PROTEIN GRANULES OF MAIZE ENDOSPERM CELLS[1]

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ABSTRACT

Cytoplasmic inclusions composed primarily of protein are found in the maize endosperm. They are roughly spherical, or soap-bubble shaped, numerous, and range in size from near the limit of resolution in the light microscope up to about 3 microns in diameter. They are largest and most numerous in the subaleurone cells, progressively decreasing in size from the outer to the inner cells of the endosperm. Their development, as followed under the light microscope, begins at 15–20 days after pollination in the region under the silk scar and spreads to the other portions of the endosperm. In a given region, enlargement of protein granules starts first in inner cells and then in cells progressively nearer the aleurone.

Histochemical tests, plus the positive correlation of period of maximum rate of growth of the protein granules with the reported period of maximum rate of increase in percent of zein during development of the endosperm and the positive correlation of the areas of greatest size of the granules with the reported areas of greatest zein concentration, suggest that protein granules are the major site of zein storage in the maize endosperm.

The growth and development in maize endosperm cells of a class of cytoplasmic inclusions termed "protein granules" has been described briefly in a previous publication (3). The present paper amplifies the descriptions given there and also relates the data to facts from other sources which deal with the protein components of the maize endosperm.

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Materials and Methods

Detailed studies were made on two distinctively different open-pollinated varieties of maize: Longfellow New England Flint and Gourdseed Southern Dent. These two varieties are representative of the two types of corn which have been proposed as the ancestors of modern Corn Belt Dents (1). The kernels of Longfellow are nondented and have a layer of horny endosperm which completely surrounds the sides and crown of the kernel. The kernels of Gourdseed are long and deeply dented, so much so, in fact, that the crown of the kernel often is completely collapsed. Gourdseed kernels have little or no horny endosperm and are composed almost entirely of floury endosperm. The small amounts of horny endosperm that occasionally do occur are located on the periphery of the side and rear (abgerminal side) of the kernel, somewhat below the midsection. Some observations also were made on several other varieties of corn, most of which were Corn Belt inbred lines or Corn Belt hybrids.

Most of the material examined was grown at the Arboretum of the Missouri Botanical Garden at Gray Summit, Missouri, in 1949 and 1950. All kernels examined were taken from hand-pollinated ears, so that the exact time from pollination to harvest was known. At intervals from pollination to kernel maturity, kernels from freshly picked ears were fixed in Craf or Zirkle-Erliki fixative (8). Hand-cut sections of fresh kernels also were examined with the aid of dissection and compound microscopes. The fixed specimens were later embedded in paraffin and sectioned. It was necessary to soak the more mature specimens in water before sectioning. The sections were stained with Heidenhain's hematoxylin (8) or were treated in other ways, as described below. Some mature unfixed kernels were soaked in water for 24 hours and then sectioned with a freezing-microtome.

Various histochemical tests were made on fixed and unfixed kernel sections, including: 1) Millon reaction for tyrosine, Serra histochemical modification (14); 2) Millon reaction, Bensley histochemical modification (2); 3) xanthoproteic test, histochemical modification (14); 4) arginine reaction, Thomas's modification (15) of the Sakaguchi reaction; 5) tryptophan test, Romieu reaction (4); 6) tryptophan test, Voisinet-Fürth reaction (14); 7) iodine in potassium iodide (I/KI); and 8) Sudan III for lipids, Jackson procedure (4).

Micro-Kjeldahl determinations of protein nitrogen were performed on various endosperm regions of a few mature kernels. The method used was essentially that described by Peters and Van Slyke (13): the Arnold-Gunning digestion method with peroxide and titration of the distilled ammonia with boric acid.

Results

In all varieties of corn examined, inspection of the sections stained with Heidenhain's hematoxylin revealed that within a week after the first appearance of starch granules in the endosperm a second class of granules (called protein granules, for reasons given below) began to enlarge. These probably corresponded to the protein granules described in mature endosperms by Mottier (11) and Harz (6). Although exact counts could not be made, the granules did not seem to increase in number in any given part of the endosperm after their first appearance there, but their enlargement made them increasingly conspicuous in any given region, with the passage of time. The protein granules were at all times easily distinguished from starch grains. In mature endosperms stained with hematoxylin, a section through a horny endosperm cell had somewhat the appearance of a section through a box of white marbles (starch grains) in which buckshot (protein granules) has been used as packing between the marbles; the whole boxful is then filled with a transparent glue (clear viscous cytoplasm) which surrounds marbles and buckshot and makes the ensemble, when dry, a rigid conglomerate.[3]

By measuring the diameter of the protein granules in equivalent cells in successively older endosperms gathered at intervals of 5 to 10 days, one may determine growth curves for the protein granules of various regions of the endosperm. Figures 1 to 4 show such curves, smoothed in from scatter plots of several measurements, for Longfellow and for Gourdseed, 1950 collections. The curves show the protein granule growth in three regions of the endosperm, in two cells of each

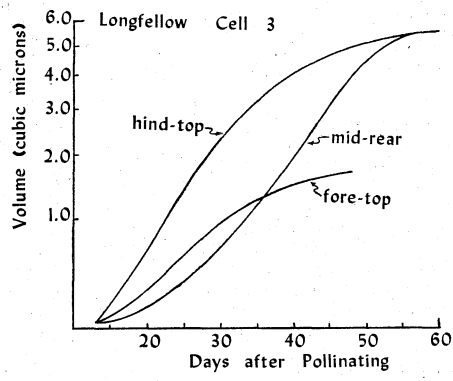

Fig. 1. Increase in volume of protein granules in cell 3 in three regions of Longfellow. Volume calculated on assumption that granules are spherical. See text for further explanation.

[3] Shown in illustrations in Duvick (3).

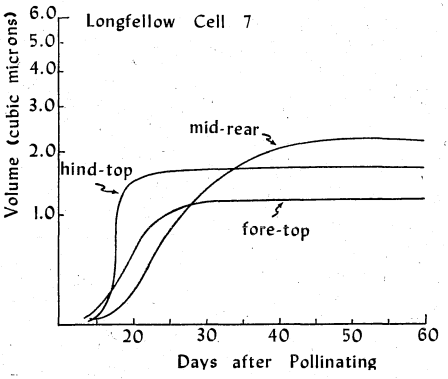

Fig. 2. Increase in volume of protein granules in cell 7 in three regions of Longfellow corn.

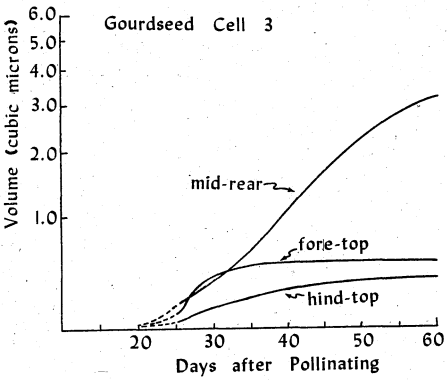

Fig. 3. Increase in volume of protein granules in cell 3 in three regions of Gourdseed corn.

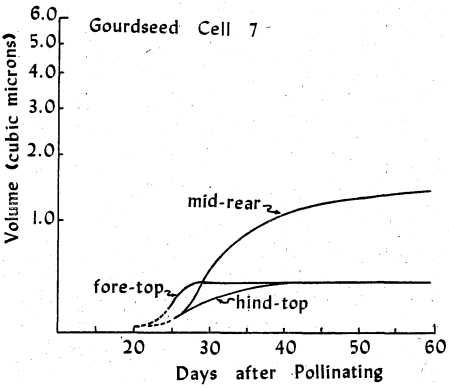

Fig. 4. Increase in volume of protein granules in cell 7 in three regions of Gourdseed corn.

TABLE I

MEAN DIAMETER OF PROTEIN GRANULES IN THREE ENDOSPERM REGIONS OF TWO VARIETIES OF MATURE CORN, 1950 CROP

VARIETY	REGION	CELL NUMBER			
		3	7	11	15
		μ	μ	μ	μ
Longfellow	Fore-top	1.6	1.6	1.3	1.0
	Hind-top	3.1	1.7	1.5	0.8
	Mid-rear	2.8	2.2	1.7	0.5
Gourdseed	Fore-top	1.0	0.7	0.4	..[a]
	Hind-top	0.8	0.7	..	..
	Mid-rear	1.9	1.7	1.4	1.1

[a] No cell.

region. The regions are: a) "fore-top," on the crown just forward of the silk scar; b) "hind-top," on the crown well to the rear of the silk scar; c) "mid-rear," in the midsection of the kernel, opposite the germ. The cells are the third and the seventh, counting in from the aleurone. Counts were not begun until the peripheral cell divisions in each region had ceased; so it is reasonably certain that equivalent cells were observed.

The curves illustrate the following general patterns, noted in all materials examined:

1. Rapid growth of protein granules begins first in cells of the crown region and progressively moves down to cells of the basal portion of the endosperm.

2. In a given endosperm region, rapid growth of protein granules begins earlier and ends earlier in inner cells.

3. The final size of protein granules in the outer cells is greater than that of protein granules in inner cells. The average final size of the protein granules is progressively smaller, going from outer to inner cells of the endosperm, until in the center, somewhere in the floury endosperm region, protein granules, as such, cannot be identified. The progressive decrease in size of protein granules, going from peripheral to central cells in two different mature endosperms, is illustrated in detail in Table I. Note, by way of contrast, that starch grains in the endosperm are progressively larger, going from outer to inner cells, until about the 15th cell after which they, too, are progressively smaller (3).

4. An exception to the size progressions described for both protein and starch granules is found in the crown region of dent corns. The curves for Gourdseed, Figs. 3 and 4, illustrate an extreme example of this in the protein granules.

The final sizes of the protein granules are insignificant in the two crown regions of Gourdseed, as compared to the midsection of the same variety or to any of the three regions of Longfellow. In Gourdseed the crown region of the kernel dents; that is, the endosperm cells in the crown remain large and vacuolate until the kernel dries down at maturity, at which time they collapse. These "permanently" vacuolate cells have little or no development of either starch or protein granules. About the usual numbers of granules start development, but their growth stops abruptly at an early stage. This phenomenon is found in any dent corn; the deeper the dent, the greater the region of "permanently" vacuolate cells in the crown.

Longfellow, a nondent, illustrates the other extreme of development of the crown region. Here, all regions of the endosperm, including the crown, are composed of horny endosperm on the periphery and floury endosperm in the inner portions. Starch and protein granules of the crown as well as of the midsection are well developed. However, even in Longfellow, as well as in Gourdseed and all other varieties examined, cells of the extreme center portion of the endosperm remain vacuolate and collapse at maturity in the same fashion as do the crown region cells of the dents. Kernels which dent are merely those in which the center region of vacuolate cells extends up to, or nearly up to, the crown.

One further modification of the region of vacuolate cells should be noted: the centermost vacuolate cells usually rupture, leaving a fluid-filled hollow during the period of maximum endosperm expansion. The regions with ruptured cells, of course, also shrink at the time the kernel dries down.

Measurements of protein granules of the 1949 samples of Longfellow and Gourdseed produced a series of curves like those shown for 1950, except that the sampling period was shorter and the curves could not be extended as far as they were in 1950.

Proofs for the proteinaceous nature of the protein granules are as follows:

1. The granules (a) gave a positive test for tyrosine with the Millon-Serra reagent, either in freezing microtome sections or after Craf-fixation and paraffin-embedding; (b) stained yellow with I/KI; (c) gave a negative reaction to the Sudan III test for lipids (fresh, freezing-microtome sections); and (d) were not birefringent in polarized light, even when starch grains nearly as small as they exhibited a clearly defined cross (3). The "clear" viscous cytoplasm, incidentally, gave a positive test with Millon and stained yellow with I/KI. It presumably is, therefore, proteinaceous.

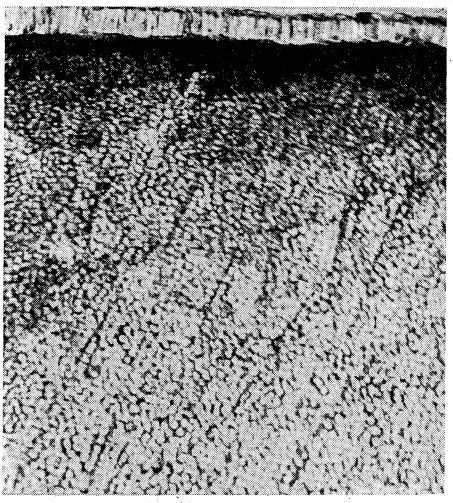

Fig. 5. Portion of crown in section of mature Longfellow endosperm, stained with Millon, to show increasing depth of color in cells in progressing from center of endosperm (bottom of picture) to aleurone layer (top of picture). White spheres are starch grains. Unfixed, freezing-microtome section. 70×.

2. When whole endosperm sections were examined with the unaided eye or under the dissecting microscope, the regions which were stained most deeply by the Millon-Serra reaction (Fig. 5), by the Bensley modification of the Millon, by I/KI (yellow), and by the xanthoproteic reaction corresponded closely to those regions in which the protein granules were known to be largest and appeared to be most numerous.

3. Micro-Kjeldahl nitrogen determinations of various endosperm regions of two mature kernels of Longfellow, shown in the table below, indicated that the regions which had the largest sizes and apparent numbers of protein granules (the subaleurone regions) also

Region	*Cell No.*	*Protein, at 14% Moisture*[a]
		%
Aleurone	1	36
Outer horny endosperm	2–7	22
Inner horny endosperm	8–14	9

[a] Mean of two determinations on each of two kernels.

had the largest percentages of protein. This minuscule experiment has been confirmed on a larger scale by Hinton (7), who has shown in another variety of flint corn that the protein percentage of the horny endosperm is greater in the subaleurone cells than it is in those horny endosperm cells nearer the center of the endosperm.

There is some evidence that the protein granules may be composed largely, or only, of the complex of proteins presently designated as zein. Evidence for this statement is given in the following numbered paragraphs.

1. The protein granules give a negative test for arginine, although aleurone cells give a strong positive test and the clear cytoplasm of the endosperm cells also gives a definite, although not a strong, test for arginine. Zein contains little arginine as compared to the glutelins which, with zein, comprise the major storage proteins of the endosperm.

2. When freezing-microtome sections of the horny endosperm region of kernels of various types of corn were shaken in 0.2% sodium hydroxide for 30 minutes or left to stand for as little as 4 hours, the starch grains and protein granules of most cells fell from the clear viscous cytoplasm in which they had been encased, and the clear cytoplasm itself largely disintegrated. In an early stage, the protein granules were thickly clustered over the surface of the starch grains. When equivalent sections were shaken for 30 minutes or left standing up to 120 hours in 70% ethyl alcohol, no change could be seen at all, either before or after staining with iodine or with Millon reagent. When sections were kept up to 120 hours in a 1:3 mixture of 0.2% sodium hydroxide and 70% ethanol, both clear cytoplasm and protein granules disappeared from most cells. This treatment was not greatly different in apparent effect, however, from that with alkali alone. A 5% sodium chloride solution had no visible effect on either clear cytoplasm or protein granules after as long as 120 hours. These differential solubility reactions are in agreement with the supposition that most of the clear cytoplasm is of the alkali-soluble glutelin type of protein and that, because it surrounds the alcohol-soluble protein granules, it protects them from dissolution by alcohol solution. In routine zein extraction alcohol may be used as the first solvent, but the grain is first ground and the grinding must reveal surfaces that are protected in the intact cell.

In a series of experiments to determine the effects of (a) sequence of treatments and (b) pretreatments, Nagy *et al.* (12) found that, when ground grain was extracted directly with ethanol, it was necessary to add sodium acetate to get maximum extraction of zein. Maximum amounts of zein could also be extracted, however, when the grain was first treated with weak alkali and then extracted with ethanol, without sodium acetate; but, if the grain in alkali was neutralized and then treated with 5% salt, which precipitated both glutelins and zein, the zein then could be maximally extracted only by again first adding weak alkali. All of these results agree with the supposition that the

zein may be in the protein granules, surrounded by clear cytoplasm made up largely of alkali-soluble proteins. Any treatment which first peptizes the clear cytoplasm will then expose a maximum number of the granules to the action of ethanol. Nagy *et al.* (12), in stating that "a part of the zein may be adsorbed on the glutenin [sic] (or on other material) as it is synthesized by the plant," presumably envisaged a more intimate union between the two classes of proteins than is supposed in the hypothesis presently advanced.

3. The time of maximum increase in size for most of the protein granules seems to coincide with the time of greatest increase in amount of zein in the endosperm. Watson (16) and Zeleny (18) have stated that zein deposition in the endosperm takes place largely in the latter portion of the endosperm maturation period. Watson's data show that the highest rate of zein deposition occurs during approximately 14 to 35 days after pollination and continues at an appreciable rate until complete grain maturation. Figures 1–4 show that maximum rates of increase in the size of protein granules in all of the endosperm regions of Longfellow began no sooner than about 2 to 3 weeks after pollination and that for Gourdseed (a much later-maturing variety of corn) maximum rates were not reached before about 4 weeks after pollination. The area of major deposition of protein granules in Gourdseed includes that represented by cells 3 and 7 of the mid-rear region, and here it is apparent that much of the size increase occurred as late as 50 days after pollination. Recent data given by Mertz (10) indicate, however, that the zein percentage of endosperm protein does not increase with time; so it may be that a re-examination of zein-accumulation data is needed.

4. The data of Hamilton *et al.* (5) indicate that the percentage of zein in horny endosperm is nearly twice that for zein in floury endosperm. As noted above, the size and apparent numbers of protein granules are progressively greater, from inner cells, in the floury endosperm, to outer cells, in the horny endosperm. The presence of identifiable protein granules apparently does not, *per se,* determine whether endosperm is horny or floury; rather, there seems to be a threshold point at which the proportion of viscous protein (clear cytoplasm) to granular inclusions (starch grains and protein granules) becomes such that the viscous protein shatters during the time the kernel is drying down. Cells in immature endosperms have a relatively high percentage of water, and the viscous protein in all cells is plastic enough and plentiful enough to encase all granules. As the endosperm dries down during the maturation process, the viscous protein loses volume and elasticity because of water loss; it shrinks and sometimes

TABLE II

MEAN DIAMETER OF PROTEIN GRANULES IN ENDOSPERM OF TWO VARIETIES OF CORN AT TWO LEVELS OF PROTEIN CONTENT

	G-94		Illinois High Protein	
	7.5% Protein	10.7% Protein	13.3% Protein	20.6% Protein
	μ	μ	μ	μ
Cell 3	0.9	1.2*	1.3	1.8**
Cell 7	0.5	1.0**	1.0	1.6*

becomes insufficient in volume to encase all granules completely; in such cases, it ruptures. This process can be followed under the microscope. The cells in which the viscous protein has been thus shattered become the floury endosperm cells; they are filled with refractive air spaces that appear white (6). As a general rule, these cells have rounded, loosely packed starch grains and small protein granules which are insufficient to pack the large interstices between starch grains. In subaleurone cells, starch grains are rounded, but interstices are filled with tightly packed protein granules, and the proportion of viscous cytoplasm to granules seems to be somewhat higher. In the horny endosperm cells farther from the periphery, the starch grains are compressed so much that the interstices are relatively small.

5. In samples of seed of varying protein percentage,[4] kernels of the hybrid G-94, grown with ample amounts of nitrogen fertilizer and with 10.7% protein in the whole grain, have larger protein granules in the mid-rear section of the horny endosperm than do kernels of the same hybrid grown without nitrogen fertilizer and which have only 7.5% protein. Kernels of Illinois High Protein, grown with ample nitrogen fertilizer and having 20.6% protein, had larger protein granules in the mid-rear section of the horny endosperm than did kernels of Illinois High Protein grown without nitrogen fertilizer and which had 13.3% protein (Table II). Numerous investigations have shown that when protein percentage is increased as a result of nitrogen fertilization, the principal increase is in the zein fraction (5).

6. The Voisinet-Fürth test for tryptophan, when used on sectioned kernels, did not show a gradation in depth of color which precisely correlated with the change in size of the protein granules, as did the Millon and xanthoproteic reaction, both of which are specific for tyrosine in proteins. Rather, the tryptophan test produced a pink color evenly over the whole endosperm except for a slight intensification of color in the outermost two or three cells. In these outer cells, it appears that the proportion of clear cytoplasm to granular inclusions is higher

[4] Kindly supplied by E. B. Earley.

than in any other part of the endosperm. The Romieu reaction for tryptophan, although used only on split rather than sectioned kernels, gave the same pattern of coloration as did the Voisinet-Fürth test on sectioned kernels. Tyrosine percentage is about the same in zein as in the glutelins; whereas it is a fact of long-standing nutritional knowledge that zein has almost no tryptophan (0.1%) but that glutelins have, by contrast, much more (about 1%) (9). Therefore, the even coloration over the endosperm indicates that the granules did not contain glutelins in proportion to their size and apparent numbers. The reactions of the tryptophan tests were too weak to allow identification of color in any specific cytoplasmic inclusions, at the high magnifications necessary for such inspection.

Discussion

Although the data given in this paper do not prove beyond all reasonable doubt the exact locations of the intracellular sites of deposition of zein and of glutelins, the information is sufficient to make further investigation seem worth while.

Implications of the hypothesis, both to those interested in methods of separating maize endosperm fractions and to breeders interested in changing the proportions and amounts of endosperm proteins, are obvious. It seems probable that study of endosperm cells of the other cereal grains will show similar cytoplasmic structures and similar relationships between inclusions and protein types. Watson *et al.* (17), for instance, have described protein granules in sorghum endosperm and have shown in photographs the similarity between these structures in corn and sorghum.

It may be pertinent to note that the earlier literature (e.g. Mottier, 11) has abundant descriptions of protein granules in various plant seeds (including maize), usually in endosperms of monocotyledonous plants and in cotyledons of dicotyledonous plants. It seems reasonable to suppose that such specialized storage organelles would each store a fairly distinct type or group of related types of protein. Thus, even though zein as extracted according to the old method of differential solubilities may not be a single molecule, a family of zeins might, nevertheless, be associated in the same cytoplasmic organelle.

It might be speculated further that in maize endosperm, the protein granules, storing mostly zein, are the variable which can most easily change to store more or less protein, depending on the genotype and the environment. The viscous cytoplasm, by comparison, probably forming its major storage proteins prior to zein accumulation in any given cell, may be a relatively stable component, quantitatively, as

influenced by either environment or heredity. It should be emphasized that these statements are, at present, conjectural.

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