INTERALLELIC COMPLEMENTATION AT THE *sh* LOCUS IN MAIZE AT THE ENZYME LEVEL[1]

PREM S. CHOUREY[2] AND OLIVER E. NELSON

Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706

Manuscript received July 5, 1978
Revised copy received September 25, 1978

ABSTRACT

EMS-induced *sh* mutants and their heterozygotes were examined for the enzyme, sucrose synthetase, which has previously been shown to be coded by the *Sh* locus. Complementing heterozygotes have a wild-type phenotype, but show no hybrid protein band after starch gel electrophoresis. The existence of a heteromeric complex, however, is inferred from the two-fold elevation in sucrose cleavage activity in the complementing heterozygotes as compared to the mutant homozygotes. The observations on complementation described here are unique, as the elevation in the activity of this reversible enzyme is noticed only in one direction (*viz*, sucrose cleavage) of the reaction and not the other (sucrose synthesis).

THE shrunken-1 (*sh-1*) mutation on chromosome *9* in maize, when homozygous, leads to a shrunken or collapsed phenotype of the kernel owing to a reduced starch content in the endosperm. This mutation is highly tissue specific in its expression and no other part of the plant is affected. SCHWARTZ (1960) observed that the *sh* mutation is also associated with a complete loss of a major protein designated as the Sh protein. Evidence was presented to show that this protein is under the control of *Sh* locus. CHOUREY and SCHWARTZ (1971) obtained six new *sh* mutants through the use of ethyl methanesulfonate (EMS); these mutants produced a protein that reacted with an antiserum against the Sh protein (were CRM$^+$) and showed the presence of Sh protein on starch gels after electrophoresis. Electrophoretic mobility alterations in this protein in two of the EMS-induced *sh* mutants support the hypothesis that the *Sh* locus is the structural gene for the Sh protein. The remaining four mutants produce Sh proteins that are not electrophoretically distinguishable from the protein present in *Sh/Sh/Sh* endosperms. When *sh-F* or *sh-C* are crossed with *sh-S*, the F_1 kernels are phenotypically normal. Restoration of the normal phenotype in the hybrids was attributed to interallelic complementation (CHOUREY 1971). The heterozygotes showed two parental protein bands, and no hybrid band was seen after starch-gel electrophoresis.

[1] The research was supported by the College of Agricultural and Life Sciences, University of Wisconsin—Madison and by Grant 15422 from the Public Health Service. This is paper No. 2249 from the Laboratory of Genetics.

[2] Present address: Department of Plant Pathology, USDA/SEA University of Florida, Gainesville, Florida 32611.

Complementation among these *sh* mutants at the phenotypic level was complete, but the extent of the interaction at the enzyme level could not be measured at that time since the enzymic deficiency of the *sh* mutation was not known. Recently we have been successful in showing that the Sh protein is the enzyme, sucrose synthetase, on the basis of the following criteria (CHOUREY and NELSON 1976): (1) Endosperms of five *sh* mutations of spontaneous independent origin were observed to have less than 10% of normal sucrose synthetase activity. More recently analyses (CHOUREY and NELSON 1978) of these five, as well as three more such *sh* mutants, using newly defined optimal assay conditions have shown that the mutants have 5% or less enzyme activity as compared to normal. Evidence was also presented to show that the residual enzyme activity in the mutant endosperms was independent of the *sh* locus. (2) A linear relationship was observed between the number of *Sh* alleles in endosperm tissue and the amount of sucrose synthetase activity, and (3) the purified Sh protein obtained from the normal endosperm possessed sucrose synthetase activity. Biochemical as well as genetic data show that both sucrose synthesis and sucrose cleavage can be catalyzed by the same sucrose synthetase protein in maize (TSAI 1974; CHOUREY and NELSON 1976). The present communication concerns analyses at the enzyme level of some of the EMS-induced *sh* mutants and their hybrids.

MATERIALS AND METHODS

The EMS-induced *sh* mutants used in this study, *sh-C*, *sh-F* and *sh-S*, have been described previously (CHOUREY and SCHWARTZ 1971). The nonmutant (*Sh*) allele assayed is that present in the inbred W22. The reference allele at the locus, *sh-R*, was also placed in the W22 background by repeated backcrossing. Developing kernels of various genotypes were harvested at 22 and 34 days after pollination, frozen on dry ice and stored at —20°. The embryo and pericarp were removed from the kernel prior to homogenization with buffer. A classification of normal and shrunken kernels from segregating 34-day-old ears was done by cutting the frozen kernels into two halves along the embryo axis. The two phenotypes were easily distinguishable as the normal kernels were filled with starch, while the mutants showed a distinct cavity filled with ice. Starch-gel electrophoresis of the endosperm proteins was done by squeezing the juice from the thawed endosperms onto a piece of filter paper to be inserted in the gel. The electrophoretic procedure, staining, and destaining of the gels was done as described by SCHWARTZ (1960). Thawed endosperms were homogenized after mixing equal parts by endosperm and chilled 0.01 M Tris maleate buffer (pH 7.0). This mixture was strained through two layers of cheesecloth and centrifuged at 30,000 × g for 20 min. The supernatant fraction was dialyzed overnight against the extraction buffer. The preparation thus obtained (referred to as a crude preparation) was used as the enzyme source for routine assays.

Enzyme activity was assayed in the direction of sucrose synthesis as well as sucrose cleavage. The reaction mixture for sucrose synthesis contained 60 μmoles of glycine-NaOH buffer (pH 9.0), 2 μmoles $MgSO_4$, 10 μmoles fructose, 2 μmoles uridine diphosphate glucose (UDPG), and 1, 5 or 10 μl of crude enzyme preparation from various genotypes in a total volume of 0.4 ml. The control tubes lacked UDPG. The reaction was carried out at 30° for 15 min, followed by the addition of 0.1 ml of 10 N NaOH. The mixture was boiled in a water bath for 12 min to destroy the unreacted fructose. The sucrose formed was then measured by the method of ROE (1934). The reaction mixture for sucrose cleavage consisted of 64 μmoles MES buffer (pH 6.0), 125 μmoles sucrose, 0.5 μmoles uridine diphosphate (UDP), and 1, 5 or 10 μl of the crude enzyme preparation from various genotypes in a total volume of 0.4 ml. Entries lacking UDP con-

stituted the control. The tubes were incubated in a 30° water bath for 15 min, and the reaction was terminated by adding NELSON's reagent (1944) for the assay of reducing sugars.

The pH optima were measured using the following buffers: Acetate (pH 4.5 and 5.5), Acetate and MES (2-N Morpholino ethane sulfonic acid) (pH 6.0), HEPES (N-2-hydroxyethyl piperazine-N-2 ethane sulfonic acid) and phosphate (pH 7.0) HEPES and Tris-HCl (pH 8.0), glycylglycine and Tris-HCl (pH 9.0) and CHES (cyclohexylaminoethane sulfonic acid) (pH 10.0). Whenever two buffers for a given pH were used, the data from the buffer giving the greater activity have been included in Figures 1A and 1B.

RESULTS

The sucrose synthetase activity in 22-day-old endosperms of various genotypes is shown in Table 1. The enzyme preparation was assayed for both sucrose synthesis and for sucrose cleavage. The mutants *sh-F* and *sh-C* showed approximately the same amount of activity in either direction, as does the *sh-R* mutant. The *sh-S* mutant also had sucrose cleavage activity similar to that of the other mutants. However, this mutant is different from all the other *sh* mutants in the sucrose synthesis reaction. It has 30% as much enzyme activity as normal. Hybrids obtained by intercrossing the EMS-induced mutants were also examined, and the results are shown in Table 1. Maize endosperm is a triploid tissue receiving two genomes from the female and one from the male. The endosperm genotypes in Table 1 and 2 are designated accordingly. The enzyme activity in the direction of sucrose synthesis in heterozygotes seems to depend upon the number of *sh-S* alleles in the endosperm. The endosperm preparation of the *sh-S* mutant shows that enzyme activity exceeds that of either complementing or noncomplementing heterozygotes. The picture, however, is entirely different when the same enzyme preparations are analyzed for sucrose cleavage. An approximate two-fold increase as compared to the parents is observed in the complementing hybrids. The noncomplementing hybrid *sh-F/sh-F/sh-C*, on the other hand, in comparison to the parents shows roughly the same level of enzyme

TABLE 1

Sucrose synthetase activity in 22-day-old endosperms of normal, EMS-induced shrunken mutants and their hybrids

Genotype	Phenotype	Synthesis (pH 9.0)		Cleavage (pH 6.0)	
		Sp. act.*	%	Sp. act.*	%
+/+/+ (W22)	normal	994.0	100	1555.0	100
sh-R/sh-R/sh-R (W22)	mutant	48.5	4.88	78.5	5.05
sh-F/sh-F/sh-F	mutant	63.9	6.43	91.9	5.91
sh-C/sh-C/sh-C	mutant	49.9	5.02	66.6	4.28
sh-S/sh-S/sh-S	mutant	301.0	30.30	73.2	4.71
sh-F/sh-F/sh-C	mutant	74.7	6.92	115.7	7.38
sh-F/sh-F/sh-S	normal	148.9	14.98	158.5	10.19
sh-S/sh-S/sh-F	normal	235.0	23.64	184.0	11.83
sh-C/sh-C/sh-S	normal	135.8	13.66	160.0	10.29
sh-S/sh-S/sh-C	normal	254.0	25.55	155.5	10.80

* Specific activity: nmoles of sucrose or reducing sugar synthesized per mg of protein per min.

TABLE 2

Sucrose synthetase activity in 34-day-old endosperms of specified genotypes

Derivation Females	Derivation Males	Genotype	Phenotype	Synthesis (pH 9.0) Sp. act.*	Synthesis (pH 9.0) %	Cleavage (pH 6.0) Sp. act.*	Cleavage (pH 6.0) %
+/+ (W22) Selfed		+/+/+	normal	1996	100	2160	100
sh/sh (W22) Selfed		*sh-R/sh-R/sh-R*	mutant	78	3.90	73	3.38
		sh-S/sh-S/sh-F	normal	316	15.80	242	11.20
sh-S/F × *sh-F/F*							
		sh-F/sh-F/sh-F	mutant	78	3.90	119	5.50
		sh-F/sh-F/sh-S	normal	209	10.50	181	8.06
sh-S/F × *sh-SS*							
		sh-S/sh-S/sh-S	mutant	668	33.40	103	4.60
		sh-S/sh-S/sh-C	normal	342	17.10	185	8.56
sh-S/C × *sh-CC*							
		sh-C/sh-C/sh-C	mutant	64	3.20	92	4.26
		sh-S/sh-S/sh-C	normal	310	15.53	190	8.80
sh-S/F × *sh-CC*							
		sh-F/sh-F/sh-C	mutant	65	4.45	105	4.68

* Specific activity: nmoles of sucrose or reducing sugar synthesized per mg of protein per min.

activity for the sucrose synthesis, but a slight elevation is noticed for the sucrose cleavage reaction. This increase in the noncomplementing hybrid was critically examined at the 34-day-old stage of development by comparing the complementing and noncomplementing heterozygotes segregating on the same ear (Table 2), thus minimizing any variation due to the genetic background. This was done in the following manner: The plants raised from the *sh-S/sh-F* heterozygote were pollinated by *sh-C* pollen. This led to a 1:1 segregation for plump *sh-S/sh-S/sh-C* and *shrunken sh-F/sh-F/sh-C* endosperms due to complementing and noncomplementing heteroallelic combinations, respectively. It was also possible to compare complementing heterozygous and parental homozygous kernels on the same ear by backcrossing the heterozygote to one of the parental types. The plump and shrunken phenotypes can be readily identified at 34-day stage, but not at 22-day stage of development. The noncomplementing heterozygote does not show the same level of sucrose cleavage activity as the complementing heterozygote (Table 2). The noncomplementing hybrid has similar levels of activity to the parental homozygotes. The complementing hybrids, on the other hand, show a consistent two-fold increase in sucrose cleavage activity as compared to the homozygous mutant endosperms segregating on the same ear. The enzyme activity in the direction of sucrose synthesis at the 34-day stage shows the same pattern as seen at the 22-day stage, *i.e.*, SSS > SSF or SSC > FFS.

Mixtures of crude preparations of various mutants were made and assayed for the enzyme activity (Table 3) to test *in vitro* complementation. Mixtures were made using two parts of crude preparation of one homozygote with one part of another, incubated overnight at 4° and analyzed for enzyme activity.

TABLE 3

Sucrose synthetase activity expressed as % of the normal 22-day-old endosperm in mixtures of crude preparations of sh-S, sh-F *and* sh-C *endosperms (designated below as S, F and C, respectively)*

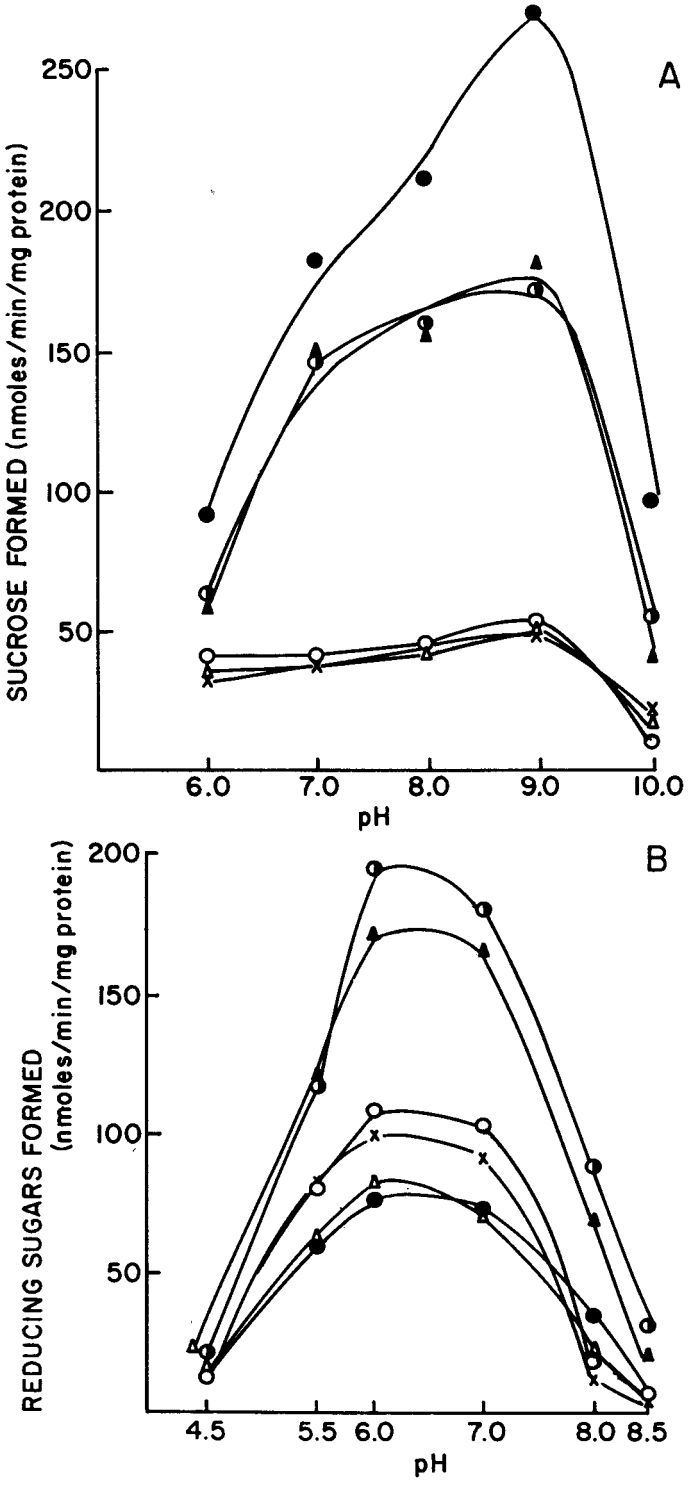
Mixture (v/v)	Sucrose synthesis	Sucrose cleavage
2S:1F	23.10	5.39
2F:1S	15.21	5.22
2S:1C	23.82	5.45
2C:1S	14.80	5.85

The levels of sucrose synthesis activity in the mixtures are similar to the corresponding hybrid (Table 1). However, the analysis of sucrose cleavage activity shows that the mixtures are similar to the donor homozygotes and no elevation in enzyme activity is observed, as in the case of hybrids.

The pH optima were determined using crude preparations of 22-day-old endosperms of various genotypes and are shown in Figure 1A and B. The highest amount of activity for sucrose synthesis and sucrose cleavage reactions was seen at pH 9.0 and pH 6.0, respectively, for all four *sh* mutants and *sh-S/F* and *sh-S/C* hybrids. The normal endosperm preparation also has the same pH optimums for the two reactions (CHOUREY and NELSON 1978).

DISCUSSION

The EMS-induced mutants, *sh-F* and *sh-C*, are similar to the reference allele, *sh-R*, as regards the amount of sucrose synthetase activity in both the forward and backward reactions. No protein has yet been identified as being coded by the *sh-R* allele, and the residual enzyme activity present in all mutants has been attributed to another locus in the genome (CHOUREY and NELSON 1976). The alleles *sh-F*, *sh-C*, and *sh-S*, on the other hand, each specify a protein that is readily identifiable by gel electrophoresis (CHOUREY and SCHWARTZ 1971). The sh-F allele codes for a protein that has a slightly faster electrophoretic mobility than the Sh protein. The protein coded by the *sh-C* allele, however, is electrophoretically indistinguishable from the Sh protein. It seems highly probable that these alleles are coding for enzymatically inactive proteins. The *sh-S* mutant is unique among the *sh* mutants analyzed so far. Though it is indistinguishable in terms of enzyme activity from the rest of the *sh* mutants when examined for sucrose cleavage, it has nearly 30% of the normal endosperm's activity for the sucrose synthesis reaction. This mutant specifies a protein with a slower electrophoretic migration rate than the Sh protein (CHOUREY and SCHWARTZ 1971). It seems clear that the mutational event creating *sh-S* has damaged the ability to catalyze the cleavage reaction more severely than the synthesis reaction. The occurrence of such a qualitative change in the enzyme of a mutant that was initially selected on the basis of its phenotype and allelism to the *sh* locus further substantiates the hypothesis that the *Sh* locus is the structural gene for sucrose synthetase. The biochemical basis of the observed alteration is not known. It is


A
SUCROSE FORMED (nmoles/min/mg protein)
250
200
150
100
50
6.0
7.0
8.0
9.0
10.0
pH
B
REDUCING SUGARS FORMED (nmoles/min/mg protein)
200
150
100
50
4.5
5.5
6.0
7.0
8.0
8.5
pH

possible that the enzyme has different active sites for synthesis and cleavage functions, and a single amino acid alteration in the enzyme has affected one site more than the other. A simpler interpretation would be that the conformation of the enzyme, due to an alteration in its primary structure, is changed. Such an alteration can lead to widely diverse effects.

Complementation at the phenotypic level in certain heteroallelic combinations is complete as the F_1 kernels are indistinguishable from the normal *Sh* kernels (CHOUREY 1971). At the enzyme level in the complementing hybrids, an increase in enzyme activity is observed only when the sucrose cleavage reaction is measured. A two-fold increase in the specific activity of the enzyme is seen in such hybrids as compared to the parental types. This constitutes nearly 10% activity in the heterozygotes as compared to the wild type endosperms at the 22-day stage of development. No such elevation in enzyme activity is seen when crude preparations of the homozygotes are mixed and assayed together. Lack of such an *in vitro* complementation using undenatured enzymes is presumably due to the fact that interallelic complementation involves, in the majority of the cases, interaction of subunits of the polymeric proteins and not the native proteins (*cf.*, FINCHAM 1966). This interaction leading to the formation of a hybrid enzyme is believed to be the molecular basis for the restoration of the normal phenotype in complementing heterozygotes (SCHLEISSINGER and LEVINTHAL 1963; CODDINGTON and FINCHAM 1965; KIDA and CRAWFORD 1974). Interallelic complementation has, however, led to varying amounts of enzyme activity in the heterozygotes. ULLMANN and PERRIN (1970) observed a range of 0.1 to 25% of the wild-type β-galactosidase activity in the complementing merozygotes of *E. coli*. Recently, GELBERT, MCCARRON and CHOVNICK (1976) also observed a range of < 1 to 16% of the wild-type xanthine dehydrogenase activity in the complementing hybrids of rosy mutants in *Drosophila melanogaster*. Speculation on the wide variation and small percent increases in the enzyme activity over mutant activity (leading to a normal phenotype in complementing heterozygotes) observed will have to take the possible labile nature of the hybrid protein into consideration. A greater deviation between the *in vitro* and *in vivo* enzyme levels is more likely in complementing heterozygotes than in the wild type. The lack of the hybrid protein band after gel electrophoresis of the complementing *sh* heterozygote extracts may be indicative of the labile nature of the heteromeric complex and has been discussed in the earlier communication (CHOUREY 1971). Sucrose synthetase in immature normal maize kernels has recently been demonstrated to be a polymeric protein, and the homomeric tetramer form is believed to be the predominant form (SU and PREISS 1978). Preliminary studies (unpublished) done by us have also suggested that maize sucrose synthetase is a tetramer. In view of these observations, the lack of a hybrid protein band is interesting, but not surprising. ULLMANN, JACOB and

FIGURE 1.—Activity-pH relationships of (A) sucrose synthesis (UDPG + fructose and (B) sucrose cleavage (UDP + sucrose) reactions catalyzed by endosperm preparations. (x) = *sh*-W22, (○) = *sh-FF*, (●) = *sh-SS*, (△) = *sh-CC*, (◒) = *sh-S/F*, and (▲) = *sh-S/C*.

MONOD (1968) observed that the enzyme produced due to a complementary interaction differs from the wild-type enzyme in tightness and stability of its tertiary folding. EDWARDS, HOPKINSON and HARRIS (1978) have recently investigated the factors that can lead to dissociation of the hybrid isozymes on electrophoresis. They demonstrated the dissociation of the heteromeric form of superoxide dismutase with increasing temperature during electrophoresis, showing that temperature has a critical influence on the appearance of the hybrid enzyme after electrophoresis.

It is interesting that the increases in the enzyme activity with complementation is observed only for the sucrose cleavage reaction and not for sucrose synthesis. The enzyme activity for the latter function shows noncomplementation, and the activity levels in the heterozygotes show a dependence on the number of copies of the S allele present. Similar levels of activity are also obtained when crude preparations of the homozygotes are mixed in the corresponding ratios.

No biochemical information concerning the heteromeric complex is available at the present time to support speculation as to how this differential effect in enzyme activity in the complementing heterozygote is attained. We have shown previously (CHOUREY and NELSON 1976) that the starch deficiency of the *sh* mutant is due to the loss of the *Sh* locus-coded sucrose synthetase activity. These observations on enzyme activity in complementing heterozygotes substantiate the hypothesis of several investigators who have suggested that *in vivo* sucrose synthetase catalyzes the first step in the transformation of sucrose to starch (see CHOUREY and NELSON 1976). The phenotypically normal kernels of the complementing heterozygotes appear to form as much starch as do nonmutant kernels. The restoration of normal phenotype in complementing heterozygotes appears to be correlated with enhanced activity for sucrose cleavage, and we interpret this observation as genetic evidence substantiating the hypothesis that the *in vivo* role of sucrose synthetase in storage tissues is the cleavage of sucrose.

LITERATURE CITED

CHOUREY, P. S., 1971 Interallelic complementation at the Sh_1 locus in maize. Genetics **68:** 434–442.

CHOUREY, P. S. and O. E. NELSON, 1976 The enzymatic deficiency conditioned by the *shrunken-1* mutations in maize. Biochem. Genetics **14:** 1041–1055. ——, 1978 Studies on sucrose synthetase in the developing endosperms and embryos of normal and shrunken genotypes. Maize Genet. Coop. Newsletter **52:** 125–127.

CHOUREY, P. S. and D. SCHWARTZ, 1971 Ethyl methanesulfonate induced mutations of the Sh1 protein in maize. Mutation Res. **12:** 151–157.

CODDINGTON, A. and J. R. S. FINCHAM, 1965 Proof of hybrid protein formation in a case of interallelic complementation in *Neurospora crassa*. J. Mol. Biol. **12:** 152–161.

EDWARDS, Y. H., D. A. HOPKINSON and H. HARRIS, 1978 Dissociation of 'hybrid' isozymes on electrophoresis. Nature **271:** 84–87.

FINCHAM, J. R. S., 1966 *Genetic Complementation.* W. A. Benjamin Inc., New York.

GELBART, W., M. MCCARRON and A. CHOVNICK, 1976 Extension of the limits of the XDH structural element in *Drosophila melanogaster*. Genetics **84:** 211–232.

KIDA, S. and I. P. CRAWFORD, 1974 Complementation *in vitro* between mutationally altered B_2 subunits of *Escherichia coli* tryptophan synthetase. J. Bacteriol. **118:** 551–559.

NELSON, N., 1944 A photometric adaptation of the Lomogyi method for the determination of glucose. J. Biol. Chem. **153:** 375–380.

ROE, J. H., 1934 A colorimetric method for the determination of fructose in blood and urine. J. Biol. Chem. **107:** 15–22.

SCHLESINGER, M. J. and C. LEVINTHAL, 1963 Hybrid protein formation of *Escherichia coli* alkaline phosphatase leading to *in vitro* complementation. J. Mol. Biol. **7:** 1–12.

SCHWARTZ, D., 1960 Electrophoretic and immunochemical studies with endosperm proteins of maize mutants. Genetics **45:** 1419–1427.

SU, J. and J. PREISS, 1978 Purification and properties of sucrose synthetase from maize kernels. Plant Physiol. **61:** 389–393.

TSAI, C. Y., 1974 Sucrose-UDP glucosyltransferase in *Zea mays* endosperm. Phytochem. **13:** 885–891.

ULLMAN, A. and D. PERRIN, 1970 Complementation in β-galactosidase. pp. 143–172. In: *The Lactose Operon.* Edited by J. R. BECKWITH and D. ZIPSER. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

ULLMANN, A., F. JACOB and J. MONOD, 1968 On the subunit structure of wild type versus complemented β-galactosidase of *Escherichia coli.* J. Mol. Biol. **32:** 1–13.

Corresponding editor: R. PHILLIPS