# Changes in the Protein Fractions of Developing Normal and opaque-2 Maize Endosperm[1,2]

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## ABSTRACT

The proteins of the endosperm of a normal inbred line of maize (W64A) and its homozygous *opaque-2* ($o_2$) counterpart, sampled at intervals after pollination, were fractionated by a modified Osborne procedure, and selected fractions were subjected to amino acid analysis and starch-gel electrophoresis. The $o_2$ gene causes a decrease in zein and an increase in glutelin, thus producing the higher lysine content characteristic of $o_2$ endosperm. The amino acid composition of the zein isolated from $o_2$ 15 days after pollination is quite unlike that of the same fraction from 15-day normal or from $o_2$ at later stages of development. The pattern of zein accumulation in $o_2$ is consistent with the suggestion that the $o_2$ gene is active only during the first 2 to 3 weeks after pollination in the maize inbred W64A. An increase in the amount of saline-soluble nitrogen in $o_2$ is largely attributable to an increase in nonprotein nitrogen, notably glutamic acid, aspartic acid, and alanine.

The work described in this paper was undertaken in an attempt to describe more precisely the changes which occur in the various classical Osborne (1) nitrogen fractions during development of the maize endosperm, and to examine how these developmental patterns are changed by the mutant gene *opaque-2* ($o_2$) so as to produce the modified amino acid and protein composition described by Mertz et al. (2). Prior to this time only two other such studies have been reported. Zeleny (3) and Bressani and Conde (4), using different methods of protein fractionation, concluded that the zein complex of proteins is the fraction synthesized most rapidly, and to the greatest extent, in the whole seed, and that it occurs at the expense of a water-soluble (3) or presumably equivalent acid-soluble (4) fraction.

## MATERIALS AND METHODS

### Material

An inbred line of maize W64A, homozygous non-opaque (normal), and the spontaneous mutant W64A, homozygous $o_2$, were grown during 1965 on adjoining plots at the Purdue University Agronomy Farm, Lafayette, Ind., and self-pollinated. The ears were harvested at 10, 15, 22, 31, 43, and 52 days after pollination. The kernels were cut from the ears in the field, immediately sealed in polyethylene bags,

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[2]Journal paper No. 4077 of the Purdue University Agricultural Experiment Station, Lafayette, Ind. The experimental data in this paper are taken from a thesis submitted by J. J. Murphy in partial fulfillment of the requirements for the Ph.D. degree. A preliminary account of part of this work was presented at the High-Lysine Corn Conference held at Purdue University in June 1966.

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and placed in dry ice. Subsequently, samples of the same age were bulked, mixed, and stored at -20°C.

#### Endosperm Preparation

Endosperm (except 10-day) was isolated by dissection in the frozen state. Because of their small size and consequent difficulty of dissection, whole 10-day-old kernels were used rather than isolated endosperm. Approximately 10-g. samples of endosperm (or 10 g. of 10-day kernels) were weighed, lyophilized, and reweighed. The lyophilized material was then crushed with a porcelain mortar and pestle and finely powdered in a miniature ball mill (Wig-L-Bug[5]) for 15 sec. This powder was the starting material for all subsequent operations.

#### Fractionation of Endosperm Preparation

All solvent extractions employed a solvent:sample ratio of 5:1 (v./w.) and were performed at 4°C. with magnetic stirring. Extracts (and washings) were separated from the undissolved sample by centrifuging at 1,300 X g for 20 min. Each sample was extracted once with the following sequence of solvents for the time indicated: n-butanol (4 hr.) (5), 5% sodium chloride solution (1 hr.), 70% (v./v.) ethanol (4 hr.), and 0.2% sodium hydroxide solution (1 hr.). Following each extraction the sample was washed once with the same solvent. Extract and washing for each solvent were combined and made to volume with the same solvent. The residual material following the n-butanol treatment was dried in a lyophilizer, weighed, and ground in the Wig-L-Bug before proceeding to the saline extraction. The final residue (after alkaline extraction) was lyophilized, weighed, and powdered in the Wig-L-Bug. Single fractionations were performed at each age.

Nitrogen was determined by a micro-Kjeldahl method (6). Protein was determined by the method of Lowry et al. (7) using bovine serum albumin as a standard. Alpha-amino-nitrogen was determined by the method of Moore and Stein (8).

#### Amino Acid Analysis

The solvent extracts were analyzed for amino acid content on a Beckman Spinco Amino Acid Analyzer (9) using the hydrolysis conditions of Nelson et al. (10). Norleucine and homoarginine were used as internal standards. The amino acid values reported here represent single hydrolysates, except when total recovery indicated that the hydrolysis should be repeated, and are reported in terms of mole percent (moles of amino acid X 100 per total moles of amino acids recovered).

#### Starch-Gel Electrophoresis

Electrophoresis was carried out in starch-gel slabs containing 15% starch and 8M urea in 0.015M aluminum lactate-lactic acid buffer, pH 3.5. The same buffer was used as the electrolyte in the buffer chambers of the gel tray. The gel tray was patterned after the one described by Cluskey (11). All samples were applied on an equal nitrogen basis. Electrophoresis was carried out at 4°C. with a constant current of 5 ma. applied for 12 hr.

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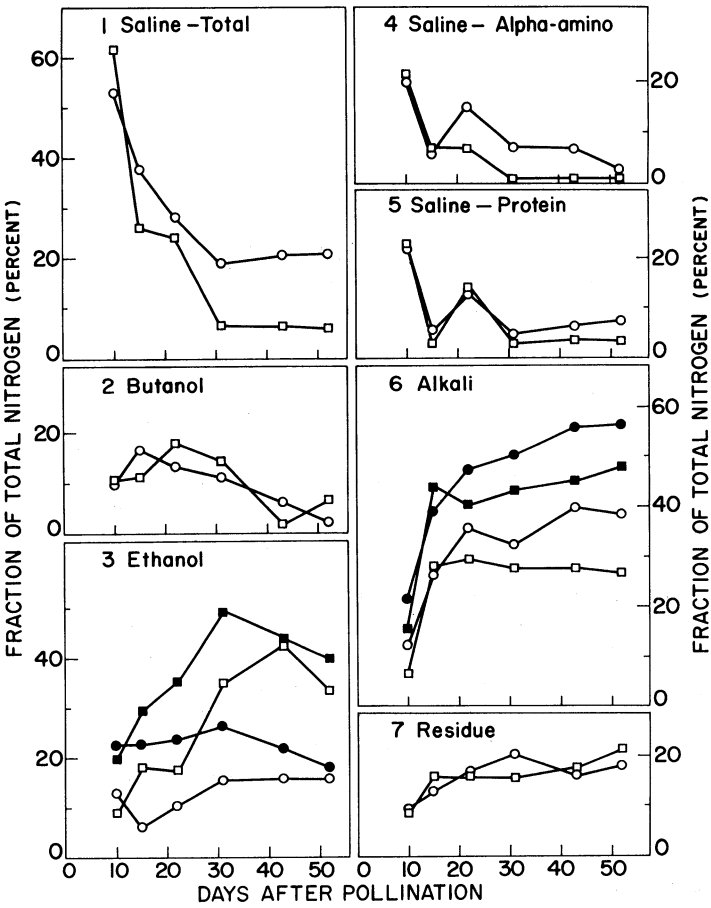
[5]Crescent Dental Mfg. Co., Chicago, Ill.




**Figs. 1 through 7. Percentage contribution to total nitrogen of various endosperm fractions of normal (open squares) and $o_2$ (open circles) maize during development (10-day values refer to fractions derived from whole kernels). Solid points in Fig. 3 indicate combined ethanol- and butanol-soluble fractions; solid squares, normal; solid circles, $o_2$. Solid points in Fig. 6 indicate combined alkali-soluble and final residue fractions; solid squares, normal; solid circles, $o_2$.**

After electrophoresis the gels were stored in a freezer for 15 min. and then sliced horizontally through the middle with a stainless-steel microtome blade. The gels were stained for protein using 0.05% (w./v.) nigrosin in 50% (v./v.) acetic acid and destained with 42% (v./v.) ethanol. The gels were also tested for lipids by the Oil Red O staining and destaining method described by Smithies (12). The gels were stained for 12 hr.

## RESULTS

### Developmental Patterns of Protein Fractions

Figures 1 through 7 show the variation in the percentage contribution of each

endosperm fraction to the total nitrogen during development. No marked difference between normal and $o_2$ was observed in either the butanol extract or the final residue (Figs. 2 and 7). In both genotypes the contribution to the total nitrogen of the butanol extract tended to decrease, and that of the final residue to increase, during the experimental period.

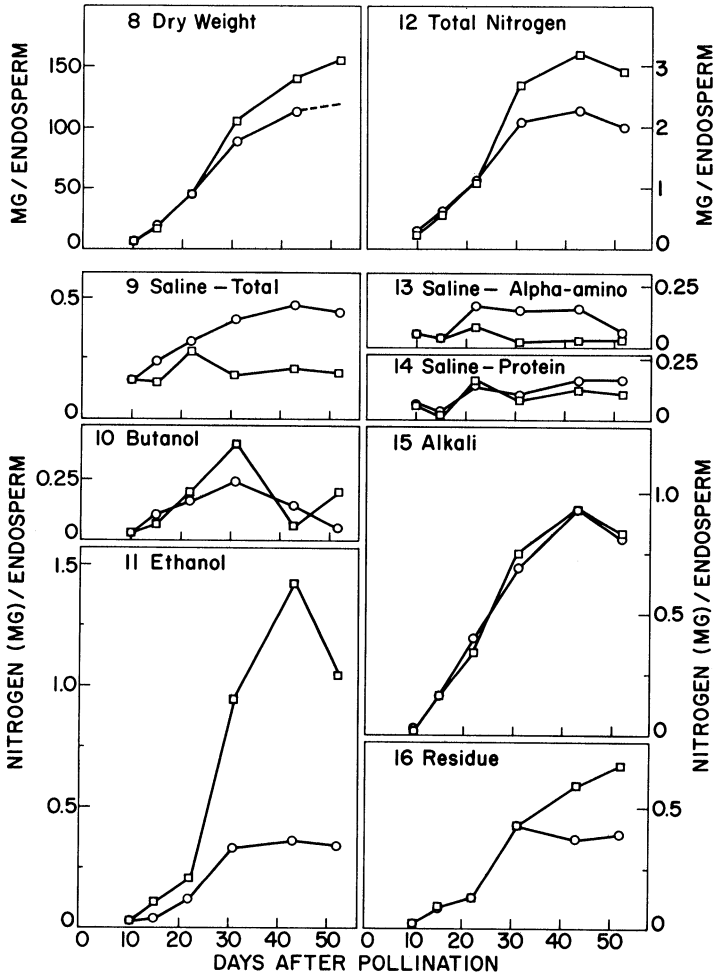
In the remaining three fractions, differences between normal and $o_2$ were apparent. Initially, the saline extract represented the largest single nitrogenous fraction in both genotypes (Fig. 1). The subsequent sharp decrease in the proportion of this fraction was far less marked in $o_2$ than normal, so that even at day 52 the saline-soluble nitrogen accounted for 21% of the total endosperm nitrogen in $o_2$, as compared with 6% in normal. Both protein and free amino acids contribute to the saline-soluble nitrogen (Figs. 4 and 5). However, a considerable and varying fraction of the nitrogen remains unaccounted for, depending upon post-pollination date. Three possibilities are amide-, small peptide-, and nucleotide-nitrogen. The first two are suggested on the basis of amino acid analyses of the saline-soluble fraction which have revealed large amounts of glutamic and aspartic acids and alanine (Table I), a considerable fraction of which is dialyzable (Murphy and Dalby, unpublished data). Any sizable contribution by nucleotide nitrogen is negated by direct analysis (Vernon and Dalby, unpublished data).

In both normal and $o_2$, the alkali-soluble glutelin fraction becomes a major nitrogenous fraction early in development and maintains its importance through to maturity (Fig. 6). The greater contribution to the total nitrogen by this fraction in $o_2$ over normal is of considerable importance in modifying the total endosperm amino acid composition (Table VII).

The ethanol-soluble zein fraction of normal endosperm continues to increase in importance almost to maturity (Fig. 3). In contrast, the zein of $o_2$ never contributes more than about 15% of the total endosperm nitrogen. During the middle and late stages of endosperm development, the butanol-soluble fraction (Fig. 2) shows a protein composition resembling the ethanol-soluble fraction both electrophoretically (Fig. 17) and in amino acid content (Table III). When these two fractions are combined (Fig. 3), a smoothing of the developmental curves results, further suggesting a complementarity between the fractions at each age. A similar complementarity is seen between the alkali-soluble fraction and the final residue in the combination curves shown in Fig. 6; these fractions also have related amino acid compositions. The decline after 31 days in the contribution of the combined alcohol-soluble fractions of both normal and $o_2$ (Fig. 3) is largely compensated for by an increase during the same period in the alkali-soluble-plus-residue combinations.

Absolute changes in the amounts of the various fractions, together with changes in dry weight and total nitrogen, are obtained by putting the results on a per endosperm basis (Figs. 8 through 16).

Figures 8 and 12 indicate that there was no difference between normal and $o_2$ in either the dry weight or total nitrogen of the endosperm prior to 3 weeks post-pollination. The subsequent divergence resulted in the 52-day $o_2$ endosperm containing only about 70% of the nitrogen found in normal. However, because of the parallel difference in dry weight, the nitrogen content per unit dry weight of the mutant was within 8 and 13% of normal at 31 and 43 days, respectively.




**Figs. 8 and 12. Changes in dry weight (Fig. 8) and total nitrogen (Fig. 12) per endosperm of normal (open squares) and $o_2$ (open circles) maize during development (10-day values represent whole-kernel data). Figs. 9, 10, 11 and 13, 14, 15, 16. Amount of nitrogen per endosperm contributed by various endosperm fractions of normal (open squares) and $o_2$ (open circles) maize during development (10-day values refer to fractions derived from whole kernels).**

The total saline-soluble nitrogen per endosperm of $o_2$ increased continuously throughout most of the period studied (Fig. 9). This is in sharp contrast to the corresponding normal curve which, with the exception of day 22, showed only slight increase with time. The exceptionally high 22-day normal value may reflect a significant metabolic event in development, since it was only at 22 days that the combined protein and $\alpha$-amino nitrogen values came near to accounting for the total saline-soluble nitrogen.

The separate $\alpha$-amino and protein nitrogen values are shown in Figs. 13 and 14,

respectively. Whereas differences in saline-soluble protein per endosperm between normal and $o_2$ appear only during later development, the $\alpha$-amino nitrogen content per endosperm of $o_2$ is elevated throughout the middle and late developmental stages, declining to near the normal value only at maturity.

Figure 15 indicates that the absolute amount of alkali-soluble nitrogen per endosperm is not conspicuously different between $o_2$ and normal at any developmental stage. This is noteworthy in view of their diverging amino acid compositions, as discussed later. Similarly, no differences in the amount of the final residue are apparent until after 31 days (Fig. 16).

The marked reduction in the net synthesis of zein in $o_2$ is apparent from Fig. 11. It may also be significant that no zein increase is apparent in $o_2$ until after 15 days, whereas in normal endosperm, zein increases continuously from 10 days post-pollination.

#### Amino Acid Analyses

The amino acid composition of the five fractions each at 15, 31, and 52 days post-pollination are presented in Tables I through VI.

When the amino acid analyses of the various saline extracts are compared directly on a mole percent basis, the picture is complex and certain relationships are obscured by the high and variable concentrations of aspartic and glutamic acids

TABLE I. AMINO ACID COMPOSITION OF SALINE EXTRACTS OF NORMAL AND OPAQUE-2 ENDOSPERM DURING DEVELOPMENT (mole percent[a])

| Amino Acid | opaque-2 | | | Normal | | |
|---|---|---|---|---|---|---|
| | Day 15 | Day 31 | Day 52 | Day 15 | Day 31 | Day 52 |
| | | | A | | | |
| Lysine | 8.6 | 9.3 | 7.3 | 9.5 | 8.5 | 6.0 |
| Histidine | 1.9 | 2.0 | 2.2 | 1.9 | 2.0 | 1.8 |
| Arginine | 4.1 | 6.7 | 8.3 | 4.7 | 7.3 | 8.3 |
| Glycine | 20.3 | 23.4 | 20.7 | 19.1 | 20.5 | 20.4 |
| Valine | 10.1 | 10.3 | 10.3 | 11.6 | 10.0 | 9.0 |
| Methionine | 6.3 | 3.4 | 2.5 | 5.4 | 4.1 | 2.9 |
| Isoleucine | 6.7 | 6.3 | 6.6 | 6.3 | 7.6 | 7.4 |
| Leucine | 11.6 | 10.0 | 11.1 | 11.2 | 11.0 | 9.7 |
| Phenylalanine | 3.8 | 3.9 | 5.1 | 4.3 | 3.5 | 4.1 |
| | | | B | | | |
| Threonine | 5.0 | 6.1 | 3.3 | 6.2 | 6.2 | 6.9 |
| Serine | 7.3 | 4.1 | 1.7 | 6.4 | 5.0 | 6.9 |
| Proline | 10.3 | 9.8 | 17.4 | 9.9 | 12.1 | 11.5 |
| Tyrosine | 1.7 | 3.0 | 2.7 | 2.6 | 2.1 | 3.1 |
| Cysteine | 2.4 | 1.7 | 0.77 | 0.84 | trace | 1.8 |
| | | | C | | | |
| Aspartic acid | 37.9 | 71.2 | 66.3 | 31.2 | 33.3 | 28.7 |
| Glutamic acid | 117 | 153 | 25.9 | 80.1 | 50.0 | 26.0 |
| Alanine | 40.3 | 48.4 | 21.0 | 34.9 | 25.1 | 22.7 |

[a]Sum of each column in A & B = 100. Values in C based on A & B total. See text.

TABLE II. AMINO ACID COMPOSITION OF ETHANOL EXTRACTS OF NORMAL AND OPAQUE-2 ENDOSPERM DURING DEVELOPMENT
(mole percent)

| Amino Acid | opaque-2 | | | Normal | | |
|---|---|---|---|---|---|---|
| | Day 15 | Day 31 | Day 52 | Day 15 | Day 31 | Day 52 |
| Lysine | 1.6 | 0.37 | 0.34 | 0.25 | 0.26 | 0.27 |
| Histidine | 0.79 | 0.50 | 0.68 | 0.76 | 0.46 | 0.77 |
| Arginine | 1.3 | 0.81 | 1.1 | 0.89 | 0.87 | 1.0 |
| Aspartic acid | 11.1 | 4.3 | 6.3 | 4.5 | 2.2 | 3.4 |
| Threonine | 2.5 | 1.7 | 2.1 | 1.7 | 0.65 | 1.1 |
| Serine | 6.0 | 2.0 | 3.3 | 2.3 | 0.66 | 1.2 |
| Glutamic acid | 30.4 | 23.9 | 21.0 | 23.9 | 24.5 | 23.2 |
| Proline | 5.8 | 10.3 | 10.8 | 10.0 | 10.3 | 10.4 |
| Glycine | 6.1 | 3.0 | 2.8 | 2.5 | 2.5 | 2.5 |
| Alanine | 13.4 | 15.4 | 14.6 | 15.7 | 17.4 | 16.1 |
| Cysteine | absent | trace | trace | trace | 0.95 | 0.55 |
| Valine | 2.0 | 3.8 | 4.2 | 4.1 | 4.7 | 4.6 |
| Methionine | 1.9 | 0.63 | 0.60 | 1.1 | 1.1 | 1.0 |
| Isoleucine | 2.8 | 4.3 | 4.0 | 4.1 | 4.1 | 4.1 |
| Leucine | 10.4 | 20.1 | 19.6 | 20.2 | 21.8 | 21.5 |
| Tyrosine | 1.4 | 3.2 | 3.0 | 2.7 | 2.4 | 3.0 |
| Phenylalanine | 2.6 | 5.8 | 5.6 | 5.2 | 5.3 | 5.5 |

TABLE III. AMINO ACID COMPOSITION OF BUTANOL EXTRACTS OF NORMAL AND OPAQUE-2 ENDOSPERM DURING DEVELOPMENT
(mole percent)

| Amino Acid | opaque-2 | | | Normal | | |
|---|---|---|---|---|---|---|
| | Day 15 | Day 31 | Day 52 | Day 15 | Day 31 | Day 52 |
| Lysine | 1.2 | 0.29 | 0.45 | 0.98 | 0.36 | 0.39 |
| Histidine | trace | 0.62 | 0.99 | trace | 1.1 | 1.3 |
| Arginine | absent | 0.45 | 0.79 | absent | 1.0 | 1.1 |
| Aspartic acid | 4.1 | 5.3 | 8.0 | 4.1 | 5.1 | 5.1 |
| Threonine | 2.3 | 3.1 | 2.6 | 2.4 | 2.6 | 2.5 |
| Serine | 3.5 | 6.3 | 6.3 | 4.4 | 6.3 | 5.6 |
| Glutamic acid | 16.6 | 22.2 | 14.9 | 13.8 | 20.6 | 19.7 |
| Proline | 8.2 | 9.0 | 15.5 | 10.1 | 10.5 | 10.3 |
| Glycine | 3.0 | 2.3 | 3.0 | 3.6 | 2.5 | 2.1 |
| Alanine | 23.7 | 18.5 | 11.8 | 22.4 | 14.7 | 13.9 |
| Cysteine | trace | trace | trace | absent | absent | absent |
| Valine | 17.0 | 5.3 | 5.0 | 15.5 | 4.3 | 6.1 |
| Methionine | 2.5 | 1.2 | trace | 3.2 | 0.71 | 0.50 |
| Isoleucine | 3.3 | 2.6 | 3.6 | 3.8 | 3.4 | 4.2 |
| Leucine | 9.7 | 15.3 | 16.9 | 9.5 | 18.4 | 18.7 |
| Tyrosine | 1.8 | 3.1 | 4.4 | 2.9 | 3.4 | 3.6 |
| Phenylalanine | 3.1 | 4.8 | 6.0 | 3.4 | 5.0 | 5.0 |

and, to a lesser extent, alanine. When these three amino acids are omitted from the calculation of mole percentages, the results presented in Table I, A and B, are obtained for the remaining amino acids. The amino acids listed in Table I, A show, at each age, close agreement in relative level between normal and $o_2$. This approximate equivalence is maintained regardless of whether the particular amino acid represents a constant, increasing, or decreasing fraction of the total during development, as exemplified by histidine, arginine, and methionine, respectively.

TABLE IV. AMINO ACID COMPOSITION OF ALKALI EXTRACTS OF NORMAL AND OPAQUE-2 ENDOSPERM DURING DEVELOPMENT (mole percent)

| Amino Acid | opaque-2 | | | Normal | | |
|---|---|---|---|---|---|---|
| | Day 15 | Day 31 | Day 52 | Day 15 | Day 31 | Day 52 |
| Lysine | 5.7 | 3.8[a] | 3.8 | 4.7 | 2.0 | 1.1 |
| Histidine | 2.2 | 3.0 | 2.9 | 2.4 | 2.9 | 2.6 |
| Arginine | 4.4 | 4.2 | 4.1 | 4.0 | 3.2 | 2.4 |
| Aspartic acid | 6.1 | 7.6 | 8.1 | 8.1 | 5.4 | 4.7 |
| Threonine | 2.6 | 4.4 | 4.2 | 3.9 | 3.9 | 3.5 |
| Serine | 1.9 | 5.8 | 5.5 | 4.7 | 5.5 | 5.6 |
| Glutamic acid | 14.2 | 15.4 | 15.0 | 14.5 | 18.7 | 19.9 |
| Proline | 7.3 | 8.8 | 8.1 | 7.7 | 11.5 | 11.9 |
| Glycine | 10.1 | 8.0 | 8.2 | 8.8 | 7.5 | 6.2 |
| Alanine | 12.1 | 9.1 | 8.8 | 10.2 | 9.7 | 10.7 |
| Cysteine | trace | 1.1 | 0.5 | trace | 1.1 | 1.0 |
| Valine | 8.5 | 6.6 | 7.1 | 6.8 | 5.7 | 5.3 |
| Methionine | 2.9 | 2.1 | 2.0 | 2.4 | 2.8 | 2.4 |
| Isoleucine | 5.1 | 4.1 | 4.6 | 4.6 | 3.4 | 3.3 |
| Leucine | 10.9 | 9.9 | 10.3 | 10.9 | 11.0 | 13.2 |
| Tyrosine | 1.8 | 2.4 | 2.3 | 2.2 | 2.6 | 2.8 |
| Phenylalanine | 4.1 | 3.7 | 4.4 | 3.9 | 3.1 | 3.6 |

[a]Values underlined differ from the next value to the left by at least 10%.

TABLE V. MOLE PERCENT RATIOS OF AMINO ACIDS IN ALKALI EXTRACTS

| Amino Acid | | | opaque-2:Normal ($o_2$/N) | | |
|---|---|---|---|---|---|
| $o_2$/N increasing with age | $o_2$/N not changing consistently with age | $o_2$/N decreasing with age | Day 15 | Day 31 | Day 52 |
| Lysine | | | 1.2 | 1.9 | 3.6 |
| | Histidine | | 0.92 | 1.1 | 1.1 |
| Arginine | | | 1.1 | 1.3 | 1.7 |
| Aspartic acid | | | 0.75 | 1.4 | 1.7 |
| Threonine | | | 0.68 | 1.1 | 1.2 |
| | Serine | | 0.40 | 1.0 | 0.99 |
| | | Glutamic acid | 0.98 | 0.83 | 0.75 |
| | | Proline | 0.95 | 0.76 | 0.68 |
| | Glycine | | 1.1 | 1.1 | 1.3 |
| | | Alanine | 1.2 | 0.94 | 0.82 |
| | Valine | | 1.3 | 1.2 | 1.3 |
| | Methionine | | 1.2 | 0.77 | 0.84 |
| Isoleucine | | | 1.1 | 1.2 | 1.4 |
| | | Leucine | 1.0 | 0.90 | 0.78 |
| | Tyrosine | | 0.80 | 0.92 | 0.82 |
| | Phenylalanine | | 1.1 | 1.2 | 1.2 |

The amino acids grouped together in Table I, B show a marked divergence from equivalence at at least one age. Thus, threonine, serine, and proline show agreement except at 52 days. A distinct lack of correlation is shown by tyrosine at both 15 and 31 days and by cysteine at all three ages. For cysteine the differences noted may be due, at least in part, to destruction during hydrolysis.

The number of moles of aspartic acid, glutamic acid, and alanine per 100 moles of total amino acids of Table I, A and B, are presented in Table I, C. In the saline

extract from normal endosperm, aspartic acid maintains an almost constant relative level at each age, whereas glutamic acid and alanine show a steady decline from 15 to 52 days post-pollination. In the $o_2$ extract, however, aspartic acid increases markedly between 15 and 31 days to a level which is then maintained through to 52 days. Glutamic acid and alanine start out in $o_2$ at 15 days at a higher relative level than the normal extract and increase further to 31 days before decreasing sharply to a level equivalent to that of normal at 52 days.

Except for histidine, aspartic acid, threonine, serine, and possibly cysteine, the ethanol-soluble proteins of normal endosperm showed marked stability in amino acid composition during development (Table II). The exceptions mentioned above (including cysteine) all showed a decline between 15 and 31 days, followed by an increase at 52 days. The 31-day $o_2$ extract closely resembled that of 15-day normal, with lysine and histidine showing the largest percentage differences. Those amino acids which increased between 31 and 52 days in normal extracts also increased in $o_2$ for the same period.

However, a significant difference in amino acid composition from any of the remaining extracts appeared in the 15-day $o_2$ extract. Here, nearly all the amino acids fall into one of two groups. The first group, comprising the basic and acidic amino acids together with threonine, serine, glycine, and methionine, are all present at elevated levels. The relative amounts of the remaining amino acids except alanine are substantially reduced. Thus, by 15 days a "typical" zein had not been produced in $o_2$[6]

At any particular age the amino acid profiles of the butanol extracts of normal and $o_2$ are similar (Table III). Likewise the changes in relative amount of any particular amino acid during development are generally found to be the same in both normal and $o_2$ The fractions as a whole are characterized by low levels of the basic amino acids and high levels of those amino acids which are preponderant in zein, i.e., glutamic acid, proline, alanine, and leucine. However, the ratios of these amino acids neither remain constant, nor are they characteristic of "typical" zein ratios. Zein-like protein bands were present in starch-gel electrophoretograms of 31- and 52-day extracts, but never in 15-day extracts (Fig. 17). It seems possible that at least part of the amino acid content of butanol extracts arises from free amino acids.

The amino acid composition of alkali extracts of $o_2$ and normal was very similar at 15 days (Table IV). The many changes which occurred in $o_2$ by 31 days were, for the most part, paralleled in normal. At 52 days the composition of the $o_2$ extract had changed little relative to the 31-day values, whereas the normal extract continued to change, mostly in the same manner as before. The relative trends in amino acid composition between $o_2$ and normal are shown by the mole percent ratios of Table V. Three distinct groups of amino acids are indicated according to

[6]The same fractionation procedure applied to 15-day material grown 2 years later (in 1967) revealed similar, but less extreme. differences between normal and o2 zein (Dalby, unpublished results). An examination of data (13,17) on the development of endosperm ribonuclease activity suggests that at 15 days post-pollination, the physiological age of the 1967 material was 2 days in advance of that for 1965. A parallel isolation of zein from the 1967 15-day material by the method of Baudet et al. (14), to the end of their second, cold-ethanol extraction, showed only two major differences between the amino acid profiles, namely in o2: normal ratios of approximately 8:1 for lysine and 2:1 for glycine (Dalby, unpublished results). Thus the pattern of differences in amino acid composition shown in Table II between 15-day zein from normal and o2 endosperm is not revealed by the procedure of Baudet et al.

TABLE VI. AMINO ACID COMPOSITION OF FINAL RESIDUE OF NORMAL AND OPAQUE-2 ENDOSPERM DURING DEVELOPMENT (mole percent)

| Amino Acid | opaque-2 | | | Normal | | |
|---|---|---|---|---|---|---|
| | Day 15 | Day 31 | Day 52 | Day 15 | Day 31 | Day 52 |
| Lysine | 6.3 | 4.9[a] | 4.8 | 5.2 | 2.5 | 2.5 |
| Histidine | 2.5 | 2.8 | 3.7 | 2.4 | 2.1 | 2.1 |
| Arginine | 5.1 | 5.0 | 4.7 | 4.3 | 2.8 | 2.9 |
| Aspartic acid | 9.4 | 8.4 | 8.0 | 8.2 | 7.3 | 6.7 |
| Threonine | 4.6 | 4.4 | 4.3 | 3.9 | 3.8 | 3.7 |
| Serine | 5.4 | 5.2 | 5.2 | 5.1 | 5.5 | 5.2 |
| Glutamic acid | 10.3 | 12.6 | 12.6 | 12.1 | 17.5 | 16.8 |
| Proline | 5.2 | 7.6 | 8.8 | 6.5 | 9.3 | 9.5 |
| Glycine | 8.6 | 8.0 | 8.0 | 7.3 | 5.4 | 5.2 |
| Alanine | 9.9 | 9.3 | 8.9 | 10.4 | 12.2 | 11.5 |
| Cysteine | 0.75 | 0.97 | 1.1 | trace | 0.82 | trace |
| Valine | 8.1 | 8.0 | 7.6 | 9.4 | 6.1 | 6.8 |
| Methionine | 2.3 | 1.9 | 1.7 | 2.0 | 1.6 | 1.8 |
| Isoleucine | 5.0 | 4.5 | 4.3 | 4.8 | 3.8 | 4.2 |
| Leucine | 9.3 | 9.5 | 9.5 | 11.5 | 13.6 | 13.9 |
| Tyrosine | 3.1 | 3.0 | 3.0 | 3.0 | 2.6 | 3.3 |
| Phenylalanine | 4.2 | 3.8 | 3.6 | 4.2 | 3.3 | 3.9 |

[a]Values underlined differ from the next value to the left by at least 10%.

whether the ratio increases, decreases, or changes inconsistently (or not at all) during development.

In both amino acid composition and compositional behavior during development, the final residues resembled the alkali extracts (Table VI). However, unlike the normal alkali extract, the normal final residue did not change appreciably between 31 and 52 days, but rather followed the $o_2$ alkali extract pattern.

#### Fractional Distribution of Lysine in Mature Endosperm

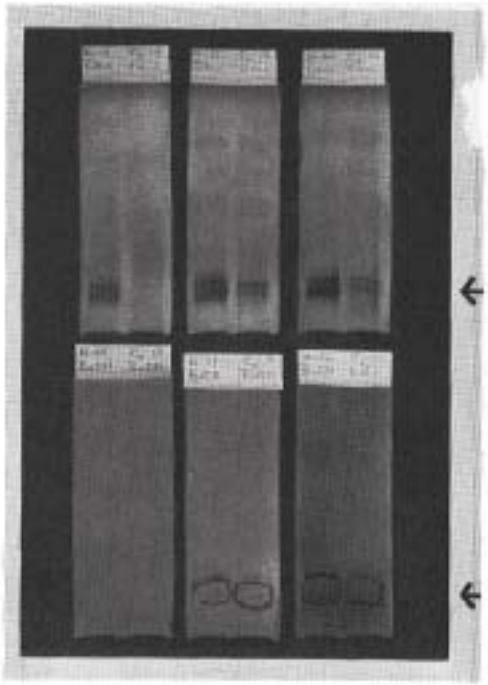
Table VII summarizes the distribution of lysine among the various fractions in 52-day $o_2$ and normal endosperm.

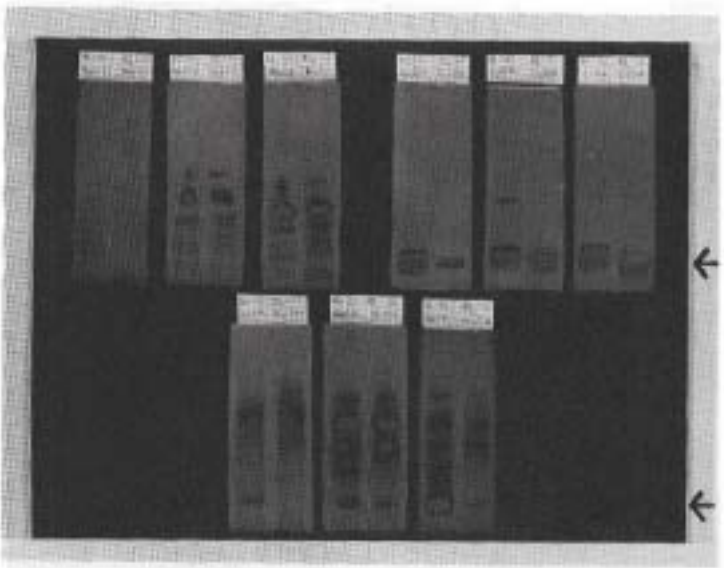
#### Starch-Gel Electrophoresis

The electrophoretic patterns of the original unfractionated endosperm powder

TABLE VII. FRACTIONAL DISTRIBUTION OF LYSINE IN DAY 52 ENDOSPERM OF NORMAL AND OPAQUE-2 MAIZE

| Extract | Fraction of Total Nitrogen | | Lysine Content | | Fractional Lysine Contribution | | | |
|---|---|---|---|---|---|---|---|---|
| | $o_2$ % | Normal % | $o_2$ g./16 g. N | Normal g./16 g. N | $o_2$ g./16 g. N | Normal g./16 g. N | $o_2$ % of total | Normal % of total |
| Saline | 21 | 6.0 | 2.1 | 1.8 | 0.43 | 0.11 | 14 | 8.5 |
| Butanol | 2.1 | 6.5 | 0.35 | 0.22 | 0.007 | 0.014 | 0.2 | 1.1 |
| Ethanol | 16 | 33 | 0.37 | 0.27 | 0.058 | 0.089 | 1.8 | 7.1 |
| Alkali | 39 | 27 | 4.1 | 1.7 | 1.6 | 0.45 | 50 | 36 |
| Residue | 18 | 22 | 6.2 | 2.8 | 1.1 | 0.59 | 35 | 47 |
| Total | | | | | 3.2 | 1.3 | | |





Figs. 17 (top) and 18 (bottom). Nigrosin-stained starch-gel electrophoretograms of the proteins of the original whole endosperm and various fractions thereof from normal and $o_2$ maize during development. Arrow (→) indicates slow-moving bands stainable with Oil Red O.

and the various solvent extractable fractions of 15-, 31-, and 52-day endosperm samples are shown in Figs. 17 and 18.

Although samples were applied on an equal nitrogen basis, the amounts of nigrosin-stainable protein apparently vary considerably between $o_2$ and normal in a number of cases. It should also be noted that areas which are ringed by stain but not deeply stained inside result from high concentrations of protein. These cause dense precipitation of stain at the gel surface and prevent further penetration of stain to the deeper layers of the gel. This precipitated material is removed during subsequent washing procedures, leaving poorly stained areas.

All the electrophoretograms shown were stained with nigrosin. The slow-moving bands in the ethanol-soluble fractions (arrowed in Fig. 18) are also stainable with Oil Red O. Similar slow-moving double-staining bands are present in the original whole endosperm, the butanol extract, and the alkali-soluble fraction (arrowed in Figs. 17 and 18), but not in the saline-soluble extract (Fig. 18). Thus it seems reasonable to suggest that where such bands occur in the butanol- and alkali-soluble fractions, they are correlatable with the presence of zein-like material. It is particularly interesting to note the extremely low concentration of these bands in the 15-day $o_2$ whole endosperm and their complete absence from the corresponding alkali-soluble fraction.

Overall, the progressive changes in the electrophoretic patterns during development correlate well with the changes in protein and amino acid composition already noted.

## DISCUSSION

The foregoing results present a reasonably consistent picture of the gross changes produced in the normal development pattern of the classical protein fractions of maize endosperm by the presence of homozygous $o_2$.

Clearly, in $o_2$, the amount of zein per endosperm is drastically reduced, in confirmation of the original observations of the effect of the $o_2$ gene on maize endosperm by Mertz et al. (2). However, this would not seem to be simply a matter of an overall reduction in the net rate of zein synthesis, since 15-day $o_2$ zein has a quite atypical amino acid composition (Table II). Furthermore, in contrast to normal, the size of the fractions does not appear to increase in $o_2$ until sometime between 15 and 22 days post-pollination (Fig. 15). Other evidence concerning the development of ribonuclease activity in normal and $o_2$ endosperm suggests that the $o_2$ gene acts only during the first 16 days after pollination in W64A (17). Thus, it seems possible that the lowered zein content of $o_2$ results, not from a lowered rate of net synthesis, but rather from a delayed *onset* of synthesis. However, this interpretation is the exact converse of the known effect of $o_2$ on ribonuclease activity, which is to change the *rate* of increase, not the timing (17). Current work is directed toward deciding between these alternatives. (The suggestion that the $o_2$ gene is active only during the first 2 to 3 weeks after pollination should be understood as referring only to the presence of absence of an effect of $o_2$ on zein or ribonuclease accumulation. Whether this effect is direct or indirect is not known. Nor is it known that $o_2$ does not affect other parameters later in development.)

The amount of "glutelin" in $o_2$ is increased. This is not immediately obvious, since the rate of increase of the alkali-soluble fraction of normal and $o_2$ is the same

(Fig. 14). However, the changes in amino acid composition (Tables IV and V) and electrophoretic data (Fig. 18) indicate that the glutelin fraction of normal maize is progressively diluted by zein-like material as development proceeds. Consequently, the non-zein (or true glutelin) part of this material must be accumulating to a lesser extent in normal than in $o_2$. Thus, the elevated lysine associated with $o_2$ results from both a lowering of zein and an absolute increase in non-zein protein as reported earlier (16), and is in agreement with the findings of Jiménez (15) on mature seed.

The differences between $o_2$ and normal in the amounts of saline-soluble nitrogen would appear to be largely accountable for in terms of low-molecular-weight nitrogenous compounds, since differences in the protein content of this fraction do not arise until late in development (Fig. 12). By assuming that all the glutamic acid and aspartic acid of this fraction are present as glutamine and asparagine, and by including alanine, it is possible to account, to a fair degree of approximation, for the differences between the total saline-soluble nitrogen and the saline-soluble protein. The fact that these three amino acids play key roles in transamination may be significant. However, it should be noted that there are differences in the relative amounts of some of the individual proteins of this fraction (Fig. 18, and Dalby, unpublished data).

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