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Some Enzymes Present in Highly Purified Invertase Preparations; a Contribution to the Study of Fructofuranosidases, Galactosidases, Glucosidases and Mannosidases

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In a preceding publication[1] from this Laboratory new methods have been described for the purification of invertase and for the preparation of highly active invertase solutions. The present communication describes the behavior of several of these yeast invertase preparations toward a series of substrates containing the β-D-fructofuranoside linkage which occurs in sucrose, and also toward a number of substrates containing other types of glycosidic linkages.

All the invertase preparations were capable of hydrolyzing all available β-D-fructofuranosides except melezitose, which is generally assumed to belong in this class although definite proof is lacking. The preparations from brewers' yeast contained also considerable amounts of an α-D-galactosidase; advantage was taken of this fact to examine a series of α-D-galactosides, and a pentoside and a heptoside with similar configurations. No evidence could be found to indicate the presence of an α-D-fructofuranosidase, a β-D-galactosidase, an α-D-glucosidase or an α-D-mannosidase in any of the invertase preparations. A small amount of a β-D-glucosidase was present in some of the preparations, and a new enzyme, a β-D-mannosidase which is capable of hydrolyzing β-phenyl-D-mannoside, was detected in preparations from both brewers' and bakers' yeasts.

Methods

Enzyme Solutions.—The five enzyme preparations which were used in this investigation are described briefly in Table I. These preparations do not represent the maximal purification possible by the methods indicated, but were selected in order to study the influence of the source of the starting material, the procedure for purification, and the extent of purification upon the relative rates of hydrolysis of different substrates.

TABLE I

YEAST INVERTASE PREPARATIONS

Source and designation	Type of purification[a]	Time value,[a] min.	Concentration,[a] units/100 ml.
Brewers' A[b]	Bentonite, picric acid	0.154	151
Brewers' B[c]	Acetic acid, ammonium sulfate	246	43.5
Brewers' C[c]	Bentonite, ammonium sulfate	172	131
Bakers' A[d]	Bentonite, picric acid	.181	166
Bakers' B[d]	Acetic acid, acetone	.193	68.4

[a] The types of purification, based upon adsorption on bentonite and upon precipitation with the other reagents, and the units for expressing the purity and concentration of the invertase preparations, have been defined in the preceding publication (ref. 1). [b] From a bottom yeast which was furnished through the courtesy of the Gunther Brewing Company, Baltimore, Maryland. [c] From a bottom yeast which was furnished through the courtesy of the Christian Heurich Brewing Company, Washington, D. C. [d] From Fleischmann's starch-free compressed yeast which was obtained from Standard Brands, Inc., Langdon, D. C.

Hydrolysis Measurements.—For comparative studies of the rates of hydrolysis by enzymes Weidenhagen[2] has recommended as "normal conditions" the use of 2.500 g. of maltose hydrate, or the equivalent amount of another substrate, at the optimal *p*H and at 30°, in a total volume of 50 ml. We have used these conditions for the hydrolysis of sucrose, raffinose and inulin. In the other hydrolyses, however, we have used one-fifth of the "normal" concentration of substrate in order to conserve rare materials and to allow the use of the less soluble substrates. In addition, the use of these dilute solutions has permitted the completion of the hydrolysis measurements within a shorter period of time, and the possibility of changes due to bacterial decomposition thus was decreased. Whenever possible, sufficient enzyme was added to complete the desired amount of hydrolysis within eight hours. Although antiseptics are known to inhibit the activity of some enzymes,[3] under the conditions used for measuring the activity of invertase as described in the preceding publication[1] we were unable to note any appreciable effect of toluene or of thymol. At the higher temperature of 30° and in the substrate concentrations which were used in this study, however, an appreciable decrease in the activity was noted upon the addition of toluene. Accordingly, toluene was added only in those

(1) Adams and Hudson, THIS JOURNAL, 65, 1359 (1943).

(2) Weidenhagen, *Z. Ver. deut. Zucker-Ind.*, 79, Tech. 71, 595 (1929); *Ergeb. Enzymf.*, 1, 201, footnote 2 (1932).

(3) See Tauber, "Enzyme Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1937, p. 13.

cases in which it was necessary to continue the experiments over a period of more than eight hours.

The *p*H during the hydrolysis studies was controlled by the use of appropriate buffer solutions, in such proportions that each 50 ml. of reaction mixture contained 5 ml. of *M* acetic acid to maintain a *p*H of 2.8, or 5 ml. of mixtures of 0.2 *M* acetic acid and 0.2 *M* sodium hydroxide to secure *p*H values between 3.2 and 6.3, or 12.5 ml. of mixtures of 0.2 *M* primary and secondary sodium phosphates when a higher *p*H was desired.

The progress of hydrolysis of the various substrates was followed, in most cases, by means of the iodometric method of Macleod and Robison[4] for the estimation of small amounts of aldose. The stoichiometric relationships involved made it possible to calculate directly the amount of aldose liberated; with phenylglycosides there was the added advantage that the liberated phenol itself consumed three molecular equivalents of iodine[5] and thus the method became still more sensitive in detecting a small amount of hydrolysis. Control experiments were carried out in all cases. Slight corrections were necessary whenever sucrose, fructose or raffinose was present in the hydrolysis mixture, or when the amount of enzyme present was large enough to show an appreciable blank. In special cases where the iodine method was not applicable, the Hanes modification[6] of the Hagedorn and Jensen ferricyanide method[7] was used. These cases will be considered in detail under the appropriate experimental section.

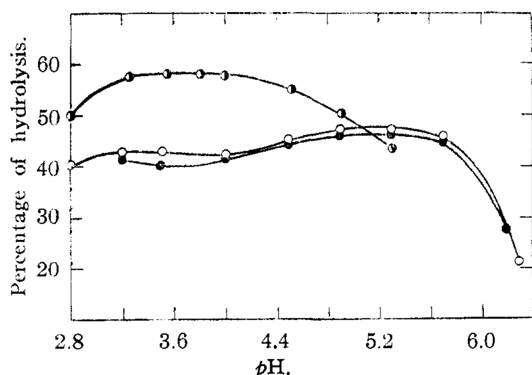


Fig. 1.—*p*H-Activity curves for the hydrolysis of sucrose, raffinose and inulin by bakers' yeast invertase preparation A: O, sucrose; ●, raffinose; ◑, inulin.

(4) Macleod and Robison, *Biochem. J.*, **23**, 517 (1929).

(5) See Helferich, Appel and Gootz, *Z. physiol. Chem.*, **215**, 282 (1933).

(6) Hanes, *Biochem. J.*, **23**, 99 (1929).

(7) Hagedorn and Jensen, *Biochem. Z.*, **135**, 46 (1923).

Fructofuranosidases

Sucrose, Raffinose and Inulin.—According to the general theory of carbohydrase specificity which has been advanced by Weidenhagen,[8] one enzyme, β-fructofuranosidase,[9] is responsible for the hydrolysis, not only of the simple β-fructofuranosides such as sucrose, raffinose, gentianose and stachyose, but also of the polysaccharides inulin[10] and irisin.[11] With our five purified invertase preparations we have made a careful study of the enzymic hydrolysis of sucrose, raffinose, stachyose and inulin in an attempt to examine critically this claim for identity of the enzymes involved.

Before any comparisons could be made, it was necessary to establish the optimal *p*H for the hydrolysis of each substrate by each enzyme preparation. In Fig. 1 are summarized the results which were obtained with one enzyme preparation, Bakers' A, acting upon sucrose, raffinose, and inulin[12] at different *p*H values. The results with the other enzyme preparations were very similar, and differed only slightly in the extent of the range of maximal activity. Accordingly, all comparative measurements of the hydrolysis of sucrose and raffinose were made at *p*H 5.0–5.5, and those of inulin[13] at 3.5–3.7. All *p*H values reported in this paper were those of the reaction mixtures; they were determined electrometrically with a glass electrode.

Michaelis and Davidsohn[14] have reported a

(8) Weidenhagen, (a) *Z. Ver. deut. Zucker-Ind.*, **79**, Tech. Tl. 115 (1929); (b) *Fermentforschung*, **11**, 155 (1930); (c) *Ergeb. Enzymf.*, **1**, 168 (1932); (d) Oppenheimer, "Die Fermente und ihre Wirkungen," Suppl. Bd. 1, Dr. W. Junk Verlag, Den Haag, 1936, p. 191; (e) Nord and Weidenhagen, "Handbuch der Enzymologie," Akad. Verlagsgesellschaft Becker und Erler Kom.-Ges., Leipzig, 1940, p. 512.

(9) Termed β-*h*-fructosidase by Weidenhagen. The names are more specific designations for the enzyme that is known usually as yeast invertase.

(10) Weidenhagen, (a) *Naturwissenschaften*, **20**, 254 (1932); (b) *Z. Ver. deut. Zucker-Ind.*, **82**, Tech. Tl. 316 (1932); (c) *ibid.*, **82**, Tech. Tl. 912 (1932).

(11) Weidenhagen, *Z. Ver. deut. Zucker-Ind.*, **83**, 376, 1042 (1933).

(12) "Purified inulin," from dahlia tubers, was recrystallized once from water according to Ohlmeyer and Pringsheim [*Ber.*, **66**, 1294 (1933)], and dried in the air; it contained 8.86% moisture and less than 0.02% ash. On a moisture-free basis, the specific rotation, $[\alpha]^{20}D$, was −40.1° in water (*c*, 1.9) in agreement with the best values of −40.1° reported by Schlubach and Schmidt [*Ann.*, **520**, 43 (1935)] and −40.2° reported by Ohlmeyer and Pringsheim. Hydrolysis measurements also were made with the air-dried material, with due allowance for the moisture content.

(13) Unless otherwise noted, all the results here reported on the hydrolysis of inulin were obtained by the ferricyanide method (ref. 6, 7). It was necessary first to determine empirical values for the reducing action, not only of fructose alone, but also of fructose in the presence of inulin and enzyme in the same proportions which existed in the hydrolysis mixtures at the time that measurements were made, so that corrections could be applied accordingly.

(14) Michaelis and Davidsohn, *Biochem. Z.*, **35**, 386 (1911).

broad optimal zone between *p*H 3.5 and 5.5 for the activity of yeast invertase toward sucrose, and most measurements which have been made heretofore have been carried out at *p*H 4.5–4.7. Our measurements with purified preparations have indicated the need of a slightly higher *p*H to obtain maximal activity under the "normal conditions" of Weidenhagen, although, as may be seen from Fig. 1, the decrease in activity on the acid side of the optimum was relatively small even at *p*H 2.8. Control experiments indicated no appreciable hydrolysis of sucrose at this acidity.[15]

For raffinose hydrolyses, Weidenhagen[16] has used the same *p*H of 4.6–4.7 which he used for sucrose, although no experimental data were given to establish an optimal *p*H for raffinose except with a "taka-saccharase" preparation.[17] With that enzyme he found that the *p*H-activity curves for sucrose and raffinose were similar. Our results (Fig. 1) with yeast invertase show a close parallelism for the two sugars also, throughout the *p*H range.

For the enzymic hydrolysis of inulin, Weidenhagen[10b,c] again has chosen a *p*H of 4.6–4.7 without citing any evidence that this is the optimum. Our results in Fig. 1 show clearly that the *p*H–activity curve for inulin differs markedly from those for sucrose and raffinose, and that the optimal *p*H for the hydrolysis of inulin by yeast invertase preparations lies between 3.2 and 4.0.[18] These marked differences between the *p*H–activity curve for inulin when compared with those for sucrose and raffinose, with each of our enzyme preparations, suggest strongly that yeast inulase is a specific enzyme that is not identical with the β-fructofuranosidase which hydrolyzes sucrose and which is generally regarded as responsible also for the hydrolysis of the sucrose union in raffinose.

It has been assumed generally that the velocity of hydrolysis of a substrate is directly proportional to the concentration of enzyme. Weidenhagen,[10c] however, has reported a lack of proportionality between the rate of hydrolysis of inulin and the concentration of inulase as larger amounts of enzyme were employed. We have studied these relationships, using sucrose, raffinose and inulin as they were hydrolyzed by the preparation Bakers' A, and have found that exact proportionality exists over a much more limited range than has been assumed generally. The results with sucrose and with raffinose were similar. At *p*H 5.3 proportionality was observed over a five-fold variation in enzyme concentration when the amounts of enzyme produced 50% hydrolysis of the substrate in from fifteen to seventy-five minutes; with smaller amounts of enzyme there was a definite decrease in the rate of hydrolysis which, with a twenty-fold dilution of enzyme, amounted to about 10% with sucrose and 20% with raffinose. This decrease in activity may be the result of inactivation of the minute amount of enzyme present in these dilute solutions. In contrast to these results, we have observed for the hydrolysis of inulin less than a 4% deviation from direct proportionality with amounts of enzyme which produced a 50% hydrolysis of this substrate in from sixteen to three hundred and twenty minutes. In comparing the enzymic hydrolysis of sucrose, raffinose and inulin, therefore, we have used sufficient amounts of enzyme to be within the range in which there is satisfactory proportionality between concentration and activity.

In Table II are presented data on the hydrolysis of sucrose, raffinose and inulin by one enzyme preparation, Bakers' A; these data are typical of a consistent series of measurements; it seems unnecessary in this and other cases to print the lengthy record of full experimental details. The values for k have been calculated on the assumption that the reactions are unimolecular. They differ noticeably for the three substrates. With sucrose, k increased throughout the course of the hydrolysis; with raffinose, k decreased slightly after being practically constant over the range of 20 to 55% hydrolysis; with inulin, k increased slightly, although the differences were small in comparison with those observed for sucrose. Entirely similar results were obtained with the enzyme preparation Brewers' A.

Similar increasing k values for the action of yeast invertase on sucrose have been reported frequently, although Willstätter, Graser and Kuhn[19] have observed both constant and increasing values of k for different invertase solutions

(15) For a recent study of the effect of acids on the activity of invertase, see Wisanski, THIS JOURNAL, **61**, 3330 (1939).

(16) *E. g.*, Weidenhagen and Dey, *Z. Ver. deut. Zucker-Ind.* **78**, Tech. Tl. 242 (1928).

(17) Weidenhagen, *ibid.*, **78**, Tech. Tl. 125 (1928).

(18) For *Aspergillus niger* inulase, a *p*H optimum at 3.8 has been reported by Pringsheim and Kohn [*Z. physiol. Chem.*, **133**, 80 (1924)]; an optimum between 3 and 4 has been found recently for the same enzyme by Pigman, *J. Research Natl. Bur. Standards*, **30**, 169 (1943).

(19) Willstätter, Graser and Kuhn, *Z. physiol. Chem.*, **123**, 1 (1922).

TABLE II

MEASUREMENTS OF THE ENZYMIC HYDROLYSIS OF SUCROSE, RAFFINOSE AND INULIN

Time, min.	Sucrose[a] % Hydrolysis	$k^d \times 10^4$	Raffinose[b] % Hydrolysis	$k^d \times 10^4$	Inulin[c] % Hydrolysis	$k^d \times 10^4$
20	26.2	66.0			22.2	54.5
22			28.7	66.9		
30	37.6	68.3	36.1	64.9		
40	48.0	71.0	44.6	64.1	39.6	54.7
46			50.1	65.6		
50	57.2	73.7			48.4	57.5
55			55.8	64.4		
60	64.6	75.2			54.8	57.5
75	74.2	78.5				
80			68.2	62.2		
90					71.6	60.7
100	85.8	84.8				
140			85.1	59.0		
150	95.0	86.7			88.2	61.9
210	99.0		93.0	56.1	94.8	61.1
300	100.0		96.9	50.5	97.4	52.8
1440			100.4		100.0	

[a] 0.0301 Mg. enzyme (Bakers' A) and 2.375 g. sucrose in 50 ml.; *p*H 5.1. [b] 0.118 Mg. enzyme (Bakers' A) and 4.125 g. raffinose pentahydrate in 50 ml.; *p*H 5.1. [c] 68.8 Mg. enzyme (Bakers' A) and 1.125 g. inulin (dry weight) in 50 ml.; *p*H 3.75. [d] Calculated in minutes and common logarithms.

even though they had been prepared from the same source of yeast and by similar methods of purification. With raffinose as the substrate, our results were similar to those of Weidenhagen and Dey[16]; they reported constant values for *k* up to 27% but have given no data for more extensive hydrolysis. With inulin as a substrate, our slightly increasing *k* values contrast with the decreasing velocity constants reported or calculated from the meager data of Weidenhagen.[10c]

Stachyose is a tetrasaccharide which was discovered first in *Stachys tuberifera*, Nd. by von Planta and Schulze.[20] The methylation procedure, as applied by Onuki,[21] has shown stachyose to be a 6-D-galactopyranosido-4-D-galactopyranosido - 2 - D -glucopyranosido - D- fructofuranoside. The glucose and the fructose molecules are linked, presumably, as in sucrose, because fructose is liberated from the stachyose molecule by hydrolysis with invertase,[22] and because stachyose gives a color reaction with diazouracil which is believed to be characteristic for the sucrose linkage.[23] The configurations of the other linkages will be discussed below under manninotriose, the trisaccharide which remains after the fructose is removed.

Only a few measurements have been published on stachyose as a substrate for purified invertase preparations,[24] presumably because of the scarcity of this tetrasaccharide. We have measured the hydrolysis of stachyose at *p*H 5.1 by the three invertase preparations Brewers' A and C, and Bakers' A.

In Table III are summarized the comparative studies of the hydrolysis of sucrose, raffinose, inulin and stachyose by our enzyme preparations. The enzyme value has been used as a basis for comparison; this value is defined by Weidenhagen[25] as the number of enzyme units in one gram of dry material, and is calculated simply as enzyme value = $k/(g \times \log 2)$ where g = grams of enzyme in 50 ml. of reaction mixture, and k = unimolecular reaction velocity constant, calculated in minutes and common logarithms, for 50% hydrolysis under the "normal conditions." With stachyose the concentration was only one-fifth of that required for "normal conditions," and the enzyme value was compared with that of sucrose determined likewise with the more dilute substrate solution. The table indicates clearly that the relative activities of the preparations toward the different substrates varied considerably with the source of the enzyme material and with the method of purification even with preparations from the same source. In all cases raffinose required more enzyme for its hydrolysis in a given length of time than did the equivalent amount of sucrose; stachyose required still larger amounts of enzyme; and inulin required very large amounts of enzyme. The results with Bakers' A′ showed that no marked change in the several ratios had been effected by partial inactivation of the enzyme preparation by heat; this would indicate that the enzymes which hydrolyze sucrose, raffinose and inulin are identical, or else that they are inactivated by heat at the same rates. We incline to the latter interpretation, on the view that one enzyme (β-D-fructofuranosidase) hydrolyzes sucrose, raffinose and stachyose, but that inulase is another enzyme.

The variations in the ratios of activities of our enzyme preparations from *different* sources acting upon the several substrates (Table III, col-

(20) von Planta and Schulze, *Ber.*, **23**, 1692 (1890).

(21) Onuki, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **20**, 201 (1933).

(22) C. Tanret, *Compt. rend.*, **134**, 1588 (1902).

(23) Raybin, This Journal, **55**, 2603 (1933); **59**, 1402 (1937); Purves and Hudson, *ibid.*, **56**, 709 (1934).

(24) *Cf.* Bourquelot and Bridel, *Compt. rend.*, **152**, 1060 (1911); Chaudun, *Bull. soc. chim. biol.*, **15**, 1117 (1933).

(25) Ref. 8c, p. 201.

TABLE III

COMPARATIVE STUDIES OF THE ENZYMIC HYDROLYSIS OF SUCROSE, RAFFINOSE, INULIN AND STACHYOSE

Enzyme preparation	Sucrose, enzyme value (I)	Raffinose,[a] enzyme value (II)	Ratio, I/II	Inulin, enzyme value (III)	Ratio I/III
Bakers' A[b]	791	182	4.35	0.282	2,800
Bakers' A'[c]	653	159	4.11	.229	2,850
Bakers' B	731	156	4.69	.225	3,250
Brewers' A[b]	885	103	8.59	.0313	28,300
Brewers' B	544	87.5	6.22	.0461	11,800
Brewers' C	884	111	7.96	.0529	16,700

	Sucrose, enzyme value for dilute substrate (IV)	Stachyose,[a] enzyme value for dilute substrate (V)	Ratio, IV/V
Bakers' A	2460	167	14.7
Brewers' C	2749	85	32.3

[a] Although the enzyme preparations from brewers' yeast contained an α-galactosidase, the small amounts of enzyme material required for the hydrolysis of the fructofuranoside linkage in raffinose and stachyose were not sufficient to cause any appreciable hydrolysis of the galactoside linkages. [b] The enzyme values reported here are somewhat lower than the original values of these preparations because of a slight loss in activity upon standing at 5° for a period of several months before these measurements were made. This decrease in activity amounted to 5% in the case of Bakers' A and 11% in the case of Brewers' A. [c] Prepared by allowing an aliquot of the enzyme preparation Bakers' A to stand at 37° for 22 days before these measurements were made.

umns 4 and 6) are similar to the results reported by other investigators.[26] But the variations in these ratios when the enzymes were prepared from the *same* source, by different methods, are in contrast to the results of Weidenhagen[10c] and of Willstätter, Graser and Kuhn,[19] who have failed to change, by purification procedures, the relative activity of enzyme preparations from the same source. On the other hand, Sreenivasaya and Iyengar[27] report that they have succeeded in effecting a partial separation of invertase and inulase by centrifugation of a frozen enzyme extract.

A considerable amount of evidence has been presented both for and against the specificity theory of Weidenhagen, and most of this material has been reviewed by Nelson[28] and by Weidenhagen.[8] Our results, particularly in view of the very small inulase activity of our most highly purified enzyme preparation, Brewers' A, suggest that inulase is different from β-fructofuranosidase, or at least that we are dealing with a group of similar fructofuranosidases rather than with a single enzyme.[29] It is necessary, however, to consider also the possibility suggested by Weidenhagen, namely, that variations in the enzyme carrier, which may be responsible for the differences observed with enzyme preparations from different sources, may also explain the differences which have been obtained with enzyme preparations purified by different methods but from the same source. The fact, now established, that the ratio of the activity of the hydrolyzing enzyme for sucrose and raffinose varies as much as from 4.11 to 8.59 with different enzyme preparations, throws grave doubt on the possibility of deciding at present whether one and the same enzyme causes the hydrolysis of both these sugars. We are inclined to the view, as mentioned previously, that one enzyme is responsible for the hydrolysis; if this view is correct, the idea of proving it by demonstrating the existence of a constant ratio appears to be an unattainable goal, at least when one is using enzyme preparations that may contain enzyme carriers or other influencing substances.

Melezitose, according to the most recent views,[30] is, in all probability, 3-α-D-glucopyranosido-β-D-fructofuranosido-α-D-glucopyranoside or, in other words, sucrose with an additional glucose molecule as a substituent group on the third carbon atom of the fructose moiety. In spite of the high concentration of β-fructofuranosidase in our preparations, no appreciable hydrolysis of melezitose was observed at a *p*H of 4.0, 5.3 or 7.0 by preparations Bakers' A or Brewers' A. The samples used for analysis were sufficiently large to enable us to detect as little as 1.5% hydrolysis; with the amount of enzyme which was used over a period of forty-eight hours, the enzyme value cannot be greater than 1×10^{-4}. This means that if melezitose is hydrolyzed at all by our enzyme preparations, it would require at least 25,000,000 times as long as does an equivalent weight of sucrose.

These results confirm the earlier work including

(26) See, for example, Pigman, *J. Research Natl. Bur. Standards,* **30**, 171 (1943).

(27) Sreenivasaya and Iyengar, *Nature,* **132**, 604 (1933).

(28) Nelson, *Chem. Reviews,* **12**, 1 (1933).

(29) The only argument in favor of the β-linkage for the fructofuranose residues in inulin is that the hydrolysis of inulin can be produced by the same yeast enzyme preparations, admittedly a mixture of enzymes, which hydrolyze sucrose. If there is present in such preparations a specific yeast inulase, as our experiments suggest, the argument for the β-linkage in inulin no longer applies. It seems to us at present that the question is completely open whether the inulin linkage is α or β [see also Haworth, "The Constitution of Sugars," Edward Arnold and Co., London, 1929, p. 81].

(30) Pacsu, Wilson and Graf, THIS JOURNAL, **61**, 2675 (1939); see also Isbell and Pigman, *J. Research Natl. Bur. Standards,* **20**, 787 (1938).

the more recent test by Weidenhagen,[31] and are in accord with Helferich's studies on 3-methyl-β-phenyl-D-glucoside,[32] on the 2-, the 3-, the 4- and the 6-tosyl-β-D-glucosides of vanillin,[33] and on the β-maltoside of vanillin,[34] none of which appears to be hydrolyzed by the β-D-glucosidase of almond emulsin even though the parent β-phenyl- and β-vanillin-D-glucosides are hydrolyzed very readily by that enzyme. Our results are thus compatible with the view that melezitose is 3-α-D-glucopyranosido - β - D - fructofuranosido- α - D - glucopyranoside; the inability of invertase to hydrolyze it is to be attributed to the presence of the glucosido substituent on the third carbon atom of the fructofuranose moiety.

The Glucose Content of Inulin.—The presence of D-glucose among the products of hydrolysis of inulin by acid has been demonstrated by Tanret, by Schlubach, and by other investigators.[35] Milder conditions, such as the enzymic hydrolysis by inulase preparations from *Aspergillus niger*, have been reported by Pringsheim and Ohlmeyer[36] also to yield glucose, to the extent of 1.5%. Weidenhagen,[10c] on the other hand, states that he was unable to detect glucose in his hydrolysis mixture from inulin. Our experiments, summarized in Table IV, are in agreement with those of Pringsheim and Ohlmeyer.[36] Although the values for iodine consumption are small, they indicate a definite and gradual liberation of an aldose, with the final hydrolysis mixture containing an average of 1.7% of aldose, which we assume to be glucose. If this glucose is not an integral part of the inulin molecule it must be an integral part of an associated molecule which is hydrolyzed at about the same rate as inulin.

Isosucrose, an α-Fructofuranoside.—Purves and Hudson have shown that neither pure α-methyl-D-fructofuranoside[37] nor pure α-benzyl-D-fructofuranoside[38] is hydrolyzed by invertase.

(31) Weidenhagen, ref. 8b. See also Kuhn and von Grundherr, *Ber.*, **59**, 1655 (1926); *cf.* Weidenhagen. *Z. Ver. deut. Zucker-Ind.*, **78**, Tech. Tl. 783 (1928).

(32) Helferich and Lang, *Z. physiol. Chem.*, **216**, 123 (1933).

(33) Helferich and Grünler, *J. prakt. Chem.*, **148**, 107 (1937).

(34) Helferich and Weber, *Ber.*, **69**, 1411 (1936).

(35) C. Tanret, *Bull. soc. chim.*, [3], **9**, 233 (1893); Hildt, *Compt. rend.*, **170**, 1505 (1920); Schlubach and Elsner, *Ber.*, **62**, 1493 (1929); Schlubach and Knoop, *Ann.*, **497**, 208 (1932); Schlubach and Schmidt, *ibid.*, **520**, 43 (1935); Pringsheim and Reilly, *Ber.*, **63**, 2636 (1930); Jackson and Goergen, *Bur. Standards J. Research*, **3**, 27 (1929); Jackson and McDonald, *ibid.*, **5**, 1151 (1930).

(36) Pringsheim and Ohlmeyer, *Ber.*, **65**, 1242 (1932); Ohlmeyer and Pringsheim, *ibid.*, **66**, 1292 (1933); *cf.* Bourquelot and Bridel, *Compt. rend.*, **172**, 946 (1921), and Pringsheim and Reilly, *Ber.*, **63**, 2636 (1930), for earlier, negative results.

(37) Purves and Hudson, THIS JOURNAL, **56**, 708 (1934).

(38) Purves and Hudson, *ibid.*, **59**, 49 (1937).

TABLE IV

THE LIBERATION OF ALDOSE, PRESUMABLY GLUCOSE, DURING THE ENZYMIC HYDROLYSIS OF INULIN[a]

Time, min.	Ml. 0.005 *N* thiosulfate[b]	Mg. glucose	% Hydrolysis, as glucose	% Hydrolysis,[c] as total reducing sugar
20	0.08	0.036	0.27	23
40	.13	.059	.44	40
50	.14	.063	.47	48
80	.27	.122	.90	66
150	.50	.225	1.67	90
1440	.47	.212	1.57	99
2880	.54	.243	1.80	99

[a] These values represent a composite of two series of experiments; 1.125 g. of inulin and 68.8 mg. of enzyme (Bakers' A) in a total volume of 50 ml.; *p*H 3.75; temperature 30°. [b] These figures represent the ml. of thiosulfate equivalent to the iodine consumed. The sample used for analysis originally contained 13.5 mg. of inulin. [c] Determined by the ferricyanide method; see refs. 6, 7. Corrections have been made as explained in ref. 13.

We have found that isosucrose,[39] which is β-D-glucopyranosido-α-D-fructofuranoside,[40] was not appreciably hydrolyzed by enzyme preparations Brewers' A or Bakers' A at a *p*H of 4.0, 5.3 or 7.0. Under the conditions of enzyme concentration and with the experimental period used, an enzyme value of 2×10^{-4} should be measurable. This result confirms the statement of Georg[40] that his invertase preparation had no effect in five days upon isosucrose.

Galactosidases

Melibiose, α-Methyl-D-galactoside and α-Phenyl-D-galactoside.—The presence in bottom yeast of an enzyme which hydrolyzes melibiose (6-α-D-galactopyranosido-D-glucose) was discovered almost simultaneously by Bau[41] and by Fischer and Lindner.[42] This enzyme, originally named melibiase, is considered by Weidenhagen[43] to be an α-D-galactosidase which hydrolyzes all α-D-galactosides. It resembles yeast invertase sufficiently in its properties to make the separation of the two enzymes difficult; Willstätter and Kuhn[44] have reported the presence of melibiase in some of their highly purified preparations.

(39) Irvine, Oldham and Skinner, THIS JOURNAL, **51**, 1279 (1929).

(40) Georg, *Helv. Chim. Acta*, **17**, 1566 (1934); Purves and Hudson, THIS JOURNAL, **59**, 1172 (1937). The recent claim of Schlubach and Middelhoff [*Ann.*, **550**, 134 (1942)], that isosucrose is a glucosidofructose rather than a glucosidofructoside, is not in accordance with the methylation studies of Irvine and Routledge [THIS JOURNAL, **57**, 1411 (1935)], and verification of that claim seems desirable.

(41) Bau, *Chem. Ztg.*, **19**, 1873 (1895).

(42) Fischer and Lindner, *Ber.*, **28**, 3034 (1895).

(43) Weidenhagen, *Z. Ver. deut. Zucker-Ind.*, (a) **77**, Tech. Tl. 696 (1927); (b) **78**, Tech. Tl. 99 (1928); (c) Weidenhagen and Renner, *ibid.*, **86**, Tech. Tl. 22 (1936).

(44) Willstätter and Kuhn, *Z. physiol. Chem.*, **115**, 183 (1921).

Although the melibiase content of our preparations from brewers' yeasts was small compared with the invertase content,[45] these "invertase" solutions have more melibiase activity than previous preparations of melibiase. Accordingly, comparative studies have been made on the hydrolysis of several α-galactosides by the two enzyme preparations Brewers' A and Brewers' C, with a view to testing the validity of Weidenhagen's claim for the existence of a single α-galactosidase. The *p*H–activity curve for melibiose shows a broad range of maximal activity between *p*H 3.5 and 5.3, similar to that found by Weidenhagen,[43b] with only a relatively small decrease in activity at *p*H 2.8 and 6.3. With α-phenyl-D-galactoside the curve indicated that there was no appreciable difference in activity between *p*H 2.8 and 5.7. For the hydrolysis of α-methyl-D-galactoside the optimal *p*H was between 3.6 and 4.8, with a marked decrease at 6.2. Accordingly 4.5 was selected as the *p*H which permitted maximal activity for all three substrates; Weidenhagen[43] used *p*H 5.0 on the basis of his melibiose experiments.

With regard to proportionality between enzyme activity and concentration, Weidenhagen[43b] found, with melibiose as the substrate, that proportionality existed over a five-fold dilution range (sixty to three hundred minutes for 50% hydrolysis). With our more active preparations, and with melibiose and α-phenylgalactoside as substrates, the proportionality existed over a ten-fold dilution (eighteen to one hundred and eighty minutes for 50% hydrolysis) with only a slight decrease in activity at a twenty-fold dilution.

Table V summarizes the results obtained on the hydrolysis of the three α-D-galactosides by enzyme preparation Brewers' C. Constant *k* values were observed both for melibiose and for α-phenylgalactoside for almost the entire course of hydrolysis. Our results with melibiose are in strong contrast to the decreasing *k* values reported by Weidenhagen[43a] for his much less active preparations. α-Methyl-D-galactoside showed decreasing *k* values; it seems probable that the methyl alcohol liberated during the hydrolysis may be responsible for these decreasing *k* values,[46] and also for the more decided effect of variations in *p*H on the enzymic activity.

TABLE V

THE ENZYMIC HYDROLYSIS OF MELIBIOSE, α-METHYL-D-GALACTOSIDE AND α-PHENYL-D-GALACTOSIDE

Time, min.	Melibiose[a]		α-Methyl-D-galactoside[b]		α-Phenyl-D-galactoside[c]	
	% Hydrolysis	$k \times 10^4$	% Hydrolysis	$k \times 10^4$	% Hydrolysis	$k \times 10^4$
25	37.5	81.6	28.8	58.9	35.4	75.9
39			39.5	55.9		
40	51.5	78.5			49.6	74.4
48			45.8	55.4		
50	61.3	82.3			56.5	72.1
60			51.5	52.4	62.1	70.7
80	76.8	79.2	61.5	51.9	73.0	71.1
110	87.0	80.5	68.9	46.1	83.7	71.6
150	94.0	81.5	76.9	42.5	92.3	74.5
210	97.8	78.5	84.5	38.8	96.8	71.2
300	100		90.0	33.3	97.4	52.8
1440			95.0	9.0	99.5	

[a] 5.24 Mg. enzyme (Brewers' C) and 0.525 g. melibiose dihydrate in 50 ml. at *p*H 4.5. [b] 100 Mg. enzyme and 0.294 g. α-methyl-D-galactoside monohydrate in 50 ml. at *p*H 4.5. [c] 4.0 Mg. enzyme and 0.355 g. α-phenyl-D-galactoside in 50 ml. at *p*H 4.5.

β-Methyl-L-arabinoside ($[\alpha]^{20}D$ +236°) has the same ring configuration as α-methyl-D-galactoside ($[\alpha]^{20}D$ +196°), and should be hydrolyzed by the α-D-galactosidase in our brewers' yeast enzyme preparations in the same way that β-ethyl-L-arabinoside[47] and β-phenyl-L-arabinoside[48] are hydrolyzed by an enzyme, presumably the α-D-galactosidase, in almond emulsin.[49] β-Methyl-L-arabinoside was found to be hydrolyzed by the preparations Brewers' A and Brewers' C, but the investigations were limited by the very slow rate of hydrolysis (40% in four weeks with undiluted enzyme[50]) and by the excessively large

(45) Weidenhagen (ref. 43b) has estimated the ratio of invertase activity to melibiase activity in bottom yeast as 83:1. Our preparations contain these enzymes in a ratio of 570:1, from which it appears that our methods for the purification of invertase have effected a considerable removal of the melibiase.

(46) See, for example, the hydrolysis experiments in 33% methyl alcohol reported by Pigman and Richtmyer, THIS JOURNAL, **64**, 373 (1942).

(47) Helferich and Appel, *Z. physiol. Chem.*, **205**, 231 (1932).

(48) Helferich, Winkler, Gootz, Peters and Günther, *Z. physiol. Chem.*, **208**, 91 (1932).

(49) In similar fashion, the β-D-galactosidase of almond emulsin is believed to be responsible for the hydrolysis of β-ethyl-L-arabinoside [Bridel and Béguin, *Compt. rend.*, **182**, 812 (1926); Helferich and Appel, ref. 47], and vicianose [Bertrand and Weisweiller, *Compt. rend.*, **151**, 325 (1910); *Bull. soc. chim.*, [4] **9**, 84 (1911)]; the synthesis of vicianose from acetobromo-L-arabinose and 1,2,3,4-tetraacetyl-D-glucose [Helferich and Bredereck, *Ann.*, **465**, 166 (1928)] shows its structure to be 6-α-L-arabinosido-D-glucose, and Helferich's naming of it as a β-L-arabinoside does not conform to the customary nomenclature. The β-D-glucosidase is believed to be responsible for the hydrolysis of the β-methyl- and β-phenyl-D-xylosides [Helferich and Appel, ref. 47], the β-D-xyloside of methyl salicylate [Robertson and Waters, *J. Chem. Soc.*, 1881 (1931)], and primeverose (6-β-D-xylosido-D-glucose) [Bridel, *Compt. rend.*, **181**, 523 (1925)]; and the α-D-mannosidase for the hydrolysis of α-phenyl-D-lyxoside [Pigman, THIS JOURNAL, **62**, 1371 (1940)].

(50) By "undiluted enzyme" is meant that the enzyme was not diluted prior to its addition to the substrate and buffer. The reaction mixture contained 1.2 ml. of dilute substrate and buffer and 0.8 ml. of the original enzyme preparation.

amounts of enzyme necessary for a more extended study. Activity measurements indicated that a *p*H of 4.1 was more favorable for the enzymic hydrolysis of this substrate than either 5.2 or 7.0.

Table VI summarizes the comparative enzyme values for the four substrates which have been discussed immediately above. The ratios of the values obtained with the two enzyme preparations show only small differences, and the ratios of melibiose to α-phenyl-D-galactoside are similar to those of Weidenhagen and Renner.[43c] From these comparisons, and in the absence of any contradictory evidence, we may conclude that the three α-D-galactosides and the configurationally related β-L-arabinoside are hydrolyzed by the same enzyme, in agreement with the general theory of specificity according to Weidenhagen.

TABLE VI

COMPARATIVE STUDIES OF THE ENZYMIC HYDROLYSIS OF MELIBIOSE AND RELATED SUBSTRATES[a]

Enzyme preparation	Melibiose, enzyme value (I)	α-Phenyl-D-galactoside, enzyme value (II)	Ratio I/II
Brewers' A	4.78	5.82	0.821
Brewers' C	4.80	5.90	0.814

Enzyme preparation	α-Methyl-D-galactoside, enzyme value (III)	Ratio I/III	β-Methyl-L-arabinoside, enzyme value (IV)	Ratio I/IV
Brewers' A	0.144	33.2	8.6×10^{-5}	56,000
Brewers' C	0.158	30.4	9.6×10^{-5}	49,000

[a] All enzyme values refer to the dilute substrate solutions.

The enzyme preparation Bakers' A was *inactive* toward melibiose, the α-phenyl- and α-methyl-D-galactosides and the β-methyl-L-arabinoside. These hydrolysis studies were carried out at *p*H 4.0, 5.3 and 7.0 under conditions which would enable us to measure an enzyme value as small as 10^{-4}. The presence of melibiase (α-D-galactosidase) has never been reported in top yeast, so the observed inactivity is not unexpected.

Manninotriose is a trisaccharide which occurs in the manna of the ash, although it is obtained usually by the partial hydrolysis of the tetrasaccharide stachyose. Onuki,[21] from methylation experiments, has shown it to be a 6-D-galactopyranosido-4-D-galactopyranosido-D-glucose. Although emulsin, which contains both α- and β-D-galactosidases, has long been known to effect, slowly, a complete hydrolysis of manninotriose to its component hexoses,[51] and although both brewers' yeast and bakers' yeast (under certain conditions) have been reported to attack manninotriose,[52] no one has examined any of these reactions closely enough to decide whether one or more enzymes is responsible for the hydrolyses, or even to hazard a guess as to the configurations of the galactoside linkages in manninotriose and stachyose.[53]

In the course of our investigations on the hydrolysis of stachyose by invertase preparations we observed that in the presence of enzyme in excess of that required for the complete hydrolysis of the fructofuranoside linkage, there remained, with bakers' A, only one equivalent of an aldehydo sugar, presumably manninotriose. However, when brewers' A or brewers' C was used, a greater amount of reducing sugar was produced; with the former the maximal hydrolysis corresponded to 98.0% and with the latter 100.4% of the theoretical amount of aldohexose, calculated for the complete hydrolysis of the manninotriose into one equivalent of glucose and two equivalents of galactose. In view of the proved presence of an α-galactosidase in our enzyme preparations from brewers' yeast, and the absence of any β-galactosidase (see below), we must conclude that both galactoside linkages, in manninotriose and in stachyose, are of the α-type. The formulas for the two sugars are thus written as I and II on the basis of Onuki's methylation results and the present inference from enzyme actions.

α-Methyl-D-manno-D-gala-heptoside[54] is a heptoside with the same ring configuration that occurs in the α-D-galactosides which are hydrolyzed by our enzyme preparations from brewers' yeast, but not by those from bakers' yeast. Pigman[55] has said "It seems probable that the heptosides which have ring configurations similar to hydrolyzable hexosides also should be hydrolyzed," and "it is to be expected that the heptosides will be hydrolyzed at a slower rate than the corresponding hexosides since it is known that an increase in the size of the atom or group attached to the fifth carbon atom decreases the rate of hydrolysis." Pigman[56] was able to confirm

(51) C. Tanret, *Bull. soc. chim.*, [3] **29**, 888 (1903); Vintilesco, *J. pharm. chim.*, [6] **30**, 167 (1909); Cheymol, *ibid.*, [8] **25**, 110 (1937); *cf.* Neuberg and Lachmann, *Biochem. Z.*, **24**, 171 (1910).

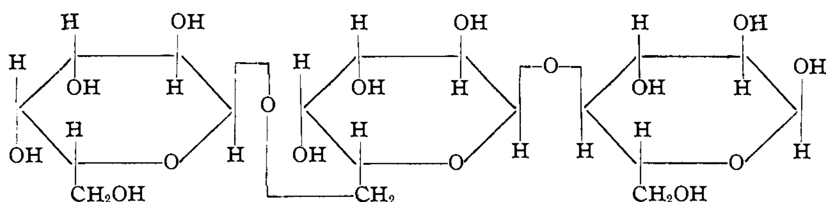
(52) C. Tanret, *Bull. soc. chim.*, [3] **27**, 957 (1902); Vintilesco, ref. 51.

(53) Armstrong and Armstrong [*J. Soc. Chem. Ind.*, **53**, 912 (1934)] wrote one α-galactoside linkage in these sugars on the assumption that raffinose is a building block in the elaboration of stachyose in nature; however, the presence of the raffinose moiety in stachyose was only a conjecture and at present the methylation data (ref. 21) exclude it.

(54) Montgomery and Hudson, THIS JOURNAL, **64**, 247 (1942).

(55) Pigman, *ibid.*, **62**, 1371 (1940).

(56) Pigman, *J. Research Natl. Bur. Standards*, **26**, 197 (1941).

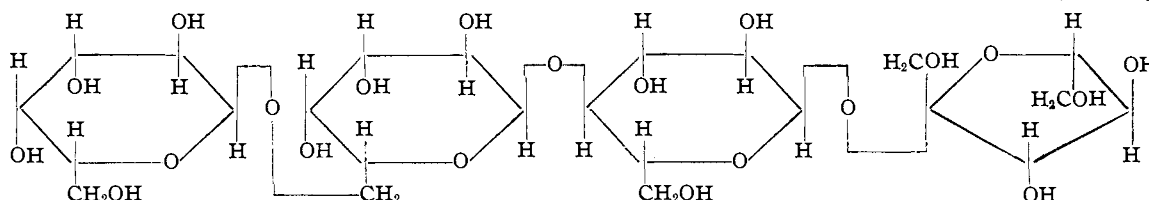


I. α-Manninotriose
(6-α-D-Galactopyranosido-4-α-D-galactopyranosido-α-D-glucopyranose)

galactoside was not attacked under the same conditions, or even in two weeks at *p*H 5.2 with undiluted[50] Brewers' A (see Table VIII).

Glucosidases

Amygdalin, Gentiobiose, Cellobiose and β-Phenyl-D-glucoside.—Extensive investigations have been carried out with the β-glucosidase component of almond emulsin,[59] but relatively little has been reported on the β-glucosidase in yeast enzyme preparations. Emil Fischer[60] discovered that an aqueous extract of dry Frohberg-type yeast would hydrolyze amygdalin (β-[*d*-mandelic nitrile]-gentiobioside) to glucose and prunasin. Later evidence showed that the reaction continued with the liberation of a second molecule of glucose, and Weidenhagen[61] has concluded that β-glucosidase alone is responsible for the hydrolysis of the two glucose molecules, consecutively rather than simultaneously, and furthermore that the β-glucosidases of almond emulsin and of yeast are identical.



II. Stachyose
(6-α-D-Galactopyranosido-4-α-D-galactopyranosido-2-α-D-glucopyranosido-β-D-fructofuranoside)

both predictions by showing that β-phenyl-D-manno-D-gala-heptoside,[57] with its β-D-galactoside ring, is hydrolyzable by sweet-almond emulsin ("Rohferment") and presumably by the β-galactosidase component, although at a very slow rate. On the other hand, even though almond emulsin contains an α-galactosidase, Pigman was unable to detect any hydrolysis of α-phenyl-D-manno-D-gala-heptoside, which has an α-D-galactoside ring, in spite of the use of a high enzyme concentration and an extended reaction period.

In our study of the closely related α-methyl-D-manno-D-gala-heptoside, we could not detect any appreciable hydrolysis either with undiluted[50] bakers' yeast enzyme preparation A in ninety-six hours at *p*H 5.2 or 7.0, or with undiluted[50] brewers' yeast enzyme preparation C, which is known to contain an α-galactosidase, in ninety-six hours at *p*H 5.2 or 7.0, or even in four weeks at *p*H 4.1. The absence of appreciable activity under the last conditions indicates that if this preparation is capable of hydrolyzing this substrate the enzyme value must be less than 2.4×10^{-6}. It is still possible that the corresponding phenyl glycoside of this heptose would be hydrolyzed by our brewers' enzyme preparations under suitable conditions.

β-Galactosides.—Lactose (4-β-D-galactopyranosido-D-glucose) was not hydrolyzed appreciably in forty-eight hours at *p*H 4.0, 5.3 or 7.0 by diluted[58] Bakers' A or Brewers' A. β-Phenyl-D-

Our purest yeast invertase preparation Brewers' A still contained a small amount of β-glucosidase which was capable of hydrolyzing amygdalin, gentiobiose and β-phenyl-D-glucoside. The rate of hydrolysis of amygdalin by undiluted[50] Brewers' A is shown in Table VII; *p*H 5.2 was found to be more favorable than either *p*H 4.1 or 7.0. That the hydrolysis did not stop after the liberation of one glucose molecule, which would correspond to 50% hydrolysis, is evident from the value of 65% which had been reached after four weeks.

It has been assumed generally[62] that one and the

(57) Known also as a *d*-α-mannoheptoside.

(58) By "diluted enzyme" is meant that the enzyme added had been diluted to one-fourth the concentration of the original preparation. The 2 ml. of reaction mixture contained 0.2 ml. of the original enzyme solution, which is equivalent to 58 mg. of Brewers' A or 75 mg. of Bakers' A in 50 ml. of reaction mixture (dilute substrate).

(59) For summaries see Helferich, *Ergeb. Enzymf.*, **2**, 74 (1933); **7**, 83 (1938).

(60) Fischer, *Ber.*, **28**, 1508 (1895).

(61) Weidenhagen, *Z. Ver. deut. Zucker-Ind.*, **79**, Tech. Tl. 591 (1929). See also Willstätter, Kuhn and Sobotka, *Z. physiol. Chem.*, **129**, 33 (1923).

(62) Helferich, *Ergeb. Enzymf.*, **7**, 83 (1938); Hofmann, *Biochem. Z.*, **285**, 440 (1936). Pigman, *J. Research Natl. Bur. Standards*, **27**, 1 (1941); also the general theory of Weidenhagen, ref. 8.

TABLE VII

HYDROLYSIS OF AMYGDALIN BY ENZYME PREPARATION BREWERS' A[a]

Time, min.	Ml. 0.005 N $Na_2S_2O_3$[b]	Mg. glucose	% Hydrolysis	$k \times 10^5$
300	0.24	0.108	5.2	7.7
2,880	1.11	.500	24.2	4.2
5,760	1.87	.842	40.6	3.9
10,080	2.32	1.04	50.2	3.0
20,160	2.59	1.17	56.5	1.8
40,320	2.99	1.35	65.2	1.1

[a] The reaction mixture contained 0.233 g. of enzyme preparation and 0.355 g. of amygdalin dihydrate in 50 ml. total volume, pH 5.2. The amount of amygdalin was slightly higher than required for one-fifth of the "normal" concentration suggested by Weidenhagen, due to the fact that the amygdalin was found later to be the dihydrate instead of the trihydrate. [b] These values represent the actual corrected volume of thiosulfate equivalent to the iodine consumed by the glucose.

same enzyme in sweet-almond emulsin, namely, β-D-glucosidase, can hydrolyze all β-D-glucosides. However, the story of yeast β-glucosidase is more complex. Oppenheimer[63] states that the true yeasts attack the gentiobiose moiety in amygdalin always, free gentiobiose sometimes, and cellobiose never. Accordingly he writes[64] that the identity of amygdalase, gentiobiase and cellobiase is still an open question. Although our enzyme preparations contained only a very small amount of β-glucosidase, we were able to measure 13.9% hydrolysis of gentiobiose and 12.0% hydrolysis of β-phenyl-D-glucoside after action of the undiluted[50] Brewers' A for four weeks (see Table VIII). No appreciable hydrolysis of gentiobiose was observed until after the methyl alcohol of crystallization had been removed by drying the sample at 70° *in vacuo* for twelve hours. With sweet-almond emulsin, β-phenylglucoside is hydrolyzed about four times as rapidly as gentiobiose.[65] We did not detect any hydrolysis of cellobiose with our invertase preparations after two weeks, yet, with sweet-almond emulsin, cellobiose is reported to be hydrolyzed twice as rapidly as gentiobiose.[44] If our preparation is capable of hydrolyzing cellobiose at all it would appear that the relative activities of yeast β-glucosidase toward it and the other substrates differ considerably from those of the β-glucosidase of almond emulsin. The question of identity of the several β-glucosidases has been thrown into greater doubt by the recent work of Zechmeister and his collaborators[66]; they report that by the application of chromatographic adsorption methods they have effected a partial separation not only of amygdalase, gentiobiase and salicinase in an enzyme preparation from the fungus *Merulius lacrimans*, but also of salicinase and cellobiase in almond emulsin.

TABLE VIII

COMPARATIVE STUDIES OF THE ENZYMIC HYDROLYSIS OF AMYGDALIN, β-PHENYL-D-GLUCOSIDE, β-PHENYL-D-GALACTOSIDE, GENTIOBIOSE AND CELLOBIOSE[a]

Time, min.	Substrate	Ml. 0.005 N $Na_2S_2O_3$[b]	% Hydrolysis	$k \times 10^5$	Enzyme value[c] $\times 10^4$
40,320	Amygdalin	2.99	65.3	1.1	4.5
40,320	β-Phenyl-D-glucoside	2.12	12.0	0.14	0.20
40,320	Gentiobiose	1.18	13.9	0.16	0.23
20,160	Cellobiose	0.02	0	..	..
20,160	β-Phenyl-D-galactoside	0.03	0	..	..

[a] With 0.233 g. of enzyme preparation Brewers' A, and 0.355 g. of amygdalin dihydrate, 0.406 g. of β-phenyl-D-glucoside dihydrate, 0.475 g. of gentiobiose, 0.475 g. of cellobiose, and 0.355 g. of β-phenyl-D-galactoside, respectively, in 50 ml. of reaction mixture, at pH 5.2. [b] The values represent the actual corrected volume of thiosulfate equivalent to the iodine consumed by the reducing hexoses which are liberated. [c] All enzyme values refer to the dilute substrate.

Although the β-D-glucosidase and the β-D-galactosidase of almond emulsin are believed to be identical,[59] the rates of hydrolysis of β-phenyl-D-galactoside, of lactose, and especially of β-methyl-D-glucoside by almond emulsin are so much lower than that of β-phenyl-D-glucoside[65] that we should not expect, nor could we detect, any appreciable hydrolysis of those three substrates by the Brewers' A preparation. Bakers' A contained still less β-D-glucosidase, and 301 mg. of it, in 50 ml. of dilute substrate at pH 5.2, caused only 15% hydrolysis of amygdalin in one week; Brewers' C (225 mg.), under the same conditions, effected no measurable hydrolysis.

α-Glucosides.—No evidence was obtained for the hydrolysis of any of the following substances by Brewers' A or by Bakers' A enzyme preparations: α-methyl-D-glucoside, α-phenyl-D-glucoside, trehalose (α-D-glucosido-α-D-glucoside), maltose (4-α-D-glucosido-D-glucose), or turanose (3-α-D-glucosido-D-fructose[30]). Because turanose is susceptible to hydrolysis by alkali,[67] the usual procedure of stopping the action of the enzyme

(63) Ref. 8d, p. 267.

(64) Ref. 8d, p. 276; see also pp. 186, 195, 266–278.

(65) Pigman, *J. Research Natl. Bur. Standards*, **27**, 1 (1941), by comparison with Helferich, ref. 59.

(66) Zechmeister, Tóth, Fürth and Bársony, *Enzymologia*, **9**, 155 (1941).

(67) Isbell, *J. Research Natl. Bur. Standards*, **26**, 35 (1941).

by adding sodium carbonate was omitted; when the iodine was added immediately before the sodium carbonate for the estimation of glucose, satisfactory measurements were possible. The crystalline α- and β-dextrins of Schardinger,[68] which are believed to contain α-glucosidic linkages exclusively, were likewise unaffected by Brewers' A and Bakers' A preparations. For all these substrates 58 mg. of Brewers' A or 75 mg. of Bakers' A was used for 50 ml. of dilute substrate. The tests were carried out at *p*H 4.0, 5.3 and 7.0 and, except in the case of β-dextrin, for a period of forty-eight hours. For the studies on β-dextrin, the ferricyanide method was used because the compound reacts with iodine. Larger amounts of enzyme (233 mg. of Brewers' A, and 301 mg. of Bakers' A) were used and the time of reaction was shortened to six hours because toluene, which was used as a preservative in all other extended reactions, precipitates β-dextrin from its aqueous solution.

Mannosidases

β-Phenyl-D-mannoside.—If ivory nut mannan and salep mannan have the β-mannoside configurations which Klages and Maurenbrecher[69] have assigned to them, the mannanase from malt extract,[70] which hydrolyzes these substances to mannose, must be considered a poly-β-D-mannosidase. The claims of Pringsheim[70] for the existence of two separate enzymes in the malt extract, one capable of hydrolyzing mannan to 4-β-D-mannosido-D-mannose, and the other a mannobiase which hydrolyzes the disaccharide, have been denied by Klages and his collaborators.[71]

The presence of a simple β-D-mannosidase[72] in our enzyme preparations from both bakers' and brewers' yeasts has been demonstrated conclusively. β-Phenyl-D-mannoside was hydrolyzed to the extent of 12.7% by Brewers' A and 9.0% by Bakers' A in four weeks at *p*H 4.0, at an enzyme concentration equivalent to 233 mg. and 301 mg. in 50 ml., respectively. These data represent enzyme values, with dilute substrate, of 2.1×10^{-5} and 1.1×10^{-5}, respectively. The presence of a β-mannosidase may be important in the removal of the yeast gums or mannans[73] during the preparation of the invertase solutions by the methods which have been described in the preceding publication.[1]

α-Mannosides.—No evidence was obtained for the hydrolysis of α-methyl-D-mannoside nor α-phenyl-D-mannoside, at *p*H 4.0, 5.3 or 7.0, in forty-eight hours with dilute substrate solutions, and with the equivalent of 75 mg. of Bakers' A or 58 mg. of Brewers' B in 50 ml. of reaction mixture.

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Summary

Five highly purified invertase preparations, obtained from both brewers' and bakers' yeasts, and by different methods of purification, have been compared in their behavior toward twenty-eight carbohydrate substrates.

The fructofuranoside linkages in sucrose, raffinose, stachyose and inulin were hydrolyzed by all five invertase preparations, but the relative rates of hydrolysis varied markedly with the source and with the method of purification of the enzyme solution. We are inclined to the view that one enzyme, β-D-fructofuranosidase, hydrolyzes the sucrose union in both sucrose and raffinose. However, the accumulated evidence from the ratios and from the optimal *p*H values suggests that the enzymic hydrolysis of inulin is brought about by a specific inulase rather than by this β-D-fructofuranosidase as postulated by Weidenhagen. The inulin molecule appears to contain 1.7% of glucose

(68) McClenahan, Tilden and Hudson, THIS JOURNAL, **64**, 2139 (1942).

(69) Klages and Maurenbrecher, *Ann.*, **535**, 175 (1938).

(70) Pringsheim and Genin, *Z. physiol. Chem.*, **140**, 299 (1924); Pringsheim, Genin and Perewosky, *Biochem. Z.*, **164**, 117 (1925).

(71) Klages and Maurenbrecher, ref. 69; Klages and Kircher, *Ann.*, **543**, 183 (1940).

(72) Sweet-almond emulsin contains no β-mannosidase; *cf.* Helferich and Winkler, *Z. physiol. Chem.*, **209**, 269 (1932).

(73) See Oppenheimer, ref. 8d, p. 516.

which is liberated gradually during the hydrolysis.

The α-D-galactosidase which is present in the brewers' yeast invertase preparations has been used in a comparative study of the hydrolysis of melibiose, α-methyl-D-galactoside, α-phenyl-D-galactoside and the configurationally related β-methyl-L-arabinoside.

The galactoside linkages in stachyose and manninotriose were hydrolyzed by the preparations from brewers' yeast which contain α-D-galactosidase but not β-D-galactosidase. Stachyose, based upon the methylation data of Onuki, can therefore be formulated completely as 6-α-D-galactopyranosido-4-α-D-galactopyranosido-2-α-D-glucopyranosido-β-D-fructofuranoside and manninotriose as 6-α-D-galactopyranosido-4-α-D-galactopyranosido-D-glucose (see formulas I and II of the text).

The purest invertase preparation, from brewers' yeast, contained a small amount of β-D-glucosidase which was capable of hydrolyzing amygdalin gentiobiose and β-phenyl-D-glucoside, but not cellobiose, or lactose.

Invertase preparations from both brewers' and bakers' yeasts contained small amounts of a new enzyme, a β-D-mannosidase which hydrolyzes β-phenyl-D-mannoside.

No evidence was obtained to indicate the hydrolysis of an α-D-fructofuranoside (isosucrose), or of any β-D-galactoside, α-D-glucoside (including the α- and β-dextrins), or α-D-mannoside by these highly purified invertase preparations. Melezitose and α-methyl-D-manno-D-gala-heptoside also were not hydrolyzed.

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The Action of *macerans* Amylase on the Fractions from Starch

BY E. JUSTIN WILSON, JR., THOMAS JOHN SCHOCH AND C. S. HUDSON

A starch fractionation recently has been reported[1] which utilizes the selective precipitation of one component of the starch by normal butyl alcohol. The fractions so obtained are characterized by marked differences in alkali lability, solubility, gelation and retrogradation tendencies. On the basis of X-ray evidence, iodine adsorption and flow polarization, Rundle and Baldwin[2] suggest that the fraction so isolated by butanol precipitation represents linear chains of glucose units, and that the material not precipitated by butanol comprises molecules of highly branched character.

Tilden and Hudson[3] have investigated the conversion of starch by the enzyme derived from *B. macerans*, and have suggested that the resulting crystalline Schardinger dextrins are derived from some basic configuration pre-existing in the starch molecule. The present study was undertaken to ascertain whether the seeming differences in the chemical nature of the butanol-separated fractions would be reflected in their behavior toward *macerans* amylase.

Isolation of Starch Fractions.—Since the butanol method for starch fractionation requires use of a continuous supercentrifuge, a procedure was developed for more general application which does not necessitate such specialized equipment. By treating a hot autoclaved starch sol with a mixture of isoamyl and normal butyl alcohols, a fraction of the starch precipitates on cooling which is readily removed by low speed centrifuging; a non-precipitated fraction is recovered from the supernate by the addition of excess methanol. While this procedure is somewhat tedious and does not lend itself to the processing of large quantities of starch, it gives substantially the same fractions as are obtained by supercentrifugal separation, and it was accordingly employed for the preparation of the starch fractions used in this study.

The yields of crude precipitated fraction from four runs of potato starch averaged 24.4 ± 0.9%; the average yield from defatted corn starch (five runs) was 24.9 ± 1.4%.[4] Waxy maize gave no precipitated fraction. Where supercentrifugal equipment is available, the use of mixed butanol and isoamyl alcohol affords somewhat improved mechanical separation of the precipitated fraction; the yields are identical with those obtained by the method herein described,

(1) Schoch, THIS JOURNAL, **64**, 2957 (1942).
(2) Rundle and Baldwin, *ibid.*, in press.
(3) Tilden and Hudson, *ibid.*, **61**, 2900 (1939).

(4) All weights and percentages in this article are corrected to the dry weight of starch substance.