Distribution and Subcellular Structure of Endosperm Protein in Varieties of Ordinary and High-Lysine Maize[1]

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ABSTRACT

All varieties of corn show a high protein content in the peripheral endosperm beneath the aleurone layer, and a decreasing gradient of protein concentration from the periphery inward. In addition to the common starchy types of endosperm cell, small amounts of two kinds of high-protein cells containing little or no starch were noted: (a) high-protein cells scattered at random among starchy cells are common in floury mutants, *floury-1* and *opaque-1;* (b) special high-protein cells occur only in the subaleurone area, usually as a fragmentary layer.

Subcellular protein bodies soluble in ethanol, dimethyl sulfoxide, and acetone are characteristic of the protein structure in endosperm of all normal maize. The bodies, ranging from 1.4 to 1.8 micron in average diameter in normal corn, were absent at the light microscope level only in the high-lysine mutants, *floury-2* and *opaque-2;* small protein granules, about 0.1 micron in diameter, were found by electron microscopy in *opaque-2* but not in *floury-2*. Demonstration of subcellular, alcohol-soluble protein bodies in endosperm cells by light microscopy provides a reliable indication that the protein composition of the variety is normal. Conversely, inability to detect alcohol-soluble protein granules indicates that the protein present is high in lysine.

Microscopic examination of normal corn endosperm shows that storage protein is deposited within the cells as two distinct components, globular bodies and an amorphous matrix in which the bodies lie embedded.

In a study of protein degradation in corn wet-milling, Cox et al. (1)

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reported a globular component of endosperm protein which was resistant to dilute sulfurous acid. They did not identify the body specifically with zein.

On the basis of certain observations, Duvick (2) suggested that the bodies were the major site of zein storage in maize. Wolf et al. (3) showed that the bodies were alcohol-soluble, providing further evidence that they were a site of zein deposition. They also reported that the high-lysine mutants, *opaque-2* and *floury-2,* were deficient in such bodies. Since zein contains little or no lysine in its amino acid make-up whereas other endosperm protein fractions have normal amounts of lysine, their observation provided structural evidence for the altered amino acid composition of storage proteins originally reported in the maize mutants by Mertz et al. (4).

All these observations emphasize that subcellular endosperm protein is a more complex structure than was originally suspected. They show that endosperm protein may vary in composition not only in different parts of the endosperm, but also in different types of corn. Detailed study is needed of the subcellular structure of storage protein in different corn varieties and mutants as a basis for improvement of grain processing and to anticipate special problems which may arise in processing new types of maize.

Light and electron microscopy were used to explore the distribution, structure, and solubility of storage protein in ordinary maize endosperm. In addition, structure of endosperm protein and distribution of alcohol-soluble protein were compared in a series of corn varieties including those with normal protein as well as high-lysine mutations.

MATERIALS AND METHODS

Corn Varieties

A commercial hybrid, Holmes Hybrid, and an inbred line, W64A, were examined as samples of normal maize. W64A is in addition a source for *opaque-2* mutant; consequently, a valid comparison can be made between the normal and *opaque-2* protein characteristics. In the floury group, Mandan and White Cuzco were studied. Mutants of similar phenotype with respect to kernel opacity included *opaque-1* (o_1), *opaque-2* (o_2), *opaque-4* (o_4), *floury-1* (fl_1), *floury-2* (fl_2), and soft starch (h_1). A popcorn, "Maiz Reventador" of Mexican origin, was included as a vitreous kernel type. Waxy corn, Illinois high-protein corn, and an amylomaize were also examined.

Specimens for Light Microscopy

Fixed or unfixed corn tissues were quick-frozen and cut at 10 to 15 microns in a cryostat at –15°C. Sections less than 10 microns thick were cut from dry corn with a glass knife at room temperature. All sections were mounted on gelatin-coated slides.

Starch granules were removed enzymatically to obtain a clear view of the interstitial endosperm protein. The starch was first gelatinized by immersion of sections for a few sec. in hot water and then digestion with alpha-amylase. A commercial fungal amylase or a salivary alpha-amylase (5) was used. Digestion was carried out in a water bath at 35° to 40°C. for 30 min. or longer.

Protein in destarched sections was stained with I_2-KI solution for visual observation and for photomicrography.

Measurements of Mean Protein Body Diameter

Approximately median longitudinal sections prepared as described above were used to measure protein granules. Protein granules from the back of the kernel at the level of the coleoptile were taken as typical of granule population. Twenty protein bodies each were measured in the second and fifth endosperm cells, counting inward from the aleurone layer in each of five kernels of a given variety. Measurements were made with a calibrated filar micrometer. With the number of measurements made, the mean protein granule diameter was measured with a precision of $\pm 5\%$. Protein body sizes in the high-lysine mutant *opaque-2* were estimated from electron micrographs.

Specimens for Electron Microscopy

Fragments of endosperm protein network isolated from destarched sections, prepared as described under "Specimens for Light Microscopy" were mounted directly on Formvar-coated copper grids and examined with an RCA EMU3F electron microscope at 50 kv.

Histochemical Tests

Protein distribution was determined in destarched sections histochemically by oxidative deamination with ninhydrin or chloramine-T, followed by treatment with Schiff's reagent (6). Control sections were treated with acetylation- and deamination-blocking agents.

Sites of alcohol-soluble protein were determined by treating destarched, unfixed sections with 70 to 80% ethanol for approximately 1 hr. at about 70°C. or for longer periods at room temperature. Other solvents included acetone (50% aqueous), 90% dimethyl sulfoxide (DMSO), and 0.1N sodium hydroxide.

Susceptibility of interstitial protein to proteolytic digestion was demonstrated by treating sections with Pronase in 0.05M Tris buffer at pH 8.0 at 35° to 40°C.

Determination of Endosperm Protein

Corn was dehulled and degermed by hand. The endosperm was ground to 40-mesh in a micro-Wiley mill. Protein nitrogen in the endosperm and in extracts of the endosperm were determined by micro-Kjeldahl analysis (7). Alcohol-soluble protein nitrogen was determined on ground endosperm previously extracted at room temperature with petroleum ether followed by 0.5M sodium chloride. A 1-g. aliquot of this material was extracted with 25 ml. of 70% ethanol (v./v.) by continuous shaking overnight at room temperature. The extract was filtered and made up to 100 ml. with 70% ethanol. A 20-ml. aliquot of this solution was analyzed for alcohol-soluble protein nitrogen.

RESULTS

Protein Distribution in Endosperm

Photomicrographs of destarched corn sections are shown in Fig. 1 to give a visual comparison of various types of corns. After treatment with

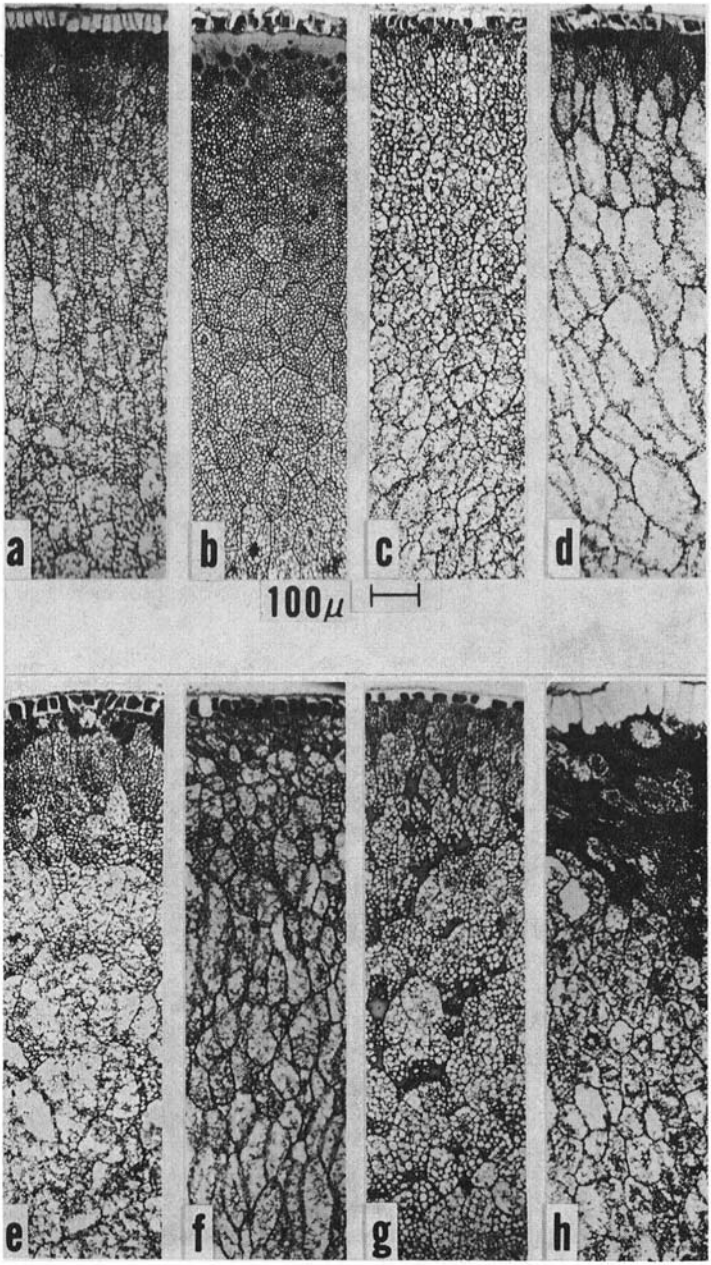

Fig. 1. Comparative endosperm structure in eight different varieties of maize, showing change in cell type and protein concentration from the aleurone layer (at top of strip) inward. All sections 7 microns thick except d, 10 microns. Sections destarched with alpha-amylase. a, Holmes Hybrid; b, Illinois High-Protein; c, soft starch, h_1; d, White Cuzco; e, opaque-1; f, opaque-2; g, floury-1; h, floury-2.

alpha-amylase to remove starch, only storage protein in the form of a fine network remains enclosed within cell walls. From these photomicrographs and microscopic observations on untreated sections, changes in cell size, protein concentration, starch granule size, and compaction of cell contents may be observed visually within a given variety and also between varieties. The photomicrographs of Fig. 1 should be studied in conjunction with the corresponding protein analyses of the samples in Table I. Protein content of the endosperm ranges between 4.3% in White Cuzco to 29.6% in Illinois High-Protein corn. Holmes Hybrid, an ordinary yellow dent corn, has 8.4% protein.

Analytical data on two varieties of maize (2, 8) and on commercial dent corn (1) show that the outer endosperm has a higher protein concentration than the inner endosperm. A pattern of protein distribution similar to that found by analysis is evident in the photomicrographs (Fig. 1). All the maize varieties illustrated, including floury types, show a dark-staining outer endosperm indicative of a relatively high protein content, and a decreasing gradient of stain density toward the center of the endosperm where the protein content is the lowest. Other general trends apparent from the photomicrographs are the increase in size of both cells and starch granules from the aleurone layer inward to the center of the kernel.

High-protein corn endosperm is unique in that it has mostly horny cells densely packed with protein (Fig. 1, b). In addition, this corn has a layer one to two cells thick adjacent to the aleurone, which contains mostly protein.

At the other extreme, flour corn may have only two or three cell layers of horny cells beneath the aleurone layer (Fig. 1, c to h); all other cells are of the floury type. In the floury mutant h_1 (Fig. 1, c), protein distribution is uniform.

TABLE I. MEAN ZEIN BODY DIAMETER AND TOTAL PROTEIN AND ALCOHOL-SOLUBLE PROTEIN IN DIFFERENT VARIETIES OF CORN

Corn Variety	Zein Bodies, Average Diameter μ	Protein[a] %	Protein[b], Alcohol-Soluble %
Holmes Hybrid	1.79	8.5	25.3
Illinois High-Protein	normal	29.6	..
Soft starch (h_1)	1.58	12.9	48.9
White Cuzco	1.50	4.3	40.2
opaque-1 (o_1)	1.39	13.6	54.1
opaque-2 (o_2)	0.1[c]	11.4	13.5
floury-1 (fl_1)	1.43	14.4	61.5
floury-2 (fl_2)	none	14.7	20.0
opaque-4 (o_4)	1.41	14.3	..
floury-2/opaque-2	<0.9[d]	13.5	25.2
Mandan	1.53	..	..
W64A	1.52	15.5	28.6
W64Ao_2	0.1[c]	13.8	15.6
Waxy	1.57	15.1	..
Pop	1.54	..	..
Amylomaize	normal	18.5	38.3

[a] Percent of endosperm; dry basis.
[b] Percent of endosperm protein; dry basis.
[c] Measurements made from electron micrographs.
[d] Protein bodies found only in some kernels of sample.

An extreme case of floury character is illustrated by White Cuzco corn (Fig. 1, d). Cells only a short distance inside the aleurone layer have a poorly developed protein network and contain little protein. Cuzco has the lowest protein content (4.3% of the endosperm, Table I) of any variety studied. Despite the open structure of flour corn endosperm, the protein content is high (exclusive of White Cuzco) relative to ordinary corn, possibly owing to a low endosperm starch content.

A variation of the usual protein distribution in flour corns is common in the mutations, *floury-1* and *opaque-1*. In endosperm of these corns there is frequently a scattering of individual high-protein cells among the characteristic floury cells of low protein content (Fig. 1, g). These high-protein cells randomly dispersed in the endosperm may account for the high protein content of *floury-1* and *opaque-1* (Table I).

Subcellular Protein

At the subcellular level, endosperm protein fills the space between starch granules and is clearly observed in destarched sections, where it appears as a two-component system made up of globular bodies embedded in an amorphous matrix (Fig. 2, a and b).

In ordinary corn, protein frequently appears as beaded strands (Fig. 2, a). Under the electron microscope, dense protein granules are readily seen in an electron-transparent matrix protein which appears as a structureless membrane (Fig. 2, b).

In flour corns that have high-protein cells randomly scattered through the starchy endosperm *(opaque-1* and *floury-1),* granules are frequently packed in dense protein masses (Fig. 2, c).

Globular protein bodies are not detectable under the light microscope in endosperm protein of the high-lysine maize mutations, *opaque-2* and *floury-2*. Protein bodies greatly reduced in size and number (Fig. 3, b) have been demonstrated by electron microscopy in *opaque-2* (3); however, the ultrastructure of *floury-2* protein is different from that of *opaque-2* and requires additional study.

Properties of Subcellular Proteins

The protein granules show no birefringence and are unaffected by alpha-amylase; consequently, the bodies are not small starch granules. Matrix protein is birefringent.

Enzymatic (Pronase) and histochemical tests (ninhydrin or chloramine-T Schiff) provide evidence that both components of the network are largely made up of protein. Solubility of the globular protein bodies in ethanol identifies the granules as a major site of zein deposition (3). Microscopic evidence for the solubility of the protein bodies in alcohol is shown in Fig. 2, d. Numerous small voids, V_z, in the matrix protein were occupied by zein bodies before alcohol treatment. A clearer picture of the effect is seen in the electron micrograph (Fig. 2, e); residual ethanol-insoluble material can

be seen in some of the voids. The zein bodies are insoluble in tissue fixed with formaldehyde. It is assumed from similar solubility experiments that the residual ethanol-insoluble matrix (Fig. 2, d and e) is largely glutelin.

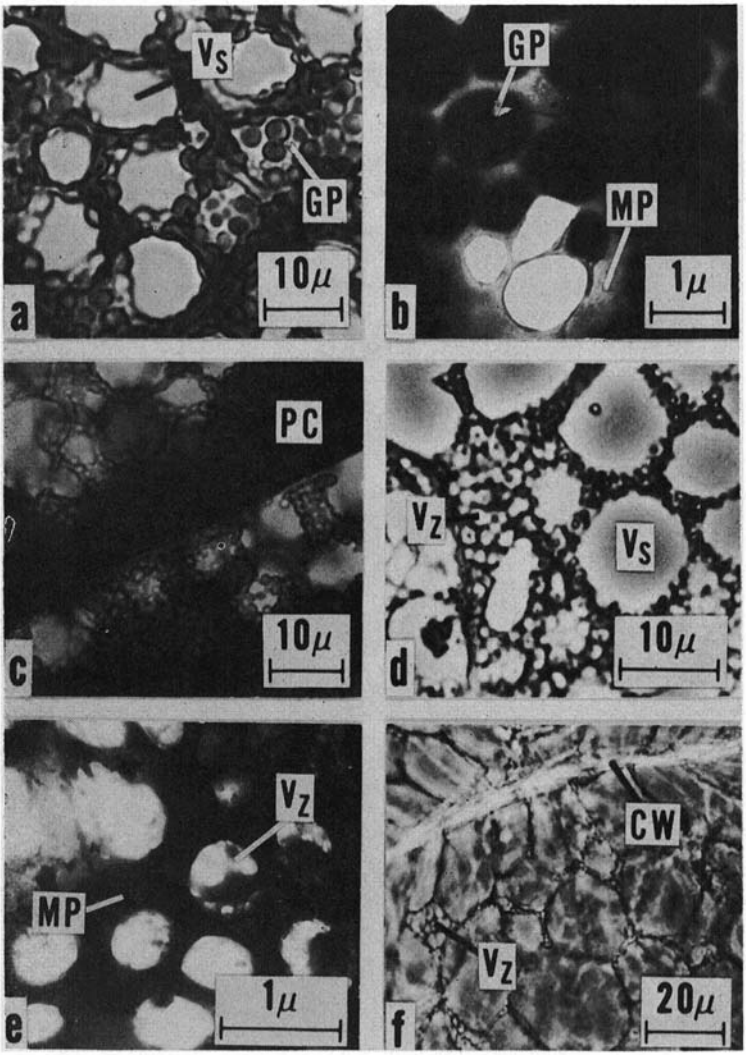

Fig. 2. Subcellular endosperm protein; all sections destarched with alpha-amylase, except f. a, Subcellular protein showing protein bodies, GP, in matrix protein. V_s, voids left by starch after digestion with alpha-amylase. W64A.

b, Electron micrograph showing protein bodies, GP, in matrix protein, MP. Holmes Hybrid.

c, Endosperm cells with dense protein masses, PC. Opaque-1 mutant.

d, Ethanol-treated section showing voids in matrix protein, V_z, after protein bodies were solubilized. W64A.

e, Electron micrograph showing voids in matrix protein, V_z, after ethanol extraction. W64A.

f, Dimethyl sulfoxide-treated section showing voids in matrix protein, V_z. CW, cell wall. Holmes Hybrid. Phase contrast.

By careful dissolving away of the matrix protein with dilute alkali, zein bodies can be isolated as shown in Fig. 3, a. In addition to ethanol, zein bodies are also soluble in aqueous-acetone solutions (50%) and in DMSO (Fig. 2, f).

A picture similar to that obtained with alcohol treatment of maize sections is noted with DMSO. This solvent is less selective than ethanol and dissolves some of the matrix protein along with the granules (Fig. 2, f).

Subaleurone Cells

Protein granules occur not only in starchy endosperm cells, but also in special subaleurone cells found in various lines of corn. These cells are densely packed with protein granules and contain few or no starch granules. They are readily distinguishable in thin sections from adjacent starchy cells by staining with iodine, by examination between crossed polarizers, or simply by their characteristic morphology. Such high-protein subaleurone cells were found in the flour corn, Mandan; in W64A, a dent type; and in opaque and floury mutants including *opaque-2* and *floury-2* (Fig. 3, c). In these varieties the special subaleurone cells form only a partial layer, ordinarily one to three cells thick, just inside the aleurone layer.

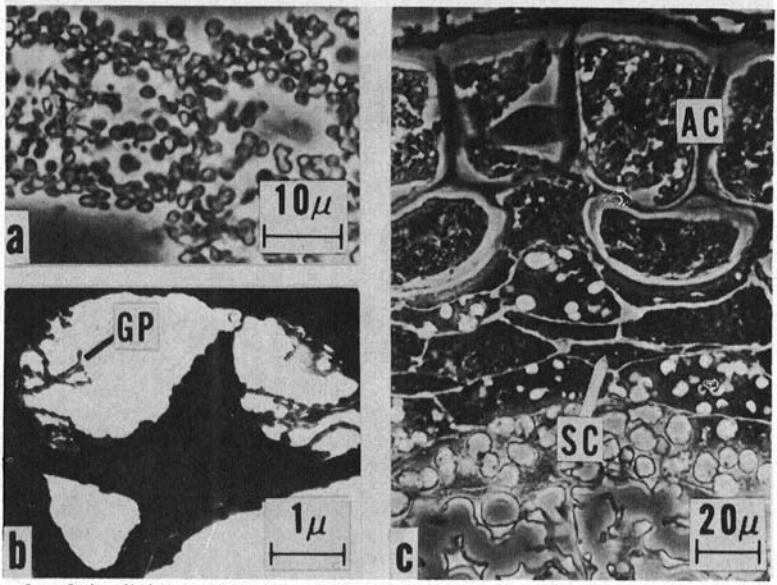

Fig. 3. Subcellular endosperm protein. Sections b and c destarched with alpha-amylase. a, Protein bodies isolated from protein matrix. Holmes Hybrid. b, Electron micrograph of endosperm protein of high-lysine corn mutant, opaque-2, with small protein bodies. c, High-protein subaleurone layer, SC. AC, aleurone cells floury-2.

Morphologically, subaleurone protein granules appear identical in size and shape to those occurring in starchy cells deeper in the endosperm.

Histochemical tests on subaleurone granules gave results similar to those on protein granules in starchy endosperm cells. However, the granules appear to be less soluble in alcohol than the zein granules of the starchy

endosperm. High-protein subaleurone cells seem to be a transition type of cell between the aleurone and starchy endosperm cells.

Size of Zein Bodies

In previous work it was shown that zein bodies in normal corn averaged about 2 microns in diameter (3). Since the presence of zein bodies provides a convenient means of judging protein quality of maize selections by microscopy, it was necessary to determine if there was any variation in zein granule size in different varieties of corn or if there were varieties of maize other than *floury-2* and *opaque-2* which lacked zein granules.

Measurements of zein granules from a number of corn varieties are summarized in Table I. Alcohol-soluble protein bodies were found in all lines of corn examined microscopically, with the exception of the high-lysine mutants *opaque-2* and *floury-2*. The data indicate that there are only two levels of zein body size. In varieties with normal lysine content, protein body diameters range between 1.4 and 1.8 micron, and in high-lysine mutants zein bodies are at the submicroscopic level.

The characteristic protein level of a corn variety in itself apparently is not correlated with protein body diameter; for example, White Cuzco with only 4.3% protein has normal-sized protein granules, similar to those found in corn with much higher protein levels.

In the cross *floury-2* × *opaque-2,* some of the kernels showed zein granules less than 0.9 micron in diameter; such protein bodies were not found in other kernels of the sample. Possibly the sample was heterozygous. Similar variability was noted in samples of *opaque-2;* occasional kernels of *opaque-2* with minute zein bodies (less than 1 micron in diameter) were generally somewhat vitreous in texture rather than floury. This variability is being studied further to determine if such selected vitreous kernels have a lower lysine content than floury kernels from the same population.

Alcohol-Soluble Protein

Alcohol-soluble protein in varieties with a normal development of zein bodies varied from 25 to 62% (Table I). There is no obvious correlation between size of protein bodies and the alcohol-soluble protein content. Various explanations may be offered for this lack of correlation, including existence of alternative sites of alcohol-soluble protein in addition to zein bodies, differences in number of protein bodies, and variation in composition of alcohol-soluble proteins.

In the mutant *opaque-2,* alcohol-soluble protein was as low as 13.5%; in *floury-2* the alcohol-soluble protein was about 20%. The value of 25% for the cross between *floury-2* and *opaque-2* is unexpectedly high and may be due to sample heterogeneity as noted above. In Holmes Hybrid, a supposedly normal corn variety, alcohol-soluble protein was also about 25% of the total protein. Microscopic examination of individual kernels of this sample showed that some kernels are deficient in zein bodies. Further evidence for this deficiency was obtained by amino acid analysis of the sample; lysine content of the endosperm protein was 2.1%, somewhat higher than the value of about 1.7% commonly found in normal corn. Apparently, the

phenomenon of kernel-to-kernel variability with respect to amino acid composition of endosperm storage proteins is widespread.

DISCUSSION

In addition to the measurements of average zein body size listed in Table I, numerous corns have been examined qualitatively with the light microscope for zein bodies, including a range of inbreds, hybrids, and exotics. The storing of alcohol-soluble protein bodies appears to be the normal condition in maize. Only *opaque-2* and *floury-2* mutants showed no zein bodies on examination under the light microscope. No examples have been found in which corn endosperm showing zein granules had a high lysine content on subsequent chemical analysis. Although more observations need to be made, it appears that microscopic identification of protein bodies, soluble in alcohol, may be taken as a reliable indication of a normal amino acid content in the maize under examination.

These observations apply to the sample as a whole. However, microscopic examination of individual kernels demonstrated appreciable heterogeneity among kernel populations with respect to endosperm protein structure. Individual kernels in both normal and high-lysine corn differ significantly from the general population with respect to presence of zein granules. This variability may be due to gene dosage effects. Individual kernel variability may account for unexpected deviations in amino acid composition sometimes noted in chemical analyses of pooled kernel samples.

When the *floury-2* and *opaque-2* mutations are excluded, the range of alcohol-soluble protein extends from 25 to about 62% (Table I). In a series of corn varieties Hansen *et al.* (9) found an increase in ratio of alcohol-soluble to total endosperm protein as the total protein increased. No correlation between alcohol-soluble and total protein was found in the present data. The percentage of alcohol-soluble protein in Holmes Hybrid and W64A is similar, despite a wide difference in total protein content (Table I). The lack of correlation is even more striking in White Cuzco and amylomaize in which the percentage of alcohol-soluble protein is similar whereas the total protein content shows a fourfold variation. Possibly moderate differences in alcohol-soluble protein in varieties may be explained on the basis of individual kernel variability as noted above.

Particularly in *opaque-2* and *floury-2,* which lack microscopically identifiable zein bodies but contain up to 20% alcohol-soluble protein (Table I), the question arises as to alternative sites of alcohol-soluble protein. One source of zein is the high-protein subaleurone common in floury mutants. Globular protein bodies occurring in these cells, even in the high-lysine mutants, are largely alcohol-soluble. Additional alcohol-soluble protein appears to be incorporated in the matrix protein in nongranular form. Qualitative evidence for this statement is based on the microscopic observation that alcohol extraction of endosperm sections not only dissolves the zein bodies (Fig. 2, e), but also appears to cause some degradation of the matrix protein, particularly on prolonged treatment. More definitive evidence is needed; however, it appears unlikely that zein bodies of the starchy endosperm are the sole site of alcohol-soluble protein.

Acknowledgments

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