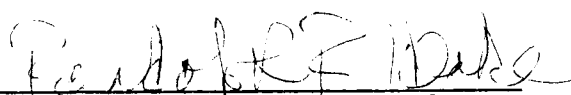
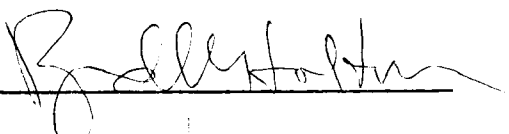
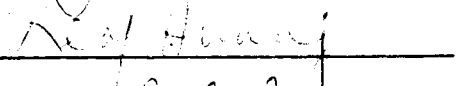


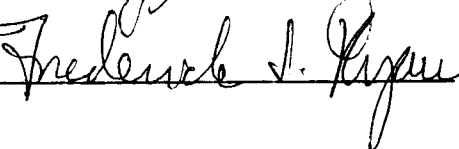
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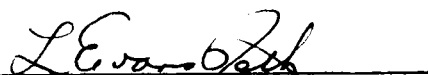

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AMINO ACID BIOSYNTHESIS IN THE DEVELOPING ENDOSPERM
TISSUE OF ZEА MAYS L.

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Rosemarie Wahnbaeck Spencer

June 1981

3051928

DEDICATION

In gratitude to my family,
especially my husband Barry,
for their patience and love
and in the memory of my father.

We dance round in a ring and suppose,
But the Secret sits in the middle and knows.

Robert Frost

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ABSTRACT

A study of the enzymes involved in the synthesis of the nutritionally essential amino acids lysine, threonine and methionine in the developing maize endosperm (Zea mays L. var. DeKalb XL43) was the subject of the major portion of this thesis. The enzymes β -aspartate kinase (EC 2.7.2.4), homoserine dehydrogenase (EC 1.1.1.3) and diaminopimelate decarboxylase (EC 4.1.1.20) have been shown to exhibit changes in activity throughout maize endosperm development. Analysis of the subcellular compartmentation of these enzymes on sucrose gradients suggests that β -aspartate kinase and diaminopimelate decarboxylase activities appear, in part, to be associated with a plastid fraction, as identified by co-fractionation with triose-phosphate isomerase, triosephosphate dehydrogenase and ribulose-1,5-bisphosphate carboxylase activities. Homoserine dehydrogenase activity, however, appears predominantly associated with the cytoplasmic fraction, or may be weakly associated with an organellar fraction.

The developmental-dependent changes in total extractable β -aspartate kinase and homoserine dehydrogenase activities suggest regulation at the gene level or level of enzyme activity. Perturbation of the free pool of the end-product effector amino acid lysine, by its injection at 200 nmoles/endosperm early in development, prevents the increase in total extractable β -aspartate kinase and homoserine

dehydrogenase activities without changing normal endosperm development, suggesting that it regulates at the gene level.

The presence of ribulose-1,5-bisphosphate carboxylase activity (EC 4.1.1.39) in the non-photosynthetic maize endosperm was demonstrated. The low specific activity suggests that the enzyme may not function in CO₂ fixation.

The non-photosynthetic maize endosperm, therefore, appears to contain some of the enzyme machinery for nitrogen and carbon metabolism.

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LIST OF ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
BAP	β -aspartylphosphate
DAP	Diaminopimelate
DEAE	Diethylaminoethyl
DTT	DL-dithiothreitol
EDTA	Ethylenediaminetetracetic acid
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NBT	Nitro blue tetrazolium
PEP	Phosphoenol pyruvate
PMS	Phenazine methosulfate
RuBP	Ribulose-1,5-bisphosphate
SDS	Sodium lauryl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane

I. INTRODUCTION

The protein nutritional value of the world's principal food crop plants is limited by deficiencies in the essential amino acids lysine, threonine, methionine and tryptophan (Mifflin, 1975; Pradilla et al., 1975; Swaminathan et al., 1970). Knowledge of the factors controlling storage protein synthesis in seeds is important in research ultimately directed to improving the nutritional value of cereal components in non-ruminant animal diets. During the course of seed development from the fertilized ovule to maturity, storage protein is laid down at a variable rate (Mifflin, 1975). The differential synthesis of proteins during development is nowhere more evident than in the developing cotyledons and developing endosperm of seeds, where, at the only time in the life of the organism, the specialized metabolic and storage proteins are produced and packaged for use in the germination of the seed. Developing endosperm tissue lends itself to a variety of experimental probes, such as following labeled precursors to their site of deposition and isolating and evaluating the kinetic properties of various enzymes involved in the utilization of carbon, nitrogen and sulfur for the production of amino acids needed for complete synthesis of the metabolic and storage proteins and their precursor pools.

II. HISTORICAL REVIEW

Seed Development

In angiosperms, seed development occurs as a result of a double fertilization; one of the male nuclei of the pollen grain fuses with the egg cell to produce a zygote, which subsequently divides and differentiates to form the diploid embryo. The second male nucleus fuses with the two polar nuclei (in the polar nucleus within the egg cell) giving rise to the triple fusion nucleus, which then develops into the triploid endosperm, the source of food for the germinating seed.

The morphology of the developing grain of maize was studied by Randolph (1939). The endosperm undergoes many nuclear divisions, passing from a free multi-nucleate to a cellular phase beginning about three and one-half days after pollination. Cell wall formation begins in the region of the endosperm surrounding the embryo and progresses to the antipodal region. Approximately four days after fertilization, the endosperm is essentially cellular. Cell division ceases around 20 to 22 days in the epidermal layer of the endosperm, but persists to maturity in the subepidermal regions.

Ingle et al. (1965) studied the changes in the chemical composition of the developing maize seeds, analyzing dry weight, water, nitrogen, total RNA and DNA and other physiological and biochemical parameters. The initial phase of endosperm development was characterized by a rapid accumulation of soluble constituents. Soluble

nitrogen, amino acids and nucleotide pools were found to increase, reaching a peak at 28 days after pollination, and subsequently decreasing to maturity. During the time period of 10 to 30 days post-pollination storage proteins were deposited.

There is considerable variation among seeds of all plants in the chemical composition and the nature of food reserves. The amino acid complement of the seed reserve proteins is also markedly different from the composition of the vegetative protein of the plant. The relative proportion of the different storage proteins affects the overall amino acid composition of the seed.

The principal reserve proteins in maize are prolamin and glutelin. Prolamin is located in subcellular structures termed protein bodies that are embedded in the endosperm matrix. Extensive research efforts over the last decade have focused on the biochemical properties of reserve proteins and on the nature of the controls that govern their deposition in the seed. However, very little attention has been given to the study of the form of nitrogen translocated and changes in the levels of nitrogen transported to the seed through development.

One of the earlier studies of the transport of nitrogen to the developing seed in maize was made by Hay et al. (1951). He investigated the extent to which nitrogen in the vegetative parts of the plant at pollination is supplied to the developing grain, by sampling different parts of the plant and analyzing chemical constituents such as total protein and nitrogen. It was found that vegetative parts lost nitrogen to the grain, principally in the form of amino acids derived from vegetative leaf protein. Small donations were made by

nitrate, ammonia, amide and α -amino nitrogen and new assimilates. No conclusions were made as to the chemical nature or route followed by nitrogen to the developing grain in this study.

The limited data on the composition and rates of nitrogen input to the developing kernel come primarily from work done in legumes, with fewer reports in cereals (McConnell, 1969; Arruda and daSilva, 1979). Conclusions drawn primarily from the legume data suggest that most of the nitrogen transported from vegetative portions of the plant to the developing seed is supplied via the phloem. The predominant forms of amino acids in the phloem sap of legumes are asparagine and glutamine (Pate et al., 1974, 1975). Other protein amino acids are present in small quantities or absent.

The composition of the vascular sap supplying the developing ears in maize was studied by Arruda and daSilva (1979) and is the only report of the composition of this transport system in maize. It was found that glutamine was the major amino acid present in the vascular sap of the maize ear peduncle that feeds the developing grain. It was present in a concentration of 30 $\mu\text{l}/100 \mu\text{l}$ (30%) at 7 to 42 days post-pollination, whereas other protein amino acids were found to be present in concentrations of 0.6 to 10 $\mu\text{mol}/100 \mu\text{mol}$ during the same developmental period. The high glutamine concentration correlates with high glutamine found in the developing maize endosperm (Sodek and Wilson, 1970) and glutamate synthase activity in the developing maize kernel (Sodek and daSilva, 1977).

If glutamine and asparagine are the principal amines of the transport stream, then tissues receiving these amino acids must possess

mechanisms for transamination to provide nitrogen for the synthesis of other α -amino acids (Mifflin and Lea, 1976). Glutamine can be converted to glutamic acid via an NAD-dependent glutamate synthase found in the developing endosperm tissues of maize (Sodek and daSilva, 1977). This, therefore, provides a means whereby nitrogen transported in the form of glutamine is made available for the synthesis of other amino acids in the developing endosperm.

Asparagine utilization is not understood, although it may be via an asparaginase which will degrade asparagine to aspartic acid. Aspartic acid, in turn, can then supply carbon for the synthesis of the aspartate family of amino acids and the formation of tricarboxylic acid cycle intermediates (Oaks, 1978). Utilization of the carbon and nitrogen from asparagine has been found for a range of amino acids in the developing lupin seed (Pate et al., 1975; Atkins et al., 1975). An asparaginase activity has been demonstrated in crude extracts of developing lupin cotyledons (Lea et al., 1976), lupin embryos (Atkins et al., 1975) and maize endosperm (Sodek et al., 1978). Double labeled asparagine ($^{14}\text{C}(\text{U})$, $^{15}\text{N}(\text{amide})$) was fed to fruiting shoots in white lupin (Lupinus albus L.) (Atkins et al., 1975). It was found that the amine nitrogen was used as a general amino donor for formation of endosperm solutes and for synthesis of protein amino acids in the developing cotyledon, especially those not arriving in sufficient quantity in the translocation stream to the developing seed.

Amino Acid Metabolism During Endosperm Development: In Vivo and In Vitro Tracer Studies

Both in vivo and in vitro studies of protein synthesis and amino acid accumulation in developing endosperm have been carried out with several cereals, such as wheat, barley, rice, corn and sorghum, as well as with legumes, for example pea and lupin. Bilinski and McConnell (1957) studied the utilization of the methyl and carboxyl carbons of acetate in the formation of amino acids in the seed proteins of wheat. The injection of ^{14}C -acetate in the top node of the stem and the subsequent isolation and hydrolysis of seed protein resulted in direct incorporation of the carboxyl carbon of acetate into the C-4 of leucine. The incorporation of label (from ^{35}S -sulfate and ^{14}C -amino acids) into storage and soluble proteins in developing wheat endosperm was demonstrated by Morton and Raison (1964). The injection of ^{14}C -labeled substrates into the top internode of the stem (McConnell, 1969) and subsequent examination of label distribution in mature kernels demonstrated the incorporation of several tracers into glutamic acid and into related amino acids arginine and proline.

Recent investigations by Atkins et al. (1975) focused on the metabolism of asparagine in legumes. Dual labeled ((U- ^{14}C) and ^{15}N -amide) asparagine was fed to the shoots through the translocation stream. Asparagine was found to be extensively metabolized on entering the seed. In 1975, Pate et al. studied the fate of xylem fed ^{14}C -labeled asparagine, glutamic acid, aspartic acid and photo-synthetically fed $^{14}\text{CO}_2$ in transport to legume seeds. Serine,

asparagine and glutamine were the main compounds labeled in the phloem sap. These experiments indicated a complex cross-transfer of solutes from xylem to phloem. Macnicol (1977) assessed the capacity of isolated developing pea (Pisum sativum L.) cotyledons for synthesis of amino acids from phloem sap precursors and their incorporation into protein, particularly the sulfur amino acids. Isolated cotyledons were fed radioactive precursors as amino acids, and when added singly, label occurred in aliphatic amino acids. His data support the proposed interconversion of amino acids in the developing endosperm tissue. All labeled amino acids were incorporated into protein.

Incorporation of ^{14}C -leucine and ^{14}C -lysine into protein of normal and Opaque-2, a high lysine, maize, was analyzed by Sodek and Wilson (1970). These precursors were injected into maize plants just below the ear at various stages of development. It was found that while about half of the injected ^{14}C -lysine in normal maize was converted to glutamic acid and proline in 30 days, almost all was recovered as lysine in the endosperm of Opaque-2 under similar conditions. Similar results were found by Brandt (1975) in barley. Brandt's experiment involved injection of ^{14}C -lysine in the top internode of the barley stem in order to study and compare its in vivo incorporation into endosperm of wild-type Bomi and high-lysine barley (1508). It was found that less lysine was converted in the high-lysine mutant; the hypothesis made that a restriction in the conversion of lysine to glutamine and proline is related to the reduced ability of the mutant endosperm to synthesize the low lysine protein, zein. Sodek and Wilson (1970) concluded that the amino acid

imbalance in endosperm proteins in maize may be a consequence of limiting amounts of amino acids, for example, lysine, in the free pool. In contrast to the results shown by Sodek and Wilson (1970), Singh and Chatterjee (1976) concluded that the free pool is not the limiting factor in the type of storage protein synthesized, by direct measurement of the free amino acid content. They propose that enzymes involved in metabolism of lysine may be more active in the normal maize endosperm than in the Opaque-2 endosperm.

Sodek (1976) investigated endosperm amino acid metabolism by injecting precursors directly into the developing endosperm tissue of maize. ^{14}C -aspartate, -alanine, -acetate and -diaminopimelate (a precursor to lysine in the aspartate pathway) were injected into the endosperm at 30 days post-pollination. The distribution of label in free and protein amino acids was investigated one week later. The results showed that diaminopimelic acid pathway functions in the biosynthesis of lysine in the developing endosperm. From the demonstration of the utilization of precursors for amino acid synthesis in the endosperm, it is indicated that de novo synthesis of amino acids occurs in the developing endosperm of maize.

When evaluating the incorporation of a single amino acid into a tissue, it is advantageous to be able to grow the isolated tissue upon a defined growth medium such that incorporation of the labeled component can be evaluated without interference from endogeneous pools of metabolites present in the surrounding tissue. In vitro studies using isolated embryo and endosperm tissues of developing cereal and legume seeds have also been conducted in order to evaluate the

contribution of exogenous amino acids to the synthesis of endosperm storage proteins. Graham et al. (1964) studied the in vivo uptake of radiolabeled sulfate, leucine and proline into detached wheat heads. After exposure, the wheat seeds were fractionated into the soluble extracts of protein bodies from the endosperm. A comparison was made of the uptake of ^{35}S -sulphate, ^{14}C -proline and ^{14}C -leucine into soluble and bound protein fractions. The specific activity of the storage protein fractions compared to the soluble protein fraction after incorporation of proline and leucine indicated that the incorporation of radioactivity from the amino acids into storage protein takes place simultaneously with, and independent of, the incorporation into soluble proteins. There may then be two independent systems for the synthesis of soluble and storage proteins and two precursor pools for the two systems in the wheat endosperm. Donovan and Lee (1977) reported that detached wheat heads grown in a liquid culture medium containing sucrose, all amino acids, salts and other organic constituents exhibited normal seed development. Bright et al. (1978) utilized the ability of growing excised wheat (Triticum aestivum L.) and barley (Hordeum vulgare L.) embryo's in culture for regulatory studies of amino acid biosynthesis. Addition of 1 mM lysine and 1 mM threonine to the culture medium effected a synergistic inhibition of embryo growth, implicating a feedback inhibition phenomenon of β -aspartate kinase and homoserine dehydrogenase activities. This system has the potential of being used for the selection of lysine overproducing mutants.

Gupta et al. (1976) used excised immature maize endosperms to study the metabolism of labeled acetate in the normal and high-lysine mutant Opaque-2 Zea mays L. The two types of tissue differed in the amount of label incorporated in free amino and organic acids. At 15 days post-pollination Opaque-2 contained 3 to 4 times the amount of label in these compounds. This trend reversed as development proceeded. Because so many parameters of development were affected by the Opaque-2 mutant, it is possible that the gene for the high-lysine mutant may affect more than just one parameter in the production of a higher-lysine-containing storage protein.

Gengenbach et al. (1977a, 1977b) pollinated maize ovules in vitro and cultured the developing kernels in a minimal medium with L-glutamine added. Approximately 5% of the fertilized ovules developed to normal maturity. This indicates that the developing kernel possesses an extensive metabolic capacity to synthesize all of its storage products from the relatively few raw materials supplied. Studies were initiated in order to determine genetic and environmental effects on the fertilization of the ovule in vitro. It was found that different factors affect fertilization more than processes involved in kernel development; therefore, providing fertilization can be enhanced, a high percentage of fertilized ovules may develop to full (normal) kernels. Thompson et al. (1977) utilized the in vitro culture of immature, developing cotyledons of soybean (Glycine max L.) to control and study the most effective nitrogen source for in vitro culture. It was found that the normal 7S and 11S storage proteins of

developing soybean cotyledons were synthesized in vitro as analyzed by sucrose density gradient centrifugation. Therefore, it appears that several systems may be available for the study of amino acid metabolism in intact tissues grown in vitro.

The Aspartate Family of Amino Acids

In plants and bacteria, the amino acids lysine, threonine, methionine and isoleucine all derive the bulk of their carbon skeletons from L-aspartate and are, therefore, members of the aspartate family of amino acids (Figure 1). Knowledge of this biosynthetic pathway and its regulation is incomplete in plants. There is also little known about the subcellular localization and the developmental variation that may occur in the synthesis and function of the key regulatory enzymes of the pathway. In vivo studies indicate that the pathway in plants is similar to that of bacteria and is under similar regulatory controls (Dougall, 1971; Henke and Wilson, 1974). The enzymes of the pathway are subject to complex regulatory controls that differ from genus to genus and in the developmental stage of the same organism (Figure 2). Extensive analysis of multi-branched biosynthetic pathways indicates that the first enzyme in the pathway and those that catalyze reactions immediately following a branch point are likely to be subject to feedback regulation. The same properties are exhibited in higher plant enzymes in the pathway. Beta-aspartate kinase (Figure 1, Key A), homoserine dehydrogenase (Figure 1, Key C) and dihydrodipicolinate synthase (Figure 1, Key F) are all subject to feedback regulation by the end-products of the pathway in higher plants.

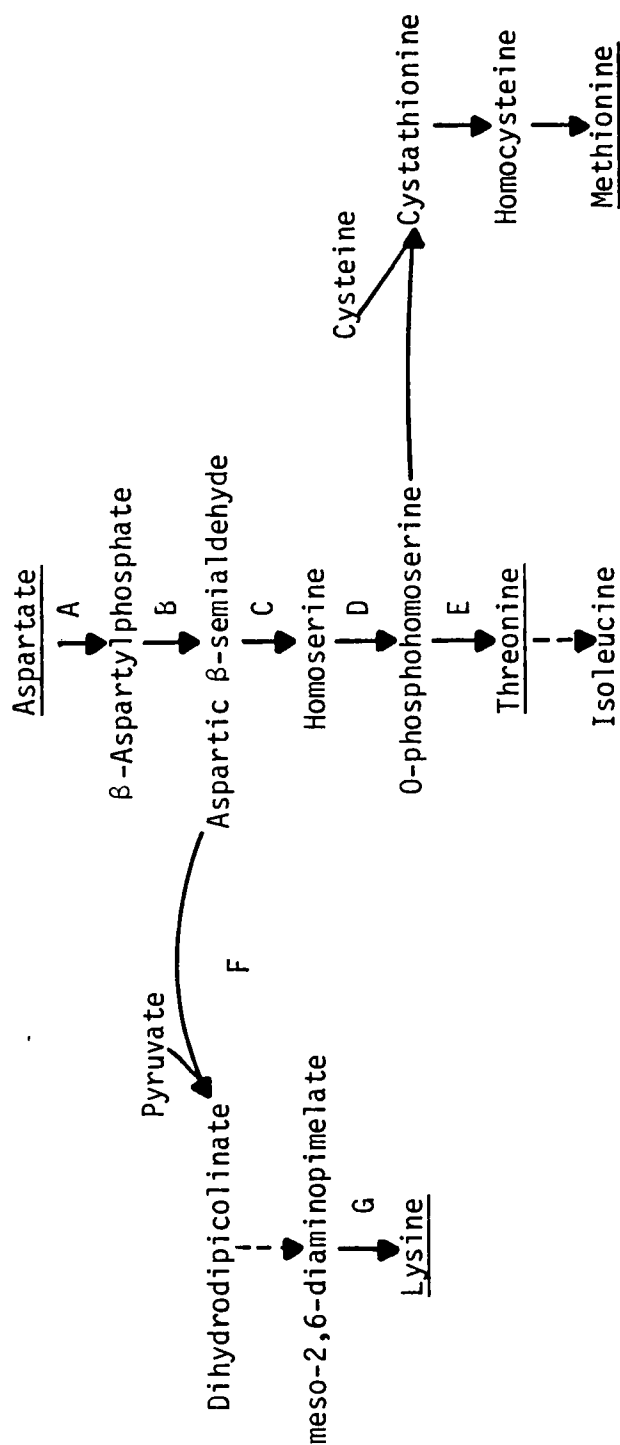


Figure 1. Biosynthetic pathway for the synthesis of lysine, threonine, methionine and isoleucine from aspartate. Listed below are enzymes which have been characterized in plants. (----- intermediate steps not shown.) (A) β-aspartate kinase (Bryan et al., 1970), (B) aspartate β-semialdehyde dehydrogenase (Bryan et al., 1970), (C) homoserine dehydrogenase (Bryan, 1969), (D) homoserine kinase (Aarnes, 1976), (E) threonine synthase (Schnyder et al., 1975), (F) dihydrodipicolinate synthase (Cheshire and Mifflin, 1975), (G) diaminopimelate decarboxylase (Mazelis et al., 1976).

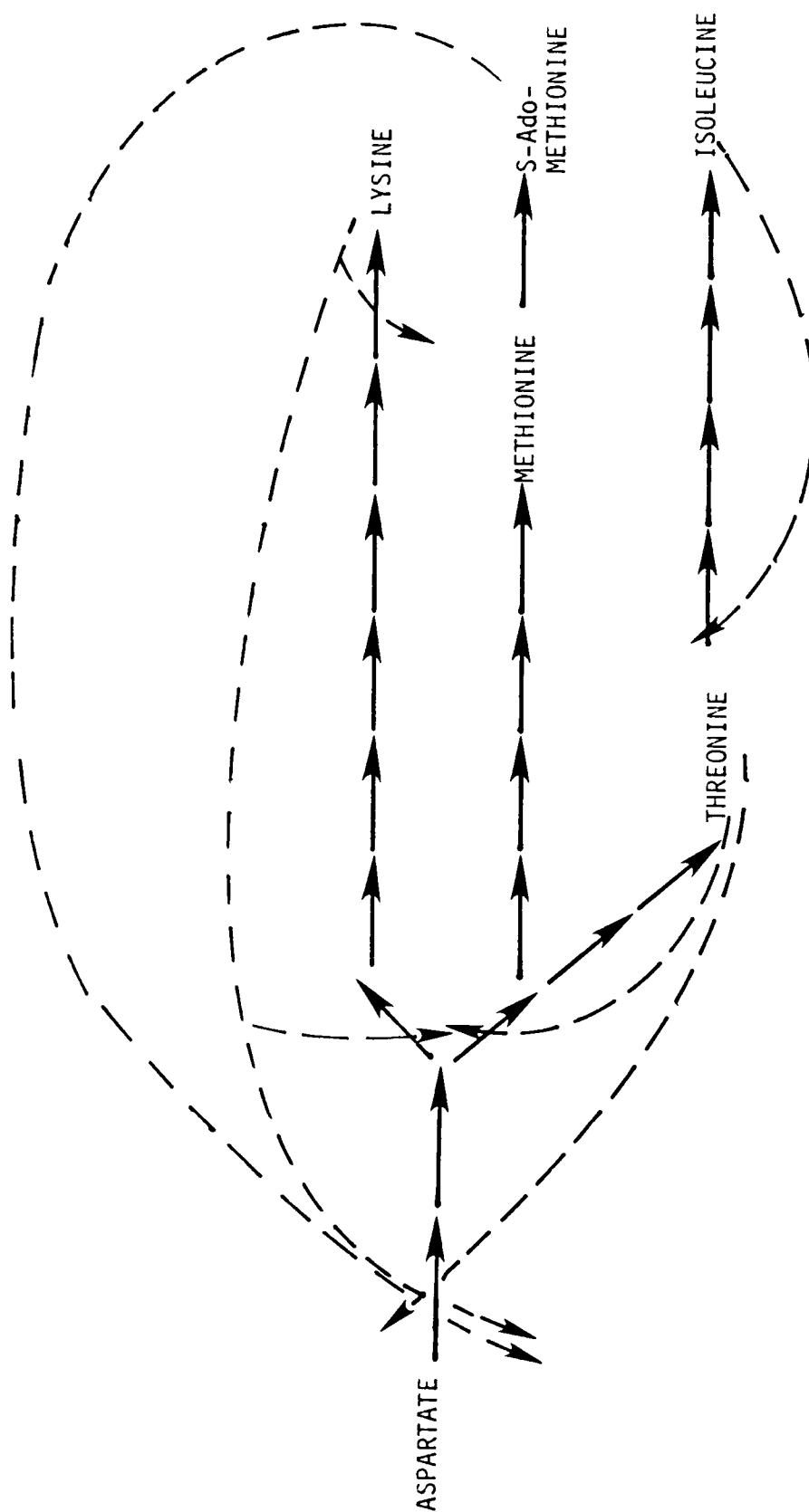


Figure 2. Complex regulatory patterns exhibited by the aspartate family of amino acids. (-----) indicates feedback inhibition of aspartate pathway enzymes by amino acids of the pathway.

Several of the pathway enzymes have been isolated from plants. The first isolation and characterization of β -aspartate kinase from plant tissue was reported by Bryan et al. (1970) in maize leaf tissue. Other laboratories have studied the pathway enzymes in crude extracts of a variety of plant tissues, generally with low levels of specific activity and often showing complex regulatory kinetic relationships between enzyme activities and substrate concentration (e.g., aspartate and β -aspartate kinase).

Tissues which have been examined for aspartate family enzyme activities include a variety of plants and plant parts, for example, shoot, root and developing endosperm of maize, pea shoots, barley seedlings and carrot callus and cell suspension cultures. The latter was used by Davies and Mifflin (1977) to isolate and characterize two β -aspartate kinase activities, one inhibited by lysine and the other inhibited by threonine. Sakano (1979) and Bright et al. (1979) attempted to demonstrate a derepression/repression of a lysine-sensitive β -aspartate kinase in the culture of carrot root and carrot cell suspension cultures, respectively. Sakano (1979) observed an increase in lysine-sensitive β -aspartate kinase activity with culture age of carrot roots slices. Upon addition of 0.1 to 1.0 mM lysine to the culture medium, he found a specific suppression of the increase in lysine-sensitive β -aspartate kinase activity with no affect in threonine-sensitive activity. Bright et al. (1979) found that during the ten-fold increase in lysine sensitive β -aspartate kinase activity with culture age of carrot cell suspensions, there was little change in total amino acid content or in amino acids derived from aspartate.

This led them to the conclusion that there is no correlation between the amount of extractable lysine-sensitive β -aspartate kinase activity and the amounts of amino acids being synthesized. Therefore, conclusive evidence of a repression/derepression phenomenon to explain the presence of a change in ratios of the threonine-sensitive and lysine-sensitive β -aspartate kinases has yet to be obtained. Since specific data on the nature of these inhibitions on β -aspartate kinase and other related enzymes is lacking, the physiological significance of these control patterns remains unclear. Additional information on the compartmentation of enzymes and metabolites and enzyme and amino acid pool sizes will shed light on mechanisms of control.

Beta-aspartate kinase activity has been demonstrated in the developing endosperm of Zea mays L. (Henke and Wahnbaeck, 1977; Gengenbach et al., 1978; Spencer, 1977) as have homoserine dehydrogenase (Gengenbach et al., 1978) and diaminopimelate decarboxylase activities (Sodek, 1978). These enzymes have been shown to undergo developmental pattern changes, with peaks in total activity at approximately 15 to 25 days post-pollination, the period of most active protein synthesis (Henke and Wahnbaeck-Spencer, 1979). These enzyme activities appear to be subject to regulatory controls and have the capacity of providing amino acids needed for storage protein synthesis in the developing maize endosperm. Research in this area will offer new potentials for selection of an improved nutritional balance of amino acids and storage proteins in the kernel.

Subcellular Localization of the Aspartate Family of Amino Acids

The subcellular localization of the biosynthetic machinery for the aspartate family of amino acids in chloroplasts and proplastids of higher plants has been suggested in recent literature. In vivo labeling studies by Mills and Wilson (1978) have shown that isolated pea chloroplasts are capable of light-driven protein synthesis as well as the incorporation of carbon-labeled CO₂ or aspartate into the aspartate family of amino acids. Mills et al. (1980) demonstrated that lysine at 2 mM could inhibit its own formation as well as that of homoserine, isoleucine and threonine in the light-dependent incorporation of ¹⁴C-aspartic acid of isolated chloroplasts from Pisum sativum L.

Several enzyme activities involved in the biosynthesis of lysine, threonine and methionine have been found to be associated with plastids. Beta-aspartate kinase activity was found to be at least partially associated with the chloroplast from spinach (Wahnbaeck-Spencer et al., 1979); homoserine dehydrogenase from maize seedlings (Bryan et al., 1977) and diaminopimelate decarboxylase from Vicia faba by Mazelis et al. (1976) have also been found to be associated with the chloroplast. These results provide more evidence for plastid involvement in nitrogen metabolism and the synthesis of lysine, threonine and methionine.

Plastids have been isolated from many plants and plant tissues. The study of the subcellular compartmentation of enzymes utilized morphological and biochemical markers for various subcellular compartments. Some of the marker enzymes used have been succinate

dehydrogenase (Zilkey and Canvin, 1972), fumarase (Sparace and Moore, 1979) and cytochrome c oxidase (Boardman and Wildman, 1962) as mitochondrial markers, catalase (Luck, 1965) as a microbody marker, and triosephosphate dehydrogenase (Heber et al., 1963), triosephosphate isomerase (Gibbs and Turner, 1964) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Nishimura et al., 1976) as plastid markers. The demonstration of these enzymes in cereal endosperm has not yet been performed. Neither has the isolation of subcellular fractions using traditional methods and markers been carried out in these tissues. The research presented in this report demonstrates that this approach can be used in the identification of subcellular organelles in the developing maize endosperm.

Plastids of developing seed cotyledons and endosperm have been found to be capable of extensive metabolism of carbohydrates and nitrogen. The "undifferentiated" plastids, termed proplastids, have been isolated by a variety of techniques. Castor bean (Ricinus communis L.) endosperm has been studied extensively for the localization of carbohydrate metabolizing enzymes of the glyoxylate, glycolytic and pentose phosphate pathways. These enzymes were found to be localized in the mitochondria and glyoxysomes. However, Zilkey and Canvin (1972) found unsaturated fatty acid synthesis associated with the plastid fraction. When this fraction was viewed with electron microscopy, the plastid was characterized by a very electron dense matrix, a double-limiting membrane, and the presence of a prolamellar body.

Sucrose metabolism was studied by Simcox et al. (1977) in castor bean endosperm. They demonstrated the presence of intermediate steps from sucrose to pyruvate, which is then incorporated into fatty acids in proplastids present in developing endosperm at 30 to 40 days after pollination. The localization of the enzymes involved in sucrose metabolism in proplastids indicates that the initial steps of glycolysis may be functioning in this compartment. This provides more evidence that most steps required for the generation of acetyl-CoA from carbohydrates may be present in the proplastid.

Nitrogen and amino acid metabolism have also been studied in relation to compartmentalization of the enzymes involved in several tissues. Miflin and Beevers (1974) reported the isolation of intact plastids from several plant tissues, including pea leaves, pea roots and castor bean endosperm. In 1974, Miflin used spinach leaves and pea and barley roots to localize nitrite reductase and other amino acid biosynthetic enzymes in the plant tissues. Root plastids were found to contain nitrite reductase, acetoacetate synthetase, glutamine synthetase and aspartate aminotransferase activities. Washitani and Sato (1978) also localized nitrogen-assimilating enzymes in the proplastids of non-green cells of cultured tobacco cells. Glutamate synthase and glutamine synthetase were found to be present in the proplastid fraction, suggesting that the glutamine synthetase-glutamate synthase pathway for primary transamination was located in the proplastid. Even though these studies suggest that the plastid is the site of extensive carbohydrate and nitrogen metabolism, little

investigation has been made into the subcellular localization of nitrogen metabolism in the developing endosperm of maize.

The occurrence of storage protein bodies in the endosperm matrix has stimulated some recent studies of the subcellular localization of organelle-specific enzymes and proteins in the maize endosperm. Khoo and Wolf (1970) followed development of protein bodies throughout the maturation of the corn endosperm. The isolated protein bodies contain, as a major component, the prolamin zein, a lysine-poor storage protein. Electron microscopy of the maize endosperm revealed the presence of membrane-bound, starch-containing proplastids in kernels 7 to 10 days after pollination. Protein bodies were found to develop from the endoplasmic reticulum vesicles and ends of the cisternae, therefore, supporting the nonplastid origin of the granules; however, no physiologic function was assigned to the proplastids.

The presence of enzymes of the aspartate family of amino acids in the plastids of many plants and in the developing endosperm of maize suggests that the pathway could be localized in the plastids of the developing endosperm. The importance of understanding the regulatory aspects of the synthesis of the essential amino acids lysine, threonine and methionine and their deposition into storage protein mandates the study of all aspects of their metabolism. The subcellular localization of the pathway enzymes and the amino acid pools may provide additional insight into the complex regulatory patterns which allow for the synthesis of the amino acids under many physiological conditions. Further studies of the de novo synthesis,

uptake and incorporation of these amino acids into free and bound pools in the endosperm will provide additional information regarding the regulation of the aspartate family of amino acids pathway in vivo.

III. ASSOCIATION OF THE ASPARTATE FAMILY ENZYMES WITH SUBCELLULAR FRACTIONS IN THE DEVELOPING ENDOSPERM OF MAIZE

Abstract

Organelles were fractionated from the developing endosperm of maize utilizing the technique that combines rate and equilibrium density separation of organelles on a combined continuous/discontinuous sucrose gradient. This technique allows separation of microbodies, mitochondria and plastids with little cross-contamination. The following marker enzymes were used to identify intact plastids: triosephosphate isomerase, triosephosphate dehydrogenase and ribulose-1,5-bisphosphate carboxylase. Intact mitochondria and intact microbodies were identified by cytochrome c oxidase and catalase activities, respectively. The subcellular distribution of three enzymes involved in the synthesis of the aspartate-derived amino acids lysine, threonine and methionine was examined. Beta-aspartate kinase (EC 2.7.2.4) and diaminopimelate decarboxylase (EC 4.1.1.20) activities were found, at least in part, to be associated with the plastid fraction at $\rho=1.23$ g/cc, whereas, homoserine dehydrogenase (EC 1.1.1.3) activity appears to be solely (98%) associated with the soluble, or cytoplasmic, fraction. These data suggest that plastids may play a role in the synthesis of amino acids, even in the nongreen endosperm.

Introduction

The localization of the enzymatic machinery for the synthesis of amino acids has been studied recently in the leaf tissue of several plant species (for a review, see Bryan, 1980). Although considerable information exists as to the subcellular distribution of enzymes involved in glycolysis, the photosynthetic carbon reduction and glyoxylate cycles (Quail, 1979), little information existed until recently on the subcellular distribution of the enzyme machinery involved in nitrogen metabolism, particularly amino acid metabolism.

The important role of the plastid in nitrogen metabolism has been pointed out by Lea et al. (1980) and Mills et al. (1980). Enzymes required for aromatic amino acid synthesis (Bickel et al., 1978) and those required for the aspartate family of amino acids (as reviewed by Bryan, 1980) have been found to be present in the chloroplasts of higher plants. Mills et al. (1980) demonstrated the light-stimulated synthesis of amino acids in the aspartate pathway from ^{14}C -labeled precursors in isolated pea chloroplasts.

Several enzyme activities involved in the synthesis of lysine, threonine, methionine and isoleucine (commonly referred to as the aspartate-derived pathway) have been observed to be present in the developing endosperm of maize. These enzymes include β -aspartate kinase (Henke and Wahnbaeck, 1977; Gengenbach et al., 1978), homoserine dehydrogenase (Gengenbach et al., 1978), aspartate β -semialdehyde dehydrogenase (Gengenbach et al., 1978) and diaminopimelate decarboxylase (Sodek, 1978).

This section reports the results of an identification of the subcellular compartments in the developing maize endosperm and association of the aspartate family of enzymes (β -aspartate kinase, homoserine dehydrogenase and diaminopimelate decarboxylase) with the subcellular fractions.

Materials and Methods

Plant Source

Seeds of maize (Zea mays L., cv. DeKalb XL43) were obtained from DeKalb AgResearch, Inc., DeKalb, Illinois. The seeds were grown in 2 gallon pots containing a 2:2:1 mixture of sand:perlite:peat under greenhouse conditions. Self-pollinated ears were collected at 15 to 25 days post-pollination, degermed and the endosperm was used fresh for the following studies.

Sucrose Density Gradient Centrifugation

Endosperm tissue was chopped with a razor blade for 15 minutes in 15 ml of isolation medium (per 100 endosperm; 5.0 gm) in a Petri dish kept on ice. The isolation medium consisted of 10 mM KCl, 1 mM $MgCl_2$, 0.1% BSA and 5 mM EDTA made to volume with 25% sucrose (w/v) in 0.1 M Tricine buffer, pH 7.5. All homogenates were filtered through several layers of cheesecloth and the filtrate was layered onto the density gradient. The sucrose gradient was that described by Mifflin and Beevers (1974). It consisted of 4 ml 60% (w/v) sucrose pad, overlaid with a 6 ml 60%-42% gradient, 5 ml 42% sucrose, 8 ml 42%-25% gradient and a final layer of 3 ml of 25% sucrose, all prepared in 0.1 M Tricine buffer, pH 7.5. Ten ml of the homogenate

were layered onto the gradient. Immediately after applying the homogenate, the tubes were placed in an SW-27 rotor in a Beckman Spinco L.5-65 centrifuge and centrifuged at 2500 g for 5 minutes, and then at 18000 g for 20 minutes. The gradients were fractionated into samples of 1.2 ml. Sucrose concentrations were determined by refractometry and protein was determined by a modified Lowry on acid-precipitated protein (Schacterle and Pollack, 1973).

Enzyme Assays

The following enzyme assays were employed in order to identify potential organelle fractions in the maize endosperm. All enzymes except ribulose-1,5-bisphosphate carboxylase* were measured spectrophotometrically at 25 C using a Beckman Model 25 double-beam spectrophotometer. Catalase (EC 1.11.1.6), used as a microbody marker, was assayed by the method of Luck (1965); cytochrome c oxidase (EC 1.9.3.1), used as a mitochondrial marker, was assayed by the method of Hackett (1964) as described by Tolbert et al. (1968); triosephosphate isomerase (EC 5.3.1.1), used as a plastid marker, was assayed by the method of Gibbs and Turner (1964) as described by Schnarrenberger et al. (1972); NADPH-triosephosphate dehydrogenase (EC 1.2.1.9), used as a plastid marker, was assayed by the method of Heber et al. (1963) and NADH-cytochrome c reductase (EC 1.6.99.3) used as an endoplasmic reticulum and protein body marker, was assayed by the method described by Larkins and Hurkman (1978). Ribulose-1,5-bisphosphate carboxylase (RuBP carboxylase) (EC 4.1.1.39) was also used as a plastid marker and

*Hereafter termed RuBP carboxylase.

was assayed by the fixation of NaHCO_3 ($^{14}\text{CO}_2$) (New England Nuclear; 6.2 mCi/mmol) into acid stable products as described by Lorimer et al. (1977). The enzyme was preincubated with 20 mM bicarbonate for 10 minutes at 30 C to insure full activation and the reaction was initiated with 0.5 mM RuBP, prepared by the method of Horecker et al. (1958).

Enzymes of the aspartate pathway were assayed as follows. Beta-aspartate kinase (EC 2.7.2.4) was assayed by the hydroxylamine method of Black and Wright (1955) as described by Henke and Wahnbaeck (1977). Homoserine dehydrogenase (EC 1.1.1.3) was assayed by following the reduction of NAD^+ in the presence of 25 mM L-homoserine, using the method of Bryan (1969). Diaminopimelate decarboxylase (EC 4.1.1.20) was assayed by following decarboxylation of ^{14}C -diaminopimelate (New England Nuclear; 105.26 mCi/mmol) by the method of Sodek (1978).

Electrophoresis

Disc gel electrophoresis of sucrose density gradient fractions was carried out using 7.5% polyacrylamide gels. One hundred μl of sample from the sucrose gradient fraction were layered onto the gel and then electrophoresed at 5 ma/tube. The gels were then removed from the tubes and placed in a reaction buffer to stain for homoserine dehydrogenase activity. The reaction buffer consisted of 60 mM Tris-HCl, pH 9.0, containing 0.15 mM EDTA, 0.21 mM 2-mercaptoethanol, 150 mM KCl, 32 mM L-homoserine, 0.266 mg/ml NBT, 0.025 mg/ml PMS and 0.96 mM NADP^+ , following the method of Matthews et al. (1975). The reaction was allowed to proceed for 30 minutes to 1 hour at 25 C.

The gels were then washed free of reaction mixture and scanned with a densitometer at 600 nm. Nonspecific activity in the gels was determined in reaction mixture minus homoserine.

Electron Microscopy

Isolated subcellular fractions were pelleted and fixed in 2.5% glutaraldehyde with 0.05 M Na cacodylate buffer, pH 7.0, overnight at 4 C. Samples were rinsed four times with the cacodylate buffer minus glutaraldehyde and post-fixed in 1% OsO₄ in 0.05 M Na cacodylate buffer, pH 7.0, for 30 minutes at room temperature. Samples were rinsed well with the cacodylate buffer and dehydrated in an alcohol series. After the final 100% alcohol rinse, samples were placed in propylene oxide (Polyscience, Inc.) at room temperature for 10 minutes. The samples were then embedded in Spurr's resin (Polysciences, Inc.) in a flat embedding mold (Ladd Industries). After sectioning on an ultramicrotome, the sections were post-stained with 0.5% uranyl acetate and 1.0% lead citrate and viewed in an Hitachi Model H-600 electron microscope.

Results

Identification of Subcellular Fractions by Sucrose Density

Centrifugation

The subcellular compartments present in the endosperm were identified by determining the distribution of traditional "marker" enzymes for organelles in fractions obtained from a combined equilibrium density and sedimentation velocity sucrose gradient

(Figures 3, 4). Three major protein bands corresponding to the soluble, microbody and proplastid fractions were identified at the top of the gradient, in the fraction containing 48% sucrose ($\rho=1.24$ at 0 C)* and the fraction containing 40% to 42% sucrose ($\rho=1.22$ at 0 C), respectively. This is similar to the observations made by Simcox et al. (1977) on a linear sucrose gradient of developing castor bean endosperm.

The distribution of traditional marker enzymes across a sucrose gradient (Figure 3) indicates that a peak of catalase activity is present in the fraction containing 48% to 50% sucrose ($\rho=1.24$ at 0 C), a peak in cytochrome c oxidase activity occurs in the fractions which contain 34% to 36% sucrose ($\rho=1.16$ to 1.18 at 0 C) designating the mitochondrial fraction, and that triosephosphate isomerase and RuBP carboxylase activities are present in substantial portion (40% to 50%) in the fraction containing 45% sucrose ($\rho=1.23$ at 0 C), designating the plastid fraction (Figure 4). [This is similar to the distribution of enzymes observed from sucrose gradients of castor bean endosperm (Mifflin and Beevers, 1974).] Analysis of the distribution of cytochrome c reductase activity (data not shown) as the marker for endoplasmic reticulum and protein bodies resulted in an even distribution of the enzyme activity across the gradient, possibly due to the varied sizes of endoplasmic reticulum pieces resulting from tissue homogenization, and the varied sizes of protein bodies.

* ρ =Density in g/cc sucrose.

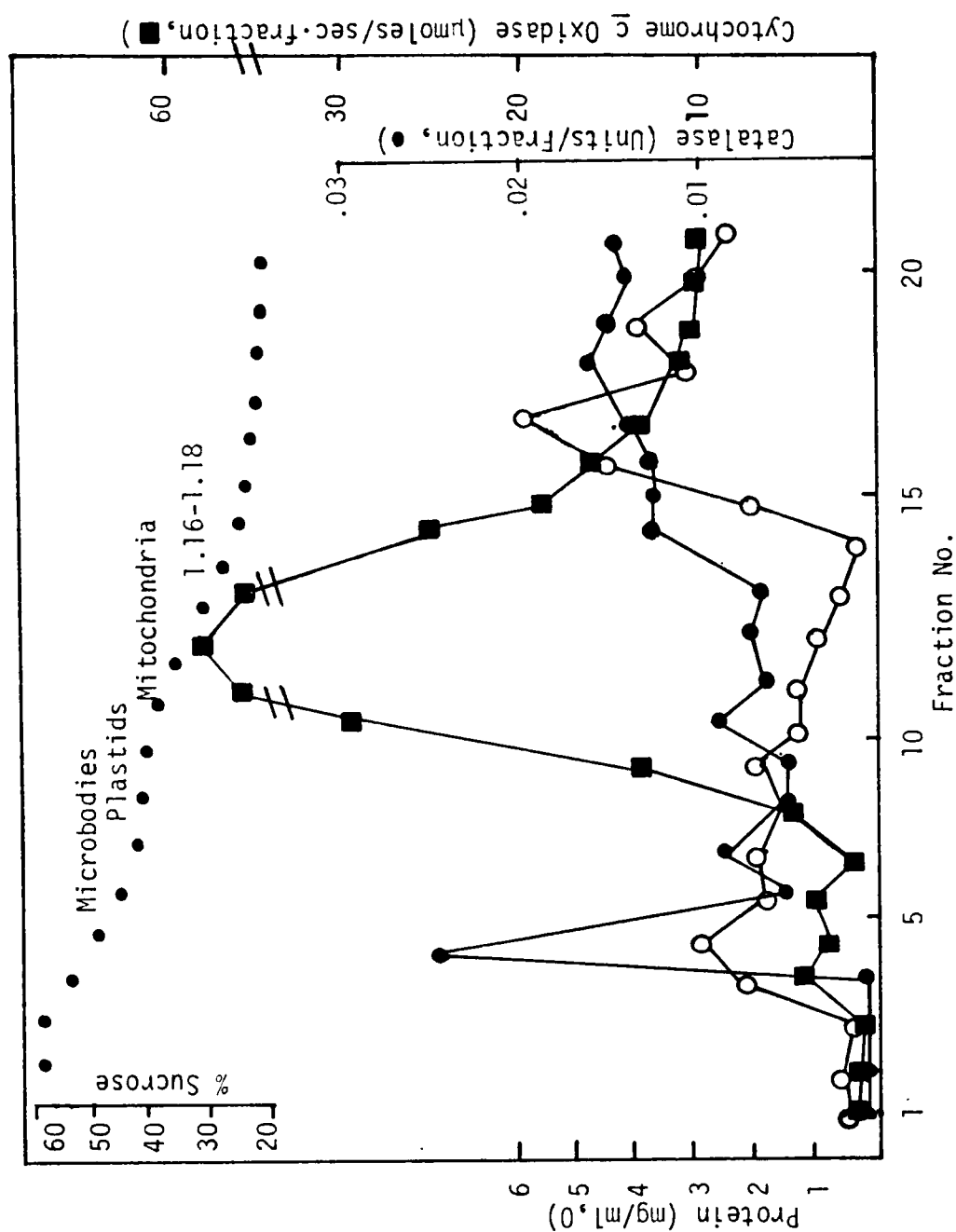


Figure 3. Distribution of enzyme activities catalase, cytochrome c oxidase and protein across a continuous/discontinuous sucrose gradient. Catalase (●), cytochrome c oxidase (■), and protein (o). Densities at which leaf organelles occur are indicated. Catalase units: Change in absorbance at 280 nm/min.

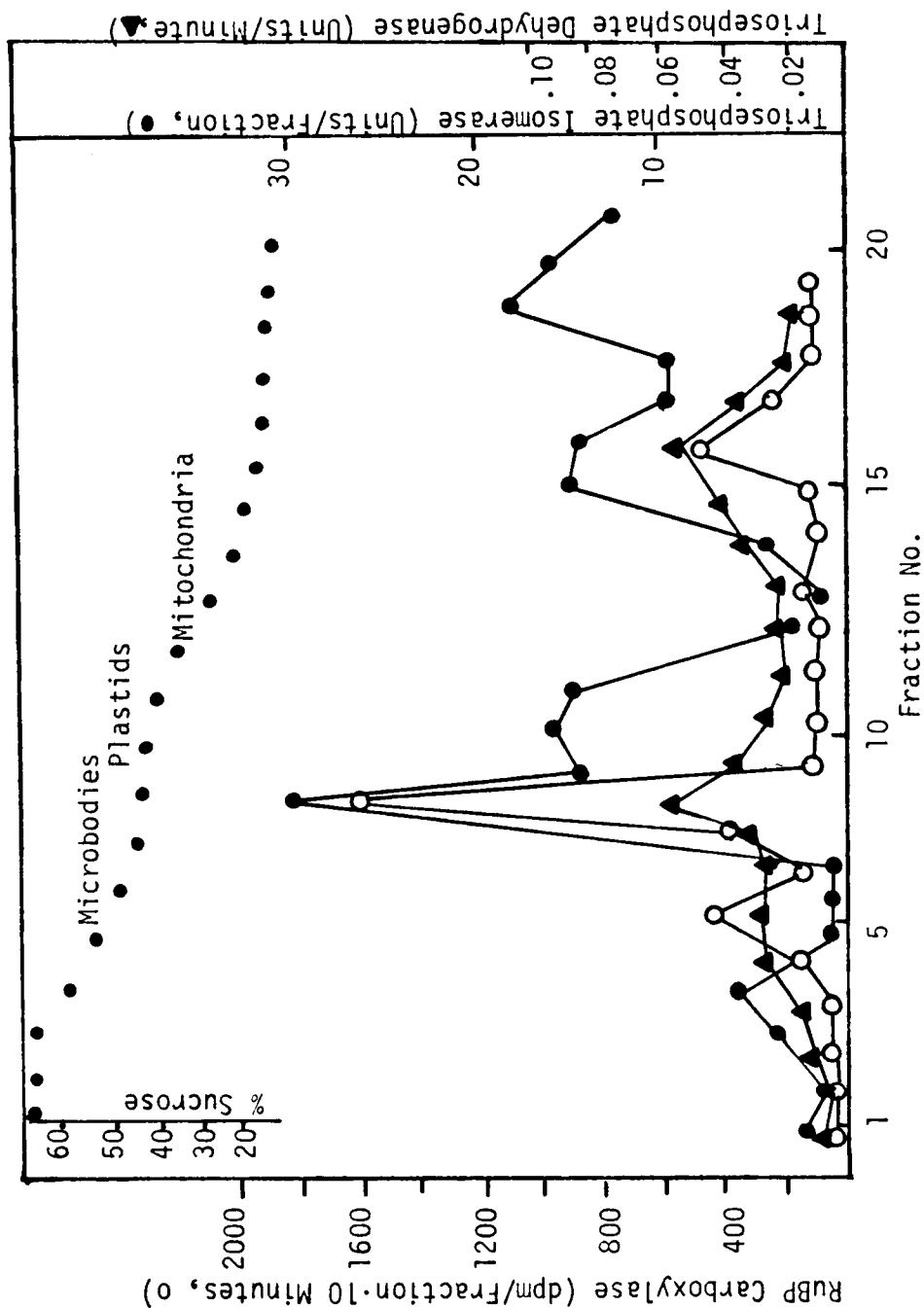


Figure 4. Distribution of triosephosphate isomerase, triosephosphate dehydrogenase and ribulose-1,5-bisphosphate carboxylase activities across a sucrose gradient. Triosephosphate isomerase (●), triosephosphate dehydrogenase (▲) and ribulose-1,5-bisphosphate carboxylase (o). Densities at which leaf organelles occur are indicated. Units of triosephosphate isomerase were and triosephosphate dehydrogenase activities are change in absorbance at 340 nm/minute.

Electron Microscopy

The crude homogenate was examined by electron microscopy. The population of organelles present in the crude homogenate (Figure 5) includes mitochondria, protein bodies, some unidentified membrane-bound vesicles and cellular debris.

Association of Aspartate Family Enzymes with Subcellular Fractions

The distribution of enzymes associated with the aspartate-derived amino acid pathway was analyzed in subcellular fractions obtained from a combined sedimentation velocity and equilibrium density gradient separation of organelles from a crude endosperm homogenate (shown in Figures 6 and 7). Distribution of traditional marker enzymes on a gradient is shown in Figure 6, with catalase, cytochrome c oxidase and RuBP carboxylase showing peaks in activity at 42% sucrose ($\rho=1.22$ at 0 C), 40% sucrose ($\rho=1.17$ at 0 C) and 45% sucrose ($\rho=1.23$ at 0 C), respectively.

Analysis of the association of the enzymes β -aspartate kinase, homoserine dehydrogenase and diaminopimelate decarboxylase activities with subcellular fractions (Figure 7) indicates that a substantial portion of β -aspartate kinase is associated with the fractions containing 43% to 45% sucrose ($\rho=1.23$ at 0 C). Diaminopimelate decarboxylase activity also shows a peak in activity in the fractions corresponding to 43% to 45% sucrose ($\rho=1.23$ at 0 C). The low specific activity of diaminopimelate decarboxylase associated with this fraction is not an artifact. The activity associated with this fraction is within the limit of sensitivity of the assay. Homoserine dehydrogenase, on the

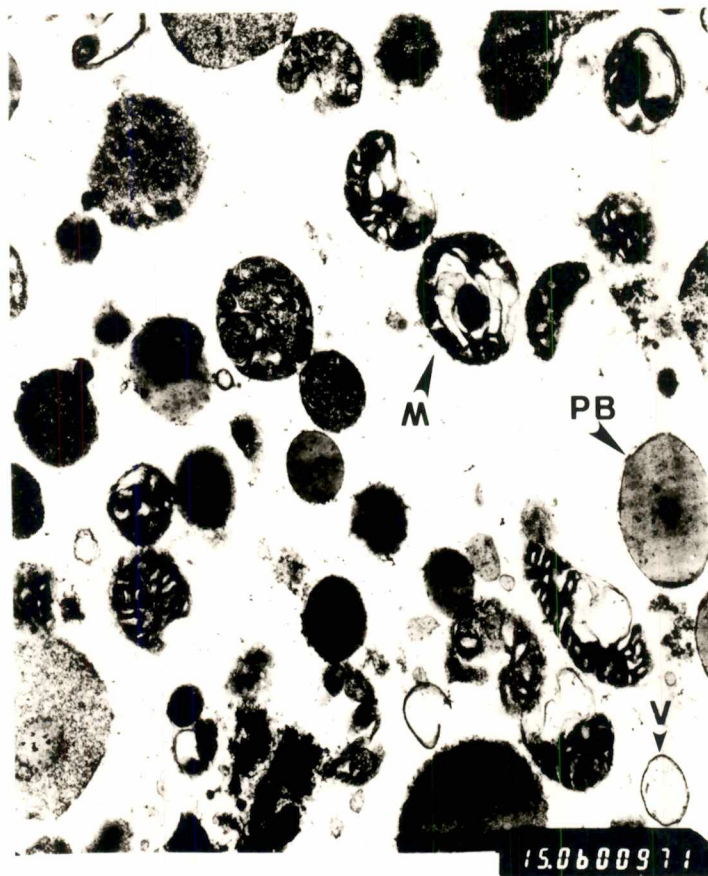


Figure 5. Electron micrograph of crude endosperm homogenate. m=mitochondrion, pb=protein body, v=membrane vesicles. Magnification x 15000.

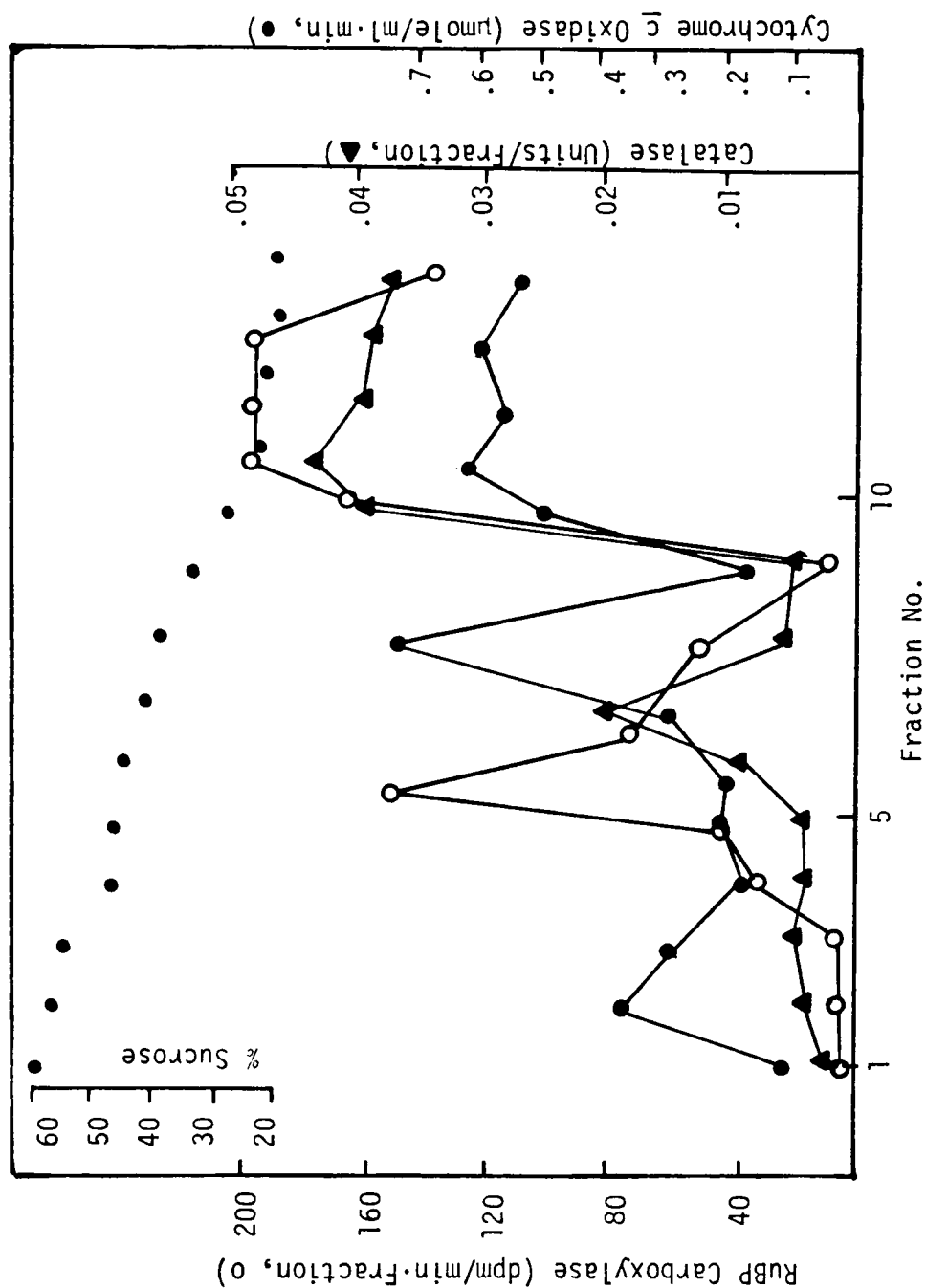


Figure 6. Distribution of enzyme activities across a second sucrose gradient. Catalase ($\blacktriangle$), cytochrome c oxidase ($\bullet$), and ribulose-1,5-bisphosphate carboxylase (o) activities were examined. Catalase units are change in absorbance at 280 nm/minute.

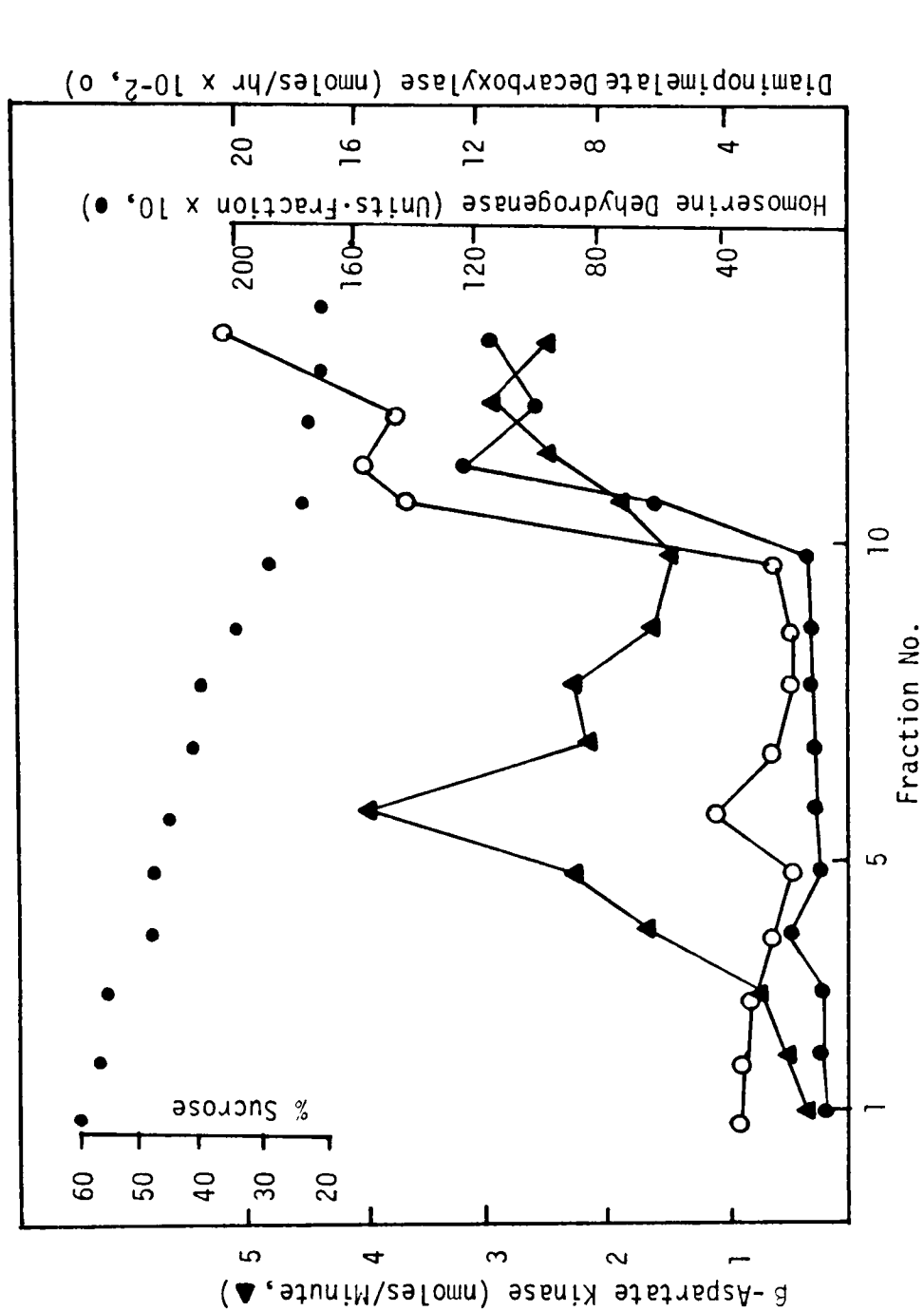


Figure 7. Distribution of aspartate family enzymes across a sucrose gradient. β-aspartate kinase (▲), homoserine dehydrogenase (●), and diaminopimelate decarboxylase (○) activities are shown. Units of homoserine dehydrogenase activity are change in absorbance at 340 nm/10 minutes.

other hand, does not appear to be associated with any organellar fraction, with the majority (greater than 98%) present in the soluble fraction. The distribution and specific activity of the enzyme activities in microbody, mitochondrial, plastid and soluble fractions (Table 1) indicates that β -aspartate kinase and diaminopimelate decarboxylase are present in the plastid fraction at 34% and 12% of the total recovered activities, respectively. The remaining activities are located in the soluble portion of the gradient. This distribution is approximately equal to the distribution of the plastid enzyme RuBP carboxylase.

Examination of the sucrose gradient fractions for isozyme forms of homoserine dehydrogenase activity following the technique described by Matthews *et al.* (1975) is shown in Figure 8. The densitometer tracings are those of gels which did stain for homoserine dehydrogenase activity in the presence of homoserine at 32 mM. The tracings represent the crude homogenate and fractions numbered 13 and 14 from the top of the gradient. Four peaks are present in each tracing, with fraction number 13 enriched with peak 1 and fraction 14 enriched for peak 2. The isozymes, therefore, all appear to be associated with the soluble fractions of the gradient.

Discussion

Enzymes which convert aspartate to lysine, threonine and methionine have previously been found in the developing maize endosperm (Gengenbach *et al.*, 1978; Sodek, 1978; Henke and Wahnbaeck, 1977; Henke and Wahnbaeck-Spencer, 1979). The results reported here suggest

Table 1. Distribution of enzyme activities across the sucrose gradient.

Enzyme Activity	Subcellular Fractions				Total Crude Activity
	Microbody	Mitochondria	Plastid	Soluble	
Catalase ^a (% Total)	35 (14)	10 (4)	14 (5.6)	190 (76)	270
Cytochrome <u>c</u> Oxidase ^b (% Total)	450 (19.6)	1270 (27.2)	750 (16.0)	2200 (47)	5184
RuBP Carboxylase ^a (% Total)	40 (5.5)	70 (9.6)	165 (23)	450 (62)	884
β-Aspartate Kinase ^a (% Total)	4.0 (7.5)	10 (18.0)	18.1 (34.0)	21.0 (39)	74
Homoserine Dehydrogenase ^a (% Total)	2.0 (<1.0)	2.0 (<1.0)	4.0 (<1.0)	425 (98)	500
Diaminopimelate Decarboxylase ^c (% Total)	2.0 (2.3)	6.0 (7.0)	8.8 (12)	67 (80)	100

^aUnits/fraction.^bnmol/min·ml.^cnmol/hr x 10⁻².

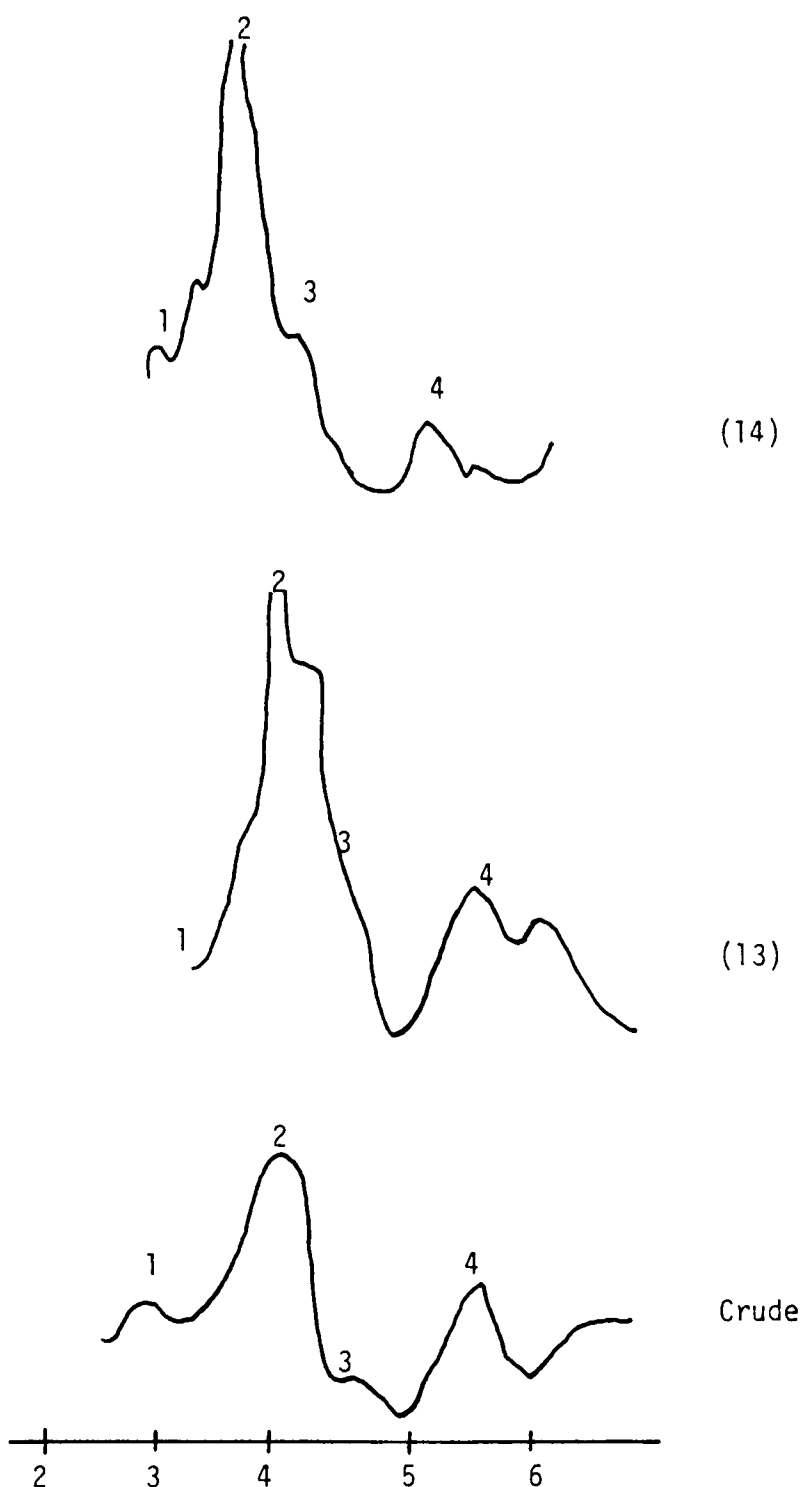


Figure 8. Densitometer scans of homoserine dehydrogenase activity on 7.5% polyacrylamide gel electrophoresis. Top: Fraction 14; Middle: Fraction 13; Bottom: Crude.

that β -aspartate kinase and diaminopimelate decarboxylase activities are associated, at least in part, with the plastid fraction of a crude endosperm extract after sucrose density centrifugation. Homoserine dehydrogenase activity, however, does not appear to be associated with any subcellular organelle, and is thus a cytoplasmic enzyme. This result conflicts with the previous localization of homoserine dehydrogenase within the chloroplast in green leaf tissue from Vicia faba (Bryan et al., 1977). Homoserine dehydrogenase activity, on the other hand, may be weakly associated with the plastid, possibly a stromal enzyme as was found for homoserine dehydrogenase in maize leaf chloroplasts (Bryan et al., 1977) and pea leaf chloroplasts (Burdge et al., 1979; Wallsgrove et al., 1979). The aspartate family enzymes studied appear to be particulate (Bryan, 1980) (with the exception of homoserine dehydrogenase in maize endosperm tissue) and may be functioning in amino acid biosynthesis in the plastids of green leaf tissue and non-photosynthetic endosperm.

The function of the pathway in the synthesis of lysine, threonine and methionine in the intact, developing endosperm was shown by Sodek (1976) by the labeling of these amino acids after incubation of the endosperm tissue with ^{14}C -aspartate and ^{14}C -acetate. Experiments in our laboratory (see Section V; see pp. 86, 88) also indicates that lysine, threonine and methionine are formed from ^{14}C -aspartate and ^{14}C -acetate after an eight hour incubation and that the pathway may be under some regulatory control.

The expression of the regulatory potential of enzymes is dependent on their interaction with substrate, effectors, etc.

Eucaryotic cells contain compartments such as mitochondria, plastids, microbodies and cytoplasm which can segregate enzymes and low molecular weight compounds. Information on the compartmentation of enzymes in organelles is gradually accumulating. A complete characterization and identification of subcellular compartments in the developing endosperm of maize had not been reported to date. Larkins and Hurkman (1978) studied the compartmentation of zein in protein bodies of maize endosperm and utilized several enzyme markers to identify this fraction and the mitochondrial fraction. Miflin et al. (1981) also examined the nature of protein bodies in developing cereal endosperms and pea cotyledons. Triosephosphate isomerase activity was found to be associated with a sucrose fraction at $\rho=1.225$ g/cc. Again, a more complete characterization of subcellular fractions was not performed. The existence of plastids in this tissue had been reported by Khoo and Wolf (1970) as a result of electron microscopy of developing endosperm tissue. The plastids contain starch grains and some vesicles and an electron dense matrix. The function of the plastids, which do not develop into chloroplasts, but undergo dessication as a result of the maturation of the maize kernel, is as yet unknown. The presence of several enzyme activities with this fraction, for example, RuBP carboxylase, triosephosphate isomerase and β -aspartate kinase and diaminopimelate decarboxylase activities, suggests that the plastid may perform a metabolic function in the developmental physiology of the immature endosperm. Other non-green tissues have been shown to contain functional plastids which

participate in active synthesis of sugars from stored fat (Benedict, 1973).

The subcellular localization of enzymes of amino acid metabolism and amino acid pools may provide additional insight into the complex regulatory patterns which allow synthesis of amino acids under many physiologic conditions and new potentials for selection of improved nutritional balance of amino acids and storage proteins in the maize kernel.

IV. PARTIAL PURIFICATION AND CHARACTERIZATION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE FROM THE DEVELOPING ENDOSPERM OF MAIZE

Abstract

The presence of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 3.1.1.4) in the developing endosperm of maize (Zea mays L.) was demonstrated in this tissue. The enzyme was detected in developing endosperm, with a peak in specific and total activities at 10 to 15 days post-pollination. A substantial portion of the activity was associated with a proplastid fraction, as identified by triosephosphate isomerase and triosephosphate dehydrogenase activities. The partially purified enzyme from sucrose density gradient centrifugation showed a specific activity of 25 nmoles (min·mg protein)⁻¹. The enzyme co-electrophoresed with tobacco ribulose-1,5-bisphosphate carboxylase on 7.5% polyacrylamide disc gels. The pattern exhibited by the partially purified enzyme on denaturing 12.5% SDS slab gels resembles that of purified tobacco carboxylase, in that there is a band at 5.5×10^4 daltons and one at 1.2×10^4 daltons. Immunologic analysis of the partially purified endosperm enzyme indicated that under conditions of a standard Ouchterlony, the protein was not cross-reactive with anti-tobacco serum. However, when tested for inhibition of immuno-precipitation between tobacco carboxylase protein and anti-tobacco serum, the maize extract prevented precipitation, indicating cross-reactivity.

Introduction

The nonphotosynthetic endosperm of developing and germinating castor bean and lupin seeds has been shown to contain many enzymes of the gluconeogenic, amino acid and lipid biosynthetic pathways. Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 3.1.1.4),* an integral component of the reductive pentose phosphate cycle in photosynthetic organisms, has also been shown to be present in the non-photosynthetic endosperm of developing castor bean seeds (Simcox et al., 1977), the endosperm and cotyledonary tissues of germinating castor bean seedlings (Benedict, 1973; Osmond et al., 1975; Gillen et al., 1976; Dockerty et al., 1977) and developing seeds of Lupinus albus (Atkins and Flinn, 1978). RuBP carboxylase is responsible for the synthesis of two molecules of 3-phosphoglyceric acid from RuBP and carbon dioxide. In addition, it also has an oxygenase activity in which phosphoglycolate and 3-phosphoglyceric acid are formed from RuBP and oxygen. The functions attributed to this enzyme in the nonphotosynthetic tissue have been postulated to be one of salvage fixation of carbon dioxide liberated when seeds respire in the light (Atkins and Flinn, 1978) or active synthesis of sugars from carbon derived from stored fat when metabolized (Benedict, 1973).

The need to obtain a suitable plastid marker for proplastids in the developing maize endosperm for localization studies of an amino biosynthetic pathway prompted the search for RuBP carboxylase activity.

*Henceforth termed RuBP carboxylase.

This paper reports, for the first time, the presence of RuBP carboxylase in the nonphotosynthetic endosperm of a developing cereal grain. It examines its presence, intracellular location and properties.

Materials and Methods

Plant Source

Seeds of maize (Zea mays L., var. DeKalb XL43) were obtained from DeKalb AgResearch, Inc., DeKalb, Illinois. The seeds were planted in a soil mixture of 2:2:1 (sand:perlite:peat) and grown under greenhouse conditions. Developing seeds were obtained from self-pollinated plants at 5 to 45 days post-pollination. Endosperm tissue was obtained after removal of the seed coat and embryo tissues.

Enzyme Isolation and Purification

Homogenates of fresh endosperm were obtained from 15 day post-pollination kernels by grinding the tissue in a Virtis homogenizer with 25 mM Tris-HCl, pH 7.5 containing 50 mM KCl, 1 mM EDTA and 1 mM DTT. The ratio of volume of buffer to weight of tissue was 2:1. The homogenate was centrifuged at 20000 x g for 15 minutes at 0 to 4 C.

The enzyme was partially purified by the one-step method described by Goldthwaite and Bogorad, 1971. Five ml of the homogenate were layered onto a linear 7% to 24% sucrose gradient (w/v), buffered with 0.02 M Tris-HCl at pH 8.0, containing 10 mM MgSO₄ and 1 mM DTT. The linear gradients were centrifuged for 14 hours at 0 to 4 C at 20000 x g in an SW 27.1 rotor in a Beckman L.5-65 ultracentrifuge. After centrifugation, the gradients were collected in 1.2 ml fractions

and protein was determined by measuring absorbance at 280 nm in a Beckman double-beam spectrophotometer. Sucrose was determined by refractometry. RuBP carboxylase activity was measured by the method of Lorimer et al. (1977).

The peak of RuBP carboxylase activity from the sucrose gradient was found to contain some contaminating proteins with nearly the same molecular weight as determined on 7.5% polyacrylamide gels. To further purify the enzyme, the pooled fractions from the sucrose gradient were dialyzed overnight against glycylglycine, 0.05 M, pH 8.0 buffer. The dialyzed fraction was added to a DEAE-A52 Cellulose column equilibrated with the same buffer. After washing with the column buffer to remove unattached protein, the bound protein was eluted from the column by a 0 to 0.5 M KCl gradient, prepared in glycylglycine, 0.05 M, pH 8.0 buffer. The fractions were collected and analyzed for protein and RuBP carboxylase activity and the conductivity was measured.

Sucrose Density Gradient Centrifugation

Association of RuBP carboxylase activity with subcellular fractions of an endosperm homogenate was determined by sucrose density gradient centrifugation. Endosperm (15 days post-pollination) tissue was sliced with a razor blade for 15 minutes in 15 ml isolation medium (per 100 endosperm) in a Petri dish kept on ice. The isolation medium consisted of 10 mM KCl, 1 mM $MgCl_2$, 0.1% BSA and 5 mM EDTA made to volume with 25% (w/v) sucrose in 0.1 M Tricine buffer, pH 7.5. All homogenates were filtered through several layers of cheesecloth and

the filtrate was layered directly onto the density gradient. The sucrose gradient was that described by Mifflin and Beevers (1974) and consisted of 4 ml of a 60% (w/v) sucrose pad, overlayers with a 6 ml 60% to 42% (w/v) sucrose gradient, 5 ml of 42% (w/v) sucrose, 8 ml of a 42% to 25% (w/v) sucrose gradient and a final layer of 25% (w/v) sucrose, all prepared in 0.1 M Tricine buffer, pH 7.5. Ten ml of the homogenate were layered onto the gradient. Immediately after application of the homogenate, the tubes were placed in an SW-27 rotor in a Beckman Spinco L.5-65 centrifuge at 2500 x g for 5 minutes, and then at 18000 x g for a further 20 minutes. The gradients were fractionated into samples of 1.2 ml. Sucrose concentrations were determined by refractometry and protein was determined by a modified Lowry on acid-precipitated protein (Schacterle and Pollack, 1973).

Enzyme Assays

All enzymes except RuBP carboxylase and PEP carboxylase were assayed spectrophotometrically at 25 C using a Beckman Model 25 spectrophotometer. Catalase (EC 1.11.1.6), used as a microbody marker, was measured by the method of Luck (1965); cytochrome c oxidase (EC 1.9.3.1), used as a mitochondrial marker, was measured by the method of Hackett (1964) as described by Tolbert et al. (1968); triosephosphate isomerase (EC 5.3.1.1), used as a plastid marker, was assayed following the method of Gibbs and Turner (1964), as described by Schnarrenberger et al. (1972), and NADPH-triosephosphate dehydrogenase (EC 1.2.1.9), used as a plastid marker, was assayed by the method described by Heber et al. (1963). NADH-cytochrome c reductase

(EC 1.6.99.3), used as a marker for endoplasmic reticulum and protein bodies, was measured by the method described by Larkins and Hurkman (1978). PEP carboxylase (EC 4.1.1.31) was measured by the method described by Wong and Davies (1973). RuBP carboxylase (EC 4.1.1.39) activity was measured as fixation of NaHCO_3 ($^{14}\text{CO}_2$) (New England Nuclear; 6.2 mCi/mmol) into acid stable products, as described by Lorimer et al. (1977). The enzyme was preincubated with 25 mM bicarbonate and MgCl_2 for 10 minutes at 30 C to activate and the reaction was initiated by the addition of RuBP (prepared by the method of Horecker et al., 1958) to a final concentration of 0.5 mM.

Polyacrylamide Gel Electrophoresis

Electrophoresis of crude, partially purified endosperm carboxylase and purified tobacco carboxylase samples was carried out on a 7.5% polyacrylamide disc gel electrophoresis, using a continuous buffer of Tris-glycine (0.05 M, pH 8.3). Electrophoresis was carried out at 15 mamps for 60 to 90 minutes. The gels were fixed and stained overnight in a solution containing 8.6% acetic acid (v/v), 43% methanol (v/v) and 0.1% Coomassie Brilliant Blue (w/v). The gels were then destained in a solution of 23% methanol and 7% acetic acid.

Electrophoresis of purified enzyme samples and standards was also carried out on 12.5% SDS-denaturing slab gels (method of Laemmli, 1970; as described by Larkins and Hurkman, 1978). The gels were 1.5 mm thick and consisted of a 9.5 cm running gel of 12.5% acrylamide in 3.75 mM Tris-HCl (pH 8.9), 0.058 mM TEMED and 0.075% SDS (w/v). The running gel was overlaid with 2.5 cm stacking gel

consisting of 5% acrylamide. Samples were applied in a buffer of 0.024 M Tris-HCl, pH 8.3, containing 1% SDS, 1% 2-mercaptoethanol, 0.002% bromophenol blue (w/v) and 10% glycerol. Samples were allowed to sit at room temperature for 30 minutes before boiling to denature the protein. Electrophoresis was carried out at room temperature with 15 mamps constant current through the stacking gel and 25 mamps constant current through the running gel until the tracking dye reached the bottom of the gel. The gels were fixed and stained overnight in a solution of 43% (v/v) methanol, 8.6% acetic acid (v/v) and 0.1% Coomassie Brilliant Blue (w/v), and destained overnight in a solution of 23% methanol (v/v) and 7% acetic acid (v/v).

Molecular weight standards included purified tobacco RuBP carboxylase (prepared by the method of Chan et al., 1972), purified RuBP carboxylase from Rhodospirillum rubrum (obtained from Dr. Fred Hartman, Oak Ridge Biology Division), cytochrome c, bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A (all obtained from Sigma Chemical Co., St. Louis, Mo.).

Immunological Analysis

Purified tobacco RuBP carboxylase was prepared by the method of Chan et al. (1972). This protein preparation was used to raise antibodies in a New Zealand White Rabbit. Serum was prepared by allowing the blood to clot and precipitating the clear liquid twice by bringing to 40% saturation with ammonium sulfate, centrifuging and resuspending the pellet in phosphate-buffered saline, pH 7.0, and dialyzing against this buffer. Ouchterlony plates were prepared with

1% Agarose A (obtained from Pharmacia Chemicals, Uppsala, Sweden) in a phosphate-buffered saline, pH 7.0. The plates were inoculated with the serum and the proteins to be studied and were incubated at 22 C for 24 to 36 hours until precipitation bands developed. The plates were then dried and stained with Coomassie blue (0.5% in 10% acetic acid and 45% methanol). The plates were then destained with 43% methanol and 7% acetic acid.

Results

Development of RuBP Carboxylase Activity with Time

The development of RuBP carboxylase activity (both specific and total) was followed in crude extracts prepared from tissue harvested 4 to 45 days after pollination (Figure 9). The activity shows a peak for both specific and total activities at 10 to 15 days post-pollination. The specific activity of the enzyme is low, therefore, the degree of error may be high and the second, smaller increase in activity at 30 days post-pollination may be an artifact. PEP carboxylase activity is approximately ten times that of RuBP carboxylase and shows a peak at 15 days post-pollination, with a steady increase to maturity. The peaks in activity precede that observed for enzymes involved in amino acid interconversions, i.e., β -aspartate kinase (Henke and Wahnbaeck-Spencer, 1979) and glutamate synthase (Sodek, 1978).

Localization of Endosperm RuBP Carboxylase

The association of RuBP carboxylase with a subcellular fraction in this tissue was determined after preliminary identification of

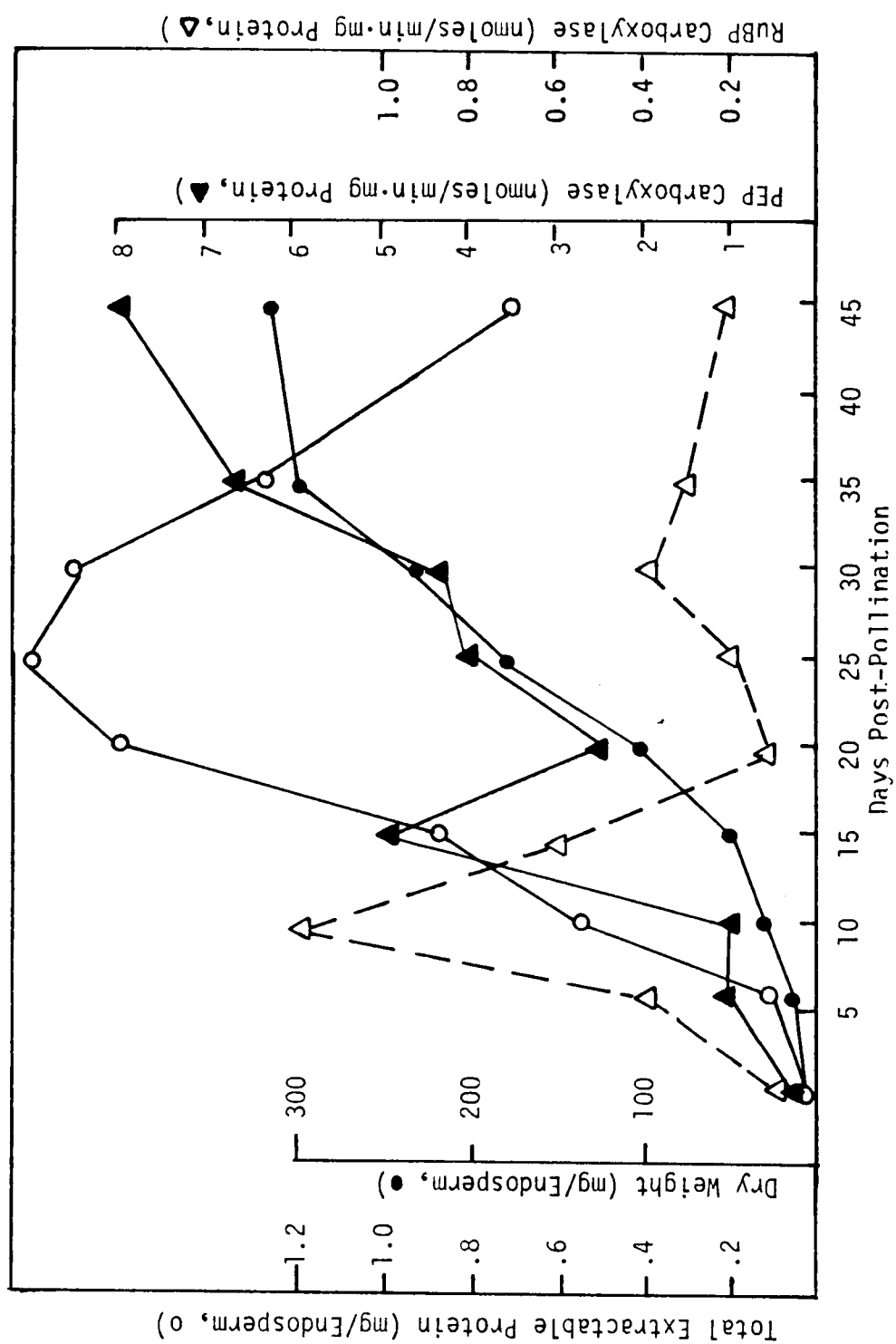


Figure 9. Development of RuBP and PEP carboxylase activities in the maturing, non-photosynthetic maize endosperm.

subcellular fractions using enzyme markers established for organelles in other tissues. The complex gradient designed by Miflin and Beevers (1974) permits rapid separation of microbodies, mitochondria and plastids. Results of organelle preparations are seen in Figure 3, page 28. Microbodies, plastids and mitochondria are at their peak densities at 1.25, 1.20 and 1.16 to 1.18 g/cc, respectively.

Results of a second gradient, where triosephosphate dehydrogenase was the plastid marker, are seen in Figure 4, page 29. Approximately 46% of the RuBP carboxylase activity applied to the gradient is associated with a plastid fraction as identified by the small peak in triosephosphate dehydrogenase activity. This fraction also represents the fraction containing the peak in triosephosphate isomerase activity and contains sucrose at a density of 1.23 g/cc. Therefore, at least part of the enzyme activity is associated with the proplastid fraction.

Partial Purification of Endosperm RuBP Carboxylase

Purification of the carboxylase by traditional column chromatography resulted in low specific activity. For this reason, the technique of Goldthwaite and Bogorad (1971) was used to concentrate quantities of enzyme in a short period of time. RuBP carboxylase was typically found in the peak corresponding to 17% sucrose ($\rho=1.08$ g/cc at 0 C) (Figure 10). The purification which resulted is seen in Table 2. Even though there was a single peak of RuBP carboxylase activity on the sucrose gradient, corresponding to a peak of protein, this protein peak resolved into several bands when electrophoresed on 7.5% disc gels. Therefore, the protein peak from the sucrose gradient

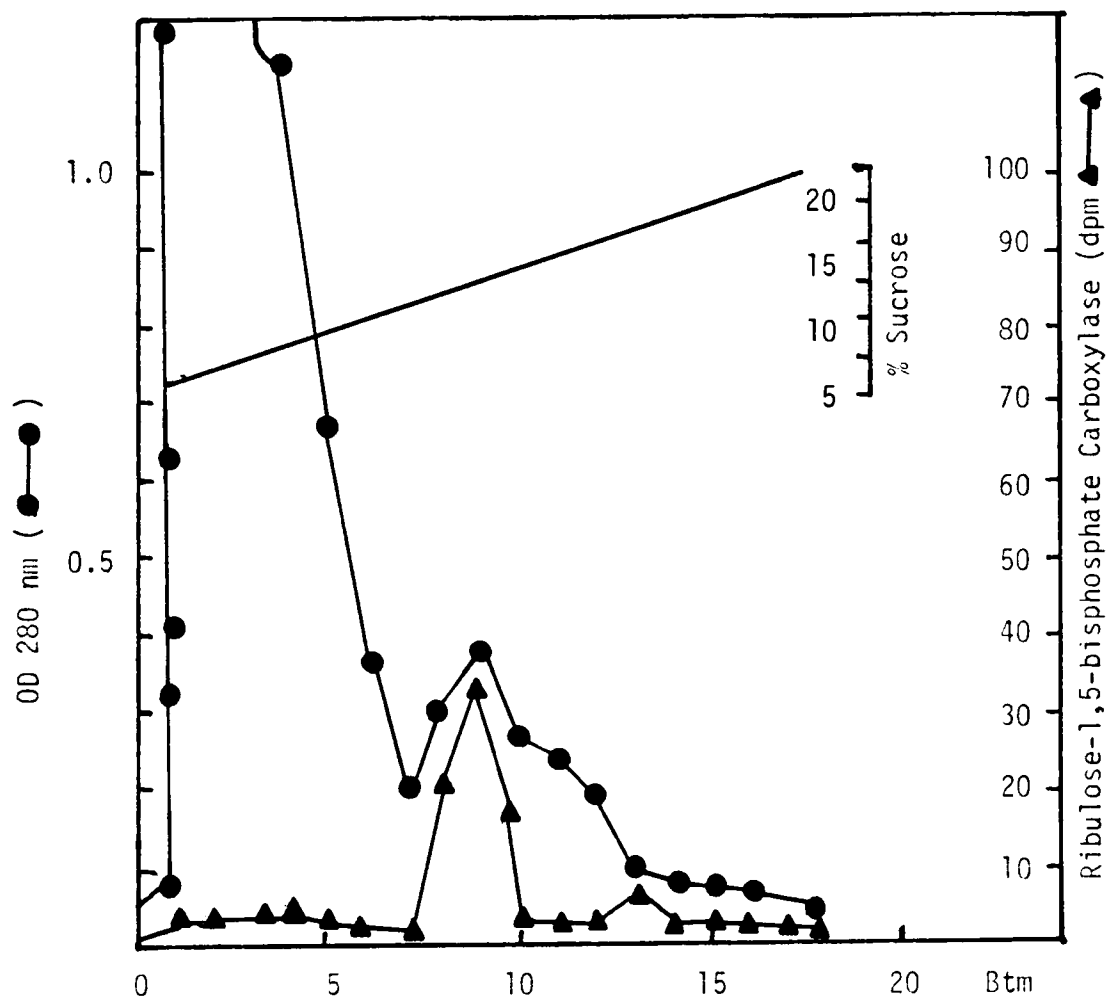


Figure 10. Partial purification of endosperm RuBP carboxylase on a linear sucrose gradient. Sample volume 5.0 ml. The centrifugation time was 14 hours at 20,000 x g in an SW-27.1 rotor.

Table 2. Purification of endosperm RuBP carboxylase.

Fraction	Volume (ml)	Specific Activity (nmoles/min·mg prot.)	Fold Purification	Total Protein (mg)
Crude	10	0.31	--	45.0
Sucrose Gradient Fraction 9	120	21.50	60	9.6
Concentrated Sucrose Fractions	3.5	25.00	60	9.6
DEAE Chromatography	3.0	25.00	60	4.0

containing RuBP carboxylase was pooled, dialyzed against glycyglycine buffer, 0.05 M, pH 8.0 and applied to a DEAE-A52 Cellulose ion-exchange column. The protein was eluted with a gradient of 0 to 0.5 M KCl and the elution profile is seen in Figure 11. One peak in absorbance at 280 nm resulted, with RuBP carboxylase eluting at approximately 10 mmho. The fractions were pooled, concentrated with ammonium sulfate precipitation, and assayed for RuBP carboxylase activity. This purification as seen in Table 2 resulted in 60 fold purification with a specific activity of 25 nmoles/min·mg protein.

Some Properties of the Enzyme

The enzyme was shown to have a reaction rate that was linear with time and enzyme concentration. Homogeneity of the RuBP carboxylase obtained from DEAE Cellulose chromatography is shown in Figure 12. The enzyme co-electrophoresed with the purified tobacco enzyme on 7.5% polyacrylamide gels. Analysis of the subunit composition of the partially purified enzyme on 12.5% SDS gels (Figure 13) indicates that the endosperm contains a small and large subunit with similar molecular weights as purified tobacco RuBP carboxylase (Figure 14).

Analysis of the cross-reactivity of the endosperm and maize leaf preparations to purified tobacco carboxylase (Figure 15) on standard Ouchterlony plates, suggests possible non-identity of the endosperm enzyme. Cross-reactivity of the maize leaf enzyme occurred with tobacco RuBP carboxylase, suggesting partial identity of antigenic determinants of the two sources of carboxylase.

Another test for cross-reactivity, or identity, was used in order to determine if the Ouchterlony test resulted in a false

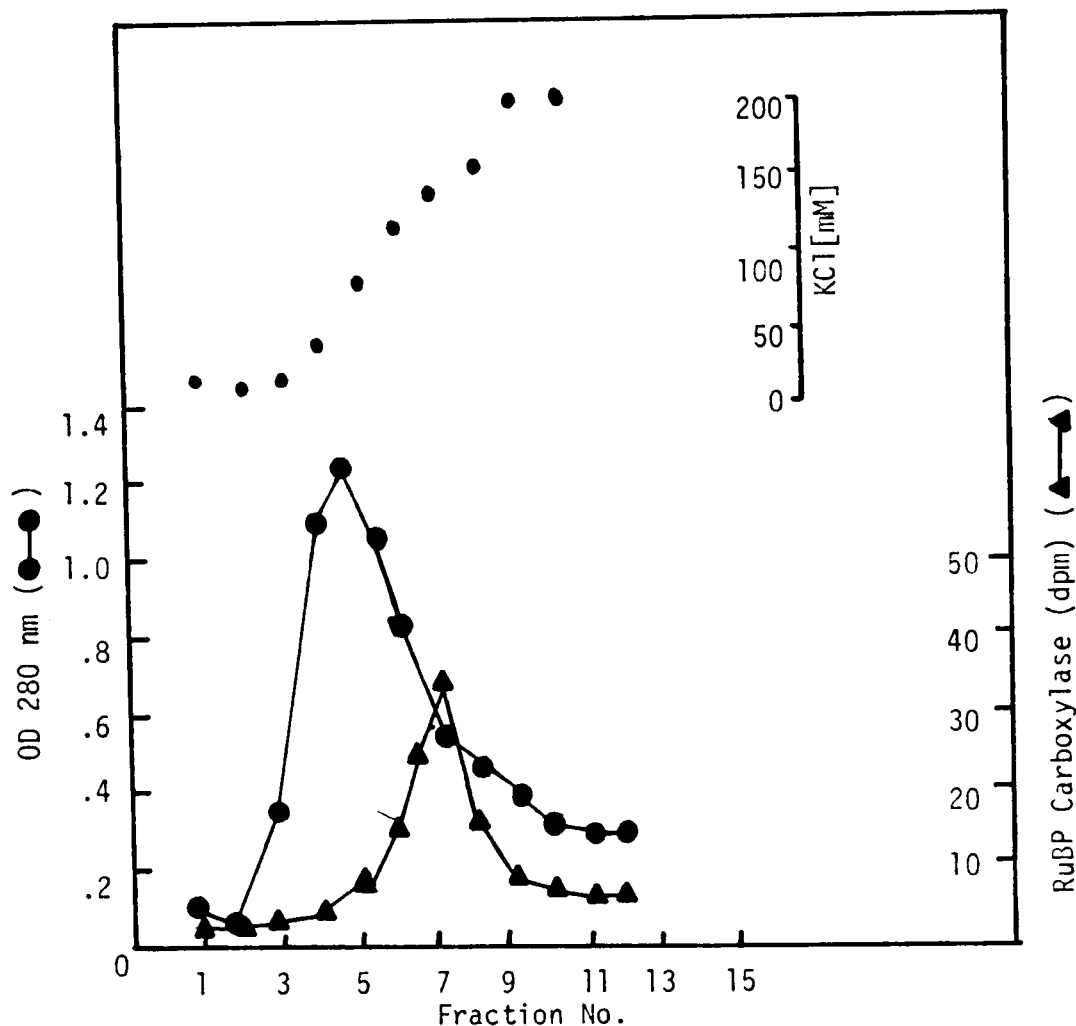


Figure 11. Partial purification of sucrose density peak by DEAE-A52 cellulose chromatography. The 7.0 mg protein was loaded onto a 6 ml bed volume column. The column was washed with glycylglycine buffer, 0.05M, pH 8.0, and protein was eluted from the column with a 0-0.5 M KCl gradient.

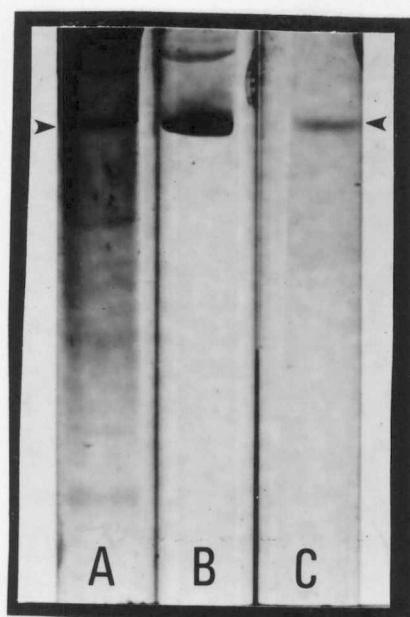


Figure 12. Polyacrylamide gel electrophoresis of crude (A), purified tobacco (B) and purified maize endosperm (C) RuBP carboxylase. 500 μ g crude, 100 μ g tobacco and 179 μ g endosperm protein were applied to 7.5% polyacrylamide gels pH 8.5. After the electrophoretic run, the gels were stained with Coomassie Brilliant Blue. Arrows designate the RuBP carboxylase protein bands.

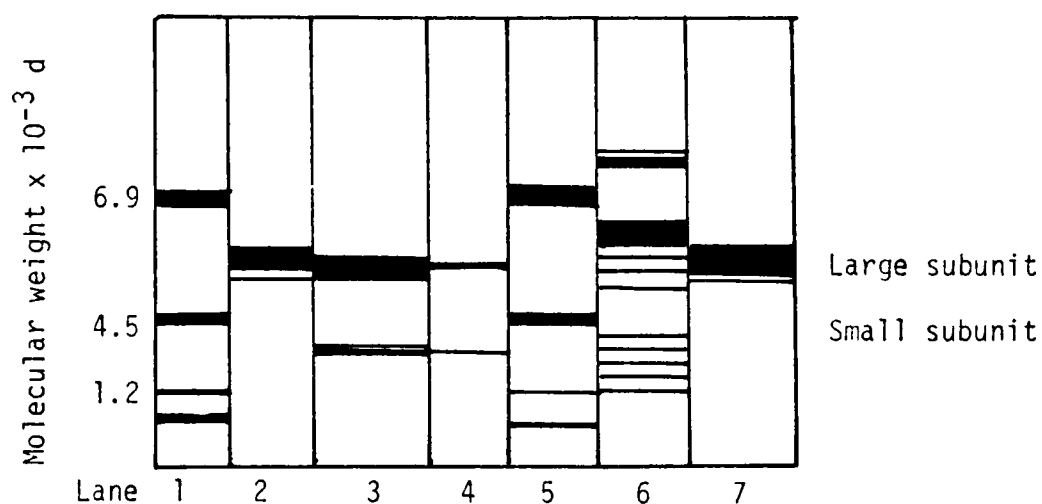


Figure 13. Results of 12.5% SDS slab gel electrophoresis. 10 μ g of protein were added to each lane and the gel was electrophoresed at 25 mamps constant current through the running gel. Samples are as follows: Lane 1 - Standards Lane 2 and Lane 7 - *Rhodospirillum rubrum*, Lane 3 - spinach RuBP carboxylase, Lane 4 - endosperm RuBP carboxylase from DEAE, Lane 5 - Standards and Lane 6 - Crude endosperm protein.

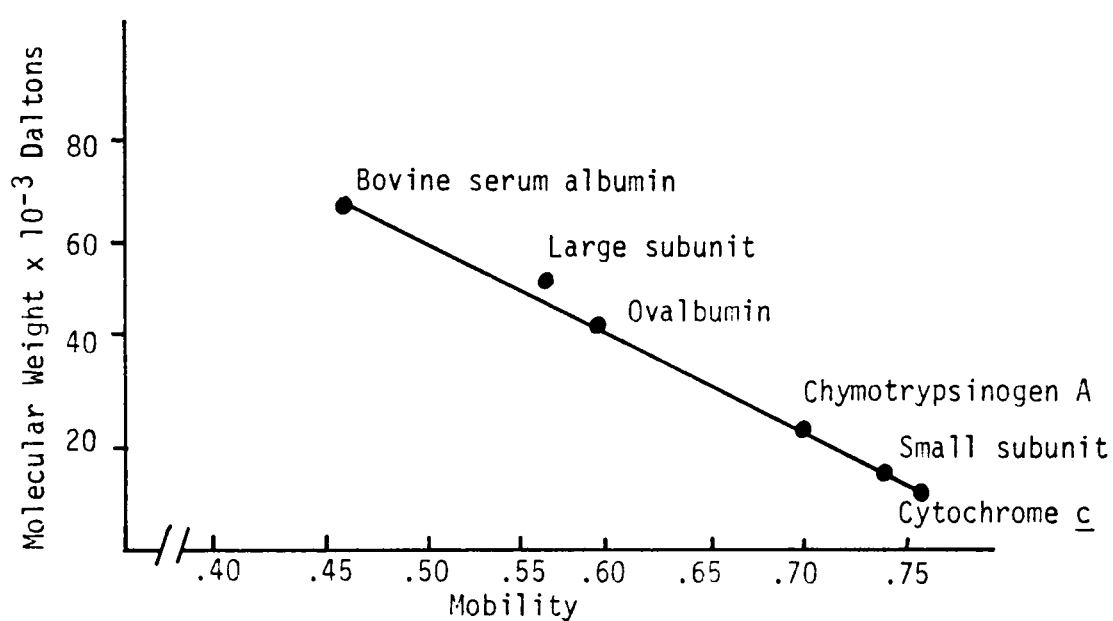


Figure 14. Mobility of endosperm RuBP carboxylase on 12.5% SDS slab gel electrophoresis.

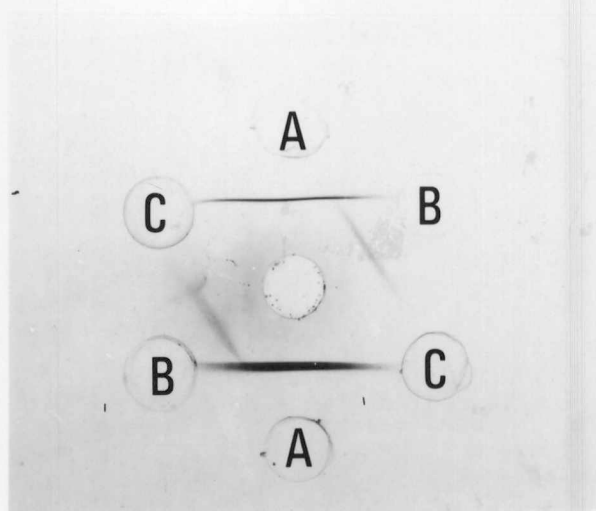


Figure 15. Immunochemical characterization of maize leaf and maize endosperm RuBP carboxylase. In the center well was placed anti-crystalline tobacco RuBP carboxylase, 65 μ g protein. The peripheral wells contain: A - tobacco RuBP carboxylase (crystalline) 3.5 μ g protein; B - maize leaf carboxylase 90 μ g protein; C - maize endosperm carboxylase 36 μ g protein.

negative, that is, if the lack of a precipitin band for endosperm carboxylase vs. anti-tobacco serum resulted from true non-identity or the lack of sufficient Ag-Ab complex to result in a visible band. The precipitin inhibition test of Kwapinski (1965) was used to determine if endosperm carboxylase would be inhibitory to precipitin band formation by anti-tobacco serum and tobacco carboxylase. As seen in Figure 16 (no. 3), the endosperm preparation of 230 μ g protein is inhibitory to precipitin band formation, while a boiled, centrifuged endosperm preparation is not inhibitory, indicating a protein is involved in the inhibition.

Discussion

The initial purpose of this investigation was to obtain a suitable plastid marker for proplastids in the developing maize endosperm. The localization of a substantial portion (46%) of the RuBP carboxylase activity with the proplastid fraction from sucrose gradients of endosperm extracts indicates that it can be used as an enzyme marker for these organelles.

The homogeneous protein was composed of a large and small subunit as evidenced by the denaturing 12.5% SDS slab gel electrophoresis (Figure 15). This enzyme is composed of the same molecular subunits as the tobacco RuBP carboxylase, with a band at 5.5×10^4 daltons and a band at 1.2×10^4 daltons. The structural similarities, however, are not mirrored by immunological identities. Under our conditions, the antiserum prepared against crystalline tobacco carboxylase was cross-reactive with maize leaf and tobacco RuBP carboxylase

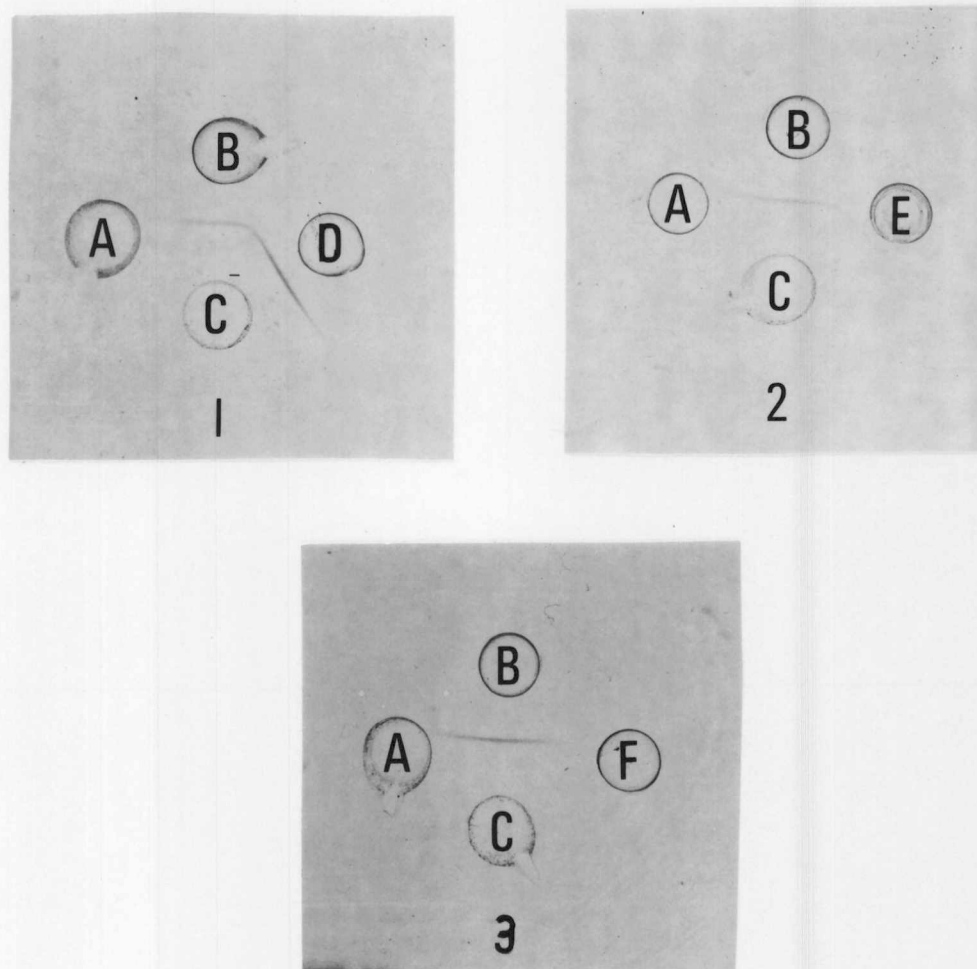


Figure 16. Precipitin inhibition test. A - Phosphate buffered saline, B - Tobacco carboxylase, 3.5 µg protein, C - Anti-tobacco carboxylase serum 65 µg protein, D - Tobacco carboxylase, 3.5 µg protein, E - Maize leaf preparation 90 µg protein, F - Maize endosperm partially purified RuBP carboxylase 230 µg protein.

(Figure 15), but not cross-reactive against endosperm carboxylase. Many experimental conditions using the standard Ouchterlony assay resulted in negative results for reactivity. This may be a false negative. If there is only a small amount of antigen-antibody complex, a visible band on an Ouchterlony plate may not be evident. Further experimentation using the precipitin-inhibition test (Figure 16) suggests that there is some serological identity of the endosperm carboxylase to the crystalline tobacco carboxylase. Additional experimentation including double antibody precipitation and immunofluorescence may provide additional evidence in favor of cross-reactivity of the nonphotosynthetic endosperm RuBP carboxylase to the tobacco carboxylase. Precedence for the immunological identity of a nonphotosynthetic RuBP carboxylase with spinach carboxylase was provided by Osmond et al. (1975) for germinating castor bean endosperm.

The development of RuBP carboxylase activity in the endosperm of developing maize seeds showed a maximum increase (under defined growing conditions) at 10 to 15 days post-pollination, with a subsequent decrease to maturity. The reason for the increase in activity has not been resolved. De novo synthesis of enzyme protein or increase in enzyme activity are both possible. The very low specific activity observed for both crude and purified preparations suggests that this enzyme may not be functional in the fixation of carbon dioxide. Primary fixation of carbon dioxide, if it occurs in the endosperm, may be performed by PEP carboxylase, which has a higher specific activity and lower K_m for CO_2 of 0.02 mM (Ting and Osmond, 1973) as compared to

the K_m for HCO_3^- of 2-4 mM commonly exhibited for higher plant RuBP carboxylase (Ryan and Tolbert, 1975).

The presence of RuBP carboxylase in non-photosynthetic tissue has been noted previously, although its function in this tissue has not been clearly defined. The presence and catalytic function of other enzymes of the reductive pentose phosphate pathway have generally not been established, and it would not seem that such tissue could in any sense be autotrophic, since energy to run the carbon fixation must come from already reduced carbon compounds. At best, the enzyme might catalyze dismutation, or may be responsible for the production of glycolate. Even these functions are difficult to establish. The overwhelming amount of PEP carboxylase activity may indicate that whatever CO_2 -salvage fixation occurs in this tissue goes through oxaloacetic acid and the gluconeogenic pathway, rather than through a pentosephosphate pathway. Until the enzyme complement of this tissue is better understood, however, this must remain speculation.

It is possible that the expression of RuBP carboxylase in non-photosynthetic tissue reflects a structural or storage function for this protein. Since the stromal space of chloroplasts contains high concentrations of protein and at least 50% of this protein is RuBP carboxylase, it is conceivable that the presence of this protein is necessary to some extent to establish a functional structure for the organelle. The large amount of RuBP carboxylase in the chloroplast has been interpreted as evidence that it is a storage protein (Huffaker and Miller, 1978). This is possible in the endosperm tissue, although the relatively small amount of the protein actually present

makes this possibility unlikely. Finally, it is possible that the protein is expressed only incidentally during the course of development, as the result of a leaky gene, although this seems unlikely in view of the energy requirement of protein synthesis.

V. IN VIVO REGULATION OF THE SYNTHESIS OF
ASPARTATE-DERIVED AMINO ACIDS IN THE
DEVELOPING MAIZE ENDOSPERM

Abstract

A sharp increase in β -aspartate kinase, homoserine dehydrogenase and diaminopimelate decarboxylase activities occurs during the development of the maize endosperm, suggesting that the levels of these enzymes are under some type of regulation. A twenty-five fold increase in extractable β -aspartate kinase activity from 12 to 19 days post-pollination was observed that coincided with or slightly preceded the onset of storage protein synthesis. However, only a three-fold increase in soluble protein levels occurred during this same period, suggesting regulation at the gene level. Sensitivity of extractable β -aspartate kinase activity to 1 and 5 mM lysine did not change at different stages of development. Injection of high levels (200 nmoles/endosperm) of lysine into the intact endosperm at 12 days post-pollination prevented the increase in β -aspartate kinase and homoserine dehydrogenase activities. These experiments suggest that in vivo regulation of enzyme activity can be studied in the intact, developing maize endosperm.

Introduction

Developing seeds receive their nutrition through the vascular connections present in the base of the seed. The supply of amino acids

and sugars present in the translocation stream are provided by the photosynthate mobilized from the vegetative tissue of the plant (Felker and Shannon, 1980) and from the root system (Oaks and Beevers, 1964). The amino acid complement of the seed reserves differs markedly from that present in the vegetative tissue and in the translocation stream that feeds the developing seed (Arruda and daSilva, 1979). Also, the complement of soluble amino acids in the developing seed change in both the level and composition during development (Arruda et al., 1978). These soluble amino acids function to some extent as precursors to the synthesis of the storage proteins. There are, however, major differences in the composition of the soluble and the protein amino acid pools.

Amino acids required for the synthesis of storage proteins in the developing endosperm can originate from two sources: 1) supplied preformed from the vascular sap, and/or 2) de novo synthesis (or transamination) in the developing endosperm tissues. The primary amino acid found to be present in the vascular sap in maize is glutamine at 30 μmol /100 μmol at 7 to 42 days post-pollination (Arruda and daSilva, 1979). Glutamine is a high nitrogen amino acid capable of participating in transamination reactions to provide nitrogen for other amino acids. Other amino acids have been found to be present in the vascular sap, but not in amounts high enough to account for all storage protein synthesis, therefore, de novo synthesis of amino acids in the developing endosperm may account for the pools of amino acids needed for protein synthesis. In fact, enzymes which participate in amino acid conversions, such as glutamate synthase

(Sodek and daSilva, 1977), β -aspartate kinase (Henke and Wahnbaeck-Spencer, 1979), homoserine dehydrogenase (Gengenbach et al., 1978), aspartate β -semialdehyde dehydrogenase (Gengenbach et al., 1978) and diaminopimelate decarboxylase (Sodek, 1978) have been found to be present in the developing maize endosperm and exhibit developmental-dependent changes in extractable levels of activity.

Few studies have been performed to determine the extent to which various sources of nitrogen contribute to the nitrogen budget of the developing seed. Observations that amino acid composition in the sap of vascular tissue entering the developing seed differs from the amino acid composition of the seed suggest that the seed possesses the biosynthetic capabilities for the interconversion of amino acids. Sodek (1976) found through tracer studies with ^{14}C -aspartic acid and ^{14}C -acetic acid injected directly into the maize endosperm, that the biosynthesis of lysine, threonine and methionine and other amino acids occurs in this tissue.

In order to gain a better appreciation of the developmental physiology of the grain and to gain insight into the importance of de novo synthesis in supplying amino acids necessary for protein synthesis, in vivo experiments were performed. These experiments involved the injection of unlabeled amino acids into the developing endosperm in order to evaluate their effects on normal endosperm development and the development of aspartate pathway enzyme activities. Labeled precursors, aspartate and acetate were injected, as well, in order to observe their fate in the absence and presence of

end-product effectors of the aspartate-derived amino acid family (lysine, threonine and methionine).

Preliminary experiments were designed following previous research by Sodek (1970, 1976). Sodek (1970) injected ^{14}C -leucine into the maize stem below the ear at various stages of kernel development. The distribution of label into free and bound amino acids was analyzed. Sodek (1976) injected ^{14}C -aspartate, ^{14}C -acetate and ^{14}C -alanine directly in the developing endosperm and the tissue was analyzed for the distribution of label into storage proteins following acid hydrolysis. These experiments provided information on the amino acid metabolism occurring in the developing maize endosperm.

Materials and Methods

Plant Source

Seeds of maize (Zea mays L. cv. DeKalb XL43) were obtained from DeKalb AgResearch, DeKalb, Illinois. The seeds were planted in two gallon pots containing a soil mixture of 2:2:1 of sand:perlite:peat and grown to maturity under greenhouse conditions. Ears were self-pollinated and harvested 5 to 55 days post-pollination. The embryo and seed coat were removed from the kernels and the endosperm was used fresh or lyophilized for subsequent analysis.

Analysis of Endosperm Development

Endosperm dry weight was determined directly from lyophilized endosperm tissue. Lyophilized endosperm taken from 5 to 55 days post-pollination kernels was ground in a chilled mortar and pestle in

Tris-HCl, 25 mM, pH 7.5 containing 1 mM KCl, 1 mM EDTA and 1 mM 2-mercaptoethanol. The homogenates were centrifuged at 20000 x g for 15 minutes. The supernatants were used to assay for enzymes and soluble protein. Beta-aspartate kinase activity was measured by the method described by Henke and Wahnbaeck (1977); homoserine dehydrogenase activity was measured by the method of Bryan (1969) and diaminopimelate decarboxylase activity was measured by the method of Sodek (1978) by following the decarboxylation of ^{14}C -diaminopimelate. Soluble protein was determined by the modified Lowry method of Schacterle and Pollack (1973) on 10% trichloroacetic acid-precipitable material from a crude endosperm extract. Bovine serum albumin was used as the standard. Total nitrogen was determined by the micro-Kjeldahl method on lyophilized endosperm tissue (Horwitz, 1970). Total protein was calculated by multiplying total nitrogen values by 6.25. Soluble amino nitrogen was determined on a 20% ethanol endosperm extract by the ninhydrin method described by Keleti and Lederer (1974).

Enzyme Mixing Experiments

Endosperm tissue was obtained from kernels harvested at 10, 25 and 40 days post-pollination. The tissue was extracted with a Tris buffer, 0.2 M, pH 8.5, containing 0.1 M KCl, 1 mM EDTA and 1.4 mM 2-mercaptoethanol at 4 C. The crude homogenates were centrifuged at 20,000 x g for 15 minutes and the supernatant was used in the mixing experiments. Beta-aspartate kinase activity was assayed as described above.

In Vitro and In Vivo Effector Studies

In vitro effector studies. Beta-aspartate kinase was partially purified from 15, 25 and 35 days post-pollination endosperm tissue and assayed as described (Henke and Wahnbaeck, 1977). L-lysine (Sigma) was added to the reaction mixture in a final concentration of 1 or 5 mM to determine the effect on β -aspartate kinase activity during a 60-minute assay. L-lysine was also added to the blank in the same final concentration.

In vivo effector studies. All experiments were performed on intact, developing endosperm tissue. Amino acid solutions were prepared in water to a final concentration of 200 nmoles/ μ l and the pH was adjusted to pH 7.0. The solutions were filter sterilized by passage through a Nalgene 0.20 micron filter (Sybron Corporation, Rochester, New York). Prior to injection, the surrounding leaf tissue on the ear was pulled back and the kernels were surface sterilized with 70% (v/v) ethanol. A sterile 10 μ l Hamilton microsyringe was used to inject 1 μ l quantities of solution into the endosperm tissue. Sterile double-distilled water was used as a control. Kernels (approximately 25 to 50 per treatment) were injected at 12 days post-pollination, the surrounding leaves were placed around the ear and the treated kernels were removed after one week incubation, at 19 days post-pollination. The endosperm was removed and was used for analysis of fresh weight, dry weight, total extractable protein, extractable β -aspartate kinase and homoserine dehydrogenase activities.

Fractionation of Endosperm Proteins

The procedure of Sodek and Wilson for the fractionation of the endosperm proteins based on solubilities in various solvents was used (1970). One hundred milligrams of lyophilized endosperm tissue was used, plus 1 ml of solvent in conical centrifuge tubes. Following incubation, the solution in the tubes was clarified by centrifugation at 1000 x g for 10 minutes. Table 3 outlines the extraction procedure. The wash in each case was 1 ml water. Protein was precipitated from each fraction with 20% trichloroacetic acid to a final concentration of 5%. Alcohol extracts were first diluted with one volume water. The acid-soluble fraction in water extracts was taken as the free amino acid fraction. This fraction was shaken four times with one volume of diethylether to remove the acid and the aqueous phase was concentrated on a rotary evaporator.

^{14}C -Tracer Experiments

Isotope source. The isotopes were obtained from New England Nuclear with the following specifications: Na acetate (^{14}C -2) (56.1 mCi/mmol) and L-aspartic acid (U- ^{14}C) (250 mCi/mmol).

Tracer feeding and analysis. Isotopes were dissolved in water to give a final concentration of 50 nmoles and 2 $\mu\text{Ci}/\mu\text{l}$ for acetate and 1 $\mu\text{Ci}/\mu\text{l}$ for aspartic acid. Each isotope was injected (1 μl) by Hamilton microsyringe directly into the surface-sterilized endosperm tissue at 10, 25 and 40 days post-pollination. At least 25 to 50 kernels were injected for each treatment and were allowed to

Table 3. Fractionation scheme of storage proteins from maize endosperm.

Fraction	Solvent, T	Extraction (min)
Free amino acids	water, 0 C	30,30,30,wash ^a
Albumins	water, 0 C	30,30,30,wash
Globulins	0.5 M NaCl, 0 C	30,30,30,wash
Zein 1	55% 2-propanol, 20 C	3,120,60,wash
Zein 2	55% 2-propanol + 0.6% 2-mercaptoethanol, 20 C	3,120,60,wash
Glutelin	0.2% NaOH, 20 C	30,120,60,wash
Residue	insoluble	30,120,60,wash

^aThe wash is 1 ml water.

metabolize the isotope for 8 hours. The kernels were then harvested, the endosperm removed and lyophilized. The lyophilized endosperm tissue was then stored frozen at -15 C until further analysis.

Analysis of Labeled Amino Acids

The free amino acid fraction obtained from the above fractionation also contains organic acids and sugars. In order to remove these substances, ion-exchange chromatography was performed.

Cation exchange chromatography. Cation exchange chromatography was used to separate organic acids and sugars from the amino acid fraction. Dowex 50 W-X-2 resin was prepared and rinsed with 2 N HCl, and washed several times with distilled water until the pH of the effluent was 7.0. The resin was then packed into a 1 x 10 cm column with a glass wool base. The mixture of amino acids, organic acids and sugars in water was added to the top of the resin and the level of liquid was allowed to drop to the resin. The effluent was collected. Five ml of water was added to the resin and was allowed to drop to the level of the resin. The effluent was collected, and this step was repeated three times. The combined effluents contain all of the organic acids and sugars added to the column. These were later separated by anion exchange chromatography. The amino acids are eluted as a group by repeating the washing steps using 20 ml of 4 N NH_4OH in place of water. The effluents were evaporated to dryness and the residue was dissolved in water or Na citrate buffer, 0.2 M, pH 2.2 for later analysis by amino acid analyzer.

Anion exchange chromatography. In order to separate the organic acids from the sugars (neutral fraction), Dowex 1-1X8-50, Cl^- form anion exchange resin was acid washed, then washed with water until the effluent was neutral and packed in a 1 x 5 cm column. The organic acid-sugar fraction was loaded onto the top of the resin and the column was washed with 20 ml water to remove the neutral fraction consisting of the sugars. The organic acids were eluted from the resin with 20 ml of 2 N Na formate/2 N formic acid solution. Organic acids which did not elute with this solution were eluted with 10 ml of 4 N Na formate/2 N formic acid solution.

Analysis of amino acids from protein hydrolysates. Acid hydrolysis of proteins was carried out in an evacuated vial with 6 N HCl at 110 C for 24 hours. The hydrolysis of proteins at high temperatures causes the destruction of tryptophan and the formation of a pigment called humin. The humin will interfere with amino acid analysis on the amino acid analyzer, and, therefore, must be removed as described below. The protein hydrolysate, after removal of particulates by centrifugation, was taken to dryness with a rotary evaporator (Büchi, Rotavapor M) and the residue was taken up in a small volume of Na citrate buffer, 0.2 M, pH 2.2. The hydrolyzed sample was then passed through a small column of Dowex-1x8, Cl^- form (about 1 ml resin) to remove the humin. The sample was eluted with the same buffer to give a concentration of 100 mg lyophilized tissue per ml buffer.

Identification and Counting of Amino Acid Fractions

Amino acids derived from free and bound hydrolysates were analyzed by thin-layer chromatography (Pillay and Mehdi, 1970) or were analyzed by the one column ion-exchange method on a JEOL Model 6-AH amino acid analyzer by Dr. Davis Lin, UTMRC following the method of Lin (1977). Fractions were collected and 10 ml of Aquasol-2 (New England Nuclear) scintillation fluid was added. The fractions were then counted in a Mark III Liquid Scintillation Counter (Searle Analytic, Inc., Des Plaines, Illinois).

Results

Variation of β -Aspartate Kinase Activity with Development

The relationship of β -aspartate kinase activity to increases in fresh weight, dry weight, total nitrogen and soluble amino nitrogen is shown in Figure 17. An increase in total β -aspartate kinase activity was found to precede the onset of storage protein synthesis. This burst in β -aspartate kinase activity began at 5 days and continued to approximately 25 days post-pollination in the development of the kernel. A period of 50 to 60 days is required for the development of mature kernels. These data suggest that one or more types of regulation may be occurring, at the level of enzyme synthesis or at the level of enzyme activity. A 25 fold increase in β -aspartate kinase activity occurred from 5 to 25 days post-pollination and then declined through the remainder of development. Since soluble protein levels increase only three fold during the same period of 5 to 25 days post-pollination, the increase in β -aspartate kinase activity appears to

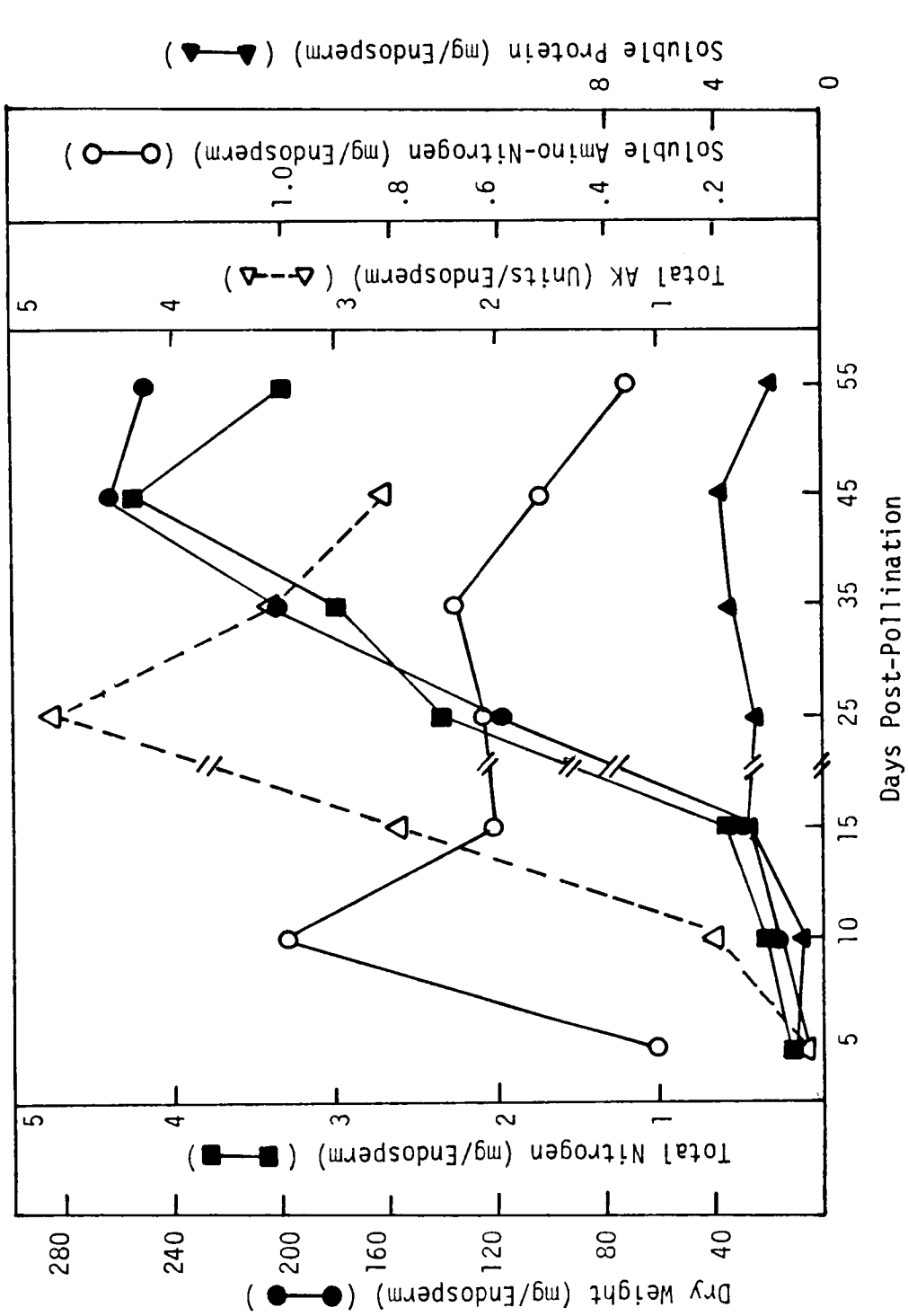


Figure 17. Development of β -aspartate kinase activity with time in development of the maize endosperm. Enzyme activity was determined in relation to dry weight (●), total nitrogen (■), soluble amino nitrogen (○) and soluble (▲) protein. AK designates β -aspartate kinase (Δ).

have some physiological significance and is not simply associated with changes in soluble protein levels. Also, the decline in β -aspartate kinase activity after 25 days post-pollination is not associated with a similar decline in soluble protein levels up to 45 days post-pollination. The same type of pattern was found to be exhibited by homoserine dehydrogenase and diaminopimelate decarboxylase activities (Figure 18). These enzymes are also involved in the synthesis of lysine, threonine and methionine and may be regulated in the same fashion as β -aspartate kinase.

Results of Enzyme Mixing Experiment with β -Aspartate Kinase

Results from the mixing experiment (Table 4) indicate that at 10 days post-pollination, enzyme activity equals $1.67 \pm .30$ units, at 25 days post-pollination, enzyme activity equals $7.50 \pm .25$ units and at 40 days post-pollination, enzyme activity equals $1.17 \pm .20$ units. When the extracts from 10 and 25 days post-pollination were mixed, the result was an additive effect of the two enzyme activities, with a combined enzyme activity of $8.83 \pm .50$ units, indicating that an inhibitor or activator is not present. Similar results were obtained with mixing of 10 + 40 and 25 + 40 endosperm extracts.

Sensitivity of β -Aspartate Kinase Activity to Lysine

The increase in total extractable β -aspartate kinase activity may be accompanied by a change in levels (or ratios) of isoenzymes sensitive to lysine, such that at the peak in extractable activity, one might expect more of an isoenzyme that is insensitive to inhibition by lysine (in vitro), whereas before the burst in activity,

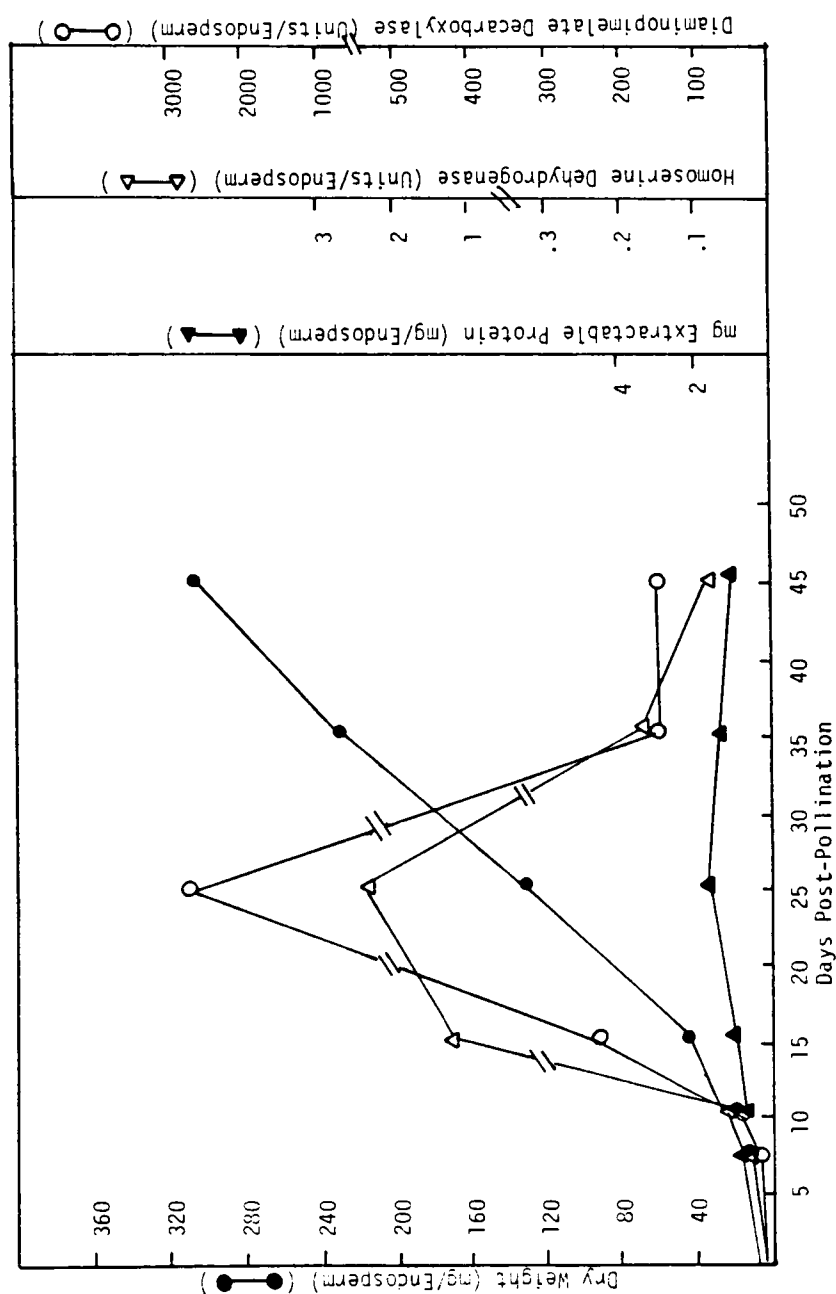


Figure 18. Development profile of homoserine dehydrogenase and diaminopimelate decarboxylase activities in maize endosperm. Dry weight (●—●), mg extractable protein (▲—▲), homoserine dehydrogenase activity (units=dpm per minute) (△—△) and diaminopimelate decarboxylase (units=dpm per minute) (○—○).

Table 4. Enzyme mixing experiment utilizing β -aspartate kinase derived from three stages of endosperm development.

Stage of Development (Days Post-Pollination)	Enzyme Activity (nmoles Product/Minute)
Alone ^a	
10	1.67 $\pm$.30 ^c
25	7.50 $\pm$.25
40	1.17 $\pm$.20
Mixing Experiments ^b	
10+25	8.83 $\pm$.50 (Σ = 9.17 $\pm$.55)
10+40	2.50 $\pm$.15 (Σ = 2.84 $\pm$.50)
25+40	7.65 $\pm$.32 (Σ = 8.67 $\pm$.20)

^a200 μ l endosperm extract was added per 1 ml assay.

^b200 μ l of each extract was added per 1 ml assay:400 μ l total enzyme.

^c $\pm$ =represents the range of the determinations.

one might expect a lysine-sensitive form of the enzyme to predominate. Upon analysis of the sensitivity of the extractable β -aspartate kinase isolated from 15, 25 and 35 days post-pollination endosperm to lysine, it was found that the enzyme did not differ in sensitivity to lysine at 1 and 5 mM (Table 5). Since the change in enzyme activity was not accompanied by changes in the sensitivity of the enzyme to feedback regulation, free pool levels of amino acids were examined to determine if pool sizes of single amino acids correlate with the change in extractable enzyme activities.

Free pool levels of amino acids in the developing maize endosperm were measured at 10, 25 and 40 days post-pollination (Table 6). The total free pool increased from 10 to 25 days post-pollination and declined to 40 days post-pollination. The increase in the free pool coincides with the onset of storage protein synthesis. This observation is consistent with the requirement for the free pools of amino acids necessary for storage protein synthesis.

The pattern of free pools of amino acids through development, especially that of the feedback inhibitor, lysine, coincides with the increase and decrease in β -aspartate kinase activity, also levels of threonine coincide with the increase and decrease in homoserine dehydrogenase activity. One would expect a negative correlation between the level of an effector amino acid and the enzyme which it inhibits. This rationale, however, does not take into account the role that compartmentation may play in the regulation of metabolite and enzyme levels.

Table 5. Lysine inhibition of β -aspartate kinase activity obtained from developing maize endosperm.

Endosperm Age Days Post-Pollination	Relative β -Aspartate Kinase Activity (% Control)*		
	Lysine mM		
	0	1.0	5.0
15	100	60	55
25	100	45	48
35	100	62	52

*The values represent the average of 6 to 9 replications with a level of activity of 1.0 to 3.5 nmoles BAP/min, using 2.0 mg protein and 25 mM L-aspartate. Standard assay conditions are: 100 mM Tris-HCl, pH 8.0, 25 mM L-aspartate, 25 mM ATP, 25 mM MgCl₂ and 400 mM NH₂OH-HCl. Standard errors ranged from 0.05 to 0.10 nmoles BAP/minute.

Table 6. Free amino acid content of developing maize endosperm at three stages in development.

Amino Acid	Amino Acid Concentration (nmoles/Endosperm)		
	Days Post-Pollination		
	10	25	40
Lysine	46	204	47
Histidine	511	688	549
OH-Lysine	37	82	31
Ornithine	8	57	11
Aspartate + Asparagine	121	227	122
Threonine	144	300	158
Serine	108	252	144
Glutamate + Glutamine	113	574	242
Proline	62	416	144
Glycine	46	100	58
Alanine	238	719	291
Valine	54	168	76
Methionine	-	-	-*
Isoleucine	8	23	22
Leucine	16	46	31
Tyrosine	10	40	24
Phenylalanine	<u>10</u>	<u>35</u>	<u>24</u>
TOTAL	1529	3924	1976

*Concentration too low to be measured by our techniques. The results are representative of three replications in two experiments.

In Vivo Effector Studies

High concentrations (200 nmoles/endosperm) of amino acid effectors lysine and the basic amino acid glutamine were injected directly into the endosperm at 12 days post-pollination and the endosperm were collected at 19 days post-pollination and analyzed. The long term experiment was performed in order to determine if high levels of exogenous amino acids injected at concentrations capable of supplying all of that amino acid needed for storage protein synthesis could inhibit the de novo synthesis of that amino acid by inhibiting the enzymes responsible for its synthesis. Lysine or glutamine at 200 nmoles/endosperm had a negligible effect on normal endosperm development as measured by fresh weight, dry weight and total extractable protein (Table 7). Upon examination of total and specific activities of extractable β -aspartate kinase and homoserine dehydrogenase (Table 8), lysine caused an inhibition in total extractable β -aspartate kinase activity by 60% and in total extractable homoserine dehydrogenase activity by 50%. Glutamine did not cause as severe an inhibition as lysine.

In Vivo Labeling of Free and Bound Amino Acid Fractions by ^{14}C Precursors

Labeling experiments were performed in order to determine if the endosperm tissue was capable of metabolizing carbon precursors into amino acids at rates which would allow study of the incorporation of label into certain amino acids in the presence and absence of effectors. The distribution of label from ^{14}C -acetate into free amino

Table 7. Effects of exogeneous amino acids on normal development of maize endosperm.^a

Treatment	Fresh Weight mg/Endosperm	Dry Weight mg/Endosperm	Total Extractable Protein mg/Endosperm
dd H ₂ O	216±16 ^b	73±1	2.14±0.10
200 nmoles Lysine/ endosperm	201±6	75±2	2.50±0.20
200 nmoles Glutamine/ endosperm	197±4	65±3	2.27±0.15

^aInjected at 12 days post-pollination and collected at 19 days post-pollination.

^b_± indicates the range of four determinations.

Table 8. Effects of exogeneous amino acids on development of β -aspartate kinase and homoserine dehydrogenase activities in maize endosperm.^a

Treatment	β -Aspartate Kinase		Homoserine Dehydrogenase	
	Units/ ^b mg Protein	Units/ ^b Endosperm	Units/ ^c mg Protein	Units/ ^c Endosperm
dd H ₂ O	1.52 $\pm$ 0.16 ^d	3.25 $\pm$ 0.15	0.113 $\pm$.003	0.242 $\pm$.004
200 nmoles Lysine/ endosperm	0.51 $\pm$ 0.05	1.27 $\pm$ 0.03	0.062 $\pm$.004	0.155 $\pm$.003
200 nmoles Glutamine/ endosperm	1.48 $\pm$ 0.16	3.35 $\pm$ 0.15	0.107 $\pm$.002	0.306 $\pm$.004

^aInjected at 12 days post-pollination and collected at 19 days post-pollination.

^bUnits=nmoles product/minute.

^cUnits-change in absorbance at 340 nm per 5 minutes.

^d $\pm$ indicates the range for three determinations in two experiments.

acid and Osbourne protein fractions, after an 8 hour incubation, is shown in Table 9. The first observation is that only 50% of the label injected is recovered in the kernels. The remainder was found in the surrounding tissue, kernels, cob and as $^{14}\text{CO}_2$. The second is that more label is present in the free amino acid fraction at 10 and 40 days post-pollination than at 25 days post-pollination. This may be attributed to the onset of storage protein synthesis and thus high metabolic activity and requirement for amino acids at 25 days post-pollination. The high incorporation of label at 25 days post-pollination into the free amino acid fraction (144100 dpm/fraction) should provide statistically reliable results when the effect of amino acids on label incorporation into the amino acids present in the free amino acid fraction is studied.

Analysis of the incorporation of label into free and bound amino acid fractions from ^{14}C -aspartate and ^{14}C -acetic acid is shown in Table 10. The amino acid fractions were analyzed by thin-layer chromatography. The spots were identified and scraped and the distribution of label in the samples was determined. The first observation is that after an 8 hour incubation, much of the label remains in the free pool of amino acids; however, all of the protein and free amino acids have incorporated some label, indicating extensive interconversions of amino acids within the developing maize endosperm at 25 days post-pollination.

Table 9. Distribution of ^{14}C into protein fractions of maize endosperm following injection of ^{14}C -acetate.

Fraction	Days Post-Pollination		
	10	25	40
	dpm/Fraction (% Total Recovered)		
Free amino acids	33570 (25.9)	144100 (18.1)	30190 (44.0)
Albumins	2710 (2.8)	74910 (9.4)	780 (1.1)
Globulins	3240 (2.5)	51550 (6.5)	3600 (5.2)
Zein 1	21720 (16.8)	111840 (14.0)	2120 (3.0)
Zein 2	1680 (1.3)	3780 (0.4)	600 (0.8)
Glutelin	30280 (23.4)	51600 (6.4)	2280 (3.3)
Residue	2820 (2.1)	15730 (1.9)	110 (0.2)
Total	96020 (74.0)	453510 (57.0)	39680 (58.0)
Organic acids and sugars	33110 (26.0)	343310 (43.0)	28930 (42.0)
Total	129130	796820	68610
Total injected	8.6×10^6 dpm	6.6×10^6 dpm	6.8×10^6 dpm
% Recovery	46	50	54
Recovered % of total respired as $^{14}\text{CO}_2$	3.2%	1.7%	2.0%

Table 10. Analysis of incorporation of labeled precursors into amino acids as analyzed by thin layer chromatography.^a

Amino Acid	dpm (% Total) (Per 100 mg Lyophilized Tissue)			
	(2- ¹⁴ C) Acetic Acid		(U- ¹⁴ C) Aspartic Acid	
	Free	Bound	Free	Bound
(Cys) ₂	2550 (0.6)	400 (0.5)	450 (0.8)	733 (3.8)
Asn	10250 (2.4)	100 (0.1)	1750 (3.1)	1099 (5.7)
Gln	290150 (70)	16100 (19.0)	7100 (12.6)	1931 (10.0)
Asp	50450 (12.1)	3700 (4.4)	7300 (13)	2031 (10.6)
His	2100 (0.5)	50100 (59.4)	1150 (2.0)	899 (4.7)
Lys	2250 (0.5)	200 (0.2)	1300 (2.3)	899 (4.7)
Ser	1500 (0.4)	200 (0.2)	650 (1.1)	999 (5.2)
Tyr	2300 (0.5)	800 (0.9)	100 (0.2)	966 (5.0)
Ala	11400 (2.7)	1300 (1.5)	2900 (5.1)	899 (4.7)
Pro	5000 (1.2)	300 (0.5)	6400 (7.6)	1399 (7.3)
Thr	1900 (0.4)	600 (0.7)	350 (0.6)	899 (4.7)
Val	750 (0.2)	500 (0.6)	Traces	899 (4.7)
Met	100 (0.03)	Traces	250 (0.5)	699 (3.6)
Phe	300 (0.07)	400 (0.5)	100 (0.2)	966 (5.0)
Leu+Ile	1600 (0.38)	2100 (2.4)	250 (0.5)	932 (4.7)
Gly	8150 (1.9)	600 (0.7)	1200 (2.1)	999 (5.2)
Glu	13350 (3.2)	400 (0.5)	23900 (43)	932 (4.7)
Arg	10200 (2.5)	500 (0.6)	6750 (12.0)	966 (5.0)

^aAfter an 8 hour incubation at 25 days post-pollination by the method of Pillay and Mehdi (1970).

Effect of Exogeneous Lysine on the Synthesis of Lysine, Threonine and Methionine in a Short Term Labeling Study

The effect of exogeneously supplied lysine on the synthesis of lysine, threonine and methionine from ^{14}C -labeled acetate was examined (Table 11). The effector amino acid, lysine, was injected directly into the endosperm at 200 nmoles/endosperm one hour prior to injection of label, in order to allow pool sizes of the amino acid to increase. Analysis of the incorporation of label into free and bound amino acids was determined by amino acid analyzer and data were interpreted following Oaks et al. (1970). There is a clear inhibition of label distribution (as Percent Total Control) into amino acids of the aspartate pathway, lysine, threonine and methionine. However, there is an increase in incorporation into free and bound fractions of neutral amino acids and amino acids derived from the branched chain biosynthetic pathway.

Discussion

The presence of all protein amino acids in the vascular sap leading into the developing maize kernel has been demonstrated (Arruda and daSilva, 1979). The major amino acid transported is glutamine, with other amino acids present in unbalanced ratios or in amounts too low in order to account for their requirement in storage protein synthesis. De novo synthesis of amino acids in the developing endosperm may overcome the imbalance of amino acids supplied by the vascular sap. The demonstration of the presence of enzymes of amino acid biosynthetic pathways (Oaks, 1979) coupled with evidence

Table 11. Effect of exogenous lysine (200 nmoles/endosperm) on incorporation of ^{14}C derived from (2- ^{14}C) acetate into free and bound amino acids (dpm/100 mg endosperm).^a

Amino Acid	Control			+ Lysine			(% Total Control)
	Free	Bound	Total	Free	Bound	Total	
Lys	6600	90	6690	180	960	1140	17
His	2250	1080	3330	150	1080	1230	37
Arg	150	270	420	180	600	780	185
Asp+Asn	6600	1125	7725	495	1440	1935	25
Thr	1500	270	1770	210	720	930	52
Ser	2100	315	2415	270	1440	1710	70
Glu+Gln	29100	4120	33220	210	4800	5010	15
Pro	1275	1855	3130	180	1400	1580	50
Gly	390	225	615	195	1080	1275	200
Ala	3750	765	4515	150	1080	1230	27
(Cys) ₂	--	--	--	--	--	--	--
Val	210	360	570	165	1080	1245	218
Met	6	180	186	--	--	--	--
Ile	120	675	795	120	1080	1280	161
Leu	120	180	300	150	1080	1130	>300
Tyr	180	225	405	240	1200	1440	>300
Phe	180	180	360	135	1080	1115	>300

^aEight hour incubation, at 25 days post-pollination. The effector was injected one hour prior to injection of label.

from in vivo labeling experiments (Sodek, 1976; Table 9; Table 10) provides evidence for the interconversion of amino acids within the endosperm tissue.

The developmental pattern exhibited by β -aspartate kinase, homoserine dehydrogenase and diaminopimelate decarboxylase activities suggests the possibility of coordinate regulation of enzymes involved in amino acid metabolism. The burst in extractable enzyme levels early in development may be due to decrease in levels of inhibitors or increase in levels of activators. Results of classic mixing experiments with β -aspartate kinase extracted from 10, 25 and 40 days post-pollination endosperm do not support the presence of activators or inhibitors as the mode of regulation of enzyme activity.

The increase in enzyme activities may also be due to decreased protein turnover or increases in the levels of enzyme protein synthesized. The injection of exogenous lysine at a concentration which should provide the endosperm with all of that amino acid needed at 25 days post-pollination in the free pool (200 nmoles/endosperm) supported normal endosperm development. At the same time, this level of lysine inhibited the increase in total extractable β -aspartate kinase and homoserine dehydrogenase activities, suggesting regulation at the gene level.

Studying the incorporation of labeled precursors in the presence and absence of a regulator can provide information about the regulation of a biosynthetic pathway at the level of enzyme activity. A comparison of the incorporation of label in an 8 hour period from ^{14}C -acetate in the presence and absence of the effector amino acid, lysine (200

nmoles/endosperm) provides data that indicate that the synthesis of acetate-derived amino acids of the aspartate pathway is subject to inhibition by lysine (Table 11). This suggests feedback regulation of the enzymes involved in the synthesis of lysine, threonine and methionine, e.g., β -aspartate kinase and homoserine dehydrogenase may be occurring during endosperm development. Addition of exogenous lysine also caused an increase in the incorporation of label into the branched-chain amino acids, perhaps by channeling acetate-derived carbon away from lysine synthesis and into this pathway. The decreased incorporation into glutamate/glutamine and proline may be explained by the degradation of lysine into these amino acid pools (Sodek and Wilson, 1970), thereby providing adequate pools for storage protein synthesis.

The isotope competition experiments described by Dougall (1971) on Paul's Scarlet Rose, by Oaks (1965) on incorporation of leucine as affected by exogenous leucine, and by Oaks et al. (1970) on the regulation of proline biosynthesis lend precedence to the inhibition of amino acid biosynthesis by exogenous amino acids. Kolderup (1979) studied the interconversions of amino acids in the developing wheat grain by injection of ^{14}C -labeled precursors and effector amino acids into the stem below the developing spike. He found that exogenous lysine inhibited conversion of aspartate to lysine. Therefore, the enzymes of the pathway appear to be capable of being inhibited by endproduct effectors in vivo as well as in vitro.

A preliminary calculation of the ability of the aspartate pathway at 25 days post-pollination to synthesize amino acids necessary for storage protein synthesis reveals that the enzyme activities (β -aspartate kinase, 9.2 nmoles product/min·endosperm; homoserine dehydrogenase, 253.4 nmoles product/min·endosperm; diaminopimelate decarboxylase, 0.889 μ moles product/min·endosperm) have the potential for producing amino acids needed for storage protein synthesis (rate of nitrogen deposition is reported as 9.5 nmoles/min·endosperm (Sodek and daSilva, 1977)), with β -aspartate kinase as the rate-limiting enzyme. Utilizing the data obtained from thin-layer chromatography for the incorporation of labeled acetate into lysine during an 8 hour incubation (2000 dpm/endosperm), calculations reveal that under the experimental conditions, approximately 0.03 to 0.10 nmoles of lysine/endosperm can be synthesized in the 8 hour period.

The endosperm contains 200 nmoles of lysine in the free pool at 25 days post-pollination (Table 6, page 80). It appears that the endosperm has the biosynthetic capacity to provide a significant portion of the lysine present in the endosperm. The exact contributions of de novo synthesis and transported lysine to the free and bound pools remains to be determined. The fact that the enzymes of the aspartate family are present in the developing maize endosperm and are subject to regulatory control in vivo provides additional evidence that the pathway may be functioning in an important capacity to provide amino acids needed for storage protein synthesis.

VI. CONCLUSIONS

The biosynthetic pathway leading to the synthesis of lysine, threonine and methionine was first established in microorganisms (Umbarger, 1978). The classic modes of regulation of amino acid biosynthesis in bacteria are allosteric feedback regulation and induction/repression phenomena of enzymes of the pathway (Umbarger, 1969). The majority of the enzymes of the aspartate family of amino acids and the pathway intermediates have been found to be present in higher plants with the exception of the enzymes and intermediates leading from dihydrodipicolinate to diaminopimelate (see Figure 1, page 12).

Over a decade of research in higher plants has revealed that the enzymes which are subject to regulation are subject to feedback regulation but not to derepression/repression mechanisms of regulation (Chu and Widholm, 1972; Oaks et al., 1970; Dougall, 1970; Mifflin and Cave, 1972). The extent to which higher plants exhibit derepression/repression mechanisms of regulation is still unresolved. Repression of enzyme synthesis in the presence of one or more endproducts of a pathway has not been demonstrated unequivocally. Sakano (1979) found that the concentration of free lysine decreased in cultured carrot root slices and coincided with an increase in total extractable β -aspartate kinase activity. Upon addition of exogenous lysine to the culture medium, the increase in lysine-sensitive β -aspartate kinase activity was suppressed. However, the exogenous lysine also

inhibited tissue growth which normally coincided with the increase in β -aspartate kinase activity.

Examples of induction of enzyme synthesis by metabolites in plants are nitrate reductase (Zielke and Filner, 1971) and ATP sulfurylase (Reuveny and Filner, 1977). Nitrate reductase activity was found to be induced by nitrate in cultured tobacco cells. The induction of enzyme protein synthesis was shown by buoyant density data indicating that the increase in nitrate reductase activity was due to de novo synthesis of enzyme protein.

An explanation of the absence of repression mechanisms in higher plants has been that normally repressed levels of enzyme activity are sufficient for sustaining plant growth. In the developing endosperm, however, the tissue is under physiological pressure to synthesize large amounts of storage protein in a short period of time, utilizing amino acids and carbon precursors supplied to it from the vascular sap. If the vascular sap is imbalanced in the amino acids necessary for storage protein synthesis, the enzymes involved in the de novo synthesis of amino acids may be induced. We found that exogenous lysine supplied to the tissue early in development suppressed the increase in β -aspartate kinase and homoserine dehydrogenase activities, therefore, indicating possible action at the gene level by repression of enzyme synthesis. This may be preliminary evidence for a derepression/repression mechanism in this unique tissue in higher plants. Conclusive evidence will come from density labeling methods with ^{18}O -labeled amino acids and radioisotope labeling coupled with enzyme

purification (Filner et al., 1969) and radioimmunoassay utilizing antibodies raised against the purified enzymes.

Higher plants may compensate for the absence of derepression/repression mechanisms of bacterial enzyme regulation by sequestering of metabolites and enzymes into 1) metabolic and storage pools and 2) subcellular compartmentation, leading to physical separation of amino acids and enzymes. The enzymes of the aspartate family, β -aspartate kinase, homoserine dehydrogenase and diaminopimelate decarboxylase have been shown to be distributed among subcellular fractions in both leaf tissue and the developing endosperm of maize. The presence of the enzyme ribulose-1,5-bisphosphate carboxylase, and its similarity to the enzyme isolated from green leaf tissue, lends additional evidence to the involvement of the plastid in the non-photosynthetic endosperm in carbon and nitrogen metabolism.

In conclusion, we have obtained a more definitive picture of amino acid metabolism and aspects of its regulation in the developing endosperm of maize. The feasibility of obtaining feedback mutations such that free pools of limiting amino acids may be increased is still in question. However, information gained on the kinetic potentials and regulatory properties of the aspartate family of amino acids enzymes yields a clearer understanding of the source and availability of these amino acids for endosperm development and the role of the endosperm in its own development.

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