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INVESTIGATIONS OF AMINO ACID METABOLISM IN PHASEOLUS
VULGARIS L. IN RELATION TO THE DEVELOPMENT OF TRANSAMINASE
ACTIVITY DURING GERMINATION AND THE PROPERTIES OF AN
AROMATIC AMINOTRANSFERASE PURIFIED FROM ROOTS.

by

JEAN CLAUDE FOREST, B. Sc. (Agr.)

A thesis submitted to the Faculty of Graduate
studies in partial fulfilment of the requirements
for the degree of Doctor of Philosophy.

Carleton University

Ottawa, Ontario

April, 1971.

c. Jean-Claude Forest, 1971

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ABSTRACT

The total nitrogen content of Phaseolus vulgaris L. seedlings grown under light or dark conditions was found to remain relatively constant during the first 2 weeks of growth, but there was considerable redistribution of nitrogen from cotyledons to the seedling tissues. Appreciable changes also occurred in the ratio between insoluble and soluble nitrogen in the different organs. The quantitative composition of the soluble nitrogen fraction, with respect to free protein amino acids, was found to differ considerably, not only between seedlings at different stages of growth, but also between organs taken from seedlings at the same stage of growth under the two light conditions. Asparagine, serine, valine, alanine and threonine were the amino acids that showed the greatest change in amounts in both types of seedlings.

The development of six aliphatic transaminase activities and of three aromatic transaminases activities was investigated in the different organs of both light- and dark-grown seedlings during the first 2 weeks of growth. Of the six aliphatic transaminase activities examined, only aspartate, alanine and asparagine aminotransferases showed high total activity. Valine aminotransferase exhibited low activity, and a serine and threonine aminotransferase could not be detected. The development of all three aromatic amino acid transaminase activities followed a very similar pattern in the different organs of seedlings grown under each experimental condition. No correlation was observed between development of the different transaminase activities and changes in concentrations of the corresponding free amino acids in these seedlings.

The intracellular distribution of aromatic aminotransferase and aspartate aminotransferase activities was investigated using roots from 8-day old etiolated seedlings. Only about 10% of these activities was found in the particulate fractions, the mitochondrial fraction containing only 4% of the total activity. On the other hand the activity of the soluble enzyme in the high-speed supernatant fraction accounted for about 90% of the total activity. The soluble aromatic aminotransferase in seedling roots was purified 570-620-fold by pH precipitation, ammonium sulfate fractionation and Sephadex chromatography and the recovery was 30-35% based on total activity. It was observed that the specific activity for aspartate- α -ketoglutarate transaminase increased proportionally to the increase in aromatic aminotransferase activities during the different steps of purification. Gel electrophoresis of the purified enzyme fraction revealed only one major protein band when stained with Amido Black, and this band corresponded to the product specific stained bands for the three aromatic aminotransferase activities assayed on other gels.

The molecular weight of the aromatic aminotransferase was found to be about 128,000. The pH optimum for the three aromatic amino acid- α -ketoglutarate and aspartate- α -ketoglutarate transaminase activities was found to be 8.5. No specific requirement for pyridoxal phosphate was detected with the purified enzyme. The enzyme was, however, inhibited by 45% by antagonists of pyridoxal phosphate, such as isonicotinic acid hydrazide, and this result suggests the presence of pyridoxal phosphate bound to the enzyme. Cations such as Ca^{++} and Mg^{++} enhanced the aromatic aminotransferase and aspartate aminotransferase activities by about 15%. Other cations such as Mn^{++} , Cu^{++} and Fe^{++}

were found to be without effect at a concentration of 3×10^{-3} M, while Zn^{++} slightly inhibited the transamination reactions tested.

The purified aromatic aminotransferase was found to exhibit rather broad substrate specificity. When α -ketoglutarate was used as amino group acceptor, the three aromatic amino acids were transaminated at a rate 10 to 15-fold less than that of aspartate. Oxaloacetate was also found to be a good amino group acceptor with the enzyme, while the aromatic keto acids were 10 to 15-fold less effective than α -ketoglutarate when aspartate was provided as the amino group donor. Pyruvate and glyoxylate and their corresponding amino acids did not serve as substrates for this aromatic aminotransferase. None of the aromatic compounds tested as possible effectors of the purified enzyme were found to have a marked effect on the phenylalanine aminotransferase activity.

The enzyme showed rather similar affinity for all four aliphatic substrates, aspartate, glutamate, α -ketoglutarate and oxaloacetate. Their apparent Michaelis constants were all found to be in the range of 2.7 to 3.1×10^{-2} M. When α -ketoglutarate was used as the amino group acceptor, the K_m value was 5.5×10^{-2} M for phenylalanine, 7.3×10^{-2} M for tyrosine and 7.7×10^{-2} M for tryptophan. When glutamate was used as the amino group donor, the K_m was 5.2×10^{-2} M for phenylpyruvate, 6.3×10^{-2} M for p-hydroxyphenylpyruvate, and 6.7×10^{-2} M for 3-indolepyruvate. When any of the aromatic amino acids was used as amino group donor, the K_m for α -ketoglutarate was 1.0×10^{-3} M and when any of the aromatic keto acids was used as amino group acceptor the K_m for glutamate was 5.0×10^{-3} M. The affinity of the enzyme for a keto acid substrate was thus found to be always slightly

higher than its affinity for the corresponding amino acid substrate.

The results obtained in this investigation have shown that all four transaminase activities studied were catalysed by a purified protein fraction isolated from bushbean roots, which was found to contain only one protein when analysed by polyacrylamide gel electrophoresis. As this protein band coincided with the product specific staining band for aromatic aminotransferase activity and since the aliphatic substrate, aspartate, competitively inhibited the phenylalanine- α -ketoglutarate transaminase reaction, it is concluded that the same enzyme is responsible for catalyzing the aromatic amino acid- α -ketoglutarate and the aspartate- α -ketoglutarate transaminase reactions in roots of bushbean seedlings.

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INTRODUCTION

A major biochemical event occurring during seed germination is the hydrolysis of food reserves in seed storage tissues and the translocation of the soluble metabolites to the growing embryo. An important aspect of these events is the breakdown of seed proteins to their constituent amino acids, which then may be further metabolized or transported to the growing embryo to sustain the synthesis of new protein. The metabolism of these amino acids, and in particular the formation of new amino acids by the process of transamination, leadsto the proper array of amino acids necessary for protein synthesis (Wilson, King and Burris, 1954; Boulter and Barber, 1963; Larson and Beevers, 1965).

The metabolism of nitrogen compounds during seed germination has already received extensive investigation (Chibnall, 1939; Street 1949; McKee, 1962; Mayer and Poljakoff-Maber, 1963), but relatively little quantitative work has been done to compare the changes occurring in light and dark-grown seedlings during the early stages of development, especially in relation to changes in the activity of enzymes concerned with the metabolism of amino acids. Furthermore, little information is available, as yet, to indicate the number of different transaminases operating in a single plant species, and this is coupled with a general lack of knowledge concerning the specificity of these enzymes for their keto-and amino-acid substrates. To date, only two transaminase enzymes have been well studied from the plant kingdom. Cruickshank and Isherwood (1958) were the first to report evidence for the presence of separate enzymes responsible for the glutamate-oxaloacetate and glutamate-pyruvate transaminase activities in

wheat germ, and these enzymes have now been purified and further studied by a number of workers (Ellis and Davies, 1961; Davies and Ellis, 1961; Fasella, Bossa, Turano and Fancelli, 1966; Wong and Cossins, 1969). Another transaminase system, the aromatic aminotransferases, has been investigated by a number of workers but no definitive answer has yet been given to the question of whether more than one enzyme is responsible for the transamination of aromatic amino acids. Gamborg and Wetter (1963) partially purified a non-specific aromatic aminotransferase from mung beans which could not only transaminate all three aromatic amino acids, but also showed activity with several aliphatic amino acids. More recently, Kretovich and coworkers partially purified (about 50-80 fold) a glutamate-phenylpyruvate aminotransferase from pea seedlings and found that this preparation also showed aspartate aminotransferase activity, which was about 15-30 fold greater than that of the phenylalanine aminotransferase activity (Red'kina, Lispenskaya and Kretovich, 1969).

The present study on the metabolism of amino acids in developing bushbean seedlings (Phaseolus vulgaris L.) was designed with the following objectives in mind; firstly, to compare the changes occurring in the composition of the free amino-acid fractions in seedlings grown under light or dark conditions, secondly, to examine the development of transaminase activity in the different seedling organs and to compare the appearance of this activity with the changing composition of the free amino acid fraction from each organ, and thirdly, to attempt the separation and characterization of the enzyme, or the different enzymes, comprising the aromatic amino acid transaminase system in bushbean seedlings.

REVIEW OF LITERATURE

I. BREAKDOWN AND METABOLISM OF SEED PROTEIN

It has long been known that the process of seed germination results in a steady decrease in the protein content of the seed and a concomitant increase in free amino acids and amides. Many of the early investigations of these biochemical changes have been well reviewed by Chibnall (1939), Street (1949), Webster (1959) and Mayer and Poljakoff-Mayber (1963). It was from Boussingault's work (1868) that botanists first gained an insight into the quantitative aspects of the changes which take place during seed germination. Boussingault allowed seeds of pea, wheat, maize and haricot bean to germinate on washed pumice and kept the seedlings either in light, or in the dark, for about a month. He showed that under these conditions, the absolute nitrogen content of the seedlings did not change, indicating that no nitrogen was excreted; but combustion analysis of the whole seeds or seedlings showed that growth in the dark was accompanied by a large diminution of total carbon, hydrogen and oxygen. Hartig (1858) was the first to suggest that during germination, the reserve proteins of the seed are broken down to provide soluble nourishment "Gleis" for the growing parts. This view was later accepted by Pfeffer (1880) who pointed out that, in so far as nitrogenous material was concerned, "Gleis" was asparagine.

Following von Gorup-Besanez's demonstration in 1874 of the presence of proteolytic enzymes in vetch seeds, there was general agreement that the breakdown of seed protein during germination was due to these agents. Schulze (1900), after many changes of opinion,

finally arrived at the general conclusion that during germination, the reserve proteins of seeds are broken down by enzymes to yield peptides and amino acids. The latter undergo further changes to give nitrogen-rich residues, possibly including ammonia, which in turn condense with organic acids to give asparagine, or in certain species, glutamine. In support of this view, Prianschnikow had shown in 1899 that part of the asparagine in seedlings must come from protein decomposition products. Then in 1906, Schulze reported the presence in germinating seeds of ten of the amino acids known to be hydrolytic products of protein, namely, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, proline, arginine, histidine and lysine.

Since these early discoveries at the end of the 19th, and beginning of the 20th century, many subsequent investigations have dealt extensively with the biochemical changes occurring during seed germination, and these are well summarized in the monograph by Mayber and Poljakoff-Mayber (1963). One of the most detailed studies was that carried out by Oota, Fujii and Osawa (1953). These authors examined the changes in dry weight, water-soluble matter, soluble sugar, insoluble polysaccharides, soluble and protein nitrogen, and changes in the nucleic acid content of the different organs of germinating Vigna sesquipedalis. The seed of this plant is characterized by large cotyledons and epigeal germination. The cotyledons showed a loss of all compounds studied, while the growing organs showed an increase in their protein and carbohydrate constituents. The data clearly indicated a marked mobility of the various seed compounds to the developing seedling and recent studies of changes

occurring in the nitrogenous constituents of several dicotyledonous seeds during germination (Lawrence, Day and Stephenson, 1959; Boulter and Barber, 1963; Cherry, 1963; Beevers and Guernsey, 1966; Olsson and Boulter, 1967; Beevers and Splittstoesser, 1968) and of those occurring in certain monocotyledonous seeds (Folkes, Willis and Yemm, 1952; Folkes and Yemm, 1957; Fukui and Nikuni, 1956; Ingle, Beevers and Hageman, 1964) have further confirmed the results of Oota et al. (1953) and other earlier workers.

II. CHANGES IN THE FREE AMINO ACID FRACTIONS DURING GERMINATION

It was not until the development of new methods of analysis, especially paper chromatography and more recently, the development of the amino acid analyzer, that it has become possible to study in detail the soluble nitrogen compounds of plants. This "non-protein" nitrogen fraction has received particular attention because it not only contains the decomposition products of proteins, but also must be the source of the substrates for protein synthesis. To date the amino acid composition of the soluble nitrogen fraction has been examined in many plants. Among the pioneer investigators in this field, the names of Steward, Virtanen, Yemm, Folkes, Fowden and their collaborators should certainly be mentioned. All 22 known protein amino acids have been found by these workers in the soluble nitrogen fractions from a wide range of plants (for review see: Steward and Thompson, 1950; Steward and Pollard, 1957; McKee, 1962; Fowden 1965) and many other non-protein amino acids such as γ -aminobutyric acid (Steward, Thompson and Dent, 1949) piperidine- α -carboxylic acid (Morrison, 1953), homoserine and citrulline (Virtanen and Miettinen, 1953), pipecolic acid (Steward and Durzan, 1965) and many others (Webster, 1959; Fowden, 1962; Dunnill

and Fowden, 1965) were also found in these nitrogen fractions.

Qualitative changes in the soluble nitrogen fraction of seeds germinating under different environmental conditions have also been extensively studied. Thus, for example, the free amino acids in pea seedlings grown under light or dark conditions have been investigated by Shirakawa and Otakara (1965) and again, more recently, by Lawrence and Grant (1963) and by Larson and Beevers (1965). The composition of the free amino acid pool in light-grown broad bean was investigated by Boulter and Barber (1968), in dark-grown soybean seedlings by Kasai, Ishikawa, Obata and Tsukamoto (1966), and in different varieties of wheat and corn by Wang (1968). Although differences were found between the results obtained in these studies, particularly with respect to the concentrations of individual amino acids, these variations could be attributed either to the different environments in which the seedlings were grown (Steward and Bidwell, 1962) or to the genotypic differences between species. It was found for example, that during germination in darkness, the accumulation of nitrogen-rich amino acids, such as asparagine, glutamine, lysine and arginine coincided with a condition of low protein synthesis. This accumulation might be considered as a means of storing amino nitrogen and/or as an ammonia detoxification process (Meiss, 1952).

The formation and accumulation of amides during seed germination has received much attention, and the results from these studies have been well reviewed by Chibnall (1939), Archibald (1945), Street (1949) and Meiss (1952). It is well known that one, or both of the amides, asparagine and glutamine, may accumulate or diminish in seedlings

growing under the influence of various factors such as light or certain inorganic nutrients (Vickery and Pucher, 1943; Meiss, 1952; Virtanen and Miettinen, 1953; Steward, Crane, Millar, Zacharius, Robson and Margolis, 1959; Weinsten and Porter, 1962). Thus, although it is apparent from the literature that qualitative changes in the free amino acid fractions have been studied in a number of plants, particularly during seed germination, relatively little quantitative work has so far been done to compare the changes occurring in the different organs of seedlings growing in light or in darkness. Shirakawa and Otakara (1956) carried out a qualitative study of this kind on germinating peas, and found that asparagine and glutamine accumulated in the soluble fractions to about 5 times greater concentration in the etiolated seedlings than that in the light-grown seedlings. They also showed that the pattern of changes in amino acid concentration during germination in the dark differed from that found during germination in the light.

Since the composition of the free amino acid fraction of seedling tissue will not only reflect the amino acid composition of the degraded seed protein, but also that of the newly synthesized protein (Steward and Bidwell, 1962; Jennings and Morton, 1963; Boulter and Barber, 1963), metabolic changes occurring in the free amino acid pool will be of great importance in supplying the cell with the proper array of amino acids necessary for the full range of its biochemical activities. One of the main reactions involved in such interconversions of amino acids is transamination.

III. TRANSAMINATION

A. Discovery and occurrence of transamination reactions:

The first demonstration of enzymic transamination was made by

Braunstein and Kritzmann (1937) using pigeon muscle preparations. Since this discovery, considerable information has been gained concerning the occurrence of transaminase enzymes in both plant and animal tissues. Early work with a number of plant preparations (Virtanen and Laine, 1938; Kritzmann, 1939; Albaum and Cohen, 1943; Leonard and Burris, 1947) established the widespread occurrence of the following transamination reactions:

Further investigation has shown that a number of other transamination reactions can be demonstrated in plants. Stumpf (1951) found transamination reactions between α -ketoglutarate and γ -aminobutyrate, leucine, isoleucine, valine and norvaline in dialyzed preparations from lima bean, lupin, pumpkin, pea seedlings and wheat germ. Miettinen and Virtanen (1953) extended the list by showing reactions between α -ketoglutarate and both γ -aminobutyrate and citrulline. Fowden and Done (1953) demonstrated transamination between glycine and α -ketoglutarate, and Wilson, King, and Burris (1954) demonstrated transamination of seventeen different amino acids when α -ketoglutarate was again used as the amino group acceptor compound. Transamination of keto analogs of valine and isoleucine with various amino acids was demonstrated by Kagan, Kretovich and Dronov, (1966) and Yu and Spencer (1969) showed the presence of a leucine aminotransferase in tomato fruit. Kretovich and Uspenskaia (1958) and Kretovich and Polyanouskii (1959) have demonstrated transamination in pea seedling homogenates by

showing the synthesis of tryptophan from indolepyruvic acid, and the synthesis of phenylalanine from phenylpyruvic acid. Similarly, Wightman and Fowden (1966) and Wightman and Cohen (1968) have demonstrated a transamination reaction between tryptophan and α -ketoglutarate in cell-free preparations from mung bean seedlings by isolating and chemically identifying the Keto product of this reaction, namely, 3-indolepyruvic acid. Cossins and Sinha (1965) demonstrated the presence of an L-amino acid: 2-glyoxylate aminotransferase in a variety of higher plants.

Transamination is also important in the metabolism of the non-protein amino acids occurring in plants. Fowden and Done (1953) demonstrated transamination of both γ -methyleneglutamate and γ -methylglutamate with α -ketoglutarate in peanut seedlings, and Virtanen and Hietala (1955) showed transamination between hydroxyglutamate and α -ketoglutarate in phlox extracts. Recently, Stinson and Spencer (1969) have demonstrated the presence of a β -alanine aminotransferase in a subcellular particulate fraction from bean cotyledons.

Although transamination is known to be the final step in biosynthesis of only about half of the protein amino acids (Fowden, 1965b) on the basis of the studies described above it is evident that plant enzymes can catalyze the transamination of nearly all protein amino acids, and many non-protein amino-acids, with α -ketoglutarate, oxaloacetate, or pyruvate. This wide occurrence of this reaction suggests that aminotransferase enzymes play an important role in the intermediary metabolism of plants.

B. Development of transaminase systems during germination.

The development of transaminase systems during germination of seeds has been investigated in a variety of plants. Smith and Williams (1951) found a marked increase in the total activity of glutamate-oxaloacetate and glutamate-pyruvate transaminases in the embryo during the first few days of germination of barley, corn, oats and squash and pumpkin seeds. Similarly, Albaum and Cohen (1943) found that the total and specific activity of glutamate-oxaloacetate transaminase increased in oat embryos for at least the first six days of germination. Furthermore, this increase coincided with an increase in protein and in soluble nitrogen. Bevilacqua and Gardella (1964) demonstrated an increase in the activity of aspartate and alanine aminotransferases in the whole seedlings of Phaseolus vulgaris L., Pisum sativum L., Triticum vulgare L. and Zea mays L. during the first five days of germination. Cossins and Wong (1969) showed that the specific activity of glutamate-oxaloacetate transaminase in pea seeds reached a peak by the fourth day of germination, and thereafter decreased. Most recently, Splittstoesser (1970) reported that an increase occurred to the total activity of aspartate aminotransferase in the cotyledons of pumpkin seedlings grown either in the light or dark during the first eight days of germination, and that thereafter, the activity rapidly decreased.

Little work has so far been done to investigate the development of other transaminases in germinating seeds. In one of the few reports on this topic, Gamborg and Keeley (1966) studied the activity of aromatic amino-acid transaminase during the germination of light-grown

mung bean seedlings. Phenylalanine-pyruvate transaminase activity, expressed in units per gram of fresh weight, increased gradually in the shoot over the first 12 days, and the high level of activity found on the twelveth day was maintained at least over the next 10 days. In the cotyledons, transaminase activity also increased but at a slower rate, and when the cotyledons senesced at about the ninth day, the transaminase activity was only one quarter that of the shoot tissues. In contrast to Gamborg and Keeley's findings, Cossins and Sinha (1965) found that the specific activity of glyoxylate transaminase decreased in the cotyledons of sunflower during germination.

Thus, it seems that changes in the activities of the transaminase systems during germination vary from one species to the other, and are also dependent upon the type of organ studied and the conditions of growth. No study has so far been made to examine the effect of those environmental factors which influence amino acid metabolism, on the development of transaminase enzymes during germination. Furthermore, no investigation has been made about the possible correlation between changes in the composition of free amino acid fractions and the development of transaminase activity. It is interesting to note here that certain animal transaminases, such as aspartate aminotransferase (Katunuma, Okada, Katsunuma, Fujimo and Matsuzawa, 1968) and tyrosine aminotransferase, are under hormonal control (Litwach and Diamondstone, 1962; Martin, Tomkins and Granner, 1969; Tomkins, Gelehrter, Granner, Martin, Samuels and Thompson, 1969; Blake, 1970) and daily rhythms of the organism in question (Black and Axelford, 1968). Similar regulation of plant aminotransferases may occur, but no examination of this

possibility has yet been made.

C. Intracellular localization of transaminase systems.

Although many studies have been made to determine the subcellular localization of transaminase systems in animal tissues, very few similar investigations have been carried out on plant tissues. The two main forms of aspartate aminotransferase in rat liver were found to be present in the soluble and mitochondrial cell fractions (Boyd, 1961). These two forms occur in a number of tissues and have been found in dog heart (Fleisher, Potter and Wakin, 1960) beef heart, pig heart (Wada and Morino, 1964) chicken heart (Bertland and Kaplan, 1968), and human tissues (Bodansky, Schwartz and Nisselbaum, 1966). The pig heart mitochondrial and soluble enzymes have different kinetic properties and immunological reactions (Martinez-Carrion and Tiemeier, 1967). Studies on the concentration of these two enzymes at various stages of growth, (Nakata, Suematzu and Sakamoto, 1964; Scheid, Morris and Roth, 1965) and on induction of these enzymes, (Katunuma, Okada and Nishii, 1966; Katunuma, Okada, Katsunuma, Fujino and Matsuzawa, 1968) suggest that the soluble and mitochondrial aspartate aminotransferases are under separate genetic control. Aspartate aminotransferase activity has been demonstrated recently in both the soluble and particulate fractions of pea cotyledons (Wong and Cossins, 1969) and of pumpkin cotyledons (Splittstoesser, 1970). Splittstoesser found that in pumpkin cotyledons the bulk of the activity (75%) was in the soluble fraction and the remaining activity was in the particulate fraction. On the other hand, he showed that less than 2% of the activity was in the particulate fraction of pumpkin hypocotyls and roots. Wong

and Cossins (1969) found that the soluble enzyme accounted for more than 90% of the total extractable aspartate aminotransferase activity in pea cotyledons.

Another class of transaminases which has been extensively studied, mainly because of their key role in brain metabolism, is that of the aromatic amino acid transaminases. Semba and Civen (1970) studied the subcellular distribution of tyrosine transaminase, 3,4-dihydroxyphenylalanine (DOPA) transaminase, tryptophan transaminase and 5-hydroxytryptophan transaminase in rat brain and found that 60-81% of the total activity of each of these enzymes was present in the crude mitochondrial fraction (in brain, this fraction contains synaptosomes). Miller and Litwack (1969), similarly, found that 95% of the tyrosine aminotransferase activity was associated with the particulate fraction in the same tissues. Fellman, Vanbellenghen, Jones and Koler (1969) have studied a soluble and mitochondrial tyrosine transaminase in human liver. They found that the lack of the soluble enzyme was associated with an increase of urinary p-hydroxyphenylpyruvate in patients with a tyrosinemia condition. This study suggested that the soluble and mitochondrial tyrosine transaminases are under separate genetic control.

In plant tissues, the localization of the aromatic amino acid transaminases seems to be quite different. For example, it has been shown (Cohen, 1968; Wightman and Cohen, 1968) that the mitochondrial fraction contained only about 5% of the total tryptophan- α -ketoglutarate transaminase activity of the original homogenate of mung bean seedling tissue, whereas the soluble cytoplasmic fraction contained over 90% of

the total activity of the transaminase.

D. Purification and characterization of plant aminotransferases.

Relatively little information is available to indicate the number of different transaminases present in a single plant species, and this is coupled with a lack of knowledge concerning the specificity of most of these enzymes for their amino and keto acid substrates.

The most extensively described transaminases in plants are aspartate aminotransferase and alanine aminotransferase. Cruickshank and Isherwood (1958) reported evidence for separate enzymes responsible for the aspartate- α -ketoglutarate and alanine- α -ketoglutarate transaminase activities in wheat-germ. Ellis and Davies (1961) purified an aspartate- α -ketoglutarate transaminase from cauliflower buds about 200-fold and showed that the enzyme would not utilize pyruvate, or transaminate many other amino acids except cysteic acid and certain substituted glutamic and aspartic acids whose molecular structure was closely similar to those of the normal substrates. Fasella et al. (1966) purified an aspartate aminotransferase from cotton seeds about 2700-fold. Their purified preparation contained three proteins showing glutamate-oxaloacetate transaminase activity and also malate dehydrogenase activity. More recently, Wong and Cossins (1969) have isolated a soluble and a particulate aspartate aminotransferase from germinating pea cotyledons and Verjee and Evered (1969) have purified the same enzyme from wheat germ. The properties of aspartate aminotransferase isolated from different plant and animal sources are given in Table 1. These data show clearly that plant aspartate- α -ketoglutarate transaminase bears close similarities to the animal aspartate- α -ketoglutarate transaminase in

Table 1: Properties of aspartate aminotransferase isolated from different animal and plant tissues.

ENZYME SOURCE	PURIFI- CATION (fold)	ISO- ENZYMES	SUB- UNITS	M.W. (daltons)	pH OPTIMUM	PYRIDOXAL-P. CONTENT (moles/mole/ Enz.)	α -KG	OXALO.	L-Asp. L-Glut.	REFERENCES
Cauliflower buds	225	--	--	--	7.8-8.0	--	7.6×10^{-4}	1.0×10^{-4}	7.5×10^{-3}	3.8×10^{-2} Ellis & Davies(1966) Davies & Ellis(1966)
Cottonseeds	2700	3	--	--	--	--	--	--	--	Fasella et al.(1966)
Pea	67	soluble	--	--	8.0	--	6.7×10^{-4}	4.8×10^{-5}	8.7×10^{-4}	4.4×10^{-3}
Cotyledons	27	Particulate	--	--	7.0-8.0	--	7.3×10^{-4}	4.5×10^{-5}	1.6×10^{-3}	6.4×10^{-3} Wong & Cossins(1966)
Wheat Germ	60	1	--	75,000	8.0-8.5	--	--	--	--	Verjee & Evered(1966)
Bovine Liver and heart	--	--	--	120,000	---	2	3.4×10^{-4}	--	2.2×10^{-3}	--
Pig heart	--	--	--	110,000	8.5	2	1×10^{-4}	4×10^{-5}	9×10^{-4}	4×10^{-3} Wada & Morino (1966) Jenkins et al.(1965) Velick & Vavra(1966)
Pig heart	--	soluble (4)	2	93,000	--	2	--	--	--	--
Pig heart	--	Mitochondrial (3)	2	91,200	--	2	--	--	--	--
Chicken heart	180	soluble (3)	--	95,000 100,000	--	2	7.9×10^{-5}	--	3.3×10^{-3}	--
	150	Mitochondrial (1)	--	95,000 100,000	--	2	1.45×10^{-3}	--	5.5×10^{-4}	--
										Bertland & Kaplan (1968) Bertland & Kaplan (1970)

terms of pH optima and Michaelis constants. It also appears clear that the plant transaminase needs more detailed studies since nothing is known yet about its subunit composition and pyridoxal-phosphate content. Furthermore, molecular weight found for the wheat germ enzyme was 75,000, whereas a molecular weight of 93,000-120,000 was reported for animal aspartate aminotransferase.

Turano, Bossa, Fasella and Fanelli (1966) purified an alanine aminotransferase from resting cotton seeds and showed by gel electrophoresis that only one protein was responsible for the activity. This represents the only advanced study to date on alanine aminotransferase in plants, and no work was carried out to determine the specificity of the enzyme. Alanine aminotransferase has been purified from many animal sources (Green, Leloir and Nocito, 1945; Turano and Riva, 1965; Saier and Jenkins, 1966) and its isozymal nature has been demonstrated (Swick, Barnstein and Stange, 1965). The soluble alanine aminotransferase was shown to be highly specific for alanine and glutamate (Saier and Jenkins, 1967).

Branched chain aminotransferases have been detected in plant tissues (Kagan et al., 1966; Wagner, Berquist and Karp, 1958; Yu and Spencer, 1969), but no attempt to isolate and characterize these enzymes has yet been made. A branched chain amino acid transaminase has been isolated from different animal sources (Ichihara and Koyama, 1966; Taylor and Jenkins, 1966; Aki, Yokojima and Ichihara, 1969) and has been shown to be specific for valine, leucine, isoleucine and α -ketoglutarate. The molecular weight of this enzyme was reported to be 75,000 (Taylor and Jenkins, 1966).

The first study on the isolation and characterization of plant aromatic amino acid transaminases was reported by Gamborg and Wetter (1963). These authors demonstrated the presence of an aromatic amino acid transaminase in mung bean plants (Phaseolus aureus) which catalyzed the transamination of phenylalanine, tyrosine and tryptophan in the presence of α -ketoglutarate. Gamborg (1965), in a more comprehensive study, demonstrated that, with a 40 to 60 fold purified enzyme preparation from mung bean, pyruvate was the best amino acceptor and the enzyme utilized a wide variety of amino acids as substrates. The selectivity of this enzyme for the amino donor appeared to be determined partly by the chain length, and partly by the substituents on the methylene carbon adjacent to the α -amino group. Red'Kina, Uspenskaya and Kretovich, (1969) purified a glutamate-phenylpyruvate transaminase from pea seedlings about 50-80 fold, and demonstrated that this preparation had an aspartate- α -ketoglutarate transaminase activity which was about 15-30 times greater than that of its glutamate-phenylpyruvate activity. Gadal, Boussou and Barthe (1969) isolated from the leaves of Quercus pedunculata an aromatic amino acid transaminase which used either p-hydroxyphenylpyruvate or phenylpyruvate as the amino acceptor, and glutamate and aspartate as the amino donor. The reversibility of the reaction was demonstrated. The properties of these aromatic aminotransferases from plant sources are reported more fully in Table 2, together with the properties of the aromatic amino acid transaminases isolated from animal tissues and bacteria.

Aromatic aminotransferases from animal sources differ from those of plant tissues mainly in their degree of specificity. Fonnum,

TABLE 2. Properties of aromatic amino acid transaminases isolated from plant and animal tissues and bacteria.

ENZYME SOURCE	PURIFICATION (Fold)	pH OPTIMUM	SPECIFICITY	MICHAELIS CONSTANTS (M)			REFERENCES
Mung bean seedlings	40-60	---	non specific	TPP 1.7×10^{-3} TY 2.7×10^{-4}	PA 4.0×10^{-4}	α -KG 7.7×10^{-4}	Gamborg and Wetter, 1963; Gamborg, 1965.
Pea seedlings	50-80	---	non specific	Glut. 9.4×10^{-3}	PPY 7.5×10^{-3}	Oxalo. 7.5×10^{-3}	Red'Kina et al., 1969
Leaves of <u>Quercus</u> <u>pedunculata</u>	180	8.5	non specific	Glut. 1.8×10^{-3}	PPY 8.0×10^{-3}	p-HPy 3.5×10^{-3}	Gadal et al., 1969.
<u>Pseudomonas</u> <u>savastanoi</u>	200	8.8	non specific	-----	-----	-----	Kuo and Kosuge, 1970.
Pig brain	900	8.0-9.0	specific	PA 5.1×10^{-2}	α -KG 7.4×10^{-4}		George and Gabay, 1968.
Bovine thyroid & liver tissue	10,000	7.25	specific	Ty -----	α -KG -----		Igo et al., 1968.
(Abbreviations: TPP: tryptophan; PA: phenalanine; Ty: tyrosine; p-HPy: p-hydroxyphenlpyruvate) Oxalo: oxaloacetate; PPY: phenylpyruvate α -KG: α -ketoglutarate;							

Haavaldsen and Tangen (1964) and Fonnum and Larsen (1965) have presented evidence for the occurrence of three aromatic aminotransferases in rat brain extracts; one transaminating 3,4-dihydroxyphenylalanine (DOPA), another transaminating both phenylalanine and tyrosine, and a third transaminating both tryptophan and 5-hydroxytryptophan. George and Gabay (1968) reported a phenylalanine aminotransferase isolated from pig brain which was highly specific for phenylalanine and α -ketoglutarate, and Igo, Mahoney and Limbeck, (1968) have isolated a specific tyrosine- α -ketoglutarate transaminase from bovine thyroid and liver tissue. Soluble and mitochondrial forms of the tyrosine aminotransferase have subsequently been studied in a variety of animal tissues (Koler, Vanbellinghen and Fellman, 1969; Fellman, Vanbellinghen, Jones and Koler, 1969). The molecular weight of this aminotransferase from rat liver was found to be 115,000, and it was found to bind approximately 4 moles of pyridoxal phosphate per mole of enzyme (Valeriote, Auriccho, Tomkins and Riley, 1969).

E. Mechanism of transamination

1. Non-enzymic transamination

An understanding of the mechanism of transamination has come mainly from the results of studies with non-enzymic systems. Early workers established that several multivalent metal ions readily catalyzed non-enzymic transamination reactions in the presence of vitamin B6 (Metzler and Snell, 1952; Meister, 1955). 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 non-enzymic transamination reactions.

The non-enzymic transamination reaction between pyridoxal and amino acids was originally discovered by Snell (1945) and is shown in the following equations:

These reactions were shown to be fully reversible (Metzler and Snell, 1952) and it was recognized that in the summation of the first two reactions, pyridoxal and pyridoxamine would act solely as catalysts which functioned respectively as amino group acceptor and amino group donor and would cancel out of the final equation to yield reaction 3, identical with enzymatic transamination, a concept which was subsequently fully verified (Snell and Jenkins, 1959). Many reactions of pyridoxal with amino acids in aqueous solution are catalyzed by appropriate metal ions (Snell, 1962a). Although at least a portion of the non-enzymatic transamination reaction proceeds through the metal chelates when metal ions are present (Longenecker and Snell, 1956) (see Fig. 1), the reaction proceeds slowly in water (Metzler and Snell, 1952) and rapidly in ethanol (Matsuo, 1957) without addition of metal ions. It is not certain whether the primary catalytic role of metal ions in promoting the reaction in aqueous solution is in catalyzing the formation and hydrolysis of the Schiff base intermediate, in promoting the requisite electronic displacements, or in both. Snell (1962a) has viewed the metal ion as playing inefficiently in non-enzymatic systems the functional role played by protein in enzymatic systems.

FIG. 1. Chemical structures showing the electron shifts during pyridoxal and metal ion-catalysed non-enzymatic transamination.

2. Enzymic transamination

Experimental support for the participation of vitamin B6 in enzymic transamination was obtained from studies on vitamin B6 deficient rats and microorganisms. Deficiency was associated with lowered levels of transaminase activity, and addition of pyridoxal phosphate to the tissue or cell preparations resulted in the restoration of enzyme activity (Ames, Sarma and Elvehjem, 1947; Lichstein, Gunsalus and Umbreit, 1945; Schlenk and Snell, 1945). Indications that mammalian aspartate aminotransferase contained pyridoxal or pyridoxamine phosphate was first reported by Schlenk and Fisher (1945). The requirement for pyridoxal phosphate in the aspartate aminotransferase reaction was later 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 B6 was required for transamination. Meister, Sober and Peterson (1954) showed that pyridoxamine phosphate could also restore aminotransferase activity. Since these early investigations, mammalian aminotransferases have been resolved into apoenzyme and coenzyme by numerous methods (Bank and Vernon, 1961; Wada and Snell, 1962; Scardi, Scotto, Iaccorino and Scarano, 1963; Churchich and Farrell, 1969) and quantitative determinations of the pyridoxal phosphate content per molecule of the holoenzyme have been made (Jenkins, Yphantis and Sizer, 1959; Saier and Jenkins, 1967; Martinez-Carrion and Tiemeier, 1967; Michuda and Martinez-Carrion, 1969; Bertland and Kaplan, 1968; 1970). The dependence of plant aminotransferase on pyridoxal phosphate has been demonstrated by Wong and Cossins (1966; 1969).

FIG. 2. Proposed scheme for catalysis of a half reaction of enzymatic transamination (Snell, 1962 b). In this illustration, the non-hydrogen-bonded aldimine structure facilitates interaction with the amino acid substrate and the liberated ϵ -amino group of lysine residue may act as a general acid-base catalyst for the electron shifts initiated by pyridoxal.

amino acceptor) occurs simultaneously at a single enzymic site. The binary mechanism postulates that the overall reaction is the sum of two separate binary reactions (i.e. Equations 4 and 5) catalyzed by one protein. The enzyme oscillates between the pyridoxal and the pyridoxamine phosphate forms (Velick and Vavra, 1962; Jenkins and Taylor, 1965; Ivanov and Karpeiski, 1969). 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. The following diagram illustrates this mechanism:

(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).

The binary reaction mechanism has been well demonstrated for glutamate-pyruvate aminotransferase isolated from animal tissues (Segal et al., 1962; Hopper and Segal, 1962; Jenkins, 1963) and for aspartate- α -ketoglutarate transaminase from mammalian tissue (Velick and Vavra, 1962; Wada and Morino, 1964; Fasella, 1967). Turano et al., (1966) have demonstrated a similar "shuttle" mechanism for an alanine aminotransferase extracted from cotton seeds and Wong and Cossins (1969) have shown such a mechanism for aspartate aminotransferase from germinating pea cotyledons.

MATERIAL AND METHODS

Germination of seedlings and sampling procedure.

Bushbean seeds (Phaseolus vulgaris L., var. Pencil Pod Black Wax) were soaked in distilled water overnight at room temperature. The seeds were planted in moist vermiculite and kept in a growth cabinet at 22-24°C either in complete darkness, or exposed to a 16 hr. daily period of 3,000 ft. candles. Seedlings were harvested every 2 days for two weeks and were divided into roots, shoots (hypocotyls plus epicotyls) and cotyledons. Growth measurements, dry weight determinations, total and soluble nitrogen determinations and analysis of the amino acid composition of the soluble nitrogen fraction were then made on each sample or organs. Ten seedlings were collected in all samples, except during the early days of the growth period when 100 and 50 seedlings were used for the 2nd and 4th day samples, respectively. All determinations were carried out on three batches of seedlings grown on separate occasions, and the results presented are the average values from the data obtained in the three experiments. For determinations of changes in total and soluble nitrogen contents, the variations observed between results from the three experiments was not greater than 15% for determination of quantitative changes in the free amino acid pool, the variations between the three sets of results was not greater than 20%.

Total nitrogen determinations.

Each sample of seedling organs was ground in distilled water using a pestle and mortar, and the homogenate was made up to a known volume. Aliquots were analyzed for total nitrogen content by a modified

microkjeldahl digestion procedure (McKenzie and Wallace, 1953) using the Nesslerization technique to measure the resulting ammonium sulfate. Results are expressed in terms of mg nitrogen per organ.

Soluble nitrogen determinations

Nitrogenous compounds soluble in 25% trichloroacetic acid are referred to as the soluble nitrogen fraction. This fraction contained free amino acids, ammonia and soluble nucleotides, and the amount of nitrogen present was again determined by the modified microkjeldahl method of McKenzie and Wallace (1953). Colorimetric measurement of the nitrogen-Nessler reagent complex was carried out using a Spectronic 20 set at a wavelength of 420 mμ. Results are expressed in terms of mg nitrogen per organ.

Extraction and analysis of free amino acids

Each sample of seedling organs was homogenized in 75% ethanol and the suspension vigorously shaken for 1 min. The alcoholic extracts were boiled for 1 min. and then stored in the cold for 24 hr. The extracts were filtered under vacuum and the residue washed with an equal volume of 75% ethanol. The filtrates were applied to small columns (12.5 x 250 mm) of cation exchange resin (Rexyn 101) which had been previously shown to retain all the protein amino acids. After thorough washing of the column, the amino acids were eluted with 100 ml of 3N NH_4OH , which is equivalent to 4 times the void volume of the column. The eluates were evaporated down to dryness and the residues redissolved in 4 ml of 70% methanol.

Separation of the amino acids was achieved by two-dimensional

paper chromatography according to the method of Dunnill and Fowden (1965). Aliquots of the methanolic amino acid fraction, equivalent to a known amount of the original seedling tissue, were applied to large sheets of Whatman 3MM chromatographic paper. The chromatograms were first developed in 80% (w/v) phenol in the presence of NH_3 vapour, and then in n-butanol: acetic acid: water (9:1:2.9, v/v/v). Identification and quantitative determination of the amino acids were made from chromatograms sprayed with a modified cadmium acetate-ninhydrin solution (Atfield and Morris, 1961). Spots of standard protein amino acids were cut from the chromatogram (Fig. 3) and the red reaction complex was eluted into 5 ml methanol for 3 hr. The intensity of the red coloration was then measured at 500 m μ using a Spectronic 20, and results were expressed in terms of μ mole amino acid per organ.

Identification of each protein amino acid in the different alcoholic extracts was achieved by co-chromatography with the authentic compound. Only the 22 amino acids known to occur frequently in plant proteins (Holley, 1965) were investigated as components of the free amino acid fractions. Other non-protein amino acids were detected on the chromatograms, but since most of these were present only in trace amounts, they were not included in the quantitative study of these fractions. Quantitative measurements of the free protein amino acids were based on standard curves obtained from the absorption values given by known amounts of the authentic amino acids, using the cadmium-ninhydrin colorimetric procedure. Linear absorption curves were obtained for each amino acid over the concentration range, 1-40 μg per spot. Four typical examples of these standard curves are shown in Figure 4. It should be

FIG. 3. Diagram showing the distribution of standard amino acids on a chromatogram developed first in 80% (w/v) phenol in the presence of NH_3 vapour, and then in n-butanol: acetic acid: water (9:1:2.9, v/v/v). The chromatogram was sprayed with a cadmium acetate-ninhydrin solution and the amino acids were identified as follow:
 1.- aspartic acid, 2.- glutamic acid, 3.- serine, 4.- cysteine, 5.- glycine, 6.- asparagine, 7.- glutamine, 8.- threonine, 9.- alanine, 10.- tyrosine, 11.- leucine, 12.- isoleucine, 13.- phenylalanine, 14.- tryptophan, 15.- valine, 16.- proline, 17.- methionine, 18.- lysine, 19.- histidine, 20.- arginine. (●) Origin.

FIG. 4. Typical standard curves of four amino acids obtained from the absorption values at $500m\mu$ of known amounts of amino acid chromatographed, using the cadmium-ninhydrin colorimetric procedure.

noted that when standard mixtures containing known amounts of authentic samples of the 22 protein amino acids were run through the above purification and colorimetric procedure, the losses obtained were consistently in the range of 10-15% for all the amino acids involved.

Preparation of the enzyme fraction for studying development of transaminase activity.

Each sample of seedling organs was ground in a Waring blender with 2 volumes of 0.05 M buffer, at pH 7.5, containing 1×10^{-4} M EDTA (Ethylenediamine-tetraacetic acid) and 1×10^{-4} M 2-mercaptoethanol. Phosphate buffer was used in the assay of the aromatic amino acid transaminases and TRIS-HCl [Tris(hydroxymethyl) aminomethane hydrochloride] for the assay of the aliphatic amino acid transaminases. The homogenate was passed through four layers of cheesecloth and then centrifuged at $1,000 \times g$ for 20 min. The sediment was discarded and 0.1% v/v Tergitol NPX detergent was added to the supernatant to facilitate the bursting of cellular organelles. After stirring for 30 min., the fraction was centrifuged twice at $27,000 \times g$ for 20 min. Each pellet was discarded and the final supernatant fraction was collected and dialyzed overnight against 50 volumes of buffer at pH 8.5. All operations were carried out in a cold room at 3°C.

Assay of Transaminase activity

The dialyzed supernatant fraction from each sample of seedling organs was assayed for transaminase activity using either aspartic acid, asparagine, alanine, valine, serine, threonine, phenylalanine, tyrosine or tryptophan as the amino acid substrate, and α -ketoglutaric acid as the amino group acceptor. All assays were carried out for 2

hours at 35°C, with a pre-incubation time of 10 min. in the presence of all assay components except α -ketoglutaric acid, which was supplied to start the reaction. A standard assay mixture was thus made up of the following components, dissolved in 0.05 M TRIS-HCl or phosphate buffer at pH 8.5, to a total volume of 1 ml: 4×10^{-2} M amino acid, 8×10^{-3} M α -ketoglutaric acid, 2×10^{-4} M pyridoxal phosphate plus the enzyme fraction. The reaction was linear with time for at least 2 hr., using the range of protein concentrations obtained in these experiments from the various supernatant fractions. The controls contained all components of the standard reaction mixture except the substrate amino acid. The reaction was stopped by adding an equal volume of hot methanol. After sedimentation of the precipitated protein by centrifugation, standard aliquots of the methanolic fraction were applied, as two inch streaks, to large sheets of Whatman No. 4 chromatographic paper. The chromatograms were developed in the descending direction using the solvent, n-butanol: acetic acid: water, 9:1:2.9 v/v/v. Identification and quantitative determination of the reaction product, glutamic acid, were made by using the cadmium acetate-ninhydrin colorimetric procedure described above.

Preparation of particulate enzyme fractions

The roots from 8-day old dark-grown seedlings were washed, blotted and ground in a mortar in 0.05 M TRIS-HCl, pH 7.5, containing 0.4 M sucrose (2.5 ml of buffer per gram of tissue), 1×10^{-4} M EDTA and 1×10^{-4} M 2-mercaptoethanol (see Fig. 5). After filtering through 4 layers of cheesecloth, the homogenate was centrifuged for 15 minutes at 1,000 x g to remove cell debris. This sediment was washed twice in grinding medium and then discarded. Centrifugation of the supernatant

All fractions were dialyzed overnight against 0.05 M TRIS-HCL, pH 8.5, containing 1×10^{-4} M EDTA and 1×10^{-4} M 2-mercaptoethanol before being tested for enzymic activity.

Figure 5: Flowsheet for the preparation of the subcellular fractions.

fraction, plus the washing, for 30 min. at 2,500 x g sedimented a particulate fraction referred to as the plastid fraction. After washing the plastid fraction twice with grinding medium, the washings were added to the supernatant and a third centrifugation was carried out for 30 min. at 10,000 x g. This latter sediment contained the mitochondrial fraction which was washed twice with the grinding medium. Membranes in the two particulate fractions were broken by stirring the sediment in 0.05 M TRIS-HCl, pH 7.5, containing 0.1% v/v Tergitol NPX detergent. After overnight dialysis, these different fractions were tested for transaminase activity using the standard reaction mixture.

Extraction and purification of the aromatic aminotransferase.

700 to 900 grams of roots of 8-day old dark-grown seedlings were ground in TRIS-HCl buffer, pH 7.5, containing 1×10^{-4} M 2-mercaptoethanol, 1×10^{-4} M EDTA, 2×10^{-4} M pyridoxal phosphate and 8×10^{-3} M α -ketoglutarate (2 ml of buffer per gram of tissue). The homogenate was passed through four layers of cheesecloth and then centrifuged at 1,000 x g for 15 min. to remove cell debris. The sediment was washed twice in grinding medium and then discarded. The washing was added to the original supernatant and this was made up to 0.1% Tergitol (using a 2.5% v/v stock solution of Tergitol) and allowed to stand for 30 min. on ice. The solution was then clarified by centrifugation at 25,000 x g for 20 min. giving the "crude total extract"; the sediment pellet contained no detectable transaminase activity.

Briefly the purification steps included: a) pH precipitation, b) ammonium sulfate fractionation, c) filtration on Sephadex G-100, d) chromatography on DEAE-Sephadex, e) filtration through Sephadex G-200, f) rechromatography on DEAE-Sephadex. All procedures were carried out at 2-4°C. The details of each purification step are given below.

a) pH precipitation

The crude total extract was adjusted to pH 5.5 with 1N HCl and stirred for approximately 1 hr. on ice. The precipitated protein fraction was collected by centrifugation at 37,000 x g for 30 minutes and was discarded since it contained no activity. The pH of the supernatant was brought back to pH 7.5 over a period of 1 hr. using 1N KOH.

b) Ammonium sulfate fractionation

The supernatant from the pH precipitation step was brought up to 40% saturation by addition of 243 grams per liter of ammonium sulfate. After stirring for 1 hr., the solution was centrifuged at 37,000 x g for 30 minutes to remove precipitated protein. The supernatant was then brought to 75% saturation by the further addition of 245 grams per liter of ammonium sulfate. After stirring for 1 hr., the precipitate was collected by centrifugation as described before. The transaminase activity was found in this protein fraction precipitated between 40-75% saturation. The precipitate was dissolved in 0.05 M TRIS-HCl pH 7.5, and dialyzed against the same buffer for 12 hr. The volume of this fraction was then reduced to about 10 ml by dehydration with Aquicide II (sodium carboxymethylcellulose). After reduction in volume, this active fraction was further dialyzed against the same buffer for about 12 hr.

c) Filtration through Sephadex G-100 column

Sephadex G-100 powder was soaked in 0.05 M TRIS buffer at pH 7.5 containing 1×10^{-4} M EDTA, 1×10^{-4} M 2-mercaptoethanol and 0.1 M NaCl at 90°C for 5 hr. with occasional stirring. After overnight

cooling at 2-4°C and removal of air from the Sephadex gel under low pressure, the column was prepared according to the manufacturer's instructions. All subsequent operations were also carried out at 2-4°C.

The column (85 x 2.5 cm) was washed with the elution 0.05 M TRIS buffer, prepared as described above, then 7-10 ml (about 265 mg protein) of the active fraction from the ammonium sulfate precipitation were carefully applied to the top of the column. The protein was then eluted with buffer at a flow rate of about 20 ml/hr. The effluent from the column was monitored at 280 m μ using an ISCO UA-2 analyser and was collected in 5 ml fractions. The fractions were assayed for transamination of aspartate, phenylalanine, tyrosine and tryptophan, using α -ketoglutarate as the amino group acceptor. The fractions showing transaminase activity were pooled.

d) DEAE Sephadex chromatography

A suspension of DEAE-Sephadex (Diethylaminoethyl-Sephadex) in 0.05 M TRIS-HCl at pH 7.5, containing 1×10^{-4} M EDTA and 1×10^{-4} M 2-mercaptoethanol, was prepared identically to the Sephadex G-100 gel. A glass column of 2.5 cm diameter was packed to a height of 45 cm and equilibrated in the cold room by washing with the suspension buffer.

The active fraction from Sephadex G-100 filtration was applied to the top of the DEAE-Sephadex column with a pipette. After the enzyme protein had been absorbed, the column was washed with 150 ml of 0.05 M TRIS-HCl at pH 7.5. Following this washing, the protein was eluted from the column using a buffer gradient produced by having 250 ml of 0.05 M TRIS-HCl in the mixing vessel and 250 ml of 0.05 M TRIS-HCl plus 0.5 M KCl in the reservoir of the gradient-making apparatus. The

flow rate of the column was about 10 ml/hr. and 5 ml fractions were collected using a LKB-ULTRORAC, model 7000, fraction collector. All fractions were routinely tested for transaminase activity and the active fractions were pooled. The volume of the pooled fraction was reduced to about 5 ml with Aquacide II, and the fraction was then dialyzed for about 12 hr. against 0.05 M TRIS-HCl at pH 7.5, containing EDTA, 2-mercaptoethanol and 0.1 M KCl.

e) Filtration through Sephadex G-200 column

A suspension of Sephadex G-200 was prepared identically to that of Sephadex G-100, except that 0.1 M KCl was used in the suspension buffer instead of 0.1 M NaCl. A column 1.5 cm in diameter was packed to a height of 85 cm. After concentration with Aquacide II, the active fraction from DEAE-Sephadex chromatography was applied to the top of the Sephadex G-200 column. The protein was eluted with the same buffer and 5 ml fractions were collected. The flow rate of the Sephadex G-200 column was about 10-15 ml/hr. All fractions were tested for transaminase activity and the fractions forming the peak of activity were pooled (see Fig. 20). This pooled fraction was dialyzed against 0.05 M TRIS-HCl at pH 7.5 containing 0.2 M KCl.

f) Rechromatography on DEAE-Sephadex

A suspension of DEAE Sephadex was prepared as previously described using 0.05 M TRIS-HCl buffer at pH 7.5 containing EDTA, 2-mercaptoethanol and 0.2 M KCl. A column 1.5 cm in diameter was packed to a height of 25 cm. The dialyzed active fraction from the G-200 filtration was applied directly to the top of the column. After the protein fraction was completely absorbed into the gel, it was eluted with the same buffer

and 2.5 ml fractions were collected. Only one protein peak was detected and this peak coincided with the peak of transaminase activity (Fig. 21).

Protein determination

The protein content of all crude preparations was determined by the biuret reaction (Beizenherz, Boltze, Bucher, Czock, Garbade, Meyer-Arendt and Pfeleiderer, 1953) while the protein in partially purified, or purified fractions was determined by the optical method (Kalckar, 1947).

Electrophoresis

Canalco equipment (Model 1200 R) was used for polyacrylamide gel electrophoresis. The gels were prepared in glass columns (0.5 x 7.5 cm, or 0.5 x 15 cm) and the procedure followed was that of Schmitt (1968) using tris-glycine buffer. The details of this method are as follows:

Solutions:

1. Separation monomer mixture

Acrylamide	28.0 g
N,N'-methylenebisacrylamide	735.0 mg
Distilled water	100 ml

2. Separation buffer pH 8.9

Trishydroxymethylaminomethane	18.3 g
1N HCl	24.0 ml
N,N,N',N'-Tetramethylethylenediamine	0.115 ml
Distilled water	100 ml

3. Catalyser

Ammonium peroxydisulfate	0.14 g
Distilled water	100 ml

This solution was not stable for more than one week.

4. Stacking gel monomer mixture

Acrylamide	10.0 g
N,N'-Methylenebisacrylamide	2.5 g
Distilled water	100 ml

5. Stacking buffer pH 6.7

Trishydroxymethylaminomethane	2.99 g
1N HCl	24.0 ml
N,N,N',N'-Tetramethylethylenediamine	0.23 ml
Distilled water	100 ml

6. Photo catylaser

Riboflavine	2.0 mg
Distilled water	100 ml

7. Electrode buffer pH 8.2

Trishydroxymethylaminomethane	6.0 g
Glycine	28.0 g
Distilled water	100 ml

This buffer was diluted 1:5 before use.

The above solutions were used in the following order and quantities:

Separating gel:

- 1 volume of solution 1
- 1 volume of solution 2
- 1 volume of glass distilled water
- 2 volumes of solution 3

Stacking gel:

- 1 volume of solution 4
- 1 volume of solution 5
- 1 volume of glass distilled water
- 1 volume of solution 6

The glass columns (7.5 cm in length) were inserted in the Cananco column holder and 1 ml of separating gel was added to each column. A drop of distilled water was added to the top to avoid the formation of a meniscus of gel. After 1 hr. at room temperature for polymerization of the separating gel, the water was shaken out of the column and 0.1 ml of stacking gel and one drop of water were added. The stacking gel was polymerized under fluorescent light for about 10 min. 0.2 ml of the purified protein fraction was then mixed with 0.1 ml of stacking gel and applied to the top of the column. When the 15 cm long columns were used, these quantities mentioned above were double. The electrophoresis was carried out using a current of 3 milliamp. per column for about 45 min. Bromophenol blue in solution in the upper buffer (solution 7) was used as marker.

Protein bands were detected on the gel by staining with 0.25% w/v naphthol blue black (Amido Schwarz) in 8% acetic acid. The gels were destained in 8% acetic acid using a reverse current of 3 milliamp. per column.

The aromatic aminotransferase activity was located on the gels by incubation at 35°C for about 30 min. in 2 ml of the following solution: 2×10^{-4} M pyridoxal phosphate, 8×10^{-3} M α -ketoglutarate, 8×10^{-2} M aromatic amino acid and 4 mg of phenazine methosulfate in 0.75 M phosphate buffer at pH 7.5, according to the technique of Valeriote et al. (1969)

Molecular weight determination

Determination of the molecular weight of the purified transaminase isolated from the roots of bushbean seedlings was carried out using the technique of gel filtration (Andrews, 1964; 1965). A column (1.5 x 85 cm) of Sephadex G-200 was prepared as described above in section e) on "Extraction and purification of the aromatic aminotransferase". The bed volume of the column was 120-124 ml and the void volume was 38 ± 1 ml. The column was calibrated using the following proteins of known molecular weight:

<u>Protein</u>	<u>Molecular weight</u>
Cytochrome C (horse heart)	12,400
Ovalbumin	45,000
Albumin	67,000
Gamma-globulin (human)	163,000
Apo ferritin (horse)	480,000

Blue dextran 2000 was used to mark the front of elution. 2 mg of each protein were dissolved in 1 ml of TRIS buffer at pH 7.5 containing 0.1 M KCl. Individual proteins, or a mixed protein fraction was applied to the top of the column and then eluted with 0.05 M TRIS-HCl

buffer containing 0.1 M KCl at a flow rate of about 10-15 ml/hr. The effluent was monitored at 280 m μ with the ISCO analyser and was collected in 2.5 ml fractions. Replicates were carried out when only one standard protein was run through the column at the time. A total of six molecular weight determinations were performed using the purified transaminase fraction.

Assay of transaminase activity of purified protein fraction

Experiments to determine the enzymic characteristics of the purified protein fraction were performed in 0.05 M TRIS-HCl buffer at pH 8.5 containing, in most instances, the following standard components: 1×10^{-4} M EDTA, 1×10^{-4} M 2-mercaptoethanol, and 2×10^{-4} M pyridoxal phosphate. The amino acid and keto acid substrates were added at the concentration and in the volume indicated in the Results section. All assays were carried out for 30 min. at 35°C. Control systems contained all the reaction mixture components except the substrate amino acid. When the product amino acid was to be determined, the reaction was stopped after 30 min. by adding an equal volume of hot methanol. The product amino acid was then isolated by paper chromatography and its quantity determined as previously described. When the product aromatic keto acid was to be determined, the reaction was stopped by adding 0.1 ml of 50% Trichloroacetic acid and the amount of the arylpyruvic acid formed was determined by the phenazine methosulfate technique of Schepartz (1969). Linear standard curves were obtained for each arylpyruvic acid at concentrations up to 1.6 μ mole/ml (Fig. 6).

One unit of aromatic aminotransferase is that amount of enzyme

FIG. 6. Standard curves for the arylpyruvic acids as determined by the phenazine methosulfate technique of Schepartz.

catalyzing transamination of the substrate amino acid to yield 1 μ mole of amino acid product per minute of reaction. The specific activity of the enzyme is the activity expressed in units of 1 mg of protein.

Chemicals.

The chemicals used in this investigation were all of analytical grade.

Chemicals	Abbreviation	Supplier
Potassium phosphate (mono and dibasic)		Fisher Scientific
Ammonium sulfate	$(\text{NH}_4)_2\text{SO}_4$	Nutritional Biochem.
Tris(hydroxymethyl)aminomethane hydrochloride		Sigma
Tris(hydroxymethyl)aminomethane	TRIS-HCl TRIS	Nutritional Biochem.
Ethylenediaminetetraacetic acid	EDTA	Fisher Scientific
2-Mercaptoethanol		Eastman Organic Chem.
Tergitol NPX		Union Carbide Corp.
Amino acids		Sigma
Aliphatic and aromatic keto acids		Sigma
Cathecols and other aromatic compounds		Sigma
Pyridoxal-5'-phosphate	PLP	Sigma
Iodoacetic acid		Sigma
Iodoacetamide		Sigma
Isonicotinic acid hydrazide		Sigma
p-Chloromercuribenzoate	p-CMB	Sigma
Phenazine methosulfate		Sigma
Sephadex G-100 and G-200		Pharmacia

Chemicals	Abbreviation	Supplier
Diethylaminoethyl-Sephadex	DEAE	Pharmacia
Acrylamide		Canalco
N,N'-Methylenebisacrylamide		Canalco
N,N,N',N'-Tetramethylethylenediamine		Canalco
Glycine		Canalco
Riboflavine		Canalco
Ammonium peroxydisulfate		Canalco
Bromophenol blue		Canalco
Naphthol blue black		Canalco
Standard protein markers		Mann Res. Lab.

RESULTS

I. GROWTH DATA

The growth data for both dark- and light-grown bushbean seedlings for the first 2 weeks of development are shown in Fig. 7. As seen from these graphs, the roots grew at almost the same rate under both conditions, whereas the two components of the shoot system, the epicotyl and hypocotyl, showed marked differences in their pattern of growth under the two conditions. In the dark, the epicotyl grew only very slowly, while the emerging portion of the hypocotyl grew very rapidly. In light, however, the emerging hypocotyl grew rapidly up to the 8th day and thereafter grew only slowly, whereas the epicotyl grew slowly in light up to the 6th day and then began to grow at a faster rate. The beginning of this rapid growth by the epicotyl coincided with the period when the early high growth rate of the hypocotyl was noticeably slowing down. With both types of seedlings, however, summation of the root, hypocotyl and epicotyl data shows that the overall growth of the seedlings under light or dark conditions was sigmoidal in character.

The dry weight data shows similar relationships between the dark and the light grown seedlings (Fig. 8). Thus, in etiolated seedlings, the total dry weight decreased steadily during the entire first 2 weeks of growth while in light-grown seedlings, the total dry weight decreased only for the first 6 days of development and then increased markedly during the rest of the period to give a 55% increase in dry by the 14th day. The decrease in dry weight of the cotyledons of light-grown seedlings was much faster than that shown by cotyledons

FIG. 7. Growth curves of bushbean seedlings during the first 2 weeks of development.
 (A) Dark-grown seedlings; (B) Light-grown seedlings.

from the etiolated seedlings. At the 6th day stage, for example, the average dry weight of cotyledons from light-grown plants was only 105 mg compared to 205 mg for the cotyledons from etiolated seedlings. It should be noted that the depleted cotyledons on the light-grown seedlings dropped off the plants by the 12th day, whereas the cotyledons on the etiolated seedlings remained attached to the plants for the entire first 14 days of growth.

II. CHANGES IN THE NITROGEN FRACTIONS DURING EARLY GROWTH

The total nitrogen content of the seedlings was found to remain relatively constant in both experimental conditions during the 2 weeks of growth (Fig 9). As might be expected, however, there was a major redistribution of nitrogen from the cotyledons to the embryo during the first week (Fig. 9). After 8 days of growth, about 60% of the total nitrogen in the cotyledons of etiolated seedlings had been transported to the embryo, whereas almost 90% had been similarly moved during the same period in light-grown seedlings. Clearly, the rate of depletion of the cotyledonary nitrogen is more rapid in light-grown than in dark-grown seedlings. As seen from Fig. 9, the shoot was the main organ accumulating nitrogen during germination under both conditions; in the dark, the hypocotyl showed the highest gain in nitrogen, whereas in the light the epicotyl showed the greatest accumulation. These changes in nitrogen content followed a pattern quite similar to those for dry weight changes (Fig. 8) in the seedlings grown under both experimental conditions.

Significant differences were observed in the distribution of insoluble and soluble nitrogen in the light and dark-grown seedlings. In etiolated seedlings, the soluble nitrogen increased steadily from

FIG. 8. Changes in dry weight of bushbean seedlings during the first 2 weeks of development. (A) Dark-grown seedlings; (B) Light-grown seedlings.

FIG. 9. Changes in total nitrogen in the different organs of bushbean seedlings during the first 2 weeks of growth. (A) Dark-grown seedlings; (B) Light-grown seedlings.

about 15% of the total in 2-day-old seedlings up to approximately 55% of the total nitrogen by the 14th day. This increase is most rapid during the first 8 days of growth and as shown in Fig. 10A, the hypocotyl was found to be the main organ accumulating soluble nitrogen. In light-grown seedlings, the soluble nitrogen content reached a peak at 8 days, when it accounted for 40% of the total nitrogen of the seedling, and thereafter the content decreased to a level of 25% of the total nitrogen by the 14th day stage (Fig. 10B).

With regard to changes in the insoluble nitrogen content in the different organs of etiolated seedlings, the data in Fig. 11A show that this nitrogen fraction decreased more rapidly in the cotyledons than it concomitantly increased in the embryo. This resulted in a net decrease of insoluble nitrogen in the seedling (Fig. 11A). In light-grown seedlings, the rate of decrease in cotyledonary insoluble nitrogen was again more rapid than the corresponding rate of accumulation of this fraction in the embryo up to the 8th day (Fig. 11B). However, during the second week of growth, unlike the situation found in etiolated seedlings, the light-grown plants rapidly accumulated insoluble nitrogen compounds, indicating an increased utilization of the soluble nitrogen pool during this period (Figs. 10 and 11).

Detailed information about changes that occur in the amino acid components of the soluble nitrogen fractions during the growth period was obtained by chromatographic analysis of the fractions, extracted at two-day intervals, from both etiolated and light-grown seedlings. Tables 3 to 8 present the quantitative data on the free protein amino acid content of these fractions from cotyledons, roots and shoots. As

FIG. 10. Changes in soluble nitrogen in the different organs of bushbean seedlings during the first 2 weeks of growth. (A) Dark-grown seedlings; (B) Light-grown seedlings.

FIG. 11. Changes in insoluble nitrogen in the different organs of bushbean seedlings during the first 2 weeks of growth. (A) Dark-grown seedlings; (B) Light-grown seedlings.

AMINO ACID	DAYS OF GROWTH							
	DRY SEED	2	4	6	8	10	12	14
ASPARAGINE	0.36	3.30	3.60	3.70	2.10	1.25	1.15	0.69
SERINE	0.14	2.73	3.20	3.00	1.00	1.00	0.52	0.34
VALINE	0.32	0.60	0.61	0.55	0.20	0.09	0.12	0.10
ALANINE	0.52	1.02	1.05	0.90	0.25	0.17	0.19	0.08
THREONINE	0.60	1.31	1.15	1.12	0.35	0.17	0.18	0.10
GLUTAMATE	0.82	2.01	2.20	2.00	1.00	0.41	0.31	0.15
GLUTAMINE	Trace	Trace	Trace	Trace	Trace	-	-	-
ASPARTATE	0.27	0.80	0.70	0.60	0.40	0.25	0.30	0.18
LYSINE	0.24	1.50	1.20	0.90	0.50	0.30	0.25	0.10
ARGININE	0.38	0.60	0.50	0.44	0.25	0.21	0.18	0.10
LEUCINE & ISOLEUCINE	0.18	0.50	0.37	0.21	0.15	0.10	0.10	0.08
TRYPTOPHAN	0.08	0.39	0.40	0.05	Trace	-	-	-
PHENYLALANINE	0.09	0.46	0.48	0.32	0.17	0.05	0.10	0.05
TYROSINE	0.12	0.25	0.24	0.22	0.09	0.04	0.05	0.02
HISTIDINE	0.10	0.31	0.33	0.50	0.31	0.17	0.09	0.08
GLYCINE	0.16	0.14	0.13	Trace	-	-	-	-

TABLE 3. Changes in the concentration of free amino acids in the cotyledons of dark-grown bushbean seedlings sampled at 2-day intervals during the first 2 weeks of growth. Results are expressed in μ moles per two cotyledons.

AMINO ACID	DRY SEED	DAYS OF GROWTH						10	12	14
		2	4	6	8					
ASPARAGINE	0.36	0.40	0.30	0.11	0.02			-	-	-
SERINE	0.14	1.70	1.60	0.89	0.10			-	-	-
VALINE	0.32	0.40	0.70	0.45	0.07			-	-	-
ALANINE	0.52	1.20	1.03	0.48	0.07			-	-	-
THREONINE	0.60	0.90	0.81	0.55	0.08			-	-	-
GLUTAMATE	0.82	3.00	1.61	0.48	0.04			-	-	-
GLUTAMINE	Trace	0.04	0.05	0.03	Trace			-	-	-
ASPARTATE	0.27	0.21	0.19	0.19	0.02			-	-	-
LYSINE	0.24	0.25	0.20	0.17	Trace			-	-	-
ARGININE	0.38	0.25	0.25	0.18	Trace			-	-	-
LEUCINE & ISOLEUCINE	0.18	0.25	1.25	0.53	0.08			-	-	-
TRYPTOPHAN	0.08	0.41	0.43	0.01	Trace			-	-	-
PHENYLALANINE	0.09	0.49	0.39	0.22	0.02			-	-	-
TYROSINE	0.12	0.16	0.16	0.17	0.03			-	-	-
HISTIDINE	0.10	0.13	0.13	Trace	Trace			-	-	-
GLYCINE	0.16	0.12	Trace	Trace	-			-	-	-

TABLE 4. Changes in the concentration of free amino acids in the cotyledons of light-grown bushbean seedlings sampled at 2-day intervals during the first 2 weeks of growth. Results are expressed in μ moles per two cotyledons.

AMINO ACID	DRY SEED	DAYS OF GROWTH						
		2	4	6	8	10	12	14
ASPARAGINE	0.36	0.80	3.70	8.90	8.00	6.70	5.00	4.50
SERINE	0.14	0.23	1.13	1.77	1.53	1.14	0.71	0.40
VALINE	0.32	0.10	0.41	1.01	0.90	0.64	0.48	0.31
ALANINE	0.52	0.20	0.89	1.12	1.04	0.90	1.35	0.20
THREONINE	0.60	0.16	0.80	1.73	1.29	1.10	0.86	0.69
GLUTAMATE	0.82	0.10	0.29	0.30	0.21	0.19	0.16	0.11
GLUTAMINE	Trace	0.04	0.06	0.04	0.04	0.03	0.04	0.04
ASPARTATE	0.27	0.07	0.20	0.15	0.19	0.11	0.10	0.12
LYSINE	0.24	0.04	0.30	0.44	0.53	0.50	0.46	0.40
ARGININE	0.38	0.06	0.16	0.32	0.20	0.25	0.25	0.23
LEUCINE & ISOLEUCINE	0.18	0.05	0.41	0.70	0.70	0.75	0.50	0.12
TRYPTOPHAN	0.08	0.02	0.12	0.12	0.10	0.10	0.10	0.08
PHENYLALANINE	0.09	0.03	0.17	0.20	0.15	0.07	0.06	0.04
TYROSINE	0.12	0.04	0.12	0.07	0.06	0.07	0.06	0.05
HISTIDINE	0.10	-	Trace	Trace	Trace	Trace	Trace	Trace

TABLE 5. Changes in the concentration of free amino acids in the roots of dark-grown bushbean seedlings sampled at 2-day intervals during the first 2 weeks of growth. Results are expressed in μ moles per root system

AMINO ACIDS	DRY SEED	DAYS OF GROWTH						
		2	4	6	8	10	12	14
ASPARAGINE	0.36	0.15	2.00	2.50	2.50	2.20	1.00	0.90
SERINE	0.14	0.17	1.81	2.50		1.14	0.53	0.60
VALINE	0.32	0.06	1.12	1.50		0.70	0.49	0.40
ALANINE	0.52	0.18	1.10	1.00		0.50	0.23	0.20
THREONINE	0.60	0.08	0.80	1.17		0.89	0.75	0.70
GLUTAMATE	0.82	0.03	0.11	0.12		0.09	0.09	0.09
GLUTAMINE	Trace	0.02	0.01	0.01	Trace	Trace	Trace	Trace
ASPARTATE	0.27	0.07	0.20	0.27		0.14	0.07	0.05
LYSINE	0.24	0.01	0.39	0.51		0.42	0.42	0.30
ARGININE	0.38	0.11	0.20	0.38		0.38	0.31	0.40
LEUCINE & ISOLEUCINE	0.18	0.06	1.10	1.10		0.50	0.32	0.30
TRYPTOPHAN	0.08	0.01	0.10	0.10		0.08	0.06	0.06
PHENYLALANINE	0.09	0.02	0.11	0.10		0.03	0.02	0.02
TYROSINE	0.12	0.01	0.17	0.10		0.05	0.04	0.04
HISTIDINE	0.10	Trace	Trace	Trace		-	-	-

TABLE 6. Changes in the concentration of free amino acids in the roots of light-grown bushbean seedlings sampled at 2-day intervals during the first 2 weeks of growth. Results are expressed in μ moles per root system.

AMINO ACIDS	DRY SEED	DAYS OF GROWTH						
		2	4	6	8	10	12	14
ASPARAGINE	0.36	1.60	4.50	4.20	24.40	36.80	39.00	40.00
SERINE	0.14	0.44	1.50	2.70	8.70	10.50	11.50	11.50
VALINE	0.32	0.16	0.40	1.16	6.00	5.40	10.30	11.50
ALANINE	0.52	0.40	0.70	2.50	6.90	8.10	10.30	10.50
THREONINE	0.60	0.32	0.67	1.90	5.00	7.20	7.00	8.10
GLUTAMATE	0.82	0.20	0.14	0.24	0.50	0.40	0.61	0.65
GLUTAMINE	Trace	0.01	0.01	Trace	Trace	Trace	Trace	Trace
ASPARTATE	0.27	0.12	0.36	0.36	1.80	2.20	3.10	2.70
LYSINE	0.24	0.01	0.25	1.03	2.30	2.60	7.10	6.00
ARGININE	0.38	0.12	0.40	0.57	2.50	2.50	3.60	4.80
LEUCINE & ISOLEUCINE	0.18	0.09	0.40	1.50	3.50	5.40	7.85	7.50
TRYPTOPHAN	0.08	0.04	0.30	0.31	0.60	1.00	0.88	0.65
PHENYLALANINE	0.09	0.05	0.48	0.82	3.90	2.40	3.80	3.60
TYROSINE	0.12	0.08	0.28	0.48	2.03	2.10	2.70	3.30
HISTIDINE	0.10	Trace	Trace	Trace	Trace	Trace	Trace	Trace

TABLE 7. Changes in the concentration of free amino acids in the shoots of dark-grown bushbean seedlings sampled at 2-day intervals during the first 2 weeks of growth. Results are expressed in μ moles per shoot system.

AMINO ACIDS	DRY SEED	DAYS OF GROWTH						
		2	4	6	8	10	12	14
ASPARAGINE	0.36	0.53	5.10	12.34	14.70	14.10	13.70	7.70
SERINE	0.14	0.19	2.40	6.35	10.10	5.50	4.30	3.00
VALINE	0.32	0.20	1.20	2.10	3.70	2.55	2.05	2.10
ALANINE	0.52	0.12	1.00	4.20	3.80	4.70	2.50	1.70
THREONINE	0.60	0.14	1.10	2.67	3.00	3.05	2.80	2.00
GLUTAMATE	0.82	0.02	1.06	0.56	0.40	0.61	0.55	0.70
GLUTAMINE	Trace	0.02	0.02	1.00	Trace	Trace	Trace	Trace
ASPARTATE	0.27	0.08	0.30	0.93	0.90	1.05	0.98	0.84
LYSINE	0.24	0.02	0.02	0.01	Trace	Trace	Trace	Trace
ARGININE	0.38	0.14	0.50	0.89	1.00	0.70	0.45	0.30
LEUCINE & ISOLEUCINE	0.18	0.04	0.50	1.60	2.60	1.07	0.80	0.60
TRYPTOPHAN	0.08	0.06	0.24	0.31	0.30	0.30	0.26	0.23
PHENYLALANINE	0.09	0.04	0.17	0.80	0.80	0.64	0.57	0.35
TYROSINE	0.12	0.11	0.47	1.10	0.54	0.48	0.16	0.11
HISTIDINE	0.10	Trace	Trace	Trace	-	-	-	-

TABLE 8. Changes in the concentration of free amino acids in the shoots of light-grown bushbean seedlings sampled at 2-day intervals during the first 2 weeks growth. Results are expressed in μ moles per shoot system.

seen from the results, the amino acid composition differed considerably not only between organs from seedlings at different stages of growth, but also between the same organs taken from seedlings grown under the two experimental conditions.

The cotyledons of dark-grown seedlings showed a large increase in free protein amino acids during the first 4-6 days of germination (Table 3) followed by a slow decrease during the rest of the growth period. On the other hand, the cotyledons of light-grown seedlings showed some increase in these amino acids during the first 2 days, but thereafter showed a gradual decrease in concentration (Table 4). The roots from both kinds of seedlings showed a peak in the concentration of free protein amino acids by the 6th day, and then the amounts of each acid, or acid amide, slowly decreased (Tables 5 and 6). The most significant differences in composition of the free protein amino acid fractions were observed in shoots grown under light and dark conditions (Tables 7 and 8). In this organ, almost all the protein amino acids were found to accumulate in dark-grown tissue during the first 2-weeks of growth, whereas in light-grown shoot tissue, the accumulation of these amino acids reached a peak by the 8th day and thereafter their amounts rapidly diminished.

As illustrated by the graphs in Fig. 12, asparagine, serine, valine, alanine and threonine, were the amino acids that showed the greatest change in concentration in both light and dark grown organs during the 2-week period of growth. These five amino acids consistently accounted for 60-90% of the soluble amino nitrogen fraction at all stages of growth. Asparagine was by far the predominant amino

FIG. 12. Quantitative changes in the major free amino acids in bushbean seedlings during the first 2 weeks of growth. (A) Dark-grown seedlings; (B) Light-grown seedlings.

compound present, and constitutes 40-50% of the total free protein amino acid fraction in both kinds of seedlings by the 14th day. In striking contrast, the other commonly found plant amide, glutamine, was present only in trace amounts at all stages of growth.

Considering each organ individually, asparagine, serine, threonine, lysine, and glutamic acid were the main free amino acids present in etiolated cotyledons, and accounted for more than 75% of the free protein amino acid fraction in this organ by the second day of growth (Table 3). Glutamic acid, serine and alanine were the predominant free amino acids in the cotyledons of light-grown seedlings and these three compounds accounted for almost 60% of the free protein amino acid fraction on the second day (Table 4). In these cotyledons, asparagine was found in extremely low concentration, which is in marked contrast to the amount of this amide found in cotyledons of etiolated seedlings.

Changes in the concentrations of free protein amino acids in the roots were found to be similar in both etiolated and light-grown seedlings, except for asparagine, which was found in much higher concentration in the roots of dark-grown plants (Tables 5 and 6). The situation in shoots was more interesting, since major differences in the concentration of amino acids were observed in this organ when grown under the two conditions (Tables 7 and 8). For example, the nitrogen-rich amino acids, asparagine, lysine and arginine, while present in very high concentration in etiolated shoots, were found in relatively low amounts in light-grown tissue.

Figures 13 and 14 present graphically the data for changes in the

FIG. 13. Changes in the free tryptophan, phenylalanine, and tyrosine contents in the shoots (■), roots (●), and cotyledons (▲) of etiolated bushbean seedlings during the first 2 weeks of development.

FIG. 14. Changes in the free tryptophan, phenylalanine, and tyrosine contents in the shoots (■), roots (●), and cotyledons (▲) of light-grown bushbean seedlings during the first 2 weeks of development.

amounts of free aromatic amino acids found in the different organs of seedlings grown under the two experimental conditions. The curves for the cotyledon and root tissues indicate that there was no appreciable difference in the respective levels of these three amino acids in each organ when the parent seedlings had been grown in either light or dark conditions. The changes observed in shoot tissue, however, were quite different from those found in the cotyledons and roots. During the first 6 days of growth, the shoots of both kinds of seedlings accumulated all three aromatic amino acids, but during the second week of growth, only the dark-grown shoots continued to accumulate these amino acids, while their levels in light-grown shoots decreased quite strikingly. Although free tryptophan, phenylalanine and tyrosine were found in relatively small amounts in all three organs examined, irrespective of whether the parent seedlings were grown in light or dark conditions, the quantitative changes observed during the two-week period followed a pattern similar to that observed for other amino acids that were found in much larger amounts, such as asparagine, valine, alanine, serine and threonine, (Fig. 12).

It should be noted that in these quantitative studies of the free protein amino acid components of the soluble nitrogen fractions, the four amino acids, proline, cysteine, cystine and methionine, were always detected on paper chromatograms of the soluble fractions from all organs. The amounts present, however, could not be measured quantitatively with the colorimetric technique used in this investigation and for this reason, it should be emphasized that the data given in Tables 3-8 do not present, therefore, a complete

quantitative analysis of the free protein amino acids occurring in the soluble nitrogen fractions isolated from bushbean seedlings.

A further point that should be noted is that summation of the values for each 2-day sample recorded in Tables 3-8 show that only about 50% of the total soluble nitrogen fraction, as previously determined by Nesslerization of the TCA-soluble fraction from comparable tissue (Fig. 10), was recovered as free protein amino acids measurable by the cadmium acetate-ninhydrin colorimetric procedure. To account for this loss, in addition to the 10-15% known to be due to the analytical procedures employed, the five protein amino acids and all the non-protein amino acids, soluble nucleotides and ammonia, which were not quantitatively analyzed, would most likely account for most of the remaining loss in soluble nitrogen that occurs during purification of these fractions.

III. APPEARANCE AND DEVELOPMENT OF ALIPHATIC AMINOTRANSFERASE ACTIVITY.

The appearance and subsequent development of aminotransferase activity for the six protein amino acids which showed the greatest quantitative changes over the 2-week period of growth, was next investigated. Figures 15 and 16 show the development of transaminase activities between aspartic acid, alanine and asparagine and the amino group acceptor, α -ketoglutaric acid, in different organs taken at 2-day intervals from both light and dark grown seedlings. In preliminary experiments with these three amino acids, α -ketoglutaric, oxaloacetic and pyruvic acids were all tested as the amino group acceptor for each transaminase reaction, and it was found that α -ketoglutarate consistently promoted the highest level of activity. Total activity for each

FIG. 15. Development of total activity of alanine- α -ketoglutarate (Δ), aspartate- α -ketoglutarate ($\blacksquare$), and asparagine- α -ketoglutarate ($\bullet$) transaminases in roots (R) and shoots (S) of bushbean seedlings grown in darkness or in light.

FIG. 16. Development of total activity of alanine- α -ketoglutarate (Δ), aspartate- α -ketoglutarate ($\blacksquare$), and asparagine- α -ketoglutarate ($\bullet$) transaminases in the cotyledons of bushbean seedlings grown in darkness (A) or in light (B).

aminotransferase is therefore expressed in terms of μ moles of glutamic acid formed per organ per hour of incubation.

As Figures 15 and 16 show, close similarity was found between the patterns of development of aspartate- α -ketoglutarate, alanine- α -ketoglutarate, and asparagine- α -ketoglutarate transaminase activities in seedlings grown under the two experimental conditions. Valine- α -ketoglutarate transaminase activity was also detected in all organs during the 2-week period of growth under both conditions, but the activity of this enzyme was consistently 15 to 20 fold less than that of the alanine aminotransferase activity in all organs. Assays for serine- and threonine- α -ketoglutarate transaminase activities were also carried out, but no evidence for the presence of enzymes catalyzing these reactions was detected in any organ during the 2-week growth period.

IV. APPEARANCE AND DEVELOPMENT OF AROMATIC AMINOTRANSFERASE ACTIVITY.

The appearance and development of the three aromatic amino acid- α -ketoglutarate transaminase activities was also examined in seedlings grown under light or dark conditions for the first 2 weeks of growth. The results obtained are presented in Figures 17 and 18. Total activity is expressed in μ moles of glutamate per organ per hour of incubation, and specific activity is expressed in μ moles of glutamate per mg protein per hour of incubation.

As seen from Figures 17 and 18, the development of all three aromatic amino acid transaminase activities followed very similar patterns in the different organs of seedlings grown under each experimental condition. For all three activities, the highest total

FIG. 17. Development of total activity (A) and specific activity (B) for the three aromatic aminotransferase systems, (■ tryptophan, ▲ phenylalanine, ● tyrosine) in shoots (S), roots (R), and cotyledons (C) of etiolated bushbean seedlings during the first 2 weeks of growth.

FIG. 18. Development of total activity (A) and specific activity (B) for the three aromatic aminotransferase systems (■ tryptophan, ▲ phenylalanine, ● tyrosine) in the shoots (S), roots (R), and cotyledons (C) of light-grown bushbean seedlings during the first 2 weeks of growth.

activity was found in the shoots of light-grown seedlings at 14 days, while the highest specific activity was observed in roots by the 8th day of growth. The total activity in cotyledons was found to decrease more rapidly in dark-grown than in light-grown seedlings, and this finding is in accord with the changes observed in non-soluble nitrogen in the cotyledons of seedlings grown under the two experimental conditions (Fig. 11). A 2-3 fold increase in specific activity was reached in the cotyledons by the 10th day in darkness, and by the 6th day in light. This increase in specific activity in cotyledons may not necessarily reflect de novo synthesis of the aromatic amino acid transaminase, since the total activity of the enzyme(s) decreased steadily during this period. The peak of specific activity could be attributed to a more rapid hydrolysis of the storage protein during this period. The development of total activity, and changes in specific activity in roots were found to be similar in both types of seedlings. Thus, a two-fold increase in specific activity and an eight-fold increase in total activity were observed during the first 8 days of growth, but during the second week, both the total and specific activities decreased. In shoots, the total activity of the aromatic aminotransferase system increased continuously during the 2-week period in both types of seedlings (Figs. 17A and 18A). The specific activity similarly increased in etiolated shoots for at least the first 10 days, whereas in light-grown shoots, the specific activity only increased up to the 4th day and thereafter steadily diminished such that by the end of the 2-week period, the specific activity of this transaminase system was only half that of the comparable system in etiolated shoots (Figs. 17B and 18B).

V. INTRACELLULAR DISTRIBUTION OF AROMATIC AMINOTRANSFERASE AND ASPARTATE AMINOTRANSFERASE ACTIVITIES.

The intracellular distribution of aromatic aminotransferase and aspartate aminotransferase activities was investigated in roots from 8-day old etiolated seedlings. Table 9 presents the mean results from four experiments of this kind. While 40% of the total protein was found in the particulate fractions, only about 10% of the aromatic aminotransferase and aspartate aminotransferase activities were found in these fractions. The fraction referred to as the mitochondrial fraction had only 4% of the total activity. On the other hand, the activity of the "soluble" enzyme, which remained in the supernatant fraction left after centrifugation at 10,000 x g, accounted for about 90% of the total activity.

The intracellular distribution of the aromatic aminotransferase and aspartate aminotransferase activities in roots of bushbean seedlings therefore resembles that found for tryptophan- α -ketoglutarate transaminase in 4-day old mung bean seedlings (Wightman and Cohen, 1968) and that found for aspartate aminotransferase in pumpkin seedlings (Splittstoesser, 1970) and in pea cotyledons (Wong and Cossins, 1969). This distribution of the aromatic and aspartic acid aminotransferase in plant cells is, however, quite different from that found in animal tissues where more than 60% of the aromatic aminotransferase activity is found in the mitochondrial fraction (Semba and Civen, 1970; Milles and Litwack, 1969).

Table 9. Intracellular distribution of aromatic aminotransferase and aspartate aminotransferase activities in roots of 8-day old etiolated bushbean seedlings as determined by differential centrifugation of root homogenates.

FRACTION	CENTRIFUGATION SPEED		PROTEIN		ACTIVITY	
	xg		mg	%	Total Units	%
Crude extract	1,000		1140	100	I	3.20
					II	2.39
					III	1.98
					IV	39.10
						100
						100
						100
						100
Plastid Fraction	2,500		340	30	I	0.19
					II	0.14
					III	0.13
					IV	2.32
						6.0
						5.8
						6.5
						5.9
						+1.0*
Mitochondrial Fraction	10,000		110	10	I	0.13
					II	0.10
					III	0.08
					IV	1.48
						4.1
						4.2
						3.9
						3.8
						+1.0
Residual Supernatant	----		690	60	I	2.82
					II	2.12
					III	1.73
					IV	35.40
						88.0
						89.0
						87.5
						91.0
						+3.0

* % variation between experiments (4 experiments were performed). I - Phenylalanine- α -ketoglutarate transaminase activity; II - Tyrosine- α -ketoglutarate transaminase activity; III - Tryptophan- α -ketoglutarate transaminase activity; IV - aspartate- α -ketoglutarate transaminase activity.

VI. PURIFICATION OF AROMATIC AMINOTRANSFERASE.

All experiments described here were performed with roots of 8 day old etiolated bushbean seedlings. Table 10 shows the main steps of a typical purification procedure. pH treatment of the "crude total extract" was the initial step of purification, and this removed a considerable fraction of inactive protein without affecting the transaminase activity. Further increases of specific activity resulted from ammonium sulphate fractionation and column chromatography using Sephadex G-100 and G-200, and also DEAE-Sephadex. The final purification obtained, calculated on the basis of specific activity, was 570-620 fold for the aromatic amino acid and aspartate aminotransferase reactions. The recovery, calculated on the basis of total units, was 30-35% for all four types of transaminase activity assayed.

The chromatographic behavior of the enzyme preparation on Sephadex G-200 is shown in Fig. 19. This fraction had two sharp and distinctive protein peaks and the transaminase activity coincided with the first protein peak. The fractions making up this small active peak were collected together and, after dialysis against 0.05 M Tris-HCl at pH 7.5, containing EDTA, 2-mercaptoethanol and 0.2 M KCl, the total volume was applied to the top of a DEAE-Sephadex column. The protein was eluted with an identical buffer and the elution pattern is shown on Fig. 20. Only one protein peak was detected and the activity of all four transaminase reactions assayed were associated with this peak. This purified fraction was dialyzed against 50 vols of 0.05 M Tris-HCl at pH 8.5, containing EDTA and

FIG. 19. Chromatography of the partially purified aromatic aminotransferase preparation of bushbean roots on Sephadex G-200.
 (●) Optical density at $280m\mu$.
 (○) Relative activity.

FIG. 20. Rechromatography of the aromatic aminotransferase from bushbean roots on DEAE-Sephadex.
 (●) Optical density at 280 $m\mu$. (○) Activity.

Table 10 Purification of the aromatic aminotransferase from the roots of 8-day old bushbean seedlings (*Phaseolus vulgaris* L.).

FRACTION	PROTEIN mg	TOTAL ACTIVITY units	SPECIFIC ACTIVITY units/mg protein	RECOVERY %	PURIFICATION fold
Crude total extract	I* II III IV	4.90 3.68 3.00 56.40	0.0028 0.0021 0.0017 0.0322	100 100 100 100	----- ----- ----- -----
pH treated fraction	I II III IV	4.57 3.57 3.12 53.50	0.0045 0.0032 0.0028 0.0480	93.5 97.0 104.0 95.0	1.6 1.5 1.6 1.4
(NH ₄) ₂ SO ₄ (40-75%) fraction	I II III IV	3.48 2.52 2.20 36.00	0.0132 0.0095 0.0083 0.1360	71.0 69.0 73.5 67.5	4.7 4.5 4.9 4.2
Sephadex G-100 fraction	I II III IV	2.95 2.24 1.92 34.50	0.0492 0.0374 0.0320 0.5750	60.0 61.0 64.0 61.0	17.5 17.8 18.8 17.8

* I: phenylalanine- α -ketoglutarate transaminase activity; II: tyrosine- α -ketoglutarate transaminase activity; III: tryptophan- α -ketoglutarate transaminase activity; IV: aspartate- α -ketoglutarate transaminase activity.

Assay conditions:

0.25 ml of protein solution was incubated with 4×10^{-2} M amino acid and 8×10^{-3} M

α -ketoglutarate for one hour at 35°C. Final volume: 0.5 ml, 0.05 M TRIS-HCL. Final pH: 8.5.

Table 10 (continued) Purification of the aromatic aminotransferase from the roots of 8-day old bushbean seedlings (*Phaseolus vulgaris* L.).

FRACTION	PROTEIN mg	TOTAL ACTIVITY units	SPECIFIC ACTIVITY units/mg protein	RECOVERY %	PURIFICATION fold
DEAE- Sephadex fraction	I* II III IV	2.60 1.79 1.65 28.20	0.3250 0.2240 0.2042 3.5200	53.0 48.0 55.0 50.0	116.0 106.0 120.0 109.0
Sephadex G-200 fraction	I II III IV	1.90 1.33 1.10 22.80	0.7600 0.5330 0.4400 3.5200	39.0 36.0 37.0 40.0	272.0 254.0 260.0 282.0
DEAE- Sephadex rechroma- tography	I II III IV	1.72 1.23 1.05 18.50	1.7200 1.2300 1.0500 18.5000	35.0 33.0 35.0 30.0	615.0 590.0 620.0 570.0

* I: phenylalanine- α -ketoglutarate transaminase activity; II: tyrosine- α -ketoglutarate transaminase activity; III: tryptophan- α -ketoglutarate transaminase activity; IV: aspartate- α -ketoglutarate transaminase activity.

Assay Conditions:

0.25 ml of protein solution was incubated with 4×10^{-2} M amino acid and 8×10^{-3} M α -ketoglutarate for one hour at 35°C. Final volume: 0.5 ml, 0.05 M TRIS-HCL. Final pH: 8.5.

2-mercaptoethanol and was diluted to a concentration of 25 μg protein/ml with this buffer. This 600-fold purified enzyme fraction was then divided into 3 ml samples and stored at -10°C . At this temperature, the enzyme was found to be stable for at least 6 months showing no loss in activity during this time. In all purification steps, the ratio of aspartate aminotransferase activity to phenylalanine aminotransferase activity was found to vary between 10 and 12. The tyrosine aminotransferase activity was about 75% that of the phenylalanine aminotransferase, and the tryptophan aminotransferase activity was 60-70% that of the phenylalanine aminotransferase.

VII. POLYACRYLAMIDE GEL ELECTROPHORESIS

25-50 μg of protein from the purified enzyme fraction was applied to the top of a polyacrylamide gel column (prepared as described previously) and electrophoresis was carried out with a current of 3 milliamp. per column. The protein from the purified enzyme fraction gave a single band when stained with amido black. This band migrated towards the anode under our experimental conditions (Fig. 21, B), and no protein band was found to migrate towards the cathode.

The aromatic aminotransferase activity was located by incubation of the gel with the substrates at the concentrations given in Materials and Methods. The green product formed by the reaction of phenazine methosulfate with the arylpyruvic acid formed was detected on the gels after only a few minutes of incubation. Fig. 21, A shows that after 30 minutes of incubation only one product-specific stained band appeared on the gel, and that this band was found at the same level of the gel for all three aromatic aminotransferase activities. Furthermore, this product-specific stained band coincided with the protein stained band.

In summary, the results presented in the last three sections have shown that all four aminotransferase activities studied were distributed

FIG. 21. Gel electrophoresis of 600-fold purified aromatic aminotransferase from bushbean roots. Polyacrylamide gels runned about 60 min. at 3 milliamp. per gel. (B) 50 μ g protein stained with Amido black. (A) Product staining of the gels by the phenazine methosulfate procedure. 50 μ g of protein. 1.- tryptophan- α -ketoglutarate transaminase activity; 2.- tyrosine- α -ketoglutarate transaminase activity; 3.- phenylalanine- α -ketoglutarate transaminase activity. S, starting point; F, front line.

identically in the subcellular fractions of bushbean seedlings, that the ratio of these transaminase activities remained the same during the different steps of purification, that only one protein band was revealed by polyacrylamide gel analysis and that this band coincided with the product-specific staining band of aromatic aminotransferase activity. These facts strongly suggest that the same enzymatic protein is responsible for catalyzing the aromatic amino acid- α -ketoglutarate and the aspartate- α -ketoglutarate transaminase reactions in roots of bushbean seedlings.

VIII. PROPERTIES OF THE AROMATIC AMINOTRANSFERASE

1. Molecular weight determination

It has been recently demonstrated that the order of elution of proteins from a Sephadex G-200 column correlates with the Stoke's radius of these proteins, but not always with their molecular weight (Siegel and Monty, 1966). However, using an extensive series of proteins selected for their globular shape, Andrews (1964; 1965) demonstrated a good correlation between elution volume from column chromatography on Sephadex G-75, G-100 and G-200, and the logarithm of the molecular weight of the proteins, as determined by other techniques. The molecular weight of mammalian aspart aminotransferase has been determined by the technique of gel filtration (Michuda and Martinez-Carrion, 1969) and by sedimentation analysis (Jenki et al., 1959; Michuda and Martinez-Carrion, 1969), and the results from both techniques were found to be quite comparable.

While recognizing that the Sephadex filtration technique can give an accurate measurement of the Stoke's radius of a protein, but does not always give a reliable estimate of its molecular weight, an estimation of the molecular weight of the aromatic aminotransferase purified from bushbean roots was carried out in this investigation using Andrews' technique of Sephadex G-200 chromatography.

The protein from the purified enzyme fraction was precipitated with ammonium sulfate and 1 mg of protein was redissolved in 1 ml of 0.05 M Tris-HCl buffer containing EDTA, 2-mercaptoethanol and 0.1 M KCl and then applied to the top of the column. The elution volume (V_e) for the proteins of known molecular weights (Fig. 22) and for the enzyme (Fig. 23) were determined from the elution profiles. By measuring the elution volumes of the proteins of known molecular weights, the corresponding K_{av} values were calculated. The K_{av} value is a constant for a given compound when chromatographed on a specific Sephadex gel. A plot of K_{av} values against the logarithm of the molecular weight of each substance is called a selectivity curve, which is linear within the fractionation range of Sephadex G-200 (cf. Sephadex calibration kit instruction manual). If the K_{av} value is determined for a protein of unknown molecular weight, then the molecular weight of that protein can be estimated directly from the selectivity curve. The K_{av} values for each protein standard can be calculated by using the following equation:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e is the elution volume for the protein, V_o is the elution volume for Blue Dextran 2000 (37-39 ml), and V_t is the total bed volume (120-124 ml). Figure 24 shows a typical selectivity curve for the standard proteins chromatographed on Sephadex G-200. By using this selectivity curve, the molecular weight of the aromatic aminotransferase enzyme was estimated on the basis of six determinations, to be approximately 128,000 daltons.

Verjee and Evered (1969) reported that the apparent molecular weight of glutamate-oxaloacetate transaminase from wheat germ was about 75,000, as measured by Sephadex G-100 gel filtration. This is the only report, to date, of a molecular weight determination on a transaminase isolated from plants. The molecular weight found in the present

FIG. 22. Elution pattern of marker proteins of known molecular weight from Sephadex G-200 column. 2.5 ml fractions were collected.

FIG. 23. Elution pattern of the purified aromatic aminotransferase from Sephadex G-200 calibrated column. 2.5 ml fractions collected.

FIG. 24. Selectivity curve for the standard proteins and the aromatic aminotransferase chromatographed on Sephadex G-200.

investigation for the aromatic aminotransferase from bushbean seedlings is comparable to that reported for tyrosine aminotransferase isolated from rat liver (Valeriote et al., (1969). These workers found that the tyrosine aminotransferase purified from rat liver had a molecular weight of 115,000 daltons as calculated from sedimentation equilibrium studies. The molecular weight of the bushbean enzyme, however, was found to be in the same order of magnitude than that of all the aspartate aminotransferases isolated from animal sources, which varied between 91,200 and 120,000 daltons (see Table 1 for details).

2. Effect of pH on the transamination reaction

The pH optima reported for the activity of plant transaminases are as follows: pH 8.0 for aspartate- α -ketoglutarate aminotransferase in cauliflower buds and pea cotyledons (Ellis and Davies, 1961; Wong and Cossins, 1969); pH 8.0-8.5 for the same enzyme in wheat germ (Verjee and Evered, 1969); pH 8.5 for aromatic amino acid- α -ketoglutarate transaminase from leaves of Quercus pedunculata (Gadal et al., 1969); pH 8.5 for tryptophan- α -ketoglutarate transaminase in mung bean seedlings (Wightman and Cohen, 1968) and pH 8.0 for the same enzyme in tomato shoots (Gibson, Barrett and Wightman, 1971).

Figure 25 shows the pH curves for the three aromatic amino acid transaminase activities of the purified enzyme fraction, and Fig. 26 shows the pH curve for the aspartate- α -ketoglutarate activity. 0.05 M citrate-phosphate buffer was used for the lower pH range 6.0 to 7.0, 0.05 M TRIS-HCl buffer for the pH range 7.0 to 8.5, and 0.05 M glycine-KOH for the pH range 8.5 to 11.0. No great variation in activity ($< \pm 10\%$) was encountered between the different buffers used at the same pH. It is evident from the curves in these two Figures, that the optimum pH was 8.5 for all four transaminase activities assayed in the

FIG. 25. pH curves for the three aromatic amino acid transaminase activities of the purified enzyme. ($\blacktriangle$) phenylalanine- α -ketoglutarate, ($\blacksquare$) tyrosine- α -ketoglutarate and ($\bullet$) tryptophan- α -ketoglutarate transaminase activity. Citrate-phosphate buffer for pH range 6.0 to 7.0; 0.05 M TRIS-HCl for pH range 7.0 to 8.5; and 0.05 M glycine KOH for pH range 8.5 to 11.0.

FIG. 26. pH curve for aspartate- α -ketoglutarate activity. 0.05 M citrate-phosphate buffer for pH range 6.0 to 7.0; 0.05 M TRIS-HCl for pH range 7.0 to 8.5; and 0.05 M glycine-KOH for pH range 8.5 to 11.0.

purified enzyme fraction. This optimum pH value for the aromatic aminotransferase of bushbean seedlings is therefore, in agreement with the values reported above for the aromatic aminotransferases isolated from other plants.

3. Pyridoxal phosphate requirement:

Mammalian aminotransferases have been resolved into their apoenzyme and coenzyme components by many workers (O'Kane and Gunsalus, 1947; Bank and Vernon, 1961; Wada and Snell, 1962) and quantitative determination of the pyridoxal phosphate content per molecule of holoenzyme has been made (Jenkins et al., 1959; Wada and Morino, 1964; Martinez-Carrion and Tiemeier, 1967; Bertland and Kaplan, 1968; 1970 (see Table 1). Aspartate aminotransferase extracted from different mammalian tissues was found to contain 2 moles of pyridoxal phosphate per mole of holoenzyme and tyrosine aminotransferase was found to bind approximately 4 moles of pyridoxal phosphate per mole of protein (Valeriote et al., 1969). Wong and Cossins (1966, 1969) resolved an aspartate aminotransferase from germinating pea cotyledons and demonstrated its absolute requirement for pyridoxal and pyridoxamine phosphate.

In the present study, resolution of the aromatic aminotransferase into its protein and coenzyme moieties was not achieved. The purified enzyme did not show appreciable increase in activity when pyridoxal phosphate was added to the reaction mixture (Table 11) which suggests that the coenzyme was tightly bound to the apoenzyme. A similar observation was made by Gibson et al. (1971) using an aromatic aminotransferase preparation from tomato shoots. After fractional purification of transaminases from wheat germ (Cruickshank and Isherwood,

Table 11. Effect of pyridoxal phosphate (PLP) upon the activity of the purified aromatic aminotransferase isolated from bushbean seedlings.

Amino acid Substrate	Keto acid Substrate	Enzyme activity (units/mg Protein)	
		Without PLP	With PLP
Phenylalanine	α -ketoglutarate	1.70	1.80
Tyrosine	α -ketoglutarate	1.22	1.21
Tryptophan	α -ketoglutarate	1.13	1.15
Aspartate	α -ketoglutarate	18.30	18.35

Assay conditions: 0.25 ml of enzyme was preincubated for 10 min. with 0.125 ml of amino acid (final concentration 4×10^{-2} M). 0.125 ml of α -ketoglutarate solution (final concentration 8×10^{-3} M) containing, or not containing pyridoxal phosphate to a final concentration of 2×10^{-4} M, was added to start the reaction. The reaction was stopped after 30 min. with 0.5 ml of hot methanol. Glutamic acid formed was determined, after chromatography, by the cadmium acetate-ninhydrin colorimetric procedure.

1958), cauliflower (Ellis and Davies, 1961) and pea cotyledons (Wong, 1968), a partial dependence upon pyridoxal phosphate could be demonstrated, although considerable transamination was still observed in the absence of added coenzyme. Thus it appears that plant transaminases differ from the animal enzymes in the much tighter binding encountered between protein and coenzyme moities.

4. Effect of inhibitors of transamination on the activity of the enzyme.

It is known that sulfhydryl reagents such as iodoacetate, iodoacetamide, and p-chloromercuribenzoate (p-CMP) inhibit enzyme reactions requiring free -SH groups (Webb, 1966). It is also known that isonicotinic acid hydrazide is an antagonist of pyridoxal phosphate and will, therefore, inhibit the activity of enzymes which have pyridoxal phosphate as their coenzyme (Webb, 1966).

In the present investigation, experiments were conducted to study the effect of such inhibitors on the purified enzyme and the results are shown in Table 12. All the compounds tested inhibited the aminotransferase activity by 30 to 45%. Since isonicotinic acid hydrazide showed a strong inhibitory effect, this observation suggests that pyridoxal phosphate is essential for optimum aromatic aminotransferase activity. Inhibition of transaminase activity by iodoacetate, iodoacetamide and p-CMP indicates that the aromatic aminotransferase from bushbean requires -SH groups for optimum activity which is similar to earlier findings with mammalian transaminases (Hopper and Segal, 1962; Turano and Riva, 1964) and other plant aminotransferase (Turano et al., 1966; Wong and Cossins, 1969).

Table 12. Effect of various inhibitors on the activity of the purified aromatic aminotransferase.

Inhibitor Tested	Amino Acid Substrate	Concentration of Inhibitor (3.3×10^{-3} M)	Concentration of Inhibitor (6.6×10^{-3} M)
		Activity %	Activity %
No inhibitor	I	100	100
	II	100	100
	III	100	100
	IV	100	100
Isonicotinic acid hydrazide	I	80	57
	II	78	58
	III	77	57
	IV	78	56
Iodoacetate	I	84	60
	II	85	58
	III	84	59
	IV	82	59
Iodoacetamide	I	90	69
	II	88	66
	III	88	67
	IV	89	65
p-Chloromercuribenzoate	I	55	57
	II	56	55
	III	58	55
	IV	56	58

Assay conditions: 0.25 ml of enzyme solution was preincubated with 0.125 ml of inhibitor solution (final conc. 3.3×10^{-3} M or 6.6×10^{-3} M) for 10 min. at 35°C. The reaction was started by the addition of 0.125 ml of a solution containing an amino acid (I. - phenylalanine, II. - tyrosine, III. - Tryptophan, IV. - aspartic acid) and α -ketoglutarate (final conc. 4×10^{-2} M and 8×10^{-3} M, respectively). Final volume: 0.50 ml, 0.05 M TRIS-HCL, pH 8.5. After one hour incubation the reaction was stopped by the addition of 0.50 ml of hot methanol. Glutamic acid was determined, after chromatography, by the cadmium acetate-ninhydrin colorimetric procedure.

5. Effect of Cations:

Although the role of metal ions in enzymic transamination reactions is not clear, there have been reports of activation of transaminases by various cations (Happold and Turner, 1957; Nadkarni and Sohoni, 1963). Patwarhan (1960) showed the participation of Fe^{++} ion as a cofactor in glutamate-oxaloacetate transaminase isolated from Dolichos lablab. More recently, Wong (1968) showed that the addition of Mg^{++} , and Mn^{++} stimulated aspartate aminotransferase activity.

Table 13 shows the effect of various cations on the aromatic aminotransferase and aspartate aminotransferase activities of the purified enzyme from bushbean seedlings. As shown in this table, all four activities were stimulated about 13% and 18% by Ca^{++} and Mg^{++} respectively, and inhibited about 5% by Zn^{++} . The three cations Mn^{++} , Cu^{++} , and Fe^{++} showed no effect on transaminase activity. Our finding that the purified aminotransferase from bushbean roots does not require Fe^{++} as a cofactor is in agreement with the results of Verjee and Evered (1969) who showed that this cation had no effect on the activity of the glutamate-oxaloacetate transaminase from wheat germ.

6. Specificity of the aromatic aminotransferase

Table 14 presents the results of an investigation on the amino acid substrate specificity of the purified aromatic aminotransferase. α -Ketoglutaric acid at a concentration of $8 \times 10^{-3}\text{M}$, was used as amino group acceptor and the substrate amino acid was supplied at a concentration of $4 \times 10^{-2}\text{M}$. Aspartic acid was found to be the best amino acid substrate and the level of the aspartate- α -ketoglutarate transaminase activity was considered as 100%. Asparagine was transaminated

Assay conditions: 0.25 ml of purified enzyme solution was preincubated with 0.125 ml of salt solution (final concentration 3.0×10^{-3} M) for 10 min. at 35°C . The reaction was started by the addition of 0.125 ml of a solution containing an amino acid (I. - phenylalanine, II. - tyrosine, III. - tryptophan, IV. - aspartic acid) and α -ketoglutarate to a final conc. of 4×10^{-2} M and 8×10^{-3} M. Final volume: 0.50 ml, 0.05 M TRIS-HCL, pH 8.5. After one hour's incubation, the reaction was stopped by the addition of 0.50 ml hot methanol. The glutamic acid formed was determined, after paper chromatography, by the cadmium acetate-ninhydrin colorimetric procedure.

Table 13. Effects of various cations on the activity of the purified aromatic aminotransferase.

Inorganic Salt added	Salt Concentration (M)	Amino Acid Substrate	units/mg Prot.	(%)
None	-----	I I	1.80	100
		II	1.27	100
		III	1.12	100
		IV	18.60	100
MgCl ₂	3.0 x 10 ⁻³	I	2.12	118
		II	1.51	119
		III	1.30	116
		IV	22.0	118
MnCl ₂	3.0 x 10 ⁻³	I	1.82	101
		II	1.27	100
		III	1.14	102
		IV	19.0	102
ZnCl ₂	3.0 x 10 ⁻³	I	1.67	93
		II	1.20	95
		III	1.08	96
		IV	17.7	95
CuCl ₂	3.0 x 10 ⁻³	I	1.76	98
		II	1.24	98
		III	1.09	97
		IV	18.4	99
CaCl ₂	3.0 x 10 ⁻³	I	2.03	113
		II	1.45	115
		III	1.23	111
		IV	22.2	112
FeSO ₄	3.0 x 10 ⁻³	I	1.75	97
		II	1.24	98
		III	1.09	97
		IV	18.4	99

Table 14. Substrate specificity of the aromatic aminotransferase isolated from bushbean roots with regard to amino acids.

Amino acid substrate	Concentration (M)	Activity	
		units/mg prot.	%
Aspartate	4×10^{-2}	18.9	100.
Asparagine	4×10^{-2}	3.2	17.
Phenylalanine	4×10^{-2}	1.8	9.5
Tyrosine	4×10^{-2}	1.3	7
Tryptophan	4×10^{-2}	1.14	6
Alanine	4×10^{-2}	0	0
Glycine	4×10^{-2}	0	0
Serine	4×10^{-2}	0	0
Threonine	4×10^{-2}	0	0
Valine	4×10^{-2}	0	0
Leucine	4×10^{-2}	0	0
Isoleucine	4×10^{-2}	0	0
Methionine	4×10^{-2}	0	0
Cystine	4×10^{-2}	0	0
Proline	4×10^{-2}	0	0
Histidine	4×10^{-2}	0.38	2.
Arginine	4×10^{-2}	0	0
Lysine	4×10^{-2}	0	0
Erythro- β -hydroxyaspartate	4×10^{-2}	13.0	70.

Assay conditions: 0.25 ml of enzyme solution was preincubated with 0.125 ml of amino acid solution (final conc. 4×10^{-2} M) for 10 min at 35°C. The reaction was started by the addition of 0.125 ml of α -ketoglutarate solution (final conc. 8×10^{-3} M). Final volume: 0.50 ml, 0.05 M TRIS-HCl containing 2×10^{-4} M pyridoxal phosphate, 1×10^{-4} M EDTA and 1×10^{-4} M 2-mercaptoethanol; pH 8.5. After one hour's incubation, the reaction was stopped by the addition of 0.50 ml of hot methanol. The glutamic acid formed was determined, after paper chromatography, by the cadmium-acetate-ninhydrin colorimetric procedure.

but at a rate 5 times less than that of aspartic acid, and the transamination rate of the three aromatic amino acids was 10-15 times less than that of aspartate- α -ketoglutarate reaction. A substituted amino acid, erythro- β -hydroxy-aspartate, was transaminated at 70% of the rate of aspartic acid.

Clearly, the aromatic aminotransferase isolated in the present study is not a specific enzyme since it can transaminate aspartic acid and asparagine in addition to phenylalanine, tyrosine and tryptophan when α -ketoglutarate is used as amino group acceptor. Thus, these results with the aromatic aminotransferase from bushbean roots confirm the lack of specificity shown by partially purified plant aromatic aminotransferases isolated by earlier workers.

Table 15 shows the results of experiments on the specificity of the purified aromatic aminotransferase with respect to substituted aromatic acids and related compounds. All three D-aromatic amino acids were not transaminated, but some substituted L-aromatic amino acids such as p-fluorophenylalanine, 3,4-dihydroxyphenylalanine (L-DOPA) and 3-iodotyrosine were able to serve as substrates. The rate of transamination of these substituted amino acids, however, except that of 3-iodotyrosine, was much less than the rates shown by the L-isomers of the three aromatic amino acids.

The results of experiments on the specificity of the aromatic aminotransferase for different amino group acceptors are given in Table 16. As the data show, when aspartic acid was provided as the amino group donor, only α -ketoglutarate supported a high level of transamination. Pyruvate and glyoxylate were completely without effect

Table 15. Substrate specificity of the aromatic aminotransferase isolated from bushbean roots with respect to aromatic amino compounds.

Aromatic compound	Concentration (M)	Activity	
		units/mg prot.	%
L-Phenylalanine	4×10^{-2}	2.0	100
L-Tyrosine	4×10^{-2}	1.5	78
L-Tryptophan	4×10^{-2}	1.3	66
D-Phenylalanine	4×10^{-2}	0	0
D-tyrosine	4×10^{-2}	0	0
D-Tryptophan	4×10^{-2}	0	0
DL-p-Fluorophenylalanine	4×10^{-2}	0.2	10
L- β -3,4-Dihydroxyphenylalanine (L-DOPA)	4×10^{-2}	0.3	15
3-Iodo-L-tyrosine	4×10^{-2}	1.6	83
Tyramine	4×10^{-2}	0	0
5-Hydroxytryptamine	4×10^{-2}	0	0
Tryptamine	4×10^{-2}	0	0
3-Hydroxytyramine	4×10^{-2}	0	0

Assay conditions: 0.25 ml of enzyme solution was preincubated with 0.125 ml of aromatic compound solution (final conc. 4×10^{-2} M) for 10 min at 35°C. The reaction was started by the addition of 0.125 ml of α -ketoglutarate solution (final conc. 8×10^{-3} M). Final volume: 0.050 ml, 0.05 M TRIS-HCl containing 2×10^{-4} M pyridoxal phosphate, 1×10^{-4} M EDTA and 1×10^{-4} M 2-mercaptoethanol; pH 8.5. After one hour of incubation, the reaction was stopped by the addition of 0.50 ml of hot methanol. Glutamic acid was determined, after paper chromatography, by the cadmium-ninhydrin colorimetric procedure.

Table 16 Specificity of the purified aromatic aminotransferase from bushbean roots with respect to the amino group acceptor when aspartic acid, glutamic acid, or phenylalanine was provided as substrate.

Amino acid substrate:	Activity	
	units/mg prot.	%
Aspartic acid		
α -Ketoglutarate	19.5	100
Pyruvate	0	0
Glyoxylate	0	0
Phenylpyruvate	1.75	9
p-Hydroxyphenylpyruvate	1.32	7
3-Indolepyruvate	1.23	6.5
Amino acid substrate:		
Glutamic acid		
Oxaloacetate	18.3	94
Pyruvate	0	0
Glyoxylate	0	0
Phenylpyruvate	1.86	9.5
p-Hydroxyphenylpyruvate	1.34	7
3-Indolepyruvate	1.15	6
Amino acid substrate:		
Phenylalanine		
α -Ketoglutarate	1.72	9
Oxaloacetate	1.63	8.5
Pyruvate	0	0
Glyoxylate	0	0

Assay conditions: 0.25 ml of enzyme solution was preincubated with 0.125 ml of amino acid solution (final conc. 4×10^{-2} M) for 10 min at 35°C . 0.125 ml of keto acid solution (8×10^{-3} M) was then added to initiate the reaction. Final volume: 0.50 ml, 0.05 M TRIS-HCl containing 2×10^{-4} M pyridoxal phosphate, 1×10^{-4} M EDTA and 1×10^{-4} M 2-mercaptoethanol; pH 8.5. After one hour of incubation the reaction was stopped by addition of 0.50 ml of hot methanol. The amino acid formed was determined, after paper chromatography, by the cadmium-ninhydrin colorimetric procedure.

as amino group acceptors in this reaction, although the three arylpyruvic acids tested were able to promote slight transamination. When glutamic acid, or any of the three aromatic amino acids were used as the amino group donor, oxaloacetate was found to support transamination at about the same rate as that found with α -ketoglutarate. Pyruvate and glyoxylate were again shown to have no effect as amino group acceptors (Table 16) and this finding for the three aromatic amino acid transaminase reactions with the bushbean enzyme differs markedly from the results of Gamborg and Wetter (1963) and Gamborg (1965) working with an aromatic aminotransferase isolated from mung bean seedlings. These investigators reported that their enzyme preparations catalyzed the transamination of aromatic amino acids at a faster rate when pyruvate was used as the amino group acceptor rather than when α -ketoglutarate or oxaloacetate was supplied in the reaction mixture.

7. Effect of various aromatic compounds on aromatic aminotransferase activity.

A number of aromatic compounds were examined as possible effectors of the purified aromatic aminotransferase by determining their action on the phenylalanine- α -ketoglutarate transaminase reaction. The results obtained are shown in Tables 17 and 18. When used at a concentration of $4.0 \times 10^{-3}M$, all the aromatic compounds had no marked effect upon the phenylalanine aminotransferase activity. When tested at $4.0 \times 10^{-2}M$, however, certain of the compounds, such as 3-indoleacetic acid, D-phenylalanine, and cinnamic acid, showed an inhibitory effect of the order of 10 to 20%. On the other hand, as shown in Table 18, compounds such as dopamine and epinephrine had an activator effect on the

Table 17. Effect of various aromatic compounds on the activity of the purified aromatic aminotransferase.

AROMATIC COMPOUND	(4.0×10^{-3} M)	(4.0×10^{-2} M)
	ACTIVITY %	ACTIVITY %
Control	100	100
p-Hydroxyphenylactic acid	97	---
DL- β -phenyllactic acid	92	---
β -Phenylethylamine	107	---
3-Indolebutyric	93	---
3-Indoleacetic acid	95	81
DL-3-Indolelactic acid	93	---
3-Indolepropionic Acid	89	---
D-Phenylalanine	100	87
D-Tyrosine	102	---
D-Tryptophan	99	---
5-Hydroxytryptamine	105	---
Tryptamine	100	103
Gramine	104	---
Tyramine	97	100
Cinnamic acid	103	88
Coumaric acid	100	95

Assay conditions: 0.25 ml of enzyme solution was preincubated with 0.125 ml of aromatic compound (final conc. 4×10^{-3} M or 4×10^{-2} M) for 10 min. at 35°C. The reaction was started by the addition of 0.125 ml of a solution containing phenylalanine and α -ketoglutarate (final conc. 4×10^{-2} M and 8×10^{-3} M, respectively). Final volume: 0.50 ml, 0.05 M TRIS-HCL containing 2×10^{-4} M pyridoxal phosphate, 1×10^{-4} M EDTA and 1×10^{-4} M 2-mercaptoethanol; pH 8.5. After one hour incubation the reaction was stopped by the addition of 0.50 ml of hot methanol. Glutamic acid was determined, after paper chromatography, by the cadmium-ninhydrin colorimetric procedure.

Table 18. Effect of various catechols and related compounds on the activity of the purified aromatic aminotransferase.

COMPOUND	(4.0×10^{-3} M)	(4.0×10^{-2} M)
	ACTIVITY %	ACTIVITY %
Control	100	100
3-Hydroxytyramine (DOPAMINE)	102	115
Normetanephrine	89	---
Epinephrine	99	118
Metanephrine	103	101
3,4-Dihydroxymandelic acid	95	---
4-Hydroxy-3-methoxy-phenylacetic acid	105	---
4-Hydroxy-3-methoxy-mandelic acid	98	---

Assay conditions: 0.25 ml of enzyme solution was preincubated with 0.125 ml of aromatic compound (final conc. 4×10^{-3} M or 4×10^{-2} M) for 10 min. at 35°C. The reaction was started by the addition of 0.125 ml of a solution containing phenylalanine and α -ketoglutarate (final conc. 4×10^{-2} M and 8×10^{-3} M, respectively). Final volume: 0.50 ml, 0.05 M TRIS-HCL containing 2×10^{-4} M pyridoxal phosphate 1×10^{-4} M EDTA and 1×10^{-4} M 2-mercaptoethanol; pH 8.5. After one hour incubation the reaction was stopped by the addition of 0.50 ml of hot methanol. Glutamic acid was determined, after paper chromatography, by the cadmium-ninhydrin colorimetric procedure.

phenylalanine aminotransferase, which results in an increase in activity of the enzyme by 15%. This finding contrasts with the data of Jacoby and La Du (1964) who found, that at similar concentration, dopamine and epinephrine completely inhibited the tyrosine transaminase activity of an aminotransferase isolated from rat liver. These workers reported that most catechols and related aromatic compounds strongly inhibited the activity of the aromatic aminotransferase from rat liver, and the results obtained in the present study indicate that these aromatic compounds do not play an important role in the regulation of aromatic aminotransferase activity in bushbean seedlings.

8. Kinetic studies on the aromatic aminotransferase

In preliminary kinetic studies, an investigation was made on the effect of enzyme concentration, time and temperature on the velocity of two of the transamination reactions catalyzed by the purified aminotransferase. Figures 27 A and 27 B show that the kinetics of the aspartate- α -ketoglutarate transaminase reaction were linear for at least the first 60 minutes when two concentrations of enzymes and a high and a low concentration of substrates were used. A similar situation was found when the kinetics of the phenylalanine- α -ketoglutarate transaminase reaction were investigated (Figs. 28 A and 28 B). Figure 29 shows the effect of temperature on the velocity of the phenylalanine- α -ketoglutarate reaction and indicates that between 30°C and 35°C, a 1.6 fold increase in velocity rate occurred. 35°C was therefore selected as the incubation temperature for all further experimentation with the purified enzyme.

a) Using Aliphatic amino acid or keto acid substrates

FIG. 27. Effect of enzyme concentration and time on the velocity of the aspartate- α -ketoglutarate transaminase reaction at 2 different concentrations of substrates. (A) Aspartate and α -ketoglutarate supplied at $1 \times 10^{-1} \text{ M}$. (B) Aspartate and α -ketoglutarate supplied at $8 \times 10^{-3} \text{ M}$. (●) 25 μg protein/ml; (■) 12.5 μg protein/ml.

FIG. 28. Effect of enzyme concentration and time on the velocity of the phenylalanine- α -ketoglutarate transaminase reaction at 2 different concentrations of substrates. (A) phenylalanine and α -ketoglutarate supplied at the concentration of $1 \times 10^{-1} \text{ M}$ and $4 \times 10^{-2} \text{ M}$, respectively. (B) phenylalanine and α -ketoglutarate supplied at $2 \times 10^{-2} \text{ M}$ and $8 \times 10^{-3} \text{ M}$. (●) 25 μg protein/ml; (■) 12.5 μg protein/ml.

FIG. 29. Effect of temperature on the velocity of the phenylalanine- α -ketoglutarate transaminase reaction. Phenylalanine and α -ketoglutarate supplied at the concentration of 4×10^{-2} M and 8×10^{-3} M, respectively.

The apparent Michaelis constants were determined according to the method of Lineweaver and Burk (1934). The K_m values for the four aliphatic substrates, aspartate, glutamate, α -ketoglutarate and oxaloacetate are given in Table 19. The enzyme shows rather similar affinity for all four substrates, although the affinity for the two keto acids is slightly greater. This is in agreement with the properties reported for aspartate aminotransferase isolated from cauliflower florets (Ellis and Davies, 1961) and from pea cotyledons (Wong and Cossins, 1969) which was found to have greater affinity for the keto acids.

The relationships between enzyme activity and substrate concentration for the four aliphatic substrates are shown in Figs. 30, 31, 32, and 33. In these experiments, the concentration of one substrate was kept constant at a concentration of 8×10^{-2} M while the second substrate was varied from 2×10^{-3} M to 4×10^{-1} M. As shown in Figs. 31 and 32, the aminotransferase activity was inhibited slightly by keto acid concentrations higher than 1×10^{-1} M. High amino acid concentrations, however, did not cause inhibition, even at 4×10^{-1} M (Figs. 29 and 30). Similar characteristics have been reported for mammalian aspartate transaminase and for aspartate transaminase from pea cotyledons (Wong and Cossins, 1969). The inhibitory effect of high concentrations of oxaloacetate and α -ketoglutarate may result from combination of the acids with the pyridoxal phosphate form of the enzyme. This would effectively reduce the interconversion of the pyridoxal phosphate to the pyridoxamine phosphate form of the enzyme (Bank *et al.*, 1963). Wong (1968) has demonstrated that such complexes can be competitively overcome by

Table 19. Apparent Michaelis constants for the different substrates of the purified aromatic aminotransferase.

Substrate concentration not varied (M)	Substrate concentration varied	K _m (M)
L-aspartate (8×10^{-2})	α -ketoglutarate	2.7×10^{-2}
L-glutamate (8×10^{-2})	oxaloacetate	2.9×10^{-2}
α -ketoglutarate (8×10^{-2})	L-aspartate	3.1×10^{-2}
oxaloacetate (8×10^{-2})	L-glutamate	2.9×10^{-2}
L-phenylalanine (8×10^{-2})	α -ketoglutarate	1.0×10^{-3}
L-tyrosine (8×10^{-2})	α -ketoglutarate	1.0×10^{-3}
L-tryptophan (8×10^{-2})	α -ketoglutarate	1.0×10^{-3}
α -ketoglutarate (8×10^{-2})	L-phenylalanine	5.5×10^{-2}
α -ketoglutarate (8×10^{-2})	L-tyrosine	7.3×10^{-2}
α -ketoglutarate (8×10^{-2})	L-tryptophan	7.7×10^{-2}
phenylpyruvate (8×10^{-2})	L-glutamate	5.0×10^{-3}
p-hydroxyphenylpyruvate (8×10^{-2})	L-glutamate	5.0×10^{-3}
3-indolepyruvate (8×10^{-2})	L-glutamate	5.0×10^{-3}
L-glutamate (8×10^{-2})	phenylpyruvate	5.2×10^{-2}
L-glutamate (8×10^{-2})	p-hydroxyphenylpyruvate	6.3×10^{-2}
L-glutamate (8×10^{-2})	3-indolepyruvate	6.7×10^{-2}

Assay conditions: 0.25 ml of purified enzyme solution was preincubated with 0.125 ml of amino acid solution for 10 min. at 35°C. 0.125 ml of keto acid solution was added to start the reaction. Final volume: 0.50 ml, 0.05 M TRIS-HCl buffer containing 1×10^{-4} M EDTA, 1×10^{-4} M 2-mercaptoethanol and 2×10^{-4} M pyridoxal phosphate. Final pH 8.5. After 30 min. of incubation the reaction was stopped by the addition of an equal volume of hot methanol. The amino acid formed was determined, after chromatography, by the cadmium acetate-ninhydrin procedure.

FIG. 30 A. Effect of various concentrations of aspartate on the aminotransferase activity when α -ketoglutarate was used as the amino group acceptor at a concentration of 8×10^{-2} M.

FIG. 30 B. Lineweaver-Burk plot for the aminotransferase assayed with various concentrations of aspartate and at one concentration of α -ketoglutarate (8×10^{-2} M).

FIG. 31 A. Effect of various concentrations of glutamate on the aminotransferase activity when oxaloacetate was used as the amino group acceptor to a concentration of 8×10^{-2} M.

FIG. 31 B. Lineweaver-Burk plot for the aminotransferase with various concentrations of glutamate and at one concentration of oxaloacetate (8×10^{-2} M).

FIG. 32 A. Effect of various concentrations of oxaloacetate on the aminotransferase activity when glutamate was used as the amino group donor at a concentration of 8×10^{-2} M.

FIG. 32 B. Lineweaver-Burk plot for the aminotransferase assayed with various concentrations of oxaloacetate and at one concentration of glutamate (8×10^{-2} M).

FIG. 33 A. Effect of various concentrations of α -ketoglutarate on the aminotransferase activity when aspartate was used as the amino group donor at a concentration of 8×10^{-2} M.

FIG. 33 B. Lineweaver-Burk plot for the aminotransferase assayed with various concentrations of α -ketoglutarate and at one concentration of aspartate (8×10^{-2} M).

increasing the concentration of the amino acid substrates.

b) Using Aromatic amino acid or keto acid substrates.

Figures 34 to 41 show the relationship between activity of the aromatic aminotransferase and different concentrations of six aromatic substrates and of α -ketoglutarate and glutamate. The apparent K_m values are reported in Table 19. The second substrate in all reaction systems was supplied at the concentration of 8×10^{-2} M, and pyridoxal phosphate was present at 2×10^{-4} M. It should be noted that solubility problems were encountered with the aromatic amino acid and aromatic keto acids at concentration higher than 2.0×10^{-1} M. The values in Table 19 show that the enzyme had a little more affinity for the aromatic keto acids than for the aromatic amino acids. When phenylalanine, tyrosine or tryptophan was used as the amino group donor, the apparent K_m value for α -ketoglutarate was 1.0×10^{-3} M. The apparent K_m for glutamate was 5.0×10^{-3} M when phenylpyruvate, p-hydroxyphenylpyruvate or 3-indoleacetate was used as amino group acceptor. This suggests that the enzyme had a much greater affinity for the aliphatic substrates than for the aromatic compounds. With regard to the affinity of the enzyme for the three aromatic amino acids, the K_m values show that the degree of affinity was similar for all three acids, although it was slightly greater for phenylalanine. A similar situation was found in the affinity of the enzyme for the corresponding arylpyruvic acids.

9. Effect of an aliphatic amino acid substrate on the aromatic aminotransferase activity of the purified enzyme.

Since the aromatic aminotransferase isolated from bushbean roots

FIG. 34 A. Effect of various concentrations of phenylalanine on the aromatic aminotransferase activity when α -ketoglutarate was used as the amino₂ group acceptor at a concentration of 8×10^{-2} M.

FIG. 34 B. Lineweaver-Burk plot for the aromatic aminotransferase assayed with various concentrations of phenylalanine and at one concentration of α -ketoglutarate (8×10^{-2} M).

FIG. 35 A. Effect of various concentrations of tyrosine on the aromatic aminotransferase activity when α -ketoglutarate was used as the amino group acceptor at a concentration of 8×10^{-2} M.

FIG. 35 B. Lineweaver-Burk plot for the aromatic aminotransferase assayed with various concentrations of tyrosine and at one concentration of α -ketoglutarate (8×10^{-2} M).

FIG. 36 A. Effect of various concentrations of tryptophan on the aromatic aminotransferase activity when α -ketoglutarate was used as the amino group acceptor at a concentration of 8×10^{-2} M.

FIG. 36 B. Lineweaver-Burk plot for the aromatic aminotransferase assayed with various concentrations of tryptophan and at one concentration of α -ketoglutarate (8×10^{-2} M).

FIG. 37 A. Effect of various concentrations of phenylpyruvate on the aromatic aminotransferase activity when glutamate was used as the amino group donor at a concentration of 8×10^{-2} M.

FIG. 37 B. Lineweaver-Burk plot for the aromatic aminotransferase assayed with various concentrations of phenylpyruvate and one concentration of glutamate (8×10^{-2} M).

FIG. 38 A. Effect of various concentrations of p-hydroxyphenylpyruvate on the aromatic aminotransferase activity when glutamate was used as the amino group donor at a concentration of 8×10^{-2} M.

FIG. 38 B. Lineweaver-Burk plot for the aromatic aminotransferase assayed with various concentrations of p-hydroxyphenylpyruvate and at one concentration of glutamate (8×10^{-2} M).

FIG. 39 A. Effect of various concentrations of 3-indolepyruvate on the aromatic aminotransferase activity when glutamate was used as the amino group donor at a concentration of 8×10^{-2} M.

FIG. 39 B. Lineweaver-Burk plot for the aromatic aminotransferase assayed with various concentrations of 3-indolepyruvate and at one concentration of glutamate (8×10^{-2} M).

FIG. 40 A. Effect of various concentrations of α -ketoglutarate on the aromatic aminotransferase activity when any one aromatic amino acid was used as the amino group acceptor at a concentration of 8×10^{-2} M.

FIG. 40 B. Lineweaver-Burk plot for the aromatic aminotransferase assayed with various concentrations of α -ketoglutarate and at one concentration of aromatic amino acid (8×10^{-2} M).

FIG. 41 A. Effect of various concentrations of glutamate on the aromatic aminotransferase activity when any one aromatic keto acid was used as the amino group acceptor at a concentration of 8×10^{-2} M.

FIG. 41 B. Lineweaver-Burk plot for the aromatic aminotransferase assayed with various concentrations of glutamate and at one concentration of aromatic keto acid (8×10^{-2} M).

was found to have relatively broad specificity, it was of interest to investigate the possible control of the transamination of aromatic amino acids by aliphatic amino acid substrates. The effect of various concentrations of aspartate on the phenylalanine- α -ketoglutarate transaminase activity was first determined. Enzyme samples (0.25 ml) were preincubated for 10 min. with 0.125 ml of various aspartate concentrations (4×10^{-5} M to 4×10^{-2} M) at 35°C: then 0.125 ml of a solution containing phenylalanine and α -ketoglutarate to give a final concentration of 4×10^{-2} M and 8×10^{-3} M respectively was added to start the reaction which was allowed to proceed for 30 min. and was then stopped by addition of 0.1 ml of 50% TCA. The keto acid product of the reaction, phenylpyruvic acid, was determined spectrophotometrically by the method of Shepartz (1969).

As indicated in Fig. 42, incubation of the enzyme with aspartate at very low concentration activated the phenylalanine- α -ketoglutarate transaminase activity. At a concentration of 4×10^{-5} M aspartate, the aromatic aminotransferase activity was enhanced by about 20%. At concentrations higher than 4×10^{-5} M aspartate progressively inhibited the phenylalanine- α -ketoglutarate aminotransferase activity so that at a concentration of 4×10^{-2} M aspartate, the aromatic aminotransferase activity was inhibited by about 85%.

In order to determine the type of competition involved in the inhibition of the phenylalanine- α -ketoglutarate transamination reaction by aspartate, an experiment was devised to test the effect of an inhibitory concentration of aspartate on the relationship between velocity of the transamination reaction and the phenylalanine

FIG. 42. Effect of various concentrations of aspartate on the phenylalanine- α -ketoglutarate transaminase activity. Phenylalanine and α -ketoglutarate supplied at a final concentration of 4×10^{-2} M and 8×10^{-3} M, respectively.

concentration provided as substrate. 0.25 ml of enzyme was preincubated for 30 min. with 0.062 ml of aspartate solution at a final concentration of 4×10^{-3} M at 35°C. In the control, where no aspartate was added, the enzyme was preincubated with 0.062 ml 0.05 M TRIS-HCl. Sequentially, 0.125 ml of phenylalanine solution and 0.062 ml of α -ketoglutarate solution (final concentration 8×10^{-3} M) were then added. The reaction was carried out for 30 min. at 35°C and was stopped by the addition of 0.1 ml of 50% TCA. The reaction product, phenylpyruvic acid, was again determined by the method of Shepartz (1969).

As shown in Fig. 43 A, at low concentration of phenylalanine, aspartate inhibited the phenylalanine aminotransferase activity by about 75%. This inhibition, however, decreased with increased concentration of phenylalanine. At the highest phenylalanine concentration tested (2×10^{-1} M), the inhibition by aspartate was only 15% compared to normal activity. The double reciprocal plot of these data (Fig. 43 B) shows clearly that aspartate inhibited the aromatic aminotransferase activity by direct competition with the aromatic substrate.

FIG. 43 A. Effect of aspartate on the velocity of the aromatic aminotransferase reaction at various concentrations of phenylalanine and at one concentration of α -ketoglutarate (8×10^{-3} M). (●) control with no aspartate present. (■) aspartate added at a concentration of 4×10^{-3} M. Units refer here to μ moles of phenylpyruvate produced per min of incubation.

FIG. 43 B. Lineweaver-Burk plot for the aromatic aminotransferase assayed with various concentrations of phenylalanine and at one concentration of α -ketoglutarate. (■) in the presence of 4×10^{-5} M aspartate. (●) in the absence of aspartate.

DISCUSSION

Since many of the experimental results have already been discussed, to a limited extent, in the results section of this thesis, the present section will be devoted to a broader discussion of the main aspects of this work with bushbean seedlings. Firstly, the changes observed in the different nitrogen fractions during early seedling growth and the development of transaminase activities in the three main organs of seedlings growing under light or dark conditions will be discussed. Secondly, the special properties of the aromatic aminotransferase purified from root tissue will be considered. As a final topic, the general metabolic significance of this aminotransferase will be discussed.

Changes in the nitrogen fractions and development of transaminase activity during early growth of bushbean seedlings.

The growth curves (Fig. 7) and the dry weight data (Fig. 8) for the different organs of seedlings grown under the two experimental conditions show that bushbean plants develop, during the first 2 weeks of growth, in a manner very similar to that already known for other dicotyledonous seedlings growing under light or dark conditions. (Mayer and Poljakoff-Mayber, 1963). The dry weight changes in the different organs (Fig. 8) show that the overall decrease in dry weight of cotyledons on dark-grown seedlings is greater than the total increase in dry weight of the young plant, so that at the end of the two-week period there was a net decrease in the total dry weight of the whole seedling. This loss of organic material undoubtedly represents the loss of carbon due to respiratory activity of the cotyledons and developing

embryo. In contrast, the light-grown seedlings showed a loss in total dry weight only during the first week of growth and by the 8th day, the dry weight began to increase rapidly, due presumably to the onset of photosynthetic activity in the seedling shoot tissues at this time.

The total nitrogen content of seedlings grown under both experimental conditions remained relatively constant throughout the two-week period. During this time, however, as the data in Figs. 9, 10, and 11 show, there was considerable redistribution of nitrogen in the seedlings and appreciable changes in the ratio between insoluble and soluble nitrogen in the different organs. The relatively constant total nitrogen content of these seedlings throughout this early period of growth is consistent with most of the classical observations on total nitrogen changes during seed germination. (Chibnall, 1939; Mayer and Poljakoff-Mayber, 1963).

Hydrolysis of the insoluble nitrogen reserves apparently occurs more rapidly in the cotyledons of light-grown bushbean seedlings than in those on etiolated plants (Fig. 11). This rapid hydrolysis of reserves in cotyledons exposed to light must be due to an enhanced rate of hydrolytic enzyme activity, but whether this is the result of a greater rate of enzyme synthesis in the light, or of greater activation of these enzymes, is not yet known. In this connection, although light has not previously been shown to have an effect on the development of hydrolytic activity in the cotyledons of germinating seeds, several workers have already demonstrated a rise in the activity of the hydrolase enzymes, protease, ribonuclease and amylase, in germinating seeds during the first few days of growth (Tazakawa and Hirokawa, 1956; Young and Varner, 1959; Beevers 1968; Beevers and Spittstoesser, 1968; Juliano and

Varner, 1969).

Qualitative changes in the soluble amino acid fraction of seedlings growing under different environmental conditions have already received considerable attention (see reviews by Steward and Bidwell, 1962; Steward and Durzan, 1965). In particular, the free amino acids in pea seedlings growing under light and dark conditions have been studied by Shirakawa and Otakara (1956) and again, more recently by Lawrence and Grant (1963) and by Larson and Beevers (1965). The composition of the amino acid pool in light-grown Vicia faba L. was investigated by Boulter and Barber (1968), in dark-grown soybean seedlings by Kasai et al (1966), and in different varieties of wheat and corn by Wang (1968). Although differences are to be found between the results obtained in these earlier studies and those presented here for bushbean seedlings, particularly with respect to the concentrations of individual protein amino acids in the different organs, these variations may be attributed either to the different environments in which the seedlings were grown (Steward and Bidwell, 1962) or to genotypic differences between the varieties or species involved in these studies.

The quantitative composition of the free amino acid fraction in the cotyledons and developing organs of the bushbean plants showed important differences. While glutamic acid was always found in low concentration in the root and shoot tissues, it was a major free amino acid in cotyledons from both light and dark-grown seedlings (Tables 3 and 4). Asparagine was always the most predominant amino acid in the tissues of both types of seedlings, except in the cotyledons of light-grown plants where the concentration was consistently low. Glutamine, on the other

hand, was detected in only low amounts at all stages of growth in the different organs (Tables 3-8). The occurrence and accumulation of these amides in developing plants have been extensively studied by many workers (Vickery and Pucher, 1943; Meiss, 1952; Virtanen and Miettinen, 1953; Weinstein and Porter, 1962; Wang, 1968) and it is now well established that one or both of these amides may accumulate or diminish in plants growing under the influence of different light conditions. It is of further interest to note from the present results that the accumulation of nitrogen-rich amino acids, such as asparagine, lysine and arginine in dark-grown seedlings coincides with conditions of low protein synthesis, and this accumulation might be considered as a means of storing amino nitrogen or as an ammonia detoxification process.

In the present study, only the protein amino acids in the soluble nitrogen fractions were examined in detail since it was felt that changes in the concentration of some of these amino acids during early growth of the seedling might be related to the development of transaminase activity. At the same time, it was recognized that the concentration of free amino acids in the soluble nitrogen fractions obtained from successive samples of seedlings represents the net result, at each 2-day stage of growth, of three biochemical events; namely, degradation of seed protein, metabolism of the derived amino acids, and utilization of many of these amino acids in protein synthesis. Although transamination is the final step in the biosynthesis of about half of the protein amino acids (Fowden, 1966; 1967; Kretovich, 1965), it is possible that all of these amino acids when present in seedling tissues at appreciable concentrations are subject, to

different degrees, to further metabolism by transaminase activity. In this connection, the properties of transaminase enzymes in a number of higher plants have already been investigated (Albaum and Cohen, 1943; Leonard and Burris, 1947; Bone and Fowden, 1960; Ellis and Davies, 1961; Gamborg and Wetter, 1963; Gamborg 1965; Pilet and Athanasiades-Mercaton, 1966; Wong and Cossins, 1969) but no study has so far been made to examine possible correlations between changes in the concentration of free amino acids in seedling tissues and the development of the transaminase system during early growth. This was one of the objectives of the present investigation, but as the results show (Tables 3-8; Figs. 12-18) no correlation was, in fact, found between the development of the transaminase activities studied and changes in the amounts of the corresponding amino acids in seedlings grown under light or dark conditions. Clearly, while development of the total activity for each transaminase was relatively similar in both light- and dark-grown seedlings (Figs. 15 to 18), changes in composition of the free amino acid fractions differed considerably between light- and dark-grown plants (Tables 3 to 8, and Figs. 12, 13, and 14). While five out of six aliphatic amino acids tested as substrates for transamination, (i.e. asparagine, alanine, valine, serine and threonine) were present in relatively high concentration in the soluble nitrogen fractions, only the aspartate, alanine, and asparagine transaminases were found to have a high total activity. Valine- α -ketoglutarate transaminase had only low total activity, and the corresponding serine and threonine transaminases were not detected in any organ.

It is concluded from this investigation of the amino acid

metabolism in light-and dark-grown bushbean seedlings, that the marked changes observed in the concentrations of the main free amino acids in the soluble nitrogen fractions are not due to differences in the rate of development of the transaminase system present in the two kinds of seedlings. While it is possible that differences in the rate of development of other enzymes capable of metabolizing amino acids could still account for some of the quantitative changes observed in the soluble nitrogen fractions, the decrease in concentrations of free amino acids in light-grown seedlings during the second week must be mainly attributed to the increased level of protein synthesis in the developing organs of these plants during this exponential period of growth.

Evidence for an aromatic aminotransferase of low specificity in roots of bushbean seedlings.

The results presented in this investigation have shown that the three aromatic aminotransferase activities, and the aspartate- α -ketoglutarate activity, were distributed identically in the subcellular fractions isolated from bushbean roots. The investigation has also shown that a soluble protein fraction extracted from the roots of 8-day old seedlings and purified about 600-fold by means of pH precipitation, ammonium sulfate fractionation and Sephadex chromatography, was able to catalyze transamination reactions between aspartate, phenylalanine, tyrosine or tryptophan and α -ketoglutarate. These reactions were all shown to be reversible and furthermore, oxaloacetate was also found to serve as an amino group acceptor for transamination of the three aromatic amino acids. Pyruvate and glyoxylate, however, and their corresponding amino acids, were not able to serve as substrates of the enzyme. The

ratio of the four primary activities were found to remain constant during the different purification steps and the final enzyme preparation was shown by gel electrophoresis to contain only one protein, which was found to have a molecular weight of 128,000 daltons. The purified preparation was shown to catalyze transamination of its aliphatic amino acid substrate (aspartate) with much greater velocity than its rate of reaction with any of the three aromatic amino acids and as expected, the affinity of the enzyme for its aliphatic substrates was greater than its affinity for the aromatic substrates (Table 19). Furthermore, from inhibition studies, aspartate was found to competitively inhibit the aromatic aminotransferase activity of the enzyme, which strongly suggests that both substrates are competing for the same active site on the enzyme molecule.

The first study of an aromatic aminotransferase from plants was carried out by Gamborg and Wetter (1963) and Gamborg (1965) who extracted an aromatic aminotransferase from acetone powders of Phaseolus aureus seedlings, which they then purified 40-60 fold. In addition to transaminating all three aromatic amino acids, this enzyme preparation was found to utilize a wide variety of amino acids such as aspartate, glutamate and methionine, and pyruvate rather than α -ketoglutarate was shown to be the best amino group acceptor. The selectivity of this enzyme preparation for the amino donor appeared to be determined partly by the chain length, and partly by the substituents in the methylene carbon adjacent to the α -amino group. Gamborg (1965) also demonstrated competitive inhibition of the phenylalanine-pyruvate transaminase activity by other amino acid substrates, such as lysine,

glutamate and methionine; the latter was shown to be the best inhibitor, followed by glutamate. Red'Kina et al. (1969) purified, 50-80 fold, a glutamate-phenylpyruvate transaminase from pea seedlings and they demonstrated that this preparation also showed aspartate- α -ketoglutarate transaminase activity, which was about 15-30 times greater than that of the phenylalanine aminotransferase activity. From these results, they concluded that two enzymes were present in their protein preparation, but in view of Gamborg's (1965) earlier results, it would seem that Red'Kina and coworkers may have been dealing with a single aminotransferase which was able to catalyze the two transamination reactions examined. Recently Gadal et al. (1969) also isolated an aromatic aminotransferase from the leaves of Quercus pedunculata, and this enzyme was found to use either p-hydroxyphenylpyruvate or phenylpyruvate as the amino group acceptor, and glutamate or aspartate as the amino group donor. The reversibility of these reactions was also demonstrated.

The present results with a purified aromatic aminotransferase from bushbean roots confirm most of the findings from these earlier studies based on experiments with a much less purified enzyme fraction, and suggest that the aromatic aminotransferase in plants has low specificity. Moreover, since the bushbean enzyme has evidently a much greater affinity for its aliphatic amino acid substrate than for any of its three aromatic amino acid substrates, when α -ketoglutarate is used as the amino group acceptor, we may, in fact, be dealing with an aspartate aminotransferase of low specificity. A similar situation was recently found with a so-called aromatic aminotransferase isolated from bacteria (Pseudomonas savastanoi) by Kuo and Kosuge (1970). These workers purified the

aminotransferase about 200-fold and found that the enzyme exhibited very low specificity and could, in fact, catalyze the transamination of 14 protein amino acids, including aspartate and the three aromatic amino acids.

It is relevant to point out here that although aspartate- α -ketoglutarate transaminase is one of the most studied aminotransferase from plants, no thorough investigation of its specificity has yet been made. This aminotransferase, purified about 250 fold from cauliflower buds, was found to be quite specific but was also able to transaminate certain substituted derivatives of aspartic and glutamic acids when either oxaloacetate or α -ketoglutarate was used as the amino group acceptor (Ellis and Davies, 1961). The three aromatic amino acids, however, were not tested as possible substrates in this study. An aspartate- α -ketoglutarate transaminase extracted from pea cotyledons and purified about 60 fold was also found to be highly specific (Wong and Cossins, 1969), but again in this study the enzyme was not assayed for activity with the aromatic amino acids.

In marked contrast to the low specificity found with plant aromatic aminotransferases, the properties exhibited by aromatic aminotransferases purified from animal tissues indicate that these enzymes are highly specific with respect to their amino acid substrate. For example, Haavaldsen and co-workers (1964) and Fonnum and Larson (1965) presented evidence for the occurrence of three aromatic aminotransferases in rat brain extracts; one catalyzing the transamination of 3,4-dihydroxyphenylalanine (L-DOPA), another for phenylalanine and tryptophan, and a third for tryptophan and 5-hydroxytryptophan. George

and Gabay (1968) isolated an aromatic aminotransferase from pig brain and showed that it was highly specific for phenylalanine and α -ketoglutarate. Similarly, Igo et al. (1968) purified an aminotransferase from bovine thyroid and liver tissue which was specific for the tyrosine- α -ketoglutarate reaction. The degree of specificity for amino acid substrates is therefore a major difference between plant and animal aromatic aminotransferases.

Metabolic significance of aromatic aminotransferase.

Transamination plays a central role in the biosynthesis and catabolism of aromatic amino acids in plants. Phenylalanine and tyrosine have been shown by tracer experiments with young wheat plants to be produced via the shikimic acid pathway and a final step involving transamination of either phenylpyruvate or p-hydroxyphenylpyruvate, respectively (Gamborg and Neish, 1959). Tryptophan has been shown to be the precursor of the important plant growth hormone, 3-indoleacetic acid, through a pathway initially involving transamination of tryptophan to indolepyruvate (Wightman and Cohen, 1968). Although the aromatic amino acids are important in protein synthesis and also as precursors of many other plant cell constituents, such as flavonoids, alkaloids and plant hormones, it seems, however, that plants have evolved in such a way that specific aromatic aminotransferases are not required to maintain the appropriate level of aromatic amino acids in plant cells to sustain normal growth and development.

The fact that the bushbean aromatic aminotransferase can also catalyze transamination reactions between the aliphatic substrates, aspartate, glutamate, oxaloacetate and α -ketoglutarate may render the

transaminase system in these seedlings very flexible and allow the possible control of the different activities by competitive substrate inhibition, as was in fact demonstrated in this study (Fig. 43). Since glutamate and aspartate are readily available during germination and normal seedling growth, through the action of this aminotransferase these amino acids can thus provide a constant source of amino groups for the formation of aromatic amino acids from the corresponding keto acids. The reverse reactions can also be facilitated by the availability of α -ketoglutarate and oxaloacetate from the Krebs cycle.

As discussed earlier, it seems that rather broad specificity may be one of the main characteristics of plant aromatic aminotransferases. This finding for the bushbean enzyme is in sharp contrast with the high degree of specificity exhibited by aromatic aminotransferases isolated from animal tissues. The biological significance of such an important difference is not clear. One possible explanation for this situation may lie in the metabolic role of aromatic amino acids in both types of organisms. While transamination in plants is involved in both biosynthesis and degradation of the aromatic amino acids, animal tissues can only catabolyze these compounds since they are essential amino acids and therefore must be supplied in the diet (Proudfit and Robinson, 1964).

One may further speculate that because of the important role played by aromatic compounds derived from the aromatic amino acids and catecholamines, in brain metabolism in particular (Guroff and Lovenberg, 1970), animals have developed a very refined aromatic aminotransferase system which may involve more rigid controls, either at the enzyme synthesis level (Holt and Oliver, 1969; Tomkins et al. 1969) or at the control of

activity level by end products and related aromatic compounds (Jacoby and La Du, 1964). The evolution of such control would likely require specific enzymes or isoenzymes. However, the derivatives of aromatic amino acids from either deamination, decarboxylation or transamination, are also very important in plant metabolism (Yoshida, 1969), but since in plants transamination can be involved in both catabolism and anabolism of aromatic amino acids, the enzyme catalyzing these reactions may not require the degree of specificity of the corresponding animal enzyme.

This study has provided an answer to the question of the number and specificity of the enzyme(s) catalyzing the transamination of aromatic amino acids in plants. The results have also opened up the further question concerning the actual number and specificity of the enzymes comprising the entire transaminase system in plants. From the present data, and from those recorded in the literature, it seems likely that plants have evolved a very different transaminase system than that found in animals. Plants appear to have only a limited number of aminotransferases, each with fairly broad specificity with respect to their amino acid substrates, whereas animals appear to have a greater number of aminotransferases each exhibiting a high degree of substrate specificity. Further investigations to test the above view by examining the properties of other aminotransferases found in bushbean roots are now in progress in this laboratory.

SUMMARY

1. The total nitrogen content of bushbean seedlings grown under light or dark conditions were found to remain relatively constant during the first 2 weeks of growth, but there was considerable redistribution of nitrogen from cotyledons to the seedling tissues, and appreciable changes occurred in the ratio between insoluble and soluble nitrogen in the different organs.
2. The quantitative composition of the soluble nitrogen fraction with respect to free protein amino acids, was found to differ considerably, not only between seedlings at different stages of growth, but also between organs taken from seedlings at the same stage of growth under the two light conditions. Asparagine, serine, valine, alanine and threonine were the amino acids that showed the greatest change in concentration in both types of seedlings.
3. The development of six aliphatic aminotransferase activities and of three aromatic aminotransferase activities was investigated in both light and dark-grown seedlings during the two weeks of growth. Of the six aliphatic activities examined, only aspartate, alanine and asparagine aminotransferase systems showed high total activities. Valine aminotransferase had low activity, and the corresponding serine and threonine aminotransferases were not detected.

4. Development of the activity of all three aromatic aminotransferases tested followed a very similar pattern in the cotyledons, roots and shoot of seedlings grown under each experimental condition. No correlation was found between the development of transaminase activities and changes in concentrations of the corresponding free amino acids in these seedling organs.
5. The intracellular distribution of aromatic aminotransferase and aspartate aminotransferase activities was investigated in roots from 8-day old etiolated seedlings. Only about 10% of both activities were found in the particulate fractions; the mitochondrial fraction containing only 4% of the total activity. On the other hand, activity of the soluble protein in the high-speed supernatant fraction accounted for about 90% of the total activity.
6. An aromatic aminotransferase was isolated from roots of 8-day old seedlings and purified by pH treatment, ammonium sulfate fractionation and Sephadex chromatography. The purification obtained was 570-620-fold and the recovery was 30-35% based on total activity. The specific activity for aspartate- α -ketoglutarate transaminase increased proportionally to changes in the aromatic aminotransferase activities during the successive purification steps.
7. Gel electrophoresis of the purified enzyme fraction revealed the presence of only one major protein when the gel was stained

with Amido black. This band corresponded to the product specific staining band obtained for all three aromatic aminotransferase activities.

8. Molecular weight determination using the Sephadex G-200 chromatographic technique indicated that the purified enzyme had a molecular weight of about 128,000 daltons.
9. pH optimum for the phenylalanine- α -ketoglutarate, tyrosine- α -ketoglutarate, tryptophan- α -ketoglutarate, and aspartate- α -ketoglutarate transaminase activities was found to be 8.5.
10. No specific requirement for pyridoxal phosphate was detected for the purified enzyme. Its aromatic aminotransferase activity, however, was inhibited by 40 to 45% by antagonists of pyridoxal phosphate, such as isonicotinic acid hydrazide. This result suggest that pyridoxal phosphate was tightly bound to the enzyme and is essential for activity.
11. Cations such as Ca^{++} and Mg^{++} enhanced the aromatic aminotransferase and aspartate aminotransferase activities of the purified enzyme by about 15%. Other cations such as Mn^{++} , Cu^{++} and Fe^{++} were found to be without effect at a concentration of 3×10^{-3} M, while Zn^{++} was found to slightly inhibit the four transamination reactions tested.
12. The purified aromatic aminotransferase was found to exhibit rather broad substrate specificity. When α -ketoglutarate was used as amino group acceptor, the three aromatic amino acids were transaminated at a rate 10 to 15-fold less than that of aspartate.

Oxaloacetate was also found to be a good amino group acceptor for the activity of this enzyme, while the aromatic keto acids were 10 to 15-fold less effective than α -ketoglutarate, when glutamate was used as the amino acid substrate. Pyruvate and glyoxylate, and their corresponding amino acids, were not substrates of this aromatic aminotransferase.

13. A number of aromatic compounds were examined as possible effectors of the purified aromatic aminotransferase by determining their action on the phenylalanine- α -ketoglutarate transaminase reaction. None of the aromatic compounds tested had a promotive effect on the phenylalanine aminotransferase activity, when used at the concentration of 4×10^{-3} M. However, when tested at 4×10^{-2} M, some compounds such as 3-indoleacetic acid, D-phenylalanine and cinnamic acid, inhibited the aromatic aminotransferase activity by 10-20%, while dopamine and epinephrine was found to activate the reaction by 15%.
14. The purified enzyme showed rather similar affinity for all four aliphatic substrates, aspartate, glutamate, α -ketoglutarate and oxaloacetate, as demonstrated by the apparent Michaelis constants which were all found to be in the range 2.7 to 3.1×10^{-2} M. When α -ketoglutarate was used as amino group acceptor the K_m value was 5.5×10^{-2} M for phenylalanine, 7.3×10^{-2} M for tyrosine and 7.7×10^{-2} M for tryptophan. When glutamate was used as amino group donor, the K_m was 5.2×10^{-2} M for phenylpyruvate, 6.3×10^{-2} M for p-hydroxyphenylpyruvate, and 6.7×10^{-2} M for 3-indolepyruvate.

When any of the aromatic amino acids was used as amino group donor, the K_m for α -ketoglutarate was 1×10^{-3} M and when any of the aromatic keto acids was used as amino group acceptor the K_m for glutamate was 5.0×10^{-3} M. The affinity of the enzyme for a keto-acid substrate was thus found to be always slightly greater than that for the corresponding amino acid substrate.

15. An experiment carried out to determine the effect of the aliphatic amino acid substrate on the aromatic aminotransferase activity of the purified enzyme showed that aspartate was able to competitively inhibit the phenylalanine- α -ketoglutarate activity.
16. The results obtained in this investigation have shown that all four transaminase activities studied were catalysed by a purified protein fraction isolated from bushbean roots, which was found to contain only one protein when analysed by polyacrylamide gel electrophoresis. As this protein band coincided with the product specific staining band for aromatic aminotransferase activity and since the aliphatic substrate, aspartate, competitively inhibited the phenylalanine- α -ketoglutarate transaminase reaction, it is concluded that the same enzyme is responsible for catalyzing the aromatic amino-acid- α -ketoglutarate and the aspartate- α -ketoglutarate transaminase reactions in roots of bushbean seedlings.

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