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The Study of the Biosynthesis of
Allylglucosinolate
Through the Use of $^{14}\text{-CO}_2$ Labeling
and the Extraction of Methionine Amino Transferase

by

Eric Glenn Russell , B.Sc.H.

A Thesis submitted to
The Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

Master of Science

Department of Biology
Carleton University
Ottawa Ontario

March 1989

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Eric Glenn Russell , B.Sc.H.
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March 1989

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of the same age of plant was exposed to $^{14}\text{CO}_2$ and allowed to metabolize . The different tissues were then isolated at different times to compare the rates of in vivo biosynthesis of allylglucosinolate to the other two tissue surveys . Again the rates of synthesis of allylglucosinolate and enzyme activity and quantity of allylglucosinolate in the tissues matched .

Since methionine amino transferase has been implicated in the biosynthesis of the ubiquitous plant growth regulator ethylene a species survey was performed (using mature leaves) and it was found to be present in all species examined , but with varying activity .

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α OG	=	<u>alpha</u> -oxoglutaric acid
α KMB	=	<u>alpha</u> -oxo- <u>gama</u> -methiobutyric acid
DNPH	=	Dinitrophenylhydrazine
Glc	=	Glucose
PPD	=	2,5-diphenyloxazole
TLC	=	Thin Layer Chromatography
HPLC	=	High Performance Liquid Chromatography
BSA	=	Bovine Serum Albumin
TCA	=	Trichloroacetic acid
PMSF	=	Phenylmethanesulfonylfluoride
PEG	=	Polyethyleneglycol
PAPS	=	3'-phosphoadenosine-5'-phosphosulfate
p-5'-p	=	pyridoxal-5'-phosphate
x g	=	gravitational force
gfw	=	gram fresh weight
mgp	=	milligram protein

Introduction

The high degree of interest in glucosinolates over the years is due mainly to their occurrence in agricultural crops which are of considerable economic importance ; particularly in Canada . Glucosinolates (see fig. 1 for examples) are found mostly in the order Capparales among the families Capparidaceae , Crucifereae , Resedaceae , Tovariaceae , and Moringaceae . They have also been found in other orders among species in the families Limnanthaceae , Salvadoraceae , Tropaeolaceae , Gyrostemonaceae , Caricaceae and Euphorbiaceae (Larsen , 1981 ; Underhill , 1980). The family Crucifereae contains a large number of important agricultural food crops such as mustard , brussels sprouts , cabbage , radish and turnip ; all of which get their characteristic flavor from the degradation of the glucosinolates they contain. (McGregor et al. , 1983). Another such crop is the canola oil crop which is harvested from species of the genus Brassica and is one of the major crops in Canada accounting for 8 - 10 % of world production of rapeseed oil (Emery et al. , 1985). The interest in glucosinolates comes not so much from the oil which is extracted from the seeds but from the seed cake that is left over after the extraction . The cake would be a good animal feed if the glucosinolate content could be reduced considerably or even eliminated (Underhill , 1980) because it is high (aprox. 80%)(Emery et al. , 1985) in good quality protein (Josefsson , 1971).

methionine

allylglucosinolate

tryptophan

indol-3-ylmethylglucosinolate

phenylalanine

p-hydroxybenzylglucosinolate

Figure # 1 : Represented are the structures of three glucosinolates and their amino acid progenitors (Underhill et al. , 1973).

Originally the seeds of the oil producing plants were also high in glucosinolates but recently plant breeders have developed a cultivar , Brassica napus cv. Bronowski , which is much lower in glucosinolate content (Josefsson , 1971). The reason why glucosinolates present a problem is not due to the chemicals themselves (see fig. 2 (a) for general structure) but due to the toxicity of their enzymatic breakdown products which include isothiocyanates , organic cyanides and thiocyanates (fig. 2)(Larsen , 1981). These breakdown products are produced in a number of different ways . One way is due to the release and action of myrosinase which results when the plant tissues are disrupted , and since myrosinase is always found accompanying but sequestered from glucosinolates this degradation always occurs (Underhill , 1980). Another way is due to bacteria found in the mammalian lower intestine which have hydrolytic enzymes resembling myrosinase (Underhill , 1980). So , even if the seed myrosinase is denatured during the oil extraction process , degradation will still occur once the glucosinolate containing pulp is ingested . It has also been shown that even in the absence of enzymes , these breakdown products will still form , even in the intact plant , although at a much slower rate (Underhill , 1980). The toxicity of these substances comes from their goitrogenic properties in mammals , including humans , and the high reactivity of isothiocyanates (Larsen , 1981). For these reasons plant breeders have tried and to a certain extent have been successful (eg. the cultivar mentioned above

Fig. 2 : Depicted are the major enzymatic breakdown products of a typical glucosinolate (a). These are sulfate and B-D-glucose (e) and one of the following ; (b) isothiocyanates , (c) organic cyanides and sulfur , or (d) thiocyanates (Larsen , 1981).

B. napus cv. Bronowski) in reducing the content of glucosinolates in the Brassica species used for oil production . There has also been a large amount of work done for the purpose of understanding the role of glucosinolates in plants (Bell , 1981) and their biosynthesis (Larsen , 1981 , Underhill , 1980 , Chisholm , 1971). One final interest in the research on glucosinolates comes from the area of biotechnology . Biotechnologists believe that the gene controlling the biosynthesis of glucosinolates can be blocked if the signals which stimulate the production of glucosinolates can be determined . To locate this gene though it would be necessary to find the controlling enzyme in the pathway to the biosynthesis of glucosinolates .

Secondary plant products are those which do not play a direct role in the primary metabolic pathways of the plant (Bell, 1981). Glucosinolates are secondary plant products which are used in defending the plant against pathogens and herbivores (Bell , 1981), and are also involved in the complex interactions with insects (Bell , 1981). Being secondary plant metabolites , their biosynthesis is an offshoot of the main biochemical pathways . All glucosinolates , of which there are over 75 to date , are presumed to be derived from certain amino acids such as methionine , phenylalanine , tryptophan etc... (fig. 1) (Larsen , 1981). The considerable variety in glucosinolate structure comes solely from the variability of the side chain (labeled 'R-' in fig. 2(a)) which is the structure that is derived from the original amino acid and has possibly been modified

before and after the formation of the glucosinolate . In this fashion for example 4-methylsulfinyl-3-butenylglucosinolate from Matthiola annua is believed to be derived from methionine (Loffelhardt and Kindl , 1975) , and 3-methylthiopropylglucosinolate and 3-methylsulfinylpropylglucosinolate from Cheiranthus kewensis are also believed to be derived from methionine (fig. 3)(Chisholm , 1972). Modification before the formation of the glucosinolate , if any at all , is limited to chain elongation (Fig. 4). This modification involves a process similar to the formation of leucine from valine (Oaks , 1965) and the formation of glutamic acid from aspartic acid (Larsen , 1981). The original amino acid is transaminated and to the 2-oxo carbon of the α -oxo acid formed is added an acetyl group via a condensing reaction (Fig. 4). This condensing step may be repeated more than once , but the final step involves the decarboxylation of the original carboxylic group and a retransamination back into an amino acid (Oaks , 1965 , Larsen , 1981). In the case of glucosinolate biosynthesis this is now a non protein amino acid .

Modifications after the formation of glucosinolates involve a variety of reactions such as oxidation , acylation , hydroxylation (Larsen , 1981), N-sulfonation and cleavage of terminal methyl-sulfur units (Underhill , 1980).

Biosynthesis of the glucosinolate itself follows the pathway outlined in fig. 5 . This pathway has been demonstrated in a number of instances (Larsen , 1981 , Underhill , 1980 , Chisholm

(a) 4-Methylsulfinyl-3-butenylglucosinolate

(b) 3-methylthiopropylglucosinolate

(c) 3-methylsulfinylpropylglucosinolate

Figure # 3 : Three glucosinolates all of which are thought to be derived from methionine (a) (4-Methylsulfinyl-3-butenylglucosinolate from Löffelhardt and Kindl (1975) , (b) 3-methylthiopropylglucosinolate and (c) 3-methylsulfinylpropylglucosinolate from Chisholm (1971).

Fig. 4 : Chain elongation of amino acids before incorporation into glucosinolates is illustrated . The original amino acid is transaminated and to the 2-oxo carbon of the α -oxo acid formed is added an acetyl group (possibly through acetyl CoA) via a condensing reaction . This condensing step may be repeated more than once , but the final step involves the decarboxylation of the original carboxylic group (*) and a retransamination back into an amino acid (Larsen , 1981).

Amino Acid

N-Hydroxyamino
Acid

Aldoxime

(a)

Thiohydroxymic
Acid

Desulfoglucosinolate

Glucosinolate

Fig. 5 : This is the generalized pathway for the biosynthesis of glucosinolates from their amino acid precursors (Larsen , 1981 , Underhill , 1980). The sulfur donor (a) is possibly cysteine .

and Matsuo , 1972) and , no matter the amino acid precursor , biosynthesis has followed this route via the aldoxime . Before the work on this thesis was started , there was no published work on the enzymes involved in the biosynthesis of glucosinolates (Since the completion of the experimental work for this thesis there has been a publication involving enzymatic work on the biosynthesis of glucosinolates by Glover et al. (1988) and this will be covered in the discussion). All of the steps in the outlined pathways have been worked out by feeding labeled intermediates into plants and tracing the products (Larsen , 1981 ; Underhill , 1980 ; Chisholm and Matsuo , 1971). The responsible enzymes are presumed to be present because enzymes catalyzing similar reactions to these proposed steps of the pathway have been isolated before . Two examples of these condensing enzymes and amino transferases are those involved in leucine and glutamic acid biosynthesis that was mentioned above . To understand the processes involved , it would be necessary to isolate the enzymes in the pathway and characterize them . In this way the metabolic controls for the biosynthesis of glucosinolates and the key controlled enzyme(s) can be determined . It was towards this end that the experiments in this thesis were designed . It was decided that by working with a plant that had predominantly one glucosinolate and an abundance of it at least in the seeds , pods , flowers and young leaves (McGregor and Love , 1987) that it would be possible to isolate some of the enzymes involved in the biosynthesis of that

glucosinolate . For this reason the plant chosen was Brassica juncea Zem and its predominant glucosinolate is allylglucosinolate (Fig. 4). B. juncea was also chosen because it is a cruciferous oilseed plant and it is related to crop plants used for the production of canola oil . Because of this and its high oil yield B. juncea is a potential canola oil crop (through crosses with other canola species) with favorable agronomic properties such as drought tolerance .

There are two logical places to start isolating the enzymes involved in the pathway to the production of allylglucosinolate ; the beginning and the end . Because of the importance of the initial step (the transamination) as a possible committed step to the pathway and because of the availability of methionine in both labeled and unlabeled forms , it was decided to start by isolating the enzymes at the beginning of the pathway . The first step involves an amino transferase reaction between methionine and some α -oxo acid to form alpha-oxo-gama-methiobutyric acid (α KMB)(fig. 4). A literature review of plant amino transferases reveals that a considerable amount of work has been done in this area (Gamborg , 1965 ; Wilson et al. , 1954 ; Wightman and Forest , 1978 ; Durham et al. , 1973 ; Cruickshank and Isherwood , 1958 ; Ellis and Davies , 1961 ; Reed and Hess , 1975 ; Cristen and Metzler , 1985). Of all of these references , only two dealt with the transamination of methionine and one of these was for the D-enantiomorph (Durham et al. , 1973) while the other involved a nonspecific amino

transferase which was found to transaminate twelve different amino acids including methionine (Gamborg , 1965). The experimental procedures followed in these works were extremely varied . Because none of the material dealt with a transaminase specific for l-methionine , the initial purification methods and reaction conditions were taken from those that were the most common and most easily performable of those found in the above literature review . The degree of purification varied from dialyzed crude preparations (Wilson et al. , 1953) to a 1100 fold purification (Reed and Hess , 1975) but most started with a desalted ammonium sulfate cut (Gamborg , 1965 ; Durham et al. , 1973 ; Cruickshank and Isherwood , 1957 ; Ellis and Davies , 1960 ; Reed and Hess , 1975). The most common pH was 7.5 (Gamborg , 1965 ; Durham et al. , 1973 ; Cruickshank and Isherwood , 1957 ; Reed and Hess , 1975 ; Wilson et al. , 1953) and of the buffers used phosphate was the most common (Gamborg , 1965 ; Durham et al. , 1973 ; Cruickshank and Isherwood , 1957 ; Ellis and Davies , 1960) except that there was some conflict as to whether phosphate ions were inhibitory or not (Wightman and Forest , 1978) ; but there were no secondary effects mentioned for Tris buffer (Reed and Hess , 1975 ; Wilson et al. , 1953). The α -oxo acids that were used most often were pyruvate (Gamborg , 1965 ; Durham et al. , 1973 ; Wightman and Forest , 1978 ; Cruickshank and Isherwood , 1957) , alpha-oxoglutarate (α OG)(Wilson et al. , 1953 ; Wightman and Forest , 1978 ; Durham et al. , 1973 ; Reed and Hess , 1975 ; Cruickshank and

Isherwood , 1957 ; Ellis and Davies , 1960) oxaloacetate (Cruickshank and Isherwood , 1957 ; Ellis and Davies , 1960 ; Wightman and Forest , 1978) and glyoxylate (Wightman and Forest , 1978). The concentrations of the α -oxo acids and amino acids varied considerably . The use of pyridoxal-S'-phosphate (p-S'-p) was found to be quite common (Wilson et al. , 1953 ; Wightman and Forest , 1978 ; Durham et al. , 1973 ; Cruickshank and Isherwood , 1957 ; Ellis and Davies , 1960) but Wightman and Forest (1978) , and Cruickshank and Isherwood (1957) found that p-S'-p had activating properties with some aminotransferases and not with others . Reed and Hess (1975) found that there was no activation with p-S'-p and aspartate aminotransferase from Avena sativa (oat) leaves . It was found that reducing agents , like beta-mercaptoethanol , were necessary to maintain activity during the isolation of the amino transferase for the D-enantiomorph of methionine (Durham et al. , 1973). They also found it was necessary to remove the reducing agent before assaying for enzyme activity because it was found to be inhibitory . The most common reaction temperature was 30 °C (Gamborg , 1965 ; Wilson et al. , 1953 ; Reed and Hess , 1975). The length of time the reaction was allowed to proceed varied from 10 min. (Cruickshank and Isherwood , 1957) to 6 hrs. (Gamborg , 1965).

Of the above choices it was decided to start the purification with a desalted ammonium sulfate cut and the buffer chosen for this was 0.1 M Tris at a pH of 7.5 . This

concentration of buffer was used because it was found to be sufficient to maintain the pH at the desired level through the various experimental conditions . The decision as to which α -oxo acids to use in the assay medium was left until those found in B. juncea could be determined and then those that were common to the above list would be used . It was decided to use p-5'-p because there were no deleterious effects mentioned . The same could be said of reducing agents so it was decided to add B-mercaptoethanol to the extraction medium and to remove it during the desalting step in case it was inhibitory as it was with the transaminase for the D-enantiomorph of methionine . The reaction temperature selected was 30 °C and a short reaction time of 30 min. was chosen ; which was thought to be sufficiently long enough to allow a detectable conversion of methionine and not long enough to create problems due to side reactions . The determination of the extent of the conversion from methionine to α KMB was determined through the use of radiolabeled methionine . The terminal methyl group of methionine which is preserved in the conversion to α KMB , had a radioactive carbon moiety . After the reaction the resultant radioactive α KMB was separated from the remaining radioactive methionine by thin layer chromatography (TLC) and counted .

Starting experimentation with the first step in the biosynthesis of allylglucosinolate has the added significance that this amino transferase(s) may also be involved in ethylene biosynthesis . Ethylene is a ubiquitous plant hormone which has

many different effects on different plant tissues at different stages in the life cycle (Ray et al. , 1983). The proposed pathway for the biosynthesis of ethylene follows a route from α KMB to methionine and then through S-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid to ethylene (fig. 6)(Kushad et al. , 1983). This then requires an aminotransferase which operates in the opposite direction to that which is sought here . The evidence for the proposed pathway comes from radio labeled tracing of precursors and not from enzymic study (Kushad et al. , 1983 , Baur et al. , 1971 , Baur and Yang , 1969) hence again there is no evidence for the transaminase for methionine . Due to the possible role of methionine amino transferase in ethylene biosynthesis , this enzyme or similar enzymes may be found in other species . That was the reasoning behind doing a species survey to see if there were such enzymes .

The inherent problem with isolating enzymes from their natural environment is that one also removes the controls that are on the enzymes and substitute an artificial set of controls due to choices in substrates , buffers , pHs' , and many other factors . Therefore the results that are obtained do not necessarily reflect rates of synthesis that are normally exhibited by these enzymes in the plant . Also the individual enzymes that are isolated , even if they are supplied with identical conditions found in vivo , will not show conversion rates indicative of those found in the plant because the other enzymes involved in the pathway will normally be supplying

Figure # 6 : The pathway from αKMB to ethylene showing the necessary transaminase step . This step is the reverse of the transaminase proposed in the biosynthesis of allylglucosinolate . (Kushad et al. , 1983).

substrates and removing products at their rates and this will affect the rate of reaction of the isolated enzyme . It was with this in mind that it was decided to follow up the isolation and analysis of the enzymes involved in the biosynthesis of allylglucosinolate with tracer studies on the rate of in vivo biosynthesis and degradation of allylglucosinolate . There has already been work done on the in vivo rate of biosynthesis of allylglucosinolate (McGregor and Love , 1987 , Glover et al. , 1988) but none of this work involved the rate of turnover nor did it deal with B. juncea . This in vivo part of the experimentation involved supplying radioactive carbon in the form of carbon dioxide for uptake by the plant (supplied in gas form in a confined space for normal photosynthetic uptake). The reason why radioactive methionine wasn't used in the tracer studies was due to the necessity that the tissues would have to be excised for labeling and a completely untampered rate of biosynthesis was desired . Analyzing tissues from the plant at various time intervals for radioactivity in the glucosinolate portion of the aqueous fraction would give an indication of the rates of biosynthesis and degradation of glucosinolates by the plant . Isolation of the glucosinolates took advantage of the sulfate group of the glucosinolate (fig. 1a). The glucosinolates were initially separated from the other anionic and neutral fractions with the use of ion exchange gels and then they were isolated from the rest of the cationic fraction by cleaving off the sulfate group with an arylsulfatase enzyme thereby rendering

it neutral (McGregor and Love , 1987). This method of isolating glucosinolates proved to be extremely selective and easy to perform because of the specificity of the arylsulfatase for sulfate groups and due to the fact that once the glucosinolate was desulfated it was simple to rinse it off of the column (Milsom et al. , 1972).

The overall purpose of the experiments in this thesis then involve the isolation of at least the initial enzyme in the pathway of glucosinolate biosynthesis and to show how its activity varies in relation to tissue , species , development and to how the glucosinolates are synthesized and degraded in vivo .

Materials And Methods

Chemicals

L-[methul- ^{14}C]-methionine was obtained from Amersham and the ^{14}C -sodium carbonate was obtained from Atomic Energy Canada . Allylglucosinolate was obtained from Sigma Chemical Co. and Desulfoallylglucosinolate was courtesy of Dr. Ian McGregor Agriculture Canada in Saskatoon . Seeds of Brassica juncea were also obtained from Dr. Ian McGregor . Sephadex G-25 medium , Sephacryl S-200 superfine , DEAE A-25 , Sephadex C-25 and DEAE A-50 were obtained from Pharmacia . Avicel Cellulose Uniplates were obtained from Mandel . The scintillation cocktail used for aqueous samples was 5 ml of toluene : triton X-100 (2:1 v/v) made up to 0.5% PPD (w/v) and the cocktail for nonaqueous samples was 5 ml of 0.5% PPD (w/v) in toluene . Otherwise reagent grade chemicals were used . The purity of all amino acids , oxo acids and glucosinolates were checked by TLC . No solution was kept any longer than 1 month and all were stored at $-10\text{ }^{\circ}\text{C}$ when not in use . The exception to this was the radioactive methionine which was stored at $-80\text{ }^{\circ}\text{C}$.

Plant Material

Seeds of Brassica juncea Zem 83-450 that were harvested in 1985 were received on 29-09-1986 courtesy of Dr. Ian McGregor from Agriculture Canada in Saskatoon . These seeds were germinated in vermiculite and 7 days after planting were

transferred to individual 6 inch standard pots in soil (sandy loam : peat moss : perlite ; 4 : 4 : 1 v/v/v). Upon transplanting , 10 ml of Nutricote slow release 14-14-14 fertilizer from Plant Products of Canada Ltd. were added to the pots . For the first 4 weeks , including the initial germination period , the plants were grown in a growth chamber . The temperature was maintained at 24 °C +/- 1 °C and they received 14 hours of light . The light period consisted of an initial hour of incandescent light supplying 200 $\mu\text{E}/\text{sec m}^2$, 12 hours of combined incandescent and fluorescent light supplying 450 $\mu\text{E}/\text{sec m}^2$, and a final hour of incandescent light identical to the first (Baillie , 1987). The light period started at 5 am and ended at 7 pm . After this 4 week period the plants were moved to the greenhouse and received sunlight augmented with fluorescent and incandescent light which was supplied from 6 am to 12 pm daily . During the entire growing period the plants were watered twice daily between 9 and 10 am , and 3 and 4 pm . Also once the plants were transferred to the green house they received , on a weekly basis , 20-20-20 fertilizer from Plant Products Canada ltd. which was added to their morning watering . Plant samples were taken between 9:30 and 10:30 am , unless otherwise stated , and were 4-5 weeks of age .

Other species used for comparative studies were obtained from those available in the green house . Their growing conditions were generally similar to that of the green house segment of the growing period for B. juncea described above .

Measurement of Plant Growth and Harvesting

Linear measurements were taken for a variety of aspects of plant growth from the point where the seedlings first appeared above the vermiculite until well after the age of the oldest plants used in any of the experiments . These measurements included the overall height of the plant , length and width of cotyledons and leaves , numbers of leaves per plant , and time of first flowering . The reasons for taking these measurements were that any plant that is harvested can be compared to the average plant to ensure consistency throughout the experiments and also any results that are obtained can be compared to the development of the plant to see if there is any correlation .

Tissues for the various experiments were excised by razor blade and the different tissue types were defined as follows :

- Flowers and Buds : all flowers whether open or not including the small stem connecting them to the main or secondary stem of the plant .

- Young leaves : all leaves above but not including the youngest of the mature leaves .

- Youngest Mature Leaf : the leaf which is most recently expanded so that it no longer appears to be crinkled .

- Mature Leaves : all leaves below and including the youngest mature leaf .

- Upper Stem : all of the stem above the youngest mature leaf .

- Lower Stem : all of the stem below the youngest

mature leaf .

Chromatography Solvents

All chromatography solvent systems were modified (ie. alterations in the ratios of the component solvents) from those found in the Handbook of Chromatography (Zweig and Sherma , 1972) and they were adjusted so that maximum separation was obtained for the particular compounds used in the experiments listed below . All solvents were tested under all of the conditions they were used in to ensure that no erroneous results were obtained due to the conditions of these experiments (ie. changes in R_f values due to pH changes etc...).

α -Oxo Acid Isolation

Leaves (2.8 Kg) and pods (0.4 Kg) were separately excised from plants of ages 4 to 6 weeks and were blended in a Waring blender in 0.2 N HCl to denature the enzymes and extract the α -oxo acids . The acidified homogenates were then centrifuged at $16,300 \times g$ for 20 min in a Sorval super speed RC2-B automatic refrigerated centrifuge . DNPH derivatives of the α -oxo acids were then prepared following the procedure of Fowden and Webb (1955) . These derivatives were then separated on Whatman # 3MM chromatography paper using n-butanol saturated with 6% ammonium hydroxide as the solvent . DNPH derivatives of the α -oxo acids : pyruvate , α OG , glyoxylate , and α KMB were also made through the procedure of Fowden and Webb (1955) and run as markers .

Amino Acid Isolation

Mature leaves from ten 4 wk. old plants (approximately 215 g) were excised and immediately boiled in 95% ethanol. These were then ground with a mortar and pestle and filtered through Whatman # 1 filter paper on a Buchner suction filter. The volume was reduced to 3-4 ml using a roto-evaporator. At this point it was necessary to centrifuge the extract due to a precipitation of aggregated pigment complexes and other aggregated particulate matter. The centrifugation was done at $480 \times g$ for 10 min. This extract was then run through a column containing 60 ml of Rexyn 101 ion exchange resin H^+ form at a flow rate of 1 ml per 100 sec. followed by 12 L of distilled water to thoroughly rinse the column, at the same rate. The amino acids were eluted by passing through 1 L of 4 M ammonium hydroxide, also at the same rate. The litre of ammonified eluate was then placed under a stream of air to drive off the ammonia and reduce the volume (3.7 ml). The final product was analyzed for amino acid content by HPLC (Waters Analyzer Model 721; RESOLVE(tm) Sum C18 3.9 mm x 15 cm column). Analysis followed the AUTO.TAG(tm) procedure which involved automated pre-column derivatization of the samples with o-phthalaldehyde (Roth, 1971) which took place in a WISP(tm) sample injector (special thanks to Dr. Joy's lab. for the performance of this procedure).

Protein Determination

The procedure that was used was a modification of Bradford's (1976) that was found to give the most stable results in the systems used. A concentrate was made up of 70 mg Serva Blue-G, 50 ml 95% ethanol, 100 ml 85% phosphoric acid and 50 ml of distilled water. This was stirred for 30 min. The concentrate was kept for no more than one month and a series of dilutions of BSA were always run along with the samples for comparison to the standard curve. Aliquots of the concentrate were then used to make up the Coomassie reagent by diluting 1:4 v/v with distilled water. This was then filtered three times with Whatman # 42 filter paper under suction. Five ml of this solution were then mixed with 0.1 ml of the protein solution to be determined and mixed by inversion. After 5 min. the samples were read at 590 nm on a Bausch and Lomb Spectronic 1001 spectrophotometer and compared to a standard curve made using BSA (fig. 6). The samples were always read in less than 20 min. of adding protein samples so that no breakdown of the dye complex occurs.

Methionine Amino Transferase Purification

The plant tissue used as the enzyme source was stem tissue unless otherwise stated. The tissue was excised and ground in a Waring blender in 0.1 M imidazole buffer pH 7.5 (or 0.1 M Tris pH 7.5 where indicated) at 0-5 °C (this temperature was maintained throughout the extraction procedure). After filtering

Figure 7 : This standard curve represents the average of 5 protein determinations of BSA each done in triplicate for the modified Bradford method (see materials and methods) used to determine protein concentrations for all experiments . For each individual assay a standard curve was also done and compared to this one . The slope of the line is $1.24\text{E-}2$ abs. units / μg protein . Absorbances from experimental samples were taken only between 0.2 and 0.5 .

through 4 layers of cheese cloth the extract was centrifuged at $48,200 \times g$ for 20 min. . At this point a 35-55% ammonium sulfate fraction was taken by first adding ammonium sulfate to 35% saturation and centrifuging and then adding sufficient ammonium sulfate to the supernatant to make it up to 55% and centrifuging again . The centrifuging was done at $12,100 \times g$ for 10 min. and there was a 15 min. wait after the addition of the ammonium sulfate to allow complete precipitation . The 35-55% pellet was resuspended in minimal (approx. 3-6 ml) 0.1 M imidazole buffer and desalted on a 15 ml Sephadex G-25 medium column prepared in the same buffer as the extract . The volume was then measured and 10 μ l of 100 mM PMSF in 95% ethanol were added per ml of desalted enzyme preparation to inhibit protease activity . The PMSF solution was made just prior to use to ensure maximum activity . This is then the standard enzyme preparation and all assays using it were done immediately after isolation .

Methionine Amino Transferase Activity Assay

Two methods of testing for amino transferase activity were used . The first method which was used for the initial trials for locating activity and enhancing it (Non-Radioactive Assay) and the second method which used radioactive methionine and was used to quantify activity (Standard Radioactive Assay). Both methods were always done in triplicate unless otherwise stated .

a) Non-Radioactive Assay

The following were mixed in the order listed :

- 38 μ l glass distilled water
- 10 μ l 1.0 M buffer pH 7.5 (for this assay the buffer was Tris)
- 5 μ l 100 mM methionine
- 2 μ l 10 mM P-S'-P
- 40 μ l enzyme preparation
- and finally to start the reaction , 5 μ l 100 mM α -oxo acid

The reaction was run at 30 °C and ran for 30 min. . The reaction was stopped by placing the reaction tubes in a boiling water bath . The α -oxo acids used were pyruvate , α -oxo-glutarate , and glyoxylate . These were neutralized first by converting them to their sodium salts . For each reaction tube there was one control which contained all of the above except instead of the α -oxo acid an additional 5 μ l of water was added .

To analyze the reaction the mixtures were chromatographed using Avicel cellulose uniplates and n-butanol : acetone : 25% ammonia : water (10:10:3:8 v/v) as the solvent . This system separates the amino acids and they were visually determined after spraying the plates with 0.1% ninhydrin in acetone and heating to 95 °C for 2 min. (Zweig and Sherma , 1972). A solution of known amino acids was used as the marker .

b) Standard Radioactive Assay

This assay was used for all of the quantitative experiments with some modifications where noted . It is identical

to the above nonradioactive assay except that 0.5 μ l of L-[methyl- 14 C]-Methionine (specific activity = 58 mCi/mmol : total activity 50 μ Ci in 100 μ l) was added along with the cold methionine to both the control and reaction tubes . Also the separation method was modified to separate methionine from its α -oxo acid α KMB . To do this acetone : 95% ethanol : 25% ammonia : water (27:10:2:1 v/v) was used as the solvent on the same TLC plates and methionine was the marker . The reaction was quantified by scraping the plates with a razor blade and counting the scrapings using a LKB Wallac 1217 Rackbeta liquid scintillation counter and the non-aqueous cocktail . Counting was done for 5 min. per sample . Breakdown of the radioactive methionine was checked periodically by chromatography .

Controls for Ensuring Methionine Amino Transferase Activity

The following series of control experiments were performed to ensure that methionine amino transferase was responsible for the obtained results .

a) Using the above nonradioactive assay different methods of stopping the reaction were tried :

1) The enzyme in the control was added before the addition of the other reaction ingredients and then boiled and both the control and the assay mixture were boiled after the assay .

2) The enzyme was added first to both the control and the assay tubes and then 50 μ l 25% TCA was added to the

control . The rest of the ingredients were then added and the reaction was stopped with TCA being added to the assay tube .

3) The control was 40 μ l of distilled water instead of enzyme .

4) The α -oxo acid or the amino acid was left out of the control and 5 μ l of water were added instead .

b) Using the nonradioactive assay conditions , the reverse of the reaction was performed . This meant that α KMB was added instead of methionine and ; glutamate , glycine and alanine were added instead of α OG , glyoxylate and pyruvate respectively .

c) The standard radioactive assay was used to determine the time course of the reaction . The assay was stopped at 10 , 15 , 30 , 45 , 60 , 90 and 120 min. .

d) The effect of varying the concentration of the enzyme was tested using the standard radioactive assay and 1 , 2 , and 4 fold dilutions of the enzyme preparation .

Additional Methionine Amino Transferase Purification Methods

a) Further Purification of Standard Enzyme Preparation Using DEAE A-50

The above standard enzyme preparation was run through a 25 ml DEAE A-50 anion exchange column chloride form in 0.1 M Tris buffer pH 7.5 and fractions were taken off using 40 ml each of the following salt concentrations : 0.1 , 0.2 , 0.3 , and 1.0

M NaCl . The flow rate was 1 ml per 30 sec. . Each of these fractions was then reduced in volume with an Amicon ultrafiltration system using a YM-10 membrane . This system is driven with compressed nitrogen and has a molecular weight cutoff of 10,000 Daltons which also results in the sample being desalted . The final fractions were then tested for enzyme activity by the non-radioactive assay .

b) Further Purification of Standard Enzyme Preparation Using Sephacryl S-200 superfine

The above standard enzyme preparation was run through a 215 ml Sephacryl S-200 superfine column (prepared by rinsing with 0.1 M Tris buffer pH 7.5) at a flow rate of 5 ml / 10 min. and collecting 50 fractions . Sephacryl separates on the basis of molecular weight . These fractions were then assayed for enzyme activity by the non-radioactive assay .

Buffer Effect on Methionine Amino Transferase Activity

For this experiment 4-5 week old stem tissue was used exclusively . The tissue was extracted by the above Amino Transferase Purification procedure with the exception that 0.01 M buffer of each of the buffers used in the assays listed below was used for the extraction (Table 1). The pH of the extraction buffer varied with the buffer (Table 1). The assay for enzyme activity was that of the Standard Radioactive Assay and it was performed for each pH of each buffer (Table 1). The entire

experiment was done in triplicate . The pH of the final reaction mixture was checked and it corresponded to those listed in table 1 within ± 0.05 pH units .

Buffer	pH of Extraction Buffer	pH's of Assays
Tris	7.5	7.5 , 8.0 , 8.5 , 9.0
HEPES	7.5	7.0 , 7.5 , 8.0
McIlvaine	7.0	5.0 , 5.5 , 6.0 , 6.5 , 7.0 , 7.5
Imidazole	7.0	6.5 , 7.0 , 7.5

Table # 1 : Listed above are the pH's for the different buffers used in the Buffer Effect on Amino Transferase Activity experiment . All buffers were 0.1 M in concentration .

Tissue Variability of Methionine Amino Transferase

The Methionine Amino Transferase Purification and the Standard Radioactive Assay procedures were used for this experiment . The following tissues were used : mature leaves , young leaves , stems , flowers and buds , pods , and roots . Both Tris (pH 7.5) and imidazole (pH 7.5) were used , although roots weren't done with Tris . Five plants were used for each tissue .

Species Distribution of Methionine Amino Transferase

The Methionine Amino Transferase Purification and the Standard Radioactive Assay procedures were used for this experiment . The following species were used : Brassica juncea , Zea mays hybrid Seneca Chief (corn) , Cucurbita pepo c.v. early prolific straight neck (squash), Lycopersicon esculentum hybrid Burpees' Big Boy (tomato) , Glucine max hybrid Maple Arrow (soybean) , and Gossypium barbadense hybrid SJ-2 (cotton). The two most recently matured leaves from each of 5 plants were used for the experiment .

Aryl Sulfatase Preparation

The crude aryl sulfatase enzyme was purified by dissolving 98 mg (2000 units) of the enzyme in 6.0 ml of water and centrifuging at $121 \times g$ for 10 min. . To the supernatant was added 6.0 ml of 95% ethanol and centrifugation was repeated . Another 18 ml of 95% ethanol was added to the supernatant and centrifuged again . After the addition of the ethanol and before centrifugation , 10 min. was allowed for precipitation of the proteins . The pellet was resuspended in 5 ml of distilled water and run through a 2 ml DEAE A-25 anion exchange column chloride form and a 2 ml Sephadex C-25 cation exchange column hydrogen form at a rate of 1 drop per second , with a 0.5 ml rinse with distilled water each (final volume 6.0 ml).

The activity of the preparation was checked by the breakdown of p-nitrocatechol sulfate . To 2 ml of 0.15 mM (0.32μ moles/

2 ml) p-nitrocatechol sulfate was added 0.5 ml of a 1 in 10 dilution of the sulfatase enzyme preparation . The breakdown of p-nitrocatechol sulfate was monitored on a Bausch and Lomb spectronic 1001 spectrophotometer at a wavelength of 325 nm . The reaction was considered over when the change in absorbance leveled off . The enzyme had an activity of 1 unit converts 1 μ mole / hr so theoretically the reaction should have taken approximately 1.1 min. . However if the reaction took less than 4 min. the enzyme was judged sufficiently active and was used .

Quantitative Isolation of Glucosinolates from Tissues

The desired tissue from a minimum of 5 plants (if < 50 g additional plants were harvested) was excised by razor blade and immediately placed in boiling 95% ethanol . This was then ground with a mortar and pestle and filtered through Whatman # 1 filter paper on a buchner suction filter . The volume was reduced to approximately 5 ml using a roto-evaporator . At this point it was usually necessary to centrifuge the extract due to a precipitation of pigments and other large macromolecules . The centrifugation was done at $480 \times g$ for 10 min. . This extract was then run through a 2 ml column containing Rexyn 101 cation exchange resin hydrogen form (removes mostly amino acids). The flow rate through the column was 1 drop per 5 sec. and an additional 2 ml was run through the column as a rinse . Next it was run through a DEAE A-25 acetate form anion exchange column (removes organic acids) at the same rate . It was necessary to

rinse the column with 50 ml of 0.02 M acetate buffer pH 4.5 to remove the last of the non-anionic compounds . The glucosinolates were then removed from the column by placing 0.5 ml of aryl sulfatase enzyme (purified as above) on the column and letting it stand over night . The desulfated glucosinolates were then removed by rinsing 5 ml of acetate buffer through the column . The purity of the end product was checked by TLC chromatography using Avicel cellulose plates and butanol : water : acetic acid (4:4:1) as the solvent . The markers were glucose , allylglucosinolate and desulfoallylglucosinolate . The compounds were detected using the silver nitrate method for detecting reducing sugars (Zweig and Sherma , 1972) .

The tissues used for this experiment were mature leaves , young leaves , flowers and buds , upper stem , lower stem and roots . The difference between the lower and upper stem is the upper stem is that which is above the most recently matured leaf and the lower stem is the rest . The final extracts were then analyzed by HPLC . This was accomplished using a Varian model 504A HPLC with a RPC18 semiprep column . The solvent was initially 100% water for the first 10 min. and then a 0 - 12% gradient of acetonitrile was washed through the column over a 20 min. time period . The glucosinolates were detected by UV (wavelength of 227 nm) and compared to standards .

Quantitative Analysis of Glucosinolates

Both glucosinolates and desulfoglucosinolates have a glucose moiety and it is possible to quantify them by measuring the amount of glucose present. The Dubois assay was used for this procedure and quantities were determined with the use of a generated standard curve (Dubois et al., 1956, glucose fig. 7, allylglucosinolate fig. 8, desulfoallylglucosinolate fig. 9).

Labeling with Radiolabeled Carbon Dioxide and Analysis of the in vivo Production of Glucosinolates

A 4-5 week old plant was placed in a fume hood supplied with water filtered incandescent light ($100 \mu\text{E}/(\text{sec m}^2)$) (Baillie, 1987). It was allowed to stabilize for 15-30 min. Then the most recently matured leaf was enclosed in a plastic bag which was sealed around the petiole with a twist tie. This was done so that the leaf wasn't touched by the bag and the petiole wasn't damaged. The label was ^{14}C -carbon dioxide produced by mixing $100 \mu\text{l}$ of ^{14}C -sodium carbonate (10.4 mCi in 10 ml 0.5 M NaOH) with an equal volume of 5 N HCl in a glass syringe and it was introduced into the bag by injecting the gas produced with the aid of the syringe. The bag was left surrounding the leaf for 15 min. and was then removed. The plant was then left for varying lengths of time to allow incorporation of the label (1, 3, 12, 24, and 48 hours). Since all labeling was started at 10 am this meant that the plants were harvested at different times of the light cycle. After 1 hour the plant was removed to

Figure 8 : This is the standard curve used in all determinations of glucose by the Dubois assay . This curve was obtained through 3 assays each done in triplicate and has a slope of 7.75×10^{-3} abs. units / μg glucose .

Figure 9 : This is the standard curve used in all determinations of Allylglucosinolate by the Dubois assay . This curve was obtained through 3 assays each done in triplicate and has a slope of $2.73\text{E-}3$ abs. units / μg Allylglucosinolate .

Figure 10 : This is the standard curve used in all determinations of desulfoallylglucosinolate by the Dubois assay . This curve was obtained through 3 assays each done in triplicate and has a slope of $3.63\text{E-}3$ abs. units / μg desulfoallylglucosinolate .

a fume hood in the green house so that it would receive normal lighting . At the end of this time , its tissues were excised and immediately immersed in boiling ethanol . The tissues were removed in the following order : labeled leaf , flowers and buds , young leaves , upper stem , mature leaves , and lower stem . The difference between the lower and upper stem is the upper stem is that which is above the most recently matured leaf and the lower stem is the rest . The tissues were then run through the procedure for Quantitative Isolation of Glucosinolates from Tissues with the exception that the desulfoglucosinolates were checked for purity by TLC and not HPLC . At the following places an aliquot was removed for counting on the scintillation counter using the aqueous cocktail : after roto-evaporation and centrifugation , after the rexin 101 column , the pool of rinses off of the DEAE A-25 acetate column , and the final rinse of desulfated glucosinolates . All samples were counted for 5 min. each .

Results

Plant Growth

B. juncea followed a very consistent growth pattern when it was supplied with stable growing conditions . At the beginning of the experimental work , when the plants were transferred to the green house after their initial germination and growing in the growth chamber they were not supplied with any additional light beyond sunlight . As the days shortened in the fall the plants became spindly and flowered up to two weeks earlier than they were in the summer . Once they were supplied with lighting (as indicated in the materials and methods) upon transfer to the green house they returned to the growth pattern typical of the summer . The summer growing season also produced its problems due to the heat that the green house could attain if not air conditioned . Once these problems were identified it was easy to produce plants that were consistent and therefore useful for experimentation .

B. juncea has an initial slow growth period for the first three to approximately four weeks after germination (week 1 : fig . 10 , week 2 : fig. 11 , week 3 : fig. 12 , week 4 : fig. 13). During this period the plant first grows , after the cotyledons , two leaves which have a different morphology than the leaves that come later . These two leaves are much smaller and don't have as much serration as the next six or seven leaves to come (fig. 13). The six or seven leaves that come next are

Figures 11 , 12 , 13 , 14 , 15 , 16 : These figures illustrate the typical growth pattern of B. juncea . The drawings depict successive weeks of age starting at one week after the seeds were planted . The leaves are numbered starting with the first to appear and the scale given represents 10 cm . The following list gives the ages :

- Fig. 11 : one week old
- Fig. 12 : two weeks old
- Fig. 13 : three weeks old
- Fig. 14 : four weeks old
- Fig. 15 : five weeks old
- Fig. 16 : six weeks old

Figure 11 :

Figure 12 .

Figure 13 :

Figure 14 :

Figure 15:

Figure 16 :

the main leaves of the plant (leaves 3 to 8 or 9 , fig. 13) because by the time the plant is ready to flower those initial two leaves have senesced (approximately 4 to 4 1/2 weeks). At this point those leaves which are called the main leaves of the plant have developed but the youngest two or three have not yet matured (fig. 13). This is also the point where the slow growth phase ends and the plant extends its height rapidly through stem elongation (fig. 16 and 14). During this time no new leaves are formed but the existing ones increase in size and at the end of this time the plant is usually in flower (fig. 14). Beyond week five the plant continues to rapidly increase in height but now the growth is of a secondary nature (fig. 15). The leaves that grow are small and less serrated and growth is restricted to the stem segments in the flowering regions . These regions start off at the leaf nodes and initially grow a few of these secondary leaves and then once they start to flower the secondary stem that they are on elongates which results in the formation of new flowering regions and the old now dead flowers which are left behind develop into pods . This is also the growth pattern of the main stem .

-Oxo Acid Composition of Leaves and Pods

This was one of the original experiments to check which α -oxo acids were more abundant so that appropriate ones were chosen for the amino acid transferase assays and also to see if α KMB was present in detectable amounts . This system detected only the

Figure 17 : Histogram of the relative heights of each week of age for B. juncea . Heights are averages from 30 plants per age group taken over a span of 6 different plantings .

Figure 17 : Growth profile

more abundant α -oxo acids (approx. > 1 n mole / g fwt) and those that were stable in 0.2 N HCL and was therefore used only for the above purpose and not to produce an α -oxo acid profile . From the leaf extracts were detected α OG , pyruvate and glyoxylate , and from the pods were detected α OG , glyoxylate and traces of pyruvate . In this system the DNPH derivative of α KMB had an R_f value which was considerably different from the three detected keto acids and it was clearly not present in detectable amounts (table 2).

It was decided to use pyruvate , α OG and glyoxylate as the initial α -oxo acids for the methionine amino transferase experiments .

α -oxo acid	R_f value
α KMB	0.76
α OG	0.15
glyoxylate	0.46
pyruvate	0.54

Table 2 : These R_f values are for the DNPH derivatives of the α -oxo acids . They were separated on Whatman # 3MM chromatography paper using n-butanol saturated with 6% ammonium hydroxide as the solvent and the solvent front ran an average of 47 cm . The values are an average of three trials .

Amino Acid Composition of Mature Leaves

This experiment like the previous one was performed so that the internal pools of the other component of the amino transferase assay , namely methionine , could be ascertained . The resultant HPLC chart (fig. 17) indicates the complex array of amino acids typically found in plants . There were 15 identified and 6 unknown amino acids and methionine was detected in barely discernable quantities . The most abundant amino acids found were serine , alanine and threonine .

Methionine Amino Transferase Purification

Since this was the first attempt at isolating the aminotransferase part of the biochemical pathway for the synthesis of allylglucosinolate an initial preparation only involved either an ammonium sulfate cut or a PEG cut in an effort to locate activity for methionine amino transferase at all . All initial experiments were plagued by high background staining on the TLC plates even after the enzyme preparation was desalted on columns that would remove amino acids . It was decided that this background activity was due to amino acids and possibly dipeptides produced due to protease activity during the reaction time of the assay and PMSF was added to hopefully inhibit this . This did in fact almost completely remove the background staining . It is of interest that no reference could be found to this problem in any earlier studies with plant amino transferases , and that no protease inhibitors were used .

Figure 19 : Time course

Figure 18 : Amino acid profile

The decision to use PMSF came from a reference involving animal proteases (Storey and Bailey, 1978). It was determined that ammonium sulfate at saturation levels between 35 and 55 % resulted in an optimal isolation of methionine amino transferase on a visual basis over any other saturation level of ammonium sulfate or any with PEG.

Methionine Amino Transferase Activity Assay

Following the isolation of the crude enzyme preparation, attempts were made to increase the amount of activity in the assay. Since these experiments were performed before the use of radio labeled methionine the determinations were made on a visual basis. Initially this was done by varying the concentrations of the α -oxo acids, methionine, buffer, and p-5'-p until those listed in the non radioactive assay were settled upon. The α -oxo acid and methionine concentrations were varied from 1 to 5 to 10 mM final concentration. The 5 mM concentration was chosen because the 1 mM concentration resulted in too little a conversion and even though the 10 mM concentration had similar conversion to the 5 mM it was found to overload the TLC plate. The range of buffer concentrations (from 10 to 100 mM to 1 M) had little effect on the conversion in the assay so the concentration that was sufficient to maintain the pH reliably (100 mM) was chosen. These changes were attempted one at a time to see which concentration for each was optimal. It was noted in the literature that some thought that it was not necessary to add

p-5'-p as a cofactor for some plant amino transferases (Wightman and Forest , 1978 , Cruickshank and Isherwood , 1957 , Reed and Hess , 1975). This was verified in the concentration check for p-5'-p , since there was activity for all concentrations used for this ingredient (0.0 , 0.2 , 1.0 , 5.0 , 10.0 mM). It was still added to the assay system because it was thought that there may have been a slight drop in activity when it was completely removed from the assay . Because this determination was on a visual basis , since activity level was on the basis of the size and intensity of color found in the stained amino acid spots found in a given assay , it was decided that it was safest to add a small amount of p-5'-p (0.2 mM) since it was not detected to inhibit the reaction .

Controls for Ensuring Methionine Amino Transferase Activity

The importance of controls , was what inspired this section of the work . The following experiments were designed in an attempt to rigidly eliminate the artifacts from the preliminary results . Due to the low activity detected in the transaminase assays any small amount of protease activity could result in erroneous results .

a) The method of stopping the reaction was picked as that which would least likely alter the control tube from the reaction tube in relation to effects on ingredients or effects on how they ran on TLC and also that which was easiest to perform . Of all of the methods of stopping the reaction only the use of

TCA appeared to produce artifacts . TCA resulted in a strong change in the R_f values of the amino acids and also resulted in the production of additional ninhydrin reactive spots on the TLC plates , and therefore was eliminated as a possibility . The effect TCA had on the assay system was not pursued since its effects were not pertinent to the study of methionine aminotransferase . The remaining methods collectively showed that the results were not produced from some ingredient in the enzyme pool or its effects on the other added ingredients . Leaving the enzyme out as the control showed that the proteases in the reaction tube still had a modicum of effect but that this was minimal (additional spots from the different α -oxo acids were minimal). Leaving out the α -oxo acid or the amino acid had an identical effect to boiling the enzyme in the control prior to the reaction . This showed that the observed reaction required the enzyme pool , methionine and one of the three α -oxo acids .

b) If the observed amino acid formation was actually due to enzyme activity then as in all known transaminases there has to be the possibility that the reaction is reversible (ie. α -KMB + amino acid $\rightleftharpoons$ methionine + α -oxo acid). This was easy to check as indicated in the methods section and resulted in definite though small amounts of methionine being detected (determination on a visual basis). The source of the methionine in this reverse reaction would have to be from the reverse activity of the transaminase enzyme as was indicated in the above controls for the forward reaction that the source of the amino

acids would have to be from α -oxo acids placed in the reaction vessels for the forward direction of the reaction .

c) The reaction would also follow a time course indicative of enzyme kinetics if an enzyme was responsible and saturation kinetics are attained . This means that the formation of the product would initially be slow during the time period where the free enzyme is used up in the formation of the enzyme-substrate complex and then would accelerate as the enzyme became saturated until the substrate concentration dropped below that required for saturation kinetics where the reaction rate would gradually taper off as the substrate runs out (Lenninger , 1979). A slow down in the rate of reaction can also be due to a breakdown of the enzyme , product inhibition or if there is a noticeable amount of reverse reaction of the enzyme . In viewing table 3 and fig. 18 the results showed the problem with all of the numeric data accumulated before the discovery of the activity attained with imidazole buffer , and that was the conversion to .KMB of methionine was so low that the accumulated error of the system was often as great as the actual value . Even though no conclusion , as to whether an enzyme was responsible , can be drawn from this particular data , it did result in a curve that resembles the above mentioned enzyme kinetics (fig. 18). Also it would appear from fig. 18 that the choice of a 30 min. reaction time does fall within the time period where the enzyme is exhibiting saturation kinetics .

d) If the enzyme in the reaction was at a concentration

Time (min.)	n moles α KMB /assay	n moles α KMB /g fwt	n moles α KMB /mgp
10	0.2 +/- 0.3	2.7	2.7
15	2.5 +/- 2.5	33.5	33.8
30	5.5 +/- 3.0	73.7	74.3
45	6.0 +/- 3.5	80.4	81.0
60	10.0 +/- 8.5	134.0	135.0
90	5.5 +/- 1.5	73.7	74.3
120	6.5 +/- 2.5	87.1	87.8

Table 3 : The enzyme time course is that for α OG using stem tissue , 0.1 M Tris buffer pH 7.5 and the standard radioactive assay . (n = 3)

Figure 19 : The curve for the enzyme time course for 0.1 M Tris buffer pH 7.5 using stem tissue . Data from table # 3 .

Figure 19 : Time course

which produced straight line kinetics then if the concentration was halved the resultant product produced for a given length of time should also be halved . Table 4 for the 30 min. reaction time does indicate that this was a possibility for the stock enzyme and the two fold dilution of the enzyme .

Additional Methionine Amino Transferase Purification Methods

Having established the presence and detectability of the methionine aminotransferase enzyme , the next step was to purify the enzyme further so that it would be more concentrated and therefore could be characterized better . Unfortunately there was absolutely no detectable enzyme activity from either the DEAE fractions or the Sephacryl fractions .

Buffer Effect on Methionine Amino Transferase Activity

The choice of which buffer should be employed was apparently critical in studying methionine amino transferase . All four buffers studied here produced different results in terms of both rates of reaction and pH optima with each of the α -oxo acids used . Peak activity with pyruvate (table 5) would appear to be at pH 7.0 . This observation is not concrete because no activity was found in McIlvaines buffer and HEPES buffer produced unreliable results (standard deviation approx. 30% of the attained value with pH's 7.0 and 7.5 , table 9). Buffers Tris and imidazole have standard deviations though that for the most part are much less than 30% of the values they correspond to (tables 8

Dilution	Time (min.)	n moles α KMB /assay	n moles α KMB /g fwt	n moles α KMB /mgp
Stock	10	0.2 $\pm$ 0.3	2.7	2.7
X 2	"	0.0	0.0	0.0
X 4	"	0.0	0.0	0.0
Stock	30	5.5 $\pm$ 1.5	73.4	74.3
X 2	"	4.0 $\pm$ 2.0	53.6	54.0
X 4	"	0.0	0.0	0.0

Fig. 4 : The effect of dilution on the enzyme assay . These dilutions are of the standard stem extraction using α OG as the α -oxo acid and 0.1 M Tris pH 7.5 as the buffer .(n = 3)

& 11). The α -oxo acid α OG had an activity peak at approximately pH 6.5 . In looking at the standard deviations for α OG in each of the buffers , imidazole buffer produces the most accurate results ; but this was mostly due to the higher activity attained in that buffer (table 11). HEPES buffer again , as with pyruvate , produced unreliable results (table 9). Glyoxylate showed the highest activity in all buffers except McIlvaines and had the lowest standard deviations (tables 8 , 9 , 10 , 11). The peak activity for glyoxylate was at approximately pH 8.0 (table 7). All three α -oxo acids had a different pH optimum but pyruvate and α OG had similar ones (7.0 and 6.5 respectively) while that of glyoxylate was different (pH 8.0 , tables 5 , 6 , 7). Though the actual values for the pH optimums are not that different the trends observed tables 5 , 6 and 7 indicate that pyruvate and α OG do have lower pH optimums than glyoxylate .

Tissue Variability of Methionine Amino Transferase

Ammonium sulfate cuts from the younger tissues and reproductive parts of B. juncea seem to show a higher activity of methionine aminotransferase than the older tissues when assayed for 30 min. in imidazole buffer and using glyoxylate as the α -oxo acid (table 12). Stems (complete stem), flowers and buds , and pods had the highest activity . The initial work done on tissue variability of methionine amino transferase was done when the buffer used was Tris . The results from this earlier work agreed with that of the latter work with imidazole buffer and the α -oxo

pH	Buffer			
	Imidazole	Tris	McIlvaines	HEPES
5.0	-----	-----	0.0	-----
5.5	-----	-----	0.0	-----
6.0	-----	-----	0.0	-----
6.5	21.5	-----	0.0	-----
<u>7.0</u>	<u>24.0</u>	-----	0.0	<u>51.5</u>
7.5	21.5	<u>21.0</u>	0.0	1.5
8.0	-----	7.0	-----	15.5
8.5	-----	2.5	-----	-----
9.0	-----	0.0	-----	-----

Table 5 : pH profile for the α -oxo acid pyruvate in different buffers . The units are n moles α KMB / mgp . The underlined numbers indicate those for the optimum pH . The enzyme source was stem tissue , the standard radioactive assay was used and the reaction time was 30 min. .

pH	Buffer			
	Imidazole	Tris	McIlvaines	HEPES
5.0	-----	-----	2.9	-----
5.5	-----	-----	28.0	-----
6.0	-----	-----	<u>42.0</u>	-----
<u>6.5</u>	<u>47.5</u>	-----	39.2	-----
7.0	43.0	-----	19.7	42.0
7.5	40.5	16.0	16.8	15.5
8.0	-----	10.5	-----	56.5
8.5	-----	5.5	-----	-----
9.0	-----	0.0	-----	-----

Table 6 : pH profile for the α -oxo acid aOG in different buffers . The units are n moles α KMB / mgp . The underlined numbers indicate those for the optimum pH . The enzyme source was stem tissue , the standard radioactive assay was used and the reaction time was 30 min. .

pH	Buffer			
	Imidazole	Tris	McIlvaines	HEPES
5.0	-----	-----	0.0	-----
5.2	-----	-----	0.0	-----
6.0	-----	-----	0.0	-----
6.5	243.0	-----	0.0	-----
7.0	374.0	-----	0.0	119.0
7.5	<u>550.0</u>	23.0	0.0	111.0
<u>8.0</u>	-----	42.5	-----	108.0
8.5	-----	<u>55.0</u>	-----	-----
9.0	-----	0.0	-----	-----

Table 7 : pH profile for the α -oxo acid glyoxylate in different buffers . The units are n moles α KMB / mgp . The underlined numbers indicate those for the optimum pH . The enzyme source was stem tissue , the standard radioactive assay was used and the reaction time was 30 min. .

pH	α -oxo Acid	n moles α KMB /assay	n moles α KMB /g fwt	n moles α KMB /mg p
7.5	Pyruvate	6.0 +/- 1.3	54.0	21.0
	α OG	4.5 +/- 1.5	40.5	16.0
	Glyoxylate	6.5 +/- 0.8	58.5	23.0
8.0	Pyruvate	2.0 +/- 0.3	18.0	7.0
	α OG	3.0 +/- 0.9	27.0	10.5
	Glyoxylate	12.0 +/- 1.0	107.5	42.5
8.5	Pyruvate	1.0 +/- 0.3	6.0	2.5
	α OG	1.5 +/- 0.3	13.5	5.5
	Glyoxylate	15.5 +/- 2.0	139.0	55.0
9.0	Pyruvate	0.0	0.0	0.0
	α OG	0.0	0.0	0.0
	Glyoxylate	0.0	0.0	0.0

Table 8 : Represented is the pH data for 0.1 M Tris buffer using stem tissue and the standard radioactive assay . Values are expressed on a n mole production of α KMB over a 30 min. reaction time . The standard d.viations are calculated for an n = 9 .

pH	α -oxo Acid	n moles α KMB /assay	n moles α KMB /g Fwt	n moles α KMB /mg p
7.0	Pyruvate	16.5 +/- 5.8	52.0	51.5
	α OG	13.5 +/- 4.4	42.5	42.0
	Glyoxylate	38.0 +/- 3.6	120.0	119.0
7.5	Pyruvate	0.5 +/- 0.2	1.5	1.5
	α OG	5.0 +/- 1.2	16.0	15.5
	Glyoxylate	35.5 +/- 4.2	112.2	110.9
8.0	Pyruvate	5.0 +/- 1.0	16.0	15.5
	α OG	18.0 +/- 4.3	57.0	56.5
	Glyoxylate	34.5 +/- 1.6	109.0	108.0

Table 9 : Represented is the pH data for 0.1 M HEPES buffer using stem tissue and the standard radioactive assay . Values are expressed on a n mole production of α KMB over a 30 min. reaction time . The standard deviations are calculated for an n = 9 .

pH	α -oxo Acid	n moles α KMB /assay	n moles α KMB /g fwt	n moles α KMB /mg p
5.0	Pyruvate	0.0	0.0	0.0
	OG	0.5 +/- 0.1	1.8	2.9
	Glyoxylate	0.0	0.0	0.0
5.5	Pyruvate	0.0	0.0	0.0
	OG	5.0 +/- 1.6	17.5	28.0
	Glyoxylate	0.0	0.0	0.0
6.0	Pyruvate	0.0	0.0	0.0
	OG	7.5 +/- 2.2	26.3	42.1
	Glyoxylate	0.0	0.0	0.0
6.5	Pyruvate	0.0	0.0	0.0
	OG	7.0 +/- 1.1	24.5	39.2
	Glyoxylate	0.0	0.0	0.0
7.0	Pyruvate	0.0	0.0	0.0
	OG	3.5 +/- 1.0	12.3	19.7
	Glyoxylate	0.0	0.0	0.0
7.5	Pyruvate	0.0	0.0	0.0
	OG	3.0 +/- 0.5	10.5	16.8
	Glyoxylate	0.0	0.0	0.0

Table 1C : Represented is the pH data for 0.1 M McIlvaines buffer using stem tissue and the standard radioactive assay . Values are expressed on a n mole production of α KMB over a 30 min. reaction time . The standard deviations are calculated for an n = 9 .

Stem tissue was also chosen because it is easily available (ie. the pods , and flowers and buds don't have that much less methionine aminotransferase but they are more difficult to obtain in quantity and also the plants then require a longer growing period).

Species Distribution of Methionine Amino Transferase in Mature Leaves

The reason why mature leaves were used was that it was the easiest way to obtain tissues from all of the species that were at the same level of development (ie. most recently matured leaf).

Of the plants used for the species distribution , only B. juncea produces glucosinolates but all of the species studied showed methionine amino transferase activity (tables 14 and 15). In the imidazole buffer (table 14) soybean and corn showed the greatest amount of activity with B. juncea and squash showing the least . With Tris buffer (table 15) B. juncea and soybean showed the greatest activity and tomato and corn showed the least (soybean had the greatest activity in both buffers). An important note here is that the tissue used for this survey was the tissue that gave the least amount of activity with B. juncea in imidazole buffer (table 12) and second to least with Tris buffer (table 13).

acid glyoxylate but not with the other two α -oxo acids (table 13). Pyruvate and α OG in Tris buffer resulted in a higher activity of methionine amino transferase in mature leaves than in young leaves . The flowers and buds and pods still had the highest activity with pyruvate and α OG but the stem tissue had high activity with only pyruvate and not α OG when Tris buffer was used (table 13).

The stem tissue in the tissue variability experiments could have been divided into two parts , as was done with the labeling experiments for in vivo biosynthesis of allylglucosinolate , namely upper and lower stem . If this were done the enzyme would most likely have been predominantly in the upper , younger tissue and not the lower , older tissue . The reasoning for this will be discussed in later sections . The other important aspect of the tissue distribution of methionine amino transferase was which tissue to use as an enzyme source for the various experiments performed . The tissue chosen for this was stem tissue because it had a more than a ten fold greater amount of methionine amino transferase than any of the other tissues and almost five times the amount of enzyme as the rest of the tissues combined on a per gram fresh weight basis (gfw). Another reason for choosing stem tissue was that a greater percentage of its protein content was methionine amino transferase so there were less contaminating proteins . This was apparent because the n moles of α KMB produced on a per mg protein (mgp) basis for stem tissue was the highest of all of the tissues .

Tissue	n moles aKMB /assay	n moles aKMB /g fwt	n moles aKMB /mg p
Young Leaves	109.0 +/- 11.3	40.0	124.5
Flowers & Buds	130.0 +/- 9.6	143.0	512.0
Stems	117.5 +/- 8.1	1930.5	635.0
Mature Leaves	60.0 +/- 4.7	15.0	77.0
Pods	123.0 +/- 3.3	147.5	434.5

Table 12 : The above represents the data for the tissue survey of B. juncea using imidazole buffer , glyoxylate and the standard radioactive assay . (n = 9)

Tissue	α -oxo Acid	n moles α KMB / assay				n moles α KMB	
		Replicate			Ave.	/g. fw.	/mg pro.
		1	2	3			
Young Leaves	Pyruvate	3.0	4.5	1.0	3.0 +/- 1.8	21.5	4.5
	α -OG	3.0	4.5	1.5	3.0 +/- 1.5	21.5	4.5
	Glyoxylate	3.5	7.0	4.5	5.0 +/- 1.8	49.5	8.0
Mature Leaves	Pyruvate	11.5	12.5	14.0	11.0 +/- 2.4	75.5	14.5
	α OG	12.5	5.5	13.5	10.5 +/- 4.4	73.0	14.0
	Glyoxylate	6.0	6.0	4.0	5.5 +/- 1.2	39.0	7.5
Stems	Pyruvate	13.0	11.5	8.0	10.5 +/- 2.6	140.5	142.0
	α OG	5.0	7.0	2.5	5.0 +/- 2.3	67.0	67.5
	Glyoxylate	8.5	5.5	7.0	7.0 +/- 1.5	93.5	94.5
Flowers & Buds	Pyruvate	8.0	6.5	7.0	7.0 +/- 0.8	168.0	23.0
	α OG	6.5	4.0	3.0	4.5 +/- 1.8	108.0	15.0
	Glyoxylate	7.0	6.0	0.5	4.5 +/- 3.5	108.0	15.0
Pods	Pyruvate	12.0	12.0	8.5	11.0 +/- 2.0	1568.0	234.0
	α OG	13.0	4.5	1.0	6.0 +/- 6.2	854.0	127.5
	Glyoxylate	0.8	0.4	0.0	0.4 +/- 2.0	285.0	42.5

Table 13 : Shown are the variations in the enzyme content (expressed as n moles α KMB produced) for each of the tissues of B. juncea using the standard radioactive assay . The buffer used is 0.1 M Tris pH 7.5 , (n = 3)

Stem tissue was also chosen because it is easily available (ie. the pods , and flowers and buds don't have that much less methionine aminotransferase but they are more difficult to obtain in quantity and also the plants then require a longer growing period).

Species Distribution of Methionine Amino Transferase in Mature Leaves

The reason why mature leaves were used was that it was the easiest way to obtain tissues from all of the species that were at the same level of development (ie. most recently matured leaf).

Of the plants used for the species distribution , only B. juncea produces glucosinolates but all of the species studied showed methionine amino transferase activity (tables 14 and 15). In the imidazole buffer (table 14) soybean and corn showed the greatest amount of activity with B. juncea and squash showing the least . With Tris buffer (table 15) B. juncea and soybean showed the greatest activity and tomato and corn showed the least (soybean had the greatest activity in both buffers). An important note here is that the tissue used for this survey was the tissue that gave the least amount of activity with B. juncea in imidazole buffer (table 12) and second to least with Tris buffer (table 13).

Species	n moles α KMB /assay	n moles α KMB /g fwt	n moles α KMB /mg p
<u>B. juncea</u>	60.0 +/- 4.7	15.0	77.0
Soybean	105.5 +/- 2.5	1537.0	373.0
Corn	102.0 +/- 6.0	922.0	323.0
Squash	19.5 +/- 3.0	117.5	49.0
Tomato	42.0 +/- 3.4	131.3	144.5

Table 14 : Shown is the species survey for glyoxylate methionine amino transferase using the most recently matured leaves from each species , imidazole buffer and the standard radioactive assay . (n = 9)

Species	α -oxo Acid	n moles α KMB / assay	n moles α KMB /g fwt	n moles α KMB / mgp
<u>B. luncea</u>	Pyruvate	11.0 +/- 2.4	75.5	14.5
	α OG	10.5 +/- 4.4	73.0	14.0
	Glyoxylate	5.5 +/- 1.2	39.0	7.5
Tomato	Pyruvate	1.5 +/- 2.0	3.0	1.0
	α OG	2.5 +/- 4.0	5.0	1.5
	Glyoxylate	2.5 +/- 1.5	5.0	1.5
Corn	Pyruvate	1.5 +/- 0.5	12.0	6.5
	α OG	4.5 +/- 2.0	35.5	20.0
	Glyoxylate	0.0	0.0	0.0
Soybean	Pyruvate	0.0	0.0	0.0
	α OG	1.0 +/- 2.0	59.0	15.5
	Glyoxylate	1.5 +/- 2.5	88.0	23.0

Table 15 : Shown is the species survey for glyoxylate methionine amino transferase using the most recently matured leaves from each species , Iris buffer and the standard radioactive assay . (n = 9)

Quantitative Isolation of Glucosinolates from Tissues

Isolation and identification of the glucosinolates from the various tissues of *B. juncea* was designed to verify that allylglucosinolate was the major glucosinolate and also to see if there was a correlation between the glucosinolate content and the amount of activity in methionine amino transferase . The tissue survey for transferase activity above showed that there was more enzyme in the younger tissues and the reproductive tissues than there was in the older tissues . In looking at figures 20 , 21 , 22 , 23 , 24 and 25 , which depict the HPLC profiles for the different tissues and the marker (fig. 19), it can be seen that the upper stem , the young leaves , and the flowers and buds have by far the highest allylglucosinolate content (labeled B). The other three tissues have very little allylglucosinolate (mature leaves , lower stem , and roots). The upper stem and the flowers and buds have the same basic profile with the exception that the flowers and buds have more of but-3-enylglucosinolate and D-2-phenylethylglucosinolate (labeled C and D respectively fig. 21 , 20). The only difference between the profile for young leaves (fig. 22) and that for mature leaves (fig. 23) is that the young leaves have considerably more allylglucosinolate . McGregor and Love (1988) also found that the content of allylglucosinolate in the leaves of *B. juncea* cv. Domo decreased as the distance from the apex increased . The lower stem and roots are almost identical (fig. 24 , 25). The glucosinolate profiles then show that there is a strong

Figures 20 , 21 , 22 , 23 , 24 , 25 and 26 : The following pages illustrate the glucosinolate profiles for the different tissues from the quantitative isolation of glucosinolate experiments . The different tissues are indicated in each figure . The first figure (fig. 19) is the desulfoallylglucosinolate marker . The glucosinolates are as listed bellow :

A , A' : artifacts

B : allylglucosinolate

C : but-3-enylglucosinolate (R = $\text{CH}_2\text{-CH=CH-CH}_2\text{-}$)

D : D-2-phenylethylglucosinolate (R = $(\text{C}_6\text{H}_5)\text{-CH}_2\text{-CH}_2\text{-}$)

X : unknown compound : most likely a glucosinolate

Figure 20 : Desulfoallylglucosinolate marker

Figure 21 : Flowers and buds

Figure 22 : Upper stem

Figure 23 : Young Leaves

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Figure 24 : Mature Leaves

Figure 25 : Lower stem

Figure 26 : Roots

Tissue	μg of glucosinolates / 5 plants
Young Leaves	996
Flowers & Buds	1146
Upper Stem	914
Lower Stem	462
Mature Leaves	318
Roots	700

Table 16 : Glucosinolate concentration is represented for the different tissues of B. juncea used in the radioactive label experiments . Plants used were the same as those used for the HPLC glucosinolate profiles . See materials and methods for the amounts of each tissue taken .

correlation between the amount of allylglucosinolate and the amount of methionine amino transferase and the age of the tissue . Also the younger tissues and reproductive parts have a greater over all content of glucosinolates than the older tissues (table 16).

Labeling with Radiolabeled Carbon Dioxide and Analysis of the in vivo Production of Glucosinolates

Glucosinolate production was measured by the amount of labeled glucosinolates that were isolated in 90% ethanol extracts from a given tissue when only the youngest mature leaf was labeled for a pulse of 15 min. and a certain amount of time was allowed to elapse before analysis . This means that any radiolabeled glucosinolates that were found in any tissue other than the labeled leaf were produced from radiolabeled compounds that were produced in the labeled leaf and then transported out of that leaf and into the other tissues . These labeled compounds could be either sugars , amino acids , organic acids or they could be glucosinolates . The radioactivity in these types of compounds from each of the tissues over the range of times used in this experiment are given in tables 17 , 18 , 19 , 20 and 21 . The patterns of the levels of radioactivity of each compound in the different tissues were quite complicated and unfortunately because of the necessity of the varying times allowed for the development of the radioactive profile in the plant before it was harvested some of the plants

Tissue	% of total Counts	% in a.a.	% in acids	% in neutrals	% in desulfo
Labeled Leaf	57.5	4.6	43.1	52.3	0.0
Flowers & Buds	2.7	0.0	51.6	48.4	0.0
Young Leaves	1.1	0.0	0.0	100.0	0.0
Upper Stem	26.6	0.0	58.8	41.2	0.0
Mature Leaves	0.0	---	---	---	---
Lower Stem	11.7	2.5	0.0	97.5	0.0

Table 17 : Distribution of radioactivity for labeling
experiments involving in vivo ^{14}C -glucosinolate biosynthesis .

Time Course : One Hour

Total Counts : 1.242E7

Percentage Glucosinolates : 0.0

Harvested at : 11 am

Tissue	% of total Counts	% in a.a.	% in acids	% in neutrals	% in desulfo
Labeled Leaf	28.4	13.3	4.8	78.2	3.7
Flowers & Buds	10.5	27.2	0.0	71.0	1.9
Young Leaves	1.1	0.0	3.5	91.3	5.2
Upper Stem	46.4	38.1	0.0	61.9	0.0
Mature Leaves	0.1	32.8	0.0	62.7	0.0
Lower Stem	13.5	9.7	0.0	88.3	2.0

Table 18 : Distribution of radioactivity for labeling experiments involving in vivo ^{14}C -glucosinolate biosynthesis .

Time Course : Three Hours

Total Counts : 3.036E7

Percentage Glucosinolates : 1.7

Harvested at : 1 pm

Tissue	% of total Counts	% in a.a.	% in acids	% in neutrals	% in desulfo
Labeled Leaf	44.5	25.1	33.4	36.2	5.3
Flowers & Buds	4.4	9.4	57.1	20.0	13.5
Young Leaves	7.0	8.3	24.4	53.3	13.5
Upper Stem	20.9	7.5	9.1	75.9	7.5
Mature Leaves	0.4	35.4	11.2	50.7	2.7
Lower Stem	22.9	12.4	2.6	82.4	3.4

Table 19 : Distribution of radioactivity for labeling
experiments involving in vivo ^{14}C -glucosinolate biosynthesis .

Time Course : Twelve Hours

Total Counts : 1.274E7

Percentage Glucosinolates : 6.8

Harvested at : 10 pm

Tissue	% of total Counts	% in a.a.	% in acids	% in neutrals	% in desulfo
Labeled Leaf	22.6	20.9	31.8	37.8	9.5
Flowers & Buds	8.2	36.5	28.9	22.6	12.0
Young Leaves	6.0	13.1	19.9	47.1	20.1
Upper Stem	62.5	2.5	0.0	93.8	3.7
Mature Leaves	0.3	26.9	22.3	44.5	5.6
Lower Stem	3.6	5.2	81.1	7.6	6.2

Table 20 : Distribution of radioactivity for labeling
experiments involving in vivo ^{14}C glucosinolate biosynthesis .

Time Course : Twenty Four Hour

Total Counts : 1.263E7

Percentage Glucosinolates : 8.1

Harvested at : 10 am

Tissue	% of total Counts	% in a.a.	% in acids	% in neutrals	% in desulfo
Labeled Leaf	25.1	19.0	30.5	42.7	7.7
Flowers & Buds	7.1	35.3	31.0	19.1	14.6
Young Leaves	3.9	27.6	27.5	29.0	16.0
Upper Stem	61.0	21.0	4.5	69.9	4.4
Mature Leaves	0.1	28.1	34.2	37.8	0.0
Lower Stem	2.4	41.6	5.8	51.9	0.8

Table 21 : Distribution of radioactivity for labeling
experiments involving in vivo ^{14}C -glucosinolate biosynthesis .

Time Course : Forty Eight Hour

Total Counts : 2.413E7

Percentage Glucosinolates . 6.9

Harvested at : 10 am

Tissue	Time (hours)				
	1	3	12	24	48
Labeled Leaf	0.00	1.05	2.36	2.15	1.93
Flowers & Buds	0.00	0.20	0.59	0.98	1.04
Young Leaves	0.00	0.06	0.95	1.21	0.62
Upper Stem	0.00	0.00	1.57	2.31	2.68
Mature Leaves	0.00	0.00	0.01	0.02	0.00
Lower Stem	0.00	0.03	0.78	0.22	0.02

Table 22 : ^{14}C -Glucosinolate profiles for the different tissues over the range of times of the ^{14}C -labeling experiments . The figures are expressed in % of total counts . Data from tables 17-21 .

were at different points in the diurnal cycle than others . The harvesting times of the one , twenty four , and forty eight hour experiments were in the morning while the three hour one was in the afternoon and the twelve hour one was at night . This was unfortunately unavoidable because they had to be either labeled at different times or harvested at different times . The basic pattern was a general drop in the amount of label in the neutral fraction and a general rise in the amino acid fraction with a continued fluctuation in the label in the acidic fraction . The data for the label in the glucosinolate fraction was the only part of the experiment that showed consistency (table 22). There was initially no labeled glucosinolate in any of the tissues . This was followed by a slow rise in each tissue which levels off at about twenty four hours for the young tissues and the reproductive parts (a slight drop off in the young leaves) and drops off by forty eight hours for the older parts (mature leaves and lower stem). As mentioned in the first part of this section , all radioactivity found in any of the tissues other than the labeled leaf had to be translocated there from the labeled leaf . In comparing the labeled leaf , which is a mature leaf , with the mature leaves there is a difference noted . The labeled leaf has a higher labeled glucosinolate content and the labeled leafs' label does not drop off by forty eight hours . Comparing the glucosinolate data with that for the other labeled compounds the impression is that the glucosinolates do turn over but at a much slower rate . This is indicated in

the limited fluctuation in the counts found in them and also by the slow drop off in the counts found in the mature tissues . The drop off could be due to a translocation of the glucosinolates from an older tissue to the younger ones ; but this unfortunately could not be substantiated .

Discussion

α -Oxo Acid and Amino Acid Content of

B. juncea

Since the first step in the biosynthesis of allylglucosinolate is probably the transamination of methionine , the internal pools of α -oxo and amino acids could give an indication as to the importance of this first step in the pathway . Methionine was detected in extracts of B. juncea in only minimal amounts (fig. 18) and homomethionine was not detected at all though it may have been one of the unknowns . Since methionine is necessary for protein and ethylene biosynthesis as well as allylglucosinolate production , there must be some amount of turn over in the pools of methionine (Baur et al. , 1971). α KMB was not detectable so presumably the transaminase for methionine does not produce α KMB faster than the other enzymes in the pathway can use it up . Current literature on the biosynthetic pathway of allylglucosinolate indicates that the initial transamination of methionine is the committal step to the production of allylglucosinolate on the merits that there is no other pathway that any of the intermediates are used in (Larsen , 1981 , Underhill , 1980 , Chisholm and Matsuo , 1972). Homomethionine and α KMB would seem to be possible exceptions to this since they are also involved in the biosynthesis of ethylene . These exceptions could mean that the transamination of methionine is not the committal step in the production of

allylglucosinolate depending on whether the two pathways are in separate compartments or not . In either case the levels of methionine and aKMB in B. juncea do not seem to give an indication of the importance of the initial step in the production of allylglucosinolate .

Purification of Methionine Amino Transferase

It was unfortunate that the activity of methionine aminotransferase attainable with imidazole buffer was not discovered before the work was done on this area . It was quite probable that the reason for the loss of activity in the subsequent steps of purification after fractionation with ammonium sulfate was due to a simple dilution of the enzyme beyond detectability . Table 4 , which shows the effect of dilution on the amount of activity in the assay system , indicates that somewhere between a two and a four fold dilution of the 30 min. assay there is a loss of detectable activity . Both of the methods chosen for the next step in the purification (DEAE and Sephacryl) would result in a greater dilution than this . This dilution is unavoidable even when the samples are reduced in volume because the fractions have to be of a minimal volume for assaying . Dilution also occurs because it would be highly unlikely that the enzyme would be contained in only one fraction .

Effect of PMSF on the Transamination of Methionine

In the above section it was mentioned that when purifying methionine aminotransferase that there was a loss of activity somewhere during the processes used . Even though PMSF was added to the ammonium sulfate extracts there was still a minimal amount of protease activity and both DEAE and Sephacryl require at least 6 hours to run ; therefore some small amount of this loss of activity was probably due to this protease activity .

Before the addition of PMSF there was a considerable production of additional amino acids during the enzyme assay . This also included a greater production of the expected amino acids . This then would result in there appearing to be more activity than was really there . So even if the conditions of the assay would produce no transamination for a given α -oxo acid the results would still show some small amount of activity . Since as mentioned in the introduction none of the literature cited made use of protease inhibitors then those that were not using pure enzyme extractions could possibly be reporting erroneous results due to the activity of proteases . This is probably the case with those that mention extremely nonspecific amino transferases .

Buffer Effect on Methionine Amino Transferase

Its already well known that buffers have a considerable effect on the activity of enzymes (Ferguson et al. , 1980 , Good et al. , 1966). This is quite clearly the case with methionine

amino transferase and the buffers used in this thesis as can be seen in tables 5 , 6 and 7 . The problem with this variability is that it is impossible to tell what activity levels are indicative of in vivo levels . There is also a beneficial side to the effects of buffers when combined with the different substrates used in the assays . Each α -oxo acid had different activity levels in each of the buffers but there were trends that lead to the possibility that there may be more than one enzyme that transaminates methionine . Pyruvate and α OG have pH optimums that were slightly acidic (tables 5 and 6). Their exact values are not as important as the patterns that they show in the buffers . Each buffer has a limited range and as a result in some of the buffers the maximum level of activity occurred at a pH which was at the limit of the buffers' range . This occurred with pyruvate in buffers Tris and HEPES , and with α OG in buffers Tris and imidazole . With both α -oxo acids and all four of the buffers this maximum activity occurred at the lower limit of the buffers' range . So this indicates with pyruvate and α OG that their pH optimums of 7.0 and 6.5 respectively are possibly a little lower . With glyoxylate on the other hand the trend was toward a more basic pH (table 7). The pH optimum of 8.0 is above the maximum pH attainable with imidazole buffer but is lower than the pH of optimum activity in Tris buffer . The most reliable results (lowest standard deviations) were with imidazole buffer and Tris buffer and these two buffers agree with the trends (tables 11 and 8). The other two buffers do not contradict the

observed trends they merely do not show it in some cases (tables 9 and 10). This difference in pH optimums would indicate that there may be two transferases involved with the transamination of methionine. One transaminase would have pyruvate and α OG for substrates and the other would use glyoxylate. The only problem with this suggestion is that the only α -oxo acid which had activity in McIlvaines buffer was α OG (table 10). This may mean that there are three enzymes involved though the data with McIlvaines buffer was both low and had high standard deviations (table 10).

Another explanation for all of the differences in activity found in the different buffers could be that there is only one enzyme and it transaminates methionine with all three α -oxo acids and the different buffers stimulate the enzyme with one or two α -oxo acids while inhibiting the use of the other substrate or substrates. If this were the case then glyoxylate would most likely be the main substrate and both pyruvate and α OG would be secondary substrates. This explanation would indicate that the enzyme is nonspecific and may be under some amount of control in the intact system. The explanation listed in the paragraph above would indicate that the transamination of methionine was the result of a number of transaminases that most likely transaminated methionine only due to their nonspecificity. This would explain the low level of activity found in most cases and also helps explain the loss of activity during the additional purification steps (ie. different enzymes could possibly separate

during the purification steps used resulting in lower overall activity).

Choosing between the two possibilities would require additional purification of the enzyme or enzymes involved . This purification should follow the glyoxylate activity in imidazole buffer . This would help prevent the loss of activity in the steps involved and would answer the question depending on whether or not its activity can be separated from that with the other α -oxo acids .

Recently some published work by Glover and associates (1988) supports the multienzyme suggestion . They found three isoenzymic forms of methionine glyoxylate amino transferase in B. carinata . Besides glycine being a good amino substrate as well as methionine , no additional work on other α -oxo acid substrates was mentioned . These isoenzymes could very well be the same or similar to that which was found in B. juncea but additional information on the other α -oxo acids would have to be known . One piece of evidence which may suggest that their enzymes are different is that the buffer they used for the enzyme assay was 0.02 M Tris pH 8.2 which would result in a loss of activity in the assay system used here .

Tissue Variability of Methionine Amino Transferase

Tissue variability of methionine aminotransferase as with the rest of the experiments on methionine amino transferase , after the discovery of the activity attainable with imidazole

buffer , was done using glyoxylate only . This resulted in the duplication of some work because some of the early work was done initially with Tris buffer . This change was made because it gave the most reliable results and also allowed a greater variety of experiments because only one third of the work was involved in each .

An interesting observation was made during the period in which B. juncea was grown in the greenhouse (ie. plants that were of experimental age) and this was that when the occasional pest would attack any of the plants they always went for the older mature leaves . A prime example of this occurred during a leaf miner infestation where the only afflicted leaves were the older of the mature leaves . It was not too surprising then that the majority of the activity of methionine amino transferase was found in the younger tissues and in the reproductive tissues (tables 12 and 13) because if this methionine amino transferase is responsible for the initial step in glucosinolate production then its activity should correspond to areas of active synthesis .

Species Variability of Methionine Amino Transferase

Since methionine amino transferase has roles in both glucosinolate biosynthesis and ethylene production and since ethylene is ubiquitous as a plant hormone , it would be likely that methionine amino transferase could be found in extracts of other species . This is of course assuming that the enzyme

isolated here is the one that is responsible for the above mentioned reactions and that this enzyme is also found in other species and is not particular to glucosinolate producing plants or just B. juncea . The results indicated that methionine glyoxylate amino transferase is found in all species studied (tables 14 , 15). The activity of this enzyme in each of these species seems to be dependant on whether Tris or imidazole buffer was used . The use of imidazole buffer results in very high activity in soybean and corn but as was the case with B. juncea the mature leaves of these plants may not be the tissue that shows optimal activity . Tris buffer showed that there was also activity in the assays using pyruvate and α -OG in all of the species except in soybean with pyruvate .

The results seem to indicate that the methionine aminotransferase enzyme(s) isolated here may also be the enzyme(s) that are involved in the ethylene production in other species .

Tissue Content of Glucosinolates in

B. juncea

For the work that has been presented here to be more than just the isolation of some unknown enzyme that transaminates methionine and the subsequent study of unrelated in vivo biosynthesis of allylglucosinolate , there must be a correlation between the two . The results indicate that the activity levels of methionine glyoxylate aminotransferase with imidazole buffer

and allylglucosinolate content in the different tissues match . The upper stem , flowers and buds , and the young leaves have the greatest content of allylglucosinolate (fig. 22 , 21 , 23) and these same tissues have the highest activity of methionine glyoxylate aminotransferase (table 12). The tissues with the least allylglucosinolate content (mature leaves and lower stem fig. 24 , 25 respectively) are also those with the least methionine glyoxylate aminotransferase activity (table 12). This indicates that methionine glyoxylate aminotransferase may be involved in the biosynthesis of allylglucosinolate .

As mentioned in the introduction , the biosynthesis of allylglucosinolate involves a single chain elongation step . The biosynthesis of but-3-enylglucosinolate (fig. 20-26) follows the same pathway as allylglucosinolate except that it undergoes two chain elongation steps so that the final glucosinolate has an additional carbon unit in the R group . Since but-3-enylglucosinolate follows basically the same biosynthesis and has the same initiating step it could be reasonably expected that its rate of biosynthesis would follow the same pattern as that of allylglucosinolate . This turns out not to be the case since the quantities of but-3-enylglucosinolate found in the tissues of B. juncea do not seem to follow the pattern of young versus old tissues . This would seem to indicate that whether the chain elongation step occurs once or twice is under some control that is tissue specific .

Labeling with Radiolabeled Carbon Dioxide and Analysis of the in vivo Production of Glucosinolates

The activity level attainable with imidazole buffer with methionine glyoxylate aminotransferase is still representable in units of μ M produced in 30 min. . This activity is easily measurable but is still low . If this activity is indicative of the in vivo biosynthetic levels of allylglucosinolate then the results from the labeling experiments should show that there is a slow production of allylglucosinolate . This did turn out to be the case . Activity in the glucosinolate fraction of the extracts from the different labeling experiments indicated that detectable amounts of glucosinolate were produced between one and three hours and the peak of activity occurred at 24 hours and this maximum level wasn't much higher than the initial levels (table 22). Also activity again is at a maximum in the younger and reproductive tissues .

The rate of turnover of the glucosinolate pool was not determined . The only indication that turnover occurred was in the drop in activity found in the non labeled mature leaves and in the lower stem . This drop may be due to a translocation of glucosinolates ; but there was no evidence that translocation occurred . A possible method for checking for turn over would be to make use of the myrosinase enzyme . This enzyme cleaves the glucosinolate molecule by removing the sulfur and glucose part of the molecule . This means that the part of the molecule that is

derived from the amino acid pools (R group) can be separated from the part of the molecule that is derived from the carbohydrate pools (glucose unit). It was shown in tables 17 to 21 that these pools are labeled at different times and therefore the radioactivity that is found in these fractions of the glucosinolate molecule may be labeled to different extents at different times. If this were the case then turnover would be indicated even though the total amount of radioactivity in the molecule may not change drastically.

Conclusion

The isolation and subsequent analysis of the activity of methionine aminotransferase is extremely dependent on the choice of buffers . The best buffer found here was imidazole ; but there may be better buffers . This result indicates the importance of the use of a variety of buffers when analyzing any enzyme . Through the use of different buffers there was found to be the possibility that there is more than one transferase that transaminates methionine . The greatest activity was found with methionine glyoxylate aminotransferase in imidazole buffer at pH 7.5 . A similar enzyme was isolated from B. carinata and it was found to have three isozymes (Glover , 1988).

The activity of methionine glyoxylate aminotransferase was found to match the quantities of allylglucosinolate that were isolated from the same tissues of B. juncea . This indicates that this enzyme is involved in the biosynthesis of allylglucosinolate . Also the levels of in vivo biosynthesis of glucosinolates in the different tissues are in the range indicated by the levels of activity of the isolated methionine glyoxylate aminotransferase from the same tissues .

Appendix

Sample Calculations :

1) Calculation for converting the CPM from a standard radioactive assay into n moles α KMB produced per assay , per g fresh weight and per mg protein (example from Table 9 , the buffer is 0.1 M HEPES using pH 7.5 and glyoxylate as the α -oxo acid) :

- average % conversion from the three replicates of each of the three extractions :

$$\begin{aligned}
 &= (\text{sum of 9 \% conversions})/9 \\
 &= (8.2 + 7.4 + 12.4 + 6.8 + 0.0 + 1.9 + \\
 &\quad 7.2 + 7.1 + 12.7) / 9 \\
 &= 7.1 \%
 \end{aligned}$$

- standard deviation of % conversion :

$$\begin{aligned}
 &= (((\text{the sum of the squares of each of} \\
 &\quad \text{the 9 differences between \%} \\
 &\quad \text{conversion and mean}) / (9 - 1)))^{1/2} \\
 &= ((8.2 - 7.1)^2 + (7.4 - 7.1)^2 \\
 &\quad + (12.4 - 7.1)^2 + (6.8 - 7.1)^2 \\
 &\quad + (0.0 - 7.1)^2 + (1.9 - 7.1)^2 \\
 &\quad + (7.2 - 7.1)^2 + (7.1 - 7.1)^2 \\
 &\quad + (12.7 - 7.1)^2) / 8)^{1/2} \\
 &= +/- 0.84
 \end{aligned}$$

- n moles of aKMB from % conversion / assay :
- = % conversion / assay
 - * final amount of methionine in assay
- = 7.1 +/- 0.84 % / assay * 0.5 n moles methionine
- = 35.5 +/- 4.2 n moles aKMB / assay

- calculation of average μg protein in Coomassie (which is from 100 μl of a *50 dilution of the desalted protein extract):

- = ((sum of(ave. absorption from
 - Coomassie / slope of
 - standard curve))
 - / # of assays)
- = ((0.171 / 1.355E-2) + (0.262 / 1.355E-2)
 - + (0.216 / 1.355E-2)) / 3
- = (12.6 μgp + 19.3 μgp + 15.9 μgp) / 3
- = 16.0 μgp

- average mg protein per gram fresh weight :
- = (((ugp in coomassie * dilution factor for assay)
 - * vol. of desalted fraction / vol. used in
 - coomassie)
 - * ave. final vol. of extract / vol. used for
 - desalting)
 - / 1000 $\mu\text{g}/\text{mg}$)
 - / ave. g fresh weight

$$\begin{aligned}
 &= (((((16.0 \mu\text{gp} * 50) \\
 &\quad * 3.5 \text{ ml} / 0.1 \text{ ml}) \\
 &\quad * 6.9 \text{ ml} / 2.0 \text{ ml}) \\
 &\quad / 1000 \mu\text{g/mg}) \\
 &\quad / 92.5 \text{ g}
 \end{aligned}$$

$$= 0.988 \text{ mgp/gfwt}$$

- mg of protein per assay from mgp/gfwt

$$\begin{aligned}
 &= ((\text{mgp/gfwt} * \text{g fresh weight}) \\
 &\quad * \text{vol. used for desalting} / \text{vol. of extract}) \\
 &\quad * \text{vol. in assay} / \text{vol. of desalted fraction}
 \end{aligned}$$

$$\begin{aligned}
 &= ((0.988 \text{ mgp/gfwt} * 92.5 \text{ gfwt}) \\
 &\quad * 2.0 \text{ ml} / 6.9 \text{ ml}) \\
 &\quad * 0.04 \text{ ml} / 3.5 \text{ ml}
 \end{aligned}$$

$$= 0.320 \text{ mgp/assay}$$

- n moles α KMB / mgp

$$= (\text{n moles } \alpha\text{KMB} / \text{assay}) / (\text{mgp} / \text{assay})$$

$$= 35.5 / 0.320$$

$$= 110.9 \text{ n moles } \alpha\text{KMB} / \text{mgp}$$

- n moles α KMB / gfwt

$$= (\text{n moles } \alpha\text{KMB} / \text{mgp}) / (\text{mgp} / \text{gfwt})$$

$$= 110.9 / 0.988$$

$$= 112.2 \text{ n moles } \alpha\text{KMB} / \text{gfwt}$$

2) Calculation for converting the counts of fractions from the ^{14}C label experiments into percentages per fraction (example from the three hour label using the labeled leaf (table 18)) :

- counts in any sample were converted to total counts for that sample by:

$$= \text{counts in } 0.05 \text{ ml of sample} \\ * (\text{vol. of sample} / 0.05 \text{ ml})$$

- % of total counts in labeled leaf

= counts in labeled leaf / the sum of all counts in all tissues

$$= 8,620,511.0 / (8,620,511.0 + 3,195,136.0 + 344,547.0 \\ + 14,087,770.6 + 16,320.0 + 4,100,040.0)$$

$$= 8,620,511.0 / 3.0364\text{E}7$$

$$= 28.4 \%$$

- counts in amino acid fraction (cationic)

= (total counts in labeled leaf

- counts through RX-101)

$$= 8,620,511.0 - 7,427,641.7$$

$$= 1,192,869.3$$

- counts in desulfoallylglucosinolate fraction

= counts off of DEAE-A25

$$= 325,344.8$$

- counts in neutral fraction (cationic)

= counts through DEAE-A25

= 6,977,446.4

- counts in acidic fraction (anionic)

= (counts through RX-101

- counts through DEAE-A25

- counts in desulfoallylglucosinolate)

= 7,427,641.7 - 6,997,446.4 - 325,344.8

= 104,850.5

- the % of total labeled leaf counts in amino acids

= counts in amino acids

/ (counts in a.a + counts in acidic fraction

+ counts in neutrals + counts in desulfo)

= 1,192,869.0 / (1,192,869.3 + 104,850.5

+ 6,997,446.4 + 325,344.4)

= 1,192,869.0 / 8,620,511.0

= 13.8 %

- similarly the percentages in the other fractions were
calculated :

- acidic fraction

= 104,850.5 / 8,620,511.0

= 1.2 %

- neutral fraction
 - = $6,997,446.4 / 8,620,511.0$
 - = 81.2 %
- desulfo fraction
 - = $325,344.8 / 8,620,511.0$
 - = 3.8 %

References

- Baillie , D. A. . 1987 . The Use of Phloem Exudate in the Study of Translocation in Cucurbita pepo . Masters Thesis . Carleton University .
- Baur , A. H. , Yang , S. F. . 1969 . Precursors of Ethylene . Plant Physiol. . 44 : 1347-1349 .
- Baur , A. H. , Yang , S. F. , Pratt , H. K. . 1971 . Ethylene Biosynthesis in Fruit Tissues . Plant Physiol. . 47 : 698-699 .
- Bell , E. A. . 1981 . The Physiological Role(s) of Secondary (Natural) Products . In : The Biochemistry of Plants ; A Comprehensive Treatise . Vol. 7 Secondary Plant Products , Editor : E. E. Conn . Academic Press . Toronto . P. 502--525.
- Bradford , M. M. . 1976 . A Rapid and Sensitive Method for the Quantitation of microgram Quantities of Protein Utilizing the Principal of Protein-Dye Binding . Anal. Biochem. . 72 : 248-254 .

- Chisholm , M. D. . 1972 . Biosynthesis of 3-Methylthiolpropylglucosinolate and 3-Methylsulfinylpropylglucosinolate in Wallflower Cheiranthus kewensis . Phytochemistry . 11 ; 197-202 .
- Chisholm , M. D. , Matsuo , M. . 1972 . Biosynthesis of allylglucosinolate and 3-Methylthiopropylglucosinolate in Horseradish , Armoracia lapathifolia . Phytochemistry . 11 : 203-207 .
- Cristen , P. , Metzler , D. E. . 1985 . Transaminases . In : Biochemistry : a Series of Monographs , Vol. 2 . Series editor : Metzler , D. E. . John Wiley and Sons . Toronto . P. 307-501 .
- Cruickchank , D. H. , Isherwood , F. A. . 1958 . Glutamic-Alanine and Glutamic-Aspartic Transaminases of Wheat Germ . Bioch. . 69 : 189-195 .
- Dubois , M. , Gilles , K. A. , Hamilton , J. K. , Rebers , P. A. , Smith , F. . 1956 . Colorimetric Method for Determination of Sugars and Related Substances . Analytical Chemistry . 28 : 350-356 .

- Durham , J. I. , Morgan , P. W. , Prescott J. M. , Lyman , C. M.
1973 . An Aminotransferase Specific for the D-enantiomorph
of Methionine . Phytochemistry . 12 : 2123-2126 .
- Ellis , R. J. , Davies , D. D. . 1961 . Glutamic-Oxaloacetic
Transaminases of Cauliflower : 1. Purification and
Specificity . Biochem. J. . 78 : 615-623 .
- Emery , W. L. . 1985 . Rapeseed . C.R.B. (Commodity Research
Bureau) Commodity Year Book . P. 21T-26T , 206-208 .
- Ferguson , W. J. , Braunschweiger , K. I. , Braunschweiger , W.
R. , Smith , J. R. , McCormick , J. J. , Wasmann , C. C. ,
Jarvis , N. P. , Bell , D. H. , Good , N. E. . Hydrogen Ion
Buffers for Biological Research . Analytical Biochemistry .
104 : 300-310 .
- Fowden , L. , Webb , J. A. . 1955 . Evidence for the Occurrence
of Gama-Methylene-Alpha-Oxoglutaric Acid in Groundnut Plants
(Arachis hypogaea) . The Biochemical Journal . 59 : 228-234.
- Gamborg , O. L. . 1965 . Transamination in Plants , The
Specificity of an Aminotransferase from Mung Bean . Canadian
Journal of Biochemistry . 43 : 723-730 .

- Glover , J. R. , Chapple , C. C. S. , Rothwell , S. , Tober , I. , Ellis , B. E. . 1988 . Allylglucosinolate Biosynthesis in Brassica carinata . Phytochemistry . 27 : 1345-1348 .
- Good , N. E. , Winget , G. D. , Winter , W. , Connolly , T. N. , Izawa , S. , Singh , R. M. M. . 1966 . Hydrogen Ion Buffers for Biological Research . Biochemistry . 5 : 467-477 .
- Isherwood , F. A. , Cruickshank , D. P. . 1954 . Chromatographic Separation and Analysis of Mixtures of Pyruvic , Oxalacetic and Alpha-Ketoglutaric Acids . Nature . 173 : 121-122 .
- Josefsson , E. . 1971 . Studies of the Biochemical Background of differences in Glucosinolate Content in Brassica napus L. I. Glucosinolate Content in Relation to General Chemical Content . Physiol. Plant. . 24 : 150-159 .
- Kushad , M. M. , Richardson , D. G. , Ferro , A. J. . 1983 . Intermediates in the Recycling of 5-Methylthioribose to Methionine in Fruits . Plant Physiol. . 73 : 257-261 .
- Larsen , P. O. . 1981 . Glucosinolates . In : The Biochemistry of Plants ; A Comprehensive Treatise . Vol. 7 Secondary Plant Products . Editor : E. E. Conn . Academic Press . Toronto . P. 502-525 .

- Lehninger , A. L. . 1979 . Biochemistry : Second Edition . Worth Publishers Inc. . New York . P. 191 .
- Loffelhardt , W. , Kindl , H. . 1975 . The Biosynthesis of 4-Methylsulfinyl-3-butenylglucosinolate (Glucoraphenin) in Matthiola Species . Z. Naturforsch. . 30 : 233-239 .
- McGregor , D. I. , Love , H. K. . 1987 . Analysis of Vegetative Tissue as a Means of Facilitating Selection for Seed Glucosinolate Composition in Brassica . Agriculture Canada Research Station , 107 Science Crescent Saskatoon , Canada . S7N 0X2.
- McGregor , D. I. , Mullin , W. J. , Fenwick , G. R. . 1983 . Review of Analysis of Glucosinolates . J. Assoc. Off. Anal. Chem. . 66 : 825-847 .
- Milsom , W. D. , Rose , F. A. , Dodgson , K. S. . 1972 . The Specific Analysis of Arylsulfatase C , a Rat Liver Microsomal Marker Enzyme . Biochem. J. . 128 : 331-336 .
- Oaks , A. . 1965 . The Synthesis of Leucine in Maize Embryos . Biochem. Biophys. Acta. 111 : 79-89 .

- Ray , P. M. , Steves , T. A. , Fultz , S. A. . 1983 . Botany .
Saunders College Publishing . Toronto . P. 212-213 , 298-
299 .
- Reed , R. E. , Hess , J. L. . 1975 . Partial Purification and
Characterization of Aspartate Aminotransferase from Seedling
Oat Leaves . The Journal of Biological Chemistry . 12 :
4456-4461 .
- Roth , M. . 1971 . Fluorescence Reaction for Amino Acids . Anal.
Chem. . 43 . 880-882 .
- Storey , K. B. , Bailey , E. . 1978 . Intercellular Distribution
of Enzymes Associated with Lipogenesis and Gluconeogenesis
in the Fat Body of the Adult Cockroach , Periplaneta .
Insect Biochem. . 8 : 125-131
- Underhill , E. W. . 1980 . Glucosinolates . Encyclopedia of
Plant Physiology New Series Vol. 8 : Secondary Plant
Products . Ed. E. A. Bell and B. U. Charlwood . Springer-
Verlag . New York . P. 493-511 .
- Underhill , E. W. , Wetter , L. R. , Chisholm , M. D. . 1973 .
Biosynthesis of Glucosinolates . Biochem. Soc. Symp. . 38 :
303-326 .

Wightman , F. , Forest , J. C. . 1978 . Properties of Plant
Aminotransferases . Phytochemistry . 17 : 1455-1471 .

Wilson , D. G. , King , K. W. , Burris , R. H. . 1954 .
Transamination Reaction in Plants . J. Biol. Chem. 208 :
863-874 .

Zweig , G. , Sherma , J. Ed. . 1972 . Handbook of Chromatography
Vol. 1 and 2 . CRC Press . Cleaveland . Ohio .

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