary state, and that after it has taken place, the proper chemical reaction makes its appearance."

The idea that water as well as sucrose combines with the enzyme makes it possible to account for the fact that the velocity of hydrolysis increases as the concentration of sucrose increases up to 5 per cent and then decreases as the concentration of the sugar is increased still more, in a way different from that advocated by Michaelis. The reaction might be heterogeneous and the velocity dependent on the relative amounts of sugar and water on the surface of the enzyme particles. This idea is in line with Freundlich's idea of this type of a reaction. He (34) states: "In this group of processes the velocity will certainly depend upon the concentrations of the two adsorbed materials at the boundary surface. Now since one substance displaces the other, we have the important conclusion that the velocity, as it varies with the concentration (or amount adsorbed as the case may be) of each of the two substances, passes through a maximum."

Michaelis and Menten failed to take into account whether the different mutameric forms of glucose and fructose retard yeast invertase action to the same extent. When Willstätter and

Kuhn (112, 113, 53) compared these different forms in this respect they found that they were quite different in their inhibiting influences. They also found that invertases prepared from material other than yeast were retarded differently by these mutamers. An invertase preparation from a Munich brewery yeast was not retarded at all by α-glucose, but was by the β-mutamer and by mutarotated fructose. On the other hand, an invertase from *Aspergillus oryzae* (taka-diastase) showed quite the opposite effect (54).

	Yeast Invertase	*Taka-invertase*
α-Glucose	No retardation	Retarded
β-Glucose	Retarded	No retardation
$\alpha\beta$-Fructose	Retarded	No retardation

These differences in the retardation of the activities of the invertases have been interpreted by Willstätter and Kuhn to mean that yeast invertase possesses no affinity for the α-glucose, and that taka-invertase can not combine with fructose but only with α-glucose. Furthermore, when an invertase combines with sucrose to form the sucrose–invertase compound, it does not attach itself to the sucrose molecule as a whole, but either to the glucose or the fructose end of the disaccharide molecule, depending on whether, as they have termed, it is a *gluco-* or a *fructo-*invertase. Yeast invertase, which is retarded by fructose, is a fructo-invertase and attaches itself to the fructose part of sucrose, while taka-invertase is a gluco-invertase and forms the sucrose-invertase compound by attaching itself to the glucose end.

As further support for this classification of invertases, Kuhn (55, 56) points out that all the higher saccharides such as the following,

Sucrose	Glucose <> fructose
Gentianose	Glucose < glucose <> fructose
Raffinose	Galactose < glucose <> fructose
Stachyose	Galactose < galactose < glucose <> fructose
Hesperonal	Phosphoric acid < glucose <> fructose

which not only have sucrose as a component but in which the fructose is present in the molecule in the same condition as in sucrose itself, are hydrolyzed by yeast invertase. On the other

hand, they found these modified sucrose sugars to be unaffected by taka-invertase. This they argued, is to be expected, because in each case the glucose end of the sucrose molecule has been modified by having attached to it some other group, such as a second glucose, a galactose, etc., leaving thereby no α-glucosidic group for a gluco-invertase, like taka-invertase, to combine with. They also point out (57) that the trisaccharide, melezitose (glucose $<>$ fructose $>$ glucose) is hydrolyzed by three different gluco-invertases, taka-invertase, and invertase preparations from *Aspergillus niger*, and from *Penicillium glaucum*, but not by yeast invertase, because in this case the fructoside group necessary for a fructo-invertase is absent.

Euler and Josephson (24) found, contrary to the German investigators, that an invertase preparation from a Swedish brewery yeast was retarded by α-glucose, as well as by β-glucose and $\alpha\beta$-fructose. This has led Kuhn and Münch (55) to repeat their experiments, using this time not only a sample of the Swedish enzyme preparation but also preparations from several other yeasts. Not only did their results turn out to agree with those of the Stockholm chemists, as far as the Swedish invertase preparation was concerned, but they also found that several of the other invertases were retarded by α-glucose. But by applying the Michaelis criteria[4] for deciding the kind of retardation, Kuhn

[4] Putting ϕ, φ and ψ to represent respectively the concentrations of total invertase, invertase combined with sucrose, and invertase combined with retardant, and S and R the concentrations of free sucrose and free retardant, then for a competitive retardant

$$S(\phi - \varphi - \psi) = K_S\varphi$$
$$R(\phi - \varphi - \psi) = K_R\psi$$

or

$$\frac{K_R}{K_S} = \frac{R}{S\left(\frac{\phi}{\varphi} - 1\right) - K_S} \tag{5}$$

Since the velocity of hydrolysis is assumed to be proportional to the concentration of the sucrose–invertase complex, if φ_0 represents the concentration of the complex in the absence, and φ the concentration in the presence of the retardant, and v_0 and V the respective velocities, then

$$\varphi_0 : \varphi = v_0 : V \tag{6}$$

(*Footnote continued on following page*)

and Münch (56) claim that the retardations by α-glucose, for all of the yeast invertase preparations affected by the hexose, were non-competitive in character and therefore different from the retardations by fructose, all of which were found to be competitive.

Nelson and Anderson (1, 78) also studied the retardation of yeast invertase action by the mutameric forms of glucose and fructose. In order to retain the hexoses in their original mutameric form as much as possible during the course of the hydrolysis, they conducted the hydrolysis at practically zero degrees.

Upon inspection of figure 5, it can be seen that the retardation of α-glucose is different from the others, and therefore, in this respect, agrees with the observations of Kuhn and Münch. But the experimental data, in the case of α-glucose, contrary to those of Kuhn and Münch, did not conform to the requirement for a non-competitive retardant, nor did the data for β-glucose and β- and $\alpha\beta$-fructose conform to the specifications for a competitive retardant (1). In other words, these results did not fall in line with the gluco-fructo-invertase theory.

Another interesting case of the influence of β-glucose on the activity of an invertase has been observed by Nelson and Sottery (80). The invertase occurring in honey does not hydrolyze raffinose, is only slightly retarded by α-methylglucoside, and hence in these two respects resembles taka-invertase (38). Instead of not being retarded by β-glucose, as Kuhn had observed in the case of taka-invertase, it actually, when present in small con-

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and, in the absence of the retardant

$$\frac{\varphi_0}{\phi} = \frac{S}{S + K_S} \qquad (7)$$

(see equation 3)

By means of equations 6 and 7, equation 5 may be written

$$\frac{K_R}{K_S} = \frac{R}{\left(S + K_S\right)\left(\frac{v_0}{V} - 1\right)} \qquad (8)$$

Retardation due to a competitive retardant will conform to equation 8, while retardation due to a non-competitive retardant, which only affects the rate of decomposition of the sucrose–invertase complex, gives constant values for $\frac{v_0}{V}$, irrespective of the concentration of the sucrose.

centrations, exerts an accelerating effect (see figure 6). This effect, however, depends not only on the concentration of the β-glucose but also on the concentration of the sucrose. When this hexose is present in higher concentrations, then it acts as a retardant.

A long series of papers by Euler and Josephson on the affinity of yeast invertase for different sugars has appeared during the past six years, mostly in the Zeitschrift für physiologische Chemie.

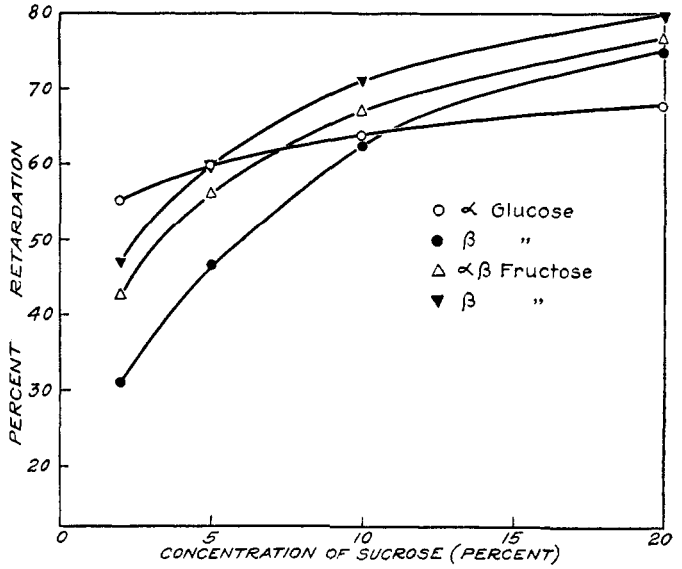
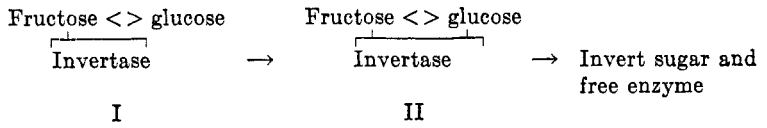


FIG. 5. PER CENT RETARDATION CAUSED BY 8 PER CENT GLUCOSE OR FRUCTOSE AT DIFFERENT CONCENTRATIONS OF SUCROSE

Nelson and Anderson: J. Biol. Chem. **69**, 443 (1926)

These investigators have proposed another theory known as the two affinities theory (14, 15, 25, 26), which, like the Willstätter and Kuhn theory, is based upon the Michaelis method for determining the affinity of an enzyme for its substrate. In this theory, they argue that invertase has two kinds of affinities, distinctly different in character. The one, which they have designated as primary, is strictly specific (in the case of yeast invertase) to fructose alone; while the other, a secondary sort of affinity, can

combine not only with glucose but with many other substances. Their idea may be represented as



in which the part played by the primary affinity is shown by I and that by the secondary by II. Compound I is formed at first and then gradually goes over to II. The latter is unstable,

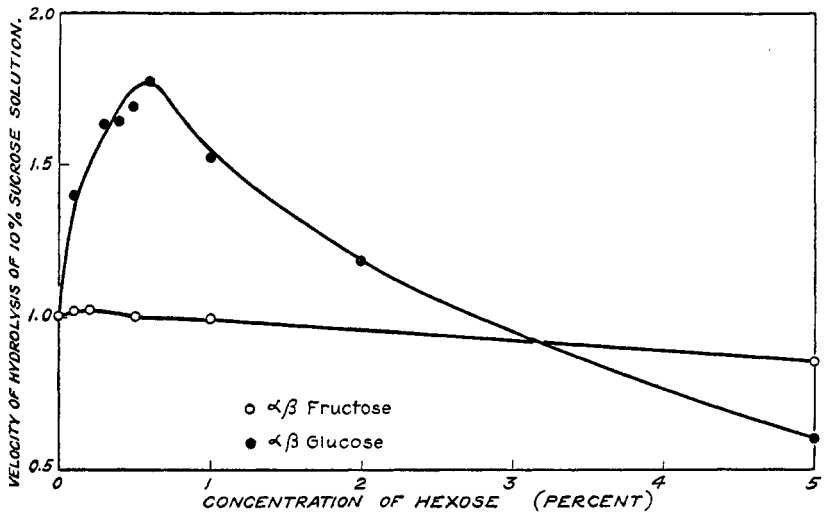


FIG. 6. INFLUENCE OF DIFFERENT CONCENTRATIONS OF GLUCOSE AND FRUCTOSE ON THE ACTIVITY OF HONEY INVERTASE

Nelson and Sottery: J. Biol. Chem. **62**, 139 (1924)

breaking down rapidly to invert sugar and free invertase, thereby causing the concentration of II to be small and the change from I to II irreversible. When a retarding substance, such as glucose, α-methylglucoside, or ethyl alcohol, is added to the hydrolyzing sucrose solution, it can unite with the less specific secondary affinity and thereby hinder the formation of the intermediate compound, II, upon the concentration of which the rate of hydrolysis of the sucrose depends (26). If a substance other than

glucose, in the form in which it occurs in the sucrose molecule, happens to be attached to the secondary affinity, it may influence the magnitude even of the primary affinity in such a way as to lower the concentration of I or the rate at which I goes over to II, and in this way exert a retarding influence (25).

The Kuhn theory that there are two types of invertase, based chiefly upon the fact that his taka-invertase preparations fail to hydrolyze raffinose and that this enzyme is retarded by α-glucose, has been threatened by several recent observations. One of the first of these observations occurred in Kuhn's own laboratory, when Miss Rohdewald (56) incidentally encountered a taka-invertase preparation which did hydrolyze raffinose. This was followed by Schlubach and Rauchalles (92) succeeding in hydrolyzing, by means of taka-invertase preparations, both the α- and β- forms of *h*-methylfructoside. Leibowitz and Mechlinski (60), repeating the Rohdewald experiment, reached the conclusion that taka-invertase preparations sometimes contained melibiase, and that the hydrolysis of the raffinose was due to the latter enzyme breaking down the trisaccharide into galactose and sucrose, and the sucrose, thus set free, then being hydrolyzed by the taka-invertase. Their explanation, however, appears to be incorrect, because Weidenhagen (104) since then has succeeded in hydrolyzing raffinose by a taka-invertase preparation which was free of melibiase. Besides showing that taka-invertase, contrary to Kuhn and Willstätter, does hydrolyze raffinose, Weidenhagen (104) has demonstrated that one of his preparations was twice as efficient as yeast invertase in catalyzing the hydrolysis of raffinose. Similarly, the classification of invertases on the basis of whether their activity is retarded by α-glucose or fructose has become doubtful. For example, Weidenhagen (104) has shown that the invertase from *Penicillium glaucum*, which Euler, Josephson, and Söderling (28) classified on the basis of retardation experiments as a gluco-invertase, hydrolyzes raffinose and hence can not be so classified. But one of the most interesting observations made by Weidenhagen (103) is the hydrolysis of sucrose by maltase, which has always been regarded as inactive towards this sugar. The reason why this has not been observed earlier is that

the pH-optimum of yeast maltase is in the neighborhood of 7, and most sucrose hydrolyses have been run in the pH range 3 to 6, the range most suitable for yeast invertase.

On the strength of observations like those mentioned above, Weidenhagen (103) claims that Kuhn's classification of the invertases into two groups, gluco- and fructo- invertases, depending on their retardation by glucose and fructose, is not justified. The carbohydrases should be classified according to the particular type of glycoside which they hydrolyse. α-*n*-Glucosides are hydrolyzed by α-*n*-glucosidase and β-galactosides, like lactose, are hydrolyzed by β-galactosidase, etc.

α-Glucosidase.....	α-Methylglucoside, maltose, sucrose, melezitose
β-Glucosidase.....	β-Methylglucoside, gentiobiose, cellobiose
α-Galactosidase....	α-Galactosides, melibiose, raffinose
β-Galactosidase....	β-Galactosides, lactose
β-*h*-Fructosidase...	β-*h*-Methylfructoside, sucrose, raffinose, gentianose

According to Weidenhagen, yeast and taka-invertase preparations consist chiefly of β-*h*-fructosidase; maltase preparations, whether prepared from yeast or malt extract are mainly α-*n*-glucosidase; while emulsin contains β-*n*-glucosidase, etc. Frequently preparations like these are mixtures of glycosidases. Crude yeast invertases contain often considerable α-*n*-glucosidase mixed with the β-*h*-fructosidase. Some taka-invertase preparations hydrolyze melezitose, owing to the presence of α-*n*-glucosidase.

Sugars which differ by means of the asymmetry of their glycosidic carbon atoms, like α- and β-*n*-methylglucosidases, or sugars which differ by the asymmetry of other carbon atoms, like α-*n*-methylglucoside and α-*n*-methylgalactoside, require specific enzymes. γ-Glycosides require different enzymes from those active towards normal glycosides. Thus β-*h*-fructosidase (yeast invertase) hydrolyzes β-*h*-methylfructoside but not β-*n*-methylfructoside (58, 33, 42), and α-*n*-glucosidase (yeast maltase) hydrolyses α-*n*-methylglucoside but not α-*h*-methylglucoside. The specificity of a glycosidase is also dependent on the length of the carbon chain in a sugar. Neither α-*n*-glucosidase (yeast maltase) nor β-*n*-glucosidase (emulsin) affect the α- or β-*n*-methylxylosides, etc. (32, 47).

Weidenhagen (103) holds that there are no enzymes specific to particular disaccharides, like sucrose, maltose, etc. These sugars, being glycosides, are acted upon by their respective glycosidases. Thus, sucrose is hydrolyzed by α-*n*-glucosidase because it is an α-*n*-glucoside and by β-*h*-fructosidase because it is also a β-*h*-fructoside, while maltose, being only an α-*n*-glucoside, is only attacked by α-*n*-glucosidase. The same holds for trisaccharides.

Melezitose: α-Glucose $<>$ α-*h*-fructose $>$ α-glucose
(α-Glucosidase; α-Glucosidase)

Raffinose: α-Galactose $<$ α-glucose $<>$ β-*h*-fructose
(α-Galactosidase; β-*h*-Fructosidase)

Amygdalin: β-Glucose $<$ β-Glucose $<$ -aglycone
(β-Glucosidase (followed by β-glucosidase);)

Weidenhagen's ideas concerning the specificity of the carbohydrases has attracted considerable interest among the workers in this field, but it is too early to predict how generally they will be adopted. Already several have come forward with cases which seem to be difficult to reconcile with the theory. Karström (48) mentions an enzyme preparation from *Bacterium coli* which hydrolyzes maltose, but which he found to be without action on sucrose. According to Weidenhagen, any enzyme which hydrolyzes maltose must also hydrolyze sucrose, because hydrolyzing maltose classifies the enzyme as α-*n*-glucosidase, and sucrose being an α-*n*-glucoside should be hydrolyzed by it. Weidenhagen (103) has repeated Karström's experiments, and states that Karström's claim is incorrect, because the enzyme does hydrolyze sucrose. On the other hand, Myrbäck (72), also repeating Karström's experiments, reaches the same conclusions as Karström. Leibowitz (60) has been unable to hydrolyze α-methylglucoside by maltase obtained from malt, and Weidenhagen (103), on the basis of his own work, claims that Leibowitz used too weak a solution of the enzyme. Since then Pringsheim, Borchardt, and Loew (89) report that their maltase preparation, from malt extract, is

inactive towards α-methylglucoside. They also find that other maltases, prepared from *Aspergillus Wentii* and from *Aspergillus oryzae* (taka-diastase) are without action on α-methylglucoside. Ivanoff, Dodonowa, and Tschastuchin (45) find a maltase in mushrooms which hydrolyzes maltose but not sucrose. Mrs. Schubert, working in the writer's laboratory, has found that an invertase preparation from honey hydrolyzes maltose but seems to be without action on α-methylglucoside. Recently Willstätter (109) announced that Grassmann, working in his laboratory, has obtained experimental data which "corrects the views of Weidenhagen concerning their (carbohydrases) specificity."

Another question which has received considerable attention from workers studying carbohydrases by means of kinetics, is whether an enzyme is specific to one particular substrate, or whether it can hydrolyze several different substrates that are closely related stereochemically. For example, are all β-*n*-glucosides hydrolyzed by the same β-glucosidase, or are yeast invertase and yeast raffinase the same enzyme? According to Weidenhagen's theory, discussed above, they are the same. But certain observations have been encountered which make it difficult to dispose of the question in such a confident manner as Weidenhagen does. Raffinase, as is well known, may be looked upon as α-galactose attached to the glucose part of the sucrose molecule. Melibiase-free yeast invertase preparations, which according to Weidenhagen contain β-*h*-fructosidase, hydrolyze the β-*h*-fructosido linkages in both of these sugars. Willstätter and Kuhn (111) found, upon comparing the rates of hydrolysis of these two sugars by invertase preparations from a series of different yeasts, all *Saccharomyces cerevisiae*, that the ratios of these two rates varied. For example, the invertase in one preparation hydrolyzed sucrose 12.3 times faster than it did raffinose, while another preparation of the enzyme hydrolyzed sucrose only 5 times faster. At first Willstätter and Kuhn were inclined to look upon this variation in the ratio of the two rates as evidence for the presence of two distinctly different enzymes, the relative concentrations of which varied in the enzyme preparations obtained from the different lots of yeast. Against this idea of two distinct enzymes, however,

was their inability to alter the ratio of the two rates for any one preparation by further purification. If the enzymes are not identical, then a more or less fractional separation ought to occur upon further purification. Since then, Kuhn (52) has tried to find a satisfactory answer to this question in a different way. In determining the dissociation constant K_s, for the invertase–sucrose compound, by the Michaelis-Menten method (illustrated in figure 2), he found that this value varied with different invertase preparations. This would mean that different invertase preparations possess different tendencies to combine with sucrose. In determining the affinity values of different preparations for raffinose by determinations which seem to draw upon the imagination to some extent, Kuhn finds that these also vary. But the dissociation constants for the sucrose–invertase compounds do not necessarily parallel the corresponding dissociation constants of the enzyme–raffinose compounds. Thus, for example, two preparations of invertase may have the same affinity for sucrose, while their affinities for raffinose might vary from each other quite widely. This variation in the affinity constants would naturally give rise to variations in the respective concentrations of enzyme–substrate compounds upon which the rate of hydrolysis is supposed to depend. By correcting the velocity values so as to correspond to these variations in affinity, Kuhn finds that the ratio of the rates of hydrolysis of the two sugars remains constant, irrespective of the particular invertase preparation used, and therefore claims that yeast invertase and yeast raffinase are identical.

Josephson (46), working in Euler's laboratory, advances another argument for claiming these two enzymes to be the same. This proof, like that of Kuhn's, rests on the Michaelis-Menten method for determining the affinity constants of the substrate–enzyme and the retardant–enzyme compounds (see equation 5). He finds that both yeast invertase and yeast raffinase have the same affinity for competitive retardants like glucose and fructose, thereby demonstrating that in this respect the two enzymes behave alike.

Workers in the writer's laboratory have met with a peculiar behavior of yeast invertase, which possibly may be related to the

variation in the ratio of the rates of hydrolysis of sucrose and raffinose observed by Willstätter and Kuhn. Nelson and Papadakis (82) found on partially inactivating yeast invertase, free from melibiase, by heat, that the loss in activity was greater if measured by the decrease in velocity of hydrolysis of raffinose, than if the loss was determined by means of sucrose. For example, a preparation suffered a loss of 58 per cent in activity with respect to the hydrolysis of sucrose, while based on the change in activity towards raffinose the loss amounted to 75 per cent. In this inactivation, the active invertase remaining did not appear to be altered in kind or in its catalytic behavior. It still continued to conform to the Nelson and Hitchcock equation (74) for normal yeast invertase, and was still retarded normally by α-methylglucoside.

Certain definite conditions seem to be necessary, judging from experience so far, for bringing about this difference in degree of activity of the enzyme preparations for the two sugars. In the first place, the pH of the invertase solution, while it is being heated, must be in the neighborhood of 5.6, and secondly, the temperature of the inactivation must not be lower than 58–60°C. Inactivation of invertase solutions at pH = 4.5 showed none of the difference.

This phenomenon does not seem to be limited to yeast invertase. Simons (93), in this laboratory, has observed the same behavior on inactivating emulsin. Here again, the enzyme lost the most activity with respect to the substrate (β-methylglucoside) which it hydrolyzes at the slowest rate. An aqueous solution (pH = 7.1) of a preparation of emulsin, from almonds, was heated for 18 minutes at 60°C. The loss in activity, calculated from the decrease in the rate of hydrolysis of salicin, amounted to 77 per cent. When the loss was calculated from the decrease in the rate of hydrolysis of β-methylglucoside, it amounted to 90 per cent. So far, however, it has not been possible to interpret these results in any way that would aid in our understanding of enzymes. It is not very probable that the emulsin contained two enzymes, β-glucosidase and salicinase, which were inactivated to different extents. This would mean that emulsin, which hydrolyzes so

many of the naturally occurring glucosides, contains a large number of different β-glucosidases, which is highly improbable.

Weidenhagen (105) mentions two instances of enzyme behavior which possibly may be related to the variation in activity with respect to different substrates. The β-glucosidase in emulsin preparations hydrolyzes salicin eight times faster than it does cellobiose, while the β-glucosidase from yeast splits cellobiose faster than it does salicin. The second case is the difference between the pH-activity (figure 1) curves for the two α-glucosidases, one occurring in malt and the other in yeast autolysate. He considers this difference to be due very likely to the colloidal carrier or material accompanying the enzymes, since these might vary in kind depending on the particular source from which the enzyme is obtained.

Many attempts have been made to derive a mathematical expression for representing the course of the reaction when cane sugar is hydrolyzed by invertase, but so far none of them has met with much success (39, 3, 101, 4, 6). Since the velocity of hydrolysis is dependent not only on the concentration of sucrose, but also on the concentrations of the several mutameric hexoses formed during the progress of the reaction, the difficulties encountered in such an attempt are obvious. Even the question as to whether all invertase preparations hydrolyze sucrose in the same way, kinetically, presents itself. Nelson and Hitchcock (74) followed the course of the reaction for a series of 10 per cent sucrose solutions, hydrolyzed by eight different yeast invertase preparations. By properly adjusting the time scales of the curves, figure 7, representing these hydrolyses, they found that five of them could be made to coincide.[5] This shows that these five preparations catalyzed the hydrolysis of the sucrose in the same way. Experience since then has shown that most preparations of invertase obtained from *Saccharomyces cerevisiae*, i.e., ordinary brewer's yeast, give hydrolysis curves that coincide, and hence such preparations have been designated as normal. The degree of purification does not seem to affect this characteristic of yeast invertase.

[5] The adjustment of the time scales was necessary because the invertase preparations used were of unequal strength or activity.

In order to recognize more easily whether a preparation is normal or not, Nelson and Hitchcock made use of the empirical equation

$$t = \frac{1}{N}\left[\log \frac{100}{100 - p} + 0.002642\, p - 0.00000886\, p^2 - 0.0000001034\, p^3\right]$$

in which p represents the per cent hydrolyzed at the time t, while N is a constant, the magnitude of which depends on the activity

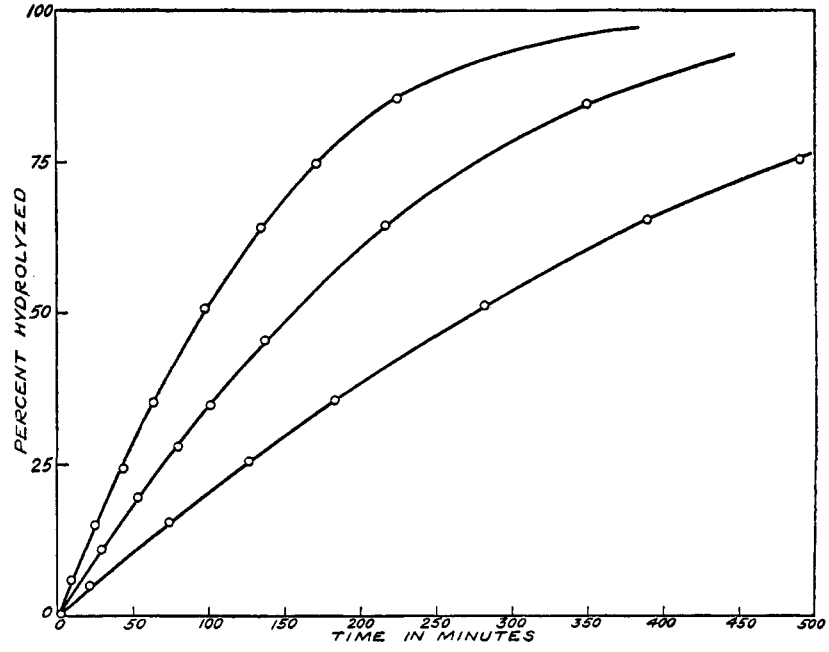


FIG. 7. PER CENT OF SUCROSE HYDROLYZED PLOTTED AGAINST TIME IN THE CASE OF NORMAL YEAST INVERTASE

Nelson and Hitchcock: J. Am. Chem. Soc. **43**, 1632 (1921)

of the preparation used. In table 2 the experimental data from two yeast invertase preparations, one normal and one abnormal, are given, as well as the corresponding values for the constant, N.

Besides the Nelson and Hitchcock equation being a characteristic property of yeast invertase, Fassnacht (29) finds in the case of normal invertase that the rate of hydrolysis of a 10 per

cent sucrose solution is lowered 73 to 80 per cent by 1 per cent of α-methylglucoside, while the retardation in the case of abnormal preparations is less, usually not over 60 per cent.

Abnormal invertase preparations, like the three observed by Nelson and Hitchcock, occur once in a while in preparing invertase from brewery yeast. Just what produces them is hard to say. Nelson and Hollander (76) found that in some instances the abnormal behavior could be attributed to instability. Invertases

TABLE 2

Data from a normal and an abnormal yeast invertase preparation

NORMAL PREPARATION*			ABNORMAL PREPARATION†		
Time	Inversion	$N \times 10^6$	Time	Inversion	$N \times 10^6$
minutes	*per cent*		*minutes*	*per cent*	
0	—	—	0	—	—
50	11.50	164	5	3.20	450
100	22.30	163	10	6.35	449
150	32.44	166	15	9.44	448
200	42.44	164	22	13.65	445
250	50.97	163	30	18.46	446
300	59.10	164	60	34.96	440
350	66.23	165	90	49.38	436
400	71.83	164	120	61.48	433
			180	78.81	431
			300	93.59	426

* Normal yeast invertase preparation. Data taken from dissertation of Miss H. Carmichael. Columbia University, 1930.

† Abnormal yeast invertase preparation. Data taken from Nelson and Hitchcock's article.

prepared from sources other than yeast do not conform to the Nelson and Hitchcock equation. In figure 8 is shown the curve (time against per cent hydrolyzed) for a 10 per cent sucrose solution hydrolyzed by an invertase preparation from honey (77). It can be seen that the first part of the curve shows an accelerating effect, which is followed by a gradual falling off in velocity as the reaction proceeds. This acceleration in the earlier part of the hydrolysis is very likely due to the influence of β-glucose mentioned on an earlier page of this paper. Weidenhagen (104) finds

that the curve (time against per cent hydrolyzed) for taka-invertase is different in shape from that of the corresponding curve for yeast invertase.

Mrs. Palmer (90) has compared, with respect to the constancy of N of the Nelson and Hitchcock equation and the extent of retardation by 1 per cent α-methylglucoside, the hydrolyses of a series of sucrose solutions by invertase preparations obtained

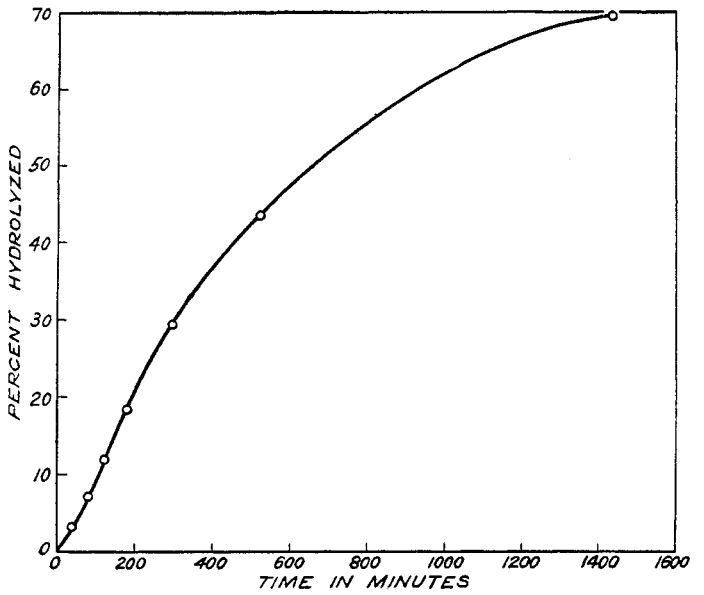


FIG. 8. PER CENT OF SUCROSE HYDROLYZED PLOTTED AGAINST TIME IN THE CASE OF HONEY INVERTASE

Nelson and Cohn: J. Biol. Chem. **61**, 193 (1927), table 3

from yeasts other than *Saccharomyces cerevisiae*, from raisins, and from taka-diastase preparations (*Aspergillus oryzae*). All of these preparations contain the enzyme (or enzymes) which, according to Weidenhagen's classification, has been termed β-*h*-fructosidase. It can be seen in table 3 that some of these hydrolyses differ quite noticeably from the hydrolysis by yeast invertase preparations (*S. cerevisiae*).

The decreases in the values for N were not nearly so pronounced for the enzyme preparations from the yeasts, *S. validus* and *Monilia* as they were for the preparations from raisins and taka-diastase (*Aspergillus oryzae*). Whether this is due to the two yeasts being more closely related genetically to the *S. cerevisiae* than the raisins or the *Aspergillus oryzae* is difficult to say.

The two characteristics of yeast invertase, its conformation to the Nelson and Hitchcock equation and the 73 to 80 per cent retardation by α-methylglucoside, are not easily affected by partially destroying the activity of the enzyme. Miss Carmichael (10) partially inactivated normal yeast invertase preparations by low concentrations of nitrous acid, but was unable to detect any

TABLE 3

Hydrolysis of sucrose solutions by various invertase preparations

	N OF THE N AND H EQUATION	RETARDATION BY α-METHYL-GLUCOSIDE
		per cent
S. cerevisiae	Constant	82
S. ellipsoidens	Constant	65
S. validus	Decreasing +	81
S. pastorianus	Constant	77
Monilia	Decreasing +	81
Raisins	Decreasing ++	Slight
Taka-invertase	Decreasing +++	6

change in these characteristics in the remaining active invertase. Likewise, when the preparations were inactivated to 60 to 75 per cent by heating at 60°C., the remaining active enzymes behave normally, at least within experimental error. Euler, Josephson, and Myrbäck (27) also report that they were unable to detect any difference in the catalytic behavior of yeast invertase preparations when these were partially inactivated by heat.

Doby (12) and coworkers have studied the effect on the invertase in *Penicillium glaucum* due to the absence of certain mineral elements in the food for the organism. They claim that the lack of potassium in the nutrient medium not only diminishes the invertase content but affects its catalytic behavior. Thus the

curve (time against per cent hydrolyzed) plotted from results obtained by hydrolyzing sucrose with the invertase prepared from the potassium-starved organism does not coincide with the corresponding curve for the enzyme preparation from the *Penicillium* grown on a complete diet. More recently (13), they find that the absence of calcium, magnesium, and phosphates also decreases the invertase content, but apparently does not influence the shape of the hydrolysis curve. The lack of potassium in the soil also, according to these investigators (11), affects the invertase in sugar beets in much the same way that it does the invertase in the *Penicillium glaucum*.

Fassnacht (29) has studied also the effect on the invertase by varying the nutrient media for yeast, by inoculating the following medium

Malt syrup, 7° Balling	3 liters
Primary ammonium phosphate	36 grams
Potassium chloride	3 grams
Magnesium sulfate	3 grams
Calcium sulfate	3 grams
Cane sugar	600 grams
Water	6 liters

with a commercial yeast, which was known to yield normal invertase preparations on autolysis. The new growth yielded preparations which behaved abnormally and were retarded only 65 per cent instead of 78 per cent by α-methylglucoside. However, when the same yeast was grown in a medium similar to the one above except that it contained 8 grams of ammonium carbonate in place of the malt syrup, an invertase preparation was obtained that was practically normal. Therefore it seems that, although it is possible to affect the catalytic behavior of yeast invertase by growing the yeast under certain conditions, we still need more data before anything definite can be said about these conditions.

So far the discussion has dealt with invertase as it exists and acts outside of the living cell. The question may be asked whether the information accumulated by the study of cell-free preparations of the enzyme also holds for the behavior of the

enzyme when it acts within the cell where conditions undoubtedly are quite different. Linderström-Lang and Holter (61) state: "It is actually only the study of the action of typically secretion enzymes, outside of the cells, that experiments *in vitro* may be considered with certainty to reproduce the processes taking place

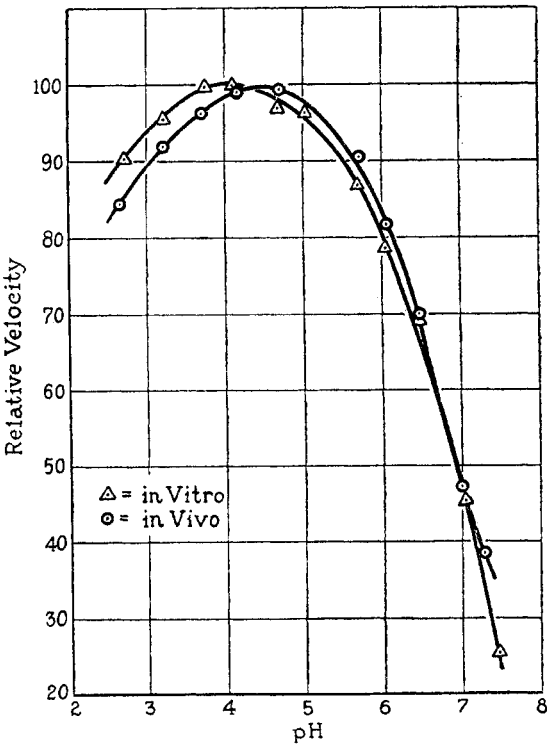


FIG. 9. COMPARISON OF THE pH-ACTIVITY RELATIONSHIPS WHEN A CELL-FREE INVERTASE PREPARATION AND LIVING YEAST CELLS WERE USED TO HYDROLYZE SUCROSE

Wilkes and Palmer: J. Gen. Physiol. **16**, 233 (1932)

in the organism." Yeast invertase is an intracellular enzyme, and is not secreted by the organism. The only way it can be separated from the cell and brought into solution is by breaking down the latter by autolytic reactions. Since, however, live yeast cells also hydrolyze sucrose, it is possible to study the kinetics of the enzyme's activity *in vivo*.

As early as 1911, Euler and Kullberg (17) attempted to compare the hydrolysis of sucrose by live cells with the reaction brought about by yeast invertase preparations, using the monomolecular constants as a measure of the enzyme's activity. Similar, though not identical, results were obtained in the two cases. Recently, Nelson, Palmer, and Wilkes (83) have compared the two reactions

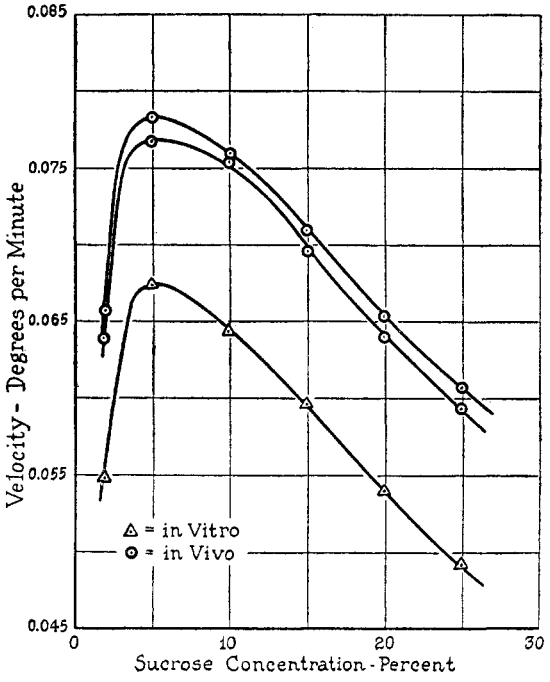


FIG. 10. COMPARISON OF THE CHANGES IN ACTIVITY WITH CHANGES IN SUCROSE CONCENTRATION WHEN A CELL-FREE INVERTASE PREPARATION AND LIVING YEAST CELLS WERE USED TO HYDROLYZE SUCROSE

by means of the Nelson and Hitchcock equation and find that the hydrolysis by the live cells also gives constant values for N, just as in the case of the cell-free preparations. In other words, as far as the course of the hydrolytic reaction is concerned, yeast invertase hydrolyzes sucrose in the same way whether it occurs in the live yeast cell or is first removed from the cell by autolysis.

The kinetics of the hydrolysis of sucrose by live cells has also

been compared (106, 84), in this laboratory, with that by invertase preparations in two other respects, viz., the pH-activity relationship and the influence of varying concentrations of sucrose and water. The results obtained are shown graphically in figures 9 and 10. In both of these comparisons it is evident that it makes practically no difference whether the enzyme is acting *in vivo* or *in vitro*; the results are the same, as is shown by the similarity of the corresponding curves.

These similarities in behavior between the invertase in the live cell and the invertase as it occurs in the cell-free preparations, brings out the interesting question as to just where in the living cell the active enzyme is located. If we regard the structure of the yeast cells as consisting of a central fluid, the cell sap, contained in various vacuoles, surrounded by a cytoplasmic layer containing protein bodies and the cell nucleus, and bound externally by a cellulosic wall, we define at least four distinct regions in which the enzyme may act, viz., in the cell sap, in the surrounding cytoplasmic layer, at the external surface of the cytoplasm, or on the inner or outer surfaces of the cell wall. According to Small (94) one would expect the pH of the cytoplasmic layer to remain fairly constant, not varying over a wider range than 1 unit in the pH scale. The results indicated in figure 9 would therefore eliminate this part of the cell as the seat of the invertase activity. The fact that when a given weight of yeast cells is placed in hypertonic sugar solutions, the weight of the yeast decreases as the sucrose concentration increases, indicates plasmolysis (35). The latter is therefore an argument against the sugar and water concentrations within the cell sap being the same as those in the outside medium in which the yeast cells are suspended. Hence the results indicated in figure 10 exclude the cell sap as being the place where the enzyme is acting on the sucrose. The elimination of the cell sap and the cytoplasmic layer leaves only the external surface of the cytoplasm or the inner or outer surfaces of the cell wall as the possible seat of the active invertase. In other words, the active invertase in the yeast cell is very likely located close to the outer surface of the cell.

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