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ABSTRACT

The experiments reported in this thesis were designed to determine the fate of ^{14}C-labelled L-phenylalanine introduced into pea seed (Pisum sativum L., var. Alaska) during imbibition. It was assumed that the labelled phenylalanine would mix with the endogenous phenylalanine pool and thereby would act as a tracer by which the pathway of the translocation and/or transformation of this amino acid could be followed.

Studies were first made to determine the uptake pattern of phenylalanine and its path of entry into the pea seeds during imbibition. The results indicated that after an initial lag period, the rate of phenylalanine uptake became more rapid than that of water. Its uptake from a dilute solution was almost complete at a time when the seeds were not fully imbibed. With increasing concentrations of phenylalanine in the medium, the seeds took up more and more, but the per cent uptake declined. The uptake of about 2 mg. of phenylalanine per seed did not have any noticeable toxic effect either on seed germination or subsequent growth at least up to a period of 72 hours. Chromatography of the imbibition medium revealed that very little or no transformation of the phenylalanine molecule occurred before it was imbibed, although some

bacteria were present in the medium. Autoradiographic studies with pea seed sections indicated that the phenylalanine molecules entered into the seeds through the micropyle or the micropylar region. The activity first went into the cotyledons and was later transported to the shoot-root axis.

Surface sterilized pea seeds, after imbibition of a dilute solution of 1-^{14}C-phenylalanine, were left to germinate in the dark. Very little of the supplied activity was respired as $^{14}CO_2$ during germination. Each day for 4 days, representative samples were separated into cotyledons and shoot-root axis. Radioactivity was found to decrease from a maximum at 24 hours in the cotyledons, while it increased gradually in the shoot-root axis. Fractionation to separate soluble and insoluble activity showed that in the cotyledons soluble activity decreased as insoluble activity increased. In the shoot-root axis both soluble and insoluble activity increased. Fractionation of water-soluble proteins through a DEAE-cellulose column to isolate a protein fraction of high specific activity was only partially successful. Suitable samples obtained from various fractions were chromatographed on paper. These samples were supplemented by samples derived from additional batches of 72-hour seedlings, fed ^{14}C-Phe during imbibition, but extracted with ethanol. Labelled phenylalanine

was recovered from both soluble non-protein fractions and from protein hydrolysates. Some activity was associated with cinnamic acid derivatives, identified as caffeic, ferulic and p-coumaric acids, and with a glycoside fraction.

The various fractionation procedures resulted in considerable loss of radioactivity. Some loss was apparently a result of non-enzymic decarboxylation of phenylalanine to phenethylamine. In an experiment using U-^{14}C-phenylalanine the activity in the ether-soluble fraction was much higher as compared to the experiments done with 1-^{14}C-phenylalanine. Evidence has been presented to show that the added phenylalanine did enter into the endogenous phenylalanine pool and was utilized indiscriminately in the new synthesis of protein.

RESUME

Nous avons étudié la translocation et le métabolisme de la phénylalanine marquée au carbone-^{14} et introduite dans les graines de pois (Pisum sativum L., var. Alaska) au cours de la période d'imbibition. Après une période initiale d'attente, le taux d'entrée de la phénylalanine devient plus rapide que celui de l'eau. A partir d'une solution diluée de phénylalanine, presque toute la phénylalanine est absorbée avant la fin de la période d'imbibition. Si on augmente la concentration de l'acide aminé plus de phénylalanine est absorbée mais le taux d'absorption diminue. L'absorption de 2 mg. de phénylalanine par graine n'a pas d'effet sur la germination ou la croissance au cours de la période étudiée.

La phénylalanine n'est pas modifiée de façon appréciable au cours de l'imbibition même en présence de bactéries. L'autoradiographie de sections de graines de pois indique que la phénylalanine est absorbée dans la région des micropyles. Elle est ensuite concentrée dans les cotylédons puis transportée dans l'axe tige-racine.

Après absorption de phénylalanine-1-^{14}-C, on laisse germer les pois à l'obscurité et chaque jours pendant 4

jours on détermine la quantité et la nature de la radioactivité qui se trouve dans les cotylédons et l'axe tige-racine. Très peu de carbone-^{14} est éliminé sous forme de $^{14}CO_2$. La radioactivité des cotylédons atteint un maximum 24 heures après le début de la période de germination et la radioactivité de la fraction soluble des cotylédons décroît alors que celle de la fraction insoluble croît. Dans l'axe tige-racine la radioactivité totale, ainsi que celle de fractions soluble et insoluble, augmente graduellement à partir de 24 heures après le début de la germination. La présence de phénylalanine-^{14}C dans la partie soluble et dans les protéines de la partie insoluble a été démontrée par chromatographie. Nous n'avons pas pu démontrer clairement la présence de fractions protéiques de très haute activité spécifique. Une partie du carbone-^{14} a été retrouvée dans certains dérivés de l'acide cinnamique, tels que l'acide caféique, l'acide férulique et l'acide p-coumarique, et dans des dérivés glycosidiques. Une partie de la phénylalanine a été aussi transformée en phényléthylamine par décarboxylation non-enzymatique. Lorsqu'on utilise la phénylalanine uniformément marquée au carbone-^{14} moins de radioactivité est perdue au cours de l'expérience. Les résultats suggèrent qu'il y avait aussi la production enzymatique de l'acide acetique par moyen de B-oxidation des dérivés de l'acide cinnamique.

GENERAL INTRODUCTION

AND

MATERIALS AND METHODS

GENERAL INTRODUCTION

Complex chemical changes begin to occur in the seed as soon as it is hydrated. Reserve food materials are degraded. For example, proteins are hydrolysed to amino acids which are then transported to the developing shoot-root axis for new syntheses. Synthesis of protein in the growing parts takes place at the expense of the amino acids thus translocated from the reserve organs (Beevers & Guernsey, 1966; Chibnall, 1939). The amino acid composition of the newly synthesized protein may differ from that of the reserve organs; and thus it can be assumed that the processes of transamination and interconversion of the carbon skeletons of the amino acids also occur (Meister, 1957; Folkes & Yemm, 1956; Chibnall, 1939).

Several workers have studied the levels of free amino acids during pea seed germination (Lawrence & Grant, 1963; Shirokawa & Otakara, 1956; Virtanen et al., 1953). However, little information is available as to the fate of the individual amino acids derived as a result of hydrolysis of stored protein. This thesis reports an attempt to provide information on the metabolic fate of one such amino acid, namely phenylalanine.

1. The Pea Seed as Experimental Material:

The pea seed has been widely used as experimental material. It has the advantage of growing to a consi-

derable size in darkness without any outside food supply and thus the process of photosynthesis is avoided. It is particularly suited for work designed to study protein degradation and synthesis because of its high protein content.

2. Structure of the Pea Seed Cotyledon:

The pea seed embryo is comprised of two relatively large cotyledons and the embryonic axis, and is covered by a testa or seed coat. It is largely composed of living parenchyma cells (Larson, 1968). Electron microscopy of the pea seed cotyledon shows that its cells contain all the structures of a typical plant cell: a nucleus, mitochondria, endoplasmic reticulum, Golgi bodies, chloroplasts and starch grains (Varner & Schidlovsky, 1963). In addition, a major portion of the cell volume is occupied by some spherical inclusions, averaging 2 microns in diameter, called protein bodies.

3. Reserve Proteins in Peas:

Twenty-five per cent of the dry weight of the pea seed is protein (Larson, 1963). The protein components of the pea seed were studied by Osborne & Campbell as early as 1898, when they described the two globulins, legumin and vicilin. These two components were isolated by Danielsson in 1951, who found each to be homogeneous, but distinctly different

from the other in physical properties and amino acid composition. By treating the globulin fraction with dodecyl sulfate, Grant & Lawrence (1964) obtained 12 well defined bands, 4 from vicilin, 6 from legumin and 2 of uncertain origin. The molecular weight of vicilin was estimated as 186,000 and that of legumin as 400,000. Weintraub & Hoffman (1961), on the other hand, suggested that vicilin contains three peptide chains, each with a molecular weight of 58,000, while legumin has 12 polypeptide chains each with an average molecular weight of 33,000.

The major portion of the seed globulins acts as storage material (Altschul et al., 1964, 1966) and is found in the protein bodies. Thus far, no enzymic activity has been attributed to this class of proteins, their function is assumed to be primarily a source of amino acids or of nitrogen for the growing seedlings (Bagley et al., 1963). Albumin probably has very little importance as a reserve protein, but this fraction contains many enzymes. The albumin is quite different from the two globulins both in chemical composition and properties (Danielsson, 1952). The proteins that become labelled by feeding ^{14}C-labelled amino acids in vivo, belong to this group (Young et al., 1960).

4. The Fate of Storage Proteins During Germination:

The process of seed germination is characterised by the hydrolysis of reserve proteins which exist in the protein bodies. In wheat endosperm, these reserve proteins have been found to occur in more or less strong chemical combinations with phytin and lipids (Morton & Raison, 1963). In bean seed, these proteins are stored in the form of some glycoprotein compounds (Jaffé & Hanning, 1965). In the early stages of germination, the proteins are separated from the complex they had formed with other substances (Ghetie, 1965) and then are split into component polypeptide chains by the breaking up of disulfide bonds (Hatch & Turner, 1960) and hydrogen bonds (Grant & Lawrence, 1964). The splitting of inter and intramolecular non-peptide bonds causes the peptide bonds that remain masked in the intact molecule to appear at the surface where they can be digested by proteolytic enzymes (Ghetie, 1965). Since no de novo synthesis of proteases takes place in germinating pea seeds (Young & Varner, 1959), they must be present in some inactive form in the seed. The activation mechanism of these enzymes has not as yet been elucidated (Ghetie 1965). The hydrolysis of reserve proteins is thus preceded by a double activation, i.e, the activation of the reserve proteins themselves as well as the activation of the proteases. This may explain the existence of a "lag period" of protein hydro-

lysis in the storage parts during the first days of germination (Ghetie, 1965).

Lawrence et al., (1959) have noticed an increase in the level of non-protein nitrogenous constituents in the cotyledons during the first 3 days of germination. A decrease was noted thereafter. In the shoot-root axis, these constituents increase, slowly at the beginning and then very rapidly. This suggests that the reserve proteins are broken down to form nitrogenous intermediates in the cotyledons which are then transported to the growing apices for the synthesis of new proteins.

It is not quite clear if the reserve proteins are degraded completely to their constituent amino acids or only to peptides which are then recombined into new proteins. Morgan & Reith (1959) noted a significant increase in peptide material in the apical cells of bean root. Further, Raacke (1951) has suggested that peptide intermediates are formed in ripening pea seeds which are used in the synthesis of new proteins. On the contrary, Boulter & Barber (1963) working with 6-day old bean seedlings did not find any significant increase in peptide material. However, they expressed doubt as to the identity of the "peptide" fraction at the present time and also mentioned the possibility that some peptides might have been precipitated by trichloroacetic acid (TCA) and determined in the protein fraction.

The protein bodies are not broken down at the same rate in all the cells. Bagley et al., (1963) working with germinating peanut cotyledons noted that the protein bodies in cells nearest to the vascular bundle were last degraded. They expressed the view that in the peanut cotyledon, the protein degradation may be due to lysosomal cathepsin activity. Furthermore, the vascular bundle might have a stabilizing effect on the lysosomes by providing oxygen or substrate for the maintenance of the integrity of the lysosome membrane. Thus, the lysosomes nearest to the vascular bundle remain intact and those furthest from the bundle break down and release their hydrolytic enzymes. On the basis of his own experimental data as well as data from the literature Ghetie (1966), is of the opinion that strong oxygenation in the vicinity of the vascular bundles prevents reduction of disulfide bonds of the protein molecules and thus hydrolysis cannot take place even if the lysosomes were to release active proteolytic enzymes. Oxygen concentration does not reach this inhibitor level in the cells situated more distantly from the vascular bundles and thus reduction of disulfide bonds can occur. Ghetie (1965) further pointed out that the hydrolytic process may also occur in the embryonic axis of exalbuminous seeds. This process would serve to deplete the small amount of reserve proteins present in this part of the seed as well.

5. Factors Controlling Synthesis & Hydrolysis of protein in the Cotyledons of Germinating Seeds:

The metabolic processes that take place in the cotyledons are triggered and controlled by some chemical factors derived from the shoot-root axis (Varner et al., 1963). Thus, the removal of axis tissue from pea seeds before imbibition makes the cotyledon tissue incapable of protein synthesis. However, removal of axis tissue after a 48 hour imbibition period does not decrease protein synthesis in cotyledons (Young et al., 1960), thus indicating that the axis factors have been already received by the cotyledons. When Ghetie (1966) followed changes in reserve proteins in comparably treated seeds, he found that excised pea cotyledons were not able to hydrolyse proteins whether excision occurred before imbibition or after 48 hours of imbibition. From this he concluded that the translocation of protein hydrolysates from the cotyledon to the axis tissue is, by itself, a control factor of protein hydrolysis. The absence of translocation inhibits protein hydrolysis.

6. Free Amino Acids During Pea Seed Germination:

The amounts of free amino acids in the dry seed are low, but increase with the germination time. Thus, Lawrence et al., (1959) noted that although they contri-

bute only about 3% to the total nitrogen of the pea seed, their contribution rises to about 10% in the 5 day seedling. When seeds germinate in the absence of an external nitrogen source, the reserve proteins are hydrolysed into their constituent amino acids. Part of these amino acids, with or without interconversions, are utilized by the growing embryonic axis, while the rest are oxidatively deaminated (Webster, 1959). The resulting carbon skeletons find their way to the various respiratory and carbon cycles. The ammonia in turn may be reincorporated into amino acids and amides or into purines and pyrimidines as was found in peas (Beevers & Guernsey, 1966) and in bean seedlings (Boulter & Barber, 1963). Thus, the free amino acids produced by hydrolysis of stored proteins are not necessarily the immediate precursors of new protein synthesis in the seedling. The work of Steward & Bidwell (1962) with tissue cultures also supports this view. In the pea seedling, there is no early mobilization of protein nitrogen until the nonprotein nitrogen of the cotyledons is depleted (Goksøyr et al., 1953; Shirakawa & Otakara, 1956). Danielsson (1951) and Lawrence et al., (1959), on the other hand, noted an increase in dialysable nitrogen in the cotyledon in the early stages of germination.

It was first reported by Virtanen et al., (1953) that growing peas contain an appreciable amount of free

homoserine, which is absent in the ungerminated seeds. They found glutamic acid to be the predominant amino acid up to 48 hours of germination. A high glutamic acid content is a general characteristic of storage protein (Block & Bolling, 1955) and reserve globulins have been shown to contain relatively large amounts of both glutamic and aspartic acids. Arginine, glycine, alanine, asparagine, aspartic acid and serine were also present in smaller amounts in the dry seeds, the levels of each of them increasing considerably by 48 hours. After 72 hours, the amount of homoserine was greatly increased and remained as the most abundant amino acid even after 15 days of germination. The precursor of homoserine is still not known, but this non-protein amino acid could be a precursor of threonine and methionine (Virtanen, et al., 1953; Lawrence & Grant, 1963; Larson & Beevers, 1965). Lawrence and Grant (1963) noted that homoserine amounted to almost 12% of the dry weight of the root and shoot shaft tissue of the 5 day old pea seedling. They further noted that next to homoserine, γ-aminobutyric acid was present in largest amount in the cotyledons and asparagine in the root and shoot shafts. Larson (1963), also working with pea seedlings, noted that in 3 day old green shoots homoserine comprised 75% of the total molar amino acid concentration, a value that fell to 11% by the twenty-fifth day. The levels of aspartic, and glutamic acid and their amides increased from 13% to 71% over the same period of growth.

7. Fate of Reserve Carbohydrates and Lipids during Pea Seed Germination:

About 45% of the dry weight of the pea seed embryo consists of starch (Larson & Beevers, 1965). Young & Varner (1959) reported the presence of an amylase activity of an undetermined nature in pea cotyledons. Swain & Dekker (1966) later characterised this to be α-amylase. They further provided evidence for the presence of β-amylase, maltase, amylopectin-1, 6-glucosidase, phosphorylase and sucrase activity in either the cotyledon, axis tissue or both. It was, therefore, suggested that both hydrolytic as well as phosphorolytic pathways are involved in the developing pea plant for the formation of glucose from starch.

Only about 2% of the dry weight of pea seeds is comprised of lipids (Mayer & Poljakoff-Mayber, 1963). Because of its low lipid content, the mechanism of lipid degradation has never been studied in pea seeds. Studies with other seeds (e.g. castor bean, soybean and peanut) generally indicate that the fats are broken down by the action of lipases to fatty acids and glycerol. Normally, these hydrolysis products do not accumulate in the seeds. The glycerol thus formed may enter into the carbohydrate pool through glycerol phosphate and triose phosphate. Beevers (1956) studied the metabolism of

^{14}C - glycerol in the intact cotyledon of germinated castor bean and found that while part of the glycerol was degraded to $^{14}CO_2$, most of the ^{14}C appeared in sucrose. The fatty acid, on the other hand, may be broken down by β-oxidation and then enter into the tricarboxylic acid cycle through acetyl CoA. The fatty acid may also undergo α-oxidation to form CO_2 (Mayer & Poljakoff-Mayber, 1963).

In many seeds disappearance of fats is accompanied by the appearance of carbohydrates. The pathway of such a conversion (Kornberg & Beevers, 1957; Bradbeer & Stump, 1959) may be represented as follows:

Fatty acid + CoA → acetyl CoA → (Glyoxalate cycle) → malate → phosphoenol pyruvate → triose phosphate → hexose

8. Purpose and Scope of the Present Investigation:

Much work has been done, as the above literature survey indicates, to show how the reserve seed proteins are broken down in the process of seed germination. Measurement of the levels of free amino acids at the various stages of pea seed germination has been the subject of several studies. Little work, however, has been carried out to determine the fate of individual amino acids derived as a result of hydrolysis of stored protein. Larson & Beevers (1965) took the initiative to study metabolism of glutamic, aspartic, leucine and two non-protein amino acids, namely homoserine and γ-aminobutyric acid in pea

seedlings. They found that, except for leucine, the usual fate of the amino acids was oxidation to carbon dioxide or transformation to homoserine. Boulter and Barber (1963) using bean seedlings arrived at an essentially similar conclusion.

^{14}C-labelled phenylalanine has been the choice of many workers as a tool to study in vitro protein synthesis. It has also been extensively used to study lignin biosynthesis in higher plants (Brown 1969). The fate of this widely used protein amino acid, which presumably originates in the seedling from the hydrolysis of stored protein, has never been studied in germinating seeds except for a preliminary study by Nozzolillo (1963).

To facilitate the study of the fate of endogenous phenylalanine in the process of pea seed germination, it was decided to introduce ^{14}C-labelled phenylalanine into the seeds during imbibition. Since information was lacking as to whether and how the labelled compound entered the seed, some preliminary studies were made to determine the uptake of phenylalanine during imbibition.

Thus, the purpose of the present investigation was two-fold:

i) To study the uptake pattern of phenylalanine by the imbibing pea seeds, and

ii) To study the metabolism of the labelled phenylalanine introduced by imbibition on the assumption that it would mix with endogenous phenylalanine pools during pea seed germination.

GENERAL MATERIALS AND METHODS USED IN ALL PHASES OF THE EXPERIMENTAL WORK

1. Surface sterilization and imbibition of the seeds :

Pea seeds (Pisum sativum L. variety Alaska, obtained from Howick Seed Company, Howick, Quebec) were surface sterilized by soaking in 2-3% sodium hypochlorite solution for 40 minutes, then rinsing thoroughly in running tap water and finally in distilled water. Batches of sixty, sound seeds were put into 9 cm. sterile Petri plates containing radioactive phenylalanine solution for imbibition. This number was found to be the maximum which a plate of this size would hold once imbibition was complete.

2. Radioactive phenylalanine :

Carboxyl labelled (^{14}C) L-phenylalanine (specific activity 50 μCi/micromole; certified radiochemical purity >99%) was obtained from California Biochemical Corporation. All of it was dissolved with 25 mg. of unlabelled L-phenylalanine (A grade) acquired from the same source, in glass-distilled water and the resultant solution was freeze-dried (specific activity 2 μCi/mg.). Stock solutions were made by dissolving 1.65 mg. into 10.0 ml. of glass-distilled water (1 mM), to obtain approximately 260,000 cpm/ml. as determined in the gas-flow (G-F) counter.

U-^{14}C-L-phenylalanine (1 μCi/millimole) was obtained from Merck, Sharp, & Dohme of Canada Limited. A stock solution, made by dissolving 0.1 mg. into 2.0 ml. of distilled water, had 4 x 10^6 c.p.m./ml. (G-F counts).

3. Bacterial counts:

Bacterial contamination was checked by plate counting of bacteria on nutrient agar. Culture medium was made by dissolving 4 g. of Nutrient Broth (Difco Lab) and 7.5 g. of Bacto Agar in 500 ml. of distilled water and autoclaving for 15 minutes at 15 lbs. pressure (Seeley & Vandemark, 1962). It was then poured into sterile plastic Petri plates. Serially diluted samples were applied onto the plates with a micropipette, spread with a sterile glass spreader and incubated at 28°C for 3 to 5 days. Bacterial colonies were counted under a hand lens.

4. Counting of Radioactivity :

Both a gas-flow (G-F) system and a liquid scintillation (L.S.) system were used for counting various radioactive samples.

a) Gas-Flow Counting :

A Nuclear Chicago gas-flow counter with a scaler Model 181-A, detector Model D-47, fitted with an automatic sample changer (Model C110-A) was used. This had a counting efficiency of 20% for carbon-14. Usually duplicate samples of 0.1 ml. were drawn with Drummonds disposable micro-pipettes (obtained from Kensington Scientific Corporation, Oakland, California) and plated onto tared disposable aluminum planchets (Laurentian Metal Products, Hull, P.Q.). A circle of uniform area was marked on the planchet and the sample was spread with a thin glass rod over the entire area enclosed by the circle. The sample was then dried in a current of cold air in a fume hood. Sample weight was determined from the differences in weights of the empty planchet and the planchet with dried sample. The total counts taken on each sample was varied according to the activity of the sample. With active samples, 5,000 counts were taken, but for samples of low activity, only 500 counts were taken. The counts were subsequently corrected for background and self absorption. The self absorption correction was based on the assumption that the self absorption error was the same as for barium carbonate and that the sample surface was perfectly smooth (Yankwich et al., 1947).

b) Liquid Scintillation Counting :

Liquid scintillation counting was performed in a Nuclear Chicago L.S. counter (Model 703, efficiency 50%). Both aqueous and ethanolic solutions of samples were counted in this system. Duplicate samples of 0.1 ml. or 0.5 ml. were put into scintillation vials to which were added 15 ml. of counting mix, composed of methyl cellusolve, toluene and ethanol (22 :44 : 33 v/v/v), containing PPO (2,5-diphenoxyoxazole) and POPOP (1,4-di, 2,5-phenyloxazolyl benzene) in the amount of 2.22 g. and 0.044 g. per liter respectively. At least two ten minute counts were taken on each sample and an average value computed. The counts were corrected for background only. A volume of 0.5 ml. of aqueous sample was completely miscible with 15 ml. of the scintillation mixture used and was without any quenching effect.

4. Paper Chromatography and Autoradiography :

a) Separation of phenylalanine and its metabolites :

The standard methods of descending paper chromatography were used with Whatman No. 1 or No. 3 MM paper (Block et al., 1958). The most commonly used solvents were n-butanol-acetic acid-water (4: 1: 5) and n-butanol-ethanol-water (4: 1: 5), (Block et al., 1958), henceforth referred to as BAW and BEW respectively. These are two phase systems, the upper layer being used in the solvent trough and the lower layer to equillibrate the chromatographic chamber. Papers were usually run first in n-butanol-acetic acid-water (4:1:5) solvent

and in one dimension only. For identification of unknown labelled compounds in various fractions, the following solvent systems were also used: water, n-butanol-2N ammonia (1:1) (Harborne & Corner, 1961); 2% acetic acid (Steck, 1967); toluene-acetic acid-water (4:1:5)(McCalla & Neish, 1959); and benzene-acetic acid-water (6:7:3) (Ibrahim & Towers, 1960).

b) Detection of phenylalanine and its metabolites:

Labelled compounds on paper chromatograms were detected by exposure to Kodak No-Screen X-ray film. The usual exposure period ranged from two weeks to three months, depending on the amount of radioactivity spotted on paper. The film was processed by a standard photographic procedure.

In some cases, unlabelled phenylalanine, run as a marker, was detected by spraying with an ethanolic solution of ninhydrin (1%, wt./v.).

UV-absorbing and fluorescing compounds were detected by scanning with a Mineralight UV lamp, Model R-51 (Ultra-Violet Production Inc., San-Gabriel, California).

c) Elution of Radioactive samples from paper chromatograms:

Rectangles large enough to cover the entire radioactive spot as detected by autoradiography were cut out and tapered at one end. The other end was slipped in between two microscope slides along with a washed inert filter paper. Water was allowed to flow by capillary action through the paper strip to elute the sample. The eluted sample was collected in a small glass test tube or on a planchet, dried, counted

and respotted (Zamecnick et al., 1951). In some cases, the eluted sample was used for taking Ultra-Violet and Infra-red spectra.

PART ONE

Uptake of L-phenylalanine by germinating pea seeds

INTRODUCTION

The tracer technique has been extensively used in the study of the intermediary metabolism of amino acids in both plants and animals. In plants, the most commonly used technique for introduction of the labelled compound has been to incubate isolated parts with the labelled compounds. For example, Lyndon & Steward (1963) used slices of potato tuber, Stephenson et al., (1956) tobacco leaf discs, Webster (1954) tissue sections from a variety of plants, Young et al., (1960) slices of pea cotyledons, Birt & Hird (1958) slices of carrot tissue and Kim & Bidwell (1967) pea root tips. The uptake of label under these conditions was generally poor. Using whole plants for the first time, Miettinen (1959) fed ^{14}C-labelled amino acids to the pea plants by placing the roots into sterile radioactive nutrient solution. Almost all the activity was taken up from the medium within 12 hours. In studies with germination inhibitors, many workers have used the imbibition process itself to introduce test materials into the seeds. However, this procedure has not been widely used in metabolic studies of amino acids in seedlings. In the study of asparagine synthesis, Swaramarkrishnan & Sarma (1954) have used this technique to feed Phaseolus radiatus seeds labelled glutamic acid and glucose, Nozzolillo (1963) in the study of utilization of phenylalanine by germinating pea seeds, and Shargool & Cossins, (1969) in the study of L-arginine biosynthesis

in germinating pea seed also used this technique.

Uptake of phenylalanine by pea seeds from an aqueous medium during imbibition would be influenced by factors affecting the process of imbibition, namely, the availability of water, the permeability of the seed coat, the chemical composition of the seed and the chemical composition of the medium.

1) Uptake of Water:

A certain amount of water must be absorbed by the seeds before germination can be initiated. Peters (1920) reported a minimum moisture requirement of 60% to enable a pea seed to germinate. Water uptake by seeds in the early stages is probably a result of physical wetting of seed tissues rather than of physiological processes. Living and dead seeds behave similarly in this phenomenon. When germination occurs, it causes an exponential increase in the rate of water uptake (Owen, 1952; Dewez, 1964).

2) Permeability of the seed coat:

The seed coat protects the young embryo against adverse physical factors. It also prevents loss of solutes, namely, monosaccharides, disaccharides, amino acids and other nitrogen containing compounds and thus helps in subsequent seedling growth (Larson, 1968).

The testa of the pea seed is composed of three distinct layers of dead cells; a layer of palisade cells, a layer of column cells and a few layers of parenchyma cells (Manohar &

Heydecker, 1964). The outer surface of palisade cells is covered with cuticle. The cells of the pea seed coat contain large lumens which hold substantial amounts of water. Manohar & Heydecker (1964) found that the testa is differentially permeable to mannitol solution, the inner parenchymatous layer is much more permeable to the solute than the outer part. They further suggested that the uptake of solute occurs only through the micropyle, an opening of about 120 micron X 80 micron limited to the outer part of the seed coat; the solute then passes through the relatively permeable inner parenchyma. Water uptake, on the other hand, is not restricted to the micropyle alone (Manohar & Heydecker, 1964; Larson, 1968). In general, permeability of the seed coat is greatest near the micropylar end where the seed coat is almost invariably thinner than in other areas (Mayer & Poljakoff-Mayber, 1963).

Permeability of the seed coat is affected by certain external factors. Heat-killed seeds often imbibe water more rapidly than the corresponding viable seeds, probably because permeability of the seed coat is increased by the heat treatment. Seed coats of various species differ with respect to permeability to water because of their differences in lipoid components. Treatment with hot water or alcohol increases the permeability of seed coats (Denny, 1917).

3) Chemical composition of the seed:

In seeds the chief component which imbibes water is the protein. Starch grains ordinarily do not add to the total swelling of the seed (Mayer & Poljakoff-Mayber, 1966).

4) Chemical composition of the medium:

The uptake of water is also dependent on the composition of the imbibition medium. Increase in solute concentration in the medium decreases imbibition. This could be largely due to osmotic effects (Mayer & Poljakoff-Mayber, 1966).

Purpose of Part I

This part of the work was done to seek information on:

i) The optimum conditions of imbibitions.

ii) The rate of uptake of phenylalanine and water by pea seeds, and

iii) The mode of entry of the phenylalanine into the seed.

MATERIALS AND METHODS USED ONLY IN THIS PHASE OF THE EXPERIMENTAL WORK

1) Measurements of water and phenylalanine uptake:

At intervals during imbibition, the seeds were picked out with forceps from the imbibition medium, shaken to remove excess liquid and quickly weighed in an aluminum pan. The increase in weight with time of imbibition was used as a measure of water uptake. The volume of fluid remaining was measured to within an accuracy of ± 0.1 ml. and two 0.1 ml. samples were removed onto tared planchets or onto vials for counting and weight determination. Thus, the specific activity (counts/ min./ ml.) and the total radioactivity of the unimbibed medium at different time intervals was easily calculated, and by difference the amount of imbibed phenylalanine could be determined. The seeds were returned to the imbibition medium as quickly as possible.

2) Cytological and autoradiographic techniques:

Surface sterilized seeds were softened by soaking in distilled water for 2 hours. Four such seeds were

transferred to 1.2 ml. of 1-^{14}C-phenylalanine solution containing a total of 374,000 c.p.m. in a 10 ml. beaker. Two seeds were picked out after 2 hours of incubation and the remaining two after 4 hours of incubation. Immediately after removal, the seeds were given a quick rinse with cold distilled water and taken separately into small boats made up of Scotch tape. O.C.T. compound (Tissue Tek, No. 4583), a gel like substance obtained from Canlab, was poured into the boat, the seed was oriented to the desired position, and the boat with its contents was slowly submerged in liquid nitrogen. The frozen block thus obtained was put in a plastic bag, sealed and stored in a freezer (-30°C). Within 2 days, the blocks were cut into sections 10 μ thick using a cryostat (British Refrigerated Service Ltd., England) at -21°C $\mp$ 1. Following this, they were put on gelatine coated slides (gelatine 5 g., chrome alum 0.5 g., dissolved in 1000 ml. of water) and stored at -21°C. The seed sections on slides were then dehydrated in a VirTis freeze-drying apparatus for a period of about 24 hours, coated with 1% collodion (in ethanol: ether, 50:50) by dipping, dried, placed in a desiccator and taken immediately to the dark-room.

The slides thus prepared were coated with Kodak NTB-3 liquid emulsion following the standard dipping technique (Rogers, 1967). This operation was carried out in a dark-room under a Wratten series 2 safety light. The emulsion was maintained in a 46°C ± 1 water bath. The slides were dipped into the liquid emulsion one by one, withdrawn slowly and held upright to drain for several seconds. The side without the sections was wiped off with a piece of cloth and placed horizontally on a glass plate to dry under a current of cold air. The dried emulsion-coated slides were put into a black plastic slide box, sealed, and stored at 4°C in a refrigerator.

After exposure periods ranging from 6 to 20 days, the slides were developed in Kodak D-19 for 6-7 minutes, dipped in distilled water for 1 minute, fixed in hardener and fixer solution for 8-10 minutes (all about 16°C) rinsed thoroughly with running water and finally air dried.

*In another procedure, based on those described by Perkins et al., (1959), Webb and Gorham (1964) and Trip and Gorham (1967), seeds incubated with labelled phenylalanine were rapidly frozen by immersion in a mixture of dry ice and

*This part of the work was done at Dr. Trip's Laboratory at the National Research Council of Canada in Ottawa, with his help and guidance.

isopentane. The frozen seeds were transferred in small pre-cooled beakers to a vacuum desiccator packed in dry ice which was subsequently evacuated to a pressure less than 0.02 mm Hg. The desiccator was stored at -40°C for 3 days, and was then allowed to warm to room temperature before the vacuum was broken. The seeds were vacuum infiltrated over-night with paraffin (Tissue-mat 54-56) in an oven at 80°C and then embedded in paraffin for sectioning. Sections 10-12 μ thick, were kept dry and mounted on Eastman Kodak NTB 10 μ plates after moistening the emulsion by gently breathing on it. The sections were then moistened in the same manner and pressed onto the emulsion with a small rubber roller. After exposure in dry boxes at room temperature for 7 days, the plates were deparaffined for 5 minutes in redistilled xylene, air dried and developed in Kodak D-19 for 5 minutes, fixed in acid fixer, rinsed in 2 baths of distilled water and air dried.

RESULTS

1. Uptake of water:

The uptake of water was determined with three batches of sixty seeds, each batch put into 30 ml. of distilled water. The results presented in Figure 1 indicate that the rate of uptake of water was very rapid up to a period of 6 hours, by which time the seed weight had increased by 10 grams. During the following 10 hours the rate of uptake of water slowed down and the seed weight further increased by only 3 grams. The water uptake was also determined in the presence of a small amount of phenylalanine. This curve, plotted as per cent of the total water uptake, is presented in Figure 2 and shows the same general pattern.

2. Uptake of phenylalanine:

The results of this section are based on six batches of seeds, sixty seeds per batch, which had been placed into 30 ml. of diluted phenylalanine solution (2 μmoles/ 30 ml.) in 9 cm. Petri plates. The specific activity (counts/ min./ ml.) was determined at different time intervals over a period of 18 hours as detailed in methods (p. 25). The results, expressed as per cent of initial counts per minute per milliliter are given in Fig. 2, curve A. This curve, which shows an overall decline with time in counts/min./ml. indicates that the uptake of phenylalanine and the uptake of water did not proceed at the same rate. If they had, the counts/min./ml. of the imbibition medium would have remained

FIGURE 1

The uptake of water as measured by the increase in fresh weight of germinating peas. Three lots of sixty seeds were placed into 30 mls of water, and removed at the intervals shown for fresh weight determinations. The data obtained from all the three batches have been plotted directly on the graph.

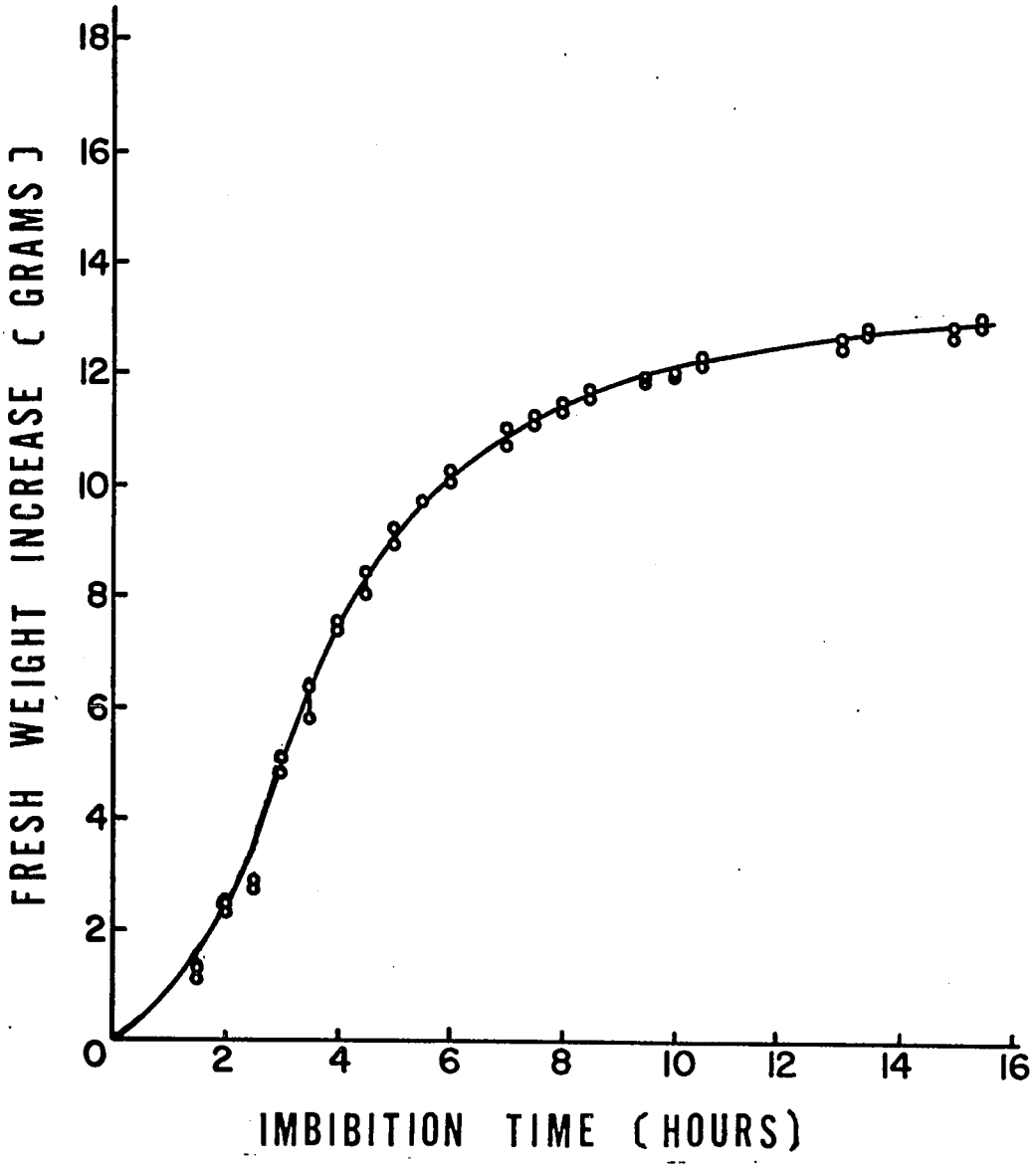
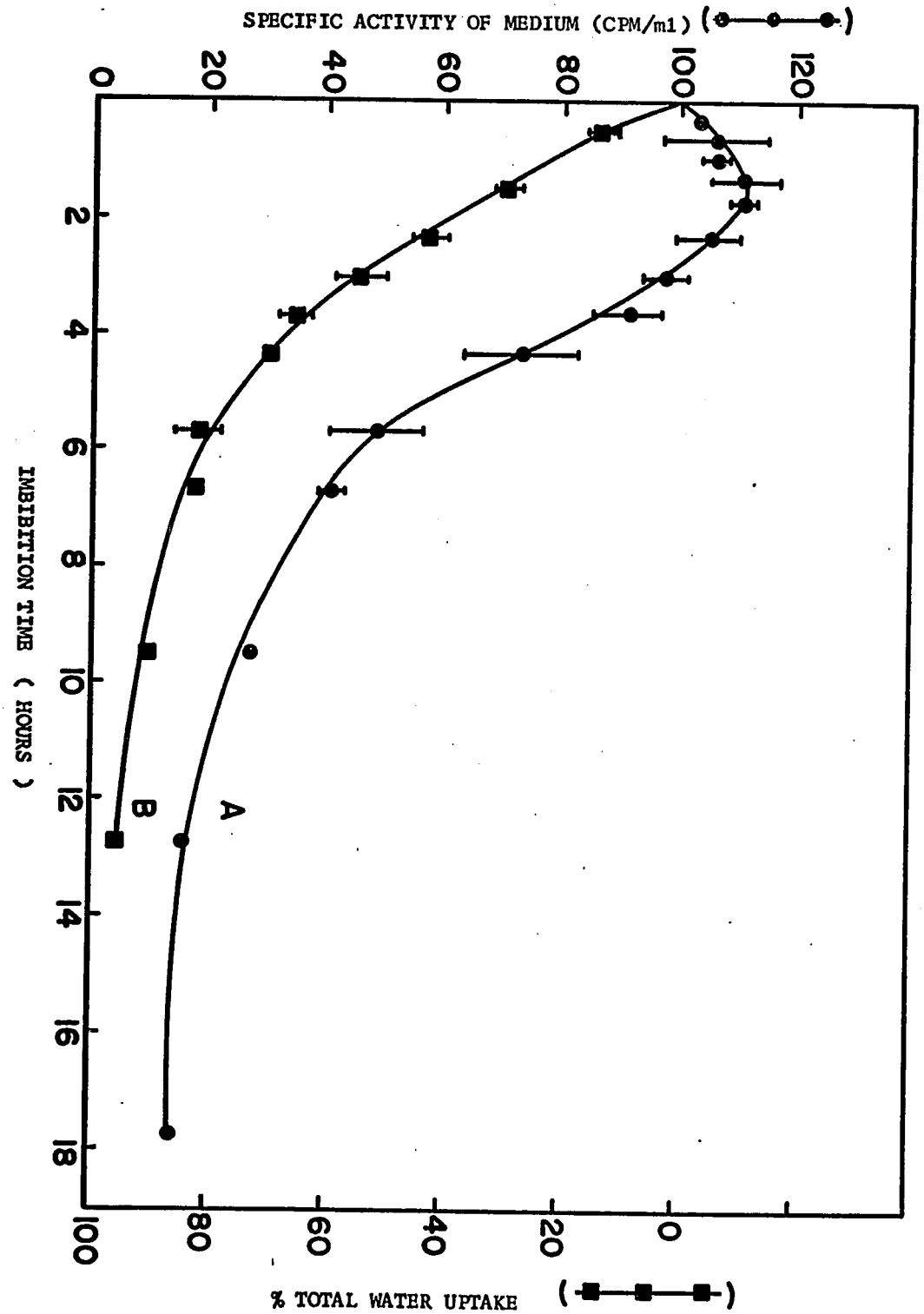

FRESH WEIGHT INCREASE (GRAMS)
0
2
4
6
8
10
12
14
16
18
IMBIBITION TIME (HOURS)
0
2
4
6
8
10
12
14
16

FIGURE 2

Rate of uptake of water, expressed as per cent of final total water uptake (curve B), and changes in specific activity (c.p.m./ml.) of the imbibition medium (curve A) during the course of imbibition by pea seeds. Each point represents a mean value obtained from 6 batches of 60 seeds ± standard deviation. Concentration of Phe in the medium was approximately 0.0001 M.

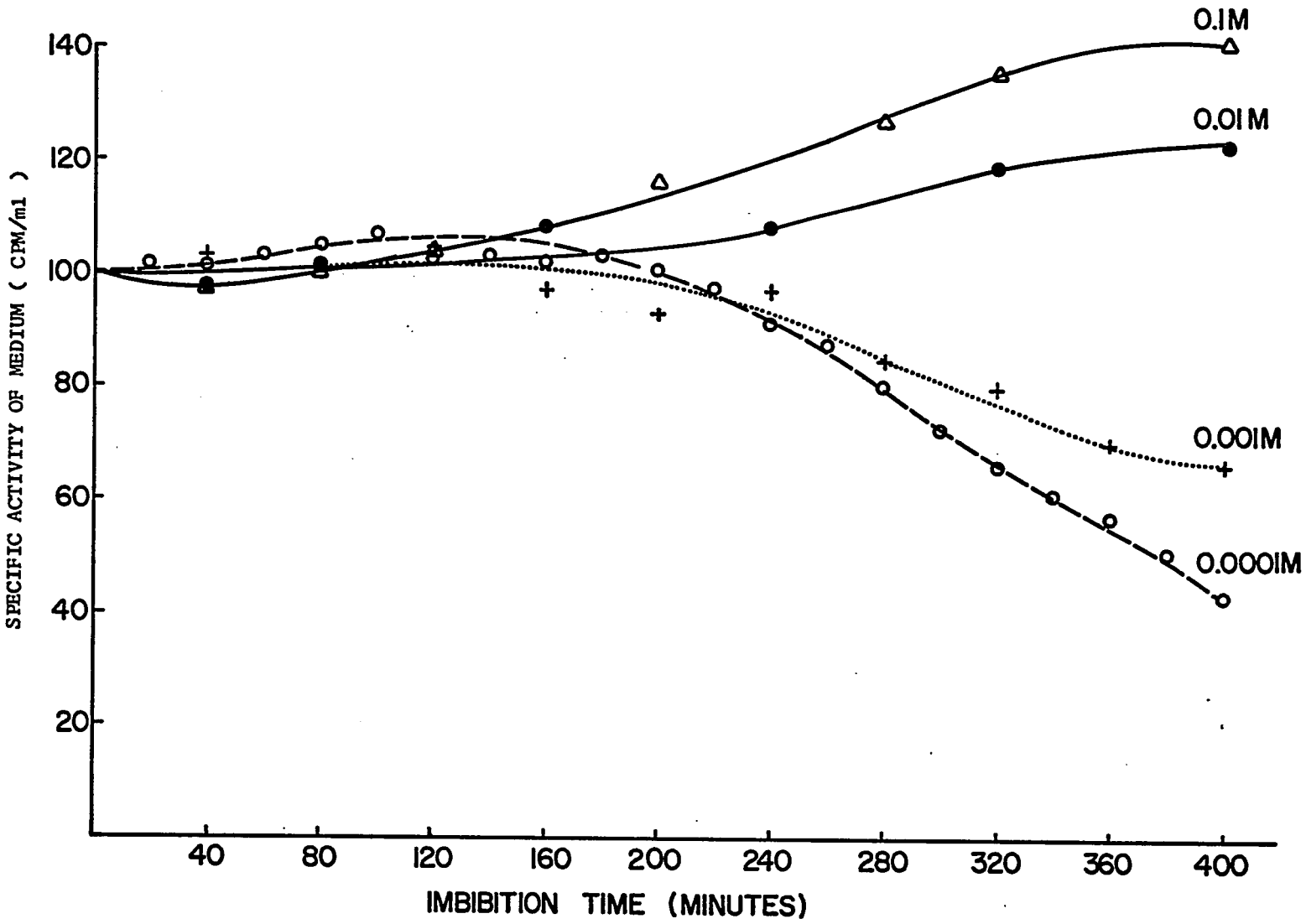

SPECIFIC ACTIVITY OF MEDIUM (CPM/ml) (●—●—●)
0
20
40
60
80
100
120
2
4
6
8
10
12
14
16
18
INHIBITION TIME (HOURS)
B
A
100
80
60
40
20
0
% TOTAL WATER UPTAKE (■—■—■)

unchanged; i.e. curve A would have been a straight line parallel to the axis at 100%. In the initial stages, the uptake of phenylalanine was apparently slower than the uptake of water, as indicated by a tendency towards an increase in counts/min./ml, but soon became more rapid than that of the water, resulting in a marked drop in the counts/min./ml. The curve for total phenylalanine uptake is not plotted separately because, expressed as total counts remaining, it almost parallels that of water uptake (curve B, Figure 2).

To test the effect of increasing concentrations of phenylalanine on its uptake pattern, the following experiment was carried out. Four batches of sixty surface sterilized seeds were each placed into a 9 cm. Petri plate. To each plate was added the same initial radioactivity but different concentrations of phenylalanine, namely 0.0001 M, 0.001 M, 0.01 M and 0.1 M in a total volume of 15 ml. The counts/min./ml. of the various incubation media were determined at 40 minute intervals for a period of 6 hours and 40 minutes. The results thus obtained are expressed as % initial counts/min./ml and are presented in Figure 3. This figure shows that there was not much change in the counts/min./ml. in any plate during the first 3 hours. Following that, the counts/min./ml. of the imbibition medium of the lowest phenylalanine concentration (0.0001 M) declined rapidly. The counts/min./ml of

FIGURE 3

Effect of phenylalanine concentration on the changes in specific activity (c.p.m./ml.) of the medium during imbibition. Each point is the mean of two samples removed for counting at each time period and the variation between the counts was within ± 5 per cent.


0.1M
0.01M
0.001M
0.0001M
140
120
100
80
60
40
20
SPECIFIC ACTIVITY OF MEDIUM (CPM/ml)
40
80
120
160
200
240
280
320
360
400
IMBIBITION TIME (MINUTES)

the 0.001 M phenylalanine solution also declined, but less rapidly. On the other hand, the counts/min./ml of the media containing 0.01 M and 0.1 M phenylalanine showed an increase which was greater in the case of the higher concentration. It should be pointed out that no statistical significance can be attached to these results since this test was made only once, and on only one batch of seeds at each concentration.

The total amount of phenylalanine unimbibed was calculated from the counts/min./ml. of the last sample and the total volume of fluid remaining. From this, the total phenylalanine imbibed was determined by difference and the per cent uptake calculated. The results of these calculations are presented in Table 1 and indicate that pea seeds imbibed added phenylalanine more completely when it was supplied in small amounts. With increasing amounts of phenylalanine in the medium, the seeds took up more and more but the per cent uptake declined. Thus, when phenylalanine was supplied at a level of 0.033 μmole per seed, the uptake was 85 per cent by 7 hours. At a level of 23.33 μmoles of phenylalanine per seed (which was almost a saturated solution), the uptake was only 45 per cent by 7 hours. Although no statistical significance can be attached to this test on the basis of a

TABLE 1

EFFECT OF INCREASING PHENYLALANINE CONCENTRATION IN THE INCUBATION MEDIUM ON PEA SEED GERMINATION

Phe conc. in the media			At 72 hours of germination		
µmole/seed		% uptake	Per cent seeds germinated	Fresh weight in g./seed	
provided	absorbed			Cotyledon	S-R axis
0.033	0.028	85	83	0.31	0.046
0.266	0.207	78	83	0.27	0.047
2.366	1.372	58	88	0.28	0.045
23.333	10.500	45	87	0.27	0.040

Four batches of sixty seeds were each placed into a different phenylalanine solution. After almost 7 hours imbibition, they were transferred to moist vermiculite for germination (defined as the appearance of the radicle) and subsequent growth. By 72 hours, the radicle averaged 3 cm. in length, but development was not uniform. No statistical significance can be attached to the results presented in this table since only one batch of seeds was tested at each concentration.

single run, nevertheless an overall picture of increasing uptake with increasing phenylalanine concentration was observed.

It thus appears that pea seeds have a great capacity to absorb phenylalanine from the external medium. In order to see if this high amount of absorbed phenylalanine had any toxic effect on the subsequent germination, the seeds were allowed to grow for 72 hours. At this time the seedlings all appeared normal. The number of seeds germinated, the fresh weight of the cotyledons and of the shoot-root axis and the length of the radicles was determined. (A seed was considered to have germinated if the radicle had emerged through the seed coat). By 3 days, the radicle averaged about 3 cm. in length, although development was not uniform. These data have been presented in Table 1. It can be seen that the high amount of absorbed phenylalanine had no apparent effect on the final per cent germination or on subsequent seedling growth as represented by the fresh weight of the shoot-root axis.

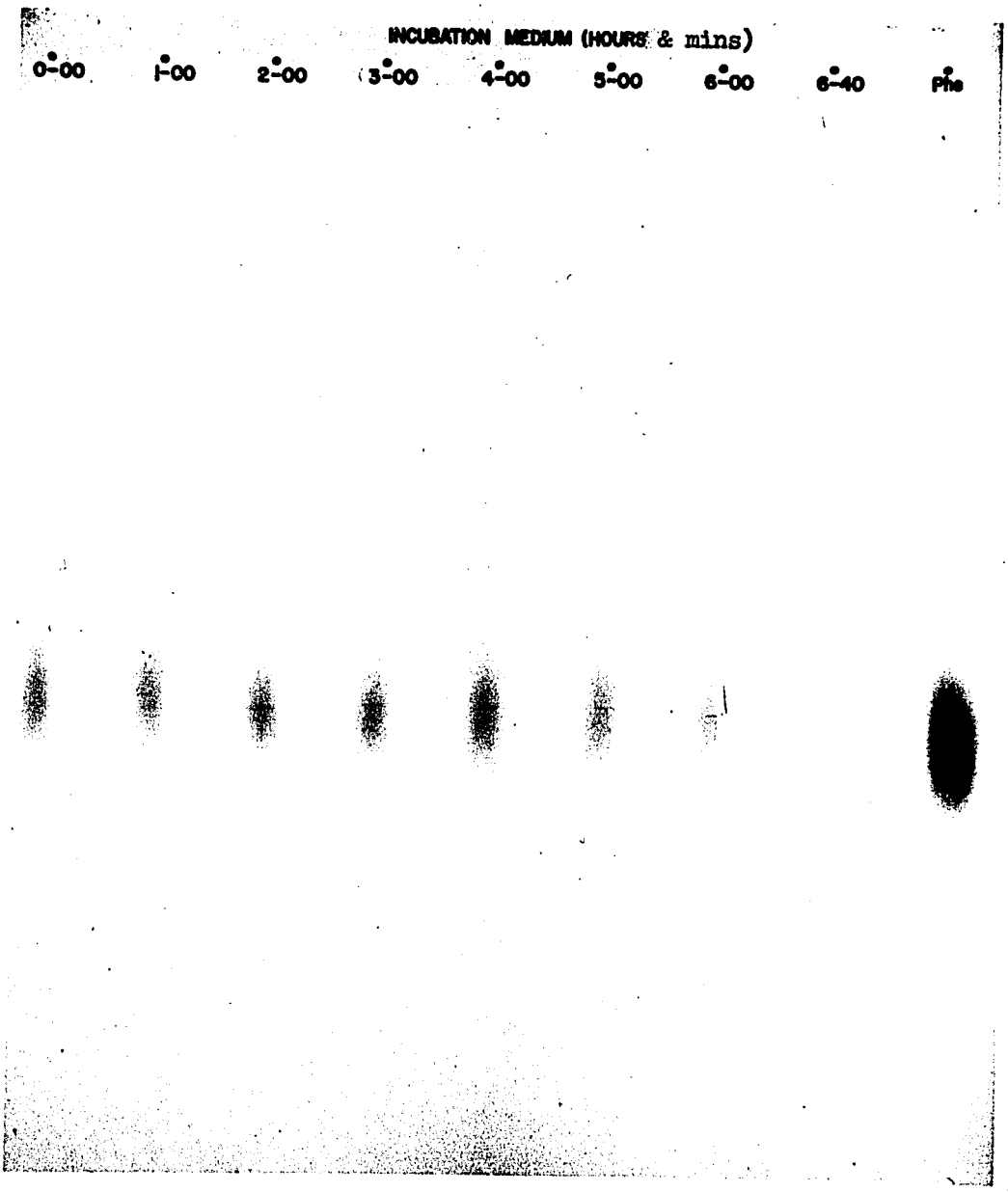
3. Bacterial populations and their effect on phenylalanine in the imbibition medium :

Since contaminating bacteria could be expected to metabolize at least some of the exogenous phenylalanine molecules and could contribute to the labelling pattern of the macromolecules in the growing tissues (Lonberg-Holm,

FIGURE 4

Representative autoradiograph of chromatogram of the imbibition medium. (as used in the phenylalanine uptake expt. described on pp 34-36).

Paper used:	Whatman No. 3 MM, developed once in BAW solvent
Rf of phenylalanine:	0.56
Approx. c.p.m. per spot:	1,200
Exposure period:	4 weeks


INCUBATION MEDIUM (HOURS & mins)
0-00
1-00
2-00
3-00
4-00
5-00
6-00
6-40
Phe

1967), the extent of bacterial contamination in the imbibition medium was checked.

It was found in the single determination made that even the zero time sample, obtained after putting the surface sterilized seeds into the imbibition medium, was not free from bacteria. The imbibition medium was perhaps not sterile or the surface sterilization procedure used was not very effective. Whatever the cause, a total of 10^4 bacteria was counted at zero time. Up to a period of 6 hours, there was no appreciable increase in the numbers of bacteria: 10^4 - 10^5 cells were counted in the 5 determinations made. By 24 hours, the numbers had increased to 10^{10} - 10^{12} bacteria as based on 7 determinations.

Chromatography of imbibition media at 6 hours or earlier showed that 10^4 - 10^5 bacteria had not caused any noticeable conversion of the added phenylalanine. Figure 4 shows that the radioactivity ran as discrete spots in the position of standard phenylalanine and no other labelled spots were detected within the limits of the method used. Imbibition media containing about 10^7 bacteria showed two or more additional radioactive spots in BAW(4:1:5) solvent. No attempt has been made to identify them. By the time the bacterial number had reached this level, most of the medium had been imbibed (Figs. 1 & 2).

4. Mode of Entry of Phenylalanine into the pea seeds:

It was shown by Manohar & Heydecker, (1964) that the testa of the pea seed is differentially permeable and that

the entry of mannitol into an intact seed occurred only through the micropyle. It was, therefore, decided to check by an autoradiographic technique whether what was true for mannitol also held true for phenylalanine. In addition, this would provide direct evidence of the actual entry of phenylalanine into the seed itself.

Most autoradiographic studies have been concerned with the detection of a radioactive isotope in water insoluble compounds, such as DNA or protein. In most methods the specimen containing the isotope is fixed in an aqueous solution before being cut into sections. It is evident that water soluble compounds will be lost in such fixing fluids or will be smeared throughout the specimen.

However, in a number of recent reports, methods of studying the distribution of soluble compounds have been discussed. One, the procedure of Trip and Gorham (1967), was tested in these experiments but was found impractical, because the compact nature of the seed tissue makes it unsuitable for sectioning. Other techniques (Fitzgerald, 1961, Greenberg & LaHam, unpublished data, 1969) however were combined and modified to suit the requirements (pp. 25-27).

A preliminary trial indicated that about 4 hours of soaking was necessary before a pea seed could be sectioned easily in a cryostat. In order to indicate the path of entry of the labelled compound by means of auto-

radiography the section should have as high a level of radioactivity as possible. On the other hand, the labelled compound should not move so as to cover the entire seed area, since the whole section area from such a seed would appear black when autoradiographed. This type of autoradiograph would fail to indicate the path of entry of the label. Thus, presoaking of pea seeds for two hours, followed by 2 and 4 hours of incubation in a radioactive phenylalanine solution was chosen. The autoradiographs presented here (Figures 5 & 6) were obtained from seeds incubated in the tracer solution for 4 hours.

In Figure 5, Nos. 1 and 2 were cut parallel to the shoot-root axis, whereas Nos. 3 & 4 were cut at right angles to the shoot-root axis. These photos show that the distribution of radioactivity is only in the peripheral area of the cotyledons extending from each end of the shoot-root axis for only two-thirds of the cotyledon circumference, i.e. the part furthest from the shoot-root axis has no activity. The inner part of the cotyledons is only poorly labelled, if at all.

Photomicrographs of plumule and radicle (Figure 6, Nos. 1 and 2) show that the labelling in these parts is generally poor. The density of silver grains both within these parts and in their surroundings is about the same.

Figure 6 (No. 3) shows the distribution of radioactivity in a section cut at right angles to the shoot-root axis, near the plumule tip. Radioactivity in the area close to the shoot-root axis is high.

FIGURE 5

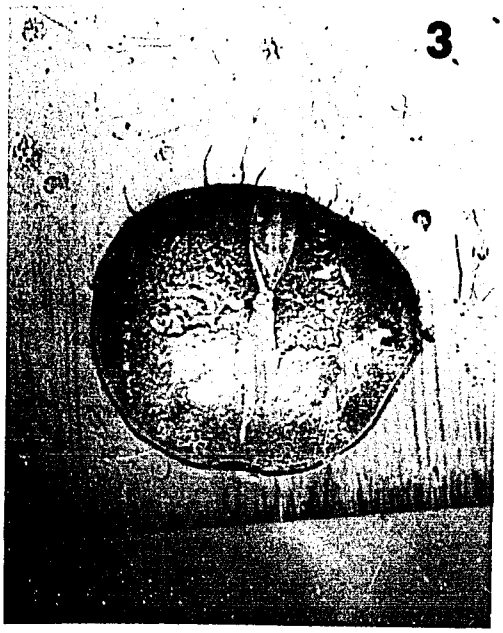
Autoradiographs of pea seed sections. The seeds were allowed to imbibe a solution of 1-^{14}C-L-phenylalanine for 4 hours. Sections 10μ thick were prepared and exposed for 6 days. Magnification 7.5X

Nos. 1 & 2: Sections cut along the sr-axis.

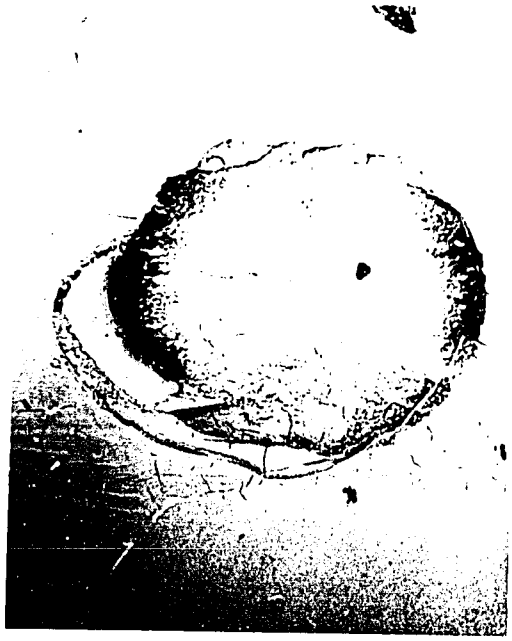
Nos. 3 & 4: Sections cut across the sr-axis.

All sections are oriented with the sr-axis uppermost, except for No. 2, where the section is unintentionally placed at an inverted position.

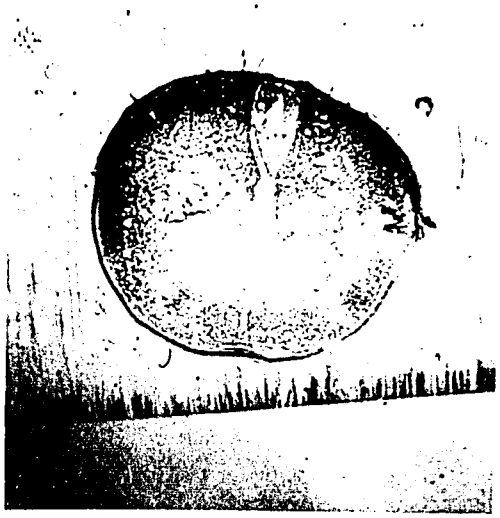

1


3


4



3



4

FIGURE 6

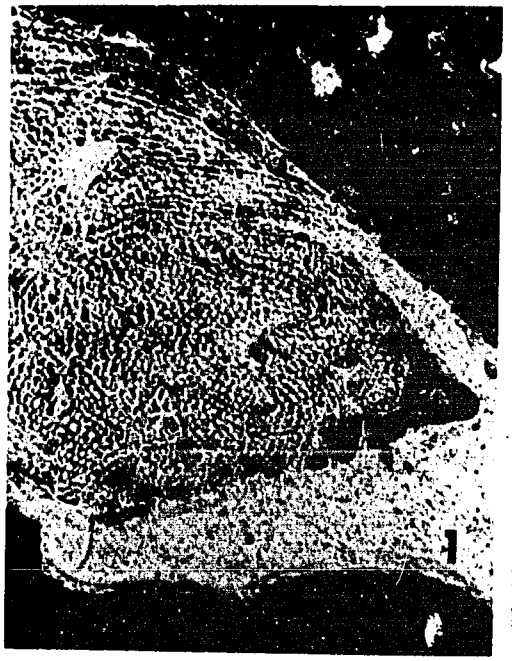
Autoradiographs of pea seed sections prepared as described in methods.

No. 1: Section cut at right angles to the sr-axis showing distribution of silver grains in and around the plumule. Exposure period: 16 days. Magnification 75X (cf No 3, Fig. 5).

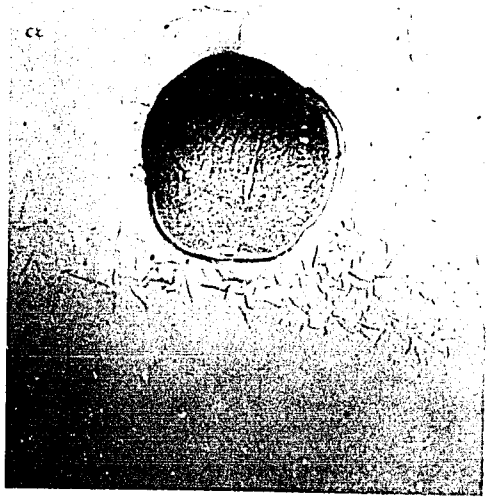
No. 2: Section cut parallel to the sr-axis showing distribution of silver grains in and around the radicle. Exposed for 10 days. Magnification 75X. (cf No. 2, Fig. 5).

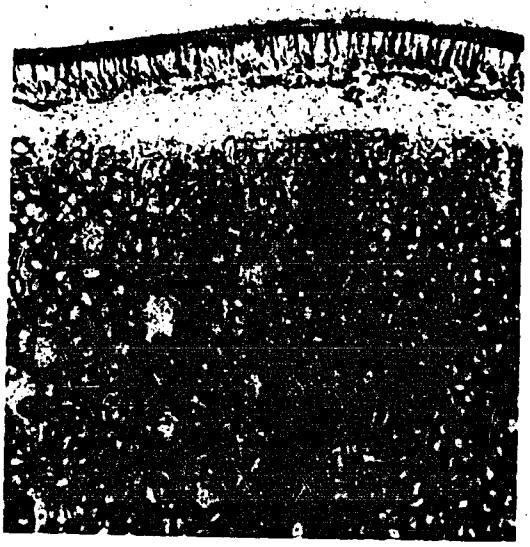
No. 3: Section cut at right angles to the sr-axis close to the plumule. Exposed for 10 days. Oriented with S-R axis uppermost. Magnification 7.5X.

No. 4: Section showing the distribution of label in the seed coat area. Exposure period: 6 days. Magnification 125X. (enlargement of the area indicated by an arrow in Fig. 5 No. 1).



3 4



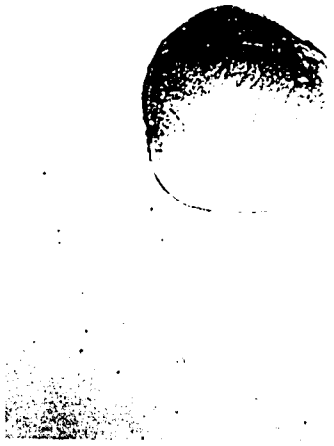


1

2

3

4



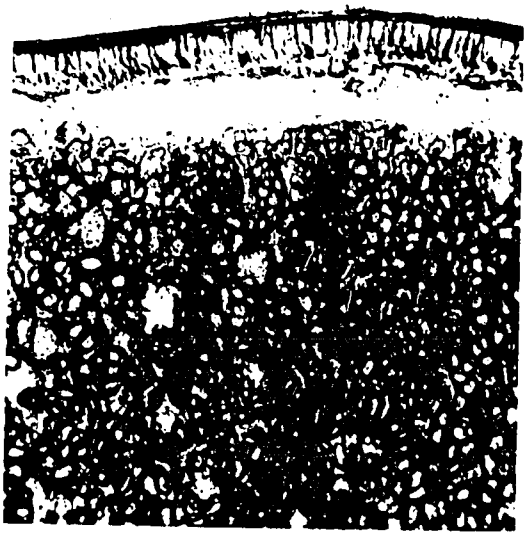


Figure 6 (No. 4) presents a photomicrograph of a section showing the distribution of radioactivity in the region of the seed coat. The density of silver grains appears to be higher on the inner surface of the seed coat and between the starch grains than it is on the outer surface of the seed, where the activity presumably is that remaining adsorbed during the washing procedure.

There was some difficulty in distinguishing those areas in the section which were dark because of reduced silver grains in the emulsion, from those which appeared dark because of light absorption by the starch grains. The distinction could easily be made at high magnification by focussing on the silver grains themselves (Figure 6, Nos. 1, 2 & 4) but at this magnification, an overall picture was difficult to visualize. Attempts were made to strip the emulsion but this proved to be impracticable. However, one section had been accidentally only half covered with emulsion (Figure 5, No. 4) and thus served to point out useful features. It can be noted that the bottom part of section No. 4 appears almost the same as the bottom of No. 3 (Figure 5) i.e., the section itself has absorbed light. The darkness in the upper half, however, is in the emulsion itself as shown by the folded flap in No. 4 (Figure 5).

DISCUSSION

The results presented in Figure 1 indicate that the uptake of water is rapid in the first 6-8 hours, but subsequently slows down; the pattern is similar to that obtained by Larson (1968). The initial rapid uptake of water may be attributed to physical wetting of the seed tissues, rather than to physiological processes (Dewez, 1967).

It would appear from the results presented in Figure 2 that the uptake of water and the uptake of phenylalanine are two separate processes. An as yet unknown process caused the phenylalanine to diffuse into the seeds at a somewhat faster rate, resulting in a drop in the counts/min./ml. of the imbibition medium. Whether or not an active process (Birt & Hird, 1958) is involved in such uptake can be resolved only by further study. However, the parallel nature of the curves for total uptake of phenylalanine, and for per cent water uptake indicates that the bulk of the phenylalanine must have been carried in with the water. The results shown in Figures 1, 2 & 3 also indicate, that when supplied at low concentration, the uptake of phenylalanine is almost complete in a period of about 7 hours. When the supply of phenylalanine in the medium is increased total uptake by the seeds also increases at least up to a concentration of 0.1M, but the per cent uptake declines. It is possible that the embryo of the pea seed has a high capacity to absorb phenylalanine while the seed coat itself limits the amount by acting as a barrier. It has been

concluded by Manohar et al., (1964) that the entry of solute into a pea seed takes place only through the micropyle. Since they find this opening to be 80μ by 120μ in area, and the maximum dimension of the phenylalanine molecule is in the order of 0.24μ (Snell et al., 1965) one would not expect any inhibition of movement of the amino acid on account of size, as long as it is in solution. However, at the higher concentrations used in the experiment described on pages 32-36, physical blocking of the pore might have occurred when the phenylalanine came out of solution as imbibition progressed.

Certain amino acids have been found to be toxic to seedling growth even at a very low concentration. Taking inhibition of root growth as an index of toxicity, Audus & Quastel (1947) noted that tryptophane was very toxic and glycine, proline and arginine were toxic to cress seedlings. On the other hand, alanine, glutamic and aspartic acids did not exert any effect on root growth even up to a concentration of 1000 p.p.m. Virtanen & Lincola (1946, 1957) noted profound morphogenetic effects of certain amino acids and amines supplied to the nutrient solution of pea plants. They found D-alanine was quite toxic, while β-alanine and phenethylamine altered the whole plant. Steinberg (1949) noted changes in tobacco seedlings with leucine and isoleucine while hydroxyproline at a very low concentration in the nutrient solution killed tobacco plants. Waris (1957) cultivated Oenenthe aquatica in a

nutrient solution containing glycine and sucrose. The plants grew normally for some time but after 3-4 months, became morbid. Results reported in Table 1 indicate that phenylalanine up to a concentration of 0.1M did not have any noticeable toxic effect on pea seed germination or subsequent seedling growth. Since the seedlings were not grown for a longer period, it is not known if phenylalanine would have had any delayed effect on the plants.

The surface sterilization procedure used in the present studies did not eliminate bacterial contamination on the seeds. Most of the Alaska pea seeds when soaked form a wrinkled surface, and thus, surface sterilizing agents may not work very effectively on them unless they are fully swollen. This might be the reason why certain workers (Swain & Dekker, 1966) soaked the seeds in water before treating them briefly with sodium hypochlorite solution. It is also possible that the imbibition media were not sterile; since they were made in glass distilled water and were not autoclaved.

The numbers of bacteria however, did not cause any detectable conversion of phenylalanine in the medium (Figure 4) when they were limited to a total number of $10^4 - 10^5$ cells or $10^3 - 10^4$ cells per ml. A count of 10^3 bacteria / ml. in a reaction mixture prepared for the study of amino acid incorporation into protein bodies, was regarded as a sterile condition (Wilson, 1966). Lonberg-Holm (1967) defined a plant tissue as "free from

bacteria" when it contained less than 10^3 bacteria/gram.

Results of the uptake studies (Figure 2 and Table 1) clearly showed that pea seeds removed 80 per cent or more of the added phenylalanine from a dilute solution in about 6 hours, or in other words, before bacterial numbers had time to increase appreciably. Thus, if the initial bacterial count is kept low in the incubation medium, the problem of bacterial degradation of the exogenous phenylalanine molecules can be overlooked.

The photos presented in Figures 5 and 6 show localization of the label mainly in the outer peripheral area of the cotyledons and especially around the shoot-root axis and micropyle. Very little or no activity was detected in the interior or bottom areas of the cotyledons. These photographs indicate that phenylalanine entered the seed through the micropyle or the micropylar region of the seed coat. The photomicrograph in Figure 6, No. 4 shows the presence of a large number of grains in between the "seed coat" and the cotyledon. The study of Manohar & Heydecker (1964) showed that the seed coat of pea seeds is separable into an "outer" and an "inner" part. The outer part is composed of cuticle, one layer of palisade cells and one layer of "column" cells, while the inner part is comprised of a few

layers of parenchyma cells. The inner part is much more permeable to solute than the outer part and the micropylar aperture is found only in the outer part. A close observation of the seed coat (Figure 6, No. 4) clearly shows all three components of the outer part, but not the parenchymatous inner part. These two parts must have been separated either during imbibition or upon sectioning and the inner part probably remained firmly bound to the cotyledon. It can be concluded that the phenylalanine solution entered through the micropyle or the micropylar region (where the seed coat is comparatively thin), travelled between the inner and the outer layers and then passed into the cotyledon through the relatively permeable inner parenchyma.

Both the plumule and the radicle were only slightly labelled (Figures 5 and 6, Nos. 1, 2) in spite of their proximity to the micropyle. This observation suggests that the labelled phenylalanine diffused only into the cotyledons at first.

The difficulties (Fitzgerald, 1961) involved in the autoradiography of water-soluble compounds have been corroborated. The procedure followed here did

cause some smearing of the radioactivity, but with proper temperature control and expert handling, this smearing could be much reduced. It is suggested that this procedure should be considered in studies involving a quick and gross localization of activity within a tissue. The results of these uptake studies have been published (Paul & Nozzolillo, 1969).

PART TWO

Metabolism of L-phenylalanine in germinating pea seeds

INTRODUCTION

The aromatic amino acids phenylalanine and tyrosine are the most abundant of the naturally occurring phenylpropanoid compounds ($C_6 - C_3$). These two primary metabolites are absolutely essential for life, but their biosynthesis has been studied in detail only in microorganisms, (Weiss et al., 1954).

It is now known that many other phenylpropanoid compounds in plants are formed from phenylalanine and tyrosine. In addition to incorporating these amino acids into proteins, higher plants have developed an alternate pathway of metabolism whereby phenylalanine and tyrosine serve as a direct source of cinnamic acid derivatives. Koukol & Conn (1961) have shown that cinnamic acid itself can arise directly from phenylalanine by the action of L-phenylalanine ammonia lyase. Similarly, p-coumaric acid can be produced directly from tyrosine by L-tyrosine ammonia lyase (Neish, 1961). Brown et al., (1959) and Higuchi & Brown (1963) postulated from the results of their experiments in which L-phenylalanine was tested as a precursor of lignin that phenylalanine can to some degree be converted to cinnamic acid through the following minor pathway:

phenylalanine → phenylpyruvic → β-phenyllactic → cinnamic

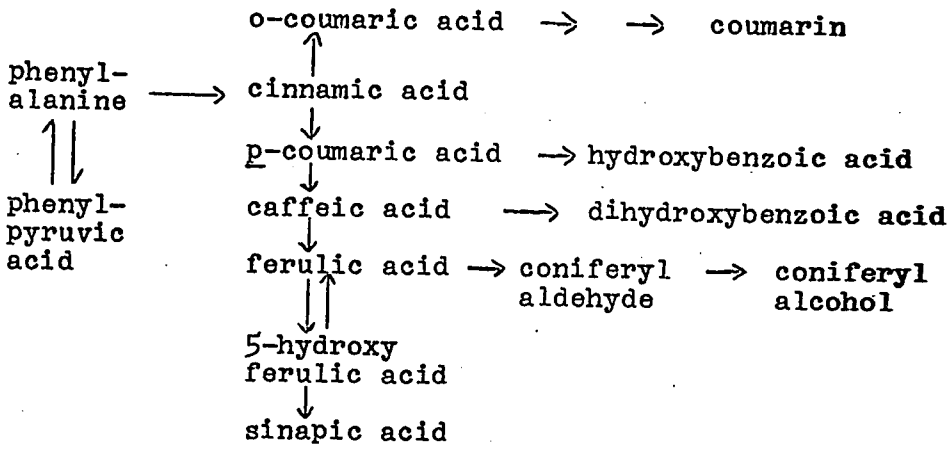
They demonstrated that both L-phenylalanine and cinnamic acids are good precursors of lignin in monocotyledons and dicotyledons, but that only the monocotyledons can convert L-tyrosine to lignin. In Salvia, McCalla & Neish, (1959) observed that very little phenylalanine was converted to tyrosine.

Reduction of cinnamic acid to its corresponding cinnamyl alcohols is believed to provide the starting materials from which lignins are made (Brown, 1969). Lignins once formed are not reutilized and thus seem to be an end product as far as higher plants are concerned. The simple phenylpropanoid compounds, however, can probably be broken down and respired in higher plants. Thus, Runeckles (1963) has observed formation of sugars from the aromatic amino acids and cinnamic acid derivatives in tobacco leaves. However, there is not sufficient evidence to show that the aromatic ring can be opened oxidatively in plants although this type of reaction is well known in bacteria (Towers, 1964). There is some proof for the view that $C_6 - C_3$ compounds can give rise to $C_6 - C_2$ and $C_6 - C_1$ compounds by the loss of side chain carbons (Neish, 1959).

The major established (Pridham, 1965) pathways of phenylalanine metabolism are shown in the following figure:

FIGURE 7

Metabolic pathways of L-phenylalanine in higher plants



The data presented in Table 2 show that only a small portion of the total free amino acids in the dry pea seed is phenylalanine. The level of phenylalanine increases slightly but steadily with the age of the seedlings from 7 µmoles per 100 seeds to 75 µmoles per 100 5-day old seedlings. Two non-protein amino acids, homoserine and γ-aminobutyric acid, increase considerably over the 5-day period. Of the main amino acids of storage protein, namely arginine, histidine, lysine, aspartic acid and glutamic acid, only the level of histidine shows a similar increase. According to Boulter & Barber (1963), these latter amino acids are

TABLE 2

Free Amino Acid Changes for Whole Pea Seedlings (var. Unica)

(After Lawrence & Grant, 1963)

	Age (Days)			
	0	1	3	5
Glutamic acid	254	320	324	332
Aspartic acid	65	45	17	37
Arginine	247	406	373	299
Histidine	5	8	35	69
Lysine	32	18	58	107
Glutamine	5	103	122	233
Asparagine	234	318	268	488
Homoserine	0	33	1360	2846
γ-aminobutyric acid	6	25	454	491
Phenylalanine	7	14	51	75
TOTAL AMINO N	1390	1710	4440	6450

These values are in μmoles per 100 seedlings, without seed coats.

extensively broken down and utilized in the synthesis of RNA templates, proteins and prosthetic groups necessary to maintain the developing respiratory and photosynthetic apparatus of the germinating seed. Other protein amino acids do not change greatly in amounts during germination; these are liberated from storage proteins and utilized more or less unchanged (Boulter & Barber, 1963).

In spite of the small amount of free phenylalanine present in the pea seedling, the importance of this amino acid should

not be underestimated. Besides being a protein amino acid, it is a key intermediate compound in lignin biosynthesis. To date no systematic studies have been conducted to determine the fate of this amino acid in germinating seeds. Therefore the present investigation was undertaken. Radioactive phenylalanine was introduced into the seeds during imbibition on the assumption that it would be integrated into the endogenous phenylalanine pools and thus would act as a tracer by which the fate of this amino acid could be followed.

The work reported in this part of the thesis was done to determine :

i) the effect of germination time on the distribution of the label in germinating pea seeds, and

ii) the fate of the phenylalanine molecule itself during the course of pea seed germination.

To accomplish these ends, the following experiments were done :

i) A time course experiment in which 1-^{14}C Phe was fed to 4 lots of 60 seeds, one lot being removed every 24 hours and fractionated as detailed in the Methods Section. The results are presented in Tables 3-8, 11, 12. These results were supplemented by two experiments in which a single lot of seeds was removed after 3 days (Tables 9, 10, 14) and by one experiment in which U-^{14}C Phe was fed to one lot of seeds (Tables 18-21).

ii) The samples obtained from the above experiments were then subjected to paper chromatography and radioautography.

MATERIALS AND METHODS

1) Germination of seeds :

Batches of sixty surface sterilized pea seeds were put into Petri plates containing 0.165 mg. (1 µmole) of phenylalanine (about 260,000 c.p.m. as 1-^{14}C-Phe or 1,145,000 c.p.m. as U-^{14}C-Phe) in distilled water. The volume of water was selected to ensure complete uptake. If the seeds were to be left in the Petri plates for the entire course of the experiment, 11.0 ml. of solution were used (cf Fig. 1). If the seeds were to be transferred to vermiculite after 6-7 hours, only 6.0 ml. of solution was used. The plates were placed in darkness in an incubator at 26°C ± 1 for imbibition, germination and growth.

In a time course experiment, four uncovered Petri plates, each containing 60 seeds, were placed in an atmosphere of high humidity. To obtain this, a desiccator was lined with moist filter paper, wrapped with black plastic cloth and placed in the incubator. Each day, i.e. after 24, 48, 72 and 96 hours, one plate was removed from the desiccator and a small amount of water was added to the remaining plates to keep the seeds moist.

2) Trapping of respiratory carbon dioxide :

The CO_2 given off by the germinating seeds was trapped in the above time course experiment by placing a 20% potassium hydroxide solution in two 50 ml. beakers in the bottom of the desiccator containing the seeds. The KOH solution was replaced with a fresh solution each day. The entrance of CO_2-free

air was permitted. The KOH solution was treated with an excess of barium hydroxide solution and the resultant precipitate collected by filtration. The $BaCO_3$ precipitate was dried in an oven to a constant weight and its radioactivity was determined in a gas-flow counter. Thus, both total CO_2 and $^{14}CO_2$ could be determined.

3) Sampling of seeds:

a) Left in Petri plates:

Both seeds and the plate were washed with cold distilled water (seed washings). The fresh weight of the washed seeds was determined and abnormal seeds were rejected (Figure 9, p. 61). The normal seeds were then dissected into seed coat, cotyledons (cots) and shoot-root (sr) axis with the help of forceps and scalpel, and the combined fresh weight of each part was determined. The seed coats were soaked for one week in concentrated hydrochloric acid (HCl) and then ground in a mortar. The HCl was evaporated in a flash evaporator and the residue was suspended in water. Samples were then removed for counting. Homogenates were prepared from the cotyledons and shoot-root axis as described below.

b) Transferred to vermiculite:

After shaking to remove adhered vermiculite, 3-day old seedlings were washed by dipping in 70% ethanol in a beaker. The normal seedlings were separated into seed coats,

cotyledons and shoot-root axes. The seed coats were discarded, and the cotyledons and the shoot-root axes were weighed and kept on ice until extracted with ethanol.

4) Preparation of extracts (each batch of sixty seeds was treated as a unit)

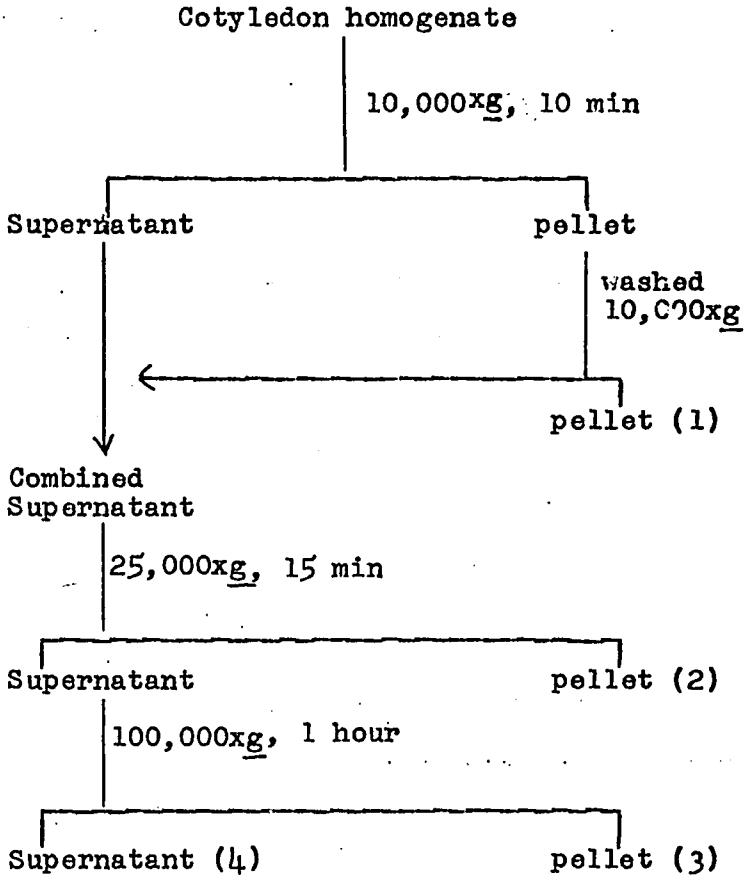
a) Extraction with water:

The cotyledons from each batch of sixty seeds were ground in a chilled Omnimix with cold distilled water (1 ml./seed) for 8 minutes, followed by homogenization in a chilled Thomas tissue grinder (A.H. Thomas Co., Philadelphia, U.S.A.) until a fine suspension was obtained. The shoot-root axes were chopped with a razor blade, ground in a chilled glass morter with distilled water, (4 ml./g. fresh weight) and homogenized in the grinder until a fairly smooth suspension was obtained. All operations were performed as close to $0^{o}C$ as possible.

The homogenates thus obtained were centrifuged at 10,000Xg for 10 minutes in an International Portable Refrigerated Centrifuge, Model PR -2 (Head 295 or 296), then at 25,000Xg for 15 minutes (Fig. 8). The 25,000Xg supernatants were then transferred to an International Refrigerated Ultra Centrifuge (Model B-20) and spun at 100,000Xg for one hour. Because of the small sample volume, centrifugation at 25,000Xg was omitted for the shoot-root axes homogenate. All the pellet fractions were suspended in small volumes of distilled water. The volumes of the sus-

FIGURE 8

Initial fractionation of cotyledon homogenate



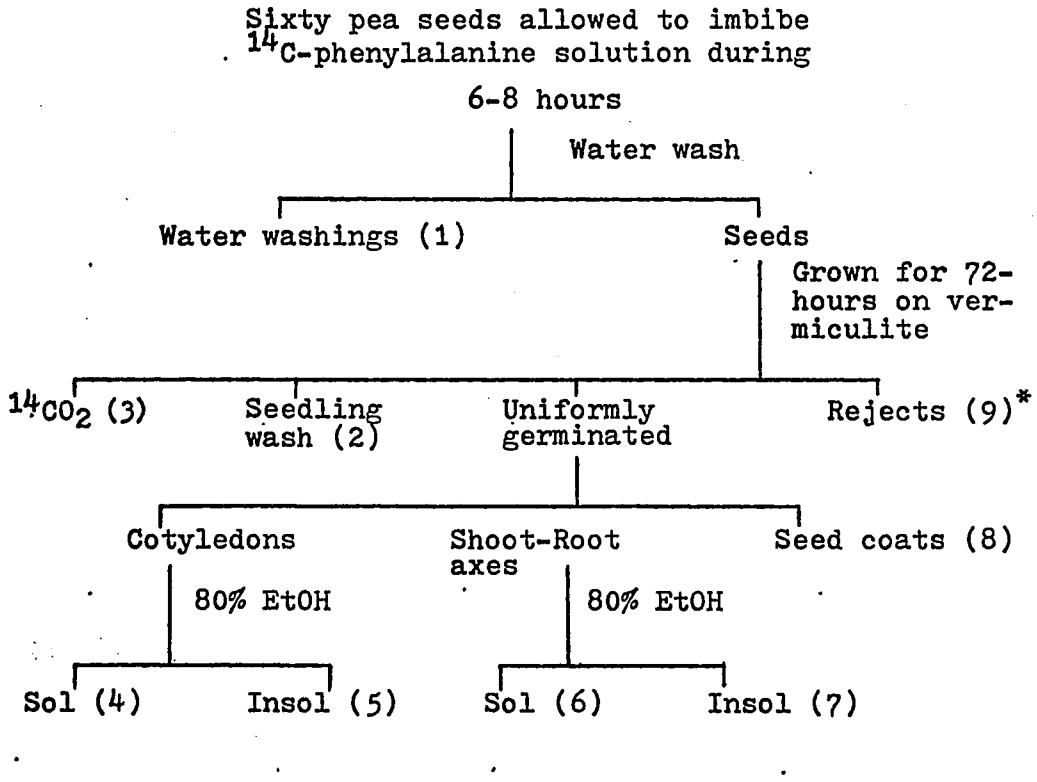
pensions were noted and samples were removed for counting.

b) Extraction with ethanol

The seeds were separated as shown in Figure 9. The cotyledons from each batch of sixty seeds were ground in an Omnimix at top speed for 2 minutes with 80% boiling ethanol (Larson & Beevers, 1965). The homogenate was decanted and the container was washed with more ethanol (about 1.5 ml. of ethanol/seedling). Shoot-root axes were ground in a glass mortar with 80% boiling ethanol (about 0.7 ml./shoot-root axis). Both homogenates were taken separately in round bottomed flasks and refluxed at about 100°C for 3 hours (1-^{14}C-Phe expt.). Refluxing was omitted in the experiment done with U-^{14}C-phenylalanine (Beevers & Guernsey, 1966). The extracts were filtered through Whatman No. 1 filter paper in a Büchner funnel under slight suction, and the residues were washed with hot ethanol. The washed residues were taken back into the flasks, refluxed again with more ethanol for 30 minutes and filtered in the same way. These ethanol washes were combined with the initial extracts. The final cotyledon and shoot-root axis extracts so obtained were filtered separately through a bed of acid washed Celite (2 X 2.5 cm.) under suction. The filtrates were evaporated to dryness in a flash evaporator at 38°C and the residues suspended in small volumes of distilled water. The extracts and the extracted residues were stored at -2°C until used.

FIGURE 9

INITIAL FRACTIONATION OF 72-HOUR OLD PEA SEEDLINGS FED ^{14}C-PHENYLALANINE DURING IMBIBITION



* Seeds and seedlings were rejected for the following reasons :

- failure to swell in size after 24 hours
- failure to germinate after three days
- obvious discoloration or obviously abnormal development of root or shoot. Differences in length were not regarded as abnormalities.

5) Extraction of protein

a) From water extracts:

Aliquots from 100,000X*g* supernatant fractions obtained from both the cotyledon and the shoot-root axis were made 5% with respect to trichloroacetic acid (TCA). The precipitate thus formed in each sample was collected by centrifugation (unwashed ppt.) and washed as described below.

b) From the Ethanol Extracted Residues:

The cotyledon residue was hydrolysed mildly with 1.4 *N* hydrochloric acid in a boiling water bath for an hour to remove starch (Pucher *et al.*, 1948). The HCl-insoluble cotyledon residue and the ethanol extracted shoot-root axis residue were extracted once with 0.1*N* NaOH (Larson & Beevers, 1965) (about 6 ml. per 100 mg. of air-dried residue) and twice with distilled water with thorough stirring.

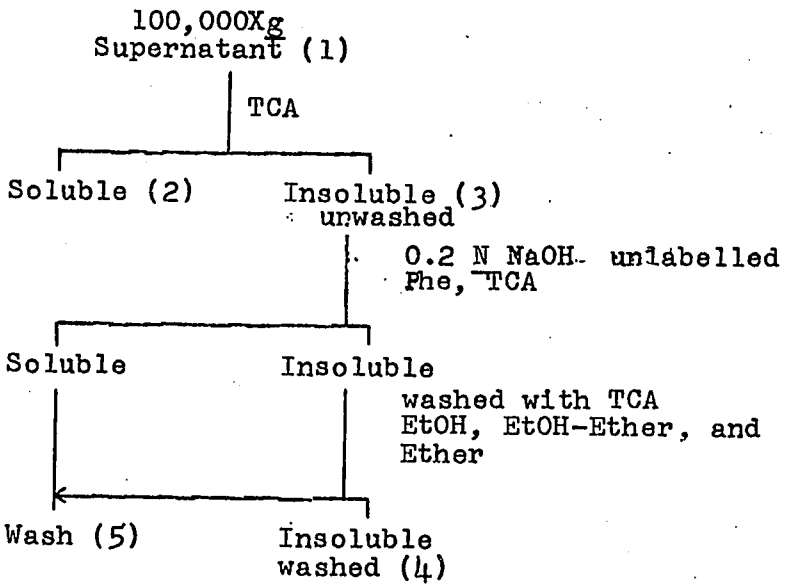
The NaOH-water extract thus obtained was neutralized with HCl, then treated with TCA following the procedure described for the water extracts.

c) Washing procedure: (Fig. 10) (modified after Siekevitz, 1952)

The TCA pellet was dissolved in 0.2 *N* NaOH containing an excess of unlabelled L-phenylalanine and reprecipitated with TCA. The precipitate was spun down and washed sequentially with cold 5% TCA solution, 80% ethanol, ethanol-ether (50:50, v/v) at 37°C for 30 minutes and finally with ether. (washed ppt.).

FIGURE 10

TCA PRECIPITATION OF THE 100,000Xg SUPERNATANT FRACTIONS OF COTYLEDON AND SR-AXIS



6) Hydrolysis of protein:

Washed TCA precipitates were hydrolysed with 6N redistilled HCl at 105°C for 20 hours in a glass tube sealed under vacuum (Fruton & Simmonds, 1953). The hydrolysates were centrifuged to remove the black humus and the supernatants were evaporated to dryness in a flash evaporator 2-3 times with the addition of water. The residues were finally dissolved in water or in 10% aqueous 2-propanol for analysis by paper chromatography.

For analysis by the Technicon amino acid analyser, dried samples (about 46 mg.) were dissolved in 2.0 ml. of 12.5 per cent sucrose solution and 0.1 ml. aliquots were applied onto the column.

7) Dialysis of the 100,000Xg supernatant (modified after Danielsson, 1951)

Twenty five ml. of the supernatant were poured into cellophane dialysis tubing (3/4 inch diameter) tied securely at one end to form a bag. The open end was then tied to leave as little air space as possible. The bag was left in 500 ml. of distilled water in a beaker at 4°C. The water in the beaker was changed at 3 hours and again at 6 hours. At 24 hours, the water was replaced by 500 ml. of DL-phenylalanine solution (12.5 mMolar) which was left for 9 hours, before it was replaced with water. Dialysis was carried out for another 41 hours with a change of water at 15 hours. After a total dialysis period of 74 hours, the bag was opened and the solution was centrifuged at 10,000Xg for 30 minutes in the International Refrigerated Centrifuge to remove the precipitated material.

8) Fractionation of dialysed samples through a DEAE-cellulose column :

The DEAE (diethylaminoethyl) cellulose column was prepared by the procedures described by Peterson & Sober, (1956, 1962) and Sober et al., (1956) with some modifications. Eleven grams of the cellulose were suspended in Tris (Hydroxymethyl aminomethane) buffer (0.01M, pH 7.8), and the non-sedimenting materials were decanted. The suspension was poured as a slurry into a glass column fitted at the bottom with a "coarse fritted" disc and allowed to settle under the flow conditions induced by gravity. The packed column was equilibrated with several volumes of Tris buffer (pH 7.8). The size of the prepared column was 2X37 cm.

The dialysed protein sample was washed into the column with several 1 ml. portions of the Tris buffer before continuous flow of buffer was begun. A NaCl gradient prepared as follows was employed for elution : two bottles were placed one above the other, the upper bottle containing 1.1M NaCl in Tris buffer (pH 7.8) and connected to the lower one, the mixing chamber, containing 300 ml. of Tris buffer only (pH 7.8). The lower bottle was connected to the column for continuous elution (modified after Varner & Schidlovsky, 1963).

Fractions of 10 ml. each were collected manually and their protein content determined by the optical density method of Keller & Block (1959). Samples were read at 280 mμ in a Beckman DU spectrophotometer. Standard curves were prepared using an aqueous solution of egg albumin.

Aliquots of eluate fractions (1 ml.) were mixed with 15 ml. of scintillation mixture for determination of radioactivity.

The adsorbants were regenerated by the procedure of Peterson & Sober (1956), and used for chromatography of additional samples.

9) Fractionation of the 10,000 Xg pellet of the shoot-root axis homogenates :

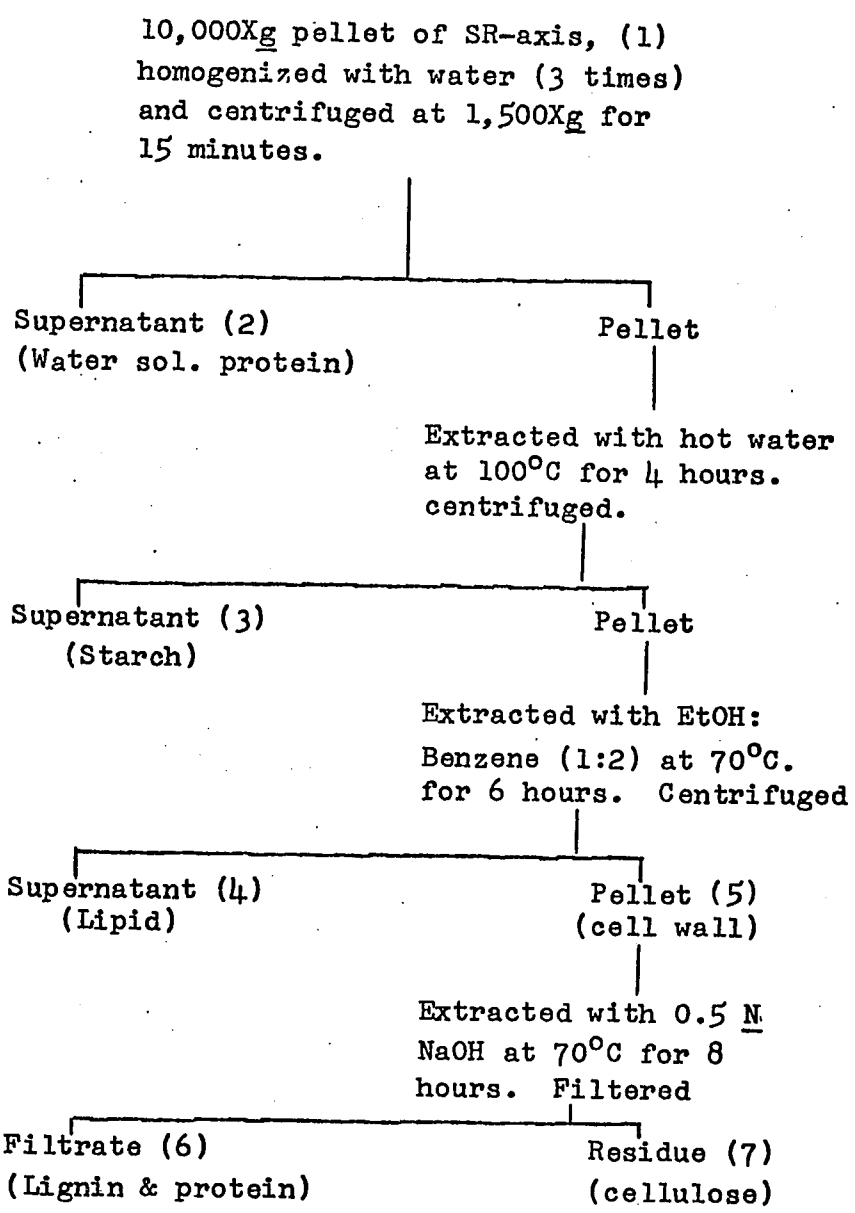
This was done to isolate a "cell wall" fraction following the scheme outlined in Figure 11.

10) Separation of amino acids on a Dowex column :

The procedures of Moore & Stein (1951) and of Thompson et al., (1959) were used with slight modifications. Dowex 50-X8 (50-100 mesh), as supplied in H^+ form was suspended in 4N HCl in a beaker, stirred well, and allowed to stand. When the resin had settled, the yellowish supernatant was decanted. After 3 washes with distilled water, the resin was resuspended in 2N NaOH. It was decanted and the resin

FIGURE 11

Fractionation of the 10,000Xg pellet obtained from a homogenate of the shoot-root axis of germinating pea seeds fed 1-^{14}C-phenylalanine (procedure modified after Bishop et al., 1958)



was resuspended in three times its volume of N NaOH and heated in a water bath for 3 hours. It was washed free of alkali with deionized distilled water and placed in a column following the usual procedure. The packed resin was then treated with six column volumes of 6N HCl. The HCl was removed with excess of deionized water until the effluent was free of chloride ions. The final column size was 1.1 X 16 cm. with about 0.5 ml. of water left at the top of the column. The sample was applied slowly with a pipette onto the column. The effluent was collected and the column washed with about 25 ml. of deionized distilled water followed by elution with 1.5N NH_4OH. The eluent was evaporated to dryness *in vacuo* and was taken in a small volume of water for further analysis.

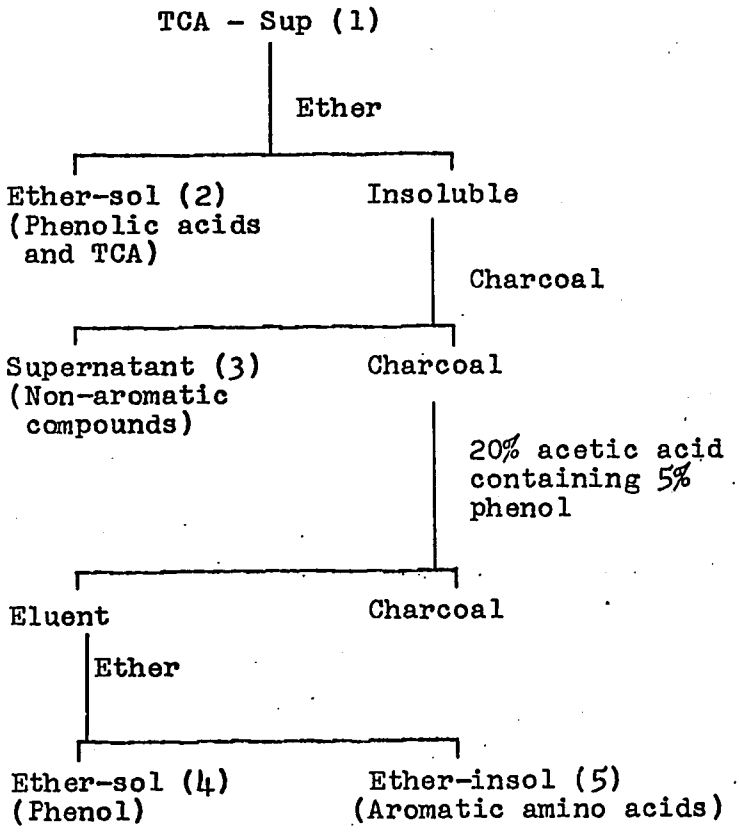
11) Isolation of aromatic compounds :

The charcoal adsorption methods of Partridge (1949) and Watkin *et al.*, (1957) were used in attempts to isolate aromatic compounds. In the former method, about 2 grams of acid washed charcoal (Norit F.Q.P.) obtained from British Drug House (Canada) Limited was added to 10 ml. of sample containing the aromatic compounds. Elution and other subsequent steps were similar to those described by Partridge (1949) and are shown in Figure 12.

In the latter method, the sample was put on a small column (2 X 1 cm.) of acid washed charcoal and Celite

FIGURE 12

ISOLATION OF AROMATIC COMPOUNDS FROM THE TCA SUPERNATANTS



(50:50, w/w) prepared in a filtering assembly and used with slight suction. The column was washed thoroughly with distilled water followed by elution of the adsorbed aromatic compounds with 60-70% ethanol.

The eluted sample obtained with both procedures was evaporated to dryness under reduced pressure. The residue was dissolved in a small volume of water, assayed for radioactivity and then chromatographed on paper.

12) Isolation of Phenolic compounds:

The fractions containing these compounds were acidified to pH 3.0-3.5 and thoroughly extracted with peroxide free ether by shaking in a separatory funnel (Bardinskaya & Shubert, 1962). The process was repeated 3 times and the combined ether extracts were dried in a current of air. The residue containing the phenolic acids was dissolved in a small volume of 70% ethanol; aliquots were removed for counting and its components were separated by paper chromatography.

13) N-terminal Analysis of protein:

The N-terminal amino acid residues of the TCA precipitates were isolated following the method of Fraenkel-Conrat et al., (1955). To 5.0 mg. of washed, dried TCA precipitate were added equal amounts of sodium bicarbonate ($NaHCO_3$), 1.0 ml. of water and 0.4 ml. of a 5% ethanolic solution of fluorodinitro benzene (FDNB). The resultant suspension was shaken

for two and a half hours. It was then acidified with 4 drops of conc. HCl and shaken three times with 15 ml. of ether each time to extract the excess FDNB. The aqueous phase was hydrolyzed by refluxing with 10 ml. of 6N HCl for 16 hours and diluted with 50 ml. of distilled water. The DNP-amino acids were extracted with three 15 ml. volumes of ether. The combined ether layers were evaporated to dryness and redissolved in 2.0 ml. of acetone. The aqueous fraction was shaken with 25 ml. of ethyl acetate to remove the peptides, if any, and the extracted residue, containing the non-N-terminal amino acids, was dried and taken in 2.0 ml. of acetone.

14) Ultra-Violet Absorption Spectrum :

The volume of samples eluted from paper chromatograms was usually adjusted to 3.0 ml. Each sample was scanned in a Perkin-Elmer spectrophotometer (Model 202) against a paper blank eluted from an equivalent size and area of the same chromatogram. The nature of the eluted sample and its concentration was determined by comparing the resultant spectrum with those of known compounds.

15) Infrared Spectrum :

Infrared spectra of samples were taken occasionally in a Beckman Infrared spectrophotometer (Model IR-8). Chloroform soluble samples were scanned against a blank cell

containing chloroform. Chloroform insoluble samples were ground with Nujol in a small mortar and pestle. The paste so obtained was applied as a thin film between two NaCl blocks before the spectrum was taken.

RESULTS

A. I. Distribution of Radioactivity in various fractions and the effect of Germination time when 1-^{14}C-phenylalanine was used:

1. In Carbon dioxide:

The results presented in Table 3 show that less than 1% of the initially added radioactivity appeared in the respiratory carbon dioxide. The production of CO_2 increased from 1.3 µmole/ seed/ hour over a period of 0 to 24 hours to 4.48 µmoles for the period of 73 to 96 hours. Production of $^{14}CO_2$, on the other hand, decreased from 2.96×10^{-5} to 0.53×10^{-5} µmole/ seed/ hour over the same length of time. Less than 0.002 per cent of the total CO_2 appeared to be labelled.

2. In the various seed parts:

The data presented in Table 4 indicate that less than 1% of the radioactivity initially provided was recovered from the seed washing and less than 2% from the seed coats. Radioactivity was primarily concentrated in the cotyledons which contained over 93 per cent of the initial amount at 24 hours and still contained 59.7 per cent at 96 hours. In the shoot-root axes, on the other hand, radioactivity gradually increased during the four day period, from 4 per cent at 24 hours

TABLE 3

CHANGES IN THE OUTPUT OF CO_2 & $^{14}CO_2$ WITH TIME

Incubation periods	CO_2 given off per seed/hour*	$^{14}CO_2$ given off per seed/hour *	$^{14}CO_2$	
			% of total CO_2	% Initial Activity
Hours	µmoles	µmoles X10 ^{5}		
0 - 24	1.30	2.96	0.0023	0.4
25 - 48	1.52	1.19	0.0008	0.2
49 - 72	2.39	0.90	0.0004	0.1
73 - 96	4.48	0.53	0.0001	0.1

Four lots of 60 seeds were germinated in Petri plates in the presence of 1-^{14}C-phenylalanine in a sealed container. CO_2 was collected in KOH and converted to $BaCO_3$. One lot of seeds was removed daily and at the same time the KOH solution was replaced with fresh. $^{14}CO_2$ was calculated from the counts present in the $BaCO_3$ precipitates and the specific activity (c.p.m./µmole) of the supplied phenylalanine.

*These values are based on carbon dioxide absorbed in the KOH.

TABLE 4

DISTRIBUTION OF RADIOACTIVITY IN GERMINATING PEA SEEDS FED 1-^{14}C-PHENYLALANINE DURING IMBIBITION

HOURS GERM.	RADIOACTIVITY (% INITIAL)					RECOVERY OF ACTIVITY (% INITIAL)
	WASH	COTS.	S.R AXES	SEED COATS	REJECTS	
24	0.5	93.4	4.0	0.7	NONE	99
48	0.7	87.7	10.3	1.3	7.1	107
72	0.5	75.0	19.2	1.2	10.0	106
96	0.9	59.7	20.9	1.6	9.0	92

Four lots of 60 seeds were germinated in Petri plates containing 11.0 ml. of water and 0.17 mg. L-phenylalanine (about 260,000 c.p.m.). One plate was removed each day for analysis. Each value is based on the average count obtained on triplicate samples, none differing more than 4 per cent from the mean.

to 20.9% at 96 hours. During this same period, the dry weights of the SR-axes of the 60 seeds increased from 60 mg to 498 mg. The figures for cotyledon and shoot-root given in Table 4 have not been corrected for the seeds rejected (Fig. 9, p. 61) and therefore slightly underestimate the actual relative distribution in these parts. All 60 seeds of the 24 hour sample were used, but 7, 6 and 8 out of 60 seeds from the 48, 72 and 96 hour samples respectively were rejected. The radioactivity present in these rejected seeds varied from 7.1% to 10% of the amount initially provided. The overall recovery of radioactivity for the 24, 48 and 72 hour batches was 99% or better and for the 96 hour batch was 92% of the amount supplied to the seeds.

3. <u>In fractions obtained by differential centrifugation of cotyledon and shoot-root axis homogenates</u> :

The cotyledon and shoot-root axis homogenates were subjected to differential centrifugation as detailed in Methods (pp. 58-69) and the radioactivity of each fraction was determined. The results are presented in Table 5 as percent of supplied radioactivity with a correction made for the rejected seeds so that the figures are directly comparable from one time period to another. This correction was made on the assumption (not verified) that each seed absorbed an equal amount of radioactivity. Thus, if 6 seeds were rejected, the value for "initial radioactivity" was reduced by 10 percent.

Differential centrifugation of the cotyledon homogenates yielded four fractions (Fig. 8). The 10,000 Xg pellet, composed mainly of unbroken cells, miscellaneous cellular debris and starch granules, had 10 to 20 per cent of the initially added radioactivity with the lowest specific activity (c.p.m./mg.) of any fraction obtained. It was separable into two layers; the upper third containing the green pigments, the lower two-thirds containing mainly the white starch grains. The 25,000 Xg pellet (presumably mitochondrial) and the 100,000 Xg pellet (presumably microsomal) fractions each had less than 5 per cent of the initially added radioactivity. The specific activities of these two fractions were 5 times higher than that of the 10,000 Xg pellet. The 100,000 Xg supernatant fractions contained most of the initially provided radioactivity with a maximum of 76.1 per cent at 24 hours and a minimum of 44.8 per cent by 96 hours. The specific activity of this fraction was initially high (107 counts/min./mg. at 24 hours) but decreased gradually with the germination time.

The shoot-root axis homogenates were divided into only three fractions : The 10,000 Xg pellet, mainly comprised of unbroken cells and cellular debris, contained 1.4 per cent of the radioactivity at 24 hours and 10.7 per cent at 96 hours. Its specific activity (c.p.m./mg.)

TABLE 5

DISTRIBUTION OF RADIOACTIVITY FOLLOWING DIFFERENTIAL CENTRIFUGATION OF EXTRACTS OF PEA SEEDLINGS FED 1-^{14}C-PHENYLALANINE

HOURS GERM.	RADIOACTIVITY (% INITIAL)				SPECIFIC ACTIVITY (c.p.m./mg. dry weight)			
	24	48	72	96	24	48	72	96
FRACTIONS								
COTYLEDONS								
ENTIRE HOMOGENATE	93.4	99.2	82.8	68.4	33	34	32	29
10,000Xg PELLET	10.1	20.0	15.4	16.7	7	17	17	15
25,000Xg PELLET	4.9	5.1	4.6	3.1	36	66	64	88
100,000Xg PELLET	2.1	4.9	3.8	3.4	65	80	80	87
100,000Xg SUP	76.1	65.8	49.7	44.8	107	87	65	57
% RECOVERY	99.8	96.9	88.7	99.4				
SHOOT-ROOT AXES								
ENTIRE HOMOGENATE	4.0	11.6	21.3	24.1	62	143	139	142
10,000Xg PELLET	1.4	4.0	12.7	10.7	63	137	136	151
100,000Xg PELLET	<0.1	0.7	<0.1	0.2	150	326	480	455
100,000Xg SUP	1.3	5.2	4.3	6.4	72	141	67	82
% RECOVERY	70.0	85.4	80.3	71.8				

Each value is based on the average count obtained on triplicate samples taken from each fraction. The standard deviation of the mean ranged from 1 to 7 per cent.

increased from 63 to 151 in the same period. The 100,000 x g pellet contained less than 1 per cent of the radioactivity, but the specific activity of this fraction, presumably microsomal, increased from 150 counts at 24 hours to 480 at 72 hours and was the highest of any obtained. The 100,000 x g supernatant contained 1.3 per cent of the radioactivity at 24 hours and 6.4 per cent by 96 hours. Its specific activity was generally the lowest of the three factions.

The overall recovery of radioactivity from the cotyledon fractions was 89 per cent or better but from the shoot-root axis fractions was only 70 to 85 per cent.

4. In various free and bound compounds:

a) Trichloroacetic acid (TCA) treatment of 100,000 x g supernatants:

Aliquots from the 100,000 x g supernatants of both the cotyledon and the shoot-root axis homogenates were fractionated with TCA as a preliminary step in the isolation of a protein fraction. The results of this fractionation calculated as per cent of activity initially supplied are shown in Table 6. It can be seen that the activity in the TCA soluble fractions parallels that of the starting material for the cotyledon, declining from a high of 51.4 per cent at 24 hours to a low of

TABLE 6

DISTRIBUTION OF RADIOACTIVITY FOLLOWING TCA PRECIPITATION OF THE 100,000Xg SUPERNATANT FRACTIONS OF PEA SEEDLINGS FED 1-^{14}C-PHENYLALANINE

HOURS GERM.	RADIOACTIVITY (% INITIAL)					RECOVERY (% 100,000Xg SUPERNATANT)	
	100,000Xg SUP	TCA FRACTIONS					
		SOL	INSOL UNWASHED	INSOL WASHED	INSOL WASH	BEFORE WASH	AFTER WASH
				COTYLEDONS			
24	76.1	51.4	16.7	12.1	2.8	89	87
48	65.8	25.5	32.6	22.2	0.9	88	74
72	49.7	12.8	34.2	20.5	1.0	94	69
96	44.8	10.9	29.7	19.6	2.6	90	74
				SHOOT-ROOT AXES			
24	1.3	0.3	0.8	0.4	NOT DETERMINED	85	-
48	5.2	1.5	2.4	1.2	"	75	-
72	4.3	2.5	1.5	0.5	"	93	-
96	6.4	3.9	2.2	1.0	"	95	-

Each value is based on average counts obtained on triplicate samples. The standard deviation of the mean ranged from 1 to 4 per cent.

only 11 per cent by 96 hours. Radioactivity in the unwashed TCA precipitate doubled from 16.7 per cent at 24 hours to 32.6 per cent by 48 hours after which it remained more or less constant. Recovery of radioactivity at this stage was about 90 per cent; but, except for the 24 hour sample, was reduced to about 70 per cent upon washing the TCA insoluble precipitate. Less than 3 per cent could be recovered from the combined washings.

In the shoot-root axis fractions, on the other hand, both the TCA soluble and the TCA insoluble activity increased with time, the former from 0.3 per cent to 3.9 per cent by 96 hours and the latter from 0.8 per cent at 24 hours to 2.2 per cent at 96 hours. Recovery of radioactivity at this stage was 75 per cent or better, but a further loss occurred when the TCA percipitate was washed.

b) Attempts to isolate a protein fraction of high specific activity from the 100,000 Xg cotyledon supernatant :

TCA separation (Table 6) indicated that the 100,000 Xg supernatant contained about 20 per cent of the supplied activity in a TCA insoluble (protein) fraction after 24 hours. These proteins were presumably albumins including some enzymes and could have been synthesized de novo (Young and Varner, 1957, 1959). Separation of these proteins might yield a fraction of high specific activity thus indicating incorporation of the supplied phenylalanine into a newly synthesized protein.

Separate aliquots of the 100,000 x g supernatant were subjected to DEAE cellulose column chromatography, after a preliminary dialysis. The results of dialysis on the radioactivity of the non-dialysable fraction are shown in Table 7. The slight precipitate which formed during dialysis had very little activity, less than 3 per cent, and is not included in this table. The 24 hour sample retained relatively little; 18.4 per cent. The sample from 48 hours, on the other hand retained about 50 per cent and those from 72 and 96 hours about 70 per cent. The specific activity of the dialysed fractions generally increased from 60 at 24 hours to 136 by 96 hours, representing an increase over the initial undialysed fraction (Table 5). As might be expected the radioactivity of the dialysed samples was very similar to that of the TCA precipitates (Table 7, last column).

The clear supernatant fractions thus obtained from the 24 and 96 hour samples were fractionated through a DEAE cellulose column and the results are presented in Figure 13. The total protein contents of both the samples were about the same (about 230 mg.). Under the conditions of separation, both the 24 and 96 hour samples yielded similar protein profiles, each showing 4 peaks (I, II, III and IV). Peaks I, II and III emerging in tubes 10, 16 and 20 respectively were poorly separated. Peak III was dominant with an O.D. of 1.06, II was only slightly lower, but I was very low. Peak IV which emerged

TABLE 7

DIALYSIS OF 100,000X*g* COTYLEDON SUPERNATANTS OBTAINED FROM SEEDLINGS HARVESTED AT FOUR DIFFERENT TIME INTERVALS

Hours of germ.	Radioactivity in 100,000X*g* supernatant		Radioactivity in dialysed sample			% activity* in unwashed TCA ppt.
	c.p.m.	%	c.p.m.	%	c.p.m./mg.	
24	59,000	100	10,830	18.4	60	22.0
48	51,900	100	26,340	50.8	118	49.5
72	38,700	100	25,853	66.8	110	68.9
96	38,500	100	27,918	72.5	136	66.4

Each value is based on the average count obtained with duplicate samples. The variation between the duplicate samples was less than ± 5 per cent of the mean.

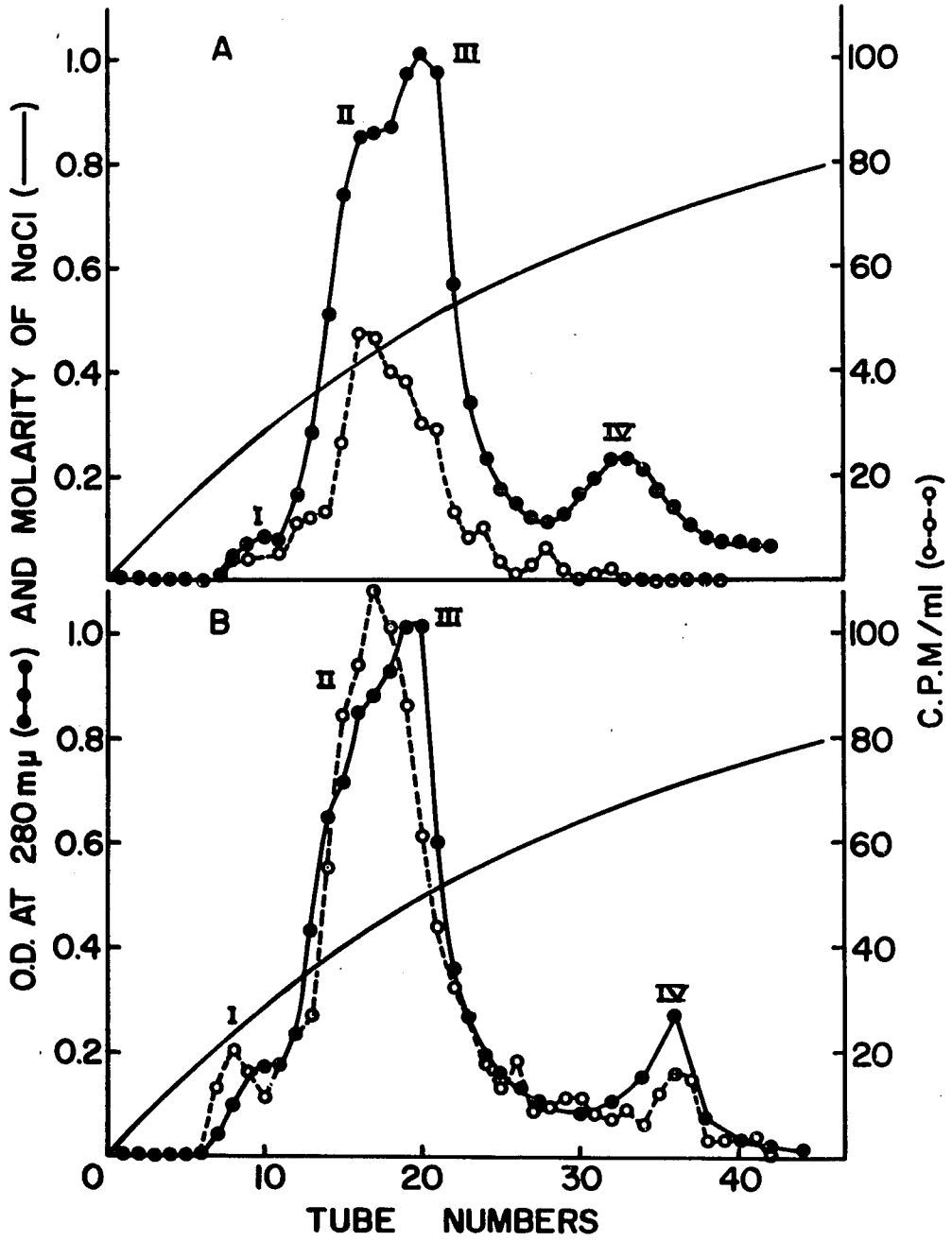
*Data taken from Table 6, but expressed as % of radioactivity present in the 100,000X*g* cotyledon supernatant for direct comparison with the dialysis results.

FIGURE 13

DEAE-cellulose column chromatography of the 100,000Xg cotyledon supernatant following dialysis. Experimental procedure and assay method are described in the text.

A. Sample obtained from 24 hour seedlings.

B. Sample obtained from 96 hour seedlings.


A
B
I
II
III
IV
1.0
0.8
0.6
0.4
0.2
0
100
80
60
40
20
4.0
10
20
30
40
O.D. AT 280mμ (●—●) AND MOLARITY OF NaCl (——)
C.P.M/ml (o-o-o)
TUBE NUMBERS

in the vicinity of tube 35 was small but well separated from the other peaks (O.D. at 280 mμ: 0.25). The pattern of radioactivity was similar for the two fractions except for the lower levels of the 24 hour sample. Both almost paralleled that of the protein except for the reversal in height of peaks II and III. This would indicate a selective incorporation but the separation was too poor to isolate this fraction.

c) Fractionation of the 10,000 x g pellet of the shoot-root axis: (see Figure 11)

The purpose of this fractionation (ref. p. 66) was to isolate a cell wall fraction and to determine its radioactivity. The results are presented in Table 8. All the fractions generally showed an increase in radioactivity with germination time with the exception of the soluble protein fraction. The radioactivity in the washed cell wall fraction increased from 26.3 per cent of the radioactivity present in 10,000 Xg shoot-root axis fraction at 24 hours to 58.3 per cent by 96 hours. The specific activity of this fraction increased much more than any other, from 30 at 24 hours to 186 by 96 hours. Sodium hydroxide treatment however, resulted in a substantial loss of radioactivity, and in all cases, there was very little if any improvement of specific activity as compared to the initial sample.

II. Distribution of Radioactivity upon Ethanol Extraction

To supplement the aqueous preparations of the time-course experiment, two additional batches of 3-day old seed-

TABLE 8

THE DISTRIBUTION OF RADIOACTIVITY IN THE VARIOUS FRACTIONS OF 10,000Xg PELLETS OF SHOOT-ROOT AXES OBTAINED AT FOUR DIFFERENT TIME INTERVALS FROM GERMINATING PEA SEEDS FED 1-^{14}C-PHENYLALANINE

Hours of germination	Radioactivity (% 10,000 Xg pellet)				Specific activity (c.p.m./mg. dry wt.)			
	24	48	72	96	24	48	72	96
FRACTIONS								
1) 10,000Xg pellet **	100.0	100.0	100.0	100.0	63.0	137.0	136.0	151.0
2) Soluble protein	22.3	15.9	14.9	8.5	38.0	54.0	59.2	65.0
3) Starch	4.6	5.0	4.0	4.3	9.1	21.0	25.2	51.0
4) Lipid	< 0.1	0.2	0.7	1.3	-	-	25.9	18.0
5) Cell walls	26.3	19.2	56.7	58.3	29.8	44.0	134.0	186.0
6) Ligno-protein	4.97	7.1	8.4	-*	6.4	16.0	33.5	-*
7) Cellulose	< 0.1	2.5	5.5	-*	-	29.0	48.0	-*

Each value is based on the average count obtained on duplicate samples. The variation between the duplicates was less than ± 5 per cent of the mean.

* Samples treated differently

**Data for the specific activity repeated here from Table 5

lings were extracted with ethanol. Table 9 shows that ethanol soluble fractions from the cotyledon and the shoot-root axis contained 10.3-10.6 and 1.8-3.1 per cent respectively of the radioactivity initially provided. The insoluble fractions had 43.5 and 6.1 per cent in the cotyledon and shoot-root axis respectively. The combined radioactivity present in all the fractions accounted for about 67 per cent (Expt 2) of the amount initially provided to the seeds. The activity associated with $^{14}CO_2$ and the seed coat was not measured, since results from the time course experiment showed very little activity in these two fractions (Tables 3, 4).

The partitioning of radioactivity between the ethanol soluble and insoluble fractions is presumably approximate to that between the 100,000Xg supernatant TCA soluble and the 100,000Xg supernatant washed TCA insoluble plus the three pellet fractions (Tables 5 and 6). Support for this assumption is given in Table 10 which shows that the amount of radioactivity in the two preparations was very similar, the only exception being the shoot-root axis insoluble fractions.

B. The Nature of the Radioactivity when 1-^{14}C-Phe was fed:

Concurrently with establishing the distribution pattern of the label, the nature of the radioactivity was also investigated, first by physical separation of the fractions into general classes of compounds, followed by paper chromatographic separations.

TABLE 9

DISTRIBUTION OF RADIOACTIVITY IN THE FRACTIONS OBTAINED BY ETHANOL EXTRACTION OF 60 72-HOUR PEA SEEDLINGS FED 1-^{14}C-PHENYLALANINE DURING IMBIBITION

Fractions	% Initial Radioactivity	
	Expt. 1	Expt. 2
1) Seed wash	3.3	3.0
2) Seedling wash	0.4	0.4
3) Carbon dioxide	N.D.	N.D.
4) Cot-EtOH-soluble	10.3	10.6
5) Cot-EtOH-insoluble	*24.9	43.5
6) SR-EtOH-soluble	3.1	1.8
7) SR-EtOH-insoluble	6.1	N.D.
8) Seed Coat	N.D.	N.D.
9) Rejects (number of)	(4)	(13)
Recovery	~49%	~67%

Each value is based on the average count obtained on triplicate samples of each fraction. Radioactivity initially supplied: 260,000 c.p.m.; results calculated for 60 seeds to make them directly comparable from one experiment to another. N.D. = not determined.

*Not determined until several fractionation steps were followed.

TABLE 10

DISTRIBUTION OF RADIOACTIVITY IN COMPARABLE FRACTIONS OBTAINED FROM 72-HOUR PEA SEEDLINGS BY EXTRACTION WITH WATER OR ETHANOL

	100,000Xg ≡ TCA SOL	ETHANOL SOL	COMBINED PELLETS & TCA INSOL (WASHED) ≡	ETHANOL INSOL
COTYLEDONS	12.8	10.3 10.6	44.3	43.5
SHOOT-ROOT AXES	2.5	3.1 1.8	13.3	6.1

1. General classes of substances:

a) Separation of aromatic substances by charcoal treatment:

The TCA supernatant fractions supposedly contained free amino acids along with other water soluble non-protein compounds. These fractions also contained fairly large amounts of radioactivity (Table 6). The intention behind the charcoal treatment, as has been outlined in Figure 12, was to isolate by adsorption onto charcoal any unchanged ^{14}C-phenylalanine along with any other aromatic compounds. Results (Table 11) indicate that the ether extraction step to remove TCA prior to charcoal treatment also extracted a small amount of radioactivity (2.2 per cent at 24 hours and 5.4 per cent at 96 hours). Less than 1 per cent of the activity was present in the charcoal non-adsorbable fractions at 24-72 hours but this increased to 10 per cent at 96 hours.

The overall recovery of activity from the various fractions accounted for 70 to 75 per cent of the activity initially present in the TCA supernatants. The remaining 25 to 30 per cent probably remained adsorbed onto the activated charcoal. (A test run with a solution of labelled phenylalanine showed that only 70 per cent recovery of activity could be expected under the conditions used).

b) Separation of free amino acids:

A few attempts were made to separate the free amino acids from other components (mainly organic acids and

TABLE 11

DISTRIBUTION OF RADIOACTIVITY IN THE VARIOUS FRACTIONS OBTAINED FROM THE COTYLEDON TCA - SUPERNATANTS FOLLOWING CHARCOAL TREATMENT

Hours of germ.	Total activity in TCA-sup(1)		Radioactivity (% TCA - sup)				Recovery (% TCA - sup.)
			Ether sol.(2)	Charcoal			
	c.p.m.	%		Non-adsorbable(3)	Adsorbable		
					Ether sol.(4)	Ether insol.(5)	
24	28,471	100	2.2	0.2	0.4	67.8	70.6
48	19,550	100	3.1	0.4	0.4	71.3	75.2
72	6,632	100	5.5	0.8	0.0	63.7	70.0
96	5,956	100	5.4	10.4	0.0	56.7	72.5

Each value is based on the count obtained on duplicate samples. The variation between the samples was within ± 4 per cent of the mean.

sugars) present in the ethanol extract, by using a Dowex-50 column (pp. 66 & 68). Radioactivity was found to accompany only the amino acid fraction. Such separation resulted in up to a 40 per cent loss of radioactivity, although the specific activity was improved 2-3 times.

c) Protein bound labelled amino acids:

The washed TCA precipitates were routinely hydrolysed by following the procedure described in methods (p. 64). The acid hydrolysis step resulted in a 30-40 per cent loss of radioactivity. There was always some formation of "humus" after hydrolysis, which contained very little or no radioactivity.

The results of N-terminal analysis of the washed TCA precipitates obtained from the 100,000 x g supernatants of the shoot-root axis homogenates show that almost all the radioactivity was in the internal amino acid fraction (Table 12). Very little or no radioactivity was detected in the ether soluble or in the N-terminal amino acid fractions. The recovery of radioactivity with such a test appeared to be only 60 per cent or slightly better.

d) Phenolic acids:

Ether extraction of the acidified cotyledon soluble and cotyledon insoluble fractions, following mild acid or alkaline hydrolysis, showed that very little activity was associated with the ether soluble (phenolic acid) fraction. Only 1.7 per cent of the initially supplied radioactivity was recovered from the cotyledon soluble and 0.4 per cent from the cotyledon insoluble fractions. Shoot-root axis fractions were not subjected to ether extraction.

TABLE 12

RESULTS OF N-TERMINAL ANALYSIS OF WASHED TCA-PRECIPITATE OBTAINED FROM SHOOT-ROOT AXIS

Hours of germ.	Radioactivity (c.p.m.) in entire sample				Recovery (% of washed TCA-precipitate
	Washed TCA-precipitate	Ether sol.*	N-term. a.a.	Non-N-term. amino acids	
24	879	6	0	525	60.4
48	2,532	8	8	1,567	62.5
72	1,149	17	0	757	67.4
96	2,441	42	27	1,425	61.2

Each value is based on the average counts obtained on duplicate samples.

*Ether extraction before hydrolysis to remove excess FDNB.

2. Attempts to identify the kinds of labelled molecules in each general class:

The paper chromatographic method was basically used in attempting to identify the various labelled metabolites. The technique used was to chromatograph in one solvent system, usually BAW, determine radioactive areas by autoradiography, elute these areas and then rechromatograph in a different solvent system.

In the course of paper chromatography of the various sample fractions, it soon became evident that the recovery of radioactivity was usually low. As shown in Figure 14, about 60 to 70 per cent of the spotted radioactivity from the cotyledon soluble and insoluble (protein hydrolysate) fractions were lost during the first two elutions. The loss was even greater for the shoot-root axis soluble fraction, although the recovery from the shoot-root axis insoluble fraction after 3 elutions was about 70 per cent. However, a considerable improvement in specific activity (c.p.m./mg.) was achieved in all the cases.

a) Chromatography of the aromatic fraction:

Chromatography and autoradiography of ether extracted TCA soluble, charcoal adsorbable fractions (Table 11) obtained at four different time intervals, indicated that the free phenylalanine did not remain as such for long. The 24, 48, 72 and 96 hour samples showed a gradual disappearance with time of the activity in the phenylalanine

FIGURE 14

Recovery of radioactivity and changes in specific activity (counts per minute/mg.) upon three successive chromatographic runs. The specific activity of material eluted from the phenylalanine area only was determined before rechromatography. Each graph represents the results obtained on a representative sample. No attempt has been made to establish the levels of confidence.

Three successive solvents were used as follows:

Cot. : BAW, BEW, and water

SR : BAW, BEW, and But./2N NH_3

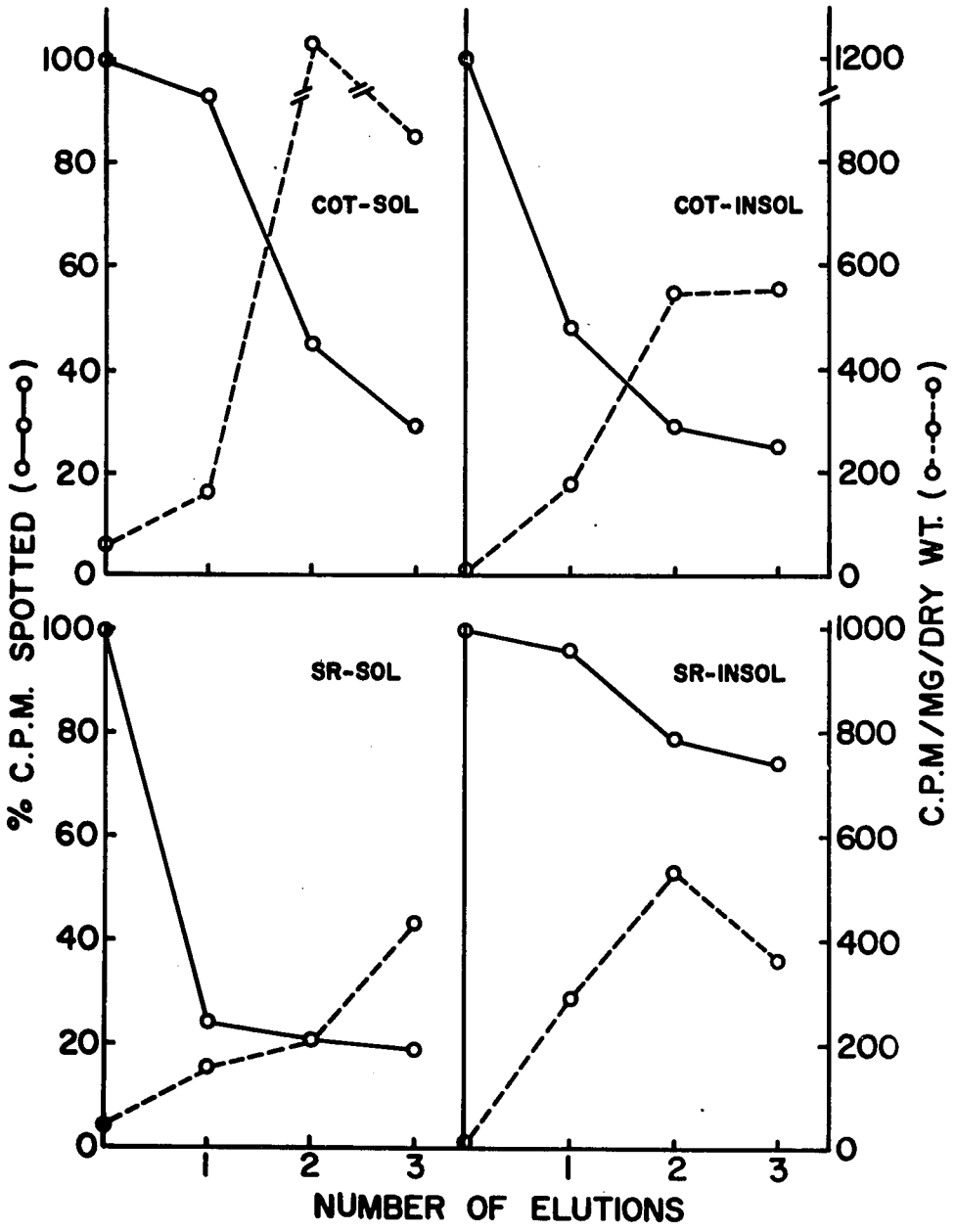
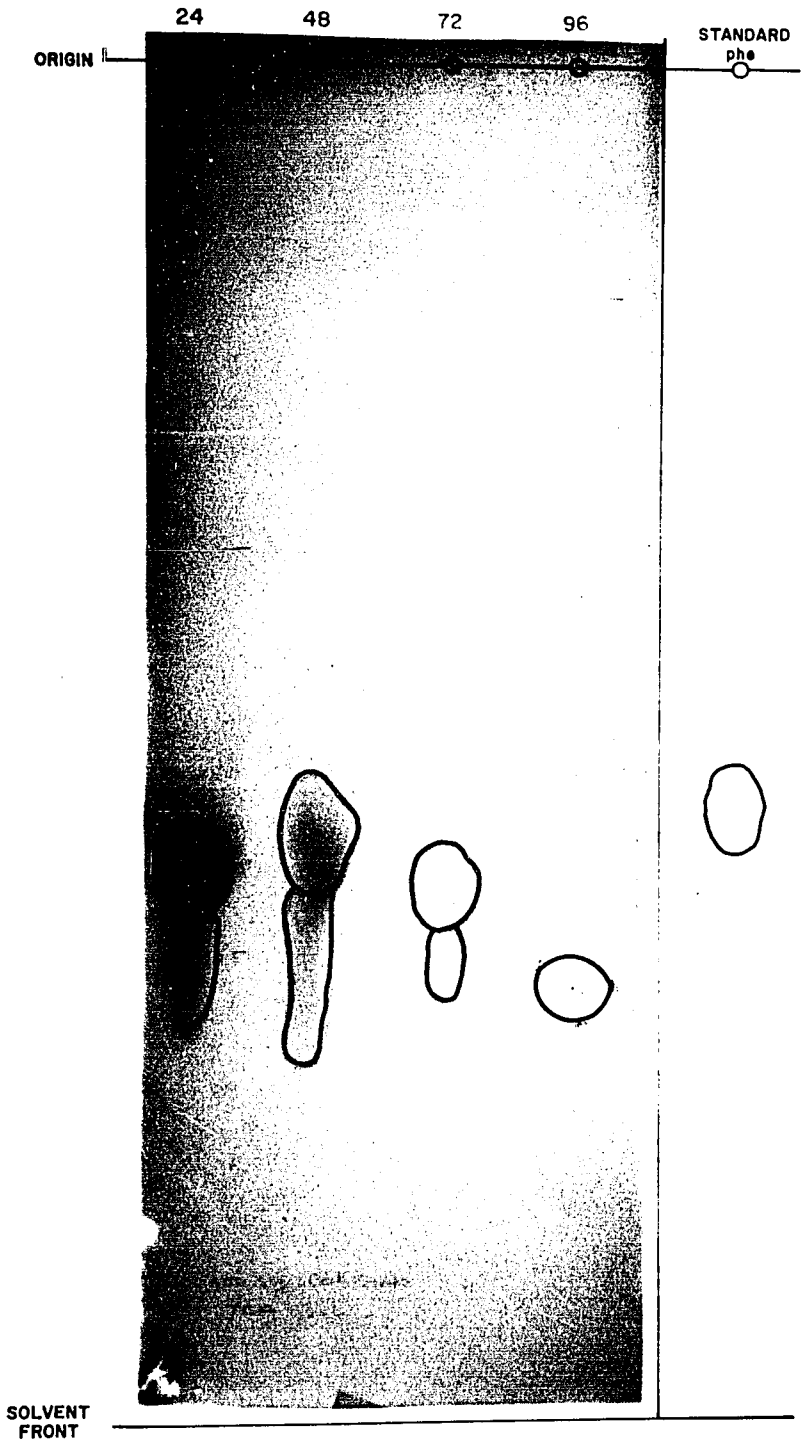

COT-SOL
COT-INSOL
SR-SOL
SR-INSOL
% C.P.M. SPOTTED (o—o—o)
C.P.M/MG/DRY WT. (o--o--o)
NUMBER OF ELUTIONS
0
20
40
60
80
100
200
400
600
800
1000
1200
1
2
3

FIGURE 15

Autoradiograph of chromatogram of the TCA-soluble fractions (ether insoluble, charcoal adsorbable) of the 100,000Xg cotyledon supernatants obtained at 4 different time intervals.

Developed once in BAW (4:1:5) solvent.

Paper used:	Whatman No 3 MM
Approx. c.p.m. per spot:	275
Exposure period:	7 weeks


24
48
72
96
ORIGIN
STANDARD
phe
SOLVENT
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area (Rf 0.55) and its gradual appearance at Rf 0.65 (Figure 15). Ether extracted cotyledon soluble fractions showed radioactivity in elongated spots covering the area of both phenylalanine (0.55) and "glycosides" (0.65), while the activity in the ether extracts ran in the free phenolic acid region near the solvent front (Rf 0.90). The typical spots obtained are shown in Fig. 16 (1) which is a radio-autograph of the cotyledon soluble fraction before ether extraction.

b) Chromatography of protein fractions following hydrolysis:

i) Soluble protein: Hydrolysates of the washed TCA precipitates obtained from both the cotyledon and the shoot-root axis usually had one radioactive spot in the vicinity of the phenylalanine area, but very rarely an additional minor spot was noted near the solvent front. Figure 16 (2,3) is typical of the results obtained.

ii) Insoluble protein: The hydrolysate of the ethanol, NaOH insoluble cotyledon fraction showed only one radioactive spot in the phenylalanine area, whereas that of the shoot-root axis had its radioactivity also in the area of free phenolic acids.

c) Chromatography of the phenolic acid fraction:

Chromatography of the substances extracted with ether from the ethanol soluble fraction following mild acid or alkaline hydrolysis, showed radioactivity only in the free phenolic acid region. (The known phenolic acids tested are listed in Table 15).

FIGURE 16

Autoradiograph of chromatograms of the cotyledon soluble and cotyledon insoluble washed precipitate.

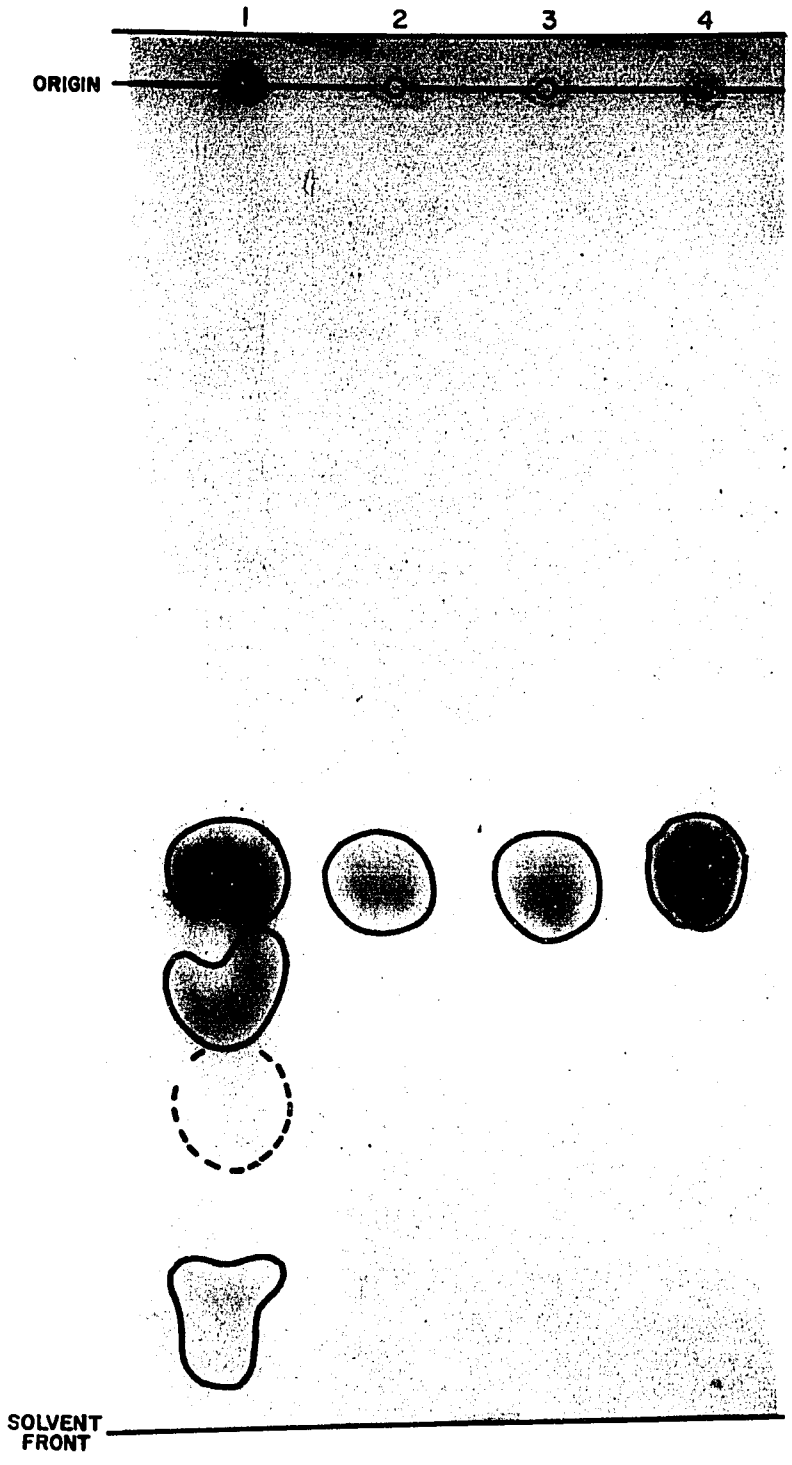
No. 1. Cotyledon soluble fraction

Nos. 2 & 3. Cotyledon insoluble (washed TCA precipitate)

No. 4. standard phenylalanine

Developed once in BEW (4:1:5) solvent

Paper used: Whatman No 1


1
2
3
4
ORIGIN
SOLVENT FRONT

In summary, then, the radioactivity was found to be associated with three regions only, which were tentatively identified as phenylalanine (Rf 0.55), phenolic glycosides (Rf 0.65) and phenolic acids (Rf 0.90). The phenylalanine area was usually the most active and gave the best recovery upon elution.

3. Attempts to isolate specific compounds for positive identification:

a) Phenylalanine (Rf 0.55 in BAW solvent):

As mentioned above, paper chromatography of all the soluble and insoluble fractions showed radioactivity primarily in the phenylalanine area. After passage through 3 or 4 papers, during which the radioactivity continued to run in the phenylalanine area (Table 13), the radioactivity present in each of the samples was determined. The amount of phenylalanine present in each sample was then calculated from the optical density at 260 mμ since direct dry weight determination of the eluted samples would also include some foreign substances. The dilution of labelled phenylalanine by the endogenous phenylalanine was calculated by dividing the specific activity of the phenylalanine initially supplied by the specific activity of the isolated sample. The results presented in Table 14 show that the recovery of radioactivity from paper chromatograms was generally poor after 3 or more elutions (between 20 to 30 per cent of the radioactivity initially spotted) except for the shoot-root axis insoluble (cell wall) fraction where

TABLE 13

CHROMATOGRAPHIC BEHAVIOUR OF THE RADIOACTIVE COMPOUND ISOLATED FROM THE VARIOUS FRACTIONS AND SUSPECTED TO BE PHENYLALANINE

Fractions	Ratio of Rf of unknown: Rf of Phe				
	BAW (4:1:5)	BEW (4:1:5)	But/2N NH_3 (1:1)	WATER	2% ACETIC
Phenylalanine	1.00	1.00	1.00*	1.00	1.00
Cot - soluble	0.85*	-	1.03*	0.92	-
Cot - insoluble	0.92	-	0.99	-	0.97
SR - soluble	-	1.09	1.02	-	0.97
SR - insoluble	1.00	1.06	-	0.90	-

*Appeared as streak

Paper used: Whatman 1 or 3 MM; Detection: Autoradiography

TABLE 14

RECOVERY OF RADIOACTIVE PHENYLALANINE FROM THE VARIOUS FRACTIONS OF SIXTY 72-HOUR PEA SEEDLINGS FED 1-^{14}C-PHENYLALANINE DURING IMBIBITION

Fractions	Recovery of radioactivity from the Phe area		Equivalent amount of Phe recovered		*Dilution with endogenous Phe
	% Ini spotted	No. of elutions	**(mg.)	S.A. c.p.m./mg.	
1. Cot sol.	20.9	4	0.565	7,113	187
2. Cot insol.					
a) NaOH sol.	27.5	3	3.94	2,396	555
b) NaOH insol.	29.5	4	0.215	7,119	187
3. SR-axis sol.	18.8	3	0.267	5,791	230
4. SR-axis insol.					
a) NaOH sol.	40.1	2	-	-	-
b) NaOH insol.	73.4	3	1.44	5,819	229

**Calculated from O.D. at 260 mμ

*S.A. (c.p.m./mg.) of phenylalanine supplied ÷ S.A. of isolated phenylalanine.

the recovery was very good (73%). The amounts of phenylalanine isolated from the soluble fractions of the cotyledon and shoot-root axis obtained from 60 seedlings were 0.565 and 0.267 mg. respectively. The NaOH insoluble (cell wall) fraction of the shoot-root axis contained 7 times more phenylalanine than the equivalent fraction from the cotyledon (1.44 and 0.215 mg.). Of all the fractions, the highest amount of phenylalanine (3.94 mg.) was recovered from the cotyledon NaOH soluble fraction. Phenylalanine in the NaOH soluble protein fraction of the shoot-root axis was not estimated because of the small amount of sample available. The dilution of labelled phenylalanine by endogenous phenylalanine in all the fractions was more or less of the same order (187 to 230) except for the cotyledon soluble protein fraction where the dilution factor was 2-3 times higher (555).

Purification of isolated phenylalanine through crystallization:

A sample of protein hydrolysate from the cotyledon was partially purified through charcoal adsorption and paper chromatography. This sample with a total weight of 18 mg. and a specific activity of 225 c.p.m. per mg. was dissolved in 1.5 ml. of 80 per cent ethanol by heating in a water bath. Crystallization was initially hampered by the presence of a yellow gummy, unlabelled substance which was not soluble in ether, and which had a maximum absorption at 271 mμ when dissolved in water. Unlabelled phenylalanine (90 mg.) was then added to the sample and dissolved in a minimal volume

(7 ml.) of 80% ethanol by heating in a water bath. Upon cooling, about three-fifths of the phenylalanine crystallized out, with the expected specific activity of about 40 c.p.m. per mg. The yellow substance remained in the ethanol supernatant. Two successive recrystallizations did not modify the specific activity of the sample. This would indicate that the label was associated only with phenylalanine.

The following tests and evidence strongly suggest that 1-^{14}C-phenylalanine was present in the "suspected phenylalanine" area.

i) Rf values in different successive solvent systems (Table 13)

ii) UV-spectra of eluted samples (Figure 17) showed a maximum absorption at 260 mμ with shoulders at 254 and 266 mμ, i.e. a typical phenylalanine spectrum.

iii) The radioactivity isolated from the phenylalanine area could be crystallized with added unlabelled phenylalanine. Further crystallization of this sample did not modify the specific activity.

iv) Spraying with ninhydrin on paper gave a positive test, accompanied by a loss of radioactivity.

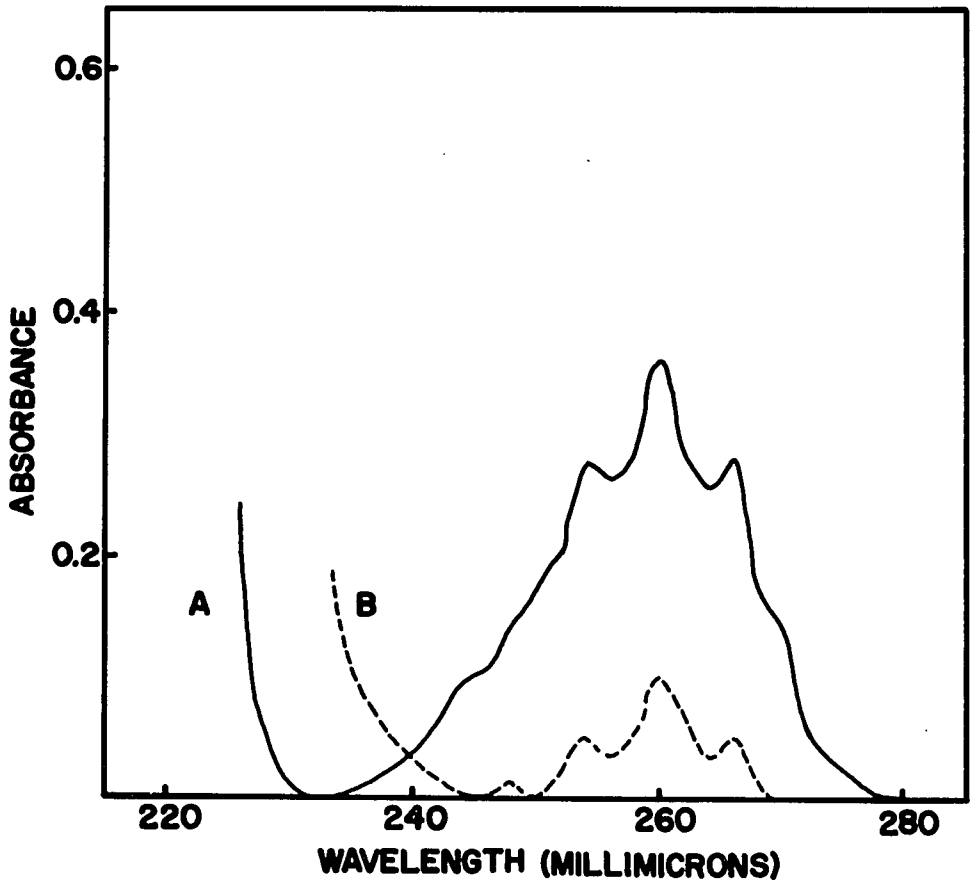
b) Phenolic glycosides (Rf 0.65 in BAW solvent):

The results obtained upon rechromatography of the sample eluted from Rf 0.65, in two other solvents (water and n-butanol -- 2N NH_3, 1:1) suggested that it was a glucoside, possibly a D-glucose ester of ferulic and/or caffeic acid or a mixture of both (Harborne and Corner 1961). Definite identification of this component was not made, because of the small size of the sample and heavy loss of radioactivity during elution and rechromatography.

FIGURE 17

Ultraviolet absorption spectra of phenylalanine (A) and of a sample eluted from a "suspected phenylalanine" area (B) from a chromatogram. The eluate from a paper blank (of equivalent size and area, cut out from the same chromatogram) was used in the reference cell for the unknown.

Solvent: water.


0.6
0.4
0.2
ABSORBANCE
A
B
220
240
260
280
WAVELENGTH (MILLIMICRONS)

c) Phenolic acids (Rf 0.90 in BAW solvent):

For identification purposes, the unknown compound or compounds from this area (Rf 0.90) was eluted and rechromatographed successively in three solvents (Butanol: 2N NH_3, BAW and benzene-acetic acid-water). Loss of radioactivity was substantial and the radioactive spots were detected only upon long exposure (2 to 3 months) to X-ray film. The results of such studies are presented in Table 15, and suggest that this unknown was comprised of at least three phenolic acids, probably caffeic, ferulic and p-coumaric acids.

In an attempt to prevent the heavy loss of radioactivity unlabelled authentic samples of caffeic, ferulic, o-coumaric, and p-coumaric acids were mixed with a phenolic acid preparation before chromatography. Radioactive areas after rechromatography were associated with spots identified as caffeic, ferulic and p-coumaric on the basis of Rf values and appearance in UV light.

The accumulated evidence listed below thus strongly suggests that the phenolic acid fraction contained labelled phenylalanine metabolites, tentatively identified as caffeic, ferulic and p-coumaric acids.

i) Radioactivity appears in the phenolic acid area upon chromatography of a crude fraction in BAW solvent

ii) Radioactivity can be extracted with ether upon acidification of a crude fraction

iii) Rf values of radioactive areas coincide with those of known phenolic acids (Table 15)

iv) Radioactivity co-chromatographs with authentic samples of known phenolic acids.

TABLE 15

CHROMATOGRAPHY OF THE UNKNOWN COMPOUND RUNNING INTO THE FREE PHENOLIC ACID AREA

Compounds	Rf values			Appearance under a UV-lamp
	But/2 N NH_3 (1:1)	BAW (4:1:5)	BenzAW (6:7:3)	
Unknown, suspected to be phenolic acids (cot-EtOH-ether sol.)	Three spots*			
	0.05	0.82	0.08	
	0.15	0.89	0.63	
	0.21	0.92	0.36	
Phenylalanine	0.20	0.64	0.10	A
Caffeic	0.04	0.86	0.08	BYF
Ferulic	0.12	0.90	0.65	BF
Cinnamic	0.40	0.95	0.90	A
O-Coumaric	0.20	0.94	0.50, 0.72	WF, YF
p-Coumaric	0.17	0.91	0.38, 0.67	A

Symbols used: B=bluish, Y=yellow, W=white, A=absorbance, F=fluorescence.

*Appeared as a streak, but was separable into three areas as indicated by their mean Rf values.

C. Attempts to account for the incomplete recovery of radioactivity:

In fractionation of samples by various means, especially in the washing procedure of TCA precipitates (Table 6), some loss of radioactivity was encountered. This loss became more evident during the paper chromatographic separation of various samples (Table 14 and Figure 14). Some of the tentatively identified components, presumably phenolic acids and their glycosides, were recovered much less completely than the phenylalanine itself. The poor recovery of these compounds could account for only a very small fraction of the total lost activity, however, because these fractions themselves contained only about 5 per cent of the initially added radioactivity.

a) Loss of activity during the drying of samples:

Estimation of radioactivity for most samples was done by counting air-dried samples on planchets in a G-F counter. Whether or not such a drying process resulted in some loss of activity was tested by counting both "wet" and "dry" samples in a L. S. counter. "Wet" samples were prepared by taking aliquots in scintillation vials and immediately adding counting mix, while for "dry" samples aliquots in vials were first air-dried, then

redissolved in the same volume of initial solvent before counting mix was added. Results shown in Table 16 generally indicate a lower count for the "dry" samples, although

TABLE 16

COUNTING OF "WET" AND "DRY" SAMPLES

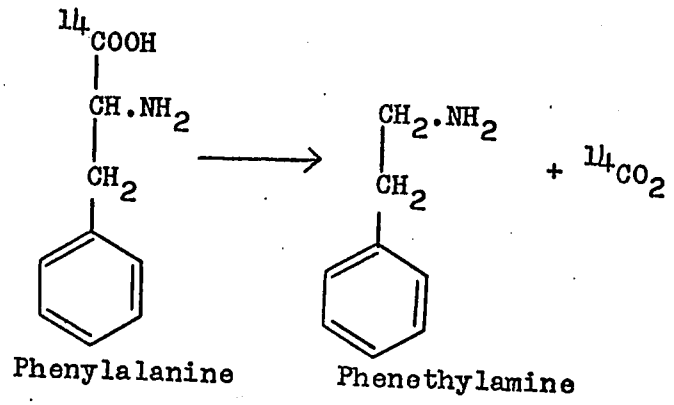
	Cotyledon			Shoot-root axis		
	Wet	Dry	%	Wet	Dry	%
NaOH-SOL	50,456	49,505	98	4,420	3,910	88
CELL WALL RES	10,320	8,080	78	10,560	8,400	79
TCA-PPT	53,120	52,320	99	4,470	4,230	94

the degree of loss varied from one sample to the other. This loss of radioactivity upon drying indicates the escape of some labelled volatile compound. However, attempts to trap such compounds in identifiable amounts were unsuccessful.

b) Was phenylalanine decarboxylated during the fractionation procedures ?

i) Evidence from paper chromatograms :

It was frequently observed on chromatograms that a yellowish, u.v.-fluorescing, non-radioactive component accompanied or ran slightly ahead of the Phe spot in BAW or BEW solvents. This observation, along with the already noted continuing loss of radioactivity from paper chromatograms suggested that phenethylamine was being formed non-enzymically from Phe.



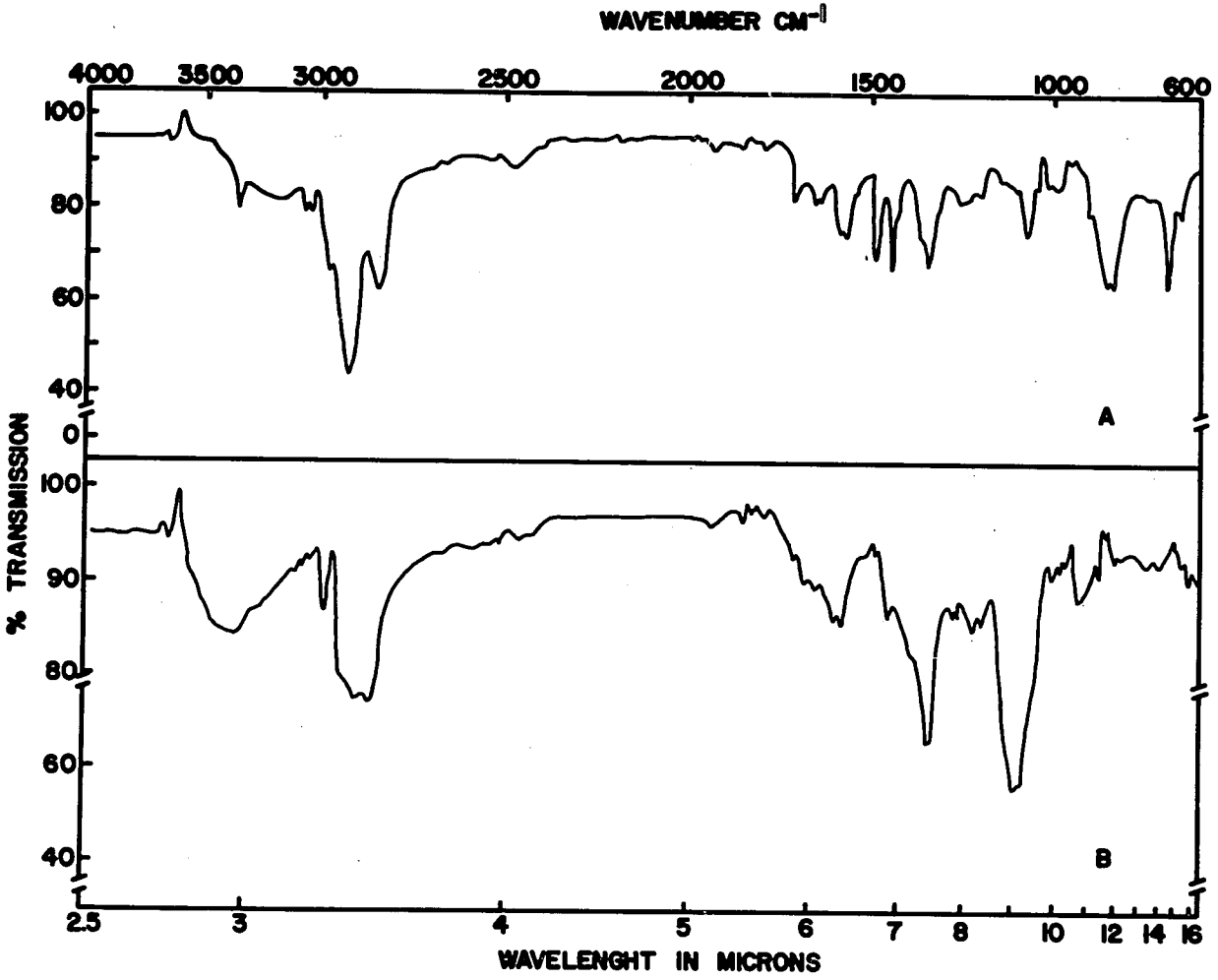
Phenylalanine Phenethylamine

Four such yellow spots were eluted from a paper chromatogram (cot.-soluble), dried, and extracted with a small volume of chloroform. Infra-red spectra of this chloroform extract and that of standard phenethylamine are presented in Figure 18. A very close scrutiny suggests that the spectrum of the unknown yellow component is similar to that of phenethylamine except that the latter had a few extra peaks (namely at 695, 840, 860, 3070). Also the relative heights of the peaks of the phenethylamine spectrum were higher than those of the unknown. These differences are probably the result of the differences in concentrations of the two samples and of some contaminations present in the isolated material.

FIGURE 18

Infrared spectra of standard phenethylamine (A) and of the "suspected phenethylamine" spots (B) eluted from a chromatogram.

Solvent: Chloroform.


WAVENUMBER CM⁻¹
4000
3500
3000
2500
2000
1500
1000
600
100
80
60
40
0
A
% TRANSMISSION
100
90
80
60
40
B
2.5
3
4
5
6
7
8
10
12
14
16
WAVELENGHT IN MICRONS

ii) Behaviour of standard phenylalanine:

Standard 1-^{14}C-phenylalanine of 4 different specific activities (c.p.m./mg.), was chromatographed and rechromatographed through 3 successive solvents. A high initial recovery of radioactivity was obtained with the BAW solvent (Table 17) but recovery decreased progressively with subsequent respotting and elution. The final recovery of radioactivity expressed as % initial spotted ranged from only 36% where specific activity was high to 61% where specific activity was low.

Freshly run phenylalanine on paper is colorless in daylight, and absorbs u.v. light slightly. Re-examination of the same paper after a month's storage showed that the phenylalanine area had turned yellow and fluoresced strongly under a UV-lamp. Presumably some decarboxylation to form phenethylamine had occurred.

iii) Behaviour of phenylalanine solution:

A saturated solution of phenylalanine in ethanol when left for several days turned yellowish and when chromatographed showed a UV-fluorescing spot in the area of phenethylamine.

These observations thus suggest the possibility that phenethylamine was formed non-enzymically from phenylalanine during storage, elution and respotting of the samples. With the 1-^{14}C-phenylalanine used in these experiments, then each molecule of phenethylamine formed would result in the complete loss of the label as $^{14}CO_2$.

TABLE 17

RECOVERY OF STANDARD 1-^{14}C-L-PHENYLALANINE OF 4 DIFFERENT SPECIFIC ACTIVITIES (C.P.M./MG.) UPON 3 SUCCESSIVE SPOTTINGS AND ELUTIONS

Phenylalanine spotted		No. of elutions & recovery of activity			Final Recovery (% Ini. spotted)
wt. in mg.	c.p.m.	1	2	3	
0.003	4,540	4,620	2,662	1,659	36.5
0.168	4,540	4,520	3,427	2,483	54.7
0.663	4,540	3,240	2,618	2,142	47.2
1.320	4,540	3,916	3,024	2,763	60.9

The solvent systems used were the following:

1: BAW, 2: BEW, 3: nBut/2NNH$_3$

D. Distribution of radioactivity when U-^{14}C-phenylalanine was used:

The results of the experiments obtained with carboxyl labelled phenylalanine had shown substantial losses of radioactivity in the various fractionation steps. It was presumed that most of the losses were due to non-enzymic decarboxylation of phenylalanine rather than to the formation of other relatively volatile metabolites (e.g. cinnamic acid derivaties) or due to handling losses. If this were true, then use of ^{14}C-phenylalanine labelled in any other position would considerably reduce the loss of radioactivity.

In an additional experiment, sixty pea seeds were allowed to imbibe a solution of U-^{14}C-phenylalanine. After imbibition, they were grown in vermiculite inside a desiccator for a period of 72 hours. Carbon dioxide was trapped in KOH solution that was replaced with fresh each day.

1) CO_2 and $^{14}CO_2$:

Time course evolution of CO_2 and $^{14}CO_2$ are shown in Table 18. Results indicate that the production of both CO_2 and $^{14}CO_2$ increased with time, although only a very small amount of the total evolved carbon dioxide was labelled. Increases in CO_2 production with time were faster than the increases in $^{14}CO_2$ production and thus $^{14}CO_2$ as per cent of total CO_2 declined with the germination time.

TABLE 18

TIME COURSE EVOLUTION OF CO_2 AND $^{14}CO_2$ BY GERMINATING PEA SEEDS FED U-^{14}C-PHENYLALANINE DURING IMBIBITION

Hours of incubation	µmoles of carbon dioxide produced / seed / hour*		$^{14}CO_2$ as % of total CO_2
	CO_2	$^{14}CO_2$ X10 ^{7}	
0 - 24	2.15	3.80	0.000018
25 - 48	4.27	5.67	0.000013
49 - 72	6.41	7.97	0.000012

*These values are based on CO_2 and $^{14}CO_2$ absorbed in the KOH.

A comparison of these results with those of the previous time-course experiment (Table 3) show that $^{14}CO_2$ as per cent of CO_2 was much less in this experiment, and the production of total CO_2 was about 2 to 3 times higher. Also in the earlier experiment with 1-^{14}C-phenylalanine, $^{14}CO_2$ production decreased with time whereas it increased in this experiment. This continued increase in labelled carbon dioxide would indicate that some of the carbons of the phenylalanine molecule other than the 1-C, were also evolved as $^{14}CO_2$ with time, a situation similar to that found in carrot tissue (Birt and Hird, 1958). However, the overall general trend remained the same; i.e., in both experiments the production of CO_2 increased with time while the per cent $^{14}CO_2$ decreased. The quantitative difference could be the result of dissimilarity in the growing conditions.

2) Distribution of radioactivity in various fractions of the seed:

Fractionation of the seedlings was carried out following the scheme outlined in Figure 9. The results shown in Table 19 indicate that less than 1% of the supplied radioactivity was associated with each of the seed coats, carbon dioxide, or the seedling wash fraction.

TABLE 19

THE DISTRIBUTION OF RADIOACTIVITY IN THE 72-HOUR PEA SEEDLING FED U-^{14}C-PHE DURING IMBIBITION

Fractions	Radioactivity	
	c.p.m.	% initial supplied to the seeds *
1) Seed washings	33,840	3.0
2) Seedling wash (at 72 hours)	7,580	0.7
3) $^{14}CO_2$	3,715	0.3
4) Cot - EtOH - Sol.	143,258	**14.2
5) Cot - EtOH - Insol.	551,604	**54.6
6) SR - EtOH - Sol.	23,611	** 2.4
7) SR - EtOH - Insol.	68,675	** 6.8
8) Seed coat	7,604	0.7
9) Rejects (7 seeds)	110,126	
Recovery	950,013	83.0

Duplicate samples were counted and an average value computed.

*Radioactivity initially provided to sixty seeds: 1,144,710 c.p.m.

**Corrected for 60 seeds to make the results directly comparable to those in Table 9.

Only 3% of the activity was left in the unimbibed seed washings obtained after 8 hours. Radioactivity in the ethanol-soluble fractions from the cotyledon and shoot-root axis was 14.2 and 2.4 per cent respectively and that in the ethanol insoluble fractions was 54.6 and 6.8 per cent respectively. Recovery of radioactivity at this stage of fractionation was about 83 per cent of the amount initially provided.

It might be of some interest to compare the data thus obtained with those from comparable experiments done earlier using 1-^{14}C-phenylalanine (Table 9). Such comparison shows that more radioactivity was recovered from the cotyledon fractions (14% versus about 10.4% in the soluble and 54.6% versus 43.5% in the insoluble) when U-^{14}C-phenylalanine was used. Distribution of radioactivity in the soluble and insoluble fractions of the shoot-root axis, however, was comparable in the three experiments. The overall recovery of radioactivity was about 16 per cent higher in the experiment done with U-^{14}C-phenylalanine, a finding which would support the conclusion that non-enzymic decarboxylation of the phenylalanine had occurred in experiments with 1-^{14}C-phenylalanine.

3. Isolation of a protein fraction

a) Ethanol extracted cotyledon residue:

Results presented in Table 20 show that mild hydrolysis with HCl to remove starch prior to protein extraction

TABLE 20

FRACTIONATION OF ETHANOL EXTRACTED COTYLEDON RESIDUE TO ISOLATE A PROTEIN FRACTION AND DISTRIBUTION OF ACTIVITY

Fractions	Radioactivity		
	c.p.m.	% Cot-EtOH-Insol.	S.A. (c.p.m./mg.)
Cot - EtOH - Insol.	551,604	100	-
A. HCl - Sol.	256,484	46.5	38
B. HCl - Insol.		53.5 (by diff.)	
1. NaOH-Water Insol.	11,805	2.1	58
NaOH-Water Sol.		51.4 (by diff.)	
2. TCA - Sol.	50,761	9.2	2
3. EtOH-Ether Sol.	73,190	13.3	215
4. Washed TCA-ppt. Hyd.	159,660	28.9	331
5. Humus	5,312	1.0	-
RECOVERY	557,212	101.0	

Each value is based on the average count obtained on duplicate samples.

also removed nearly half of the radioactivity (46.5%) present in the cotyledon residue. Following extraction with NaOH and water to remove soluble proteins, the residue (unwashed cell wall fraction) retained only 2 per cent of the radioactivity. The TCA-soluble fraction obtained from the material dissolved by NaOH contained 9.2 per cent of the radioactivity, while washing of the TCA insoluble portion with ethanol and ether removed 13 per cent of the radioactivity initially present. Only 29 per cent was left associated with the washed protein fraction following acid hydrolysis. The specific activities of the ethanol-ether wash and the protein hydrolysate were high, 215 and 331 c.p.m./mg. respectively.

Recovery of radioactivity was 100 per cent at this stage of fractionation, in contrast to the poor recovery (about 70 per cent) obtained in earlier experiments with 1-^{14}C-phenylalanine (Tables 6 and 9). In the experiment with U-^{14}C-phenylalanine, the amount of radioactivity present in the washings, TCA-soluble plus ethanol-ether soluble fractions, was 22.5 per cent, while that found in the washed TCA precipitate after hydrolysis was 28.9 per cent. The corresponding figures from an experiment done with 1-^{14}C-phenylalanine (Table 6) were one per cent and 20.5 per cent. Thus it is concluded that some decarboxylated product of phenylalanine was present in the washings, a product which would not be radioactive in experiments with 1-^{14}C-phenylalanine.

b) Ethanol Extracted shoot-root axis residue:

Although radioactivity present in this fraction was rather low (6% of Initial, Table 19), nevertheless, valuable information was obtained upon fractionation of this sample. (Fig. 11). Table 21, which is a summary of the results of this fractionation shows that the NaOH-water insoluble (unwashed cell wall) residue contained slightly more than one third of the total radioactivity (37.5%) present in the entire fraction, while about 46 per cent was present in the protein hydrolysates. About 10 per cent was associated with the ethanol-ether wash fraction. The specific activity (c.p.m./mg.) of the starting sample was moderately high (335); only the protein hydrolysate (514) showed an improvement over this value.

The overall recovery of activity from this fractionation procedure was slightly better than 97 per cent, in marked contrast to the poor recovery obtained from fractionation of the 10,000Xg pellet from the shoot-root axis, as shown in Table 8.

4. Chromatography of Soluble and Insoluble Samples:

Chromatography of cotyledon and shoot-root axis soluble fractions in BAW solvent showed a major radioactive spot in the area of phenylalanine, but only the cotyledon soluble sample showed an additional prominent spot in the phenolic acid region. There was a radioactive streak all the way from the origin to the solvent front which was accompanied by a visible light yellowish coloration.

TABLE 21

THE FRACTIONATION OF ETHANOL EXTRACTED SHOOT-ROOT AXES RESIDUE TO ISOLATE A PROTEIN FRACTION AND TO SHOW DISTRIBUTION OF RADIOACTIVITY

Fractions	Radioactivity		
	c.p.m.	% SR-EtOH-Insol.	S.A. (c.p.m./mg.)
SR - EtOH - Insol.	68,675	100	335
1. NaOH-Water Insol.	25,753	37.5	220
2. TCA - Sol.	1,923	2.8	1
3. EtOH-Ether Sol.	7,005	10.2	266
4. Washed TCA-ppt. Hyd.	31,522	45.9	514
5. Humus	549	0.8	-
RECOVERY	66,752	97.2	

Duplicate samples were counted and an average value computed.

Both the cotyledon and the shoot-root axis insoluble hydrolysates were chromatographed in two solvent systems simultaneously (BAW and 2% Acetic acid). For each sample, the radioactivity ran as a single spot in the area of phenylalanine. Each spot had a faint yellow non-radioactive streak.

5. Amino acid composition :

The ethanol soluble fractions and the protein hydrolysates were analysed by a Technicon amino acid analyser. The data obtained have been expressed in terms of μmoles of amino acids per 60 seedlings (72 hours old). The amino acid peaks were identified by comparing their emergence time from the column with that for the standards. The main objective of this part of the work was to obtain a quantitative measurement of phenylalanine in the various fractions of this particular variety of pea seedlings. Therefore, no attempts were made to identify and estimate the doubtful peaks and these are not included in the Tables.

a) Free amino acid composition :

Aspartic, glutamine, asparagine, threonine and serine emerged as a large single peak followed by another large peak of homoserine and glutamic acid. The peaks for these combined amino acids were almost off the scale for the

cotyledon soluble fraction, and hence were not estimated. In the shoot-root axis, on the other hand, the first peak was relatively smaller than the homoserine-glutamic acid peak (2nd peak), but both were out of the scale. However, the results indicate that glycine, cystine, methionine and tyrosine were present in the cotyledon soluble fraction at a very low concentration. These amino acids were not detected in the shoot-root axis soluble fraction under the conditions of analysis. Leucine and isoleucine were also present in small quantity in the cotyledon, but only isoleucine could be detected in the shoot-root axis. Concentrations of all other amino acids were moderately high in both the fractions except that the levels of arginine in the cotyledon and lysine in the shoot-root axis were relatively high. The concentration of phenylalanine in the cotyledon and shoot-root axis soluble fractions was 14 and 3 µmoles respectively (Table 22).

Semiquantitative analyses by paper chromatography and quantitative estimation of free amino acids in the pea seedling cotyledons and shoot-root axis tissue at various growth stages have been reported (Virtanen, 1953; Lawrence & Grant, 1963). Lawrence & Grant, (1963) noted that glutamic acid, arginine and asparagine were the predominant amino acids in the dry pea seeds and after one day of germination, but homoserine became the pre-

TABLE 22

Free Amino Acid Composition of Cotyledon and Shoot-Root Axis Soluble Fractions Obtained From 72-hour Pea Seedlings

Amino Acids	µmoles per 60 seedlings	
	Cotyledons	Shoot-Root
Aspartic Threonine Serine Glutamic Homoserine Proline	Not estimated	
Glycine	3.2	0.0
Alanine	65.3	10.0
Valine	32.5	6.7
Cystine	4.9	0.0
Methionine	2.2	0.0
Isoleucine	6.6	1.2
Leucine	7.9	0.0
Tyrosine	4.9	0.0
Phenylalanine	14.0	3.0
Ammonia	31.0	12.9
Ornithine	0.0	0.0
Lysine	20.7	29.8
Histidine	23.0	6.2
Arginine	186.5	16.2

Each value for the cotyledon is the average of two determinations made at 2 levels of dilution. The values for the shoot-root axis are based on only one determination.

dominant amino acid as germination progressed (Virtanen, 1953). The data obtained in the present study also support their findings. However, on a quantitative basis, the value of phenylalanine obtained for the cotyledons and shoot-root axis was only 53 and 72 per cent respectively of that obtained by Lawrence et al., (1963). This could be due to varietal difference and difference in the extraction and fractionation procedures.

b) Amino acid composition of ethanol insoluble fractions:

Isolated protein fractions (washed TCA precipitate) obtained from the ethanol insoluble residue of cotyledons and shoot-root axis and the entire ethanol insoluble shoot-root axis residue were hydrolysed and aliquots were used for analysis. The results of such studies are presented in Table 23. All the common protein amino acids and ammonia emerged as separate and identifiable peaks, except that cystine, cysteine and tryptophan were not detected. Tryptophan presumably was destrcyed in the process of acid hydrolysis (Fruton and Simmonds 1953).

In the cotyledons, levels of aspartic, glutamic, leucine, isoleucine and valine were very high followed by lysine, alanine, glycine and phenylalanine. The concentration of phenylalanine was estimated to be 127 µmoles per sixty seedlings.

TABLE 23

Amino Acid Composition of Cotyledon and Shoot-Root Axis Insoluble Fractions Obtained From 72-hour Pea Seedlings

Amino Acids	μmoles per 60 seedlings		
	Cot-NaOH* Sol	Shoot-Root Axis	
		NaOH-Sol*	NaOH-Insol**
Aspartic	230.0	17.2	49.6
Threonine	72.8	16.0	24.8
Serine	106.0	14.1	31.7
Glutamic	195.9	27.4	31.9
Proline	82.4	0.0	0.0
Glycine	125.0	24.7	33.1
Alanine	146.4	19.8	31.9
Valine	173.3	26.9	29.9
Cystine	0.0	0.0	0.0
Methionine	12.4	1.3	0.8
Isoleucine	174.3	17.6	21.6
Leucine	233.0	25.7	38.0
Tyrosine	52.6	6.6	5.7
Phenylalanine	126.6	12.4	11.0
Ammonia	110.9	32.2	101.7
Lysine	149.6	22.0	31.0
Histidine	36.0	6.5	8.4
Arginine	74.3	11.1	13.0
TOTAL	2,101.5	281.5	464.1

Each value is the average of two determinations, each made at different dilution.

*Ethanol insoluble, NaOH-soluble, TCA-insoluble washed precipitate hydrolysate.

**Derived by difference between the analysis obtained on the entire ethanol insoluble residue and the NaOH-soluble residue in column 2.

There seemed to be little difference between the analysis obtained on the cot-entire-ethanol-insol residue and the NaOH soluble fraction in column 1, hence no comparable column is included for the cotyledons.

Concentrations of amino acids in the shoot-root axis protein fraction were of the order of 20 to 30 μmoles per sixty seedlings, except that the levels of methionine, tyrosine and histidine were quite low and proline was not detected. About 13 μmoles of phenylalanine were present in this fraction.

The levels of amino acids in the ethanol extracted shoot-root axis residue were also determined to obtain an estimation of the bound phenylalanine in the NaOH insoluble cell-wall protein. This was derived by difference between the values obtained for the entire residue and the NaOH-soluble proteins.

Results (Table 23) indicate that the concentration of aspartic acid was about 3 times higher in the cell wall protein than in the NaOH-soluble protein. The amounts of threonine, serine, alanine, leucine and ammonia were also about two times higher in the cell wall protein. The concentrations of other amino acids including phenylalanine were more or less equal in the two fractions.

The levels of phenylalanine thus estimated in the NaOH-soluble and NaOH-insoluble fractions obtained from the shoot-root axis show a positive correlation with the amount of radioactivity present in them. The NaOH-soluble fraction had a higher amount of phenylalanine (12.4 μmoles) and a higher per cent of the radioactivity (45.9) than did

the NaOH-insoluble fraction (11.0 μmoles and 37.5% of the activity)(cf Tables 23 and 21). Chromatographic evidence revealed that the only labelled amino acid was phenylalanine.

DISCUSSION

The results obtained from the studies reported in this thesis indicate that pea seeds imbibed about 97% of the available phenylalanine from the imbibition medium in about 7 hours (Tables 9, 19). Although the seeds exuded some substances into the imbibition medium, very little if any of the imbibed activity was exuded. After 3 days of germination less than 1 per cent of the supplied radioactivity was recovered from the seedling wash fraction.

Larson (1968) working with the same variety of pea seeds as used in the studies noted that loss of total diffusate, carbohydrate and total nitrogen was higher during the first 4 hours of imbibition than during subsequent 4 hour periods. He detected at least 12 amino acids in the diffusate but did not identify them. Agnihotri & Vaartaja (1967) also detected 13 amino acids in the root exudate of red pine, but phenylalanine was not amongst them. However, because of the low level of free phenylalanine present in the ungerminated pea seeds (Lawrence et al., 1963), it is unlikely that phenylalanine would be exuded in any identifiable quantity.

It is not known whether the small amount of activity found in the seedling wash was exuded by the seeds or represented unabsorbed activity. Whichever might be the case, the activity was not in the form of phenylalanine alone. Chromatography of this sample in BAW solvent showed at least two

other spots besides phenylalanine, one at the origin and the other in the area of free phenolic acid. Whether this conversion was caused by bacteria on the exterior surface of the seeds or by the seeds themselves can only be resolved by further study.

Besides exudation, another possible way of losing absorbed phenylalanine would be decarboxylation to yield CO_2. This is a common phenomenon found with many amino acids. Larson (1963) injected uniformly labelled (^{14}C-) glutamic, aspartic, γ-aminobutyric, homoserine and leucine into germinating pea seedlings and noted that appreciable amounts of the carbon skeletons of the supplied amino acids were respired as $^{14}CO_2$. Working with excised barley embryos, Joy and Folkes (1965) noted a substantial loss of supplied aspartic and glutamic acids by way of respiration. The experiments reported in this thesis show that unlike the amino acids mentioned above, only a negligible amount of supplied phenylalanine (either 1-^{14}C-phenylalanine or U-^{14}C-phenylalanine) was decarboxylated to $^{14}CO_2$ (Tables 3, 18). This is not unique to phenylalanine alone: Steward *et al.*, (1958) working with carrot tissue noted that only about 4% of the assimilated proline was respired per day. Using excised barley embryos, Joy and Folkes (1965) could trace only 2 per cent of the radioactive carbons of lysine into $^{14}CO_2$. However, Birt & Hird (1958) noted some metabolic destruction of phenylalanine in carrot tissues.

It thus appears that the radioactive phenylalanine taken up by the seeds was neither exuded nor degraded to yield CO_2 to any appreciable extent. In fact, better than 98 per cent of the supplied radioactivity could be detected in the seed parts until up to 72 hours of germination (Table 4). The time-course distribution pattern of the label suggests that the labelled compound first found its way to the cotyledons. This view is supported by the autoradiographic studies reported in Part I of the thesis. As germination progressed, the radioactivity was transported gradually to the growing parts: i.e. radioactivity in the shoot-root axis increased while that in the cotyledon decreased (Table 4).

Of the radioactivity present in the cotyledons, most was found in the 100,000 X g supernatant fractions (Table 5). This fraction contained the water soluble proteins as well as free amino acids.

TCA treatment of the 24-hour sample showed that the radioactivity in the TCA-soluble or the "amino acids" fraction was as much as three times higher than the TCA-insoluble or the "protein" fraction (Table 6). This indicates that up to a period of 24 hours after the seeds were hydrated, the level of free labelled phenylalanine in the cotyledon remained quite high. This TCA-soluble radioactivity was not entirely transported to the shoot-

root axis in the following days, some was incorporated into the protein fraction of the cotyledons. Similar results were reported for leucine in pea seedlings (Larson, 1963). It was rather interesting, though not unexpected, to note incorporation of radioactivity into the protein fraction at a time when rapid hydrolysis of storage protein was presumably taking place. Incorporation of labelled amino acids into protein and de novo synthesis of certain enzymes in the cotyledons of germinating peas has been reported (Young & Varner, 1959, Young et al., 1960). Young (1957) has observed that the enzymic activity of an adenosine triphosphatase of pea seed cotyledons increased ten to fifteen fold during the first 5 days of germination at 23°C. De novo synthesis of α-amylase has also been demonstrated in barley endosperm (Varner, 1964). The synthesis of new enzymes in the cotyledons is not very surprising in view of the fact that the entire apparatus necessary for protein synthesis has been found to be functional in the cotyledon of the ungerminated peanut (Marcus & Feeley, 1964).

The proteins of the 100,000 X g supernatant fraction of the cotyledons presumably include all the albumins to which most of the enzymes belong. Thus the result presented in this thesis, is in agreement with the findings of Young et al., (1960) that the proteins which become labelled in vivo from ^{14}C-labelled amino acids belong to

the albumin fraction. This soluble protein fraction was separated into four peaks by DEAE cellulose chromatography. The labelling pattern indicated that by 24 hours only one peak was moderately labelled, while all peaks were labelled by 96 hours or earlier (Fig.13). A picture similar to the 96 hour sample probably would have been obtained with the 48 hour or the 72 hour sample, since the per cent distribution of radioactivity in all 3 was more or less the same. Whether any new protein synthesis took place in the cotyledons after a period of 48 hour or whether there was a rapid turnover of protein without any net synthesis after that period is not known.

It is very difficult to obtain a clean preparation of protein from a plant source (Loomis & Battaile, 1966). This difficulty might lead one to suspect that the activity in the soluble protein fraction was not a result of incorporation of phenylalanine into the protein molecule, but was a result of adsorption. N-terminal analysis of the washed TCA-precipitate obtained from the shoot-root axis fraction showed that the bulk of the activity was in the non-N-terminal amino acid fraction. Similar results have been reported by Nozzolillo (1963) with TCA precipitates from both the cotyledon and the shoot-root axis. The results show that there was indeed some incorporation of the label into proteins.

After 48 hours the radioactivity of the cotyledon protein fraction declined slightly an indication perhaps of a turnover of some of the newly synthesized proteins. Larson (1963) has suggested a similar possibility with leucine.

A major part of the radioactivity in the developing shoot-root axis was traced into the unwashed cell wall fraction (Table 5). The radioactivity in the washed cell wall fraction gradually increased with the germination time. The cell wall thus isolated was presumably mainly composed of cellulose and structural protein, since the leguminous plants remain soft and non-ligneous until the last stages of their development (Bondi & Meyer, 1948), and that the dark-grown seedlings lack lignin. Separation of proteins and lignin, if any, from the cellulose achieved with NaOH treatment (Bondi & Meyer, 1948) showed that the activity in the protein fraction was twice as much as that in the cellulose fraction. Considerable loss of activity upon NaOH treatment became evident, a phenomenon also noted by Nozzolillo (1963).

There was also a slow but steady increase in the TCA-soluble activity. Most of the recovered radioactivity was in the form of phenylalanine in all the fractions obtained whether soluble or insoluble.

The level of free phenylalanine has been estimated to be 14 μmoles in the cotyledon and 3 μmoles in the shoot-root axis per sixty 72-hour old seedlings. No estimation was made at 0-day and thus the changes of phenylalanine level during the 3-day period is not known. However, the work of Lawrence & Grant (1963) shows this level to be 4.2 and 0.06 μmoles per 60 seeds for the cotyledon and the shoot-root axis respectively on 0-day, which rose to 26.4 and 4.3 by 72 hours, values of the same order as obtained with Alaska despite their use of a different variety (Unica). It seems likely that the level of free phenylalanine increased to some extent with germination, but how much of this increase was derived from protein hydrolysis, interconversion or due to new synthesis, is not known. However, no conversion of phenylalanine to tyrosine has been noted in the experiments reported here. The possibility that there was some formation of tyrosine which was immediately converted to *p*-coumaric acid is very unlikely. Although grasses are good source of tyrase, the enzyme that converts tyrosine to *p*-coumaric acid, legumes contains very little of it, if anyat all (Neish, 1965).

Interconversion of amino acids is a common phenomenon in germinating seeds and seedlings (Meister, 1957; Folkes & Yemm, 1956; Larson & Beevers, 1965; Chibnall, 1939). However, there is not much evidence to show any appreciable conversion of phenylalanine to other amino acids in plants. Nozzolillo (1963), working with pea seeds and McCalla & Neish, (1959) with Salvia, have indicated some conversion of phenylalanine to tyrosine. The enzyme phenylalanine hydroxylase that converts phenylalanine to tyrosine (Kaufman, 1961) is absent from plant systems, although Gamborg and Neish (1959) noted some conversion of the intermediates of the phenylalanine pool to tyrosine. This suggests the possibility that tyrosine is formed in plants, as in microorganisms, primarily from prephenic acid rather that by p-hydroxylation of phenylalanine. The latter route probably exists in plants as a minor alternate pathway (Neish, 1965).

Although phenylalanine was not converted to other amino acids, all of it did not remain in the free form as such for long. There was some conversion to cinnamic acid derivatives, the level of which increased with the germination time as indicated by chromatographic evidence. These derivatives were mainly caffeic, ferulic and p-coumaric acids, the common phenolic acid intermediates of lignin biosynthesis and were detected both in the cotyledon and in the shoot-root axis.

Phenylalanine was presumably converted first to cinnamic acid by the action of L-phenylalanine ammonia lyase (Koukol & Conn, 1961). This step was followed by a series of hydroxylation and methylation reactions (McCalla & Neish, 1959) to form the other derivatives. It has been reported that the activity of phenylalanine ammonia lyase is increased greatly by exposure of the tissue to white light (Zucker, 1965). The results presented in this thesis show only a small conversion of phenylalanine to cinnamic acid derivatives. This could be due to the fact that the seedlings were grown in darkness, with very little exposure to light.

Phenolic compounds are abundant in higher plants and they occur mostly as glycoside derivatives. Whether or not phenols and phenolic glycosides are translocated to any significant level is still in dispute (Pridham 1965). It is not clear from the results reported in this thesis whether the cinnamic acid derivatives detected in the shoot-root axis were transported from the cotyledon or were the results of deamination in the shoot-root tissue itself. There was some indication of the presence of labelled glycosides in the cotyledon soluble fractions, but these were not detected in the shoot-root axis fractions. Since the activity in the shoot-root axis fractions was very small, it is possible that even if some glycosides were formed, they were not detectable by the methods used.

Labelled phenylalanine was introduced into the seeds with the assumption that it would be integrated into the endogenous phenylalanine pool. Isolation of phenylalanine from the various fractions and determination of specific activity would indicate the extent to which the supplied phenylalanine had been diluted by the endogenous phenylalanine. This result might provide an insight into the utilization of endogenous phenylalanine by the germinating seeds.

The dilution factors reported in (Table 14) for the various fractions were all about 200 except that for the cotyledon-NaOH soluble (protein) fraction which was almost 3 times greater. This more or less uniform value at least shows that the labelled phenylalanine was used indiscriminately with the endogenous phenylalanine in the tissues. The higher dilution of phenylalanine in the Cot-NaOH-soluble fraction leads to the conclusion that the amount of newly synthesized protein in the cotyledon was proportionately less than the protein already present in the fraction.

The results reported in this thesis indicate substantial loss of radioactivity in almost every step of fractionation. In vivo decarboxylation would have resulted in much more $^{14}CO_2$ production than the amount reported. It is possible that some phenylalanine was decarboxylated to form phenethylamine in vitro. Since only carboxyl labelled

phenylalanine had been employed in the initial experiments this one step reaction would be enough to explain the loss of activity at many fractionation steps. The presence of phenethylamine in a cotyledon soluble fraction has been suggested (p. 109). The fact that chromatography and re-chromatography of standard 1-^{14}C-phenylalanine of 4 different specific activities resulted in a progressive loss of radio-activity with each respotting and elution also supports the view that phenylalanine was decarboxylated in vitro (Table 17). The observation that samples of low specific activity gave a better recovery throughout the successive elutions can be explained on the assumption that decarboxylation of labelled and unlabelled molecules occurred indiscriminately. Thus decarboxylation of some of the phenylalanine in the samples of low specific activity would not affect the total radioactivity to as great an extent as it would that of samples of high specific activity.

Thus some losses of radioactivity, especially those occurring during paper chromatography, can be accounted for by in vitro reactions. This, however, is not sufficient to account for all the losses observed, and some other source must be postulated.

One possible avenue to account for loss of radioactivity would be the formation of volatile metabolites in vivo which would remain in the extracts until driven off by various treatments. There was apparently some loss of radioactivity

simply by boiling in ethanol, since the recovery of radioactivity in ethanol fractions was at best only 83 per cent, in contrast to the 100 per cent recovery obtained with cold water extraction. Treatment of protein fractions with NaOH resulted in some loss of radioactivity (Table 6) and recovery following solubilization of starch was usually not complete (Table 8). The fact that some radioactivity was lost upon drying of samples (Table 16) is also a support for this possibility.

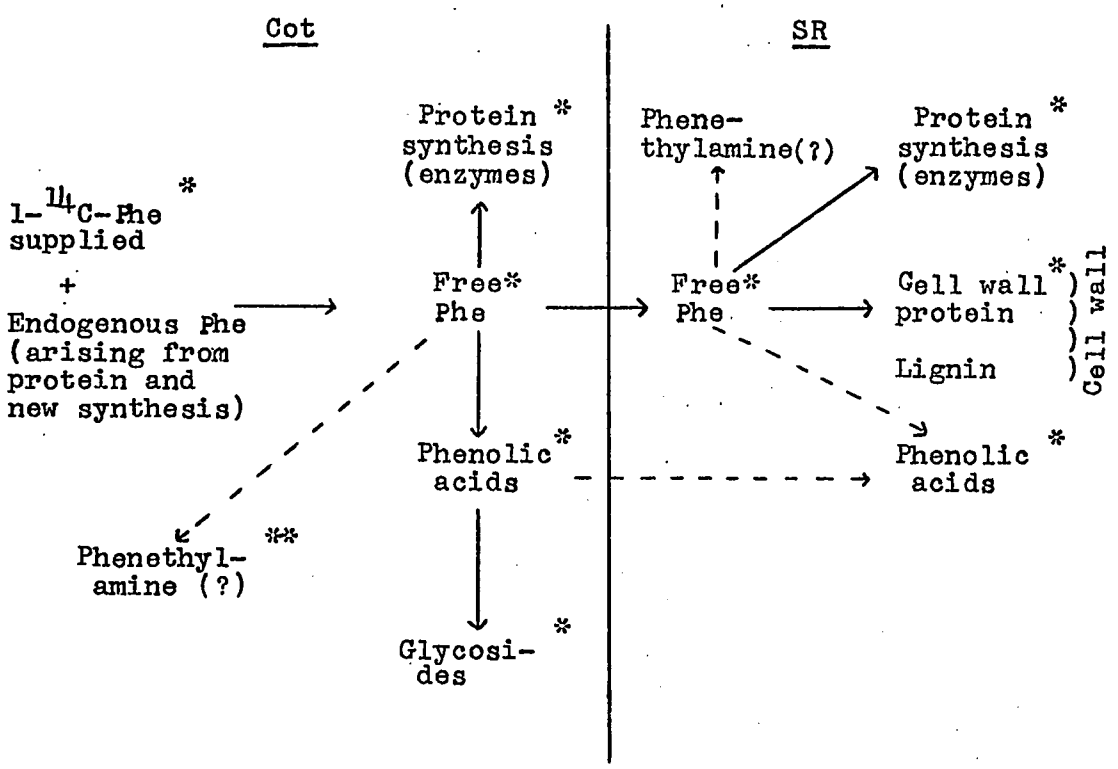
The possible nature of such metabolites can at the moment only be suggested since all attempts to trap such compounds in an identifiable form were unsuccessful. The demonstration that cinnamic acids were formed from the phenylalanine, however, leads to several suggestions. El-Basyouni and Neish (1965) have shown that the B-oxidation of cinnamic acids is a widespread activity in plants. This would lead to the formation of a volatile 2 carbon fragment which would contain all the radioactivity of 1-^{14}C-phenylalanine. Similarly, it would be reasonable to expect that reduction reactions leading to the formation of the volatile lignin intermediates from cinnamic acids (Brown, 1969) would be present in the tissues of the pea seedling. The possibility that a volatile substance such as coumarin might be formed (Brown et al., 1964) also should not be overlooked. Thus several types of volatile compounds can be postulated on the basis of well established reactions of plant tissues. The fact that the ether soluble washings of the protein fractions of the experiment with U-^{14}C-phenylalanine contained more radio-

activity than did those obtained from experiments with 1-^{14}C-phenylalanine (Tables 20.& 21; 6 & 11), would lend some support to the suggestion. Benzoic acids formed by B-oxidation would be found in this fraction, but they would be radioactive only in extracts made from pea seeds fed U-^{14}C-phenylalanine. Chromatography of this fraction in the benzene solvent for phenolic acids (Ibrahim & Towers 1960) indicate that indeed additional phenolic acids are present in the U-^{14}C-preparations, but they were not obtained in sufficient quantity for positive identification.

Phenylalanine is otherwise not readily degraded in plants, although it is readily metabolized to fumaric and acetoacetic acid via tyrosine and homogentisic acid in animals (Greenberg, 1954). Hydroxylation of many aromatic compounds, including phenylalanine can be effected in non-enzymic systems in the presence of O_2, ascorbic acid and Fe^{2} ions (Udenfriend et al., 1954). Since labelled tyrosine was not detected in any of the fractions, it is unlikely that the carboxyl carbon was lost as $^{14}CO_2$ in the step involving the formation of homogentisic acid from tyrosine.

The overall fate of phenylalanine in the germinating pea seeds can be represented schematically as follows:

A scheme showing the fate of phenylalanine in pea seeds after 72 hours of germination



Enzymic decarboxylation to form CO_2 was very small.

* Activity was detected.

** Probably mainly originated non-enzymically (as an artifact).

SUMMARY AND CONCLUSIONS

To study the metabolism of endogenous phenylalanine in the germinating pea seeds, labelled phenylalanine was introduced into the seeds during imbibition. The concept behind such an approach was that the labelled amino acid would intimately mix with the endogenous phenylalanine pool and thus would indicate its pathway of translocation and/or transformation in the germinating seeds.

1. Uptake of phenylalanine

Studies were conducted to determine some aspects of phenylalanine uptake by the imbibing pea seeds. The uptake of phenylalanine and the uptake of water did not proceed at the same rate. The uptake of phenylalanine was initially slow as compared to water, but soon became more rapid. The seeds imbibed phenylalanine almost completely from a dilute solution in about 7 hours. With increasing concentrations of phenylalanine in the medium, the seeds took up more and more, but the per cent uptake declined. The uptake per seed from a concentrated solution of phenylalanine (0.1M) was 2.2 mg., yet this did not have any noticeable toxic effect on germination and growth of the seedlings at least up to a period of 72 hours.

Contaminating bacteria in the medium might be regarded as a serious threat against introducing a compound

into the seeds through imbibition. The results indicated that if the initial bacterial counts were kept low (about 10^4-10^5/ml.), the danger of bacterial degradation of added phenylalanine in the medium can be overlooked, since the uptake of phenylalanine was complete before the bacteria had multiplied.

Autoradiographic studies with the pea seed sections indicated that the labelled phenylalanine did enter into the seeds. The compound entered through the micropyle (or the micropylar region), travelled in between the inner and the outer layers of the seed coat before diffusing into the cotyledons through the relatively permeable inner layer. From the cotyledons the radioactivity was later transported to the shoot-root axis. Autoradiographic studies with tissue sections involving water-soluble labelled compounds have always been very tricky (Fitzgerald, 1961). For a quick and gross localication of radioactivity in tissues, especially for compact tissues like seeds where paraffin impregnation is relatively difficult, the procedure described in the thesis might be worthy of trial.

2. Metabolism of phenylalanine

In the initial experiments, 1-^{14}C-phenylalanine was introduced into the seeds through imbibition. The radioacti-

vity was taken in by the seeds almost completely. Very little of it accompanied the seed wash, seedling wash, carbon dioxide or the seed coat fraction.

A time-course experiment showed that the activity in the cotyledons was initially very high. As the seedlings grew, the activity from the cotyledons slowly moved to the shoot-root axis. In the cotyledons, the activity was primarily associated with the 100,000Xg supernatant fraction while in the shoot-root axis, it was mainly in the 10,000Xg pellet (unwashed cell wall) and to a lesser extent, in the 100,000Xg supernatant fraction.

TCA treatment of the 100,000Xg supernatants showed that the TCA-soluble activity decreased with time in the cotyledon, while that in the shoot-root axis increased. TCA-insoluble activity, on the other hand, increased up to a period of 48 hours and thereafter remained more or less unchanged. Some loss of radioactivity was noted upon washing the TCA precipitates.

Dialysis of the 100,000Xg cotyledon supernatants yielded fractions which had comparable activity to that of the TCA precipitates. Separation of the dialysed sample through a DEAE cellulose column to obtain a protein fraction of high specific activity met with only partial success.

Ethanol extraction of seedling parts showed that the amount of activity present in the ethanol soluble fraction and that in the TCA-soluble fraction were comparable. In this regard, the ethanol insoluble residue was found comparable to the combined pellets and the TCA-precipitate. Of the activity present in the soluble fraction, most of it was charcoal adsorbable (i.e., still in an aromatic molecule) and only a small percentage was ether soluble (primarily phenolic acids).

The radioactivity in the ether-insoluble fraction was primarily associated with phenylalanine. Moreover, this fraction from the cotyledon indicated the presence of a labelled glycoside. The ether soluble fraction had at least three labelled phenolic acids, namely caffeic, ferulic and p-coumaric acids.

The radioactivity in the washed TCA-insoluble precipitate was associated only with phenylalanine. Standard protein washing procedure removed very little activity from the TCA-insoluble fraction. This and the result of N-terminal analysis indicated that the label was well incorporated into the protein fraction.

Lawrence & Grant, (1963) have reported an increase in the level of free phenylalanine in pea seedlings during the first 3 days of germination. The hydrolysis

of stored protein presumably resulted in such an increase. The experimental evidence reported in this thesis indicated that the supplied phenylalanine had mixed intimately with the endogenous phenylalanine pool and was utilized by the seedling indiscriminately in the new synthesis.

The various fractionation steps and chromatographic procedures resulted in a substantial loss of radioactivity. It is suggested that some of the loss was due to non-enzymic breakdown of phenylalanine to phenethylamine. Other losses could occur through evaporation of volatile metabolites of phenylalanine produced, for example, by B-oxidation of the cinnamic acids produced through the action of phenylalanine ammonia lyase on phenylalanine. However, this point had not been substantiated by further work.

3. Concluding Remarks

The work reported in this dissertation was done primarily to provide answers to the questions raised by Nozzolillo (1963), who initiated the work in our laboratory. Her preliminary work was repeated in a move to answer the related questions, although the experimental conditions and the fractionation procedures differed greatly. The results of the two studies agreed very well, except that the present work has failed to confirm any

conversion of phenylalanine to tyrosine. Using U-^{14}C-phenylalanine, she noted poor recovery of radioactivity. With 1-^{14}C-phenylalanine used in the initial studies reported here, the recovery was also poor. The experiment with U -^{14}C-phenylalanine was not taken as far but here also considerable losses were incurred.

It is believed, the questions raised by Nozzolillo (1963), have been fully answered by the present work. Added phenylalanine did enter into the cell, mixed well with the endogenous phenylalanine pool and was utilized indiscriminately. The loss of radioactivity presumably occurred as a result of non-enzymic decarboxylation of phenylalanine to phenethylamine and of metabolic formation of volatile C_6-C_3, C_6-C_1 and C_2 compounds. The presence of a small number of bacteria in the imbibition media did not seem to affect the results of the experiments. In addition, this work has established the path of entry of phenylalanine and at least a part of its pathway of metabolism in the germinating pea seeds.

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