ions appear to have an unfavorable influence. In this respect again gluc amylase resembles beta rather than alpha amylases.[18,19]

Influence of Hydrogen Ion Activities of Its Substrates upon the Action of Gluc Amylase.—The data summarized in Fig. 1 show the influence of hydrogen ion activities of its substrates upon the action of highly purified gluc amylase in hydrolyses at 40° of 120 minutes for maltose and of 30 minutes for a number of other substrates. The substrates included Lintner soluble potato starch; a linear fraction[20] from corn starch; a branched fraction[20] from corn starch, glycogen[21]; maltose.

For this work each substrate except maltose was dissolved in molar potassium hydroxide, diluted with water, neutralized with hydrochloric acid and all were adjusted to a final concentration of 1% substrate, 0.01 *M* acetate and 0.05 *M* chloride.

The data given in Fig. 1 show that the optimal action of gluc amylase under the conditions used here was obtained with each of the substrates at hydrogen ion activities that included *p*H 4.5. Similar results were obtained in hydrolyses of 24 hours; reaction mixtures adjusted to *p*H 4.5 again favored the action of the amylase. Therefore, it is recommended that the action of gluc amylase under the other conditions used here be studied at *p*H 4.5.

The data given in Fig. 1 compare the influence of hydrogen ion activity upon the hydrolysis of the different substrates by gluc amylase. They do not necessarily represent the relative rates of the hydrolysis of the different substrates. Such comparisons are discussed elsewhere.[16]

In addition to establishing the conditions which favor the action of gluc amylase at 40°, the data given in Fig. 1 show that gluc amylase produces glucose from starch, from its components, and from glycogen under conditions that prevent or inhibit the formation of glucose from maltose. Therefore, these data offer additional strong evidence that gluc amylase forms glucose directly from starch or its components without the preliminary formation of maltose.

In the course of these experiments it was found that concentrations of potassium chloride up to 0.1 *M* and of acetate up to 0.05 *M* did not change appreciably either the hydrogen ion activities that favor the action of gluc amylase or the activity of the amylase under the other conditions used here. These findings are of interest because the use of potassium hydroxide to dissolve starches and their components and glycogen sometimes results in the presence of different concentrations of potassium chloride in the final reaction mixtures.

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(20) The authors wish to thank Dr. T. J. Schoch for the gift of the linear and of the branched fractions from corn starch.

(21) The authors wish to thank Dr. James McBride for the gift of highly purified glycogen.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

A Study of the Action of Gluc Amylase, a Glucose-producing Amylase, Formed by the Mold, *Rhizopus delemar*[1]

BY LOUISE LANG PHILLIPS[2] AND M. L. CALDWELL

The action of highly purified gluc amylase, treated to free it from traces of alpha amylase, has been investigated with a number of different substrates. Given sufficient time for the hydrolyses and sufficiently high concentrations of amylase, such preparations of gluc amylase produce 95 to 96% of the theoretical glucose from the linear and from the branched fractions from corn starch and from defatted waxy maize starch; 92% from glycogen; 89% from residual beta dextrins, 100% from maltose. Glucose is the sole reducing product formed in these hydrolysates at least until approximately 90% of the theoretical glucose has been produced. Gluc amylase appears to hydrolyze glucose from the non-aldehydic ends of the glucosidic chains of its substrates in a manner similar to the hydrolysis of maltose from its substrates by beta amylase, but with gluc amylase, there is no evidence of the formation of high molecular weight non-reducing residual dextrins. Neither highly purified gluc amylase nor the alpha amylase that accompanies it in the crude extracts has any detectable influence upon the alpha or the beta Schardinger dextrins, upon a dextran, a polymer of α-D-glucopyranose, having predominantly 1,6-α-D-glucosidic linkages; or upon isomaltose (brachiose). The extensive hydrolysis of the branched substrates shows that gluc amylase either hydrolyzes the 1,6-α-D-glucosidic linkages of these substrates or passes by them in some manner. However, the failure to reach 100% hydrolysis with the branched substrates such as is attained with maltose and the failure of the gluc amylase to hydrolyze the dextran or isomaltose suggest that gluc amylase does not hydrolyze the 1,6-α-D-glucosidic linkages of its substrates. This point has not been established conclusively and is being investigated further. In the hydrolysis of the linear substrate by gluc amylase, a straight line relationship is obtained between the blue values and the glucose values of the hydrolysates. A similar relationship between the blue values of a linear hydrolysate and its maltose values has been reported previously for beta amylase. Gluc amylase appears to resemble beta amylases rather than alpha amylases in its action and in certain of its properties. The Michaelis or affinity constants of gluc amylase for linear and for branched fractions from corn starch and for maltose have been determined and are discussed.

Introduction

A glucose-producing amylase formed by the mold *Rhizopus delemar*[3] has been prepared in highly purified form[4] and freed from all detectable traces of alpha amylase activity.[4] The work reported here deals with a study of the action of such highly purified preparations of gluc amylase upon a number of substrates.

Experimental

Substrates.—The substrates investigated included: a

(1) The authors wish to thank the Takamine Laboratory, Inc., for generous grants in aid of this investigation.

(2) The data reported here are taken from a dissertation submitted by Louise Lang Phillips in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry under the Faculty of Pure Science of Columbia University.

(3) J. Corman and A. F. Langlykke, *Cereal Chem.*, **25**, 190 (1948).

(4) L. L. Phillips and M. L. Caldwell, THIS JOURNAL, **73**, 3559 (1951).

TABLE I

A STUDY OF THE RATE AND EXTENT OF THE HYDROLYSIS OF LINEAR FRACTION FROM CORN STARCH AND OF THE EXTENT OF THE HYDROLYSIS OF CERTAIN OTHER SUBSTRATES BY PURIFIED GLUC AMYLASE

Hydrolysis[a] time, minutes	Relative concentrations of amylase: 1-fold[b] Total reducing values[c] as glucose equivalents	1-fold[b] Glucose[d] produced	1-fold[b] Reducing products other than glucose	10-fold[b] Total reducing values[c] as glucose equivalents	10-fold[b] Glucose[d] produced	10-fold[b] Reducing products other than glucose	25-fold[b] Total reducing values[c] as glucose equivalents	25-fold[b] Glucose[d] produced	25-fold[b] Reducing products other than glucose
	Expressed as per cent. of theoretical glucose								
	A. Linear fraction from corn starch								
5				15	14	1	27	30	
10				24	26		44	46	
15				32	33		54	54	0
30	11	11	0	50	53		71	69	2
60	16	17		66	66	0	79	78	1
120	26	28		79	78	1	89	85	4
180	35	37		84	83	1	92	85	7
240	40	43		86	79	7	89	83	6
1440	78	76	2	93	89	4	95	86	9
	B. Branched-chain fraction from corn starch								
1440	88	89		90	90	0	95	89	6
	C. Waxy maize starch								
1440	89	84	5	96	90	6	96	85	11
	D. Glycogen								
1440	72	70	2	91	87	4	92	83	9

[a] Substrate 1%; 0.10 *M* potassium chloride; 0.05 *M* acetate; *p*H 4.5; 40°. [b] Amylase preparation; 0.057 mg. 0.57 mg. or 1.43 mg. per 100 mg. substrate. [c] Determined by iodometric method.[12] [d] Determined by copper acetate method.[16]

linear fraction from corn starch[5]; a branched fraction from corn starch[5]; waxy maize starch[6]; glycogen[10]; maltose[11]; a bacterial dextran,[13] formed by the action of the bacteria, *Leuconostoc mesenteroides;* residual beta dextrins; alpha and beta Schardinger dextrins[14] and isomaltose (brachiose).[15]

For use, the substrates were dissolved in potassium hydroxide, neutralized with hydrochloric acid and adjusted to give 1% substrate, 0.05 *M* acetate, 0.1 *M* potassium chloride and *p*H 4.5. The hydrolyses were carried out at 40°. These conditions had been found to favor the action of gluc amylase.[4] The total reducing values of the hydrolysates were determined by an iodometric method.[12] This method gives a stoichiometric measure of the glucosidic linkages broken in the substrate. These total reducing values were calculated to their equivalents of glucose and are reported as such. Glucose was determined by a modification[16] of the method of Zerban and Sattler.[17] Any difference between the total reducing value, calculated as glucose, and the reducing value actually due to glucose represented the reducing value of products other than glucose.

Maltose was included as a substrate because all of the evidence[4] so far obtained indicates that gluc amylase produces glucose from maltose as well as from starches, their components and their hydrolysis products.

Hydrolysis of Linear Substrate, Reducing Values.—The data summarized in Table I, section A, are typical of the results obtained when highly purified gluc amylase, freed from detectable traces of alpha amylase,[4] reacted with the linear fraction from corn starch. Increasing concentrations of gluc amylase increased the rate and the extent of the hydrolysis of the substrate. However, complete, 100% hydrolysis was not attained in hydrolyses of 24 hours even with a relatively very high concentration of the amylase, 25 times that used for activity measurements in 30 minute hydrolyses of Lintner soluble potato starch.[4] The small difference between the 95% theoretical glucose repeatedly attained with the highest concentration of amylase in 24 hours, and 100% was probably due in part to retrogradation of unhydrolyzed substrate. That some retrogradation had occurred was indicated by the fact that hydrolysates of the linear substrate invariably became cloudy even in the presence of the highest concentrations of gluc amylase investigated.

The data given in Table I show close agreement between the total reducing values and the glucose values of the hydrolysates of the linear fraction until the very late stages of the hydrolyses, when approximately 90% of the theoretical glucose has been formed. Therefore, glucose is the sole reducing product formed from the linear substrate until the very late stages of its hydrolysis by gluc amylase.

In the very late stages of the hydrolyses of the

(5) The authors wish to thank Dr. T. J. Schoch who kindly furnished the linear and the branched fractions from corn starch.

(6) The waxy maize starch was kindly furnished by the National Starch Products Co., Inc. It was defatted by treatment with methanol[7] and washed repeatedly with water. It gave no evidence of the presence of linear components either by potentiometric titration[8] or by precipitation procedures.[9]

(7) T. J. Schoch, THIS JOURNAL, **64**, 2954 (1942).

(8) F. L. Bates, D. French and R. E. Rundle, *ibid.*, **65**, 142 (1943).

(9) T. J. Schoch, "Advances in Carbohydrate Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1945, p. 260.

(10) The authors wish to thank Dr. James McBride for the highly purified glycogen.

(11) Maltose $[\alpha]^{25}D$ 130.1; reducing value 95% by iodometric method.[12]

(12) M. L. Caldwell, S. E. Doebbeling and S. H. Manian, *Ind. Eng. Chem., Anal. Ed.*, **8**, 181 (1936).

(13) The authors wish to thank Dr. Allene Jeanes for the dextran. The dextran is described as Dextran A or Dextran C, Table II, A. Jeanes, C. A. Wilham and J. C. Miers, *J. Biol. Chem.*, **176**, 603 (1948).

(14) The authors wish to thank Dr. Evelyn Tilden who kindy supplied the Schardinger dextrins.

(15) The authors wish to thank Miss Edna Montgomery who kindly supplied the isomaltose.

(16) L. L. Phillips and M. L. Caldwell, *Ind. Eng. Chem., Anal. Ed.*, in press.

(17) F. W. Zerban and L. Sattler, *ibid.*, **10**, 669 (1938).

linear substrate, small but perhaps significant differences are observed between the total reducing values and the glucose values of the hydrolysates, Table I. These differences suggest the presence of low concentrations of low molecular weight reducing products other than glucose that are hydrolyzed slowly or with difficulty by gluc amylase. Presumably, such residual products would be characterized by 1,4-α-D-glucosidic linkages because they are obtained from a linear substrate. It is hard to reconcile the presence of such residual products in the hydrolysates of a linear substrate with the finding, discussed later, that gluc amylase produces 100% glucose from maltose under the same conditions and in the presence of the same relative concentrations of amylase and substrate, see Fig. 4, beyond. Therefore, it is suggested that the presence of low molecular weight reducing products during the very late stages of the hydrolyses of the linear substrate may indicate synthetic action by gluc amylase, similar to that reported for other amylases from mold sources.[18,19] This possibility is being investigated.

Hydrolysis of Linear Substrate, Blue Values.—With the linear fractions from starches, the hydrolysis of the substrate can be followed quantitatively by determinations of blue values.[20,21] These determinations depend upon the fact that the linear components of starches absorb approximately 19% of their weight of iodine[7] to form complexes[8,22,23,24] that can be determined quantitatively by spectrophotometric methods.[20] For this part of the work, aliquots were removed from the hydrolysates of the linear fraction of corn starch at suitable time intervals and measured for glucose[16] and for blue values according to Hassid.[20] The results are summarized in Fig. 1 and compared with data reported by Bourne, *et al.*,[25] for similar measurements of the hydrolysis of a linear fraction from starch by beta amylase from soy beans and by salivary amylase, an alpha amylase. Although the slopes are not the same, straight lines are formed by the data for beta amylase[25] and by those for gluc amylase, Fig. 1. This linearity indicates a similarity in the action of gluc amylase and of beta amylase. Beta amylases are believed to split maltose from the non-aldehydic ends of the glucosidic chains of their substrates.[26] It is suggested that gluc amylase splits glucose similarly from the non-aldehydic ends of the glucosidic chains of its substrates. This suggestion is supported by the repeated observation that glucose appears to be the sole reducing product formed by gluc amylase from its substrates at least until the very late stages of the hydrolyses, when very low concentrations of low molecular weight residual dextrins or other sugars may also be present, Table I.

Hydrolysis of Branched Chain Substrates, Production of Glucose.—Close agreement between the total reducing values[12] and the glucose values[16] of the hydrolysates until the very late stages of their hydrolyses, corresponding to approximately 90% theoretical glucose, also was observed with all the other substrates so far studied with purified gluc amylase that had been treated to free it from traces of alpha amylase.[4] Substrates in addition to the linear substrate for which this agreement was found include a branched fraction from corn starch; waxy maize starch; glycogen; residual beta dextrins obtained from the above waxy maize starch; Lintner soluble potato starch.[4] The close agreement in these values with each of these substrates leads to the conclusion that glucose is the sole reducing product formed from its substrates by purified gluc amylase at least until the very late stages of their hydrolyses, when approximately 90% of the theoretical glucose has been formed. Because of their similarity to the data presented in Table I, section A, for the linear substrate, the detailed data for similar measurements with the other substrates are omitted for the sake of brevity. However, results obtained in 24-hour hydrolyses of several of these substrates with increasing concentrations of purified gluc amylase are included for comparison in Table I, sections B, C and D.

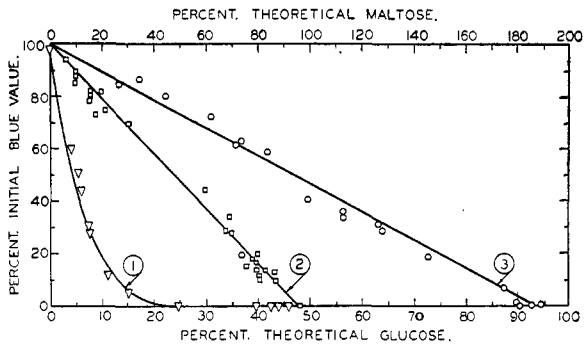

Fig. 1.—A comparison of the hydrolysis of a linear substrate by purified gluc amylase with similar measurements, reported by Bourne, Macey and Peat,[25] for beta amylase from soy beans and for salivary amylase, an alpha amylase: curve 1, salivary amylase[25]; curve 2, beta amylase[22]; curve 3, gluc amylase.

Hydrolysis of Branched Substrates, Residual Products.—As shown in Table I, sections B, C and D, the hydrolysates of the branched substrates also failed to reach 100% theoretical glucose even with a relatively high, 25-fold, concentration of gluc amylase acting for 24 hours. Again, there is an indication in the later stages of the hydrolyses of the presence of small concentrations of low molecular weight residual reducing products other than glucose. On the other hand there was no indication in any of the hydrolysates of the formation of high molecular weight non-reducing residual dextrins such as are obtained in hydrolysates of starches and of their branched components with beta amylases.[27-30] These data suggest that gluc amylase either can break the 1,6-α-D-glucosidic linkages at the branching points of these substrates or can by-pass them in some manner. Evidence to be presented later supports the latter explanation.

A Comparison of the Rates and of the Extents of the Hydrolyses of Different Substrates.—The data given in Figs. 2, 3, 4 and 5 compare the rates and the extents of the hydrolyses of different substrates with increasing concentrations of purified gluc amylase that had been treated to free it from traces of alpha amylase.[4] These data and those given in Table I show that the branched fraction from corn starch and waxy maize starch were hydrolyzed at very nearly the same rate and to very nearly the same extent when treated under the same conditions with the same concentrations of purified gluc amylase. This similarity is not surprising because these two substrates are probably very similar in architecture.[26,31] The comparisons given

(18) S. C. Pan, L. W. Nicholson and P. J. Kolochov, THIS JOURNAL, **73**, 2547 (1951).

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(20) R. M. McCready and W. Z. Hassid, THIS JOURNAL, **65**, 1154 (1943).

(21) Blue value is defined as follows: B. V. = $k \log 100/T$, where T is the per cent. transmission of a solution of 5 mg. of a linear fraction in 500 ml. of a solution of 0.002% iodine and 0.02% potassium iodide and where $k = 1/\log 100/T^*$, and T^* is the per cent. transmission of a solution of a pure linear fraction of the same concentration. In the present case, $k = 2.69$ and $T^* = 42.5\%$. The per cent. transmission was read in a Lumetron with a no. 660 filter.

(22) R. R. Baldwin, R. S. Bear and R. E. Rundle, THIS JOURNAL, **66**, 111 (1944).

(23) R. E. Rundle, J. F. Foster and R. R. Baldwin, *ibid.*, **66**, 2116 (1944).

(24) S. Lansky, M. Kooi and T. J. Schoch, *ibid.*, **71**, 4066 (1949).

(25) E. J. Bourne, A. Macey and S. Peat, *J. Chem. Soc.*, 882 (1945).

(26) K. H. Meyer, "Advances in Colloid Science," Vol. I, Interscience, New York, N. Y., 1942, p. 172.

(27) E. Ohlsson, *Z. physiol. Chem.*, **189**, 17 (1930).

(28) G. A. van Klinkenberg, *ibid.*, **209**, 253 (1932).

(29) C. O. Beckmann and Q. J. Landis, THIS JOURNAL, **61**, 1495 (1939).

(30) K. H. Meyer, P. Bernfeld, R. A. Boissonnas, P. Gürtler and G. Noelting, *J. Phys. Colloid Chem.*, **53**, 319 (1949).

(31) H. H. Schopmeyer, G. E. Felton and C. L. Ford, *Ind. Eng. Chem.*, **35**, 1168 (1943).

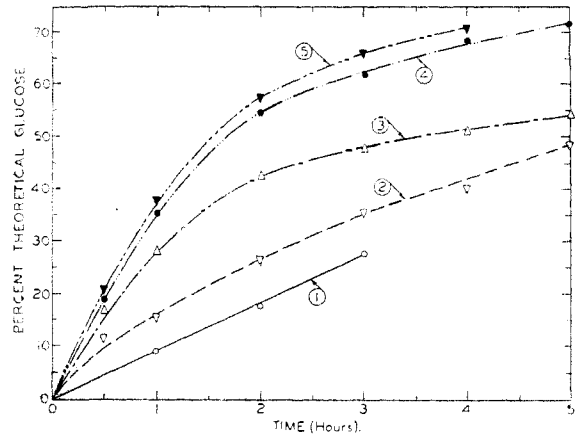

Fig. 2.—A comparison of the hydrolysis of several substrates by purified gluc amylase that had been treated to free it from traces of alpha amylase. Substrates 1%; 0.05 *M* acetate; 0.10 *M* KCl; *p*H 4.5; 40°. Gluc amylase preparation, 0.057 mg. per 100 mg. substrate. Curve 1, maltose; curve 2, linear substrate; curve 3, glycogen; curve 4, branched fraction from corn starch; curve 5, defatted waxy maize starch.

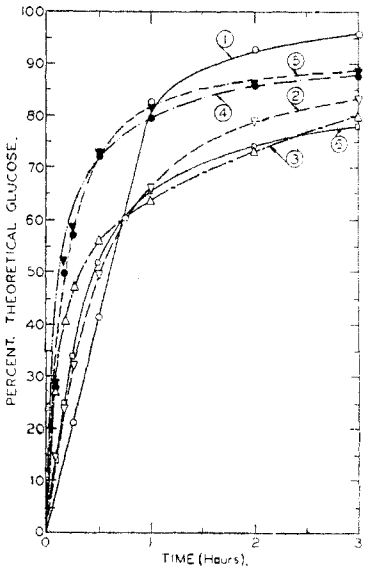

Fig. 3.—A comparison of the hydrolysis of several substrates by purified gluc amylase. Conditions, the same as those for Fig. 2 except for a 10-fold increase in the concentration of gluc amylase: curve 1, maltose; curve 2, linear substrate; curve 3, glycogen; curve 4, branched fraction from corn starch; curve 5, defatted waxy maize starch; curve 6, residual beta dextrins from the waxy maize starch.

in Figs. 2, 3, 4 and 5 also show that the branched fraction from corn starch and waxy maize starch were hydrolyzed more rapidly by the same concentrations of gluc amylase than the linear fraction from corn starch. This difference in the rate of the hydrolyses of these substrates suggests that gluc amylase finds more points for attack in the more numerous non-aldehydic ends of the branched substrates than in the linear substrate.

Glycogen, also, was hydrolyzed more rapidly than the near component from corn starch during the earlier stages of their comparable hydrolyses, Figs. 2, 3, 4 and 5, but less rapidly than the other branched substrates studied. These differences suggest that the branches in the highly branched

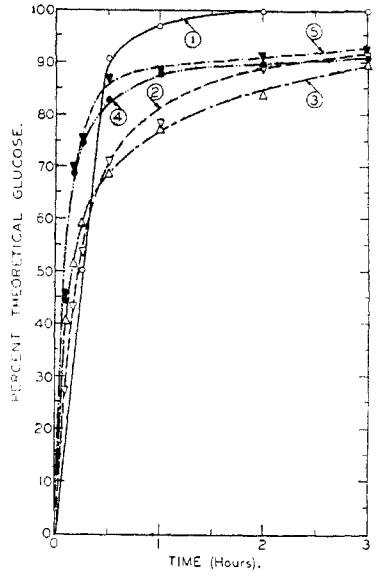

Fig. 4.—A comparison of the hydrolysis of several substrates by purified gluc amylase. Conditions, the same as those for Fig. 2 except for a 25-fold increase in the concentration of gluc amylase: curve 1, maltose; curve 2, linear substrate; curve 3, glycogen; curve 4, branched fraction from corn starch; curve 5, defatted waxy maize starch.

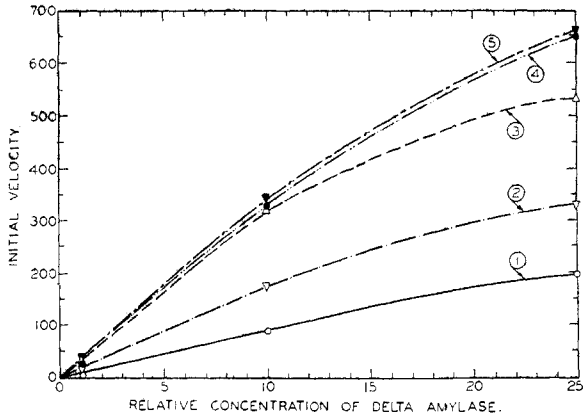

Fig. 5.—A comparison of the initial velocities of the hydrolyses of several substrates by purified gluc amylase that had been treated to free it from detectable traces of alpha amylase. Substrates 1%; 0.05 *M* acetate; 0.10 *M* KCl; *p*H 4.5; 40°. Initial velocity, taken from early linear part of hydrolysis—time curves and expressed as per cent. hydrolysis per hour. Curve 1, maltose; curve 2, linear substrate; curve 3, glycogen; curve 4, branched fraction from corn starch; curve 5, defatted waxy maize starch.

glycogen[32] may be too short to be as favorable to the action of gluc amylase as the longer branches[26] of the other branched substrates studied here. This point is being investigated further.

Hydrolysis of Maltose.—The data given in Figs. 2, 3, 4 and 5 also show that maltose was hydrolyzed more slowly by highly purified gluc amylase than the other substrates in the comparison. However, eventually maltose was hydrolyzed completely, yielding 100% glucose in 24 hours even under the influence of the relatively low, 1-fold, concentration of gluc amylase. This finding, repeatedly observed, indicates that the failure to reach 100% hydrolysis with the other substrates may be significant. Additional evidence is being sought on this point.

(32) K. H. Meyer, "Advances in Enzymology," Vol. 3, Interscience Publishers, Inc., New York, N. Y., 1943, p. 109.

Michaelis Constants.—The Michaelis or affinity constants[33,34] were determined for the action of gluc amylase upon the linear and the branched fractions from corn starch and upon maltose. The glucose produced by the action of gluc amylase upon each of several different concentrations of each of these substrates was plotted against time and the initial velocities calculated for each of the substrate concentrations. Values for the Michaelis constants were then determined by both graphical methods recommended by Lineweaver and Burk[34] and averaged for each substrate.

Preferably, the affinity constants of an enzyme for different substrates should not be compared in terms of per cent. concentrations. Therefore, the molar concentrations of the substrates were calculated from the best available data for their molecular weights. That for the linear fraction from corn starch was taken as 150,000[35] and that for the branched fraction from corn starch as 1,500,000.[35] Using these values, the Michaelis constants (K_s) for the three substrates are 0.23% or 6.6×10^{-3} M for maltose, 0.575% or 4.4×10^{-5} M for the linear substrate and 0.062% or 4.1×10^{-7} M for the branched substrate. The values calculated for the affinities, the reciprocal of the Michaelis constant ($1/K_s$), of gluc amylase for the three substrates are 150 for maltose, 22,000 for the linear substrate and 2,400,000 for the branched substrate.

A study of the data given in Fig. 5 shows that the relative initial velocities of the action of gluc amylase on the three substrates are in the approximate ratios of 0.5 for maltose to 1.0 for the linear substrate to 2.0 for the branched substrate. On the other hand, the affinity constants given above for the action of gluc amylase on these three substrates are in the approximate order of 0.01 for maltose to 1.0 for the linear substrate to 100 for the branched substrate. These relationships suggest that the relative rates of the hydrolysis of different substrates by gluc amylase are doubled as the affinity constants of the amylase for these substrates increase 100-fold. A more comprehensive study of these relationships for gluc amylase and for several other amylases will be reported elsewhere.[36]

Hydrolysis of Residual Beta Dextrins from Waxy Maize Starch.—Residual beta dextrins formed by the exhaustive action of beta amylase on waxy maize starch also were used as substrate for gluc amylase action. Beta amylase reacted at pH 4.5 and 0.01 M acetate upon 2% waxy maize starch. The hydrolysate reached a limit of hydrolysis at 49% theoretical maltose. This limit was not changed by prolonging the time of hydrolysis or by the addition of more beta amylase. The hydrolysate was boiled to inactivate the beta amylase, dialyzed until free from maltose, adjusted to 1% dextrins, 0.05 M acetate and pH 4.5 and treated with gluc amylase at a concentration of 10 times that used in 30 minute activity measurements with starch.

The comparison given in Fig. 3 shows that the beta residual dextrins were hydrolyzed more slowly under comparable conditions than the waxy maize starch from which they were formed. However, eventually they were hydrolyzed to very nearly the same extent as the original waxy maize starch.

The data for the hydrolyses of the residual beta dextrins shows 14% theoretical glucose in 5 minutes; 52% in 30 minutes; 66% in 60 minutes; 78% in 180 minutes; 82% in 24 hours and 89% in 96 hours. A total of 89% theoretical glucose for the residual beta dextrins corresponds to a total of 94% theoretical glucose, calculated[37] for the original waxy maize starch by way of the beta dextrins. This value of 94% compares favorably with the 96% theoretical glucose obtained directly from the waxy maize starch in comparable measurements by the action of gluc amylase alone.

Failure to Cause the Hydrolysis of Dextran or of the Schardinger Dextrins.—The dextran[13] conforms to the following description. It is a polymer of α-D-glucopyranose. The "molecule" has a branched structure in which the predominant glucosidic linkage is 1,6- while 1,4-linkages occur at points of branching.[38] The alpha and beta Schardinger dextrins[14] are cyclic α-D-glucopyranosides having 6 and 7 glucose units, respectively, joined by 1,4-α-D-glucosidic linkages.[39-42]

Attempts to cause the hydrolysis of the dextran and of the Schardinger dextrins were made with highly purified gluc amylase and also with a preparation of gluc amylase that was contaminated with considerable alpha amylase. No evidence of activity was obtained in any of these reaction mixtures even after 96 hours at 40° in the presence of concentrations of gluc amylase that produced 90 to 98% of the theoretical glucose from Lintner soluble potato starch in control hydrolyzates.

In order to make sure that the negative results were not due to inactivation of the amylases by unknown impurities, the reaction mixtures in which no hydrolysis had been detected were measured for amylase activity. Portions of the reaction mixtures that gave no evidence of hydrolysis of dextran or of Schardinger dextrins in 24 hours were added to an equal volume of 2% Lintner soluble potato starch substrate and incubated at 40°. The Lintner soluble potato starch was hydrolyzed. It yielded 87 to 97% theoretical glucose in 24 hours. The higher values for theoretical glucose were obtained with the reaction mixtures that had contained alpha amylase as well as gluc amylase. These results show that active amylase remained in the inactive reaction mixtures and that the negative results obtained with dextran and with the Schardinger dextrins were not due to inactivation of the amylases. The results lead to the conclusion that neither the gluc amylase nor the alpha amylase from *Rhizopus delemar* hydrolyzes 1% dextran or 1% alpha or beta Schardinger dextrins under the conditions of these experiments.

It is interesting to note in passing that quantitative measurements showed that the purified gluc amylase had lost approximately 30% of its activity upon being incubated with dextran or with Schardinger dextrins for 24 hours at 40°. These findings are not surprising as the amylase apparently did not form protecting enzyme–substrate complexes with these substances.[43]

Isomaltose or Brachiose.—Attempts to cause the hydrolysis of isomaltose or brachiose,[15] 6-[α-D glucopyranosyl]-D-glucose, also were not successful. No increase in reducing value was detected when a 2% solution of isomaltose, adjusted to 0.01 M acetate and pH 4.5, was incubated at 40° for 24 hours with highly purified gluc amylase at a relative concentration of 2.5. This concentration of gluc amylase was 2.5 times that required to cause the formation of 15% theoretical glucose from 1% starch in 30 minutes or from 1% maltose in 2 hours or of 100% theoretical glucose from maltose in 24 hours, under otherwise the same conditions. Measurements with Lintner's soluble potato starch showed that 78% of the gluc amylase activity remained after the 24 hours of incubation with the isomaltose. Therefore, the failure to hydrolyze the isomaltose cannot be ascribed to inactivation of gluc amylase by some unknown impurities.

Discussion

Glucose, Sole Reducing Product Formed at Least until the Very Late Stages of the Hydrolyses.—That glucose is the sole reducing product formed until the very late stages of the hydrolyses of starches, of their components and of glycogen by gluc amylase is strongly indicated by the close agreement observed between the total reducing values and the glucose values of the hydrolysates until approximately 90% of the theoretical glucose has been formed. This close agreement also indicates that gluc amylase hydrolyzes glucose from the non-aldehydic ends of the glucosidic chains

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(37) From outside branches, 49% theoretical maltose; from resulting residual beta dextrins, 89% of 51% or 45%, a total of 94% theoretical glucose from the waxy maize starch by way of the beta dextrins.

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and thus increases only slightly the reducing value attributable at any one time to the dextrins formed.

Influence of 1,6-α-D-Glucosidic Linkages.—The hydrolysis of the branched fraction from corn starch to 95% theoretical glucose; of waxy maize starch to 96%; of glycogen to 92% and of residual beta dextrins to 89% proves that gluc amylase either hydrolyzes the 1,6-α-D-glucosidic linkages of its substrates or by-passes them in some manner. On the other hand, the failure of gluc amylase to hydrolyze isomaltose or dextran strongly suggests although it does not prove that gluc amylase does not hydrolyze 1,6-α-D-glucosidic linkages.

Additional indirect evidence also appears to support the conclusion that gluc amylase either does not hydrolyze the 1,6-α-D-glucosidic linkages of its substrates at all or does so slowly, with difficulty, and perhaps only in certain positions in the molecule. Thus, the data given in Table I and in Figs. 2 to 4 show that all of the branched substrates that were hydrolyzed by gluc amylase appeared to reach a limit of hydrolysis at approximately 92 to 96% theoretical glucose under the same conditions that yielded 100% theoretical glucose from maltose. It is possible that these values of 92 to 96% do not represent limits of hydrolysis but rather stages of low rates of change in the reactions and that eventually 100% hydrolysis might have been attained with these branched substrates as well as with maltose. It is also possible that the differences between 100% and 92% to 96% theoretical glucose represent experimental error or are due to unknown impurities in the substrates. However, these differences may have significance. They have been repeatedly obtained in about the same magnitude and always in the same direction, below the 100% hydrolysis attained with maltose. Moreover, there probably is significance in the small differences observed consistently between the total reducing value and the glucose values of the hydrolysates during the very late stages of the hydrolyses. These differences suggest the presence in the hydrolysates of small residual dextrins that are hydrolyzed with difficulty by gluc amylase, presumably because they contain 1,6-α-D-glucosidic linkages. Taken all together, the evidence appears to indicate that gluc amylase does not hydrolyze the 1,6-α-D-glucosidic linkages of its substrates. Because this evidence is not entirely conclusive, the study is being continued with other glucosides that contain 1,6-α-D-glucosidic linkages. It is recognized that many factors in addition to the kind of linkage influence the action and affinity of an enzyme.

It is also recognized that the reducing products other than glucose that appear in the late stages of the hydrolyses of its substrates may represent products of synthetic action of gluc amylase similar to that reported for other amylases of fungus origin.[18,19] This possibility is being investigated.

Influence of Other Factors.—The failure of gluc amylase to hydrolyze the cyclic alpha and beta Schardinger dextrins suggests but does not prove that gluc amylase requires end groups for its action. This interpretation is in accord with the higher affinity of gluc amylase for its branched than for its linear substrates which indicates that additional end groups in its substrates favor the action of gluc amylase. On the other hand, the hydrolysis of residual beta dextrins to 89% theoretical glucose shows that gluc amylase does not require chains of more than 3 or 4 glucose units for its action.[44]

Comparison of Gluc Amylase and of Beta Amylases.—Gluc amylase resembles beta amylases in certain aspects of its action and in certain of its properties. Like beta amylase, gluc amylase appears to attack the non-reducing ends of the glucosidic chains of its substrates but unlike beta amylase, it produces glucose rather than maltose and gives no evidence of the formation of high molecular weight residual dextrins.

Products that give color with iodine persist in the hydrolysates throughout the hydrolyses of linear substrates with beta amylases and until the very late stages of the hydrolyses with gluc amylase and make it possible to detect traces of alpha amylase impurities in beta amylase and also in gluc amylase preparations.[4]

Like beta amylases,[45] a greater inactivation takes place with gluc amylase solutions at unfavorable temperatures in the presence of calcium ions than in their absence.[4] Again, with both beta and gluc amylases a linear relationship is obtained between the per cent. of theoretical maltose on one hand or of glucose on the other and the per cent. of the initial blue value given by the linear substrate that remains unhydrolyzed during the action of these amylases.

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