The stability of polymyxin to heat, acid, alkali and proteolytic enzymes has been discussed in earlier reports.[2,7]

Summary

The preparation of polymyxin hydrochloride assaying 1000–1400 units per mg. is described in detail. The preferred method is by adsorption on Darco G-60, elution with acid methanol and precipitation with acetone. Purification of the crude hydrochloride and certain properties of polymyxin hydrochloride and picrate are described.

Related isolation studies and alternative methods of isolation are briefly discussed.

(7) Stansly and Ananenko. *Arch. Biochem.*, **15**, 473 (1947).

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[CONTRIBUTION FROM THE DIVISION OF PLANT NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA]

Starch. II. Molecular Weights of Amyloses and Amylopectins from Starches of Various Plant Origins

BY A. L. POTTER AND W. Z. HASSID

It is now recognized that starch is not homogeneous but can be separated into two fractions, amylose and amylopectin, each having a different chemical constitution. The amylose consists of long unbranched chains, the amylopectin of chains with branches that are relatively short. There is no adequate information at present regarding the molecular weights of the two starch components. Much of the available information concerning molecular weights was derived from work on partly degraded acetylated or methylated starch, employing viscosity measurements.[1,2,3] The molecular weights inferred from such viscosity determinations can at best be considered only as rough approximations. Carter and Record,[4] using osmotic pressure measurements on whole starches, which were partly degraded with hot alcoholic hydrochloric acid and then methylated, obtained molecular weights ranging from 40,000 to 124,000. Osmotic pressure measurements of acetylated corn amylose and corn amylopectin in tetrachloroethane by Meyer, *et al.*,[5] gave molecular weights of about 78,000 and 300,000, respectively. Caesar, *et al.*,[6] applying Barger's method for the determination of the molecular weights of the nitric triester derivatives of starches in ethyl acetate, obtained a molecular weight of 360,000 for the potato amylose derivative and of 64,000 for the corn amylose derivative.

Inasmuch as amylose readily retrogrades from water solution and amylopectin forms a highly colloidal solution, molecular weight determination of these substances cannot be made in this solvent. It is therefore necessary to use the acetylated or methylated derivatives which are soluble in organic solvents. However, it is recognized that during the process of acetylation of starch with acetic anhydride at 60° in the presence of pyridine some degradation of the molecule occurs due to the elevated temperature. Similarly, partial degradation of starch occurs during methylation when this process is carried out in an alkaline medium, especially since it is necessary to repeat the process about eight times in order to completely methylate the product.

The purpose of this investigation was to determine the molecular size of the amylose and amylopectin fractions of starch under conditions of minimum degradation. The mildest possible treatment was therefore employed in the isolation of starch and the separation into its components. Intrinsic viscosity in 1 *N* potassium hydroxide was used as a criterion of degradation. Only starches with the highest viscosities were used.

In the preparation of the acetylated derivatives elevated temperatures were avoided. Using formamide as a dispersion medium[7] for the starch fractions, acetylation with a mixture of acetic anhydride and pyridine could be carried out at room temperature.

Periodate oxidation data[8] showed that the amylopectins from a number of starches of different plant sources ranged from 22 to 27 glucose units per end-group, while the chain lengths of the corresponding amyloses ranged from 420 to 980 glucose residues. In this connection it was of interest to find out whether these amyloses and amylopectins differed in their total molecular size. The determination of the molecular weight of amylose in conjunction with an end-group determination should also answer the question as to whether or not a single amylose molecule constitutes one chain, or whether several chains are combined to form the molecule.

The molecular weights of the starch fractions were determined by osmotic pressure measurements of the acetylated products in chloroform. Within the range of concentration used, the relationship of the osmotic pressure and concentration[9] could be expressed by $\pi/C = aC^n + b$, where

(1) E. L. Hirst and G. T. Young, *J. Chem. Soc.*, 1471 (1939).
(2) H. Staudinger, *Naturwissenschaften*, **25**, 673 (1937).
(3) H. Staudinger and E. Husemann, *Ber.*, **71**, 1057 (1938).
(4) S. R. Carter and B. R. Record, *J. Chem. Soc.*, 664 (1939).
(5) K. H. Meyer, P. Bernfeld and W. Hohenemser, *Helv. Chim. Acta*, **23**, 885 (1940).
(6) G. V. Caesar, N. S. Gruenhut and M. L. Cushing, THIS JOURNAL, **69**, 617 (1947).
(7) J. F. Carson and W. D. Maclay, *ibid*, **68**, 1015 (1946).
(8) A. L. Potter and W. Z. Hassid, *ibid.*, **70**, 3488 (1948).
(9) I. S. and E. S. Sockolnikoff, "Higher Mathematics for Engineers and Physicists," McGraw-Hill Book Co., New York, N. Y., 1941, p. 533.

π = osmotic pressure, C = concentration, a and b are constants and average values of $n = 1.39$ and 2.25 for acetylated amyloses and amylopectins, respectively. Plotting π/C against C^n when these n values are used, straight lines are obtained. Assuming this relationship holds in solutions approaching zero concentration and that the intercept of the ordinate is the value obtained from van't Hoff's law,[10] the equation $\pi/C = aC^n + RT/M$ may be written, from which the molecular weight M can be calculated.

The "number-average" molecular weight[11] for six acetylated amyloses from different plant sources (tapioca, potato, wheat, corn, sago, Easter lily) varied from 180,000 to 370,000. The molecular weights of the acetylated amylopectins from the same plant sources are much larger than those of the amyloses. The values obtained varied from approximately 2,000,000 to 10,000,000. It is recognized that the molecular weights of the amylopectins are averages of mixtures of branched polymers of higher and lower molecular weights. Similarly, the values obtained for the molecular weights of the amyloses probably represent averages of larger and shorter chains.

A fair correlation is found to exist between the intrinsic viscosities and the molecular weights of the amyloses (Table III). With the amylopectins the correlation is not good. However, since the starch fractions constitute mixtures of polymers of various sizes, no strict correlation should be expected. This agrees with Wagner's[12] results obtained with fractionated polyvinyl acetate polymers. A better correlation between the intrinsic viscosities and the molecular weights would be expected if the starch components were subfractionated into entities of uniform size. Similar investigations are in progress on the subfractions from various amyloses, prepared by differential precipitation procedures.

The molecular weights obtained by osmotic pressure measurements in conjunction with the periodate oxidation data indicate that amylopectin is highly branched. Acetylated corn amylopectin, for example, which has a molecular weight of 10,000,000 and an average of 26 glucose residues per end-group, contains approximately 1300 branches per molecule. It is observed that with potato and Easter lily amyloses there is a fair agreement (Table III) between the molecular weights obtained by osmotic pressure measurements and those obtained by the periodate end-group method. It can therefore be concluded that for each of these amyloses a single chain represents one molecule. With the other amyloses the molecular weights derived from osmotic pressure measurements are higher than those calculated from the periodate end-group method. The data indicate that branching in amylose, if it exists, occurs only to a slight degree. With the samples examined there can be not more than two or three chains linked together to form one amylose molecule.

(10) G. N. Lewis and M. Randall, "Thermodynamics and the Free Energy of Chemical Substances," McGraw-Hill Book Company, New York, N. Y., 1923, p. 235.

(11) The term "number-average" molecular weight for heterodisperse polymers was introduced by W. D. Lansing and E. O. Kraemer, THIS JOURNAL, **57**, 1369 (1935)). Most of the polymers, whether natural or synthetic, consist of an assemblage of molecules of different molecular weights.

(12) R. H. Wagner, *J. Polymer Sci.*, **2**, 21 (1947).

Experimental

Starch Samples.—The starch samples used in this investigation were supplied by Dr. Thomas J. Schoch of the Corn Products Refining Company. These starches were used in previous work dealing with the end-group determinations of amylose and amylopectin fractions by periodate oxidation.[8]

Acetylation.—Carson and Maclay[7] recommended the use of formamide as a dispersing agent at 65° for acetylation of starch. We found that freshly precipitated starch can be easily dispersed in this reagent at room temperature.

Two grams of amylopectin was dissolved in 100 ml. of hot water and precipitated by the addition of 250 ml. of 95% ethanol. The precipitate was collected on a Buchner funnel, washed with ethanol and ether; the slightly moist amylopectin was transferred to a flask and stirred with 30 ml. of formamide until dissolved. Fifty ml. of pyridine was added slowly with continuous stirring, followed by the addition of 40 ml. of acetic anhydride in small portions over a period of one hour. Stirring was continued for several hours and the mixture was allowed to stand overnight. The solution was filtered through a fine cotton cloth into a beaker containing approximately 1200 ml. of water. If the solution was too viscous to filter it was diluted with glacial acetic acid. The acetylated product was filtered on a Pyrex sintered glass filter, washed with an excess of water until the filtrate gave a neutral reaction and then dried *in vacuo* at 80°.

Most of the amylopectins were not completely acetylated by one such procedure. Analysis of the product after the first acetylation showed an acetyl content of approximately 40% (calculated $COCH_3$ content for the triacetate, $(C_6H_7O_5(CH_3CO)_3)_n$, 44.8%).

The process was repeated by dissolving the partially acetylated product in 50 ml. of pyridine and acetylating with 40 ml. of acetic anhydride as before. After the second acetylation at room temperature, the amylopectins were completely acetylated giving the theoretical acetyl value. The specific rotations in chloroform (c, 1) of the acetylated amylopectins ranged from $[\alpha]_D$ + 163 to +175°.

Acetylation of Amylose.—The amylose samples were reprecipitated as follows: a 2-g. sample was dissolved in 67 ml. of 3% potassium hydroxide, neutralized with dilute acetic acid and precipitated by the addition of an equal volume of ethanol. The precipitate was filtered, washed with ethanol and ether. The freshly precipitated amylose was dispersed in formamide and acetylated in the presence of pyridine with acetic anhydride at room temperature as with amylopectin. Similarly, two acetylations were required to completely acetylate the amylose. The specific rotations of acetylated amyloses in chloroform (c, 1) ranged from $[\alpha]_D$ +170 to +178°.

Molecular Weight Determination by Osmotic Pressure Measurement.—The molecular weights of the acetylated starch fractions were determined from osmotic pressure measurements, using an osmometer identical with that described by Zimm and Myerson[13]. This osmometer proved to be very satisfactory for osmotic pressure measurements when organic solvents were used.

Membranes were prepared from solutions containing 72% C. P. Merck collodion, 14% ether and 14% ethanol according to Carter, Scott and Magat[14] with the modification that chloroform was substituted for toluene in the aging of the membrane. After the iron ring was disen-

(13) B. H. Zimm and I. Myerson, THIS JOURNAL, **68**, 911 (1946).

(14) W. C. Carter, R. L. Scott and M. Magat, *ibid.*, **68**, 1480 (1946).

gaged the membrane was allowed to remain in water overnight and then placed successively in water–ethanol, ethanol and chloroform for two hours each. Before the membrane was used, it was aged by letting it remain for about a week in pure chloroform. These membranes proved very satisfactory for polymers of high molecular weights.

The filled osmometer was placed in a glass cylinder containing chloroform, which in turn was placed into a constant temperature bath at 27° controlled within ±0.01.

The dynamic method was used for measurement of the osmotic pressure. Measurements were made, using a cathetometer, at intervals of about twenty minutes. Equilibrium was approached from both above and below. The height in centimeters was plotted against time and the mean value between the two curves was taken as the osmotic pressure. The pressure was converted to grams per square cm.

The osmotic pressure was measured for each acetylated starch fraction at several different concentrations (Tables I, and II) and π/C was plotted against C^n (π = osmotic pressure in g. cm.$^{-2}$; C = concentration of acetylated fraction in grams per liter; n = 1.39 for acetylated amylose and n = 2.25 for acetylated amylopectin). The molecular weight was calculated from the intercept of the ordinate (Figs. 1 and 2), which is equal to RT/M where M = molecular weight, R = gas constant (84.7 lit.-g. cm.$^{-2}$ degree^{-1} mole^{-1}) and T = 300°K.

TABLE I

OSMOTIC PRESSURE DATA FOR ACETYLATED AMYLOSES

Concn., C, g. per l.	$C^{1.39}$	Osmotic pressure, π (g. per sq. cm.)	π/C
	Tapioca, T-3/4-A		
2.54	3.7	0.235	0.093
5.18	9.8	.714	.138
7.52	16.5	1.35	.180
10.2	25.2	2.40	.235
12.5	33.4	3.64	.291
	Potato, P-3/4-A		
2.56	3.7	0.310	0.121
5.74	11.4	.973	.169
7.23	15.7	1.42	.196
12.8	34.6	4.00	.312
	Wheat, W-1/2-A		
2.50	3.6	0.315	0.126
5.00	9.4	.821	.164
7.50	16.4	1.58	.211
10.0	24.6	2.67	.267
10.2	25.2	2.76	.270
	Corn, C-107/111-A		
5.71	11.3	1.04	0.182
12.4	33.1	4.03	.325
14.8	42.3	5.48	.371
20.1	64.5	10.3	.512
22.1	73.8	12.5	.566
	Sago, S-1/2-A		
.50	3.6	0.353	0.141
5.00	9.4	.878	.176
5.19	9.9	.926	.178
7.50	16.4	1.66	.221
10.0	24.6	2.73	.273
	Easter Lily, L-3-A		
2.50	3.6	0.426	0.170
2.86	4.3	.500	.175
5.00	9.4	1.07	.214
7.50	16.4	2.05	.273
10.0	24.6	3.32	.332

TABLE II

OSMOTIC PRESSURE DATA FOR ACETYLATED AMYLOPECTINS

Conc., C, g. per l.	$C^{2.25}$	Osmotic pressure, π (g. per sq. cm.)	π/C
	Corn, C-141-B		
12.0	268	0.238	0.0198
15.3	463	.428	.0280
17.7	643	.684	.0387
19.9	837	1.03	.0517
22.2	1050	1.46	.0657
25.4	1450	2.19	.0862
31.0	2270	4.29	.1390
	Corn, C-109-B		
10.0	177	0.128	0.0128
10.4	194	.150	.0144
13.9	373	.335	.0241
15.7	490	.499	.0318
17.6	635	.677	.0385
20.0	846	1.03	.0515
24.9	1390	2.00	.0803
	Wheat, W-2-B		
15.0	443	0.392	0.0261
20.0	846	.946	.0473
25.0	1400	1.88	.0752
	Easter Lily, L-3-B		
10.6	203	0.237	0.0224
12.5	294	.369	.0295
15.0	443	.640	.0427
17.4	618	1.04	.0598
19.9	837	1.52	.0764
	Tapioca, T-3-B		
12.5	294	0.270	0.0216
17.1	581	.653	.0382
20.1	856	1.08	.0538
	Sago, S-1-B		
10.1	182	0.202	0.0200
12.2	278	.303	.0248
15.2	456	.530	.0349
17.3	610	.681	.0394
20.1	856	1.06	.0527
24.9	1390	1.93	.0775

Determination of the Number of Chains per Molecule of Amylose.—The periodate oxidation data[3] were used in conjunction with the osmotic pressure data for the determination of the number of chains present in the amylose molecule. It is assumed that if one amylose molecule represents a single chain, the molecular weight obtained from the osmotic pressure measurements should agree with that calculated from the periodate oxidation end-group method. If the amylose molecule consists of more than one chain, the molecular weight would be greater than that obtained from the periodate oxidation data.

The moles of formic acid obtained from one mole of amylose, minus two moles of formic acid due to the terminal reducing glucose unit, should give the average number of non-reducing terminal glucose residues or the average number of chains. The following formula was used for

TABLE III

MOLECULAR WEIGHTS OF AMYLOSE AND AMYLOPECTIN FRACTIONS

Plant source	Intrinsic viscosity[a] in 1 *N* potassium hydroxide	π/C from intercept of ordinate	Molecular weight of acetylated product	Calculated molecular weight of deacetylated product	Average no. of glucose units per molecule	Average no. of glucose units per molecule from periodate oxidation	Average no. of non-reducing terminal glucose units per molecule
			Amyloses				
Tapioca, T-3/4-A	2.25	0.069	370,000	210,000	1300	980	2.0
Potato, P-3/4-A	1.95	.098	260,000	150,000	930	980	0.8
Wheat, W-1/2-A	1.54	.102	250,000	140,000	860	540	2.8
Corn, C-107/111-A	1.23	.113	230,000	130,000	800	490	2.9
Sago, S-1/2-A	1.13	.116	220,000	120,000	740	420	3.3
Easter Lily, L-3-A	1.06	.143	180,000	100,000	620	640	0.9
			Amylopectins				
Corn, C-141-B	1.35	0.0025[b]	10,000,000	6,000,000			
Corn, C-109-B	1.24	.0030	8,000,000	5,000,000			
Wheat, W-2-B	1.22	.0035	7,000,000	4,000,000			
Easter Lily, L-3-B	1.26	.0045	6,000,000	3,000,000			
Tapioca, T-3-B	1.27	.0045	6,000,000	3,000,000			
Sago, S-1-B	0.82	.0115	2,000,000	1,000,000			

[a] The intrinsic viscosity data were furnished by Dr. Thomas J. Schoch. [b] Inasmuch as the intercepts of the ordinate of the amylopectins are small, the error of the molecular weights may be considerable; it becomes smaller as the molecular weights decrease.

calculating the average number of chains: No. of chains = (equiv. of formic acid × M)/W −2, where M is the molecular weight and W is the number of grams of amylose.

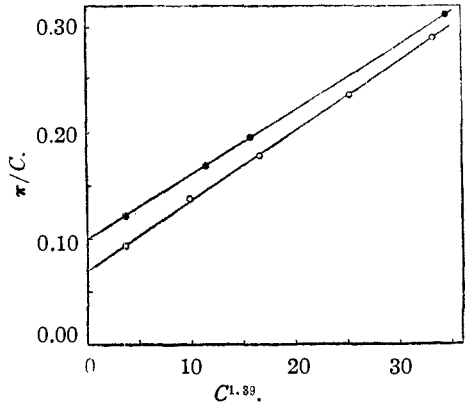

Fig. 1.—Osmotic pressure–concentration relationship of amylose: O, tapioca; ●, potato.

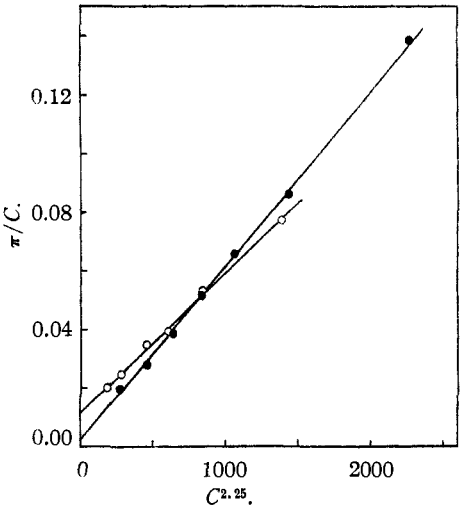

Fig. 2.—Osmotic pressure relationship of amylopectin: O, sago; ●, corn, C-141-B.

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Summary

The number-average molecular weights of six amylose and five amylopectin components of starches from different plant sources were determined by osmotic pressure measurements.

The number-average molecular weights of the amyloses ranged from 100,000 to 210,000; the amylopectins ranged from approximately 1,000,000 to 6,000,000.

Comparison of the molecular weights determined by osmotic pressure measurements with those estimated from the chain-length values found by the periodate end-group method indicate that in some amyloses (Table III, potato and Easter lily) single molecules constitute single chains. In other amyloses, two or three chains may be linked to form one amylose molecule, indicating the possibility of a slight degree of branching.

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