450. *The Soluble Polyglucose of Sweet Corn* (Zea Mays).

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The two water-soluble polyglucoses of sweet corn (*var.* Golden Bantam) have now been investigated. The view cannot be substantiated that these amylaceous polysaccharides (which are separated by their relative solubilities in 67% acetic acid) are structurally different. Evidence is given which shows each to have the characteristics of a glycogen rather than of a starch. Further, their properties are consistent with the view that 67% acetic acid effects an arbitraty fractionation of a polymer-homologous series of glycogen-type molecules for which it is proposed that the name " phytoglycogen " be reserved.

It is demonstrated that extensive amylolytic degradation occurs during the aqueous extraction of the grain and that when enzyme action is inhibited by Hg^{++} during extraction no degradation products of starch or glycogen are found.

THE older literature contains many references to the presence in starch-bearing plants of water-soluble, iodine-staining polysaccharides (" dextrins ") which were assumed to be amylolytic degradation products of granular starch. *Zea mays* (sweet corn) has a relatively high proportion of such water-soluble polysaccharides and Parker [1] suggested that these " dextrins " constitute the units from which the starch grains of maize are synthesised. The evidence of Morris and Morris [2] showed, however, that the water-soluble polysaccharide of the seeds of *Zea mays* (*var.* Golden Bantam) is more closely related to animal glycogen than to starch or the starch dextrins. Repeating this work, with some modifications of extraction procedure, Hassid and McCready [3] reported the presence of two water-soluble, amylaceous polysaccharides in sweet corn which were separable by their relative solubilities in 67% acetic acid. The fraction which was precipitated by this reagent and had been discarded by Morris and Morris [2] was described as a " soluble starch " since it gave a blue stain with iodine and had a basal-chain length [4] of 25 glucose units (methylation assay). The fraction soluble in 67% acetic acid, corresponding to the glycogen of Morris and Morris, had the same basal-chain length as had animal glycogen, namely, 12 glucose units. Sumner and Somers [5] also isolated two water-soluble polysaccharides from sweet corn, namely, " glycoamylase " (Hassid's soluble starch) and " phytoglycogen " (Morris' glycogen). Glycoamylose appeared to be distinguished from granular maize starch and animal glycogen by the faint blue colour it gave with iodine, although Cameron [6] observed both red and blue particles in iodine-stained glycoamylose. Dvonch and Whistler [7] showed, in contrast to Hassid and McCready, that glycoamylose and photoglycogen had almost the same basal-chain length, *viz.*, 12 and 11 units respectively. There is thus an indication that both maize polysaccharides have glycogen-like structures.

It appeared possible that the water-soluble polysaccharides of sweet corn might represent intermediates in the starch metabolic cycle but to examine this question it was first necessary to ensure that no enzymic degradation occurred during the extraction of the various carbohydrate fractions. It being established that 0·01N-mercuric chloride inhibited all the carbohydrases likely to cause autolytic changes in the carbohydrates of maize, a comparison was instituted between the products extracted from sweet corn in the presence and absence of mercuric ions. The lengthy extraction procedure was essentially that of Hassid and McCready.[3] For comparison, the glycogen of immature sweet corn was prepared by the method of Meyer and Fuld.[8]

There is reason to believe (see below) that amylose is not a significant constituent of

[1] Parker, *Plant Physiol.*, 1935, **10**, 713.
[2] Morris and Morris, *J. Biol. Chem.*, 1939, **130**, 535.
[3] Hassid and McCready, *J. Amer. Chem. Soc.*, 1941, **63**, 1632.
[4] Peat, Whelan, and Edwards, *J.*, 1955, 355.
[5] Sumner and Somers, *Arch. Biochem.*, 1944, **4**, 7.
[6] Cameron, *Genetics*, 1947, **32**, 459.
[7] Dvonch and Whistler, *J. Biol. Chem.*, 1949, **181**, 889.
[8] Meyer and Fuld, *Helv. Chim. Acta*, 1949, **32**, 757.

either of the water-soluble polysaccharides from the mature grain and that the names "soluble starch" or "glycoamylose" are misleading. For convenience of reference, we propose in this paper to name the soluble polysaccharides, phytoglycogen-A and -B, the former being insoluble and the latter soluble in 67% acetic acid. The phytoglycogens, isolated as described, were freed from protein by Sevag's method [9] and from phytin (in phytoglycogen-B) by precipitation with methanol from the acidified solution. Table 1 records the properties of the polysaccharides of *Zea mays*.

TABLE 1. *Properties of the polysaccharides from sweet corn.*

	From mature grain *						From immature grain.†
	Phytoglycogen-A		Phytoglycogen-B		Granular starch		
Polysaccharide Method of extraction	$+Hg^{++}$	H_2O	$+Hg^{++}$	H_2O	$+Hg^{++}$	H_2O	Phyto-glycogen-A
Crude yield (g./100 g. of seed)	41	11·1	3·3	3·9	22·7	20·6	—
Carbohydrate (%) after purification (by acid hydrolysis)	94·2	95·1	94·7	93·8	97·4	97·7	98·8
Blue value	0·018	0·014	0·008	0·010	0·350	0·356	0·019
Basal-chain length	13·2	9·7	7·3	5·8	24·0	23·8	9·4
Conversion (%) by							
(i) β-amylase	50	28	40	19	62	64	48
(ii) β-amylase + R-enzyme	52	28	46	44	72	75	48
Relative mol. wt. ($\times 10^{-6}$) (ultracentrifuge)	10·5	3·1	1·2	0·06	—	—	—

* Hassid and McCready's procedure.[3] † Meyer and Fuld's procedure.[8]

The much greater yield of phytoglycogen-A when extracted in the presence of mercuric chloride shows this fraction to be enzymically degraded during water-extraction to products which were then soluble in 67% acetic acid. Further evidence of enzymic degradation came from an examination of the extracted sugars. The sugar yields in the presence and absence of mercuric ion were respectively 12·9 and 73·5 g. from 400 g. of seed. When the enzyme-inhibitor was present, the sugars, separated by chromatography on charcoal,[10] were identified as glucose, fructose, sucrose, and raffinose. Except for a trace of maltose, amylolytic degradation products of starch were not found. When, however, no mercuric ions were present the sugar fraction contained glucose, fructose, and, significantly, maltose, maltotriose, and higher maltodextrins, but no sucrose or raffinose. It appeared from chromatographic data that maltulose was also present and further work on this aspect is proceeding. The smaller yield of phytoglycogen-A when the extraction was by water alone is reflected in the lower value of its average basal-chain length, as determined by periodate oxidation. This suggests partial degradation by β- and/or α-amylase, during extraction, a view confirmed by the observation that water-extracted phytoglycogens were hydrolysed to the extent of only 18—30% by crystalline β-amylase whereas values of 40—50% were obtained with (Hg^{++}) extracts. Enzymic degradation during extraction may account for the low β-amylolysis limit (20%) of maize glycogen reported by Morris.[11] Both α- and β-amylase, as well as phosphorylase and glucose-1 phosphatase, were shown to be present in an aqueous extract of immature sweet corn. The α-amylase was detected by the Wijsman agar plate technique [12] and by the fact that the extract partly hydrolysed amylose glycollate, a substrate which resists β-amylolysis almost completely.[13] Bernstein [14] failed, however, to find α-amylase in developing maize endosperm. A consideration of the isolation procedures used by the various investigators of the phytoglycogens suggests that enzymically degraded products would only result from application of the methods of Morris and Morris,[2] and Hassid and McCready.[3] Other workers have either

[9] Sevag, *Biochem. Z.*, 1934, **273**, 419.
[10] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677; Whelan, Bailey, and Roberts, *J.*, 1953, 1293.
[11] Morris, *J. Biol. Chem.*, 1944, **154**, 503.
[12] Wijsman, *Rec. Trav. chim.*, 1890, **9**, 1.
[13] Peat, Bourne, and Thrower, *Nature*, 1947, **159**, 810.
[14] Bernstein, *Amer. J. Bot.*, 1943, **30**, 517.

extracted the ground seed with dilute trichloroacetic acid [5, 7] or have added the acid to an extract prepared by rapid maceration of the seeds in water.[8]

It has been noted that 67% acetic acid, even at 0°, effects some hydrolysis of the polysaccharide. The extent of acid degradation in 18 hours (the usual period of contact) is not, however, significant.

The Phytoglycogens.—Table 1 shows that in basal-chain length, β-amylolysis limit, and iodine-stain, undegraded phytoglycogen-A and -B are more closely related in structure to animal- or yeast-glycogen than to amylopectin. We are in agreement with Dvonch and Whistler,[7] but not with Hassid and McCready,[3] as to the basal-chain length of phytoglycogen-A. Professor Hassid kindly supplied us with Golden Bantam seed of the type he had used and the basal-chain lengths of the " A " and " B " fractions, isolated in the presence of mercuric ions, were found to be 11 and 9, respectively, agreeing with our previous results (Table 1). Meyer and Fuld [8] have obtained phytoglycogen from immature sweet corn and, finding the basal-chain length of the unfractionated material to be 10, inferred that the immature corn contained only Hassid's " glycogen " (basal-chain length 12) and not the " soluble starch " (basal-chain length 25). We have repeated this work and agree with Meyer's estimate of the basal-chain length (Table 1). We also agree that the phytoglycogen from immature grain is not the usual mixture of A and B fractions, its complete insolubility in 67% acetic acid showing it to be phytoglycogen-A, which corresponds to the " soluble starch " fraction of Hassid and McCready.

The similarity of undegraded phytoglycogen-A and -B to animal glycogen is further emphasised by their behaviour with R-enzyme. This enzyme, which readily debranches amylopectin, is without action on either animal glycogen [15] or phytoglycogen, as is shown by the insignificant increase in the β-amylolysis limit after treatment with R-enzyme. On the other hand, the water-extracted, and therefore amylolytically degraded, phytoglycogen-B was substantially debranched by R-enzyme (Table 1) and it is known that rabbit-liver glycogen is attacked by R-enzyme only after fragmentation of the molecule by α-amylase.[15] A possible explanation is given later of the fact that water-extracted phytoglycogen-A is not attacked by R-enzyme.

Their properties show that there is no marked structural difference between phytoglycogen-A and -B and the evidence is consistent with the view that 67% acetic acid merely effects a more or less arbitrary fractionation of a polymer-homologous series of highly ramified molecules of glycogen type. (It will be shown in a later paper that the branch linkages in each phytoglycogen have the usual α-1 : 6-configuration.) A systematic fractionation was therefore carried out by the stepwise addition of acetic acid to an aqueous solution of a mixture of undegraded phytoglycogen-A and -B, extracted from the same sample of maize. No precipitation occurred until the acetic acid concentration reached 55%; thereafter fractions were collected within the acid concentration limits given in Table 2. Relative molecular weights were determined (by Dr. C. T. Greenwood) from ultracentrifugal data, the assumption being made that the values of the diffusion constant and partial specific volume were the same as for animal glycogen. Basal-chain length was determined by periodate oxidation. The data in Table 2 show that a continuous series

TABLE 2. *The fractionation of phytoglycogen with acetic acid.*

Acetic acid concn.; limits (%)	Yield * (g.)	Relative mol. wt. ($\times 10^{-6}$) †	Basal-chain length (periodate)
55—60	4·25	3·25	10·3
60—65	37·8	3·2	11·0
65—70	2·36	1·9	9·7
>70	1·05	0·18	7·8

* From 62 g. of total phytoglycogen. † See text.

of fractions of decreasing molecular weight and basal-chain length are obtained by a simple procedure based upon solubility. It is seen, however, that the bulk of the phytoglycogen lies within narrow ranges of molecular weight and mean basal-chain length.

It is now possible to understand why the yield of phytoglycogen-A is lower, and that

[15] Peat, Whelan, Hobson, and Thomas, *J.*, 1954, 4440.

of phytoglycogen-B slightly higher, when enzyme action during extraction is not inhibited by the addition of mercuric ions (Table 1). Amylolysis of phytoglycogen-A would diminish its molecular size and thus increase its solubility in aqueous acid. Less would be precipitated at 67% concentration and more found in the phytoglycogen-B fraction. The increased yield of the B fraction is not commensurate with the lower yield of phytoglycogen-A because fragments of both A and B appear in the oligosaccharide fraction. The observation that degraded phytoglycogen-B is attacked by R-enzyme whereas degraded phytoglycogen-A is not (Table 1) is also explicable on the view that although the A fraction is degraded during extraction, the residue precipitated by 67% acetic acid still consists of molecules sufficiently large to retain its glycogen characteristics, which include inaccessibility of the branch links to R-enzyme.

The conclusion is reached that the water-soluble polysaccharides of *Zea mays* constitute a single polymer-homologous series (as do glycogen [16] and the components of starch [17]) and although the maize polysaccharide is structurally indistinguishable from glycogen of animal or microbial origin, it will be convenient to retain the name " phytoglycogen " to indicate its source. The A fraction of phytoglycogen contains a small amount (3%) of a polyglucose which is stained blue by iodine and the investigation of this component is proceeding.

Experimental

Analytical Methods.—Blue values were determined as by Bourne *et al.*[18] Total phosphorus was determined as by Peat *et al.*;[19] inorganic and 7-minute hydrolysable ester-phosphorus as by Bailey and Whelan.[20] The Somogyi copper reagent[21] was used for the estimation of reducing power; when R-enzyme was present, the reducing solutions were first deproteinised.[22] The carbohydrate content of a polysaccharide was determined by acid hydrolysis, as by Pirt and Whelan.[23] β-Amylase activity was determined as by Bourne *et al.*[24] The basal-chain length (*i.e.*, proportion of terminal non-reducing end groups) of a branched polysaccharide was estimated by periodate oxidation, the liberated formic acid being determined iodometrically by one of two methods: (i) In the semimicro-method, the polysaccharide (250 mg.) was dissolved in 3% sodium chloride (25 ml.) and 0·37M-sodium metaperiodate (25 ml.) was added; the reaction was allowed to proceed at room temperature in subdued light, and, at suitable intervals, aliquot parts (5 ml.) were removed and added to ethylene glycol (1 ml.); after 10 min., potassium iodide (0·2 g.) was added and the liberated iodine titrated with neutral 0·005N-sodium thiosulphate with starch glycollate as indicator. (ii) When the amount of material available was more limited, a micro-method was used: the reaction mixture contained the polysaccharide (25 mg.) and 0·37M-sodium metaperiodate (5 ml.) in a total volume of 10 ml.; the formic acid in an aliquot sample (1 ml.) was titrated iodometrically in an atmosphere of nitrogen, an " Agla " micrometer syringe being used as burette. Control experiments with rabbit-liver glycogen, by the two methods, gave basal-chain lengths of 13·7 and 13·6.

Paper-chromatographic fractionation of sugars was carried out with butan-1-ol–acetic acid–water (4 : 1 : 5 by vol.) and Whatman No. 54 filter paper.

Preparation of Enzymes.—Crystalline β-amylase of sweet potato was kindly provided by Dr. A. K. Balls.[25] R-Enzyme was prepared as by Hobson *et al.*[22]

Fractionation of the Carbohydrates of Zea mays.—The method was essentially that of Hassid and McCready.[3] (a) *In presence of mercuric chloride.* Mature *Zea mays* (Golden Bantam; 400 g.) was finely ground and steeped in 0·01N-mercuric chloride (1 l.) under toluene for 18 hr. The solids were re-extracted five times for 2-hourly periods with portions (1·5 l.) of 0·001N-mercuric chloride. The extracts were combined with the steep liquor, filtered through a Celite pad, and concentrated over calcium carbonate to a volume of 1·2 l. The coagulated protein was filtered off, and the soluble polysaccharides were precipitated by the addition of ethanol

[16] Meyer, *Adv. Enzymology*, 1943, **3**, 109.
[17] Kerr, *Arch. Biochem.*, 1945, **7**, 377; Greenwood and Robertson, *J.*, 1954, 3769.
[18] Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924.
[19] Peat, Thomas, and Whelan, *J.*, 1952, 722.
[20] Bailey and Whelan, *J.*, 1950, 3573.
[21] Somogyi, *J. Biol. Chem.*, 1945, **160**, 61; Hobson, Whelan, and Peat, *J.*, 1950, 3566.
[22] Hobson, Whelan, and Peat, *J.*, 1951, 1451.
[23] Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **5**, 224.
[24] Bourne, Macey, and Peat, *J.*, 1945, 882.
[25] Balls, Walden, and Thompson, *J. Biol. Chem.*, 1948, **173**, 9.

(1·5 vol.) at 0°, and collected in a centrifuge. The supernatant liquid, which contained the sugars, was concentrated over calcium carbonate to small volume, mercuric ions were removed by hydrogen sulphide, and the solution was evaporated to a syrup.

The polysaccharide fraction was dissolved in warm water (10 parts), cooled to 0°, and treated with acetic acid (2 vol.). After being kept for 18 hours at 0°, the precipitate (phytoglycogen-A) was centrifuged off, washed with alcohol and ether, and dried. A second fraction (phytoglycogen-B) was isolated from the supernatant liquid by the addition of ethanol (0·5 vol.) at 0°. After 18 hr. it was centrifuged off, washed, and dried as before. Granular starch was obtained by kneading the maize-meal residue in a cloth in 0·001N-mercuric chloride (2 l.). The starch which passed through the cloth was washed by decantation with 0·001N-mercuric chloride, washed with ethanol, and dried. Partial purification of the phytoglycogen fractions was carried out by the method of Hassid and McCready.[3]

(b) *In absence of mercuric chloride.* The quantities and procedure used were as described under (a) except that all mercuric chloride solutions were replaced by water only.

(c) *Phytoglycogen from immature maize.* Immature *Zea mays* cobs (Golden Bantam; 95 g.) were extracted as by Meyer and Fuld.[8] Practically the whole of this phytoglycogen was insoluble in 67% acetic acid, pointing to the absence of the " B " fraction.

Purification of the Phytoglycogen and Starch Fractions.—All the polysaccharides contained protein impurities, and, in the case of the phytoglycogen-B fractions, 10—30% of phytin. They were purified as follows. Phytoglycogen-A and -B were treated by Sevag's method[9] to remove protein, and the phytin and inorganic matter were then removed by precipitation of the polysaccharide from solution at 0° with methanol (2 vol.) immediately after addition of hydrochloric acid to 1% concentration. The precipitate (*ca.* 50—60%) was washed with ethanol and ether. The whole starch (15 g.) was suspended in 0·1% aqueous sodium chloride (1 l.) and boiled for 1 hr. After cooling, the gelatinous residue was removed and the starch precipitated from the solution by ethanol (2 vol.) at 0°. Some protein still remained, and this was removed by extraction of the starch with 33% chloral hydrate solution at 80° for 1 hr., in which reagent starch, but not protein, is soluble.[26] The insoluble residues were removed and the starch was precipitated from solution by acetone (2 vol.).

Examination of the Fractions.—(a) *The sugar fraction.* The sugar syrups (6 g.) were fractionated on charcoal–Celite columns (100 × 4 cm.), the columns being irrigated successively with water, 5%, 10%, 15%, 18%, 20%, 23%, and 50% aqueous ethanol. Serial groups of fractions were combined, concentrated, and refractionated, when necessary. The sugars so obtained were tentatively identified by paper chromatography. When extraction was carried out in the presence of Hg^{++}, the following sugars were detected: fructose, identified as 2 : 3-4 : 5-di-*iso*propylidenefructose, m. p. 89—90°, $[\alpha]_D^{18}$ −32·0° (*c* 1·0 in $CHCl_3$); glucose, as the β-penta-acetate, m. p. 129—130°, $[\alpha]_D^{18}$ +3·70° (*c* 1·0 in $CHCl_3$); sucrose, as the octa-acetate, m. p. 86—88°, $[\alpha]_D^{18}$ +58·1° (*c* 2·0 in $CHCl_3$); maltose, present in traces, by R_F value only; raffinose by R_F value and by mild hydrolysis to fructose and melibiose, identified by paper chromatography. When mercuric chloride was not present during extraction, fructose was identified as 2 : 3-4 : 5-di*iso*propylidenefructose, m. p. 89—90°, $[\alpha]_D^{18}$ −32·3°; glucose as the β-penta-acetate, m. p. 129—131°, $[\alpha]_D^{18}$ +3·72° (*c* 1·2 in $CHCl_3$), and maltose as the β-octa-acetate, m. p. 158—159°, $[\alpha]_D^{18}$ +62·2° (*c* 1·0 in $CHCl_3$). Maltulose was tentatively identified by R_F value, by hydrolysis with purified yeast maltase to glucose and fructose, and by its non-hydrolysability by invertase. Maltotriose was identified by R_F value, acid hydrolysis to glucose only, and slow hydrolysis by crystalline β-amylase to a mixture of glucose and maltose; maltotetraose by R_F value, acid hydrolysis to glucose, and by its rapid hydrolysis by β-amylase to maltose. Higher maltodextrins were also present, and were characterised as such by acid hydrolysis to glucose and by their rapid β-amylolysis to maltose and maltotriose. Sucrose and raffinose were not found, even in traces. (b) *The polysaccharide fractions.* The properties of the fractions are listed in Table 1. Enzyme action was studied on digests made as follows. The polysaccharide (25 mg.) was dissolved in warm water (10 ml.), or, in the case of the starches, in 0·25N-sodium hydroxide (10 ml.) and neutralised. 0·2M-Acetate buffer (3 ml.) was added, followed by the enzyme solution and water to 25 ml. For β-amylolysis, the digest pH was 4·8 and 1000 enzyme units were used. In studying R-enzyme action, the enzyme (50 mg.; 1 ml.) was allowed to act for 18 hr. at pH 7 in a similar digest (23 ml.). 0·2M-Acetate buffer (pH 3·6; 1 ml.) was added, thereby adjusting the digest pH to 4·8, followed by β-amylase (1000 units; 0·2 ml.) and water to 25 ml. All digests were incubated at 35°, and the course of reaction was

[26] Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 875.

followed by determination of reducing power. The conversion limits given in Table 1 were measured after 24 hr.

Fractionation of Phytoglycogen (*Table* 2).—Phytoglycogen (62 g.), obtained by extraction of mature corn with aqueous mercuric chloride, was fractionated by a continuous addition of acetic acid at 0° to an aqueous solution (800 ml.). The precipitated fractions (Table 2) were kept in contact with the supernatant liquid for varying periods (10—16 hr.) before being separated, washed, and dried. The fourth fraction was obtained by the final addition of ethanol (0·5 vol.). The fractions were purified as described for phytoglycogen-A.

Detection of Carbohydrases in Immature Zea mays.—Immature corn cobs (1050 g.) were macerated with water (500 ml.) and centrifuged. To the supernatant solution was added 50% ammonium sulphate (pH 7·0; 4 vol.), and the precipitate centrifuged, redissolved in 0·1M-citrate buffer (pH 7·0, 100 ml.), and freeze-dried. The dried powder (14 g.) was used as the test source of enzyme activity. Phosphorylase and phosphatase activities were determined by a modification of the method of Green and Stumpf,[27] the phosphatase activity being measured in absence of starch primer.[28] The respective activities were 23·8 and 19·9 units per g. The presence of α-amylase was shown by incubating the enzyme preparation (102 mg.) with sodium amylose glycollate (25 mg.) [13, 19] at pH 4·8 in a total volume of 25 ml. After 22 hr. the percentage conversion (as maltose) was 24·9. Under identical conditions β-amylase (500 units) effected a conversion of 3·1%, whereas with human saliva (50% by vol.; 2 ml.) the conversion was 94·8%. Both α- and β-amylolytic activities were detected by Wijsman's [12] agar-plate technique.

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[27] Green and Stumpf, *J. Biol. Chem.*, 1942, **142**, 355; Whelan and Bailey, *Biochem. J.*, 1954, **58**, 560
[28] Whelan, "Methods in Enzymology," Academic Press Inc., New York, 1955, Vol. I, p. 193; Porter, *J. Exp. Bot.*, 1953, **4** 44.