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ACID HYDROLYSIS OF (1→4)-α-D-GLUCANS

PART I. ANALYSIS OF PRODUCTS BY QUANTITATIVE PAPER CHROMATOGRAPHY*,**

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ABSTRACT

The acid hydrolysis of linear (1→4)-α-D-glucans can be described by using two rate constants; a constant for the glucosidic bond at one end of the chain and a smaller constant for each of the remaining glycosidic bonds. Polymers having an average degree of polymerization of 26 or more appear to be able to protect some of their glycosidic bonds and decrease the effective concentration of carbohydrate. This protection is not afforded by conventional helical segments in the chain. The helical conformation, necessary to bind iodine, is not adopted at the temperatures of hydrolysis, as shown by a hysteresis in heating and cooling curves of the amylose–iodine complex.

INTRODUCTION

The acid hydrolysis of amylose does not proceed by simple first-order kinetics, although the chemical bonds linking the polymers are all α-D-(1→4) bonds. BeMiller[1] has reviewed this problem and has stated some of the various explanations: (*a*) the two terminal bonds in the chain are hydrolyzed at a higher rate than the rest; (*b*) only one terminal bond is hydrolyzed at a higher rate; (*c*) the rate of hydrolysis progressively decreases from one or both terminal bonds; and (*d*) the rate of hydrolysis is the same for all bonds in a given polymer but varies with chain size. The data presented here can be viewed as supporting explanation *b*.

MATERIALS

Amylose.— Commercial, defatted potato starch was fractionated by the Schoch procedure[2]. The amylose fraction was reprecipitated twice with butanol and stored as the butanol complex. Immediately before amylose was to be hydrolyzed the complex was dissolved in water and boiled. Boiling was continued for 10 min after the odor of butanol had disappeared and the hot solution was filtered through glass wool

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to remove retrograded material. The volume was kept nearly constant during boiling to limit retrogradation.

Amylodextrin. — A Nägeli amylodextrin was available in our laboratory. The preparation of this material is described elsewhere[3]. The degree of polymerization, determined by the Nelson reducing-sugar method, was 26.2. Paper chromatography showed only a spot at the origin, giving a lower limit of molecular size greater than 11. The amylodextrin stained purple with iodine.

Oligosaccharides. — Maltose and maltotriose were donated by Dr. M. Abdullah. Maltohexaose was isolated from a hydrolyzate of β-Schardinger dextrin by descending paper chromatography with 65% 1-propanol.

Schardinger dextrins. — α- and β-Schardinger dextrins were donated by Dr. J. Robyt.

METHODS

Determination of carbohydrate. — The phenol–sulfuric acid method was used to determine total carbohydrate[4].

Determination of reducing power. — Reducing power was determined by the Nelson method[5].

Determination of D-*glucose.* — The D-glucose oxidase method described by Fleming and Pegler[6] was used in determining concentration of D-glucose.

Ascending paper chromatography. — Whatman No. 3-MM chromatography paper was cut into sheets 13″ high × 14″ wide and rinsed with 2M acetic acid and ethanol to remove some of the material positive to phenol–sulfuric acid that can be eluted from the paper. The quantitative procedure is described by Dimler *et al.*[7]. The chromatogram was irrigated three times with hot 70% 1-propanol[8]. The digests of Schardinger dextrin were separated by using 3:2:2 1-butanol–pyridine–water. Schardinger-dextrin guide-strips were first developed by staining with iodine (20 ml, 20mM KI and 10 mM I_2 in 200 ml of methanol). The spots were circled and the iodine removed by repeated washings with acetone. Reducing sugars were developed by the alkaline silver dip method[9]. Digest spots and blanks were eluted and analyzed by the phenol–sulfuric acid method.

EXPERIMENTAL

Determination of rate constants. — Method I. Determination from digest aliquots. Hydrolysis of 0.1–0.4% substrate in 5 mM sulfuric acid was performed at 95°. The concentration of substrate was determined by the phenol–sulfuric acid method. The degree of hydrolysis was determined from aliquots of the digest by the reducing-power analysis of Nelson for amylose, amylodextrin, and the Schardinger dextrins. The D-glucose oxidase method was used in the hydrolysis of maltose. The first-order requirements necessary for determining the initial, average, rate-constants are met by obtaining straight lines in the kinetics plots. The method of least squares was used throughout for the plotting of straight lines.

Method II: Determination of products. Carbohydrate substrates were digested

with acid as described above. The hydrolysis was quenched by placing the reaction tube into a beaker of ice–water. Aliquots were removed and diluted for determination of D-glucose and reducing value. The remaining digest was neutralized by adding a calculated amount of barium carbonate or pyridine. When amylose or amylodextrin were the substrates, the digests were concentrated 3-fold to diminish the volume needed for paper chromatography.

Reducing sugars that overlapped the Schardinger dextrins on chromatotograms were removed by digestion with β-amylase or corrected by reducing-power analysis of a separate chromatogram. Aliquots of 100 μl were applied to each sample strip for quantitative chromatography. Average rate-constants were calculated for the Schardinger dextrins and maltohexaose from the initial and final concentration of substrate. The products of hydrolysis were used, as well, for maltose and maltotriose. For amylose and amylodextrin the average rate-constants were determined by the Sillen equations (Eqns. *11* and *12*), which take the change in rate constant into account.

Effect of temperature on the amylose–iodine complex. — The amylose–iodine complex was formed by pipetting 5 ml of an amylose solution (164 or 328 μg/ml) into a clean Klett–Summerson colorimeter tube and adding 50 μl of a 10 mM triiodide solution (10 mM I_2 and 100 mM KI). The tubes were sealed, placed in a water bath, and the temperature raised and lowered between 25–85°. The tubes were removed from the bath after reaching thermal equilibrium and the absorbancies were measured quickly. Each tube was taken through the heating–cooling cycle at least twice.

RESULTS AND DISCUSSION

Determination of rate constants. — If we assume as a first approximation that all the bonds in α-(1→4)-linked D-glucans have the same hydrolytic rate constant, the number of bonds, B, remaining at any time can be described by a first-order rate equation.

$$B = B^0 e^{-kt} \qquad (1)$$

The bonds remaining can be calculated from the total carbohydrate concentration, N, and the concentration of reducing groups, R.

$$B = N - R \qquad (2)$$

In the case of maltose, where the concentration of liberated D-glucose, C_1, was determined, the equation is:

$$B = 1/2(N - C_1). \qquad (3)$$

Results from the hydrolysis of amylose, amylodextrin, and maltose are shown in Figs. 1 and 2 and Table I.

The rate constant for maltose was determined from quantitative paper chromatography data by using Eqns. *3* and *4*.

$$C_2 = C_2^0 e^{-kt} \qquad (4)$$

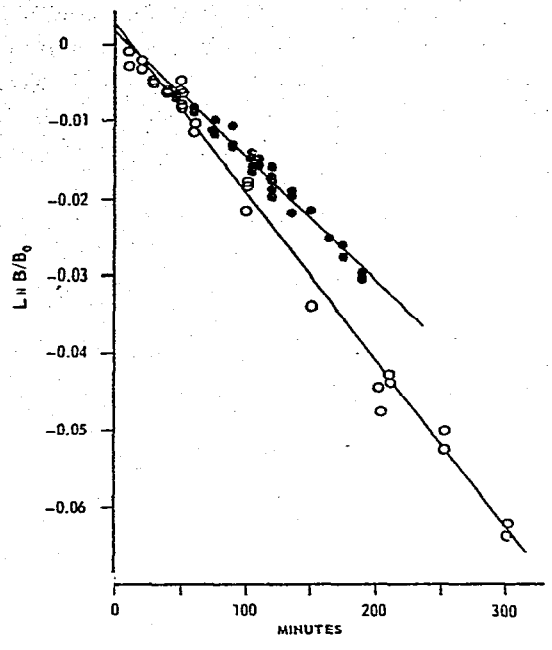

Fig. 1. Kinetic plot. Natural logarithm of mole fraction of uncleaved bonds against time in min. Amylodextrin, open circles; amylose, filled circles.

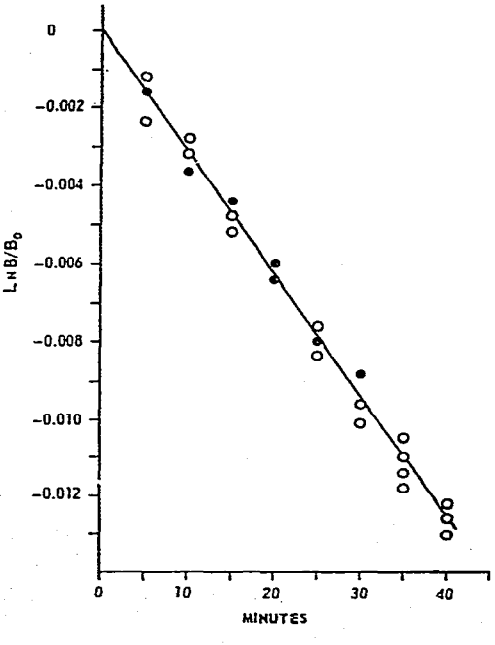

Fig. 2. Kinetic plot of hydrolysis of maltose. Natural logarithm of mole fraction of uncleaved bonds against time in min. Filled circles show two coinciding points.

TABLE I

AVERAGE RATE CONSTANTS FOR HYDROLYSIS OF GLYCOSIDIC BONDS IN D-GLUCANS AT 95.0° IN 5 mM SULFURIC ACID

Substrate	*Amylose*	*Amylodextrin*	G_2[a]	G_3[a]	G_6[a]	α[b]	β[b]
	k $\pm\sigma$[c] $\times 10^4$ *min*$^{-1}$						
Method I	1.63 ±0.04	2.21 ±0.04	3.12 ±0.07				
Method II	1.64 ±0.07	1.94 ±0.15	3.14 ±0.45	2.42 ±0.24	2.08 ±0.18	0.647 ±0.057	0.628 ±0.062

[a] G_2 = maltose, G_3 = maltotriose, *etc.* [b] α = α-Schardinger dextrin, *etc.* [c] σ = Standard deviation. [d] Hydrolyzed for 1440 min; except amylodextrin, 1284 min.

Average rate constants for the glycosidic bonds were calculated for α- and β-Schardinger dextrins, maltohexaose and maltotriose, from the following equations.

For α-Schardinger dextrin:

$$C_\alpha = C_\alpha^0 e^{-6kt} \tag{5}$$

For β-Schardinger dextrin:

$$C_\beta = C_\beta^0 e^{-7kt} \tag{6}$$

For maltohexaose:

$$C_6 = C_6^0 e^{-5kt} \tag{7}$$

For maltotriose:

$$C_3 = C_3^0 e^{-2kt} \tag{8}$$

$$C_2 = 2C_3^0(e^{-kt} - e^{-2kt}) \tag{9}$$

$$C_1 = C_3^0(3 + e^{-2kt} - 4e^{-kt}) \tag{10}$$

The rate constants from these experiments are given in Table I.

Data from the amylose and amylodextrin digests, analyzed by quantitative paper chromatography, can be interpreted by using the equations of Sillen[10]. These equations are based on the assumption that hydrolysis of one bond at the end of a polymer chain has a rate constant that differs from all the rest.

$$C_n = N(1-x)^2 x^{n-1+p} e^{p(1-x)} \tag{11}$$

$$n_o \gg n > 1$$

$$C_1 = N[1-(2-x)x^{1+p}e^{p(1-x)}] \tag{12}$$

where: $k_1 = (1+p)k_2$; k_1 = rate constant for one end-bond; k_2 = rate constant for all other bonds; n_0 = chain length of substrate; n = chain length of product; $x = e^{-k_2 t}$; C_n = concentration of product; N = total concentration of carbohydrate, as D-glucose. Taking the common logarithm of both sides of Eqn. *11* yields:

$$\log C_n = \log N + p(1-x)\log e + 2\log(1-x) + (n-1+p)\log x \tag{13}$$

The slope of $\log C_n$ *vs* n is:

$$\frac{\Delta \log C_n}{\Delta n} = \log x = -\frac{k_2 t}{2.303}. \tag{14}$$

Because of the restrictions of Eqn. *11*, D-glucose may not be on this line. The $\log C_n$ *vs.* n plots are shown in Fig. 3, the data are given in Tables II and III, and the rate constants are given in Table I.

The average rate-constant for the glycosidic bonds in maltotriose is 2.42×10^{-4} min^{-1}. The rate constant for maltotriose is then 4.84×10^{-4} min^{-1}. The sum of the rate constant for maltose and k_2 for amylose is 4.78×10^{-4} min^{-1}, in good agreement

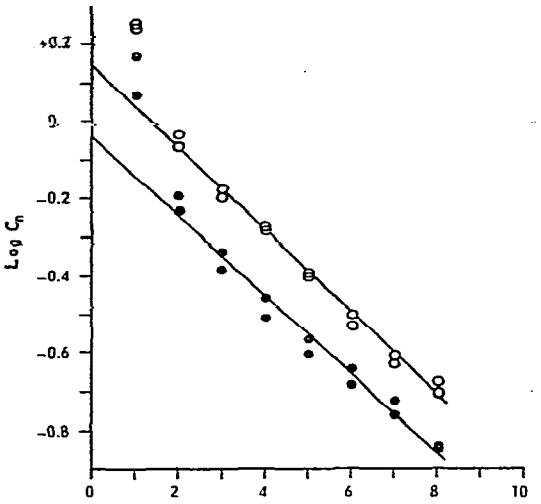

Fig. 3. Common logarithm of moles of product against degree of polymerization of the product. Amylodextrin, open circles; $\log x \pm\sigma = -0.1079 \pm 0.0083$, $Y \pm\sigma = 0.1464 \pm 0.0412$. Amylose, filled circles; $\log x \pm\sigma = -0.1027 \pm 0.0043$, $Y \pm\sigma = -0.0457 \pm 0.0230$.

with the above value. The rate constant for maltohexaose is 10.4×10^{-4} min^{-1}. Adding 1 rate constant for maltose to $4k_2$ gives 9.70×10^{-4} min^{-1}, which is a fair agreement. These results also agree well with radioactive-tracer work[11]. We may draw the conclusion that the rate constants for maltose and one end bond of (1→4)-α-D-glucans are the same. Also the rate constants for all other glycosidic bonds in this homologous series are the same, and are less than the value for maltose.

TABLE II

PRODUCTS OF HYDROLYSIS OF AMYLOSE AT 95.0° IN 50 mM SULFURIC ACID FOR 1440 MINUTES

n	*Digest No 1.*		*Digest No. 2*	
	C_n' (μg/ml)	C_n (μmole/ml)	C_n' (μg/ml)	C_n (μmole/ml)
1	211	1.17	265	1.47
2	210	0.583	232	0.643
3	223	0.413	246	0.454
4	223	0.309	250	0.347
5	224	0.249	244	0.271
6	222	0.206	245	0.227
7	216	0.171	235	0.187
8	201	0.139	221	0.140
HMW[a]	1783		1802	
Total[b]	3513		3740	
Total[c]	4320		4580	
Total[d]	2966		3020	

[a]Products of higher molecular-weight. [b]From sum of products. [c]From phenol–sulfuric acid analysis of digest. [d]Calculated from $N = a/(1-x)^2 + \Delta a$.

TABLE III

PRODUCTS OF HYDROLYSIS OF AMYLODEXTRIN AT 95.0° IN 5.0 mM SULFURIC ACID FOR 1284 MINUTES

n	*Digest No. 1*		*Digest No. 2*	
	C_n' (μg/ml)	C_n (μmole/ml)	C_n' (μg/ml)	C_n (μmole/ml)
1	311	1.73	313	1.74
2	308	0.856	328	0.911
3	346	0.639	357	0.66a1
4	380	0.528	373	0.581
5	351	0.390	362	0.402
6	317	0.293	341	0.315
7	297	0.236	306	0.243
8	285	0.198	294	0.204
HMW[a]	2203		2235	
Total[b]	4798		4909	
Total[c]	4910		5040	
Total[d]	4178		4180	

[a–d]See footnotes, Table II.

Determination of p. — Since $k_1 = (1+p)k_2$, a p-value of about 0.9 would be expected. This value can be obtained from hydrolytic data on amylose or amylodextrin by solving the Sillen equations for p. The degree of precision in the data and the nature of the equations combine to allow only a rough approximation of p. For amylose, the average value of p is 1.43 and for amylodextrin 1.00 (see Appendix 1).

Determination of N. The values of p depend upon the total carbohydrate concentration, N. This number can be determined by analysis of the digest with phenol–sulfuric acid, by summation of the products eluted from the quantitative paper chromatograms, or by calculation from Eqn. *15* (Derived in Appendix 2).

$$N = \frac{a}{(1-x)^2} + \Delta a \qquad (15)$$

The term x is equal to $(C_n+1)/C_n$ and is determined from the slope of the line of $\log C_n$ *vs.* n. The first term in the series, a, is equal to C_1 taken from this line. The difference between the experimental D-glucose concentration and the extrapolated value (a) is Δa. Total carbohydrate concentrations given as weight of D-glucose appear in Tables II and III.

Values for N, determined by summing the eluted products, were expected to be low because of retrogradation and the limited solubility of high-molecular-weight polymers. Calculated N values were the lowest of the three methods, and the only ones that gave p values from Eqns. *17* and *18* that were in agreement. The authors cannot readily explain this discrepancy. However, if we suppose that part of the polymer chain were protected from hydrolysis, thereby decreasing the effective concentration of the substrate, we can account for the data. It was first thought that such protection

might be afforded by helical conformations in the chain. The small hydrolytic rate-constants for the Schardinger dextrins (Table I) support this idea.

Effect of temperature on amylose–iodine complexes. — Heating and cooling curves of the amylose–iodine complexes strongly suggest that there is no helical component in amylose (that is the conventional compact helix having 6 D-glucose residues per turn) at the temperature of hydrolysis. The iodine is tightly held within the core of the amylose helix, producing an intensely blue color[12]. The color disappears upon heating and is restored with cooling (Fig. 4). The hysteresis effect

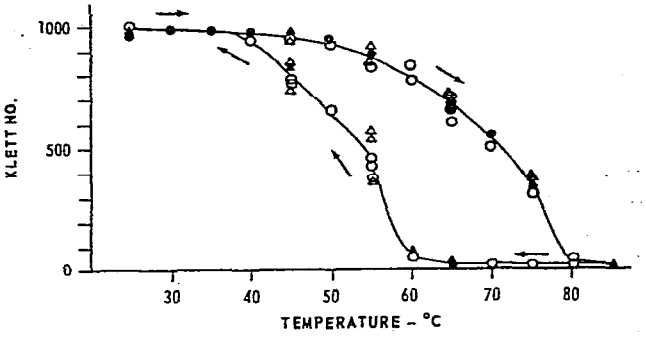

Fig. 4. Absorbance of amylose–iodine complex in Klett–Summerson units (No. 54 filter) against temperature. Circles, 328 μg/ml of amylose, 0.10 mM I_3^-. Triangles, 162 μg/ml of amylose, 0.10 mM I_3^-. Filled symbols signify more than 1 point.

observed can be explained by the slow release of iodine as the helix uncoils. At lower temperatures, where helical formation is again possible, the complex reforms. Peticolas has noted similar changes upon heating and cooling, but without the hysteresis effect[13]. Hysteresis is difficult to observe with high concentrations of iodine. It does not appear that the discrepancies in N value can be explained by inhibition of hydrolysis by helical segments.

APPENDIX I

The log C_n intercept (Y) from Eqn. *13* is:

$$Y = \log N + p(1-x)\log e + 2\log(1-x) + (p-1)\log x. \qquad (16)$$

Solving for p gives:

$$p = \frac{Y + \log x - \log N - 2\log(1-x)}{\log x - (1-x)\log e}. \qquad (17)$$

A value for p can also be calculated from the amount of D-glucose produced by rearranging Eqn. *12*, taking the logarithm of both sides and solving for p.

$$p = \frac{\log[1-(C_1/N)] - \log(2-x) - \log x}{\log x - (1-x)\log e} \qquad (18)$$

APPENDIX 2

The series of hydrolytic products forms a geometric progression whose sum is:

$$S_1 = C_1+C_2+C_3+C_4+ \ldots = a(1+x+x^2+x^3+ \ldots) = \frac{a}{1-x}. \qquad (19)$$

The number of D-glucose residues in the products is then:

$$S_2 = C_1+2C_2+3C_3+4C_4+ \ldots = a(1+2x+3x^2+4x^3+ \ldots) = \frac{a}{(1-x)^2}. \qquad (20)$$

This sum can be calculated from the plot of log C_n *vs.* n. However, since D-glucose is not on that line, a correction term (Δa) must be added (see Eqn. *15*).

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