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STUDIES OF THE ASPARTATE AMINOTRANSFERASES IN GERMINATING PEA COTYLEDONS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies of the aspartate aminotransferases in germinating pea cotyledons" submitted by Fook Kwan Wong in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

This investigation was undertaken to illustrate the presence and the properties of aspartate aminotransferase in germinating pea cotyledons. Pulse feeding experiments using acetate-2-^{14}C showed that a large percentage of the ^{14}C was incorporated into the amino acids and organic acids. Of the acidic amino acids, glutamate was found to be highly labeled. During the post-pulse period the labeling in aspartic acid was found to decrease while that present in the neutral and basic amino acids increased.

Examination of pea cotyledon extracts for aspartate aminotransferase (E.C.2.6.1.1.) indicated that 3 and 4 day old tissues contained the greatest amounts of enzyme activity. Aminotransferase activity was found in the cytoplasmic and particulate fractions when mannitol buffered extracts were centrifuged at 10,000 x g for 15 min. In addition, glutamate dehydrogenase activity was present in these fractions and was found to increased during germination reaching maximal levels in 3 day old cotyledons.

Various treatments including $(NH_4)_2SO_4$ fractionation; DEAE-cellulose, Sephadex G-200 and hydroxylapatite column chromatography were used to isolate the cytoplasmic enzyme. Using these techniques the specific enzyme activity was increased more than 150 times. Fractionation of the particulate protein by $(NH_4)_2SO_4$ precipitation followed by gel filtration on columns of Sephadex G-200 gave preparations of the particulate enzyme having specific enzyme activity approximately 26 times greater than the starting material.

Studies of the general catalytic properties of these partially purified enzymes were carried out using a sensitive spectrophotometric assay. Both partially purified enzymes had similar K_m values for the four substrates attacked. Data from kinetic experiments suggested that both enzymes have a binary mode of action. Resolution studies of the cytoplasmic enzyme indicated that catalytic amounts of pyridoxal phosphate or pyridoxamine phosphate were required in the transamination reaction.

The properties and possible relationships of these two aminotransferases in the intermediary metabolism in germination pea cotyledons are discussed.

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LIST OF ABBREVIATIONS

CPM:	Counts per minute
Tris:	Tris (Hydroxymethyl) aminomethane
PPO:	2,5-diphenyloxazole
POPOP:	P-bis 2-(5-phenyloxazaloyl)-benzene
EDTA:	Disodium ethylene diamine tetra-acetic acid
CM-cellulose:	Carboxylmethyl cellulose
DEAE-cellulose:	Diethylaminoethyl cellulose
BSA:	Bovine Serum Albumin
TCA cycle:	Tricarboxylic Acid Cycle
E.C.:	Enzyme Commission
NAD^+:	Oxidized Nicotinamide-adenine dinucleotide

INTRODUCTION

1. Discovery of Transamination Reactions

Since the first demonstration of enzymic transamination by Braunstein and Kritzmann in 1937 using pigeon muscle preparations (1937) considerable information has been documented regarding the nature, distribution and properties of aminotransferases in living organisms. Early work reported that α-ketoglutaric acid and oxaloacetic acid were capable of accepting the L-amino groups of a large number of amino acids. However, subsequent work by Cohen (1949) suggested that enzymic transamination was mainly limited to reactions between alanine, glutamate, aspartate and their α-keto analogs. This limited transamination led biochemists to believe that these reactions were of relatively minor significance in metabolism. This conclusion, however, left a number of observations concerning the amination of α-keto acids unexplained. For example, the rapid incorporation of ^{15}N into amino acids of plant proteins (Vickery, Pucher, Schoenheimer and Rittenberg, 1940) and the conversion of α-keto acids (other than pyruvate, α-ketoglutarate, and oxaloacetate) to the corresponding amino acids, and the ability of α-keto acid analogs of certain amino acids to replace these amino acids in supporting the growth of rats and microorganisms (Meister, 1965).

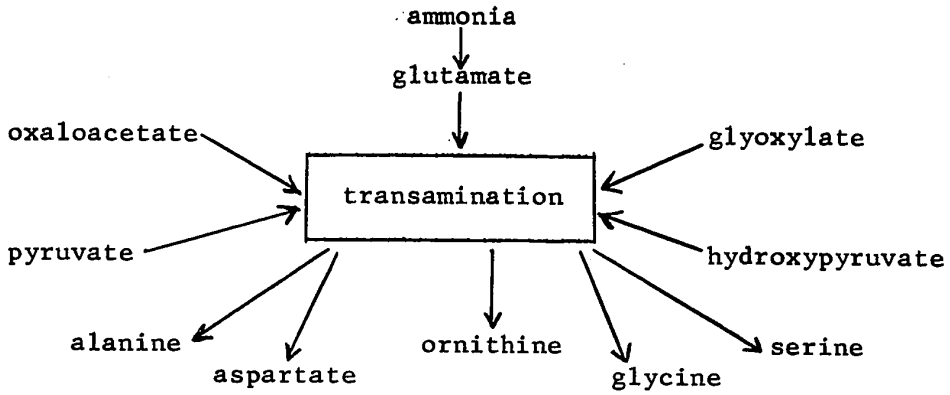
Since 1950, studies have shown that animals, plants and bacteria have in fact a wide range of amino group donors and amino group acceptors (Cammarata and Cohen, 1950; Feldman and Gunsalus, 1950). Cammarata and Cohen showed that twenty-two amino acids in addition to alanine, aspartate and glutamate were able to participate in transamination

reactions using aqueous extracts of pig heart, liver and kidney. Feldman and Gunsalus were able to show that extracts from Escherichia coli, Pseudomonas fluorescens and Bacillus subtilis catalyzed the formation of glutamate from α-ketoglutarate using nine different amino acids as amino group donors. Stumpf (1951) using dialyzed extracts of lima bean, lupine, pumpkin, pea seedlings, pumpkin leaves and wheat germ was able to demonstrate transmination between α-ketoglutarate and a number of aliphatic amino acids. Similarly, using a soluble and particulate preparations of white lupine and barley seedlings, Wilson, King and Burris (1954) showed that transamination occurred between α-ketoglutarate and seventeen different amino acids. On the basis of these studies transamination reactions may involve all of the naturally occurring amino acids, giving aminotransferases unique and important roles in the intermediary metabolism of animals, plants and microorganisms.

2. Importance of Transamination Reactions

The metabolic importance of transamination reactions is illustrated by the fact that a large number of amino acids readily undergo transamination with α-ketoglutaric acid resulting in glutamate formation. Furthermore, the ability of α-keto acids to replace the corresponding α-amino acids to support growth of some microorganisms illustrates the importance of these reactions in intermediary metabolism. As early as 1939 it was realized that the transamination of α-ketoglutarate by a number of amino acids represented an important step in oxidative deamination reactions involving glutamate

dehydrogenase for oxidative deamination (Braunstein and Bychkov, 1939; Braunstein and Asarkh, 1945). Such coupled reactions are clearly significant in the metabolism of amino acids and provide a pathway for the conversion of α-amino groups to ammonia and other nitrogen containing products. The following scheme emphasizes the key role aminotransferases can play in the biosynthesis of many amino acids.



3. Individual Aminotransferases Catalyzing Distinct Reactions

The early conclusion that transamination reactions were limited to alanine, glutamate, aspartate and their corresponding α-keto acids suggested that the glutamate-alanine and glutamate-aspartate reactions were the major systems for amino group transfer. Later work, however, emphasized the diversity of transamination reactions and so suggested the involvement of several distinct enzymes.

Further support for the existence of separate enzymes came

from studies of induction and repression. When various amino acids such as glutamate, aspartate, arginine, valine, leucine, isoleucine,alanine and phenylalanine were added to the growth medium of Neurospora crassa Fincham and Boulter,(1956)found that glutamate-alanine (L-alanine: 2-oxoglutarate aminotransferase EC 2.6.1.2) and glutamate-aspartate (L-aspartate: 2-oxoglutarate aminotransferase EC 2.6.1.1) transaminase activities were not affected. Furthermore, the addition of pyridoxal phosphate together with the amino acids did not affect the levels of these two enzymes. However, increases were observed in the levels of ornithine transaminase when these amino acids were added. Stimulation was also observed in the transmination of α-ketoglutarate with valine, leucine, isoleucine, phenylalanine, tryptophan and methionine. The addition of pyridoxal phosphate also stimulated these latter reactions.

Studies with young male rats showed that the administration of anterior pituitary hormone increased the activity of alanine aminotransferase but not aspartate aminotransferase. However, in adult male rats alanine aminotransferase activity decreased while glutamate-aspartate activity remained the same when this hormone was administered. (Beaton, Ozawa, Beaton and McHenry, 1953).

Direct evidence for the existence of separate aminotransferases is now available from studies with highly purified proteins. Although many different transaminase reactions are known, relatively few of these enzyme proteins had been completely purified.

4. Purification of Aspartate Aminotransferase

One of the first aminotransferase to be completely purified and studied in great detail was the aspartate aminotransferase from mammalian tissues. Although purification of this enzyme had been attempted since 1945 (Green, Leloir, and Nocito, 1945) preparations approaching homogeneity were not achieved until 1958 (Lis, 1958). According to Lis's method, fractionation of the pig heart homogenate with acetone, CM-cellulose column chromatography and zone electrophoresis yielded an enzyme preparation having a specific activity 50 fold greater than the crude extract. Only one protein was detected by electrophoresis.

Highly purified preparations of aspartate aminotransferase have also been obtained by Jenkins, Yphantis and Sizer, (1959). Again using pig heart as the source of enzyme, they were able to purify the aminotransferase by using heat treatment, ammonium sulphate fractionation, and hydroxylapatite column chromatography. Although their final preparation had a specific activity 18.7 times greater than the crude extract, ultracentrifugation, electrophoresis and spectroscopic studies showed that the preparation was 80-85% pure.

By modifying the methods of Lis (1958) and Jenkins et al. (1959), Polyanovskii (1962) obtained a preparation of the enzyme having a purity of 100%. His method involved heat treatment, ammonium sulphate precipitation, acetone precipitation, CM-cellulose column chromatography and finally zone electrophoresis. The criteria used to determine purity were coenzyme content (pyridoxal phosphate content), specific enzyme activity and specific optical density of

the characteristic absorption maxima (360 mμ in weakly alkaline solution, 430 mμ in weakly acidic solution).

Employing essentially the method of Jenkins et al. (1959), Wada and Morino (1964) crystallized the enzyme from mitochondria and cytoplasmic fractions of beef liver, beef heart and pig heart. After heat treatment, ammonium sulphate fractionations and hydroxylapatite column chromatography, crystallization of the mitochondrial enzyme was possible using ammonium sulphate in the presence of α-ketoglutarate at pH 6.0. The cytoplasmic enzyme was similarly crystallized except for the omission of α-ketoglutarate and an increase of pH to 8.0.

Although transamination reactions in plants had been known for many years, there have been no reports of homogeneous protein preparations containing aspartate aminotransferase activity. However, numerous investigators had reported the isolation and partial purification of this enzyme. Cruickshank and Isherwood (1958) partially purified this enzyme from wheat germ by ammonium sulphate precipitation. Using extracts of Dolichos lablab Patwardhan (1960) purified the aminotransferase 23 fold after subjecting the crude extract to anionic resin, alumina-C γ gel, calcium phosphate gel, and ammonium sulphate. Using a combination of ammonium sulphate fractionation, calcium phosphate gel treatment and DEAE-cellulose column chromatography, Ellis and Davies (1961) purified the enzyme from cauliflower florets over 250 fold. By far the greatest purification for any plant aspartate aminotransferase was reported by Fasella, Bossa, Turano and Rossi Fanelli (1966). The enzyme from dormant cotton seeds was purified

2,700 times by ultracentrifugation, DEAE-cellulose column chromatography and hydroxylapatite column chromatography. However, starch gel electrophoresis showed that the final preparation contained 8 different proteins, only 3 of which displayed aspartate aminotransferase activity.

5. Multiple Forms of Aspartate Aminotra sferase

Different authors have used different terms to designate various molecular forms of an enzyme. However, difficulties in finding an acceptable term have arisen because these molecular forms are highly variable both in their catalytic and physical characteristics. For a summary of currently used terms the reader is referred to the recent article by Bossa (1966). In the present work, the term isoenzyme is used to refer to protein molecules catalyzing a common reaction but differing in physico-chemical characteristics.

The existence of multiple forms of this enzyme was originally observed by Green et al. (1945). These workers subjected their preparations to electrophoresis and noted three distinct bands having enzyme activity.

With the development of ion-exchange chromatography for enzyme purification, Fleisher, Potter and Wakim (1960) were able to successfully isolate a cationic and an anionic form of the aminotransferase using CM and DEAE-cellulose. Electrophoretic analysis on paper also confirmed the presence of these two forms of the enzyme.

Since the work of Fleisher et al. (1960) several investigators

including Borst and Peeters (1961), Boyd (1961), Hook and Vestling (1962) have confirmed the existence of aspartate aminotransferase isoenzyme in numerous mammalian tissues. Although it was generally assumed that the anionic form of the enzyme is localized in the cytoplasm and that the cationic form is in the mitochondria, evidence for such a localization was not conclusive until Boyd reported his findings in 1961. He further showed that activity of the mitochondrial enzyme could be increased by treatments such as osmotic shock, high speed blending, sonication and Triton X-100. Studies with other subcellular particles showed that aspartate aminotransferase activity were also detected in the nuclear fraction, and in the microsomal fraction.

In addition to finding isoenzymes in different subcellular fractions, multiple forms of the supernatant and mitochrondrial aspartate aminotransferses had been reported by Nisselbaum and Bodansky (1964, 1966); Martinez-Carrion, Riva, Turano and Fasella (1965) and by Martinez-Carrion, Turano, Chiancone, Bossa, Giartosio, Riva and Fasella (1967). In every case, these forms of the enzyme had been separated by starch gel electrophoresis.

Questions have been raised whether the different charges and mobility of aspartate aminotransferase on ionic exchange materials and starch gel electrophoresis are biologically determined or a reflection of the isolation procedures. Early attempts to differentiate between the two isoenzymes were based on criteria such as difference in charge, pH effects and Michaelis constants. Boyd (1961) found the mitochondrial enzyme to have a broad pH optimum ranging from 7-9. On the other hand, the cytoplasmic enzyme displayed a sharp

optimum at pH 8.5. Studies on the effects of substrate concentration showed that the mitochondrial isoenzyme differed from the cytoplasmic isoenzyme in Michaelis constants. In all tissues examined, canine heart (Fleisher et al., 1960), rat liver (Boyd, 1961), human heart and liver (Nisselbaum and Bodansky, 1964), pig hear, bovine liver and heart (Wada and Morino, 1964), the mitochondrial isoenzyme had a greater apparent affinity for L-aspartic acid but a lower affinity for α-ketoglutaric acid than the cytoplasmic isoenzyme. Immunological studies with rabbit antisera (Nisselbaum and Bodansky, 1964) and beef antisera (Wada and Morina, 1964) both showed that the mitochondrial and cytoplasmic aspartate aminotransferases were distinct proteins.

Further support for the existence of two distinct isoenzyme proteins came from a comparison of their chemical characteristics (Martinez-Carrion and Tiemeier, 1967). For example, differences were observed in their amino acid and peptide compositions thus resulting in different electrophoretic mobilities. Although both isoenzymes were similar in molecular size and polypeptide chains, they differed in their N-terminal amino acids.

6. Properties of Aspartate Aminotransferase

Both the supernatant and the mitochondrial aspartate aminotransferases from pig heart and bovine liver and heart have been highly purified and crystallized by Wada and Morino (1964). The possibility of obtaining homogenous preparations of the enzyme has led to studies of its physical and chemical properties. Most of this

work has concentrated on the properties of the supernatant isoenzyme. In view of the difficulties in achieving homogenous preparations from plant tissues, it is not surprising that the majority of the known properties of this enzyme relate to studies with the mammalian enzyme. Sedimentation analysis on the pure enzyme (Jenkins, et al., 1959) give sedimentation coefficients of 5.39 to 5.55. Determination of molecular weight by the Archibald method give a value of 110,000 $\pm$ 11,000. On the basis of an 81% pure preparation, Jenkins et al. (1959) calculated that the enzyme contained 2 molecules of pyridoxal phosphate per molecule of apoenzyme. Due to the presence of this coenzyme the enzyme behaves as a typical pH indicator, changing from bright yellow at pH values below 7 to a colorless form at pH 8.0. At pH 12, the enzyme turns yellow again because of the liberation of pyridoxal phosphate from the denatured protein.

The molecular weight of the plant aminotransferase had not been determined due to difficulties in achieving homogenous preparations. For this reason the content and identity of the coenzyme has yet to be determined.

Even with highly purified preparations of aspartate aminotransferase specificity studies showed that catalysis is not restricted to L-aspartate, α-ketoglutartate, L-glutamate and oxaloacetate (Meister, 1965). For example, the enzyme will attack mesoxalic acid (replacing oxaloacetate) and cysteic acid (replacing L-aspartate), but in these cases enzyme activity was much lower than with the true substrates. On the other hand, it has been found that the enzyme catalyzed a transamination between cysteinesulfinate and

α-ketoglutarate more rapidly than with L-aspartate and α-ketoglutarate. In addition, the enzyme also catalysed a reaction between α-methylglutamate and oxaloacetate. In the case of the plant enzyme, cysteate and cysteine sulphinate also supported transamination with oxaloacetate (Davis and Ellis, 1961) but with a much lower affinity than was shown for L-glutamate. It appears, therefore, that highly purified aspartate aminotransferase will catalyze transamination reactions between substrates having similar structures to aspartate, glutamate and oxaloacetate.

Studies on the effect of pH on activity of the mammalian enzyme showed an optimum at 8.5 (Jenkins et al., 1959). However, the optimum pH for the plant enzyme range from 7.0 - 8.5 (Davies and Ellis, 1961; Cruickshank and Isherwood, 1958).

Determination of Michaelis constants for the mammalian and plant enzymes have shown that both have greater affinity for the keto acids than for the amino acids. Furthermore, the keto acids inhibit enzyme activity at high concentrations (Davies and Ellis, 1961; Velick and Vavra, 1962).

Some properties of aspartate aminotransferase from various sources are summarized in the following table.

Highly purified aspartate aminotransferase from pig heart has been shown to contain six thiol groups per molecule of protein (Polyanovskii, 1962). Titration of the first thiol group with p-CMB did not affect enzyme activity but titration of the next two groups resulted in 90% inhibition. It can, therefore, be concluded that two of the six thiol groups are essential for activity.

Some Properties of Cytoplasmic Aspartate Aminotransferases

Property	Bovine liver and heart[1]	Pig heart	Cauliflower florets[4]
Molecular weight	120,00	110,000[2]	--
Absorption maxima (mμ)			
pH 5	430	430[2]	
pH 8	362	362[2]	
pH optimum	--	8.5[2]	7.0-8.0
Pyridoxal phosphate content (moles/mole enzyme)	2	2[2]	--
Michaelis constants (M)			
Pyridoxal phosphate	5×10^{-7}	--	--
α-ketoglutarate	3.4×10^{-4}	1×10^{-4}[3]	7.6×10^{-4}
oxaloacetate	--	4×10^{-5}[3]	1×10^{-4}
L-aspartate	$2\text{-}2.5 \times 10^{-3}$	9×10^{-4}[3]	7.5×10^{-3}
L-glutamate	--	4×10^{-3}[3]	3.8×10^{-2}

[1]Data from Wada and Morino (1964).
[2]Data from Jenkins et al. (1959).
[3]Data from Velick and Vavra (1962).
[4]Data from Davies and Ellis (1961).

No such detailed studies had been performed with the plant enzyme although sulfhydryl reagents such as iodoacetate and p-CMB inhibit enzyme activity (Cruickshank and Isherwood, 1958; Nadkarni and Sohonie, 1963).

An understanding of the mechanism of transamination has largely been the result of studies with nonenzymic systems. Early workers established that several multivalent metal ions readily catalyzed

nonenzymic transamination reactions, especially in the presence of vitamin B_6 (Metzler and Snell, 1952). For this reason the role of metals in enzymic transamination reactions was examined. Happold and Turner (1957) reported that magnesium ions activated sheep heart aspartate aminotransferase by as much as 100%. Patwardhand, (1960) and Nadkarni and Sohonie (1963) found that plant aspartate amino-transferase was activated by ferrous and manganese ions. These findings have to be repeated with the purified enzyme. However, with the availability of a pure mammalian enzyme and methods of trace metal analysis Fasella, Hammes and Vallee (1962) re-examined the role of metals in enzymic transamination. The metals analyzed for were Ca, Sr, Mg, Al, Ba, Cr, Fe, Zu and Mn. With the exception of calcium (0.3 mole/mole of coenzyme), all other metals were present at concentrations of less than 0.1 mole/mole of coenzyme. Addition of these metals did not increase enzyme activity. This evidence supports the now accepted view that metal ions are not involved in the reaction catalyzed by this particular enzyme.

7. Role of Vitamin B_6 in Nonenzymic Transamination

There is now overwhelming evidence to support the metabolic role played by vitamin B_6. Evidence for the existence of vitamin B_6 arose originally from nutritional studies on rat pellagra, a disease due to the lack of vitamin B_6.

The natural occurrence and commercial availability of the aldehyde and amine forms of this vitamin allowed Snell (1958) to suggest and illustrate the involvement of pyridoxal in nonenzymic

transamination reactions. Such reactions were shown to involve many amino acids at different physiological pH values. Data from a model system which was examined for the ability of various aldehydes to undergo transamination contributed much knowledge regarding the mechanism and structural requirements for pyridoxal phosphate (Snell, 1958). The role played by metals in nonenzymic transamination is still not clearly understood. It has been suggested that the metal might facilitate the formation of a Schiff base and contribute stability to enzyme substrate complexes (Snell and Jenkins, 1959). In enzymic transamination the protein moiety of the holoenzyme had been speculated to play a role similar to that of the metal in the nonenzymic reaction.

8. Role of Vitamin B_6 in Enzymic Transamination

The role played by vitamin B_6 in nonenzymic transamination between amino acids and keto acids led to an understanding of enzymic transamination. That the mechanism of enzyme transamination involved vitamin B_6 was proposed by the work of Snell (1944), Schlenk and Snell (1945) and Schlenk and Fisher (1945). These workers found that rat tissues deficient in vitamin B_6 had lower levels of aspartate aminotransferase activity. However, if pyridoxal and ATP were added, the rate of transamination was raised to almost the normal level. Streptococcus faecalis grown on a medium deficient in vitamin B_6 failed to catalyze the reaction between glutamate and oxaloacetate but would do so if pyridoxal was added to the growth medium (Lichstein, Gunsalus and Umbreit, 1945).

Indications that mammalian aspartate aminotransferase contained pyridoxal or pyridoxamine phosphates was initially reported by Schlenk and Fisher (1945). Upon purification of the enzyme, the pyridoxal phosphate content increased.

The requirement for pyridoxal phosphate in the aspartate aminotransferase reaction was shown by O'Kane and Gunsalus (1947), who resolved the enzyme into protein and coenzyme. Although the coenzyme was not identified, restoration of enzyme activity by pyridoxal phosphate supported the concept that vitamin B_6 was required for transamination. Further work by Meister, Sober and Peterson (1954) showed that pyridoxamine phosphate could also restore aminotransferase activity.

Since the first report of the resolution of mammalian aspartate aminotransferase by O'Kane and Gunsalus (1947), there have been numerous reports of resolution of this enzyme by various methods (Bank and Vernon, 1961; Wada and Snell, 1962; Scardi, Scotto, Iaccarino and Scarano, 1963).

Not until the late 1950's was aspartate aminotransferase purified sufficiently to allow a quantitative determination of the pyridoxal phosphate content per molecule of the holoenzyme (Jenkins et al., 1959). Although the enzyme from plants has not been purified to such an extent, activation with pyridoxal phosphate and inhibition with isonicotinic acid hydrazide suggest that the plant enzyme is also vitamin B_6 dependent. Such dependence has been demonstrated by the work of Wong and Cossins (1966).

9. Mechanism of Transamination

Following confirmation that pyridoxal and pyridoxamine phosphates are the coenzymes of aspartate aminotransferase detail studies of the mechanism of enzymic transamination were carried out. Two principal types of mechanisms have been proposed. These are the ternary and the binary mechanisms. The ternary mechanism assumes that interaction occurs simultaneously at a single enzymic site among all three reactants (viz. amino donor, amino acceptor and coenzyme) (Nisonoff and Barnes, Jr., 1952). Evidence, however, did not support this hypothesis (Jenkins, 1963). The binary mechanism postulates that the overall reaction is the sum of two separate binary reactions as illustrated below.

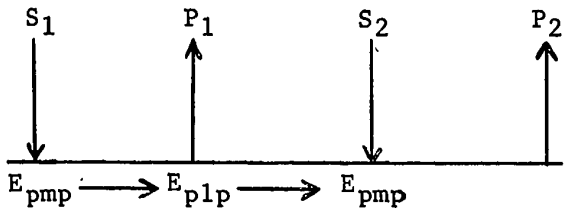
(i) amino $acid_1$ + plp-enzyme $\rightleftarrows$ keto $acid_1$ + pmp-enzyme

(ii) keto $acid_2$ + pmp-enzyme $\rightleftarrows$ amino $acid_2$ + plp-enzyme

plp-enzyme refers to pyridoxal phosphate form of the enzyme; and, pmp-enzyme refers to pyridoxamine phosphate form of the enzyme.

In principle these two binary reactions could be catalyzed by 2 distinct enzymes, one specific for amino $acid_1$ and pyridoxal phosphate and the other specific for keto $acid_2$ and pyridoxamine phosphate. However, there is no experimental evidence to support the existence of two enzymes. For example, Meister et al., (1954) found that binding of pyridoxal phosphate to the apoenzyme was very slow, binding of pyridoxamine phosphate was even slower and prolonged dialysis of the holoenzyme did not remove the coenzyme. Furthermore, there have been no reports in the literature of the isolation of two distinct enzymes required to catalyze these two half reactions.

A more acceptable hypothesis is that the mechanism involves the sum of the 2 binary reactions but catalyzed by one protein. The enzyme oscillates between the pyridoxal phosphate and the pyridoxamine phosphate forms. The substrates are bound and the products are released one at a time in sequence. Cleland (1963) referred to this as the Ping Pong Bi Bi mechanism. A diagramatic representation of this mechanism is illustrated below.



S refers to substrate and P refers to product;

E_{plp} and E_{pmp} refer to pyridoxal phosphate and pyridoxamine phosphate forms of the enzyme, respectively.

Much evidence had been gathered to support this hypothesis.

Experimental evidence from spectrophotometric studies showed that at pH 8.5, the enzyme exhibited maximum absorbancy at 360-362 mμ. On complexing with its substrate (L-aspartate or L-glutamate) this maximum absorbancy shifted to 335 mμ which is characteristic of free and enzyme-bound pyridoxamine phosphate. Pyridoxamine phosphate was then identified after liberation from the denatured enzyme. (Lis, Fasella, Turano and Vecchini, 1960). If the pyridoxamine enzyme was mixed with oxaloacetate or α-ketoglutarate the maximum absorbancy at 335 mμ shifted back to 360 mμ, characteristic of the pyridoxal form. At pH 5.0 the pyridoxal enzyme was yellow having a maximum absorbancy at 430 mμ. But at pH 8.4 the absorbancy at 430 mμ was

lost, being replaced by maximum absorbancy at 360-362 mμ . Absorbancy at 430 mμ was due to a complex between α-ketoglutarate and enzyme (Jenkins et al., 1959). Other nonsubstrate dicarboxylic acids such as glutarate, adipate and malate also formed this complex with the enzyme. These findings are interpreted as showing that the aldehyde group of pyridoxal phosphate is linked to protein through the amino group of lysine to form a Schiff base. That this is the case was confirmed by borohydride reduction and chymotrypsin digest (Hughes, Jenkins and Fischer, 1962).

Equilibria studies between enzyme, coenzyme, substrates and inhibitors showed that two half-reactions occur between the non-dissociable enzyme-bound coenzyme and substrates. The four substrates are mutually competitive and therefore occupy a single site on the enzyme in sequence. Equilibria constants for the overall and the half-reactions and the dissociation constants for enzyme-substrate complexes showed that the association reaction between the enzyme and substrates was not rate-limiting. The rate-limiting steps are intramolecular transformations involving the reversible isomerization of the pyridoxal phosphate enzyme-aspartate complex into the pyridoxamine phosphate enzyme-oxaloacetate complex (Velick and Vavra, 1962). Such reversible transformations are supported by data from studies of exchange transamination (Jenkins et al., 1959; Jenkins and Taylor, 1965).

Although spectrophotometric studies with the plant enzyme have not been possible due to impurities, kinetic data support the contention that plant aminotransferases catalyze transamination reactions by a binary mechanism (Davies and Ellis, 1961).

10. The Present Investigation

Background

Experiments with pea cotyledons have indicated that during the early stages of germination there are marked changes in the concentrations of certain amino acids (Lawrence and Grant, 1963; Larson and Beevers, 1965). During the first day of germination glutamic acid and glutamine increase markedly while the levels of aspartic acid decline. However, by the third day large increases in the amounts of glutamine and homoserine occur. Such changes are clearly indicative of amino acid synthesis and metabolism during the earliest stages of seedling development.

Studies of ethanol, acetaldehyde and acetate metabolism in pea cotyledons (Cameron and Cossins, 1967) indicated that at early stages of germination, the TCA cycle is highly active. During short feeding experiments the organic acids were rapidly labeled followed by glutamate. As the amount of ^{14}C labeling in the organic acids decreased there was a concomitant increase in the labeling of glutamate, thus indicating that this amino acid was labeled at the expense of the organic acids. Such data implies that glutamate must have arisen from α-ketoglutarate. In experiments with ^{14}C-carbonate, a rapid turnover in the labeling of aspartic acid while the glutamate pool was increasing in radioactivity indicates that aspartate was utilized for the production of oxaloacetate and glutamate. For such conversions to occur, aspartate aminotransferase must be active in the tissue.

General Objectives

In order to examine the biosynthesis of glutamic and aspartic acids in more detail experiments in which acetate-2-^{14}C was pulse fed were conducted. Due to the importance of aspartate aminotransferase in the biosynthesis of glutamic and aspartic acids this enzyme was studied in detail. It was hoped that these studies would elucidate the nature and importance of this enzyme in intermediary metabolism.

MATERIALS AND METHODS

Chemicals

All chemicals used in this investigation were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio, and from Fisher Scientific Company, Edmonton, except where specified otherwise.

Ammonium sulphate, special enzyme grade was purchased from Mann Research Laboratory Inc., New York. Sephadex was purchased from Pharmacia, Canada Ltd., Montreal. POPOP and PPO were purchased from Nuclear-Chicago, Des Plaines, Illinois. α-ketoglutarate-5-^{14}C was purchased from Atomic Energy of Canada Limited, Ottawa. Dowex anion and cation resins (analytical grade) were purchased from Bio. Rad Laboratories, Richmond, California. Hydroxylapatite was prepared according to the method of Tiselius, Hjertén and Levin (1956).

All reagents used were of the highest analytical grade available. Solutions were, in all cases, prepared with glass-redistilled demineralized water.

Germination of Peas

Seeds of Pisum sativum L. C.V. 'Homesteader' were purchased from Steel Robertson Ltd., Edmonton. They were soaked in distilled water for 24 hours at room temperature. The seeds were quickly rinsed in 0.1% $HgCl_2$ and then germinated in moist vermiculite at 28°C for periods up to 5 days in darkness. Under these conditions the approximate state of germination on each day can be described as follows:

1 day old - radicles completely enclosed by the testas, seeds fully imbibed.

2 day old - radicles beginning to emerge.

3 day old - radicles now 3 - 4 cm long.

4 day old - radicles now 5 - 6 cm long, epicotyls approximately 2 cm long.

5 day old - radicles now 7 - 8 cm long, epicotyls 3 - 4 cm long.

Extraction and Partial Purification of the Cytoplasmic Enzyme

All procedures were carried out at 4°C. For extraction of aspartate aminotransferase, the testas of the 3 day old peas were removed and the cotyledons (250 gm) ground with 500 ml of 0.05 M Tris-HCl (pH 7.8) in a Waring Blendor for 1 min. at full speed. The homogenate was passed through 6 layers of cheese cloth and the resulting extract was then centrifuged at 10,000 x g for 20 min. The supernatant was brought to 40% of saturation by addition of saturated $(NH_4)_2SO_4$ solution. After centrifugation at 10,000 x g for 20 minutes to remove precipitated protein, the supernatant was brought to 60% of saturation by further addition of saturated $(NH_4)_2SO_4$ solution. After 20 min. the precipitate was removed by centrifugation as before. When DEAE-cellulose column chromatography was employed, this precipitate was dissolved in 50 - 75 ml of 0.005 M Tris-HCl (pH 7.8) and dialyzed against the same buffer for 15 hours. For hydroxylapatite column chromatography this 40 - 60% $(NH_4)_2SO_4$ fraction was dissolved in 50 ml of a 0.05 M Tris-HCl

containing 0.001 M α-ketoglutarate at pH 7.8.

Column Chromatography of the Cytoplasmic Aspartate Aminotransferase

1. DEAE-cellulose Column Chromatography

A suspension of DEAE-cellulose in 0.005 M Tris-HCl (pH 7.7) was packed by gravity in a glass column of 2.8 cm diameter to a height of 50 cm. The column was then equilibrated at room temperature by washing with the same buffer until the pH remained constant.

A sample of the dialyzed enzyme preparation containing approximately 300 mg of protein was then applied to the column. After the enzyme protein had been adsorbed, the column was washed with 400 ml of 0.005 M Tris-HCl (pH 7.8). Following this washing the column was eluted using a gradient produced by having 200 ml of 0.005 M Tris-HCl in the mixing vessel and 0.2 M Tris-HCl in the reservoir, both at pH 7.8. Fractions of 5 ml were collected every 2.5 min. using a refrigerated Buchler fraction collector equipped with a circulating pump. Assay of enzyme activity showed that fractions 158 - 167 contained approximately 90% activity recovered. These fractions were then pooled for kinetic studies and for isolation of the apoenzyme. For further purification using columns of Sephadex G-200, the six fractions containing the highest enzyme acitivity were pooled, dialyzed in glass-redistilled demineralized water, and lyophilized. The dried enzyme protein was then redissolved in approximately 10 ml of glass-redistilled demineralized water.

2. Sephadex G-200 Column Chromatography

Sephadex G-200 from the supplier was soaked in distilled water

at room temperature for 3 days with occasional stirring. After removal of the fine material, the gel was packed by gravity into a column to give a bed of 2.8 x 50 cm. The Sephadex was then washed with 0.005 M Tris-HCl buffer (pH 7.7) until the pH remained constant.

Three millilitres of the enzyme from the above step, containing approximately 85 mg of protein were applied very carefully to the top of the column using a Pasteur pipette. The enzyme solution was allowed to sink into the bed before commencing the elution with 0.005 M Tris-HCl buffer at pH 7.8. Three millilitre fractions were collected at $4^{o}C$ every 9 min. Enzyme assay showed that the activity was located from fractions 51 - 64.

3. Hydroxylapatite Column Chromatography

The hydroxylapitite was prepared according to the method of Tiselius, et al.,(1956). The material was aged in 0.001 M NaH_2PO_4 (pH 6.0) at $4^{o}C$ for at least one month before use.

A sample of the 40 - 60% saturated $(NH_4)_2SO_4$ fraction dissolved in 0.05 M Tris-0.001 M α-ketoglutarate solution (pH 7.8) was carefully immersed in a boiling water bath and the temperature of the protein solution allowed to rise to $60^{o}C$. The solution was then quickly cooled in ice. After centrifugation at 10,000 x g for 20 min. the clear supernatant containing the enzyme was dialyzed in 0.005 M Tris-HCl buffer (pH 7.8) for 15 hours.

A sample of this dialyzed extract, containing approximately 250 mg of protein, was then applied to a 2 x 20 cm column of

hydroxylapatite previously equilibrated with 0.005 M Tris-HCl, pH 7.8. The enzyme was readily washed from the column with 200 ml of 0.005 M Tris-HCl buffer, also at pH 7.8. The effluent was collected at 4°C in 3 ml fractions at a flow rate of 0.2 ml/min. Each fraction was assayed for enzyme activity and their optical densities determined at 280 mμ. It was found that fractions 39 - 44 contained all of the enzyme activity.

In another experiment, a column of hydroxylapatite 2.8 x 50 cm was equilibrated with 0.005 M Na_2HPO_4 at pH 7.8. A sample of the 40 - 55% saturated $(NH_4)_2SO_4$ fraction containing approximately 800 mg of protein which had been previously dialyzed against 0.005 M Na_2HPO_4 (pH 7.8) was applied to the column. The enzyme was still adsorbed by the column after washing with 300 ml of 0.005 M Na_2HPO_4. Enzyme activity was subsequently eluted from the column using a gradient produced by placing 200 ml of 0.005 M Na_2HPO_4 in the mixing vessel and 0.2 M Na_2HPO_4 in the reservoir, botn at pH 7.8. Each fraction of 3 ml was collected at 4°C every 6 min. The whole process took approximately 20 hours. The fractions collected were assayed for enzyme activity and their optical densities determined at 280 mμ. It was found that fractions 230 - 236 contained most of the enzyme activity.

Isolation and Purification of the Particulate Aspartate Aminotransferase

1. Preparation of the Particulate Fraction

The particulate fraction from pea cotyledons was prepared

using a slight modification of the method described by Ikuma and Bonner (1967). Three hundred grams of 3 day old pea cotyledons were ground at 4°C in a handmill with 600 ml of a grinding medium consisting of 0.3 M mannitol, 0.1% bovine serum albumin (BSA), 0.05% cysteine, and 0.001 M EDTA (pH 7.2). The homogenate was further ground by hand in a cold mortor and pestle for 30 seconds. After passing the crude homogenate through 6 layers of cheesecloth, the extract was centrifuged at 1500 x g for 15 min. The supernatant was then further centrifuged at 10,000 x g for 15 min. Enzyme activity in the supernatant is designated as 'cytoplasmic'. The pellets from this centrifugation were washed in a medium consisting of 0.3 M mannitol, 0.1% BSA and 0.001 M EDTA (pH 7.2). After a further centrifugation at 10,000 x g for 15 min. the pellets were again washed in the same medium and centrifuged as before. The pellets were then suspended either in the wash medium or in 0.05 M Tris-HCl buffer (pH 7.8). The whole isolation process was carried out at 4°C.

2. Extraction of Aspartate Aminotransferase From the Particulate Fraction

Various methods were used in an attempt to release the aspartate aminotransferase from the particulate fraction. The 10,000 x g fraction suspended in 0.05 M Tris-HCl buffer was subjected to high speed blending either in a Waring Blendor or in a Virtis homogenizer for 2 - 5 min. at 4°C. Other preparations in the same buffer were subjected to ultrasonic distintegration using a Raytheon Sonic Oscillator, Model DF-101, operated at 0.96 amperes for 4 min.

For osmotic shock treatment the pellets were suspended in 0.005 M Tris-HCl (pH 7.8). Such suspensions were left in the cold for 15 hr. before assaying for enzyme activity. Further attempts to release the enzyme involved treatment with sodium deoxycholate solution. The twice-washed pellets were suspended in a 0.05 M Tris-HCl solution containing 1% sodium deoxycholate at pH 8.0. After stirring in the cold for 30 min. the suspension was centrifuged at 30,000 x g for 20 min. The supernatant was then assayed for aminotransferase activity and subsequently subjected to further purification procedures.

3. Purification of the Particulate Aspartate Aminotransferase

Since deoxycholate appears to be the most effective treatment, it was routinely included to solubilize the enzyme before further purification. To the deoxycholate treated supernatant, cold saturated $(NH_4)_2SO_4$ solution was added until 40% saturation was reached. After standing for 20 min. the suspension was centrifuged at 30,000 x g for 20 min. The supernatant was again treated with saturated $(NH_4)_2SO_4$ solution until 60% saturation was reached. This suspension was left standing in the cold overnight (15 hr.) to allow complete precipitation. This protein, having been collected after 20 min. of centrifugation at 30,000 x g was dissolved in a small volume (15 ml) of 0.05 M Tris-HCl buffer (pH 7.8).

The 40 - 60% saturated $(NH_4)_2SO_4$ fraction was subjected to Sephadex G-50 column chromatography in an attempt to remove excess $(NH_4)_2SO_4$. To a Sephadex G-50 column (35 x 1.5 cm) previously

equilibrated with 0.05 M Tris-acetate buffer (pH 6.0) 5 ml of the sample containing approximately 16 mg of protein, was carefully applied. After the enzyme solution had settled into the gel bed the column was eluted with the same buffer at a rate of 1 ml/min. Fractions of 1.6 ml were collected at 4°C each being assayed for enzyme activity and protein content.

For partial purification the particulate enzyme was treated with Sephadex G-200. To a 2.8 x 50 cm Sephadex G-200 column previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.8), 12 ml of the 40 - 60% $(NH_4)_2SO_4$ fraction, containing approximately 24 mg protein, were carefully added to the gel bed by a Pasteur pipette. The protein solution was allowed to sink into the gel bed before the column was eluted with 0.05 M Tris-HCl buffer (pH 7.8). Fractions of 3 ml were collected at 4°C using a flow rate of 0.8 ml/min. Enzyme activity was found in fractions 45 - 58.

Preparation of the Apotransaminase

The apoenzyme of aspartate aminotransferase was routinely prepared from the holoenzyme preparations purified on DEAE-cellulose columns according to a modification of the method described by Banks and Vernon (1961). A solution of the holoenzyme in Tris-HCl (pH 7.8) containing approximately 38 mg of protein was precipitated by addition of saturated solution of $(NH_4)_2SO_4$ to give 80% of saturation. The protein collected after centrifugation at 10,000 x g for 20 min. was redissolved in 10 ml of 1.0 M K_2HPO_4, pH 6.0. After heating the enzyme solution at 60°C for 30 minutes it was rapidly cooled in an

ice bath. After sedimenting the denatured protein by centrifugation at 10,000 x g for 20 min. the clear supernatant containing the apoenzyme was dialyzed against water at 4°C for 15 hr. or longer.

Reconstitution of the Apotransaminase

A sample of the apotransaminase (approximately 0.14 mg of protein) was incubated with pyridoxal phosphate or pyridoxamine phosphate and 100 μ moles of Tris-HCl buffer (pH 8.0) at 37°C for periods up to 50 min. After these incubation periods, 20 μ moles of L-aspartate were added. After a further incubation (5 min. at 37°C), 10 μ moles of α -ketoglutarate were added to initiate the reaction.

In another series of experiments a solution of the apoenzyme containing 1.4 mg of protein and 300 mg of pyridoxal phosphate, pyridoxamine phosphate, pyridoxal-HCl, pyridoxamine-HCl and pyridoxine phosphate, respectively, in a total volume of 8 ml of 0.005 M Tris-HCl (pH 7.8) was incubated at 37°C for 30 min. Each of these mixtures was then dialyzed thoroughly for 40 hr. with 5 changes of water. After dialysis each mixture was assayed for aspartate aminotransferase activity without further addition of vitamin B_6 derivatives.

Assay of Aspartate Aminotransferase Activity

A slightly modified method of Jenkins et al., (1959) for the standard assay of cytoplasmic aspartate aminotransferase activity was used in the present investigations. This assay involved incubation of the enzyme with 20 μ moles of L-aspartate and 100 μ moles of Tris-HCl (pH 8.0) at 37°C for 5 min. After this incubation period,

10 µmoles of α-ketoglutarate were added to initiate the reaction. The final pH was 8.0 and the final volume was 3 ml.

For particulate aspartate aminotransferase activity, the enzyme was incubated with 40 µmoles of L-aspartate and 100 µmoles of Tris-HCl (pH 8.0) at 37°C for 10 min. After this incubation period, 10 µmoles of α-ketoglutarate were added to initiate the reaction. The final pH was 8.0 and the final volume was 3 ml.

In both cases reaction was followed spectrophotometrically (Green, et al., 1945) using Beckman recording spectrophotometers (Models DB and DB-G) equipped with potentiometric recorders. The reference cuvette contained all of the reactants with the exception of α-ketoglutarate which was replaced by distilled water. The changes in optical density at 280 mµ were followed continuously and expressed as µmoles of oxaloacetate reference to a standard curve. One unit of enzyme activity is defined as the amount of enzyme which will produce or consume 1 µmole of OAA/min. at 37°C. Specific enzyme activity is expressed as units of enzyme activity/ mg protein.

Assay of Glutamate Dehydrogenase Activity

Glutamate dehydrogenase activity was assayed by a modification of the method described by Strecker (1955). The crude extract was incubated with 100 µmoles of K_2HPO_4 (pH 8.0) and 30 µmoles of L-glutamate at 37°C for 10 min. After the incubation period, 7.5 µmoles of NAD^+ were added to initiate the reaction. The reference cuvette contained all of the components with the exception of NAD^+

which was replaced by distilled water. The final volume was 3 ml and the final pH was 8.0.

Replicate enzyme assays were carried out in all investigations and the data presented in the text are the average of duplicate determinations.

Determination of Protein

Protein was routinely determined by the colorimetric method of Lowry, Rosebrough, Farr and Randall (1951). Reference curves were constructed using crystalline egg albumin.

Feeding Experiments Using Tissue Slices

All experiments with tissue slices were carried out in duplicate, the results presented being the average of such experiments.

After removal of the testas, 3 day old pea cotyledons were carefully sliced (0.3 mm thickness) using a sharp razor blade. The slices were then washed several times in distilled water at room temperature followed by blotting dry with paper towels. For pulse feeding experiments 1 gm samples of the slices were placed in Warburg flasks containing a solution of 1 ml of 0.1 M Na H_2PO_4 (pH 5.5) and 2.5 μc (0.5 μmole) of sodium acetate-2-^{14}C. A small piece of filter paper containing 20% KOH was placed in the center well to absorb CO_2 evolved during the experimental period. The tissues were incubated for 30 min. after which they were transferred to a sintered glass funnel and washed thoroughly with distilled water. The washed tissue was then quickly transferred to another Warburg

flask containing 1 ml of 0.1 M NaH_2PO_4 (pH 5.5). The center well again contained a piece of filter paper with 20% KOH. This washing and transferring process was accomplished in less than 5 min. Samples of the tissues were removed at various intervals and killed in boiling 80% ethanol for 3 min. The filter papers were counted directly for radioactivity in a polyether counting fluor system as described in the appropriate section.

Analytical Methods

After boiling in 80% ethanol the tissues were ground thoroughly in a glass hand grinder. The resulting suspension was then centrifuged at 1,000 x g for 20 min. and the supernatant collected. The insoluble residue from this centrifugation was successively washed with 20 ml of 50% ethanol and 20 ml of distilled water. The supernatants were combined and then evaporated to dryness under reduced pressure at 40°C in a Buchler flask evaporator. The dried sample was washed with 10 ml of diethylether and this ether solution was in turn washed with three 10 ml portions of distilled water. Substances soluble in the ether solution are referred to as the lipid fraction. The ethanol/water soluble materials were further fractionated using ion exchange chromatography (Canvin and Beevers, 1961).

Preparation of Ion Exchange Resins

Glass columns (1.5 cm in diameter) were filled with an aqueous slurry of cation exchange resin in the hydrogen form (Dowex Ag 50 W-X8, 200-400 mesh) to a depth of 4 cm. The columns were washed with distilled water until the pH of the effluent remained constant at 5.5.

The anion exchange resins in the chloride form was converted to the formate and acetate forms before use. This was achieved by passing 1 M solutions of sodium formate or sodium acetate through the resin until the effluent no longer contained chloride ions. The resin was then washed with 50 ml of 0.1 N formic or acetic acid, followed by distilled water until the effluent had a constant pH value of 5.5. Columns (1.5 x 4 cm) of these resins were routinely used in fractionation of the labeled extracts as described below.

Fractionation of the Ethanol/Water Soluble Fraction

The ethanol/water soluble materials in 10 ml (see above) were carefully applied to a column of cation exchange resin in the hydrogen form. Uncharged and negatively charged compounds were washed from the column in 50 ml of distilled water. Positively charged compounds such as amino acids, were subsequently eluted from the column using 50 ml of a 4N NH_4OH solution.

Further separation of various fractions was achieved using anionic resins either in the formate or in the acetate form. The effluent from the hydrogen column (negatively charged and neutral compounds) which has been concentrated to 5 ml under reduced pressure, was applied to a column of anion exchange resin in the formate form. Neutral compounds were washed from the column with 50 ml of distilled water. Negatively charged compounds, mainly organic acids, were eluted from the column with 50 ml of 8 N HCOOH solution.

The eluate from the hydrogen column (positively charged compounds) was evaporated to dryness under reduced pressure, redissolved

in 5 ml of distilled water and then passed through a column of anion exchange resin in the acetate form. The basic and neutral amino acids including amides were removed by washing the column with 50 ml of distilled water. The acidic amino acids was eluted from the column with 50 ml of 8 N CH_3COOH solution.

Further fractionation of the neutral and basic amino acids was achieved by hydrolyzing the amides to their corresponding acidic amino acids. The hydrolysis was carried out in 2N HCl at 97°C for 4 hours. Glutamic and aspartic acids released by this treatment were then separated using anion exchange resins in the acetate form. Before measuring ^{14}C content or further chromatographic separation each fraction from the columns was evaporated to dryness, neutralized and then redissolved in distilled water. The amino acid fractions were further separated and the major radioactive components identified by paper chromatography.

Paper Chromatography of Amino Acids

For separation of amino acids the technique of descending paper chromatrography was employed (Cameron and Cossins, 1967). Radioactive samples were applied to sheets of Whatman no. 1 chromatographic paper and followed by development in the appropriate solvent system. Labeled compounds were detected on the chromatograms by autoradiography using Kodak no-screen X-ray film. Radioactive compounds were identified by reference to the mobilities of authentic compounds in the various solvent systems.

Aliquots of the neutral and basic amino acid fraction were

spotted on paper chromatograms and developed in n-butanol: glacial acetic acid: water (4:1:5 by vol.) for 20 hours at 25°C. The markers were sprayed with a 0.1% (v/v) solution of ninhydrin in n-butanol.

The acidic amino acids were separated in a phenol:water (8:3 v/v) system for 24 hours at 25°C. The markers were detected with the ninhydrin reagent.

Radioactive areas on the chromatograms were carefully cut out and placed directly in the counting fluor. In measuring the radioactivity of aqueous samples, aliquots were pipetted into counting vials followed by addition of the counting fluor.

Scintillation Counting

In the present investigation radioactive samples were counted in a Nuclear-Chicago Unilux I liquid scintillation counter. A polyether counting fluor containing 9.6 gm PPO and 0.4 gm POPOP in a 800 ml mixture of dioxane:anisole:1,2 - dimethoxyethane (6:1:1 by vol.) was routinely used. Efficiency of counting was determined by the channels ratio method. All samples were counted several times for 4 min. periods at the balance point. Under these conditions, aqueous solutions of ^{14}C samples were counted at an efficiency of approximately 68%. Radioactive samples present on filter paper were counted with an efficiency of 54% in this system. In all cases the counts were corrected for background.

RESULTS

The importance of aspartic and glutamic acids for biosynthesis of other amino acids in plants has been well documented by Fowden (1965). More recently Cameron and Cossins (1967) emphasized the role of the TCA cycle for the production of glutamic acid in germinating pea cotyledons. They suggested that aspartic and glutamic acids were compartmented so that they can serve as precursor pools for the synthesis of their corresponding amides. To further examine compartmentation of aspartic and glutamic acids the metabolism of acetate-2-^{14}C was followed by means of pulse feeding experiments.

During the 30 minute pulse feeding very little $^{14}CO_2$ was released (Table 1). As expected much of the ^{14}C was incorporated into the organic acid and amino acid fractions. During the post-pulse period the total radioactivity recovered from the tissues continued to increase indicating that an endogenous pool of labeled acetate was being further utilized during this experimental period. As the post-pulse period was extended the amount of radioactivity in CO_2, neutral and basic amino acids and glutamine continued to increase. The ^{14}C content of aspartic and glutamic acids increased up to 1 hr. and then decreased while that present in the organic acids decreased after 2 hr. (Table 1, Figure 1).

In agreement with Cameron and Cossins' work, the conversion of acetate-2-^{14}C to CO_2 was relatively minor. The rapid labeling of glutamic acid from acetate suggests that glutamate dehydrogenase may play an important role in the synthesis of this amino acid. Furthermore, the pronounced decrease in the labeling of glutamic acid while the neutral and basic amino acid fraction was increasing in radioactivity (Table 1) suggests a flow of carbon from glutamate

Table 1

Incorporation of acetate-2-^{14}C into the soluble fractions of pea cotyledons (Expressed as c.p.m.)

Fraction	Pulse feeding 0.5 hr.	Post pulse incubation 0.5 hr.	1 hr.	2 hr.	3 hr.
Carbon dioxide	1760	3340	5230	14,550	22,200
Neutral fraction	45,690	50,790	13,130	15,410	25,090
Neutral and basic amino acids	73,800	144,060	294,180	308,980	391,240
Glutamine	6390	12,960	19,760	34,760	38,550
Glutamic acid	293,000	314,540	484,990	398,660	274,600
Aspartic acid	31,360	28,260	46,860	33,620	9,700
Organic acids	304,780	472,090	755,740	1,018,470	907,510

Cotyledon slices (1 g. fresh weight) were incubated for 30 min. at 30°C with 0.5 µmole of acetate-2-^{14}C containing 2.5 µc of ^{14}C and 100 µmoles of sodium phosphate buffer (pH 5.5). The tissues were then washed thoroughly and incubated at 30°C with 100 µmoles of sodium phosphate buffer (pH 5.5) for periods up to 3 hr. as indicated.

Figure 1

Changes in the radioactivity of soluble compounds following a 30 min pulse feeding with acetate-2-^{14}C.

1 gm samples of cotyledon slices were incubated for 30 min at 30°C with 2.5 µc of acetate-2-^{14}C (0.5 µmoles) and 100 µmoles of NaH_2PO_4 (pH 5.5). The tissues were then washed thoroughly and incubated at 30°C with 100 µmoles NaH_2PO_4 (pH 5.5) for periods up to 3 hr as indicated.

x-x-x Aspartic acid

o-o-o Glutamic acid

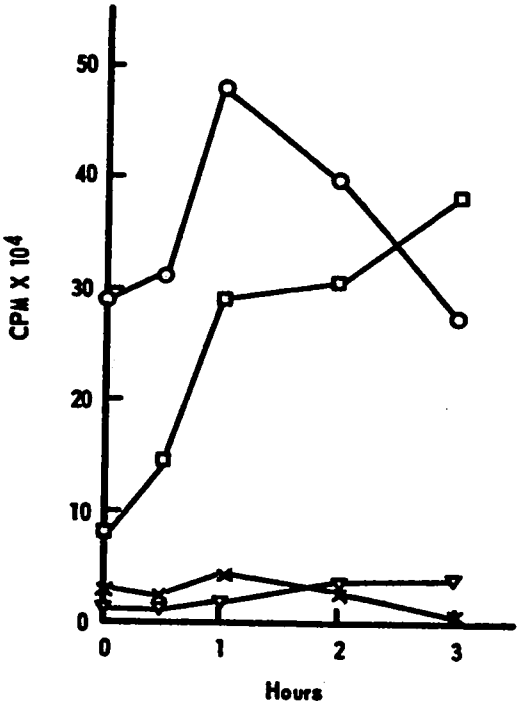
□-□-□ Neutral and basic amino acids

■-■-■ Organic acids

▲-▲-▲ Carbon dioxide

●-●-● Neutral fraction

△-△-△ Glutamine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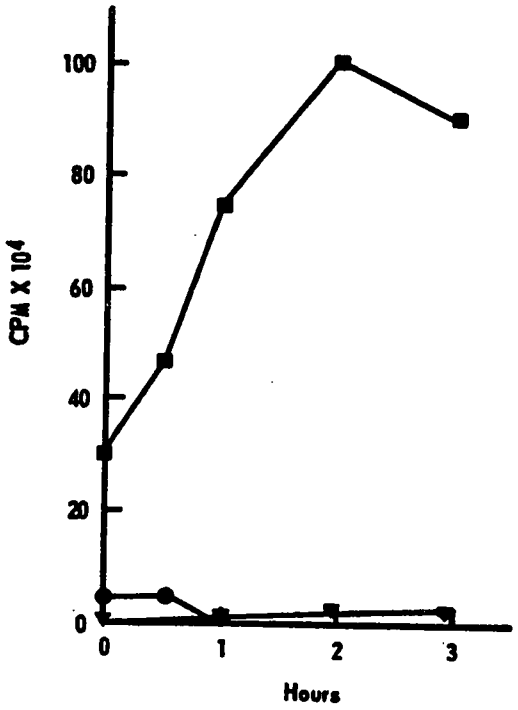
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80
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3
Hours

to the neutral and basic amino acids. The fact that relatively little aspartic acid was labeled implies that the pool of this amino acid is very small and is possibly in a state of rapid turnover. This latter possibility is supported by the considerable decrease in the labeling of aspartate during the post-pulse period (Table 1). Such turnover may be related to the utilization of aspartate for homoserine biosynthesis (Larson and Beevers, 1965). Furthermore, equilibrium of this aspartate pool with oxaloacetate in the TCA cycle would produce a similar turnover of ^{14}C. Such an equilibrium would probably be affected by aspartate aminotransferase.

It is clear that glutamate dehydrogenase and aspartate aminotransferase play important roles in intermediary metabolism. Due to their biochemical relationships with the TCA cycle, it is possible that these two enzymes are localized in the mitochondria. In addition, the general importance of glutamic acid for the synthesis of other amino acids by transamination reactions suggests that enzymes catalyzing these reactions may also exist in other parts of the cell. For these reasons the possible intracellular localization of glutamate dehydrogenase and aspartate aminotransferase in pea cotyledons was examined in further experiments.

Changes in Aspartate Aminotransferase Activity During Germination

1. Cytoplasmic Aspartate Aminotransferase

Crude homogenates were assayed for aspartate aminotransferase activity as described in the Methods section. Cytoplasmic aspartate aminotransferase activity was detected at all of the stages of germination studied (Figure 2). Total enzyme activity was relatively low in one and two day old tissues. On the basis of fresh weight and protein content, three and four day old cotyledons contained the greatest amounts of cytoplasmic aspartate aminotransferase activity.

2. Particulate Aspartate Aminotransferase

Pea cotyledons show a pronounced development of subcellular organelles during germination (Bain and Mercer, 1966). Experiments were, therefore, conducted to follow possible changes in the levels of particulate aspartate aminotransferase activity in germinating pea cotyledons. The particulate fraction was isolated and treated with deoxycholate as described in the Methods section. As is evident from Figure 3, total enzyme activity was very low initially but reached a maximum after three days. After this, total enzyme activity slowly declined. However, specific enzyme activity reached a maximum on the fourth day and then declined. During the five day period examined, the activity of the particulate fraction represented approximately 4 - 5% of the total extractable aspartate aminotransferase activity.

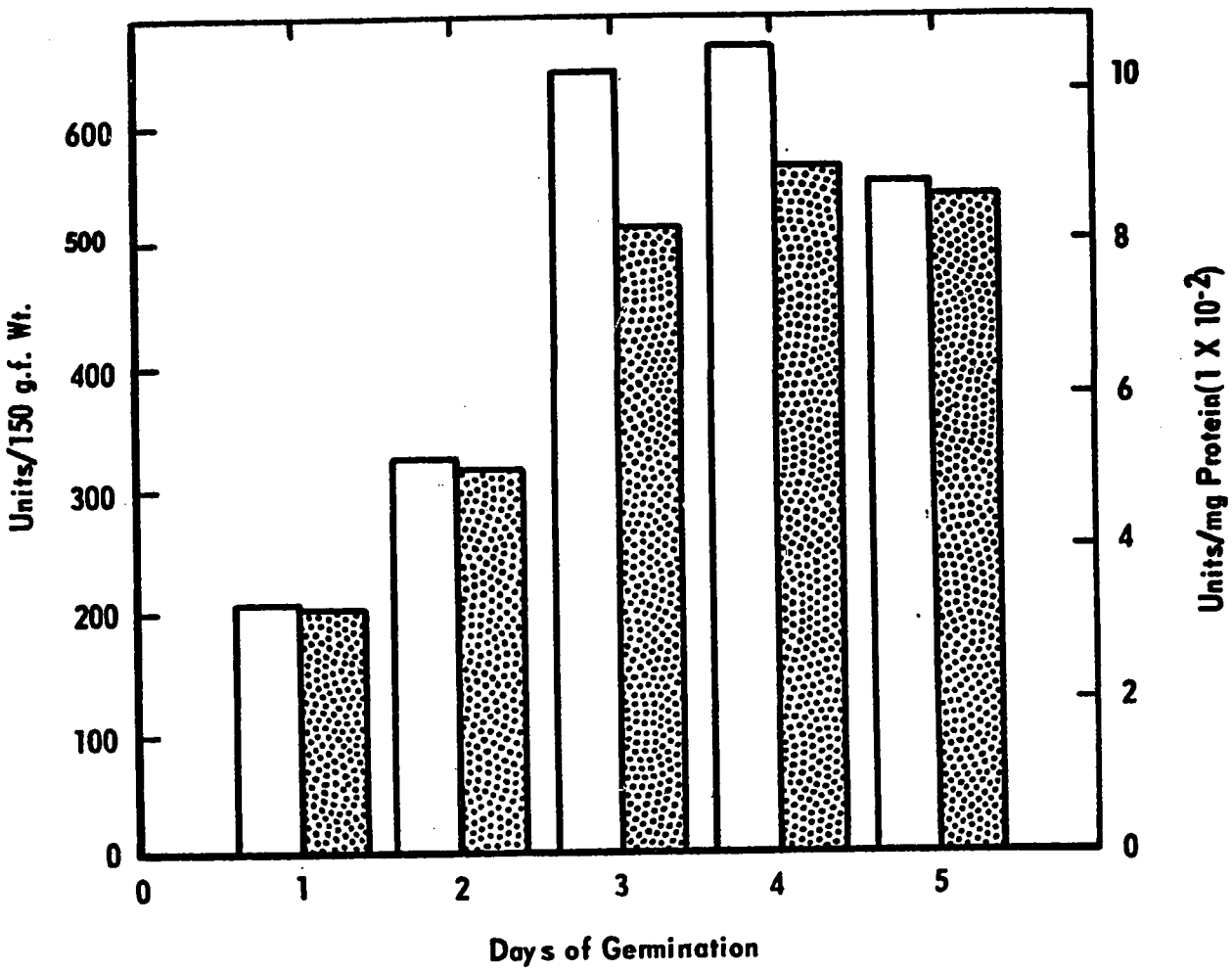
Figure 2

Changes in cytoplasmic aspartate aminotransferase activity during germination

Assay conditions: samples of the 10,000 x g supernatant were pre-incubated with 100 µmoles of Tris-HCl (pH 8.0) 40 µmoles of L-aspartate and 10 µg of pyridoxal phosphate for 10 min. at 37°C. 10 µmoles of α-ketoglutarate were then added to initiate the reaction. The final volume was 3 ml; the final pH was 8.0.

Stippled bar represents units/150 g.f.wt.

Open bar represents units/mg protein


Units/150 g.f. Wt.
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500
400
300
200
100
0
0
1
2
3
4
5
Days of Germination
10
8
6
4
2
0
Units/mg Protein(1 X 10-2)

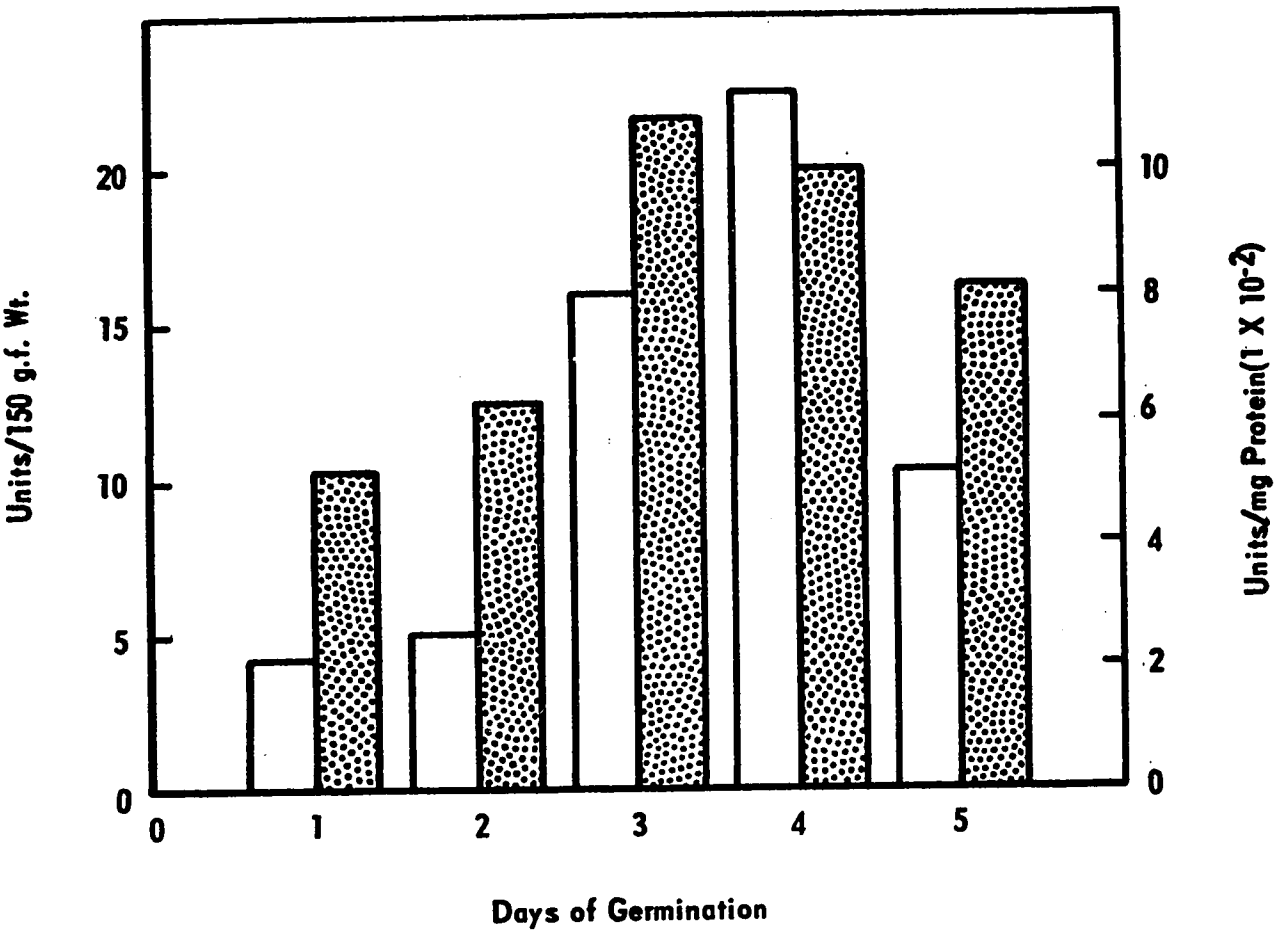
Figure 3

Changes in particulate aspartate aminotransferase activity during germination

Samples of the 10,000 x g particulate fraction were treated with 1% deoxycholate and assayed for enzyme activity as described in Figure 2.

Stippled bar represents units/150 g.f.wt.

Open bar represents units/mg protein


Units/150 g.f. Wt.
20
15
10
5
0
0
1
2
3
4
5
Days of Germination
10
8
6
4
2
0
Units/mg Protein(1 X 10-2)

Changes in Glutamate Dehydrogenase Activity in the Particulate Fraction During Germination

Since glutamate dehydrogenase also plays an important role in glutamate biosynthesis, the particulate fraction was examined for this enzyme during germination. As illustrated in Figure 4, enzyme activity increased with age, reaching a maximum in three days and then decreasing. The specific enzyme activity reached a maximum on the fourth day. The particulate fraction was found to contain approximately 4% of the total extractable glutamate dehydrogenase activity of the tissue. Preliminary studies indicated that the cytoplasmic fraction also contained appreciable levels of glutamate dehydrogenase. However, in contrast with the other enzymes, activity was rapidly lost on storing the preparations for short periods at 4°C. It was, therefore, found impossible to obtain accurate data on the levels of this enzyme in the cytoplasmic fraction.

The changes in aspartate aminotransferase activity during germination (Figures 2 and 3) suggest that the enzyme is synthesized. This suggestion was supported by studies with chloramphenicol and cycloheximide. At a concentration of 1×10^{-4}M, chloramphenicol inhibited the characteristic increase in enzyme activity during the second day of germination by 14%. At the same concentration, cycloheximide gave an inhibition of 45%.

There have been several reports in the literature of increases in enzyme activity following treatment of tissues with gibberellic acid (Varner, 1965). In the present work, the possible

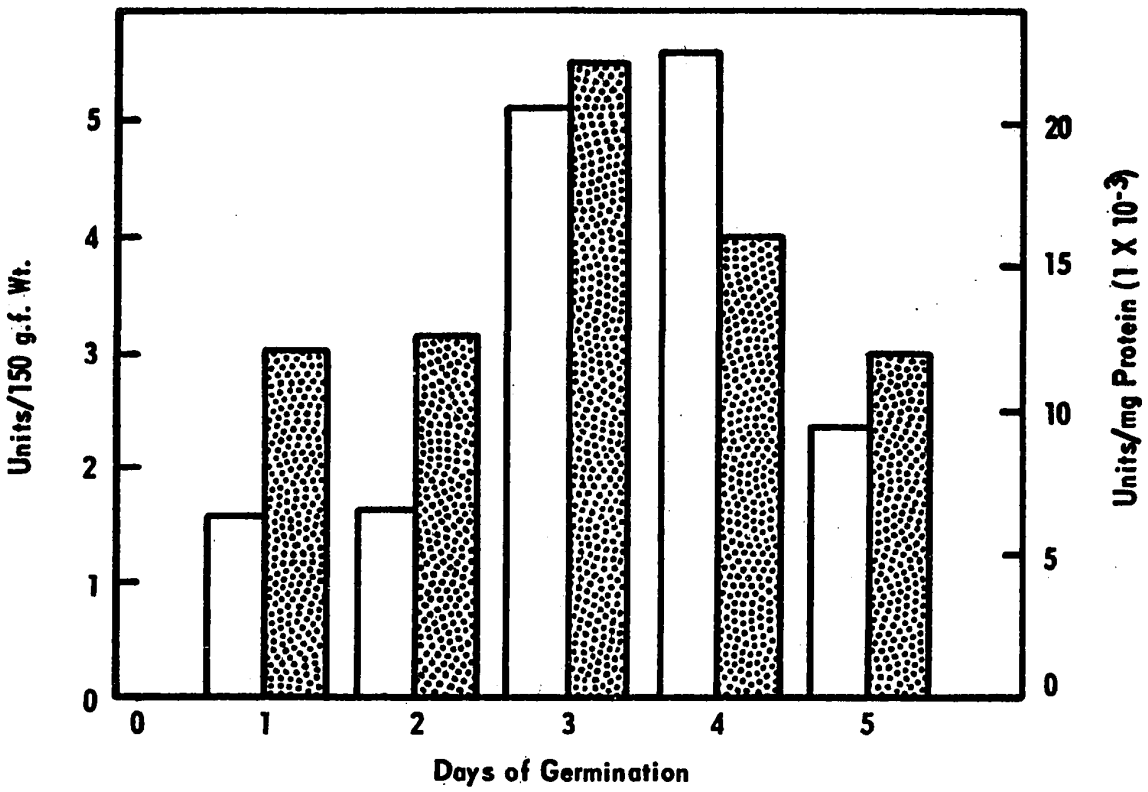
Figure 4

Changes in particulate glutamate dehydrogenase activity during germination

Assay conditions: the crude particulate suspension (deoxycholate treated) was pre-incubated with 100 µmoles of K_2HPO_4 (pH 8.0) and 30 µmoles of L-glutamate at 37°C for 10 min. 7.5 µmoles of NAD^+ were then added to initiate the reaction. The reference cuvette contained all of the components with the exception of NAD^+. The final volume was 3 ml; the final pH was 8.0.

Stippled bar represents units/150 g.f.wt.

Open bar represents units/mg protein


Units/150 g.f. Wt.
Units/mg Protein (1 X 10⁻³)
Days of Germination
0
1
2
3
4
5
0
5
10
15
20

effect of gibberellic acid on the synthesis of aspartate aminotransferase in the presence and absence of aspartic and glutamic acids was investigated. Although the hormone doubled the length of the epicotyl, aspartate aminotransferase activity was not affected.

Partial Purification of the Aspartate Aminotransferases

In an attempt to characterize the cytoplasmic and the particulate aspartate aminotransferases, these enzymes were partially purified and their kinetic properties were studied. The methods employed for enzyme purifications included $(NH_4)_2SO_4$ fractionation; DEAE-cellulose, Sephadex G-200, and hydroxylapatite column chromatographies as detailed in the Methods section.

Partial Purification of the Cytoplasmic Enzyme

1. DEAE-cellulose column chromatography

Most of the cytoplasmic enzyme activity was precipitated when the $(NH_4)_2SO_4$ concentration was raised to 40 - 60% of saturation. When a sample of this fraction was applied to a column of DEAE-cellulose, much of the protein was immediately eluted by 0.005 M Tris-HCl buffer (pH 7.8). Enzyme activity was subsequently eluted from the column using a gradient of Tris-HCl buffer as described in the Methods section. This indicated that the cytoplasmic aminotransferase from pea cotyledons displayed a negative charge under these experimental conditions. It is interesting to note that the corresponding enzymes from mammalian heart (Borst and Peeters, 1961), and cauliflower florets (Ellis and Davies, 1961)

both displayed a similar charge at pH 7.8. Total recovery after column chromatography was approximately 70%. From Table 2, it is clear that chromatography on DEAE-cellulose resulted in an increase in specific activity of approximately 8 times over the $(NH_4)_2SO_4$ fraction and approximately 23 times over the crude extract. Similar to the enzyme from mammalian and cauliflower tissues (Nisonoff and Barnes, Jr., 1952; Ellis and Davies, 1961), the enzyme from pea cotyledons after DEAE-cellulose chromatography lost little activity when stored for several months at -15°C. Once activity was completely lost (After storage at -15°C for approximately 1 year) additions of pyridoxal phosphate and mercaptoethanol did not restore enzyme activity.

2. Sephadex G-200 column chromatography

For Sephadex G-200 column purification the enzyme from DEAE-cellulose was lyophilyzed and redissolved in demineralized redistilled water. The solution displayed a bright yellow color, there was no indication of denaturation or lost of enzyme activity by this treatment as 100% of the enzyme activity was recovered after freeze drying.

When the DEAE-cellulose purified enzyme was applied to the Sephadex column and was subsequently eluted with 0.005 M Tris-HCl buffer (pH 7.8), the bulk of the protein was eluted in the void volume. Enzyme activity recovered from the column had a specific activity approximately 67 times greater than the crude extract (Table 2). Furthermore, all of the activity applied to the column

Table 2

Purification of the cytoplasmic aspartate aminotransferase

Fraction	Total enzyme activity (units)	Total protein (mg)	Specific activity	Purification
Crude extract	455	11,050	0.40	---
40-60% saturated $(NH_4)_2SO_4$	182	1,654	0.11	2.8
DEAE-cellulose*	24.5	26.6	0.92	23.0
Sephadex G-200	22.3	8.3	2.70	67.5

Assay conditions: the enzyme was pre-incubated with 200 µmoles of L-asparate and 100 µmoles of Tris-HCl at 37°C for 5 min. 10 µmoles of α-ketoglutarate were then added to initiate the reaction. Final volume: 3 ml; final pH: 8.0.

* 35 units from the $(NH_4)_2SO_4$ fraction were applied to the DEAE-cellulose column. After chromatography on Sephadex G-200 approximately 26% of the initial enzyme activity was recovered.

was recovered. After Sephadex G-200 purification the enzyme was active for several weeks at -15°C.

3. Hydroxylapatite column chromatography

Although the routine purification procedure, as outlined in Table 2, did not include the use of hydroxylapatite some fractionation studies were carried out with this material in attempts to achieve further purification of the cytoplasmic enzyme. In these experiments the hydroxylapatite columns were equilibrated and then eluted with 0.005 M Tris-HCl (pH 7.8). Under these conditions the cytoplasmic aspartate aminotransferase was not adsorbed by hydroxylapatite. Maximum enzyme activity and specific activity occurred in fractions having the smallest amount of protein. The best purification achieved was approximately 150-200 times over the crude extract. However, the enzyme lost approximately 60% of its activity after being stored for 24 hours at 4°C. Additions of pyridoxal phosphate or mercaptoethanol to such preparations failed to restore its original enzyme activity.

In experiments where hydroxylapatite columns were equilibrated and eluted with Na_2HPO_4 buffer (pH 7.8), the enzyme was adsorbed by the ion exchanger. Enzyme activity was, however, readily eluted off the columns when a gradient of phosphate buffer at pH 7.8 was applied (see Methods section). The instability of the enzyme after hydroxylapatite column chromatography is one feature which is different from the mammalian enzyme. This is

perhaps one reason why the mammalian aspartate aminotransferase can be so highly purified. Although, hydroxylapatite columns highly purified the pea enzyme, the instability of such preparations prevented the routine use of this treatment.

Extraction and Partial Purification of the Particulate Asparate Aminotransferase

1. Extraction of the Particulate Enzyme

Since aspartate aminotransferase activity was detected in the particulate fraction, various physical methods were attempted to release the enzyme into solution. Table 3 shows that enzyme activity was increased by high speed blending and by sonication. However, the enzyme was not completely solubilized for much of the enzyme activity remained in the sedimented material after centrifugation at 30,000 x g. However, after treatment with 1% deoxycholate, most of the enzyme activity was solubilized. It appears, therefore, that the particulate aspartate aminotransferase is membrane bound and it is released only when the lipids were solubilized by the bile salt. Deoxycholate at the concentration used had no effect on the aminotransferase activity.

2. Partial Purification of the Particulate Aspartate Aminotransferase

Fractionation of the crude suspension with n-butanol and acetone was attempted but resulted in denaturation of the enzyme protein. Partial purification of the particulate aspartate aminotransferase was, however, achieved by $(NH_4)_2SO_4$ fractionation

Table 3

The effects of blending and sonication on the enzyme activity of the particulate fraction

Treatment	Enzyme activity	
	μmoles OAA/min	% of control
Control (0.05 M Tris-HCl)	0.037	100
High speed blending (2 min)	0.056	151
Sonication (0.96 amperes)	0.056	151

Aliquots (0.2 ml) of the particulate fraction in 0.05 M Tris-HCl buffer (pH 7.8) were assayed for enzyme activity before (control) and after blending and sonication, respectively. The enzyme was pre-incubated with 100 μmoles of Tris-HCl, 40 μmoles of L-aspartate and 10 μg of pyridoxal phosphate for 10 min at 37°C. The reaction was then initiated by addition of 10 μmoles of α-ketoglutarate. Final volume, 3 ml; final pH, 8.0.

followed by Sephadex G-200 column chromatography (Table 4). After the Sephadex G-200 step 45% of the original activity was recovered and the specific activity was increased 26 times.

Properties of the Cytoplasmic Aspartate Aminotransferase

Due to its stability the enzyme after DEAE-cellulose column chromatography was routinely used for kinetic studies. At this stage of purification the enzyme had a specific activity of 0.90, 22 times greater than that of the crude extract. Under the experimental conditions described in the Materials and Methods section, aspartate aminotransferase activity was found to be linear with protein concentrations up to 2 mg.

1. Specificity

The strict limitation of the action of an enzyme to one substrate or to a small number of closely related substances is one of the most important properties of a biological catalyst. It is now known that the aspartate aminotransferases from mammalian tissues and from cauliflower florets have a high degree of substrate specificity. Experiments were conducted to determine whether the enzyme from pea cotyledons also displayed similar substrate specificity. As indicated in Table 5, only α-ketoglutarate and L-glutamate supported transamination with L-aspartate and oxaloacetate, respectively.

2. Effects of Certain Buffering Systems

The effects of glycine, phosphate, Tris-maleate, Tris-HCl and Tris-acetate buffers on aspartate aminotransferase activity

Table 4
Purification of the particulate aspartate aminotransferase

Fraction	Total activity (units)	Total protein (mg)	Specific activity	Purification
Crude suspension*	46.5	1178.0	0.04	---
40-60% saturated $(NH_4)_2SO_4$	36.4	48.5	0.61	15.2
Sephadex G-200**	8.1	7.6	1.07	26.8

Assay conditions: the enzyme was pre-incubated with 40 µmoles of L-aspartate, 100 µmoles of Tris-HCl and 10 µg of pyridoxal phosphate for 10 min. at 37°C. 10 µmoles of α-ketoglutarate were then added to initiate the reaction. Final volume, 3 ml.; final pH, 8.0.

*10,000 x g fraction treated with deoxycholate as described in the Materials and Methods.

**14 Units from the $(NH_4)_2 SO_4$ fraction was applied to the Sephadex G-200 column. After this chromatography approximately 45% of the initial enzyme activity was recovered.

Table 5

Specificity of the cytoplasmic aspartate aminotransferase

Substrate	Concentration (µmoles/3 ml)	Enzyme activity (µ mole OAA/min)
a) Amino acceptors		
pyruvate	1	0
hydroxypyruvate	10	0
glyoxylate	10	0
α-ketoglutarate	10	0.18
b) Amino donors		
L-aspartate	20	0
D-aspartate	20	0
L-asparagine	20	0
L-serine	20	0
L-leucine	20	0
glutamine	20	0
γ-aminobutyrate	20	0
L-alanine	20	0
glycine	20	0
L-glutamate	20	0.14

Assay conditions
(a) with amino acceptors: enzyme (0.4 mg of protein) was preincubated with 100 µmoles of Tris-HCl and 20 µmoles of L-aspartate for 5 min at 37°C. The amino acceptor as then added to initiate the reaction. Final volume 3 ml; final pH, 8.0.
(b) with amino donors: enzyme (0.6 mg protein) was pre-incubated with 100 µmoles of Tris-HCl and 20 µmoles the amino donor for 5 min at 37°C. 1 µmole of OAA was then added to initiate the reaction. Final volume, 3 ml; final pH, 8.0.

was examined as shown in Table 6. Enzyme activity appeared to be lower when glycine, phosphate and Tris-maleate buffers were used. For this reason Tris-HCl buffer was used for all experiments. The only exceptions to this being experiments where a wider range of pH was required. Banks, Lawrence, Vernon and Wootton (1963), had reported that the enzyme reaction produces oxaloacetate in the keto form and in order to measure this compound at 280 mμ , it must be converted to the enol form. It appears that the buffering systems have the ability to catalyze this conversion (Henson and Cleland, 1964). On the basis of these findings it is possible that glycine and phosphate buffers used in the present work decreased the enolization of oxaloacetate and, therefore, gave apparently lower reaction velocities. In addition, phosphate buffer may inhibit enzyme activity by causing dissociation of the coenzyme. Since the enzyme was incubated with aspartic acid, it was presumably converted into the pyridoxamine phosphate form. Studies by Scardi et al., (1963) indicated that the effectiveness of phosphate buffer in resolving the enzyme was greatest when the enzyme was in the pyridoxamine phosphate form.

The inhibitory effect of Tris-maleate may be due to the action of maleate on the enzyme. As shown by Velick and Vavra (1962), maleate competes with the substrates for the amino and aldehyde forms of the enzyme-coenzyme complex.

3. Effects of pH

From the previous section on the effects of some buffers,

Table 6

Effects of some buffering systems on cytoplasmic aspartate aminotransferase activity

Buffer used	Enzyme activity (μmole OAA/min)	% of control
Tris-HCl (control)	0.134	100
Tris-acetate	0.134	100
Tris-maleate	0.040	30.0
Glycine	0.072	53.7
K_2HPO_4	0.111	83.0

Assay conditions: enzyme (0.3 mg of protein) was pre-incubated with 100 μmole of the buffer (pH 8.0) and 20 μmole of L-aspartate at 37°C for 5 min. 10 μmoles of α-ketoglutarate were then added to initiate the reaction. Final pH, 8.0; final volume, 3 ml.

it is clear that one buffering system must be used so as to avoid variations in keto-enolization and to avoid other specific effects. Since Tris-acetate was found to be capable of buffering in a wide range of pH, it was utilized. Standard curves for oxaloacetate at different pH values were used to correct for the changes in absorbance as a function of pH. From Figure 5, it is clear that the optimal pH for enzyme activity was 8.0. Studies using the radiometric assay over the same pH range showed that maximal conversion of α-ketoglutarate to glutamate also occurred at pH 8.0. The optimum pH of 8.0 is, therefore, similar to those reported for mammalian and plant aminotransferases (Jenkins et al., 1959; Nadkarni and Sohonie, 1963). It was not surprising to find a decrease in enzyme activity at acid pH since α-ketoglutarate has a greater tendency to inhibit the mammalian enzyme at low pH values (Velick and Vavra, 1962). It was difficult to study the effects of pH on enzyme activity above 8.5 because of the rapid spontaneous breakdown of oxaloacetate to pyruvate (Jenkins et al., 1959).

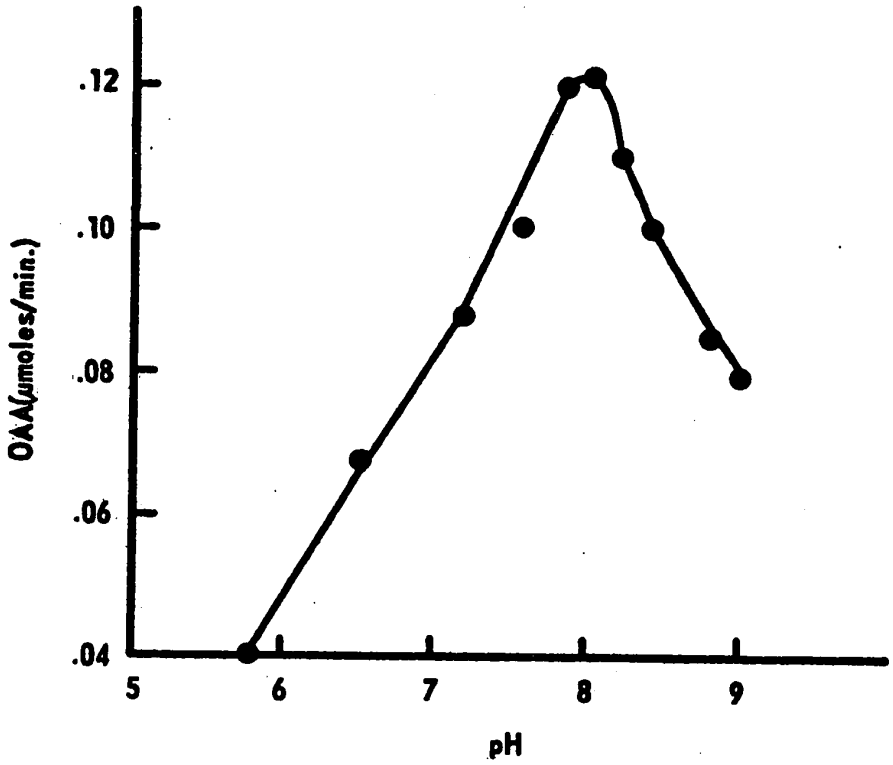
4. Effects of Inhibitors

It is known that sulfhydryl reagents such as iodoacetamide, iodoacetate and p-chloromercuribenzoate (p-CMB) inhibit enzyme reactions requiring free -SH groups. It is also known that isonicotinic acid hydrazide and KCN inhibit enzymes which have pyridoxal phosphate as their coenzyme. In the present investigation, experiments were conducted to study the effects of such

Figure 5

Effects of pH on the activity of the cytoplasmic enzyme.

Assay conditions: samples of enzyme (0.3 mg protein) were pre-incubated with 100 μmoles of Tris-acetate (pH as indicated) and 20 μmoles of L-aspartate at 37°C for 5 min. 10 μmoles of α-ketoglutarate were then added to initiate the reaction. The final pH was determined after the reaction had continued for 3 min.


.12
.10
.08
.06
.04
OAA(μmoles/min.)
5
6
7
8
9
pH

inhibitors on aspartate aminotransferase activity. Table 7 shows that all of the compounds tested inhibited aminotransferase activity. Since isonicotinic acid hydrazide is an antagonist of pyridoxal phosphate its inhibitory effect strongly suggests that the aminotransferase is pyridoxal phosphate dependent. The inhibitory effect of KCN also supports this contention, although its action is less specific. Inhibition by iodacetate, iodoacetamide and p-CMB indicates that the enzyme requires -SH groups for activity.

In experiments with iodoacetate 30 µg of pyridoxal phosphate or 60 µmoles of mercaptoethanol added to the reaction system before the inhibitor completely prevented inhibition. However, if the inhibitor was incubated with the enzyme before the addition of mercaptoethanol or pyridoxal phosphate inhibitory effect was not relieved.

Similar to the mammalian enzyme the aminotransferase from pea cotyledons was also inhibited by high keto acid concentrations. Figure 6 indicates that concentrations of oxaloacetate and α-ketoglutarate higher than 7.0 µm and 5 µm respectively inhibited transaminase activity. However, amino acid concentrations above saturation did not cause inhibition. The inhibitory effects of the keto acids may result from combination with the pyridoxal phosphate form of the enzyme. This would effectively reduce interconversions of the pyridoxal phosphate to pyridoxamine phosphate forms of the enzyme (Banks et al., 1963). However, such complexes were competitively overcome by increasing the concentration of the amino

Table 7

Effects of various inhibitors on cytoplasmic aspartate aminotransferase activity

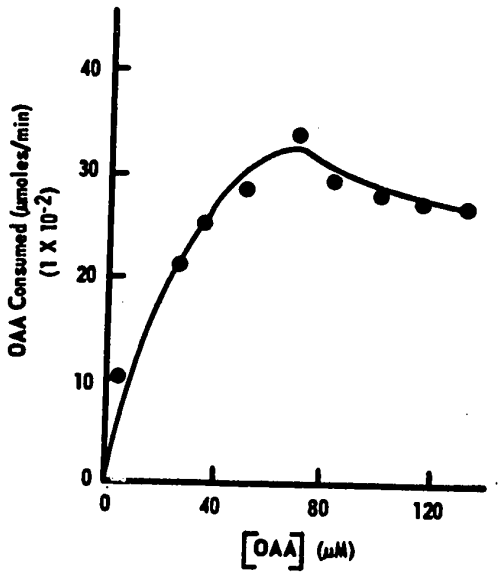
Inhibitor	Concentration (M)	Inhibition (%)
KCN	1.7×10^{-2}	100
Isonicotinic acid hydrazide	3.3×10^{-3}	100
Iodoacetate	3.3×10^{-3}	40
Iodoacetamide	1.7×10^{-2}	100
p-CMB	2.7×10^{-4}	23

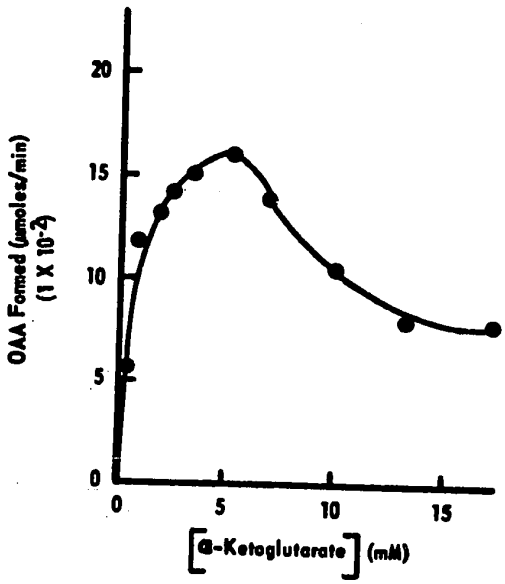
Assay conditions: enzyme (0.4 mg protein) was incubated with the inhibitor and 100 μmoles of Tris-HCl at 37°C for 10 min. Then 20 μmoles of L-aspartate were added and the mixture incubated for a further 5 min. After this, 10 μmoles of α-ketoglutarate were added to initiate the reaction. Final volume, 3 ml; final pH, 8.0.

Figure 6

Effects of varying keto acid concentrations on cytoplasmic aspartate aminotransferase activity.

Assay conditions: samples of the enzyme (0.3 mg of protein) were incubated with either 20 μmoles of L-aspartate or L-glutamate and 100 μmoles of Tris-HCl (pH 8.0) at 37°C for 5 min. After this incubation α-ketoglutarate or oxaloacetate were added to initiate the reaction. The final volume was 3 ml and the final pH was 8.0.


OAA Consumed (μmoles/min)
(1 X 10⁻²)
40
30
20
10
0
0
40
80
120
[OAA] (μM)


OAA Formed (μmoles/min)
(1 X 10⁻²)
20
15
10
5
0
0
5
10
15
[α-Ketoglutarate] (mM)

acid substrates as illustrated in Figures 7 and 8.

5. Effects of Cations

Although the role of metal ions in enzymic transamination reactions is not clear, there had been reports of enzyme activation by various cations (Happold and Turner, 1957; Nadkarni and Sohoni, 1963). Table 8 shows that addition of $MgCl_2$, $CaCl_2$ and $MnCl_2$ stimulated transamination. This stimulation was also confirmed by measuring the production of radioactive glutamate. In test for possible nonenzymic reactions boiled enzyme preparations were incubated with the substrates and the cations. In the presence of $CaCl_2$ and $MgCl_2$ no production of oxaloacetate could be detected. However, in the presence of $MnCl_2$ some absorbance at 280 mμ was observed but this was considerably lower than the readings obtained with the unboiled enzyme. This may be the reason for different percentage of stimulation shown by $MnCl_2$ and $MgCl_2$ as measured by the production of oxaloacetate and ^{14}C-glutamate (Table 8). Pyridoxal phosphate was omitted from these reaction mixtures as it is known to react nonenzymically with certain metals (Metzler and Snell, 1952). Monovalent cations such as Na^+, K^+, and NH_4^+ had no effect on enzyme activity.

6. Michaelis Constants

The apparent Michaelis constants were determined according to the method of Lineweaver and Burk (1934), and from the slope term. The apparent Michaelis constants for the four substrates attacked are given in Table 9. In agreement with the data reported for the

Table 8
Effects of cations on cytoplasmic aspartate aminotransferase activity

Addition	Concentration (M)	Enzyme activity (µmole OAA/min)
none	---	0.14
$MnCl_2$	3.3×10^{-3}	0.67
$MgCl_2$	3.3×10^{-3}	0.20
$CaCl_2$	3.3×10^{-3}	0.20
NaCl	3.3×10^{-3}	0.14
KCl	3.3×10^{-3}	0.14
NH_4Cl	3.3×10^{-3}	0.14

Assay conditions: for spectrophotometric assay enzyme (0.4 mg protein) was pre-incubated with the cation, 20 µmoles of L-aspartate and 100 µmoles of Tris-HCl at 37°C for 5 min. 10 µmoles of α-ketoglutarate were then added to initiate the reaction. Final volume, 3 ml; final pH 8.0.

Stimulation of enzyme activity by $MnCl_2$ and $MgCl_2$ was also confirmed by measuring ^{14}C-glutamate produced from radioactive α-ketoglutarate. The enzyme reaction was carried out as for the spectrophotometric assay except that 0.3 µc (9 µmoles) of ^{14}C-α-ketoglutarate was added to initiate the reaction. The reaction was allowed to proceed for 30 seconds and then terminated with 4 ml of 4N HCl. The ^{14}C-glutamate was separated on a cation resin column (H^+ form) and counted for radioactivity. The ^{14}C-glutamate produced contained 1770, 2950 and 4330 cpm in the control, $MnCl_2$ and $MgCl_2$ treatments, respectively.

Table 9

Michaelis constants for the substrates of the cytoplasmic aspartate aminotransferase

Substrate concentration not varied (M)	Substrate concentration varied	Km (M) Lineweaver-Burk plot	Km (M) Slope term
L-aspartate 6.7×10^{-3}	α-ketoglutarate	6.7×10^{-4}	7.2×10^{-4}
L-glutamate 6.7×10^{-3}	oxaloacetate	4.8×10^{-5}	4.6×10^{-5}
α-ketoglutarate 3.3×10^{-3}	L-aspartate	8.7×10^{-4}	9.4×10^{-4}
Oxaloacetate 3.3×10^{-4}	L-glutamate	4.4×10^{-3}	4.1×10^{-3}

Assay conditions: enzyme (0.3 - 0.4 mg protein) was pre-incubated with the amino acid and 100 μmoles of Tris-HCl for 5 min. at 37°C. After this pre-incubation, the keto acid was added to initiate the reaction. Final volume, 3 ml; final pH, 8.0.

enzyme from mammalian tissues and cauliflower florets, the enzyme from pea cotyledons displayed a higher apparent affinity for the keto acids. The experimental data also indicates that the enzyme reaction is freely reversible.

7. Double Reciprocal Plots of Initial Reaction Velocities As A Function of One Substrate Concentration at Fixed Concentrations of the Second Substrate

Studies of the mammalian aspartate aminotransferase have indicated that a double reciprocal plot of initial velocity against one substrate concentration at different fixed concentrations of the second substrate yielded parallel lines. To explore this possibility the pea aminotransferase was examined in this way. As illustrated in Figures 7, 8, 9 and 10, the data obtained from such experiments gave parallel lines when plotted in this way. It can be seen that the enzyme has a different Michaelis constant for different fixed concentrations of the second substrate. In the case of the amino acids, the values for aspartic or glutamic acids was higher when the keto acids were at a higher fixed concentration. This can be attributed to the higher inhibitory concentration of the keto acids. However, in the case of the keto acids higher fixed concentrations of the amino acids gave lower Km values.

The parallel lines obtained from the double reciprocal plots are in good agreement with the mathematical relationships derived by Velick and Vavra (1962) for the binary mechanism. Although, these data do not exclude the operation of the ternary

Figure 7

Double reciprocal plot of initial reaction velocities versus α-ketoglutarate concentrations at two fixed concentrations of aspartate.

Assay conditions: aliquots of the cytoplasmic enzyme (0.1 mg protein) were pre-incubated for 10 min at 37°C with 100 μmoles of Tris-HCl and a fixed concentration of L-aspartate as indicated. Various concentrations of α-ketoglutarate were added to initiate the reaction. The final volume was 3 ml and the final pH was 8.0.

Note that at high α-ketoglutarate concentrations the reaction is inhibited as indicated by the upward inflection of the data points.

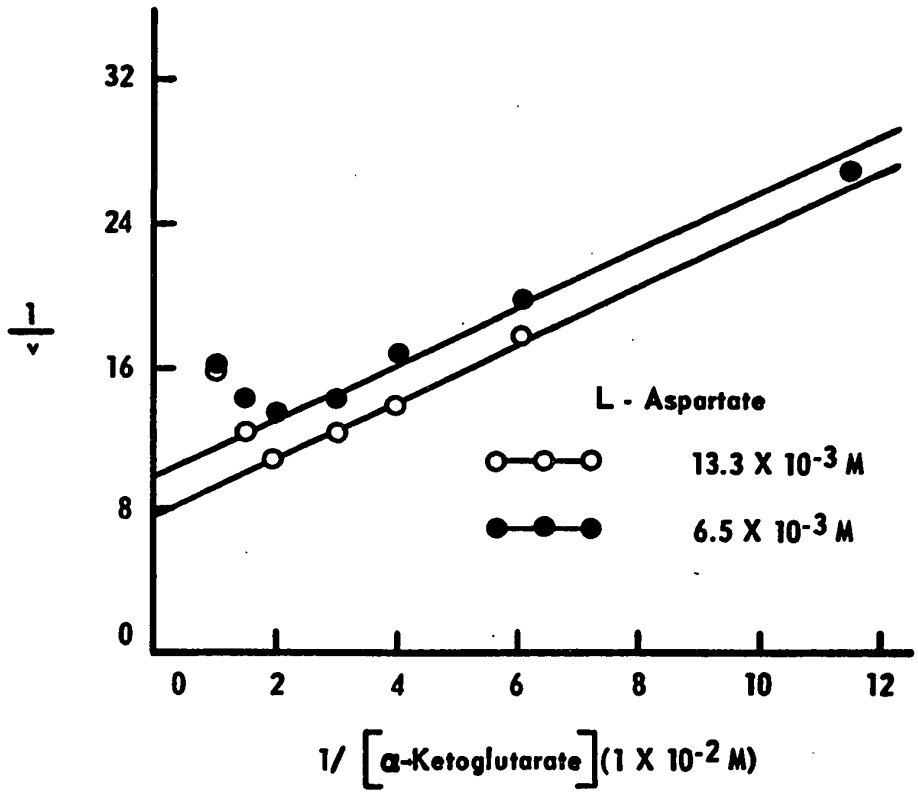

32
24
16
8
0
1/v
0
2
4
6
8
10
12
L - Aspartate
13.3 X 10-3 M
6.5 X 10-3 M
1/ [α-Ketoglutarate] (1 X 10-2 M)

Figure 8

Double reciprocal plot of initial reaction velocities versus oxaloacetate concentration at two fixed concentrations of glutamate.

Assay conditions: aliquots of the cytoplasmic enzyme (0.1 mg protein) were pre-incubated for 10 min. at 37°C with 100 μmoles of Tris-HCl (pH 8.0) and a fixed concentration of L-glutamate as indicated. Various concentrations of oxaloacetate were added to initiate the reaction. The final volume was 3 ml and the final pH was 8.0.

Note that at high OAA concentrations the reaction is inhibited as indicated by the upward inflection of the data points.

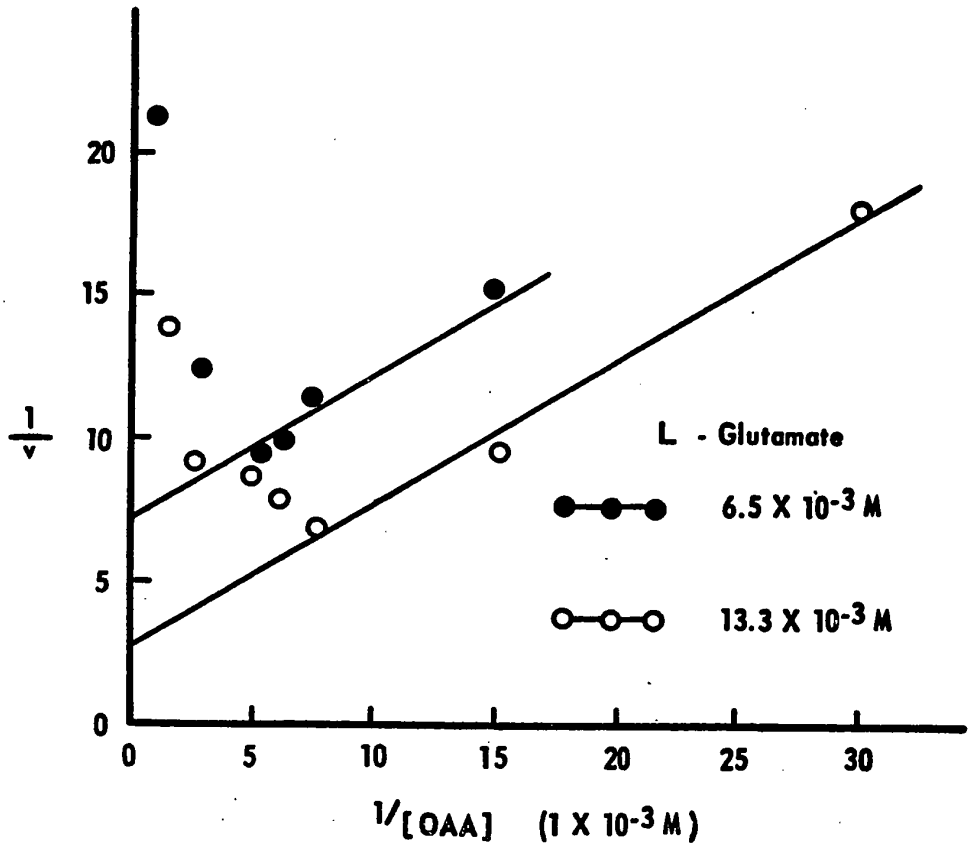

20
15
10
5
0
1/v
0
5
10
15
20
25
30
1/[OAA] (1 X 10-3 M)
L - Glutamate
6.5 X 10-3 M
13.3 X 10-3 M

Figure 9

Double reciprocal plot of initial reaction velocities versus aspartate concentration at two fixed concentrations of α-ketoglutarate.

Assay conditions: aliquots of the cytoplasmic enzyme (0.1 mg protein) were pre-incubated for 10 min at 37°C with 100 μmoles of Tris-HCl and various concentrations of L-aspartate as indicated. α-Ketoglutarate was then added to give a final concentration of 1.7×10^{-3} M and 6.7×10^{-3} M as indicated. The final volume was 3 ml and the final pH was 8.0.

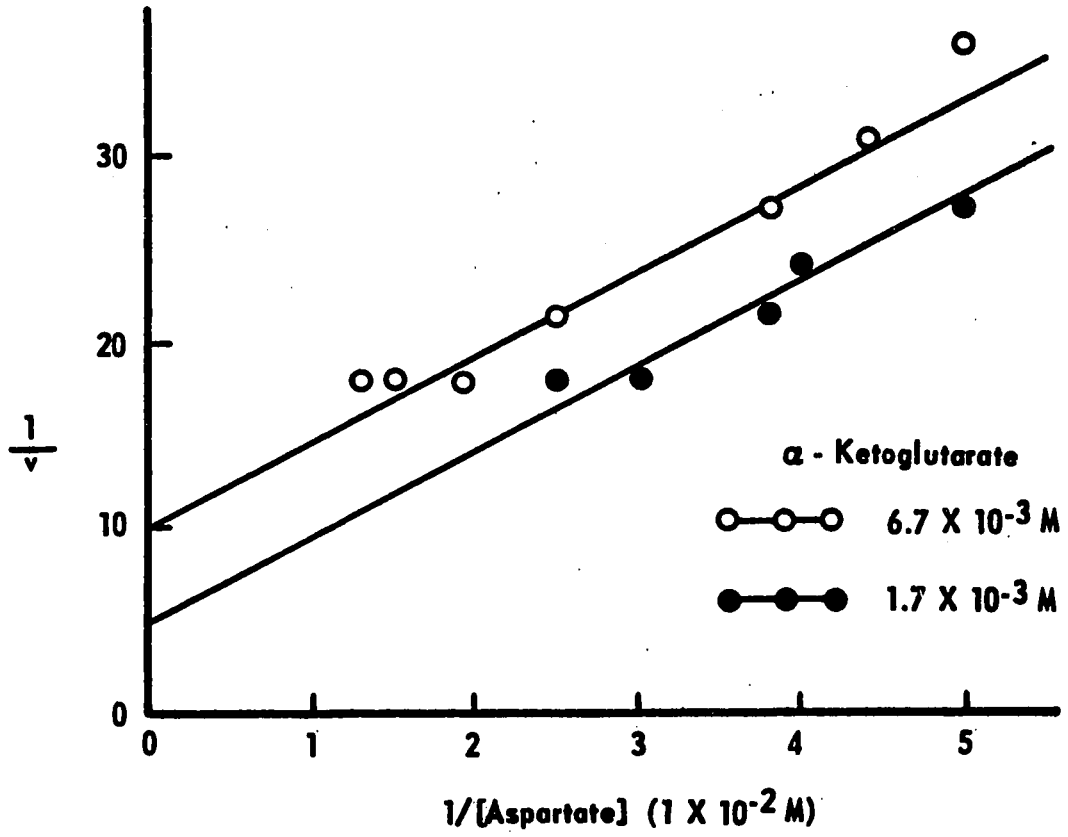
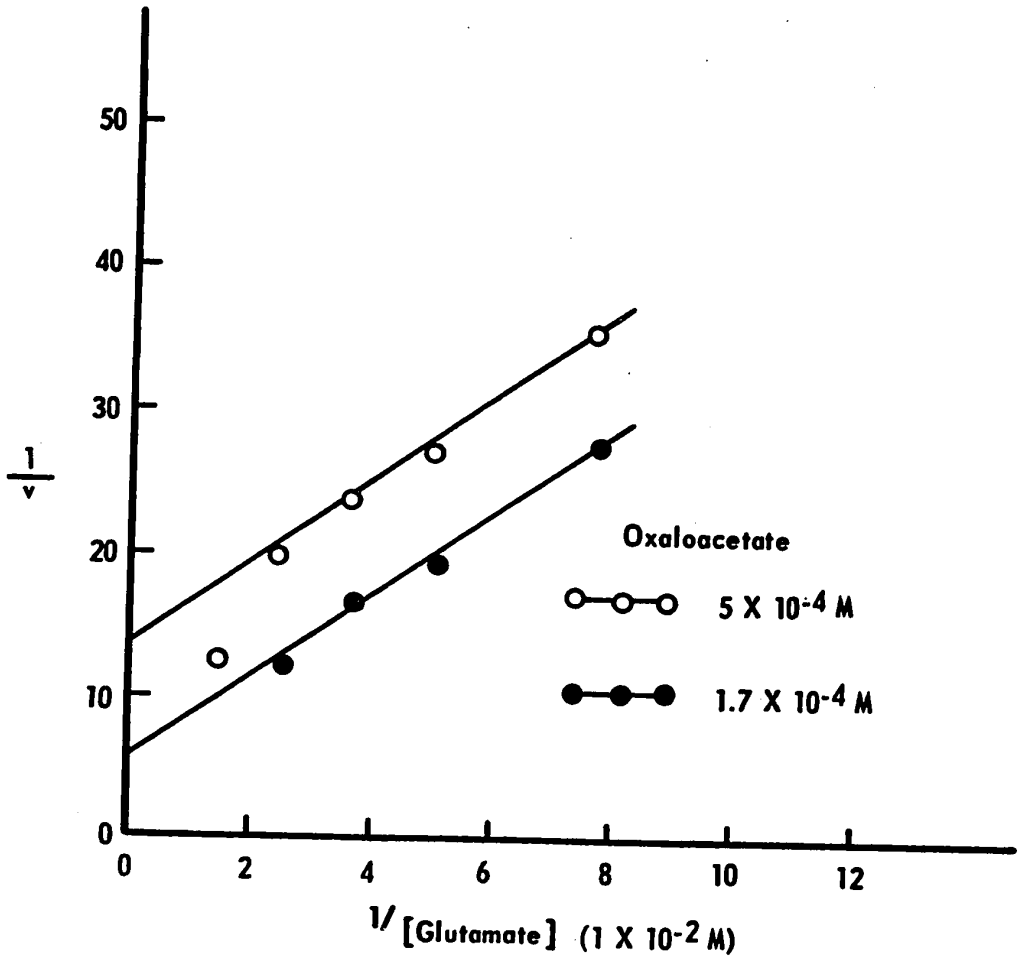

30
20
10
0
1/v
0
1
2
3
4
5
1/[Aspartate] (1 X 10-2 M)
α - Ketoglutarate
6.7 X 10-3 M
1.7 X 10-3 M

Figure 10

Double reciprocal plot of initial reaction velocities versus glutamate concentration at two fixed concentrations of oxaloacetate.

Assay conditions: aliquots of the cytoplasmic enzyme (0.1mg protein) were pre-incubated for 10 min at 37°C with 100 μ moles of Tris-HCl (pH 8.0) and various concentrations of L-glutamate as indicated. Oxaloacetate was then added to give a final concentration of 1.7×10^{-4} M and 5×10^{-4} M as indicated. The final volume was 3 ml and the final pH was 8.0.


50
40
30
20
10
0
1/v
0
2
4
6
8
10
12
1/ [Glutamate] (1 X 10-2 M)
Oxaloacetate
5 X 10-4 M
1.7 X 10-4 M

mechanism, they do, however, suggest that the kinetic behaviour under the experimental conditions can be best explained in terms of the binary mechanism (Banks et al., 1963).

Resolution and Reconstitution of the Cytoplasmic Aspartate Aminotransferase

Since isonicotinic acid hydrazide inhibited aminotransferase activity further experiments were conducted to examine the possible requirement for pyridoxal phosphate and related compounds. Partial and complete resolution and an absolute requirement for pyridoxal or pyridoxamine phosphates is shown in Table 10. When pyridoxal-HCl, pyridoxamine-HCl or pyridoxine phosphate were added to the holoenzyme or to the reconstituted apoenzyme, the amount of product formed was not changed. In addition, when the apoenzyme was incubated with pyridoxal-HCl, pyridoxamine-HCl or pyridoxine phosphate and then dialized thoroughly for 70 hours against 0.005 m Tris-HCl buffer (pH 7.8) pyridoxal or pyridoxamine phosphates were still required for the reaction. However, in the absence of pyridoxal or pyridoxamine 5-phosphates, pyridoxal-HCl, pyridoxamine-HCl and pyridoxine phosphate did not support transamination with the apoenzyme. After resolution and reconstitution of the apoenzyme with pyridoxal phosphate approximately 50 - 60% of the initial enzyme activity was recovered. The reconstituted apoenzyme was stable enough to allow at least two further repeated resolution and reconstitution treatments. The synthetic coenzyme was not dissociated from the

Table 10

The effect of pyridoxal phosphate and chemically related compounds on cytoplasmic aspartate aminotransferase activity

Addition to reaction system	Specific enzyme activity	
	holoenzyme	apoenzyme
none	0.46	0
pyridoxal phosphate	0.50	0.81
pyridoxamine phosphate	0.51	0.81
pyridoxal-HCl	0.50	0
pyridoxine phosphate	0.50	0
pyridoxamine-HCl	0.51	0

Assay conditions: 20 μmoles of L-aspartate, 100 μmoles of Tris-HCl and 50 μg of the vitamin B_6 derivative were incubated with 0.37 mg holoenzyme protein or 0.14 mg apoenzyme protein at 37°C for 5 min. The reaction was then initiated by the addition of 10 μmoles of α-ketoglutarate. Final volume, 3 ml; final pH, 8.0

apoenyzme by prolonged dialysis against distilled water at 4°C for periods of at least 70 hours.

When approximately 0.14 mg of apoprotein was incubated with 50 µg of pyridoxal or pyridoxamine phosphates enzyme activity reached a maximum after 5 to 10 minutes of incubation at 37°C. However, if 10 µg of these derivatives were used, pyridoxal phosphate gave maximal activity after 5 to 10 minutes at 37°C, whereas pyridoxamine phosphate, required 50 minutes in order to reach maximal activity.

Under the defined experimental conditions, the Michaelis constants for pyridoxal phosphate and pyridoxamine phosphate were 5.8×10^{-7}M and 5.2×10^{-7}M, respectively (Table 11). Coenzyme concentrations above saturation were not inhibitory. The similarities in the Michaelis constants for the two coenzymes suggests that the enzyme can be interconverted from the aldehyde to the amine form. Such interconvertibility would add further support for the binary mechanism of enzyme action (Jenkins, 1963; Jenkins and D'Ari, 1966).

As in the case of the holoenzyme, the reconstituted apoenzyme was also inhibited by K_2HPO_4 buffer, iodoacetate, KCN, and p-CMB and it is also stimulated by $MnCl_2$, $CaCl_2$ and $MgCl_2$.

Properties of the Particulate Aspartate Aminotransferase

The enzyme after Sephadex G-200 column chromatography (see Methods) was routinely used for more detailed studies. At this stage of purification the enzyme had a specific activity of 1.1,

Table 11
Michaelis constants for pyridoxamine phosphate and pyridoxal phosphate

Coenzyme	Km (M) (Lineweaver-Burk Plot)
Pyridoxal phosphate	5.8×10^{-7}
Pyridoxamine phosphate	5.3×10^{-7}

Assay conditions: the apoenzyme (0.14 mg protein) was pre-incubated with 20 µmoles of L-aspartate, 100 µmoles of Tris-HCl and the coenzyme at 37°C for 5 min. 10 µmoles of α-ketoglutarate were then added to initiate the reaction. Final volume, 3 ml; final pH, 8.0.

which was 28 times greater than the crude extract.

1. Stability of the Enzyme

When the 10,000 x g particulate fraction was suspended in 0.05 M Tris-HCl (pH 7.8) and kept at 4°C the enzyme remained active without appreciable loss of activity for 2 weeks. After fractionation with $(NH_4)_2SO_4$ and Sephadex G-50 column chromatography the partially purified enzyme in 0.05 M Tris-acetate buffer (pH 6.0) was completely inactivated when stored at -15°C for 48 hours. However, after $(NH_4)_2SO_4$ fractionation and Sephadex G-200 column chromatography the partially purified enzyme in 0.05 M Tris-HCl (pH 7.8) remained active for 2 weeks when stored at 4°C, but lost all of its activity if stored at -15°C for one week.

2. Effects of pH

The relationship between pH and enzyme activity is shown in Figure 11. The enzyme was inactive at pH below 4.5. Maximum activity was observed from pH 6.8 to 8.5, but then decreased at higher pH values. This range of pH for optimal enzyme activity is similar to that for the mitochondrial enzyme of beef liver (Morino and Wada, 1963).

3. Effects of Inhibitors and Activators

Since certain sulfhydryl reagents were inhibitory to the cytoplasmic enzyme these reagents were also tested with the particulate aspartate aminotransferase. As indicated in Table 12,

Figure 11

Effects of pH on particulate aspartate aminotransferase activity.

Assay conditions: aliquots of the enzyme (0.14 mg protein) were pre-incubated with 10 µg of pyridoxal phosphate, 40 µmoles of L-aspartate and 133 µmoles of Tris-acetate buffer (pH as indicated) at 37°C for 10 min. 10 µmoles of α-ketoglutarate were then added to initiate the reaction. Each pH value was determined after the reaction had proceeded for 3 min. The final volume was 3 ml.

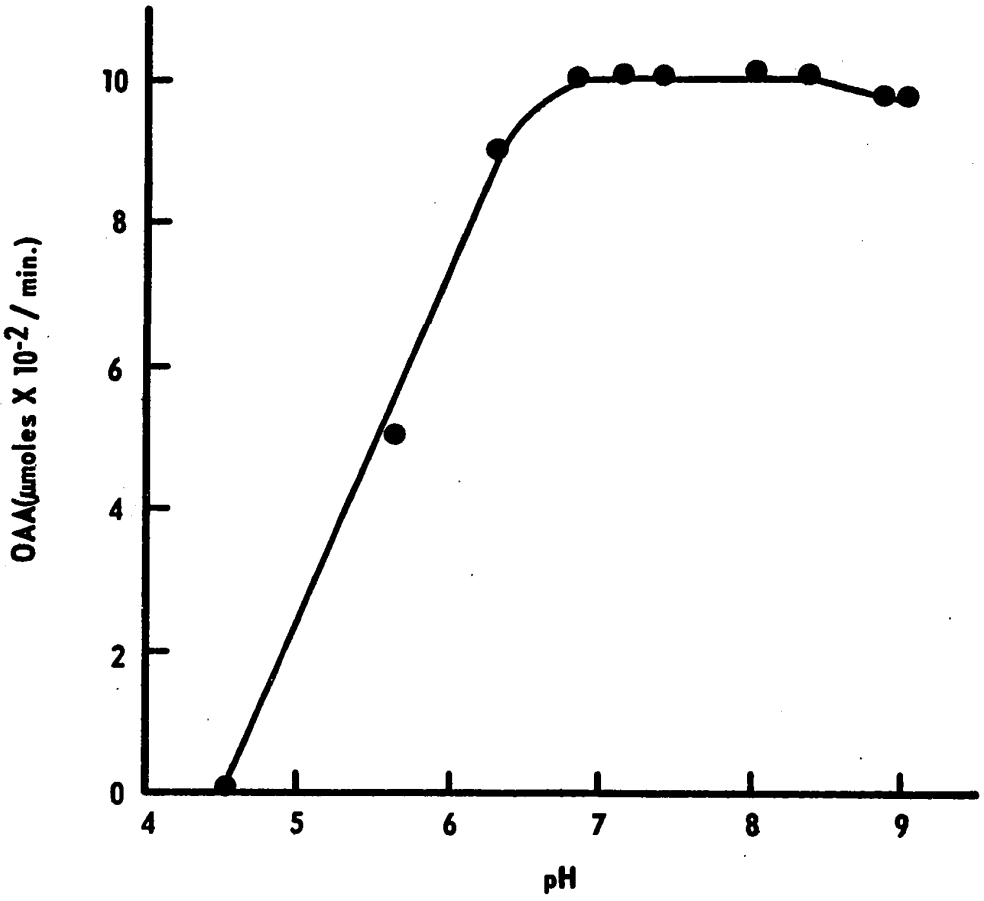

OAA(μmoles X 10-2 / min.)
10
8
6
4
2
0
4
5
6
7
8
9
pH

Table 12
Effects of various inhibitors on particulate aspartate aminotransferase activity

Inhibitor	Concentration (M)	Inhibition (%)
KCN	3.3×10^{-3}	100
Isonicotinic acid hydrazide	3.3×10^{-3}	100
Iodoacetate	3.3×10^{-3}	40
Iodoacetamide	3.3×10^{-3}	40
p-CMB	1.9×10^{-4}	40

Assay conditions: aliquots of the enzyme (0.05 mg protein) were pre-incubated with the inhibitors and 100 µ moles of Tris-HCl at 37°C for 10 min. 40 µmoles of L-aspartate were then added and the mixture incubated for a further 10 min. at 37°C. After this, 10 µmoles of α-ketoglutarate were added to initiate the reaction. Final volume, 3 ml; final pH, 8.0.

iodoacetate, iodoacetamide and p-CMB inhibited enzyme activity by 40%. In addition, KCN and isonicotinic acid hydrazide at concentrations of 3.3×10^{-3}M completely inhibited the reaction. As in the case with the cytoplasmic enzyme the particulate aminotransferase was stimulated by $MnCl_2$, $CaCl_2$ and $MgCl_2$. Monovalent cations such as Na^+, K^+, and NH_4^+ had no effect (Table 13). Pyridoxal phosphate was omitted from these reaction mixtures for the reasons given earlier.

In common with the cytoplasmic enzyme, the particulate aminotransferase was also inhibited by high concentrations of oxaloacetate (Figure 12). However, unlike the cytoplasmic enzyme, the particulate aspartate aminotransferase was not inhibited by α-ketoglutarate, even at concentration of 6.7×10^{-3} M.

4. Michaelis Constants

The apparent Michaelis constants for the four substrates were determined from Lineweaver-Burk plots and from the slope term. These data are shown in Table 14. The particulate aminotransferase displayed a higher apparent affinity for the keto acids than the amino acids. Similar data was obtained for the cytoplasmic enzyme (Table 9). Both enzymes appeared to have a greater apparent affinity for oxaloacetate than for α-ketoglutarate.

5. Double Reciprocol Plots of Initial Reaction Velocities as A Function of One Substrate Concentration at Fixed Concentrations of the Second Substrate

Table 13
Effects of various cations on particulate aspartate aminotransferase activity

Addition	Final concentration (M)	Enzyme activity (μ mole OAA/min)
none		0.05
$CaCl_2$	3.3×10^{-3}	0.08
$MgCl_2$	3.3×10^{-3}	0.06
$MnCl_2$	3.3×10^{-3}	0.14
NaCl	3.3×10^{-3}	0.05
KCl	3.3×10^{-3}	0.05
NH_4Cl	3.3×10^{-3}	0.05

Assay conditions: aliquots of the enzyme (0.05 mg protein) were pre-incubated with 100 μ moles of Tris-HCl, 40 μ moles of L-aspartate and the cation at 37°C for 10 min. 10 μ moles of α-ketoglutarate were then added to initiate the reaction. Final volume, 3 ml; final pH, 8.0.

Figure 12

Effects of varying oxaloacetate concentrations on particulate aspartate aminotransferase activity.

Assay conditions: samples of the enzyme (0.08 mg protein) were pre-incubated with 100 μmoles of Tris-HCl (pH 8.0) and 40 μmoles of L-glutamate at 37°C for 10 min. Oxaloacetate was then added to initiate the reaction. The reference cuvette contained all of these components with the exception of oxaloacetate which was replaced by distilled water. The final volume was 3 ml and the final pH was 8.0.

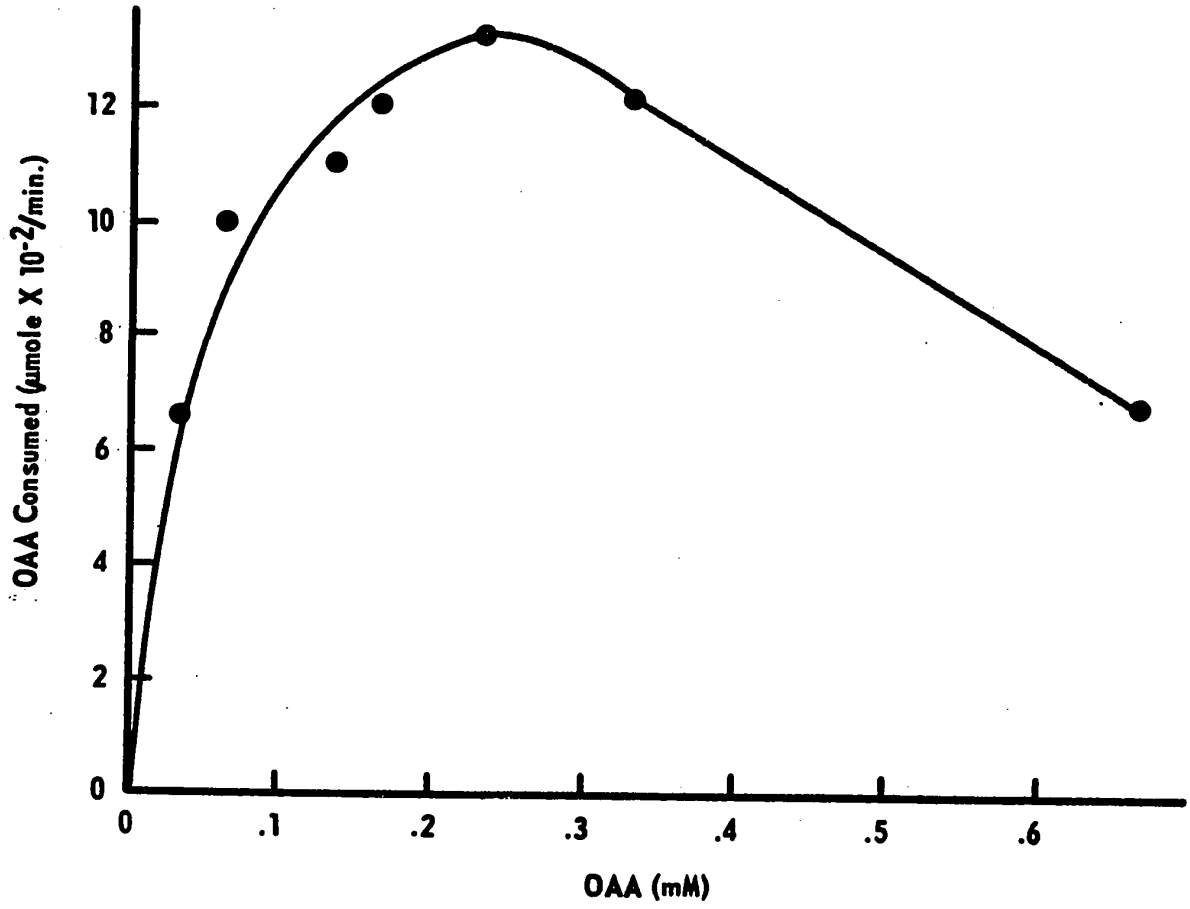

OAA Consumed (μmole X 10-2/min.)
12
10
8
6
4
2
0
0
.1
.2
.3
.4
.5
.6
OAA (mM)

Table 14

Michaelis constants for the substrates of the particulate aspartate aminotransferase

Substrate concentration not varied (M)	Substrate concentration varied	Km (M) Lineweaver-Burk plot	Km (M) Slope term
L-aspartate 13.4×10^{-3}	α-ketoglutarate	7.3×10^{-4}	7.7×10^{-4}
L-glutamate 13.4×10^{-3}	oxaloacetate	4.5×10^{-5}	4.3×10^{-5}
α-ketoglutarate 3.3×10^{-3}	L-aspartate	1.6×10^{-3}	1.2×10^{-3}
oxaloacetate 0.33×10^{-3}	L-glutamate	6.4×10^{-3}	6.6×10^{-3}

Assay conditions: enzyme (0.08 mg protein) was pre-incubated with 100 μmoles of Tris-HCl and the amino acid at 37°C for 10 min. The keto acid was then added to initiate the reaction. Final volume, 3ml; final pH, 8.0.

When the reciprocals of initial velocities were plotted against the reciprocals of substrate concentration at fixed concentrations of the second substrate (Figure 13, 14, 15 and 16) parallel lines were obtained. These results are, therefore, indicative of a binary mechanism for the reaction catalyzed by the particulate enzyme.

The enzyme showed a lower apparent affinity for L-aspartate, oxaloacetate and α-ketoglutarate when the fixed concentrations of α-ketoglutarate, L-gluamate and L-asparate, respectively were increased (Figures 13, 14, 15 and 16). However, where L-glutamate concentrations were varied increases in the fixed oxaloacetate concentration increased the apparent affinity of the enzyme for L-glutamate (Figure 15). The reason for such an effect is that oxaloacetate, at a concentration of 5×10^{-4}M was found to be inhibitory.

Heterogeneity of the 10,000 x g Preparations

The twice washed 10,000 x g preparations were examined under the electron microscope after negative staining with phosphotungstic acid. Scanning of these preparations under the electron microscope revealed particles resembling mitochondria and other subcellular bodies. Unfortunately clear photographs of these particles were not obtained despite several exposures at different magnifications.

The heterogeneous nature of the preparation was also evident after centrifugation on a sucrose density gradient. A discontinuous sucrose gradient consisting of 5 ml of 60%, 10 ml of 45% and 15 ml of 32% was used. Aliquots of the 10,000 x g fraction, containing approximately 25 mg of protein, were carefully layered onto the gradient and centrifuged at 46,000 x g for 4 hr. in an

Figure 13

Double reciprocal plot of initial reaction velocities versus α-ketoglutarate concentration at two fixed concentrations of aspartate.

Assay conditions: aliquots of the particulate enzyme (0.1 mg protein) were pre-incubated for 10 min at 37°C with 100 μ moles of Tris-HCl (pH 8.0) and a fixed concentration of L-aspartate as indicated. Various concentrations of α-ketoglutarate were added to initiate the reaction. The final volume was 3 ml and the final pH was 8.0.

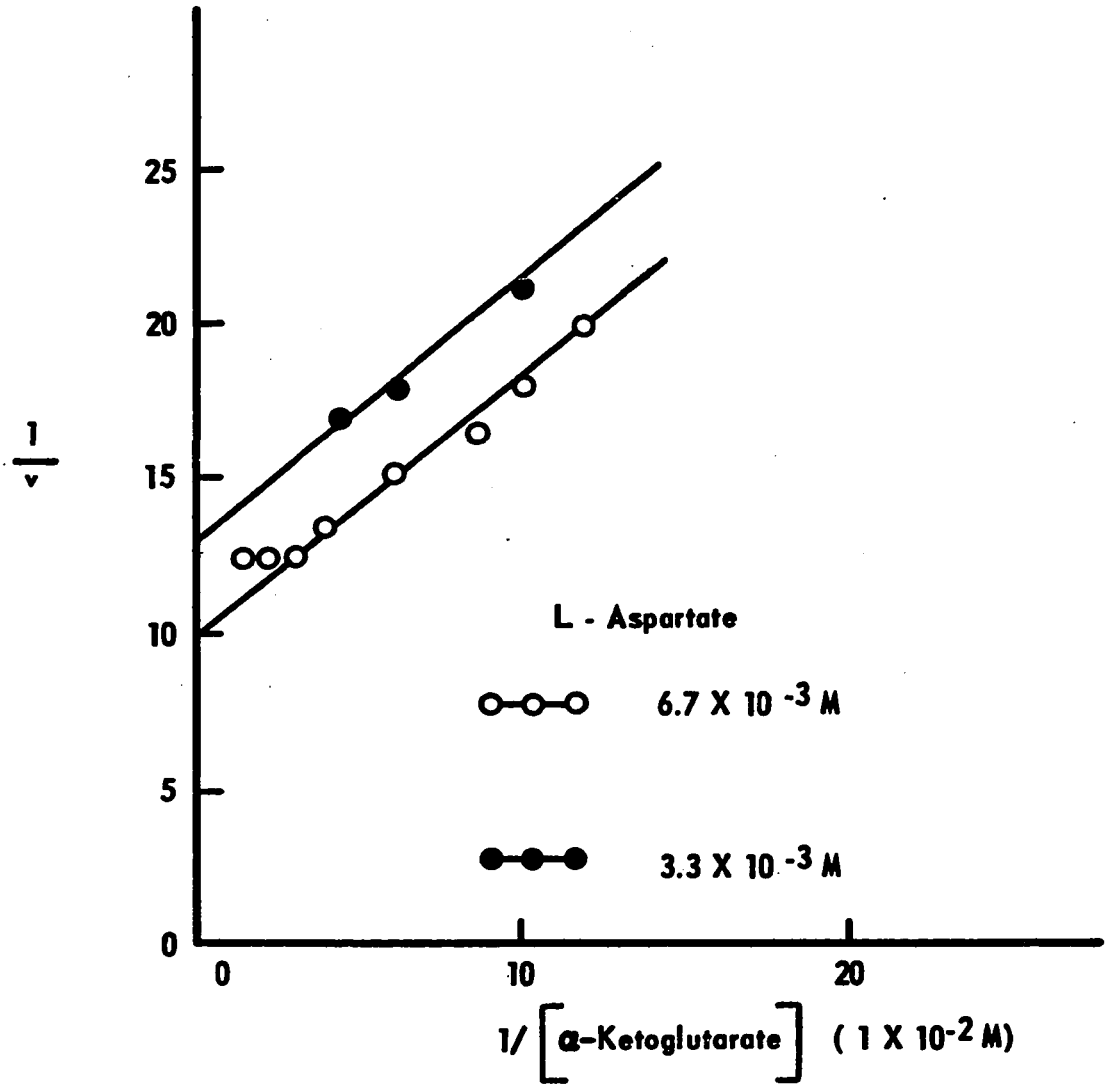

25
20
15
10
5
0
1/v
L - Aspartate
6.7 X 10 -3 M
3.3 X 10 -3 M
0
10
20
1/ [α-Ketoglutarate] (1 X 10-2 M)

Figure 14

Double reciprocal plot of initial reaction velocities versus oxaloacetate concentration at two fixed concentrations of glutamate.

Assay conditions: aliquots of the particulate enzyme (0.1 mg protein) were pre-incubated for 10 min at 37°C with 100 μ moles of Tris-HCl (pH 8.0) and a fixed concentration of L-glutamate as indicated. Various concentrations of oxaloacetate were added to initiate the reaction. The final volume was 3 ml and the final pH was 8.0.

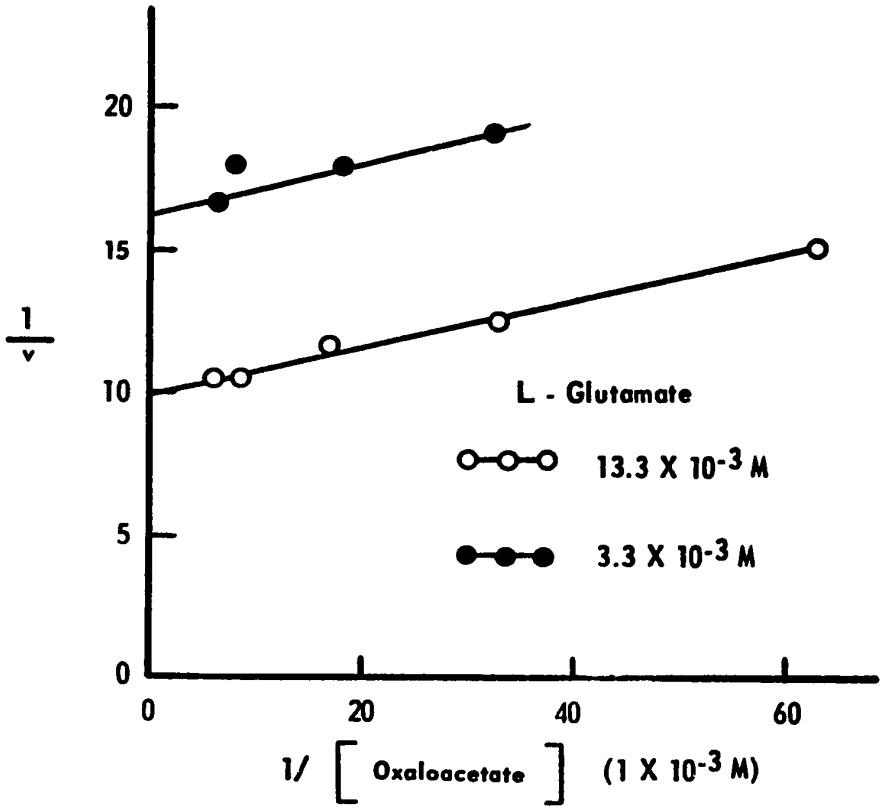

20
15
10
5
0
1/v
L - Glutamate
13.3 X 10-3 M
3.3 X 10-3 M
0
20
40
60
1/ [Oxaloacetate] (1 X 10-3 M)

Figure 15

Double reciprocal plot of initial reaction velocities versus glutamate concentration at two fixed concentrations of oxaloacetate.

Assay condition: aliquots of the particulate enzyme (0.1mg protein) were pre-incubated for 10 min at 37°C with 100 μ moles of Tris-HCl (pH 8.0) and various concentrations of L-glutamate as indicated. Oxaloacetate was then added to give a final concentration of 1.7×10^{-4} M and 5.0×10^{-4} M as indicated. The final volume was 3 ml and the final pH was 8.0.

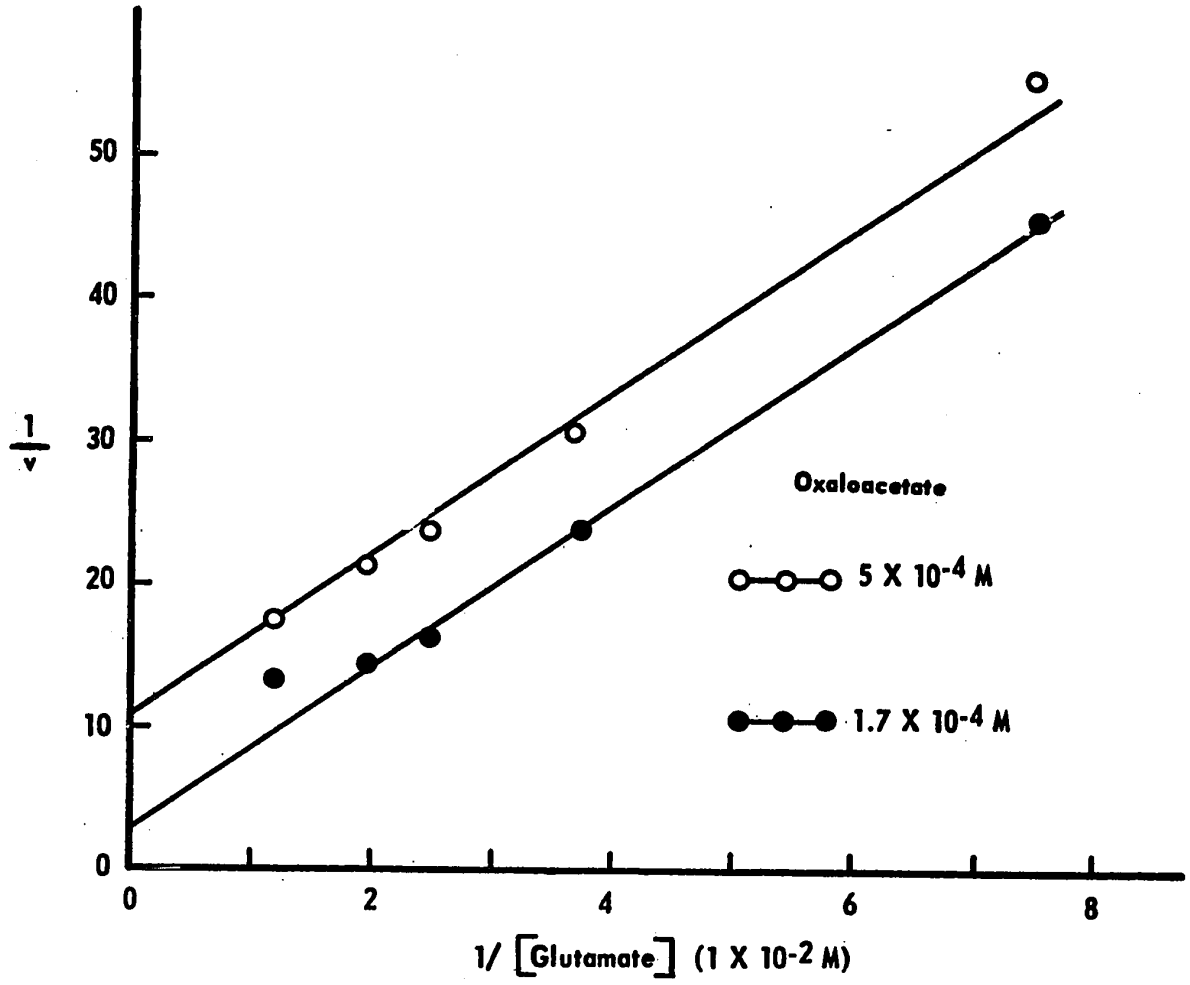
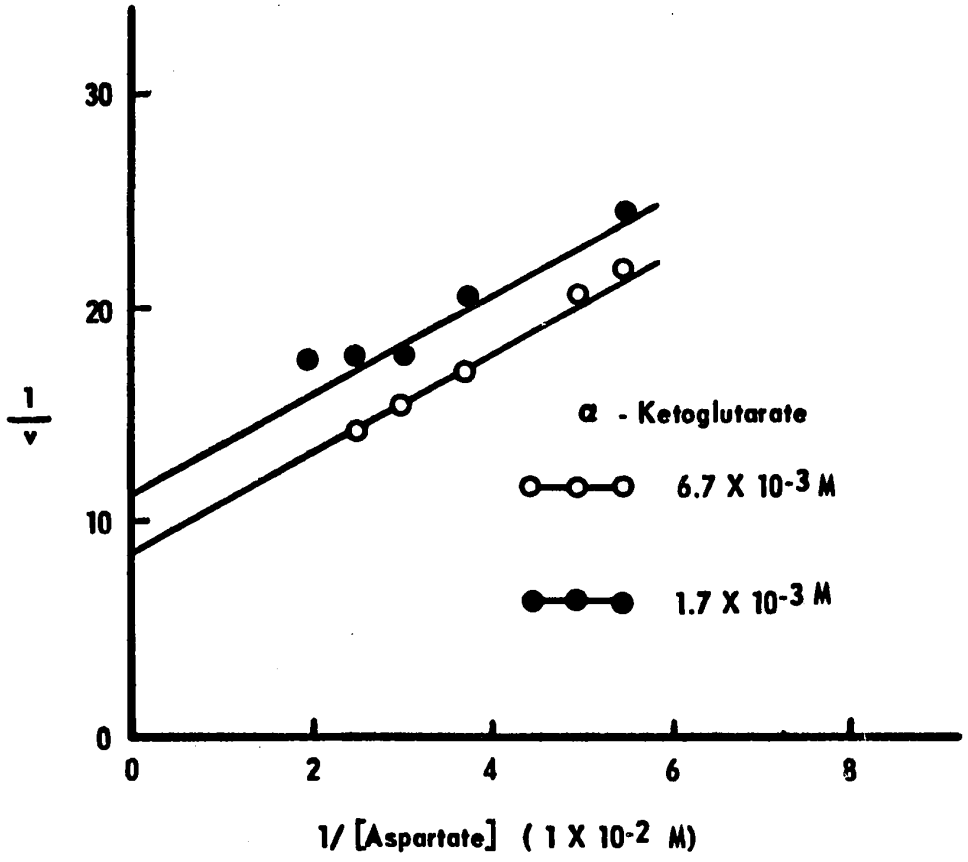

50
40
1/v
30
20
10
0
0
2
4
6
8
Oxaloacetate
5 X 10-4 M
1.7 X 10-4 M
1/ [Glutamate] (1 X 10-2 M)

Figure 16

Double reciprocal plot of initial reaction velocities versus aspartate concentration at two fixed concentrations of α-ketoglutarate.

Assay conditions: aliquots of the particulate enzyme (0.1 mg protein) were pre-incubated for 10 min at 37°C with 100 μmoles of Tris-HCl (pH 8.0) and various concentrations of L-aspartate as indicated. α-Ketoglutarate was then added to give a final concentration of 1.7×10^{-3} M or 6.7×10^{-3} M as indicated. The final volume was 3 ml and the final pH was 8.0.


30
20
10
0
1/v
α - Ketoglutarate
6.7 X 10-3 M
1.7 X 10-3 M
0
2
4
6
8
1/ [Aspartate] (1 X 10-2 M)

International refrigerated centrifuge (model B-20). After such treatment four distinct bands of material were observed (Figure 17). The contents of each band were then carefully removed by suction. The four samples collected in this manner were then assayed for aspartate aminotransferase activity after being suspended in the pre-incubation reaction mixture for 30 min. at 37°C as described in the Materials and Methods section. Enzyme activity was observed in material collected from bands A and D.

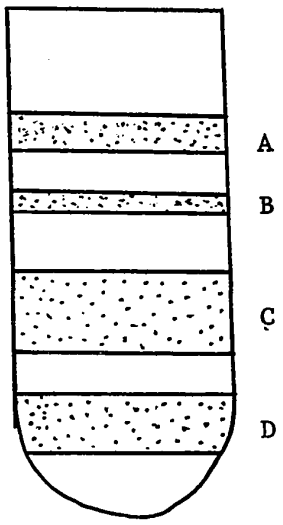


Figure 17

Sucrose density gradient pattern of crude 10,000 x g fraction.

Samples of the 10,000 x g fraction were centrifuged on a sucrose density gradient and assayed for aspartate aminotransferase activity as described on page 80 .

GENERAL DISCUSSION

Although much of the experimental data has been discussed in the appropriate section of this thesis, the present section is devoted to a broader discussion of the enzyme and its possible involvement in the nitrogen metabolism of plant tissues.

Evidence for Cytoplasmic and Particulate Aspartate Aminotransferase

The present investigation has indicated that aspartate aminotransferase activity is present in both cytoplasmic fraction and the 10,000 x g fraction of germinating pea cotyledons. Although the 10,000 x g fraction was not characterized, the speed at which the fraction was sedimented from the isotonic medium is in the range used by other workers for the isolation of plant mitochondria (Ikuma and Bonner, 1967; Olson and Spencer, 1967; Stinson and Spencer, 1967). On the basis of electron microscopy and sucrose density gradient studies it is clear that this aminotransferase may be associated with more than one type of subcellular particle in pea cotyledons. Alternatively aminotransferase activity may be associated with particles having similar morphological characteristics but which show different degrees of sedimentation on the sucrose density gradients used in this study. Further studies will have to be conducted in order to determine the morphological and biochemical nature of these particles. Since completion of the present work several reports have appeared describing similar heterogeneity of mitochondrial preparations (Breidenbach and Beevers, 1967; Yamasaki and Tolbert, 1968). It is clear from these latest studies that mitochondria, peroxisomes and glyoxysomes have

characteristic enzymes and by implication have distinct metabolic roles in the tissues in which they occur.

Although the particulate fraction was not purified it is unlikely that the presence of aspartate aminotransferase activity in this fraction was simply due to contamination from the cytoplasmic enzyme. Support for this conclusion comes from a consideration of the methods used to prepare the particulate fraction, to extract enzyme activity from it and a comparison of the kinetic properties of the two enzymes.

When the twice-washed 10,000 x g fraction was suspended in the wash medium and an aliquot of this was assayed, very little enzyme activity was detected even after three minutes of reaction. However, if the particulate fraction was suspended in 0.05 M Tris-HCl and then assayed, enzyme activity was recorded. Furthermore, the increase in enzyme activity after high speed blending and sonication, also, the solubilization of the enzyme after deoxycholate treatment indicate that the enzyme must be membrane bound.

A notable difference between the cytoplasmic enzyme and the particulate enzyme was their stability under similar experimental conditions. The cytoplasmic aminotransferase was exceedingly stable whereas the particulate aminotransferase was relatively unstable. It is possible that the extraction of the particulate enzyme from its associated membranes results in enzyme molecules which are rapidly denatured in solution.

The two aspartate aminotransferases in pea cotyledons also differ in certain of their kinetic properties. As illustrated in Figures 5 and 11, the two enzymes are affected by pH in a somewhat different way. These differences are also characteristic of the cytoplasmic and mitochondrial aminotransferases of beef liver. This difference may be related to structural differences in the protein molecules.

On the basis of the Michaelis constants the decreasing order of affinity for the two aspartate aminotransferases was found to be oxaloacetate, α- ketoglutarate, aspartate and glutamate. This is similar to that displayed by the enzymes from beef liver (Morino, Itoh and Wada, 1963). However, when comparing the apparent affinity for each substrate the notable difference between the two enzymes is their affinity for aspartic and glutamic acids. The cytoplasmic enzyme has a higher apparent affinity for the two amino acids than does the particulate enzyme. Although the cytoplasmic and the particulate enzymes displayed different affinities for the 4 substrates these differences are very small. Henson and Cleland (1964) arrived at the same conclusion from their studies on the aspartate aminotransferase isoenzymes from pig heart.

The experiments summarized in Figures 6 and 12 show that at high oxaloacetate concentrations both enzymes were inhibited. In addition the cytoplasmic aspartate aminotransferase was inhibited by high concentrations of α- ketoglutarate . This keto acid did not inhibit the particulate enzyme at a comparable concentration. As suggested for the mammlian enzyme (Velick and Vavra, 1962) the two

keto acids must form abortive complexes with the cytoplasmic enzyme, thereby inhibiting enzyme activity. Such inhibitory effects have been interpreted as indicating that the substrates occupy the same catalytic site one at a time in sequence. The inhibitory effect of high oxaloacetate and α- ketoglutarate concentrations has also been observed in studies with an aspartate aminotransferase purified from cauliflower by Davies and Ellis (1961).

Recent work by Bhargava and Sreenivasan (1968) on the mitochondrial aspartate aminotransferases of rat liver have drawn attention to the existence of two forms of this enzyme. They found that considerable enzyme activity was only released when mitochondrial preparations were treated with n-butanol. This released activity had an electrophoretic mobility which was distinct from that shown by enzyme protein released from the mitochondria by ultrasonic treatment. These workers concluded that the two forms of aspartate aminotransferase are probably proteins having different degrees of membrane binding. Although this point was not examined extensively in the present work some evidence for differences in release of enzyme activity from the particulate fraction were observed (Page 49). For example when the particulate fraction was suspended in 0.05 M Tris-HCl (pH 7.8) for 15 hr. at 4°C some enzyme activity was solubilized but considerable amounts of activity were still associated with material sedimented at 30,000 x g. This latter activity could be released only partially by a subsequent ultrasonic treatment but more extensively by treatment with deoxycholate. Clearly extensive studies

are required on these fractions before they can be characterized as mitochondrial isoenzymes.

Metabolic Significance of Aspartate Aminotransferase

Studies with germinating pea cotyledons (Larson and Beevers, 1965) indicated that while the protein content declined, there was a concomitant increase in free amino acids. During these early stages of germination the predominant amino acids were glutamate and homoserine. The aspartic acid content showed a small increase. From these studies it can be concluded that the increase in free amino acid content is mainly due to the hydrolysis of storage protein.

Studies with ^{14}C labeled glutamate (Larson and Beevers, 1965) showed that this amino acid was incorporated into homoserine in germinating pea cotyledons. The ^{14}C-glutamate appeared to be deaminated giving rise to α-ketoglutarate which was then cycled through the TCA cycle with the production of oxaloacetate. This oxaloacetate would then be transaminated resulting in the labeling of aspartic acid which would give rise to labeled homoserine. In this connection aspartate aminotransferase could be of importance in the transamination of oxaloacetate.

In the majority of plant tissues the main source of nitrogen is nitrate. However before this nitrate can be utilized it must be reduced to ammonia. The sequence of reactions involved in such a reduction is not clearly established, but it appears that nitrate is reduced to nitrite and finally to ammonia by a pathway involving postulated intermediates. It is now known that the reductive amination of

α-ketoglutaric acid followed by transamination reactions is the major pathway for the synthesis of several amino acids in plants. In this connection, the TCA cycle plays an important role in supplying α-ketoglutarate for the amination reaction. As glutamic acid is a well known reactant in many different reactions (Fowden, 1965) it is clear that this amino acid is important for the synthesis of many different amino acids. Thus glutamate dehydrogenase occupies a central position in the nitrogen metabolism of plant tissues undergoing growth. In pea cotyledons on the other hand where mobilization of storage reserves is prominent, glutamate dehydrogenase is apparently of less importance as compared to the major roles assumed by various amino-transferases. These enzymes would be active in catalyzing extensive interconversion of protein amino acids and would also produce keto acids. The latter compounds would then be utilized through established pathways of intermediary metabolism.

In pea seedlings carbon dioxide fixation has been shown to occur in both the cytoplasmic and the mitochondrial fractions (Mazelis and Vennesland, 1957). The amount of oxaloacetate produced in the mitochondria could control the operation of the TCA cycle. It is known that high concentrations of oxaloacetate in mammalian and plant mitochondria will inhibit succinate oxidation (Lehninger, 1965; Bone and Fowden, 1960). However mitochondrial oxaloacetate could be removed by the operation of the cycle and by transamination with glutamate. The presence of aspartate aminotransferase in the mitochondria would suggest a possible role for this enzyme in the removal of oxaloacetate.

Such a role had been proposed for the aspartate aminotransferase of rat liver mitochondria (Lardy, Paetkau and Walter, 1965). This possibility directs attention to many interesting areas for further research.

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