or ions within narrow ranges of *p*H and ionic strength.

The gels obtained with barium hydroxide are highly oriented. The specificity of the barium ion for their formation has not been demonstrated. The divalence of the barium ion and its size may both be important in the formation of oriented chains or networks of protein molecules. This possible explanation has been suggested by Dr. John T. Edsall (private communication).

In both series of crystalline preparations the birefringence of the crystals ($\gamma - \alpha$) decreases on drying. The general decrease in birefringence is accompanied by a marked increase in the absolute values of the refractive indices of the dried crystals.

A similar increase in refractive indices on drying has been reported by Jones[5] for lysozyme. The increase in the absolute values for the refractive indices of both series of dry protein crystals is to be expected on the basis of the calculated value for the refractive index of anhydrous human serum albumin.[14] The presence of water ($n^{20°} = 1.333$) and methanol ($n^{20°} = 1.328$) in the crystals would be expected to reduce the refractive indices of the crystal below that of the anhydrous protein molecule.

The intrinsic optical anisotropy of the serum albumin molecule may be represented by the optical anisotropy of the dry crystal. The optical anisotropy of the wet crystals may reflect the distribution of the alcohol–water layers in the crystal structure. The dilution effect in both series of serum albumin crystals is itself anisotropic, and appears to conceal and reverse the anisotropy of the molecule. It thus differs from the dilution effects assumed in methaemoglobin crystals.[4]

The behavior of these crystals upon drying indicates that at a certain stage the edges of the crystal bounding the main face are "dry" before the center of the crystal has reached equilibrium. Subsequent liquid loss must take place, therefore, at the air–crystal interface of the plate.

Acknowledgments.—We wish to thank Professor E. J. Cohn for suggesting this research and for his encouragement and interest. We wish also to thank Professor J. T. Edsall for his stimulating discussion and criticism at all stages of the work and during the preparation of this manuscript. We also benefitted from discussion with Professor C. Frondel.

BOSTON, MASS. RECEIVED JANUARY 8, 1951

[CONTRIBUTION FROM THE GEORGE M. MOFFETT RESEARCH LABORATORIES, CORN PRODUCTS REFINING COMPANY]

The Action of Amylo-glucosidase on Amylose and Amylopectin

BY R. W. KERR, F. C. CLEVELAND AND W. J. KATZBECK

The hydrolysis of corn amylose by an *Aspergillus niger* enzyme preparation was studied by determination of total reducing substance, of glucose and of the change in wave length for peak light absorption of the iodine–hydrolysate complex. Two actions were apparent; an endwise attack on some amylose molecules to produce relatively large percentages of glucose early in the reaction, and a random hydrolysis of the others. By treatment of the enzyme preparation with acid at lower *p*H levels, the α-amylase function was inactivated more in proportion than the amylo-glucosidase; at a level of *p*H 2.2, substantially only amylo-glucosidase activity remained. Amylo-glucosidase was found to produce glucose at the same rate, in weight per unit time, from equimolar solutions of amyloses differing widely in original chain length. The unconverted polymer residue at different times during the first half of the hydrolysis was found not to change materially in DP_n, as measured by iodine-spectrophotometry, and glucose was the only sugar found in the hydrolysate by paper chromatography. At equal in weight concentrations, the initial rate of hydrolysis for corn amylopectin (B-fraction) was found to be 4.5 times the initial rate for corn amylose (A-fraction), but the rate for the amylopectin decreased abruptly to a lower value at a level of 60% hydrolysis to glucose. The hydrolysis of a highly linear substrate, corn crystalline amylose, was found to be a first order reaction. Amylo-glycosidase action can be interpreted by assuming a terminalwise hydrolysis of starch molecules to glucose and, in the case of linear ones at least, according to the "single-chain" mechanism. The wave length for peak light absorption by the iodine complexes of a series of amyloses was correlated against DP_n.

The α-glucosidases, as a class, are able to hydrolyze α-glucoside linkages in glucosides, producing glucose. Members of this class of enzymes which are able to operate on higher polysaccharides were recognized in early work by Kerr and co-workers[1,2] because of their ability to produce large yields of glucose from starch and starch products. One fungal preparation at least was found able apparently to hydrolyze even the limit dextrins remaining after an extended hydrolysis of starch by malt diastase, materially increasing the glucose yield and decreasing the percentage of residual dextrins.[2] Glucosidases which split 1–6 α-glucoside linkages have been called limit dextrinases.[3,4] The α-glucosidases have also been grouped under the general heading of maltases.[5] Other names have been proposed and of the names suggested it would now appear appropriate, following Cori and Larner,[6] to designate all of these starch-splitting, glucose-producing enzymes by the general group name of amylo-glucosidases.

Work in recent years with very pure enzyme preparations has shown, however, that the α-amylases are also able to produce glucose from starch. Thus, Bernfeld and Studer-Pecha,[7] in a study of the action of crystalline α-amylases, believe that the final limit of hydrolysis of amylose is attained in a prolonged second phase of the reaction with complete conversion of substrate to glucose and mal-

(1) R. W. Kerr and N. F. Schink, *Ind. Eng. Chem.*, **33**, 1418 (1941).
(2) R. W. Kerr, H. Meisel and N. F. Schink, *ibid.*, **34**, 1232 (1942).
(3) E. Kneen and J. M. Spoerl, *Am. Soc. Brewing Chemists, Proc.*, 28 (1938).
(4) T. M. Back, W. H. Stark and R. E. Scalf, *Anal. Chem.*, **20**, 56 (1948).
(5) J. Corman and A. F. Langlykke, *Cereal Chem.*, **25**, 190 (1948).
(6) Gerty Cori and Joseph Larner, *Federation Proc.*, **9**, 163 (1950).
(7) P. Bernfeld and H. Studer-Pecha, *Helv. Chim. Acta*, **30**, 1895 (1947).

tose in a ratio of 13% of the mono- to 87% of the disaccharide. It seemed desirable therefore to learn how amyloglucosidases differ, if at all, from α-amylases.

Furthermore, very little is known of the mechanism by which amylo-glucosidases operate. Kerr and co-workers[8,9,10] have proposed that β-amylase, which operates on the non-aldehydic terminal of starch chains such as amylose by splitting off successive maltose units, completes its task on one molecule before attacking another. In contrast to this "single-chain" mode of attack, Swanson[11] found that α-amylase is less discriminating in its action, breaking all substrate molecules into large fragments, which in turn are reduced to progressively smaller sections, a view which is shared by many workers in this field.[12] It is of interest to learn whether other sugar-producing enzymes act in the unique manner postulated for β-amylase or whether the action is more random as exhibited by the α-amylases.

An enzyme preparation containing amylo-glucosidase was selected for this study, which was prepared from *Aspergillus niger* N.R.R.L.-330 #1,[13] and which was already known to produce exceptionally large yields of glucose[5]; approximate yields from starch were reported as 92% glucose and only 2% maltose. The substrate chosen for most of our studies was the butanol precipitable fraction of corn starch (corn A-fraction) so as to lessen possible complications which might arise from the presence of more than one type of α-glucosidic linkage. Other amylose samples and an amylopectin were used in several experiments.

The action of the *Aspergillus niger* enzyme was studied, (a) by comparing the wave length of minimum light transmission for the iodine–hydrolysate complex with the extent of hydrolysis of amylose as measured by ferricyanide oxidation, (b) by comparing the ratio of glucose formed to the total yield of reducing substance, (c) by comparing the rates at which glucose was formed from equimolar solutions of amyloses of different chain lengths, and (d) by noting the change in these relationships as the enzyme preparation was inactivated, or partially inactivated, by acid.

Experimental

Preparation of the corn A-fraction sample (our preparation No. 27) has already been described[8]; the potato and tapioca amyloses (A-fractions) were prepared by the same procedure. Preparation of corn crystalline amylose has been described by Kerr and Severson.[14] The acid-hydrolyzed amylose sample was prepared from a commercial grade of 90-fluidity corn starch by butanol precipitation and recrystallization from aqueous butanol.

For the hydrolysis, 2.500 g., dry basis, of the amylose was dissolved in 12.5 ml. of 2 *N* KOH at room temperature. Within 2 hours the solution was adjusted to *p*H 5.0 with dilute HCl, adjusted to a final volume of 625 ml. at 45°, including the addition of 10 ml. of the enzyme preparation. The temperature was maintained at 45° and 5-ml. portions of the hydrolysate were withdrawn for analysis by alkaline ferricyanide oxidation according to the method previously outlined[8] with the exception that recrystallized glucose (anhydrous basis) was used as a standard instead of maltose. The results at stated intervals as shown in the tables were calculated on the basis of per cent. by weight of amylose hydrolyzed to glucose.

Several aliquots were analyzed for actual glucose content by a modification of the method of Sichert and Bleyer.[15]

At stated times during the hydrolyses, aliquots were diluted, stained with iodine and light absorption values determined as follows: 5 ml. of the hydrolysate was pipetted into a liter flask containing about 650 ml. of water and 4 ml. of iodine–iodide solution. The latter contained 5 g. of iodine and 7.5 g. of KI per liter. The volume was quickly adjusted to 1 liter, 13-mm. cuvettes were filled with the solution and fractional light transmission values were determined immediately in a Coleman, Model 14, spectrophotometer. At different times during hydrolysis minimum per cent. light transmission values were estimated and the corresponding wave lengths noted.

The procedures used in obtaining the wave length of minimum light transmission for the iodine complexes of the several amyloses listed in Table IV were the same as those given above, with the exception that no enzyme was added to the solution. Number average molecular weights, expressed as DP_n values, were determined from osmotic pressure measurements of the acetates in chloroform solution by procedures essentially as given in earlier reports.[16,17] The corn amylose sample (no. 27) was acetylated by the formamide dispersion method[17] and the value obtained, DP_n 480, is measurably higher than the value of DP_n 455, obtained after acetylation in aqueous pyridine.[16] Values obtained for the potato amylose fraction were substantially the same by both procedures, DP_n 850. The corn crystalline amylose sample (from hot water extraction) was acetylated by dispersion in the formamide.

In one of the experiments wherein the hydrolysis of corn A-fraction was carried substantially to completion and glucose was determined by the copper acetate oxidation method (data in Table III), the hydrolysate was also analyzed by paper chromatography. Droplets (0.010 ml.) of the hydrolysate were applied to strips of Schleicher and Schuell paper (No. 507), 7.5 mm. wide and 584 mm. long at a distance of 100 mm. from one end. These drops spread out into a band on either side of the origin totaling approximately 25 mm. After drying, the ends were immersed in a solvent mixture consisting of butanol (6 parts), pyridine (4 parts) and water (3 parts), and suspended in a closed chamber at room temperature for 24 hours. At this time the solvent boundary was noted and the strips were removed to air-dry. After spraying the strips with a solution consisting of 1 g. of 3,5-dinitrosalicylic acid in 100 ml. of *N* KOH, air-drying again and heating in an oven at 115° for 15 min., the positions of the red-brown colored bands on the paper strips were determined. From these, the per cent. migration (R_f values) of the solute in the bands was calculated in reference to the solvent.

The hydrolysate gave only one band, 24 mm. wide, approximately the width of the droplet as applied to the paper. The R_f value was 0.310. Glucose applied from a 0.4% solution gave an R_f value of 0.304, whereas the value for maltose was 0.240 and for amyloheptaose, 0.040. The latter, a 7-membered homolog of maltose, was supplied by Dr. Dexter French. Amylodextrin, an acid-hydrolyzed linear starch polymer of approximately DP_n 25, failed to move from its original position at the origin; $R_f = 0$.

The sugar present in the enzyme hydrolysate, therefore, is obviously glucose, no other sugars being present in an amount large enough to be detected. It seems possible that the remaining reducing materials consisted of infinitesimal amounts of all of the possible chain lengths between that of glucose and amylose. The hydrolysate after 3 hours reaction time (36.3% hydrolyzed to glucose) was similarly analyzed. This solution, estimated as being 0.16% con-

(8) F. C. Cleveland and R. W. Kerr, Am. Assoc. of Cereal Chemists, Abstract of Papers for the 32nd Annual Meeting at Kansas City, Mo., May, 1947, p. 24; see also *Cereal Chem.*, **25**, 133 (1948).

(9) R. W. Kerr, *Nature*, **164**, 757 (1949).

(10) R. W. Kerr and F. C. Cleveland, manuscript in preparation.

(11) M. Swanson, *J. Biol. Chem.*, **172**, 825 (1948).

(12) For an extended discussion of the several phases of α-amylase action, see K. Myrbäck, *Advances in Carbohydrate Chem.*, **3**, 269 (1948).

(13) This preparation was kindly supplied by Dr. H. M. Tsuchiya of the Northern Regional Research Laboratory, Peoria, Illinois.

(14) R. W. Kerr and G. M. Severson, THIS JOURNAL, **65**, 193 (1943).

(15) K. Sichert and B. Bleyer, *Z. anal. Chem.*, **107**, 328 (1936).

(16) F. C. Cleveland and R. W. Kerr, THIS JOURNAL, **71**, 16 (1949).

(17) R. W. Kerr and F. C. Cleveland, *ibid.*, **71**, 3455 (1949).

centration in reference to glucose, gave one band 21 mm. in width with an R_f value of 0.292 and another band at the origin, confirming the fact that glucose does form early in the hydrolysis and that it is the only lower sugar formed in significant amount.

Discussion

Table I shows the course of hydrolysis induced by the *Aspergillus niger* enzyme preparation on corn A-fraction. The very marked reduction in wave length of minimum light transmission for the iodine–hydrolysate complex as hydrolysis progresses closely resembles the results for α-amylase reported by Swanson,[11] rather than those for β-amylase. From this we may conclude either that the very rapid production of reducing substance, calculated as glucose, is by random hydrolysis, perhaps in the manner shown by α-amylase, or that the glucosidase operates in an orderly fashion but is admixed with an α-amylase enzyme.

TABLE I

HYDROLYSIS OF CORN A-FRACTION WITH *Aspergillus niger* ENZYME

Hydrolysis time, hr.	Wave length, minimum % light transmission of iodine complex, mμ	Corn A-fraction hydrolyzed; total reducing sugar as glucose, %
0	645	0
0.5	625	5.97
1.0	610	13.40
2.0	595	27.73
3.0	555	44.24
4.5	465	62.88
24.0	No color	96.05

Table II shows the course of hydrolysis after the enzyme preparation had been treated by the classical method of adjusting the *p*H value to 3.2 at 5° for 6 days to inactivate α-amylase. The method has been described in detail by Bernfeld and Gürtler.[18] It will be observed that, using an equivalent amount of the treated enzyme, no activity was lost whatsoever, neither in respect to saccharification nor in respect to typical α-amylase action as measured by iodine-spectrophotometry.

TABLE II

HYDROLYSIS OF CORN A-FRACTION WITH *Aspergillus niger* ENZYME AFTER TREATMENT AT *p*H 3.2

Hydrolysis time, hr.	Wave length, minimum % light transmission of iodine complex, mμ	Corn A-fraction hydrolyzed; total reducing sugar as glucose, %
0	645	0
0.5	625	5.76
1.0	610	13.33
2.0	595	27.87
3.0	555	43.87
4.5	465	64.38
24.0	No color	95.90

Table III shows the course of hydrolysis induced by the acid-treated enzyme after it had been stored at *p*H 4.9 and 5° for 18 months. During this time a considerable portion of the solids had precipitated and these were discarded. It will be noted now that saccharifying activity had been completely preserved, but there was a slight diminution in α-amylase activity during the period of storage.

(18) P. Bernfeld and P. Gürtler, *Helv. Chim. Acta*, **31**, 106 (1948).

This hydrolysis was investigated in greater detail; the amount of glucose actually produced was measured and compared against the amount of total reducing substance calculated as glucose. The data show that 90% or more of the reducing substance formed during hydrolysis can be accounted for as glucose and that approximately 95% of the amylose was finally hydrolyzed to this sugar. (This result is equivalent to a weight yield of 104%.) Of equal significance is the fact that relatively large percentages of glucose were formed early in the hydrolysis. That glucose is the only lower sugar formed in measurable quantities, both in the early stages as well as the latter phases of hydrolysis, was established by paper chromatography.

TABLE III

HYDROLYSIS OF CORN A-FRACTION WITH *Aspergillus niger* ENZYME AFTER STORAGE AT 5° FOR 18 MONTHS

Hydrolysis time, hr.	Wave length, minimum % light transmission of iodine complex, mμ	Corn A-fraction hydrolyzed Calculated as glucose, %	Determined as glucose, %
0	645	0	
0.5	630	6.14	5.85
1.0	610	13.05	11.25
2.0	600–05	27.78	
3.0	590	40.83	36.30
4.5	570	63.71	
24.0	(No color)	98.24	94.50

TABLE IV

WAVE LENGTH OF MINIMUM PER CENT. LIGHT TRANSMISSION FOR AMYLOSES OF DETERMINED DP_n VALUES

Amylose	DP_n (by osmotic pressure)	Wave length, mμ
Tapioca A-fraction[a]	1050	650–55
Potato A-fraction	850	650
Corn A-fraction	480	645
Corn crystalline amylose[b]	230	620
Acid-hydrolyzed amylose	130	600–05

[a] It may be possible that the amyloses of highest DP_n, such as tapioca, do not show a proportionately higher wave length because their structure is branched to some extent and that their average chain length is less than their DP_n. [b] Swanson concluded[11] that the low value of 610 to 620 mμ reported for corn crystalline amylose by Kerr and Trubell (*Paper Trade J.*, 117, No. 15, 25 (1943)) compared to the higher value found for corn A-fraction, was due to impurities in the corn crystalline amylose sample. It appears that the discrepancy is due to a difference in chain length.

This effect is definitely not typical of α-amylase activity; an abundant number of reports have established that glucose is produced in lesser yields, and then mostly in the latter stages of α-amylase hydrolyses. These results with the *Aspergillus niger* enzyme preparation suggest that glucose is liberated directly from the amylose molecules; the reduction noted in the chain length of the high polymer fraction of the hydrolysate may have been brought about concomitantly by another mechanism. The interpretation proposed becomes more evident as the fact by reference to the data in Table IV wherein the wave length of minimum per cent. light transmission for iodine complexes is related to the molecular weight of several amyloses of known DP_n. Thus, if we select, for example, the figures in Table III for a hydrolysis time of 60 minutes when the wave length for the iodine complex of the high

polymer fraction (of chain length long enough to form an iodine complex[19]) is 610 mμ, reference to Table IV shows that the chain length of this fraction is near the level of DP_n 200. A reduction from DP_n 480 to 200 indicates that an average of about 1.2 glucosidic linkages per molecule had been hydrolyzed to produce the high polymer fraction, or approximately 2.5 glucoside linkages per thousand. However, it is evident that for 11% of the original polymer to have been hydrolyzed to glucose, approximately 110 glucoside linkages per thousand were hydrolyzed. If this amount of glucose had been produced entirely by random hydrolysis, then obviously the high polymer fraction would have been of much lower DP_n than 200 and if the glucose had been produced entirely by an endwise attack on all amylose molecules, then the chain length of the high polymer residue would have been 425. It is probable, therefore, that the action is twofold; the major action is an endwise attack to produce glucose directly (from about 10% of the molecules in the example taken) and a random attack (on the other 90%) by a second factor, possibly an unusually acid resistant α-amylase.

In order further to confirm and elucidate the mechanism proposed, the *Aspergillus niger* preparation was additionally treated with acid at lower *p*H levels. The data in Table V show the course of hydrolysis for corn A-fraction by the enzyme after it had been subjected to a second acid treatment, at *p*H 2.7, for 7 days at 5°. Although somewhat over 50% of total hydrolytic activity had been lost by this treatment, the residual activity was measurably less proportionately that of an α-amylase. Thus, for example, comparing the data in Table III and V, it will be observed that before the acid treatment at the lower *p*H, the wave length of minimum light transmission of the iodine complex was 610 mμ when 13% of the amylose had been hydrolyzed to reducing substances, estimated as glucose. After the treatment at *p*H 2.7, 21% of the amylose was hydrolyzed by the enzyme before a wave length minimum of 610 mμ was reached.

TABLE V

HYDROLYSIS OF CORN A-FRACTION WITH *Aspergillus niger* ENZYME AFTER TREATMENT AT *p*H 2.7

Hydrolysis time, hr.	Wave length, minimum % light transmission of iodine complex, mμ	Corn A-fraction hydrolyzed; total reducing sugar as glucose, %	Rate of hydrolysis as % amylose per hr.
0	645	0	
0.5	635–40	2.46	4.92
1.0	630–35	5.08	5.08
2.0	625	8.62	4.31
3.25	620	14.77	4.55
4.5	610	20.77	4.60

Table VI shows the course of hydrolysis after the enzyme had been held for 7 days at 5° and *p*H 2.2 instead of at *p*H 2.7. In this case, a large part of the total activity was lost. However, the remainder now was substantially free from α-amylase activity as indicated by the spectrophotometer values, at least during the first part of the hydrolysis. Therefore, it would appear to be confirmed that α-amylase and amylo-glucosidase activities are two distinguishable functions.

It would appear furthermore from this experiment that an alternative hypothesis proposed for the action was not a very likely one. This hypothesis proposes that the enzyme makes a multiple attack at some distance from the ends of a substrate molecule, cutting out several glucose units but leaving two relatively large residues.

A second less obvious hypothesis proposes that some of the amylo-glucosidase molecules, possibly less perfect than the others,[10] cease hydrolysis before completing their task on an individual amylose molecule and perhaps dissociate from the reaction complex to begin the hydrolysis of some nearby molecule. This would give the effect, in part at least, that the enzyme had operated by a "multi-chain" mechanism. French and co-workers[20] have shown a possible analogous effect with β-amylase wherein the enzyme, under very unfavorable conditions of high temperature or low *p*H, was apparently unable to complete the hydrolysis of amyloheptaose molecules and amylopentaose, an intermediate product, accumulated during the hydrolysis.

There are several additional considerations, however, which favor the view that *Aspergillus niger* contains an exceptionally acid resistant α-amylase and confirm the proposal that a second enzyme present, amylo-glucosidase, operates not only in a terminalwise fashion to produce glucose but also very likely by the unique "single-chain" mechanism previously shown for β-amylolysis. Amyloses of quite different molecular weights were hydrolyzed by the acid-treated enzyme preparation, but using equimolar concentrations of the substrates. The results are shown in Table VI. Comparing the rate of glucose production, in milligrams per unit of time, in these equimolar amylose solutions, it will be observed that the amount of the sugar formed per unit time was substantially the same for each amylose solution although the carbohydrate concentration by weight varied over a wide range. It would appear therefore that the effective substrate concentration is the number of terminals of amylose molecules per unit volume and that the total number of glucoside bonds per unit volume is not necessarily a determinant for the rate of hydrolysis. With the terminalwise manner of attack confirmed, the spectrophotometric data in Table VI, showing that the unconverted polymer retains the chain length of the original amylose, is fairly convincing evidence that the amylo-glucosidase completes its attack on one molecule before beginning the hydrolysis of another.

If the rate at which amylose is hydrolyzed to glucose is compared in the several tables, an unusual effect is to be observed which is obviously related to the treatment of the *Aspergillus niger* enzyme with acid. Thus, for example, the data in Table V show that the percentage of amylose hydrolyzed per hour, up to various times of hydrolysis, is very

(19) According to the work of Swanson, *J. Biol. Chem.*, **172**, 815 (1948), all chain lengths greater than DP_n 30 give bluish colors with iodine. It is to be noted at this point that our chromatographic analyses of the hydrolysates showed no chain lengths less than this value except glucose.

(20) D. French, D. W. Knapp and J. H. Pazur, THIS JOURNAL, **72**, 1866 (1950).

TABLE VI

HYDROLYSIS OF EQUIMOLAR SOLUTIONS OF AMYLOSES[a] WITH *Aspergillus niger* ENZYME AFTER TREATMENT WITH ACID AT *p*H 2.2

	Corn A-fraction (DP_n = 480)				Corn crystalline amylose (DP_n = 230)				A-fraction (DP_n = 130) From acid-treated corn starch			
Hydrol. time, hr.	Wave length iodine complex, mμ	Amylose hydrolyzed %, calcd. as glucose	Glucose produced in mg. per 100 ml. Calcd.	Detd.	Glucose produced in mg. per 100 ml. Calcd.	Detd.	Amylose hydrolyzed %, calcd. as glucose	Reaction constant k_1[b]	Glucose produced in mg. per 100 ml. Calcd.	Detd.	Amylose hydrolyzed %, calcd. as glucose	Reaction constant k_1[b]
0	645		0		0				0			
3	650	1.65	7.3		6.1		3.36	0.0115	8.1		6.80	0.0235
4.5	650	2.76	12.3		10.3		5.29	.0119	14.3	15.0	11.90	.0281
21	645	10.51	46.7	44.3	43.0	42.5	20.67	.0110	46.9	40.0	39.09	.0235
45	645	18.42	81.8	78.0	85.0	85.5	40.38	.0115	80.1	66.5	66.57	.0243
69	645[c]	26.55	117.9	108						Retrograded		
120	640[c]	35.90	159.4	153	157.4	155	74.36	.0113				
144	640[c]	47.61	211.4	198		Retrograded		.0114				
168		50.42	223.8					±.0002				
239		61.78	274.3	262								

[a] In the hydrolysis of the A-fraction from acid-treated starch, retrogradation became pronounced after about 45 hours, with corn crystalline amylose, at about 120 hours and for the corn A-fraction, occasionally not until after 200–240 hours.
[b] Calculated from the monomolecular reaction equation, $k_1 = \log_e (100/100 - x)/t$ where x is % hydrolyzed at time t in hr.
[c] These values were run, increasing the concentration of the hydrolysate in proportion in the % amylose hydrolyzed.

TABLE VII

HYDROLYSIS OF CORN B-FRACTION, 0.4 G. PER 100 ML. WITH *Aspergillus niger* ENZYME AFTER TREATMENT AT *p*H 2.2

Hydrolysis time, hr.	Corn B-fraction hydrolyzed; total reducing sugar as glucose, %	Glucose produced in mg. per 100 ml. Calcd.	Detd.
0	0	0	
1.5	2.55	11.3	
3.0	4.18	18.4	
4.5	5.72	25.3	25.3
21	22.70	100.7	100.4
45	43.69	193.9	188.5
69	56.70	252	252
93	62.38	277	273
120	65.54	...	...
168	73.15	325	326
239	80.16	356	349

nearly constant as would be demanded were the reaction of zero order. This is after the enzyme preparation had been treated at *p*H 2.7. Prior to this treatment, however, hydrolysis rates (calculated from the data in Table I to III) show a definite acceleration in per cent. per hour, or a reaction of lower order than zero, indicating that the substrate concentration was actually tending to increase as hydrolysis progressed. This unusual result becomes understandable if the hydrolytic effect measured, such as the production of sugar, is the result of amylo-glucosidase operating terminalwise according to the "single-chain" mechanism and if the enzyme preparation contained a supplementary factor, operating as an α-amylase to create an increased number of terminals for the amylo-glucosidase to operate upon. The effect is not satisfactorily explained by an alternative hypothesis, mentioned above: that the amylo-glucosidase operates in part by a "multi-chain" mechanism, the reduction in chain length of the high polymer fraction in the hydrolyzate having been brought about by a piece-meal removal of glucose units; in this case there would be no increase in the number of end-groups upon which the amylo-glucosidase could operate and for this reason no acceleration in the reaction rate.

The data shown in Table VI for hydrolysis with an amylo-glucosidase preparation in which the α-amylase function had been inactivated by acid treatment at *p*H 2.2, indicate a de-accelerating rate, or a reaction of higher than zero order. The hydrolysis now approaches a first order reaction, which, indeed, it should, if the amylo-glucosidase operates by the "single-chain" mechanism. The kinetics of the reaction are most readily demonstrated with the highly linear substrate, corn crystalline amylose. The data in Table VI clearly indicate a first order reaction, just as was found to be the case for the analogous action of β-amylase on this crystalline amylose by Kerr and Severson.[14] These results are convincing evidence that the amylo-glucosidase acts terminalwise by the "single-chain" mechanism on linear starch molecules.[21]

At equal weight concentrations, the rate of hydrolysis for amylopectin (corn B-fraction) is initially very much greater than for corn amylose. From the data shown in Table VII, the initial rate for corn B-fraction was graphically calculated and found to be 4.5 times the initial rate similarly determined from the data in Table VI for corn A-fraction. A much higher value for amylopectin than for amylose would be expected if, as proposed, the glucosidase operated in a terminalwise fashion since there are possibly 10 or more times the number of molecular terminals per unit weight of corn amylopectin than per unit weight of corn A-fraction. The relatively high rate for the B-fraction was not maintained, however, possibly because the terminal branches are short in relation to the size of the molecule, thus offering a physical hindrance to the alignment of substrate and enzyme molecules, secondly, because the enzyme may experience some difficulty as points of branching are approached, and lastly, because of the probable much lesser diffusion rate of the large amylopectin molecule, com-

(21) Very recently, and since this work was completed, D. French and D. W. Knapp, *J. Biol. Chem.*, **187**, 463 (1950), reported that a maltase obtained from *Clostridium acetobutylicum* hydrolyzed polysaccharides only approximately according to the first order law.

pared to amylose. The hydrolysis of amylopectin and amylose is compared in Fig. 1, which graphically shows that the hydrolysis rate for the amylopectin decreased abruptly to a definitely lower value as the level of 60% conversion was approached, a striking analogy to the well known behavior of β-amylase on this substrate. These results may be taken as further evidence that the amylo-glucosidase does not operate at random but, rather, by a terminalwise attack on the non-reducing end of starch molecules, and that the rate of hydrolysis for anomalous linkages, at points of branching, is very much less than the rate for normal glucoside linkages.

It appears quite possible in light of these experiments, that the limit-dextrinase activity noted in early work for some relatively crude enzyme preparations may have been due in part at least to the combined action of an amylo-glucosidase and an α-amylase.

The very large and no doubt slightly branched amyloses, such as tapioca A-fraction, gave hydrolysis rates intermediate between that of corn amylose and amylopectin. Using *equimolar* concentrations, the rate of hydrolysis for these larger amylose molecules, as mg. of glucose produced per unit time, was initially greater than the rate for corn amylose, but decreased to values approaching that of the corn samples as hydrolysis progressed. These experiments will be detailed in a future communication.[22]

It is possible that the final amylo-glucosidase preparation, which was used to obtain the data in Tables VI and VII and for Fig. 1, still contained a trace of α-amylase activity. Even a trace of α-amylase would modify the results and may account for the slight drop in peak light absorption values during the corn A-fraction hydrolysis as well as a part, at least, of the secondary phase of the amylopectin hydrolysis. However, it is believed that the data given in Tables VI and VII, when considered together with the data in preceding tables, showing the manner in which the kinetics of the hydrolysis tend to change as the enzyme preparation was progressively freed of α-amylase activity and the residual activity became more exclusively that of the amylo-glucosidase factor, clearly indicate the general pattern of the action of this latter enzyme and distinguish it from other carbohydrases.

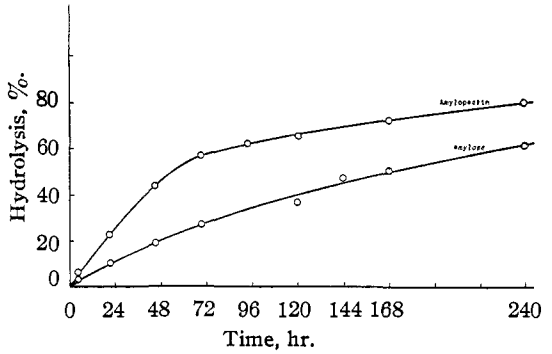

Fig. 1.—Hydrolyses of amylose and amylopectin at equal weight concentration by amylo-glucosidase.

(22) R. W. Kerr and F. C. Cleveland, manuscript in preparation.

ARGO, ILLINOIS RECEIVED JANUARY 31, 1951

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, TEXAS AGRICULTURAL EXPERIMENT STATION TEXAS AGRICULTURAL AND MECHANICAL COLLEGE SYSTEM]

The Synthesis of C^{14}-Labeled Glycerol[1,2]

BY HERMANN SCHLENK AND BERNICE WALLACE DEHAAS

Glycerol-1,3-C_2^{14} has been prepared in over-all yield of 19%, by condensing nitromethane with paraformaldehyde-C^{14}, followed by reduction and diazotization.

Glycerol, labeled with carbon-14, was needed for projected studies in this Laboratory on the metabolism of glycerides. Although there are several synthetic routes[3] which might have been employed, the scheme shown in Fig. 1 was chosen because of the availability of both nitromethane-C^{14} [4] and formaldehyde-C^{14}.[5]

Experimental

Sodium-2-nitro-1,3-propanediol (I).—According to the procedure of Schmidt and Wilkendorf,[6] 13.47 g. of paraformaldehyde-C^{14} (0.449 mole)[7] was allowed to react with 8.54 g. of nitromethane (0.14 mole) in 110 ml. of absolute methanol by the addition of 8–10 drops of 50% KOH. When Eastman Kodak Co. paraformaldehyde was used, the reaction began immediately upon heating on a steam-cone, and with mechanical stirring, the solution became clear within ten minutes. When the reaction was performed with the isotopic paraformaldehyde, there were still some unreacted particles after 45 minutes heating. After decanting from the insoluble particles the mixture was cooled to −5°. A solution containing 4.02 g. of sodium dissolved in 65 ml. of absolute methanol was added during a period of 20 minutes with mechanical stirring. After standing for six hours at −5°, the precipitate was filtered off. The yield of the sodium salt of 2-nitro-1,3-propanediol, containing 2 molecules of methanol of crystallization, was 27 g. or 58%. The filtrate was neutralized with 7.8 *N* HCl in methanol, mixed with the unreacted paraformaldehyde and the whole procedure was repeated by adding 3.05 g. (0.05 mole) of nitromethane. The yield in this recycling was 8 g. A second recycling yielded 1 g. The total yield was 36 g. or 77% based on the paraformaldehyde.

2-Amino-1,3-propanediol Hydrochloride (II).—Hydrogenation of I was carried out in two batches of about 18 g. each, using an apparatus similar to the Parr apparatus. Each batch was dissolved as completely as possible in 225 ml. of absolute methanol, and 2.1 equivalents of glacial acetic acid was added, followed by 4 g. of Raney nickel. The pressure was kept between 37 and 20 lb./sq. in. Hy-

(1) This investigation was supported in part by a grant from the United States Atomic Energy Commission.

(2) Presented at the 118th Meeting of the American Chemical Society, Chicago, Illinois, September, 1950.

(3) Since the writing of this manuscript the preparation of glycerol-1-C^{14} was reported with a yield in radioactivity of 12.3%: A. P. Doerschuk, THIS JOURNAL, **73**, 821 (1951).

(4) L. G. Sowden, *J. Biol. Chem.*, **180**, 56 (1949).

(5) A. R. Jones and W. J. Skraba, *Science*, **110**, 332 (1949).

(6) E. Schmidt and R. Wilkendorf, *Ber.*, **52**, 395 (1919).

(7) Isotopes Division, U. S. Atomic Energy Commission, Oak Ridge, Tenn. The material was lumpy and sticky, whereas Eastman paraformaldehyde is powdery. Its activity was specified as approximately 5 microcuries per millimole.