ASPARAGINE SYNTHETASE IN *ZEA MAYS*

By

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ASPARAGINE SYNTHETASE IN *ZEA MAYS*

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ABSTRACT

In legume cotyledons, asparagine is synthesized by a transfer of the amide nitrogen of glutamine to aspartate. The asparagine synthetase reaction requires ATP-Mg^{2+}, aspartate and glutamine as substrates, although NH_4Cl can act as a rather inefficient nitrogen donor.

In this project, asparagine synthetase was studied in extracts from several maize tissues. Enzyme activity (in nmoles asparagine per 20 minutes per gram fresh weight) was about 30 in developing endosperm and root tips, 60 in developing embryos, and 150 in scutella and mature root of seedlings.

Asparagine (2.0 mM) resulted in a 50% inhibition of the reaction in endosperm extract; 10 mM asparagine on the other hand inhibited the mature root and scutellar reactions less than 30%. The potential end products AMP and ADP inhibited the mature root reaction more strongly than the scutellar reaction. Glutamate (10 mM) did not inhibit the scutellar reaction.

The addition of 1.0% bovine serum albumin to a crude extract from mature roots did not affect enzyme activity; however albumin doubled enzyme activity in mature root extract that had been filtered through Sephadex.

Km values obtained for glutamine in embryo, scutellar, mature root and soybean cotyledon extracts were 1.6, 0.49, 0.59, and

0.18 mM, respectively. Km values for NH_4Cl were 4.0, 2.6, 2.6 and 2.9 mM. These values indicate that the soybean enzyme reacts much more efficiently with glutamine than with NH_4Cl, but that the enzyme from various maize tissues reacts only slightly better with glutamine.

Glutamine protected the enzyme from heat inactivation in extracts from four maize tissues; however, its effect on mature root enzyme was much less pronounced than on embryo, scutellar or endosperm enzyme. NH_4Cl had little effect on the rate of inactivation in any of the four extracts.

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INTRODUCTION

The element nitrogen is needed in substantial amounts throughout the life cycle of a plant, for it is an integral part of all genetic material, structural proteins and enzymes. Because the above materials are continuously degraded and rebuilt, a continuous supply of nitrogen is essential. Nitrogen is usually assimilated by the roots in the form of nitrate or ammonium or through fixation by symbiotic bacteria of atmospheric N_2. This nitrogen must be transported in some form to other parts of the plant. Developing seeds have a particularly high demand for nitrogen because large amounts of storage protein are made there. During germination, this storage protein is degraded to amino acids, the carbon and nitrogen of which can be used by the young seedling.

A. Metabolism of Asparagine

Asparagine and arginine have higher nitrogen:carbon ratios (2:4 and 4:6 respectively) than any other amino acids, and are thus ideal amino acids for the storage and transport of nitrogen in the plant. When nitrogen in the form of nitrate is taken up by plant roots, it is sometimes transported to the shoot as nitrate (4,24), but is often reduced to ammonium and then incorporated into other compounds (41). Ammonium and cyanide are toxic to the plant in high concentrations. Asparagine and glutamine are major compounds into which ammonium is

incorporated, while cyanide can be detoxified by incorporation into asparagine (9).

In legumes, asparagine is the chief compound used for transporting nitrogen (26). Atkins *et al.* showed that during lupine seed development, 40-50% of the amino acid composition of the phloem was asparagine and 15-20% was glutamine (2). All other amino acids were found in much smaller amounts. Glutamate and glutamine composed 10-20% of the amino acids in the seed protein but asparagine composed only about 10%. These results suggest that not all the asparagine is incorporated directly into protein but that some is metabolized to other amino acids before incorporation.

Herridge *et al.* found that the ureides allantoin and allantoic acid constituted 60-80% of xylem-borne nitrogen in cowpea (19). Glutamine, asparagine and other amino acids transported much smaller amounts of nitrogen. In corn, glutamine, asparagine, aspartate and glutamate are the four most important nitrogen transport compounds, in that order (23). Asparagine and arginine are the major transporters of nitrogen during the spring mobilization of storage protein in apple trees (51).

The storage function of asparagine was illustrated in the 1920's by Prianischnikov, who found an accumulation of asparagine in cotyledons of young lupin seedlings which coincided with protein degradation (12). Up to 85% of protein nitrogen released was found in asparagine. Due to a carbohydrate deficiency in the young seedling, amino acids derived from storage protein are respired and the nitrogen is recovered as asparagine. In legumes, asparagine is an important storage compound

but is not a major component of storage protein itself. Arginine and proline are present in high amounts in the storage protein of legumes and cereals, respectively (30).

Several workers have attempted to elucidate the path of carbon into asparagine by using carbon-14 as a tracer. For example, Mitchell and Bidwell fed pea slices with aspartate labelled individually in several positions and with ^{14}C-labelled four-carbon organic acids plus or minus ^{12}C-aspartate (31). From the labelling patterns found in asparagine and other compounds, the authors concluded that externally supplied ^{14}C-aspartate was converted only indirectly to ^{14}C-asparagine through intermediates of the Krebs Cycle. They concluded that if aspartate were the direct precursor of asparagine, the aspartate used for asparagine formation was compartmented away from externally supplied aspartate.

Streeter found that ^{14}C-aspartate was poorly metabolized when fed to soybean cotyledon slices and that extremely little was converted to asparagine (46). However, exogenously added ^{14}C-succinate was extensively metabolized and converted, in the following order, to fumarate, malate, aspartate and asparagine (based on the timing of peak radioactivity readings). Streeter concluded that asparagine is formed from succinate via the intermediates fumarate, malate, and aspartate; he suggested that only succinate can move freely between the two compartments.

Oaks and Johnson incubated excised corn root tips in a Hoagland's salts solution for various periods of time (34). The roots were then

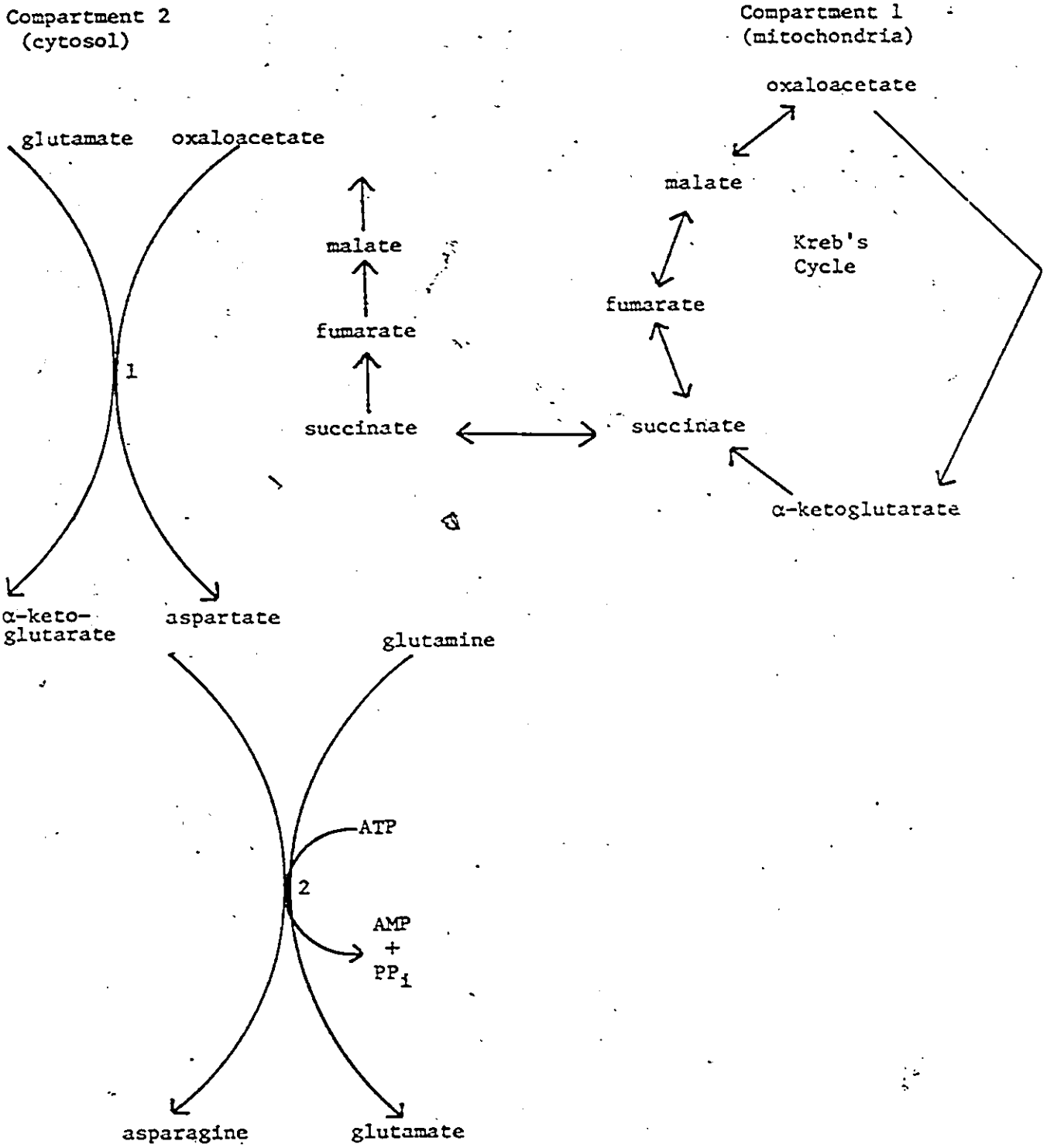
transferred to fresh media containing acetate-2-^{14}C and incubated for another two hours. They found that incorporation of ^{14}C from acetate into asparagine increased with total incubation time. In a competition experiment, succinate was more effective than aspartate in reducing the labelling of asparagine. Thus it appears that added succinate is more accessible than added aspartate to the pathway of asparagine formation in corn root tips.

A diagrammatic outline of the currently accepted pathway of carbon into asparagine is shown in Figure 1. Carbon which is to be used for asparagine formation first enters the Krebs Cycle in the mitochondrial compartment. In the form of succinate it moves into the cytosol, where it can be converted to asparagine carbon.

The entry of nitrogen into asparagine has been studied using ^{15}N-ammonium. Yemm and Willis fed nitrogen-deficient barley roots with ^{15}N-ammonium and found that it was quickly assimilated into the amide nitrogen of glutamine (53). Incorporation of ^{15}N into asparagine occurred much more slowly, but continued long after incorporation into glutamine had levelled off. Kretovitch found that when ^{15}N-ammonium was fed to lupine or vetch seedlings, much more was incorporated into the amide nitrogen of asparagine than into the amino nitrogen (25). These observations are consistent with a model where ammonium is first incorporated into glutamine and then into asparagine. An asparagine synthetase has been extracted from several plants which requires aspartate, ATP-Mg^{2+} and either glutamine or NH_4Cl as substrates (27,42, 43,46,48,49). When glutamine is a substrate, the amide nitrogen is

Figure 1. Entry of Carbon into Asparagine. (Modified after Mitchell and Bidwell, 1970).

Enzymes: (1) Glutamate: α-ketoglutarate amino transferase
(2) Asparagine synthetase


Compartment 2
(cytosol)
Compartment 1
(mitochondria)
oxaloacetate
glutamate
oxaloacetate
malate
malate
Kreb's
Cycle
fumarate
fumarate
1
succinate
succinate
α-ketoglutarate
α-keto-
glutarate
aspartate
glutamine
ATP
2
AMP
+
PPi
asparagine
glutamate

transferred to aspartate. For most of the plant enzymes studied, the Km value for glutamine is much lower than the Km value for ammonium; it has been postulated that glutamine is the true physiological substrate (46).

A diagrammatic outline of the currently accepted pathway of ammonia assimilation is shown in Figure 2. Ammonium first reacts with glutamate to become the amide nitrogen of glutamine. Glutamine is a nitrogen donor in several reactions, including transamidations with α-ketoglutarate to form two glutamate molecules, or with aspartate to form asparagine. Glutamate is used in the synthesis of many amino acids. Lea and Fowden have suggested that asparagine accumulates when levels of reduced nitrogen exceed those of α-keto acids available for synthesis of amino acids (26). At times of carbohydrate excess, nitrogen would be shunted from glutamine to glutamate and henceforth into a variety of amino acids. In both situations, glutamine can be maintained at the physiological concentration required for its various other functions.

The accumulation of asparagine at times of carbohydrate deficiency is consistent with the observations of Mothes, who found that much more asparagine accumulated when detached leaves were cultured on water than when they were cultured on glucose (12). Also, the inclusion of glucose in a mineral salts solution greatly reduced the formation of asparagine in young corn seedlings (33), and glucose inhibited the formation of asparagine from acetate-2-^{14}C in excised corn root tips (34).

A second pathway of asparagine synthesis has also been found. In 1963, Blumenthal-Goldschmidt *et al.* fed K^{14}CN to *Sorghum* seedlings

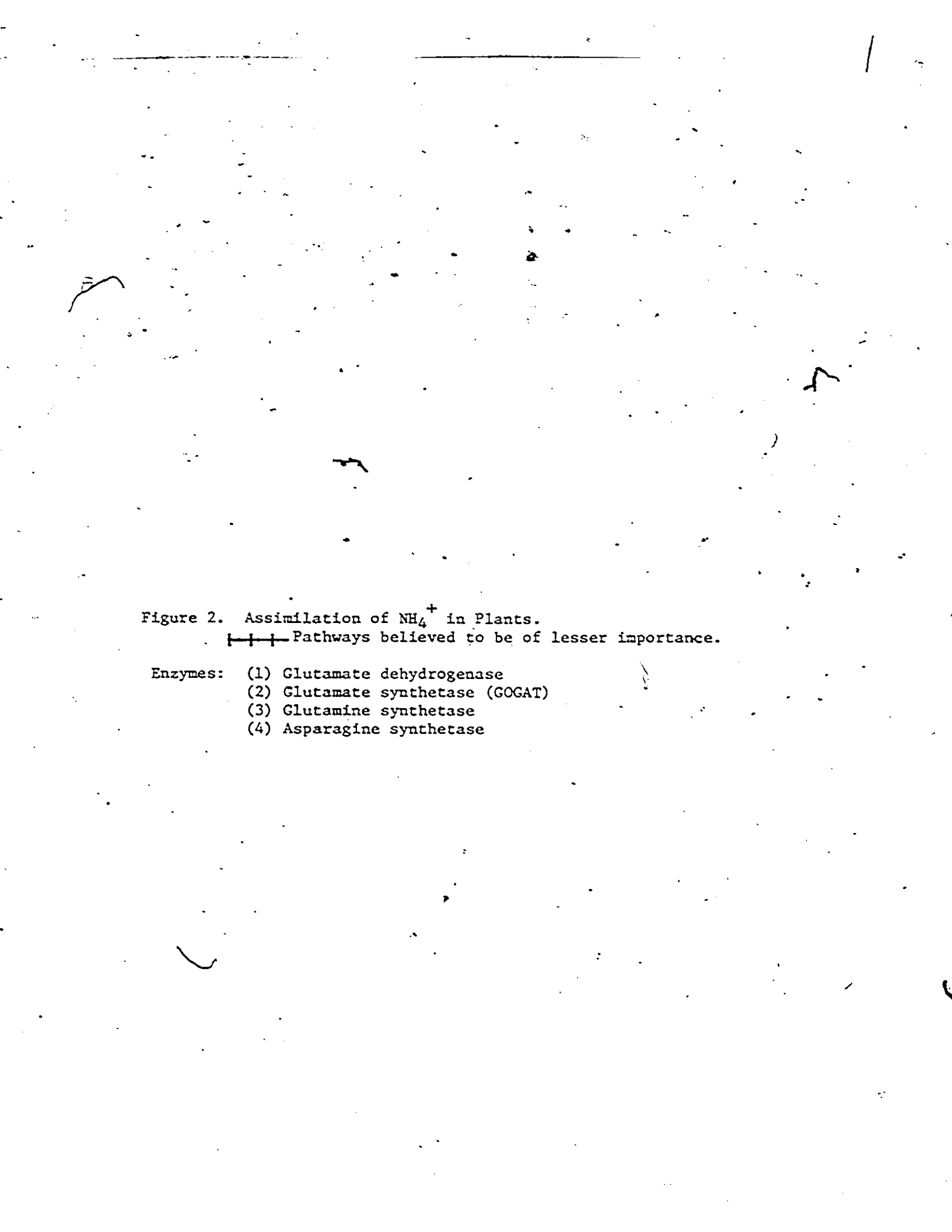
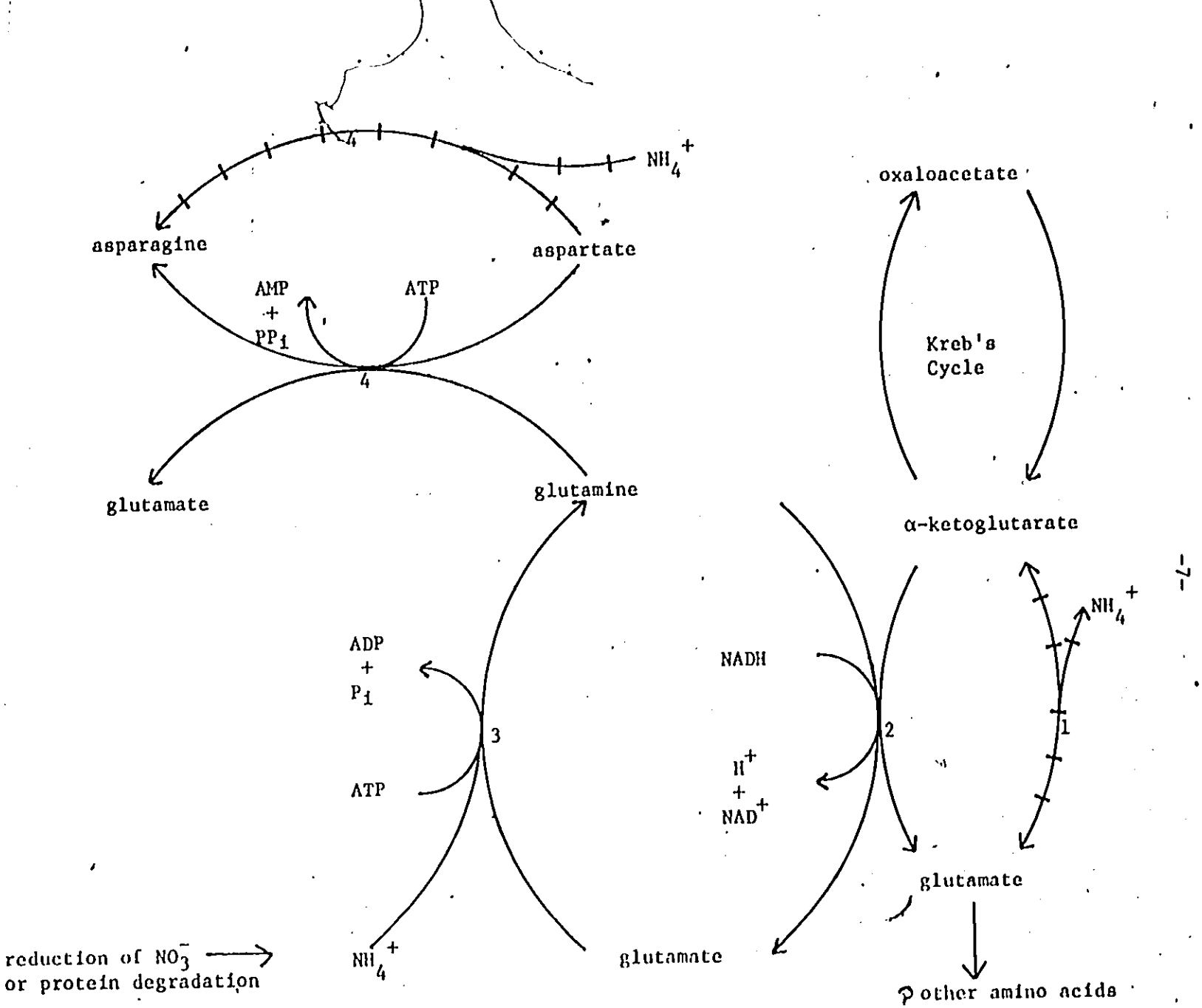


Figure 2. Assimilation of NH_4^+ in Plants.
⊢⊣⊢⊣⊢ Pathways believed to be of lesser importance.

Enzymes: (1) Glutamate dehydrogenase
(2) Glutamate synthetase (GOGAT)
(3) Glutamine synthetase
(4) Asparagine synthetase


4
NH_4^+
asparagine
aspartate
AMP
+
PP_i
ATP
4
glutamate
glutamine
oxaloacetate
Kreb's
Cycle
α-ketoglutarate
NH_4^+
NADH
ADP
+
P_i
3
2
1
H^+
+
NAD^+
ATP
glutamate
reduction of NO_3^-
or protein degradation
NH_4^+
glutamate
other amino acids

and found much of the ^{14}C in the amide carbon of asparagine (5). The incorporation of cyanide into asparagine has since been observed in many plants and tissues. Two enzymes have been extracted from plants which together can convert the cyanide carbon to the amide carbon of asparagine:

1. Cysteine + HCN $\xrightarrow{\text{β-cyanoalanine synthase}}$ β-cyanoalanine + H_2S

2. β-cyanoalanine + H_2O $\xrightarrow{\text{β-cyanoalanine hydrolase}}$ asparagine

β-cyanoalanine synthase and β-cyanoalanine hydrolase were first extracted from blue lupins (9,18). β-cyanoalanine synthase has also been extracted from corn root tips (49). In excised corn root tips, label from exogenously-added $K^{14}CN$ was rapidly incorporated into asparagine, but ^{14}C-cysteine was not converted to ^{14}C-asparagine unless it was added together with KCN (35). These results suggest that cyanide is not normally present in corn root tips, and that the cyanide pathway of asparagine formation is present but non-functional. However, cyanide is present as cyanogenic glycosides in some plants such as *Lotus* (8). The cyanide pathway is probably important in removing cyanide from cells where cyanogenic glycosides are being degraded. In other instances it probably has no physiological role.

Although it has been claimed that asparagine is a dead-end metabolite, it must be metabolized if the nitrogen and carbon stored as asparagine are to be liberated. Pate showed that the carbon of

asparagine could be metabolized by feeding ^{14}C-asparagine to pea plants when they were flowering (a time when nitrogen would be in demand) (41). After 48 hours, 40% of the ^{14}C remaining in the soluble fraction had been converted to other compounds, particularly homoserine, proline, glutamate and serine. When Atkins *et al.* fed ^{14}C, ^{15}N(amide)-asparagine to lupin plants during the period of maximum seed protein synthesis, over 60% of ^{15}N was found in protein amino acids while over 65% of ^{14}C remained as aspartate (2). Thus after the amide nitrogen had been transferred to other compounds, the carbon was metabolized more slowly. Because asparagine is an excellent nitrogen source for soybean cotyledons grown in tissue culture (50), asparagine nitrogen must be metabolized in this system as well.

The enzyme asparaginase, which hydrolyzes the amide bond of asparagine, was extracted first from germinating barley roots in 1927 by Grover and Chibnall (17). Asparaginase activity has since been demonstrated in several plants and tissues (30); activity is high in soybean root nodules (47). The aspartate formed by asparaginase could be transformed into Krebs Cycle intermediates by glutamate-α-ketoglutarate aminotransferase.

Several workers have found that transamination of asparagine also occurs in plants. For example, Streeter has shown that in soybean leaves, asparagine transaminase is much more active than asparaginase (47).

1. Asparagine + glyoxylate / pyruvate $\xrightarrow{\text{asparagine transaminase}}$ α-ketosuccinamate

2. α-ketosuccinamate $\xrightarrow{\text{α-ketosuccinamate deamidase}}$ oxalacetate + NH_3

Soybean leaves contain high levels of α-ketosuccinamate deamidase as well. These observations suggest that this pathway is also important in the degradation of asparagine.

B. Asparagine Synthetase

Asparagine synthetases have been isolated from a variety of bacterial, animal and plant sources. A bacterial enzyme from *Escherichia coli* requires ammonium, aspartate and ATP-Mg^{2+} as substrates but cannot use glutamine (10). On the other hand, asparagine synthetase from chick liver embryo requires glutamine and aspartate, but cannot use ammonium (1). Other asparagine synthetases from animal sources and all plant asparagine synthetases studied thus far can use either glutamine or ammonium and also require aspartate and ATP-Mg^{2+} (20,27,42, 43,46,48,49).

Plant and animal asparagine synthetases can be classified as amidotransferases: enzymes which catalyze the transfer of the amide group of glutamine to another substrate. The glutamine analogues azaserine, albizziine, and 6-diazo-5-oxo-L-norleucine (DON) bind irreversibly to the glutamine active site of amidotransferases (7). These analogues have been used extensively for exploring the active site.

The first successful extraction of a plant enzyme which synthesizes asparagine from aspartate was by Rognes in 1970 (42). For this enzyme obtained from lupin cotyledons, maximum activity with glutamine was four to ten times the maximum activity with NH_4Cl; the

ratio varied with the pH. The Km for glutamine was 0.5 mM. Streeter extracted an asparagine synthetase from soybean cotyledons (46). In this case the maximum activity with glutamine was four times that with NH_4Cl and the Km values were 0.12 mM glutamine and 3.1 mM NH_4Cl; this represents a 25-fold difference. He concluded that glutamine was the physiologically important substrate.

Working with extracts from lupin cotyledons, Lea and Fowden obtained a 100-fold purified enzyme by ammonium sulphate precipitation and filtration through Sephadex G-75 and G-200 columns (27). The ratio of maximum reaction rates with glutamine and NH_4Cl was 10:3 and the Km values were 0.04 mM glutamine and 2.1 mM NH_4Cl, a 50-fold difference. An experiment in which tissue exudate was centrifuged through a sucrose gradient indicated that the enzyme is located in the soluble portion of the cell. Asparagine (2 mM) inhibited the reaction 50%. Because 2.5 mM AMP inhibited the enzyme reaction 50% and 2.5 mM ADP did not inhibit the reaction, the authors concluded that AMP and inorganic pyrophosphate are the actual products of ATP cleavage by asparagine synthetase.

Because azaserine and albizziine (5 mM) almost completely inhibited the reaction, Lea and Fowden concluded that the reaction did indeed involve the transfer of the amide nitrogen of glutamine to another molecule. The reaction was also inhibited by β-hydroxy-L-aspartate and β-methyl-L-aspartate. From this the authors concluded that the β-carbon atom of aspartate was not involved in the binding to the enzyme.

Further work with lupin cotyledon extracts has enabled Rognes to purify asparagine synthetase over 500-fold (43). He was able to

identify AMP as a reaction product and did some initial velocity kinetic studies of the glutamine-dependent reaction. His results suggest that the enzyme operates according to a ping pong mechanism involving the formation of an adenylylated enzyme intermediate.

Stulen and Oaks extracted asparagine synthetase from corn seedling roots and found that on a fresh weight basis, activity was about five times higher in the mature section of the root than in the root tip (48). However, when excised root tips were preincubated for five hours in a 1/10 strength Hoagland's solution, enzyme activity rose to about two-thirds the level in the mature section. Preincubation of the mature section led to a decrease in activity. Because the development of activity in the root tip was sensitive to cycloheximide, an inhibitor of protein synthesis, and cordycepin, an inhibitor of RNA synthesis, they concluded that the new activity is dependent on the *de novo* synthesis of enzyme. Relatively high concentrations of albizziine (50 mM) and azaserine (100 mM) inhibited the enzyme reaction 62%. Azaserine inhibited competitively with either ammonium or glutamine (Stulen and Oaks, unpublished results).

The enzyme from both root sections could react twice as fast with glutamine as with NH_4Cl; the Km values were 1.0 mM glutamine and 2.5 mM NH_4Cl (49). These results indicate that the corn root enzyme reacts only slightly better with glutamine than with NH_4Cl. Similar heat-denaturation curves were obtained for the glutamine- and NH_4Cl-dependent activities in root tip and mature root sections. This suggests that asparagine synthetases from root tip and mature root preparations are essentially the same.

More extensive work has been done on asparagine synthetases from animal and bacterial sources. Horowitz and Meister purified an asparagine synthetase from mouse leukemia cells 173-fold (20). Like the plant enzymes that have been studied, it can use either glutamine (Km=1.0 mM) or NH_4Cl (Km=9 mM) as nitrogen donors. The enzyme exhibits glutaminase activity as well as asparagine synthetase activity; the chloride ion is essential for the glutaminase and glutamine-dependent activities, but not for the NH_4Cl-dependent activity. Preincubation of the extract with the glutamine analogue L-2-amino-4-oxo-5-chloropentanoic acid for 20 minutes led to almost complete decay of the glutamine-dependent activity and almost no decay of the NH_4Cl-dependent activity.

Working with asparagine synthetase from mice, Cooney *et al.* found that it could react with both nitrogen donors, having a preference for NH_4Cl at pH 7.6 or lower, and for glutamine at pH 8.0 or higher (13). They found that mucochloric acid inhibited competitively with respect to glutamine and non-competitively with respect to NH_4Cl. However, it inhibited the NH_4Cl-dependent activity more strongly than the glutamine-dependent activity. The authors suggested that there were two binding sites on asparagine synthetase--one for NH_4Cl and one for glutamine. The inhibitor was behaving as a glutamine analogue but interacted with both sites on the enzyme. On the other hand, DON inhibited the glutamine-dependent activity much more strongly than the NH_4Cl-dependent activity.

Cedar and Schwarz purified asparagine synthetase from *Escherichia coli* 370-fold and found that it required aspartate, ATP-Mg^{2+} and NH_4Cl as substrates, but could not use glutamine (10,11). Asparagine, AMP and

inorganic pyrophosphate were produced stoichometrically as reaction products. Asparagine (5 mM) inhibited the reaction completely. The Km for NH_4Cl was about 0.1 mM and concentrations above 0.5 mM were inhibitory. Studies on reaction mechanism indicated that aspartate and ATP first bind to the enzyme in random order with the formation of a β-aspartyl adenylate intermediate. Pyrophosphate then leaves; this is followed by the binding of ammonium to the enzyme. Asparagine and AMP then separate from the enzyme in random order. The addition of the substrates aspartate, ATP and magnesium acetate separately to the extract did not protect the enzyme from heat inactivation, but addition of all three substrates together resulted in substantial protection of enzyme activity.

The purpose of the present study is to look at asparagine synthetase levels in maize tissue at various stages of development and to partly characterize the enzyme from four different tissues: developing endosperm, developing embryo, scutellum of the young seedling and primary root of the young seedling. Particular emphasis is placed on examining the possibility of the existence of isozymes in the different tissues by studying heat denaturation of asparagine synthetase, nitrogen donor Km values and product inhibition.

MATERIALS AND METHODS

A. Materials

Corn seeds (variety W64A X W182E) were obtained from the Wisconsin College of Agriculture, Madison, Wisc.; soybean seeds (*Glycine max* (L.) Merr.) were a gift from J.G. Streeter.

Other chemicals and materials were obtained from the following sources: Trizma base, aminooxy acetic acid, L-aspartic acid, adenosine-5'-triphosphate (ATP), adenosine-5'-diphosphate (ADP), adenosine-5'-monophosphate (AMP), L-asparagine, L-glutamic acid, cycloheximide, methionine sulfoximine and methionine sulfone, Sigma Chemical Co., St. Louis, Mo., puromycin, Nutritional Biochemicals Corporation, Cleveland, Ohio; ammonium sulfate, 2-mercaptoethanol and magnesium chloride, Baker Chemical Co., Phillipsburg, N.J.; L-glutamine for routine assays and 1,2 naphthoquinone-4-sulfonic acid, Eastman Kodak Co., Rochester, N.Y.; L-glutamine for Km experiments and azaserine, Calbiochem, San Diego, Calif.; ammonium chloride and potassium phosphate, Fisher Scientific Co., Fair Lawn, N.J.; Bacto-agar, Difco Laboratories, Detroit, Mich.; Sephadex G-100, Pharmacia, Uppsala, Sweden; Dowex ion-exchange resins, Bio-Rad Laboratories, Richmond, Calif.; and L-[^{14}C(U)]-aspartate, L-[^{14}C(U)]-glutamine and Omnifluor, New England Nuclear, Boston, Mass.

B. Preparation of Tissue

1. Young Seedlings

Corn seeds (W64A X W182E) were rinsed in distilled water and planted on 0.9% Bacto-agar made up in one-tenth strength Hoagland's solution which contained 10 mM $(NH_4)_2SO_4$ and 10 mM KNO_3. Seeds were germinated and allowed to grow in the dark for 66-72 hours at 25°C.

Two sections of the primary root were studied: the root tip (0-10 mm from tip) and the mature section (20-35 mm from the tip). When scutella were used, the root, embryonic axis and endosperm were first removed. One experiment used endosperm tissue from seedlings 70 hours after imbibition.

After excision, root tips were preincubated for five hours under gentle aeration in a solution of 1/10 strength Hoagland's, 10 mM $(NH_4)_2SO_4$ and 10 mM KNO_3. Usually about 0.45 grams of tissue were added to 100 ml of solution. Roots were washed with distilled water before extraction.

Soybean seed was surfaced sterilized in 0.05% hypochlorite solution for 15 minutes, washed with 0.01 N HCl and rinsed six times in distilled water. Seeds were planted in a moist vermiculite-sand mixture and germinated in the dark for eight days at 27°C. Cotyledons were then harvested.

With the exception of root tips, prepared tissue was chilled on ice before being homogenized.

2. Developing Seeds

Corn seeds (W64A inbted or W64A X W182E) were germinated in soil and grown indoors under fluorescent lighting at 23°C with a 16 hour light period. Plants were cross-pollinated by hand when silks first appeared. Cobs were collected at various times after pollination and kernels were removed. When kernels were older than 15 days post-pollination, the embryonic axis-scutellum (referred to in this thesis as "developing embryo") was removed from the endosperm. Both tissues were frozen immediately in liquid nitrogen and subsequently stored at -20°C for up to 10 days before use.

C. Preparation of Materials

Before use, Dowex-AG-1-X10 resin (200-400 mesh, chloride form) was converted to the acetate form by the standard procedure described by Barnard (3). After use, resin was stored in dilute acetic acid to prevent microbial growth and was regenerated in bulk. Dowex-AG-50W-X8 resin (200-400 mesh, hydrogen form) was cleaned with hydrochloric acid and water according to standard procedures (3).

L-[^{14}C(U)]-aspartate was passed through a Dowex-1-acetate column to remove other neutral and basic contaminants. The final ^{14}C-aspartate fraction was taken up in H_2O to give a concentration of approximately 11.0 uCi/ml. The pH was checked for neutrality using pH paper. L-[^{14}C-(U)]-glutamine was passed through a Dowex-1-acetate column only, in order to remove acidic amino acids. Final concentration was about

13.3 uCi/ml.

For preparation of scintillation cocktail, four grams of Omnifluor were first dissolved in one liter of toluene. For early experiments, two volumes of the above solution and one volume of Triton X-114 were mixed and used as scintillation cocktail. This cocktail was used in determining radioactivity in ^{14}C in aqueous solution. Because this cocktail mixes poorly with water, an improved cocktail, containing equal volumes of Triton and toluene-Omnifluor, was used in later experiments. Similar radioactivity readings were obtained using each cocktail.

D. Extraction and Assay of Asparagine Synthetase

Using a mortar and pestle and sea sand, tissue was homogenized at 5°C in 0.1 M potassium phosphate buffer, 25 mM 2-mercaptoethanol, 10.0 mM $MgCl_2$, 1.0 mM ATP and 0.80 mM aspartate (extraction buffer). The volume of buffer per gram fresh weight varied with the type of tissue. Extract was strained through miracloth and centrifuged for 20 minutes at 12,000 xg; the supernatant was saved and constituted the crude extract.

In most experiments, the protein fraction containing asparagine synthetase was precipitated with $(NH_4)_2SO_4$ at 45% saturation (embryo and scutellar extracts) or 50% saturation (mature root, endosperm and soybean extracts). After standing on ice for 30 minutes, this was centrifuged at 27,000 xg for 10 minutes. The supernatant was discarded

and the pellet was suspended in a small amount of extraction buffer (less than 2.0 ml). This suspension was layered on a 200 X 14 mm Sephadex G-100 column pre-equilibrated with extraction buffer; this buffer was also used to elute the protein. All procedures were carried out at 0-5°C. The major purpose of passing the extract through the column was to remove small molecules that could interfere with the enzyme reaction. The first ml of protein-containing eluant was discarded; the next 3-10 ml were collected and assayed. This constituted the Sephadex-filtered extract.

All substrates were dissolved in 0.08 M Tris buffer, pH 8.0, containing 1.0 mM 2-mercaptoethanol. Assays were usually run in 12 ml glass centrifuge tubes. For all tissue extracts except scutellar, each assay tube contained routinely 1.90 mM ATP, 10.0 mM glutamine, 13.2 mM $MgCl_2$, 1.00 mM aminooxy acetic acid (a transaminase inhibitor)[1], 1.06 mM aspartate, approximately 0.22 uCi L-[^{14}C-(U)]-aspartate, and 0.2 ml of tissue extract, in a total volume of 0.62 ml[2]. Most experiments with scutellar extract routinely used 25 mM glutamine and 2.30 mM ATP. When NH_4Cl was used in place of glutamine, routine concentration for all tissues was 10 mM. For blank values the extract was boiled for approximately 60 seconds before the incubation.

Enzyme reaction was started by transferring tubes from ice to a

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[1] Aminooxy acetic acid is a specific inhibitor of all transaminases (6). In maize tissue there is a considerable activity which converts aspartate to alanine. This reaction is inhibited by the addition of aminooxy acetic acid (Stulen and Oaks, unpublished results).

[2] Calculation of substrate concentrations takes into account contributions from the extraction buffer.

35°C water bath; standard incubation time was 20 minutes. The reaction was stopped by the addition of one ml of 95% ethanol. Tubes were centrifuged for 20 minutes at 2000 r.p.m. on a table top centrifuge to remove precipitated protein. The supernatant was poured off and evaporated. A 0.5 ml aliquot of water was added to each tube and passed through a 50 X 5 mm Dowex-1-acetate column into a scintillation vial. Columns were eluted with an additional 1.0 ml of water in early experiments or an additional 1.5 ml of water in later experiments. Unreacted aspartate remains on the column whereas asparagine passes through with the water. Fifteen ml of scintillation cocktail was added to each vial and the counts per minute in asparagine were determined using a Beckman LS-250 liquid scintillation counter. The nanomoles of asparagine formed was calculated for each assay tube from counts per minute. One nanomole corresponded to about 700 counts per minute.

The concentration of total protein in the extracts was determined by the method of Lowry *et al.* (29).

Alterations from routine procedure are explained in the "Results" section.

E. Demonstration of Identity of Radioactive Product as Asparagine

Three routine assays were run using crude extracts of mature roots. Water eluants from Dowex-1-acetate columns were lyophilized. One sample was treated with 2 N HCl at 100°C for four hours to hydrolyze

asparagine to aspartate and the others served as controls. All samples were spotted on a paper chromatogram and cold asparagine and aspartate were spotted as standards. The two amino acids were separated by descending chromatography using butanol:acetic acid:water (3:1:1 by volume) as solvent, and were developed with 0.02% 1,2-naphthoquinone 4-sulfonic acid in acetone. Chromatogram strips were cut up into small pieces and radioactivity in each was determined by liquid scintillation counting. Toluene-omnifluor was used as scintillant.

Asparagine chromatographed six to nine cm from the origin and aspartate was found 10-14 cm from the origin. If asparagine were indeed the ^{14}C product of enzyme reaction, radioactivity in the samples not treated with HCl should co-chromatograph with asparagine, and radioactivity in the HCl-treated samples should be found with aspartate. Of the total radioactivity detected in the control samples, 68% was found with asparagine and 16% with aspartate. In the HCl-treated sample, 11% of radioactivity co-chromatographed with asparagine and 82% with aspartate. Some radioactivity chromatographed between the positions of the two amino acids. It was concluded that the majority of the radioactive product was indeed asparagine.

RESULTS

A. Dependence of Enzyme Reaction on Volume of Extraction Buffer Used in Homogenizing Tissue

In the first few experiments, tissue was homogenized in two ml of extraction buffer per gram fresh weight. It soon became apparent that the reaction was not linear for longer than 20 minutes, the standard incubation time. By making a more dilute extract or by passing the crude extracts through Sephadex, linear kinetics and higher activities on a gram fresh weight basis were usually obtained. In this section, experiments to determine the ideal volumes of extraction buffer for each maize tissue are described.

Root tips were homogenized in two, four and ten ml of extraction buffer per gram fresh weight and the reaction was allowed to run for periods up to 90 minutes. After 20 minutes incubation, activity per gram fresh weight was similar in four and ten ml per gram extracts but lower in the two ml per gram extract. The reaction was almost linear in the four ml per gram extract for periods up to 60 minutes (Figure 3). In a second experiment, tissue was homogenized in four and six ml of buffer per gram fresh weight. After 20 minutes incubation, activity per gram fresh weight was higher in the six ml per gram extract (103 nmoles asparagine per gram) than in the four ml per gram extract (83 nmoles asparagine per gram). It was decided to homogenize root tips

in six ml of extraction buffer per gram fresh weight.

On a fresh weight basis, activity in a four ml per gram extract of mature roots was slightly higher than activity in a two ml per gram extract after 20 minutes incubation. After 60 minutes, the rate of asparagine formation fell off in both cases (Figure 4). It was decided to homogenize mature roots in two ml of extraction buffer per gram fresh weight because activity per assay tube was much higher in the two ml per gram extract than in the four ml per gram extract after 20 minutes incubation.

With developing embryos, activity per gram fresh weight in a four ml per gram extract was about 20% higher after 20 minutes incubation than in a two ml per gram extract after 25 minutes incubation. The reaction in the four ml per gram extract was linear for 60 minutes (Figure 5). In the two ml per gram extract, the reaction did not continue after 25 minutes. It was decided to homogenize embryo tissue in four ml of extraction buffer per gram fresh weight.

With scutellar tissue, activity per gram fresh weight in a six ml per gram extract was about 25% higher than activity in a four ml per gram extract after 20 minutes incubation (Figure 6). Activity in the six ml per gram extract was linear for at least 40 minutes; activity in a four ml per gram extract from a separate experiment was linear for only 20 minutes. It was decided to homogenize scutella in six ml of extraction buffer per gram fresh weight.

In developing endosperm, a much higher activity was recovered when ten rather than two or four ml of extraction buffer was used per

Figure 3. Time Course of Asparagine Synthetase Reaction in Crude Extracts from Root Tips.

*nmoles asparagine/g. fresh weight

Root tips were preincubated for five hours in 1/10 strength Hoagland's solution + 10 mM $(NH_4)_2SO_4$ + 10 mM KNO_3. Tissue was homogenized in: O two, ◑ four or ● ten ml of extraction buffer per gram fresh weight. The mean (circles) ± range (bars) of two replicate assays is shown for each point. Extracts were assayed using 10 mM glutamine and routine concentrations of other substrates (see "Methods").

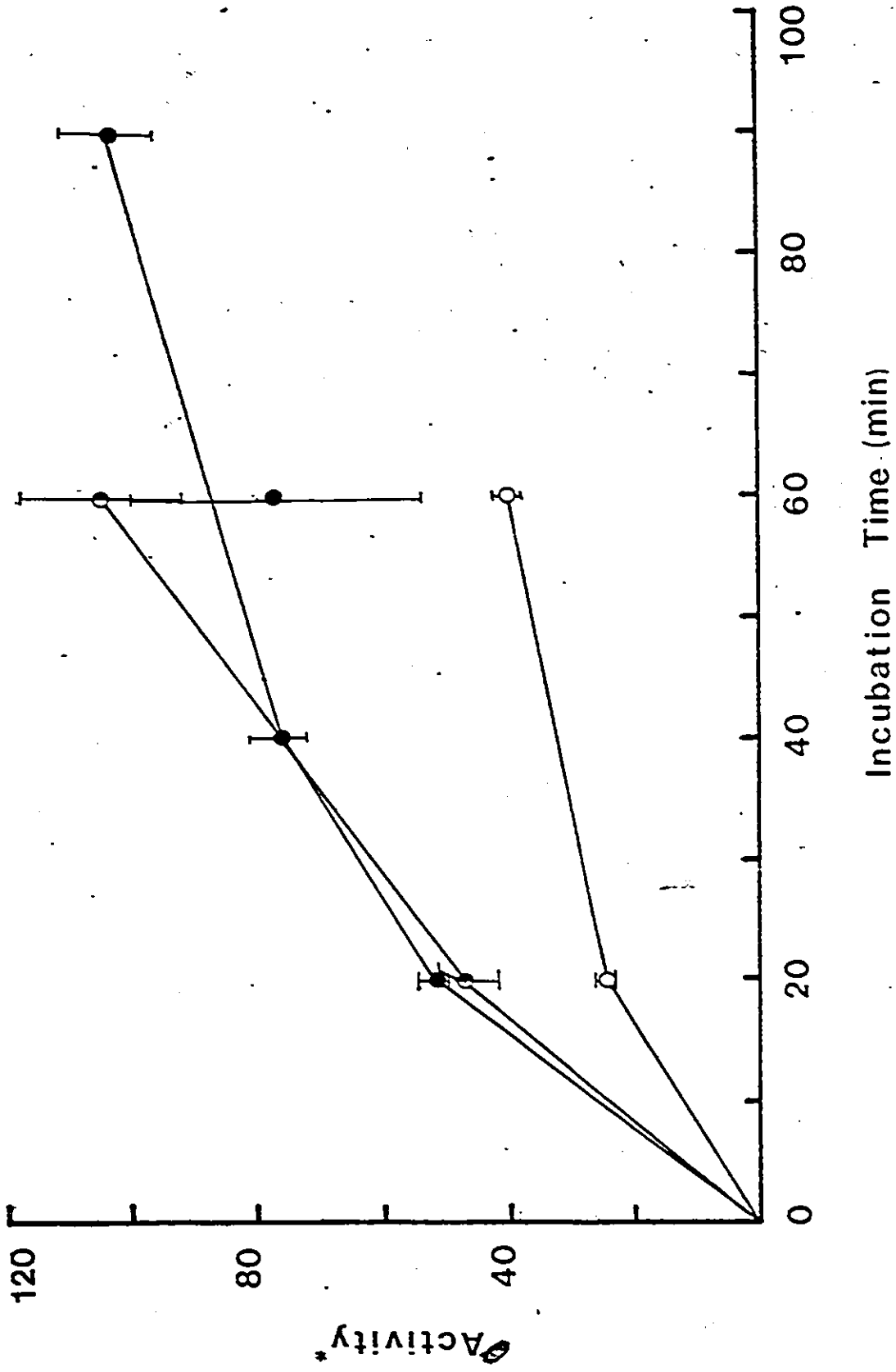

120
80
40
0
Activity*
0
20
40
60
80
100
Incubation Time (min)

Figure 4. Time Course of the Asparagine Synthetase Reaction in Crude Extracts from Mature Root.

*nmoles asparagine/g. fresh weight

Tissue was homogenized in: O two or ● four ml of extraction buffer per gram fresh weight. The mean (circles) ± range (bars) of two replicate assays is shown for each point. Extracts were assayed using 10 mM glutamine and routine concentrations of other substrates (see "Methods").

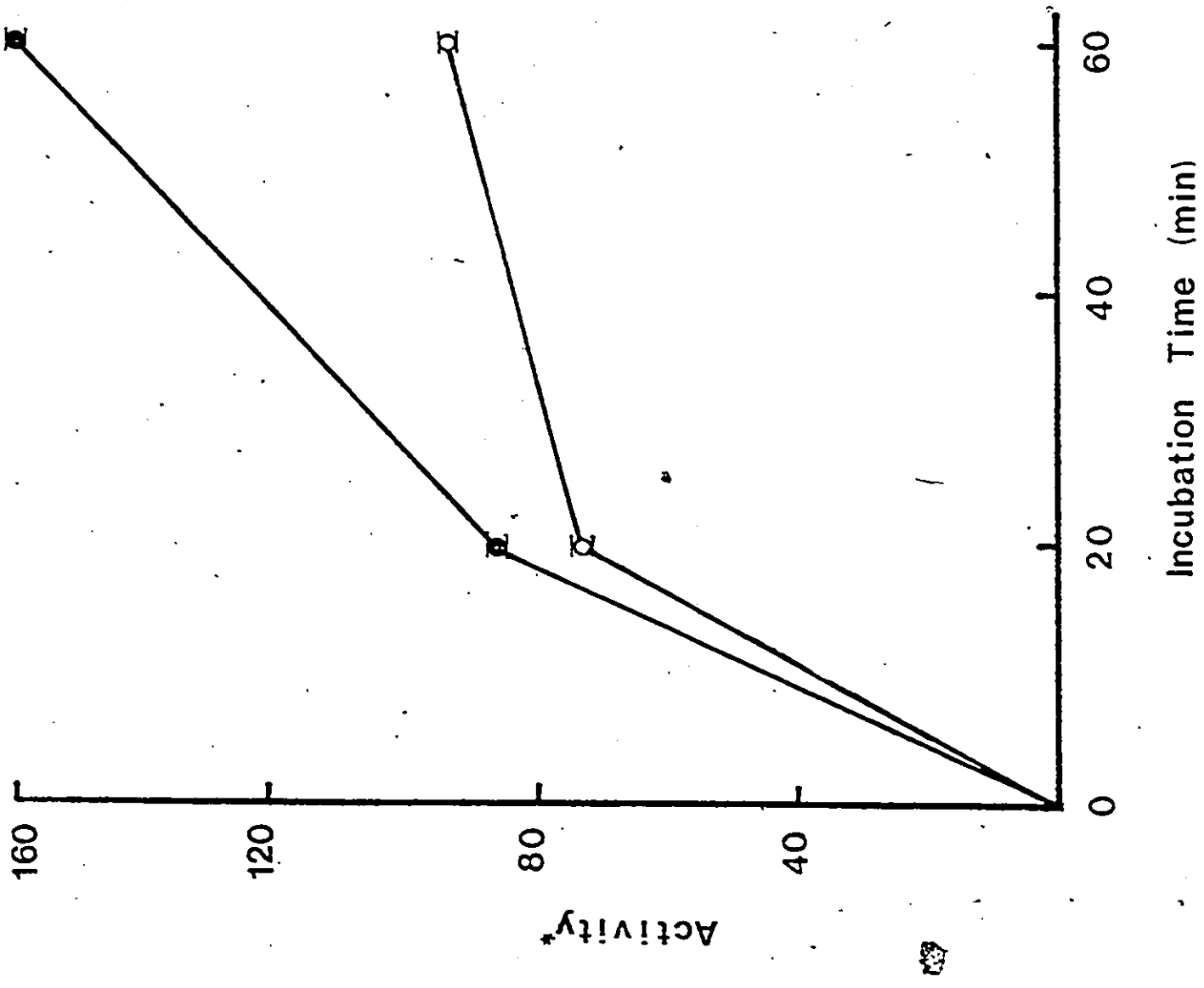

160
120
80
40
0
Activity*
0
20
40
60
Incubation Time (min)

Figure 5. Time Course of the Asparagine Synthetase Reaction in Crude Extracts from Developing Embryos.

*nmoles asparagine/g. fresh weight

Tissue was homogenized in O two or ● four ml of extraction buffer per gram fresh weight. The mean (circles) ± range (bars) is shown for assays done in duplicate. Extracts were assayed using 10 mM glutamine and routine concentrations of other substrates (see "Methods").
Embryo tissue (W64AxW182E) was harvested 20 days after pollination.

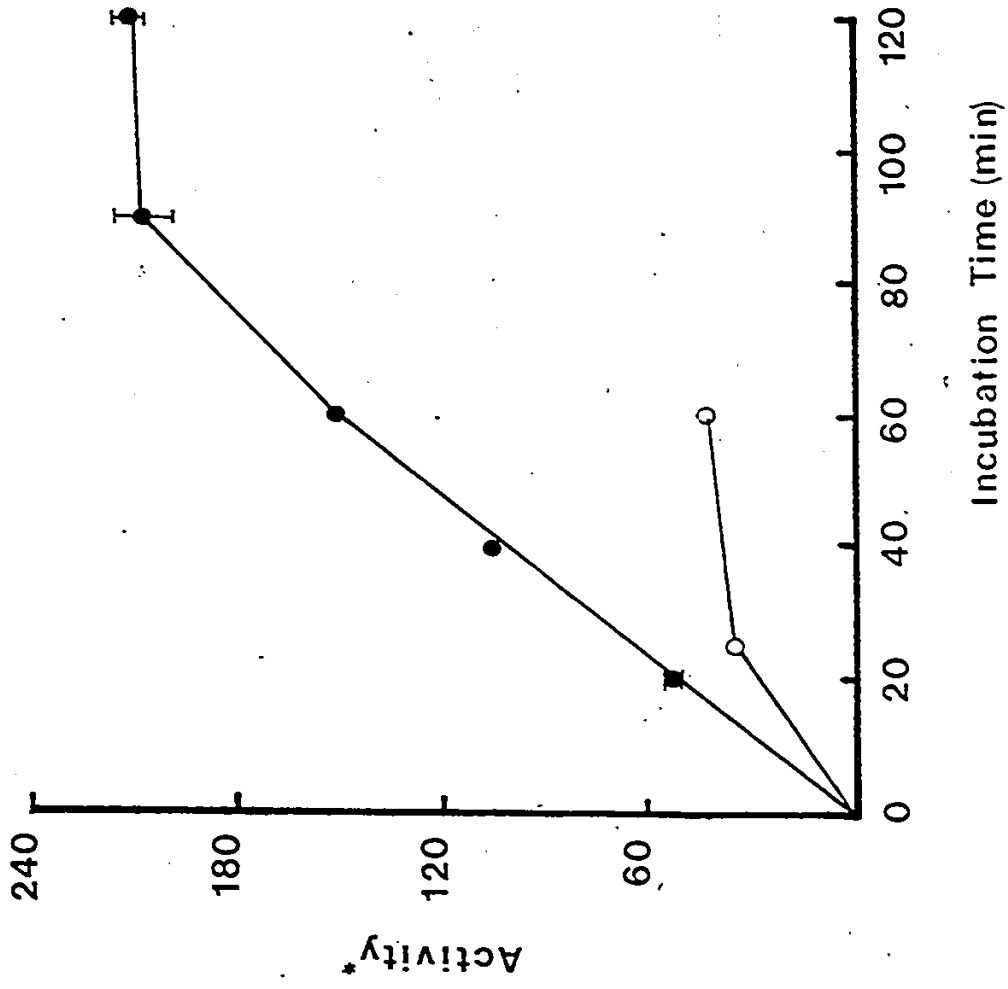
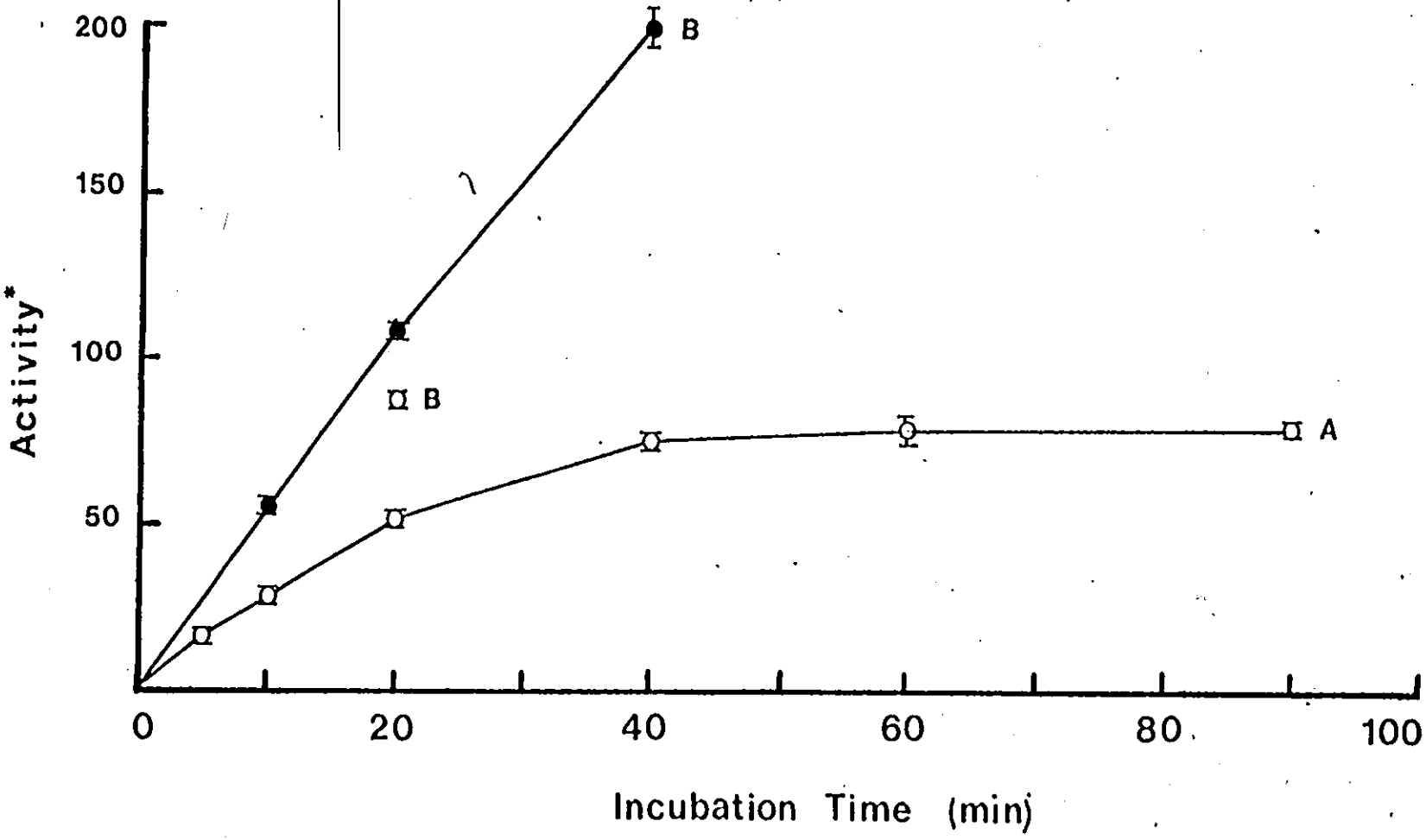

Activity*
240
180
120
60
0
20
40
60
80
100
120
Incubation Time (min)

Figure 6. Time Course of the Asparagine Synthetase Reaction in Crude Extracts from Scutella.

*nmoles asparagine/g. fresh weight

Tissue was homogenized in four or six ml of extraction buffer per gram fresh weight. The mean (circles) ± range (bars) of two replicate assays is shown for each point. Extracts were assayed using 10 mM glutamine and routine concentrations of other substrates.

Data are from two separate experiments ("A" and "B"). In experiment "B", the four ml/g. extract was assayed using a 20 minute incubation only. The single point is presented so that a comparison of enzyme activities in four and six ml/g. extractions of the same batch of scutella can be made.


200
150
100
50
Activity*
B
B
A
0
20
40
60
80
100
Incubation Time (min)

gram of tissue (Figure 7). In addition, with ten ml of extraction buffer the reaction was linear for 60 minutes. In the four ml per gram extract, the reaction rate fell off considerably after 20 minutes and in the two ml per gram extract, activity after 60 minutes incubation was actually lower than activity after 20 minutes incubation. These results suggest that some factor reduces asparagine synthetase activity in the more concentrated extracts. It was decided to homogenize endosperm tissue in ten ml of buffer per gram fresh weight, when crude extract only was to be assayed. If extract was to be filtered through Sephadex, endosperm would be homogenized in four ml of buffer per gram fresh weight.

B. Effect of Passing the Crude Extract Through Sephadex

1. Elution Pattern of Total Protein and Asparagine Synthetase Activity

A protein fraction (which included asparagine synthetase) in the crude extract was often precipitated with ammonium sulphate and filtered through a Sephadex G-100 column in order to remove small molecules from the extract (see "Methods"). Figure 8 shows the elution of total protein and asparagine synthetase activity when mature root extract was filtered through Sephadex. The first ml after the void volume contained almost one-half of the total protein eluted but only 15% of total activity--most asparagine synthetase eluted after the first ml. When embryo extract was filtered through Sephadex, the first 1.6 ml after void volume contained 29% of total protein eluted but only nine

Figure 7. Time Course of the Asparagine Synthetase Reaction in Crude Extracts from Developing Endosperm.

*nmoles asparagine/g. fresh weight

Tissue was homogenized in O two, O four or ● ten ml of extraction buffer per gram fresh weight. The mean (circles) ± range (bars) of two replicate assays is shown for each point. Extracts were assayed using 10 mM glutamine and routine concentrations of other substrates (see "Methods"). Endosperm tissue (W64AxW182E) was harvested 20 days after pollination.

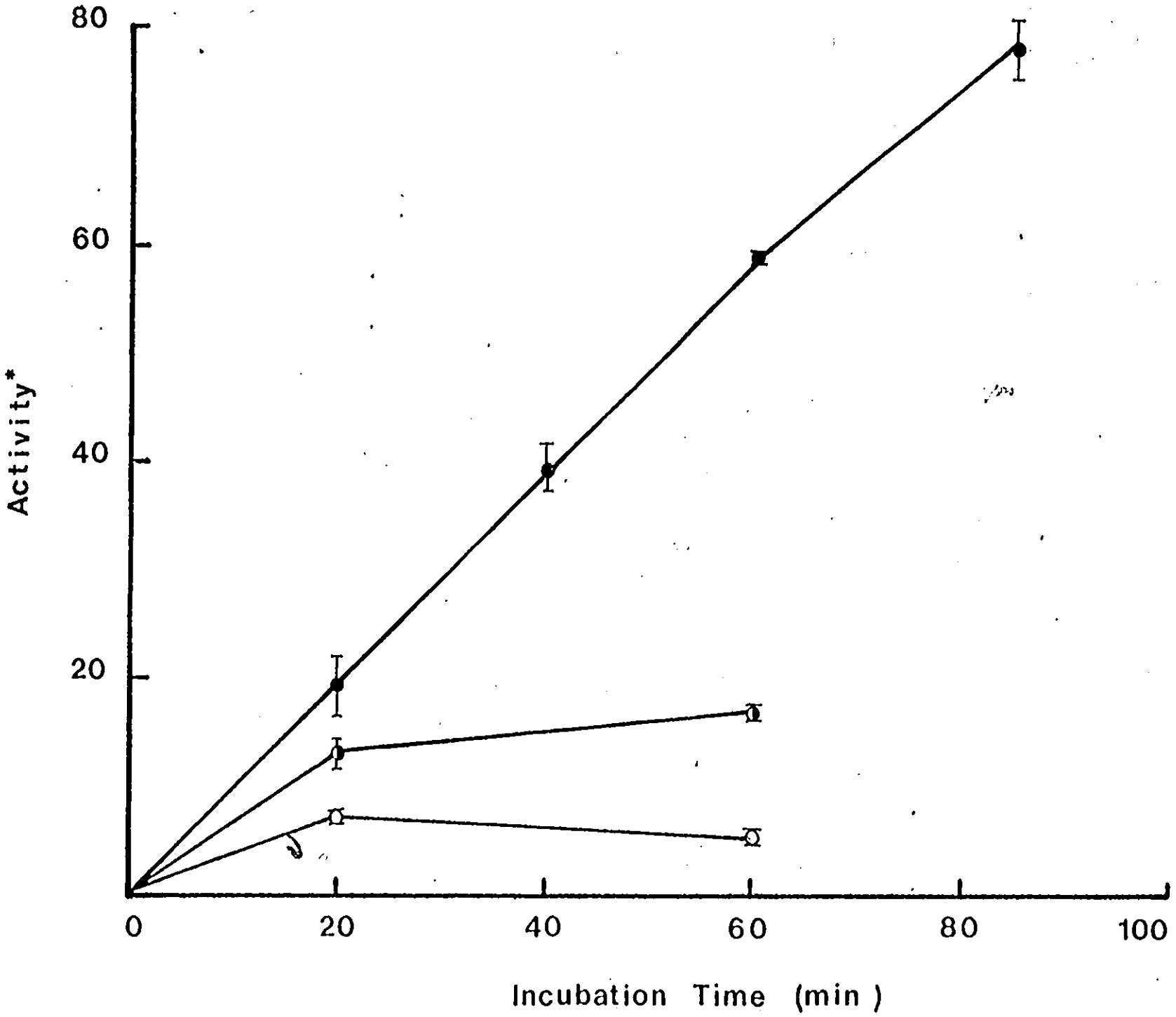
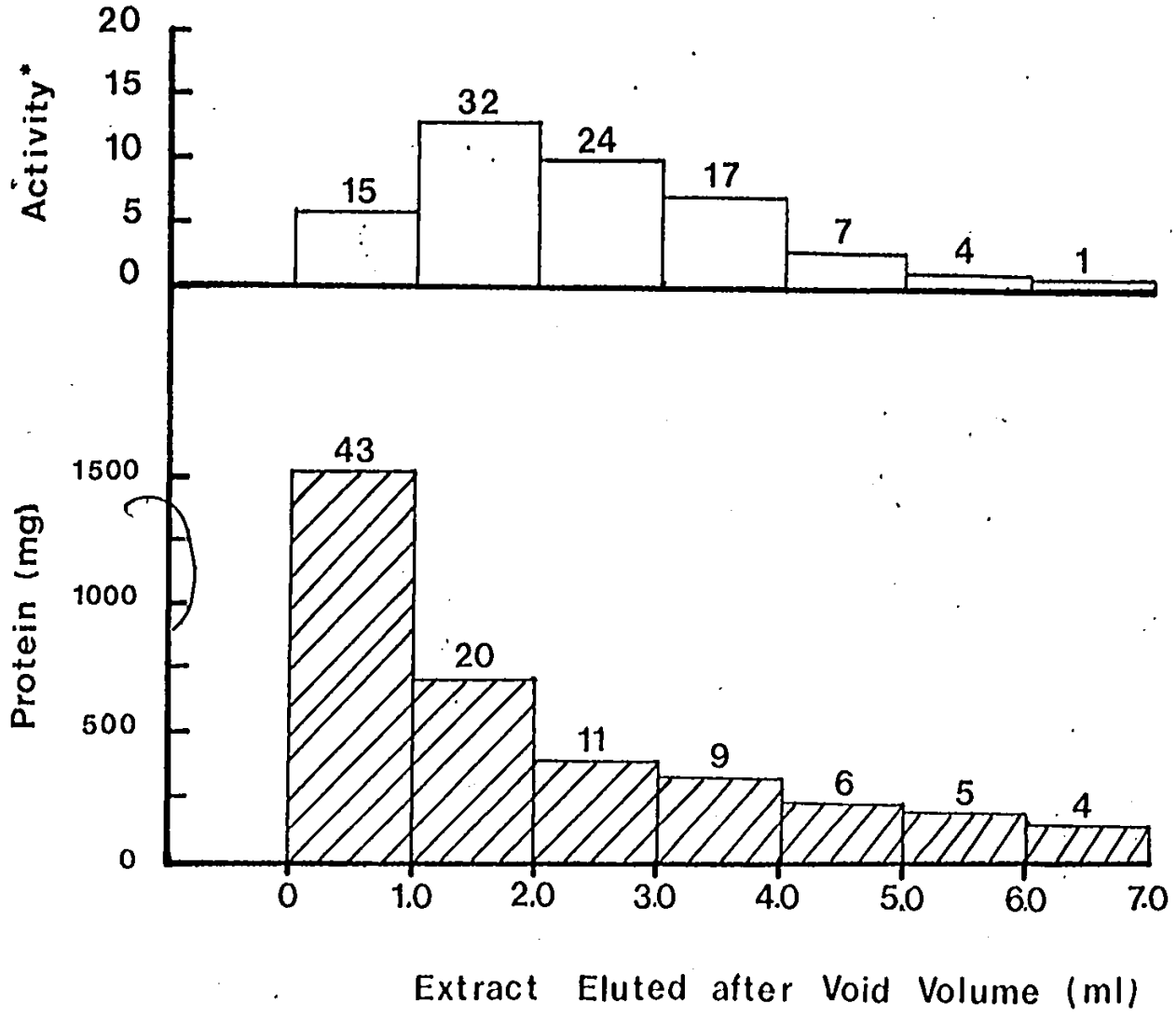

80
60
40
20
Activity*
0
20
40
60
80
100
Incubation Time (min)

Figure 8. Elution of Total Protein and Asparagine Synthetase Activity When Mature Root Extract is Filtered Through Sephadex.

*nmoles asparagine/20 minutes/fraction

Figures above histogram bars show protein or activity as % total collected.
Protein in the crude extract was precipitated with $(NH_4)_2SO_4$ and filtered through a Sephadex G-100 column (see "Methods"). Fractions were assayed in routine fashion using 10 mM glutamine. The void volume was 8.1 ml.


Activity*
20
15
10
5
0
15
32
24
17
7
4
1
Protein (mg)
1500
1000
500
0
43
20
11
9
6
5
4
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Extract Eluted after Void Volume (ml)

per cent of the total activity (Figure 9). Almost half the total protein and activity was found in the following 1.2 ml fraction. It was decided that when any extract would be filtered through Sephadex, the first ml which contained protein would be discarded.

2. Recovery of Asparagine Synthetase Activity

Table 1 summarizes the percent recovery of total activity when crude extracts were filtered through Sephadex, and the change in specific activity. More than 65% of total activity was recovered after filtration of crude scutellar or endosperm extract through Sephadex, but only about 40% of activity was recovered after filtration of crude mature root section extract. Total activity in embryo and soybean extracts was higher after filtration through Sephadex; this suggests that a factor in the crude extracts is inhibiting enzyme activity. The increase in specific activity when crude extract was filtered through Sephadex ranged in maize tissue from none (mature root) to four fold (embryo). The increase in soybean was eight fold.

3. Kinetics of Sephadex-treated Enzyme

Asparagine synthetase activity in Sephadex-filtered scutellar, endosperm and mature root extracts was linear for 90 minutes when assayed with glutamine (Figures 10 and 11). However, when extract from mature root section was assayed with 10 mM NH_4Cl, the reaction was faster during the initial ten minutes of incubation than during subsequent incubation. Between 20 and 90 minutes, the reaction was linear but much slower than

Figure 9. Elution of Total Protein and Asparagine Synthetase Activity When Extract from Developing Embryo is Filtered Through Sephadex.

*nmoles asparagine/20 minutes/fraction

Figures above histogram bars show protein or activity as % total collected.
Protein in the crude extract was precipitated with $(NH_4)_2SO_4$ and filtered through a Sephadex G-100 column (see "Methods"). Fractions were assayed in routine fashion using 10 mM glutamine. The void volume was 7.6 ml.

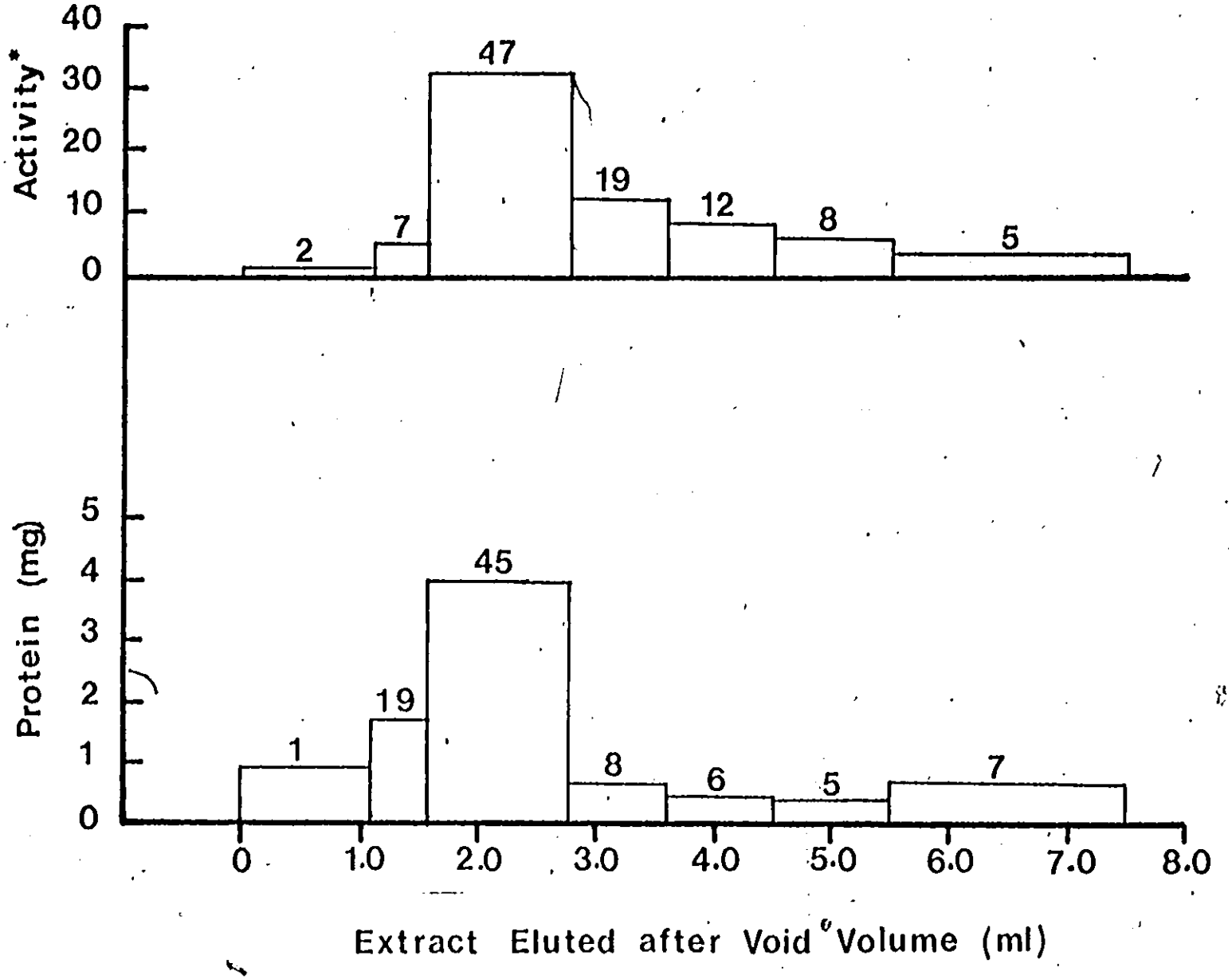

Activity*
40
30
20
10
0
2
7
47
19
12
8
5
Protein (mg)
5
4
3
2
1
0
1
19
45
8
6
5
7
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Extract Eluted after Void Volume (ml)

Table 1

Asparagine Synthetase Activity in Crude and Sephadex-filtered Extracts

Tissue	Trial	Total Activity Crude[1]	Total Activity Sephadex-filtered[1]	% Recovery	% Recovery (mean)	Specific Activity Crude[2]	Specific Activity Sephadex-filtered[2]	Purification	Purification (mean)
Developing embryo, W64A, 20-30 days post pollination	1	65.0	98.8	152	130	1.99	7.72	3.9	3.9
	2	101	124	122		1.08	4.92	4.6	
	3	60.9	72.4	119		3.22	10.2	3.2	
Developing endosperm, W64A, 25 days post pollination	1	100	93.0	93	81	1.72	3.32	1.9	1.8
	2	75.6	61.0	81		1.58	3.29	2.1	
	3	313	213	68		2.17	3.04	1.4	
Scutellum	1	652	571	87	66	6.33	14.0	2.2	1.9
	2	737	422	57		5.54	9.31	1.7	
	3	416	221	53		3.40	6.50	1.9	
Mature Root	1	450	203	45	39	18.7	25.9	1.4	1.0
	2	485	184	38		25.9	21.9	.85	
	3	713	243	34		21.1	18.0	.85	
Soybean Cotyledon	1	2360	3760	159		5.35	43.7	8.2	

Continued...

Table 1 (continued)

[1] nmoles asparagine/20 minutes/total volume of extract.

[2] nmoles asparagine/20 minutes/mg protein.

[3] Data are reported from the three experiments which yielded highest recoveries of activity. Data for soybean are from one experiment only.

Extracts were assayed using 25 mM glutamine (scutellar) or 10 mM glutamine (all others) and routine concentrations of other substrates (see "Methods").

Figure 10. Time Course of the Asparagine Synthetase Reaction in Sephadex-Filtered Endosperm and Scutellar Extracts.

*nmoles asparagine/assay tube

O Endosperm Extract (W64A, 25 days post-pollination)

● Scutellar Extract

Activity was assayed using 10 mM glutamine (endosperm extract) or 25 mM glutamine (scutellar extract) and routine concentrations of other substrates (see "Methods"). The mean (circles) ± range (bars) of two replicate assays is shown for each point.

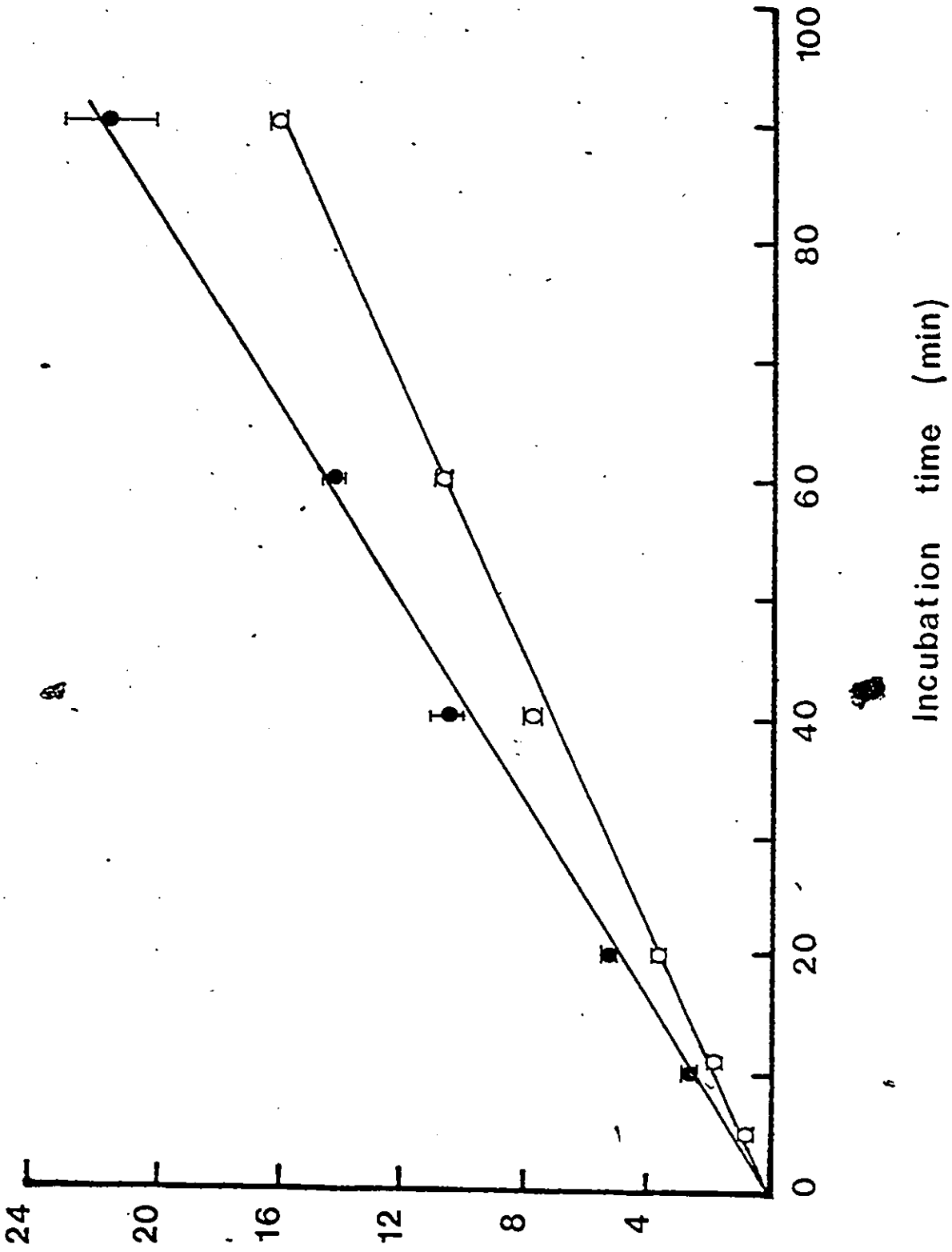
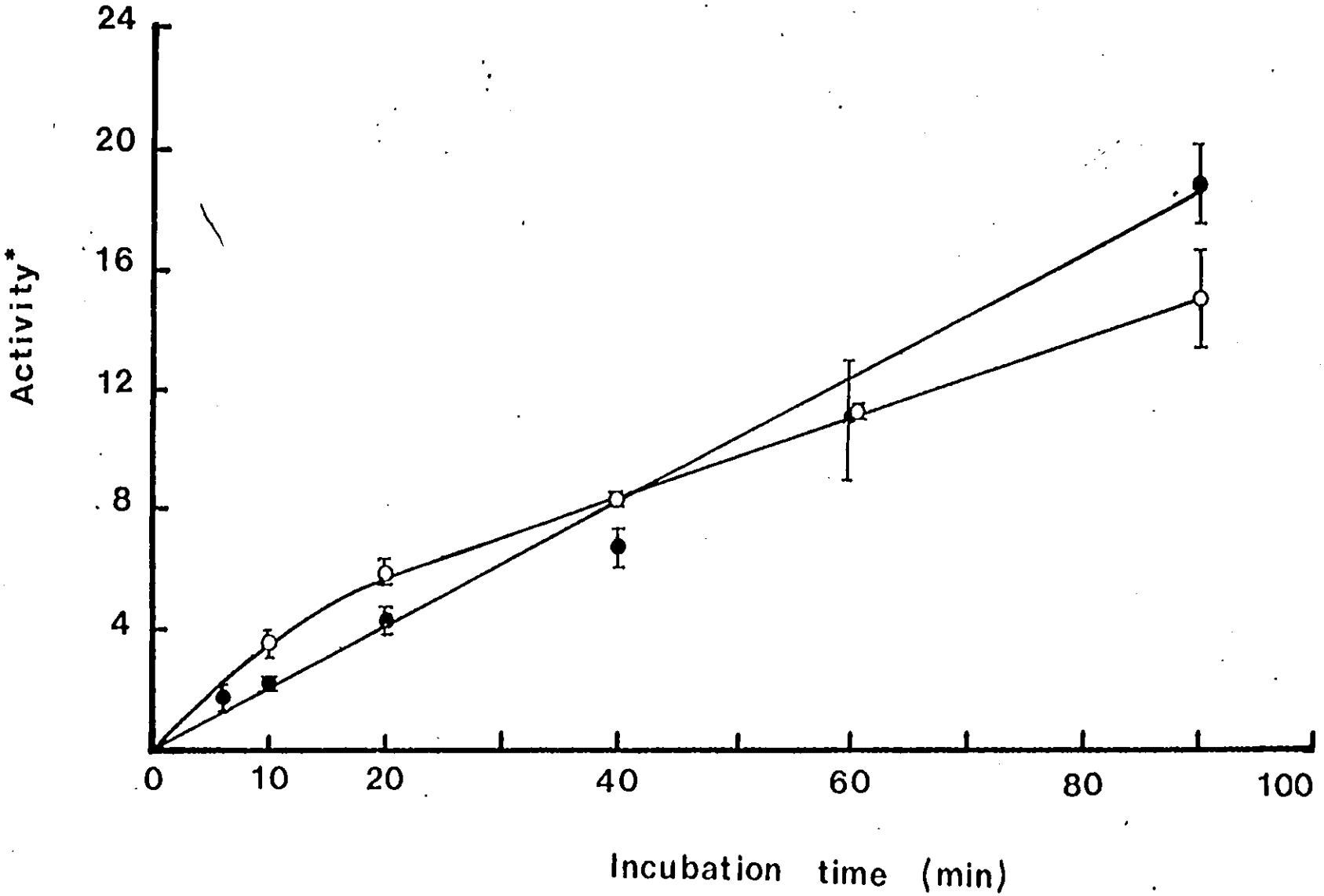

Activity*
24
20
16
12
8
4
0
20
40
60
80
100
Incubation time (min)

Figure 11. Time Course of the Asparagine Synthetase Reaction in Sephadex-Filtered Mature Root Extract.

*nmoles asparagine/assay tube

Extracts were assayed using O 10 mM NH_4Cl or ● 10 mM glutamine and routine concentrations of other substrates (see "Methods"). Data are from two experiments. The mean (circles) ± range (bars) of two replicate assays is shown for each point.


24
20
16
12
8
4
Activity*
0
10
20
40
60
80
100
Incubation time (min)

during the first 20 minutes of incubation.

4. Dependence of Enzyme Activity on Volume of Extract Assayed

In a crude extract of developing endosperm homogenized in four ml of extraction buffer per gram of tissue, activity per assay tube decreased with increasing volume of extract (Table 2). This result again suggests the presence of some inhibiting factor. Activity in Sephadex-filtered scutellar and developing endosperm extracts was linear in the range 0.1-0.3 ml of extract per assay tube.

C. Optimizing the Reaction

1. Optimizing Substrate Concentrations

Optimal substrate concentrations were determined for asparagine synthetase in crude scutellar extracts (Table 3). Activity was much higher when enzyme was assayed with 2.67 mM aspartate than with 1.06 mM aspartate. However, counts per minute in asparagine were about 25% lower when the higher concentration of aspartate was used (blank-corrected cpm/20 minutes with 1.06 mM aspartate were 3430, and with 2.67 mM aspartate, 2560). Activity was over 20% higher when extract was assayed with 13.2 mM $MgCl_2$ than with 5.2 or 33.2 mM $MgCl_2$. Activity was essentially the same with 1.92, 2.32 and 4.32 mM ATP. A higher concentration of ATP (10.32 mM) inhibited the reaction. Slightly more activity was obtained with 25 mM glutamine than with 10 mM glutamine. It was decided that future assays of scutellar extract (but not extracts from other tissues) would employ routinely 25 mM glutamine, 2.32 mM ATP,

Table 2

Effect of Volume of Extract Assayed on Asparagine Synthetase Activity

Extract	ml Extract/Tube	Activity/Tube[1]	Activity/ml Extract[1]
Crude Endosperm (W64A, 25 days post-pollination)	0.1	0.895 ± .005	8.95
	0.2	1.18 ± .04	5.90
	0.3	1.10 ± .01	3.67
Sephadex-filtered endosperm (W64A, 25 days post-pollination)	0.1	1.24 ± .13	12.4
	0.2	3.72 ± .09	18.6
	0.3	4.96 ± .28	16.5
Sephadex-filtered scutellar extract	0.1	6.36 ± .16	63.6
	0.2	13.8 ± .3	69.0
	0.3	19.8 ± .6	66.0

[1] Enzyme activities are expressed as nmoles asparagine formed/20 minutes. The mean ± range of two replicates is shown for Activity/tube.

Assays were run in a total volume of 0.62 ml using 25 mM glutamine (scutellar or extract) or 10 mM glutamine (endosperm extract) and routine concentrations of other substrates (see "Methods").

Table 3

Effect of Substrate Concentrations on Asparagine Synthetase Activity in Crude Scutellar Extract

Substrate[1]	Concentration (mM)	Activity (nmoles asparagine/20 minutes/tube)	Activity as % Control
ATP	1.92	4.39 ± .05	(100)
	2.32	4.56 ± .00	104
	4.32	4.38 ± .04	100
	10.32	1.74 ± .15	40
Aspartate	0.66	3.02 ± .02	69
	1.06	4.39 ± .05	(100)
	2.67	8.27 ± .08	188
Glutamine	1.0	3.43 ± .01	78
	5.0	4.14 ± .08	94
	10.0	4.39 ± .05	(100)
	25.0	4.84 ± .02	110
$MgCl_2$	5.2	3.55 ± .06	81
	13.2	4.39 ± .05	(100)
	33.2	3.43 ± .01	78

[1] Routine concentrations were 1.92 mM ATP, 1.06 mM aspartate, 10 mM glutamine and 13.2 mM $MgCl_2$. Other assay conditions are described under "Methods".

The mean ± range of two replicate activities is shown. Tissue was homogenized in four ml extraction buffer per gram fresh weight.

13.2 mM $MgCl_2$, and 1.06 mM aspartate.

2. Preparing the Sephadex-filtered Extract

Table 4 shows the effects of omitting ATP or Aspartate from the extraction buffer. Activity was similar in a routine crude extract from mature roots (ATP, aspartate and $MgCl_2$ included) and in crude extracts minus ATP or aspartate. There was a similar recovery of activity when "routine" or "minus aspartate" extracts were filtered through Sephadex (almost 20 %) but an almost complete loss of activity when a "minus ATP" extract was filtered through Sephadex. It was concluded that inclusion of ATP is essential for recovery of enzyme activity during the filtration of extracts through Sephadex.

3. The Effect of Bovine Serum Albumin on Enzyme Activity

During the course of experiments reported in this thesis, bovine serum albumin was added to several extracts to adjust total protein concentration. The effect of albumin on enzyme activity in several extracts is shown in Table 5. In crude extracts from root tip or mature root sections, 0.3% albumin had no significant effect on glutamine-dependent enzyme activity. However 0.1 or 0.3% albumin doubled glutamine-dependent activity in Sephadex-filtered mature root extracts. On the other hand, 0.3% albumin increased the NH_4Cl-dependent activity by only 27% (one experiment only). In a Sephadex-filtered scutellar extract, 0.08% albumin did not stimulate activity; it may have caused a slight inhibition.

Table 4

Effect of Omitting Aspartate or ATP from the Extraction Buffer on Activity in Mature Root Extract

Substrates in Extraction Buffer			Activity[1] in Crude Extract	Activity[1] in Sephadex-filtered Extract	Percent Recovery of activity
$MgCl_2$	Aspartate	ATP			
+	+	+	113	18.4	16.3
+	-	+	93.9	18.1	19.3
+	+	-	125	3.04	2.4

[1] nmoles asparagine/20 minutes in total extract.

All substrates were added to extraction and assay buffers in routine concentrations (see "Methods"). Total volume of each crude and Sephadex-filtered extract was 3.5 and 2.0 ml, respectively.

Table 5

Effect of Bovine Serum Albumin on Asparagine Synthetase Activity

Extract	Nitrogen Donor in Assay (10 mM)	% Albumin in Extract	% Albumin in Assay	Activity -Albumin	Activity +Albumin	$\frac{+\text{Albumin}}{-\text{Albumin}} \times 100\%$
Root Tip, Crude[1]	Glutamine	1.0	0.32	3.12±.09	3.01±.06	96
	Glutamine	2.0	0.65	3.12±.09	2.77±.08	89
Mature Root, Crude[2]	Glutamine	1.0	0.32	9.64±.41	9.10±.05	94
Mature Root[2], Sephadex-filtered	Glutamine	0.10	0.032	4.57±.44	6.17±.44	135
	Glutamine	0.30	0.097	6.24±.03	12.0 ±.3	192
	Glutamine	0.30	0.097	6.13±.01	12.8 ±.3	209
	Glutamine	1.0	0.32	4.57±.44	8.35±.07	183
	NH_4Cl	1.0	0.32	5.81±.35	7.38±.36	127
Scutellar, Sephadex-filtered[3]	Glutamine	0.080	0.026	6.97	2.89±.23	41

Procedures followed before assaying were:

[1] Extract was diluted to 4/5 strength with H_2O or albumin in H_2O.

[2] Extract was diluted to 9/10 strength with extraction buffer or albumin in extraction buffer.

[3] Control was extract containing 10 mM glutamine. This extract was then diluted to ½ strength with extraction buffer containing 0.5% albumin and 10 mM glutamine, and assayed again. Thus if albumin had no effect, activity in the diluted extract should be 50% control activity.

All extracts were assayed in routine fashion (see "Methods"). Mean ± range of two replicates is shown for most assays.

4. Effect of Potential End Products on the Rate of Reaction

Enzyme reactions are often inhibited by their end products. This inhibition can result from a product's competition for a substrate's binding site or a change in conformation of the protein caused by a product binding to a separate site. Inhibition by the potential end products of the glutamine-dependent asparagine synthetase reaction was tested using Sephadex-filtered endosperm, scutellar and mature root extract, in order to find out how enzyme activity might be regulated in each tissue.

Asparagine inhibited the reaction in endosperm extract more strongly than in mature root or scutellar extracts; two mM was required for 50% inhibition in endosperm while in mature root or scutellar extract 10 mM inhibited only 20-30% (Table 6). AMP and ADP, which are possible alternate products resulting from ATP hydrolysis, inhibited reaction in scutellar extract similarly and more strongly than asparagine; 10 mM was required for about 45% inhibition. ATP (10 mM) also inhibited the reaction (Table 3). In mature root extract, AMP and ADP inhibited the reaction more strongly than in scutellar extract; 10 mM AMP and ADP caused 70 and 80% inhibition respectively. ADP inhibited the mature root enzyme reaction more strongly than AMP. Ten mM glutamate increased the activity in scutellar extract about 20%. However, when mature root enzyme was assayed with 10 mM glutamate in the absence of glutamine, some apparent activity resulted (11% of control activity). Thus the stimulation of activity by glutamate may be a result of some glutamate-requiring reaction which converts ^{14}C-aspartate to a

Table 6

Effect of Potential End Products on the Asparagine Synthetase Reaction

End Product Added	Concentration (mM)	Endosperm Extract Trial 1	Activity[1] Mature Root Extract Trial 1	Mature Root Extract Trial 2	Scutellar Extract Trial 1	Scutellar Extract Trial 2
None (control)	-	2.44±.27	5.56±.06	8.45±.28	6.32±.28	7.84±.17
	0.1	2.52(103)	-	-	-	-
	0.5	1.64 (67)	-	7.71 (91)	-	7.16 (91)
Asparagine	2.0	1.15 (47)	-	7.00 (83)	-	6.57 (84)
	10.0	1.02 (41)	-	7.05 (83)	-	5.58 (71)
	0.5	-	-		5.03 (80)	-
AMP	2.0	-	-	6.28 (74)	4.57 (72)	7.13 (91)
	10.0	-	1.79 (32)	2.45 (29)	3.67 (58)	4.31 (55)
	0.5	-	-	-	5.26 (83)	-
ADP	2.0	-	-	4.72 (56)	5.30 (84)	6.68 (85)
	10.0	-	1.07 (19)	1.75 (21)	3.44 (54)	3.67 (47)
Glutamate	10.0	-	-	-	7.59(120)	-

[1] nmoles asparagine/20 minutes/g fresh weight. For control assays, mean ± range of two replicates is shown. Replicate assays were run with end products except with endosperm extract. In brackets is activity as % control.

All extracts were filtered through Sephadex before being assayed. All assays included 10 mM glutamine and routine concentrations of other substrates (see "Methods").

non-acidic product. These results provide no evidence that glutamate has a direct effect on asparagine synthetase activity. In summary, no inhibitor caused more than 50% inhibition of activity at a concentration of 2.0 mM or less. Thus the results suggest that none of the products is a strong regulator of the glutamine-dependent asparagine synthetase activity in any tissue studied. However, it does appear that asparagine is a weak regulator of the enzyme in developing endosperm.

D. Asparagine Synthetase Levels in Maize

1. Enzyme Levels in Various Tissues

Table 7 compares asparagine synthetase activity in all tissues studied. Endosperm and embryo tissue was studied at several stages of development; the data reported here are for the stage when highest activity was found. On a fresh weight basis, activity was much higher in soybean cotyledons than in any maize tissue studied. In corn, activity in the root tip was about one-fifth the mature root activity but rose two and one-half fold if root tips were preincubated in Hoagland's solution. Activity in developing endosperm was even lower than in the root tip. Activity in developing embryo reached a level close to three times that reached in the developing endosperm on a fresh weight basis.

Specific activity was about four times higher in the mature root than in the preincubated root tip or scutellum; activity per mg protein in developing tissues was 25-45% the scutellar level.

Table 7

Asparagine Synthetase Levels in Various Tissues of Maize and in Soybean Cotyledons

Tissue	Age of Tissue	Extraction Ratio (ml buffer/g tissue)	Activity/g fresh weight[1]	Specific Activity[2]
Developing Endosperm, W64A x W182E	20 days post pollination	10	19*	1.8
Developing Endosperm, W64A	25 days post pollination	4	26	1.9
Developing Embryo, W64A x W182E	20 days post pollination	4	54*	3.0
Developing Embryo, W64A[3]	25 days post pollination	4	68*	-
Mature Root	70 hours post imbibition	2	151	31
Root Tip, fresh	70 hours post imbibition	6	31	3.4
Root Tip, Preincubated for Five Hours	70 hours post imbibition	6	77	7.2
Scutellum	70 hours post imbibition	6	162	6.6
Soybean Cotyledon[3]	8 days post imbibition	4	621*	-

[1] nmoles asparagine/20 minutes/g fresh weight.

[2] nmoles asparagine/20 minutes/mg protein.

Continued...

Table 7 (continued)

[3] Data are from assay of Sephadex-filtered extract. All other results are from assay of crude extract.

* Figure is based on one experiment only. For all other tissues, data from the three experiments which produced highest activities have been averaged.

Extracts were assayed using 25 mM glutamine (scutellar) or 10 mM glutamine (all others) and routine concentrations of other substrates (see "Methods").

2. Effect of Age on the Level of Asparagine Synthetase

a. Endosperm

Developing endosperm (W64A X W182E) were homogenized in ten ml of buffer per gram fresh weight. In each extract, enzyme reaction was close to linear for 60 minutes. Endosperm increased in weight about eight fold between nine and 20 days after pollination; after 20 days, endosperm grew very little (Table 8). Enzyme levels per endosperm also rose about eight fold between nine and 20 days, but by 32 days had declined to one-half the 20 day level. When dry seeds were germinated, no detectable enzyme activity was found in endosperm extracted 70 hours after imbibition.

b. Developing Embryo and Scutellum from Young Seedlings

Developing embryo tissue was homogenized in four ml of buffer per gram fresh weight. The embryo increased in weight about six fold between 20 and 43 days after pollination. When calculated on a gram fresh weight basis, enzyme activity fell from 54 to 22 nmoles asparagine formed per 20 minutes during this time (Table 9). However, enzyme levels per embryo actually rose two and one-half fold between 20 and 43 days after pollination. More activity might have been seen if these extracts had been filtered through Sephadex.

Scutellar tissue from young seedlings was homogenized in six ml of buffer per gram fresh weight. The total enzyme level found in a fully developed scutellum was about one-half the level found in an embryo (embryonic axis and scutellum) harvested 43 days after pollination. The scutellum increased in weight about four-fold during the first 24

Table 8

Asparagine Synthetase Levels in Endosperm During Development

Days after Pollination	Activity[1]/g fresh weight 20 minute assay	60 minute assay	Activity[1]/endosperm 20 minute assay	Average Weight of endosperm(g)
9	18.5 ± 1.3	15.1 ± 1.1	0.46	0.025
20	19.3 ± 3.1	19.7 ± 0.1	3.85	0.199
26	11.7 ± 3.6	11.8 ± 0.5	2.90	0.248
32	8.4 ± 1.4	9.9 ± 0.0	1.92	0.228
70 hours post imbibition	0	-	0	-

[1] nmoles asparagine/20 minutes. For activity/g fresh weight, mean ± range of duplicates is shown.

All results are from assays of crude extracts of endosperm, W64A x W182E. Developing and post-imbibition endosperm were homogenized in ten or four ml buffer per gram fresh weight, respectively. Extraction and assay procedures have been described under "Methods".

Table 9

Asparagine Synthetase Levels in the Developing Embryo and in Scutellar Tissue of Young Seedlings

Developing Embryo: Days after Pollination	Scutellum: Hours after Imbibition	Activity[1] per g. fresh weight	Activity[1] per embryo or scutellum	Average Weight piece (g)
20	-	54.0 ± 0.8	0.38	0.0070
43	-	22.0 ± 0.7	0.98	0.045
-	0	40.8 ± 3.1	0.45	0.011
-	24	48.3 ± 2.5	2.07	0.043
-	48	89.7 ± 2.9	4.24	0.047
-	72	78.6 ± 1.9	3.82	0.049

[1] nmoles asparagine/20 minutes.

"Developing embryo" includes scutellar tissue and embryo tissue. Embryo tissue was removed from scutella harvested 0-72 hours after imbibition. Developing embryos and scutella were homogenized in four and six ml extraction buffer per gram fresh weight, respectively. All data are from assay of crude extracts. The mean ± range of two replicates is shown for developing embryo; mean ± standard deviation of three replicates is shown for scutella. Activity in developing embryo and scutellar extracts was assayed using 10 and 25 mM glutamine, respectively. All tissue was W64A x W182E.

hours after imbibition and grew very little after that time. The enzyme level per scutellum increased about nine-fold between zero and 48 hours after imbibition; enzyme activity per gram fresh weight increased about two fold (Table 9). Seventy-two hours after imbibition, the enzyme level had levelled off.

Several other experiments employed scutella which were harvested 70-72 hours after imbibition. The enzyme levels that were measured varied from 78.6 to 178 nmoles asparagine formed per 20 minutes per gram fresh weight. Hence it is not certain that there was an actual drop in asparagine synthetase from day 43 post pollination to dry seed.

E. Experiments with Inhibitors

1. Effect of Inhibitors on the Development of Asparagine Synthetase

When root tips were excised and preincubated for five hours in a solution of 1/10 strength Hoagland's, 10 mM $(NH_4)_2SO_4$ and 10 mM KNO_3, asparagine synthetase activity increased about two and one-half fold (Table 7 and (48)). This increase could be due to an activation of pre-existing enzyme molecules, or it could result from the synthesis of new enzyme. If the latter were the case, synthesis of new messenger RNA might or might not be required. In order to characterize the cause for the increase in activity, root tips were preincubated with a variety of potential inhibitors of protein or RNA synthesis. Cycloheximide inhibits protein synthesis (40); if *de novo* synthesis of asparagine synthetase were responsible for the increase in enzyme activity, cycloheximide should

inhibit this increase. Puromycin is also an antibiotic which is often used as an inhibitor of protein synthesis (39). Azaserine is a glutamine analogue which can also inhibit nucleic acid synthesis (48). Methionine sulfoximine and methionine sulfone are glutamate analogues which inhibit the glutamine synthetase reaction. Both analogues caused an inhibition of asparagine formation from ^{14}C-acetate in both root tip and mature root sections (38). This inhibition could be due to a lack of glutamine needed for asparagine biosynthesis. However, the analogues might also interfere with the formation of asparagine synthetase.

The effect of these compounds on the development of asparagine synthetase in root tips is shown in Table 10. Cycloheximide (3.6 μM) inhibited the development of asparagine synthetase almost completely. This observation suggests that new enzyme is being synthesized during the incubation period. Oaks and Johnson have shown that cycloheximide at this concentration inhibits protein synthesis almost completely in corn root tips (36). Azaserine (100 μM) inhibited enzyme development about 50%. In experiments similar to those reported here, Stulen and Oaks found that both cycloheximide and azaserine inhibited development of asparagine synthetase activity in root tips (48).

Methionine sulfoximine (2.78 mM) caused a slight inhibition of the development of enzyme activity, whereas methionine sulfone (2.76 mM) caused a slight stimulation; it is doubtful that either had a significant effect. At these concentrations, both compounds caused an inhibition of asparagine formation from ^{14}C-acetate in both root tip and mature

Table 10

The Effect of Several Potential Protein or RNA Synthesis Inhibitors on the Development of Asparagine Synthetase Activity in Excised Root Tips

Inhibitor	Concentration (mM)	Activity Which Developed During the Five Hour Preincubation[1] Trial 1+	2	3	4	5	6
none (control)	-	41.2±1.2 (100)*	45.5±9.1 (100)*	34.2±7.3 (100)*	61.0±18.8 (100)*	50.2±3.6 (100)*	38.0±2.2 (100)*
Cycloheximide	0.0036	≈ 0	3.7±1.3 (8)	-	-	-	-
Azaserine	0.100	16.3±0.4 (40)	23.3±0.9 (51)	-	-	-	-
Methionine sulfoximine	2.78	32.8±0.4 (81)	40.2±2.4 (88)	18.2±2.2 (53)	-	-	-
Methionine sulfone	2.76	59.6±2.7 (144)	59.2±4.2 (130)	39.1±4.2 (115)	-	-	-
Puromycin	0.100	44.5±0.3 (110)	12.3±1.4 (27)	27.3±3.4 (80)*	43.5±5.5 (71)*	63.9±7.6 (127)*	33.9±12.9 (89)*
Activity in the initial root preparation		-	21.8±1.5*	32.4±6.7*	21.9±1.2*	32.1±1.7*	27.9±2.5*

Continued...

Table 10 (continued)

[1] All activities are expressed as nmoles asparagine/20 minutes/g. fresh weight. The activity which developed during the preincubation period was determined by subtracting the activity in the initial root preparation from the total activity found for each sample. In brackets are activities as percent control.

* Two sets of roots were preincubated and homogenized; mean ± standard deviation of four assays is shown. For other samples, mean ± range of two replicates is shown.

+ Activity in the initial root preparation was not determined; activity in roots preincubated with cycloheximide (31.8 nmoles/20 min) was subtracted from activity in other samples.

The roots were preincubated for five hours in 1/10 strength Hoagland's solution plus 10 mM $(NH_4)_2SO_4$, 10 mM KNO_3 and appropriate inhibitor. Roots were homogenized in four (Trials, 1, 2, 3) or six ml buffer per gram fresh weight (Trials 4, 5, 6). Extraction and assay procedures are described under "Methods".

root sections (38). The six trials with puromycin (100 μM) were variable; however, they suggest at most a minor inhibition of the development of asparagine synthetase activity.

An experiment with a crude extract of mature roots showed that puromycin, methionine sulfoximine and methionine sulfone do not significantly inhibit asparagine synthetase activity at the concentrations used in the preincubation solutions. Incubation with each resulted in 95% control activity.

2. Cycloheximide as an Inhibitor of Enzyme Activity

More extensive tests were conducted with cycloheximide since other workers have suggested that it might act in some systems as a glutamine analogue (36). Competition between glutamine and cycloheximide for asparagine synthetase was tested by using several concentrations of each and Sephadex-filtered mature root extract. The results in Table 11 show that high concentrations of cycloheximide cause only minor inhibitions of asparagine synthetase activity, regardless of the concentration of glutamine. By contrast azaserine, a *bona fide* glutamine analogue, inhibited asparagine synthetase completely with respect to glutamine (Stulen and Oaks, unpublished). Hence cycloheximide does not appear to be acting as a glutamine analogue in this case.

F. Glutamine and Ammonia as Alternate Nitrogen Donors

1. Glutamine Hydrolysis During Incubation

Because significant glutamine hydrolysis during incubation could

Table 11

Effect of Cycloheximide on the Glutamine-Dependent Mature Root Enzyme Reaction

Concentration of Cycloheximide (mM)	Enzyme Activity[1] Concentration of Glutamine (mM)		
	0.5	1.0	2.0
0	2.43 ± 0.53(100)	4.14 ± 0.02(100)	5.52 ± 0.18(100)
2.0	2.32 ± 0.86(95)	3.89 ± 0.06(94)	4.49 ± 0.37(81)
5.0	1.41 ± 0.06(58)	2.59 ± 0.88(62)	5.01 ± 0.24(91)
10.0	2.05 ± 0.54(84)	2.94 ± 0.40(71)	3.10 ± 0.88(56)

[1] nmoles asparagine/20 minutes/assay tube. The mean ± range of two replicates is shown; in brackets are activities as percent control (-cycloheximide).

Extract was filtered through Sephadex and was assayed using routine concentrations of all substrates except glutamine (see "Methods"). The reaction was started after the addition of extract to the substrates plus or minus cycloheximide.

Enzyme activity using 10 mM glutamine was 6.24 ± 1.43 nmoles asparagine/20 minutes.

lead to erroneous determination of the Km for glutamine, the extent of glutamine hydrolysis was determined using Sephadex-filtered mature root and scutellar extracts. Routine assay conditions were used except that ^{14}C-glutamine replaced ^{14}C aspartate in the assay. In these experiments the concentration of glutamine was varied by altering the content of ^{12}C-glutamine. Dowex-1-acetate columns were eluted with 12 ml of water to remove unhydrolyzed glutamine. The glutamate-containing fraction was eluted with 3.0 ml 6N acetic acid into scintillation vials and scintillation fluid was added. About 19,000 counts per minute were found in this fraction in samples incubated with boiled extract; this value was subtracted from counts per minute in other samples to give counts per minute in glutamate synthesized by enzymatic hydrolysis of glutamine.

The extent of glutamine hydrolysis during 20 minutes incubation is shown in Table 12. When the concentration of glutamine was 0.333 mM, the lowest concentration used in these experiments, six percent was hydrolyzed by mature root extract, and nine percent by scutellar extract. Total glutaminase activity was much higher than asparagine synthetase activity in both extracts. In the mature root extract, for example, asparagine synthetase activity with 10 mM glutamine was 8.52 nmoles asparagine formed per 20 minutes per assay tube, while total glutaminase activity with 10 mM glutamine was 78.5 nmoles glutamine hydrolyzed per 20 minutes per assay tube. Since nmoles of glutamine hydrolyzed by asparagine synthetase should equal nmoles of asparagine formed, asparagine synthetase would account for only 11% of the observed

Table 12

Hydrolysis of Glutamine by Mature Root and Scutellar Extracts

Concentration of Glutamine (mM)	Mature Root Extract Glutamate formed[1] c.p.m.[2]	nmoles	% conversion[3]	Scutellar Extract Glutamate formed[1] c.p.m.[2]	nmoles	% conversion[3]
10	7155	78.5	1.3	9305	102	1.7
3.33	13050	47.7	2.3	-	-	-
2.00	16670	36.6	3.0	23500	51.6	4.2
1.00	24120	26.5	4.3	35500	39.0	6.3
0.667	26400	19.3	4,7	-	-	-
0.333	35000	12.8	6.2	51700	18.9	9.2

[1] All activities are on a "per 20 minutes per assay tube" basis.

[2] Counts per minute in glutamate, blank-corrected.

[3] Glutamate formed as % glutamine added.

Routine asparagine synthetase assay conditions were employed (see "Methods") except that ^{14}C-glutamine (565,000 c.p.m./assay) was included in each assay and ^{14}C-aspartate was omitted. Incubations with boiled extract (blank) resulted in 19,000 c.p.m. in acidic product. Asparagine synthetase activity (using 10 mM glutamine) in the mature root and scutellar extracts was 8.52 and 15.1 nmoles asparagine/20 minutes/assay tube, respectively.

hydrolysis of glutamine.

2. Km Values for Glutamine and NH_4Cl

Km values for glutamine and NH_4Cl were determined using Sephadex-filtered extract from mature roots, scutellum and developing embryo. Km values for these two substrates have been reported previously for asparagine synthetase from soybean cotyledons (46). To demonstrate that any differences between Km results reported here for corn and reported previously for soybean are real and not a result of differences in experimental procedure, glutamine and NH_4Cl Km values for enzyme from soybean cotyledons were also determined.

Enzyme activity was assayed using several concentrations of glutamine or NH_4Cl and routine concentrations of all other substrates. Km values were determined from Lineweaver-Burk plots. Lines of best fit and correlation coefficients were then calculated using a pocket calculator.

Km values for scutellar and mature root enzyme were also redetermined from a Lineweaver-Burk plot of inverse activity versus inverse estimated final glutamine concentrations obtained from Table 12. Because the original plot ignores the lessening glutamine concentration during the incubation period, it leads to an overestimation of the apparent Km for glutamine. The second plot leads to an underestimation of Km because it takes into account only the concentration of glutamine remaining at the end of the incubation period.

Typical Lineweaver-Burk plots for glutamine and NH_4Cl are shown

in Figures 12-15. A summary of Km values for glutamine is shown in Table 13. Taking into account the problem of glutamine hydrolysis reduced the calculated Km for glutamine for mature section and scutellar enzyme by about 10%; this reduction is quite insignificant. Km values for mature sections (about 0.57 mM) and scutellum (about 0.45 mM) are very similar, but the calculated Km for glutamine for developing embryo enzyme is about three times higher. The Km value obtained in this project for enzyme from soybean cotyledons (0.18 mM) is significantly lower than any value for maize enzyme. It should be noted that 29% of 1.0 mM glutamine was hydrolyzed during the 20 minute incubation with soybean extract, and hydrolysis was probably more extensive when lower concentrations of glutamine were used. Thus 0.18 mM glutamine could be a substantial overestimation.

The apparent Km's for NH_4Cl were calculated for asparagine synthetase in the four tissues and as seen in Table 14 the values are similar. However, the ratio of NH_4Cl Km to glutamine Km was much greater for soybean cotyledon enzyme (16.0) than for enzyme from maize tissue (2.5 - 5.3).

Km values determined in this project for soybean asparagine synthetase (0.18 mM glutamine and 2.9 mM NH_4Cl) compare favourably with Streeter's values of 0.12 mM glutamine and 3.1 mM NH_4Cl (46).

3. Comparison of Maximum Reaction Rates Using Glutamine and NH_4Cl

In many experiments, an enzyme extract was assayed using each nitrogen donor substrate at a concentration of 10 mM. Hypothetical Vmax

Figure 12. Lineweaver-Burk Plots for the Asparagine Synthetase Reaction in Extracts from Developing Embryo.

*(nmoles asparagine/20 minutes/assay tube)$^{-1}$

The substrate varied in the assay was ● glutamine or O NH_4Cl; routine concentrations of other substrates were used (see "Methods"). Circles are results from single assays. Extract from developing embryo (W64A, 25 days post-pollination) was filtered through Sephadex before use. For glutamine, the equation of line of best fit is $\frac{1}{v} = 0.27529\ (\frac{1}{[s]}) + 0.20414$; $r^2 = 0.816$ and Km = 1.35 mM. For NH_4Cl, the equation of line of best fit is $\frac{1}{v} = 1.1658\ (\frac{1}{[s]}) + 0.28930$; $r^2 = 0.900$ and Km = 4.0 mM.

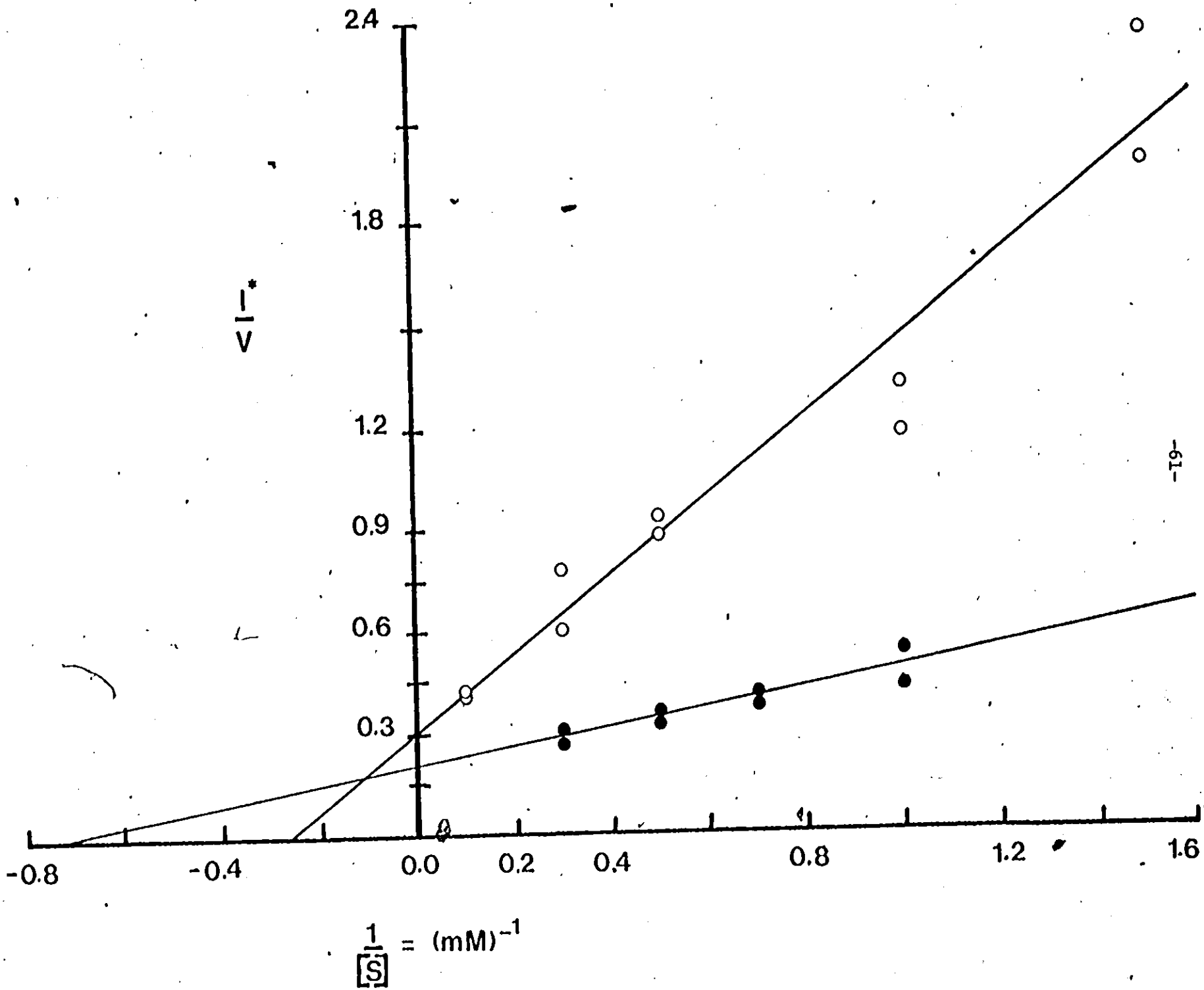

2.4
1.8
1.2
0.9
0.6
0.3
$\frac{I^*}{V}$
-0.8
-0.4
0.0
0.2
0.4
0.8
1.2
1.6
$\frac{1}{[S]} = (mM)^{-1}$

Figure 13. Lineweaver-Burk Plots for the Asparagine Synthetase Reaction in Scutellar Extract.

*(nmoles asparagine/20 minutes/assay tube)$^{-1}$

The substrate varied in the assay was ● glutamine or O NH_4Cl; routine concentrations of other substrates were used (see "Methods"). Circles are results from single assays. Extract was filtered through Sephadex before use. For glutamine, the equation of line of best fit is $\frac{1}{v} = 0.033584\ (\frac{1}{[s]}) +$ 0.073316; $r^2 = 0.996$ and Km = 0.46 mM. For NH_4Cl, the equation of line of best fit is $\frac{1}{v} = 0.36285\ (\frac{1}{[s]}) +$ 0.14265; $r^2 = 0.969$ and Km = 2.6 mM.

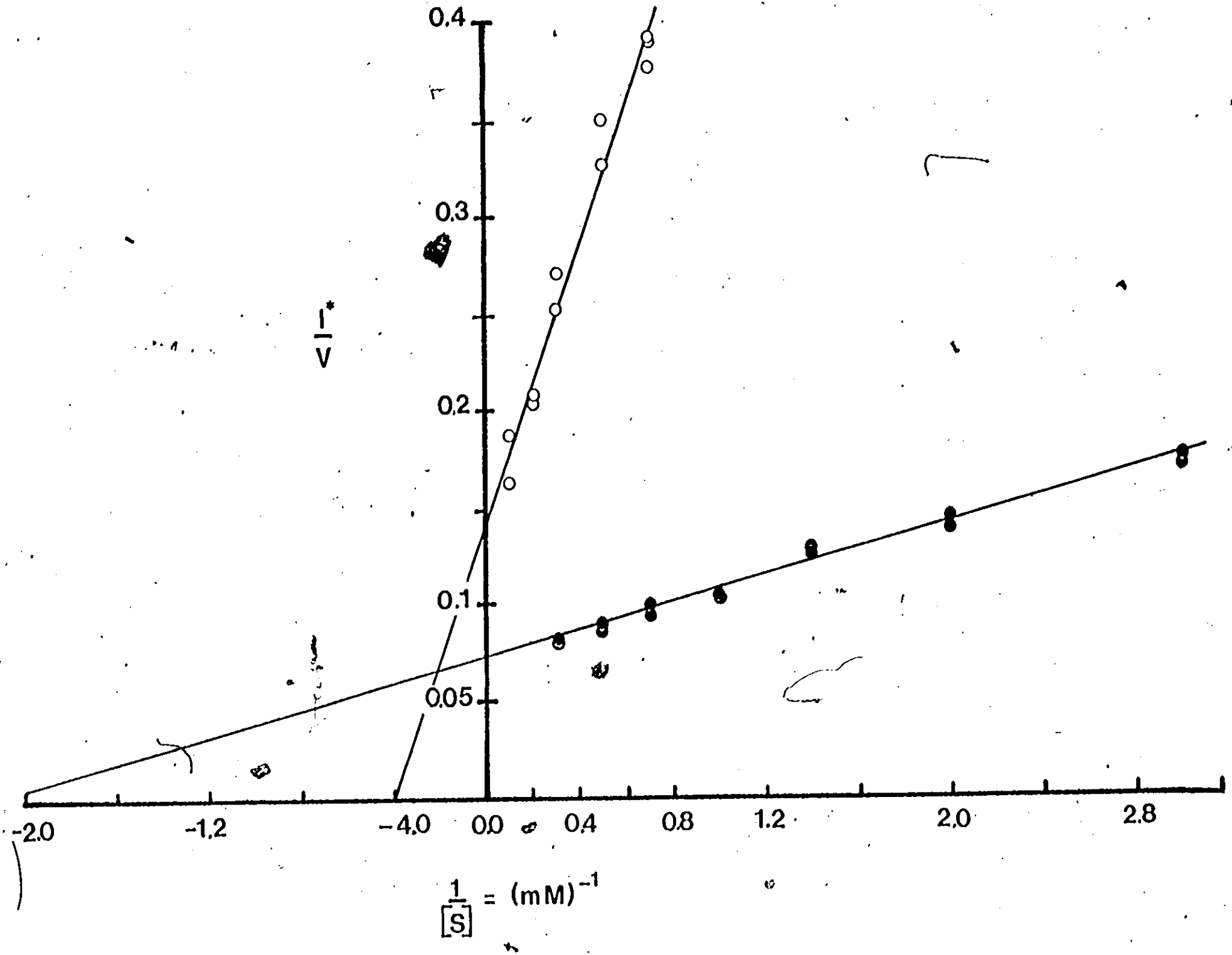

0.4
0.3
0.2
0.1
0.05
$\frac{1}{V}$
-2.0
-1.2
-4.0
0.0
0.4
0.8
1.2
2.0
2.8
$\frac{1}{[S]} = (mM)^{-1}$

Figure 14. Lineweaver-Burk Plots for the Asparagine Synthetase Reaction in Mature Root Extract.

*(nmoles asparagine/20 minutes/assay tube)$^{-1}$

The substrate varied in the assay was ● glutamine or O NH_4Cl; routine concentrations of other substrates were used (see "Methods"). Circles are results from single assays. Extract was filtered through Sephadex before use. For glutamine, the equation of line of best fit is $\frac{1}{v} = 0.067403\ (\frac{1}{[s]}) + 0.15129$; $r^2 = 0.930$ and Km = 0.45 mM. For NH_4Cl, the equation of line of best fit is $\frac{1}{v} = 0.25400\ (\frac{1}{[s]}) + 0.096838$; $r^2 = 0.983$ and Km = 2.6 mM.

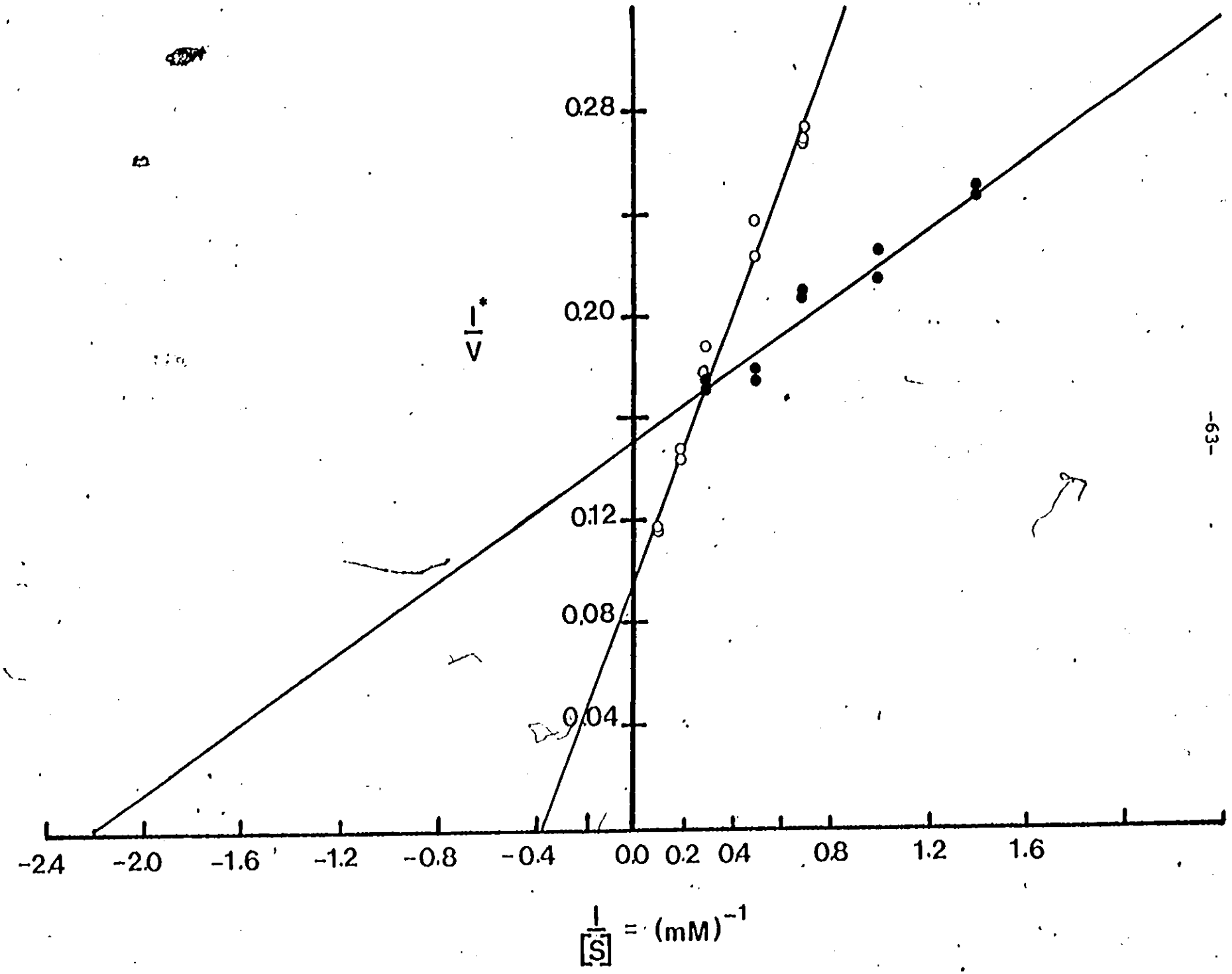

0.28
0.20
0.12
0.08
0.04
$\frac{1}{V}^*$
-2.4
-2.0
-1.6
-1.2
-0.8
-0.4
0.0
0.2
0.4
0.8
1.2
1.6
$\frac{1}{[S]} = (mM)^{-1}$

Figure 15. Lineweaver-Burk Plots for the Asparagine Synthetase Reaction in Soybean Cotyledon Extract.

*(nmoles asparagine/20 minutes/assay tube)$^{-1}$

The substrate varied in the assay was ● glutamine or O NH_4Cl; routine concentrations of other substrates were used (see "Methods"). Circles are results from single assays. Extract was filtered through Sephadex before use. For glutamine, the equation of line of best fit is $\frac{1}{v} = 0.0015499 \left(\frac{1}{[s]}\right) + 0.0084174$; $r^2 = 0.981$; Km = 0.18 mM. For NH_4Cl, the equation of line of best fit is $\frac{1}{v} = 0.070151 \left(\frac{1}{[s]}\right) + 0.023982$; $r^2 = 0.952$; Km = 2.9 mM.

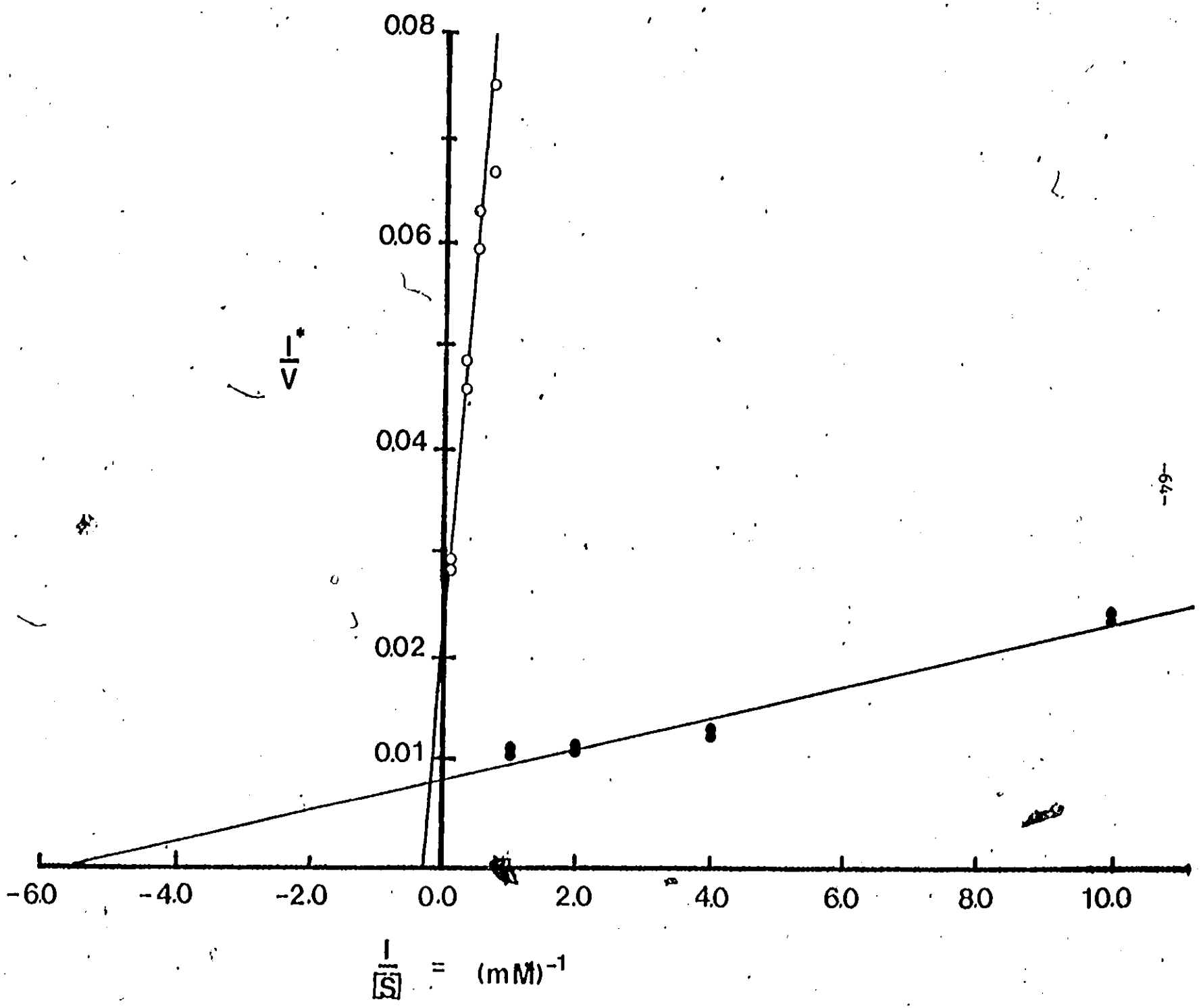

0.08
0.06
0.04
0.02
0.01
I*/V
-6.0
-4.0
-2.0
0.0
2.0
4.0
6.0
8.0
10.0
I/[S] = (mM)-1

Table 13

Km Values for Glutamine in the Asparagine Synthetase Reaction

Tissue	Are Glutamine Concentrations Corrected for Glutamine Hydrolysis?[1]	Km for Glutamine (mM) Trial 1	Trial 2	Trial 3	Mean	r^2[2] Trial 1	Trial 2	Trial 3
Developing Embryo, W64A, 20-25 days post pollination	No	1.3*	2.0	-	1.6	0.816	0.949	-
Mature Root	No	0.63	0.70	0.45*	0.59	0.936	0.900	0.930
	Yes	0.58	0.64	0.42	0.55	-	-	-
Scutellum	No	0.46*	0.53	-	0.49	0.966	0.998	-
	Yes	0.41	0.47	-	0.44	-	-	-
Soybean Cotyledon	No	0.18*	-	-	0.18	0.981	-	-

[1] See Results, Section E, Part 1.

[2] r^2 = Correlation Coefficient for the line of best fit in Lineweaver-Burk plots. Km values were calculated from the equations of these lines.

* Results are presented graphically in Figures 12-15. Routine extraction and assay conditions were used (see "Methods" and "Results, Section A") except that the assay was run using several concentrations of glutamine (0.333-3.33 mM for maize extracts and 0.10-1.0 mM for soybean cotyledon extract).

Table 14

Comparison of Km Values for NH_4Cl and Glutamine in the Asparagine Synthetase Reaction

Tissue	Km for NH_4Cl (mM) (one trial only)	r^2 (1) (for NH_4Cl)	Km for Glutamine (from Table 12)	Km Ratio $\frac{NH_4Cl}{Glutamine}$
Developing embryo, W64A, 25 days post pollination	4.0	0.932	1.6	2.5
Mature Root	2.6	0.983	0.59	4.4
Scutellum	2.6	0.969	0.49	5.3
Soybean Cotyledon	2.9	0.952	0.18	16

1. r^2 = Correlation Coefficient for the line of best fit in Lineweaver-Burk plots. Km values were calculated from the equations of these lines. Results are presented graphically in Figures 12-15. Routine extraction and assay conditions were used (see "Methods" and "Results, Section A") except that the assay was run using several concentrations of NH_4Cl (0.667-10.0 mM for developing embryo extract, and 1.43-10.0 mM for other extracts).

values were calculated using the rearranged Michaelis-Menten equation $V_{max} = v(1 + K_m/[S])$ where S = 10 mM. Table 15 compares maximum rates of enzyme activity using glutamine and NH_4Cl. The results indicate that enzyme from mature root sections but from no other tissue reacted faster with NH_4Cl than with glutamine. Enzyme from developing endosperm, scutellum and soybean cotyledon reacted at a relatively higher rate with glutamine than with NH_4Cl.

G. Heat Denaturation Studies

1. Introduction

When an enzyme in aqueous medium is subjected to an adequately high temperature for an extended period of time, it undergoes a change in conformation with a concomitant loss of enzyme activity. This process occurs at a constant rate, therefore, a plot of log (enzyme activity) versus time of exposure to high temperature should be a straight line. The rate of denaturation increases with temperature.

At a particular temperature and environment, no two enzymes will denature at exactly the same rate. Enzyme or total protein concentration and pH can also affect the rate of denaturation. The inclusion of substrates or inhibitors in the extract can greatly stabilize or destabilize an enzyme by binding to it and changing its conformation.

Heat denaturation studies on asparagine synthetase were undertaken for two reasons:

1. To examine the possibility of isozymes by comparing denaturation rates in extracts of several tissues.

Table 15

Comparison of the Glutamine- and NH_4Cl-dependent Asparagine Synthetase Reactions

Tissue	Trial	Enzyme Activity[1,2] glutamine	Enzyme Activity[1,2] NH_4Cl	Activity Ratio[2] (NH_4Cl/glutamine)		V_{max}[1,3] glutamine	V_{max}[1,3] NH_4Cl	V_{max} Ratio[3] (NH_4Cl/glutamine)	
Mature Root	1	7.70	8.34	1.08		8.14	10.5	1.29	
	2	4.30	2.24	0.54		4.55	2.82	0.62	
	3	7.11	8.57	1.21	1.03	7.52	10.8	1.44	
	4	5.56	7.81	1.41		5.88	9.84	1.67	
	5	6.26	6.00	0.96		6.62	7.56	1.14	
Scutellum	1	5.95	2.53	0.43		6.06	3.19	0.53	
	2	10.4	4.92	0.47	0.44	10.6	6.20	0.58	0.54
	3	13.6	5.78	0.42		13.9	7.28	0.52	
Endosperm, W64A, 25 days post pollination	1	2.44	1.17	0.48		-	-	-	
Embryo, W64A, 25 days post pollination	1	4.00	2.40	0.60		4.64	3.36	0.72	
	2	2.23	2.08	0.93	0.77	2.59	2.91	1.12	0.92
Soybean Cotyledons	1	99.0	34.7	0.35		101	44.8	0.44	

Continued...

Table 15 (continued)

1 Activities are expressed as nmoles asparagine/20 minutes/assay tube.

2 Activity was assayed using 10 mM NH_4Cl (all extracts) and 25 mM glutamine (scutellar extract) or 10 mM glutamine (all other extracts). Other routine assay conditions were used (see "Methods").

3 V_{max} values were calculated using the rearranged Michaelis-Menten equation $V_{max} = V(1 + \frac{Km}{[S]})$ where "V" isobserved enzyme activity and "[S]" is concentration of glutamine or NH_4Cl.

All values are means of two replicates.

2. To compare the effects of the alternate nitrogen donor substrates, NH_4Cl and glutamine, on the stability of the enzyme to heat.

2. Routine Procedure

It is important to note that all extracts contained the substrates ATP, $MgCl_2$ and aspartate and were filtered through Sephadex. Extract (0.2 ml aliquots) was added to glass test tubes on ice. These tubes were preincubated for appropriate time periods at 40°C or 45°C and replaced on ice. All non-radioactive substrates, dissolved in Tris buffer, were added in a 0.4 ml aliquot; ^{14}C-aspartate was added in 20 µl of H_2O to bring each tube's total volume to the routine 0.62 ml. Tubes were then incubated at 35°C for 20 minutes in the routine way.

The log of enzyme activity was plotted against time of heat treatment and the line of best fit was calculated. Unless otherwise indicated, the control activity (no preincubation) was not included in this calculation. In many experiments, rate of decay of activity decreased considerably after most of the activity had decayed. Points chosen during the time of slower decay were never included in the calculation of line of best fit. Rate of denaturation is expressed as the half-life of enzyme activity (the time required for a 50% reduction in activity).

3. Denaturation at 40°C

A loss of activity curve for preincubation of Sephadex-filtered scutellar extract at 40°C is shown in Figure 16. In addition, a

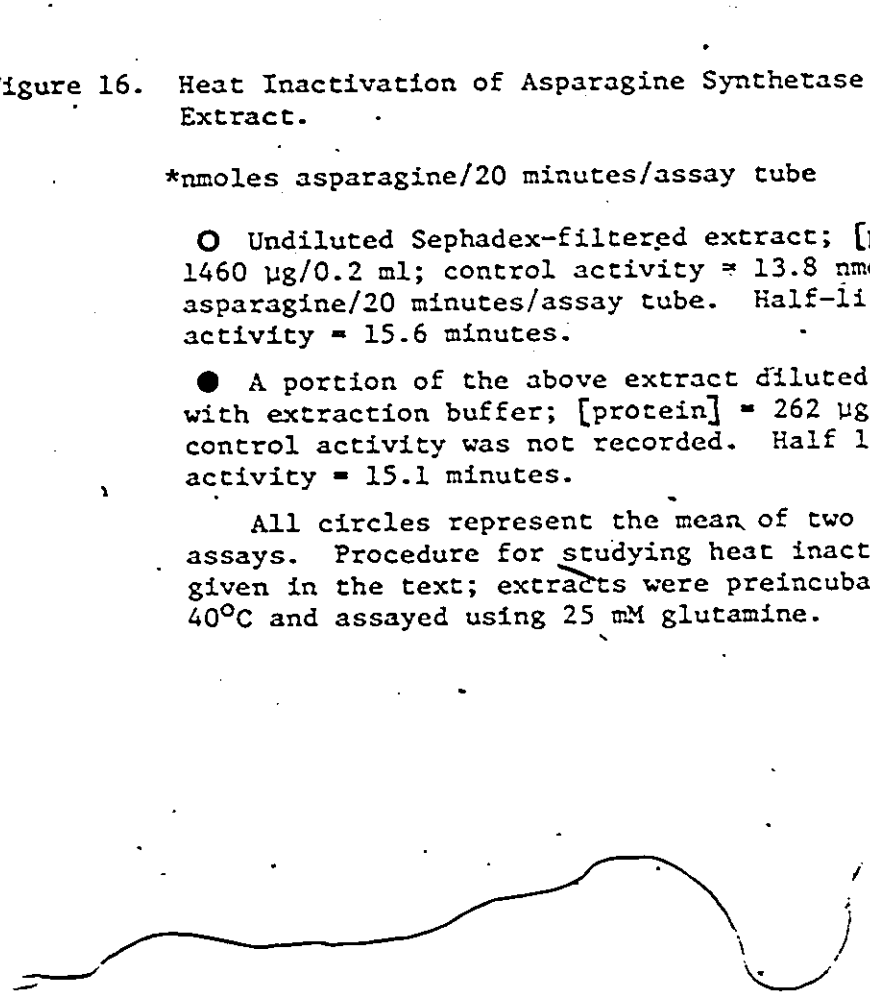


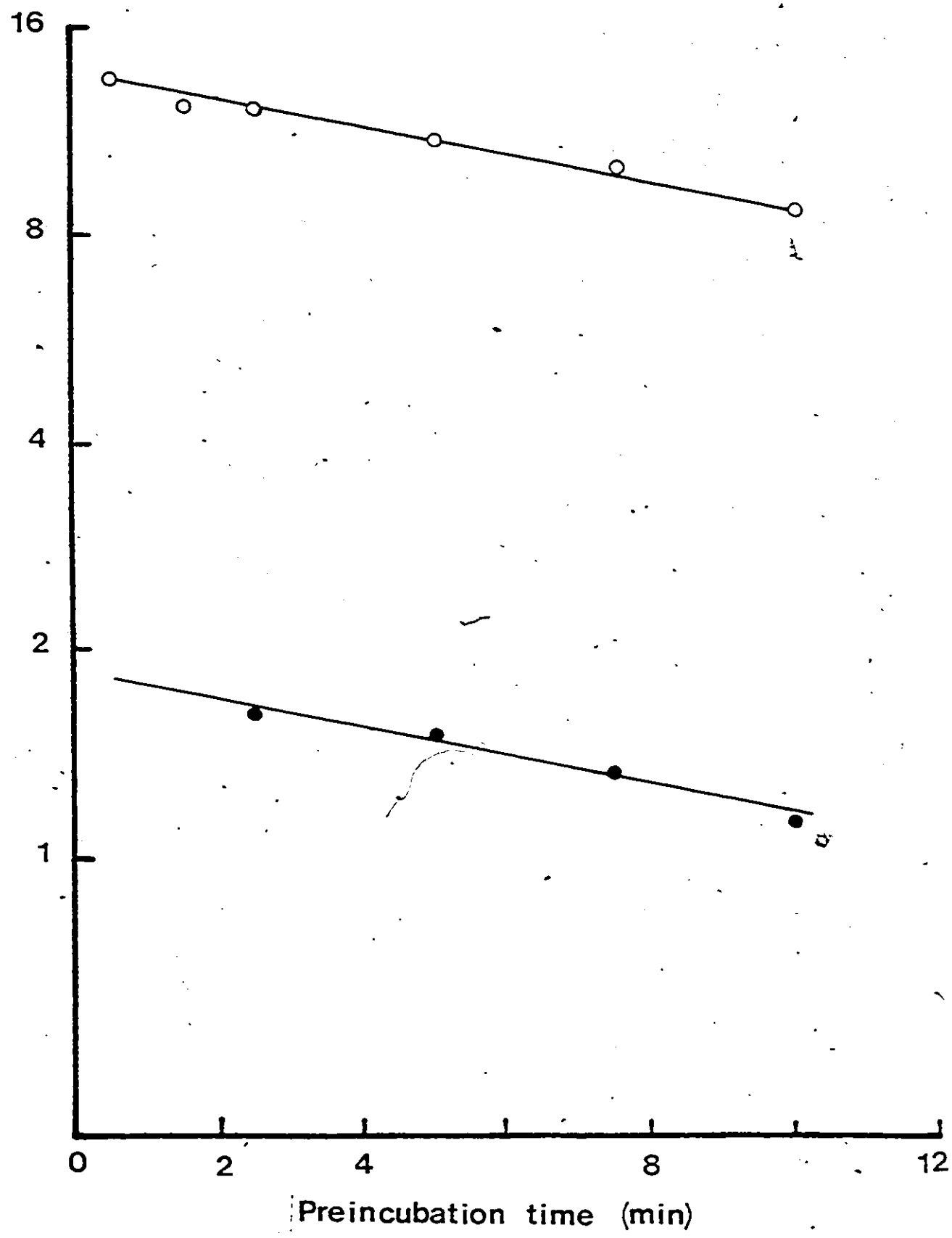
Figure 16. Heat Inactivation of Asparagine Synthetase in Scutellar Extract.

*nmoles asparagine/20 minutes/assay tube

○ Undiluted Sephadex-filtered extract; [protein] = 1460 µg/0.2 ml; control activity = 13.8 nmoles asparagine/20 minutes/assay tube. Half-life of activity = 15.6 minutes.

● A portion of the above extract diluted five fold with extraction buffer; [protein] = 262 µg/0.2 ml; control activity was not recorded. Half life of activity = 15.1 minutes.

All circles represent the mean of two replicate assays. Procedure for studying heat inactivation is given in the text; extracts were preincubated at 40°C and assayed using 25 mM glutamine.


16
8
4
2
1
Activity*
0
2
4
8
12
Preincubation time (min)

portion of the same extract was diluted one in five with extraction buffer, preincubated at 40°C and assayed. Asparagine synthetase activity decayed with a half-life of about 15 minutes in each case. For scutellar enzyme, the rate of denaturation at 40°C appears to be independent of total protein concentration in the range 260-1460 µg per 0.2 ml.

The half-life of asparagine synthetase activity at 40°C was similar (about 12 minutes) in extracts from mature root, scutellum and embryo (Table 16). The average half-life for the two trials for endosperm extract is 6.6 minutes, which is considerably lower than values calculated for the other tissues. The results suggest that asparagine synthetase from endosperm differs in some parameter from asparagine synthetase in the other tissues studied. However, the faster rate of enzyme denaturation could also result from some factor unique to the endosperm extract which makes asparagine synthetase less stable at high temperature.

4. Denaturation at 45°C

a. Comparison of Extracts from Several Tissues

In an experiment employing Sephadex-filtered scutellar extract (protein concentration, 1110 µg per 0.2 ml), enzyme activity in a five fold dilution of this extract decayed with a half-life of about 2.5 minutes; for scutellar enzyme, the rate of denaturation at 45°C can depend on the concentration of the extract.

Enzyme in scutellar extract was much more stable at 45°C than

Table 16

Heat Inactivation of Glutamine-dependent Asparagine Synthetase Activity

Tissue	Half-life of Enzyme Activity (minutes)		Range of Time Points[1] (minutes)		µg protein/0.2 ml extract	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Mature Root	12.8	-	2.5-15.0	-	460	-
Scutellum	10.3	15.6*	2.5-10.0	0.5-10.0	856	1460
Developing endosperm, W64A	7.92	5.27	2.5-15.0	2.5-13.5	606	1310
Developing embryo, W64A	10.3	-	2.5-15.0	-	288	-

[1] Shown are the shortest and longest times of preincubation used in calculating the lines of best fit for decay of activity (semi-log plot). Half-lives were calculated from the equations of these lines.

* Result is presented graphically in Figure 14.

Procedure for studying heat inactivation is given in the text. The extracts were preincubated at 40° C and assayed using 25 mM glutamine (scutellar) or 10 mM glutamine (all others).

enzyme in the three other tissue extracts (Table 17, column 1). Whereas the relative instability of the endosperm enzyme at 40°C suggests that it differs from the enzyme in other tissues, the result here suggests that scutellar asparagine synthetase is a different protein than endosperm, embryo or mature root asparagine synthetase. Total protein in the endosperm extract was only 30% lower than in the scutellar extract. Hence the large difference between enzyme activity decay rates in endosperm and scutellar extracts cannot be attributed to the difference in protein concentration.

In order to test whether the relative instability of the mature root enzyme was due to the relatively low protein concentration, bovine serum albumin was included in one portion of mature root extract in order to bring protein concentration in line with that in scutellar extract. Albumin improved the stability of asparagine synthetase in mature root extract only slightly (Figure 17). Thus the different decay rates of scutellar and mature root enzyme activities cannot be attributed simply to differences in total protein concentration in the two extracts.

b. Effect of Glutamine and NH_4Cl on the Stability of Asparagine Synthetase

Extract was divided into three portions: one portion was diluted to 9/10 strength with extraction buffer, the second was diluted similarly with extraction buffer containing glutamine and the third was diluted to 9/10 strength with extraction buffer containing NH_4Cl. The final

Table 17

Effects of Glutamine and NH_4Cl on Heat Inactivation of Asparagine Synthetase

Tissue	Half-life of Enzyme Activity (minutes) Glutamine Dependent[1]		NH_4Cl-Dependent[2]		µg protein/0.2 ml extract
	-Glutamine	+Glutamine	-NH_4Cl	+NH_4Cl	
Mature Root Trial 1	2.10	3.66	1.22	1.30	460
Trial 2	1.70	2.21			590
Scutellum	5.03*	20.9*	4.04*	4.55*	1110
Developing Endosperm, W64A	1.59	6.17	1.28	1.86	850
Developing Embryo, W64A	1.38+	5.77	2.34+	2.07+	290

1 Extracts plus or minus 10 mM Glutamine were preincubated at 45°C and assayed using 25 mM Glutamine (Scutellar extract) or 10 mM Glutamine (other extracts).

2 Extracts plus or minus 10 mM NH_4Cl were preincubated at 45°C and assayed using 10 mM NH_4Cl.

* Results are presented graphically in Figures 15A and 15B.

\+ Control activities were included in calculations because after 2.0 minutes preincubation (shortest time tested) a large fraction of activity had already decayed. Procedure for studying heat inactivation is given in the text.

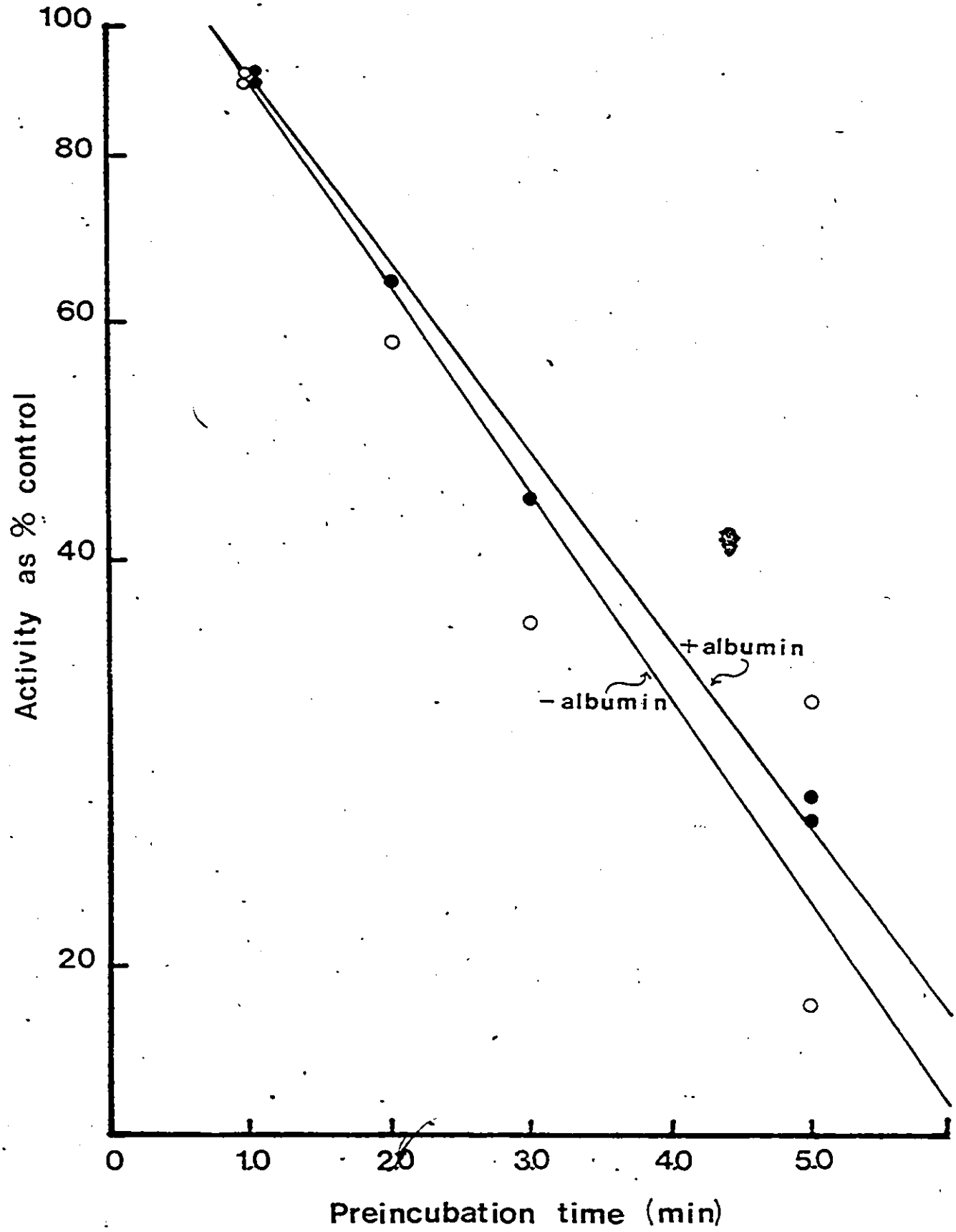
Figure 17. The Effect of Bovine Serum Albumin on the Heat Inactivation of Asparagine Synthetase in Mature Root Extract.

After elution of extract from a Sephadex column, one portion was diluted to 9/10 strength with extraction buffer and a second portion was diluted to 9/10 strength with albumin in extraction buffer. Aliquots of each portion were preincubated at 45°C in routine fashion (see text) and assayed using 10 mM glutamine.

O Extract minus albumin; [protein] = 460 µg/0.2 ml; control activity = 6.24 nmoles asparagine/20 minutes/ assay tube. Half life of activity = 2.10 minutes.

● Extract plus albumin; [protein] = 1190 µg/0.2 ml; control activity = 12.0 nmoles asparagine/20 minutes/ assay tube. Half life of activity = 2.23 minutes.

Circles represent single assays.


100
80
60
40
20
Activity as % control
+albumin
-albumin
0
1.0
2.0
3.0
4.0
5.0
Preincubation time (min)

concentration of glutamine and NH_4Cl in the extract was 10 mM. Each portion of extract was preincubated at 45°C in routine fashion. The first portion (control extract) was assayed with each nitrogen donor, the second with glutamine only and the third with NH_4Cl only.

Figures 18A and 18B show graphically the effect of glutamine and NH_4Cl on the rate of denaturation of asparagine synthetase in scutellar extract. The glutamine-dependent activity decayed much more slowly when glutamine was included in the extract, whereas the inclusion of NH_4Cl in the extract had no significant effect on the decay rate of the NH_4Cl-dependent activity. Table 17 summarizes the effects of glutamine and NH_4Cl on decay of enzyme activity in four different tissue extracts. The inclusion of glutamine protected the glutamine-dependent asparagine synthetase reaction in all tissues tested but the effect was much greater in scutellar, endosperm and embryo extracts than in mature root extracts. The inclusion of NH_4Cl in these extracts did not protect asparagine synthetase from heat denaturation. These results suggest that glutamine is binding to the enzyme and as a result is changing its configuration to a more stable form, but provide no evidence that NH_4Cl binds to asparagine synthetase.

c. Heat Denaturation of Enzyme in Mixed Mature Root and Scutellar Extract

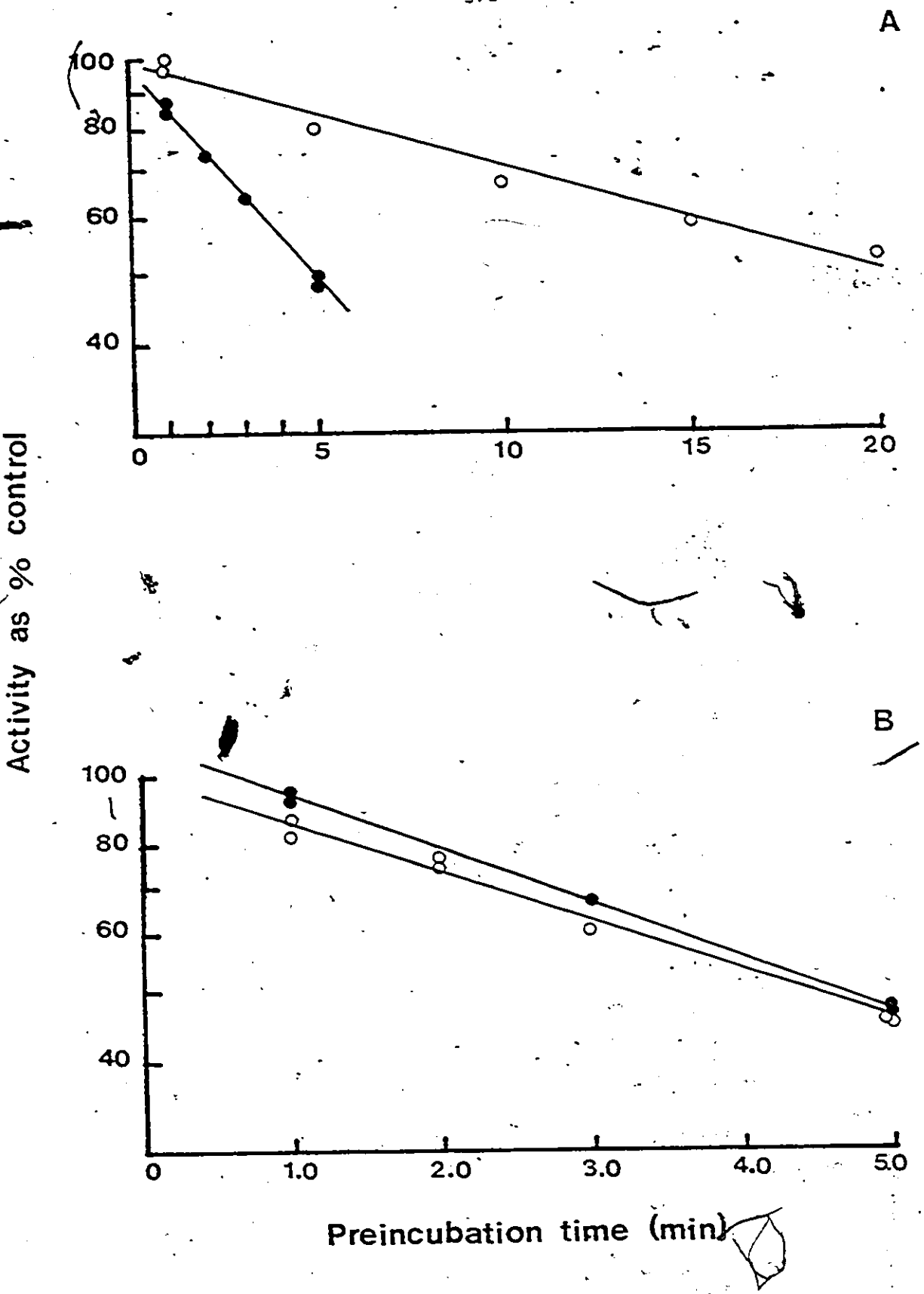
Figure 17 outlines the design of an experiment to show whether the different decay of activity rates found in mature root and scutellar extracts could be attributed to a factor in one of the

Figure 18A. The Effect of Glutamine on Heat Inactivation of Asparagine Synthetase in Scutellar Extract.

Extracts O included or ● did not include 10 mM glutamine and were assayed using 25 mM glutamine and routine concentrations of other substrates (see "Methods"). Circles represent single assays. Procedure for studying heat inactivation is given in the text. Control activity was 10.4 nmoles asparagine/20 minutes/assay tube.

Figure 18B. The Effect of NH_4Cl on Heat Inactivation of Asparagine Synthetase in Scutellar Extract.

Extracts O included or ● did not include 10 mM NH_4Cl and were assayed using 10 mM NH_4Cl and routine concentrations of other substrates (see "Methods"). Circles represent single assays. Procedure for studying heat inactivation is given in the text. Control activity was 4.92 nmoles asparagine/20 minutes/assay tube.


A
100
80
60
40
0
5
10
15
20
B
100
80
60
40
0
1.0
2.0
3.0
4.0
5.0
Activity as % control
Preincubation time (min)

extracts which stabilizes or destabilizes asparagine synthetase. In previous experiments, the difference in decay rates was greatest when the extracts contained glutamine: at 45°C, the half-life of enzyme activity was about three minutes in mature root extract and 20 minutes in scutellar extract. In this experiment, 45° heat denaturation curves were compared for scutellar extract plus glutamine, mature root extract plus glutamine (which were both control extracts) and a mixture of the two. As seen in Figure 19, the two control extracts were diluted to one-half strength, in order that any unknown factor would be present in equal concentrations in control and mixed extracts. By using albumin, protein concentration was made roughly equivalent in all three extracts; the amounts of albumin to be added were estimated from a knowledge of protein concentration in extracts used in previous experiments.

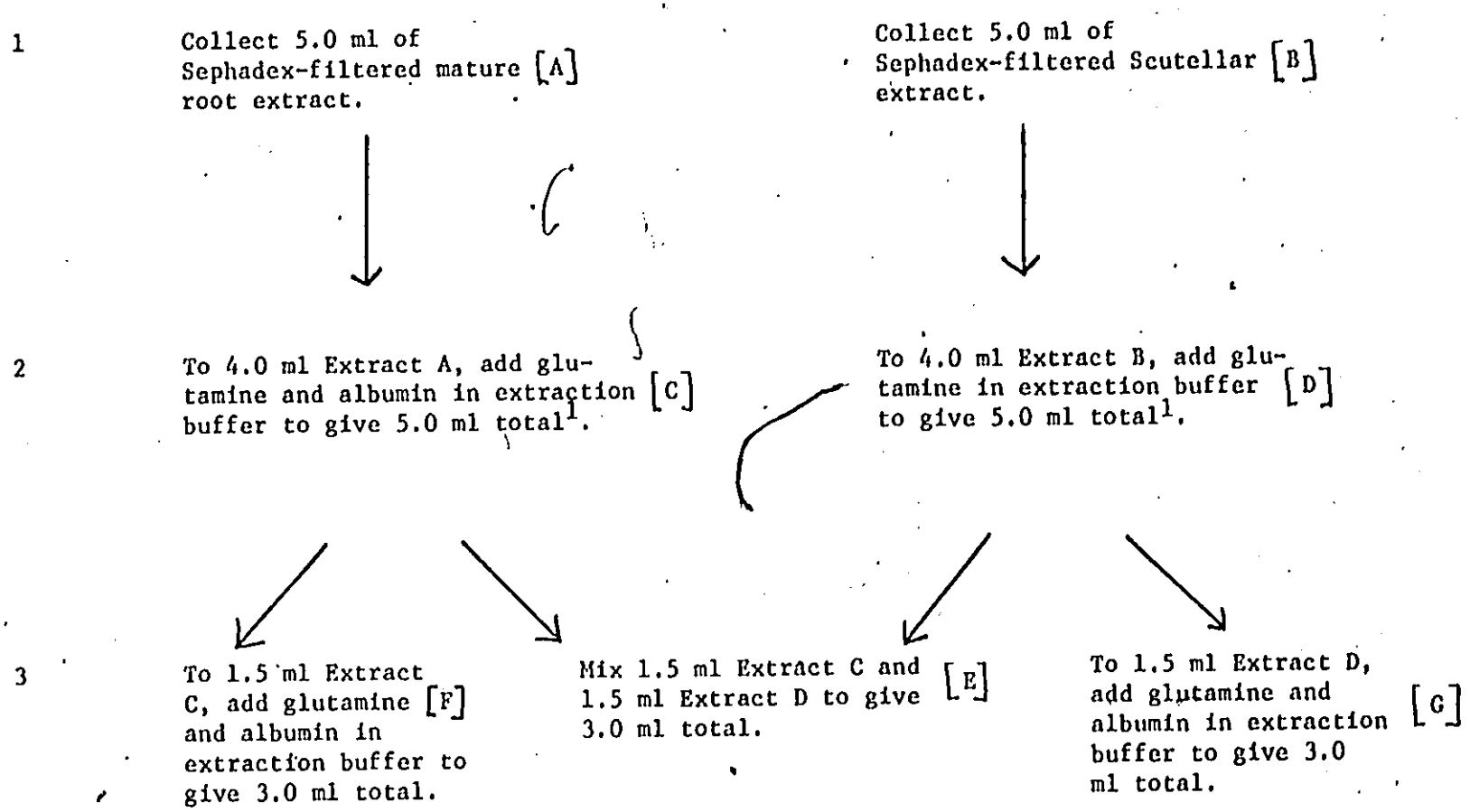
If the existence of two isozymes constitutes the sole reason for the large difference in rates of decay for mature root and scutellar enzyme, the sum of enzyme activity in both control extracts should equal activity in the mixed extract for all preincubation times. If a destabilizing factor exists in the mature root extract, activity in the mixed extract should decay at the same rate as in the mature root extract. If a stabilizing factor exists in the scutellar extract, activity in the mixed extract should decay at the same rate as in the scutellar extract.

Control activity in the mixed extract was 116% the sum of individual control activities, but after five minutes preincubation

Figure 19. Experimental Design for Heat Inactivation in Mixed Scutellar and Mature Root Extracts.

[1]Sephadex-filtered mature root extract generally has a much lower protein content than Sephadex-filtered scutellar extract. Albumin is added to mature root extract to make total protein concentration similar in each extract. Extracts C-F include 10 mM glutamine and the protein concentrations are adjusted to 900 µg/0.2 ml.

Figure 19



Aliquots of Extracts E, F and G are preincubated at 45°C and assayed using 10 mM glutamine.

at 45°C, activity in the mixed extract fell to 76% the sum of activities in the individual extracts (Table 18 and Figure 20). This suggests that some factor from mature root extract destabilizes asparagine synthetase, a conclusion which is confirmed by examining the similar rate of activity decay in mature root and mixed extracts (Figure 20). In both, the half-life of activity decay was 4.0 minutes between one and five minutes preincubation. Between 5.0 and 15 minutes preincubation, activity in the mixed extract had a half-life of 8.9 minutes while activity in mature root extract had a half-life of 6.7 minutes; this difference may be significant. Activity in the scutellar extract was stable for five minutes at 45°C and then decayed with a half-life of about ten minutes. In summary, the results suggest the presence of a destabilizing factor in the mature root extract which is not present in the scutellar extract, but do not completely rule out the existence of isozymes. This experiment has illustrated that different components in different maize extracts can affect the thermolability of asparagine synthetase. It is therefore unwise to conclude that isozymes exist in two different tissues, merely because rates of activity decay differ in Sephadex-filtered extracts from those tissues.

Table 18

Effect of Mixing Scutellar and Mature Root Extracts on the Heat Inactivation of Asparagine Synthetase

Row	Extract	Enzyme Activity (Rows A-D) Time of Preincubation (minutes) 0	1	2	3	5	10	15
A	Mature Root (½ Strength)	4.12±0.12	4.01±0.26	3.82	2.57	1.98	1.09	0.68
B	Scutellar (½ Strength)	2.89±0.23	2.98±0.11	2.87	3.01	3.16	2.05	1.52
C	Sum of Activities (A + B)	7.01	6.99	6.69	5.58	5.14	3.14	2.20
D	Mixed[2]	8.12±0.18	7.83	6.51	5.70	3.91	2.82±0.12	1.80
E	Mixed as % Sum $\left(\frac{D}{C} \times 100\%\right)$	116	112	97	102	76	90	82

[1] nmoles asparagine/20 minutes/assay tube

[2] An equal mixture of full strength mature root and scutellar extracts.

Most assays were not done in duplicate. Where applicable, mean ± range of two replicates is shown.

Experimental design is given in the text and in Figure 17. All extracts were assayed using 10 mM glutamine. Protein concentrations were adjusted using bovine serum albumin. In each extract, protein concentration was 870 µg/0.2 ml extract.

Figure 20. Effect of Mixing Scutellar and Mature Root Extracts on the Heat Inactivation of Asparagine Synthetase.

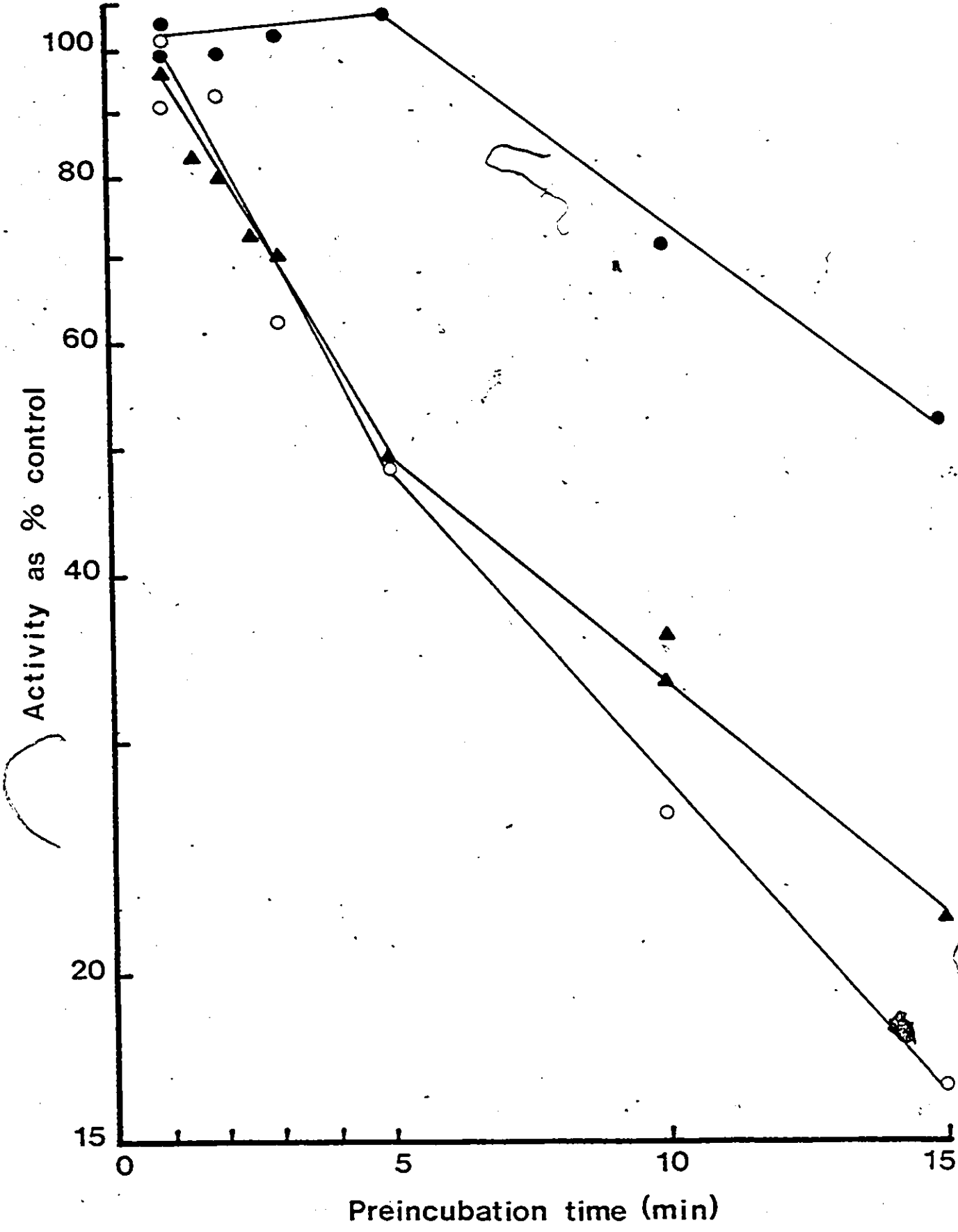
Experimental procedure is described in the text and in Figure 17. Extracts were preincubated at 45°C and assayed using 10 mM glutamine.

O Mature Root extract; control activity = 4.12 nmoles asparagine/20 minutes/assay tube.

● Scutellar extract; control activity = 2.89 nmoles asparagine/20 minutes/assay tube.

▲ Mixed extract; control activity = 8.12 nmoles asparagine/20 minutes/assay tube.

All points on graph represent individual assays.


100
80
60
40
20
15
Activity as % control
0
5
10
15
Preincubation time (min)

DISCUSSION

A. Nitrogen Metabolism

1. Seed Development

The maize endosperm serves an important function in the storage of nitrogen, which will eventually be made available to the embryo upon germination. Nitrogen is stored as protein amino acids; there are three major categories of storage protein in maize (4):

1. Globulins, which are rich in glutamate, glutamine, aspartate, asparagine and arginine.
2. Prolamines, which are rich in proline and glutamine but low in arginine and lysine.
3. Glutelin, which contains a similar amount of proline as the prolamines, but has higher amounts of arginine and lysine (15).

During development of the endosperm, there is a rapid increase in RNA, DNA and protein between 15 and 30 days after pollination (22), followed by a period of much slower increases. About 40 days after pollination, a second rapid increase in protein levels occurs and continues until maturity. The two periods of rapid protein increase could represent the deposition of different types of storage protein (4). Throughout the development of the embryo, protein increases at a fairly constant rate (22).

Nitrogen and carbon of the seed protein could originate from the

degradation of senescing leaf protein. A second alternative would be a primary synthesis of amino acids within the seed itself. In the latter case, the carbon would be obtained from leaf photosynthate and the nitrogen from the soil. In both cereals and legumes, the amino acid composition of the vegetative protein, soluble amino acid pool of the endosperm and endosperm reserve protein are all quite different (4). It is therefore logical to conclude that extensive interconversion of amino acids occurs before their incorporation into reserve protein.

The scant amount of work done on corn xylem sap analysis has indicated that glutamine is the most predominant amino acid, followed by asparagine, aspartate, glutamate, valine and leucine (23). Thus the developing endosperm probably receives substantial amounts of these amino acids. More extensive analysis has been done on wheat, which like corn is a cereal. Kirckman and Miflin have shown that in maturing wheat, asparagine and glutamine are the two predominant amino acids in the xylem (24). During ear formation, glutamine and asparagine levels vary considerably at the stem base, but asparagine always constitutes 58-90% of the xylem amino acids at the top internode. The predominance of asparagine in the xylem and the relative enrichment of the xylem sap with asparagine as it ascends the plant has also been observed in legumes (2,24). Nitrate is also found in the xylem sap of wheat, and increased nitrogen nutrition increases xylem nitrate more than it increases amino acid nitrogen (24).

There is no *in vivo* evidence that indicates that asparagine is synthesized in the endosperm. However, Sodek and Wilson found that

^{14}C-lysine was partly converted to glutamate and proline in maize endosperm (45). This shows that the endosperm is capable of amino acid degradation and resynthesis.

In this study, it was found that on a "per endosperm" basis, asparagine synthetase levels rise substantially between nine and 20 days after pollination. Oaks *et al.* found that in maize endosperm, both asparagine synthetase and glutamine synthetase levels rise dramatically between 10 and 15 days after pollination (37).

Although both amides are important components of the storage protein, it seems unlikely that substantial synthesis of the amides in the endosperm is usually necessary, because high levels of each are entering from the xylem. Atkins *et al.* found that during most stages of lupine seed development, less than 20% of asparagine entering the seed is needed for synthesis of protein asparagine (2). Kirckman and Miflin found that asparagine and aspartate accounted for 72% of amino acids in the top internode xylem but only 4.2% of grain protein in wheat during the grain-filling stage (24).

Two observations in this project cast doubt on whether the levels of extractable asparagine actually reflect *in vivo* asparagine synthesis:

1. Asparagine inhibits the endosperm reaction moderately: 2.0 mM asparagine inhibited over 50%. Thus high levels of asparagine in the endosperm would inhibit asparagine synthesis. More recent experiments have shown that the embryo asparagine synthetase is inhibited even more strongly by asparagine (Ross and Oaks, unpublished results).

2. Extractable enzyme levels per endosperm in a crude extract are much lower when tissue is homogenized in two rather than ten ml of extraction buffer per gram of tissue (Fig. 7). This could reflect the higher concentration of asparagine or some other inhibitor in the more concentrated extract.

It seems likely that conversion of asparagine to other amino acids needed for reserve protein synthesis should be a more important function of the endosperm than asparagine synthesis. However, synthesis of asparagine for the purpose of nitrogen storage could become important if the supply of nitrogen to the endosperm exceeded the supply of carbon available for the synthesis of amino acids. This might happen in a nitrogen-rich soil, which might lead to a relatively high flow of nitrate in the xylem, as has been observed in wheat. This project has demonstrated that the enzymatic machinery for asparagine formation does exist in both the developing endosperm and in the developing embryo.

2. Germination

When a desiccated mature seed is exposed to appropriate conditions of temperature, moisture and aeration, germination can occur (4). This is characterized by imbibition of water by the seed and later by a transport of reserve material to the embryonic axis.

Ingle *et al.* studied changes in nitrogenous components of the endosperm, scutellum and embryonic axis during germination and early seedling growth of maize (21). The level of protein containing ethanol-insoluble nitrogen rose in the axis and remained constant in the

scutellum during the period studied (0-5 days after imbibition). Levels of free amino acids were low in all three tissues before imbibition and increased substantially only in the axis. These results suggest that the growing axis uses nitrogenous materials derived from storage protein in the endosperm; these materials must travel through the scutellum.

Generally in plants, the amino acid composition of the reserve protein is very different from the soluble amino acid composition of the reserve tissue or the axis (4). Folkes and Yemm found that in barley grain, the storage protein contained much larger amounts of glutamate, glutamine and proline than did the protoplasmic protein, and that the storage protein was deficient in several other amino acids such as arginine and lysine (16). They concluded that during germination, nitrogen from glutamine and proline is used for the synthesis of the amino acids which are deficient in the storage protein. Sodek and Wilson found that in maize, the amino acid composition of scutellar and axis protein is fairly similar, but differs substantially from the composition of the endosperm protein (45). Ingle *et al.* suggest that the scutellum may be a major site of amino acid interconversion (21).

Oaks found that when ^{14}C-acetate was fed to maize scutellum, most of the ^{14}C recovered in the scutellum as alcohol soluble amino acids was found in glutamate and glutamine; 1.3% of soluble ^{14}C was in asparagine (32). However, asparagine accounted for 35% of soluble carbon recovered in the root tip. She concluded that asparagine is a major component of the transport system leading from the scutellum to

the root tip, and that most other amino acids in the scutellum were not as accessible to the transport stream. When ^{14}C-acetate was fed to a mature section of the root, considerable ^{14}C-asparagine was recovered there; when ^{14}C-acetate was fed to the root tip region, extremely little ^{14}C was recovered as asparagine.

Oaks' observations are consistent with results from this project, which have shown that on a fresh weight basis, asparagine synthetase activity is higher in the scutellum or mature root than in the root tip, developing endosperm or developing embryo. Since no asparagine synthetase activity was detected in endosperm tissue three days after imbibition, it appears that the final step in the conversion of protein nitrogen to asparagine nitrogen begins in the scutellum and continues in the mature root. In the scutellum, this conversion reaches its maximal rate about two days after imbibition (Table 9). The asparagine synthesized in probably used to transport nitrogen from the scutellar region to the root or shoot of the plant. During this period of the seedling's growth, photosynthetic activity is just beginning, so it is particularly important to use an amino acid with a low C:N ratio (such as asparagine) as a nitrogen transport compound. In the root tip or shoot tip, transported asparagine could be reconverted to other amino acids needed for enzymatic protein synthesis; asparagine nitrogen could also be used for nucleic acid synthesis. Unfortunately, no work was done on older seedlings, which would obtain relatively more nitrogen from the soil as endosperm and scutellar nitrogen became depleted. One might expect asparagine synthetase levels

to decrease in the scutellum and remain high in the mature root.

On a fresh weight basis, asparagine synthetase activity in the cotyledons of young soybean seedlings was four times higher than in maize scutellar tissue. This difference reflects the even more dominant role of asparagine as a transport compound in soybean that in corn. Asparagine constitutes up to 60% of the soluble nitrogen of soybean sap and is the dominant amino acid at all times of development (26). In corn, glutamine is more predominant than asparagine as a transport compound (23).

Product inhibition studies indicated that in contrast to the 50% inhibition of the endosperm enzyme reaction by 2.0 mM asparagine, the scutellar and mature root enzyme reactions were inhibited less than 30% by 10 mM asparagine (Tables 6,19). The regulation of asparagine synthesis in the latter two tissues by asparagine is probably not advantageous to the seedling; nitrogen is transported from there to other areas of the plant, and carbon can be conserved if much of the nitrogen is transported as part of a low carbon molecule such as asparagine. Asparagine inhibited the lupine cotyledon reaction more strongly than the maize scutellar reaction. In both these tissues, asparagine is synthesized for export; perhaps the need for moderate regulation of asparagine synthesis in soybean cotyledons exists only because enzyme levels are so high.

B. Characterization of Asparagine Synthetase

1. The Amidotransferases

As mentioned before, asparagine synthetase is a member of the

Table 19

End Product Inhibition of Asparagine Synthetases from Several Tissues

Organism	Tissue	Reaction Studied	Activity as % Control										Reference
			Asparagine[1]			Glutamate[1]			AMP[1]		ADP[1]		
			2.0	5.0	10.0	10.0	20	150	2-2.5	10-12	2-2.5	10	
Escherichia coli		NH_4^+-dependent		2						20*			10
Mouse	leukemia cells	Gln-dependent	34		9		84		75				20
Lupinus albus	etiolated shoots	Gln-dependent		64									42
	cotyledon	Gln-dependent	50					50	50		100		27
Zea mays	mature root	Gln-dependent	83		83				74	30	56	20	This project
Zea mays	scutellum	Gln-dependent	84		71	120			82	57	85	50	
Zea mays	developing endosperm	Gln-dependent	47		41								
Zea mays	developing embryo	Gln-dependent	37		19								Ross and Oaks, unpublished

[1] Concentrations of potential end products are given in mM.

* Using 1.0 mM ATP

amidotransferase group of enzymes, which catalyze the transfer of the amide group of glutamine to another molecule. Work has been done on the structure and function of other amidotransferases which may have a relationship to the structure and function of asparagine synthetase. Trotta *et al.* have found that carbamyl phosphate synthetase contains two polypeptide chains of unequal molecular weight (52). The heavy chain will catalyze an ammonium-dependent synthesis of carbamyl phosphate while the light chain catalyzes glutamine hydrolysis only. Together, the two chains can catalyze a glutamine-dependent synthesis of carbamyl phosphate. It has been proposed that in the complete reaction, glutamine binds to the light chain and donates the amide nitrogen to the heavy chain, where the remainder of the reaction takes place. Because other amidotransferases such as anthranilate synthetase (54) seem to operate in a similar fashion, Trotta has proposed that many amidotransferases may have evolved from the union of ammonium-dependent synthetase and glutaminase activities.

2. Asparagine Synthetase: General Considerations

Although there is no concrete evidence that asparagine synthetase operates in the above mentioned fashion, at least one observation has been made which is consistent with that model. Plant and animal asparagine synthetases have been shown to exhibit glutaminase activity which is independent of asparagine synthesis (20,43).

The most detailed study on a plant asparagine synthetase has

been done by Rognes on lupine cotyledon enzyme (43). He has shown that the enzyme catalyzes the formation of equimolar amounts of AMP, PPi and asparagine. The initial velocity kinetic patterns of the glutamine-dependent reaction indicate that glutamine or ATP-Mg^{2+} add first to the enzyme, followed by the release of PPi. Aspartate then binds to the enzyme, followed by the release of glutamate and asparagine.

When the enzyme was filtered through Sephadex G-200 in the absence of $MgCl_2$ and ATP, it eluted as one band with a molecular weight of 160,000. When eluted in the presence of ATP and $MgCl_2$, it had a molecular weight of approximately 320,000. Rognes concluded that the lupine asparagine synthetase is an oligomer in the absence of substrates but associates to a dioligomer in the presence of ATP-Mg^{2+}. There is no evidence from Rognes' results that the two oligomers of lupine asparagine synthetase are in any way equivalent to the two unequal subunits of carbamyl phosphate synthetase described by Trotta (52).

3. Effect of Substrates on Stability

Rognes also found that the lupine enzyme was extremely thermolabile in the absence of substrates at 37°C, but that the reaction was linear for 60 minutes at the same temperature (43). He concluded that substrate binding must result in a much more stable conformation. He also found that the enzyme was stable when stored in the presence of all substrates at -25°C, and states that the protecting effect appeared to be due to ATP and $MgCl_2$.

In this project, it was found that when ATP was omitted from the extraction buffer, almost all the activity was lost after filtration through Sephadex. This suggests that asparagine synthetase irreversibly denatures during the enzyme preparation in the absence of ATP. This is in agreement with Rognes' statement.

Heat denaturation studies have indicated that glutamine also protects asparagine synthetase, but in a much different fashion from ATP. It is not necessary to extract the enzyme and pass it through Sephadex in the presence of glutamine, in order to retain activity. However, glutamine protected asparagine synthetase from heat denaturation at 45°C in at least four maize tissues: mature root, scutellum, developing embryo and developing endosperm. This suggests that glutamine binds to the enzyme and changes its conformation to a more stable form. The alternate substrate NH_4Cl did not affect the rate of inactivation, indicating that it has a much less dramatic effect than glutamine on the enzyme conformation.

4. Effect of Potential End Products on the Enzyme Reaction

A comparison of end product inhibition of asparagine synthetase reactions in several tissues is shown in Table 19. The effect of asparagine on plant enzyme reactions has been discussed in relation to seed development and germination. In general, asparagine inhibited the reaction in developing maize tissue more strongly than in young seedling tissue. Asparagine inhibited the mouse leukemia enzyme reaction more strongly than any plant tissue reaction that was studied.

By far the strongest inhibition by asparagine was in the *E. coli* reaction. However, this enzyme cannot use glutamine and large differences between it and animal or plant asparagine synthetases are to be expected.

The negligible inhibition of the scutellar reaction by 10 mM glutamate is consistent with Lea and Fowden's observation that 150 mM glutamate inhibited the lupine cotyledon reaction only 50%. Glutamate is, perhaps, a poor candidate for regulation of asparagine synthetase, because it is involved in a variety of reactions not related to asparagine metabolism, and high levels of glutamate might be needed at the same time that asparagine synthesis is required.

There is little difference between the amount of inhibition by 2.0 mM ADP and 2.0 mM AMP in either the scutellar or mature root reaction (less than 45% inhibition in all cases). This differs from the results of Lea and Fowden, who found a much stronger inhibition of the lupine enzyme reaction by AMP than by ADP (27). It seems unlikely that AMP or ADP would be useful regulators of asparagine synthetase *in vivo*, because they are products of so many other enzyme reactions.

5. Effect of ATP on the Enzyme Reaction

Rognes found that free ATP inhibited the lupine enzyme reaction, but that when Mg^{2+} was added in molar excess of ATP, no inhibition was observed (43). He concluded that an ATP-Mg^{2+} complex was the actual substrate. In this project, when a crude scutellar extract was used, 10 mM ATP inhibited the reaction 60%, while maximal activity was obtained with 1.92-4.32 mM ATP (5.32 mM $MgCl_2$ was used in all cases). The low

activity observed with 10 mM ATP could have resulted from one or both of the following reasons:

(1) The use of 10 mM ATP meant that ATP was in excess of $MgCl_2$ by 4.7 mM. Inhibition could have been caused by free ATP, in which case it could have been eliminated by using greater than 10 mM $MgCl_2$.

(2) Since ATP was not pH-adjusted before addition to the assay mix, the higher concentration of ATP (which is acidic) could have lowered the pH of the reaction mixture, and thus lowered the reaction rate of asparagine synthetase. A more recent experiment has shown that pH-adjusted 10 mM ATP inhibits the mature root reaction in a Sephadex-filtered extract by only 13% (Ross and Oaks, unpublished data).

6. Effect of Bovine Serum Albumin on Enzyme Reactions

The observation in this project that albumin doubles asparagine synthetase activity in Sephadex-filtered mature root extract is similar in some ways to observations by Schrader *et al.* (44). When albumin or casein was included in extracts of several different plants, up to 15-fold higher nitrate reductase activity was obtained. They also found that the added protein protected the enzyme from decaying at 0°C. Schrader *et al.* suggested that a diversion of protease activity from nitrate reductase could account for the added protein's protective effect, and also suggested three possible explanations for the increase in activity:

(1) A grinding medium containing albumin decreases binding of nitrate reductase to broken plastids and organelles (originally suggested

by Dalling *et al.* (14).

(2) The added protein prevents an inhibitor from binding to nitrate reductase.

(3) A higher concentration of protein in the extract may prevent dissociation of the nitrate reductase molecule.

In this project, it seems unlikely that a protease is active against asparagine synthetase, because the reaction is linear for 90 minutes (Figure 11). It is also unlikely that any of the remaining three explanations can account for the observed effect of albumin in this project. Whereas Schrader extracted the enzyme in the presence of added protein, the albumin was added after collection of the Sephadex-filtered extract in this project. Therefore, the albumin could not be affecting the binding of enzyme to organelles, preventing an inhibitor from binding to the enzyme during extraction, or preventing dissociation of the protein during extraction. Addition of albumin to a purified nitrate reductase from *Chlorella* increased the activity several fold (Yamaya and Oaks, unpublished results). This observation can also not be accounted for by the above explanation.

The effect of albumin on the asparagine synthetase reaction could be related to the structure of asparagine synthetase. It is possible that the enzyme has separate binding sites for glutamine and ammonium; these sites may even be on different subunits if asparagine synthetase is similar in structure to other amidotransferases (52,54). During the preparation of the Sephadex-filtered extract, the relative position of the two binding sites could be shifted due to some minor

change in the conformation of the enzyme. This would lead to a less efficient transfer of the amide nitrogen of glutamine to the ammonium binding site during the reaction. Albumin could be acting to restore the enzyme to its original conformation and thus allow the reaction to go more quickly. Perhaps the effect of albumin results from some interaction between it and asparagine synthetase, or from a change in viscosity of the reaction medium.

The above explanation is consistent with the observations that albumin has a much less pronounced effect on the ammonium-dependent activity than on the glutamine-dependent activity, and that albumin has no effect on the glutamine-dependent activity in crude mature root extracts. Albumin had no effect on enzyme activity in Sephadex-filtered scutellar extract. Possibly the higher concentration of protein in the scutellar extract protects the enzyme from the reversible change in conformation hypothesized above for mature section enzyme. It is interesting to note that only 39% of activity was recovered when mature root extract was filtered through Sephadex and assayed minus albumin. If the Sephadex-filtered extract is assayed in the presence of albumin, the percentage recovery rises to about 80%. The latter figure is comparable to the recovery of activity when scutellar or endosperm extracts are filtered through Sephadex (81% and 66%, respectively).

7. NH_4Cl and Glutamine as Alternate Substrates

Although the glutamine-dependent asparagine synthetase reaction

in Sephadex-filtered mature root extract is linear for at least 90 minutes (0.20 nmoles asparagine/min.), the NH_4Cl-dependent reaction exhibits a different pattern (Figure 11). During the first ten minutes, asparagine is formed at the rate of 0.36 nmoles/min., while between 20 and 90 minutes, the reaction is linear but has slowed down to 0.13 nmoles/min. These results are consistent with the existence of two asparagine synthetase enzymes. One can use either glutamine or NH_4Cl and is stable for 90 minutes at 35°C in the presence of substrates. The second can use NH_4Cl only and denatures completely within 20 minutes.

A summary of the Km values and activity ratios obtained for glutamine and NH_4Cl in various asparagine synthetase reactions is shown in Table 20. Asparagine synthetase from lupine and soybean exhibits a strong preference for glutamine over NH_4Cl; the NH_4Cl:glutamine Km ratio is at least three to one in favour of glutamine (27,42,46). (The activity ratio of 2.2 obtained for the soybean reaction in this project is, however, much lower than Streeter's value of four). The Km and activity ratios are much lower for the maize enzyme. It is interesting to note that the Km for NH_4Cl does not vary much in any of the plant reactions studied. The lupine and soybean enzymes can, however, react using a much lower glutamine concentration than the maize enzymes. This observation is consistent with the relative importance of asparagine as a nitrogen transport compound in legumes compared with corn. In legumes, asparagine is the dominent transport compound (2), while in corn, more nitrogen is transported as glutamine than as

Table 20

Comparison of Km values and V_{max} Ratios for Glutamine and NH_4Cl for Asparagine Synthases from Several Plant Tissues and from Mouse Leukemia Cells

Plant	Tissue	Km value (mM) Glutamine	Km value (mM) NH_4Cl	Km Ratio (NH_4Cl/Glutamine)	V_{max} Ratio (Glutamine/NH_4Cl)	Reference
Lupinus albus	etiolated shoot	0.5	-	-	4-10	42
Lupinus albus	cotyledon	0.04	2.1	50	3.3	27
Soybean	cotyledon	0.12	3.1	25	4	46
Soybean	cotyledon	0.18	2.9	1.6	2.2	This project
Zea mays	root tip	1.0	2.5	2.5	2	49
	mature root	1.0	2.5	2.5	2	
Zea mays	mature root	0.59	2.6	4.4	0.8	This project
	scutellum	0.49	2.6	5.3	1.9	
	developing embryo	1.6	4.0	2.5	1.0	
Mouse	leukemia cell	1.0	9.0	9	1.5-2.0	20

asparagine (23). The Km values and nitrogen transport results suggest that legumes are more efficient than corn in incorporating nitrogen into asparagine for transport or storage.

8. Subcellular Location of Asparagine Synthetase

In a sucrose gradient experiment employing corn root tip extract, 96% of asparagine synthetase activity was found in the soluble fraction of the cell (P. Gadal, personal communication). This result is consistent with two experiments on legume extracts, which have both indicated that asparagine synthetase is a soluble enzyme (27,46). Gadal's result is also consistent with *in vivo* work which suggests that asparagine is synthesized outside the mitochondria (31,46).

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