GENETIC INTERACTIONS AFFECTING MAIZE PHYTOGLYCOGEN AND THE PHYTOGLYCOGEN-FORMING BRANCHING ENZYME[1]

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Received October 27, 1965

THE study of the effects of gene mutations on the regulation of metabolic processes in maize promises to increase our understanding of gene action in higher organisms. Developing maize endosperm is particularly well suited for studies of genetic interaction because of the existence of a wide variety of specific genes which cause qualitative and quantitative changes among the carbohydrates synthesized in this tissue. The goal of such studies is to establish the metabolic effects of the various genes while at the same time gaining a better understanding of the metabolic processes themselves.

Among the materials influenced by the endosperm mutations in maize is a highly branched molecule resembling animal glycogen. This starch-related material, termed phytoglycogen, is found in large quantities in the water soluble polysaccharide (WSP) fraction of sweet corn endosperm which contains the mutant gene sugary-1 (su_1) on chromosome 4. CREECH, MCARDLE and KRAMER (1963) observed that the mutant genes amylose-extender (*ae*) on chromosome 5, dull (*du*) on chromosome 10, and waxy (*wx*) on chromosome 9, singly or in combination with su_1, also exert a major influence on the WSP content of the endosperm. These mutants have been of primary interest to maize geneticists in the past because of their influence on the types of starches deposited in the starch granules.

The purpose of this paper is to report on the nature of a number of genetic interactions affecting phytoglycogen synthesis in maize. Both the phytoglycogen-forming branching enzyme, which produces phytogylcogen from amylose (LAVINTMAN and KRISMAN 1964; HODGES, CREECH and LOERCH 1965a), and the phytoglycogen fraction itself were isolated from the various genotypes and characterized. A relationship is established in this study between the occurrence of phytoglycogen and the presence of the branching enzyme. The possible mode of action of these genes in the pathways of phytoglycogen metabolism is discussed in light of the genetic interactions revealed in this study.

A literature review relating to the characterization of sweet corn WSP (phytoglycogen) has been presented in an article by PEAT, WHELAN and TURVEY (1956).

[1] This investigation was supported in part by National Science Foundation Research Grant GB-1876, National Science Foundation Institutional Funds, and USDA Regional Research Funds. Paper No. 3068 in the Pennsylvania Agricultural Experiment Station journal series.

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MATERIALS AND METHODS

The maize mutants used in these studies were grown on the research plots at the Pennsylvania State University during the 1964 growing season. The planting date was the same for all genotypes. All of the ears were hand pollinated to insure genetic purity. Ears were harvested 20 and 24 days after pollination and frozen in liquid nitrogen within 30 minutes of removal from the plant. The kernels were shelled and stored in plastic bags at —25°C until ready for analysis. All of the mutants, with the exception of Golden Cross Bantam sweet corn, had been incorporated into a background related to the single cross W23/L317. The backgrounds were not isogenic and this fact must be kept in mind when the data for specific genotypes are compared.

The genetic mutations which were included in this investigaiton were *ae*, *du*, su_1 and *wx*. The double and triple recessive gene combinations were also studied. The normal genotype was included as a basis of comparison.

Water soluble polysaccharide isolation techniques: The primary isolation technique for WSP used in this investigation was the mercuric chloride extraction method described by GREENWOOD and DAS GUPTA (1958). This method inactivates the polysaccharide degrading enzymes present in the extract. The kernels were homogenized for 1 minute in a Waring Blendor with 1.5 volumes of cold (2°C) 0.01 M mercuric chloride solution. The homogenized kernels were centrifuged at $2000 \times g$ for 10 minutes and the supernatant filtered through cheeescloth. The supernatant was then recentrifuged at $10{,}000 \times g$ for 20 minutes to remove the starch which remained in suspension. The solution was deproteinized by bringing it to 0.1 M in sodium chloride and shaking for 12 hours in a separatory funnel with one-tenth volume of toluene (redistilled). This step was repeated until the toluene-water interface no longer showed a precipitate. At this point the WSP was precipitated by adding 2 volumes of 95% ethanol to the solution. The precipitated material was then defatted by refluxing with 80% methanol in a Soxholet apparatus for 24 hours.

Quantitative analysis of WSP: Ten-gram samples of frozen kernels were blotted dry of all ice and external moisture and weighed. Each sample was taken from a single ear. After drying in a Virtis freeze-dryer, the samples were reweighed and the WSP isolated by the mercuric chloride extraction described above. The isolated WSP was dried and weighed. Since some non-polysaccharide material was found to be extracted by this technique (BLACK 1965), the actual phytoglycogen content was determined by hydrolyzing a known weight of dried material for 2 hours in 3 ml of 2 N H_2SO_4 at 100°C and measuring the hydrolyzed glucose by the Nelson-Somogyi reducing sugar method (NELSON 1944).

Qualitative test for phytoglycogen: The isolated WSP fraction was treated in part with the $CaCl_2$-I_2 reagent of KRISMAN (1962). The development of a red color having an absorbancy maximum at 460 mμ was a reliable indicator for the presence of phytoglycogen. Other starch-like materials have different characteristic absorbancies with this reagent but were never detected in the WSP fraction. All spectra were obtained on the Cary 14 recording spectrophotometer.

Molecular structure determinations: A combination of the periodate oxidation method of GREENWOOD and THOMSON (1962) and the β-amylolysis method of COWIE, FLEMING, GREENWOOD and MANNERS (1957) was used to determine the average internal and external chain lengths of the phytoglycogen obtained from the various genotypes.

Phytoglycogen branching enzyme: Samples consisting of 20 g of frozen kernels of 20 day maturity were extracted as described by LAVINTMAN and KRISMAN (1964). Each sample was taken from a single ear. The supernatant from the extraction was passed through a DEAE-cellulose column and analyzed by the procedures of HODGES, CREECH and LOERCH (1965b). The column was eluted with a continuous sodium chloride concentration gradient which varied from 0 to 1 molar. The material eluted from the column was collected in 10 ml fractions and assayed for the branching enzyme by incubating 2 drops of the contents of each fraction collected with 0.2 ml of amylose at a concentration of 1 mg per ml for 12 hr at 30°C. After the incubation period, 1.2 ml of the $CaCl_2$-I_2 reagent described above was added to each reaction tube and the absorbancy maximum of the polysaccharide present was determined on the Cary 14 to obtain evidence for the conversion of amylose to phytoglycogen. In these studies the formation of material absorbing at 500 mμ or below with the reagent was indicative of the presence of the

branching enzyme. The Q-enzyme, which appeared in different fractions, produced a material (amylopectin) which absorbed at 530 to 540 mμ with the reagent.

RESULTS

From the outset of this investigation, two important facts regarding the water soluble polysaccharide fraction became apparent. A so-called WSP fraction could be isolated from all of the mutants by the aqueous extraction procedure, but only in certain genotypes did this material or any portion of it have a structure related to starch or phytoglycogen. Secondly, all of the starch-related material which could be isolated from the genotypes in which it was found to be present had an absorbancy maximum of 460 to 465 mμ with the $CaCl_2$-I_2 reagent. This value corresponds solely to that expected for phytoglycogen.

The results of the quantitative analysis of the mutants for phytoglycogen and dry matter are presented in Table 1. No material capable of forming glucose upon acid hydrolysis or a colored complex with the iodine reagent could be isolated from the WSP fractions of the mutants listed as having zero percent phytoglycogen, although several percent of total dry matter was present in each of the WSP fractions analyzed. No correlation was found between the dry matter content of the kernels and the percent phytoglycogen isolated in the WSP fraction. The gene *wx* is associated with a high dry matter content when present alone and

TABLE 1

The quantities of phytoglycogen and total dry matter in entire kernels of 15 maize genotypes at 24 day maturity†*

Genotype (homozygous)	Phytoglycogen (percent)	Dry matter (percent)
normal	0.0	22.8
ae	0.0	27.4
du	0.0	24.4
su_1§	24.9	28.4
wx	0.0	27.8
ae du	0.0	‡
ae su_1	0.0	25.0
ae wx	0.0	‡
du su_1	17.9	21.6
du wx	1.9	28.7
su_1 *wx*	19.0	28.2
ae du su_1	6.6	22.3
ae du wx	trace	‡
ae su_1 *wx*	7.3	26.8
du su_1 *wx*	27.9	29.0
Least significant difference	.05 = 3.0 .01 = 4.2	.05 = 2.3 .01 = 3.1

* Percent of dry matter.
† Three replications (three randomly selected ears).
‡ Insufficient material for quantitative analysis.
§ Golden Cross Bantam background.

when in combination with other genes. The lowest dry matter content appears to be associated with *du* except when it is in combination with *wx*.

The highest phytoglycogen content was found in *du* su_1 *wx*. This finding would be expected since the interaction of *du* and *wx* with su_1 has been reported to increase the content of WSP over that found in su_1 alone (ANDREW, BRINK and NEAL 1944; MANGELSDORF 1947). In this study the mutation su_1 in a Golden Cross Bantam sweet corn background was found to have a greater phytoglycogen content than either the *du* su_1 or su_1 *wx* genotypes in our standard background. This exaggerated effect of the su_1 gene in Golden Cross Bantam sweet corn is in contrast to earlier results (CREECH *et al.* 1963) and may be explained by the fact that Golden Cross Bantam is a commercial line which has a higher WSP content than is normally found in the su_1 mutant. It was used in place of the su_1 from the related background in this instance because of shortages of material. The su_1 line did not germinate in 1964.

The gene *ae* appears to be epistatic to su_1 in the synthesis of phytoglycogen. It also sharply reduces the amount of phytoglycogen which accumulates when in combination with *du wx*. In the genotype *ae* su_1, *ae* appears to block completely the formation of phytoglycogen. In the triple mutant combinations *ae du* su_1 and *ae* su_1 *wx*, the phytoglycogen content has been reduced by almost two-thirds from the level found in *du* su_1 and su_1 *wx*. In these genotypes, the gene *ae* is apparently unable to overcome completely the effects of the interaction of *du* and *wx* with the gene su_1 although it interferes markedly with its accumulation.

The results obtained with *du wx* were somewhat erratic. In some samples there was no evidence of phytoglycogen being present while in others it was present at a level of several percent of the dry weight. The fact that trace quantities of phytoglycogen were present in *ae du wx* supports the finding that the *du wx* combination causes the accumulation of phytoglycogen, although at a lower level than is caused by su_1.

ERLANDER (personal communication) has reported that he has been able to demonstrate the presence of soluble phytoglycogen in normal corn. This was accomplished by boiling freshly harvested kernels in 80 percent methanol for 30 minutes followed by extracting the residue with cold water. He suggests that the hot methanol treatment is required to break the plastids and allow the phytoglycogen to be released, and the homogenization in water is not sufficient to release the material from the plastids. Repeated attempts in this investigation to isolate phytoglycogen from fresh normal maize kernels by this technique were unsuccessful.

The results of periodate oxidation and β-amylolysis of the material isolated from mutants in which phytoglycogen was present in quantity are shown in Table 2. The β-limit data represent the percent of the total phytoglycogen hydrolyzed by β-amylase. These give a direct measure of the average external chain lengths of the molecules, appropriately converted to glucose units. The average overall chain lengths, expressed in glucose units, were obtained by the periodate value for the total number of external branches present in a given quantity of material, and the average internal chain lengths were obtained from the differ-

TABLE 2

*The β-amylolysis limits and mean overall, internal and external chain lengths of phytoglycogen from maize mutants**

Genotype (homozygous)	β-limits (percent)	Mean chain length, glucose units		
		Overall	External	Internal
su_1	40.5	14.5	8.4	6.1
du su_1	39.7	14.1	8.1	6.0
su_1 *wx*	40.6	14.1	8.2	5.9
ae du su_1	40.1	13.5	7.9	5.6
ae su_1 *wx*	41.0	14.7	8.5	6.2
du su_1 *wx*	38.4	14.1	7.9	6.2

* Mean of duplicate determinations.

ence between the values for external chain length and average overall chain length.

The values for su_1 phytoglycogen reported in the literature (GREENWOOD and DAS GUPTA 1958; LAVINTMAN and KRISMAN 1964) agree very closely with the data in Table 2, indicating the lack of structural differences between phytoglycogens from different genotypes and different sources. The interaction of the gene *ae* with *du* su_1 and su_1 *wx* caused a significant decrease in phytoglycogen, but there were apparently no qualitative effects on the phytoglycogen structure. Neither was there any indication of the presence of a starch-related material other than phytoglycogen in the WSP fraction of any of the mutants.

The results of the assay for the phytoglycogen forming branching enzyme in different maize genotypes are presented in Table 3. In addition to the branching enzyme the assay reveals the presence of the Q-enzyme which produces amylopectin (absorbancy maximum at 520–540 mμ with the $CaCl_2$-I_2 reagent) but not phytoglycogen from amylose, and a mixture of unidentified degradative

TABLE 3

*Interactions of maize endosperm mutations on the synthesis of phytoglycogen and related enzymes in various genotypes**

Genotype (homozygous)	Phytoglycogen	Branching enzyme	Q-enzyme	Degradative enzymes
normal	—	—	+	+
ae	—	—	+	+
du	—	+	+	+†
su_1	+	+	+	+
wx	—	+	+	+†
ae su_1	—	—	+	+
du wx	+	+	+	+†
su_1 *wx*	+	+	+	+
ae su_1 *wx*	+	+	+	+

* The symbol (+) denotes the presence of phytoglycogen or the corresponding enzyme; (−) denotes its absence.
† These degradative enzymes appeared much earlier in the column fractionation sequence than usual, indicating a possible deviation in their nature from those found in the other genotypes.

enzymes which leave only non-staining reaction products. These enzymes are also reported.

The data of Table 3 indicate that the branching enzyme was isolated from all of the mutants in which the presence of phytoglycogen could be demonstrated. The genotypes normal, *ae*, and *ae su*$_1$ do not appear to contain either phytoglycogen or the phytoglycogen-forming branching enzyme. In these mutants, as well as all of the others investigated, enzyme fractions corresponding to Q-enzyme were found.

The results obtained for *du* and *wx* are somewhat contradictory in that the phytoglycogen-forming branching enzyme was found in these mutants, but phytoglycogen could not be demonstrated to be present. Another difference associated with these mutants, as well as with *du wx*, was that the degradative enzymes were eluted with the first several fractions, overlapping with the branching enzyme, rather than later in the fractionation procedure as was the case in all of the other mutants studied.

DISCUSSION

The complexity of the genetic control of metabolic pathways in higher organisms is evident in the interactions of the many genes responsible for carbohydrate type and quantity in maize. The present study demonstrates that the mutation *su*$_1$ is not in itself responsible for the production of either phytoglycogen, with which it had been closely associated, or the branching enzyme which converts amylose to phytoglycogen. In the presence of the *ae* gene, *su*$_1$ produces neither phytoglycogen nor the branching enzyme. The presence of either *du* or *wx* in triple combination with *ae* and *su*$_1$ restores both the branching enzyme and phytoglycogen in the endosperm. The *du* and *wx* mutations alone produce no detectable phytoglycogen but contain the branching enzyme and an anomalous complement of degradative enzymes. The *du su*$_1$ and *su*$_1$ *wx* combinations contain the branching enzyme and have been shown to contain greater quantities of phytoglycogen (CREECH *et al.* 1963) than *su*$_1$ alone. The ability of the genotype *du wx* to produce phytoglycogen and the branching enzyme in the absence of the *su*$_1$ gene, and the lack of these in the *ae su*$_1$ genotype indicate that the *su*$_1$ gene is not the controlling factor in the production of either.

Phytoglycogen may be of much greater significance in the normal pathways of starch metaoblism than previously supposed; however, ERLANDER (1960) has postulated that phytoglycogen is an intermediate in starch synthesis. It appears to accumulate in those genotypes which produce severe disruption of the normal deposition of starch granules. It remains to be established whether phytoglycogen is a shunt product of hindered starch metabolism or a starch precursor which accumulates in these metabolically disturbed systems. The ability of *ae* to prevent phytoglycogen accumulation is complete with the *su*$_1$ gene alone, but is less effective when in combination with *du wx*, *du su*$_1$ and *su*$_1$ *wx*.

The production of the branching enzyme is similarly independent of the *su*$_1$ mutation. The enzyme has been found in all genotypes studied which accumulate phytoglycogen. This enzyme was also found in the single genotypes studied

which enhance the production of phytoglycogen in the presence of each other and the su_1 gene. The lack of phytoglycogen accumulation in these genotypes may be due to the presence of degradative enzymes which differ from those found in the phytoglycogen-accumulating genotypes. Some evidence to support this possibility was obtained. The branching enzyme itself may be inducible. This would presuppose the presence of a common inducer (derepressor) in those genotypes which accumulate phytoglycogen or enhance its accumulation in combination with other genes. This material would be absent in the other genotypes.

The finding that all the polysaccharide-related materials isolated in the WSP fractions had the same structure as phytoglycogen indicates the likelihood that a common phytoglycogen synthetic pathway exists in the various genotypes studied. No straight chain or other water soluble polysaccharides were isolated from the normal or *ae* genotypes which might serve as intermediates in starch synthesis, leaving open the possible pathways by which this material is produced. The endosperm fraction commonly described as WSP has been shown with these genotypes to give no color with iodine and not to be a glucose-containing polymer. Care must be exercised in differentiating between the gross WSP fraction of maize endosperm, which may not contain any starch-like materials, and phytoglycogen which appears only in certain genotypes.

SUMMARY

The influence and interactions of the *ae*, *du*, su_1 and *wx* maize endosperm mutations on the synthesis and structure of phytoglycogen have been determined. The phytoglycogen-forming branching enzyme was assayed in these genotypes to provide evidence for genetic control of its synthesis. The su_1 gene was implicated in the production of both phytoglycogen and the branching enzyme. The *du* and *wx* genotypes, while containing the branching enzyme, do not produce detectable quantities of phytoglycogen. These genes in combination with each other or with su_1 cause phytoglycogen to accumulate. The gene *ae* is epistatic to su_1 in that no phytoglycogen or branching enzyme is produced in the double mutants. The *ae* gene appears to reduce the phytoglycogen content in combination with *du* su_1, su_1 *wx* and *du wx* in maize endosperm, although the branching enzyme is present in these genotypes. It was concluded that the gene su_1 is not the controlling factor either in the formation of phytoglycogen or the branching enzyme. Presence of the branching enzyme in all of the mutants in which phytoglycogen was found indicates a probable role for the enzyme in the *in vivo* synthesis of phytoglycogen and suggests that it may be inducible. The possible role of phytoglycogen in the pathways of starch synthesis was discussed.

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