GLUTAMINE AND ASPARAGINE METABOLISM IN DEVELOPING ENDOSPERM OF CORN

By

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GLUTAMINE AND ASPARAGINE METABOLISM IN DEVELOPING ENDOSPERM OF CORN (*ZEA MAYS* L.)

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TITLE: Glutamine and asparagine metabolism in developing corn endosperm (*Zea mays* L.)

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ABSTRACT

Metabolism of glutamine and asparagine was examined in developing endosperm of maize with emphasis on:

1. Characterization of the enzymes of amide metabolism.
2. A comparison of the levels of activity of enzymes of nitrogen assimilation in the endosperm of a normal variety of maize (W64A) and its isogenic high lysine mutants opaque-2 and floury-2.
3. Investigations of the metabolism of (UL-^{14}C) glutamine in the endosperm of cultured corn caryopsis.

Results showed that glutamate synthase (GOGAT), glutamine synthetase (GS), glutamate dehydrogenase (GDH), asparagine synthetase (AS), asparaginase (ASnase) and asparagine transaminase activities were present in the developing endosperm. The enzymes increased in activity just prior to the onset of zein biosynthesis, reached a maximum between 15 and 35 days after pollination and then declined. Two forms of GOGAT with molecular weights, estimated to be 171,000 and 270,000 for ferredoxin- and NADH-GOGAT, respectively, were identified. Almost equal levels of each enzyme were present at every stage of development. Either of these enzymes could mediate the transfer of the amide nitrogen of glutamine to α-amino nitrogen of glutamate. Glutaminase activity was not detected in the endosperm tissue.

The pattern of developmental changes in activity, and the actual levels of activity of GDH, GS, AS, and ASnase were similar in the control and the mutant varieties. In the mutants, the level of GOGAT activity was higher at all developmental stages examined. Twenty days after pollination the activity was 13 ± 0.6, 29 ± 0.5 and 18 ± 0.07 nmol NADH oxidized·min^{-1}·$endosperm^{-1}$ in normal, opaque-2 and floury-2, respectively.

To estimate the capacity of the endosperm tissue to metabolize glutamine, (UL-^{14}C) glutamine was injected into the endosperm of cultured caryopses. After 168 hr of incubation, 21% of the total label incorporated was recovered in starch, 38% in zein, 8% in glutelin and 13% in the free amino acid fraction. In the zein hydrolysate, glutamine accounted for 14% of the total label in this fraction. The results from tracer studies as well as the observations on the presence of enzymes lead to the conclusion that corn endosperm tissue is fully capable of metabolizing glutamine. The presence of label in the amino acid fraction as well as in carbohydrates suggests the presence of an enzyme system for the extensive metabolism of glutamine in the endosperm tissue.

PREFACE

The experiments described in this thesis were carried out in the Department of Biology, McMaster University, from September, 1977 to December, 1982. Except where others are specifically mentioned, this thesis consists entirely of my own work.

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LIST OF ABBREVIATIONS

Asnase	Asparaginase
AS	Asparagine synthetase
Asp	Aspartic acid
Asn	Asparagine
AOA	Amino-oxyacetic acid
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BAPNA	α-N-benzoyl-DL-arginine-p-nitroanilide
cpm	counts per minute
DT	Dithionite
DAP	Days after pollination
EDTA	Ethylene diamine tetraacetic acid
Fd	Ferredoxin
Fl-2	Floury-2
GOT	Glutamate-oxaloacetate transaminase
GOGAT	Glutamate synthase
GS	Glutamine synthetase
GDH	Glutamate dehydrogenase
glu	Glutamic acid
gln	Glutamine
HEPES	N-2-hydroxyethyl piperazine-N'-2 ethanesulfonic acid
HSA	α-hydroxysuccinamic acid
KF	Potassium fluoride
KSA	α-keto succinamic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
MDH	Malate dehydrogenase
MV	Methyl viologen
OPA	o-phthaldialdehyde

LIST OF ABBREVIATIONS (cont'd)

OAA	Oxaloacetic acid
op-2	Opaque-2
PMSF	Phenyl methyl sulfonyl fluoride
SDS	Sodium dodecyl sulfate
TCA	Tricarboxylic acid cycle
Var.	Variety

INTRODUCTION

The cereal grain, a single seeded fruit called a caryopsis, is composed predominantly of an endosperm and embryo which account for approximately 90% and 5%, respectively, of the seed dry weight. These two structures are surrounded by the testa (seed coat) and the pericarp (fruit coat) (MacLeod, 1969). During development of the grain, large amounts of storage reserves (carbohydrates, lipids and proteins) are deposited, mainly in the endosperm. The seedling draws on these reserves after germination. In corn, protein reserves make up about 10-16% of the dry weight of the seed. The endosperm typically contains about 80% of the total protein of the cereal seed (Earle *et al.*, 1946).

A. Endosperm Protein Reserves

Osborne (1897) separated and characterized the major seed proteins on the basis of their solubility. Proteins soluble in water were regarded as albumins, those soluble in salt-water as globulins. In terms of absolute amounts, saline and water soluble proteins are of limited significance contributing less than 10% of the total endosperm protein (Wall and Paulis, 1978). However, these proteins are of major importance functionally because of their enzyme content. A major protein fraction extracted in aqueous alcohol is called prolamine, or zein in corn. A fourth fraction, glutelin, can be extracted

with dilute alkali. The amino acid composition of each of these fractions in corn is shown in Table 1.

Albumins and globulins are relatively rich in lysine, arginine, threonine, histidine, aspartic acid and have a good balance of other amino acids. Zein and glutelins are the storage proteins of corn and together may account for up to 80% of the total endosperm protein in mature kernel (Dalby, 1966; Tsai *et al.*, 1978). The latter two classes of protein differ in their amino acid composition. Zein is rich in glutamine, leucine, proline and alanine but is essentially devoid of lysine and methionine. Glutelins have a more balanced amino acid composition (Dalby, 1966; Murphy and Dalby, 1971; Sodek and Wilson, 1971; Wall and Paulis, 1978). The two types of proteins also differ in their localization (Christiansen *et al.*, 1974) and pattern of synthesis in developing grain (Dalby, 1966; Murphy and Dalby, 1971). Glutelin accumulation begins 10 days after pollination in maize, continues at a steady rate until 42 days and then ceases. In contrast, zein synthesis is low during the initial 20 days (Dalby, 1966; Murphy and Dalby, 1971; Tsai, 1979; Misra and Oaks, 1981). Between 20-42 days, the rate of zein accumulation is about 3-fold higher than that of glutelins. In mature normal maize, zein accounts for 60% of the total endosperm protein and hence is the major storage protein (Hensel *et al.*, 1973). The amino acid composition of the corn kernel is, thus, to a large extent a reflection of zein amino acid composition. For example, zein contains a low level of lysine, methionine, and tryptophan. These amino acids are also the ones which limit the

Table 1

Amino Acid Composition of "Osborne Fractions" from a Normal Maize Variety*

Amino acid	Albumin	Globulin	Prolamine	Glutelin
	(percent of total protein fraction nitrogen)			
Lysine	5.4	5.0	0.1	2.6
Histidine	1.9	2.0	0.7	2.7
Arginine	5.3	5.5	1.0	3.0
Aspartic acid	8.2	8.6	5.4	5.7
Threonine	5.0	4.9	2.9	3.7
Serine	5.6	5.6	5.7	5.1
Glutamic acid	12.3	11.6	21.0	16.4
Proline	6.9	7.4	10.2	10.9
Glycine	10.1	11.3	2.1	7.0
Alanine	11.0	10.4	14.2	10.2
Half-cystine	0.2	1.2	0.1	1.5
Valine	7.1	7.0	4.8	5.8
Methionine	1.3	1.2	0.3	3.4
Isoleucine	4.4	4.1	4.2	3.5
Leucine	9.3	8.1	19.5	11.6
Tyrosine	2.5	2.6	2.9	3.4
Phenylalanine	3.3	3.6	4.8	3.4
Percent of grain Total nitrogen	0.9	1.5	36.9	41.4

* Data adapted from Sylvester-Bradley and Folkes, 1976.

nutritional quality of corn proteins for humans and monogastric animals. This suggests a negative correlation between the zein content and the nutritional quality of the grain (Mertz, 1972, 1976).

In normal varieties of corn, the production efficiency and yield of the crop has increased significantly. This is achieved mainly due to selection of varieties which respond to application of higher levels of nitrogen fertilizer. However, addition of nitrogen fertilizers enhances the synthesis of zein type protein and hence only exaggerates the amino acid deficiencies in corn. Non-zein proteins which have a more uniform amino acid content remain more or less unaffected by fertilization (Keeny, 1970; Hensel *et al.*, 1973; Rendig and Jimenez, 1978; Tsai *et al.*, 1980). Data shown in Table 2 illustrate this point. Normal maize grown with low levels of nitrogen contain 1.07 mg zein·endosperm^{-1}, whereas those grown on higher levels of nitrogen contain 2.38 mg zein·endosperm^{-1}. This represents an increase of 122% relative to the control. Other protein fractions, i.e., albumins, glutelins and globulins show only slight increases (20-40% relative to the control).

Several endosperm mutations have been identified that both suppress the synthesis of zein and improve the nutritional quality of grain (Mertz *et al.*, 1964; Nelson *et al.*, 1965). For example, by screening maize genotypes for lysine content, Mertz *et al.* (1964) and Nelson *et al.* (1965) identified mutations opaque-2 and floury-2, respectively. The opaque-2 mutant contains twice as much lysine as that of normal corn. Floury-2 also contains higher than normal lysine

Table 2

Effect of Application of Nitrogen Fertilizer on Zein and Non-zein Protein Content in Developing Endosperm of Normal and Opaque-2

Fraction	Normal			Opaque-2		
	(-)N	(+)N	% increase	(-)N	(+)N	% increase
	mg·endosperm^{-1}			mg·endosperm^{-1}		
Zein	1.07	2.38	122	0.52	0.7	34
Glutelin	1.45	1.76	21	1.2	1.5	25
Albumin	0.12	0.15	25	0.24	0.30	24
Globulin	0.17	0.24	41	0.25	0.32	28
Total protein	2.81	4.53	61	2.19	2.80	28

Data taken from Tsai *et al.* (1980).

(-)N = no nitrogen fertilizer added.

(+)N = nitrogen fertilizer added.

in endosperm. In addition, the methionine content is also higher in the floury-2 mutant (Hansel *et al.*, 1973). Comparison of amino acid composition of endosperm and germ proteins shows that effect of opaque-2 is restricted to endosperm (Dalby, 1966; Table 3). The improved amino acid composition of the endosperm is due to a shift in protein class distribution rather than to changes in amino acid composition of individual proteins. In mutant varieties, the level of zein is reduced and as a result, the relative contribution of other proteins which contain normal amounts of lysine, is higher (Paulis and Wall, 1978). It has been clearly established that zein synthesis is both slower and terminates at an earlier stage of development in opaque-2 lines of maize relative to the normal isogenic lines (Murphy and Dalby, 1971; Tsai, 1979; Misra and Oaks, 1981). The rate of zein accumulation is also diminished in the other high lysine mutants, floury-2 (Tsai and Dalby, 1975; Misra and Oaks, 1981) and opaque-7 (Tsai and Dalby, 1975). These mutant alleles reduce zein synthesis to a different degree.

The reduction of zein synthesis in the mutants, at least in opaque-2, may be related to a decreased "availability" of zein mRNA in the mutant endosperm (Jones *et al.*, 1977). During endosperm development, zein is synthesized on polysomes bound to membranes (Jones *et al.*, 1976; Jones *et al.*, 1977; Larkins *et al.*, 1980). These membrane-bound polyribosomes vary in size. Large size membrane-bound polysomes increase in number during endosperm development. The ratio of large polysomes to total membrane-bound polysomes is highest when zein is being rapidly synthesized. The membrane-bound polyribosomes

Table 3

Amino Acids in Endosperm and Germ of Normal and High Lysine Kernels of Corn

Amino acid	Endosperm		Germ	
	opaque-2 (g/100 g protein)	normal	opaque-2 (g/100 g protein)	normal
Lysine	3.4	2.0	5.9	6.1
Histidine	3.4	2.6	2.9	2.9
Amide NH_3	3.4	3.3	2.1	2.2
Arginine	5.1	3.8	9.2	9.1
Aspartic acid	8.5	6.2	9.2	8.2
Glutamic acid	19.1	21.3	13.9	13.9
Threonine	3.9	3.5	3.7	3.9
Serine	5.0	5.2	5.0	5.5
Proline	9.4	9.7	5.3	4.8
Glycine	4.0	3.2	5.5	5.4
Alanine	7.0	8.1	5.8	6.0
Valine	5.0	4.7	4.4	4.3
Cysteine	2.4	1.8	0.9	1.0
Methionine	2.0	2.8	1.5	1.7
Isoleucine	3.9	3.8	2.5	3.1
Leucine	11.6	14.3	5.6	6.5
Tyrosine	4.7	5.3	2.2	2.9
Phenylalanine	5.0	5.3	3.6	4.1

Data adapted from Dalby (1966).

of opaque-2 endosperm lack the larger polyribosomes present in normal corn (Jones *et al.*, 1977a). When Jones *et al.* (1977) compared the *in vitro* protein synthesis by membrane-bound polysomes of normal and opaque-2 mutant, they found that approximately 50% of the labelled protein synthesized by the membrane-bound polyribosomes of normal maize was ethanol soluble. In contrast, in opaque-2 only 34% of the total protein was soluble in ethanol. As revealed by SDS-polyacrylamide gel electrophoresis, there are two major molecular weight components of zein; 23,000 or Z_1 and 19,000 or Z_2, both of which are synthesized in approximately equal amounts in endosperm of normal corn (Gianazza *et al.*, 1977). In opaque-2 mutant, the synthesis of Z_1 component is drastically reduced both *in vivo* and *in vitro* (Jones *et al.*, 1976; Jones *et al.*, 1977b; Lee *et al.*, 1976; Larkins *et al.*, 1980).

In endosperm of high lysine mutants, in addition to the reduced synthesis of zein, levels of ribonuclease activity (Wilson and Alexander, 1967), trypsin inhibitor (Reed and Penner, 1978) and certain free amino acids are much higher at maturity (Sodek and Wilson, 1971; Mertz, 1976), while levels of carbohydrates are lower (Tsai *et al.*, 1978). Little is known concerning the metabolic relationship of these disturbances. Due to their lower yield, the commercial production of high lysine corn is limited (Paulis and Wall, 1978). Unlike normal corn, the level of zein in opaque-2 does not increase with an increasing level of fertilizer (Tsai *et al.*, 1980; Rendig and Jimenez, 1978; Table 2). The increase in protein components due to high nitrogen fertilizer in the homozygous opaque-2 mutant appears to be proportionately

distributed in all protein fractions (Table 2). Thus, the fact that application of nitrogen fertilizer does not stimulate all protein fractions equally, and does not stimulate zein synthesis in certain mutants, suggest that there is some genetic constraint on the biosynthesis of specific endosperm proteins. The uptake of NO_3^- from soil by corn and its assimilation into organic compounds has been studied extensively by Hageman's group (Hageman, 1979; Beevers and Hageman, 1980; Reed *et al.*, 1980) and several other laboratories (Beevers and Hageman, 1980). However, the metabolism of nitrogen transported to the developing corn endosperm has not been studied extensively and needs to be characterized in detail. In this regard, mutants with an altered response to fertilizer level and a reduced synthesis of zein provide an important tool for studies related to metabolic control.

B. Nitrogen Metabolism in the Developing Seed

During seed development, a rapid synthesis of protein takes place, resulting in a net deposition of storage proteins (Millerd *et al.*, 1973). This rapid synthesis of proteins creates a heavy demand for nitrogen and carbon which are supplied to the developing seed from roots and leaves via xylem and phloem (Pate, 1973; 1980). Since developing seeds transpire slowly, it is believed that phloem contributes more to nitrogen and carbon demands than does the xylem. Analysis of the phloem sap shows that NO_3^- and ammonia tend to be absent or are present in low amounts. Therefore, the developing seed must rely on an organic source of nitrogen to maintain protein

synthesis. The major nitrogenous compounds used for transport are asparagine, glutamine, arginine and the ureides (allantoin and allantoic acid (Pate, 1973; 1980).

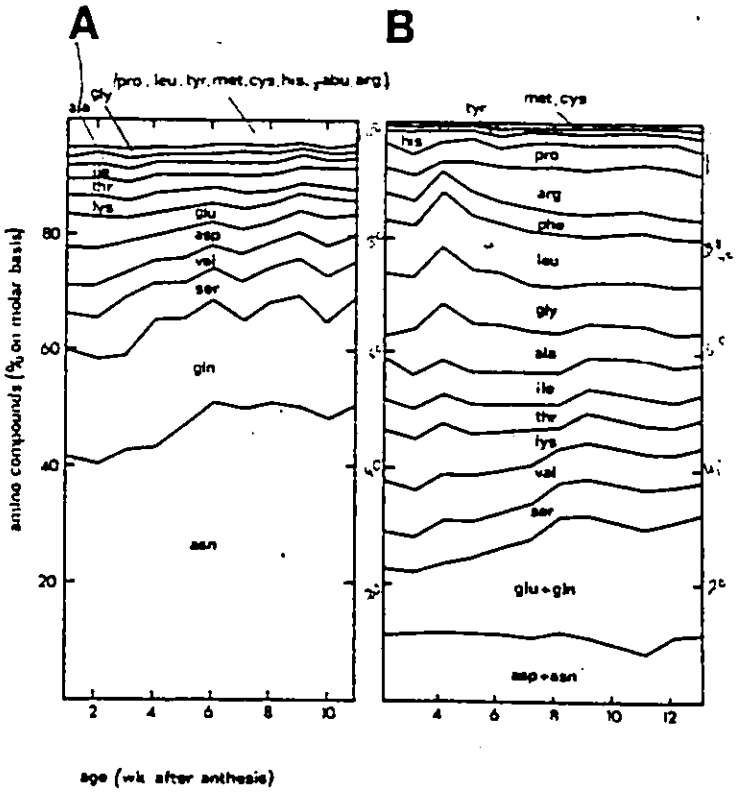
In legumes, the bulk of the nitrogen is supplied in the form of amides (temperate legumes) and ureides (tropical legumes). Other amino acids are either absent or are present in amounts too low to meet the requirement in protein synthesis. For example, arginine is a major amino acid in the storage proteins of legumes. However, it is absent from the supply to the developing seed of pea and lupin. Asparagine is the major amino acid of the sap, accounting for as much as 60% of the total amino acids supplied (Atkins *et al.*, 1975) yet aspartyl residues in seed protein represent only 6-10%. The excess asparagine is probably utilized for the synthesis of arginine and other amino acids supplied in low amounts (Fig. 1). Indeed (^{15}N) labelling studies have shown that amide nitrogen of asparagine is utilized for synthesis of a variety of amino acids. Atkins *et al.* (1975), with the use of asparagine labelled with (UL-^{14}C) and the (^{15}N) in the amide position, followed the fate of both the carbon skeleton and amide nitrogen of asparagine in the developing seeds of *Lupinus albus*. During the period of maximum protein synthesis, over 68% of the nitrogen was found in protein amino acids other than asparagine while over 65% of the ^{14}C remained as aspartate. The wide distribution of (^{15}N) into all the amino acids suggested that there was an extensive *de novo* synthesis.

Figure 1. Composition of amino acids in phloem exudate and seed proteins of *Lupinus albus*.

(Adapted from Atkins *et al.*, 1975).

(A) Percentage composition of amino acids in fruit phloem exudate of *Lupinus albus*.

(B) Percentage composition of amino acid residues in seed protein of *Lupinus albus*.


A
B
ala
gly
(pro . leu . tyr . met . cys . his . γ-abu . arg)
ile
thr
lys
glu
asp
val
ser
gln
asn
tyr
met . cys
his
pro
arg
phe
leu
gly
ala
ile
thr
lys
val
ser
glu + gln
asp + asn
amino compounds (% on molar basis)
80
60
40
20
2
4
6
8
10
12
age (wk after anthesis)

In corn plants also, analysis of the xylem sap shows a general imbalance of amino acids (Arruda and Dasilva, 1979). Although proline accounts for up to 10% on molar basis of total amino acids of storage protein zein (Oaks and Beevers, 1964), it is completely absent from the vascular supply. Glutamine is the major form of nitrogen supplied, followed by asparagine, aspartate, and glutamate in that order (Ivanko and Ingeversen, 1971; Arruda and Dasilva 1979; Zelenic *et al.*, 1966). This is also true in barley (Tully and Hensen, 1979) and wheat (Kirkman and Miflin, 1979). Thus, cereals in general seem to use glutamine as the preferred transported form of nitrogen, however it is also a major amino acid of the storage protein.

The ratio and amount of nitrogenous compounds transported is affected to a certain extent by the amount of nitrogen fertilizer applied to the roots. Increased amounts of nitrate applied to wheat plants increased the total amount of nitrogen transported in the xylem and also the ratio of nitrate nitrogen to amino nitrogen. Analysis of xylem exudate showed that the major products of nitrate assimilation from roots were glutamine and asparagine (Kirkman and Miflin, 1979). In corn plants, addition of nitrate to nitrogen starved plants rapidly increased the total nitrogen content of the sap (Ivanko and Ingeversen, 1971). However, nitrate nitrogen accounted for as much as 63% of the total nitrogen. The addition of NH_4^+ to the roots caused a rapid synthesis of the two amides, glutamine and asparagine. After six hours of application of NH_4^+, organic nitrogen

accounted for 93% of the total nitrogen in the sap. Glutamine alone accounted for 37% of the total amino acids transported (Ivanko and Ingaversen, 1971). Arruda and Dasilva (1979) have shown that the levels, and proportions of amino acids supplied through the xylem to the maize ear during development, remain constant with glutamine as the predominant amino acid.

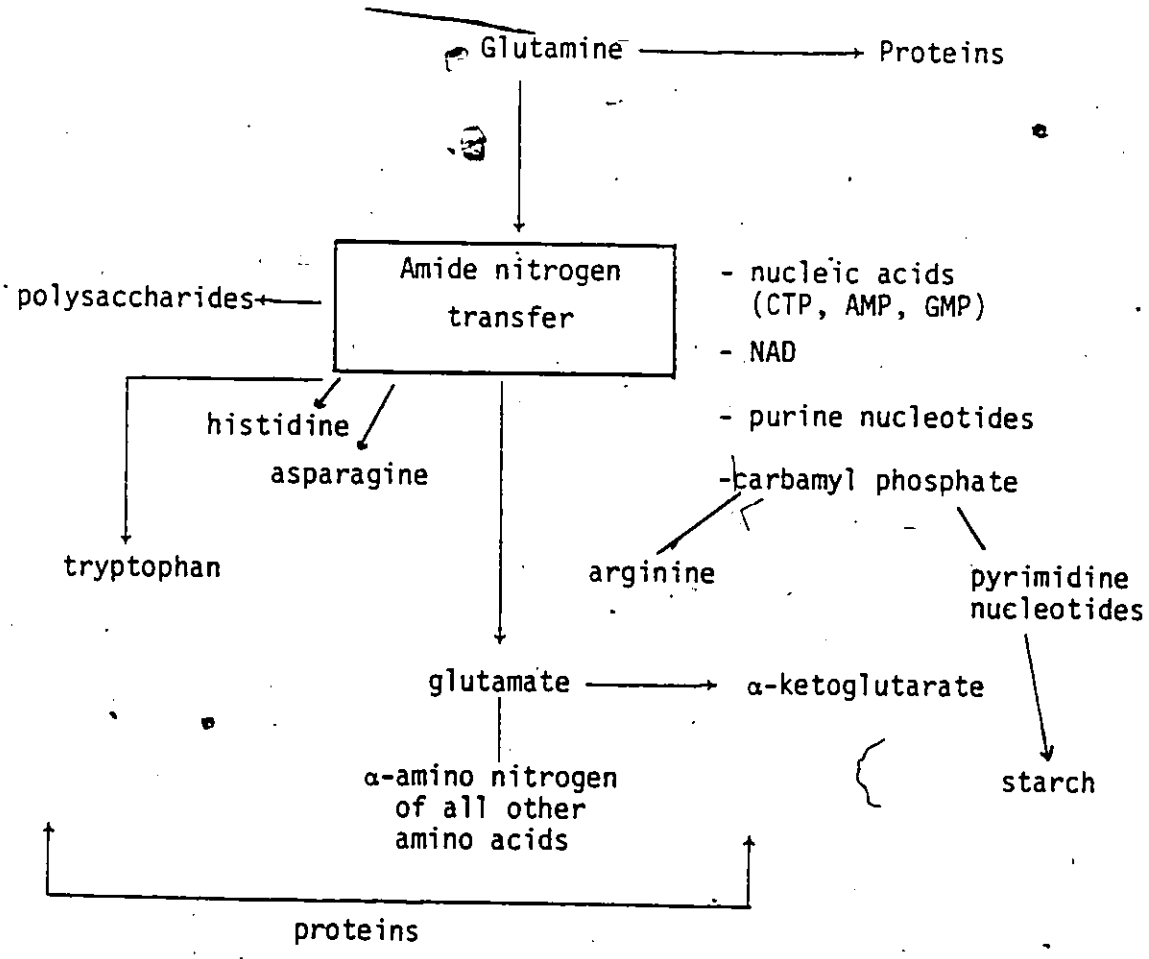
1. Enzymes of Primary Nitrogen Assimilation

Glutamine plays a central role in the metabolism of higher plants (Miflin *et al.*, 1980; Miflin *et al.*, 1981). It is the major port of the entry of inorganic nitrogen incorporated into the plant. It can be used unaltered in protein synthesis, particularly in cereals, where prolamine contains high levels of this amide (Dalby, 1966; Murphy and Dalby, 1971). Glutamine also provides nitrogen for a number of biosynthetic reactions (Fig. 2): For example; in the synthesis of various amino acids, for biosynthesis of purine and pyrimidine nucleotides and glucosamine-6-phosphate. The indole nitrogen of tryptophan, and an imidazole nitrogen atom of histidine are derived from the amide group of glutamine. The amide nitrogen of glutamine can also be transferred directly to asparagine. The enzymes that catalyze the synthesis and degradation of this amino acid are, therefore, of major importance in nitrogen metabolism.

Fig. 3 illustrates the currently held view of nitrogen metabolism in plants. Nitrate is converted to nitrite and ammonium by the enzymes nitrate reductase and nitrite reductase, respectively.

Figure 2. Molecules requiring glutamine in their biosynthesis.

(Adapted after Prusiner and Stadtman, 1973, on p. 1.)


Glutamine
Proteins
Amide nitrogen transfer
polysaccharides
- nucleic acids (CTP, AMP, GMP)
- NAD
- purine nucleotides
-carbamyl phosphate
histidine
asparagine
tryptophan
arginine
pyrimidine nucleotides
glutamate
α-ketoglutarate
α-amino nitrogen of all other amino acids
starch
proteins

Glutamine synthetase catalyzes the formation of glutamine from glutamate and ammonia. One mole of ATP is hydrolyzed in this process to yield ADP and inorganic phosphate. Glutamate, a substrate for glutamine formation, is generated by the glutamate synthase reaction. In this reaction, the amide nitrogen of glutamine can be directly transferred to α-ketoglutarate to yield two molecules of glutamate (Miflin and Lea, 1976; Miflin and Lea, 1977). One of the two molecules of glutamate formed is cycled via glutamine synthetase to glutamine and the other molecule can be used in biosynthetic reactions. Together the glutamine synthetase/glutamate synthase (GS/GOGAT) is referred to as the glutamate synthase cycle. These two enzymes have been isolated from higher plant tissues and are thus known to be widely distributed (Miflin and Lea, 1980).

The activity of glutamate synthase was first discovered in the bacteria *Aerobacter aerogenes* where the enzyme used NADPH as the electron donor (Tempest *et al.*, 1970). In pea leaves however, the activity was shown to be dependent on reduced ferredoxin (Lea and Miflin, 1974). About the same time, an NAD(P)H dependent glutamate synthase activity was described in cultured carrot root cells (Dougall, 1974) and in pea roots (Fowler *et al.*, 1974). Recently, the two types of activity, ferredoxin dependent and pyridine-nucleotide dependent, have been detected in etiolated shoots (Matoh *et al.*, 1980; Suzuki *et al.*, 1982; Wallsgrove *et al.*, 1982), roots (Matoh *et al.*, 1979; Suzuki *et al.*, 1982) and seeds (Matoh *et al.*, 1979).

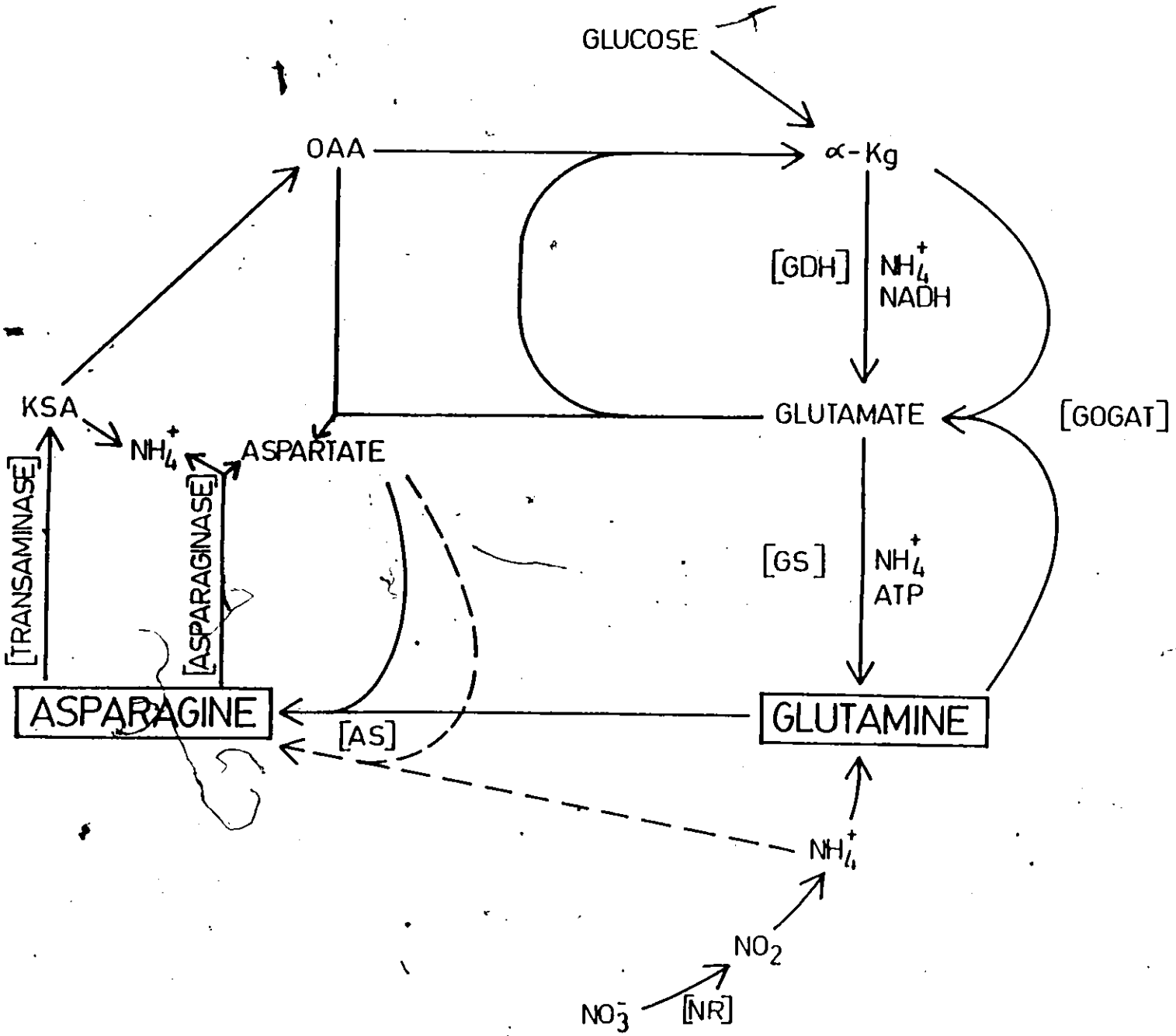
Figure 3. Enzymes of primary nitrogen assimilation.

Glutamine can be considered the primary port of entry of reduced nitrogen into metabolism.

The transfer of glutamine amide nitrogen to α-amino nitrogen is mediated by glutamate synthase (GOGAT): glutamine + α-ketoglutarate (α-Kg) → 2 glutamate. Asparagine could be hydrolyzed by asparaginase to aspartate and ammonia or it could be transaminated to give α-ketosuccinamic acid (2-KSA).

2-ketosuccinamate may be metabolized to oxaloacetic acid (OAA) and ammonia. The ammonia produced could be reassimilated by glutamate dehydrogenase (GDH) to produce glutamate or by glutamine synthetase (GS) reaction to produce glutamine.

Ammonia could also be derived from the reduction of NO_3^- to NO_2^- to NH_4^+ mediated by the enzymes nitrate reductase (NR) and nitrite reductase (NiR). Glutamine and/or NH_4^+ could then be utilized in the asparagine synthetase (AS) reaction.


GLUCOSE
OAA
α-Kg
[GDH]
NH_4^+
NADH
KSA
GLUTAMATE
[GOGAT]
NH_4^+
ASPARTATE
[TRANSAMINASE]
[ASPARAGINASE]
[GS]
NH_4^+
ATP
ASPARAGINE
GLUTAMINE
[AS]
NH_4^+
NO_2^-
NO_3^-
[NR]

In pea leaves, subcellular localization studies have shown that glutamine synthetase is equally distributed between chloroplast and cytosol, whereas, glutamate synthase is located solely within the chloroplast (Wallsgrove *et al.*, 1979). In corn roots, glutamine synthetase is mainly soluble while glutamate synthase is localized in plastids (Suzuki *et al.*, 1981). In barley leaves, two forms of glutamine synthetase designated GS_1 and GS_2 have been described (Guiz *et al.*, 1979; Mann *et al.*, 1979). These two forms do not differ in their molecular weights but differ in pH optima, stability, and kinetic properties. Subcellular localization studies show that GS_1 is the cytosolic form while GS_2 is present in chloroplast. Enzyme extracts prepared from barley roots and developing seeds show a single peak of GS activity which coincides with GS_1 when eluted on a DEAE-Sephacel column. Thus, GS_1 appears to be present in leaves, roots and seeds while GS_2 is present in green tissue only (Mann *et al.*, 1979).

GS of *E. coli* is regulated by several mechanisms including repression and derepression of its synthesis, rapid reversible inactivation and inhibition by potential end products of glutamine metabolism (Woolfolk and Stadtman, 1967; Stadtman, 1973). Similar regulation of enzyme from higher plants have not been found (Lea and Miflin, 1980). In *Lemna minor*, changes in activity of glutamine synthetase, glutamate dehydrogenase and glutamate synthase have been examined under a range of conditions. Glutamine synthetase and glutamate synthase activities are high under conditions of low ammonia availability. Increasing the NH_4^+ concentration results in a

decrease in the level of these two enzymes and an increase in the glutamate dehydrogenase level.

Glutamine synthetase has a high affinity for ammonia. For example, in *Lemna minor*, the Km for ammonia is 1.2-1.5 $x10^{-5}$ M, whereas, that of NAD-linked glutamate dehydrogenase is 33 mM (Stewart and Rhodes, 1977). In this organism, the intracellular concentration of ammonia does not exceed 0.2 to 1.0 mM, whether the plants are grown on NO_3^- or low levels of ammonia. Thus, glutamine synthetase could efficiently assimilate ammonia under these conditions. When external ammonia concentrations are high, the intracellular concentration of ammonia can increase to 30 mM. Under these conditions GDH could mediate a reductive amination of α-ketoglutarate to produce glutamate.

The activity of GDH is inhibited by ATP, a substrate for GS. On the other hand, GS is inhibited by ADP and 5'-AMP. The inhibition of GS activity by these nucleotides is competetive with respect to ATP. Similar results related to nucleotide inhibition of GS activity have been reported in pea leaf (O'Neal and Joy, 1975) and in rice root (Kanamori and Matsumoto, 1972). The inhibition of GS activity by ADP and 5'-AMP and of GDH by ATP suggests that activity of these enzymes is regulated by the energy charge.

In pea leaves (O'Neal and Joy, 1975) and in rice roots (Kanamori and Matsumoto, 1972) GS is also activated by the presence of α-ketoglutarate, indicating regulation by carbon precursors. In rice roots, a stimulation as high as 50% has been observed.

Thus, it has been suggested that glutamine synthetase would catalyze the primary reaction for assimilation of ammonia when energy charge is high and carbohydrate supply is plentiful. However, when the energy charge is low and the ammonia concentration is high, glutamate dehydrogenase may be involved and may, in fact, be the more important reaction in assimilation of ammonia (Stewart and Rhodes, 1977). Similar results are observed in corn root tissue (Oaks *et al.*, 1980). Incubation of excised root of corn in presence of glucose, enhances the synthesis of nitrate reductase, stabilizes the levels of GS and GOGAT and reduces the synthesis of glutamate dehydrogenase. It is believed that in addition to the supply of carbon, glucose may enhance the energy charge and thus could be important in regulating the synthesis or activity of enzymes of nitrogen assimilation.

Asparagine synthetase catalyzes the formation of asparagine. This enzyme was first extracted from bacterial systems. The reaction required L-aspartate, ammonium ion and ATP as substrates. Asparagine, adenosine monophosphate and inorganic pyrophosphate are the products formed by the enzyme (Cedar and Schwartz, 1969). In higher plants and animals, the asparagine synthetases are classified as amidotransferases (Buchanan, 1973). It is the amide nitrogen of glutamine rather than free ammonia that serves as the nitrogen source. This is supported by the fact that the Km for glutamine is much lower than the Km for ammonia. Glutamine-dependent asparagine synthetases have been isolated from the cotyledons of lupin (Rognes, 1975; Lea and Fowden, 1975) and soybean seeds (Streeter,1973). The enzyme is 4 to 10 times more

active with glutamine than with ammonia. In soybean cotyledon extracts, the Km is 0.12 mM and 3.9 mM for glutamine and ammonia respectively, suggesting that glutamine is the physiological substrate. Enzyme from corn roots, embryo and scutellar extracts have Km's of 1.6, 0.49 and 0.59 mM for glutamine and 4.0,2.6 and 2.6 mM for ammonia, respectively (Ross, 1979). Therefore, the enzyme from corn roots reacts only slightly better with NH_4^+ than glutamine.

Since asparagine and glutamine are supplied in excess in developing seeds, it is likely that the degradation rather than synthesis of these amides is the dominant reaction. In fact, activity of AS and GS could be inhibited as a result of the high concentrations of these amino acids in the endosperm. In corn endosperm extracts, the asparagine synthetase is inhibited by 2 mM asparagine *in vitro*. The enzyme from scutellum or roots is much less sensitive to inhibition by asparagine (Ross, 1979). Asparagine synthetase activity in extracts from lupin cotyledons, is also sensitive to asparagine (Lea and Fowden, 1977).

In many respects, events that occur in developing seeds are the reverse of what happens in the roots. For example, it has been well documented that asparagine or glutamine support growth of legume cotyledons (Millerd *et al.*, 1975; Thompson *et al.*, 1977; Lea *et al.*, 1979; Skokut *et al.*, 1982) and of corn endosperm tissue (Straus, 1960; Reddy and Peterson, 1977; Shannon and Liu, 1977) in culture. Thus, instead of being a product of metabolism, glutamine or asparagine represent the major source of nitrogen (Farrar and Ganugapati, 1970). Thus, GOGAT, a glutaminase, an asparaginase or

asparagine transaminase can all be important in supply of NH_4^+ for other biosynthetic reactions. Glutamine synthetase and glutamate dehydrogenase may be involved in secondary assimilation of ammonia liberated as a result of the degradation of the amides in the seeds.

2. Asparagine metabolism

In legumes, the metabolism of asparagine has been studied extensively. Two routes of asparagine metabolism have been identified (Sodek *et al.*, 1980; Ireland and Joy, 1981). The enzyme asparaginase catalyzes the hydrolytic deamidation of asparagine, liberating ammonia and aspartate as the products. The presence of the enzyme was first reported in plant tissue by Grover and Chibnall (1939) and Kretovich (1958). Lees and Blakeney (1970) demonstrated the enzyme activity in crude extracts of *Lupinus* and *Dolichos* roots and in nodules.

In developing seeds, Atkins *et al.*, (1975) reported the presence of the enzyme in crude extracts of maturing seeds of *Lupinus albus*. In a time course study, the (^{15}N) from amide group of asparagine was traced to free ammonia, glutamate + glutamine, and alanine in the soluble endosperm. The results suggested that the initial hydrolysis of the amide group of asparagine was followed by reassimilation of ammonia into glutamine and alanine. *In vitro* activity of asparaginase was up to 5 µmoles of aspartate formed per hour per gram fresh weight which more than accounted for the estimated rate of asparagine utilization *in vivo*. Recently, Sodek *et al.* (1980)

have demonstrated the presence of this enzyme in a large number of legume and cereal seeds.

Streeter (1977) demonstrated that asparagine was transaminated in soybean leaf extracts, suggesting that in this tissue, a transamidase rather than asparaginase was present. The enzyme was most active with glyoxylic acid as the ketoacceptor, followed by pyruvate, oxaloacetate and α-ketoglutarate. Lloyd and Joy (1978) have extended these experiments to examine the fate of (^{14}C)-asparagine in growing pea and soybean leaves. Following a 30 minute incubation period in light, label appeared in aspartate, glutamate, homoserine and alanine. In a 270 minute incubation period, 75% of the labelled asparagine was metabolized and more than half of the metabolized carbon skeleton was present as 2-hydroxy-succinamate. When ^{14}C-ketosuccinamate (KSA), the immediate product of transamination of asparagine was fed to the pea leaves, an efficient conversion to 2-hydroxysuccinamate was observed. After two hours of labelling with (^{14}C)-KSA, amino acids became labelled (aspartate, glutamate, homoserine), but two-thirds of the metabolized carbon was recovered as 2-hydroxysuccinamate (2-HSA). Thus, 2-KSA was deaminated to some extent but the majority was reduced to 2-HSA which tends to accumulate. Based on these results, they suggested that in pea and soybean leaves, reduction of 2-ketosuccinamate to 2-hydroxysuccinamate provided the major pathway for the utilization of 2-ketosuccinamate. The 2-hydroxysuccinamate was slowly metabolized in the dark to liberate ammonia and malate.

In soybean cotyledons, (^{15}N) labelling studies show that both asparaginase and asparagine-transaminase activities are present. The pattern of labelling suggests that in early stages of development, asparagine-transaminase is mainly responsible for metabolizing asparagine α-amino group nitrogen for protein synthesis (Skokut *et al.*, 1982).

3. Glutamine metabolism

In developing seeds, the activity of glutamate synthase is high and the increase in activity coincides with period of rapid protein synthesis (Beevers and Storey, 1976; Storey and Reporter, 1978 Storey and Beevers, 1978, Sodek and Dasilva, 1979; Oaks *et al.*, 1979; Misra and Oaks, 1981). The reaction is of primary importance in the developing seeds as the enzyme could serve to transfer the amide nitrogen of glutamine to other compounds.

Storey and Beevers (1978) followed the glutamate synthase activity in the pods and cotyledons of pea during development and maturation. The activity increased rapidly as the pod elongated. Cotyledonary GOGAT activity also increased during the period of active growth and maximum protein synthesis and later declined. The *in vitro* activity of the enzyme was several fold higher than the measured rate of protein synthesis and was more than sufficient to account for the metabolism of all of the accumulated nitrogen in the seed.

Skokut *et al.*,(1982) examined the fate of (^{15}N) amide nitrogen of glutamine in cotyledons of soybean grown in sterile cultures, with glutamine as the sole source of nitrogen. Analysis of protein

revealed approximately equal amounts of (^{15}N) and (^{14}N) incorporated into seed protein. This result is consistent with the operation of glutamate synthase activity. The amide nitrogen (^{15}N) of asparagine on the other hand, appeared as ($^{15}NH_4^+$) in the medium, an observation consistent with an asparaginase activity *in vivo*.

Sodek and Dasilva (1979) have reported that activity of glutamate synthase reaches significant levels in the maize endosperm at the time of rapid synthesis of storage proteins. Oaks *et al.*, (1979) have shown that, in addition to glutamate synthase, activities of other enzymes of nitrogen metabolism i.e. GS, GDH, asparagine synthetase also increase in the endosperm and their appearance coincides with the onset of zein biosynthesis.

Injection of (^{14}C) labelled precursors directly into the developing endosperm of maize indicates that pathways of amino acid biosynthesis are functional in developing endosperm (Sodek, 1976). Thus potentially, the amide nitrogen of glutamine or asparagine could be utilized for synthesis of various amino acids *in situ*. However, the understanding of the degradation of asparagine and glutamine in corn is not complete.

In the present study, the metabolism of asparagine and glutamine was examined in the developing endosperm of maize with emphasis on:

1. a) Characterization of the enzymes of nitrogen metabolism, particularly asparaginase, glutamate synthase, glutamine synthetase and glutaminase.

b) A comparative assessment of the levels of activity of the enzymes involved in synthesis and degradation of amides and of reassimilation of the released ammonia in the endosperm of a normal variety of maize (W64A) and its isogenic high lysine mutants opaque-2 and floury-2.

2. Investigation of the synthesis and degradation of glutamine in the endosperm of cultured caryopsis of hybrid variety W64A x W182E using [2-^{14}C]-acetate and [UL-^{14}C]-glutamine.

MATERIALS AND METHODS

A. Materials

Maize kernels *Zea mays* (L), inbred W64A, and its isogenic homozygous mutants opaque-2 and floury-2 were supplied by Dr. O. E. Nelson of the University of Wisconsin, Madison, WI, USA. Samples of the hybrid variety W64A x W182E were purchased from the Wisconsin Seed Foundation, Madison, WI. Cobs of field grown commercial variety Dekalb 14A were kindly supplied by Dr. S.F.H. Threlkeld, McMaster University, Hamilton, Ontario.

The following materials were obtained from Sigma Chemicals Co., St. Louis. HEPES (N-2-hydroxyethyl piperazine-N'-2 ethanesulfonic acid), tricine (N-tris(hydroxymethyl)methyl glycine), aminooxyacetic acid, ferredoxin, L-glutamate dehydrogenase, catalase, alcohol dehydrogenase, malate dehydrogenase, asparaginase, nicotinamide adenine dinucleotide (reduced form), nicotinamide adenine dinucleotide phosphate (reduced form), L-aspartic acid, L-glutamic acid, α-ketoglutarate (monosodium salt), trypsin, yeast RNA (type III), cacodylic acid (dimethyl arsenic acid), phenyl methyl sulfonyl fluoride (PMSF), L-glutamate-oxaloacetate transaminase, blue dextran, o-phthaldialdehyde, adenosine 5'-triphosphate, α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA), streptomycin sulfate, penicillin.

Pronase, carboxypeptidase A, L-glutamine and L-asparagine were obtained from Calbiochem, San Diego, California.

Tris-(hydroxymethyl)amino methane, sodium dodecyl sulfate, Dowex-resins, and protein-dye reagent were obtained from Bio-Rad Laboratories, Richmond, California.

Two-mercaptoethanol, titanium trichloride, potassium dihydrogen phosphate, sodium borate, ammonium sulfate, ammonium nitrate, magnesium chloride and ferrous sulfate were obtained from Baker Chemicals Co., Phillipsburg, N.Y.

Sodium molybdate, zinc sulfate, copper sulfate, disodium ethylene diamine tetraacetic acid were obtained from Fisher Scientific Co., Fair Lawn, N.Y.

Sucrose, ninhydrin reagent, potassium chloride, potassium nitrate, magnesium sulfate, potassium iodide, calcium chloride, methanol, manganese sulfate and cobalt chloride were obtained from British Drug House Chemicals, Toronto, Canada.

Potassium phosphate was obtained from Mallinckrodt Inc. Paris, Kentucky. Bacto-agar was obtained from Difco Laboratories, Detroit, Michigan. Sephadex resins were purchased from Pharmacia, Uppsala, Sweden. [UL-^{14}C]-glutamine (40 mCi/mmol), [2-^{14}C]-acetate (40-60 mCi/mmol) were obtained from Amersham, IL., USA.

(UL-^{14}C) aspartate (200 mCi/mmoles) and Omnifluor, were obtained from New England Nuclear, Boston, Mass.

B. Growth Conditions for Developing Seeds

Corn caryopses were planted in a soil-peat sand and manure mixture (3:2:1:1) in disposable pots, and were grown under greenhouse conditions (22°C, natural light) for 2 weeks. The seedlings plus

pots were then transferred to the field, or else were grown under growth chamber conditions of 26°C, 16 h light (about 150 μEinsteins·$m^{-2}.sec^{-1}$). Flowers were cross-pollinated by hand when silks first appeared. Developing caryopses were harvested at 5 day-intervals starting at 5 days after pollination. After removal of the embryo, the endosperm was frozen in liquid nitrogen, transferred to freezer bags and stored at -20°C until further use. For tissue culture, freshly harvested 5-7 day-old caryopses were used.

I. Enzyme Extraction and Assay Procedures

A. General methods

For the enzymes of nitrogen assimilation, i.e., glutamate synthase, glutamate dehydrogenase, glutamine synthetase, asparagine synthetase and asparaginase, the extraction was carried out at 0-4°C. The endosperms were ground in a mortar and pestle with 2 volumes of extraction buffer per gram fresh weight. The extracts were filtered through one layer of Miracloth before centrifugation (15,000 g for 20 min). The supernatant was treated with $(NH_4)_2SO_4$ (0.315 g/ml = 50% saturation). After 20 min on ice, the extract was centrifuged again (15,000 g for 10 min) and the pellet was redissolved in a minimum volume of extraction buffer. It was then filtered through a Sephadex G-75 column (20 x 1.5 cm) which had been equilibrated with elution buffer. Protein fractions were collected and pooled. The final activity of each enzyme was calculated on the basis of the amount of product produced or substrate utilized per endosperm, or as specified in the text.

B. Glutamate synthase (GOGAT)

1. Extraction and assay

The extraction buffer contained 0.2 M HEPES (pH 7.5, neutralized with NaOH), 12.5 mM 2-mercaptoethanol, and 2 mM EDTA. The elution buffer contained 25 mM HEPES (pH 7.5), 1 mM EDTA, and 12.5 mM 2-mercaptoethanol. The assay mixture developed by Oaks *et al.* (1979) contained 20 mM HEPES (pH 7.5), 15 mM KCl, 1 mM EDTA, 1 mM α-keto-glutarate, and 20 mM glutamine in a total volume of 1.0 ml. As electron donor, 0.16 mM NAD(P)H or 40 μmoles ferredoxin were used per assay. When ferredoxin was used, 1.8 mg sodium dithionite plus 1.8 mg sodium bicarbonate were also added. The reaction was monitored by either following the oxidation of NAD(P)H at 340 nm in a Gilford spectrophotometer or by assaying glutamate formation after separation on a reverse-phase HPLC column (see under HPLC methodology).

2. Sephadex G-200 column chromatography

Step 1. Extraction: For Sephadex G-200 filtration of GOGAT, crude extracts were prepared according to the method of Matoh *et al.* (1979). Phosphate buffer 200 mM (pH 7.5) containing 25 mM 2-mercaptoethanol, 2 mM EDTA, and 1 mM PMSF* was used. The homogenization was performed in an electric blender for 2 min at maximum speed. The homogenate was filtered through four layers of gauze and centrifuged at 15,000 g for 20 min. All operations were performed at 0-4°C.

* Stock solution of PMSF was prepared by dissolving 35 mg of PMSF in 10 ml of isopropyl alcohol, this was diluted to 60 ml with 25 mM phosphate buffer (pH 7.5) to give a final concentration of 4 mM.

Step 2. Ammonium sulfate fractionation: The supernatant was brought to 25% saturation with solid ammonium sulfate (8.64 g/60 ml). After stirring for 20 min on ice, the sample was centrifuged at 15,000 g for 20 min. Ammonium sulfate was added to the supernatant to give a final concentration of 60% (13.5 g/60 ml). The resulting precipitate was collected by centrifugation and suspended in 50 mM potassium-phosphate buffer (pH 7.5) containing 12.5 mM 2-mercapto-ethanol, 2 mM EDTA, 1 mM PMSF and 2 mM α-ketoglutarate. It was dialyzed against the same buffer for 16 hr. The dialyzed enzyme was concentrated using an immersible CX-30 ultrafiltration unit (Millipore Corp., Bedford, Mass.), brought to 50% glycerol (1:1 v/v) and stored at -20°C overnight.

Step 3. Filtration on Sephadex G-200: The concentrated enzyme solution was subjected to gel filtration using a Sephadex G-200 column (1.8 x 82 cm) previously equilibrated with the dialysis buffer, supplemented with 100 mM NaCl. Fractions of 2.1 ml were collected and assayed for NAD(P)H- and Fd-dependent GOGAT.

3. Molecular weight estimation

The molecular weights of the enzymes were estimated by filtration through Sephadex G-200 column, using the method of Andrews (1970). The column was equilibrated with 50 mM phosphate buffer (pH 7.5) containing 12.5 mM 2-mercaptoethanol, 100 mM NaCl. It was calibrated with yeast alcohol dehydrogenase (150,000), catalase from bovine liver (250,000), and bovine serum albumin (BSA) (fraction V, Sigma) (68,000). These marker proteins were

located by measuring absorbance of eluted fractions at 280 nm. The results were plotted as Ve/Vo *vs* the log of molecular weight.

C. Glutamate Dehydrogenase (GDH)

The extraction buffer contained 0.2 M HEPES (pH 7.5), 12.5 mM 2-mercaptoethanol and 2 mM EDTA. The elution buffer contained 25 mM HEPES (pH 7.5), 1 mM EDTA and 12.5 mM 2-mercaptoethanol. The assay mixture contained 0.8 M $(NH_4)_2SO_4$, 1 mM α-ketoglutarate, 0.16 mM NADH, 0.1 M tris-HCl buffer (pH 7.9) in a total volume of 1.0 ml. The disappearance of NADH at 35°C was measured with a Gilford spectrophotometer.

D. Asparagine Synthetase (AS)

A radiotracer method adapted from Stulen and Oaks (1977) with slight modifications was used for the asparagine synthetase assay. Tissue was homogenized in 200 mM HEPES buffer (pH 7.5), 12.5 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM ATP, and 10 mM $MgCl_2$. This buffer was also used for the elution of the enzyme from Sephadex column.

The assay mixture contained 100 mM tris-HCl buffer (pH 8.0), 1 mM 2-mercaptoethanol, 1.9 mM ATP, 10 mM glutamine, 1 mM α-aminooxyacetate, 13.2 mM $MgCl_2$, 1.1 mM aspartate (0.22 mCi of (UL-^{14}C)-aspartate) and 0.2 ml of enzyme in a total volume of 0.62 ml. The assay was run for 20 min at 35°C and was terminated by the addition of 1.0 ml of ethanol (95%).

The product [^{14}C]-asparagine was separated from aspartate on Dowex-1-acetate column (50 x 5 mm). The product was identified

as asparagine or aspartic acid by paper chromatography before and after hydrolysis by 1 N HCl or by a commercial asparaginase. Effluents from the column were mixed with 15 ml of a toluene-triton X-114 (1:1 V/V) scintillation cocktail. The samples were then counted in a Beckman LS-250 scintillation counter. Values were corrected for radioactivity in the asparagine fraction from assays run in the absence of a nitrogen source.

E. Glutamine Synthetase (GS)

1. Extraction and assay

The method used was a modification of the one described by O'Neal and Joy (1973). The extraction and elution buffer contained 50 mM tris-HCl (pH 7.9), 20 mM $MgSO_4$, and 1 mM EDTA. The reaction buffer contained 10 mM tricine (pH 7.8), 20 mM $MgSO_4$ and 1 mM EDTA. The assay mixture also contained glutamic acid (88 mM), hydroxylamine (6 mM), and ATP (5 mM). The pH was adjusted to 7.8 with KOH and the total volume was 1.0 ml. The assay was routinely run for 20 min at 35°C. The reaction was stopped by addition of 1.0 ml of $FeCl_3$ reagent (0.37 M $FeCl_3$ + 0.2 M TCA in 0.67 N HCl). The tubes were spun at top speed in a clinical centrifuge for 20 min to remove the proteins. Formation of γ-glutamylhydroxamate iron complex was monitored at 540 nm.

2. Ion-exchange chromatography of GS

For characterizatipn of isoforms of GS, the method of Hirel and Gadal (1980) was employed.

a. Enzyme extraction: Ten grams of endosperm were homogenized in 50 ml of 25 mM imidazole-HCl buffer (pH 7.8) containing 1 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, and 10 mM 2-mercaptoethanol in a Waring blender for 2 min. The brei was filtered through a single layer of Miracloth and centrifuged at 20,000 g for 30 min. The supernatant was assayed for GS activity.

b. DEAE-Sephacel column chromatography: The crude extract was loaded on a DEAE-Sephacel column (20 x 2 cm) previously equilibrated with extraction buffer. A linear gradient of 0-0.5 M NaCl was used to elute the enzyme. The flow rate was adjusted to 10 $ml \cdot h^{-1}$ and 2 ml fractions were collected. Each fraction was assayed for enzyme activity.

3. Immunotitration analysis

The antibodies raised against GS_1 (cytosolic GS) from etiolated leaves of barley were kindly supplied by Dr. B. Hirel. Immunotitration was performed by incubating antibodies with enzyme for 12 h at 4°C. The antigen-antibody complex was removed by centrifugation at 10,000 g for 10 min and glutamine synthetase activity was assayed in the supernatant solution.

F. Asparaginase (Asnase)

1. Extraction and assay

The activity was measured by a coupled spectrophotometric assay developed during the course of this study. The extraction and elution buffers contained 25 mM Na-HEPES (pH 8.0), 12.5 mM 2-mercapto-

ethanol, 1 mM EDTA and 30 mM KCl. The enzyme was assayed using Na-HEPES (25 mM, pH 8.0), asparagine (100 mM), KCl (20 mM), and 0.2 ml of enzyme in a total volume of 0.8 ml. Asparagine used in this assay was purified by passing it over a Dowex-1-acetate column. The water-eluant was concentrated under vacuum at 30°C and was then diluted to the appropriate concentration. The incubation was carried out at 30°C and the reaction was stopped by placing the tubes in boiling water for 5 min. The precipitate was removed by centrifugation at top speed in a clinical centrifuge. The supernatant was assayed for total aspartate formed by adding 20 µg glutamate-oxaloacetate transaminase (GOT), and 1.27 µg malate dehydrogenase (MDH) to an appropriate aliquot of supernatant, also included were 0.1 ml of NADH (0.16 mM), 0.1 ml of α-ketoglutarate (1 mM), and tris-HCl (0.1 M, pH 7.8). The total volume was adjusted to 1 ml. The total oxidation of NADH was followed at 340 nm with a Gilford spectrophotometer.

2. Product identification

The production of aspartate was confirmed by descending paper chromatography in butanol:acetic acid:water (12:3:5) or phenol:water (80:20 w/v), solvent system. An analysis with a Beckman model amino acid analyzer also confirmed the presence and amount of aspartate. This latter test was performed by Dr. K.W. Joy, of Carleton University, Ottawa.

Ammonia produced in the reaction mixture was assayed in Conway dishes. The NH_4^+ was liberated from the reaction mixture by

adding a saturated solution of potassium carbonate (3 ml/ml of reaction mixture). It was allowed to distill overnight into 0.0143 N sulfuric acid. The sulfuric acid sample was then assayed for NH_4^+ by the phenol-hypochlorite reaction (Russel, 1944).

G. Asparagine Transaminases

Frozen endosperms were ground in 50 mM Tris-HCl, pH 7.8, with a mortar and pestle. The tissue buffer ratio was 1:2.5. The brei was passed through cheesecloth, centrifuged at 12,000 g for 10 min, and the supernatant was passed through Sephadex G-25, equilibrated and eluted with the extraction buffer. Enzyme (1 ml) was added to 0.1 ml of reaction mixture at pH 7.8 to give the following concentrations: asparagine 10 mM; pyruvate or α-ketoglutarate 10 mM; pyridoxal phosphate 0.02 mM. The mixture was incubated at 30°C and after 20 and 40 min, 0.5 ml aliquots were removed and added to 25 mg of 5-sulphosalicylic acid. Precipitated protein was removed in a micro-centrifuge and amino acid content of the supernatant was determined using a Beckman 119 BL amino acid analyzer. This test was performed by Dr. K.W. Joy of Carleton University, Ottawa.

H. Endopeptidase

The endosperm pieces were homogenized in 100 mM tris-HCL buffer, pH 7.5. Two milliliters of buffer were used per gram of fresh weight of tissue, and the homogenate was centrifuged at 28,000 g for 20 min at 0°C. The supernatant was used as crude enzyme. Protease activity was determined by the method of Melville and Scandalios (1972). The assay mixture contained α-N-Benzoyl-DL-

arginine-p-nitroanilide (BAPNA) as substrate (120 µg/assay), tris-HCL buffer (0.1 M pH 7.5), and 50 µl of crude enzyme preparation in a total volume of 1.12 ml. The reaction was monitored at 35°C by following changes at 410 nm with a Gilford spectrophotometer. Trypsin assays were also performed with known amounts of trypsin and from these values, the endopeptidase activities could be expressed as microgram equivalents of trypsin.

I. Ribonuclease

Tissue was homogenized with 5 volumes of 50 mM tris-HCl (pH 7.5) and centrifuged at 500 g for 20 min. The crude preparation was then assayed for ribonuclease activity by the method of Wilson (1967). A 2.5 ml reaction mixture containing 50 mM cacodylate buffer (pH 5.8), 160 mM KCl, 5 mg of dialyzed yeast RNA, and 0.2 ml of crude enzyme preparation, was prepared and the incubation was carried out at 38°C for 10 min. The reaction was stopped by the addition of 0.5 ml of 25% perchloric acid containing 0.75% uranyl acetate. The reaction was chilled on ice for 20 min and then centrifuged to remove the precipitate. The supernatant was diluted 20 times before reading at 260 nm.

II. Radioactive Labelling of Endosperm Reserves

A. Preparation of Labelled Substrate

(UL-^{14}C) glutamine was purified by passage through Dowex-1-acetate column to remove acidic amino acids. The final (^{14}C) glutamine fraction was taken up in water to give a concentration of

approximately 12.5 µCi/ml. Just before use the solution was sterilized by filtration through a disposable Millipore filter. Appropriate amounts of sterilized solution was then added to the media. Solutions of (2-^{14}C) acetate (10 µCi/ml) were sterilized in a similar manner just before use.

B. Incubation of Excised Endosperm

Cobs were harvested 20 days after pollination. The caryopses were surface-sterilized in 1% hypochlorite solution for 1 min and rinsed in distilled water several times. The embryo was dissected out under sterile conditions and either whole or sliced (5 slices/endosperm) endosperm were transferred to 25 ml Erlenmeyer flask containing 3 ml of Hoagland's salt solution, 5 µCi (2-^{14}C)-acetate or 0.8 µCi (^{14}C) glutamine. Penicillin (10 µg/ml), and streptomycin (250 µg/ml) were included to minimize bacterial contamination. The flasks were incubated at 26°C ± 1, with constant shaking. After the required incubation time aliquots of incubation media were plated on nutrient agar to test for contamination. At the end of the experiment the endosperms were rinsed with distilled water, frozen in liquid nitrogen and stored at -20°C. The incubation medium was also frozen immediately and stored at -20°C.

C. Caryopsis Culture

For caryopsis culture, the technique of Gengenbach (1977) as modified by Shimamoto and Nelson (1981) was used. Ears of maize were harvested 5-7 days after pollination and were cut into blocks

containing 10 caryopses/block. The blocks were surface-sterilized with 1% sodium hypochlorite for 1 min and then rinsed thoroughly with sterile distilled water. Five of these blocks were placed on the nutrient media (50 ml) in a 125 ml Erlenmeyer flask in such a way that only cob tissue was in contact with the medium. They were grown for 7 days on the standard medium and then transferred onto (^{14}C) glutamine-containing medium (5 µCi/20 ml) or (2-^{14}C) acetate-containing medium (1 µCi/ml), in a 128 ml baby food jar. In some later experiments, (^{14}C) glutamine was injected directly into the endosperm and the caryopses were incubated for 7 days at 28°C. At the end of the experiment, the blocks were harvested, endosperm, embryo and cob tissues were frozen separately in liquid nitrogen, and stored at -20°C until further analysis. After the caryopses were harvested, the agar medium was eluted overnight with 50 ml of water at 4°C. Fifty microlitre aliquots of the eluted medium were assayed for radioactivity and the amount of label left in the medium at the end of the incubation period was estimated.

D. Preparation of the Cultured Tissue

Frozen samples were freeze dried for two days and the dry weight of each sample was recorded. Tissue was ground to a fine powder in a Prolabo (Dangomau) ball mill. The total radioactivity in each sample was assayed according to the method of Shimamoto and Nelson (1981). Two to five mg of each tissue were mixed with 10 ml scintillation cocktail, shaken well, left for 24 h at 4°C and then counted in a Beckman LS-250 liquid scintillation counter.

E. Fractionation of Endosperm Reserves

A modified method of Sodek and Wilson (1971) was employed to progressively extract the various endosperm fractions, according to their solubility, i.e., total salt-soluble nitrogen, starch, zein and glutelin. Ten millilitres of solvent was used per gram of tissue. Two replicate samples of 100 mg each were extracted in the following manner:

Extraction in 0.1 M NaCl for 1 hour at 4°C, was followed by centrifugation for 10 min at top speed in a clinical centrifuge. The pellet was washed 3 times with water and the washings were combined with the original supernatant solution. This constituted the combined water-soluble and salt-soluble fractions, i.e., albumins, globulins, free amino acids, organic acids, and sugars. This combined fraction was lyophilized and then resuspended in 70% ethanol (v/v), which dissolved the amino acids, sugars, and organic acids. The albumin and globulin proteins were precipitated and collected by centrifugation. The pellet was washed twice with 70% ethanol and the washings were combined with the ethanol-soluble fraction. The residue remaining after the extraction of salt-soluble compounds, was treated with 0.1 N HCl for 1 h at 100°C in order to hydrolyze the starch. After centrifugation, the pellet was washed 3 times with water and the washings were combined with the supernatant. The residue remaining from the HCl treatment was shaken with 70% ethanol containing 1 mM 2-mercaptoethanol for 1 h at 60°C. After incubation, the suspension was centrifuged at 12,000 g for 5 min to recover the supernatant. The

pellet was washed twice with ethanol and the washings were combined with the ethanol supernatant solution. Finallly, the glutelins were extracted by incubating the pellet with 0.1 N NaOH for 30 min at 40°C. The supernatant was recovered by centrifugation and the washings were combined with the supernatant. The pellet remaining after extraction of the major protein fractions was digested in 6 N HCl at 115°C and 15 lbs pressure for 12 h. Protein in each fraction was determined colorimetrically using the BioRad protein dye reagent with bovine serum albumin as the standard. To determine radioactivity in each fraction, 50 µl aliquots were mixed with 10 ml of scintillation cocktail. The samples were then counted in a Beckman LS-250 scintillation counter.

F. Hydrolysis of Endosperm Proteins

1. Enzymatic hydrolysis

To recover glutamine in the protein digests, a modified method of Winkler and Schön (1979) was used for the enzymatic hydrolysis of proteins.

a. Preincubation: Fifty to one hundred mg of endosperm powder was pretreated for 1 h at 40°C with 0.1 N NaOH (1.8 ml) plus 15% SDS (0.4 ml), stirring continuously. After this preincubation, the pH of the premix was adjusted to 8.3 using 2 N HCl and 2.0 ml of sodium borate buffer (pH 8.3) was added.

b. Dialysis of pronase: One hundred mg of enzyme was dissolved in 1.0 ml of 0.1 M tris-buffer (pH 7.0). This enzyme solution was dialyzed for 40 h against 3-4 litres of 0.1 mM tris buffer at 40°C.

c. Incubation: To the buffered premix, 20 mg of dialyzed pronase and 2.5 mg of carboxypeptidase A were added. Ethanol at a final concentration of a 10% and 20 µl of chloroform were also added to avoid microbial contamination during incubation period. The mixture was then incubated at 40°C for 48 h in a shaking water bath. At the end of the experiment, the samples were spun for 10 min at top speed in a clinical centrifuge. The supernatant and the residue were stored frozen at -20°C.

G. Amino Acid Analysis

1. Preparation of samples

The soluble fraction containing amino acids, sugars and organic acids was first separated by use of ion-exchange resins (Dowex-50 (H^+), 1 x 5 cm). Elution of these columns with 50 ml of water removed sugars and organic acids. Amino acids were then eluted with 50 ml of 2 M NH_4OH. Each fraction was taken to dryness under vacuum at 40°C and made up to a known volume. Radioactivity in each fraction was assessed as described previously. A reverse-phase high performance liquid chromatography column was used to separate glutamine, glutamate, asparagine and aspartate. The other amino acids were collected in bulk.

2. HPLC-methodology

a. Equipment: A model 332 gradient liquid chromatography (Beckman, Altex Scientific Inc., Berkeley, Ca.), a model 334 dual pump solvent delivery system with a model 210 sample injection valve,

equipped with a 20 µl sample loop were employed in this study. Two Altex model 110 A pumps and Altex model 421 microprocessor were used for generation of elution gradients. Chromatographic separations were performed using an ultrasphere ODS (particle size 5 µm) packed reverse-phase column (250 x 4.6 mm). The absorbance of the column eluants was monitored with a Gilson model spectra/GLO fluorometer with excitation filter of 360 nm. The emission filter peaked at 455 nm. The chromatographic peak areas were integrated by an Altex C-IRA recording data processor. All chemicals were of HPLC grade and solvents were degassed and filtered through 0.45 µm Millipore filters before use.

b. Derivatization: The derivatization was performed according to Lindroth and Mopper (1979). Twenty microlitres of amino acid standard (1.0 mM each) or sample were reacted with 100 µl of o-phthaldialdehyde (OPA) reagent at room temperature. Samples were then spun for 2 min in a Brinkman Eppendorf centrifuge. Seventy-five microlitres of reacted sample were injected using a 25 µl micro-syringe or else a model 500 automatic sampler (Beckman).

Preparation of derivatizing solution: Fifty-four mg of OPA were dissolved in 1.0 ml of pure methanol. To this solution, 9 ml of borate buffer (400 mM, pH 9.5) and 0.2 ml of 2-mercapto-ethanol were added. The solution was allowed to stand in the dark for half an hour before use.

c. Chromatographic procedure: OPA derivatives were eluted from the column by isocratic elution as follows: solvent was a 20 mM sodium phosphate buffer (pH 6.8) : methanol (70:30), pumped

at a flow rate of 0.8 ml/min. In the amino acid analysis where a gradient was employed, the concentrations were as follows: 0.02 M phosphate buffer (pH 6.8) + methanol gradient starting at 10% methanol and increasing to 33% over a 20 min period following by an isocratic step at 33% for 20 min duration. This was followed by increasing the methanol to 50% over a 10 min period. An isocratic step with 50% methanol was maintained for 10 min. At the end of the run, initial conditions were restored by running a reversed methanol gradient at a rate of 2%/min so that final concentration of methanol was 10%. This gradient worked well for the separation of acidic amino acids and their amides. For radioactive assays, the column eluants were collected, taken to dryness in scintillation vials and after addition of 10 ml of scintillation fluid, counted in a liquid scintillation counter.

For the complete separation of the amino acid mixture, sodium acetate buffer and methanol were used in the following manner: solvent A: 0.05 M Na-acetate buffer pH 5.9 + tetrahydrofuran + methanol (80:1:19, v/v/v), solvent B: 0.05 M sodium acetate buffer pH 5.9 + methanol (40:60, v/v). The solvent was pumped at a flow rate of 0.8 ml/min and the gradient program was as follows: 100% A for the initiation of the program, a linear step to give 25% B in 3 min, an isocratic step at 25% B for 10 min, a linear step to 80% B in 20 min, an isocratic step at 80% B for 15 min, a linear step to 100% B in 15 min, and an isocratic step at 100% B for 5 min. At the end of the experiment, initial conditions were restored by running a reverse methanol gradient at a rate of 2.4%/min.

III. Analytical Determinations

A. Total Nitrogen and Zein Protein Nitrogen

For quantitative extraction of zein, the method of Dalby (1974) was adopted. One hundred mg of endosperm powder and 0.4 ml of 70% ethanol were heated in a sealed tube at 60°C for 90 min with constant shaking. The tubes were centrifuged for 3 min at top speed in a clinical centrifuge, shaken on a vortex-mixer and recentrifuged for 5 min. One hundred microlitres of supernatant solution was transferred to Whatman No. 3 filter paper discs. To remove non-zein impurities, discs were washed in bulk through a series of solvents as follows: 5% NaCl for 15 min (2 times), distilled water, 15 min (1 time), anhydrous ethyl ether for 10 min (1 time), cold acetone for 10 min (1 time), room temperature acetone for 10 min (1 time), anhydrous ethyl ether for 10 min (2 times). The discs were air dried after the last step and were hydrolyzed in concentrated sulfuric acid. For total nitrogen lyophilized-endosperm powder was digested with concentrated sulfuric acid. In each case, the resulting NH_4^+ was measured by Nesslerization (Ballantine, 1957).

B. Free Amino Acid Content of Endosperm

Frozen endosperm powder was ground in 2 volumes of HEPES buffer (0.05 M pH 7.5). After filtration through one layer of Miracloth, crude extracts were obtained by centrifugation at 12,000 g for 20 min. To the supernatant an equal volume of HPLC grade methanol was added. After 20 min on ice, the precipitate was removed by centrifugation. An appropriate aliquot of the supernatant

was used for quantitative analysis of the free amino acid pool of the endosperm by reverse-phase high performance liquid chromatography. Some samples were also assayed with a Beckman (model 119 BL) amino acid analyzer by Dr. K.W. Joy of Carleton University, Ottawa.

C. NH_4^+

The crude homogenates prepared as described above were used for estimation of diffusible ammonia. After distillation in Conway dishes into 0.0143 N sulfuric acid for 12 h, the ammonia was assayed according to the modified method of Berthelot (Kaplan, 1965) or by Nesslerization (Ballantine, 1957).

D. Enzyme protein

Enzyme protein was measured by the method of Lowry *et al.* (1951) and for this test, bovine serum albumin (BSA, fraction V, Sigma) was used as the standard. In later experiments, the BioRad protein dye reagent method was employed (Bradford, 1976).

RESULTS

I. Characterization of the Enzymes of Nitrogen Assimilation

The method for extraction and assay of asparaginase activity was developed during the course of the present study. The properties of asparaginase from corn endosperm were characterized. The extraction and assay for GOGAT and GDH were adapted from literature (Wallace, 1973; Oaks *et al.*, 1979). The conditions for maximum enzyme activities in corn endosperm were optimized. In addition to NADH-dependent activity, a ferredoxin-dependent GOGAT activity was identified in endosperm tissue and the physical and kinetic properties of both enzymes were characterized in detail. The optimum conditions for corn endosperm GDH activity were worked out by Karen Jones. Additional information obtained in this investigation dealt with the effect of Ca^{2+} on enzyme activity. Results showed that GDH from corn endosperm was not responsive to Ca^{2+} (0-7.5 $mM \cdot assay^{-1}$) and as a result it was omitted from the assay mixture. Both the extraction buffer for asparagine synthetase and the assay conditions were as modified by Ross (1979).

The glutamine synthetase assay was partially adapted from O'Neal and Joy (1973). The antigenic properties of the enzyme from endosperm and embryo were studied. Antibodies raised against the cytosolic form of GS (GS_1) from etiolated barley leaves were supplied

by Dr. B. Hirel. Glutaminase activity was assayed according to a radioactive assay (Prusinger and Milner, 1970), a coupled assay involving glutaminase and glutamate decarboxylase (Chiu and Boecker, 1979) and by conventional methods, i.e., measuring production of ammonia by Nesslerization and glutamate by paper chromatography. None of these methods gave positive results for glutaminase activity in the endosperm tissue. In the experiments described in this section, I will concentrate on asparaginase, glutamine synthetase and glutamate synthase, those reactions which have been examined in detail in this study.

A. Asparaginase (Asnase)

1. Developing an assay for asparaginase

Asparaginase activity can be measured by determining the production of ammonia or aspartic acid (Wriston and Yellin, 1973). In my assay method, I have taken advantage of the fact that aspartate, a product of the asparaginase reaction, can be transaminated in the presence of α-ketoglutarate to yield oxaloacetate and glutamate. The oxaloacetate is then reduced to malate in the presence of NADH and malate-dehydrogenase (MDH) according to the following reaction:

$$\text{L-asn} + H_2O \xrightarrow[\text{Asnase}]{} \text{L-asp} + NH_3 \qquad (1)$$

$$\text{L-asp} + \alpha\text{-ketoglutarate} \xrightarrow[\text{GOT}]{} \text{L-glu} + \text{OAA} \qquad (2)$$

$$\text{OAA} + \text{NADH} + H^+ \xrightarrow[\text{MDH}]{} \text{malate} + NAD^+ \qquad (3)$$

The total decrease in concentration of NADH followed at 340 nm should

equal the aspartate concentration of the reaction mixture if there are no interfering reactions. A calibration curve was prepared using various concentrations of aspartic acid. Fig. 4 shows that there was a linear relationship between NADH oxidized and the amount of aspartate added to the reaction mixture (0-100 nmoles·assay^{-1}).

Table 4 illustrates the requirements for the assay of asparaginase in extracts prepared from maize endosperm (var. W64A). The reaction required asparagine and endosperm extract in the preincubation mixture. The endosperm extracts had been passed through Sephadex G-75 to remove endogenous aspartate. In the coupled reaction glutamate-oxaloacetate transaminase (GOT), malate dehydrogenase (MDH), α-ketoglutarate, NADH and aliquots from the "complete" preincubation mixture were required. The addition of α-aminooxyacetate (AOA), a transaminase inhibitor (Brannstein, 1973), to the coupled reaction assay completely inhibited the oxidation of NADH, suggesting that there was not interference from GDH in the reaction. Also, the addition of α-ketoglutarate and NADH alone did not support the oxidation of NADH.

a. Linearity of the assay: With increasing time of incubation, there was an increase in the amount of NADH oxidized in the coupled reaction (Fig. 5). The amount of aspartate formed was calculated from the standard curve of NADH oxidized *vs.* aspartate concentration. The reaction was linear with time for at least 90 min (Fig. 6). The reaction was also linear with enzyme concentration (Fig. 7).

b. Identification of the reaction products: The production of aspartate was confirmed by descending paper chromatography in

Figure 4. Calibration curve for asparaginase

Varying concentrations of aspartate (10-100 nmoles) were assayed quantitatively by measuring a coupled transaminase and MDH reaction.

The total oxidation of NADH was followed at 340 nm. Based on values for nmoles of NADH oxidized, the recovery of aspartate varied between 90%-98%.

The reaction mixture contained 0.1 ml NADH (0.16 mM), 0.1 ml α-ketoglutarate (1 mM), MDH (1.27 µg protein) and GOT (20 µg protein) in a total volume of 1 ml. Values shown are the average of two separate experiments ± range (bars).

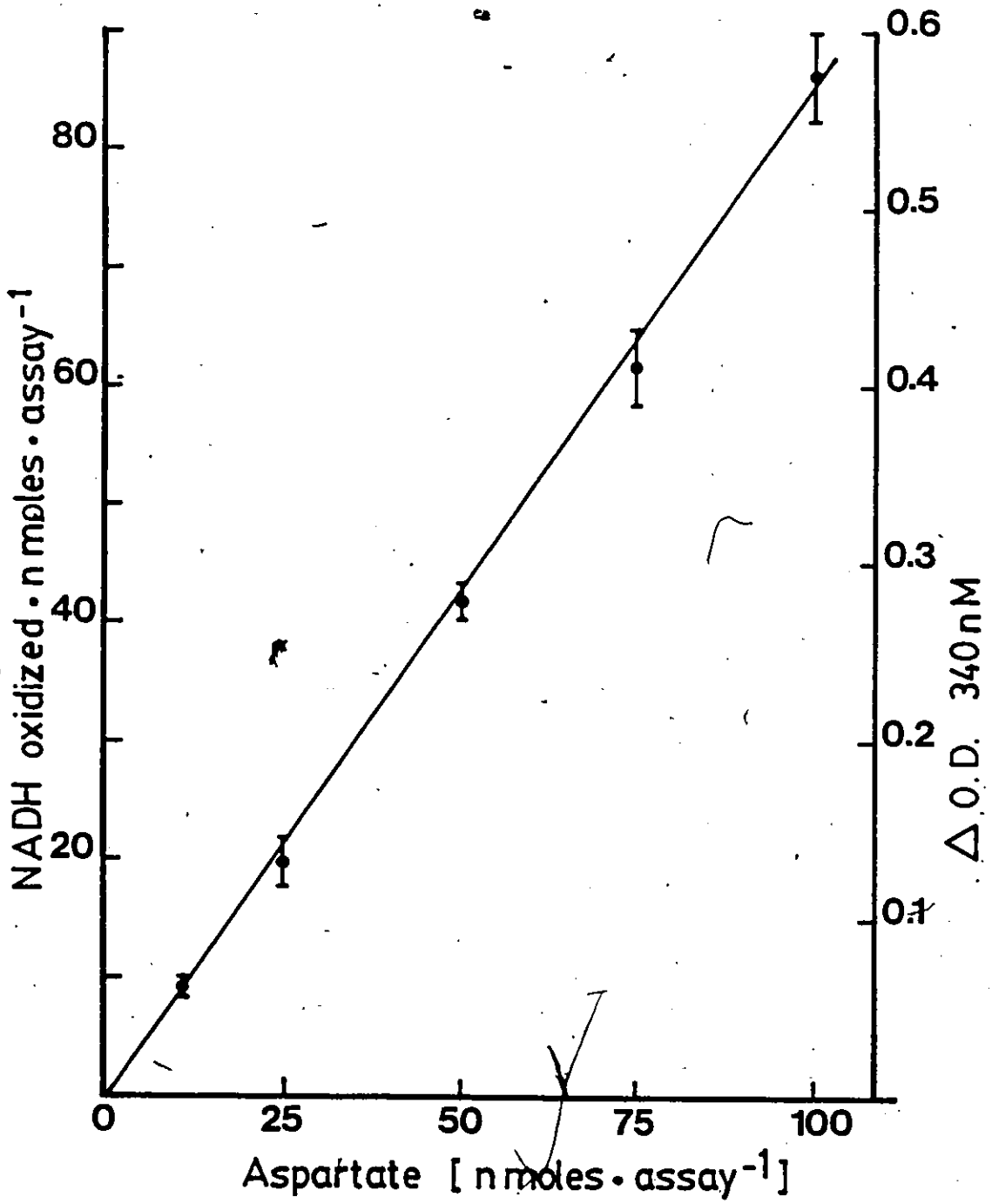

NADH oxidized • n moles • assay⁻¹
80
60
40
20
0.6
0.5
0.4
0.3
0.2
0.1
Δ O.D. 340 nM
0
25
50
75
100
Aspartate [n moles • assay⁻¹]

Table 4

Requirements for the Assay of Asparaginase in Extracts Prepared from Maize Endosperm (W64A) 20 Days Post-Pollination

Assay conditions	Activity nmoles NADH·assay^{-1}
a. Preincubation mixture	
complete-zero time	5.00
- complete	116.00
- KCl	46.40
- Enzyme extract	0.80
- Asn	0.20
b. Coupled reaction	
- complete + AOA (1 mM)	no activity
- transaminase - MDH	no activity
- Asn + α -kg + NADH	0.2

a. The complete preincubation mixture contained 100 mM Asn, 20 mM KCl, 25 mM Na HEPES (pH 8.0) enzyme 0.2 ml in a total volume of 0.8 ml, incubated for 60 min before addition of GOT and MDH.

b. The coupled reaction contained supernatant from pre-incubation mixture (see Methods). 0.1 ml of NADH (0.16 mM) 0.1 ml α-ketoglutarate (1 mM) MDH (1.27 µg protein/assay) and GOT (20 µg protein/assay).

Figure 5. Oxidation of NADH measured at 340 nm with increasing time of preincubation of endosperm extracts with asparagine.

In blank samples, asparagine or enzyme was omitted from the reaction mixture.

The concentration of asparagine was 100 mM and 0.2 ml of Sephadex G-75 filtered endosperm extract (var. W64A) was used.

The coupled reaction contained 0.1 ml NADH (0.16 mM), 0.1 ml α-ketoglutarate (1 mM), GOT (20 µg protein), MDH (1.27 µg protein) and an appropriate aliquot of the supernatant in a total volume of 1.0 ml.

(0,25,50,55 - time of preincubation in minutes.)

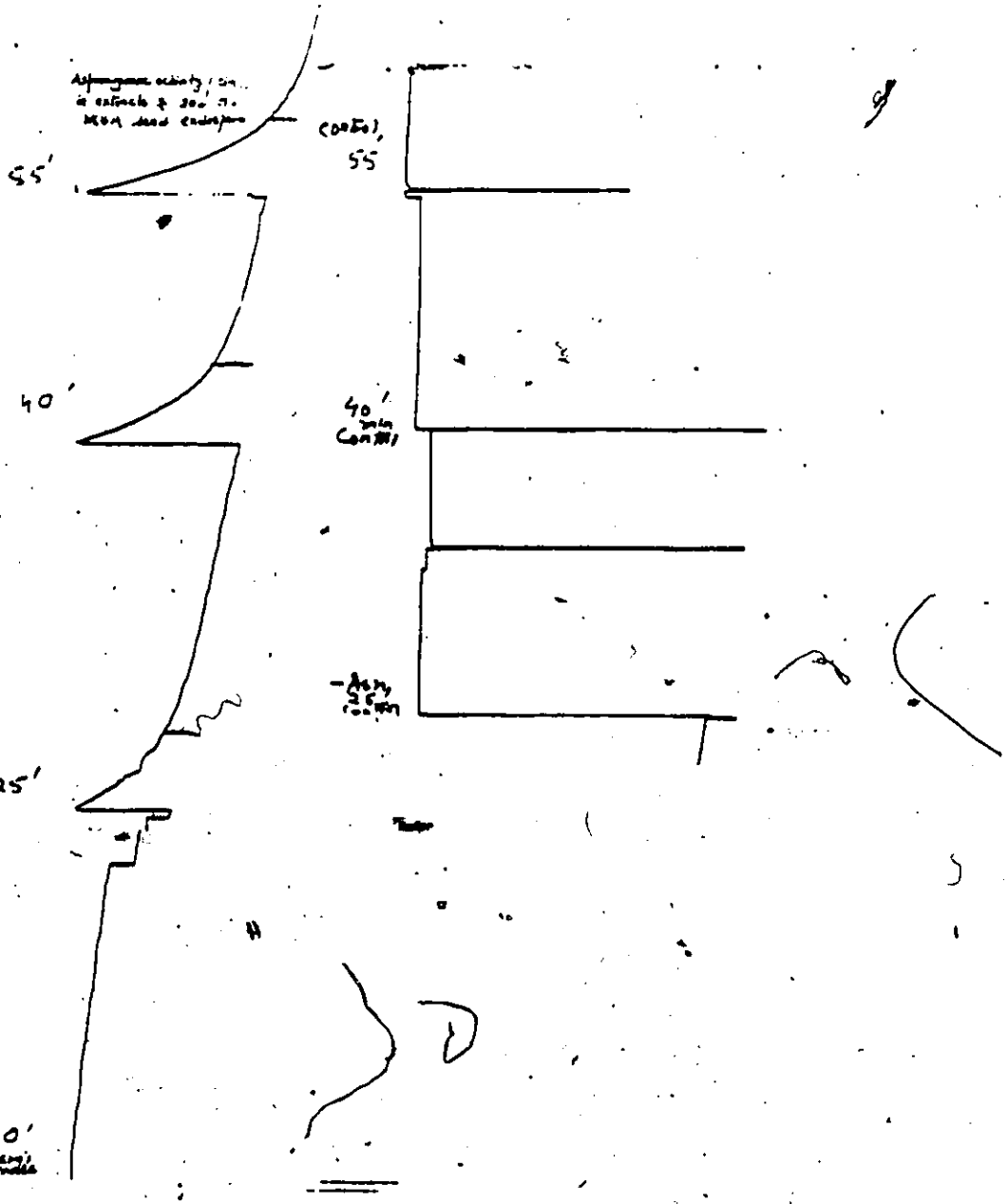

55'
40'
25'
0'
(control)
55
40'
min
control

Figure 6. The effect of increasing time of preincubation on the activity of asparaginase isolated from 20-day old sample (var. W64A).

The complete preincubation mixture contained 100 mM asparagine, 20 mM KCl, Na-HEPES (pH 8.0) and 0.2 ml of enzyme in a total volume of 0.8 ml. The coupled reaction was as described in Methods.

The mean ± range (bars) of two replicate assays is shown.

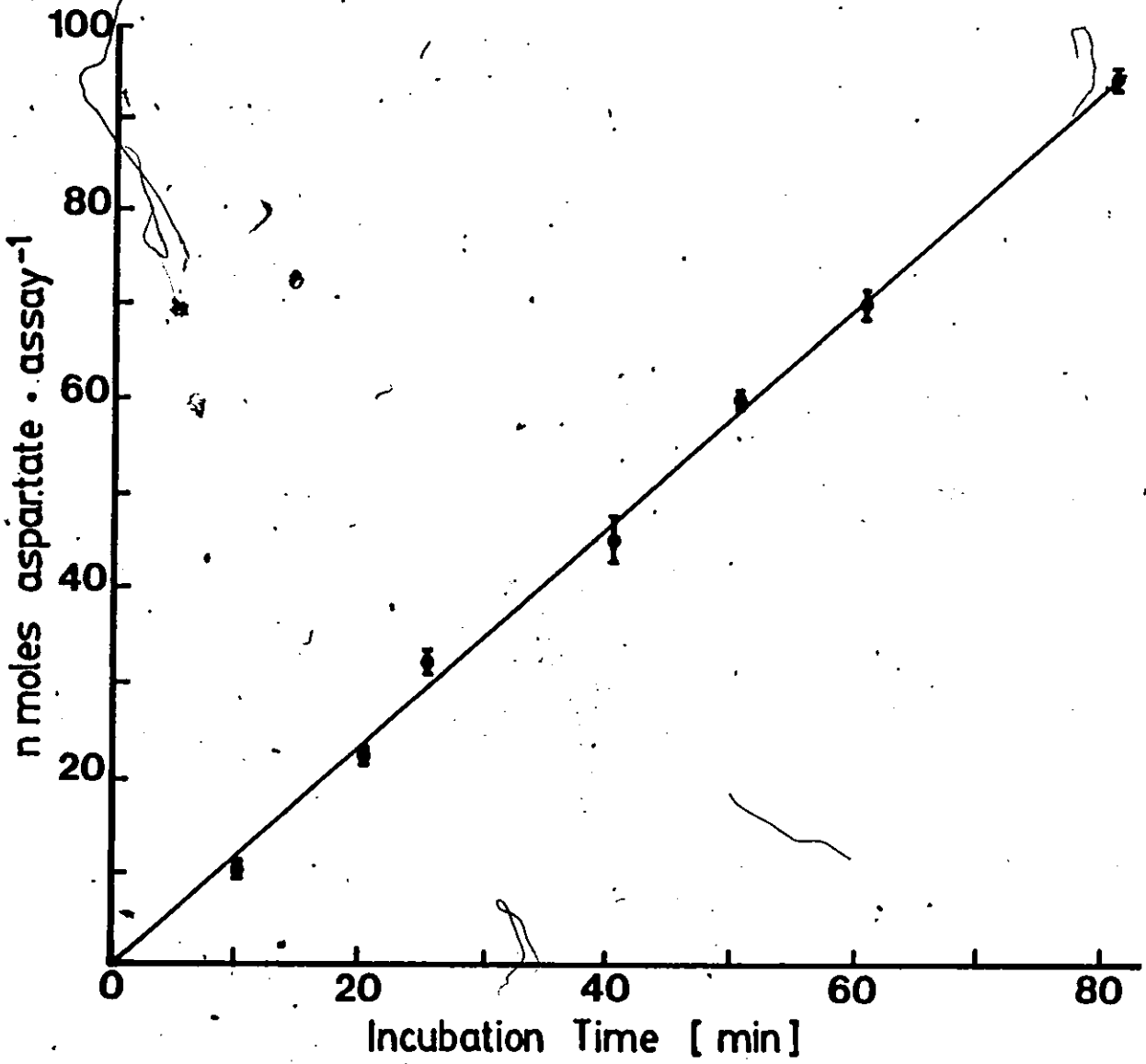
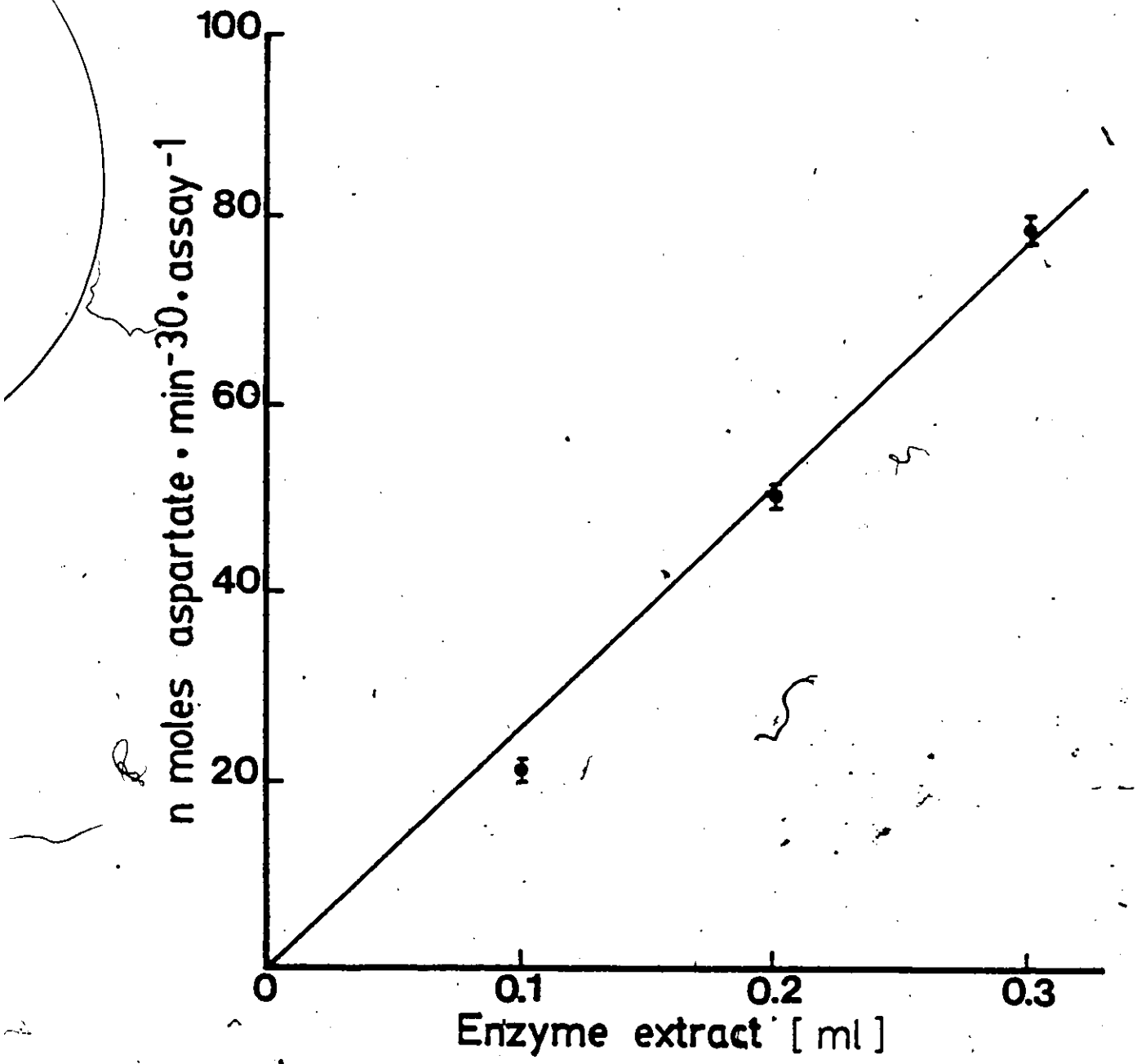

100
80
60
40
20
n moles aspartate · assay⁻¹
0
20
40
60
80
Incubation Time [min]

Figure 7. The effect of increasing the enzyme content on the activity of asparaginase.

Endosperm extracts were prepared from inbred maize (var. W64A), harvested 20 days after pollination. Time of preincubation was 30 min and the production of aspartate was measured as described in Methods.


100
80
60
40
20
n moles aspartate • min-30 • assay-1
0
0.1
0.2
0.3
Enzyme extract [ml]

butanol:acetic acid:water (12:3:1) or phenol:water solvent system (80:20). After chromatography of the reaction mixture minus GOT and MDH, a ninhydrin positive spot with an Rf similar to aspartate was detected (Fig. 8). The increasing colour intensity of the aspartate spot with increasing time of incubation suggests an increasing amount of aspartate produced during the reaction. No aspartate was detected when either enzyme or asparagine was omitted from the reaction.

c. Stoichiometry of the asparaginase reaction: Table 5 illustrates the quantitative correlation between NADH oxidation, aspartate and ammonia produced in the reaction. The levels of aspartate were quantitatively estimated with an amino acid analyzer (by Dr. K.W. Joy). There was a good correlation in the levels of aspartate formed whether aspartate levels were determined by the oxidation of NADH or by direct analysis. Ammonia, the other product of asparaginase activity was first distilled into dilute sulphuric acid in Conway diffusion dishes and then measured by phenol-hypochlorite reaction (Russel, 1944). The amount of ammonia also increased with time.

2. Properties of asparaginase from corn endosperm

a. Effect of KCl on enzyme activity: The asparaginase activity in corn endosperm extracts was stimulated by K^+ ions and hence is similar to the asparaginase system described for legume cotyledons (Sodek *et al.*, 1978; Lea *et al.*, 1978). Fig. 9 shows the effect of various concentrations of the KCl added to the assay media. In "minus-KCl" samples, activity was 20 nmoles aspartate·min^{-50}. assay^{-1}. On addition of 6 mM KCl to the assay mixture, activity

Figure 8. Identification of aspartate.

The chromatogram shows asparagine and aspartate in the asparaginase reaction mixture after 20, 40, 60 and 80 min incubation period.

The amino acids were separated using descending paper chromatography in the butanol, acetic acid, and water solvent (12:3:5). Chromatogram was run for 18 hr, dried and run again in the same solvent for another 13 hr.

Table 5

Stoichiometry of the Asparaginase Reaction

Time of incubation (minutes)	NADH (nmoles/assay)	Asp* (nmoles/assay)	NH_4^+ (nmoles/assay)
0	10.68	21.6	17.2
20	46.8	45.6	54.0
40	83.6	87.6, 84.4	95.09
60	116.8	116.4, 114.8	124.58
80	143.6	162.0	155.44
80 (-asn)	0 - 0.8	0.08	not detectable

Complete reaction as described in Table 4.

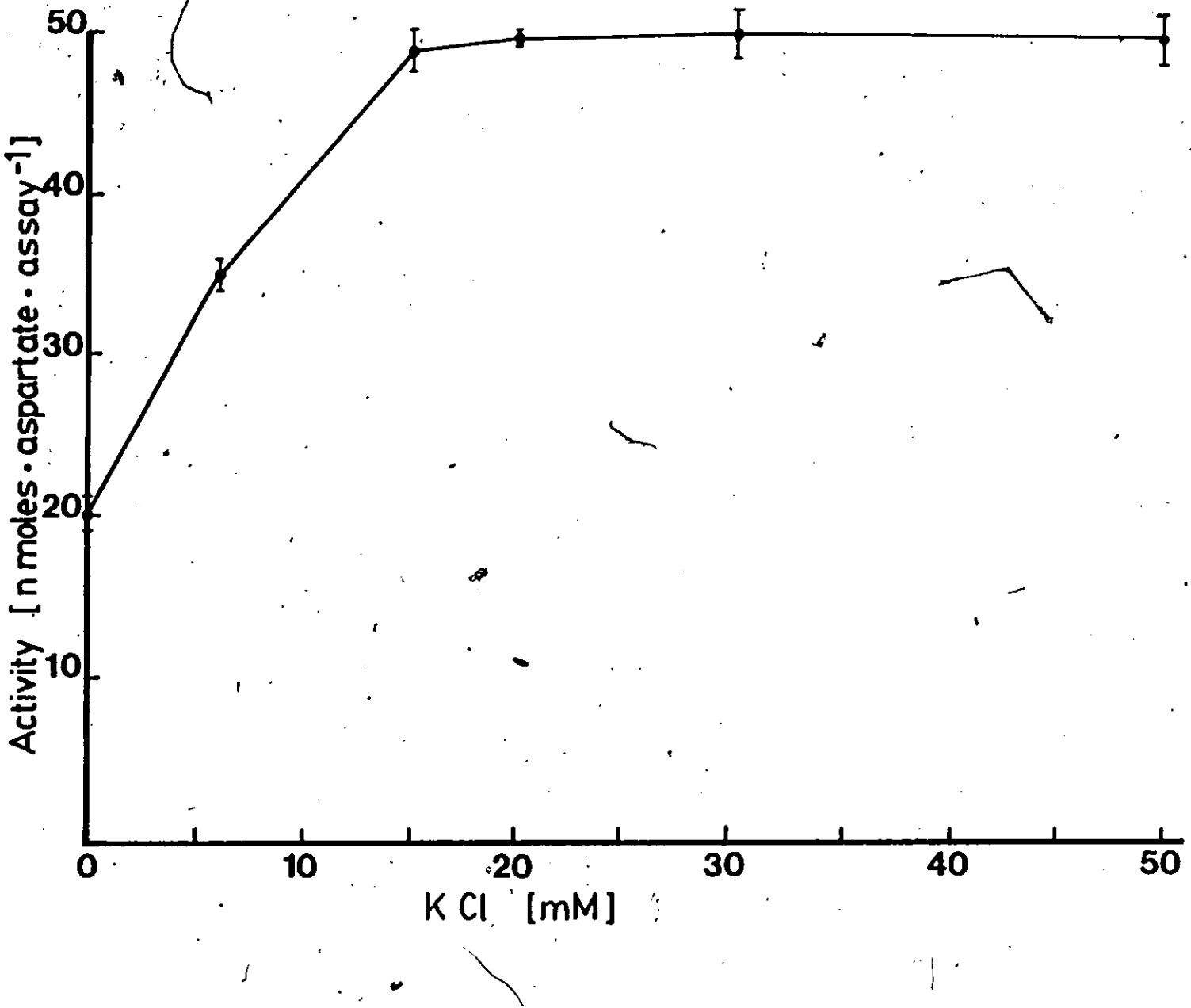
*Aspartate levels were determined by amino acid analyzer. At 60 min the nmoles of asp/endosperm were 349 and per mg protein were 290.

Figure 9. Effect of increasing concentration of potassium-chloride on asparaginase activity isolated from 20-day endosperm tissue.

Assay conditions were as described in Methods. KCl was omitted from the elution buffer.

The mean values ± range (bars) of two replicate assays are shown.

Time of preincubation was 50 minutes.


50
40
30
20
10
Activity [n moles • aspartate • assay^{-1}]
0
10
20
30
40
50
K Cl [mM]

increased to 35 nmoles aspartate·min^{-50}·assay^{-1}. The reaction was saturated at a concentration of 20 mM of KCl. Routinely, 20 mM KCl was added to the asparaginase assay preincubation mixture.

Sodek *et al.* (1980) have demonstrated that K^+ affects both the activity and the stability of the enzyme in solution. In the absence of KCl, the enzyme from developing cotyledons of pea was unstable in solution and almost 90% of the activity was lost after 10 min at 30°C, whereas, very little activity was lost in the presence of K^+ (50 mM). This stabilizing effect was shown to be separate from its activating effect. When assayed under conditions where the enzyme showed a reasonable degree of stability in the absence of K^+ (i.e., at 20°C for 20 min) the enzyme still required K^+ for maximal activity.

b. Substrate saturation kinetics: Fig. 10 shows the substrate saturation kinetics of asparaginase. There is an increase in activity with increasing concentration of asparagine. The reaction is not fully saturated even at a concentration of 100 mM asparagine.

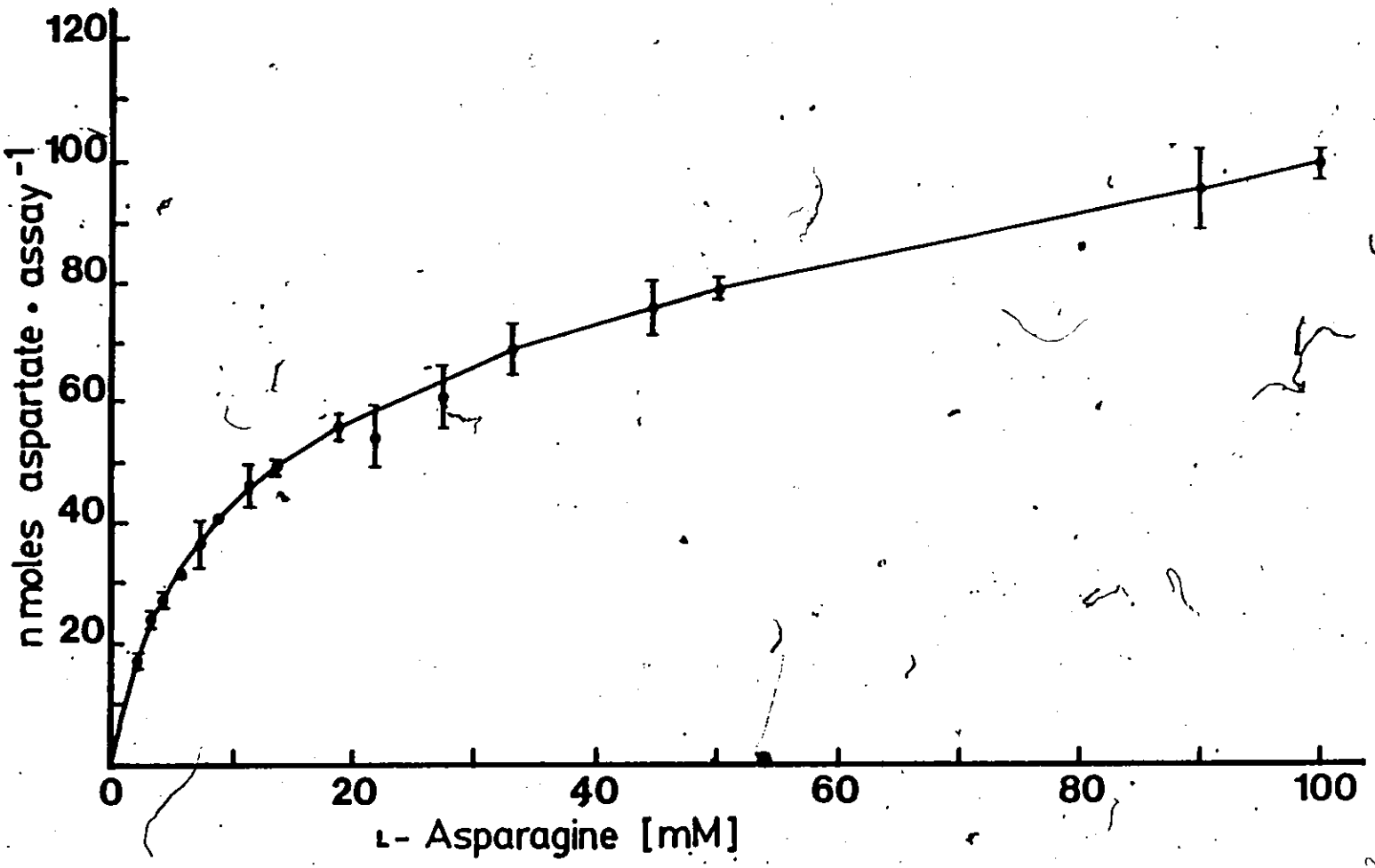
Routine assay for enzyme activity

Based on the above results, in all future assays, 100 mM asparagine, 20 mM KCl and 0.2 ml of Sephadex-treated enzyme were used per assay. Under optimum assay conditions, the maximum asparaginase activity in the developing corn endosperm was in the order of 5.6 nmol·min^{-1}·mg protein^{-1}. In bacteria and animal tissues where level of asparaginase can be quite high (50-500 nmol·min^{-1}·mg protein^{-1}) a direct spectrophotometric procedure has been employed (Cooney and

Figure 10. The effect of increasing concentrations of asparagine on asparaginase activity in corn endosperm extracts.

The enzyme extract was filtered through a Sephadex G-75 column before use. The preincubation mixture contained 20 mM KCl and 0.2 ml of enzyme extract in a total volume of 0.8 ml. The incubation period was 30 min. The coupled reaction was as described in Methods.

The average values ± range (bars) for replicate assays are shown for each point.


120
100
80
60
40
20
n moles aspartate • assay⁻¹
0
20
40
60
80
100
L- Asparagine [mM]

Handschumacher, 1968; Kojima and Wacker, 1969). However, due to relatively low levels of asparaginase activity in corn endosperm tissue, it was necessary to preincubate the asparagine with enzyme and then couple it to GOT and MDH systems. In routine assays, therefore, asparagine was first hydrolyzed by asparaginase to aspartic acid and ammonia during a preincubation of the endosperm extract with the substrate asparagine. The reaction was stopped by placing the tubes in boiling water bath for 5 min. The precipitated protein was removed by centrifugation and the supernatant was assayed for total aspartate by the coupled reaction.

The good stoichiometry of the reaction plus the lack of oxidation of NADH in the minus asparagine treatment show that there is no interference from GDH and other NADH-consuming reactions and that the method is, therefore, a reliable assay for asparaginase. The method can also be adapted for measurement of asparagine and aspartate levels in the tissues.

ç. Km value for asparagine: The Km value for asparagine was determined using the Sephadex-filtered enzyme. Enzyme activity was assayed using several concentrations of asparagine and Km values were determined from Lineweaver-Burk plots. The line of best fit was then calculated. A typical Lineweaver-Burk plot is shown in Fig. 11. The Km value obtained for asparagine was 8.93 mM. Km values for asparagine show a wide range depending on the plant origin of asparaginase, e.g., pea seed (3 mM) (Sodek *et al.*, 1980; Ireland and Joy, 1981), *Lupinus sp.* (12.2 mM) (Atkins *et al.*, 1975; Lea *et al.*, 1978).

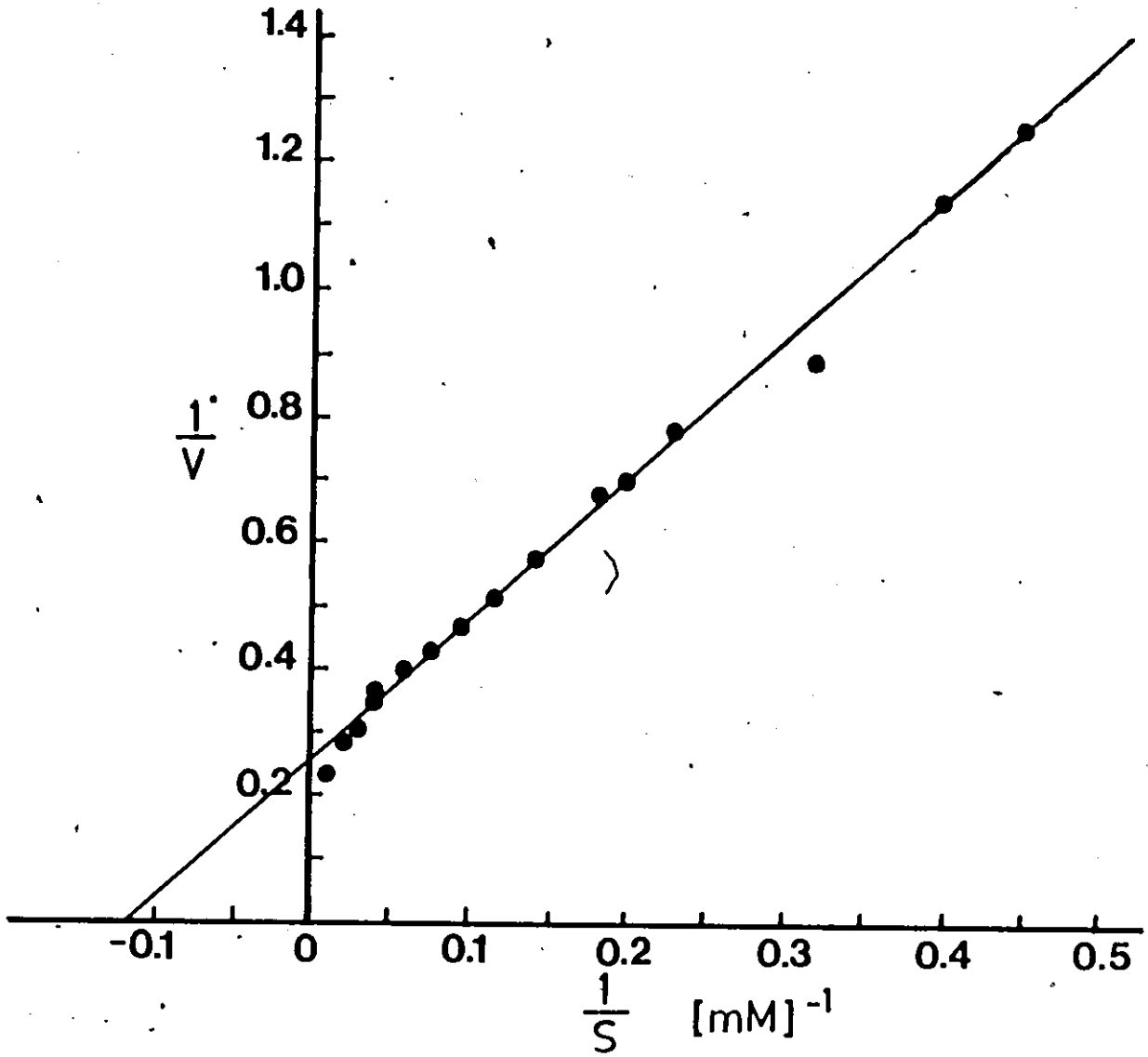
Figure 11. Lineweaver-Burk plot for asparaginase reaction in corn endosperm.

*(nmoles Asp·min^{-30}·assay^{-1}).

The substrate varied in assay was asparagine. The equation for line of best fit is

$$\frac{1}{V} = 0.1086 \left(\frac{1}{S}\right) + 0.0122$$

$$Km = 8.93\ \mu M$$


1.4
1.2
1.0
0.8
0.6
0.4
0.2
1/V
-0.1
0
0.1
0.2
0.3
0.4
0.5
1/S [mM]⁻¹

B. Glutamate Synthase (GOGAT)

1. Effect of Sephadex-filtration on NADH-GOGAT activity

Since the method for assaying GOGAT involved oxidation of NADH, preliminary experiments were performed to make sure that glutamine-dependent oxidation of NADH was due specifically to the glutamate synthase activity. Crude extracts prepared from developing endosperms showed a high rate of glutamine-independent oxidation of NADH (Table 6). The substrate independent oxidation was lost after treatment with Sephadex. This indicated the presence of some interfering reactions in crude extracts. One possibility is that aspartic acid is present in crude extracts. As a result, the α-ketoglutarate and NADH required for GOGAT are used instead of a glutamate-oxaloacetate transaminase and malate dehydrogenase reactions.

Addition of α-aminooxyacetate, a specific inhibitor of transaminase (Brannstein, 1973) would inhibit a transaminase-dependent NADH oxidation but not that catalyzed by NADH-GOGAT. Addition of 1 mM α-aminooxyacetate (AOA) to the assay mixture inhibited the activity in the crude extract by 73%. After Sephadex filtration, however, the AOA no longer had an effect on the GOGAT reaction showing that substrates for the transaminase reaction had been removed. It is interesting to note that activity seen in crude extracts plus AOA equals the value obtained for GOGAT-dependent NADH oxidation in gel-filtered extracts.

2. Characteristics of Sephadex-treated NADH-GOGAT

In Sephadex-treated extracts glutamate synthase activity was strictly dependent on L-glutamine as the N donor (Table 6).

Table 6

Effect of Sephadex-filtration on Activity of NADH-Glutamate Synthase Isolated from 20 day Post-Pollination Endosperm of Hybrid Maize (var. W64A x W182E)

Reaction mixture	Activity nmoles NADH·min^{-1}· endosperm^{-1}	
	crude extract	Sephadex-filtered extract
A. Complete system*	71.9	15.9
-Gln	52.4	0.9
-α-Kg	8.0	0.64
-α-Kg-Gln	8.2	0.65
-Enzyme	6.0	0.60
Complete system + AOA	15.2	15.0
B.-Gln + NH_4^+ (20 mM)	-	2.30
-Gln + NH_4^+ (40 mM)	-	1.39
-Gln + Glu (40 mM)	-	1.10
-Gln + Asn (20 mM)	-	0.98

* Complete system is as described in Methods.

20 mM HEPES (pH 7.5), 20 mM glutamine, 15 mM KCl, 1 mM α-keto-glutarate and 0.16 mM NADH in a total volume of 1.0 ml.

Addition of $(NH_4)_2SO_4$ (20 mM/assay^{-1}) at a concentration equivalent to that of glutamine used for assay of GOGAT gave values close to blank samples showing that NADH oxidation was not caused by a GDH reaction which could use ammonia released by glutaminase. With 40 mM $(NH_4)_2SO_4$ slightly higher rates of oxidation of NADH are obtained. Maximum activities for GDH endosperm tissue are seen with 0.8 M $(NH_4)_2SO_4$. Asparagine or glutamate did not replace glutamine in the reaction.

a. Linearity of the assay: Fig. 12 shows that with 0.1 ml of enzyme per assay, the reaction was linear for at least 13 min as measured by oxidation of NADH at 340 nm. At this time, the change in absorbance was 0.44 units. The activity was also linear in the range of 0.05-0.15 ml of Sephadex-treated extract per assay (Fig. 13). In all subsequent assays, 0.1 ml of enzyme was used. The reaction was recorded for the initial 5 min and from this the rate of oxidation of NADH was calculated.

b. Stoichiometry of the NADH-GOGAT reaction:

L-glutamine + NADH + α-ketoglutarate → 2-glutamate + NAD^+

The stoichiometry of the NADH oxidized and glutamate produced in the GOGAT reaction was examined. The amount of NADH oxidized in the enzyme reaction was measured by following the change in absorbance at 340 nm. For quantitative analysis of glutamate, a reverse-phase high performance liquid chromatography (HPLC) procedure was employed.

Fig. 14 shows the elution pattern of o-phthaldialdehyde (OPA) derivatives of standard glutamate and glutamine. Figs. 14b, 14c

Figure 12. Effect of increasing time of incubation on NADH-GOGAT activity.

Sephadex G-75 filtered extracts of hybrid maize (var. W64A x W182E) harvested at 20 days after pollination were used.

Enzyme was assayed using 0.15 ml of extract, 20 mM glutamine, 1 mM α-ketoglutarate, 0.16 mM NADH, and 15 mM KCl in a total volume of 1.0 ml.

Activity was measured by following the oxidation of NADH at 340 nm.

The mean ± range (bars) of two replicate assays is shown for each point.

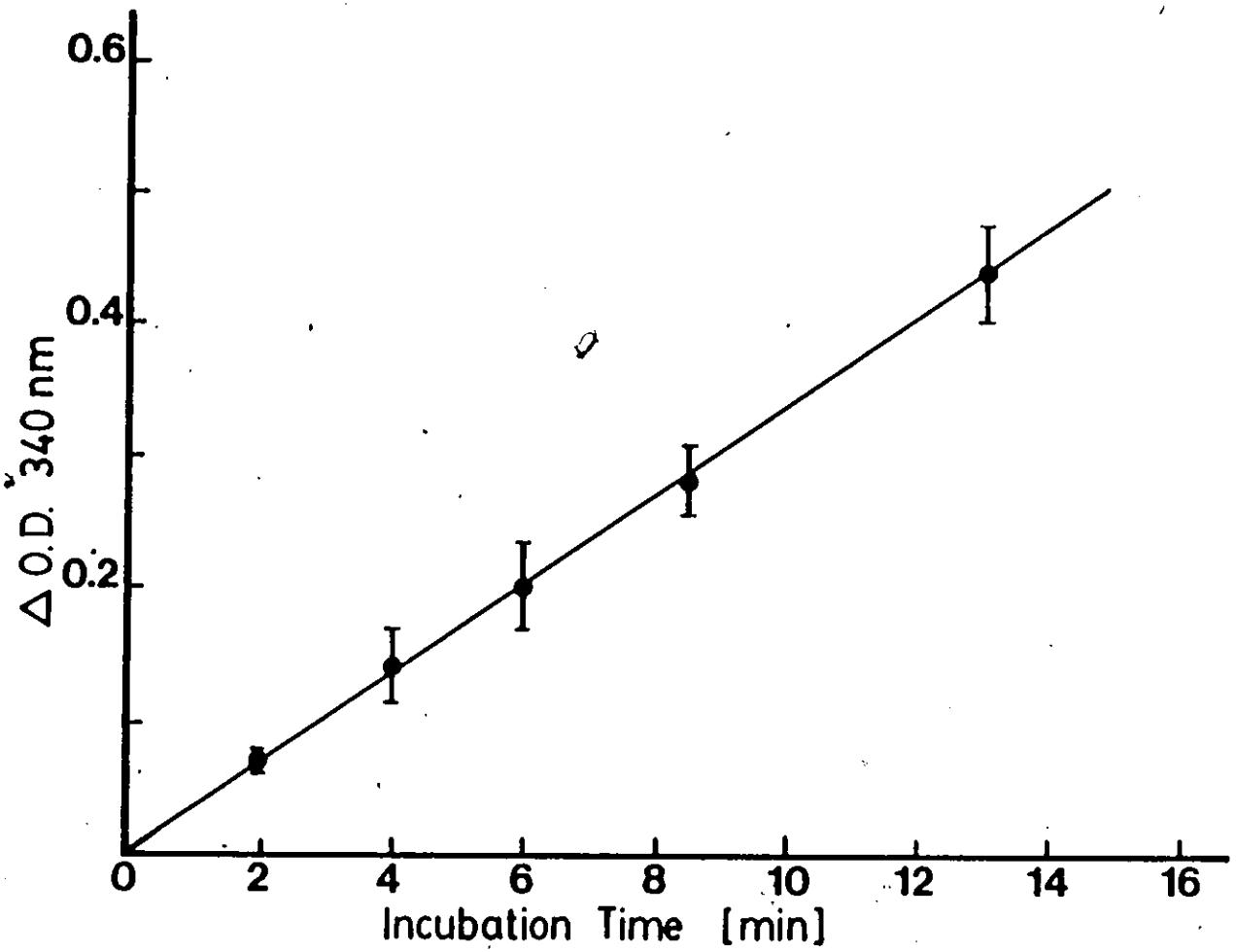

0.6
0.4
0.2
Δ O.D. 340 nm
0
2
4
6
8
10
12
14
16
Incubation Time [min]

Figure 13. Effect of enzyme concentration on activity of NADH-glutamate synthase.

Activity was measured by following oxidation of NADH at 340 nm. Extracts were prepared from 20-day endosperm tissue of hybrid maize (var. W64A x W182E).

Enzyme was assayed using routine concentrations of substrates (see Methods).

The mean ± range (bars) of two replicate assays is shown for each point.

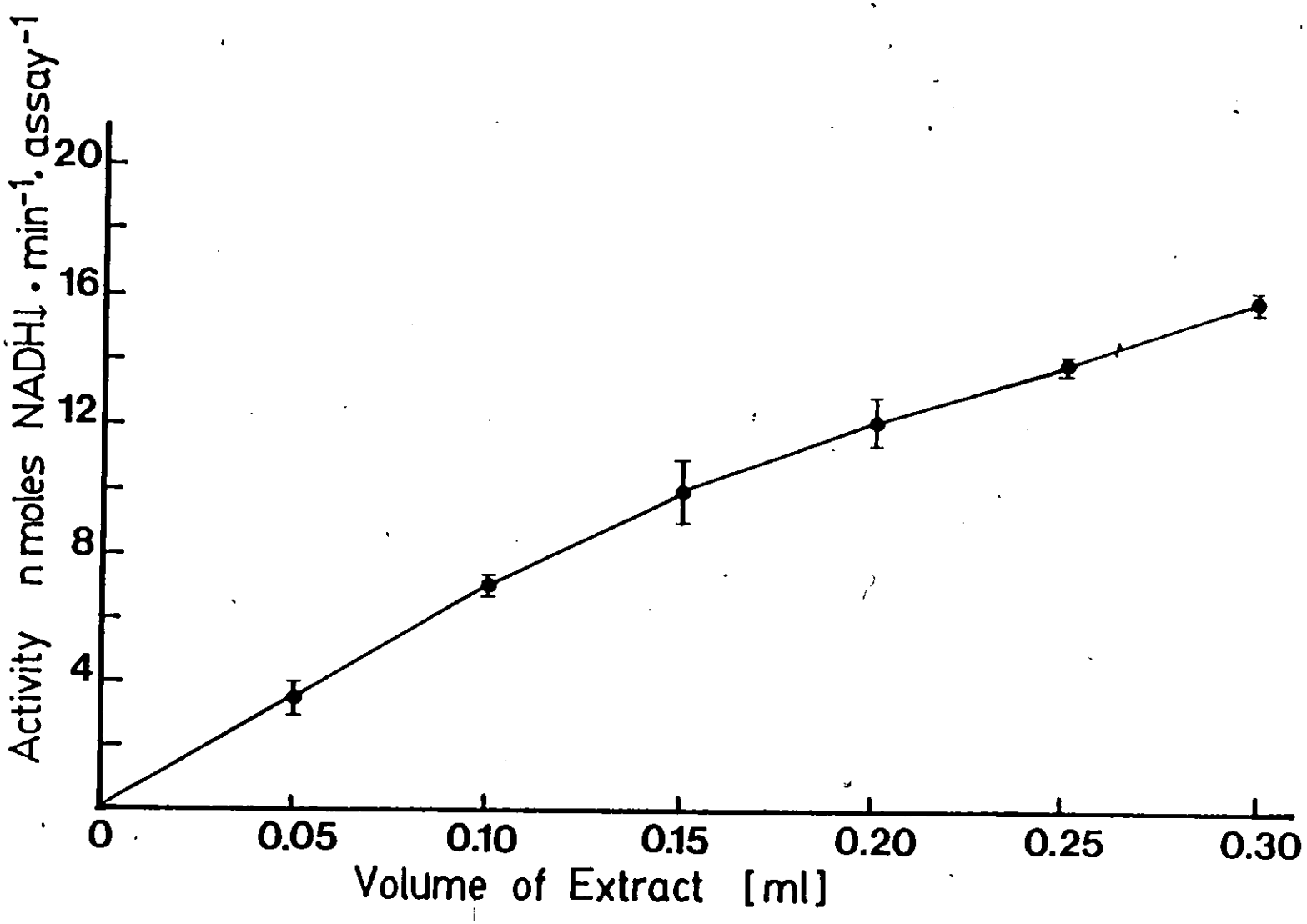

Activity n moles NADH. $\cdot min^{-1} \cdot assay^{-1}$
20
16
12
8
4
0
0.05
0.10
0.15
0.20
0.25
0.30
Volume of Extract [ml]

Figure 14 (a-c).: Elution profile of amino acids on reverse-phase high performance liquid chromatography (HPLC) column.

Chromatograms of o-phthaldialdehyde derivatives of glutamic acid and glutamine.

a. Standards; glutamate and glutamine.

b. Reaction mixture of NADH-GOGAT.

c. Reaction mixture of Fd-GOGAT.

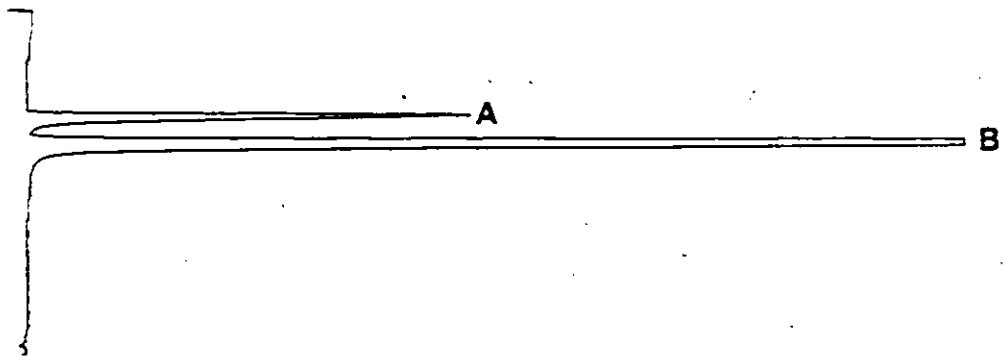
Derivatives were eluted from the reverse-phase column by using a 20 mM sodium phosphate buffer (pH 6.8):methanol (67:33) solvent. The flow rate was 0.8 ml/min.

A = glutamate

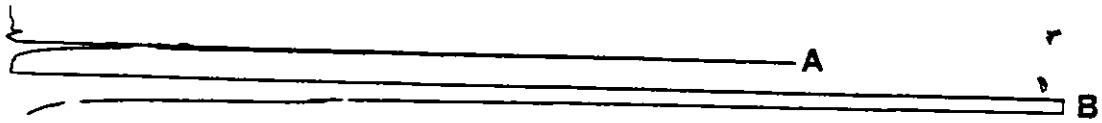
G = glutamine

X = tris buffer

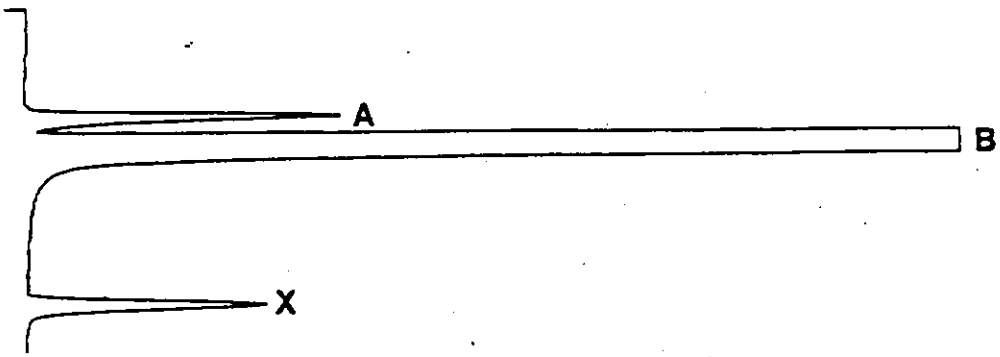
a



b



c



show the elution pattern of OPA derivatives of reaction products. Two major peaks corresponding to glutamic acid and glutamine were obtained in the reaction mixture. Glutamic acid eluted at 5 min and glutamine at 15 min, after injection of derivatized samples (the elution conditions are described in the Figure legends). In assays which include ferredoxin instead of NADH, an additional peak eluting at 23 min was found and it corresponded to tris-buffer. The amount of glutamate produced in the reaction mixture was calculated from the corresponding values of the peak area obtained with known concentrations of glutamate.

Table 7 illustrates the stoichiometry of the NADH oxidation and glutamate formation in GOGAT reaction. A ratio of 2.1, 1.75 and 2.08 (Glu/NADH) was obtained in three separate readings. A stoichiometry of 2.0 is predicted from the reaction equation (see p. 64). Thus, NADH oxidation is the result of glutamate synthase.

c. Effect of inhibitors on NADH-GOGAT reaction

1. End product inhibition

Enzyme reactions are often inhibited by their end products. The effect of end products of glutamate synthase on enzyme activity is shown in Table 8. Increasing the concentration of glutamate from 5 to 10 mM resulted in slightly higher inhibition of enzyme activity (from 11% to 21% inhibition of enzyme activity relative to the control).

NAD^+, a product of NADH oxidation, also caused a minor inhibition of the reaction. These results show that the end products of glutamate synthase reaction do not regulate the enzyme activity rigorously in corn endosperm.

Table 7

Stoichiometry of NADH Oxidized and Glutamate Produced in GOGAT Reaction

Assay time (min)	NADH (nmoles/assay)	Glutamate (nmoles/assay)	Ratio Glu/NADH
10	37	80	2.1
20	80	140	1.75
30	120	250	2.08

NADH oxidation was monitored at 340 nm using a spectrophotometer and glutamate was assayed by reverse phase high performance chromatography using a glutamate standard. Assay conditions were as described in Methods. The extracts were filtered through Sephadex G-75 column.

Table 8

Effect of Potential End Products and Other Inhibitors on the Glutamate Synthase Activity in Endosperm Preparations

Treatment	Activity relative to control
Control	100.0 (20.3)
+ NADH + NAD (0.16 mM)	90.0
+ Glu (5 mM)	89.0
+ Glu (10 mM)	79.0
+ methionine sulfoximine (2.7 mM)	52.0
+ methionine sulfone (2.7 mM)	33.0
+ azaserine (0.5 mM)	15.0
+ azaserine (1.0 mM)	0.0
+ albizziine (1 mM)	88.5
+ albizziine (25 mM)	47.7

Value in brackets represents the actual rate in nmoles NADH oxidized·min^{-1}·mg protein^{-1}. The enzyme extracts were passed through a Sephadex G-75 column. Standard assays included 20 mM KCl in the endosperm extracts prepared from hybrid maize (var. W64A x W182E). α-Ketoglutarate and glutamine concentrations were 1 and 20 mM, respectively.

2. Analogues of glutamine and glutamate

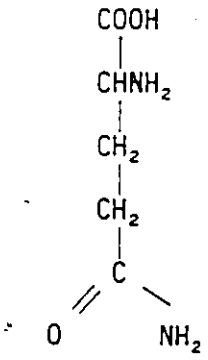
The glutamine analogues azaserine (o-diazoacetyl-L-serine) and albizziine (L-2-amino-3-ureido-propionic acid) are inhibitors of glutamine amide transfer reactions (Miflin and Lea, 1980; Fig. 15). These analogues are also known to inhibit the glutamate synthase reaction in pea root (Miflin and Lea, 1975) and in pea leaf (Wallsgrove *et al.*, 1977). For example, the enzyme activity in each case is 50% inhibited by 0.2 mM azaserine and 2 mM albizziine.

In corn endosperm extracts, azaserine at a concentration of 1 mM resulted in a complete inhibition of glutamate synthase activity (Table 8). It is believed that, because of the structural similarity between glutamine and azaserine, they compete for binding to the active site. However, once bound, the diazo-acetyl group of the analogue reacts with the enzyme and the analogue becomes irreversibly attached (Miflin and Lea, 1980). Albizziine, another analogue of glutamine, was less effective as an inhibitor and at a concentration of 25 mM gave only 50% inhibition of enzyme activity. GOGAT was also sensitive to a lesser degree to the glutamate analogues methionine sulphoximine and methionine sulfone, each causing 50% and 67% inhibition of activity, respectively, at a concentration of 2.7 mM.

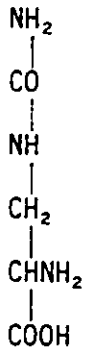
3. Electron donor specificities of GOGAT

In Sephadex G-75 filtered extracts or in dialyzed extracts prepared from endosperm, the enzyme could use NADH, NADPH, ferredoxin (Fd) and methyl viologen (MV) as the electron donor (Table 9). The NADPH-dependent activity at equimolar concentrations, was 50% of that

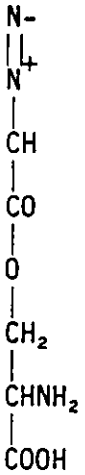
Figure 15.. Structure of glutamine and glutamine analogues.



(L-Glutamine)



(Albizziine)



(Azaserine)

observed with NADH as the electron donor. Sodek and DaSilva (1979) have also demonstrated NADH- and NADPH-dependent GOGAT activities in developing endosperm extract of corn, but in their preparations, the NADPH-dependent activity was variable and ranged between 20-100% relative to NADH-GOGAT activity. Similar results of dual specificity for pyridine nucleotide linked GOGAT activity have been reported in etiolated rice leaf and in roots (Suzuki *et al.*, 1982), soybean cotyledons (Storey and Reporter, 1978), etiolated pea leaf and roots (Wallsgrove *et al.*, 1982; Matoh *et al.*, 1982).

Two generalizations can be made from these reports: a) The enzyme is active with NADH and NADPH but NADPH-dependent-GOGAT activity is generally lower than NADH-GOGAT activity and b) in purer preparations, the enzyme appears to be NADH-specific. In desalted-extracts of developing soybean cotyledons, it has been suggested that NADPH-dependent activity is an artefact in the assay system owing to the presence of pyridine nucleotide phosphatase activity which would convert NADPH to NADH:

$$\text{NADPH} \xrightarrow[\text{(phosphatase)}]{} \text{NADH} + \text{Pi} \xrightarrow[\text{GOGAT}]{} \text{NAD}^+$$

The phosphatase reaction is inhibited by potassium fluoride (KF) and for this reason, KF was used to determine the specificity of the pyridine nucleotide-linked activities (Wells and Hageman, 1974; Dailey *et al.*,1982). In the assay with corn nitrate reductase, 2 µmoles/ml (2 mM) of KF reduced NADPH-NR activity to 10-15% of the control and phosphatase activity (assayed directly) to 3% of

Table 9

Substrate Requirements of Glutamate Synthase from Developing Endosperm of Corn var. W64A x W182E

Assay conditions	nmoles glutamate · min^{-1} $mg\ protein^{-1}$
Complete system (NADH)	25.09
- NADH + NADPH	12.50
- Enzyme	2.20
- Glutamine	2.20
- α-ketoglutarate	1.50
- NADH	1.72
Complete (ferredoxin)	17.00
- DT + Fd	2.00
- Enzyme	-
- Glutamine	2.01
- Fd + MV/DT	8.50
- α-ketoglutarate	1.50

Each assay was run for 30 min at 30°C.

Dialyzed extracts were used for enzyme assays and the reaction was stopped by the addition of 1.0 ml of methanol. Glutamate was assayed by reverse phase HPLC.

the control. There was no effect on NADH-NR activity. Similar results were obtained with soybean extracts and with barley leaf NR (Wells and Hageman, 1974) except that higher concentrations of KF (40 mM, 250 mM, respectively) were required.

Fig. 16 shows the effect of addition of KF in assay mixture on GOGAT activity of enzyme from corn endosperm. It can be seen that at a concentration of 10 mM, KF had little effect on the NADPH-GOGAT activity whereas 250 mM KF completely inhibited the NADPH-GOGAT reaction. There was no effect on the NADH-dependent activity at any concentration of KF. Thus, it appears that in extracts from corn endosperm, the observed NADPH-GOGAT activity may be due to conversion of NADPH to NADH by phosphatase. In this regard, my results differ from those of Sodek and DaSilva (1979) in corn endosperm. They report that NADPH-GOGAT activity was insensitive to KF, although they do not indicate the concentration of KF used.

4. Optimizing conditions for Fd-GOGAT assay

a. Linearity of assay: The conditions for Fd-dependent GOGAT were optimized in Sephadex G-75 filtered endosperm extracts. The reaction was linear with time (Fig. 17) and with increasing concentrations of the enzyme (0-0.76 mg protein·assay^{-1}) (Fig. 18). The reaction saturated at 40 µg Fd·assay^{-1} 0.5 mM α-ketoglutarate and 2 mM L-glutamine. In all routine assays, 40 µg Fd, 1.0 mM α-ketoglutarate and 5 mM L-glutamine were used. Incubation was carried out for 30 min at 30°C using 0.2 ml of enzyme.

Figure 16. Effect of potassium-fluoride upon NADH- and NADPH-dependent glutamate synthase activities in extracts of corn endosperm.

Sephadex G-75 filtered extracts of hybrid maize (var. W64A x W182E) harvested at 20 days after pollination were used.

Activity was measured by following oxidation of NAD(P)H at 340 nm.

Values are expressed as percentage of activity relative to the control samples.

●——● NADPH-dependent GOGAT.

■——● NADH-dependent GOGAT.

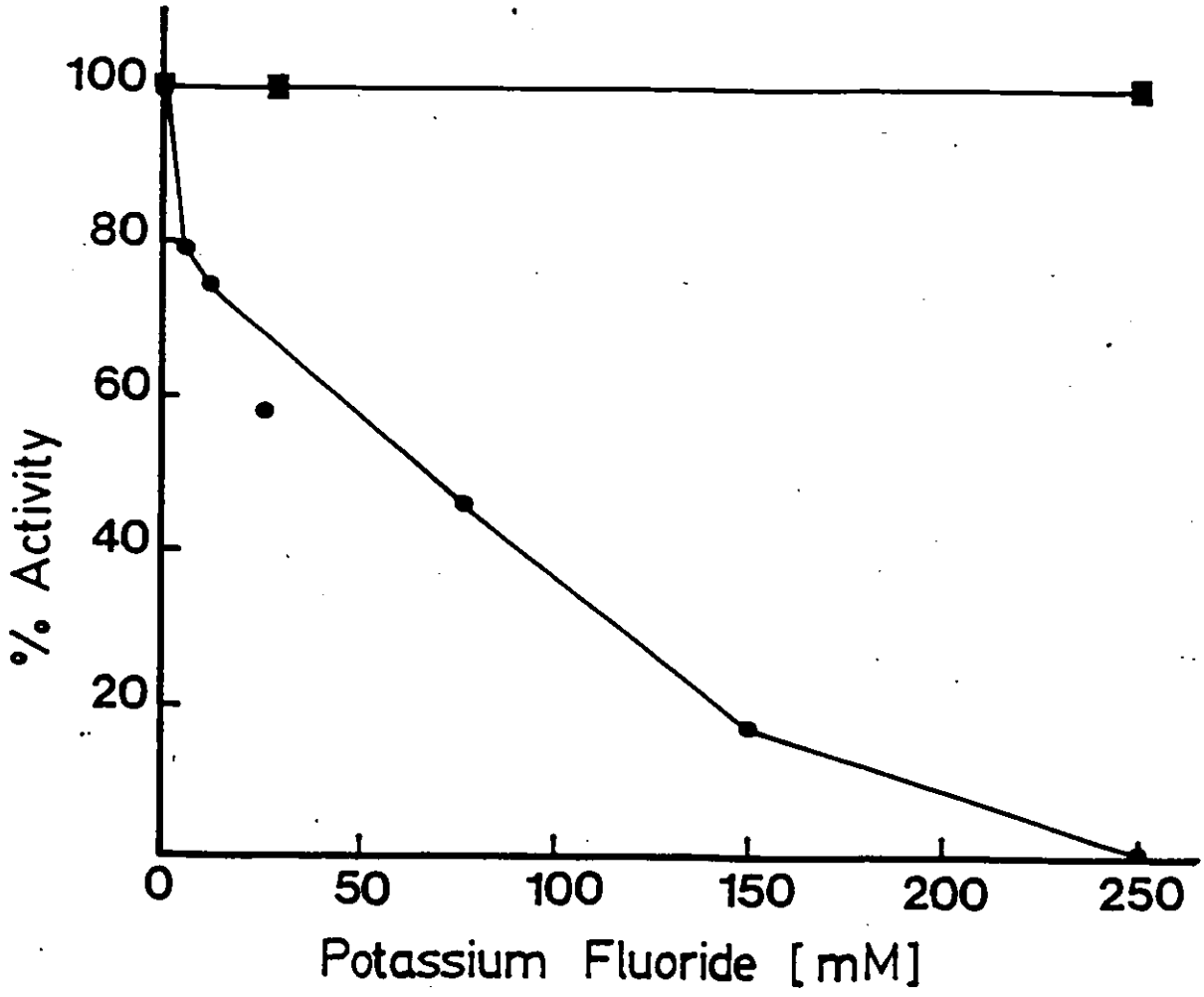

100
80
60
40
20
% Activity
0
50
100
150
200
250
Potassium Fluoride [mM]

Figure 17. Effect of increasing time of incubation on Fd-GOGAT activity in Sephadex G-75 filtered extracts of hybrid maize (var. W64A x W182E).

The reaction was stopped by addition of an equal volume of methanol, aliquots of supernatant were assayed for glutamate on HPLC. Assay was performed using 0.15 ml of enzyme extract, 1.0 mM α-keto-glutarate, 5 mM glutamine, 40 µg ferredoxin, 1.6 mg sodium dithionite, 1.6 mg sodium bicarbonate and 20 mM HEPES buffer in a final volume of 1.0 ml.

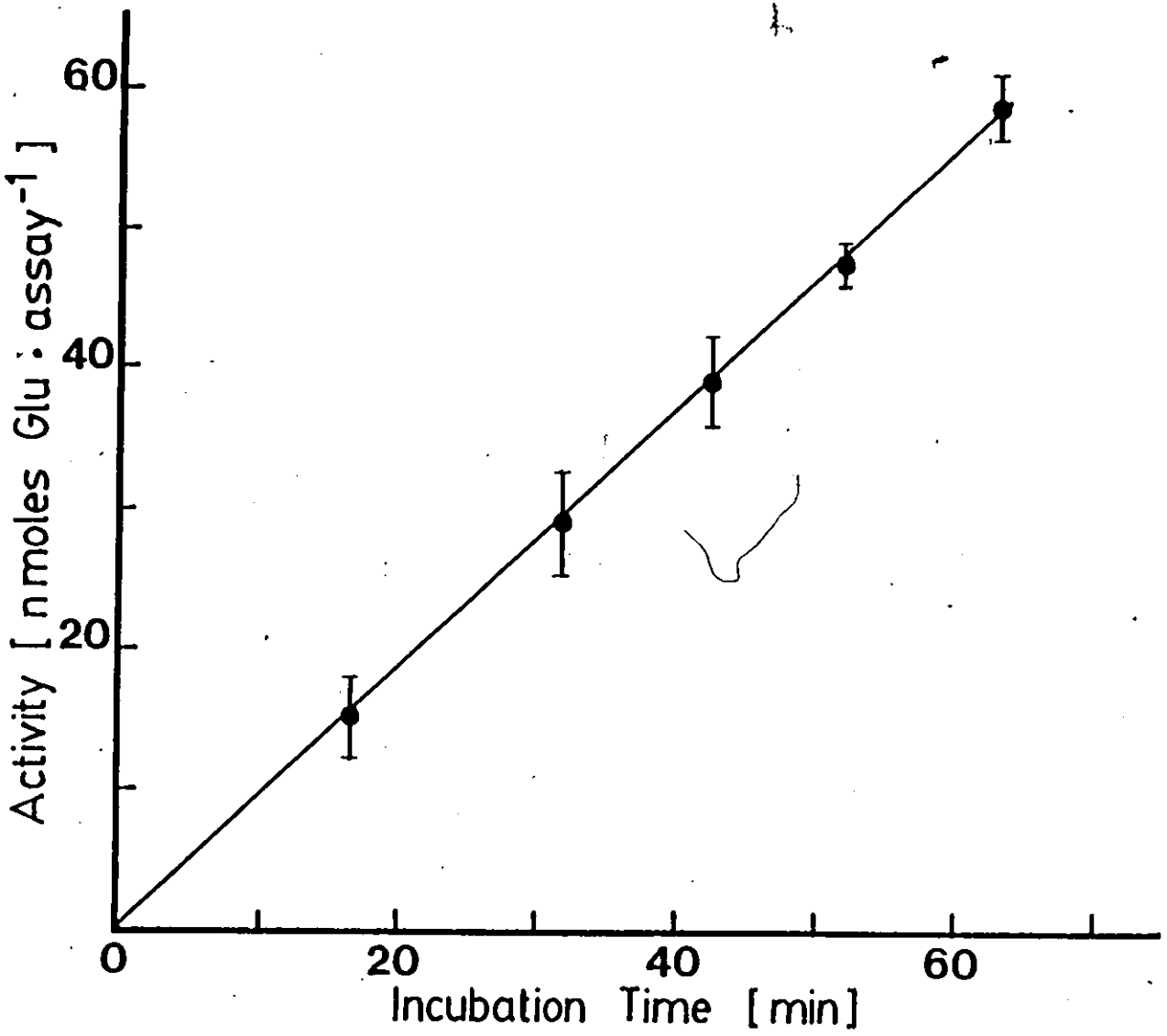
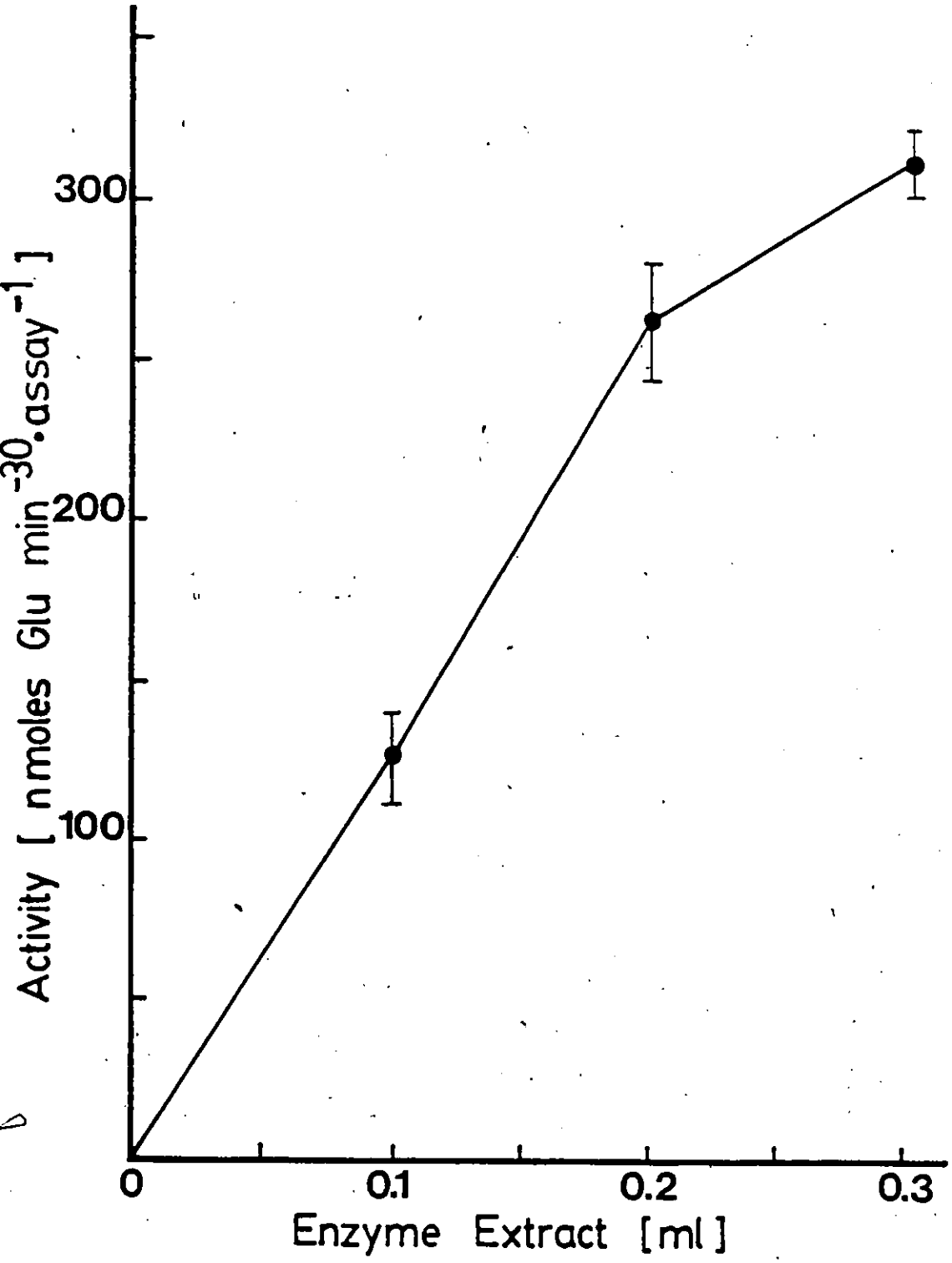

Activity [nmoles Glu · assay^{-1}]
60
40
20
0
20
40
60
Incubation Time [min]

Figure 18. Effect of increasing the enzyme volume on activity of Fd-glutamate synthase.

Sephadex G-75 filtered extracts from 20 day post-pollination endosperm of hybrid maize (var. W64A x W182E) were used. Assay conditions were as described in Figure 17.


300
200
100
Activity [nmoles Glu min-30.assay-1]
0
0.1
0.2
0.3
Enzyme Extract [ml]

5. Sephadex G-200 column chromatography

In developing cotyledons of pea, Matoh *et al.* (1979) have shown that NADH-dependent and ferredoxin-dependent glutamate synthases are two distinct enzymes as defined by separation on Sephadex G-200 column. To examine this possibility in developing endosperm of corn, the extracts were passed through a Sephadex G-200 column (2.7 x 82 cm). The elution profile of enzyme is shown in Fig. 19. Three milliliter fractions were collected and the absorbance of each fraction was monitored at 280 nm. Protein content was also estimated by BioRad protein test (Bradford, 1976). Each fraction was assayed for NADH, NADPH and ferredoxin-dependent GOGAT activity. NADH and ferredoxin-dependent activities appeared as two distinct peaks but the NADPH-dependent activity was lost. The fractions showing NADH- and ferredoxin-dependent activities were pooled separately and their kinetics and physical properties were characterized.

6. Substrate requirements of NADH- and Fd-GOGAT

The substrate specificities of each enzyme after filtration through Sephadex G-200 are illustrated in Table 10. The specific activity of NADH-GOGAT (111 nmoles·glu·min^{-1}·mg protein^{-1}) and of Fd-GOGAT (77 nmoles·glu·min^{-1}·mg protein^{-1}) was 4- and 3-fold higher respectively than in dialyzed extracts. The Fd-dependent activity was strictly dependent on the presence of glutamine, reduced ferredoxin and α-ketoglutarate in the assay mixture. The NADH-GOGAT activity was also dependent on the presence of α-ketoglutarate, NADH, and glutamine in the assay mixture.

Figure 19. Elution profile of GOGAT from developing endosperm of corn on a Sephadex G-200 column.

The column (2.7 x 82 cm) was equilibrated and eluted with the phosphate buffer containing 200 mM NaCl. The enzyme sample (54 mg in 2.0 ml) was applied to the column and 3 ml fractions were collected. Three hundred µl aliquots from each fraction were assayed for NADH-(●——●), Fd-(⊙——⊙), and NADPH-(▲——▲) dependent activity.

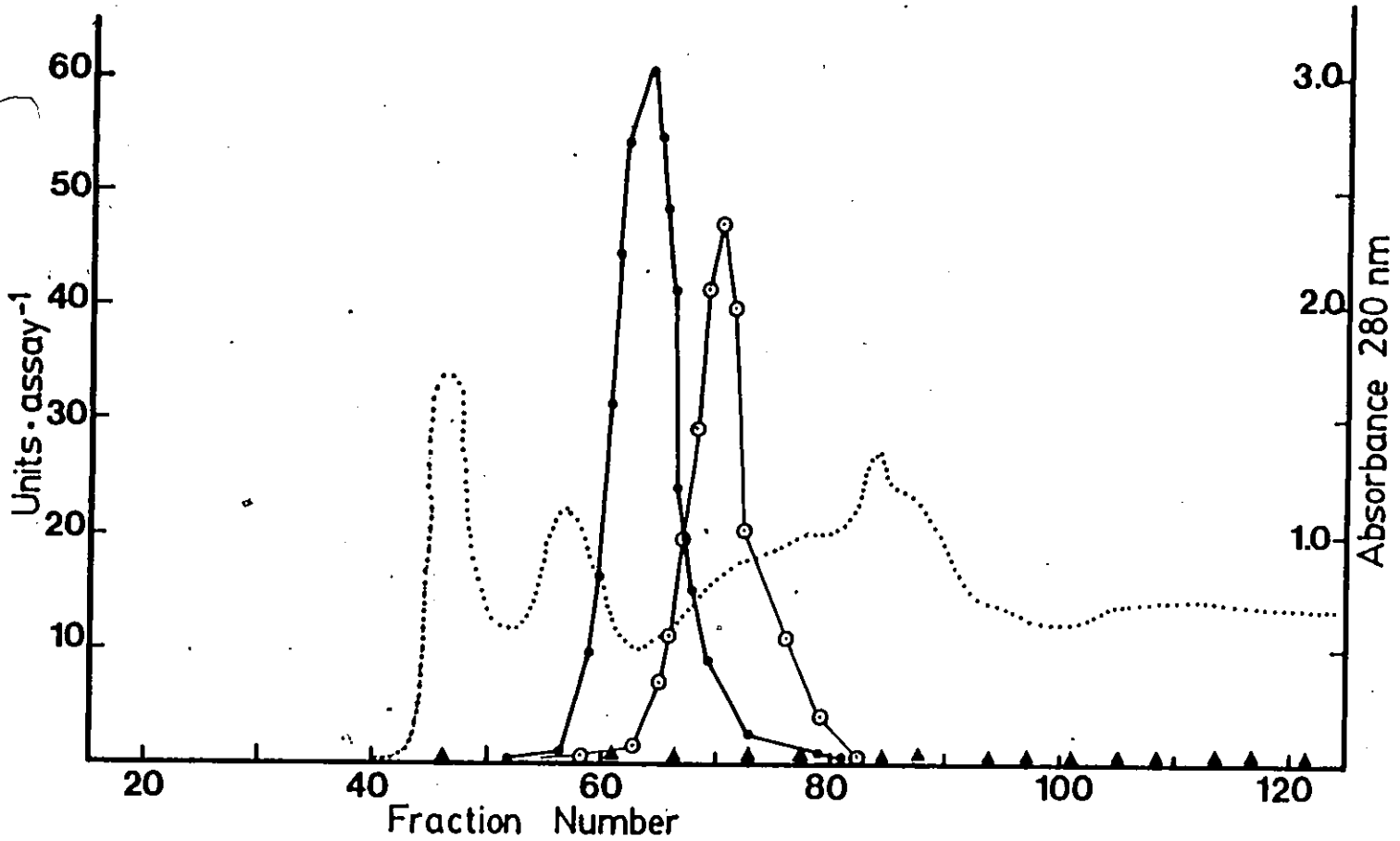

Units • assay⁻¹
60
50
40
30
20
10
3.0
2.0
1.0
Absorbance 280 nm
20
40
60
80
100
120
Fraction Number

Table 10

Substrate Requirements of the Two Forms of Glutamate Synthase from Developing Endosperm after Sephadex G-200 Filtration

Assay conditions	nmoles glu·min^{-1}·mg protein^{-1}
Complete(NADH)	111.00
-NADH + NADPH	11.00
-NADH + Fd/DT	14.00
-NADH + MV/DT	8.00
-NADH	2.00
-α-ketoglutarate	8.00
-Gln-α-ketoglutarate	2.00
-enzyme	2.50
Complete(ferredoxin)	76.66
-Fd + NADH	11.00
-Fd + NADPH	3.90
-Fd + MV/DT	8.00
-Gln	0.00
-Fd	0.00
-α-ketoglutarate	7.40
-enzyme	2.7

Enzyme extracts were prepared from 20 day post-pollination endosperm (var. W64A x W182E). Glutamate production was assayed on reverse phase HPLC.

7. Estimation of molecular weights

For determination of molecular weight by gel-filtration, a Sephadex G-200 column was calibrated using standard proteins of known molecular weight. The void volume of the column (Vo) was calculated from elution pattern of Blue-Dextran and values were plotted as Ve/Vo *vs* log molecular weight. The concentrated enzyme sample (54 mg/2 ml) (25%-60% $(NH_4)_2SO_4$ fraction), was applied to the column and enzyme activities in the eluants were localized by their appropriate assays. The estimated molecular weight of ferredoxin-GOGAT was 171,000 and of NADH-GOGAT was 280,000 (Fig. 20).

8. Km value for each substrate

To compare the apparent catalytic efficiency of each enzyme in developing endosperm tissue, Km values for glutamine, α-ketoglutarate and NADH/Fd were determined using Sephadex-treated enzyme.

Enzyme activities were measured using several concentrations of the variable substrate in the reaction and routine concentrations of all other substrates. Km values were determined using double reciprocal plot of velocity *vs* substrate concentrations. Lines of best fit and correlation coefficients were then calculated.

Typical Lineweaver-Burk plots for glutamine, α-ketoglutarate and NADH for NADH-glutamate synthase reaction are shown in Figs. 21-23 and those of glutamine, α-ketoglutarate and ferredoxin for Fd-glutamate synthase reaction are shown in Figs. 24-26. A summary of Km values for each substrate is shown in Table 11. Km values for L-glutamine, α-ketoglutarate and NADH for NADH-GOGAT are 1.068, 0.539

Figure 20. Estimation of molecular weight of glutamate synthase of corn endosperm by gel filtration.

The Sephadex G-200 column (2.7 x 82 cm) was equilibrated with 200 mM phosphate buffer (pH 7.5) containing 100 mM NaCl and was calibrated with:

1. BSA (68,000)
2. Alcohol dehydrogenase (150,000)
3. Catalase (250,000)

I NADH-GOGAT
II Fd-GOGAT

were identified by appropriate enzyme assays

*Ve/Vo is the ratio of the relative elution volume (Ve) for the particular protein to the column void volume (Vo).

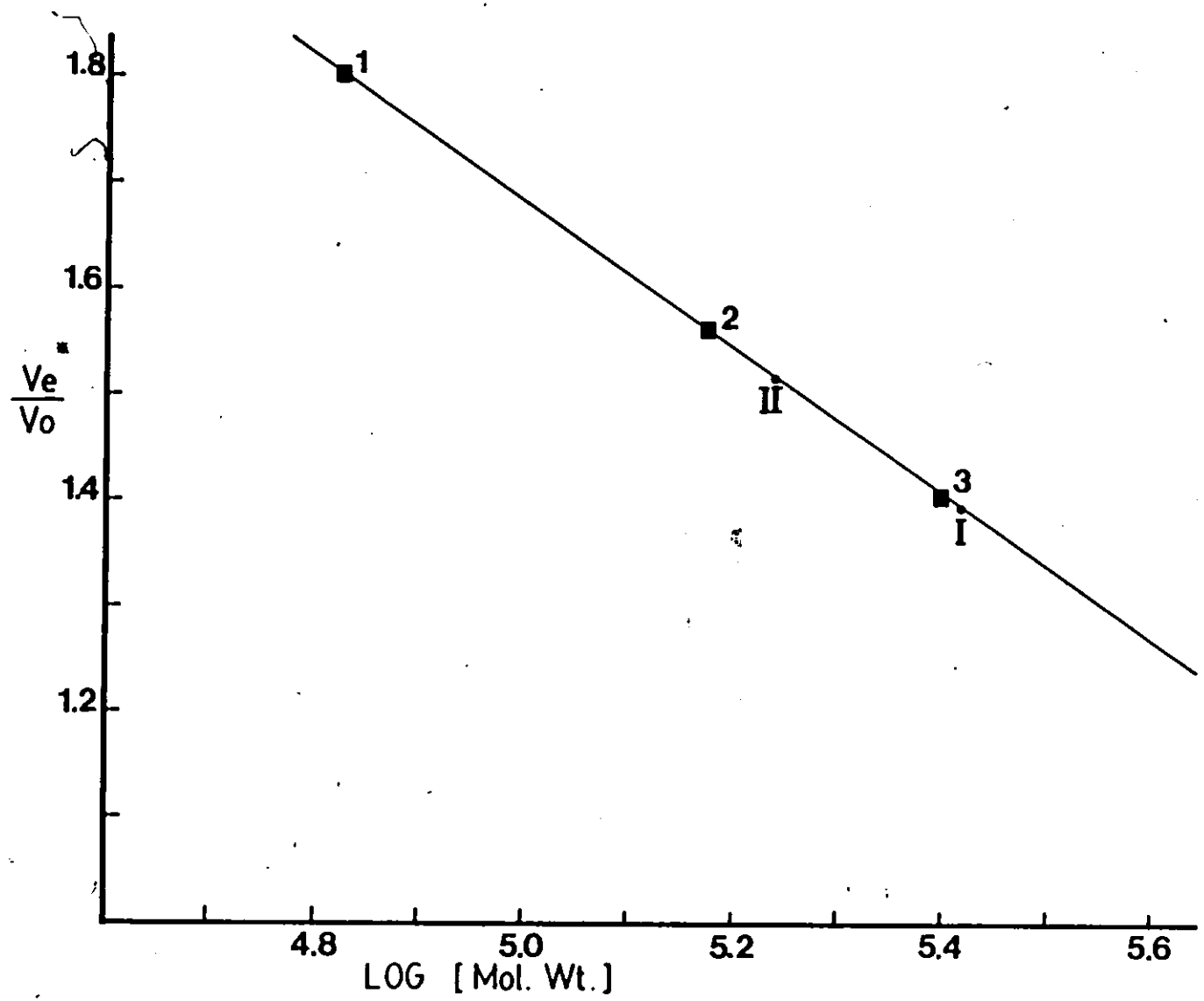

1.8
1.6
1.4
1.2
Ve/Vo
1
2
3
II
I
4.8
5.0
5.2
5.4
5.6
LOG [Mol. Wt.]

Figure 21. Substrate saturation kinetics and Lineweaver-Burk plot of NADH-glutamate synthase reaction in corn endosperm extracts.

$1/V$ = nmoles glu·min^{-30}·assay^{-1}.

The substrate varied in the assay was L-glutamine. Concentrations of other substrates were as described in Methods.

For L-glutamine the equation of line of best fit

$$1/V = 0.0333679 \left(\frac{1}{S}\right) + 0.0314508$$

$r^2 = 0.998$

Km = 1.068 mM

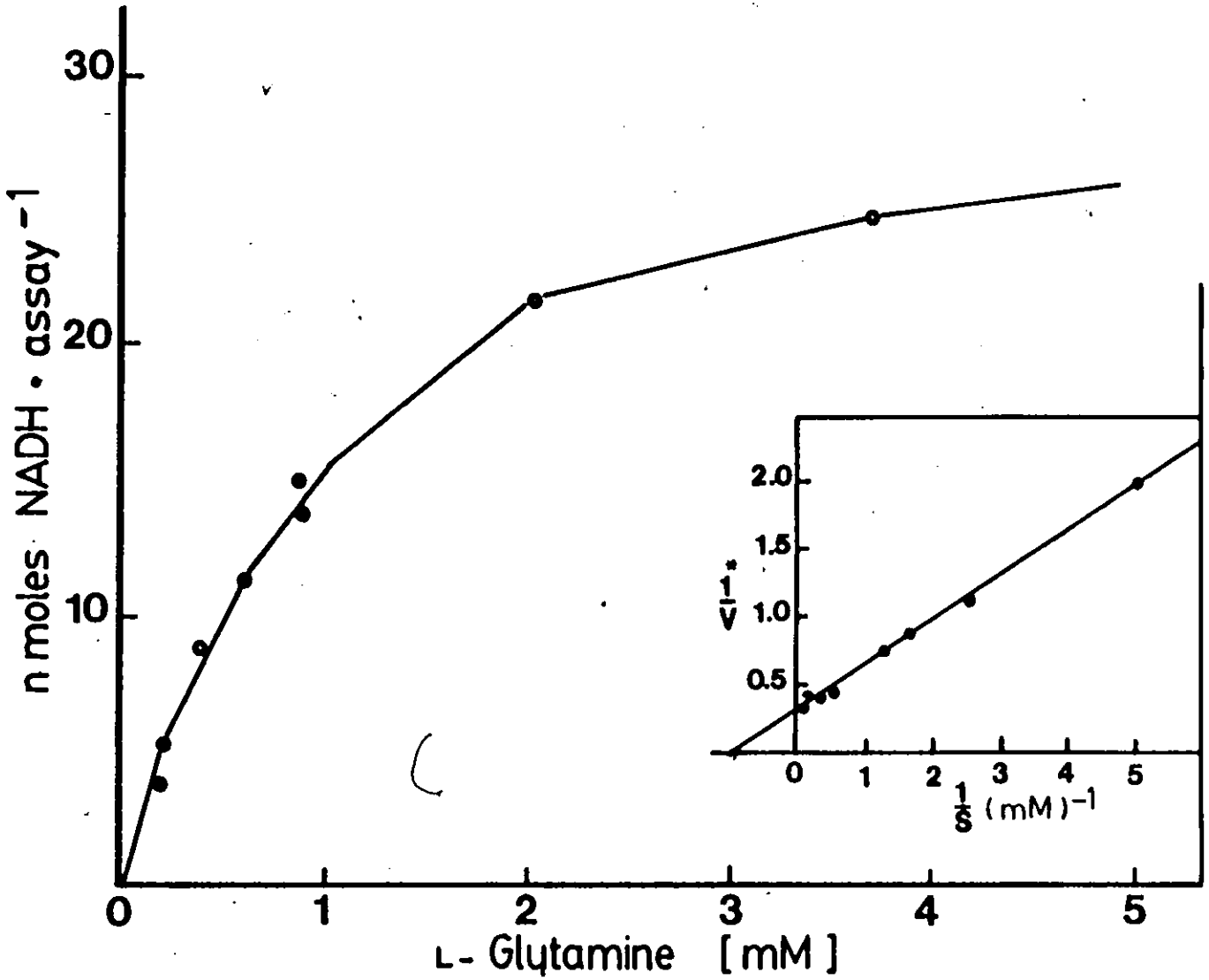

30
20
10
n moles NADH • assay⁻¹
0
1
2
3
4
5
L- Glutamine [mM]
2.0
1.5
1.0
0.5
1/V*
0
1
2
3
4
5
1/S (mM)⁻¹

Figure 22. Substrate saturation kinetics and Lineweaver-Burk plot for NADH-glutamate synthase reaction in corn endosperm extracts.

$1/V$ = nmoles glu·min^{-30}·$assay^{-1}$.

The substrate varied in the assay was NADH. Concentrations of other substrates were as described in Methods.

For NADH, the equation of line of best fit:

$$1/V = 0.000410 \left(\frac{1}{S}\right) + 0.0483541$$

$r^2 = 0.931$

Km = 8.06 μm.

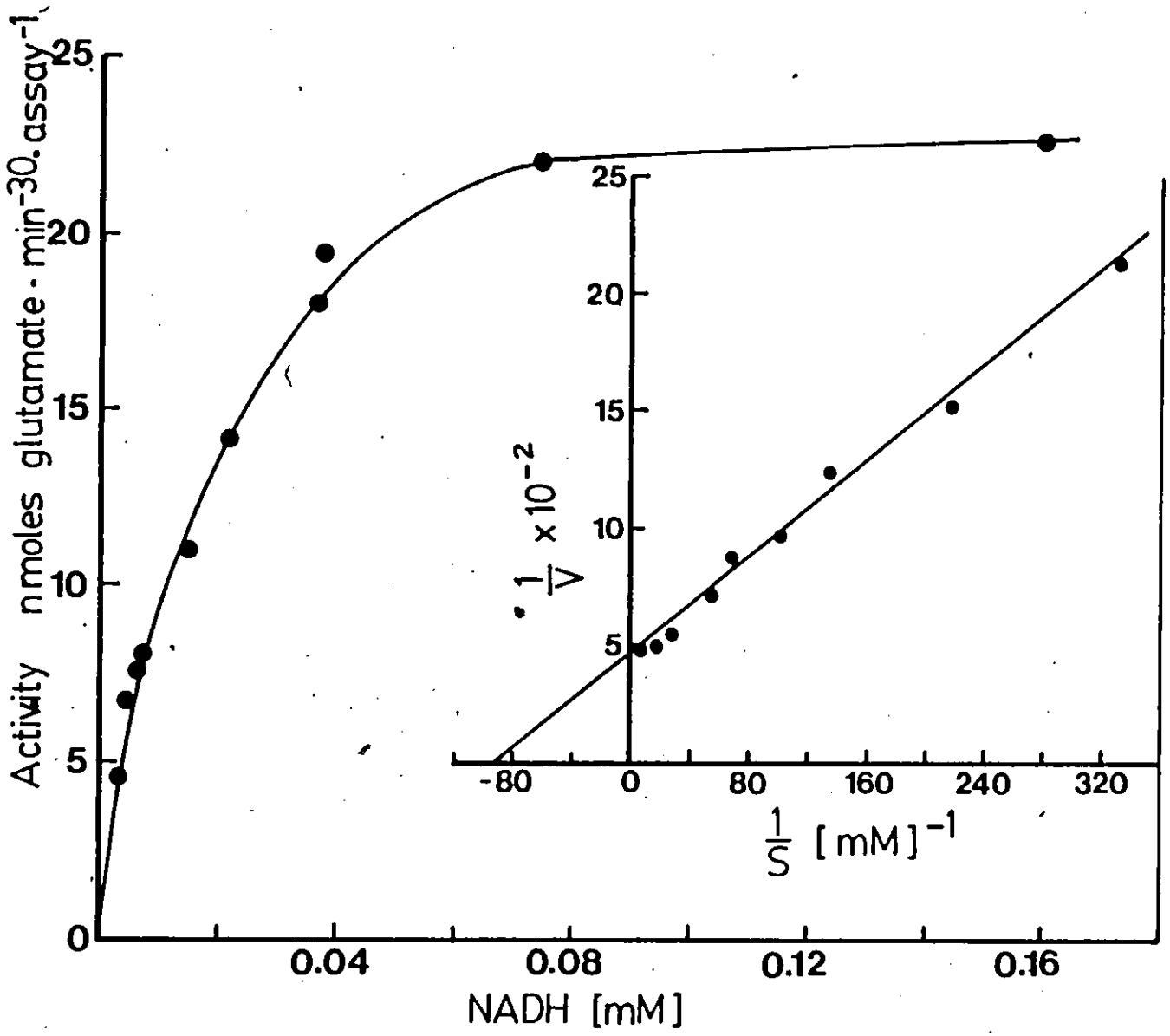

Activity nmoles glutamate · min-30. assay-1
25
20
15
10
5
0
0.04
0.08
0.12
0.16
NADH [mM]
25
20
15
10
5
-80
0
80
160
240
320
1/V x 10-2
1/S [mM]-1

Figure 23. Substrate saturation kinetics and Lineweaver-Burk plot for NADH-glutamate synthase reaction in corn endosperm extracts.

$1/V$ = nmoles glu·min^{-30}·$assay^{-1}$.

The substrate varied in the assay was α-keto-glutarate. Concentrations of other substrates were as described in Methods.

For α-ketoglutarate, the equation of line of best fit

$$1/V = 0.0092495 \left(\frac{1}{S}\right) + 0.0171315$$

$r^2 = 0.9772$

Km = 0.539 mM.

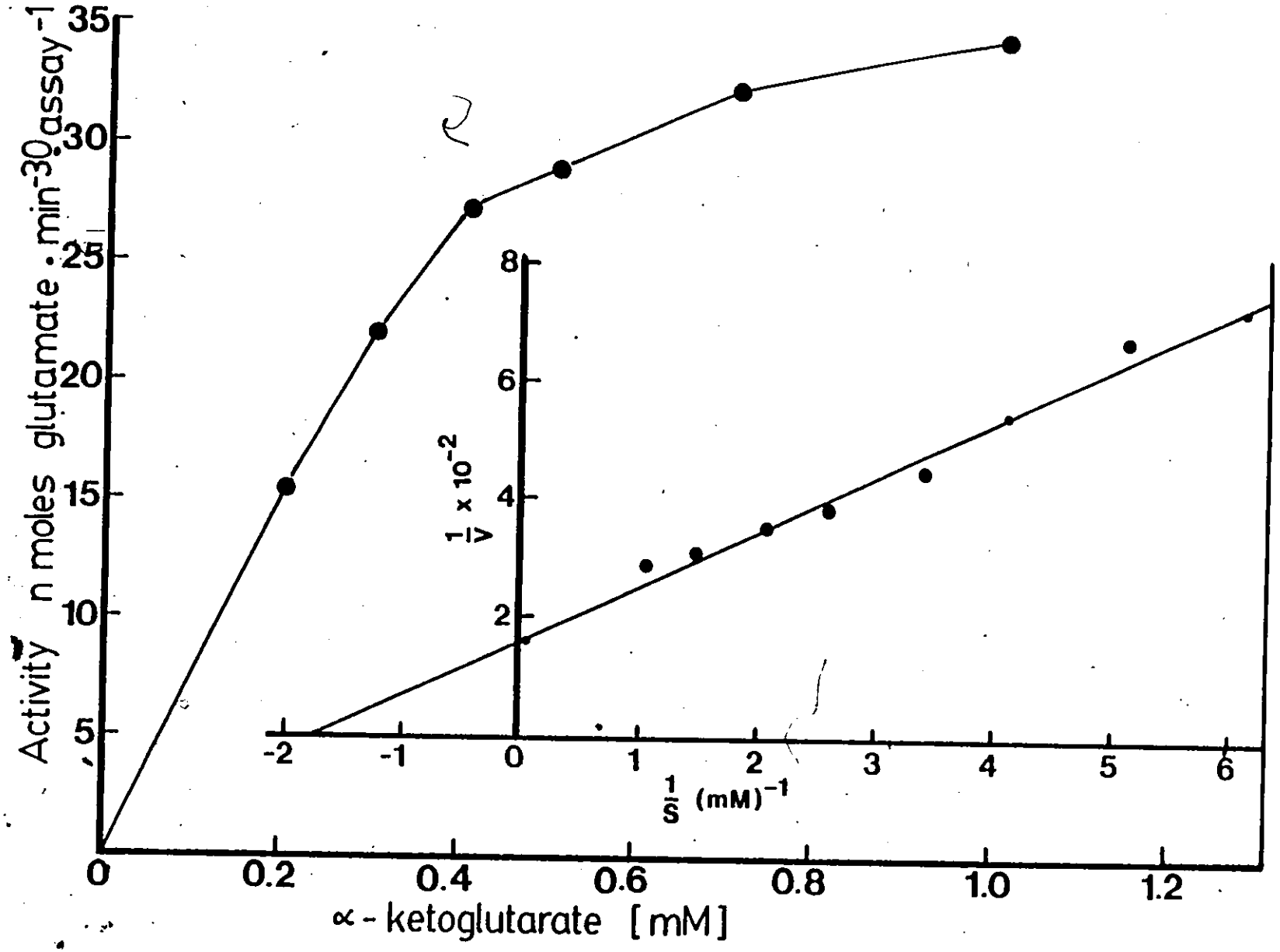

Activity n moles glutamate · min-30assay-1
35
30
25
20
15
10
5
0
0.2
0.4
0.6
0.8
1.0
1.2
∝ - ketoglutarate [mM]
1/V x 10-2
8
6
4
2
-2
-1
0
1
2
3
4
5
6
1/S (mM)-1

Figure 24. Substrate saturation kinetics and Lineweaver-Burk plot for Fd-glutamate synthase reaction in corn endosperm extracts.

The substrate varied in the assay was L-glutamine.

$1/V$ = nmoles glu·min^{-30}·$assay^{-1}$.

For L-glutamine, the equation of line of best fit

$$1/V = 0.0057102 \left(\frac{1}{S}\right) + 0.0093437$$

$r^2 = 0.997$

Km = 0.617 mM.

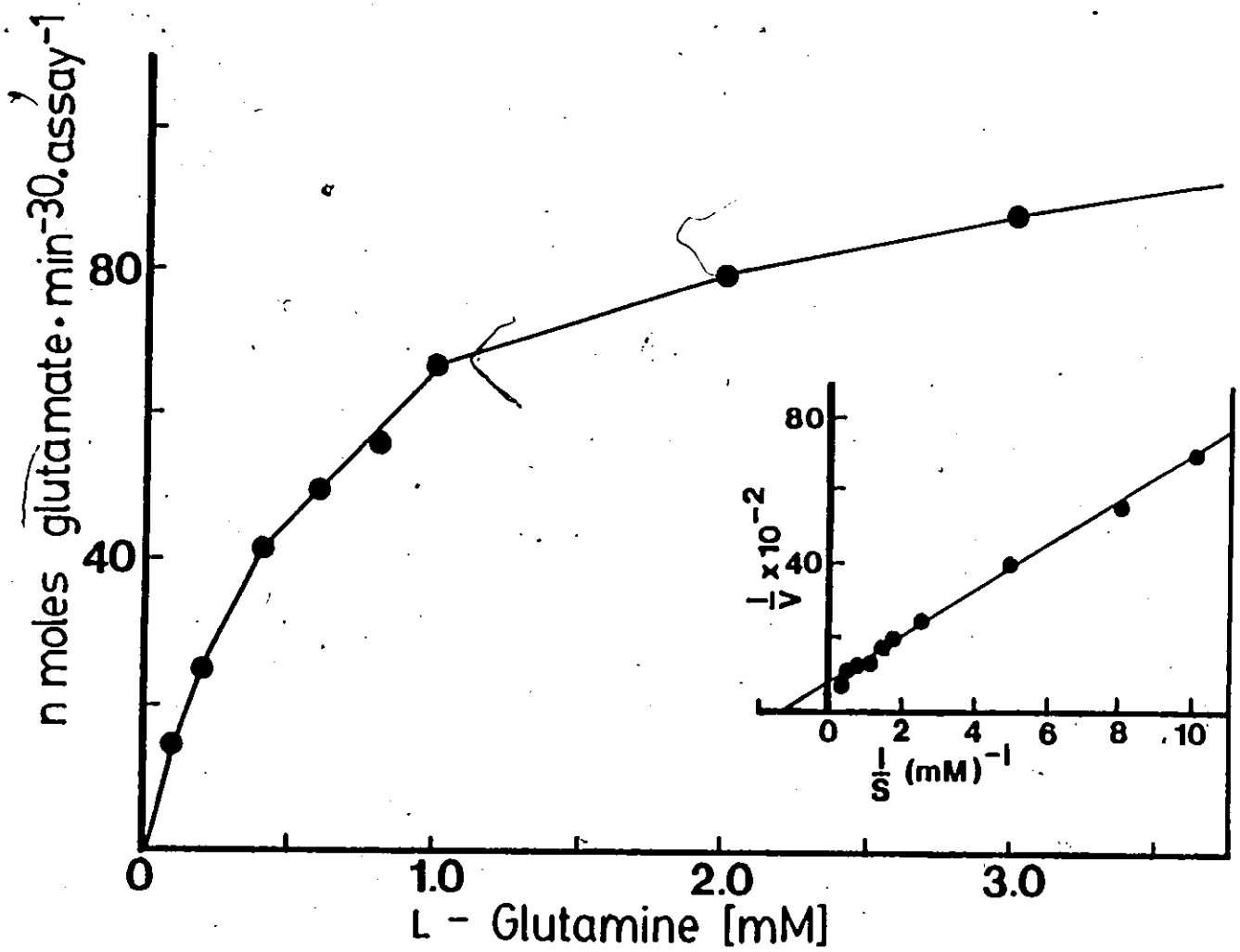

n moles glutamate · min-30.assay-1
80
40
0
1.0
2.0
3.0
L - Glutamine [mM]
80
40
1/V x10-2
0
2
4
6
8
10
1/S (mM)-1

Figure 25. Substrate saturation kinetics and Lineweaver-Burk plot for Fd-glutamate synthase reaction in corn endosperm extracts.

*1/V = nmoles glu·min^{-30}·assay^{-1}.

The substrate varied in the assay was α-keto-glutarate. Concentrations of other substrates were as described in Methods. For α-ketoglutarate the equation of line of best fit:

$$1/V = 0.0125498 \left(\frac{1}{S}\right) + 0.0484607$$

$r^2 = 0.988$

Km = 0.258 mM.

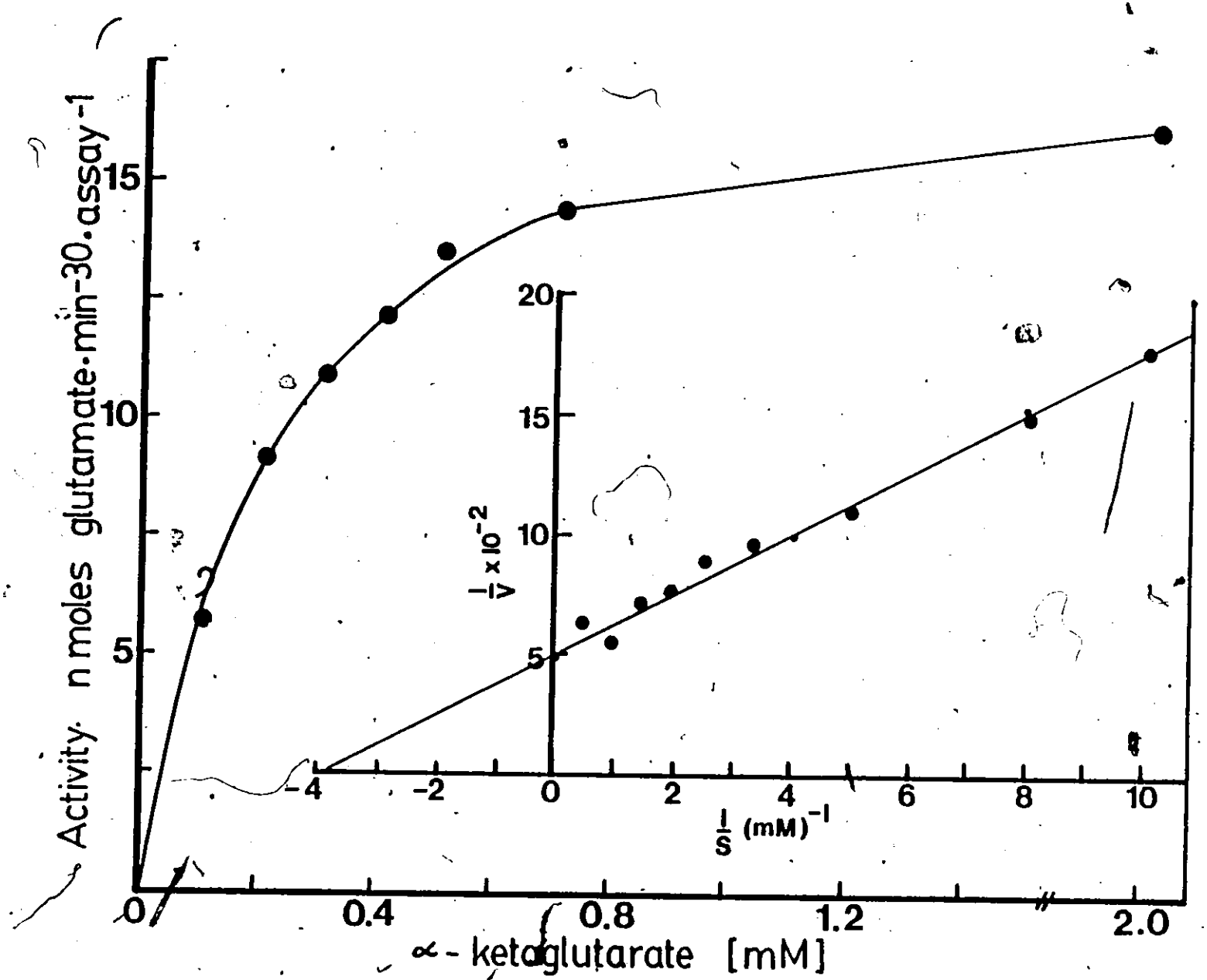

Activity n moles glutamate·min-30·assay-1
15
10
5
0
0.4
0.8
1.2
2.0
∝ - ketoglutarate [mM]
20
15
10
5
$\frac{1}{V} \times 10^{-2}$
-4
-2
0
2
4
6
8
10
$\frac{1}{S}$ (mM)$^{-1}$

Figure 26. Substrate saturation kinetics and Lineweaver-Burk plot of Fd-glutamate synthase reaction in corn endosperm extracts.

*1/V = nmoles glu·min^{-30}·assay^{-1}.

The substrate varied in the assay was ferredoxin. Concentrations of other substrates were as described in Methods.

For ferredoxin the line of best fit:

$$1/V = 0.0058861 \left(\frac{1}{S}\right) + 0.0031565$$

$$r^2 = 0.975$$

$$Km = 1.864\ \mu M.$$

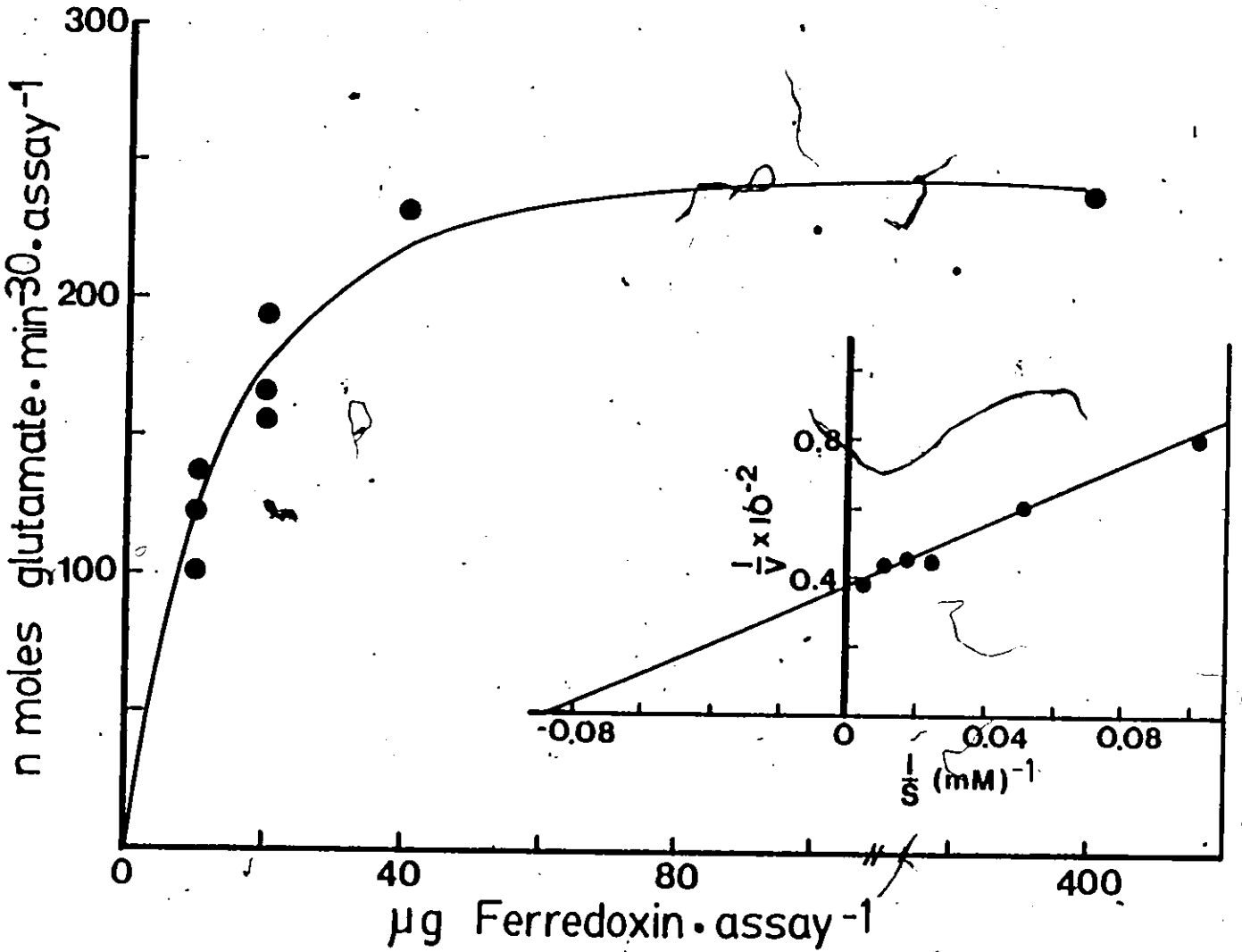

300
200
100
n moles glutamate • min-30 • assay-1
0
40
80
400
µg Ferredoxin • assay-1
0.8
0.4
1/v x 10-2
-0.08
0
0.04
0.08
1/S (mM)-1

Table 11

Km Values for Different Substrates in the NADH- and Ferredoxin-dependent Glutamate Synthase Reactions

Substrate	NADH-GOGAT		Fd-GOGAT	
	Km (μM)	r^2	Km (μM)	r^2
Glutamine	1068	0.998	617	0.997
α-ketoglutarate	539	0.997	258	0.998
NADH	8.06	0.931	-	-
Ferredoxin	-	-	1.86	0.975

Extracts were prepared from 20 day post-pollination endosperm (var. W64A x W182E), glutamate was assayed by HPLC.

r^2 = correlation coefficient for the line of best fit in Lineweaver-Burk plots, Km values were calculated from the equation of these lines.

and 0.0086 mM respectively. These values are in close agreement with published results of Sodek and DaSilva (1979). The Km values for L-glutamine, α-ketoglutarate, and ferredoxin for Fd-GOGAT are 0.617, 0.258 and 0.00186 mM respectively.

The Km values obtained for glutamine (0.617 mM) and α-ketoglutarate (0.258 mM) in Fd-GOGAT reaction are significantly lower than glutamine and α-ketoglutarate Km values (1.068 mM and 0.539 mM respectively) for NADH-GOGAT reaction. Hence, if appropriate reductant were available, this enzyme could be physiologically more important in the formation of glutamate.

C. Asparagine Synthetase (AS)

ATP + aspartate + glutamine $\xrightarrow{Mg^{2+}}$ asparagine + glutamate + AMP + PP_i.

1. Optimizing the extraction conditions

a. Effect of omitting ATP, aspartate and $MgCl_2$ from the extraction buffer: Routinely Sephadex G-75 filtered extracts from developing endosperms were used. Conditions were optimized for extraction and Sephadex-filtration of the enzyme. In experiments with roots of corn, it has been shown that addition of enzyme substrates (ATP, aspartate and $MgCl_2$) to the extraction and elution buffers protected the enzyme activity (Stulen and Oaks, 1977; Ross, 1979). The effect of each of these ingredients was tested on the activity of the enzyme in endosperm extracts. Activity was similar in a standard extraction and elution buffer and in extracts lacking aspartate (Table 12). When ATP was omitted from the buffer, 30% of

Table 12

Effect of Omitting Aspartate, ATP, or $MgCl_2$ from the Extraction and Elution Buffers on Asparagine Synthetase Activity in Developing Endosperm Extract

Extraction conditions	Activity ($cpm \cdot min^{-20} \cdot assay^{-1}$)	Average ± mean variation	% control
1. *Standard extraction buffer	1. 571 2. 527 3. 555	551 ± 16	-
2. - Aspartate	1. 548 2. 580 3. 577	568 ± 13	103
3. - ATP	1. 391 2. 357 3. 390	379 ± 15	69
4. - $MgCl_2$	1. 540 2. 454	497 ± 50	91

Sephadex filtered extracts of endosperms harvested 20 days after pollination were used.

Extracts were assayed as described in Methods.

The mean ± range of three replicates is shown for treatments 1-3.

The values obtained for $MgCl_2$ were from two separate assays.

*Standard extraction buffer: 0.1 M phosphate buffer (pH 7.5) + 15% glycerol, 12 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM ATP, 10 mM $MgCl_2$ and 0.8 mM aspartate.

the activity relative to the control was lost; in absence of $MgCl_2$, 10% of the activity was lost.

b. Comparison of phosphate buffer *vs* HEPES extraction buffer: In the initial experiments, phosphate buffer + 15% glycerol containing 1 mM EDTA, 10 mM $MgCl_2$ and 12.5 mM 2-mercaptoethanol was used. Later, the tissue was homogenized in 200 mM HEPES buffer (pH 7.5) containing 1 mM EDTA. Enzyme extract was eluted from Sephadex G-75 column with 25 mM HEPES buffer (pH 7.5) + 1 mM EDTA, in the presence or the absence of ATP (1 mM) and $MgCl_2$ (10 mM). The activity in HEPES buffer (+ $MgCl_2$ + ATP) was 30% higher than that in complete phosphate buffer (Table 13). As with the phosphate buffer, the omission of ATP and $MgCl_2$ from the HEPES buffer resulted in a loss of asparagine synthetase activity. Based on these observations, the extraction and elution buffers for asparagine synthetase were modified to contain 200 mM HEPES buffer (pH 7.5) containing 1 mM EDTA, 1mM ATP, 12 mM 2-mercaptoethanol and 10 mM $MgCl_2$.

2. Linearity of the assay

Fig. 27 shows the effect of increasing incubation time on asparagine synthetase activity. The activity was linear with time of incubation up to 65 min. The activity was also linear with volume of Sephadex-filtered extract over the range of 0.1-0.3 ml of enzyme extract per assay (Fig. 28). Routinely, 0.2 ml of enzyme extract was used and the incubation time was 20 min.

Table 13

Effect of Extraction Buffer on Activity of the Asparagine Synthetase in Endosperm Extract: Comparison of Phosphate Buffer *vs* HEPES buffer

Extraction buffer	Volume of extract (ml)	Activity			Average ± mean variation
		$cpm \cdot min^{-20} \cdot assay^{-1}$	Average ± mean variation	nmoles $Asn \cdot min^{-20} \cdot endosperm^{-1}$	
Phosphate buffer + ATP (1 mM) and + $MgCl_2$ (10 mM)	0.2	1. 712 2. 742	725 ± 15	3.56	3.79 ± 0.33
	0.3	1. 1203 2. 1240	1226 ± 23	4.02	
HEPES buffer + ATP (1 mM) and + $MgCl_2$ (10 mM)	0.2	1. 923 2. 1017	970 ± 47	4.85	4.84 ± 0.025
	0.3	1. 1453 2. 1440	1446 ± 7	4.82	
HEPES buffer - ATP - $MgCl_2$	0.2	1. 455 2. 396	425 ± 30	2.125	2.36 ± 0.24
	0.3	1. 787 2. 780	783 ± 4	2.61	

Extracts were prepared from endosperms collected 20 days after pollination. Maize var. (W64A) was used. Extracts were filtered through a Sephadex G-75 column. Assays were performed in duplicate. The complete phosphate buffer contained 0.2 M phosphate (pH 7.5), 1 mM EDTA, 1 mM ATP, 10 mM $MgCl_2$, 12.5 mM 2-mercaptoethanol, 0.8 mM aspartate and 15% glycerol. The complete HEPES buffer contained 200 mM HEPES (pH 7.5), 1 mM EDTA, 12.5 mM 2-mercaptoethanol, 1 mM ATP and 10 mM $MgCl_2$.

Figure 27. Effect of incubation on asparagine synthetase activity.

Sephadex G-75 filtered extracts of hybrid maize (var. W64A x W182E), harvested at 25 days after pollination were used.

The assay mixture contained 1.0 mM ATP, 10 mM glutamine, 1 mM α-aminooxyacetate, 13.2 mM $MgCl_2$, 1.1 mM aspartate, 0.22 mCi of (^{14}C)-aspartate and 0.2 ml of the enzyme extract in a total volume of 0.62 ml. Two assays were run at each time point.

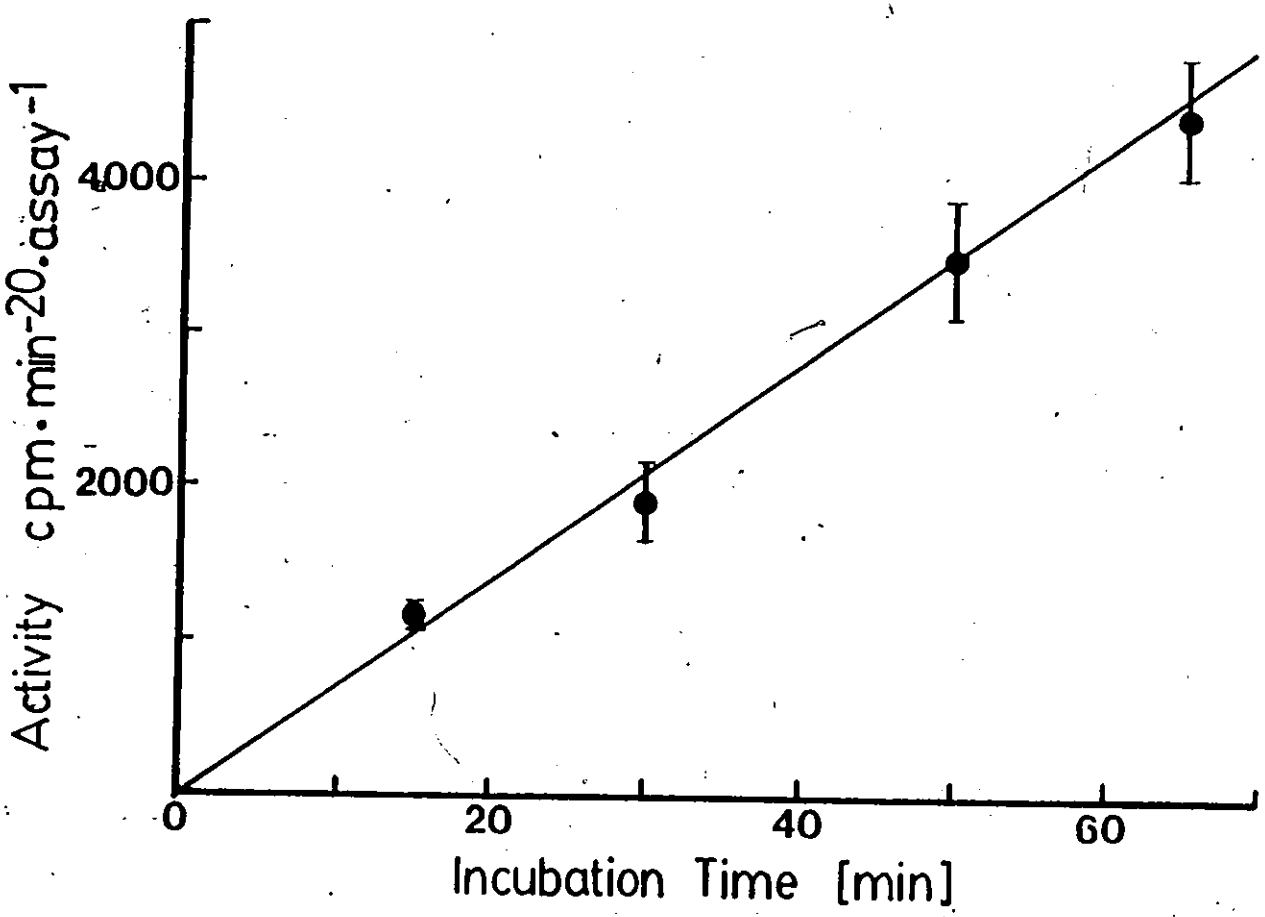
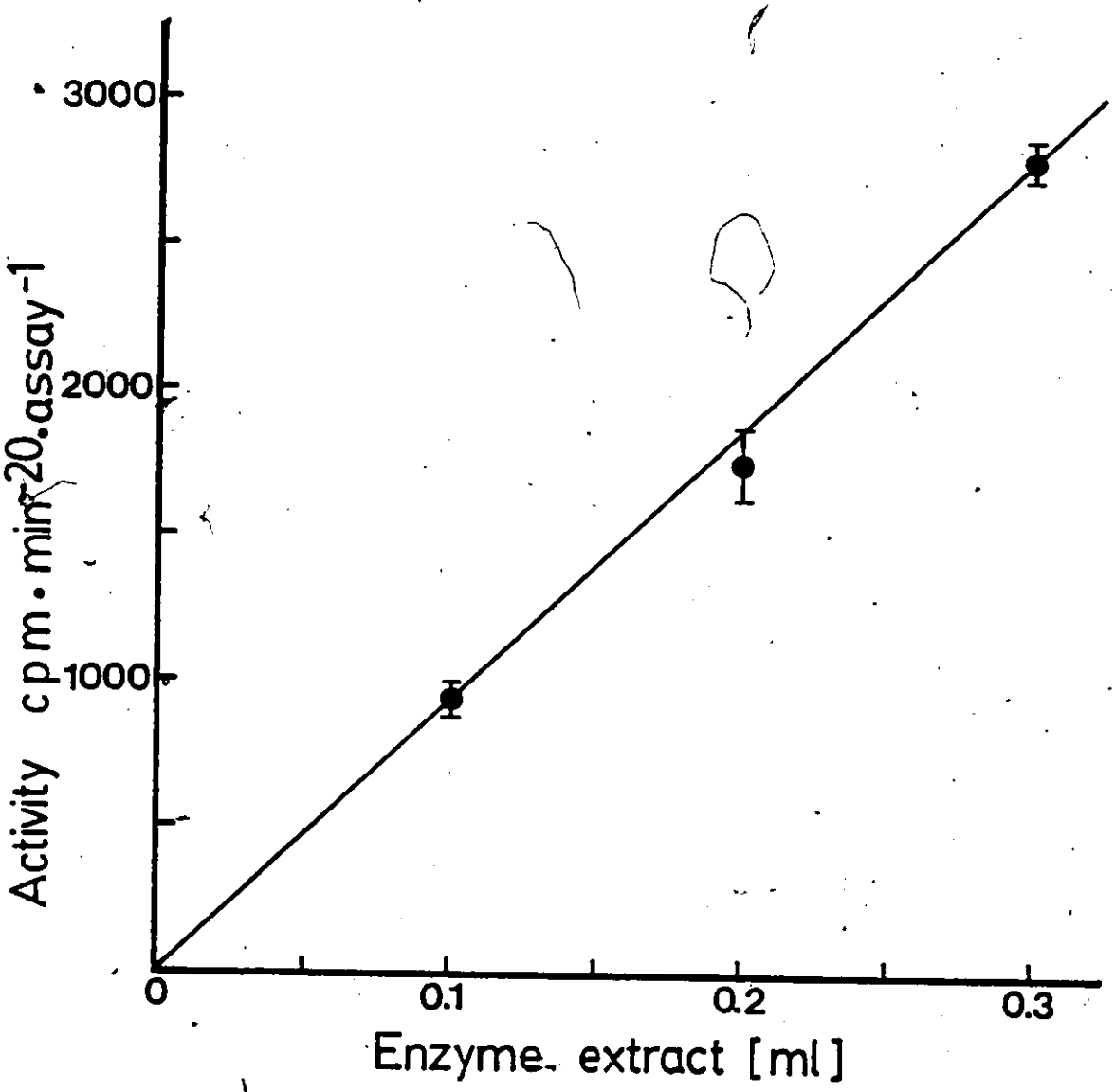

Activity cpm·min-20.assay-1
4000
2000
0
20
40
60
Incubation Time [min]

Figure 28. Effect of increasing the enzyme volume on asparagine synthetase activity.

Extracts were prepared from endosperm of inbred maize (var. W64A), harvested 25 days after pollination.

The assay mixture contained 1.0 mM ATP, 10 mM glutamine, 1 mM α-aminooxyacetate, 13.2 mM $MgCl_2$, 1.1 mM aspartate, 0.22 mCi of (^{14}C)-aspartate in a total volume of 0.62 ml.

The mean values ± range (bars) of two replicate assays are shown for each point.


3000
2000
1000
0
0.1
0.2
0.3
Activity cpm • min⁻²⁰.assay⁻¹
Enzyme extract [ml]

D. Glutamine Synthetase (GS)

$$\text{L-glutamate} + \text{ATP} + NH_3 \xrightleftharpoons{Me^{2+}} \text{L-glutamine} + \text{ADP} + P_i$$

1. Linearity of the assay

Fig. 29 shows the effect of increasing time of incubation on activity of glutamine synthetase in crude extracts and in Sephadex-filtered extracts obtained from maize endosperms. In either case, the activity is linear with increasing time of incubation and with increasing volume of enzyme extract (Figs. 29, 30). The activity in crude extracts was lower than that in Sephadex-treated extracts.

2. Recovery of glutamine synthetase activity

Table 14 illustrates the percent recovery of total activity when crude extracts were filtered through Sephadex G-75 and the change in specific activity. The percent recovery of total activity in all cases was higher after filtration through Sephadex column (117-156% in W64A and 183-190% in opaque-2 and 169-172% in floury-2) relative to the crude extract values. The activity on a per endosperm basis and the specific activity was higher after filtration through Sephadex. The increase ranged from two (W64A) to three and a half fold (opaque-2).

3. DEAE-Sephacel column chromatography of glutamine synthetase

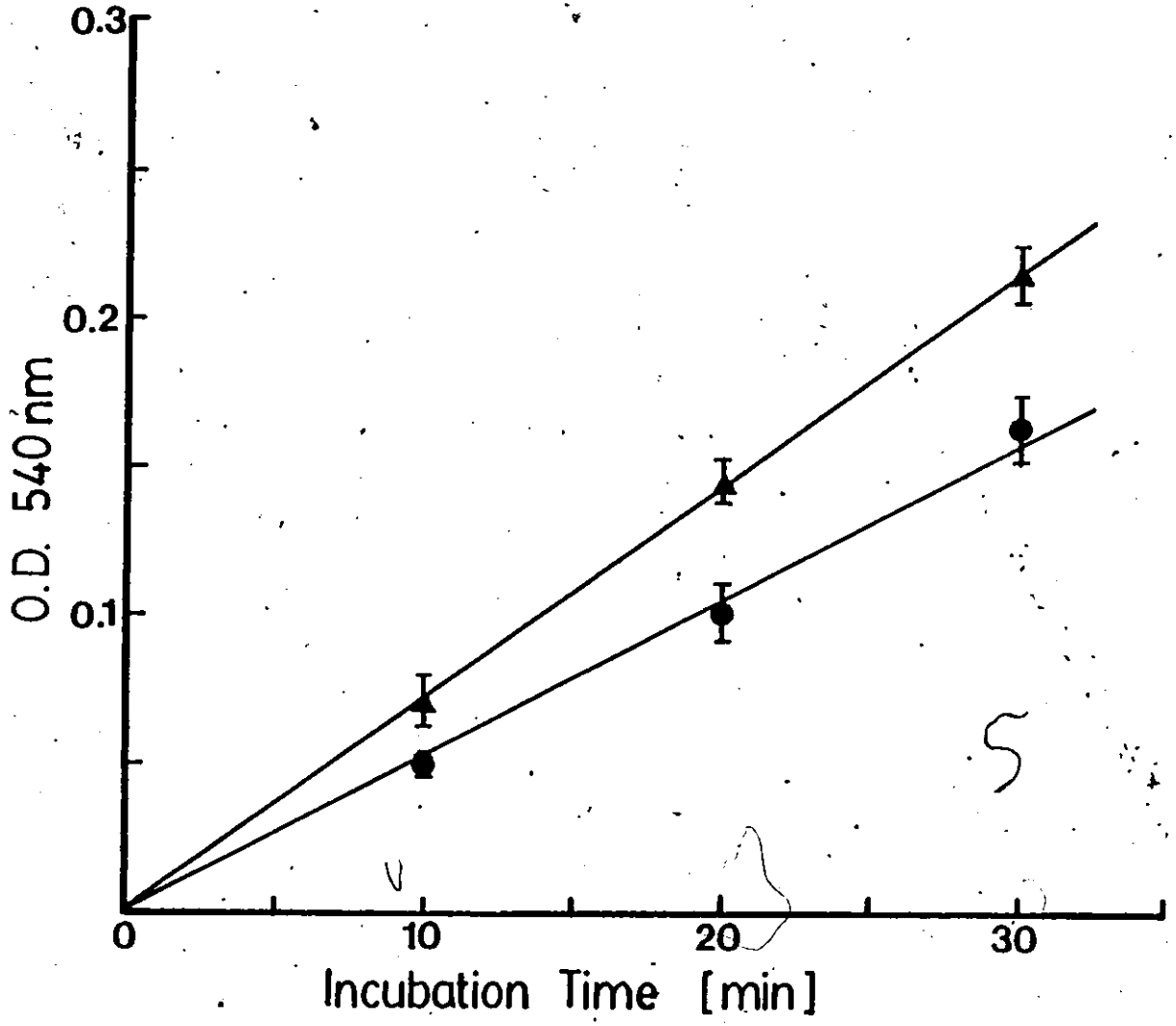
The chromatographic properties of glutamine synthetase in corn endosperm and embryo extracts were examined by DEAE-Sephacel column chromatography. Sixty fractions of 2 ml each were collected from the column. Fractions were assayed for glutamine synthetase

Figure 29. Effect of increasing incubation time on glutamine synthetase in crude (●——●), and in Sephadex-filtered (▲——▲) extracts.

Enzyme extracts were prepared from hybrid maize (var. W64A x W182E) harvested at 25 days after pollination.

The assay mixture contained glutamic acid (88 mM), hydroxylamine (6 mM), ATP (5 mM), $MgSO_4$ (20 mM) and EDTA (1 mM), and 0.2 ml of enzyme in a total volume of 1.5 ml.

Formation of γ-glutamyl hydroxamate was monitored at 540 nm.


0.3
0.2
0.1
O.D. 540 nm
0
10
20
30
Incubation Time [min]

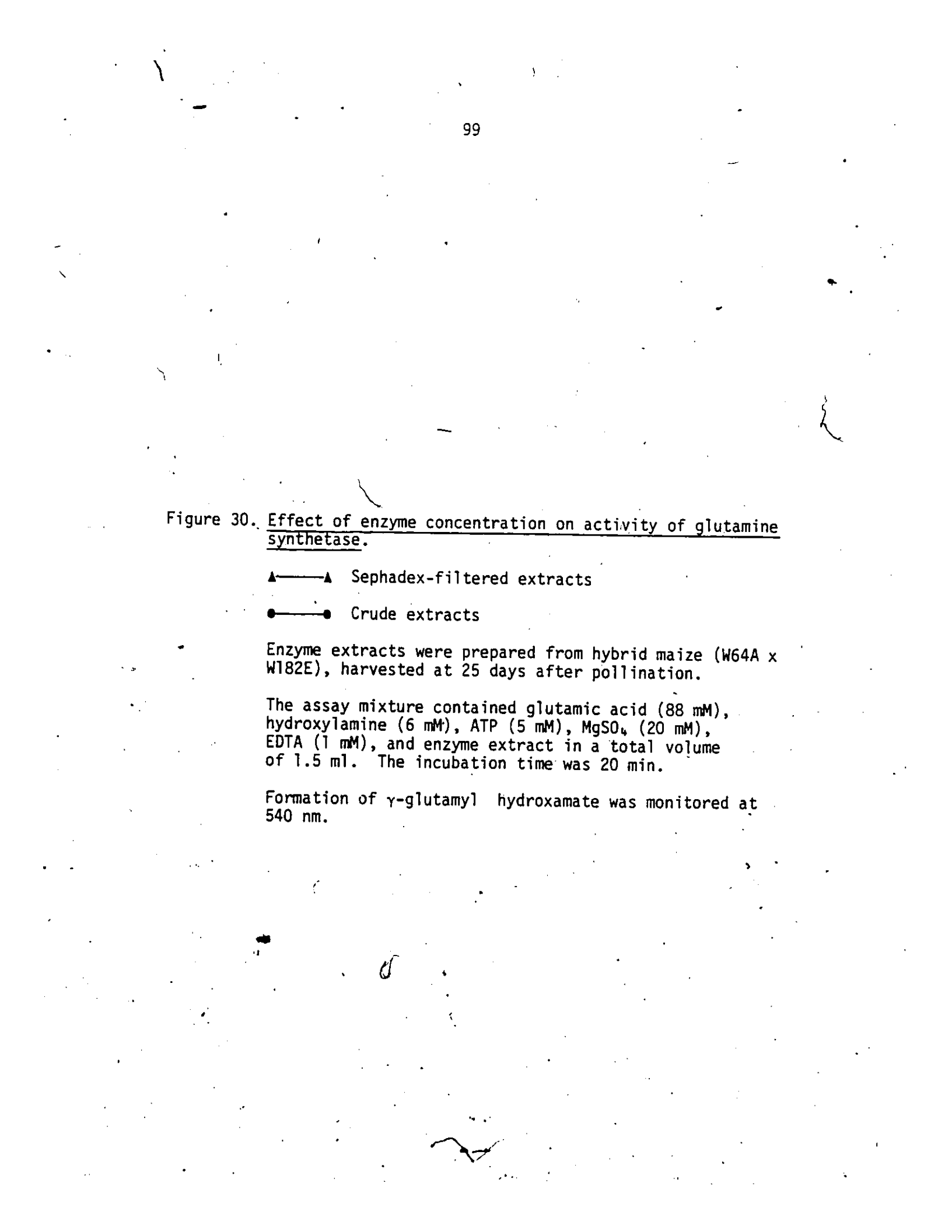


Figure 30. Effect of enzyme concentration on activity of glutamine synthetase.

▲——▲ Sephadex-filtered extracts

●——● Crude extracts

Enzyme extracts were prepared from hybrid maize (W64A x W182E), harvested at 25 days after pollination.

The assay mixture contained glutamic acid (88 mM), hydroxylamine (6 mM), ATP (5 mM), $MgSO_4$ (20 mM), EDTA (1 mM), and enzyme extract in a total volume of 1.5 ml. The incubation time was 20 min.

Formation of γ-glutamyl hydroxamate was monitored at 540 nm.

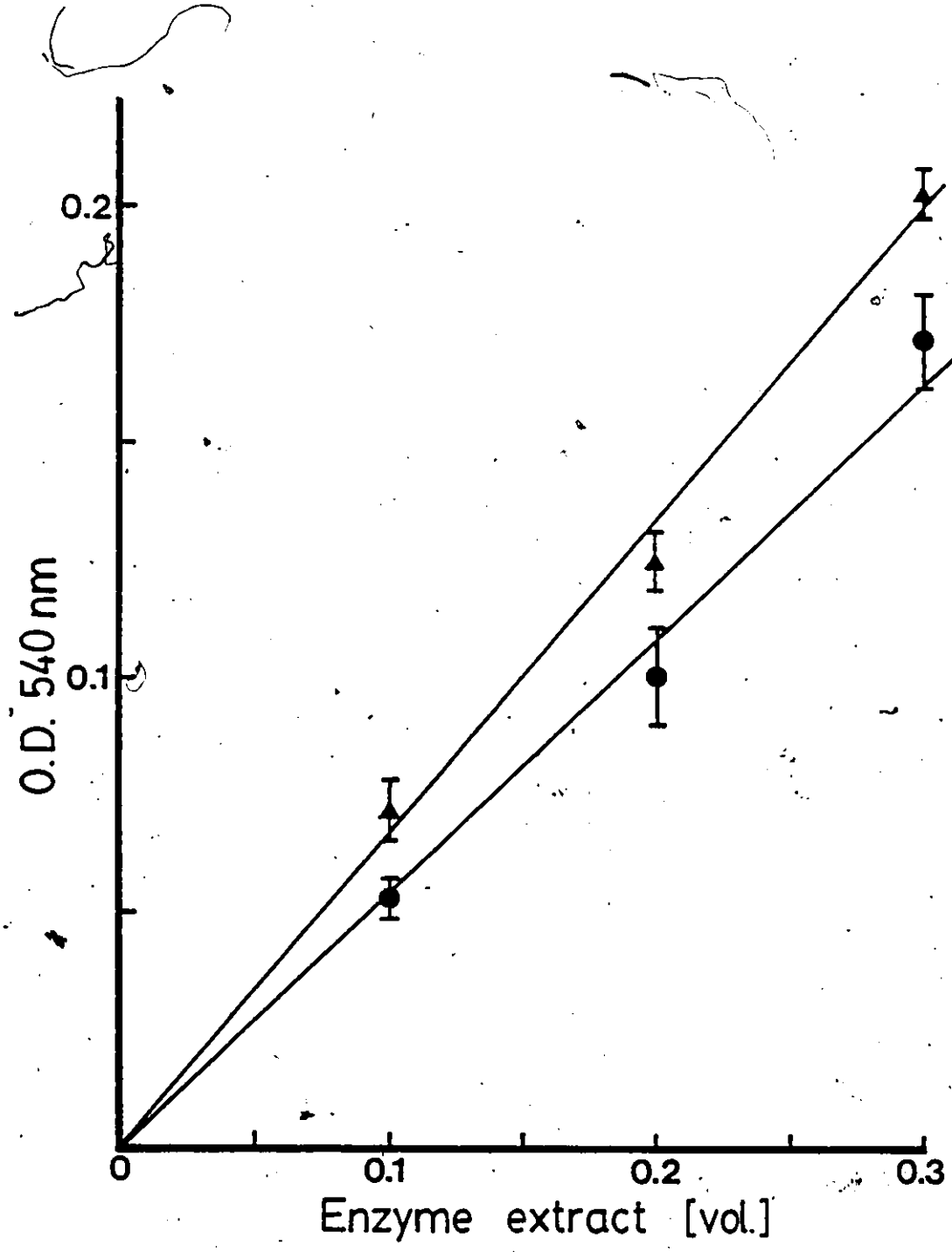

0.2
0.1
O.D. 540 nm
0
0.1
0.2
0.3
Enzyme extract [vol.]

Table 14

Glutamine Synthetase Activity in Crude and Sephadex-filtered Extracts of 30 day old Endosperm of W64A, Opaque-2 and Floury-2

Plant material		Total activity		% Recovery		Activity per endosperm		Specific activity	
		Crude[1]	Sephadex[1]			Crude[2]	Sephadex[2]	Crude[3]	Sephadex[3]
W64A	1	64.95 ± 1.51	75.99 ± 2.91	117.0	1	2.82 ± 0.02	4.42 ± 0.01	1.52 ± 0.4	3.17 ± 0.02
	2	57.8 ± 0.01	90.74 ± 0.02	156.0	2	2.93 ± 0.73	3.63 ± 0.27		
Opaque-2	1	62.21 ± 0.41	118.40 ± 3.6	190.0	1	2.85 ± 0.01	5.21 ± 0.24	1.25 ± 0.02	4.21 ± 0.13
	2	57.06 ± 0.01	104.26 ± 4.79	183.0	2	3.45 ± 0.02	6.57 ± 0.20		
Floury-2	1	49.16 ± 1.11	65.12 ± 1.24	169.0	1	2.59 ± 0.06	4.45 ± 0.08	1.27 ± 0.02	3.63 ± 0.06
	2	84.64 ± 1.54	110.32 ± 2.01	172.0	2	2.83 ± 0.05	4.79 ± 0.09		

[1] µmoles HA·h^{-1}·total volume of extract.

[2] µmoles HA·h^{-1}·$endosperm^{-1}$.

[3] µmoles HA·h^{-1}·mg·$protein^{-1}$.

Data are reported from 2 separate experiments. Three replicates were performed on each treatment. Percent total recovery in all 3 cases was higher after filtration through Sephadex.

activity by monitoring γ-glutamylhydroxamate formation in the presence of NH_2OH. Fig. 31 shows that each extract had only one GS peak. Activity of GS from endosperm was 0.0062 umoles γ-glutamylhydroxamate (HA)·min^{-1}·g·fresh wt^{-1}. Activity of GS from embryo was 0.0071 umoles HA·min^{-1}·g·fresh wt^{-1}.

4. Reactivity of GS towards the anti-GS IgG

Glutamine synthetase, GS_1, is the cytosolic isoform of the enzyme, the plastid form is designated as the GS_2 (Guiz *et al.*, 1979; Mann *et al.*, 1963). Antibodies were raised against GS_1 obtained from etiolated barley leaves. Behaviour of glutamine synthetase towards the anti-glutamine synthetase IgG was examined by immunotitration analysis. Fig. 32 shows the immunotitration analysis of glutamine synthetase from corn endosperm and embryo. The activity in the supernatant decreased with the increasing concentration of the IgG (ranging between 0-50 µl). With 20 µl of the IgG, activity in endosperm extract was completely precipitated. For glutamine synthetase extracts from the embryo, only one concentration of the anti-GS_1 IgG was tried (25 µl). At this concentration of the IgG, the GS activity was completely inactivated. Therefore, the enzyme from both endosperm and embryo was susceptible to precipitation by anti-GS_1 IgG suggesting that each corresponds to the cytosolic form of GS-GS_1.

E. Reproducibility of Enzyme Extraction and Assay Procedures

Table 15 illustrates the reproducibility of extraction and assay procedures of enzymes of nitrogen assimilation, i.e., glutamate

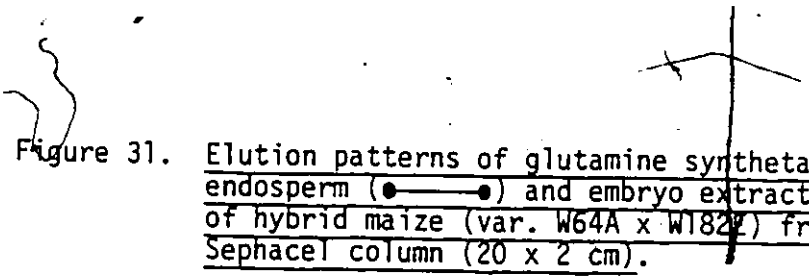


Figure 31. Elution patterns of glutamine synthetase of corn endosperm (●——●) and embryo extracts (o——o) of hybrid maize (var. W64A x W182E) from a DEAE-Sephacel column (20 x 2 cm).

Elution of proteins was performed by progressively mixing 50 ml of 25 mM imidazole-HCl buffer (pH 7.8) containing 1 mM DTT, 1 mM EDTA, 10 mM 2-mercapto-ethanol and 1 mM $MgCl_2$ with 50 ml of the same buffer which also contained 0.5 M NaCl.

Two ml fractions were collected and 200 µl aliquots were assayed.

Maximum activities for GS (endosperm) and GS (embryo) were 0.0062 µmoles and 0.0071 µmoles γ-glutamyl-hydroxamate·min^{-1}·g fresh wt^{-1}, respectively.

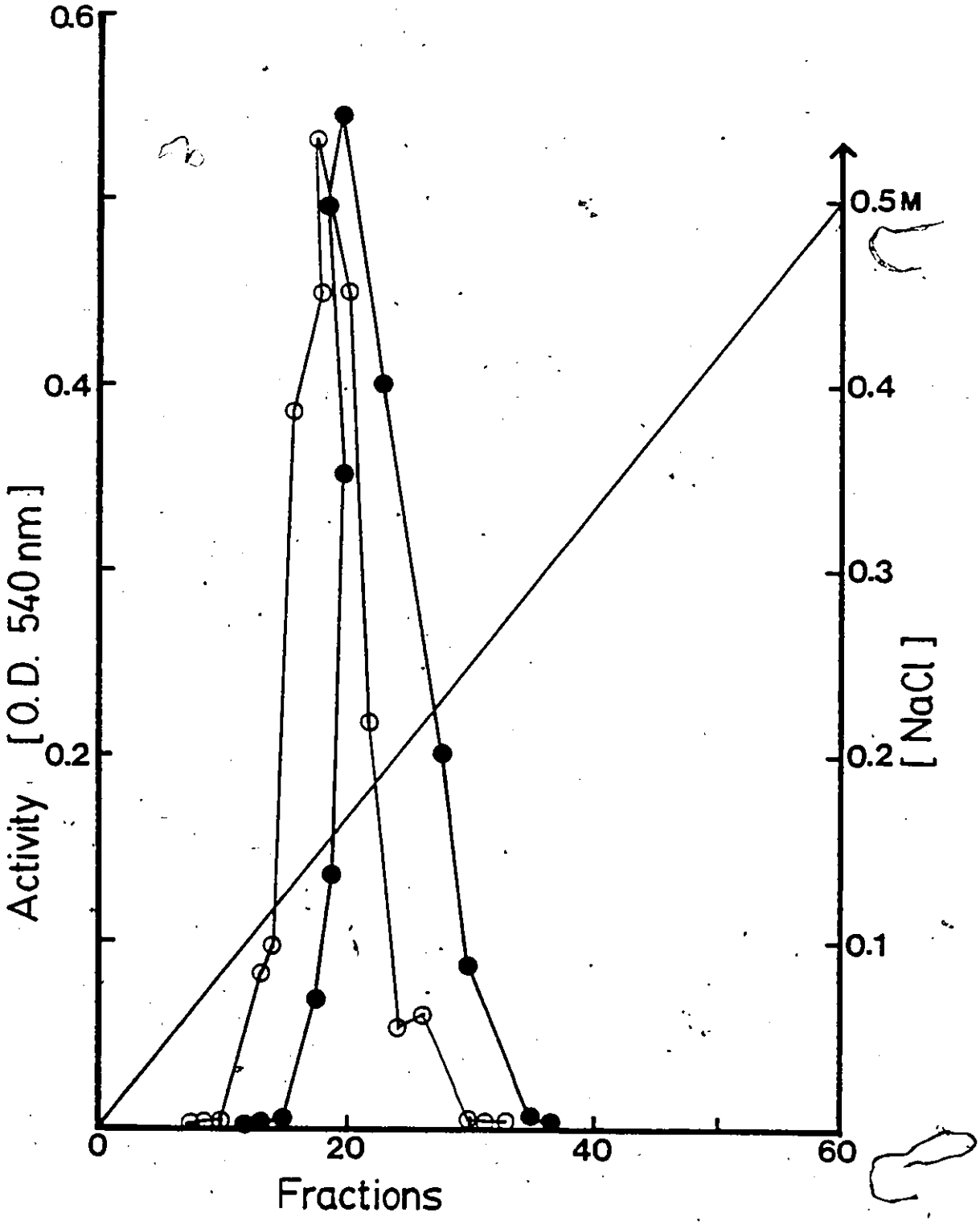
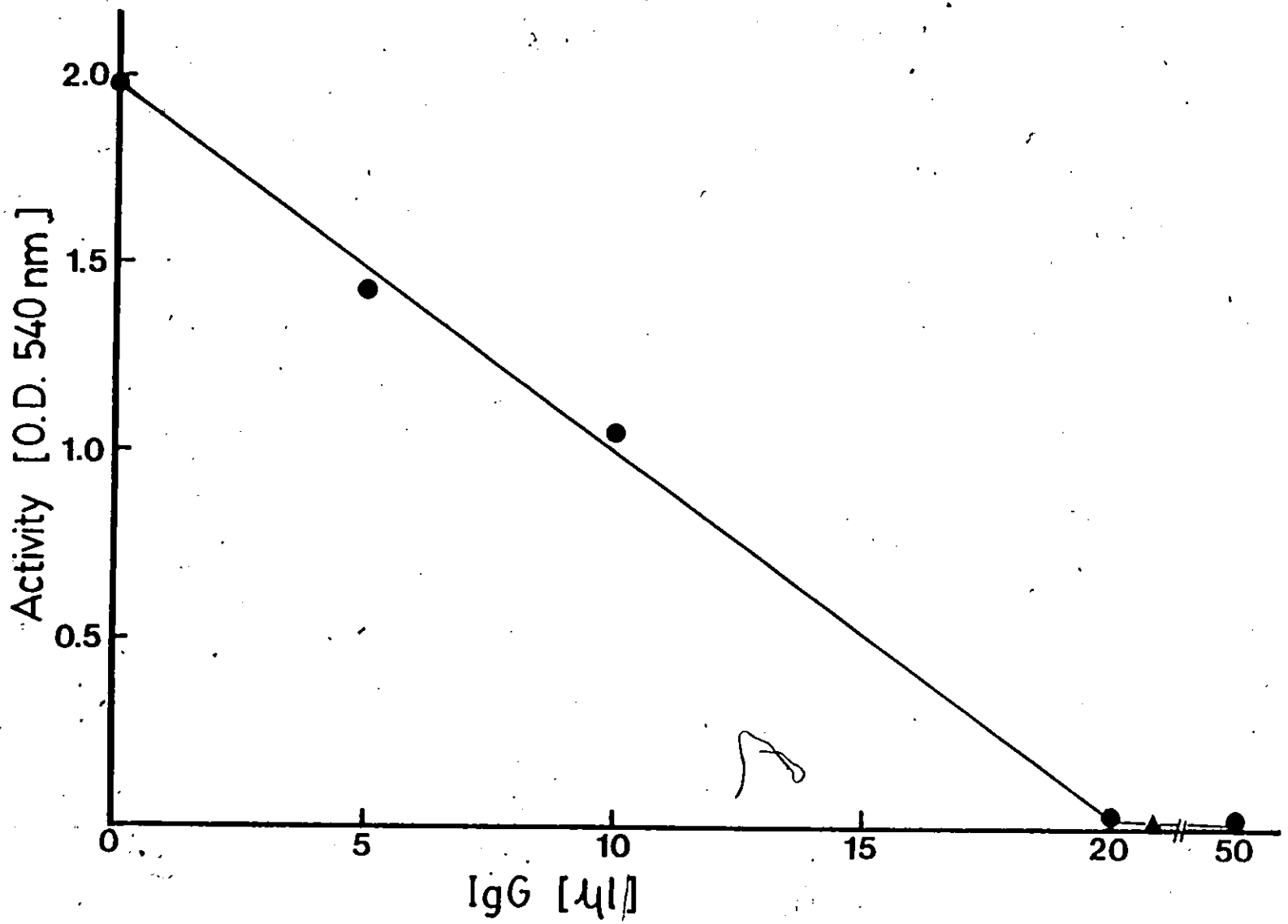

0.6
0.4
0.2
0
20
40
60
Fractions
Activity [O.D. 540 nm]
0.5M
0.4
0.3
0.2
0.1
[NaCl]

Figure 32. Immunotitration curves of glutamine synthetase from endosperm (●——●) and embryo (▲——▲) of developing corn kernels.

The antibody (anti-IgG-GS_1) from etiolated barley leaf (kindly supplied by Dr. B. Hirel) was used. One hundred µl of enzyme was incubated with an increasing amount of anti-GS_1-IgG. For the extract from embryo, only one concentration of antibody (25 µl) was used.


2.0
1.5
1.0
0.5
0
5
10
15
20
50
Activity [O.D. 540 nm]
IgG [µl]

synthase, glutamate dehydrogenase, glutamine synthetase, asparagine synthetase and asparagine in developing endosperm of corn. To check the reproducibility of the enzyme assays, duplicate assays were performed on 3 separate extracts. Values were averaged and percentage variation was calculated as:

$$\frac{\text{mean variation}}{\text{average}} \times 100$$

Standard deviation values were also calculated and are given within the brackets for each enzyme activity. As can be seen from the results, the variability of assays was low and the reproducibility of extraction was also good as judged by the values for 3 separate extracts. The mean variation was within ± 10% of the average activity and standard errors of the mean values were in the range of (±0.056 - ± 0.311). Therefore, the extraction and assay procedures were considered satisfactory.

F. Stability of Enzymes under Storage Conditions (-20°C)

In these experiments, developing kernels were harvested, the endosperm tissue was separated from the embryo, frozen in liquid nitrogen and subsequently stored in freezer bags at -20°C. The stability of the enzymes of nitrogen assimilation as a function of time in storage was checked and the results are illustrated in Table 16. The enzyme activities of glutamate synthase (NADH-dependent), glutamine synthetase, asparaginase, asparagine synthetase and glutamate dehydrogenase were assayed at various time intervals ranging between 0-300 days in storage. Initial enzyme activities were recorded within 5 days of harvesting the endosperm tissue.

Table 15

Reproducibility of Extraction and Assay Procedures of Enzymes of Nitrogen Assimilation in Developing Endosperm in Corn

Material	Enzyme assayed (units)	Extract #	Activity	Average activity ± mean variation	Mean variation as % of average activity
W64A-op-2 20 days post-pollination	GOGAT (nmoles $NADH \cdot min^{-1} \cdot endosperm^{-1}$)	1	25.5 26.5		
		2	30.0 29.0	27.8 ± 1.4 *(± 0.05)	5.3
		3	27.0 29.0		
W64A 15 days post-pollination	GOGAT (same as above)	1	5.5 5.8		
		2	6.0 6.0	6.13 ± 0.5 (± 0.09)	8.3
		3	6.8 7.0		
W64A-op-2 20 days post-pollination	Asparaginase (nmoles $Asp \cdot min^{-1} \cdot endosperm^{-1}$)	1	5.7 5.6		
		2	5.6 5.6	5.70 ± 0.2 (± 0.31)	3.73
		3	6.2 5.2		

Table 15 (continued)

Material	Enzyme assayed (units)	Extract #	Activity	Average activity ± mean variation	Mean variation as % of average activity
W64A-op-2 25 days post-pollination	Glutamate dehydrogenase (nmoles NADH·min^{-1}· endosperm^{-1})	1	29.3 28.7		
		2	28.3 28.9	29.7 ± 1.38 (± 0.06)	4.65
		3	33.4 29.0		
W64A 15 days post-pollination	Glutamate dehydrogenase (same as above)	1	30.37 28.30		
		2	22.3 20.3	26.07 ± 3.00 (± 0.15)	11.5
		3	26.2 29.10		
W64A 20 days post-pollination	Glutamine synthetase (µmoles HA·h^{-1}· endosperm^{-1})	1	5.22 4.22		
		2	5.35 5.76	5.22 ± 0.55 (± 0.1)	10.5
		3	5.99 5.77		
		4	5.77 6.12		
W64A-op-2 10 days post-pollination	Glutamine synthetase (µmoles HA·h^{-1} endosperm^{-1})	1	2.47 2.47		
		2	2.39 2.59	2.495 ± 0.15 (± 0.07)	6.05
		3	2.20 2.85		

Table 15 (continued)

Material	Enzyme assayed (units)	Extract #	Activity	Average activity ± mean variation	Mean variation as % of average activity
W64A-op-2 20 days post-pollination	Asparagine synthetase ($nmoles \cdot min^{-2}0 \cdot endosperm^{-1}$)	1	2.84 2.45	2.677 ± 0.11 (± 0.10)	4.27
		2	3.04 2.84		
W64A 20 days post-pollination	Asparagine synthetase (same as above)	1	2.159 2.46	2.179 ± 0.08 (± 0.08)	3.7
		2	2.19 2.19		

To check the reproducibility of enzyme assay system, duplicate assays were performed on two or three separate extracts.

*Values within brackets represent standard deviation.

Table 16

Stability of GOGAT, Asparaginase, GS, GDH and AS under Storage Conditions (-20°C)

Enzyme (units)	Time in storage after harvest (days)				
	0 - 5	40 - 60	130 - 150	170 - 200	240 - 300
Glutamate synthase (nmoles $NADH \cdot min^{-1} \cdot endosperm^{-1}$)	[a] 10.855 ± 0.361	[a] 10.87 ± 0.01	[a] 10.79 ± 0.4	-	-
	-	9.95 ± 0.75 (W64A, op-2 15 DAP*)	9.5 ± 0.5 (W64A, op-2 15 DAP)	-	5 ± 0.02
Asparaginase (nmoles $ASP \cdot min^{-1} \cdot endosperm^{-1}$)	[a] 5.762 ± 0.166	[a] 5.782 ± 0.015	[a] 5.789 ± 0.09	-	-
	-	-	-	6.13 ± 0.08 (W64A, f1-2 20 DAP)	5.65 ± 0.23 (W64A, f1-2 20 DAP)
Glutamine synthetase (µmoles $HA \cdot hr^{-1} \cdot endosperm^{-1}$)	[a] 7.006 ± 0.16	[a] 7.69 ± 0.078	[a] 7.58 ± 0.11	-	-
	-	-	5.22 ± 0.25 (W64A, 20 DAP)	-	5.98 ± 0.7 (W64A, 20 DAP)
Glutamate dehydrogenase (nmoles $NADH \cdot min^{-1} \cdot endosperm^{-1}$)	[a] 21.05 ± 0.963	[a] 22.97 ± 1.67	[a] 20.95 ± 1.53	-	-
	-	42.00 ± 2.0 (W64A, 20 DAP)	41.88 ± 0.05 (W64A, 20 DAP)	-	32.36 ± 0.05 (W64A, 20 DAP)
Asparagine synthetase (nmoles $ASN \cdot min^{-20} \cdot endosperm^{-1}$)	-	2.485 ± 0.01 (W64A, 20 DAP)	2.71 ± 0.23 (W64A, 20 DAP)	-	1.2 ± 0.08 (W64A, 20 DAP)

Table 16 (continued)

[a] Corn kernels (W64A x W182E) were planted in a soil, peat, sand and manure mixture (3:2:1:1) and were grown in growth chambers at 26°C with a 16 hr light period. Developing seeds were harvested at 22 days after pollination. The embryos were removed and the endosperms were used either directly or were frozen in liquid nitrogen and subsequently stored at -20°C until further use. Hybrid kernels (W64A x W182E) were used unless otherwise indicated. W64A, opaque-2 and floury-2 samples were from field-grown plants. Storage conditions were the same as for hybrid seeds.

* DAP = days after pollination

Results show that activity of each of these enzymes was stable within 150 days in storage. After this period, the activity of NADH-glutamate synthase and asparagine synthetase started to decline and by 300 days, activity of these enzymes was reduced by 50% relative to the initial activity. Glutamate dehydrogenase activity declined by only 25%, whereas asparaginase and glutamine synthetase activities remained fairly constant. Since all the enzyme activities remained constant for at least 5 months of storage, it is safe to compare enzyme activities within this time period.

II. Changes in Nitrogen Fractions in Developing Endosperm

Before comparing the activities of enzymes of nitrogen assimilation in the normal and the mutant varieties, it was necessary to define the system in terms of basic changes occurring in total protein, soluble α-amino nitrogen, and amounts of NH_4^+. The action of the enzymes involved in the primary assimilation of nitrogen were also examined in the endosperm of a hybrid (W64A x W182E) and an inbred line (W64A). It proved difficult to get good seed set with W64A under the growth chamber conditions (as described in Methods). As a result, much of the initial enzyme work was done with the hybrid variety. Samples of developing endosperm were analyzed at 5,10,15, 20,25 and 30 days after pollination. During this period, the dry weight of the endosperm increased about 10-fold in each variety (Table 17).

A. Changes in Levels of Proteins

The results of Fig. 33a illustrate the changes in level of

Table 17

Changes in Dry Weight of Developing Endosperm of Hybrid Maize (W64A x W182E) and Inbred Maize (W64A)

Days after pollination	Average dry weight ($mg \cdot endosperm^{-1}$)	
	W64A x W182E*	W64A*
10	10.5	10
15	32	18
20	53.7	45
25	95.8	70
30	105	110
40	-	120

A sample of 25 endosperms were analyzed at each stage of development. The samples were lyophilized and the dry weight of each sample was recorded. The average dry weight of the endosperm ($mg \cdot endosperm^{-1}$) was obtained by dividing the total dry weight by the number of endosperms.

*The hybrid variety (W64A x W182E) was grown in the growth chamber and the inbred variety (W64A) was field grown.

Figure 33a. Changes in protein fractions and total nitrogen levels during development in the maize endosperm var. (W64A).

T = total nitrogen

Z = zein nitrogen

A = albumin + globulin

T/2 = total nitrogen values are reduced by 1/2.

AX10 = protein (albumin + globulin) values are increased 10-fold.

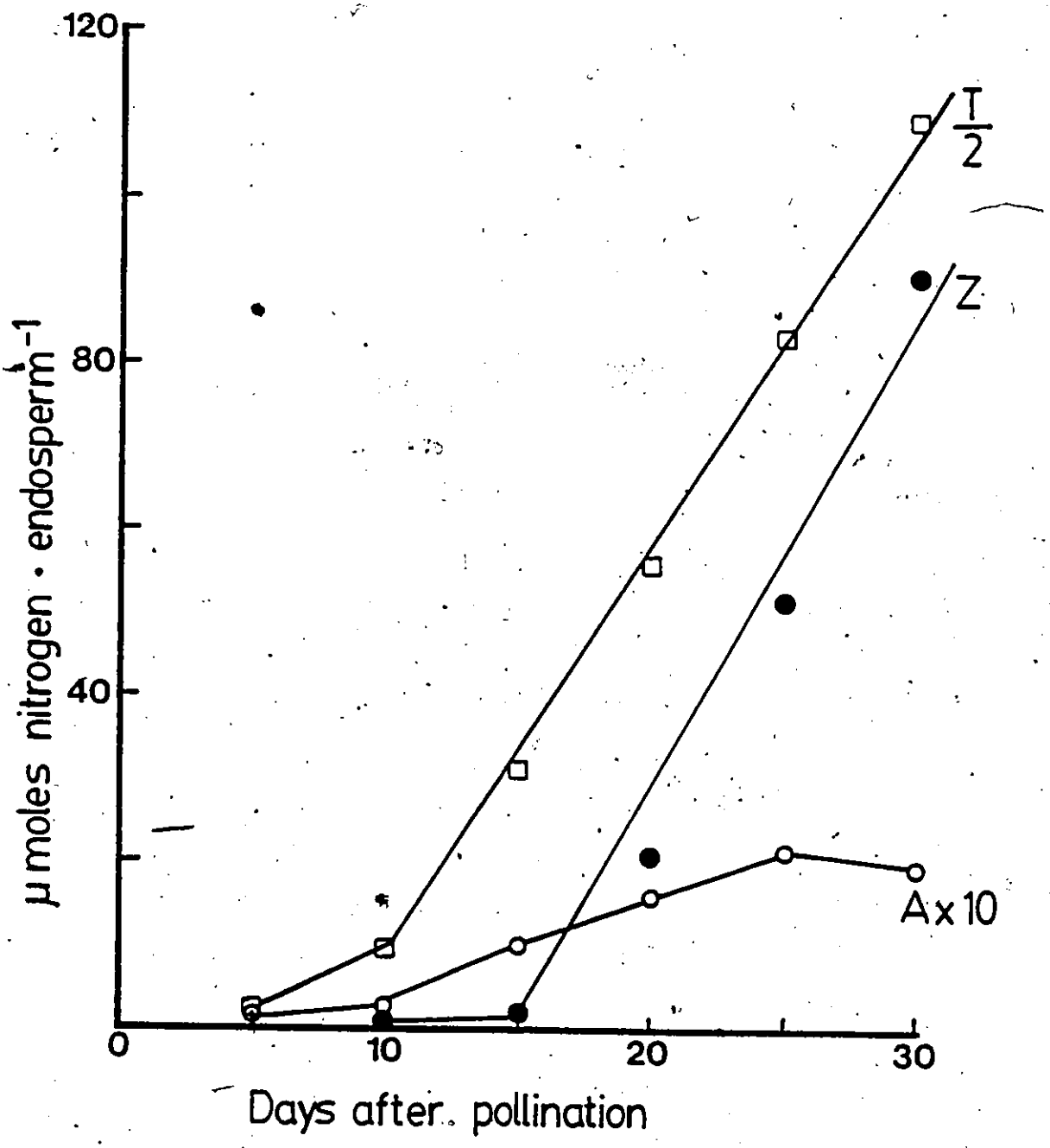

120
80
40
0
10
20
30
μ moles nitrogen · endosperm⁻¹
Days after pollination
T/2
Z
A x 10

Figure 33b. Changes in protein fractions during development in corn endosperm (W64A x W182E).

T = total nitrogen

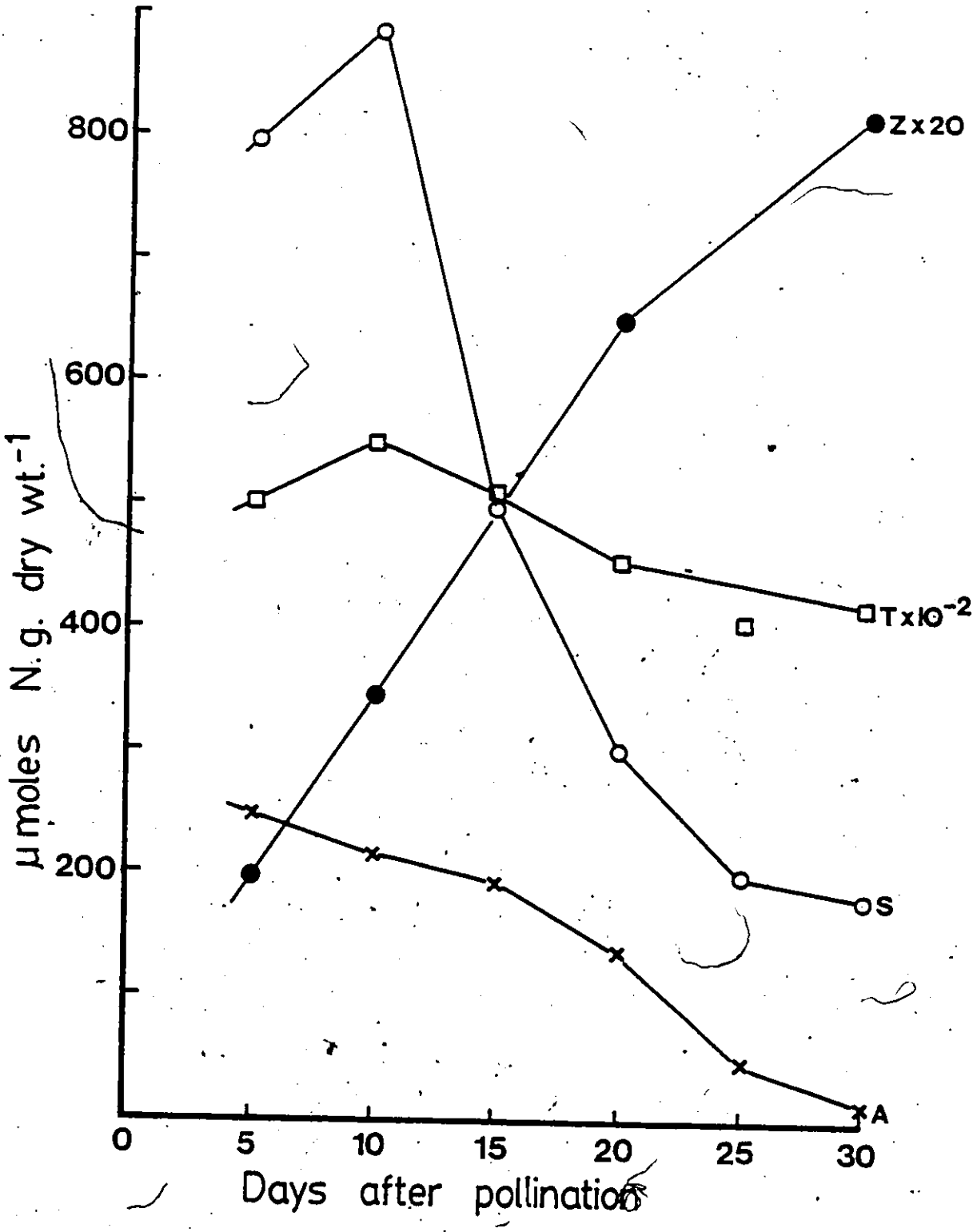
Z = zein nitrogen

S = soluble nitrogen

A = albumin

Results are expressed on a dry weight basis (μmoles N·g·dry wt^{-1}).

Adapted from Oaks *et al.* (1979).


800
600
400
200
0
5
10
15
20
25
30
µ moles N.g. dry wt.$^{-1}$
Days after pollination
Z x 20
T x 10^{-2}
S
A

endosperm proteins. There was a major shift in protein synthesis between day 10 and day 15. The accumulation of total nitrogen and zein was initiated at 10 and 15 days after pollination, respectively. There was an increase in these two components throughout the experiment. The soluble proteins also showed an abrupt increase 15 days after pollination but no large change after that. The trends observed are very similar to those observed in hybrid corn (Oaks *et al.*, 1979) and also agree with results of Murphy and Dalby (1971) and Tsai (1979).

In Fig. 33b, the changes in level of various proteins are expressed on a dry weight basis. The level of soluble proteins decline rapidly as the endosperm matures. Zein is the only protein that increases with time relative to increase in dry weight. Since the initiation of zein synthesis represents a major metabolic shift in the process of endosperm development, Oaks *et al.* (1979) suggested that it could be used as a marker for examining changes in the levels of specific enzymes in the developing tissue, i.e., enzymes of amide metabolism and those of reassimilation of ammonia.

B. Changes in the Level of Soluble Amino Acids

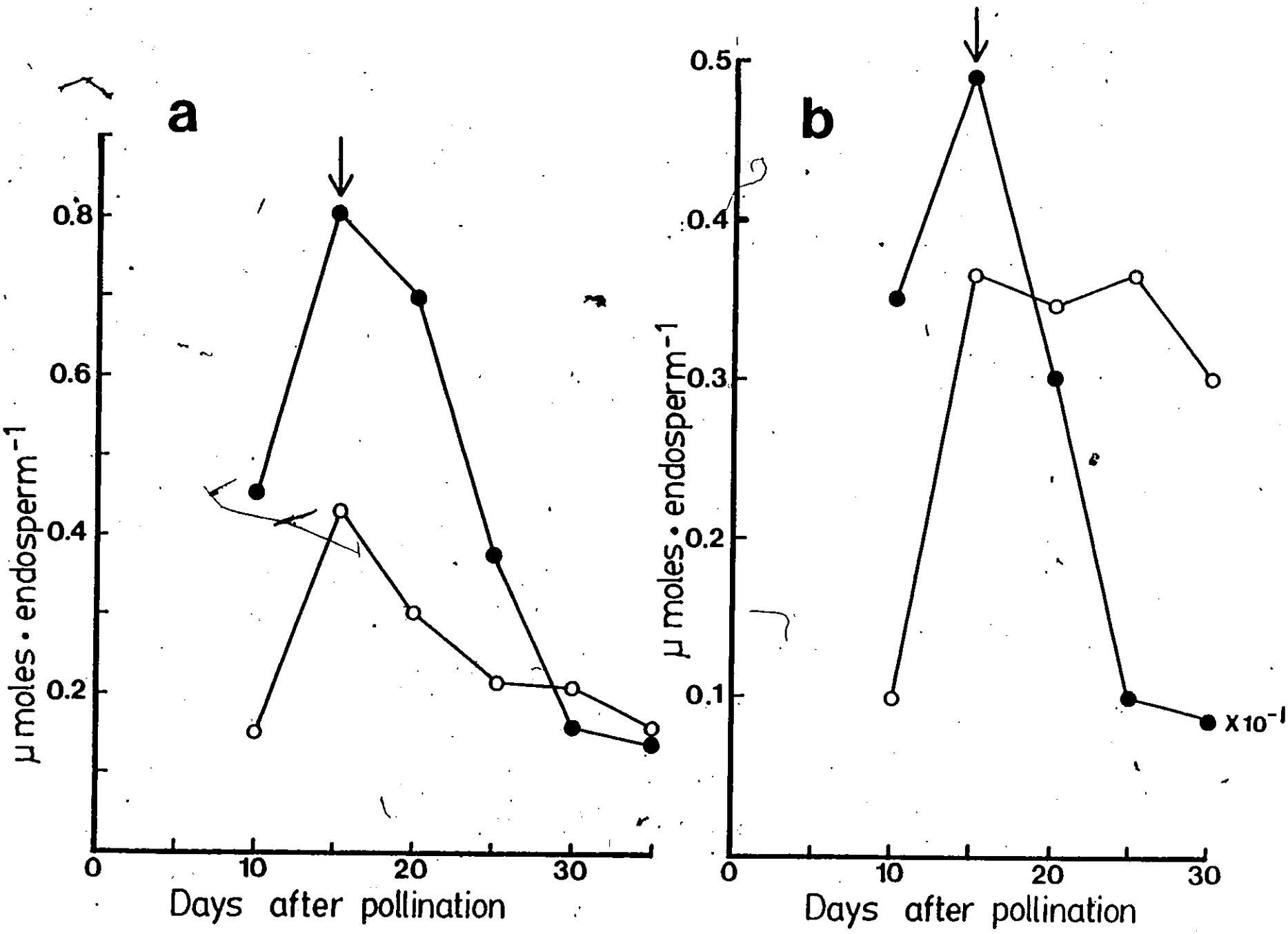
Fig. 34 (a,b) shows the changes in level of free glutamine, glutamate, asparagine and aspartate in the endosperm with age. The levels of soluble glutamine and glutamate started to increase at about 10 days after pollination. Thereafter, the level of glutamate remained high, whereas levels of glutamine declined rapidly. Levels of soluble asparagine and aspartic acid also started to increase at

Figure 34 (a,b). Changes in the level of free amino acid content in the developing endosperm (var. W64A).

Arrow indicates the onset of zein biosynthesis.

a. Changes in the level of asparagine (●——●) and aspartic acid (o——o).

b. Changes in the level of glutamine (●——●) and glutamic acid (o——o).


a
b
0.8
0.6
0.4
0.2
0.5
0.4
0.3
0.2
0.1
µ moles • endosperm⁻¹
0
10
20
30
Days after pollination
X 10⁻¹

10 days after pollination, peaked at about 15 days and then declined. Asparagine levels were twice those of aspartate up to 20 days after pollination. Almost equal levels of asparagine and aspartate were found after that time. In each case, the concentration of glutamine was about 5 to 10 times higher than other amino acids. The maximum concentration of glutamine in the endosperm was 30 mM (15 days after pollination) and that of asparagine was 9 mM (20 days after pollination). The disappearance of these amino acids at later stages of development reflects either a relatively faster incorporation into proteins, a faster rate of catabolism or a lower rate of supply.

C. Accumulation of Ammonia

Ammonia levels increased abruptly about day 10, reached a maximum level at day 20 and then declined. Similar accumulation of ammonia is observed in the hybrid variety (Oaks *et al.*, 1979). In each variety, the accumulation of ammonia preceded the maximum accumulation of albumin and the peak in endopeptidase activity (Fig. 35).

D. Changes in Levels of Enzymes of Nitrogen Assimilation

The activities of enzymes involved in nitrogen assimilation, i.e., glutamate synthase, glutamine synthetase, glutamate dehydrogenase, asparagine synthetase, asparaginase and asparagine-transaminase appear to rise just prior to or about the time zein biosynthesis is initiated in the endosperm (Fig. 36). The levels of these enzymes were significantly higher at a time when rapid synthesis of zein

Figure 35. Changes in NH_4^+, albumin and endopeptidase levels in developing endosperm of inbred (W64A).

x——x albumin (mg protein·endosperm^{-1} , values increased 7-fold)

□——□ NH_4^+ (µmoles N·endosperm^{-1}, values multiplied x 10)

●——● endopeptidase (trypsin equivalents·min^{-1}·endosperm^{-1}).

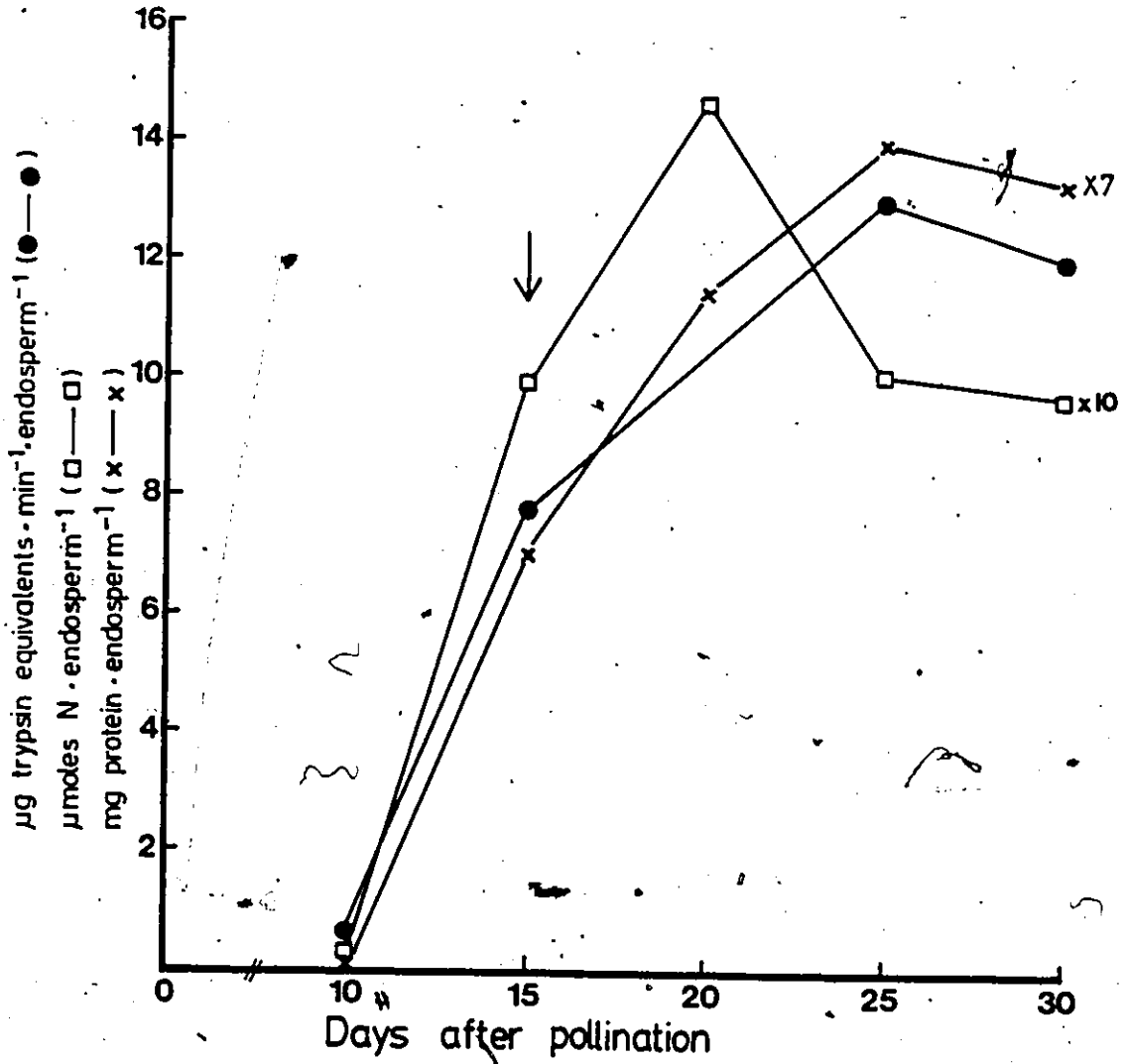

µg trypsin equivalents · min⁻¹ · endosperm⁻¹ (●—●)
µmoles N · endosperm⁻¹ (□—□)
mg protein · endosperm⁻¹ (×—×)
16
14
12
10
8
6
4
2
0
10
15
20
25
30
Days after pollination
X7
X10

Figure 36. Changes in the level of enzymes of nitrogen assimilation during endosperm development (var. W64A).

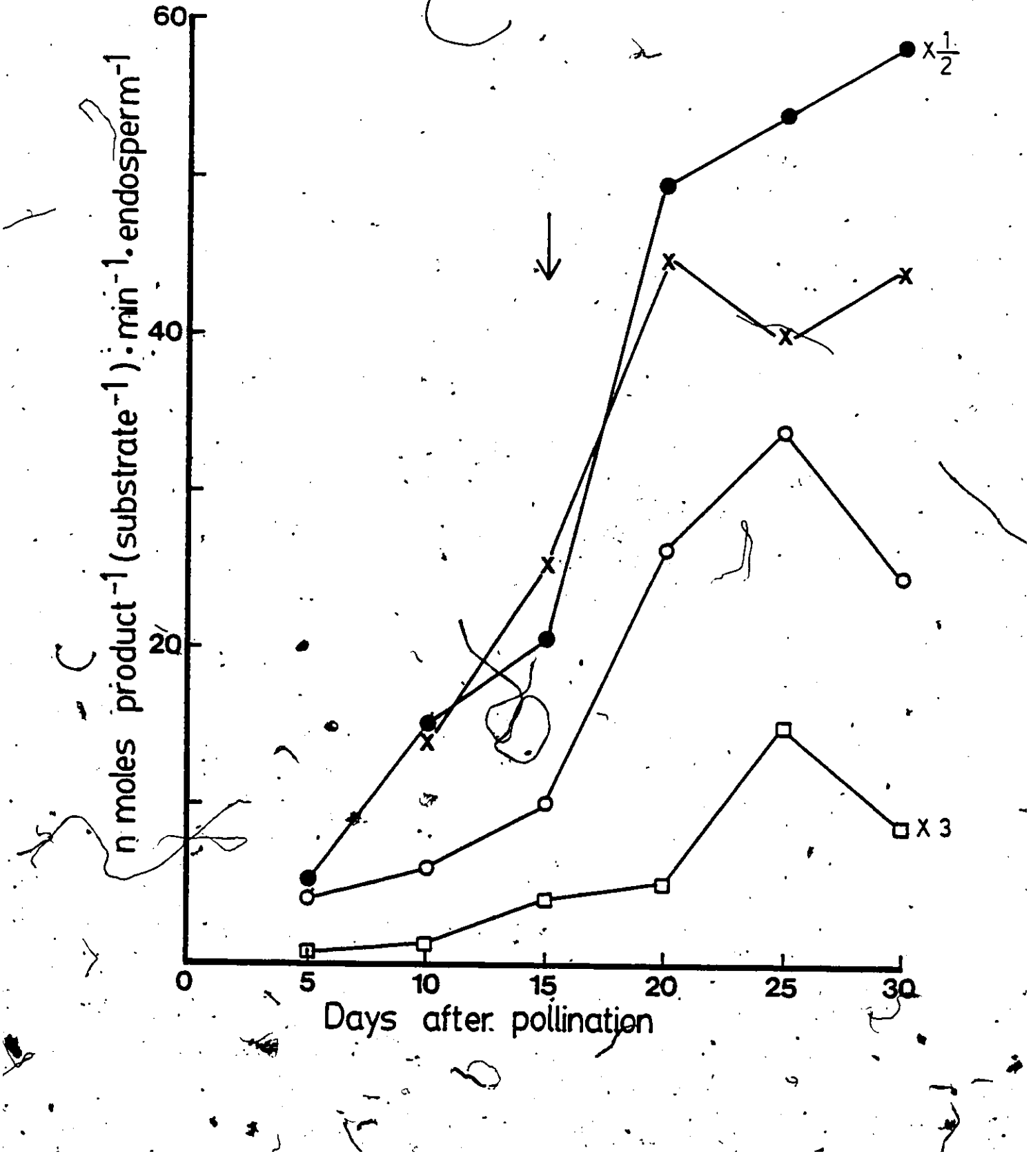
Arrow indicates the onset of zein biosynthesis.

o——o glutamate synthase

●——● glutamine synthetase (values reduced by 1/2)

□——□ asparagine synthetase (values multiplied X 3)

x——x glutamate dehydrogenase.


60
40
20
0
5
10
15
20
25
30
n moles product⁻¹ (substrate⁻¹) · min⁻¹ · endosperm⁻¹
Days after pollination
X 1/2
X 3

takes place in the endosperm. Table 18 illustrates the relative levels of enzyme in the inbred and hybrid variety of maize at 20 days after pollination. All enzyme assays were performed on Sephadex G-75 filtered extracts. Glutamate synthase, glutamate dehydrogenase and glutamine synthetase were present in high amounts in the endosperm of each variety. Activity of glutamine synthetase was 2- to 3-fold higher than glutamate synthase activity in each variety.

The peak of glutamate synthase activity is reached 5-10 days after the peak accumulation of glutamine and coincides with the period of active zein accumulation. An enzyme capable of hydrolyzing glutamine in a reaction similar to that of asparaginase was not detected in the endosperm tissue.

1. Changes in levels of NADH- and Fd-GOGAT

Table 19 shows the changes in level of NADH- and ferredoxin-dependent glutamate synthase activity with developmental stages. Almost equal levels of activity of these two enzymes were present in the endosperm at each stage of development examined. At day 15, the level of each enzyme was low in the endosperm (3.02 nmoles glu·min^{-1}·endosperm^{-1} and 3.62 nmoles glu·min^{-1}·endosperm^{-1} for NADH and Fd-GOGAT, respectively). Thereafter, a parallel increase in activity of each enzyme was observed and levels per endosperm rose about 5-fold between day 15-20. Thus, equal levels of activity during endosperm development suggest that either of these enzymes could be important in glutamine degradation in developing endosperm. In fact, the lower Km values for glutamine and α-ketoglutarate in the ferredoxin-dependent

Table 18

Enzymes of Nitrogen Assimilation in Developing Endosperm

Enzyme	µmoles product (or substrate)·mg protein^{-1}. h^{-1}	
	I*	II
Glutamate synthase:		
1. NADH-dependent	1.54	0.90
2. Fd-dependent	1.02	-
Glutamate dehydrogenase	1.82	2.64
Glutamine synthetase	4.08	3.17
Asparagine synthetase	0.020	0.015
Asparaginase	0.164	0.33
Asparagine transaminase:		
1. Asn-α-ketoglutarate	0.200	-
2. Asn-pyruvate	0.220	-

Samples were harvested 20 days after pollination.

All enzyme assays were performed on Sephadex G-75 filtered extracts.

I* hybrid var. (W64A x W182E)

II inbred var. (W64A).

Table 19

NADH and Fd-dependent GOGAT activities in Endosperm with Stages of Development

Days post-pollination	nmoles glu·min^{-1}·endosperm^{-1}	
	NADH-GOGAT	Ferredoxin-GOGAT
15	3.02	3.63
20	15.8	10.03
25	13.0	10.00
30	16.0	12.13

Samples of hybrid maize (W64A x W182E) were harvested at various times after pollination.

Enzyme assays were performed on Sephadex G-75 filtered extracts.

GOGAT reaction suggest that if reduced ferredoxin was available, this could be the physiologically more important reaction.

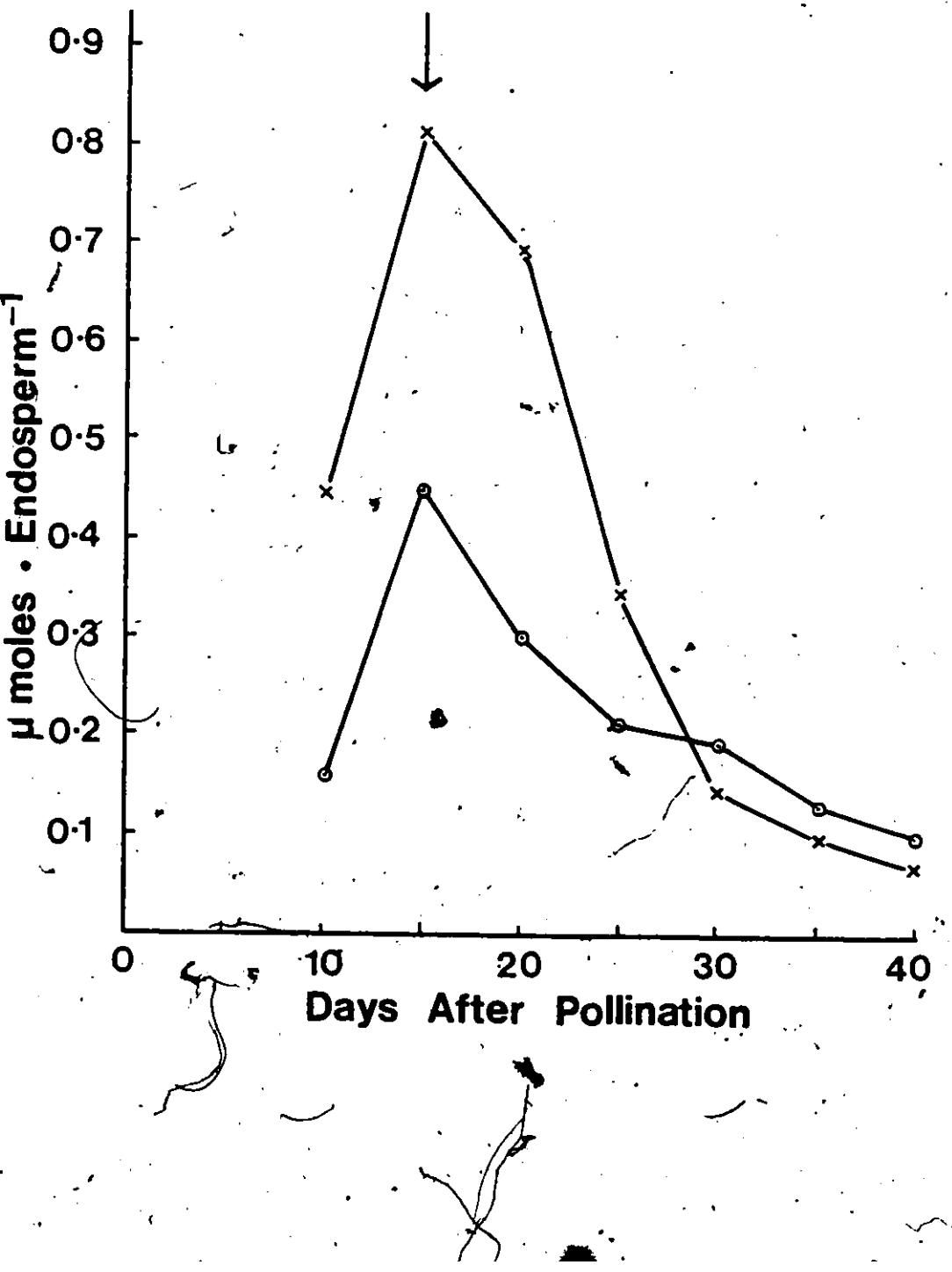
2. Asparagine degrading enzymes

Two known routes of breakdown of asparagine in higher plant tissues are: hydrolytic deamidation of asparagine catalyzed by the enzyme asparaginase (Wriston and Yellin, 1973) and the direct transamination of the amino group of asparagine to a ketoacceptor (Bauer *et al.*, 1977; Streeter, 1977; Ireland and Joy, 1981). Activity of both enzymes could also be detected in the developing endosperm of corn. Fig. 37 shows the changes in activity of asparaginase with age. Asparaginase activity was apparent 5 days after pollination, increased until 25 days post-pollination and then declined. The maximum asparaginase activity per endosperm was 0.349 $\mu mol \cdot asp \cdot h^{-1}$. Asparaginase activities were lower than GOGAT activities when maximal activities were compared under optimum assay conditions. The cereal endosperms appear to have much less asparaginase than legume cotyledons (Sodek *et al.*, 1980). The maximum asparaginase activity in developing pea seed is in the order of 3.6 $\mu moles \cdot h^{-1} \cdot seed^{-1}$ in the testa tissue and 1.2 $\mu mole \cdot h^{-1} \cdot seed^{-1}$ in the cotyledons.

Asparagine transaminase activity was found with both pyruvate and α-ketoglutarate as amino acceptors. During development, the level of asparagine-pyruvate transaminase activity varied and showed a maximum at 15-20 days after pollination (Fig. 38). The asparagine-α-ketoglutarate transaminase activity was somewhat higher initially.

Figure 37. Changes in activities of asparaginase (o——o) and glutamate synthase (x——x) during development in the maize endosperm (W64A).

Arrow indicates the onset of zein synthesis. For all assays Sephadex G-75 filtered extracts were used, assay conditions were as described in Methods.


0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
µ moles • Endosperm⁻¹
0
10
20
30
40
Days After Pollination

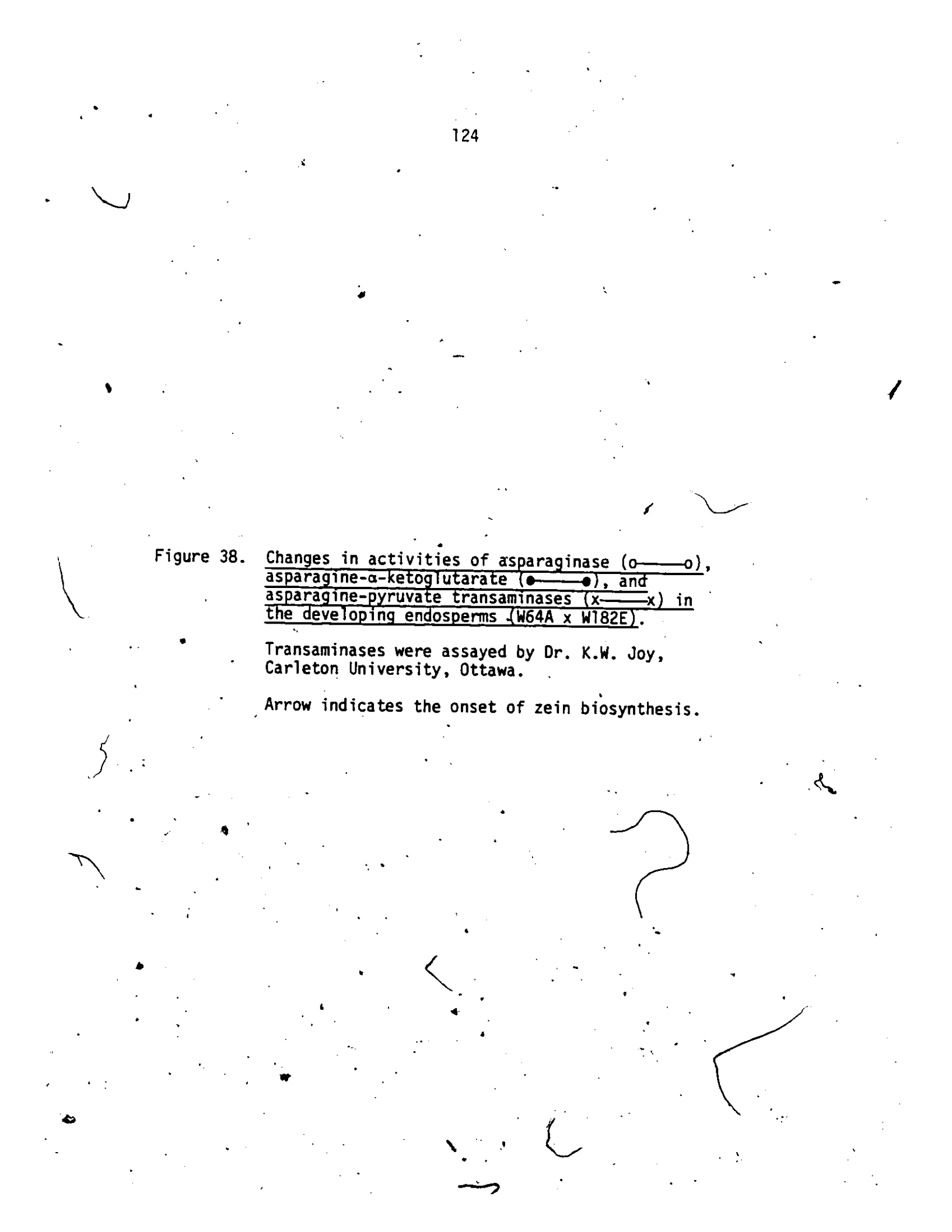
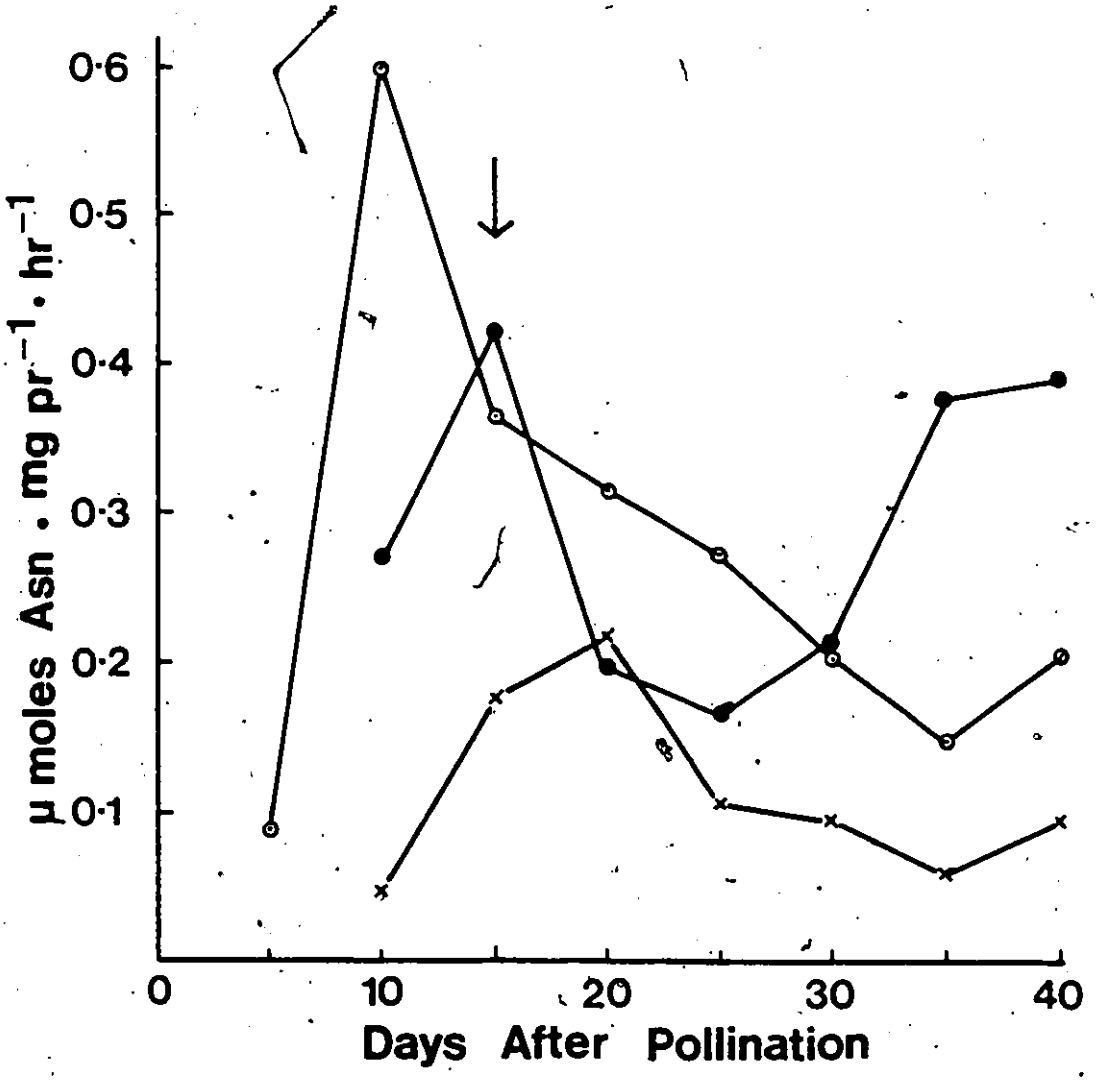


Figure 38. Changes in activities of asparaginase (o——o), asparagine-α-ketoglutarate (●——●), and asparagine-pyruvate transaminases (x——x) in the developing endosperms (W64A x W182E).

Transaminases were assayed by Dr. K.W. Joy, Carleton University, Ottawa.

Arrow indicates the onset of zein biosynthesis.


0·6
0·5
0·4
0·3
0·2
0·1
μ moles Asn . mg pr^{-1} . hr^{-1}
0
10
20
30
40
Days After Pollination

The different pattern of activities during endosperm development suggests that separate proteins could be responsible for the two activities. The peaks in these activities (Asn-α-ketoglutarate transaminase, 4 nmoles Asn·mg protein^{-1}·h^{-1}, Asn-pyruvate transaminase, 3.5 nmoles Asn·mg protein^{-1}·h^{-1} at 15 and 20 days after pollination, respectively) were later than the peak for asparaginase activity (5.8 nmoles Asn·mg protein^{-1}·h^{-1} at 10 days after pollination). The asparagine-transaminase activities were substantially lower than some of the other transaminases. For example, it was only a few percent of the glutamate-pyruvate or glutamate-oxaloacetate transaminase activity (Table 20). Unlike developing pea cotyledons (Ireland and Joy, 1981), the levels of the asparagine transaminase in maize endosperm tissue were of the same order of magnitude as those of asparaginase.

In extracts from freshly harvested endosperm, the Km for asparagine (in the asparagine-α-ketoglutarate transaminase reaction) was in the range of 4-9 mM. Some activity appeared to be lost during storage of endosperm at -20°C and it was subsequently found that the Km for asparagine increased during storage. For example, after 3 months of storage, Km for asparagine was 10 mM. As endosperm samples had been frozen for some weeks before assay, it is likely that the value presented in Fig. 38 is an underestimate of the levels which would have been measured in the fresh tissue.

The approximate concentration of free asparagine in the endosperm tissue was found to be 9 mM. Thus, although asparaginase and

Table 20

Transaminase Activity in Developing Maize Endosperm Tissue

Transaminase reaction	Age (days after pollination)		
	21	30	32
	Activity ($nmoles \cdot h^{-1} \cdot endosperm^{-1}$)		
Asn → α-ketoglutarate	223	315	229
Asn → pyruvate	241	-	77
Glu → pyruvate	25,600	27,530	18,510
Asp → α-ketoglutarate	11,637	14,210	7,890
Asp → pyruvate	680	-	215
Asparaginase	164	-	58

Sephadex-treated extracts were used in all assays and the assays were analyzed by amino acid analyzer. For asparaginase, K^+ was present throughout the enzyme extraction procedure. (W64A x W182E, growth chamber-grown seed.)

asparagine transaminase are present in the developing endosperm, their low level of activity, combined with high Km for asparagine (~10 mM) suggest that they are probably not capable of efficiently degrading the endosperm asparagine.

III. Comparative Study of Inbred W64A and its Mutants Opaque-2 and Floury-2

A. Nitrogen Content in Developing Endosperm

1. Dry matter accumulation and total nitrogen content

The dry weight and total nitrogen of the normal (W64A), opaque-2 and floury-2 endosperms are shown in Fig. 39A,B. The changes were recorded at 5 day intervals for 40 days after pollination. Dry weights and total nitrogen contents in the three genotypes were similar over the initial 25 day period. However, after this time, the normal endosperm had more dry weight, and total nitrogen than the mutants. At 40 days post-pollination, dry weights of the endosperms were 137 mg, 128 mg, and 124 mg in normal, floury-2 and opaque-2, respectively. The rate of nitrogen accumulation calculated on a daily basis during the period of rapid accumulation of nitrogen was 10.7 μmoles·N·day^{-1}·endosperm^{-1}, 9.2 μmoles·N·day^{-1}·endosperm^{-1} and 7.0 μmoles·N·day^{-1}·endosperm^{-1} in normal, floury-2 and opaque-2, respectively.

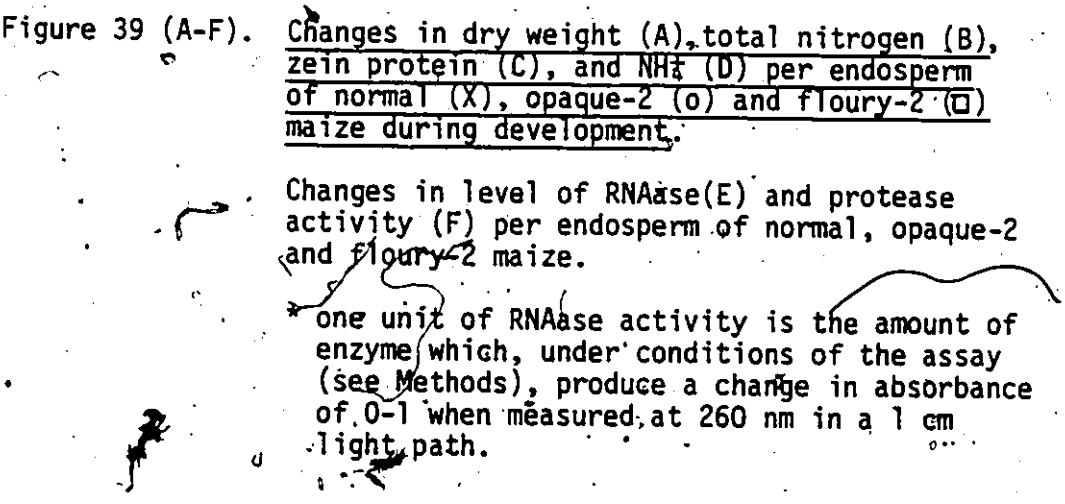
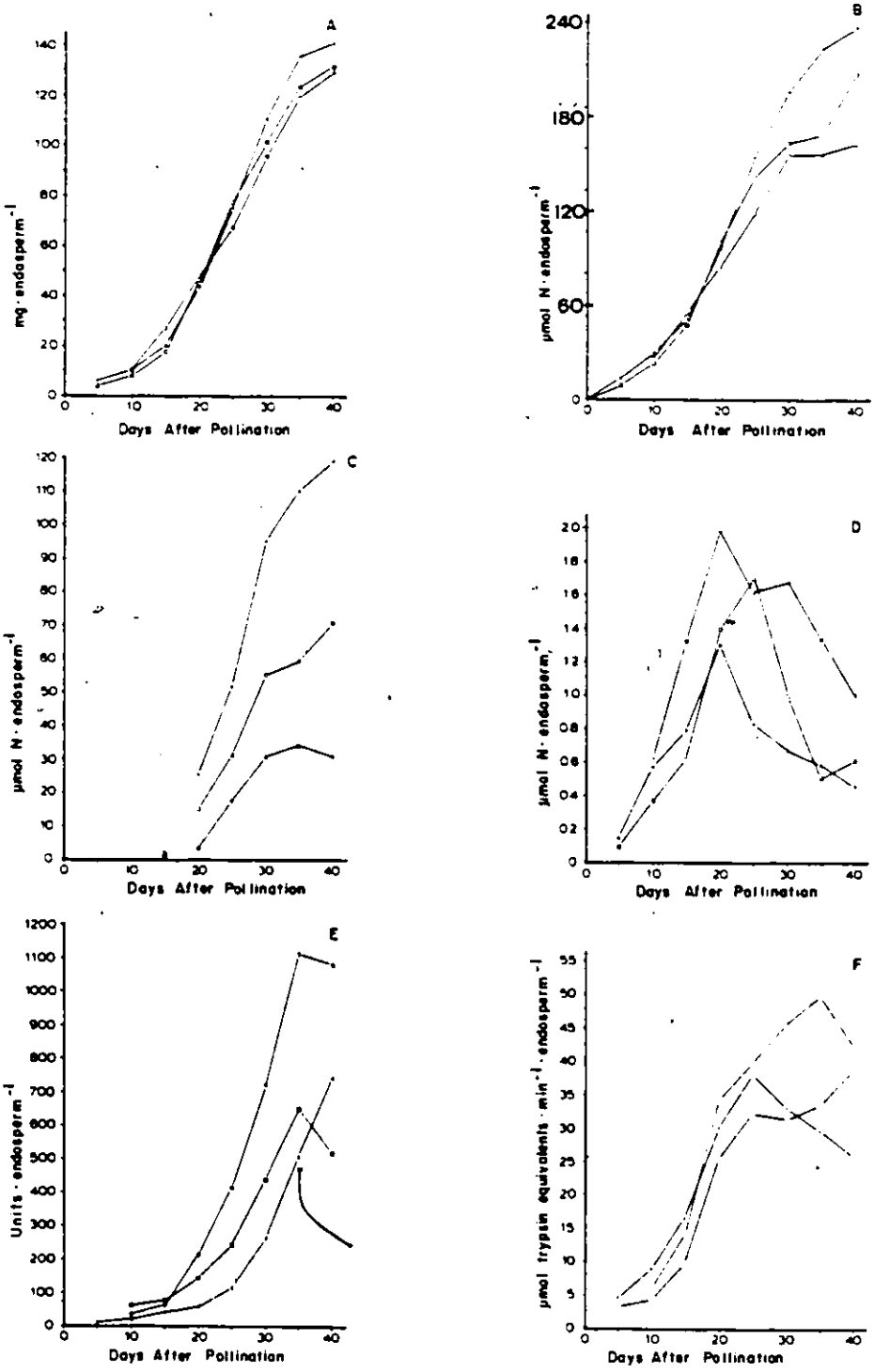


Figure 39 (A-F). Changes in dry weight (A), total nitrogen (B), zein protein (C), and NH_4^+ (D) per endosperm of normal (X), opaque-2 (o) and floury-2 (□) maize during development.

Changes in level of RNAase(E) and protease activity (F) per endosperm of normal, opaque-2 and floury-2 maize.

* one unit of RNAase activity is the amount of enzyme which, under conditions of the assay (see Methods), produce a change in absorbance of 0-1 when measured at 260 nm in a 1 cm light path.


A
mg · endosperm⁻¹
Days After Pollination
B
240
180
120
60
μmol N · endosperm⁻¹
Days After Pollination
C
μmol N · endosperm⁻¹
Days After Pollination
D
μmol N · endosperm⁻¹
Days After Pollination
E
Units · endosperm⁻¹
Days After Pollination
F
μmol trypsin equivalents · min⁻¹ · endosperm⁻¹
Days After Pollination

2. Zein nitrogen

A modified method of Dalby (1974) was used to extract zein protein from dry powders of lyophilized endosperms. In this method, the zein is first extracted by treating dry powder with 70% ethanol + 1 mM 2-mercaptoethanol. Any contaminants in this extract are sequentially removed by extraction in a series of solvents. The reproducibility of extraction by this procedure was examined.

Table 21 shows the results obtained in two replicate determinations made on two separate extractions of endosperm powder of normal and opaque-2 varieties. The values were averaged and percent variation calculated. The variability between replicate samples was 2-3% for both samples. The method of extraction was, therefore, considered satisfactory. In control plants and floury-2 mutants, synthesis of zein was initiated 15-20 days after pollination and it continues to increase for at least 40 days when expressed on a per endosperm basis (Fig. 39C). Floury-2 mutants had lower than normal rates of zein accumulation throughout the developmental sequence. For example, between 25-30 days after pollination, the rate of zein accumulation in floury-2 is 4.8 μmoles $N \cdot day^{-1}$ compared with a rate of 9.2 μmoles $N \cdot day^{-1}$ in the control endosperm (Table 22). In opaque-2 mutant, zein synthesis was also initiated 15-20 days after pollination. However, in addition to reduced rate of accumulation (5 μmoles $N \cdot day^{-1}$ between 25-30 days after pollination), zein synthesis was terminated at about 30 days after pollination. In each case, the increase in zein nitrogen closely parallels the

Table 21

Reproducibility of Extraction and Estimation of Zein Nitrogen

Sample	Extract #	Zein content µmoles N. endosperm^{-1}	Average ± mean variation	Mean variation as % of average activity
W64A 20 DAP	1	21.31 21.31	20.95 ± 0.55	2.65
	2	19.85 21.32		
W64A-op-2 30 DAP	1	24.30 21.26	22.50 ± 0.6	2.76
	2	23.28 21.26		

Zein was extracted from lyophilized endosperm powders according to the method of Dalby (1974). Duplicate assays were performed on two separate extractions.

Table 22

Rate of Accumulation of Zein in Developing Endosperm of Control (var. W64A) and Mutant (op-2 and fl-2) Plants

Days post-pollination	µmoles N·day^{-1}		
	$W64A_{+}$	$W64A_{op-2}$	$W64A_{fl-2}$
15 - 20	-	-	-
20 - 25	5.0	2.4	3.2
25 - 30	9.2	5.0	4.8
30 - 35	2.8	0.4	0.8
35 - 40	2.0	0.4	2.4

The values for each time interval were calculated from graph 39-C. (µmoles zein nitrogen·endosperm^{-1}·day^{-1}).

increase in total nitrogen in the endosperm. At 40 days post-pollination, zein accounted for 45%, 36% and 18.7% of total nitrogen in normal, floury-2 and opaque-2, respectively.

When zein accumulation in maize endosperm is expressed on a dry weight basis (Fig. 40), there is no apparent increase in zein beyond 30 days after pollination (in either the normal inbred or the mutants opaque-2 and floury-2). In opaque-2, around 30 days, the dry weight increase is higher than increase in zein. Similar patterns of zein accumulation in normal and in the mutants opaque-2 and floury-2 are reported by Tsai and Dalby (1975).

3. Accumulation of NH_4^+

NH_4^+ levels in the control endosperm increased just prior to the onset of zein biosynthesis, reached a maximum level at about 25 days after pollination and then declined (Fig. 39B). In mutant endosperms also the level of ammonia rose from low level at day 5 to significant levels at 20 and 25 days after pollination in opaque-2 and floury-2, respectively, and then declined. In opaque-2, NH_4^+ levels were higher than the control levels initially and remained high throughout the experimental period. Floury-2 endosperm had intermediate levels of ammonia. At 20-25 days after pollination, the level of ammonia in the endosperm was 1.3, 1.7 and 1.98 μmoles$\cdot$endosperm^{-1} in the normal, floury-2 and opaque-2, respectively.

B. Ribonuclease

Fig. 39E illustrates the changes in level of ribonuclease activity in the three varieties with time after pollination. Higher

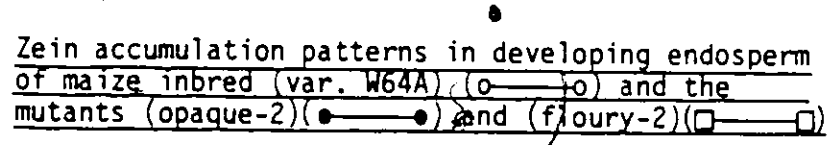
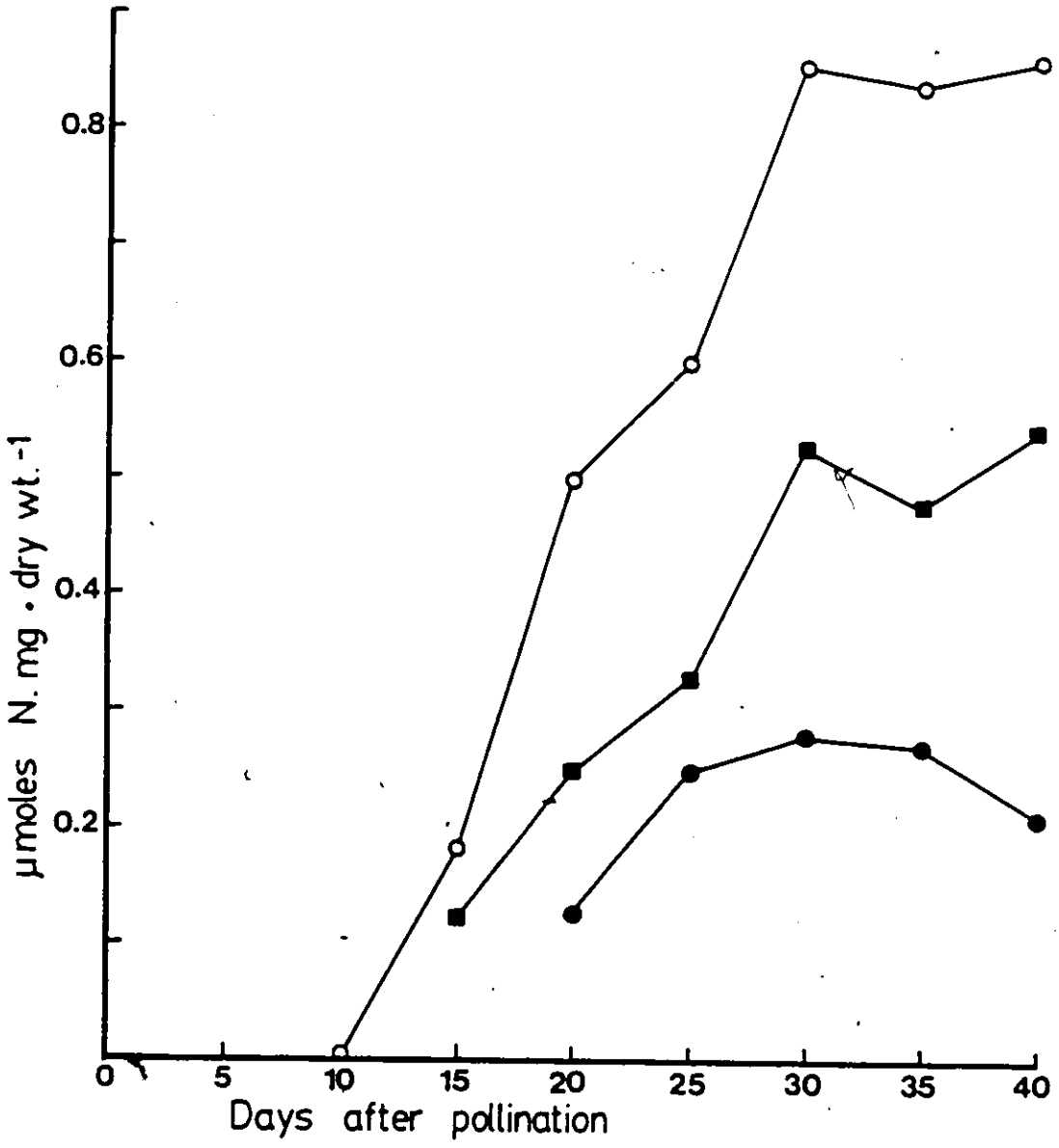


Figure 40. Zein accumulation patterns in developing endosperm of maize inbred (var. W64A) (o——o) and the mutants (opaque-2) (●——●) and (floury-2) (□——□)

Zein accumulation is expressed on a dry weight basis (μmoles N·mg·dry wt^{-1}).


0.8
0.6
0.4
0.2
µmoles N. mg • dry wt.-1
0
5
10
15
20
25
30
35
40
Days after pollination

levels of ribonuclease activity were seen in the mutants throughout the developmental sequence. The increase was more apparent in the opaque-2 mutant. Similar increases in ribonuclease activity in mutant endosperm have been reported earlier (Wilson, 1967; Dalby and Cagampang, 1970).

C. Protease

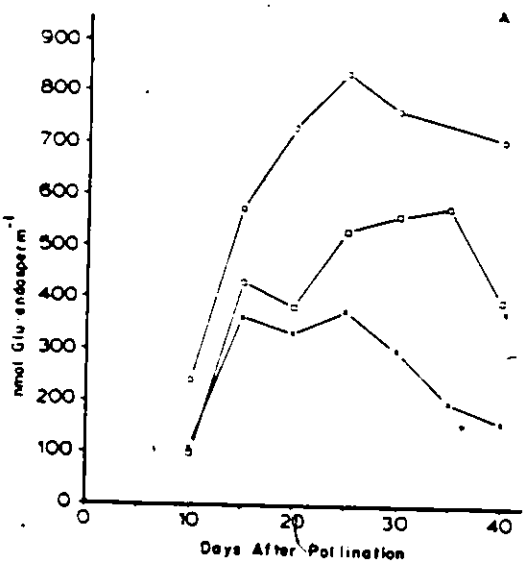
A neutral endopeptidase measured in the endosperm increased between 5-10 days after pollination in each variety (Fig. 39F). In the control (W64A), the highest level is reached at 25 days after pollination (38 μmoles trypsin equivalents·min^{-1}·endosperm^{-1}) followed by a decline. In the mutants, there is no decline in activity so that by later stages of development, protease activities are somewhat higher in the mutants.

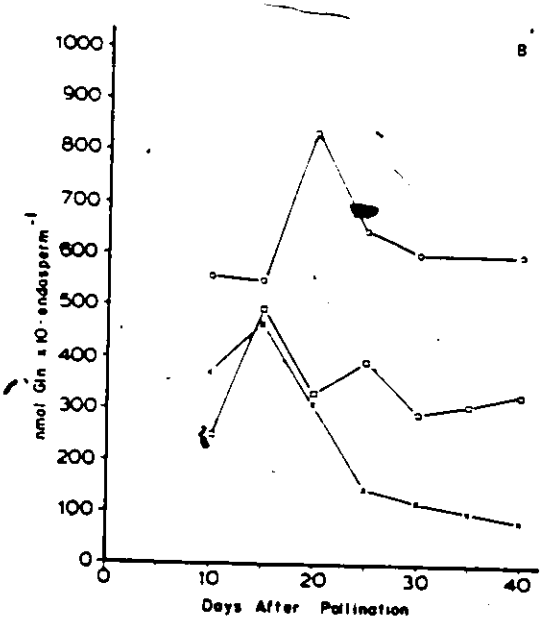
D. Free Amino Acid Content

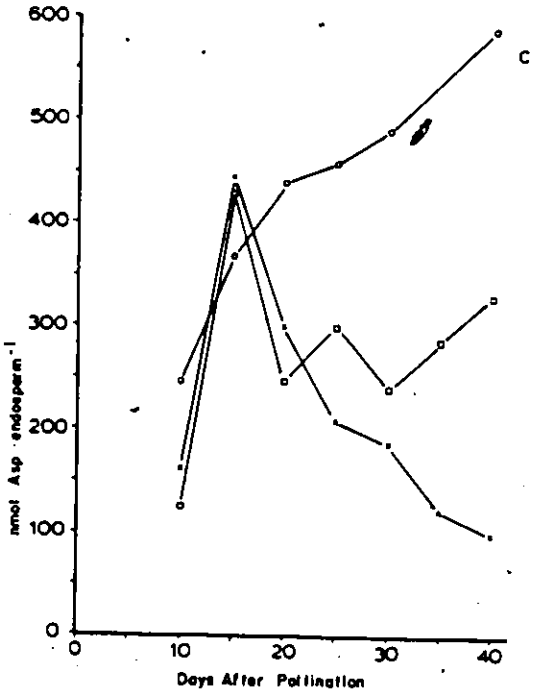
The concentrations of glutamate and aspartate and their amides were determined in the developing endosperm of the three varieties. The patterns of change of each amino acid, i.e., aspartate, glutamate, asparagine and glutamine are described below.

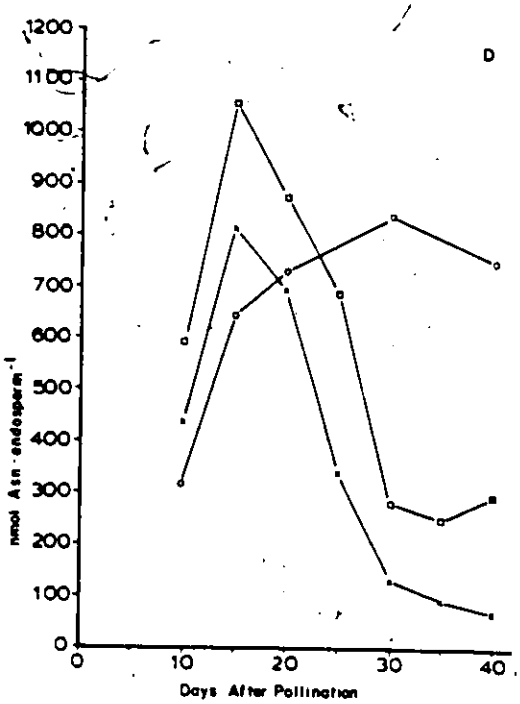
The concentration of glutamine was highest at 15 days after pollination in normal and floury-2 endosperm tissues (4.8 and 4.9 μmoles·endosperm^{-1}, respectively), and at 20 days after pollination in opaque-2 mutant (8.0 μmoles·endosperm^{-1}) (Fig. 41B). In the controls, the level of glutamine began to decline rapidly after 15 days after pollination. In the mutants, there is an initial decline

Figure 41 (A-D). Changes in level of free glutamic acid (A), glutamine (B), aspartic acid (C), and asparagine (D) per endosperm of normal (x), opaque-2 (o) and floury-2 (□) maize during development.


A
nmol Glu · endosperm⁻¹
Days After Pollination


B
nmol Gln ÷ 10 · endosperm⁻¹
Days After Pollination


C
nmol Asp · endosperm⁻¹
Days After Pollination


D
nmol Asn · endosperm⁻¹
Days After Pollination

followed by a levelling off at later stages of development. At 40 days after pollination, the level of glutamine was 0.8 µmoles, 4.2 µmoles and 6.8 µmoles·endosperm^{-1} in normal, floury-2 and opaque-2, respectively.

The asparagine concentration in the endosperm also reached a maximum at 15 days after pollination in control and floury-2 endosperms (Fig. 41D) and then declined over the next 25 days. In opaque-2, asparagine level showed a gradual increase up to 30 days after pollination and then showed a slight decrease over the next 10 days. At 40 days after pollination, the level of asparagine was 0.05, 0.3 and 0.8 µmoles·endosperm^{-1} in normal, floury-2 and opaque-2, respectively.

The level of glutamate increased rapidly initially in all three varieties (Fig. 41A). In W64A, the level of glutamate was more or less constant between 15-25 days after pollination and then decreased over the next 15 days. In opaque-2, it continued to increase over the initial 25 days after pollination and then levelled off. In floury-2, there is a gradual increase for 35 days followed by a slight decline. At 40 days, glutamate concentration per endosperm was 0.18, 0.45 and 0.75 µmoles in normal, floury-2 and opaque-2, respectively (Table 23).

The aspartate levels reach a maximum at 15 days after pollination in control and floury-2, followed by a decline (Fig. 41c). In opaque-2, aspartic acid continued to increase throughout the experimental time. In the floury-2 mutant also, the level of

Table 23

Levels of Free Amino Acids in the Developing Endosperm of W64A, Opaque-2 and Floury-2 at 40 Days After Pollination

Amino acid $\mu moles \cdot endosperm^{-1}$	Sample		
	W64A	W64Aop-2	W64Afl-2
Glutamine	0.80	6.8	4.2
Asparagine	0.09	0.8	0.3
Glutamate	0.18	0.75	0.45
Aspartate	0.10	0.60	0.35

(Data taken from graph 41 A-D.)

aspartic acid shows a gradual increase between 30 and 40 days after pollination. The level of aspartate at 40 days after pollination was 0.1, 0.35 and 0.6 μmoles·endosperm^{-1} in normal, floury-2 and opaque-2, respectively (Table 23).

E. Enzymes of Nitrogen Assimilation

For comparisons of enzyme activities in the three varieties, an extraction buffer was developed such that activities of asparagine synthetase, glutamate synthase, and glutamate dehydrogenase, asparaginase and glutamine synthetase could be extracted in one buffer system and then assayed. For this purpose, a HEPES buffer, 0.2 mM (pH 7.5) containing 2 mM EDTA, 12.5 mM 2-mercaptoethanol (routine GOGAT buffer) + 1 mM ATP and 10 mM $MgCl_2$ (to protect asparagine synthetase activity) was tested. The activity of each enzyme extracted and eluted in standard buffers for each enzyme (see Methods) as compared to those extracted and eluted in modified buffer are shown in Table 24.

Activities of glutamate synthase and glutamate dehydrogenase were similar in each buffer. The activity of asparagine synthetase was a little higher (32.8%) in HEPES buffer, whereas activity of glutamine synthetase was lower (66% relative to that in standard buffer). AS, GDH, and GOGAT reactions were also linear with enzyme concentration and with time of incubation. Therefore, it was decided to assay asparagine synthetase, GDH, and GOGAT in the extracts prepared with HEPES buffer. Glutamine synthetase and asparaginase were prepared separately by the methods described earlier (see pages 32, 33).

Figure 42E shows the changes in activities of the enzymes of nitrogen assimilation with age in the three varieties. Glutamate

Table 24

Effect of Extraction and Elution Buffer on Activity of Various Enzymes of Nitrogen Assimilation in Endosperm Extracts

Enzyme	Standard[I] extraction & elution buffers	HEPES[II] extraction & elution buffers
GOGAT	40.21 ± 2.88 (nmoles $NADH \cdot min^{-1} \cdot ml \cdot xt^{-1}$)	38.6 ± 2.90
GDH	116.0 ± 4.7 (nmoles $NADH \cdot min^{-1} \cdot ml \cdot xt^{-1}$)	118.0 ± 3.6 (nmoles $NADH \cdot min^{-1} \cdot ml \cdot xt^{-1}$)
Asparagine synthetase	3.63 ± 0.23 (nmoles $Asn \cdot min^{-20} \cdot ml \cdot xt^{-1}$)	4.94 ± 0.025
GS	9.0 ± 0.064 (μmoles $HA \cdot h^{-1} \cdot ml \cdot xt^{-1}$)	6.0 ± 0.078
Asparaginase	6.30 ± 1.0 (nmoles $Asp \cdot min^{-1} \cdot ml \cdot xt^{-1}$)	n.d.

I: See Methods.

II: HEPES buffer 0.2 mM (pH 7.5), 2 mM EDTA, 12.5 mM 2-mercaptoethanol, 1 mM ATP, and 10 mM $MgCl_2$. Samples were harvested 25 days after pollination.

All enzyme assays were performed on Sephadex G-75 filtered extracts of inbred var. W64A.

Figure 42 (A-E). Changes in level of enzymes of nitrogen assimilation in developing endosperm of normal (x), opaque-2 (o) and floury-2 - (□).

*Activity of each enzyme is expressed on the basis of the amount of product produced or substrate utilized per endosperm per unit time.

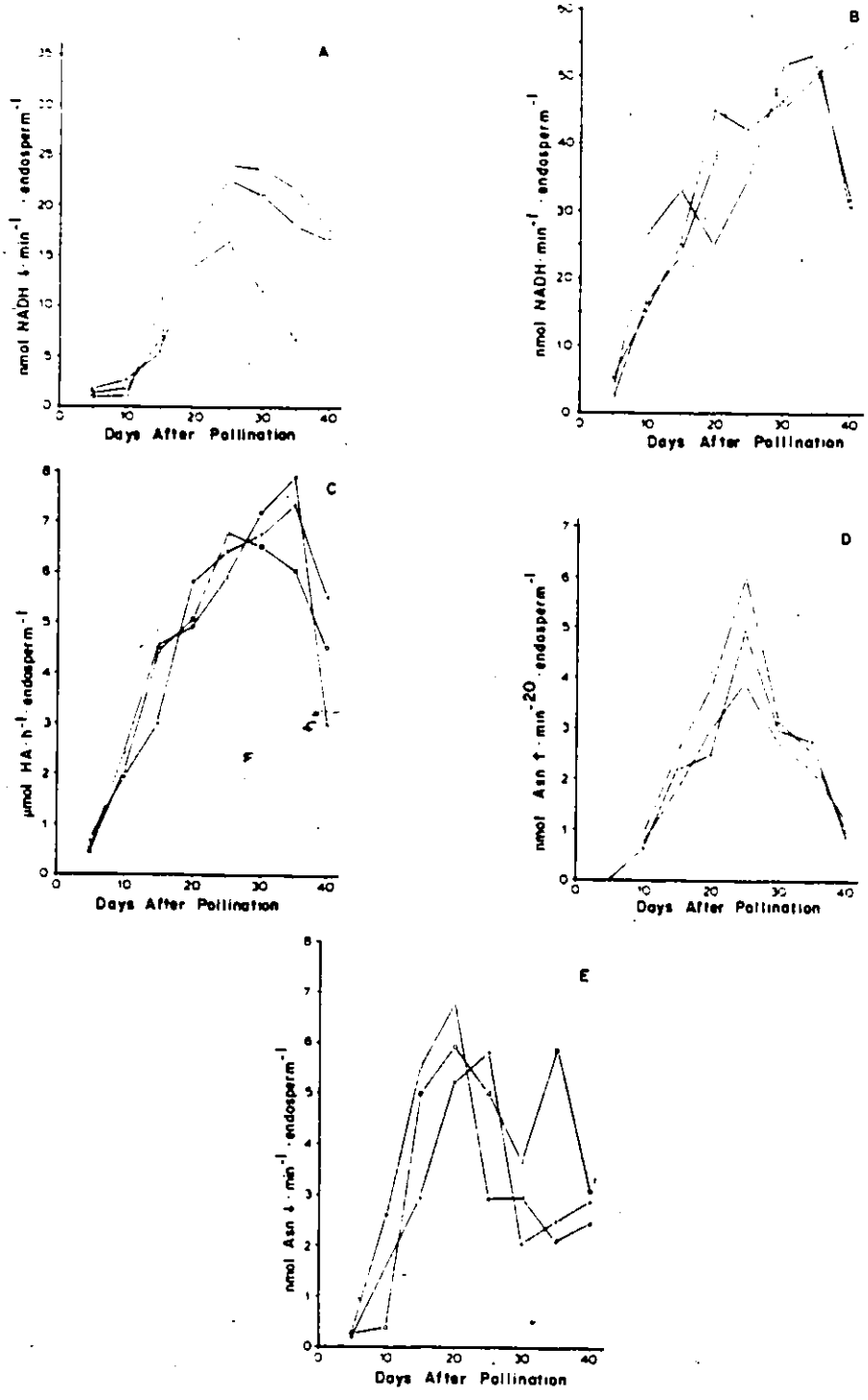
A - glutamate synthase

B - glutamate dehydrogenase

C - glutamine synthetase

D - asparagine synthetase

E - asparaginase.


A
nmol NADH ↓ · min⁻¹ · endosperm⁻¹
Days After Pollination
B
nmol NADH · min⁻¹ · endosperm⁻¹
Days After Pollination
C
µmol HA · h⁻¹ · endosperm⁻¹
Days After Pollination
D
nmol Asn ↑ · min⁻²⁰ · endosperm⁻¹
Days After Pollination
E
nmol Asn ↓ · min⁻¹ · endosperm⁻¹
Days After Pollination

synthase (NADH-dependent), glutamate dehydrogenase, glutamine synthetase, asparagine synthetase and asparaginase were present in the immature endosperm of each variety (Table 25). There is a general increase in activity in each case just prior to the initiation of zein biosynthesis.

Asparagine synthetase in three varieties showed a similar pattern of development (Fig. 42D). Calculated on a per endosperm basis, enzyme activity rises from a low level at day 10 to a peak at 25 days after pollination and then declined in later stages of development. The level of maximum activity was 4,5 and 6 nmoles Asn produced·min^{-20}·$endosperm^{-1}$ in normal, floury-2 and opaque-2, respectively.

Asparaginase activity in each variety started to increase early in development, reached a maximum at day 25 in control and at day 20 in the mutant varieties. In floury-2, the enzyme activity showed a second peak around 35 days after pollination. In each case, levels of activity in the endosperm were low and no significant differences were observed between the three varieties (Fig. 42E).

The level of NADH-GOGAT activity examined in each variety started to increase at day 10 and reached a maximum level at 25 days after pollination in floury-2 and W64A, and at 20 days after pollination in opaque-2 (Fig. 42A). Thereafter, the enzyme levels decreased gradually (in the mutant varieties opaque-2 and floury-2). There was a more rapid loss in activity in the wild type. The enzyme level was higher in the mutant varieties relative to the control throughout the developmental sequence. At day 20, the measured values are significantly higher in opaque-2 (29 ± 0.45 nmoles NADH·min^{-1}·$endosperm^{-1}$),

Table 25

Enzymes of Nitrogen Assimilation in Developing Endosperm of Normal and Mutant Varieties

Enzyme	Sample		
	$W64A_{+}$	$W64A_{op-2}$	$W64A_{fl-2}$
GOGAT	13.65 ± 0.25	28.75 ± 0.875	17.9 ± 0.48
GDH	44.00 ± 1.25	29.85 ± 0.45	34.5 ± 1.99
GS	5.80 ± 0.238	4.69 ± 0.04	4.77 ± 0.93
Asparagine synthetase	2.465 ± 0.035	3.79 ± 0.06	3.01 ± 0.83
Asparaginase	5.25 ± 0.046	6.17 ± 0.078	5.01 ± 0.00

Samples were harvested 20 days after pollination.

Each value is an average of 2 duplicate assays performed on 2 separate extracts.

floury-2 (18 ± 0.7 nmoles NADH·min^{-1}·endosperm^{-1}), than in the wild type (13 ± 0.58 nmoles NADH·min^{-1}·endosperm^{-1}).

Activity of glutamate dehydrogenase began to increase 5 days after pollination in each variety, and continued to increase until 30 days in the normal and opaque-2 varieties (Fig. 42B). In floury-2, the peak of activity was at day 25 after pollination.

The activity of glutamine synthetase also started to increase 5 days after pollination and continued to increase until day 25 in floury-2 and day 35 in normal and opaque-2 varieties (Fig. 42C). Thereafter, the activities declined in each variety. Although samples show minor differences in the time course for the development of GDH and GS activities, the mutant varieties are not clearly different from the wild type. Tsai (1979) has reported previously that GS was much lower in the opaque-2 endosperm throughout the experiment and that GOGAT was higher and, in fact, continued to increase throughout a 40 day post-pollination period. The differences between results described here and his could be due to his use of crude extracts. Results presented earlier (Table 16) showed that GS activities are higher with Sephadex-treated extracts, and with this treatment, the activities of GS are comparable in each variety (at least until 35 days after pollination).

As mentioned earlier, aspartic acid is present in crude extracts and as a result, the α-ketoglutarate and NADH substrates for GOGAT can also be used as substrates in the glutamate oxaloacetate transaminase and malate dehydrogenase reactions (Miflin and Lea, 1975; Misra and Oaks, 1980). In fact, aspartic acid is much higher in the opaque-2 mutant than in the wild type and it continues to rise

throughout the developmental sequence. Thus, the much higher level of GOGAT in Tsai's experiments probably reflects the increase in aspartic acid. In my experiments with Sephadex-treated extracts, GOGAT activity is about 2-fold higher in the mutant opaque-2 at 20 days after pollination.

IV. Glutamine Metabolism in Endosperm

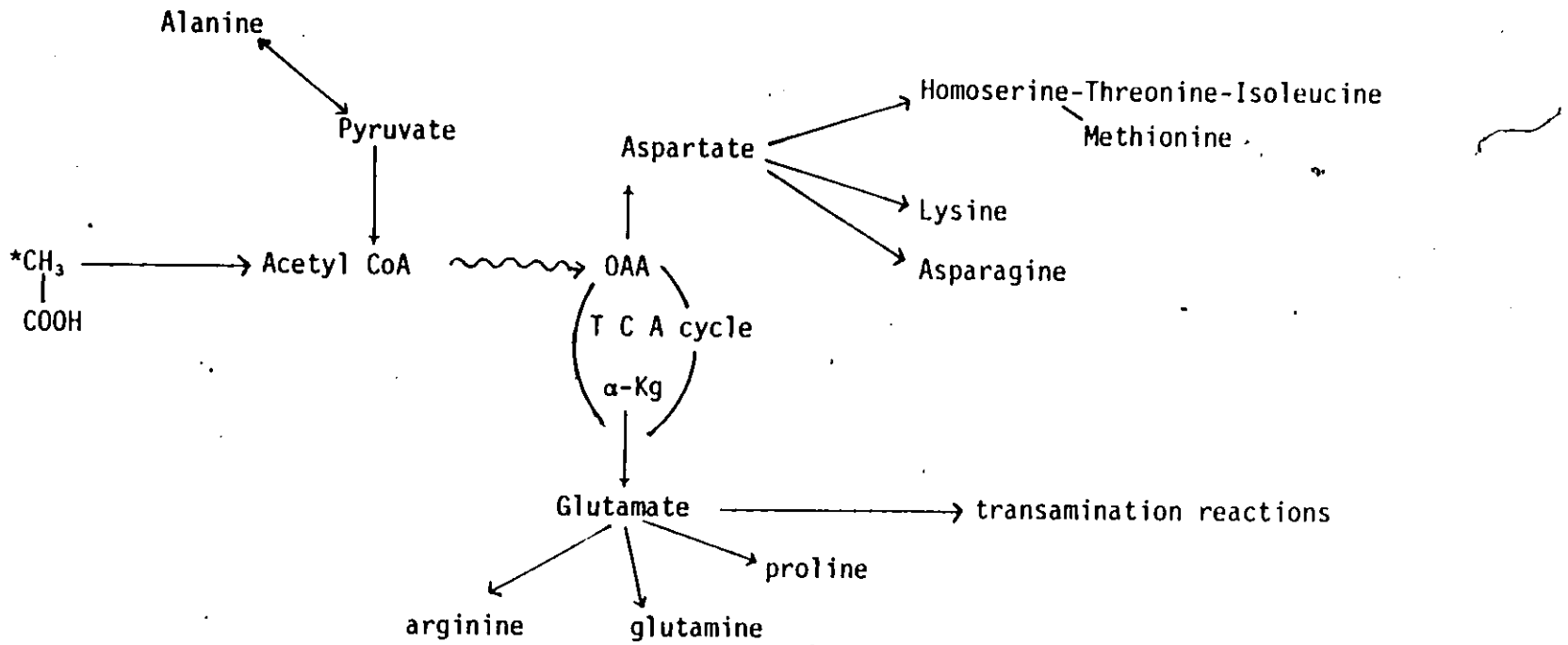
The *in vivo* metabolism of glutamine was examined by feeding (UL-^{14}C)-glutamine and (2-^{14}C)-acetate to endosperm tissue. If the major fate of glutamine in the endosperm tissue was its incorporation into zein then a large proportion of label in zein should be recovered as glutamine. On the other hand, extensive metabolism of glutamine should result in a recovery of (^{14}C) in many different constituents.

A. Establishing Conditions for Glutamine Feeding

1. Acetate-2(^{14}C)

(2-^{14}C)-Acetate was used for characterization of the optimum experimental conditions. Acetate is readily incorporated into various components of the cell (Fig. 43). It is especially useful for labelling dicarboxylic acids via TCA cycle (MacLennan *et al.*, 1963) and amino acid derivatives of the TCA cycle. The dicarboxylic acids serve as precursors for biosynthesis of a number of amino acids including glutamate and glutamine (Oaks, 1965; Oaks *et al.*, 1970). Two types of tissue were examined: (1) Whole endosperm or sliced endosperm pieces obtained from the standard laboratory var. W64A x W182E ; and (2) Cultured corn caryopses of a commercial variety Dekalb 14A and of our standard hybrid variety W64A x W182E.

Figure 43. Metabolism of acetate via TCA cycle into the amino acid derivatives.


Alanine
Pyruvate
Aspartate
Homoserine-Threonine-Isoleucine
Methionine
Lysine
Asparagine
*CH3
COOH
Acetyl CoA
OAA
T C A cycle
α-Kg
Glutamate
transamination reactions
proline
arginine
glutamine

a. Incubation of excised endosperm

Experimental design: Endosperms obtained from the maize hybrid W64A x W182E were harvested at 20-25 days after pollination. At this stage of development, there is a rapid synthesis of storage proteins and the activities of enzymes involved in nitrogen assimilation are high (Table 18; Fig. 36). The synthesis of starch is also rapid at this time. Using aseptic conditions, the endosperm tissue was dissected out. Incubation was carried out in 1/10 Hoagland's salt solution which contained acetate (5 μCi/3 ml). When endosperm slices were used, each endosperm was sliced into 5 parts of approximately equal thickness. The uptake of label was measured by total label accumulated in the tissue. Endosperm fractions were extracted as described in Methods.

i. Whole endosperm *vs* endosperm slices

Table 26 shows the incorporation of (2-^{14}C)-acetate into protein and non-protein fractions, when whole endosperm tissue or sliced endosperm were used as experimental material. The total uptake of (^{14}C)-acetate by slices over a 90 min incubation period is 2.5 times greater than uptake into the whole endosperm. However, the percentage distribution of label into three major fractions, i.e., water soluble proteins, water soluble non-protein fractions and storage proteins, is similar in each case. Based on this observation, it was decided to use endosperm slices in further experiments.

ii. Kinetics of uptake with time and amount of tissue

The kinetics of uptake of (^{14}C)-acetate with time and with amount of tissue used per assay is shown in Fig. 44a-b). The incorporation of acetate carbon increases more or less linearly for

Table 26

Incorporation of ^{14}C-acetate into Various Endosperm Fractions After Incubation of Whole Endosperm and Endosperm Slices

Fraction	Whole endosperm (cpm·endosperm^{-1})	Endosperm slices (cpm·endosperm^{-1})
Water soluble (non-protein)	30,800 (65.5)*	79,060 (65.0)
Water soluble (protein)	7,240 (15.1)	19,800 (16.3)
Water insoluble residue	9,780 (20.4)	22,680 (18.66)
TOTAL	47,820	121,540

Excised endosperm from maize hybrid (W64A x W182E) were incubated for 90 min in Hoagland's salt solution + (2-^{14}C) acetate (5 µCi/3 ml media). Extracts were prepared as described in Methods.

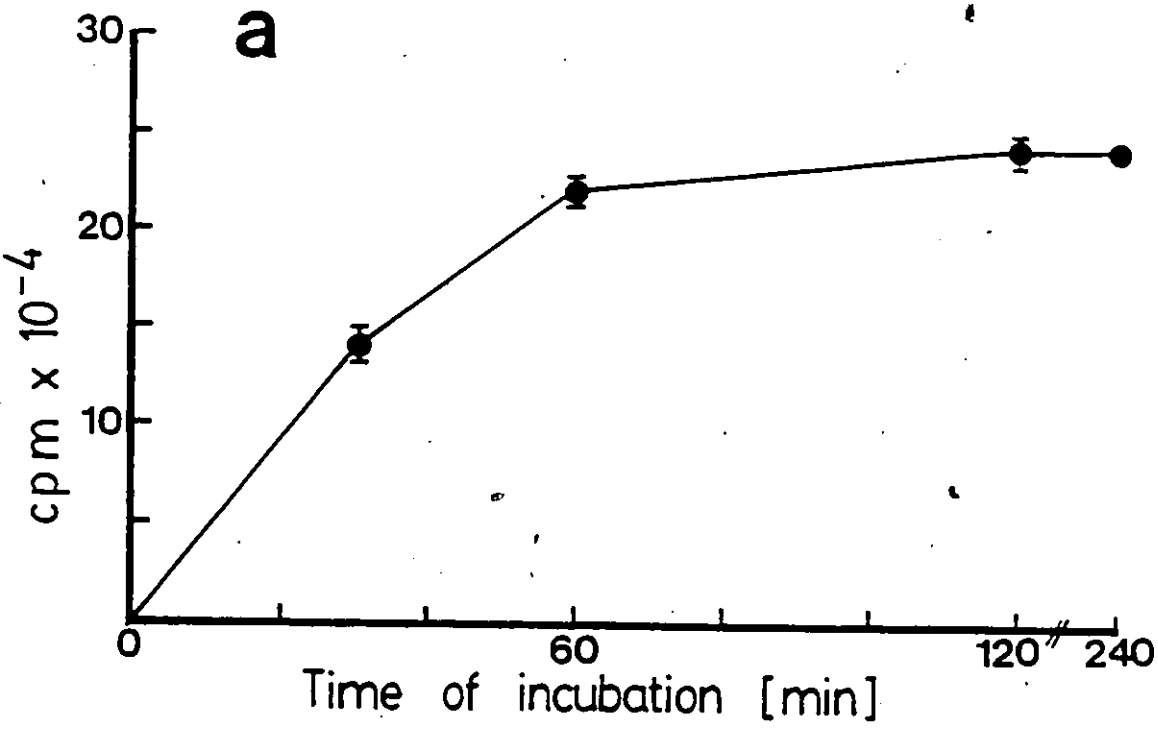
* Figures in brackets represent % of total in each fraction.

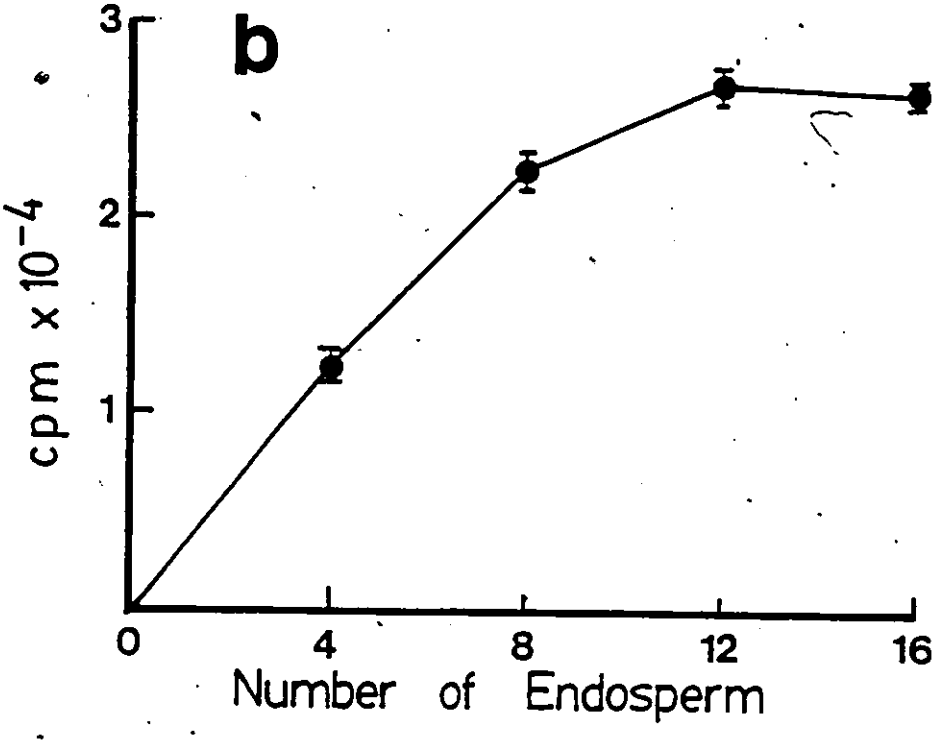
Figure 44 (a,b). Kinetics of uptake of (^{14}C)-acetate with time (a) and amount of tissue (b).

Endosperm tissue of the hybrid variety W64A x W182E was harvested 20 days after pollination and was incubated in 1/10th Hoagland's solution containing 5 μCi (2-^{14}C)-acetate per 3 ml of incubation media. In treatment (a), 5 endosperms were incubated per flask for various time intervals.

Values for each treatment are the means from duplicate samples.

In treatment (b), the incubation time was 120 min.


a
30
20
10
0
cpm x 10⁻⁴
60
120
240
Time of incubation [min]


b
3
2
1
0
cpm x 10⁻⁴
4
8
12
16
Number of Endosperm

60 min and then ceases. The incorporation of label also increased with increasing amount of endosperm tissue per incubation. For example, increasing the number of endosperm pieces from 20 to 40 per flask resulted in increase in incorporation of label from 1.25×10^{-4} to 2.25×10^{-4} cpm in 30 min. With more than 50 endosperm pieces per flask, no further increase was observed (Fig. 44b).

b. Fate of (^{14}C)-acetate in endosperm

i. Table 27 shows the distribution of acetate carbon into various fractions of the endosperm. After 2 hr of incubation, most of the label was in the water soluble fraction, i.e., amino acids, sugars, organic acids, albumins and globulins. This fraction accounted for 60-70% of the total label incorporated during the 2 hr incubation period. Of the total label in this fraction, about half of the radioactivity was in the free amino acid fraction. The organic acids + sugars and albumins + globulins contained 22% and 5% of the total label, respectively. Thus, amino acids account for a substantial proportion of total radioactivity. Label in starch accounted for 16-20% but only minor amounts of the total label was recovered from the storage proteins, i.e., zein (2.76%) or glutelin (2.0%).

ii. Analysis of the amino acid fraction: The o-phthaldialdehyde (OPA) derivatized amino acids in the free amino acid fraction of the endosperm were analyzed by liquid chromatography using a reverse-phase column. The peaks for aspartate, asparagine, glutamate and glutamine were identified by comparison with standard samples of these amino acids. The total fraction of each amino acid was collected and then assayed for radioactivity. The remaining amino

Table 27

Incorporation of (2-^{14}C)-acetate into Endosperm Slices of Hybrid Maize var. (W64A x W182E)

Fraction	12 endosperms·flask^{-1}		16 endosperms·flask^{-1}	
	cpm/100 mg	% of total	cpm/100 mg	% of total
Lipid	45,920	12.1	29,521	8.33
Starch	72,250	19.1	57,986	16.4
Albumin + Globulin	16,830	4.4	18,634	5.2
Zein	12,474	3.30	8,060	2.2
Glutelin	7,700	2.03	7,150	2.0
Amino acids	139,738	36.92	151,231	42.7
Sugars + organic acids	67,695	17.89	65,840	18.6
Residue	15,780	4.17	15,050	4.25
TOTAL	378,387		353,472	

The cobs were harvested 20 days after pollination. Endosperms were treated as described in Methods and were incubated in a Hoagland's salt solution which contained 5 µCi/3 ml of (2-^{14}C)-acetate in addition to the standard ingredients. The samples were incubated for 2 hr.

acids were collected in bulk and the radioactivity in this fraction was also assessed. An aliquot of the incubation media taken at the end of the experiment was also assayed for amino acids and for radioactivity.

Fig. 45a-c shows the elution profile of standard amino acids (a), amino acids from endosperm extract (b) and from the media (c). As can be seen, large amounts of free amino acids were also present in the incubation media. Since tests for microbial contamination of the incubation media were negative, the amino acids were probably leaking from the endosperm tissue during the course of experiments.

Table 28 shows the distribution of (^{14}C)-acetate carbon in various amino acids analyzed in the free amino acid fraction of the endosperm and those present in the media. In each case, label was found in the acidic amino acids, their amides and the neutral and basic amino acid fraction. Of the total counts in the free amino acid fraction of the endosperm, glutamine accounted for 10% of the total counts. Presence of labelled amino acids in the incubation media suggests that acetate entered the endosperm tissue and that metabolites of acetate moved out.

B. Caryopsis Culture

1. Optimizing conditions for [^{14}C-glutamine] feeding

a. Experimental design: Fig. 46 illustrates the technique for caryopsis culture. All procedures were performed under sterile conditions. Sections of cob containing 10 kernels were removed from the developing cob 5-7 days after pollination. The sections were

Figure 45 (a-c). Chromatogram of o-phthaldialdehyde derivatized amino acids.

a) standard aspartate (A), glutamate (B), asparagine (C) and glutamine peaks (D).

b) elution profile of the amino acid fractions of the endosperm extract.

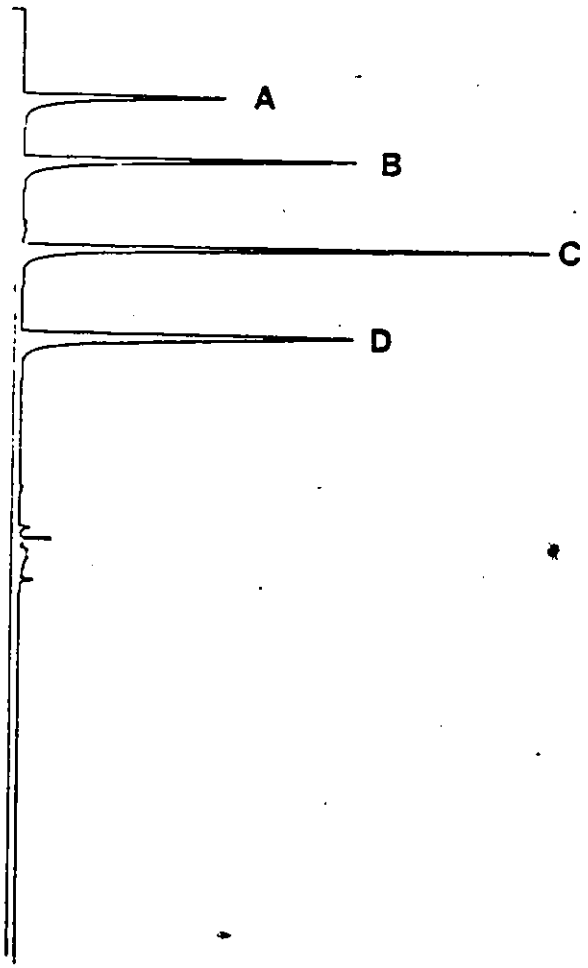
c) elution profile of the amino acids in an aliquot of incubation media, taken at the end of the experiment.

Endosperm tissue (hybrid var. W64A x W182E) was harvested 20 days after pollination.

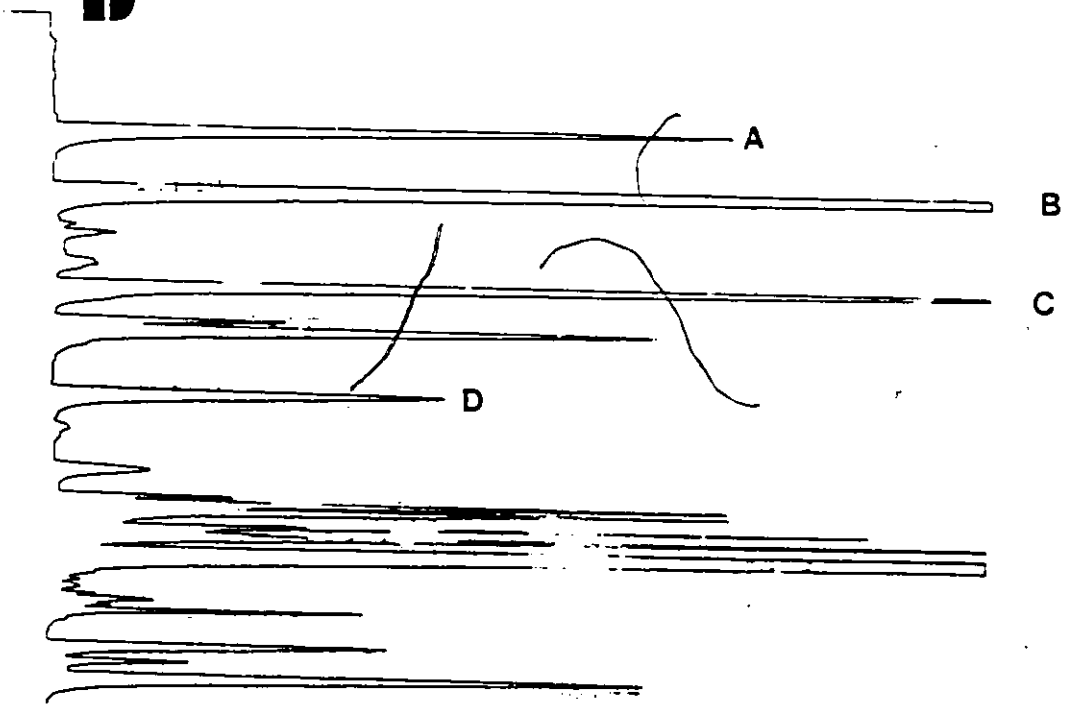
Incubation was performed in 1/10th Hoagland's salts solution containing 5 μCi (2-^{14}C)-acetate per 3 ml of incubation media.

At the end of the experiment, amino acid fractions were prepared and eluted from reverse-phase high performance liquid chromatography column using the gradient described in Methods.

a



b



c

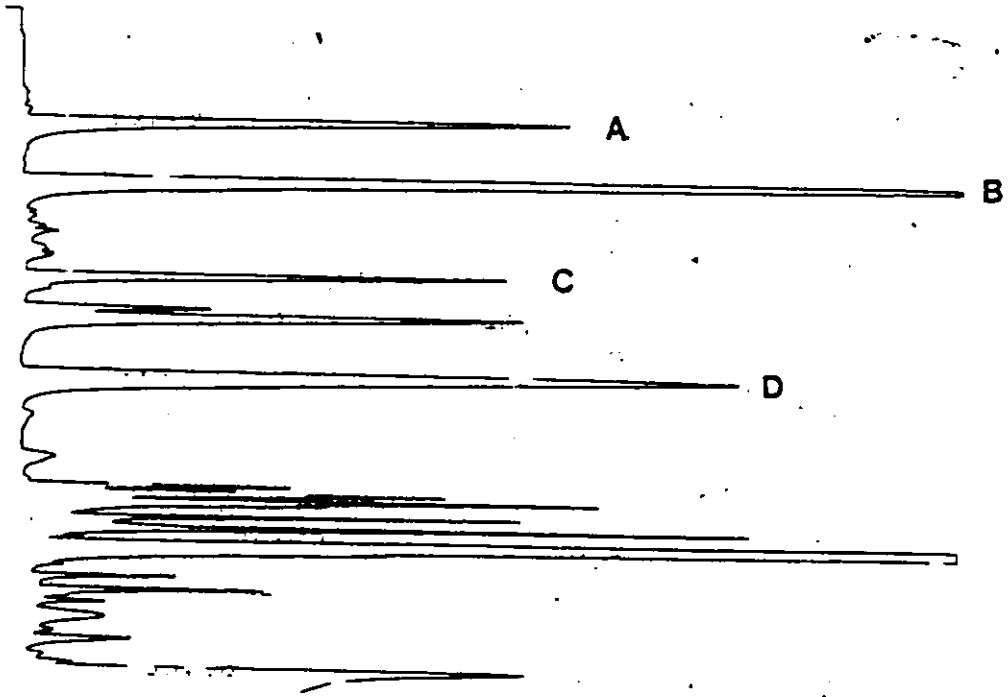


Table 28

Distribution of (^{14}C) among Various Amino Acids in Free Amino Acid Fraction of Endosperm Tissue and Those Present in the Incubation Media at the End of the Experiment

Amino acid fraction	Incubation media		Endosperm extract	
	cpm	µmoles·endosperm^{-1}	cpm	µmoles·endosperm^{-1}
Glutamine	578	1.77	921	2.24
Glutamate	474	7.50	1,819	12.00
Asparagine	289	1.60	313	4.57
Aspartate	470	1.33	920	4.57
Neutral + Basic	1,395	-	4,090	-

Endosperm tissue from hybrid maize was incubated in Hoagland's solution containing (2-^{14}C)-acetate (5 µCi/3 ml).

After a 2 hr incubation period, analysis of the amino acid fraction of the endosperm and of incubation media was performed as described in Methods.

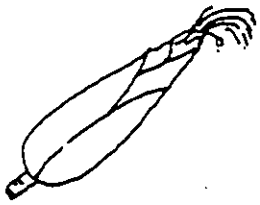
Figure 46. Caryopsis culture technique.

Samples of maize were harvested 5-7 days after pollination.

Sections of cob containing 10 kernels, were surface-sterilized in 1% hypochlorite solution for 1 min and were thoroughly rinsed with sterile distilled water. Incubation on nutrient media was performed according to the method of Gengenbach (1977).

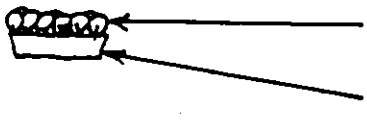
O/N = overnight.

1.



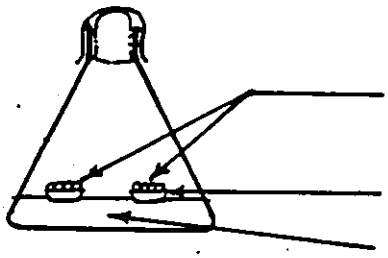
5-7 days after pollination

2.



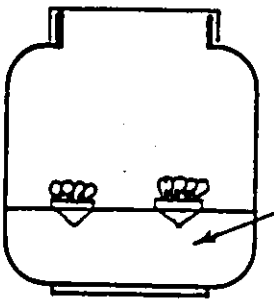
Cob blocks [10 caryopses each]

3.



Incubate 28 ± 1°C (10 days)

4.



Incubate 28 ± 1°C (O/N : 7 days)

placed on nutrient agar and were allowed to grow for 10 days (step 3). They were then transferred to media containing (2- ^{14}C)-acetate (1 μCi/ml) or (UL-^{14}C)-glutamine (0.25 μCi/ml) (step 4). The sections were placed on media in such a way that only the cob tissue was in contact with the media. At the beginning of the transfer process (step 3), a large number of kernels aborted. The percentage of kernels surviving (step 1), was about 25% whether the cobs were obtained from field or from growth chamber-grown materials. Cobs harvested 7 days after pollination gave a better growth response than those harvested 5 days after pollination. Even though the cob blocks were surface sterilized with 1% hypochlorite prior to their transfer on nutrient media, some samples were lost due to microbial contamination. The frequency of microbial contamination was higher in field-grown than in growth chamber-grown cobs.

b. Kinetics of uptake of label from the media: A time course experiment with the system described above, showed a continuous uptake of (^{14}C) substrate over a period of several days (Fig. 47a,b). Samples of endosperm tissue analyzed after 168 hr of incubation showed that in each case significant label was incorporated into starch and zein (Table 29). With (2-^{14}C)-acetate as the precursor, 16.6% of the label was in starch, 42.1% in zein, 16.2% in glutelin and 12.6% remained in the soluble fraction of the endosperm (amino acids, sugar, organic acids). When (UL-^{14}C)-glutamine was the precursor, 19% of total label appeared in starch, 33% in zein, 26% in glutelin and 9.0% remained in the soluble fraction.

Figure 47 (a,b). Kinetics of uptake of label from nutrient media.

a. (2-^{14}C)-acetate containing media.

b. (UL-^{14}C)-glutamine containing media.

The samples of var. Dekalb 14A were cultured on nutrient media according to the method of Gengenbach (1977).

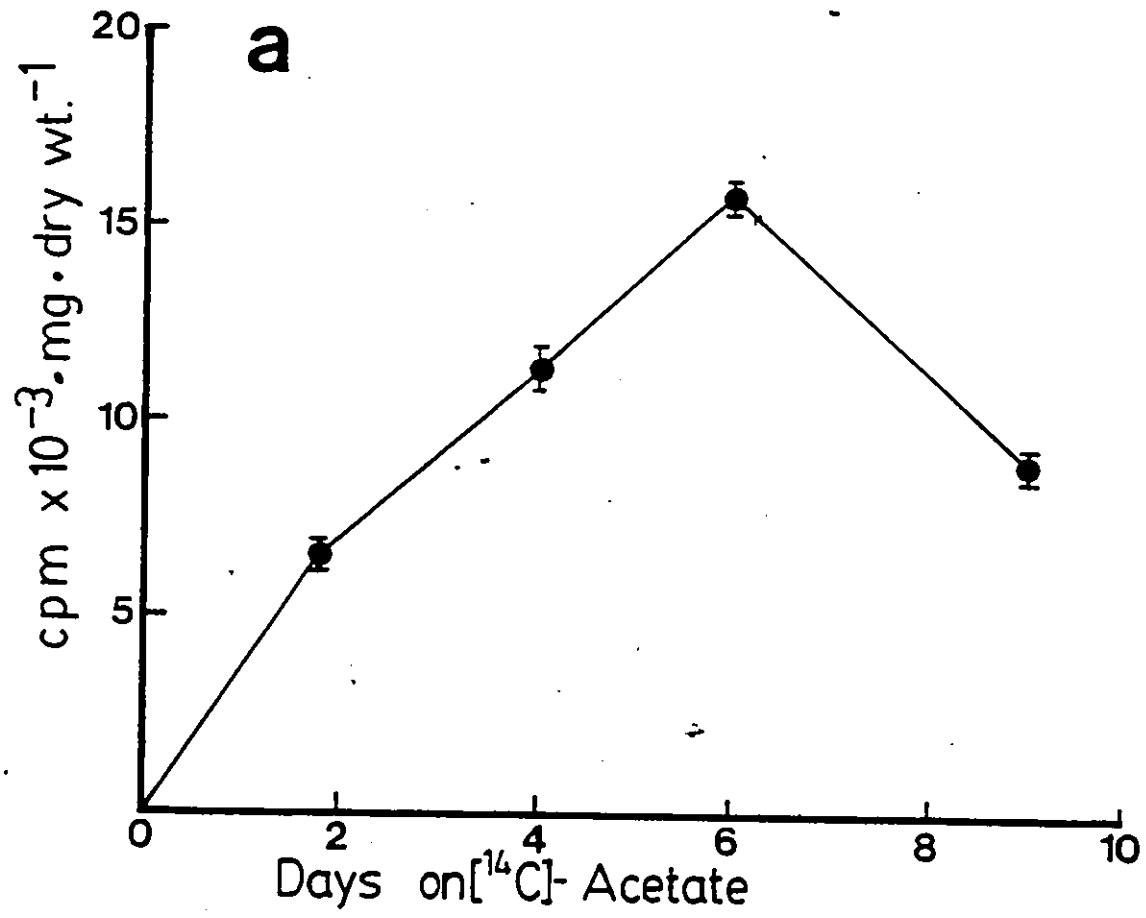
Developing caryopsis were harvested at the times indicated in figures a and b.

Each treatment had 5 flasks.

Each flask contained 5 caryopses.

At the end of the experiment, samples from all 5 flasks were pooled and uptake of label/mg of tissue was measured.

Each point on the graph is the mean value obtained from 25 caryopses.


a
20
15
10
5
0
2
4
6
8
10
cpm x 10⁻³. mg. dry wt.⁻¹
Days on [¹⁴C]- Acetate

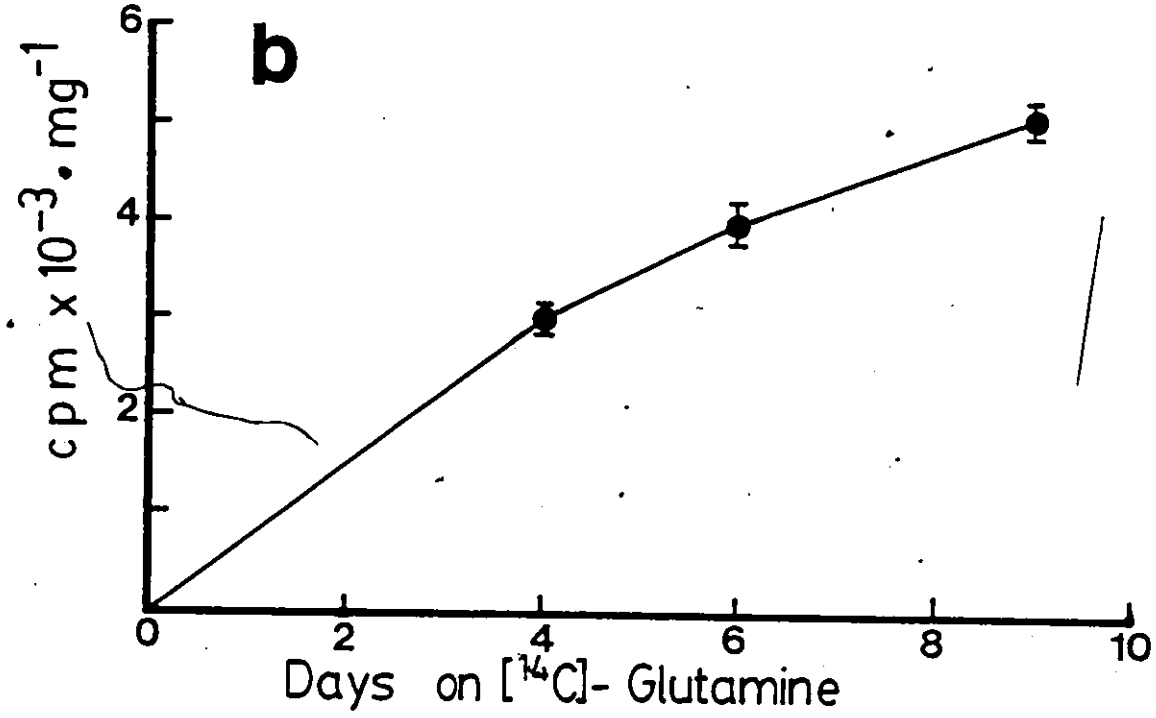

b
6
4
2
0
2
4
6
8
10
cpm x 10⁻³. mg⁻¹
Days on [¹⁴C]- Glutamine

Table 29

Incorporation of (2-^{14}C)-acetate and (UL-^{14}C)-glutamine into Maize Endosperm

(var. Dekalb 14A)

Fraction	(2-^{14}C)-acetate		(UL-^{14}C)-glutamine	
	cpm	%	cpm	%
Lipid	26,040	4.2	4,461	3.2
Starch	703,560	16.6	26,520	19.0
Total soluble (water)	78,480	12.6	72,480	9.03
Zein	261,512	42.14	46,080	33.3
Glutelin	101,000	16.20	37,000	26.0
Residue	5,122	8.20	9,540	7.2
TOTAL	662,814		138,081	

The cobs were harvested 5 days after pollination, cultured on Gengenbach's nutrient media for 10 days and then transferred to (^{14}C) containing media ((2-^{14}C)-acetate, 1 μCi/ml and (UL-^{14}C)-glutamine, 0.3 μCi/ml of media). The samples were harvested after a 9-day-incubation on labelled media. For extractions, 100 mg of lyophilized, pulverized endosperm powder was used. Extractions were performed as described in Methods.

Analysis of the medium at the end of the experiment showed a single peak which corresponded to glutamine (Fig. 48a,b). This shows that there was no diffusion of amino acids from the endosperm back into the media. Incorporation of ^{14}C in zein suggested that this system mimicked normal kernel development. Therefore, this technique was used for the definitive experiments on the fate of (^{14}C)-glutamine in hybrid maize variety W64A x W182E.

2. Definitive experiment on the fate of [^{14}C] glutamine in W64A x W182E

a. Increase in dry weight with time of incubation: Table 30 shows the increase in dry weight of endosperm, embryo and cob tissue during a 7 day period in culture. The average weight of endosperm increased from 26 ± 2.36 mg at day one to 45.41 ± 3.6 mg at day 7. The average weight of embryo also increased from 0.8 ± 0.13 mg to 2.286 ± 0.256 mg, whereas, the dry weight of cob tissue remained more or less constant (175 ± 23.3 mg to 187 ± 15 mg). The dry weight increase observed in endosperm tissue was comparable to that observed in kernels developing on normal cob in growth chamber conditions. For example, 20 days after pollination, the dry weight of endosperm from growth chamber-grown plants was 53.0 mg·endosperm^{-1} (Table 17). At 19 days after pollination, the average dry weight of the cultured endosperm was 45.41 ± 3.6 mg.

b. Total uptake of (^{14}C)-glutamine: The samples were harvested at 15,24,48,96 and 168 hr after incubation on (UL-^{14}C)-glutamine. Each treatment had five replicates containing 7 caryopses each. The total uptake increased over a 7 day incubation period.

Figure 48 (a,b). Chromatogram of OPA derivatized glutamine standard (a) and an aliquot of the incubation media (b).

The standard glutamine (4 mM) was reacted with o-phthaldialdehyde reagent and 20 µl of derivatized sample were injected.

Elutions were performed by reverse-phase chromatography.

For assay of the incubation media, the agar nutrient media was extracted with 25 ml of distilled water at 4°C, overnight.

An aliquot of this extract was reacted with OPA reagent and 20 µl sample was injected.

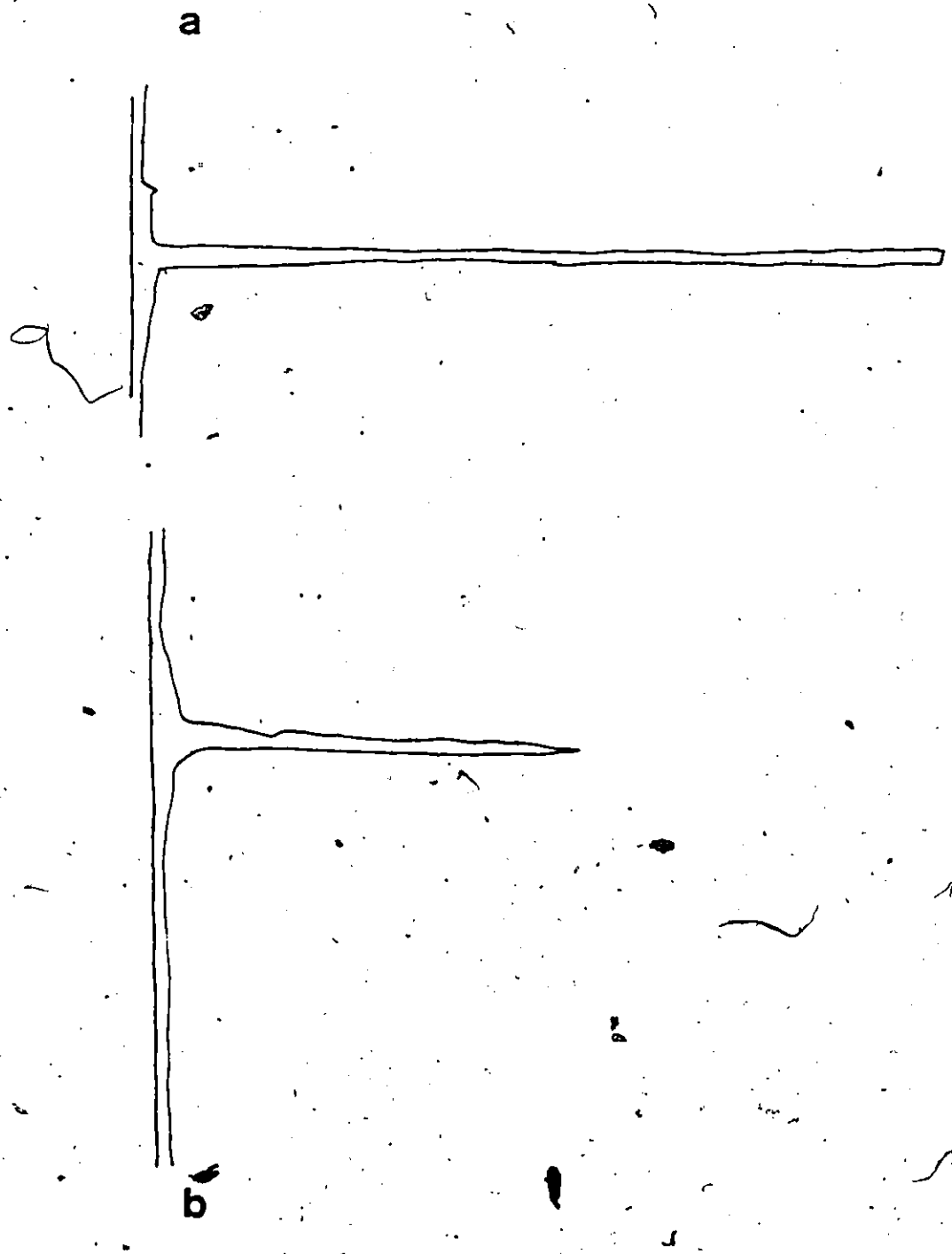

a
b

Table 30

Changes in Dry Weight of Endosperm, Embryo and Cob Tissue During a 7 Day Growth Period

Time of incubation (hr)	Sample #	Endosperm *mg·endosperm^{-1}	$\bar{n}$	Dry weight embryo mg·embryo^{-1}	$\bar{n}$	Cob tissue mg	$\bar{n}$
24	1	27.10	26	1.0	0.8	190	175
	2	27.58	±	1.0	±	140	±
	3	22.58	2.36	0.7	0.13	195	23.3
48	1	27.1	27	1.0	0.895	140	147.5
	2	30.0	±	0.85	±	215	±
	3	30.0	2.7	0.85	0.067	135	33.5
	4	22.1		0.88		100	
96	1	32.8	32.61	1.71	2.91	160	197
	2	27.4	±	1.42	±	150	±
	3	38.50	3.04	2.42	0.675	250	34.25
	4	32.0		1.85		210	
168	1	30.18	45.41	1.8	2.286	200	187
	2	45.15	±	2.0	±	210	±
	3	43.63	3.6	2.36	0.256	190	15
	4	46.36		3.09		175	
	5	53.43		2.18		163	

After harvesting the caryopsis, endosperm, embryo and cob tissue were separated, frozen in liquid nitrogen, freeze-dried for 2 days in lyophilizer and their weight recorded.

*Average weight of 7 endosperms.

$\bar{n}$ Average of replicate samples ± mean variation.

There was a parallel decrease in the (^{14}C) content of the media (Fig. 49).

c. Distribution of [^{14}C] from glutamine in cob endosperm and embryo: Fig. 50 shows the distribution of (^{14}C) in various tissues of the cultured cob blocks both as total ^{14}C content and as ^{14}C content per mg dry weight. Estimation of label in different tissues showed a gradient of decreasing concentration of ^{14}C in order: cob ⟶ endosperm ⟶ embryo. However, at later stages of incubation, i.e., 96 and 168 hr, embryo and endosperm had almost equal amounts of label on a $cpm \cdot mg^{-1}$ dry weight. In general, the pattern of uptake is similar to the patterns reported by Shimamoto and Nelson (1981) for leucine, phenylalanine, and proline in the cultured caryopsis.

3. Fate of (^{14}C) derived from (^{14}C)-glutamine in cultured endosperm tissue

a. Distribution of label in various fractions of endosperm: The metabolism of glutamine and its incorporation into protein amino acids was examined. Kinetic studies on the incorporation of label into various endosperm fractions showed that after 15 hr of incubation, most of the label was in the water soluble fraction, but as the incubation period increased, label appeared in the zein fraction. The incorporation into this fraction was greatest between 96 and 168 hr. At 168 hr, 31% of the total label was in zein fraction, 15% in glutelin, 24% in starch and 19% remained in the free amino acid fraction pool (Table 35, Figure 51).

Figure 49. Kinetics of uptake of (^{14}C)-glutamine by cultured cob blocks of var. W64A x W182E

Samples were harvested 5-7 days after pollination.

Incubation on nutrient media containing (^{14}C)-glutamine (0.25 µCi/ml) was performed as described in Methods.

●——● (cob tissue + endosperm + embryo)

o——o (media)

Mean values of 5 replicate samples ± range (bars) are shown for each point.

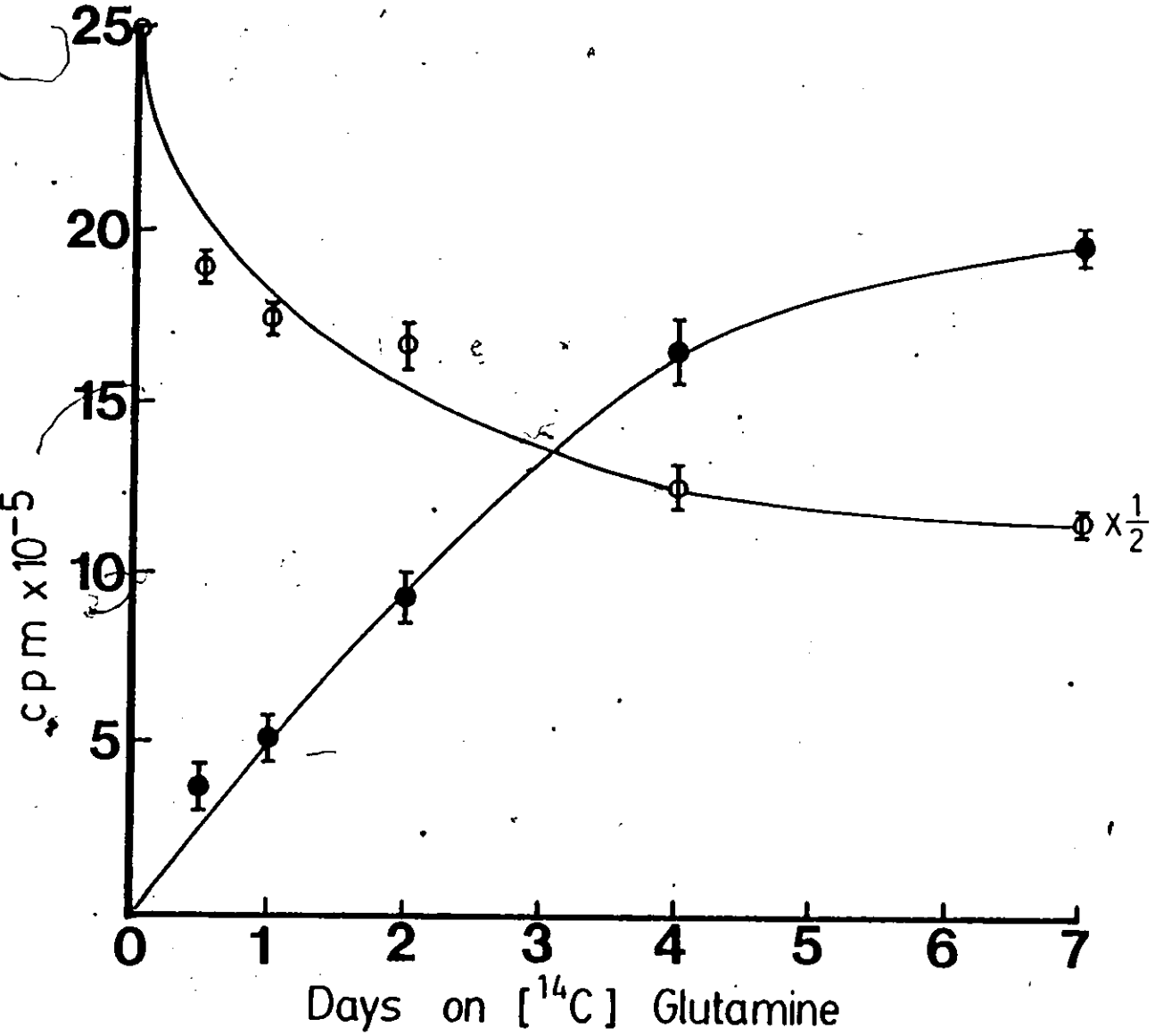

25
20
15
10
5
cpm x10⁻⁵
Φ
Φ
Φ
Φ
Φ X 1/2
0
1
2
3
4
5
6
7
Days on [14C] Glutamine

Figure 50. Incorporation of (^{14}C)-glutamine into cultured cob tissue, endosperm and embryo with time of incubation.

Samples of maize hybrid W64A x W182E were harvested 5-7 days after pollination.

Incubation on nutrient media containing (^{14}C)-glutamine (0.25 µCi/ml) was performed as described in Methods.

●——● cob tissue

o——o endosperm

□——□ embryo

Mean values of 5 replicate samples ± range (bars) are shown for each point.

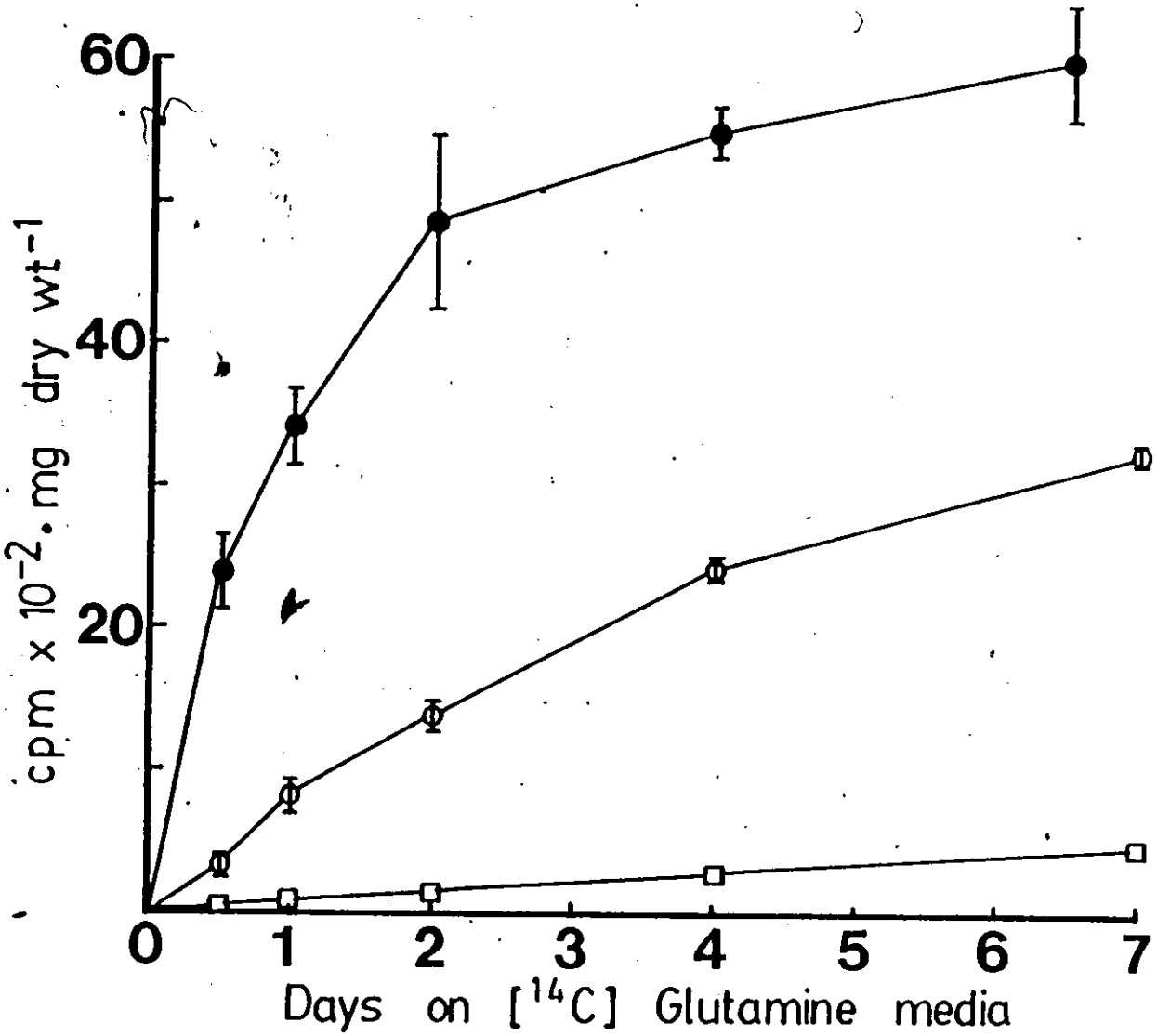

60
40
20
0
1
2
3
4
5
6
7
cpm x 10^{-2}. mg dry wt^{-1}
Days on [^{14}C] Glutamine media

Figure 51. Distribution of label in the various fractions of endosperm extracts with time of incubation.

Samples of maize hybrid W64A x W182E were harvested 5-7 days after pollination. Incubation on nutrient media containing (^{14}C)-glutamine (0.25 µCi/ml) was performed as described in Methods.

■——■ amino acids

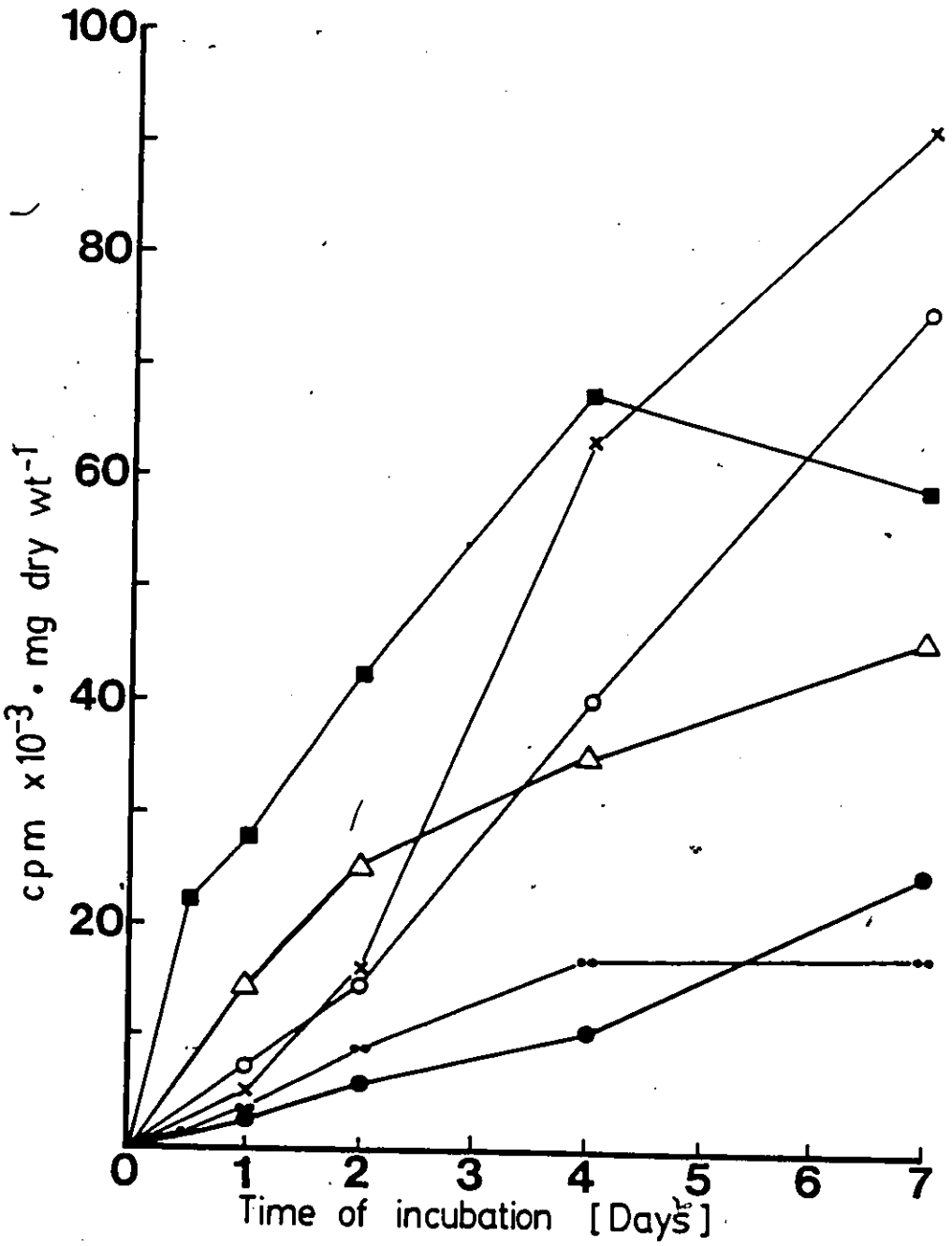
x——x zein

o——o starch

▲——▲ glutelin

●●——●● albumins and globulins

Δ——Δ organic acids and sugars


100
80
60
40
20
0
1
2
3
4
5
6
7
cpm x10⁻³ • mg dry wt⁻¹
Time of incubation [Days]

b.) Enzymatic method for hydrolysis of endosperm proteins

In order to digest proteins, lyophilized and pulverized endosperm powders were treated with commercial proteases: a mixture of pronase and carboxypeptidase A. The kinetics of the hydrolysis are shown in Fig. 52a. The values for release of total nitrogen and α-amino nitrogen in the supernatant are represented as a percent of total values measured on acid hydrolyzed powders. After 21 hr, 90% of the total nitrogen is released into the supernatant solution, whereas only 60% of the total α-amino nitrogen is released. After 60 hr, about 86% of the total α-amino nitrogen (relative to that obtained in acid hydrolyzed powders) is released. This was the maximum value obtained in these experiments. Estimation of ammonia on these samples showed that the amount of diffusible ammonia increased in the samples with the longer periods of incubation (Fig. 52a,b). For example, in the enzyme hydrolysates, after 30 hr of incubation, ammonia contributed 2.5% of total nitrogen, after 54 hr, 6%. This suggested some breakdown of amides with longer periods of incubation. To reduce the time of incubation, and to achieve maximum hydrolysis of proteins, the method was modified according to Winkler and Schön (1979). The dry endosperm powders were pretreated with sodium dodecyl sulphate (SDS) to solubilize the proteins according to the procedure described in Methods; this was followed by enzymatic hydrolysis. At time zero (after 30 min in SDS at 40°c), all the nitrogen was recovered in the medium. Thirty-five percent of the total nitrogen was present as α-amino nitrogen. After 48 hr, the level of α-amino nitrogen in medium was almost

Figure 52 (a,b). Kinetics of enzymatic hydrolysis of endosperm powders.

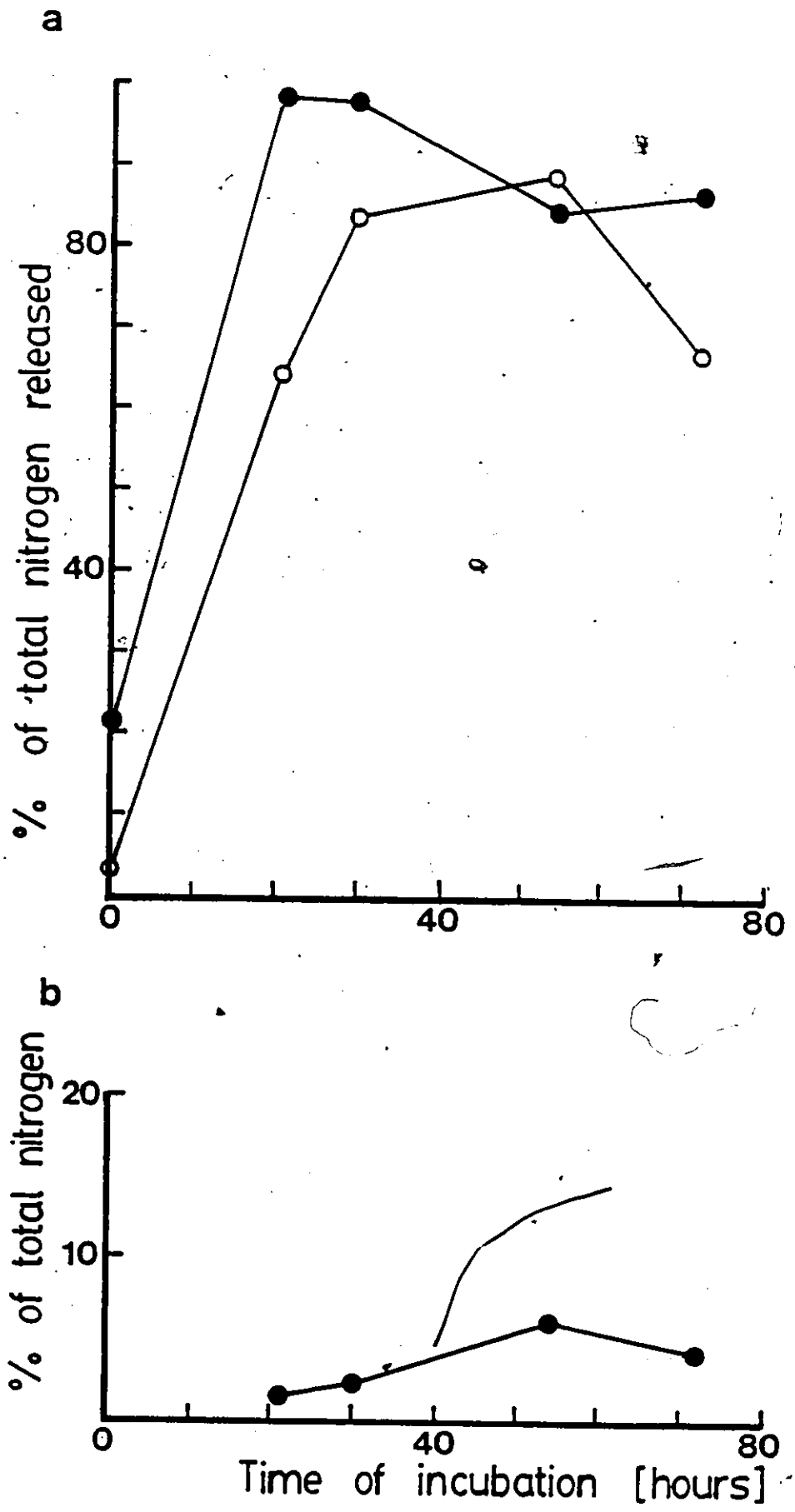
a) Release of total nitrogen and α-amino nitrogen from endosperm powders *vs* time of incubation.

●——● total N

o——o α-amino N

b) Release of ammonia in enzymatic hydrolysates of endosperm powders incubated for various time periods.

Fifty mg of dry endosperm powder were incubated with 100 mg pronase + 2.5 mg carboxypeptidase A in 2.0 ml of borate buffer (pH 8.3). To avoid microbial contamination, 20 µl of chloroform was added. Incubation was performed at 40°C.


a
% of total nitrogen released
80
40
0
40
80
b
% of total nitrogen
20
10
0
40
80
Time of incubation [hours]

equal to that obtained after acid hydrolysis of the dry powders..

The amino acid analysis of the acid hydrolysates and the enzymatic hydrolysate of the 30 day post-pollination powder yielded similar results, except that proline was not detected in the enzymatic hydrolysates (Tables 31,32). Also, the values for (Glu + Gln) and (Asn + Asp) in enzymatic hydrolysates are somewhat larger than those obtained for glutamate and aspartate in the acid hydrolysates, respectively. On the other hand, the level of arginine, valine, isoleusine, leucine and tyrosine are only slightly higher in the enzyme hydrolysed samples.

The supernatant solutions after enzyme hydrolysis were also subjected to acid hydrolysis. Prior to acid hydrolysis, the samples were brought to 50% with methanol, incubated at -20°C overnight and then centrifuged. The resultant supernatant solution was hydrolyzed in 6 N HCl for 12 hr. Amides recovered by enzyme hydrolysis were converted to glutamate or asparatate after acid hydrolysis. Proline, was also released after acid hydrolysis.

Therefore, it may be that some of these amino acids are released as peptides and that presence of proline in primary structure is inhibiting the activity of proteases (Oaks *et al.*, 1982). Since most of the glutamine was recovered by enzymatic treatment, the method was considered satisfactory for digestion of proteins.

c.. Distribution of label in amino acids derived from zein:

After 168 hr of incubation, the zein fraction accounts for

Table 31

Total Nitrogen Content and the α-amino N Content of Acid Hydrolysate and Enzymatic Hydrolysate of 30 Days Post Pollination Endosperm Powder

Treatment	0' time		48 hr	
	Supernatant	Residue	Supernatant	Residue
	µmoles/assay		µmoles/assay	
Enzymes + powder				
Total N	69.30	5.78	74.66	-
α-amino N	33.60	-	63.57	-
Enzymes alone				
Total N	25.58	1.41	25.00	1.5
α-amino N	19.00	-	27.00	-
[Enz + Powder - Enz only]				
Total N	48.08	-	47.16	-
α-amino N	14.60	-	36.57	-
Acid digests				
Total N	44.50	-	-	-
α-amino N	39.14	-	-	-

Fifty mg of endosperm powder + 20 mg dialyzed pronase (Calbiochem), 2.5 mg carboxypeptidase A (Calbiochem), 2 ml of borate buffer (pH 8.3) (20 λ of pure chloroform and 0.5 ml of 95% ethanol were added to avoid microbial contamination). Incubation was performed at 40°C. The powder was pretreated with 0.1 N NaQH and 15% SDS to solubilize the proteins. For acid hydrolysis 2 ml of 2N HCl and 40 mg of powder were autoclaved overnight. The tubes were evacuated with N_2 and then sealed, before autoclaving.

Table 32

Amino Acid Composition of Endosperm Powder Hydrolysates

Amino acid	Enzyme hydrolysates nmoles/100 mg	Acid hydrolysates nmoles/100 mg
Lysine	0.448	0.361
Histidine	0.575	0.479
Arginine	1.095	0.709
Aspartate	0.416	2.392
Threonine	1.301	1.425
Serine	2.222	2.470
Glutamate	0.613	8.320
Proline	N.D.	4.870
Glycine	1.686	1.832
Alanine	5.600	5.300
Valine	2.617	2.132
Methionine	1.780	2.200
Isoleucine	2.285	1.420
Leucine	7.300	6.710
Tyrosine	1.860	1.340
Phenylalanine	1.830	1.600
Asn	1.300	-
Gln	5.000	-
[Asp + Asn]	1.700	2.400
[Glu + Gln]	5.613	8.300

Amino acid analyses were performed by Dr. K.W. Joy, Carleton Univ., Ottawa, Ontario.

Table 33

Amino Acid Composition of Endosperm Powders Digested with Acid, Enzymes and Enzymes + Acid

Amino acid	nmoles·mg powder^{-40}		
	I*	II[a]	III
Lysine	178.9	173.0	144.0
Histidine	230.0	153.0	191.0
Arginine	624.0	249.0	238.0
Aspartic acid	669.0	719.0	957.0
Threonine	520.0	346.0	510.0
Serine	899.0	584.0	988.0
Glutamic acid	245.0	2,036.0	3,328.0
Proline	not detected	1,003.0	1,948.0
Glycine	717.0	674.0	733.0
Alanine	2,109.0	1,666.0	2,119.0
Valine	1,055.0	774.0	853.0
Isoleucine	913.0	518.0	566.0
Leucine	2,929.0	1,810.0	2,684.0
Tyrosine	744.0	431.0	534.0
Phenylalanine	734.0	521.0	641.0
Asparagine	505.0	-	-
Glutamine	1,194.0	-	-
TOTAL	15.05	11.65	16.43

I* Enzymatic digestion.

II Enzymatic digestion followed by acid digestion.

III Acid digestion only.

Endosperm powders prepared from samples harvested 30 days after pollination were used.

The hydrolysis was essentially as described in Methods.

II[a]: In supernatant solution of enzyme hydrolyzed powders, the proteins were first precipitated with ethanol and then the supernatant was hydrolyzed with 6 N HCl.

up to 37% of the total counts incorporated into the endosperm powders. Zein was then hydrolysed by pronase and carboxypeptidase A, in order to determine the incorporation of glutamine into this fraction. Fig. 53a,b shows the elution pattern of OPA-derivatized amino acids in these hydrolysates. The glutamine concentration is higher than that of glutamate. Radioactivity in aspartate, asparagine, glutamine, glutamate and in the neutral + basic amino acids was measured (Tables 34b, 36). The results show that the acidic amino acids and their amides together accounted for 33% of the total label in zein fraction and of these, glutamine alone accounted for 13% of the label. The values for glutamine in the zein hydrolysate may be an underestimate because of the somewhat lower recovery of Glu + Gln in enzyme hydrolysates relative to that in acid hydrolysate.

d. Analysis of the cob tissue: As all the label entering the kernel must pass through the cob tissue, the cob tissue was also examined for (^{14}C). Results in Table 43a show that glutamine was extensively metabolized to sugars and organic acids in cob tissue. These two fractions accounted for 37% of the total label incorporated into cob tissue. Of the free amino acid fraction, glutamine was the major amino acid and it alone accounted for about 40% of the total label. In contrast to the cob tissue, in the free amino acid fraction of the endosperm, only 12-16% of total label was in glutamine (Table 34b). Thus, is appears that in the cob tissue,

Figure 53 (a,b). Elution pattern of amino acids in zein hydrolysate (b).

The zein fraction was hydrolyzed with commercial pronase and carboxypeptidase as described in Methods.

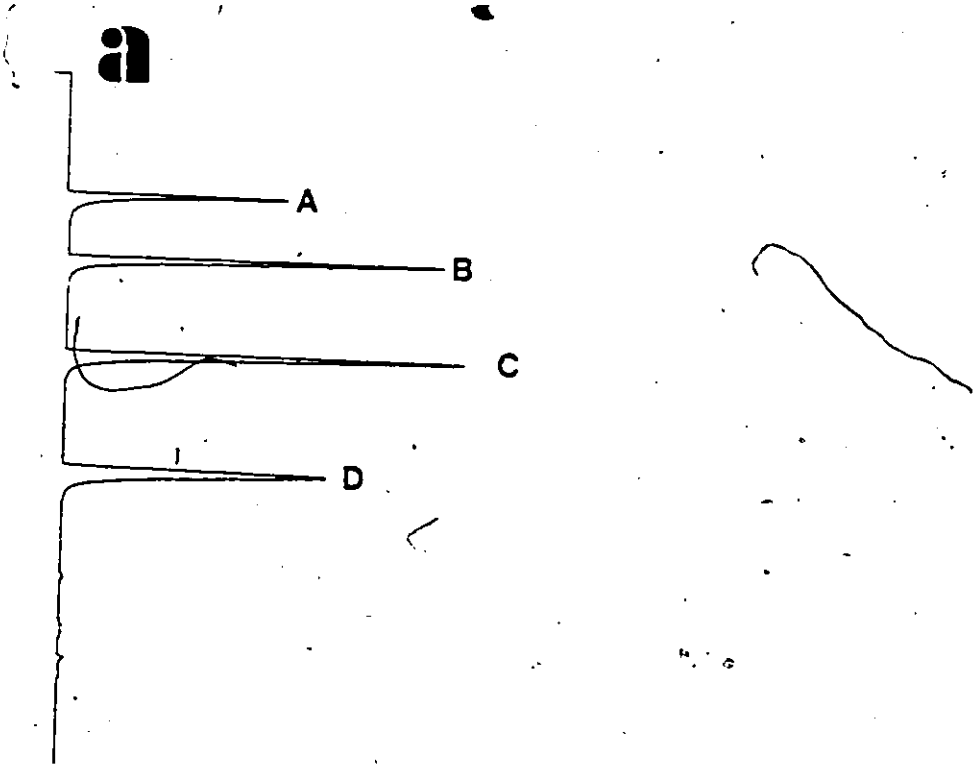
Appropriate aliquots were lyophilized and derivatized with o-phthaldialdehyde and 2-mercaptoethanol.

Aspartate, glutamate, asparagine and glutamine were identified by running derivatized standards(a). Other amino acids were collected in bulk.

Conditions

A phosphate buffer (0.02 mM, pH 6.8) + methanol gradient starting at 10% methanol and increasing to 33% over a 20 min period at a rate of 1.65%/min, isocratic step at 33% for 30 min, followed this by 50% methanol for 10 min, increasing at a rate of 0.48%/min. Flow rate was 0.8 ml/min.

A ASPARTATE
B GLUTAMATE
C ASPARAGINE
D GLUTAMINE


a
A
B
C
D

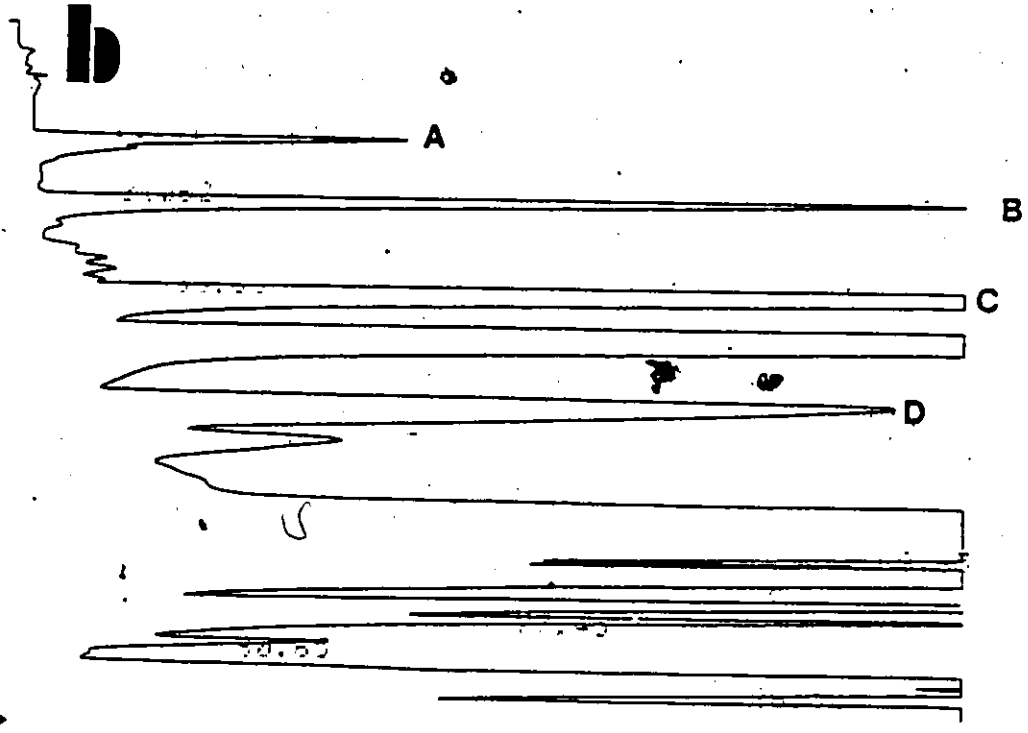

b
A
B
C
D

Table 34a

Distribution of ^{14}C-glutamine in Tissue Extracts from Cultured Caryopsis

Fraction	Cob		Endosperm	
	cpm x 10^{-3}	%	cpm x 10^{-3}	%
Starch	62.19	20	39.60	35.0
Zein	-	-	37.00	33.0
Glutelin	-	-	8.46	7.5
Albumin + Globulin	1.13	0.36	8.83	7.8
Amino acids	103.15	33.2	11.76	10.5
Organic acids + sugars	120.17	38.7	4.94	4.4
Residue	-	-	2.24	2.0
Total recovered	310.24	-	112.83	-
% recovery	94.00	-	87.00	-

Fifty mg powder of freeze dried sample was used for extraction of starch, proteins and "TCA soluble" fractions. Extraction of various components was performed according to the modified method of Sodek and Wilson (1971). Radioactivity is expressed in cpm/50 mg of endosperm or cob tissue powder.

Table 34b

Distribution of the ^{14}C-label Among Various Amino Acids of the Free Amino Acid Fraction and the Zein Hydrolysate of the Endosperm, After 7 Days of Culture on [^{14}C] Glutamine Media

Amino acids	[I] Amino acids (Dowex-50 H^+ fraction)		[II] Zein hydrolysate (Dowex-50 H^+ fraction)
	Cob	Endosperm	Endosperm
	cpm x 10^{-2}	cpm x 10^{-2}	cpm x 10^{-2}
Glutamine	388.80 [38.0]*	13.00 [12.00]	27.80 [10.3]
Glutamate	132.00	17.60	17.80
Aspartate	147.20	13.94	19.60
Asparagine	163.20	7.79	22.70
Neutral and basic amino acids	200.30	57.20	179.95
Total recovered	1,031.50	110.93	267.85
Total counts	1,101.50	117.60	292.00
Recovery	93	94	91.7

[I] Amino acids were eluted from the Dowex-H^+ column by 6 N NH_4OH, and redissolved in 30% methanol. The samples were then injected into HPLC columns. Counts eluted in each fraction were recorded according to the methods outlined in Methods.

[II] Zein fraction was hydrolyzed using commercial pronase and carboxypeptidase A preparation.

* Values in brackets are the % of Gln present

glutamine is extensively metabolized. Also, it suggests that metabolic products of glutamine produced in the cob may be translocated to the endosperm. For example, a large amount of (^{14}C) in the carbohydrate of the endosperm could result from translocation of (^{14}C) in sugars derived from the metabolism of glutamine in the cob tissue. To rule out this possibility, glutamine (0.05 µCi/µl/endosperm) was injected directly into the endosperm of caryopses, 10 days after the initiation of the cultures.

e. Fate of [^{14}C]-glutamine injected into the endosperm: An analysis of the various endosperm fractions obtained 168 hr after injection, confirmed that glutamine was not only used for the synthesis of amino acids in the endosperm but also that a large fraction was converted into carbohydrates. The distribution of label in various fractions after 168 hr of incubation was 21% in starch, 38% in zein, 8% in glutelin and 13% in the free amino acid fraction (Table 35). In zein hydrolysates, glutamine accounted for 14% of the total label (Table 36). This distribution pattern is similar to the pattern obtained when ^{14}C-glutamine was supplied through the nutrient media. In both experiments, label in glutamine of the endosperm tissue accounted for only about 12-14% of the total label incorporated into this tissue. The percentage recovery of (^{14}C)-glutamine in whole endosperm powders after enzymatic hydrolysis of proteins was similar to the values obtained on zein hydrolysates. Table 37 shows the percentage recovery of glutamine in whole endosperm powders digested

Table 35

Distribution of Label Among Various Fractions of Endosperm Extracts of Cultured Caryopsis W64A x W182E

Fraction	Supplied via media[I]		Supplied via injection[II]	
	cpm x 10^{-3}/100 mg	% of total	cpm x 10^{-3}/100 mg	% of total
Starch	74.17	24.7	20.0	21.3
Amino acids	57.8	19.3	12.7	13.0
Sugars + organic acids	24.6	8.0	6.6	7.1
Albumin + globulin	17.9	5.9	7.57	8.1
Zein	91.68	30.66	37.56	40.21
Glutelin	45.04	15.00	7.30	8.0
Residue	0.96	0.32	0.58	0.6
Total	299.93	-	92.48	-

[I] Label was supplied in the media as (UL-^{14}C)glutamine (0.25 µCi/ml media).

[II] With a microsyringe, approximately 0.05 µCi/µl of (UL-^{14}C)-glutamine was injected into the endosperm of cultured caryopses.

Seeds were analyzed 7 days later for incorporation of label into various fractions.

Table 36

Distribution of Label in Zein Hydrolysates of Cultured Endosperm Powders

Fraction (amino acids)	Supplied by media[I]		Supplied by injection[II]	
	cpm x mg^{-100}	% of total	cpm x mg^{-100}	% of total
Aspartate	3,506	4.2	2,090	6.8
Glutamate	3,918	4.7	3,020	9.9
Asparagine	2,231	2.7	1,100	3.6
Glutamine	9,150	11.0	4,990	16.4
Neutral + basic	63,750	77.0	18,724	61.6
Total	82,550	-	30,354	-
Recovery	-	90	-	83

Label was supplied in the media as (UL-^{14}C)-glutamine (0.25 µCi/ml), or by injection into the endosperm of cultured caryopsis (0.05 µCi/µl/seed). Endosperms were analyzed 7 days later for incorporation of label into zein fraction. Zein was hydrolyzed with commercial pronase and carboxypeptidase A. Amino acids were separated on reverse-phase HPLC column and eluants were assayed for radioactivity. Recovery is expressed as percentage of total counts injected onto HPLC column.

Table 37

Distribution of Glutamine-Carbon in

the Endosperm Amino Acids

Amino acid fraction	cpm·sample	
	*I	II
Asp	80	121
Asn	n.d.***	n.d.
Glu	91	102
Gln	176 (16.7)**	153 (13.4)
Others	698	761
TOTAL	1,049	1,137

* Two replicate samples of *in vitro* cultured caryopses. Each sample contained 7 caryopses.

** (^{14}C)-glutamine as percentage of total counts.sample.

*** n.d. = not detected.

Whole endosperm powders of cultured caryopses on a (^{14}C) glutamine-containing media were hydrolyzed with commercial proteases as described in Methods.

Amino acids were fractionated by HPLC and radioactivity was recorded.

with commercial proteases. In two experiments, the percentage value of glutamine was 13-17% of the total (^{14}C) in the amino acid fraction.

DISCUSSION

The developing kernels of cereals depend upon vegetative tissue for organic nitrogen and other nutrients. Assimilated nitrate is reduced in the roots and leaves and is thought to be transported as amino acids or amides. Amino acids are also mobilized from senescing leaves to developing kernels, (Pate, 1980).

In young corn seedlings, glutamine is a major transport compound and in fact may account for as much as half of the total amino acids and amides transported from the roots to the upper parts of the plant (Ivanko and Ingeversen, 1971). Asparagine also contributes to the nitrogen imported into the developing seed. Kirkman and Miflin (1979) have measured the amino acid levels of bleeding sap of wheat plants during the growing season and they found that glutamine was the major amide present. However, its relative abundance declined with increasing age. Arruda and Da Silva (1979) have also shown that glutamine is a major source of nitrogen supplied to developing corn cobs. Results of this study also indicate a relatively high level of glutamine in endosperm extracts. The concentration of free glutamine in the endosperm of inbred variety (W64A) was calculated to be in the order of 30 mM. The levels of glutamic acid plus glutamine represent 22 percent of the amino acid content of zein, the major storage protein in maize. Aspartic acid plus asparagine, on the other hand,

represent only 5-6 percent of the amino acid content (Dalby,1966). The major fate of imported glutamine (or asparagine) could be its direct incorporation into endosperm proteins. Because maize endosperm tissue will grow in sterile cultures with either asparagine or glutamine or NH_4^+ salts as a source of nitrogen, it is clear that this tissue has the capacity to catabolize either amide and to use the resultant NH_4^+ derived from the amides. Nitrate salts do not support growth of corn endosperm tissue in culture and hence are probably not important as nitrogen precursor in developing endosperm (Straus,1960; Farrar and Ganugapati,1970; Reddy and Peterson,1977; Shannon and Lin,1977).

A. Enzymes of Asparagine Metabolism

The two known major routes for asparagine degradation in plants are as follows:

I Asparaginase:

Asparagine + water $\longrightarrow$ Aspartate + NH_3

II Asparagine transaminase:

Asparagine + α-ketoacid $\longrightarrow$ 2-ketosuccinamate + amino acid

IIa 2-ketosuccinamic acid + water $\longrightarrow$ oxaloacetate + NH_3

IIb i 2-ketosuccinamic acid $\xrightarrow{2H}$ 2-Hydroxysuccinamate

ii 2-Hydroxysuccinamate $\xrightarrow[H_2O]{}$ Malate + NH_3

In pea seed, asparagine transaminase activity is low (Ireland and Joy, 1981). However, high asparaginase activity (76 nmoles aspartate. min^{-1} . $seed^{-1}$) combined with a low Km for asparagine (~3 mM) suggests the presence of an efficient system for the utilization

of the amide nitrogen of asparagine (Sodek *et al.*, 1980; Ireland and Joy, 1981). The activity of the enzyme develops initially in the tests, subsequently the activity appears in the cotyledons. The maximum level of activity of the cotyledon enzyme was sufficient to allow the synthesis of up to 5 mg protein per cotyledonary pair per day from asparagine. This compares with a maximum calculated rate of cotyledonary protein synthesis of 3.08 mg/seed/day during the period of rapid protein accumulation. An asparaginase with an apparent Km of 12.2 mM has been described for *Lupinus sp.* (Lea *et al.*, 1978) but in this case, the concentration of asparaginase in the phloem stream supplying the developing seed is in the order of 50 mM (Atkins *et al.*, 1975). Hence, this enzyme too could also have an important function *in vivo*.

In corn endosperm extracts, asparaginase activity was assayed using a coupled spectrophotometric assay, developed during the course of this work (Misra and Oaks, 1980). The asparaginase activity was detected in the developing endosperm at all stages examined. As with the pea enzyme, the asparaginase from maize endosperm was stimulated by K^+. Asparagine at a concentration of 100 mM and KCl at 20 mM saturated the reaction. Under optimum assay conditions, asparaginase activity per endosperm was 0.349 $\mu moles \cdot h^{-1}$. Levels of asparaginase peaked in the endosperm about 20 days after pollination. The approximate concentration of asparagine, when maximum levels of asparagine were found in the endosperm tissue, was 9 mM. Since asparaginase from corn endosperm has an apparent Km of 10 mM, the potential capacity of the maize asparaginase for degrading asparagine

in vivo, is much less than that of the pea enzyme.

Asparagine transaminases have also been reported in a number of plants, most recently in soybean (Skokut *et al.*,1982) and pea (Ireland and Joy, 1981). (^{15}N) amide and (^{15}N) amino-nitrogen labeling studies performed in developing cotyledons of soybean show that the transaminase reaction is of major importance in the utilization of asparagine for synthesis of storage proteins. During the seven days in culture with asparagine as the sole source of nitrogen, the amino nitrogen donated approximately twice as much nitrogen to proteins as did the amide nitrogen. However, the use of the amide nitrogen increased with longer periods of incubation (Skokut *et al.*,1982).

In developing maize endosperm tissue, the levels of asparagine-transaminases were low. The peaks for transaminase activities (asparagine-pyruvate, 241 nmoles.h^{-1}.endosperm^{-1} and asparagine-α-ketoglutarate,223 nmoles.h^{-1}.endosperm^{-1}) were 5-10 days later than the peak in asparaginase activity (349 nmoles.h^{-1}.endosperm^{-1}.). The initial increase coincides with increases in zein protein in the endosperm. Both the levels of activity at each stage of development and Km for asparagine (~10 mM) suggest that either of these reactions could be responsible for the degradation of asparagine in maize endosperm. However, the low levels of activity of enzymes of asparagine catabolism, a low affinity for substrates and low concentration of free asparagine in endosperm extracts suggest that the dominant fate of asparagine is probably its direct incorporation into endosperm

proteins rather than its catabolism. Tracer studies done on wheat seem to support this suggestion. For example, Kolderup (1979) has shown that asparagine supplied externally to developing wheat spikes is not metabolized extensively and most of it is incorporated intact into storage proteins.

B. Enzymes of Glutamine Metabolism

An enzyme (glutaminase) capable of hydrolyzing glutamine in a reaction similar to that of asparaginase, was not detected in the endosperm tissue. On the other hand, glutamate synthase activity is high enough in the developing endosperm of corn to supply glutamic acid for the synthesis of other amino acids and hence could be the enzyme responsible for the degradation of glutamine in the seed. The peak of glutamate synthase activity is reached 5-10 days after the peak accumulation of glutamine and coincides with the period of active zein accumulation. Sodek and Da Silva (1977) have also shown that the glutamate synthase activity is related to the rate of nitrogen accumulation in the endosperm and that the level of enzyme activity is high enough to account for the observed rate of nitrogen accumulation. For example, when the rate of nitrogen accumulation was 9.5 nmoles.min^{-1}, the GOGAT activity was in the order of 56 nmoles glutamate.min^{-1}. Similar increases in GOGAT activities are also observed in developing cotyledons of pea (Storey and Beevers, 1978; Murray and Kennedy, 1980). However, the primary role of the enzyme in the pea tissue is reassimilation of ammonia (via GS/GOGAT) released from asparagine (Miflin and Lea, 1980; Miflin *et al.*, 1981).

In crude preparations from corn endosperm, the enzyme appears to be active with reduced ferredoxin as well as NAD(P)H. In pea cotyledons, it has been shown that NAD(P)H and ferredoxin-dependent GOGATs are distinct proteins (Matoh *et al.*, 1979). More recently, similar results have been obtained in etiolated pea shoots (Matoh *et al.*, 1980), *Chlamydomonas* (Cullimore and Sims, 1981) and in etiolated rice shoots (Suzuki *et al.*, 1982). In the latter tissue, immunologic studies showed that Fd-GOGAT from green and etoliated leaves are antigenically identical whereas Fd-GOGAT from roots is only partially related to the leaf enzyme. The antibodies raised against ferredoxin-GOGAT from green leaves of rice did not cross react with NAD(P)H-dependent glutamate synthases in etiolated leaf and root tissues.

In green tissues, ferredoxin reduction is mediated by the activity of photosystem I. In non-green tissues, it is believed that NAD(P)H-ferredoxin reductase mediates the reduction of ferredoxin and that the NAD(P)H is supplied by the activity of pentose phosphate pathway in the plastids (Miflin and Lea, 1980). The situation is analogous to the reduction of nitrite in roots and in cultured cells. In these tissues, the nitrite reductase activity is dependent on ferredoxin. Nitrite reductase, like glutamate synthase, is located in the plastids and reduction of nitrite is closely linked to the activity of the pentose phosphate pathway (Dalling *et al.*, 1972; Miflin, 1974; Emes and Fowler, 1979; Oaks and Gadal, 1980; Dry and Wallace, 1981).

In developing endosperm of corn, there is also an NADH-GOGAT and Fd-GOGAT activity. The estimated molecular weight of ferredoxin-GOGAT

was 171,000 and of NADH-GOGAT was 270,000. In higher plants, the molecular weight of ferredoxin-GOGAT ranges from 145,000 in *Vicia faba* leaves to 224,000 in *Chlamydomonas reinhardii* (Table 38). Where two forms of GOGAT have been described in one tissue, the molecular weight of NADH-GOGAT is higher than that of ferredoxin-GOGAT. It is believed that association of flavin-moities results in slightly higher molecular weight of NADH-GOGAT than Fd-GOGAT. Since studies with flavo-enzyme inhibitors (e.g., tryptamine, acridine) show that NADH-GOGAT is inhibited by these inhibitors whereas Fd-GOGAT remains unaffected (Matoh *et al.*, 1979). Spectrophotometric absorption studies also show lack of flavin in ferredoxin-GOGAT (Matoh *et al.*, 1980; Tamura *et al.*, 1980).

Almost equal levels of activity of these enzymes were present in the endosperm of corn at each stage of development examined. The Km values for glutamine, α-ketoglutarate and NADH for NADH-GOGAT are 1.068, 0.539, and 0.0086 mM, respectively. The Km values for L-glutamine, α-ketoglutarate and ferredoxin for ferredoxin-GOGAT are 0.617, 0.258, and 0.0086 mM, respectively. The high affinity of each enzyme for their respective substrates and almost equal levels during development suggest that each enzyme has the potential to metabolize glutamine provided that sufficient reductants are available. In fact, the lower Km values for glutamine and α-ketoglutarate in ferredoxin-GOGAT reaction relative to that of NADH-GOGAT reaction suggest that this enzyme could be physiologically more important in the formation of glutamate.

Table 38

Properties of Glutamate Synthases Isolated from Various Sources

Source (enzyme)	Molecular weight	Km (μmoles)				
		NADH	NADPH	Fd	α-Kg	Gln
Escherichia coli	800,000	-	7.7	-	7.3	250
Lupin root nodule	235,000	1.24	-	-	23.0	500
Phaseolus root nodule*	220,000	7.10	-	-	19.0	222
Pea shoots[1]	220,000	4.00	-	-	37.0	400
Chlamydomonas reinhardii I	165,000	-	-	0.01	170.0	190
Chlamydomonas reinhardii II	240,000	13.00	-	-	7.0	900
Pea developing cotyledon	n.d.	13.30	-	-	960.0	1,430
Corn developing endosperm	n.d.	7.0	-	-	570.0	1,350
Corn developing endosperm I*	170,000	-	-	1.8	258.0	617
Corn developing endosperm II*	270,000	8.06	-	-	539.0	1,068
Pea cotyledons I[2]	220,000	-	-	-	-	-
Pea cotyledons II	155,000	-	-	-	-	-
Corn leaves[3]	160,000	-	-	1.7	240.0	1,100
Spinach leaves[4]	180,000	-	-	-	-	-
Rice leaves[5]	224,000	-	-	5.5	330.0	270 - 570

(Adapted after Miflin *et al.*, 1981.)

[1]Matoh *et al.* (1980).

[2]Matoh *et al.* (1979).

[3]Matoh *et al* (1979).

[4] Tamura *et al.* (1980).

[5] Suzuki *et al.* (1982).

* My data.

In developing pea leaves, the relative level of NADH and ferredoxin-GOGAT seem to vary with age (Takahashi *et al.*, 1982). In immature leaf, NADH-GOGAT is much higher than the ferredoxin-GOGAT. During maturation of the leaf, the ferredoxin-GOGAT becomes relatively more important. Further it has been shown that chloroplasts from immature leaves mediate a light independent conversion of glutamine to glutamate, when the appropriate carbohydrate supply is available. In mature leaf chloroplasts, GOGAT activity was dependent on light. The predominance of NADH-GOGAT in the immature tissue might account for the light independence of glutamine utilization in this tissue. Thus, the changes in the relative level of these enzymes in leaf tissue appear to be integrated with the changes in reductant supply (NAD(P)H or ferredoxin).

In corn seeds, the physiological significance of the coexistence of the two enzyme forms is not clear. However, the two enzyme forms may differ in their distribution within the seed tissue. For example, in developing seeds of barley, ferredoxin-GOGAT activity, measured in crude extracts, is localized exclusively in the testa and pericarp, whereas, NADH-GOGAT activity is predominant in the endosperm tissue (Miflin and Shewry, 1979). Unlike corn seed, testa and pericarp in immature barley seeds are chlorophyllous.

C. Comparative Assessment of Nitrogen Metabolism in Normal and High Lysine Mutant Varieties

Prolamine and starch are the major storage products of cereals. Zein, an ethanol soluble protein fraction may account for up to 60% of

the total protein at maturity (Mertz, 1972). It is relatively high in glutamine, proline, alanine and leucine, whereas lysine is poorly represented (Nelson *et al.*, 1965, Murphy and Dalby, 1971). Also, the zein fraction increases with increasing levels of nitrogen fertilizers, while non-zein proteins show only minor responses (Keeny, 1970; Rendig and Jimenez, 1979; Tsai *et al.*, 1980). In high lysine mutants of corn, opaque-2 and floury-2, the synthesis of zein is reduced. As a result, the relative contribution of other endosperm proteins which contain significant amounts of lysine is higher (Murphy and Dalby, 1971; Sodek and Wilson, 1971; Tsai *et al.*, 1980).

In barley, the high lysine trait is associated with a block in starch synthesis and an accumulation of soluble sugars and amino acids in the endosperm (Kreis and Doll, 1980). Direct evidence for blocked starch synthesis has not yet been demonstrated in high lysine corn. Although a lower dry weight (which is probably related to a lower level of carbohydrate) of kernels at maturity compared with the normal variety, is characteristic of the opaque-2 and floury-2 seeds (Murphy and Dalby, 1971; Dalby and Tsai, 1975; Tsai *et al.*, 1980).

Tsai *et al.* (1978) showed a positive correlation between levels of starch, kernel weight, zein content and germ size of normal variety of maize. This suggests that levels of starch are correlated in some way with rates of zein biosynthesis. Some starchy mutants of corn do, in fact, have a lower zein content (Tsai and Dalby, 1975). Dalby and Tsai (1975) reported that mutation at the opaque-2 locus, which reduces zein synthesis, results in a reduction of kernel

weight and yield. The results are explained on the basis that zein may serve as a functional nitrogen sink in the kernel and thus may affect starch accumulation and kernel weight. The results of my study show that up to 35 days, the differences in dry weight of normal and mutant varieties are minor although the zein content is clearly reduced from 20 days post pollination. At 40 days, the control kernels had the highest level of zein and marginally higher levels of total nitrogen and dry weight.

Besides affecting prolamine synthesis, major differences exist in the metabolism in normal and high lysine mutant varieties. For example, it has been shown that high lysine mutant varieties of barley as well as corn have an altered amino acid metabolism (Sodek and Wilson, 1970; Brandt,1975; DaSilva and Arruda,1979). In the normal endosperm, a reduction in lysine concentration is seen during maturation, whereas, the high lysine mutants maintain a constant level of lysine in the endosperm throughout the developmental period (Brandt,1975; DaSilva and Arruda, 1979). Sodek and Wilson (1970) demonstrated a lower rate of catabolism of lysine to proline and glutamate and this could be the cause of the higher lysine content in mutant varieties at maturity. In their experiments (^{14}C) labeled leucine and lysine were injected below the ear into the shank of the corn plant and the kernels were analyzed at maturity. Appreciable levels of ^{14}C derived from lysine was found in zein. Analysis of (^{14}C) in individual amino acids of zein indicated that lysine had been converted to glutamic acid and proline in the endosperm of normal variety but to a lesser extent in opaque-2. Similar results related to the catabolism

of lysine have also been reported in barley (Brandt,1975).

Results of my experiments show higher accumulations of glutamate, glutamine, aspartate and asparagine in opaque-2 and floury-2 mutants than in the wild type. Similar observations have been made previously (Dalby,1966; Murphy and Dalby,1971; Sodek and Wilson,1971; Dierks-Ventling,1981). The accumulation of these amino acids could result from a normal transport coupled to a reduced utilization in the synthesis of zein. Glutamine would be an example of this mechanism. Other neutral and basic amino acids should also accumulate. For example, proline, alanine and leucine which are abundant in zein, might be expected to accumulate in the soluble fraction. A complete analysis of amino acid fractions of normal and opaque-2 endosperms has shown that all amino acids do not accumulate in the endosperm (Sodek and Wilson,1971). For example, in opaque-2, the level of arginine, histidine, valine and isoleucine remain more or less unchanged. This may indicate that the synthesis of these amino acids is regulated by end product inhibition. One might predict that if the synthesis of these amino acids were regulated by end product inhibition, there would be a reduction in the conversion of transported glutamine into other amino acids and an enhanced tendency for the accumulation of glutamine.

Oaks (1963) and Oaks *et al.* (1970) have shown that synthesis of proline and a number of other amino acids is regulated by end product inhibition in roots of corn. The extent of regulation of amino acid biosynthesis in endosperm tissue is not well understood. Recently, Kolderup (1979) in a limited series of experiments has examined this

phenomenon in developing spikes of wheat. In his experiments, arginine supplied externally to developing wheat spikes inhibited arginine synthesis but not proline. Proline supplied externally did not inhibit its own biosynthesis. Glutamate is the precursor for proline and arginine (McConnell, 1959). Proline is abundant in storage proteins of cereals and is absent from vascular supply, therefore, its synthesis must occur entirely *de novo*. Higher levels of proline relative to the wild type are observed in endosperm tissue from opaque-2 (Dierks-Ventling, 1981). This observation agrees with results of Kolderup (1979) and suggests that proline biosynthesis is not stringently regulated in endosperm tissue.

Enzymes involved in nitrogen assimilation, i.e., glutamate synthase, glutamine synthetase, glutamate dehydrogenase and asparagine synthetase appear just prior to or coincident with zein biosynthesis in the endosperm. Based on these observations, Oaks *et al.* (1979) suggested that the time of increase may be characteristic of the biosynthesis of the enzymes of nitrogen metabolism. This contrasts with enzymes involved in carbohydrate metabolism (Tsai *et al.*, 1970).

The level and pattern of development of glutamine synthetase, asparagine synthetase, glutamate dehydrogenase and asparaginase did not differ significantly in mutant and normal varieties. Glutamate synthase, on the other hand, was slightly higher in the opaque-2 mutants than in the wild type throughout the experimental period. Miflin and Shewry (1979) have also found that changes in GDH and GS were similar in normal (BOMI) and high lysine (1508) barley. As with corn, the activities of glutamate synthase were slightly higher in the mutants.

Thus, it appears that the mutations at the opaque-2 and floury-2 locus appears to alter the zein synthesis significantly but do not appreciably affect the enzymes of nitrogen assimilation.

In each of the mutant varieties, the activity of glutamate synthase is several times higher than the observed rate of nitrogen accumulation. For example, when the rate of accumulation of nitrogen is maximum, GOGAT activity per endosperm is in the range of 30,40 and 60 nmoles of glutamate·min^{-1}·$endosperm^{-1}$ in normal, floury-2 and opaque-2 respectively. This compares to a nitrogen accumulation rate of 6.7, 6.0 and 5.2 nmoles·min^{-1}·$endosperm^{-1}$, respectively. There seems to be a negative correlation between GOGAT activity and the rate of nitrogen accumulation. Thus, levels of GOGAT activity alone cannot serve as an indicator of the capacity for protein synthesis (Table 39).

There is an accumulation of NH_4^+ in the endosperm which reaches a peak about 20 days after pollination in both normal and high lysine varieties. The actual levels of ammonia are higher in the mutant varieties. Similar observations relating to accumulation of ammonia in developing seeds have been made in hybrid corn (Oaks *et al.*, 1979), barley (Duffus and Rosie, 1979), and pea (Sodek *et al.*, 1980). In pea, asparagine is the major transport form of nitrogen, and there are high levels of asparaginase. Thus, the accumulation of ammonia probably results from the hydrolysis of asparagine. In cereals, the reason for the accumulation of ammonia is not apparent. The accumulation of ammonia precedes the maximum accumulation of albumin and the peak in endopeptidase activity (Oaks *et al.*, 1979). On the other hand, the

Table 39

Relative Activity of GOGAT, Zein, Ammonia, Total N and Glutamine Accumulation in Normal and Mutant Varieties (20 days post-pollination)

Parameter	Normal	Floury-2	Opaque-2
GOGAT ($nmoles \cdot min^{-1} \cdot endosperm^{-1}$)	17.0	22.0	30.0
Glutamine ($\mu moles \cdot endosperm^{-1}$)	3.5	3.6	9.0
Ammonia ($\mu moles \cdot endosperm^{-1}$)	1.3	1.7	2.0
Zein ($\mu moles$ zein $N \cdot endosperm^{-1}$)	30.0	15.0	5.0
Rate of N accumulation ($nmoles \cdot N \cdot min^{-1}$ $endosperm^{-1}$)	6.7	6.0	5.2

accumulation of NH_4^+ does coincide with the appearance of enzymes of nitrogen assimilation. It may, therefore, be that glutamine and/or asparagine are the major precursors of this NH_4^+. However, glutaminase activity is not present in the endosperm. Enzymes of asparagine catabolism although present are much less active than GOGAT. The source of ammonia in cereals could be the coupled action of GOGAT and GDH. It is believed that the GDH may function primarily in a deaminating direction in higher plants, i.e., oxidizing glutamate to α-ketoglutarate and NH_4^+. The activity of this enzyme increases significantly in senescing leaf (Thomas, 1978) and root tissue (Oaks *et al.*, 1980). Labelling studies with (^{15}N) in the amide-nitrogen of glutamine have provided evidence for the catabolic activity of GDH in developing soybean cotyledons (Skokut *et al.*, 1982). For example, consistent with the operation of glutamate synthase activity, approximately equal amounts of ^{15}N- and ^{14}N-labelled glutamate were formed in the cotyledons. Some of the (^{15}N) glutamate was subsequently metabolized to $^{15}NH_4^+$.

My experiments have confirmed the early termination of zein biosynthesis in the mutants (Mertz, 1971; Murphy and Dalby, 1971) and the dramatic increase in RNAse reported earlier by others (Wilson and Alexander, 1967; Dalby and Davies, 1967). There is also a minor increase in GOGAT activity and a delay in the loss of endopeptidase activity in the mutants relative to the isogenic control. Accumulation of amino acids, particularly of aspartate and glutamine and of NH_4^+ reflects a basic disturbance in the supply and utilization of the soluble precursors

of protein synthesis. This disturbance is more pronounced in the opaque-2 than the floury-2 mutants.

D. [UL-^{14}C] Glutamine Metabolism

McConnell (1969) injected (^{14}C) glutamine into the top internode of the wheat plant stem during late stages of growth. He showed that glutamine contributed significantly to the synthesis of starch (48% of total label in meal) and gluten (34% of total label), the major storage protein of the wheat. In gluten, a large proportion of label was recovered in glutamine. However, label was also found in a range of other amino acids, i.e., proline, arginine, aspartic acid, threonine, glycine, serine and alanine. On the other hand, when labelled arginine was supplied, it remained largely as arginine and was incorporated as such into the storage proteins. More recently, Kolderup(1979) has confirmed these results.

Harvey (1973) demonstrated a considerable conversion of both acetate and proline carbon into starch in developing maize as well as an extensive incorporation into storage proteins. The distribution of (^{14}C) in protein amino acids was the same whether the caryopses had been fed with proline or acetate. In wheat also (^{14}C) proline fed to developing spikes was metabolized to glutamate and contributed significantly to synthesis of starch and gluten (McConnell, 1969). In gluten, the specific activity of glutamate (glutamate + glutamine) was higher than proline thus indicating a ready metabolism of glutamate and proline in the cereals. The ratio of specific activity of gluten to starch was 13:1, in contrast, with arginine as precursor the ratio was 65:1.

Thus, it appears that glutamine and proline could support *de novo* synthesis of amino acids in cereals and contribute carbon for starch. However, since the protein precursors used in these studies were supplied via the stem and since incubations were carried out over several weeks, nothing can be said concerning their rates of conversion or the sites of conversion.

In maize kernels, there are no vascular connections between endosperm and maternal tissue. All translocated substances enter the developing seed through specialized transfer cells at the base of the endosperm and then diffuse into the rest of the tissue (Felker and Shannon, 1980). Shannon (1972) has also shown that (^{14}C) photosynthates fixed in leaves of *Zea mays*, translocated to developing kernels pass through these specialized basal cells prior to movement into the starchy endosperm. Detailed analysis of the movement pattern showed that sucrose contained three-fourths of the radioactivity in the kernel base 1-6 hours after ($^{14}CO_2$) treatment of plant. However, in basal endosperm tissue three-fourths of the label were in glucose and fructose. Based on these observations, it was suggested that, in the case of (^{14}C) assimilates, metabolism can occur prior to entry into the endosperm followed by reassimilation. Thus, translocated sucrose may be cleaved to glucose and fructose during entry into the endosperm and the monosaccharides diffuse throughout the endosperm.

Little information is available concerning the translocation of amino acids from maternal tissue to the kernel. The relative efficiency of movement of amino acids from the maternal tissue to the endosperm is much lower than for sugars (Shimamoto and Nelson, 1981).

Studies done on proline, leucine and phenylalanine show that each amino acid is metabolized in the cob tissue and contributes significantly to other amino acids and sugars in this tissue. For example, when (^{14}C) proline was fed to caryopsis developing in sterile cultures, only 18% of the (^{14}C) recovered from the cob tissue was in proline. On the other hand, sugar and various amino acids, accounted for 60% of total label. In endosperm extracts, a relatively high percentage of total label (30%) was recovered in proline. Other amino acids accounted for 34% of the label and sugars for 21%. Thus, an accurate evaluation of proline metabolism by endosperm tissue has not yet been accomplished.

Sodek (1976) injected (^{14}C) precursors (aspartic acid, alanine, acetate) into the developing endosperm of maize. In each case, all the protein amino acids became labelled. Label was also found in sugars and organic acids. This experiment demonstrated the *de novo* biosynthesis of amino acids in the endosperm tissue.

In present study, an *in vitro* technique for growth of corn caryopsis (Gengenbach, 1977) was also adopted to study the metabolism of glutamine. Results showed that when (^{14}C) glutamine was supplied in the media, extensive metabolism of glutamine did occur in the cob tissue. Glutamine, in this tissue, was metabolized mainly to sugars, organic acids and amino acids. These three fractions accounted for 71% of the total label incorporated into cob tissue. Of the free amino acid fraction, glutamine was the major amino acid and it alone accounted for about 40% of the total label. Therefore, it was possible

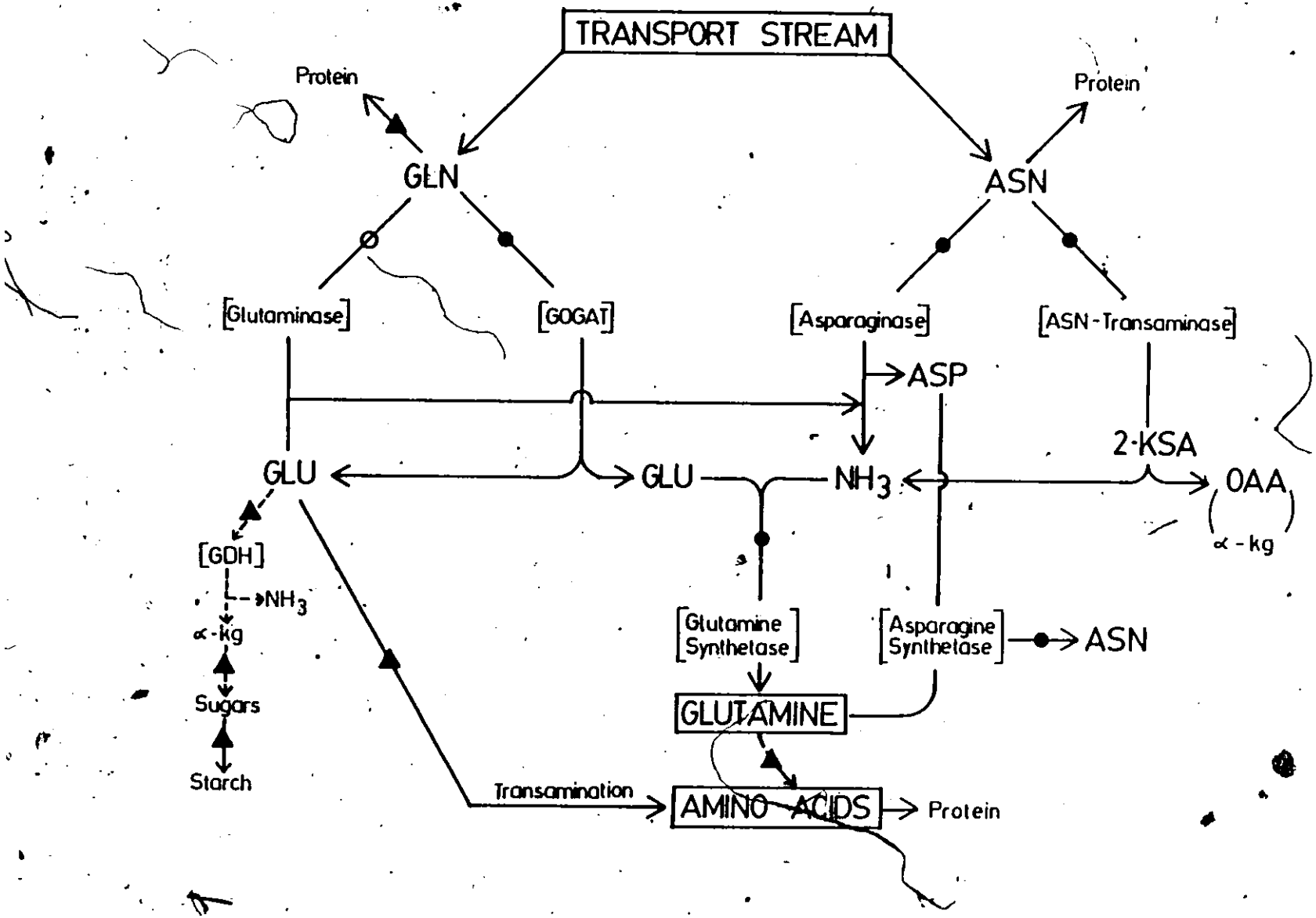
that products of glutamine metabolized in cob tissue could enter the endosperm and contribute to the apparent recovery of ^{14}C glutamine of various fractions in the endosperm. When (^{14}C) glutamine was injected directly into the immature endosperm of cultured caryopsis, analysis of the tissue showed that of the total label incorporated, 21% was in starch, 38% in zein, 8% in glutelin and 13% in free amino acid fraction. In zein hydrolysate, glutamine accounted for 20% of the total label. This distribution was similar both when (^{14}C) glutamine was supplied through the media or was injected directly into the endosperm. Glutamine accounted for only about 15% of the total radioactivity incorporated into the endosperm in each type of experiment. Thus, it is apparent that glutamine is extensively metabolized in the endosperm tissue.

The results from tracer-studies, as well as observations on the presence of enzymes of glutamine metabolism in the endosperm of corn kernel lead to the conclusion that corn endosperm tissue is capable of metabolizing glutamine. The metabolic products of glutamine not only contribute to carbon and nitrogen of various amino acid residues of storage proteins, but also to carbon for the synthesis of carbohydrates. The pathways involved in the metabolism of glutamine are summarized in Fig. 54. Glutamine supplied by the transport system may be incorporated directly into proteins or be metabolized via GOGAT to glutamate and then via GDH to α-ketoglutarate of the tricarboxylic acid (TCA) cycle. Biosynthesis of sugars could be by gluconeogenesis or by the participation of the glyoxylate pathway (Beevers, 1981). Synthesis of

Figure 54. Summary of pathways of glutamine and asparagine degradation identified in developing endosperm of corn.

Activities of enzymes identified by *in vitro* assay.

- ● present
- o absent
- ▲ pathways predicted from tracer studies only.


TRANSPORT STREAM
Protein
Protein
GLN
ASN
[Glutaminase]
[GOGAT]
[Asparaginase]
[ASN-Transaminase]
ASP
2-KSA
GLU
GLU
NH3
OAA
∝-kg
[GDH]
NH3
∝-kg
Sugars
Starch
[Glutamine Synthetase]
[Asparagine Synthetase]
ASN
GLUTAMINE
Transamination
AMINO ACIDS
Protein

sugars from TCA cycle intermediates is found in only a few specialized tissues. For example, synthesis of sugars from stored fat during germination of fat storing seeds (Beevers,1981) and also in maize scutellum (Oaks and Beevers,1964) and synthesis of sugars from phosphoglyceric acid in photosynthesizing tissues. In cereal endosperm, amino acids which are metabolized to precursors of TCA cycle or glycolytic pathway, for example; alanine, glutamate, glutamine, and proline generally contributed significantly to the synthesis of starch (McConnell 1969; Harvey,1973; Sodek,1976).

Sylvester-Bradley and Folkes (1976)have suggested earlier that little or no interconversion of amino acids occurs in developing cereal seeds and as a result, the abundance of amides, specially glutamine, favours the production of the glutamine rich prolamine fraction in the endosperm. However, on the basis of observations related to general imbalance of amino acid composition of the xylem sap and that of grain (Arruda and Da Silva,1979; Kirkman and Miflin,1979), the presence of enzymes of nitrogen metabolism (Duffus and Roberta, 1978; Da Silva and Arruda, 1979) and the data on tracer studies on cereal seeds (McConnell 1959, 1969; Sodek, 1976), other workers have disregarded his hypothesis as an over simplification. My data on the presence of label in all the amino acid fractions as well as carbohydrates also suggests the presence of an enzyme system for the extensive metabolism of glutamine in the endosperm tissue. The results of this study are more conclusive in this respect than the results obtained from tracer studies with intact plants,

since the involvement of maternal tissue in the metabolism of glutamine during translocation to the kernel can not be avoided in such experiments.

The system of caryopsis culture adopted in this study permitted a realistic evaluation of the fate of the glutamine in the developing endosperm of maize under controlled growth conditions, without perturbing the synthesis of storage proteins. Since the composition of media in such a system can be altered with ease, the system could be of potential use in studying the effect of various nutrients and hormones on the growth and metabolism of the developing kernel and the regulation of amino acid biosynthesis.

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