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THE METABOLISM OF PARTS OF SEEDS DURING EARLY GERMINATION

by

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CONVENTIONS

1. In this thesis botanical names are used for two of the seeds studied and common names for the other two. This is done to simplify the comparison between the seeds used for this thesis and similar seeds whose metabolism has been investigated in other studies. The botanical name of *Phaseolus vulgaris* is used because the seeds of many different cultivars (each with its own common name) of this species have been studied. Barley, on the other hand, is the common name for what are often considered as different species of *Hordeum*. The common name is used because the seed metabolism of the different species could be expected to be similar. The common name of castor oil is used because it is shorter than the botanical name, while *Pinus radiata* is both the botanical and common name for this species in New Zealand.

2. In accordance with a common biochemical practice, the unionised form of an acid is shown in biochemical equations, but it is referred to by its ionic name (e.g. malate for malic acid).

3. The numbers shown in brackets after the name of an enzyme are taken from the list given by Dixon and Webb.[216] This list is that of the Enzyme Commission with the addition of provisional numbers ending in a letter in those cases for which the commission had not allotted numbers.

ABBREVIATIONS

Abbreviations used in the text include the following:

ADP, ATP	Adenosine di- and triphosphate
Ci	Curie
CoA	Coenzyme A
NAD	Nicotinamide adenine dinucleotide
$NADH_2$	Reduced form of NAD
NADP	Nicotinamide adenine dinucleotide phosphate
$NADPH_2$	Reduced form of NADP
NAD(P)	NAD or NADP
-P	Phosphate group
Pi	Inorganic phosphate
PPi	Inorganic pyrophosphate
UDP, UTP	Uridine di- and triphosphate
UDPG	Uridine diphosphate glucose
rep	roentgen equivalent physical
RNA	Ribonucleic acid

Abbreviations used in the illustrative plates are:

AB	4-Aminobutyrate
Ala	Alanine
Asp	Aspartate
Cit	Citrate
EtOH	Ethanol
Gln	Glutamine
Glu	Glutamate
Hr	Hour(s)
Lac	Lactate
Mal	Malate

Min	Minutes
Phos	Sugar phosphates
Suc	Succinate
Sucr	Sucrose
THO	Tritiated water

ABSTRACT

There have been few studies of the metabolism of germinating seeds during the first minutes and hours of imbibition. This is partly because most biochemical techniques are unsuitable for studying these biological systems. One technique that has been developed for studying such systems involves using tritiated water as a tracer. In this technique the biological material is placed in tritiated water for a known time and then extracted. The extracts are chromatographedon paper and the tritium labelled compounds are detected by scintillation radioautography. The technique has already been successfully applied to the study of intact seeds of two species of dicotyledons. In this present work the technique was used to study the metabolic development during the first six hours of imbibition of the different parts of seeds from four species: a monocotyledon (barley), two dicotyledons (*Phaseolus vulgaris* and castor oil), and a gymnosperm (*Pinus radiata*). The seeds of these species are representative of the majority of seeds in the plant world. The results show that performing experiments on excised parts of seeds provides additional information to that obtained from using intact seeds. The most important conclusion of this work is that the axes and embryos of all seeds develop the same basic metabolic pathways during the first hours of imbibition and that these pathways develop in a similar order. Amino acid metabolism begins during the first few minutes of imbibition, followed by the metabolism of Krebs cycle acids and, after two hours, sucrose metabolism. Ten different labelled

compounds were identified and labelled groups of compounds described as lipids and sugar phosphates were also present. Detailed conclusions are made about the metabolism based on the tritium labelled compounds formed, together with any other relevant information available. A number of additional experiments, including ^{14}C tracer experiments, were performed on parts of two of the seeds. These experiments enabled more definite conclusions to be made about the metabolism of sucrose and 4-aminobutyrate, two of the compounds labelled in the tritiated water experiments. Most and in some cases all the labelled compounds formed in the axes and embryos were also found in the storage organs. However, the rate of metabolic development of the storage organs was slower than that of their corresponding axes or embryos, and a comparison of water absorption to metabolism indicated that initially this may have been due to their slower water absorption. The metabolic development was not as uniform among the storage organs as it was among the embryos and axes. In particular, the barley endosperm appeared to have a much simpler metabolism than the castor oil endosperm and it was shown that its metabolism occurred entirely in the aleurone layer of cells on its surface. It is concluded that the metabolic development of seed storage organs depends more on their morphology and function than on their embryological origin.

CHAPTER 1

INTRODUCTION

1-1 SEED GERMINATION

1-1.0 DEFINITION

The definition of germination used in this thesis is that of Mayer and Poljakoff-Mayber (p. 12, ref. 1): "Germination of the seed of the higher plant we may regard as that consecutive number of steps which causes a quiescent seed, with a low water content, to show a rise in its general metabolic activity and to initiate the formation of a seedling from the embryo. ... we identify germination by the protrusion of some part of the embryo from the seed coat ..." This definition of germination is not universally accepted, many authors regarding growth of the seedling after protrusion as part of the germination process.[2, 3, 4]

To initiate germination a seed must be placed in an environment from which it can absorb water. The process of absorbing water is referred to as imbibition. The word is often used in this thesis when referring to the time for which a seed had been placed in conditions under which it could germinate, as in the phrase: "after one day of imbibition". This is preferable to the expression: "after one day of germination". Germination is not defined in most scientific papers on the subject and there is sometimes little indication as to whether phrases such

as "after one day of germination" refer to the situation in seeds one day after the start of imbibition, one day after the seed was transferred from water to some solid medium, or one day after radicle protrusion. Despite this ambiguity, some authors continue to use such expressions. [3, 5]

1-1.1 LITERATURE

The published literature on seed germination is vast. One bibliography of seed publications contains more than 20,000 abstracts of papers published before 1965. [6] Approximately 3,000 of these papers refer directly to germination and many thousands of others have some application to this subject. Yet even in the period with which the bibliography deals, it does not contain abstracts to all the relevant papers on the subject of seed germination. Of the papers that have been published on this subject only a small fraction deals with metabolism, but this fraction represents a considerable number. Even after the exclusion of papers which deal wholly with seedlings (and yet have titles containing the word germination) many hundreds of papers remain. This is a reflection partly of the importance of the subject and partly of the large number of different seeds available for investigation.

A number of general reviews have been published which contain sections about the metabolism of germinating seeds. [1, 7, 8, 9, 10, 11]

Such reviews give a broad general picture of the subject, but obscure the metabolism that occurs in any particular seed because of the tendency to assume that, in the absence of any experimental proof, similar metabolism occurs in dissimilar seeds. In this thesis there is no such general review. Instead the literature was reviewed for each particular seed studied. In some cases, where there was some justification for believing that the metabolism would be similar, papers about seeds of closely related species were also reviewed.

1-2 AIMS OF THIS INVESTIGATION

1-2.0 THE PROBLEM

The statement made eight years ago by Varner (p. 782, ref. 9) that "in spite of the large volume of literature on the metabolism of germinating seeds it is not possible to specify the order in which metabolic reactions are initiated during imbibition" is not as true to-day. With the use of the tritiated water technique developed by Wilson [12] some information is now available about the order in which metabolic reactions are initiated during the imbibition of intact *Sinapis alba* seeds. [13, 14] Although this information requires some interpretation it represents a valuable advance, an advance that would have been difficult to make using more established techniques. The purpose of this thesis was, at least in part, to determine whether seeds that are embryologically and

morphologically different from *Sinapis alba* have a similar metabolic development during imbibition.

Varner (p. 782, ref. 9) also stated that " ... the germinating seed consists of a collection of tissues which have vastly different functions and fates. Ordinarily, cell division occurs only in the shoot-root axis. The endosperm and cotyledons are primarily, if not solely, reserve tissues ... To obtain meaningful data about the metabolism of seed germination one must therefore study the metabolism of these tissues separately." This statement raised the question of whether or not different parts of the same seed have a similar metabolic development during imbibition. The work for this thesis was designed to answer this question also.

1-2.1 SCOPE OF THIS INVESTIGATION

As outlined in Section 1-2.0, this work was begun with the aim of using the tritiated water technique (described in Section 1-3) to investigate the metabolic development of imbibing seeds. It was planned to extend the work performed on the seed of *Sinapis alba* [13, 14] (which is a member of the dicotyledon class of the angiosperms) to other unrelated seeds, and also to study the metabolic development of different parts of the same seed. Since the tritiated water technique, unlike most other techniques, is particularly suited to studying the metabolism of seeds during the first minutes and hours of

of imbibition, and since the work on *Sinapis alba* had shown that an extensive metabolism was developed over such a time span, it was planned to restrict this present work to the first six hours of imbibition. Because the investigation of any particular seed, even over only the first six hours of imbibition, would occupy a considerable amount of time, it was clearly advisable to carry out both of the planned aspects of investigation in each particular seed studied. Accordingly, it was necessary to procure samples of seeds which were embryologically and morphologically different, and which could be easily dissected into their constituent parts. If a particular species of seeds fulfilled these two criteria, the sample of seeds obtained were required to have a high germination percentage, as it would not be possible to determine the viability of the individual seeds dissected for each experiment.

The embryology and morphology of seeds have been studied for many years, and the following discussion incorporates many, generally agreed conclusions. [1, 7, 15, 16] Seeds can be broadly categorised on the basis of a number of common features. While there are numerous exceptions, which make the distinctions between the different categories somewhat blurred, the differences between most seeds are such that they can be easily placed in one category or another. These broad categories of seeds are

associated with the categories into which plants are classified. Seed bearing plants are divided into two branches, angiosperms and gymnosperms, and a suggested evolutionary relationship between them is shown in Figure 1.1 [17] All viable seeds of both these branches of plants contain an embryo, which develops into the growing plant during germination, and with a few exceptions a seed-coat or testa. In gymnosperm seeds the embryo is usually surrounded by a storage tissue, the female gametophyte. Angiosperms are divided into two classes, monocotyledons and dicotyledons. The seeds of many families in both classes contain a storage tissue, the endosperm. Unlike the storage tissue of gymnosperm seeds, which is formed only from the ovule, the endosperm is formed by a combination of ovule and pollen nuclei during fertilisation. In the mature seeds of some species of dicotyledons there is little or no endosperm, and in such cases the reserves are stored in the two cotyledons of the embryo. As well as these two main types of dicotyledon seeds, the seeds of some species contain, as well as the embryo and endosperm, additional storage tissues. The seeds of the majority of monocotyledon species consist of an embryo, which is differentiated into an axis and a scutellum, and an endosperm. However, one large order (Orchidales) of the monocotyledons has seeds

FIGURE 1.1

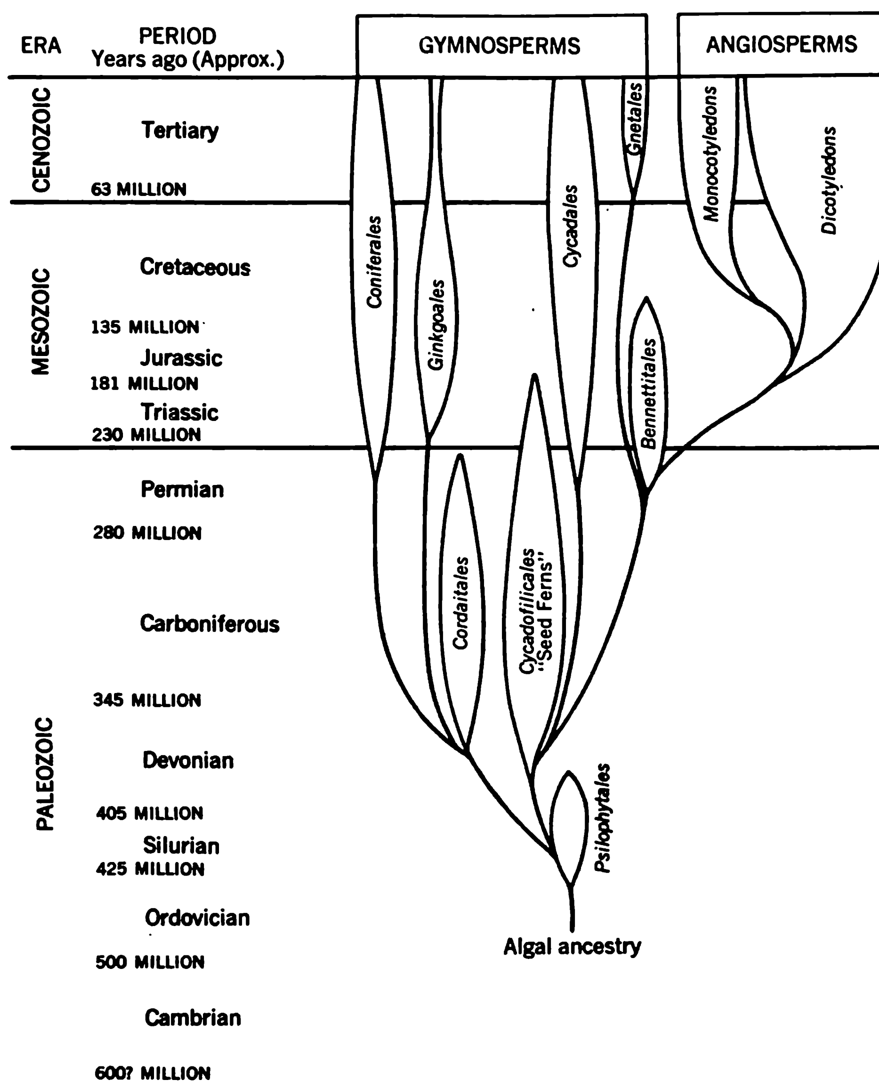


Chart of possible relationships and evolutionary history of the seed plants according to Porter.[17]

which have undifferentiated embryos and which contain little or no storage tissue.

Seeds can thus be divided into five main categories. The seeds of the Orchidales are extremely small, do not consist of differentiated parts, and were not readily available. It was therefore decided to investigate a typical seed from each of the other four categories: a gymnosperm seed with a female gametophyte, a monocotyledon seed with an endosperm, a dicotyledon seed with an endosperm, and a dicotyledon seed without an endosperm.

1-3 MATERIALS

Seeds

Dwarf French Seminole bean (*Phaseolus vulgaris*) seeds were a gift from F. Cooper, Limited, Wellington, N. Z.; they are dicotyledon seeds without an endosperm.

Barley (*Hordeum vulgare*) seeds were a gift from Farmers Co-op Auctioneering Company Limited, Hamilton, N. Z.; they are monocotyledon seeds with an endosperm.

Castor oil (*Ricinus communis*) seeds were packaged by Dessert Seed Company, California, U.S.A., and were purchased from Zenith Seeds Limited, Wellington, N.Z.; they are dicotyledon seeds with an endosperm.

Pinus radiata seeds were a gift from Forest Pesearch Institute, Rotorua, N. Z.; they are gymnosperm seeds with a female gametophyte.

Tritiated water

A 5 ml (5 Ci/ml) supply of tritiated water was obtained from the Radiochemical Centre, Amersham, England.

^{14}C labelled compounds

50 μCi amounts were obtained from The Radio-Chemical Centre, Amersham, England.

Chromatography paper

Whatman No. 4 paper, cut into 27 x 15 cm rectangles was used.

Water

All the water used had been purified by double distillation in a glass still.

Chromatography solvents

Unless otherwise stated solvents were monophasic and were prepared immediately prior to chromatography from reagent grade chemicals.

All extracts from imbibition experiments were chromatographed in two dimensions in the pair of solvents:

(a) 100 g redistilled phenol:29 ml water; stored in the dark at $4^{o}C$. [18]

(b) (1) 3750 ml n-butanol:253 ml water.

(2) 1760 ml propionic acid:2240 ml water.

Solvent (b) was prepared by mixing equal volumes of the stock solutions (1) and (2).[19] More (2) was added dropwise if necessary for production of a one-phase system.

A large number of solvents were tested for co-chromatography. The following solvents were found to be the most satisfactory and were the ones mainly employed.

For two dimensional co-chromatography of amino acids:

(a) n-Butanol:pyridine:water 1:1:1.[20]

(b) n-Butanol:acetone:diethylamine:water 10:10:2:5.[20]

For two dimensional co-chromatography of organic acids:

1. (a) Ethanol:0.920 ammonia:water 16:1:3.[20]

   (b) n-Propanol:eucalyptol:98% formic acid 5:5:2; upper phase.[20]

Solvent (b) was saturated with water and was used two days after preparation.

2. (a) Ethanol:0.920 ammonia:water 16:1:3.[20]

   (b) 2-Ethoxyethanol:eucalyptol:88% formic acid 5:5:2; upper phase.[21]

For one dimensional co-chromatography of sucrose: Ethyl acetate:n-propanol:water 1:7:2.[20]

For two dimensional co-chromatography of glucose and fructose:

(a) Phenol:water 100:29. [18]

(b) Ethyl acetate:pyridine:water 12:5:4. [20]

Chromatography location reagents

A number of location reagents were tested and the following were found to be most suitable: For amino acids a 0.2% solution of ninhydrin in acetone was used.[20] Chromatograms were dipped in this solution, hung at room temperature for 5-10 minutes, and then heated at 105-110° C for 3-5 minutes.

For organic acids an aniline-xylose reagent was used. [20] 1 g of xylose was dissolved in 3 ml of water, 1 ml of aniline was added and the solution was made up to 100 ml with methanol. Chromatograms were dipped in this solution, hung at room temperature for 5-10 minutes, and then heated at 105-110°C for 5-10 minutes.

Ketose sugars (including sucrose, due to release of fructose by phosphoric acid hydrolysis) were located with a naphthoresorcinol reagent. [20] This was prepared by mixing five volumes of 0.2% naphthoresorcinol (1,3-dihydroxynaphthalene) in acetone, with one volume of 9% phosphoric acid in water. After being dipped in this mixture

chromatograms were placed for 5-10 minutes in an oven at 95°C in which a beaker of hot water had been standing for some time.

Aldose sugars were located with an aniline-phthalate reagent. [22] This was prepared by dissolving 0.93 g of aniline and 1.66 g of phthalic acid in 100 ml of water-saturated n-butanol. Chromatograms were dipped in this reagent, hung at room temperature for 5-10 minutes and then heated to 105-110°C (for 5-10 minutes).

Scintillating liquids

For scintillation radioautography, 6 g of p-diphenylbenzene was dissolved in 2.5 l of purified toluene. [23] The purified toluene was obtained by passing technical grade toluene slowly through a 3.5 x 75 cm column of chromatographic alumina, covered by a 5 cm layer of finely powdered silica gel. Sulphur compounds which would have quenched fluorescence of the scintillator were thereby removed. For scintillation counting the following mixture was used: five volumes of analytical grade toluene containing 6 g of 2,5-diphenyloxazole (PPO)/l to three volumes of 2-ethoxyethanol. [24]

Photographic film

The film used was Kodak Royal Blue, R.B. 54 Duplitized X-ray, 35.6 x 43.2 cm sheets. The manufacturers developer and fixer were used.

Reference compounds for co-chromatography

Amino acids: Shandon 0.01 M solutions in 10% isopropanol:water were used.

Sugars and organic acids: 0.2 M solutions in 10% isopropanol:water were used.

1-4 METHODS

1-4.0 GERMINATION PROCEDURES

At intervals of 2-3 months during the periods in which experiments were being performed on a particular species of seed, samples of seeds were tested for their ability to germinate. In the case of the seeds of *Phaseolus vulgaris*, barley and *Pinus radiata* the following procedure was used. Two samples, each of twenty seeds, were placed on two thicknesses of filter paper in separate petri dishes. Enough water was added so that the level was 1-2 mm above the surface of the filter paper. The seeds were left to germinate at room temperature (21±2°C) without other variables being controlled.

In the case of the castor oil seed this procedure was found to be unsatisfactory. Both purchased and locally collected seeds failed to germinate under these conditions, or exhibited less than 10% germination. In the case of these seed the germination procedure used was that advocated by Hawker.[25] The seed-coats were removed from samples of ten seeds and the seeds were placed on filter paper in a large glass beaker. The filter

paper was supported in the bottom of the beaker by an upturned petri dish which was half immersed in water. The edges of the filter paper were bent over the side of the petri dish into the water. With this arrangement the filter paper became moist by capillary action but was not covered by surface water. Evaporation was prevented by covering the beaker with aluminium foil. The beaker was kept at room temperature (21 ± 2°C) without controlling other variables.

1-4.1 DISSECTION

The seeds used were not sterilized before dissection for the reasons outlined below of which the third is of greatest significance.

1. Because of the experimental procedures used, contamination by micro-organisms would have been insignificant, In most of the experiments described in this thesis the seed parts were allowed to imbibe water for six hours or less, while in a few experiments the imbibition time was 12 hours. Contaminating micro-organisms would not be expected to have a significant effect on the metabolism at times such as these.[29] Most of the micro-organisms would be expected to be on the seed-coat which, except in the case of the barley endosperm tissue, was always removed from the seed parts before the experiments were performed.

2. Because seeds are difficult to sterilize, the effective procedures that have been devised involve protracted treatment of the seeds with physiologically powerful chemicals whose effects on the metabolism of germinating seeds have been inadequately studied. Most treatments involve immersing the seeds in aqueous solutions of various compounds, including antibiotics and oxidising agents. For the sterilization of barley seeds, Miflin [26] advocated soaking the seeds in an aqueous solution of sodium hypochlorite for one hour, followed by a thorough water rinsing; while Paleg [27] advocated a six stage process which included the dehusked seeds being soaked in calcium hypochlorite for one hour, and which involved the seeds being in contact with aqueous solutions for a total period of more than two hours. Renwick et al [28] found it impossible to sterilize *Phaseolus vulgaris* seeds by immersing them for short periods (1-15 min) in aqueous solutions of sodium hypochlorite, mercuric chloride, organo-mercury compounds or antibiotics. They were successful only with a process that involved dipping the seeds in 70% ethanol followed by a 20 minute shaking in sodium hypochlorite and then four water rinses.

3. If the seeds had been sterilized, their metabolic development during early imbibition could not have been studied. The sterilization procedures outlined

above involve immersing the seeds in aqueous solutions for periods of more than 20 minutes and sometimes more than two hours. If such sterilization procedures had been used, either before or after dissection ,the seed parts would have been imbibing water for more than 20 minutes (and even longer with some of the procedures) before they were placed in tritiated water. As well as this, if they had been sterilized before dissection, their metabolism might have continued to develop while they were being dissected. Clearly, if such procedures had been used, it would have been impossible to investigate the metabolism during the first 30 minutes of imbibition.

The seeds were dissected under clean, but not sterile conditions: all the instruments used for dissection were carefully cleaned before use, each seed was dissected on a fresh piece of filter paper, and polythene gloves were always worn. That these procedures did not lead to significant contamination of the seed parts by micro-organisms was shown in control experiments with dead seeds. Samples of each of the four seeds used in this thesis were killed by autoclaving them for one hour at a temperature of 120° C. When dissected parts of such seeds were imbibed for six hours in tritiated water, not even trace amounts of labelled compounds were formed.

1-4.2 IMBIBITION IN TRITIATED WATER

From the bulk supply of tritiated water 1 ml quantities were transferred to small, tightly stoppered glass vials.

When required, tritiated water was removed from a glass vial using a disposable micro-pipette attached to a calibrated glass syringe. All manipulations were performed on plastic trays in a fume cupboard , and polythene gloves were always worn in the handling procedures.

For an imbibition experiment, the seed parts were placed in the conical tube of an all-glass tissue homogenizer. The specified amount of tritiated water was added and the tube was sealed with self-gripping transparent plastic film. The tube was left for the specified time at room temperature (21 ± 2°C).

1-4.3 EXTRACTION OF METABOLITES

After the specified period of imbibition, 1 ml of water was added to the imbibing seed parts, the tube was shaken gently for five seconds, and the solution was decanted into a small pear-shaped flask. This process was repeated, and the second solution was added to the first. The combined solutions are referred to as "the water rinse". The time taken to produce the water rinse was not more than one minute. Approximately 4 ml of absolute ethanol was then added to the seed parts and they were ground to a homogeneous suspension (4-5 min). This suspension was centrifuged and the clear ethanolic supernatant was decanted into a pear-shaped flask. The ground seed parts were extracted with a

further 4 ml of absolute ethanol and this second extract was centrifuged and combined with the first. The ground seed parts were then extracted once with 4 ml of water and after centrifugation this extract was decanted into a pear-shaped flask. The three extracts, water rinse, ethanol extract and water extract, were then freeze-dried.

Previous users of the tritiated water technique,[13, 14] extracted seeds with ethanol and water but did not make an initial water rinse. The present author found this initial water rinse to be most useful. In the case of the water extracts and sometimes the ethanol extracts, only a fraction of the extract could be applied to a single chromatogram before overloading of the chromatogram occurred, probably because of dissolved solutes in the extract. In the case of the water rinse the entire amount if necessary, could be applied to a single chromatogram. Possibly because of this difference, the labelled compounds were particularly well resolved on the water rinse chromatograms. During the first minutes of imbibition, most of the activity in the compounds that were labelled was present in the water rinse. For these reasons, the radioautograms shown in the illustrative plates are mainly those of the water rinse chromatograms. All the labelled compounds that were found in the water rinses were also found at the same or a later time in either the

ethanol or water extracts. The appearance of so many of the tritiated compounds in the water rinses should not be regarded as surprising. Exudation of compounds is a normal process in intact seeds during imbibition,[30, 31, 32] and the process is facilitated by removal of the seed-coat.[32] Among other compounds, most of the protein amino acids and a number of common sugars, including sucrose, have been identified from the medium in which seeds were imbibed. The amount of exudation appears to increase when dissected parts are imbibed: one study showed that more than half the amount of ^{14}C-pyruvate absorbed by cotyledons which had been dissected from seedlings, was metabolised to alanine and exuded back into the medium.[33]

1-4.4 CHROMATOGRAPHY OF EXTRACTS

The freeze-dried extracts were dissolved in the minimum amount of the appropriate solvent (ethanol for the ethanol extracts and water for the water rinses and water extracts). The solution of each extract was applied to the origin of two paper chromatograms using a fine glass capillary tube. The solvent was allowed to evaporate from the origin after each application before a further application was made. As much of the solution as possible was applied to the first chromatogram and only a small number of applications were made on the

second chromatogram. Graded loading of chromatograms in this way enabled trace amounts of activity to be identified on the heavily loaded chromatograms, while heavily labelled compounds with similar R_f values were resolved on the lightly loaded chromatograms.

The chromatograms were developed in two dimensions using descending chromatography. The solvents used were phenol: water 100:29 followed by butanol:propionic acid:water 15:7:10 prepared as described in Section 1-3. These two solvents are particularly suitable for separating from plant extracts, groups of compounds having widely different solubilities and occurring in greatly varying concentrations.

1-4.5 DETECTION OF LABELLED METABOLITES

The radioactive compounds on the chromatograms (apart from those travelling with high R_f values in both solvents on the ethanol extract chromatograms) were located by scintillation radioautography. This technique developed by Wilson [12], is the photographic equivalent of scintillation counting. Four chromatograms were stapled onto a used sheet of X-ray film and three pieces of filter paper, impregnated with a sufficient quantity of some tritiated toluene-insoluble compounds, were stapled onto three of the corners to locate the detector film after development and fixing. A shallow metal tray, 1-2 cm deep, was three quarters

filled with scintillating liquid. A used X-ray film was placed on the bottom of the tray, the film with the chromatograms was placed over this, and the chromatograms were covered by a sheet of unexposed X-ray film. A metal cover was placed over the tray and held down by a lead brick. This prevented evaporation of toluene and helped protect the unexposed film from stray light. The whole assembly was covered with two thicknesses of black Italian cloth and stored in a dark-room at room temperature. The chromatograms were usually exposed to film for 1-2 weeks, although in the case of weakly labelled chromatograms and co-chromatograms the exposure time was extended to four weeks. After exposure, the detector film was removed and the toluene on it allowed to evaporate; it was wiped over both surfaces with damp cotton wool to remove any adhering p-diphenylbenzene and developed and fixed using twice the time specified by the manufacturers of the film. After washing and drying, the developed film was located over the chromatograms using the three radioactive pieces of filter paper as markers. The X-ray film used was coated with photographic emulsion on both sides, but because tritium emits only very low energy β particles, the light emitted by the scintillation liquid was too weak to expose the upper photographic emulsion (the emulsion on the side of the film further away from the chromatogram).

To visualise traces of activity more clearly this unexposed layer of emulsion was sometimes removed using a dilute sodium hydroxide solution.

It was known that any lipids extracted in these experiments would be found in the ethanol extracts and would run as an unresolved mass with an R_f value approaching 1.0 in both the phenol:water and butanol:propionic acid:water solvents used.[34] The corners of the ethanol extract chromatograms which contained these compounds, were removed before scintillation radioautography as any such compounds would have been soluble in the toluene scintillator. These corners were placed in scintillation vials and counted for ^3H activity in a Beckman L.S. 233 Liquid Scintillation System. In the tables of results the intensity of labelling in these fractions has been expressed in the same notation as that of the other compounds. This was achieved by comparison of the count rates of the lipid corners to the count rates of a number of excised areas of chromatograms that had previously exhibited varying intensities of labelling on radioautograms.

1-4.6 IDENTIFICATION OF LABELLED METABOLITES

All the compounds that occurred as tritiated metabolites were ones that had been identified by

previous workers. They were tentatively identified from their relative positions on chromatograms by comparison with those on standard chromatographic maps.[35] This tentative identification was verified or refuted by the process of co-chromatography. In this process a strip of paper containing part or all of the radioactive compound being identified was cut from the chromatograms. An easily detectable amount of the pure compound that this radioactive metabolite was suspected to be was applied to this strip. All the material on the strip was then eluted onto the origin of a chromatogram using the technique of Wilson and Calvin.[19] The strip was cut to a point and stapled to one end of a paper wick and the other end of the wick was placed into a trough filled with a suitable solvent. For strips from ethanol extract chromatograms, the solvent used was 95% ethanol:water, while for strips from water rinse and water extract chromatograms, 30-50% ethanol:water solvents were used. The point of the excised strip was placed on the origin of a chromatogram held between glass plates. Both plates had central holes 5 cm in diameter and the origin of the chromatogram was located in the centre of these. The elution apparatus was covered to prevent the wick drying by evaporation and the material on the strip was eluted continuously onto the origin for a period

of 2-3 hours. The elution solvent was evaporated off the chromatogram through the hole in the lower glass plate, with the assistance of a hair dryer when necessary. The chromatogram was developed in two dimensions (one dimension in the case of sucrose) with the solvents described in Section 1-3, and the radioactive spot was revealed by scintillation radioautography. The unlabelled compound which had been added to the strip was detected by dipping the chromatogram in a suitable, location reagent. The spot revealed by this reagent was compared with the spot on the radioautogram. If these coincided in every detail, chemical identity was inferred. (In the case of the compound identified as citrate, some isocitrate may have been present as these two compounds could not be separated by any of the chromatography solvents used).

No individual constituents of the compounds described as lipids and sugar phosphates were identified by co-chromatography. The compounds which had high R_f values (0.9-1.0) in both phenol:water and butanol:propionic acid:water solvents, were assumed to be lipids as lipids are known to behave in this way.[34] The compounds which had low

R_f values (less than 0.25) in both the above solvents were assumed to be sugar phosphates. They could also have been nucleotides, as all phosphate containing compounds have low R_f values in these solvents.[35] The intensities of labelling in the groups of compounds described as lipids and sugar phosphates, were included in the tables of results to show a complete picture of the labelling patterns.

1-4.7 DETERMINATION OF WATER CONTENT.

The rates of water absorption of the various parts of the seeds that were investigated, are given in the experimental sections. These were determined by measuring the water content of each seed part at intervals during imbibition. A seed part was immersed in water for the required time, superficially dried on filter paper, and weighed in a pre-weighed vial. The weighing was accomplished as quickly as possible, because the seed parts began to lose water by evaporation as soon as they had been superficially dried. The vial containing the seed part was heated in an oven at 105° C for 24 hours, cooled in a desiccator and re-weighed. Triplicate determinations of the water content were made at each particular time; the mean of these three values was used for the water absorption graphs in the experimental sections.

1-4.8 HYDROLYSIS OF SUCROSE WITH INVERTASE.

Samples of labelled sucrose, formed in the imbibition experiments with tritiated water, were hydrolysed with invertase (3.2.1.26) to determine whether or not the label was present in both the glucose and fructose moieties. Strips, similar to those used for co-chromatography were cut from areas of chromatograms containing labelled sucrose. A solution of invertase in water (1 mg/ml) was prepared. Some difficulty was experienced in treating the sucrose on these strips with this invertase solution. The strips had of necessity come from chromatograms that had been exposed to film in a scintillation bath. The chromatograms appeared to have become impregnated with p-diphenylbenzene during this process, and the strips could not be wet with the invertase solution. In an attempt to remove the impregnating substance, the strips were eluted with toluene at a rate of 0.5 ml/hr for 24 hours. This treatment was partially successful but there were still areas on the strips which did not absorb water. It was therefore decided to elute the sucrose from each strip using 95% ethanol :water. Approximately 1 ml of ethanol solution was collected in a vial from each strip.

The ethanol was evaporated from the vial under vacuum at room temperature, and approximately 0.01 ml of invertase solution was added. The solution in the vial was applied with a glass capillary tube to the origin of a two dimensional chromatogram which was then developed as described in Section 2-4.11.

1-4.9 TREATMENT OF ^{14}C TRACER COMPOUNDS

The ^{14}C tracer compounds were obtained as solids in 50 μCi amounts, and were stored until required at -10°C. To obtain manageable amounts of activity, the stock quantity of each compound was first dissolved in 5 ml of 20% isopropanol:water. A micro-pipette attached to a graduated syringe was used to transfer a specified amount of the compound, usually 1 μCi, to a small glass tube. The solution in each tube was evaporated under vacuum at room temperature; the tube was sealed with self-gripping plastic film and stored until used in a sealed container over silica gel at -10°C.

The ^{14}C labelled tracer compound to be used was purified by chromatography. The compound was dissolved in 0.1 ml of 20% isopropanol:water and applied to the origin of a one dimensional chromatogram.

The chromatogram was developed with the solvent butanol:propionic acid:water 15:7:10. The position of the pure radioactive compound was identified by radioautography, the chromatogram being exposed to film for one day. The strip containing the compound was cut from the chromatogram and stapled to a paper wick whose other end was placed in a solvent trough. The compound was eluted into a small glass vial over a period of 2-3 hours, using an ethanol:water solvent. The solvent was evaporated under vacuum at room temperature, and the purified radioactive compound was dissolved in the amount of water required for the experiment. In each ^{14}C tracer experiment a small amount of the solution containing the ^{14}C compound was treated as a blank. It was left under the same conditions as those used for the tracer experiment, and was chromatographed in two dimensions in the same way as the extracts from the tracer experiment.

1-5 PRINCIPLES AND FEATURES OF BIOLOGICAL STUDIES WITH TRITIATED WATER

1-5.0 ADVANTAGES OF THE METHOD

There are a number of advantages in using tritium in the form of tritiated water as a tracer

in biochemical systems.

1. By using tritiated water, the metabolism of a biological system can be studied *in vivo*. In many biochemical studies, experiments are carried out with cell-free extracts of various kinds. Such experiments are performed in an environment vastly different from that of the cell. The metabolism of the intact cell is then inferred from the results of these experiments. While such techniques are indispensable in elucidating the metabolism of all biological systems, they are by themselves inadequate. Tritium in tritiated water readily penetrates the intact cell wall and its internal membranes, and by its incorporation into various compounds provides an *in vivo* indication of the cell's metabolism.

2. The method is applicable to certain biochemical problems which are unable to be easily studied by other means. The metabolism of seeds in the first minutes of imbibition can be studied by allowing the seeds to imbibe tritiated water. It is difficult to study this situation using other techniques.

3. The method is cheap and simple. Provided small amounts of tissue are use, only a small amount of tritiated water is required and this is cheap compared to the cost of other radiochemical tracers.

No complicated apparatus is needed when using this method.

4. The method is sensitive. The extremely short range of the β radiation emitted in tritium decay allows high autoradiographic resolution. Using the technique developed by Wilson [12] a radioactive spot whose area on a paper chromatogram is 1 cm^2, and whose activity is 0.1 μCi can easily be detected after 50 hours exposure to film in a scintillation bath.

1-5.1 DISADVANTAGES OF THE METHOD

There are two main disadvantages in using tritium as a tracer, the isotope effect and radiation damage.

Isotope effect:

The isotope effect of tritium is larger than that of any other isotope because the mass of tritium is three times that of protium. The isotope effect of tritium in a biochemical reaction depends on the mechanism of the reaction and on the site or sites in which the substrates are or can be labelled. In some cases the effect is very large [36] but in others it is negligible.[37] However, in tritiated water of specific activity 5 Ci/ml, only 0.15%

of the hydrogen atoms are tritium. Hence, while tritium atoms participating in reactions would affect the rates of some reactions, their presence in such low concentrations would not be expected to alter the operation of metabolic sequences or have any effect on metabolic pool sizes. Since the work involved in this thesis is qualitative rather than quantitative, any changes in the rates of enzymic reactions are unlikely to affect the interpretation of results.

Radiation damage:

Radiation damage is much less important than the isotope effect in most techniques that use tritium as a tracer. This is because quantitative results are usually being sought, because tritium emits only a very low energy radiation (0.018 Mev. β particle) and because tritium of relatively low specific activity is used. In the experimental technique used in this thesis, radiation damage is more important than the isotope effect . This is because the results sought are not quantitative and because high specific activity tritiated water (5 Ci/ml) is used.

Previous workers who used this technique were aware of this disadvantage. Both Mann [23] and Spedding[38] made calculations based on many assumptions, of the radiation dose to which *Sinapis alba* seeds would be

exposed while they were imbibing tritiated water of specific activity 5 Ci/ml. Spedding concluded that each seed received 36,000 rep/day. Mann calculated that the dose was an order of magnitude higher than this but concluded that the actual dose was probably somewhere in between the two values. Baillie [39] showed that the dry *Sinapis alba* seed is able to withstand a dose of 1×10^6 rep (actually 1×10^6 rad but the difference is minor compared to the assumptions already involved) of γ radiation without any effect on its ability to germinate; although the seeds took longer to germinate, the germination percentage remained the same. On this basis, Mann [23] and subsequent investigators [18,40] concluded that radiation damage would probably be insignificant in their experiments.

Spedding[38] was the only user of this technique to make any attempt to justify this conclusion empirically. He allowed ten *Sinapis alba* seeds to imbibe tritiated water for one hour before placing them in non-tritiated water. They germinated normally. He did not unfortunately perform any further such investigations. In a recent paper Durzan et al [41] reported that the germination of

Pinus banksiana seeds was inhibited by 96% when the seeds were left to germinate in tritiated water of specific activity 100 mCi/ml. Their definition of germination excludes any seeds which take more than four days to germinate. The tritiated water may therefore only be causing the seeds to take longer to germinate (an effect observed in the case of *Sinapis alba* seeds exposed to γ radiation [39]). Nevertheless these results cast doubt on the non-empirical conclusions of Mann.

In this thesis four relatively large seeds were studied. The quantity of water required to germinate (radicle protrusion) these seeds is also relatively large. To attempt to germinate any of these seeds in tritiated water would therefore involve the handling of large quantities of radioactivity and this would be expensive. Despite this expense it was clearly desirable in light of the above results to carry out some such attempt.

Accordingly, four samples, each of ten barley seeds, were allowed to germinate on filter paper that had been moistened with 2 ml of water. The germinating seeds were kept in tightly sealed cylindrical glass containers at room temperature. The containers were tightly sealed to prevent tritiated water vapour

escaping, and they were cylindrical so that a small amount of water was sufficient and yet a large quantity of oxygen was available. The first sample of seeds was placed in 2 ml of 5 Ci/ml tritiated water, the second sample was allowed to imbibe 5 Ci/ml tritiated water for six hours before being placed in 1.5 ml of non-tritiated water to germinate, the third sample was placed in 2 ml of 0.5 Ci/ml tritiated water, and the fourth sample, a control, was placed in 2 ml of non-tritiated water. The second, third and fourth samples all had the same germination percentage (80%) and germinated over the same period of time (2-4 days). In the first sample the radicles protruded slightly from two seeds, but otherwise there was no sign of germination even after four weeks.

It is clear from the above germination trials that when barley seeds are exposed for long periods of time to high specific activity tritiated water the biological damage is sufficient to prevent radicle protrusion occurring. However, in the experiments in this thesis the seeds were rarely exposed to tritiated water for more than six hours and clearly, at least in the case of barley, this would not have been deleterious to the seeds.

It would be desirable to investigate much more fully the ability of seeds to germinate in tritiated water. Such investigation lies outside the scope of this thesis. It will require considerable expenditure in procuring sufficient amounts of tritiated water and stringent precautions will be needed to avoid the resulting increased radiation hazard. As regards this latter point the author wishes to note that, when he was performing regular experiments in fume cupboards with relatively small amounts of tritiated water (0.5 Ci), the tritium activity in his body rose to a significant level above background. Calculations from saliva activity showed that it could have been as high as 0.2 of the allowable body burden, which was 1 mCi.

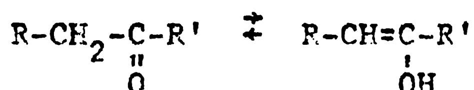
1-5.2 BASIS FOR INTERPRETATION OF RESULTS

In considering the labelling of metabolic compounds in tritiated water, it is useful to distinguish three types of bound hydrogen atoms.

1. Labile Hydrogen. The hydrogen atoms of the groups -OH, $-NH_2$, =NH, -COOH, -SH are readily ionisable and exchange rapidly with free protons in solvents such as water. In tritiated water,

compounds containing these groups rapidly become labelled whether or not there is any metabolism. However, when these compounds are subsequently placed in non-tritiated aqueous solvents, as occurs during extraction and chromatography, the label is rapidly lost

2. Semi-labile hydrogen. When there is a carbonyl group in a compound, hydrogen atoms on adjacent groups can be rendered labile by keto-enol tautomerism:



$$R\text{-}CH_2\text{-}\underset{\underset{O}{\|}}{C}\text{-}R' \rightleftarrows R\text{-}CH{=}\underset{\underset{OH}{|}}{C}\text{-}R'$$

The rate of exchange of such hydrogen atoms varies greatly depending on other attached groups. In compounds where the rate of exchange is sufficiently rapid, the situation will be the same as in 1., and the label will be lost during extraction and chromatography. In some cases the rate of exchange is slow enough for a compound labelled in such a position to retain its label when placed in a non-tritiated aqueous solvent. One example of such a case is the hydrogen atom attached to C-2 of glucose-6-P[42]. Clearly in cases such as this the label can only be incorporate metabolically.

Since the rate of exchange is too slow for the label to be lost non-metabolically it will also be too slow for it to be acquired non-metabolically.

3. Non-labile hydrogen. Hydrogen atoms bound directly to a carbon atom and not involved in keto-enol tautomerism can be exchanged in a biological system only through a biochemical (or chemical)reaction . If a tritium atom is incorporated into such a position it can be stated with certainty that the compound concerned has been involved in such a reaction. This is the essential feature underlying the technique used in this thesis. (The possibility of chemical reactions being responsible for the compounds labelled in this present work was considered unlikely because when seeds were imbibed in tritiated water after being autoclaved for one hour at 120°C (Section 1-4.1) no labelled compounds were formed). All references in this thesis to compounds "labelled" with tritium **refer**, unless otherwise qualified, to compounds containing tritium in a non-labile position.

In studying biological systems, there is an essential difference between the information gained from using tritiated water and that gained from using compounds labelled with ^{14}C. If,when a ^{14}C tracer

compound is added to a biological system, another compound becomes labelled, it can be stated that there is a metabolic pathway operating between the two compounds. If the two compounds are adjacent members of a metabolic sequence common to other biological systems, this is strong evidence that the reaction between the two compounds in that sequence is occurring. If however, two adjacent compounds in a well known metabolic sequence become labelled when a biological system is placed in tritiated water, this demonstrates only that both compounds were involved in some metabolism. They may or may not have become labelled in the reactions of that particular sequence. Such information by itself cannot show whether or not there is a metabolic pathway operating between the two compounds in that system. If a compound is present in a seed but unlabelled at an early stage of imbibition, and it then becomes labelled at a later stage of imbibition, this is an indication that a reaction involving that compound has begun to operate to a significant extent at some time between the two stages. As before however, the particular reaction involved cannot be identified with certainty, but it must be one in which tritium is incorporated into a stable position.

The question remains as to how the results of the experiments performed with tritiated water are to be interpreted. Clearly, no matter what compounds are labelled, such experiments can never provide conclusive evidence for the existence of any particular metabolic reaction or pathway. A number of previous workers have used tritiated water to investigate the metabolism of seeds during the first hours of imbibition.[18, 23, 38, 40] They studied in some detail the possible reactions of those compounds that they had found to be labelled with tritium. They eliminated those reactions which would not, or were unlikely to form labelled compounds in the presence of tritiated water. It transpired that among the possible reactions that remained were those of a few, almost universal, interrelated metabolic pathways. They suggested that the labelled compounds they had found were formed by the reactions of these pathways, and that therefore these pathways were active at an early stage of imbibition. They were aware of the uncertainties involved in this conclusion.

In this thesis, the interpretation of results has, of necessity, also been based on this approach. Wherever possible however, the results of the experiments have been interpreted in the light of any

other relevant information available. This was often the case, as the four seeds used for the experiments reported in this thesis have been better studied during the first day of imbibition than most other seeds. This was in fact one reason for choosing them. In the case of barley and *Phaseolus vulgaris* seeds a number of additional experiments were also performed, and the results of these were of some assistance in interpreting the results of the imbibition experiments with tritiated water.

1-5.3 CONCLUSIONS FROM PREVIOUS WORK.

The tritiated water technique developed by Wilson[12] has been used to investigate the metabolism of intact seeds during the first hours of imbibition. Investigations have been made under aerobic [18, 23, 38] and anaerobic [18, 40] conditions, and at 0°C [23] and room temperature.[18, 38, 40] These studies have been confined almost entirely to the *Sinapis alba* seed, although there has also been a very limited study of the *Latuca sativa* seeds.[38] Both *Sinapis alba* and *Latuca sativa* are members of the dicotyledon class of the angiosperms. The results and conclusions from the experiments in which *Sinapis alba* seeds were imbibed in tritiated

water under air at room temperature, are summarised below, and it was claimed that similar results were obtained for the *Latuoa sutiva* seed. [38] This summary is based mainly on the paper by Spedding and Wilson, [13] but takes account of the later identification of some compounds reported by Missen and Wilson.[14] Even allowing for these compounds that were later identified, the results reported in these two papers are not entirely in agreement with each other, although the same general conclusions are reached.

The first compounds to be labelled, after five minutes of imbibition were the amino acids 4-aminobutyrate and aspartate. Glutamate and alanine were labelled after ten minutes, malate and citrate after 15 minutes, succinate and sucrose after 30 minutes, lactate and lipids after three hours and sugar phosphates after six hours. On the basis of these results it was concluded:

1. That the amino acids glutamate, alanine and aspartate probably became labelled by the action of deaminases and transaminases.

2. That the lebelling of these amino acids was an indication that their 2-oxo acids were being formed. It was suggested that these unstable oxo acids could

be stored as amino acids in the resting seed and then released when required by deamination and transamination reactions.

3. That labelled 4-aminobutyrate was formed from glutamate either by decarboxylation or transamination.

4. That the Krebs cycle was operating in the seed at an early stage of imbibition. This was based on the fact that from 30 minutes onwards, citrate, malate and succinate were all labelled, as well as the amino acids glutamate, alanine and aspartate.

5. That the degradation of lipid reserves was probably occurring after three hours and supplying acetyl-CoA to the Krebs cycle.

1-5.4 INTERPRETATION OF RESULTS IN DETAIL

In the experiments reported in this thesis ten different labelled compounds were identified.
The amounts of labelling in the groups of substances that behaved during chromatography like lipids and sugar phosphates were tabulated, but no compounds in either of these classes of substances was actually identified. The same ten unidentified compounds, and the same two classes of identified compounds, were also found to be labelled in *Sinapis alba* seeds. It is not surprising, therefore, that the

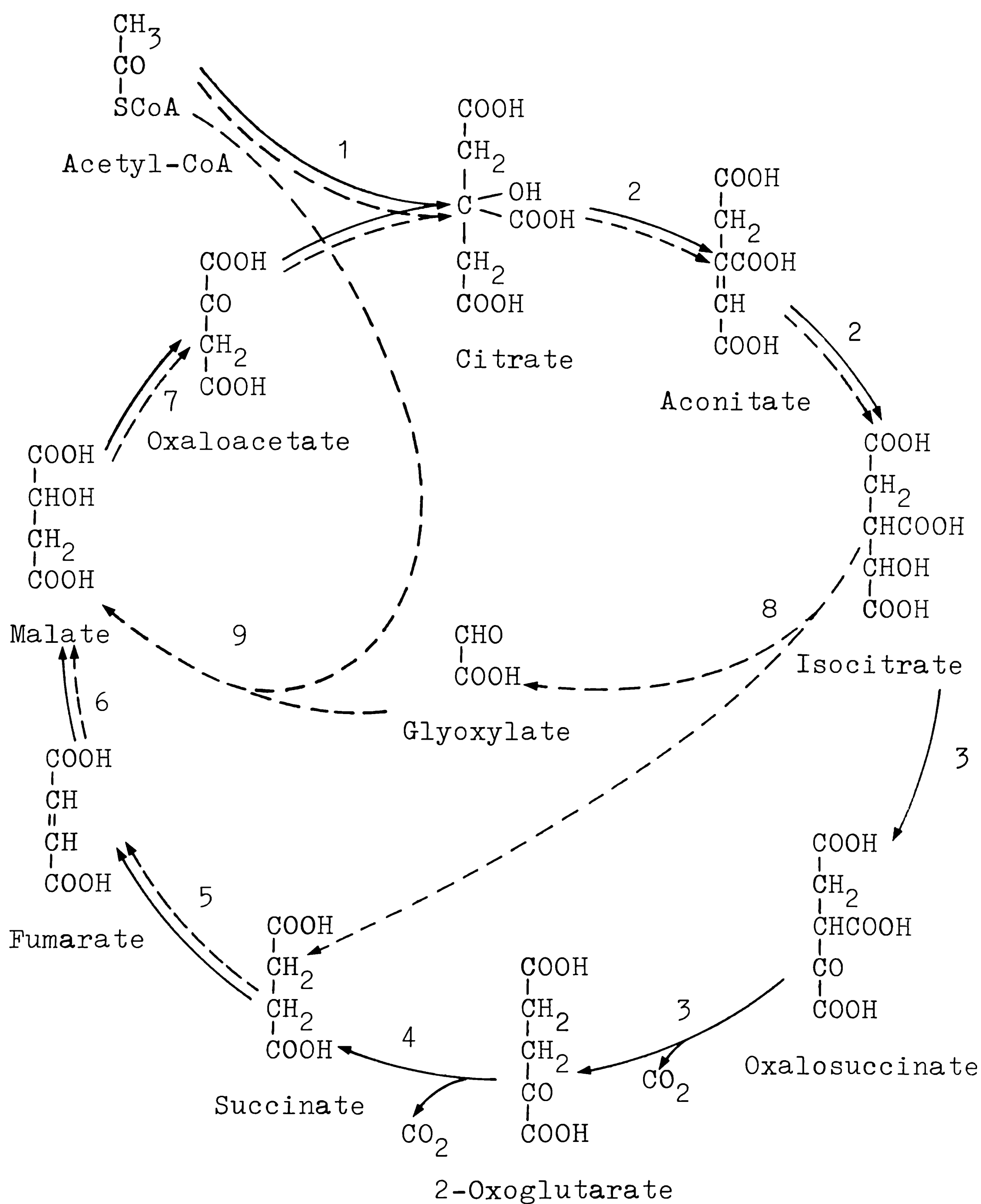
interpretations made in this section of the labelling that occurred in each of these compounds bears much similarity to the interpretations made by previous workers.[18, 23, 38]

Krebs cycle:

It was suggested in the work on *Sinapis alba* seeds,[13] and it is suggested for most of the seed parts investigated in this thesis, that the Krebs cycle (Fig.1.2) is active during early imbibition. This is based mainly on the fact that six compounds that are related to, or members of, the Krebs cycle, become labelled when these seed parts are imbibed in tritiated water. The six compounds concerned are: aspartate, glutamate, alanine, citrate, malate and succinate.

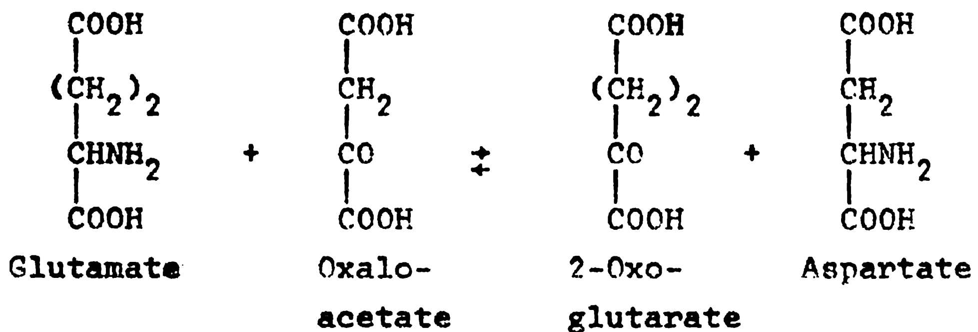
Aspartate, glutamate and alanine can be converted by either transamination or deamination to respectively, oxaloacetate, 2-oxoglutarate and pyruvate. The first two of these oxo acids are members of the Krebs cycle (Fig.1.2) Pyruvate combines with CoA to form acetyl-CoA so it is closely related to the Krebs cycle. Transaminases and at least one deaminase that would carry out these conversions have been found in resting seeds.[43, 44, 45]

FIGURE 1.2

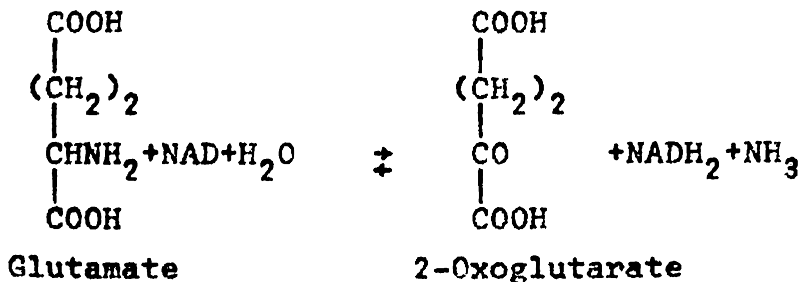


Krebs(—) and glyoxylate(--) cycles: 1, citrate synthase; 2, aconitase; 3, isocitrate dehydrogenase; 4, 2-oxoglutarate dehydrogenase; 5, succinate dehydrogenase; 6, fumarase; 7, malate dehydrogenase; 8, isocitrate lyase; 9, malate synthase.

The three amino acids involved would become labelled by the action of their transaminases in tritiated water. This can be asserted from the results of experiments with deuterated water.[46, 47] A typical reaction is that catalysed by aspartate aminotransferase (2.6.1.1):



In the case of the deaminase that has been found in seeds, glutamate dehydrogenase (1.4.1.2), labelling of glutamate would occur only if the reduced cofactor $NADH_2$ was itself labelled.[48] However, this enzyme might be required to provide the initial oxo acid for the operation of the transaminases. The reaction catalysed by this enzyme is:

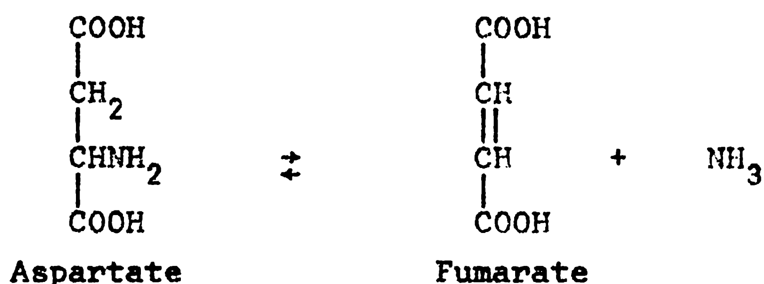


Citrate, malate and succinate are all members of the Krebs cycle. All three acids would become labelled by the action of the Krebs cycle in tritiated water. This was shown conclusively in experiments with the Krebs cycle enzymes fumarase (4.2.1.2),[49] reaction 6 of Fig. 1.2; succinate dehydrogenase (1.3.99.1),[50] reaction 5 of Fig. 1.2; and also aconitase (4.2.1.3),[51] reaction 2 of Figure 1.2; and citrate synthase (4.1.3.7),[52] reaction 1 of Fig. 1.2.In deuterated water these enzymes caused malate, succinate and citrate respectively to become labelled with deuterium.

The fact that these six compounds become labelled does not definitely establish that the Krebs cycle is operating. The interconversion of the three amino acids with their corresponding oxo acids could also occur in the absence of the Krebs cycle. Citrate, malate and succinate could possibly become labelled in tritiated water by the action of the glyoxylate cycle (also shown in Fig. 1.2): malate in the reaction catalysed by malate synthase (4.1.3.2), reaction 9 of Fig. 1.2 ; succinate and isocitrate (which could not be distinguished from citrate by the chromatography solvents used in this thesis) in the reaction catalysed by isocitrate lyase

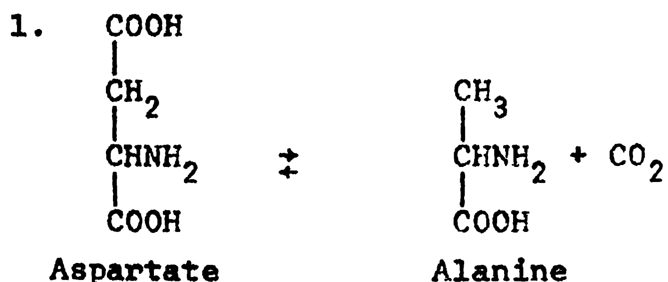
(4.1.3.1), reaction 8 of Fig. 1.2.

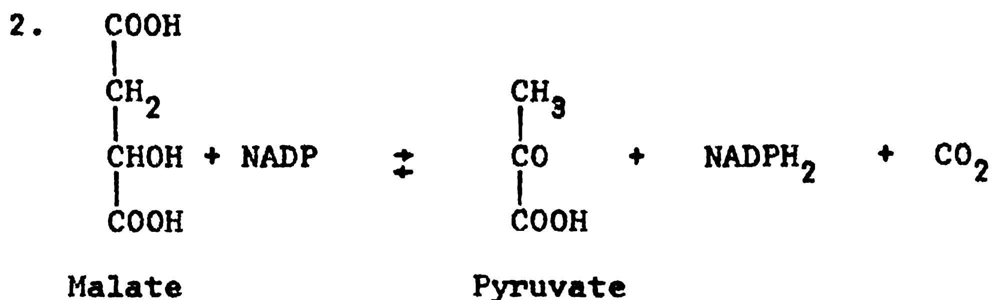
These six compounds could also become labelled in reactions that are not closely associated with each other. Experiments with deuterated water have shown that aspartate would be labelled in tritiated water by the action of the enzyme aspartase (4.3.1.1).[53] The reaction is:



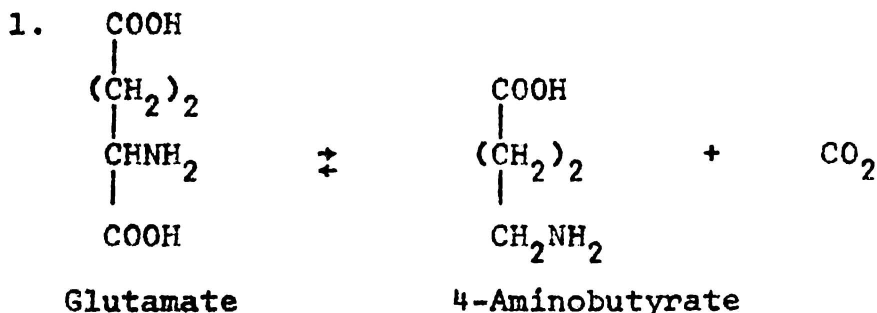
Since a proton is apparently incorporated from water into (1.) alanine by the action of aspartate 4-decarboxylase (4.1.1.12), and into (2.) malate by the action of malate dehydrogenase (decarboxylating) (1.1.1.40), these compounds could become labelled if those reactions were occurring in tritiated water. The reactions are:

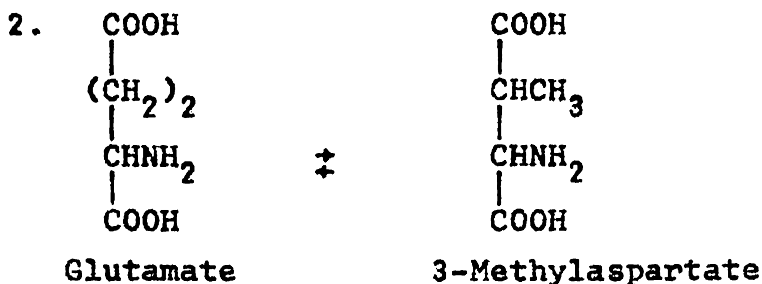
1.





There are some reactions where it can be stated that compounds will not become labelled if that reaction is occurring in tritiated water. Experiments with deuterated water have shown that glutamate would not become labelled by the action of either (1.) glutamate decarboxylase (4.1.1.15)[54] or (2.) glutamate mutase (5.4.99.1)[55] in tritiated water. The reactions catalysed are:



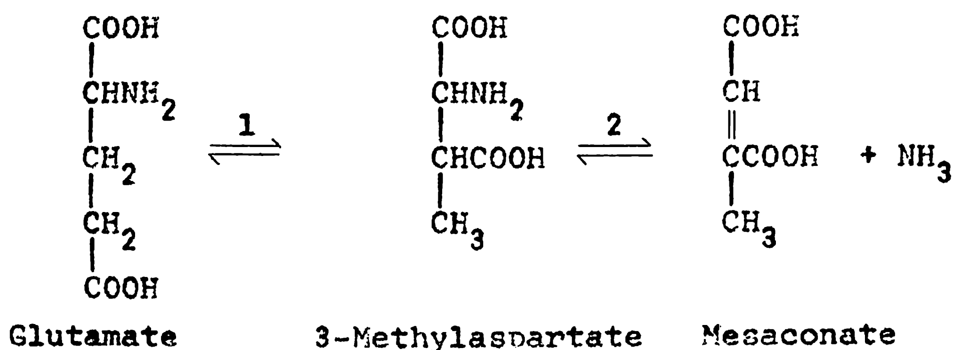


Any other reactions in which the mechanism indicates that a carbon to hydrogen bond is not broken or formed, would also not be expected to cause compounds to become labelled in tritiated water. In the case of the three amino acids this includes those reactions such as protein hydrolysis, in which only a peptide or amide bond is broken or formed.

There are a large number of other reactions in which these six compounds could be involved. In some cases there is some information available about the mechanism of these reactions and a prediction could be made about the possibility of labelling in tritiated water. In other cases, reactions and whole pathways are known or believed to exist, but have not as yet been studied in any detail. As will become apparent in the following discussion, little is to be achieved by considering all the possible reactions, no matter how improbable, that might or might not have been responsible for the labelling of compounds that occurs when seeds are imbibed in tritiated water.

There is a further complicating factor in interpretation. As well as compounds which incorporate tritium directly from tritiated water in an enzymic reaction, there is also the possibility that tritium could be incorporated indirectly. A tritium label

could be incorporated into a compound in one reaction of a metabolic sequence and this label could be retained in subsequent steps of the sequence. Because of different pool sizes of the various members of the sequence, the label might eventually be detected in a compound which is metabolically remote from that into which the label was first incorporated. This is a well known phenomenon in ^{14}C tracer experiments. The same situation could sometimes occur for tritium, especially if the tritium were in a position that was not involved (and therefore not discriminated against by isotope effects) in the further enzymic reactions of the sequence . A simple example of such a system is that provided by the two step pathway between glutamate and mesaconate. The two enzymes are (1) glutamate mutase (5.4.99.1) and (2) methylaspartate ammonia-lyase (4.3.1.2). The sequence is:



It has been shown that 3-methylaspartate becomes labelled by the action of methylaspartate ammonia-lyase (4.3.1.2) in tritiated water, and that this label is then passed on to glutamate in the reaction catalysed by glutamate mutase (5.4.99.1).[56] Even though glutamate does not incorporate a label directly from tritiated water by the action of glutamate mutase (5.4.99.1), it can become labelled if both the enzymes of the sequence are present.

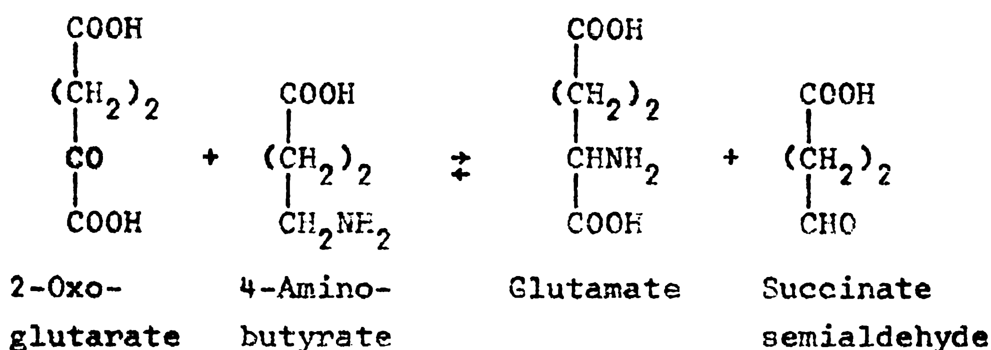
From the preceding discussion it can be seen that the number of ways in which a compound could become labelled in tritiated water is theoretically large. In a situation such as this the question must be asked as to what is the simplest and most likely explanation that would explain the labelling of these compounds, after having taken account of all the information that is at present known about seed metabolism. With respect to the labelling in the three amino acids and three organic acids being discussed here, the answer is the Krebs cycle and the transamination and deamination reactions associated with it. Although the glyoxylate cycle would explain the labelling almost as well as the Krebs cycle, its existence in seeds during early imbibition can be discounted on the basis of the further information presented in the experimental sections.

To establish beyond doubt that an organism has an active Krebs cycle the accepted criteria are the presence of all enzymes of the cycle and a net flow of compounds through the cycle *in vivo*. Clearly, the evidence presented in the preceding discussion does not fulfil either of these criteria. The amount of information available about the metabolism of seeds during the first hours of imbibition has been severely limited because of the difficulty of applying standard biochemical techniques to this biological material. The tritiated water technique used in this thesis provides information about the metabolism of seeds during early imbibition. This information indicates the possible and often the probable metabolism, but it never provides definitive evidence that a particular reaction is occurring. In the absence of definite proof, the most probable explanation must be presented. It is on this basis that it was suggested above that the Krebs cycle is occurring and it is on this basis that the interpretation of the labelling is made in the following discussion.

4-Aminobutyrate:

There is only one well established pathway involving 4-aminobutyrate in higher plants (see Section 6.1). In this pathway glutamate by decarboxylation

forms 4-aminobutyrate which then undergoes transamination with 2-oxoglutarate to form succinate semialdehyde. The two enzymes are respectively, glutamate decarboxylase (4.1.1.15) and 4-aminobutyrate aminotransferase (2.6.1.c). The equation for the first reaction was presented previously (p. 46). The second reaction is:

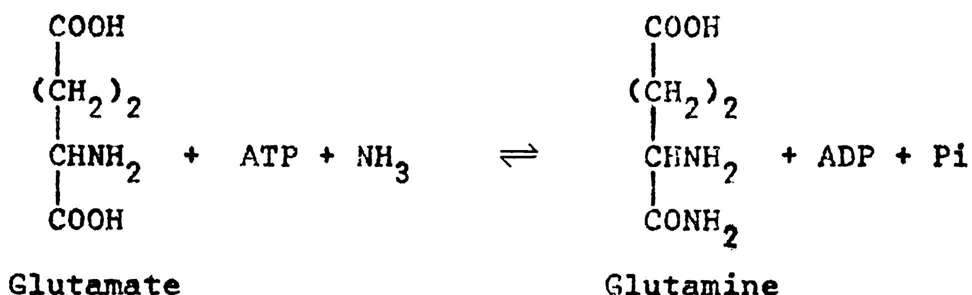


Labelled 4-aminobutyrate would be formed if either of these reactions were occurring in tritiated water. In the case of glutamate decarboxylase (4.1.1.15), this has been shown in experiments with deuterated water.[54] The other enzyme is a transaminase, and the accepted transaminase mechanism, based on experiments with deuterated water, involves incorporation of a proton from solution.[46, 47]

Glutamine:

In all the experiments reported in this thesis and in the work on *Sinapis alba* seeds in which glutamine was labelled with tritium,

glutamate was also labelled. The enzyme glutamine synthetase (6.3.1.2) has been found in seeds,[57] and would form labelled glutamine if labelled glutamate was the substrate. For these reasons it is suggested in this thesis that the labelled glutamine is formed in the reaction catalysed by this enzyme. The reaction is:



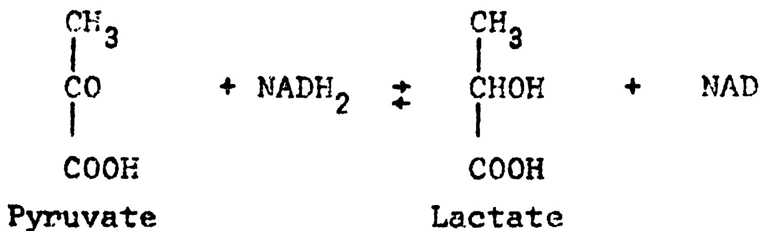
Glutamate Glutamine

Clearly this would be an example of indirect incorporation of a label: since the mechanism apparently only involves the formation of an amide bond, tritium would not be expected to be incorporated directly from tritiated water in this reaction. Labelled glutamine could only be formed in this reaction if labelled glutamate was present.

Labelled glutamine would also be formed in the presence of the enzyme glutamine-oxoacid aminotransferase (2.6.1.15). In this reaction tritium would be incorporated directly from tritiated water by the usual transaminase mechanism.

Lactate:

Lactate is almost certainly labelled in the reaction catalysed by lactate dehydrogenase (1.1.1.27). This is the only established reaction in plants in which lactate is involved, and experiments with deuterated waterhave shown that lactate would become labelled with tritium if the reduced cofactor $NADH_2$ was labelled with tritium in the right position.[58] As well as this, Missen [18] and Reynolds[40] both reported increases in the amount of labelled lactate that was formed when seeds were imbibing tritiated water under anaerobic conditions. It would be expected that under these conditions, as opposed to aerobic conditions, more of the glycolytic flow would be divertedthrough the reaction catalysed by lactate dehydrogenase (1.1.1.27). The reaction is:



Sucrose and sugar phosphates:

The labelling of sucrose was the subject of further investigation in this thesis. The interpretation of this labelling is presented in Section 2-4.

The compounds described as sugar phosphates were never identified as such. Their existence was only inferred from the fact that during chromatography some labelled compounds had low R_f values, characteristic of sugar phosphates, in both the phenol:water and butanol:propionic acid: water solvents. In the experiments reported in this thesis, the intensity of the label in these compounds appeared to be associated with the intensity of the label in sucrose. For this reason it was tentatively suggested that the labelled compounds with low R_f values, might be those which are immediate precursors of sucrose.

Lipids:

In the work on *Sinapis alba* seeds and the seeds studied in this thesis, the lipid fraction (defined as compounds with high R_f values in both the phenol:water and butanol:propionic acid:water solvents) became labelled in the first few hours of imbibition in tritiated water. These labelled lipid fractions were not analysed to determine their constituents. However, it is tentatively suggested that this label is introduced during lipid synthesis. Most of the lipid fraction of seeds is composed of fatty acids, either free or astriglycerides (p. 42, ref. 7), and a study of

the reactions involved shows that labelled fatty acids would be formed if they were being synthesized in tritiated water. The breakdown pathway for fatty acids in plants is most probably β-oxidation, although other pathways cannot be definitely excluded as possibilites. [59, 60] Individually, the reactions of the β-oxidation system are reversible, [60] and since some of the reactions would be expected to incorporate tritium from tritiated water, labelled lipids could theoretically be formed. However, this is unlikely as *in vivo* β-oxidation rapidly converts long chain fatty acids to acetyl-CoA without any intermediates being released. [60, 61]

A labelled lipid fraction would also be formed if the seeds were synthesizing triglycerides from labelled glycerol 3-phosphate. This compound is formed in the reaction catalysed by glycerolphosphate dehydrogenase (1.1.1.8). The reaction is:

$$\begin{array}{l} CH_2OH \\ | \\ CO \\ | \\ CH_2OP \end{array} + NADH_2 \rightleftarrows \begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OP \end{array} + NAD$$

Dihydroxyacetone phosphate — Glycerol 3-phosphate

Labelled glycerol 3-phosphate would be formed in this reaction if the reduced cofactor $NADH_2$ had been labelled in other reactions occurring in tritiated water.

CHAPTER 2

EXPERIMENTS ON *PHASEOLUS VULGARIS* SEEDS

2-1 INTRODUCTION

2-1.0 GENERAL

The seed of *Phaseolus vulgaris* was the first seed studied for this thesis. The particular variety was described by its suppliers as the "dwarf French bean". This is a member of the tribe Phaseoleae, of the sub-family Lotoideae (the pea sub-family), of the family Leguminosae, also named the Fabaceae (p. 308, ref. 17). The Leguminosae family belongs to the dicotyledon class and is one of the three largest families of the Angiosperms.

The most interesting characteristic of seeds of the Leguminosae is the lack of an endosperm in the mature seed. During fertilisation the endosperm is formed, as in other angiosperms, by fusion of the two polar nuclei with the second sperm nucleus; but during subsequent development of the seed the endosperm is absorbed by the embryo. (p. 2, ref. 7). The mature seed is composed only of an embryo and a seed-coat. The cotyledons are large, take up most of the volume of the seed and act as the storage organs.

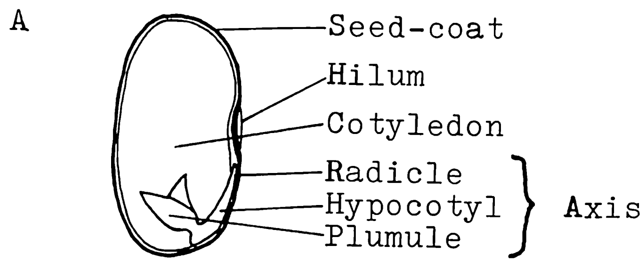
This seed was selected for study because:

1. It had a high germination percentage (above 95%).
2. It was big enough to dissect yet small enough for several axes to be imbibed together in a small amount of tritiated water.
3. It was easy to dissect: the seed-coat was firmly attached to the cotyledons but once it was cut away the cotyledons almost fell apart and the exposed axis could be gently prised out.
4. It is a seed whose storage reserves are mainly starch and whose storage organs are the cotyledons.

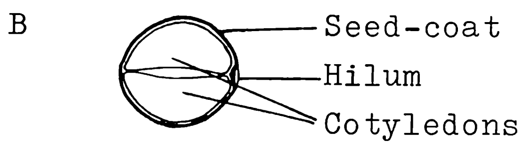
2-1.1 DESCRIPTION

In the variety of *Phaseolus vulgaris* used, the average seed was 1 cm long and 0.05 cm in diameter. The seeds were hard and the seed-coat thin but tough. The seeds had a mottled brown and light purple colour. The average seed weighed 0.3 g. As shown in Fig. 2.1 most of a *Phaseolus vulgaris* seed is composed of the cotyledons. These together with the embryonic axis form the embryo. This axis, which comprises only 1% of the seed, is the part which develops into the growing plant. It is situated between the cotyledons and is firmly attached to the base of each cotyledon. The axis consists of a plumule and a hypocotyl. In the intact seed the tip of hypocotyl, the radicle, is directed towards the hilum (which is a scar that indicates where the seed was attached to the pod). The plumule develops into the

FIGURE 2.1



1 cm



Dwarf French bean(*Phaseolus vulgaris*) seed shown in (A) longitudinal and (B) transverse sections.

first true leaves of the seedling, the hypocotyl becomes the stem, and the radicle becomes the root. Germination is indicated by the radicle protruding through the seed-coat near the hilum. In the seeds used for this present work, radicle protrusion began to occur after 24 hours of imbibition at room temperature. Half the seeds had germinated after 36 hours and the remainder by 60 hours.

Phaseolus vulgaris has an epigeal seedling. This means that during seedling growth the cotyledons are carried above ground.

2-1.2 STORAGE

While seeds generally remain viable when kept at low humidities and temperatures, there are many exceptions[62]. The situation with *Phaseolus vulgaris* does not seem to be at all clear [62]. As the temperature and humidity are lowered there is an increase in the percentage of hard-seeds. In hard-seeds the coat is impermeable to water and must be scarified before the seed will germinate. This is a trait of seeds of the Leguminosae and is believed to be one of the causes of their long-lived viability. However, it is thought that other forms of dormancy are also induced by storage at low temperatures and humidity. To avoid these complications the seeds used for this present work were stored at room temperature and humidity. Under these conditions a germination percentage of greater than 95% was sustained during the

the 18 months in which the experiments were performed.

2-1.3 COMPOSITION

A comprehensive investigation of the composition of *Phaseolus vulgaris* seeds was carried out by Eichelberger.[63] He reported the following figures: starch, 35.22%; protein, 22.69%; moisture, 10.08%; insoluble hemicelluloses, 8.9%; dextrins, 3.23%; total sugar, 2.61%; ether extract (lipids), 2.36%; ash, 3.43%; crude fibre, 3.62%; and other (by difference), 7.86%. His results are supported by other less comprehensive investigations. These give the following ranges of values for the three main classes of substances: carbohydrate, 50.6%-53.7%;[64, 65]; protein, 21.4%-28.2%;[64, 65]; lipids, 0.89%-2.6%;[64, 65, 66, 67]

There have been more detailed studies on some of the fractions. Duperon[68] reported that free sugars comprise 8% of the dry weight of the cotyledons and a smaller percentage of the axis. The main sugars present are sucrose, raffinose and stachyose. There are only trace amounts of hexoses.[69] Small amounts of all the protein amino acids as well as some non-protein ones are present in the free state in the seed.[70, 71] Glutamate and arginine are particularly abundant in both the free state and in proteins. Half the proteins in the seed are classified as globulins and there are smaller amounts of albumins and basic proteins.[72] Malonate, citrate and malate are present in small amounts in both the axis and cotyledons.[73]

About 4-5% of the lipid fraction is composed of steroids, and these are also present in both the axis and cotyledons. [66, 67]

There is some variation among the values because different varieties were used by different authors and the seeds were produced in widely different localities. However, it is clear that the main storage compound is starch and that, from a quantitative point of view, lipids can only be of minor importance as respiratory reserves.

2-1.4 METABOLISM

There have been a number of papers published on the metabolism of the seeds of *Phaseolus vulgaris* and other closely related species during early germination. Duperon studied the metabolism of *Phaseolus vulgaris* seeds by using ^{14}C labelled compounds. After three hours of imbibition, isolated cotyledons were placed in solutions containing ^{14}C-citrate for 45 minutes. [74] Krebs cycle acids including 2-oxoglutarate became labelled. The only amino acid which became well labelled was glutamate. Since 2-oxoglutarate and glutamate cannot become labelled by the action of the glyoxylate cycle, Duperon concluded that the Krebs cycle was operating. This accords with the conclusion of Beevers [75] that the glyoxylate cycle occurs only in those tissues where lipids are relatively abundant. There is only a low concentration of lipids in *Phaseolus vulgaris* seeds (section 2-1.3).

At least two of the Krebs cycle acids, citrate and malate, are present in the resting seed,[73] so some labelling could occur even before the Krebs cycle is fully operative.

Duperon found[69] that after 3-4 hours of imbibition, whole seeds were incorporating the label from ^{14}C-glucose into alanine and glutamate as well as into lactate and the Krebs cycle acids. He also found[76] that $^{14}CO_2$ was incorporated mainly into malate, citrate and malonate. CO_2 was incorporated after as little as one hour of imbibition and the axis was more active in this respect than the cotyledons.

The malonate formed during carboxylation probably came from the action of acetyl-CoA carboxylase (6.4.1.2) and malonate CoA-transferase (2.8.3.3) in the sequence: Acetyl-CoA + CO_2 → malonyl-CoA → malonate. This pathway appears to be the one responsible for forming malonate in nine day old *Phaseolus vulgaris* seedlings,[77] so it is quite possible that it also accounts for the malonate formed during the first hours of imbibition.

The malate was probably formed by β-carboxylation reactions. In seeds, at least three enzymes have been found which will carboxylate pyruvate or phosphoenol-pyruvate to malate or oxaloacetate.[78] These latter two acids and citrate are members of the Krebs cycle and the fact that labelled citrate was formed from feeding $^{14}CO_2$ would be expected if the Krebs cycle was operating.

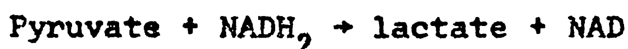
Opik [79] studied respiration in the cotyledons of *Phaseolus vulgaris* . After three hours of imbibition, respiration as measured by oxygen uptake was negligible, but by six hours it was rising steeply and continued to do so for the first 24 hours. After 18 hours he separated out the mitochondrial fraction and discovered that this was responsible for much of the respiration in the cotyledons. Stinson and Spenser [80] showed that at 24 hours the respiration in the mitochondria of *Phaseolus vulgaris* was fairly tightly coupled to phosphorylation; they made no measurements earlier than that.

On the basis of the work of Duperon and the last two papers cited it would appear that very early in germination sugar reserves are being respired through glycolysis and the Krebs cycle; the reduced pyridine nucleotides from this are being oxidised by the electron transport system of the mitochondria, and this transport system is coupled with phosphorylation.

Opik [79] noted that not all the respiration of the cotyledons was due to the mitochondria. He suggested that some of the oxygen uptake was due to the action of oxidases, enzymes which are common in seeds. On the basis of the effect on respiration of adding various compounds he suggested that some oxygen uptake was due to the action of ascorbate oxidase. (1.10.3.3) He proposed that the pathway first suggested by Mapson and Moustafa [81]

for *Pisum sativum* occurred also in *Phaseolus vulgaris* cotyledons. In this pathway, electrons from reduced NADP are passed through glutathione to ascorbate where they are oxidised by molecular oxygen.

When *Phaseolus vulgaris* seeds were germinated under largely anaerobic conditions (a state that occurs when seeds are imbibed under water or in very wet sand) large amounts of lactate were produced. [82] This was produced even in the first three hours of imbibition. Presumably, as in other organisms, this lactate came from the glycolytic sequence via pyruvate. Under anaerobic conditions large quantities of reduced pyridine nucleotides would accumulate and production of lactate would free some of these for further metabolism in the reaction:

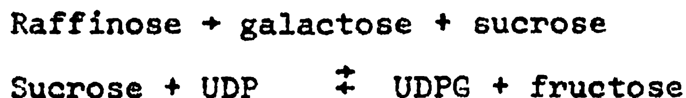


$$\text{Pyruvate} + NADH_2 \rightarrow \text{lactate} + NAD$$

In his tracer experiments with ^{14}C-glucose Duperon [69] found (in addition to the results cited previously) that sucrose and to a lesser extent fructose, raffinose, and stachyose became labelled. All these sugars are present in varying amounts in resting *Phaseolus vulgaris* seeds [69]. It thus appears that the enzymic pathways that interconvert these sugars are in operation at an early stage of imbibition. Some of the label from ^{14}C-glucose also appeared in the lipid fraction. However, after saponification,

neither the fatty acids nor the unsaponifiable fraction was labelled

It is interesting that Duperon found raffinose to be involved in metabolism so early in germination. Apart from starch, raffinose is the most common form of carbohydrate reserve in seeds. Pridham et al [83] studied the metabolism of this sugar in the seed of *Vicia fabia* (broad bean). This belongs to the same sub-family as *Phaseolus vulgaris* (p. 14, ref. 84) and like *Phaseolus vulgaris* the major reserve polysaccaride of the seed is starch and the seed contains only small amounts of lipids (p. 234, ref. 84). They found that the enzymes α-galactosidase (3.2.1.22) and sucrose synthetase (2.4.1.13) were present even in the resting seed and that their concentration rose during the first day of imbibition. These two enzymes respectively catalyse the reactions:



$$\text{Raffinose} \rightarrow \text{galactose} + \text{sucrose}$$

$$\text{Sucrose} + \text{UDP} \rightleftharpoons \text{UDPG} + \text{fructose}$$

The second enzyme, sucrose synthetase (2.4.1.13) is inhibited by glucose and fructose. Pridham et al suggested that raffinose is the first reserve to be metabolised and that it is used until starch degradation begins and the associated glucose and fructose produced stop the operation of the raffinose pathway. The galactose produced from raffinose is probably metabolised

to galactose-1-P as galactokinase (2.7.1.6), the enzyme which catalyses this, is present in the resting seed. This would account for the absence of free galactose in the seed.

The enzyme α-galactosidase (3.2.1.22) has also been found in *Phaseolus vulgaris* seeds.[85, 86] It is present in both the axis and cotyledons. Initially, its activity is high in the axis but low in the cotyledons. The high activity in the axis may indicate that the pathway suggested by Pridham et al [83] is occurring early in germination, supplying the sugar needed for respiration before translocation from the cotyledons begins. Some support for this statement comes from the report that, in the very closely related seed of *Phaseolus radiatus*, raffinose and other galactose containing sugars are metabolised even in the first six hours of imbibition. [87]

Oota [88] studied the respiration, water content and changes in the level of carbohydrates in excised axes of the bean *Vigna sesquipedalis*. This belongs to the same tribe as *Phaseolus vulgaris* (p. 27, ref. 84) and like *Phaseolus vulgaris* contains large amounts of starch and only small amounts of lipids (p. 234, ref. 84). He found that there was active sugar metabolism even in the first two hours of imbibition. Some of the sugar

was exuded, some was respired, and some was converted to starch. Unfortunately, the sugar involved was not identified, but presumably it was mainly sucrose as this is the only free sugar found in the resting seed,[89] and it is the usual sugar that is translocated in plants.

Sucrose is certainly involved in the carbohydrate metabolism of the cotyledons of the *Vigna sesquipedalis* seed. After 17 hours of imbibition Oota et al [90] showed that the cotyledons had begun to metabolise their starch reserves, respiration was occuring and the amount of sucrose was increasing at the same time. The lipid concentration was low in the cotyledons of the resting seed but it decreased even further in the first 17 hours of imbibition.

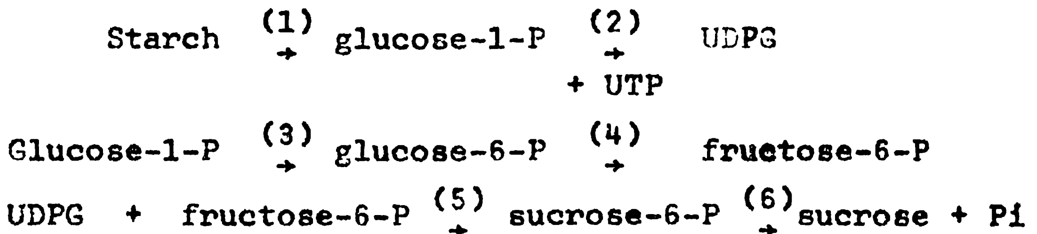
No further studies appear to have been done on the metabolism of the seeds of *Phaseolus vulgaris* and its closely related species during the first day of imbibition. However, there have been a number of papers on the metabolism after one or more days of imbibition, from which some knowledge of the differences in function of the cotyledons and axis is gained.

Oota et al [2] in another study on *Vigna sesquipedalis*, followed changes in the cotyledons and parts of the axis of total sugars, polysaccharides, protein, nucleic acids, and soluble nitrogen at day intervals from the start of imbibition. They showed that there was little change in

any part of the seed during the first day. After this the cotyledons started to translocate all five classes of their constituents to the growing axis. All parts of the axis began to grow. Initially, the hypocotyl (the stem or middle part of the axis) grew fastest and exhibited the largest increase in levels of translocated compounds, other than soluble polysaccharides. The plumule had the largest increase in soluble polysaccharides of any part of the axis, and this was out of all proportion to its increase in dry weight. The plumule develops into the first true leaves of the seedling, so eventually it possesses a photosynthesising system. It appears that even in the first days of imbibition it has already developed the efficient starch producing system it will later need to store the products of photosynthesis.

There appear to have been no studies on the breakdown of starch in *Phaseolus vulgaris* seeds during germination, although it is the major storage compound. However, Fekete [91] studied starch breakdown in the cotyledons of the seeds of *Vicia fabia*. As mentioned before, this species is related to *Phaseolus vulgaris* and both seeds contain large amounts of starch. It could, therefore, be expected to have a similar starch metabolism. Fekete assayed the cotyledons after one day of imbibition for fourteen of the common enzymes involved in sugar and starch metabolism.

On the basis of the activities of these enzymes she proposed that the starch is first degraded to glucose-1-P by the enzyme (1) phosphorylase (2.4.1.1). The glucose-1-P is then either metabolised to UDPG, the enzyme involved being (2) glucose-1-P uridylytransferase (2.7.7.9); or it is metabolised through glucose-6-P to fructose-6-P, the two enzymes involved being respectively (3) phosphoglucomutase (2.7.5.1) and (4) glucosephosphate isomerase (5.3.1.9). Fructose-6-P and UDPG then combine to form sucrose-6-P which loses its phosphate group in a further reaction to form sucrose. The enzymes responsible for these last two reactions are respectively (5) sucrose-6-P synthetase (2.4.1.14) and (6) a phosphatase. The direction of the pathway and the reactions involved are:



This combination of reactions is interesting because no free glucose is formed and amylases are not involved. Fujii [89] suggested, on the basis of sugar analyses, that this pathway might be occurring in the cotyledons of the *Vigna sesquipedalis* seed.

Swain and Dekker [92] studied starch breakdown in germinating seeds of *Pisum sativum* (the common pea).

This belongs to the same tribe as *Vicia fabia* (p. 28, ref 84) and like *Vicia fabia* and *Phaseolus vulgaris* the seed contains large amounts of starch but small amounts of lipids (p. 234, ref. 84). They assayed both the cotyledons and axis for a number of enzymes and also carried out experiments with ^{14}C labelled sugars. On the basis of that work they suggested that the phosphorylase pathway shown above, was occurring at an early stage of germination and that it accounted for the small amount of starch that was broken down in the cotyledons during the first day of imbibition. However, they concluded that the hydrolytic pathway was responsible for the major amount of the starch breakdown that occurred in the cotyledons in the later days of seedling growth. In this hydrolytic pathway the starch is broken down to oligosacharides in the cotyledons by the action of α - amylase (3.2.1.1). These oligosacharides are then broken down further, either in the cotyledons, or (after translocation)in the axis; the enzymes involved are β-amylase (3.2.1.2) and α-galactosidase and the final product is glucose.

Phaseolus vulgaris cotyledons, like the storage organs in other seeds, contain large amounts of proteins which, during germination and subsequent seedling development, are broken down and translocated to the growing axis. [45]

There have been a number of studies of the amino acids in *Phaseolus vulgaris*. Zacharius [70] and Jones and Boulter [71] measured the levels of free and total amino acids in whole seeds. Their results show the importance of the nitrogen rich amino acids lysine, asparagine, and arginine. They found only small amounts of glutamine. Glutamate, as well as being a major free amino acid, was present in large amounts in proteins and peptides. However, by themselves, analyses of amino acids in resting seeds give little idea of possible metabolism in germinating seeds.

Forest and Wightman [45] measured the levels of free amino acids in the cotyledons, roots and shoots of *Phaseolus vulgaris* seeds in the resting state and at two day intervals during germination and subsequent seedling growth. The two analyses above [70, 71] were much less extensive but are generally consistent with their results. They found that while glutamate was a major free amino acid in the cotyledons, it was present in only moderate amounts in the roots and shoots. Asparagine was a major free amino acid in all parts of the seed whereas glutamine was only a minor constituent. When the seeds were germinated in the dark there was an increase in the nitrogen rich amino acids, asparagine, lysine and arginine. The authors suggested that this was associated with a low protein synthesis.

Forest and Wightman also tried to correlate the levels of the six amino acids present most often in largest amount, with the activity of their corresponding 2-oxoglutarate transaminases. While they could find no correlation, their studies on the enzyme activities are interesting. Using the acceptor 2-oxoglutarate they measured transaminase activity for the amino acids serine, threonine, valine, alanine, aspartate and asparagine. Alanine and aspartate transaminases were present in largest amount and asparagine transaminase in smaller amounts. There was a very small transaminase activity for valine and none at all for serine and threonine. This applied to both axis and cotyledons. The enzyme activities were present even in extracts from resting seeds. The activity rose in the roots and shoots with time and fell continuously in the cotyledons. So it appears that these transaminases are never synthesized in the cotyledons of the germinating seed but are present in relatively large amounts during early germination.

Jones and Boulter [3] studied the metabolism of arginine in *Vicia fabia.* They found evidence for the presence of a urea cycle in both the axis and cotyledons. Although they did not definitely state this, it appears that they were studying six day old seedlings; so their system was far removed from early germination.

2-1.5 IMBIBITION

The seed of *Phaseolus vulgaris,* in common with other seedsthat have been studied, appears to have a three phase imbibition. Walton [93] studied water uptake in excised axes of *Phaseolus vulgaris* at 26°C. He found an initial rapid increase in water content lasting about three hours. This appeared to be a purely physical process since it occurred even in axes from dead seeds and was independent of protein synthesis. This was followed by a lag phase of about four hours in which there was only a very slow increase in water content. In the third phase, water content increased linearly and this coincided with axis elongation. The rate of oxygen uptake also increased markedly at this time. Cell divisions began to occur after 11 hours of imbibition (i.e. after four hours of the third phase).

In the intact seed, radicle protrusion occurred at 24 hours. The axis at this stage had a water content comparable to that of an excised axis at 13 hours and an oxygen consumption comparable to that of an excised axis at 15 hours. Excised axes therefore absorbed water and consumed oxygen at approximately twice the rate of attached axes.

Opik and Simon [94] studied water uptake and oxygen consumption of the cotyledons of *Phaseolus vulgaris* in the intact seed at 25°C. They also found a three-phase

system; the first lasting 10-16 hours, the second lasting 3-8 hours, and the third until senescence set in after about six days. As with the axis, the first phase appeared to be a physical process and in the second phase there was no increase in water content or oxygen consumption. In the third phase both water content and oxygen consumption increased slowly until the cotyledons began to shrivel after having translocated most of their stored nutrients to the growing axis. The times over which the three phases occurred were similar for both cotyledons and axes in intact seeds. However, on a weight basis, the rise in water content and oxygen consumption during these phases was always less in the cotyledons.

The changes that occur in the cotyledons of *Phaseolus vulgaris* during imbibition, germination and senescence were studied by Opik.[95] He found three types of tissue in the cotyledons: the storage tissue which makes up nearly all of the cotyledons, the thin layer of epidermal cells around the outside of the cotyledons, and the vascular bundles which conduct the reserves to the axis. After 24 hours of imbibition the cotyledons had lost little or none of their weight and the storage tissue was densely packed with starch and protein granules, and what appeared to be a few small lipid granules. During the second day the seed germinated, the axis began a period of rapid growth, and the storage materials in the

cotyledons were being broken down and translocated to the axis. After five days this process was well advanced and the cotyledons had been reduced to almost half their initial weight. Many areas in the storage tissue now contained empty cells and the only filled cells were packed around the vascular bundles. There was no gradation of digestion from tip to base of the cotyledons. If the seedlings were being grown in the light the cells adjacent to the epidermis and vascular bundles produced green plastids by the fifth day. Even so the storage cells continued to die, the cotyledons shrivelled and after eight days they started to fall off the growing seedling.

In accord with the fact that the cotyledons act as a storage tissue Opik [79] noted that during germination and subsequent seedling growth there was neither cell division nor cell expansion in the cotyledons.

Opik and Simon[94], and Walton[93] studied the effect of temperature on imbibition. At 16°C the first phase was extended by about one hour while the second, or lag, phase became much longer. In the axis the lag phase lasted for more than 10 hours, and in the cotyledons it became indistinguishable from the third phase. The situation is similar to that in the *Vigna sesquipedalis* seed studied by Oota.[29] He found that lowering the temperature had little effect on the first phase, but that the second phase became longer, and the eventual rise in water content and oxygen consumption of the third phase became slower.

The way in which seeds are imbibed is crucial to their germination. For many years it has been known that *Phaseolus vulgaris* seeds are damaged when they are imbibed under water.[96] Under some conditions this injury is so severe that almost no germination occurs.[97] It is known too that small amounts of various compounds are exuded when seeds of *Phaseolus vulgaris* and other species are imbibing (see Section 1-4.3), and at one stage it was believed that loss of some essential compound in this way caused damage to the seeds.[98] However, in a more recent paper by Orphanos and Heydecker [99] it was concluded that the injury was due to lack of oxygen in the centre of the seed during early imbibition. When the seed was imbibed under water the cavity between the cotyledons became flooded with water. The seed became excessively swollen and under these conditions the entry of oxygen was greatly retarded. The injury could be avoided by removing the seed-coat either before or after immersing the seed for 24 hours, or by cutting the end of the seed off to allow the water to escape.

These conclusions are supported by the results of Sherwin and Simon [82] who imbibed *Phaseolus vulgaris* seeds in sand containing different amounts of water. They found that as the water in the sand increased so did the amount of anaerobic metabolism. This was

particularly shown by the great increase in the amount of lactate. Germination took longer as the amount of water was increased and there was some failure to germinate when the seeds were completely immersed. They found that removal of the seed-coat allowed more oxygen to enter the seed. Doireau and Duperon [100] carried out similar work on *Phaseolus vulgaris* and *Pisum sativum* seeds. They also concluded that seeds imbibing under water or with an intact seed-coat had a more anaerobic metabolism than those without a seed-coat or with just a thin layer of water on the surface. They too found that imbibition was fastest with the seed-coat removed and the seed totally immersed.

Another complicating factor in the study of *Phaseolus vulgaris* imbibition is the wide variation in the amount of water absorbed by intact seeds. Preston and Scott [101] found a range of 23-91% water content in the seeds imbibed for 24 hours. They studied the effect of the micropyle (the small hole in the seed-coat near the hilum) on imbibition and found that contrary to expectations, this accounted for only a small proportion of the water absorbed. This variability in water absorption is not mentioned by some authors [82, 94] and one can only surmise that they scarified the seeds before use, used seeds where the variability was much less, or simply discounted the extreme results.

2-2 IMBIBITION OF PARTS OF SEEDS IN TRITIATED WATER

2-2.0 EXPERIMENTAL

The axis of *Phaseolus vulgaris* is attached to the cotyledons through only two small connections and it can be easily separated from them. However, because the *Phaseolus vulgaris* seed contains large amounts of starch and only small amounts of lipids, the resting seed is very hard and the axis is brittle. For this reason sometimes as many as a dozen seeds had to be dissected before two undamaged axes were obtained. The pieces of cotyledon were cut from about the middle of the cotyledon and the section of seed-coat adhering to the outside of each piece was cut off. Two axes or two pieces of cotyledon were imbibed for each experiment. The experiments were carried out as outlined in Section 1-4.

During the experiments at shorter times the axes and pieces of cotyledon did not imbibe enough water to make them soft. For this reason grinding took as much as five minutes longer in these short-time experiments. The times shown for these experiments can only be regarded as approximate, as the metabolism might not have been inactivated until several minutes after the slow grinding. Also since grinding of these hard pieces of seed might not have been very efficient, the labelled metabolites might have been incompletely extracted.

In the case of the three minute experiment, the ethanol extract and water rinse were combined. This was done to ensure that there was enough radioactivity on the chromatograms to be detected. In all other cases the three extracts (ethanol, water and water rinse) were chromatographed separately.

2-2.1 RESULTS AND DISCUSSION

The results are given in Table 2.1, where the labelling in the three extracts has been combined for presentation. Lactate and succinate have similar R_f values in the chromatography solvents used and when there was only a trace of activity that could have been either compound this is indicated in the table by placing the symbol in a position between the names of the two compounds and in the plates by the abbreviation Lac/Suc. The radioautograms of the water rinse chromatograms are shown in Plates 2.1 and 2.2. The abbreviations used in the plates are Asp, aspartate; Glu, glutamate; Gln, glutamine; Ala, alanine; AB, 4-aminobutyrate; Cit, citrate; Mal, malate; Suc, succinate; Lac, lactate; Sucr, sucrose; THO, tritiated water.

At the earliest times the metabolism of the axis was quite different from that of the cotyledon. After three minutes only amino acids were labelled in the axis and this was so even at five minutes. In the cotyledon there was little labelling at three minutes and at five minutes citrate was the only compound

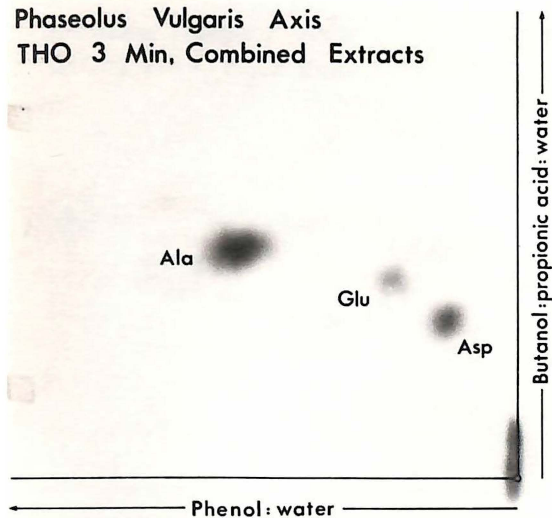
Table 2.1 Compounds labelled when *Phaseolus vulgaris* axes and pieces of cotyledon imbibed tritiated water.

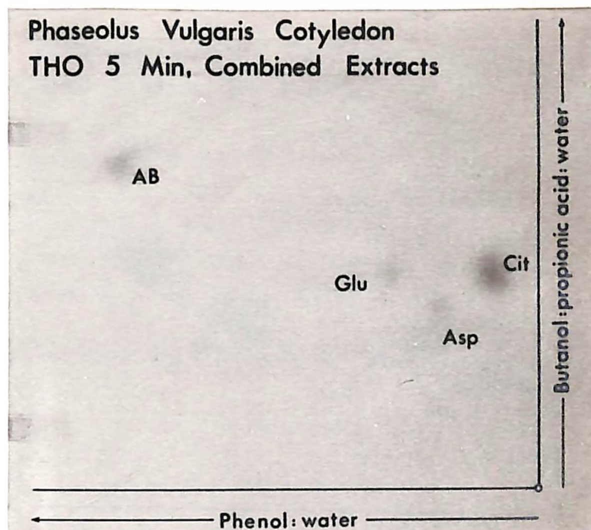
Compounds labelled	Time (min)							
	3	5	15	30	60	120	180	360
(a) Axis								
4-Aminobutyrate		t	+	++	++	++	++	+
Aspartate	+	+	+	+	+	+	+	++
Glutamate	+	+	+	+	+	+	+	+++
Alanine	+	+	+	+	++	++	++	++
Citrate			+	+	t	++	++	+++
Malate				+	+	++	++	+++
Succinate				+	t	+	+	t
Lactate					++	t	++	
Sucrose						+	+++	+++
Glutamine						+	++	++
Lipids ?			t	+	++	++	++	+++
Sugar phosphates ?				t	t	+	++	+++
(b) Cotyledon								
4-Aminobutyrate		t	+	++	++	++	++	++
Aspartate		t	+	+	+	++	+	++
Glutamate	t	t	+	+	++	++	++	++
Alanine			+	+	++	++	++	t
Citrate	t	+	+	++	++	++	++	++
Malate					+	++	++	++
Succinate					t	+	+	+
Lactate						+	++	t
Sucrose						t	+	++
Glutamine						+	++	t
Lipids ?			t	t	+	++	++	++
Sugar phosphates ?						t	t	+

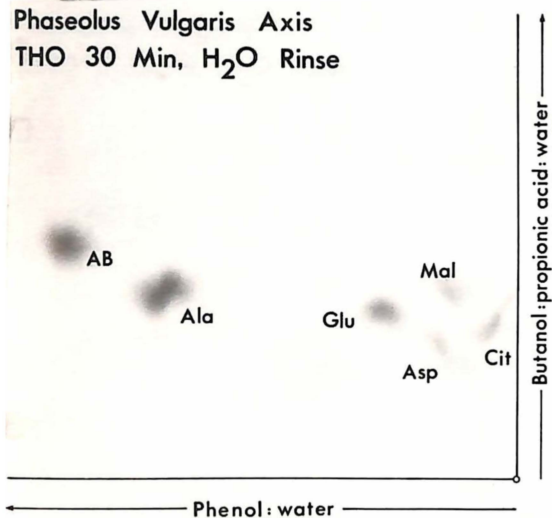
Key: +, ++, +++ = increasing relative intensity;

t = trace.

PLATE 2.1







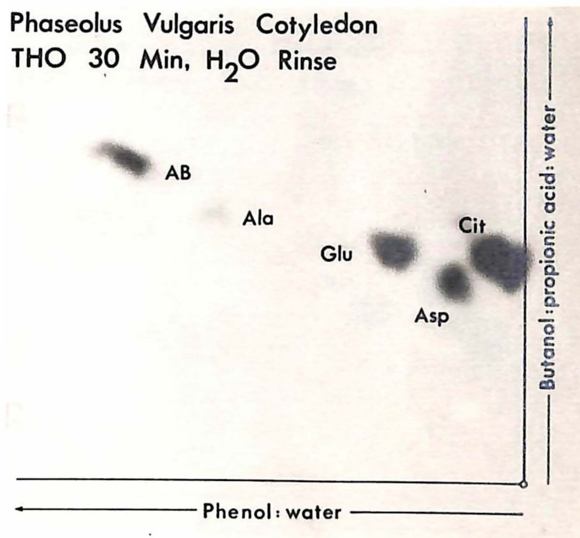
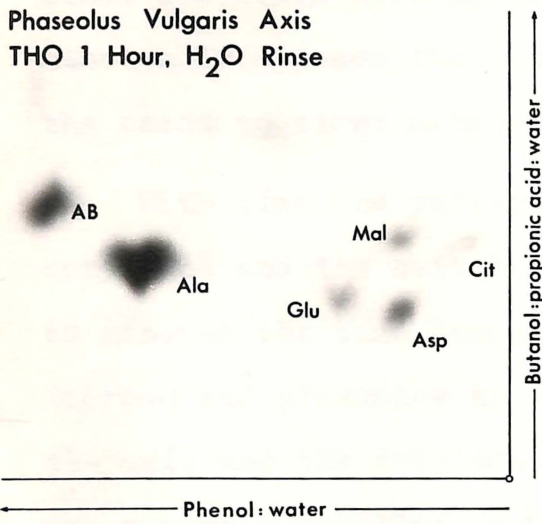
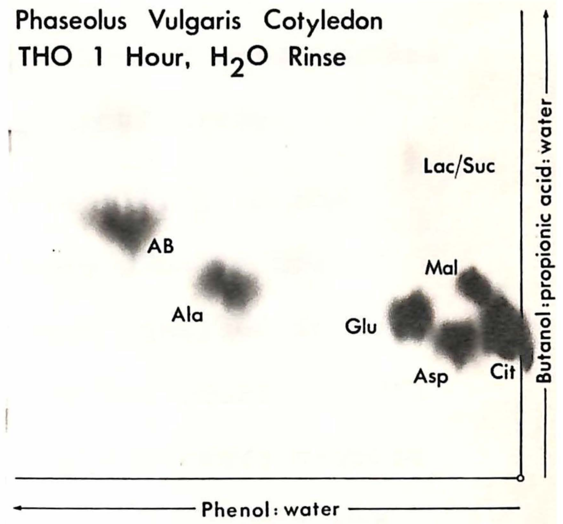
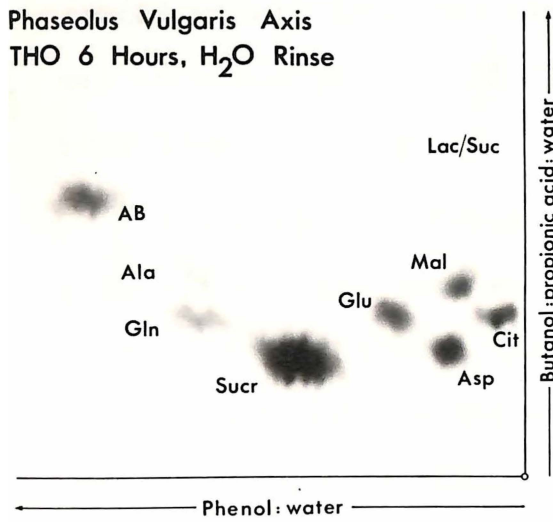
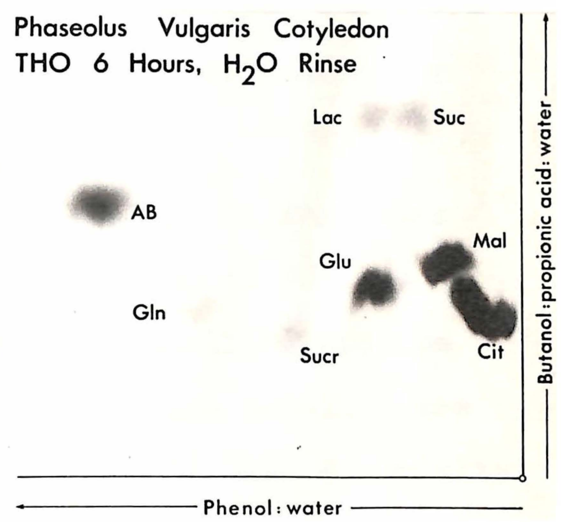


PLATE 2.2









strongly enough labelled to be positively identified. Since compounds were labelled even as early as three minutes it appears that the addition of water causes the seeds to start metabolising immediately.

With time the patterns of labelling in the cotyledon and the axis became more similar and by 60 minutes the same compounds were labelled in both. Sucrose and glutamine appeared at two hours in both the axis and the cotyledon and by six hours sucrose was strongly labelled in the axis. Succinate was labelled at 30 minutes in the axis and at 60 minutes in the cotyledon. The amount of labelled lactate varied widely from one experiment to another and there was no clear pattern of its increasing or decreasing with time.

The compounds found to be labelled in *Phaseolus vulgaris* are the same as those labelled in *Sinapis alba*, [13, 18] although the order of labelling and the time at which different compounds became labelled are not the same. Spedding, [38] Missen, [18] and especially Mann, [23] examined in some detail the possible reactions that might be involved in forming these labelled compounds. Their conclusions were summarised in Section 1-5.3. The reasoning behind the suggested reactions for the labelling of amino and organic acids was presented in Section 1-5.4. In the discussion that follows this reasoning is incorporated without much elaboration.

Amino acids

The three amino acids that were labelled in the three minute experiment on the axis, alanine, aspartate and glutamate, are all closely related to the Krebs cycle and would have become labelled if they were being formed from their corresponding 2-oxo acids in the presence of tritiated water. A label would be incorporated by the action of transaminases, and two such enzymes, aspartate aminotransferase (2.6.1.1) and alanine aminotransferase (2.6.1.2) have been found in resting seeds of *Phaseolus vulgaris*.[45] The reactions catalysed are respectively:

Aspartate + 2-oxoglutarate $\rightleftarrows$ oxaloacetate + glutamate

Alanine + 2-oxoglutarate $\rightleftarrows$ pyruvate + glutamate. Since the reactions are reversible they could account for the labelling of all three of the amino acids involved. Oxaloacetate and 2-oxoglutarate, the corresponding 2-oxo acids of aspartate and glutamate, are both members of the Krebs cycle. The 2-oxo acid corresponding to alanine is pyruvate. By decarboxylation and condensation with CoA, pyruvate forms acetyl-CoA which, in the Krebs cycle, donates its acetyl group to oxaloacetate in the reaction forming citrate. Thus the labelling of the amino acids probably indicates that a number of the Krebs cycle intermediates are also present.

Labelled 4-aminobutyrate was present in both the axis and cotyledon after five minutes. It could have been formed by transamination (its corresponding 2-oxo acid is succinate semialdehyde), but it appears more likely that it was formed by decarboxylation of glutamate. This suggestion is based on the following three points: the enzyme glutamate decarboxylase (4.1.1.15) has been found in many resting seeds;[102, 103] 4-aminobutyrate is formed by the action of this enzyme in ground powders made from resting seeds of one species;[104] and in another species of seed this enzyme was shown to be active at an early stage of imbibition.[105] It is now widely believed (Section 6-1) that 4-aminobutyrate is an intermediate in a pathway known as the 4-aminobutyrate bypass. The presence of 4-aminobutyrate as a labelled metabolite in the tritiated water experiments with *Phaseolus vulgaris* seeds, raised the possibility that this bypass might be occurring in these seeds during early imbibition. Experiments were performed to investigate this possibility and the results of these are reported in Section 6-2.

Glutamine was labelled im both the axis and cotyledon from two hours onwards. It would be labelled if it were formed from labelled glutamate. Labelled glutamate was present and the enzyme glutamine synthetase (6.3.1.2) has been found in seeds.[57] The labelling of glutamine may indicate that after

two hours of imbibition, amino acids were being broken down and the ammonia formed was being used in synthesizing glutamine. Spedding and Wilson [13] suggested that deamination of amino acids was occurring during early germination to provide 2-oxo acids for the Krebs cycle. Ammonia for the synthesis of glutamine could be provided by such deaminations.

Krebs cycle acids

In the cotyledon citrate was labelled within five minutes, while malate and succinate were not labelled until after 60 minutes. In the axis all three acids were labelled after 30 minutes. Although the presence of these labelled acids cannot be taken as definitely establishing that the Krebs cycle was operating, it is a reasonable supposition that this was the case. This is supported by the following observations:

1. It is likely that the 2-oxo acids needed for the operation of the Krebs cycle were present, as their corresponding amino acids were labelled. Transaminases that interconvert these amino acids and oxo acids have been found in resting *Phaseolus vulgaris* seeds, [45] and the amino acids would become labelled by the operation of these enzymes in tritiated water (Section 1-5.4).

2. The evidence presented by Duperon [74] indicated that the Krebs cycle was operating in

Phaseolus vulgaris seeds after 3-4 hours of imbibition. He did not perform any experiments at times earlier than that, but if the Krebs cycle were operating after 3-4 hours it must have started before then. The suggestion based on the work in this thesis that the Krebs cycle began to operate in the axis after 30 minutes and in the cotyledon after 60 minutes fulfils this requirement.

3. The only other pathway in which all three acids could become labelled is the glyoxylate cycle. It is most unlikely that this exists in *Phaseolus vulgaris* seeds as they have only a low lipid content. [75]

4. It seemed possible that most of the Krebs cycle was working but that the reaction step between 2-oxoglutarate and succinate had been replaced by the 4-aminobutyrate bypass. This pathway is discussed in Section 6-1. While it would provide an attractive explanation for the labelling of 4-amin-butyrate, it appears an unlikely possibility on the basis of the experiments which are described in Section 6-2.

Lactate

Lactate was labelled quite strongly in some experiments but barely at all in others. It seems almost certain that this reflects small variations in the amount of tritiated water in which the seed parts

were immersed. As much care as possible was taken to add the same amount of tritiated water in each experiment, but because there was only a small amount of liquid involved there was inevitably some variation. As well as this, the size and shape of the two axes or pieces of cotyledon varied somewhat from one experiment to the next. They were therefore covered with more water in some experiments than in others. As explained in Section 2-1.5, the more water *Phaseolus vulgaris* seeds are imbibed in, the more anaerobic their metabolism becomes and the more lactate they produce.[82] Missen and Wilson[14] showed that when seeds were imbibing tritiated water under completely anaerobic conditions, large amounts of labelled lactate were produced. It is clear that the amount of labelled lactate formed in these present experiments, was an indication of the size of the lactate pool in the seed parts, and that the size of this pool was indicative of the degree of anaerobic metabolism. The formation of lactate was probably due to restriction of oxygen transport by the water around the seed parts. However, there are a number of facts which suggest, that despite the formation of this lactate, the oxygen tension in the seed parts was still sufficiently high for the metabolism to be mainly aerobic. These are:

1. Missen and Wilson[14] reported that when

Sinapis alba seeds were imbibed in tritiated water under a completely anaerobic atmosphere, the patterns of labelling differed substantially from those when the seeds were imbibed in tritiated water under air. The patterns of labelling in *Phaseolus vulgaris* seeds more closely resemble those of *Sinapis alba* seeds imbibed under air than those of the seeds imbibed under completely anaerobic conditions.

2. The amount of water covering the seed parts was at most a few millemetres and while this would restrict oxygen transport to the seed parts it would not be expected to completely prevent it.

3. The seed parts were not surrounded by a seed-coat and the absence of this would considerably enhance the transfer of oxygen into them. [82]

Sucrose

Although sucrose was labelled in the 2-6 hour experiments in both axis and cotyledon it was always more strongly labelled in the axis. Sucrose is the usual sugar translocated in plants, but it is not obvious why it should be labelled so early in seed germination. Duperon [69] showed that in *Phaseolus vulgaris* seeds sucrose was being synthesized from glucose during the first four hours of imbibition. His experiments were performed only on whole seeds. He suggested that the synthesis of sucrose could only

be of minor importance at this time as the amounts of free hexoses in the seed were so low, and the breakdown of other sugars was negligible. However, Missen,[18] who found that sucrose was labelled in *Sinapis alba* even after one hour of imbibition, suggested that it was being formed from the breakdown of raffinose. Oota et al [2] studied the seed of *Vigna sesquipedalis*. They found evidence to suggest that there was a starch to sugar conversion in the hypocotyl during the first days of imbibition. Since this sugar was then translocated to the plumule, it could be expected to be sucrose. There was also the unlikely possibility that sucrose was being synthesized from glucose and fructose formed by reverse of the glycolytic sequence. To try to differentiate between these possibilities further experiments were done and these are described in Section 2-4.

Sugar Phosphates

While the only evidence for presence of labelled sugar phosphates was their R_f values in the chromatography solvents phenol:water and butanol:propionic acid:water, it is of interest to note that the amount of labelling in these compounds can be correlated with that in sucrose. As sucrose became more heavily labelled so did the sugar phosphates. Sucrose was never labelled as strongly in the cotyledon as in the axis and neither

were the sugar phosphates. Since sucrose was being synthesized the labelled sugar phosphates may be intermediates (such as glucose-1-P and fructose-6-P) of this synthesis.

Lipids

The axis was more active in labelling lipids than was the cotyledon. Resting seeds of *Phaseolus vulgaris* contain only small amounts of lipids (Section 2-1.3). These could have only a limited food storage function. Labelling probably occurs therefore during the lipid synthesis which would be needed to make the cell membranes required for future growth of the seedling. This is supported by the results of Macey and Stumpf [106] who found that in the seed of *Pisum sativum* there was active lipid synthesis during the first day of germination. *Pisum sativum* is closely related to *Phaseolus vulgaris*, and the seeds of both species have low levels of lipids.

Comparison between Cotyledon and Axis

Although the same compounds were labelled in both the axis and cotyledon, the labelling occurred later in the cotyledon and was less intense. Thus apart from citrate, all the labelled compounds either appeared first in the axis or appeared in both the axis and cotyledon at the same time. Apart from a few exceptions at particular times the compounds were more heavily labelled in the axis. In the axis the labelling

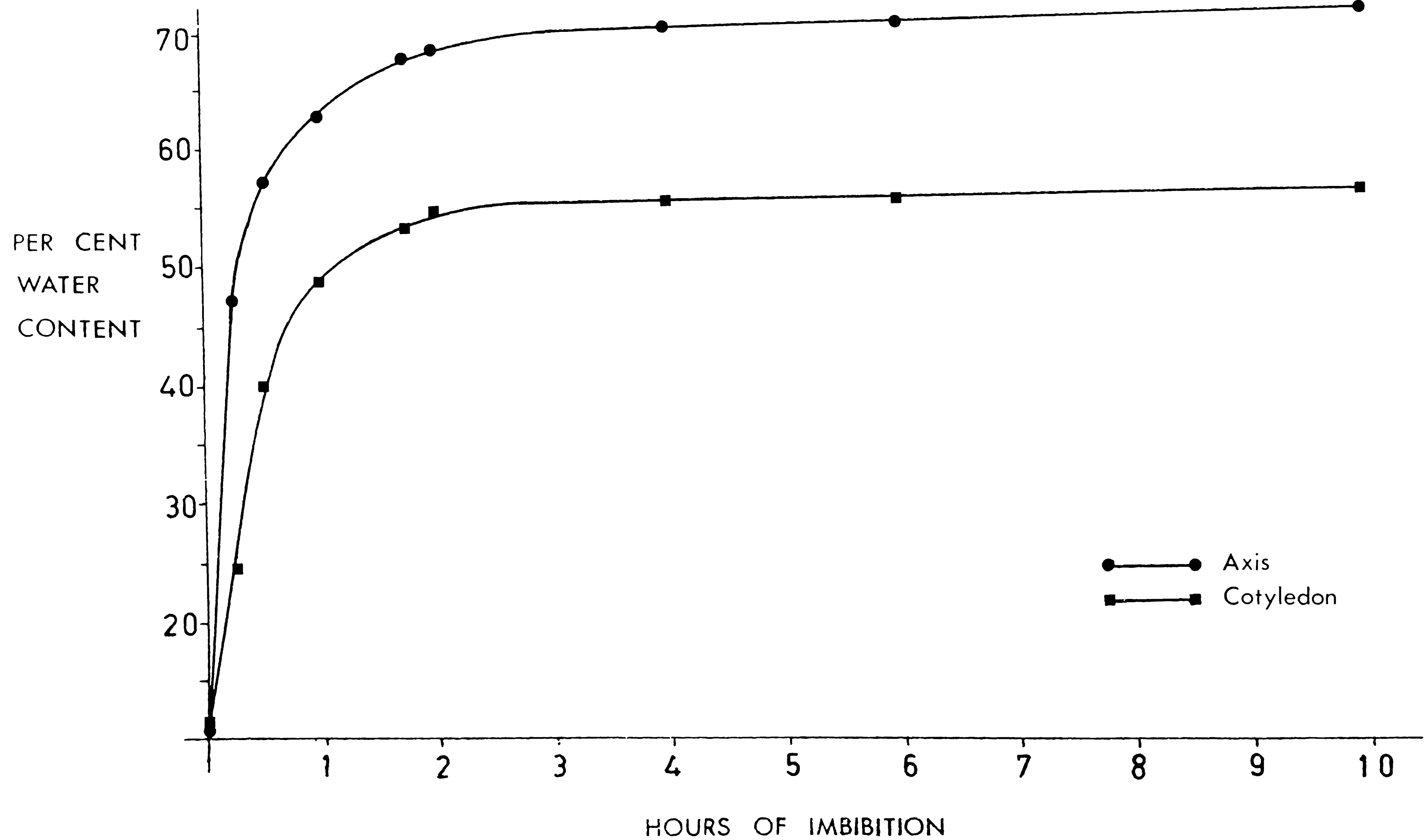
became progressively more intense, till by six hours some of the compounds were very intensely labelled. By contrast, in the cotyledon there was little change in the amount of labelling after the first two hours.

The above differences are reflected to some extent in the differences in water absorption. A comparison between the axis and cotyledon of this absorption during the first ten hours of imbibition is shown in Fig. 2.2. Both tissues absorbed water rapidly to begin with, but the absorption was always faster in the axis. When the absorption levelled off after two hours the axis had a water content of almost 70% compared to 55% in the cotyledon. These results are in general agreement with those of Walton.[93] It is interesting to note that all the labelled compounds found in the first six days of imbibition were present after two hours, the same time at which the rapid water increase levelled off. The results suggest that during the first two hours the increase in water content was linked with the increase in metabolism.

The differences in labelling between the axis and cotyledon in the three and five minute experiments may be directly attributed to the differences in water absorption. A histochemical study of the axis of *Phaseolus vulgaris* by Sato [107] after 20 hours of

FIGURE 2.2

Water absorption by parts of Phaseolus vulgaris seeds



imbibition, showed that it has a definite structure with inner and outer layers. Mitochondria and succinate dehydrogenase (1.3.99.1) are present in some of these layers but not in others. Conversely, the cotyledon absorbs water more slowly but is a more or less homogeneous mass of cells and certainly does not have a layered structure like the axis. [95] It has been shown that different enzymes are activated by different amounts of water in wheat germ. [105] Since the cotyledon and axis of *Phaseolus vulgaris* have different structures, and since they absorb water at different rates, enzymes might be activated in a different order in each tissue. This could explain why, despite the fact that the axis had a faster water absorption, citrate was labelled first in the cotyledon. It is a reasonable assumption that the outside cells of the axis will become hydrated before the inside ones, as happens for the cotyledon. [94] If this is so, it is quite possible that at five minutes the central region of the axis could have less water than the surface region of the cotyledon, even though the water percentage over the whole axis would be greater than that of the whole cotyledon. If it is postulated postulated that the only enzyme system capable of forming citrate in the axis during early germination is in the central region, then at five minutes the water content in the axis could be too low to activate the system; at the same time it

could be high enough to activate the system in the surface region of the cotyledon.

2-2.2 CONCLUSION

These experiments show that additional information about the metabolism of seeds can be obtained by imbibing different parts of the seeds separately. The metabolism of the axis during the first six hours of imbibition was similar to, but not identical with that of the cotyledon. In both cases amino acid metabolism dominated the first 15 minutes of imbibition followed by organic acid metabolism and, after two hours, sucrose metabolism. During the first two hours of imbibition both the metabolic development and the water absorption of the cotyledon were slower than for the axis and it is therefore suggested that water absorption was linked to metabolic development during that time. After two hours the same compounds were labelled in the cotyledon as in the axis. This may be because both tissues have a common embryonic nature, or alternatively, because the compounds were labelled in basic metabolic pathways that operate in the tissues of all germinating seeds. In support of this latter possibility it is noted that although the order in which compounds became labelled in the *Sinapis alba* seed is not identical to that in either

the *Phaseolus vulgaris* axis or cotyledon, it is broadly similar, and the same compounds were eventually labelled in both seeds.

In *Phaseolus vulgaris* the cotyledons are the storage organs and during the later stages of germination and subsequent seedling growth supply large quantities of nutrients to the axis. However, the experiments reported here indicate that no significant amount of the cotyledon reserves are converted to translocatable compounds in the first six hours of imbibition.

2-3 EFFECT OF DISSECTION

2-3.0 INTRODUCTION

The reasons for dissecting the seeds and imbibing the parts separately have already been discussed. It was decided that the results that might be obtained justified this rather drastic procedure. However, parts of dissected seeds cannot be expected to have exactly the same metabolism as parts in intact seeds. It has so far been assumed that the differences between the two are not sufficient to invalidate the work on excised parts, but it appeared desirable to find out more information about the differences to see if this assumption was reasonable. Dissection of the seed can be

divided into two parts:

1. Removal of the seed-coat.
2. Separation of the axis from the cotyledons.

Experiments were done to find the effect of each of these processes.

2-3.1 IMBIBITION OF WHOLE SEEDS

2-3.10 INTRODUCTION.

A seed without a seed-coat absorbs water faster and has a less anaerobic metabolism during imbibition than an intact seed (Section 2-1.5). However, the most important effect of removing the seed-coat, at least in the case of *Phaseolus vulgaris*, is that it eliminates variations in water absorption. The absorption of water is not uniform over the surface of the seed, and it is clear both from the literature 101 and from observations made in the course of this present work, that not all seeds start absorbing water at the same time. The slowness and variation of water absorption during imbibition of a seed with an intact seed-coat, should be reflected in its metabolism. To see if this were so, two intact seeds were imbibed in tritiated water.

2-3.10 EXPERIMENTAL AND RESULTS

Each seed was imbibed at 21°C in 0.1 ml of tritiated water in a conical tube. While this is a relatively large amount of tritiated water (0.5 Ci), it is only enough to half immerse a seed. To make

conditions as similar as possible, each seed was imbibed on its side and the conical tube was at an angle of about 45°. This ensured that both ends of the seed were immersed to the same extent in the tritiated water. One seed was imbibed for six hours and the other for 13 hours. These are long times compared with the times for which the excised parts were imbibed but they were chosen because it was known that imbibition would be slower in the intact seeds.

After the period of imbibition, the seed was rinsed twice with water and the rinses combined. The seed-coat was removed and the naked seed was again rinsed with water. The seed was dissected and the axis and three pieces of cotyledon were then ground and extracted separately and the extracts treated in the usual way. The three peices of cotyledon came respectively from the axis end, the middle of the seed, and the non-axis end.

The results are given in Table 2.2 and the radio-autograms of the water extracts of the pieces of cotyl-don from both experiments are shown in Plate 2.3. The same abbreviations are used in Plate 2.3 as in plates 2.1 and 2.2 with the addition of Phos, for sugar phosphates.

2-3.12 DISCUSSION

The results of the six hour experiment are very

revealing. From the compounds labelled and the intensity of labelling it is apparent that the water entered the seed from the non-axis end. The trend of decreasing water content in going from the non-axis to the axis end is clearly illustrated down the left hand side of Plate 2.3. This trend was also apparent from visual observation of the variation in softness of different parts of the seed during dissection. The labelling in the piece of cotyledon from the non-axis end was similar to that in an excised cotyledon at 3-6 hours. The labelling in the piece of cotyledon from the middle of the seed was similar to that in an excised cotyledon at 15 minutes. The piece of cotyledon from near the axis had even less labelling than this but the pattern was still similar to that in an excised cotyledon at 15 minutes.

There was no labelling in the axis at all. Two possible explanations for this are advanced below.

1. Since the water entered the seed from the non-axis end the axis would be the last part of the seed to receive any water. In six hours it might not have been able to absorb enough water to allow its enzymes to operate.

2. As the tritiated water moved through the seed its specific activity would decrease due to exchange with ionisable hydrogen atoms of the seed material. The tritiated water which initially reached the axis

Table 2.2 Compounds labelled in the axis and different parts of the cotyledons when intact *Phaseolus vulgaris* seeds imbibed tritiated water for six and 13 hours.

<table>
<tr><th></th><th colspan="4">6 hours</th><th colspan="4">13 hours</th></tr>
<tr><th>Compounds labelled</th><th>A</th><th>AC</th><th>MC</th><th>NAC</th><th>A</th><th>AC</th><th>MC</th><th>NAC</th></tr>
<tr><td>4-Aminobutyrate</td><td></td><td>t</td><td>+</td><td>++</td><td>++</td><td>+</td><td>+</td><td>+</td></tr>
<tr><td>Aspartate</td><td></td><td>t</td><td>+</td><td>+</td><td>+</td><td>+</td><td>++</td><td>++</td></tr>
<tr><td>Glutamate</td><td></td><td>t</td><td>+</td><td>++</td><td>+</td><td>++</td><td>++</td><td>++</td></tr>
<tr><td>Alanine</td><td></td><td>+</td><td>++</td><td>+++</td><td>+</td><td>+</td><td>++</td><td>t</td></tr>
<tr><td>Citrate</td><td></td><td></td><td>t</td><td>++</td><td>++</td><td>++</td><td>++</td><td>++</td></tr>
<tr><td>Malate</td><td></td><td></td><td>+</td><td>++</td><td>+</td><td>++</td><td>++</td><td>++</td></tr>
<tr><td>Succinate</td><td rowspan="2"></td><td rowspan="2">t</td><td rowspan="2">t</td><td>+</td><td rowspan="2">t</td><td rowspan="2">t</td><td rowspan="2">t</td><td rowspan="2">t</td></tr>
<tr><td>Lactate</td><td>+++</td></tr>
<tr><td>Sucrose</td><td></td><td></td><td></td><td>+++</td><td>+++</td><td>+++</td><td>++</td><td>+++</td></tr>
<tr><td>Glutamine</td><td></td><td></td><td></td><td>+</td><td>++</td><td>++</td><td>+</td><td>+</td></tr>
<tr><td>Lipids ?</td><td></td><td>t</td><td>+</td><td>++</td><td>++</td><td>++</td><td>++</td><td>+++</td></tr>
<tr><td>Sugar phosphates ?</td><td></td><td></td><td></td><td>+</td><td>t</td><td></td><td></td><td>+</td></tr>
</table>

Key: +, ++, +++, = increasing relative intensity;

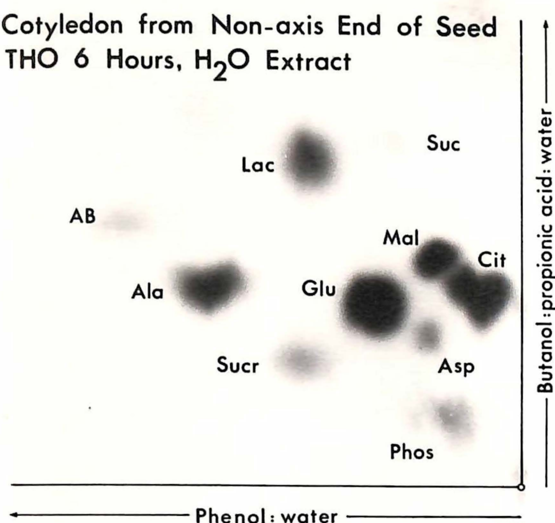
t = trace. A = axis

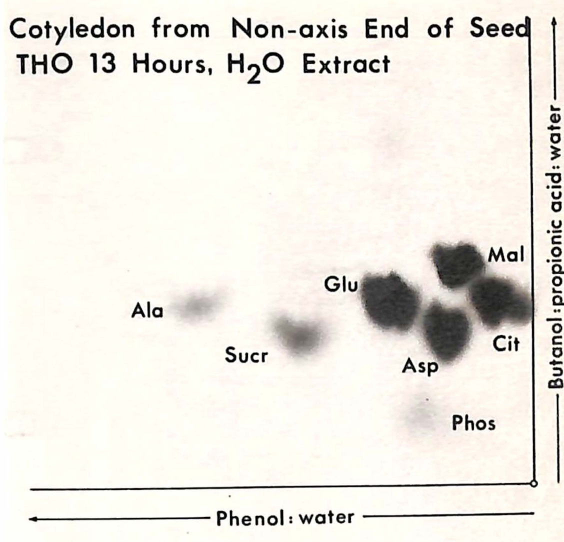
AC = piece of cotyledon from axis end of seed.

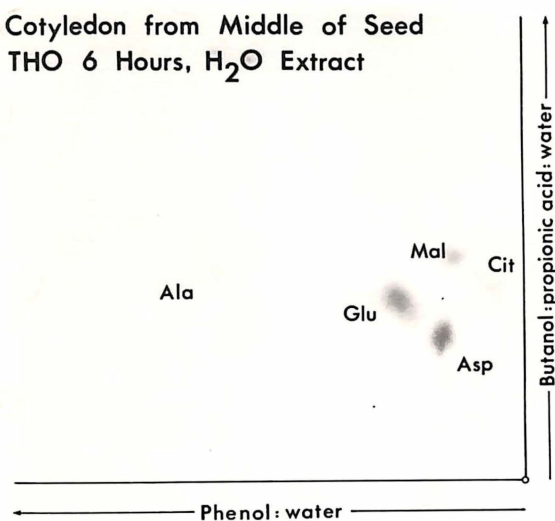
MC = piece of cotyledon from middle of seed.

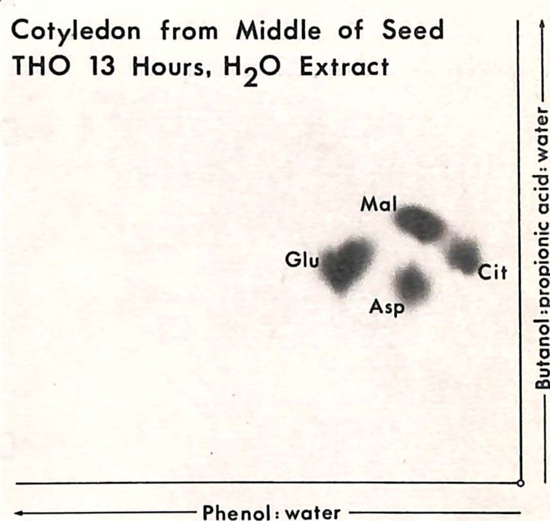
NAC = piece of cotyledon fron non-axis end of seed.

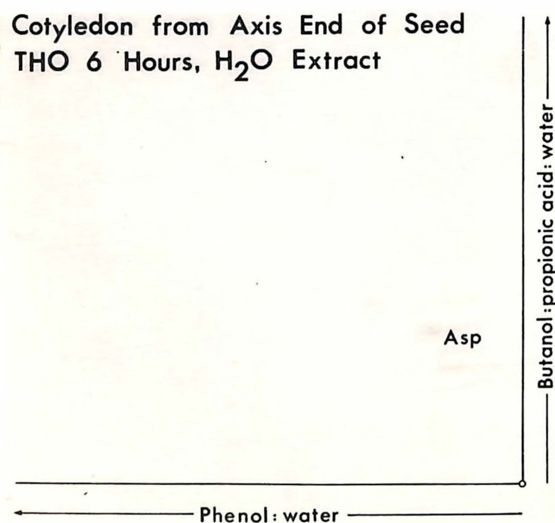
PLATE 2.3

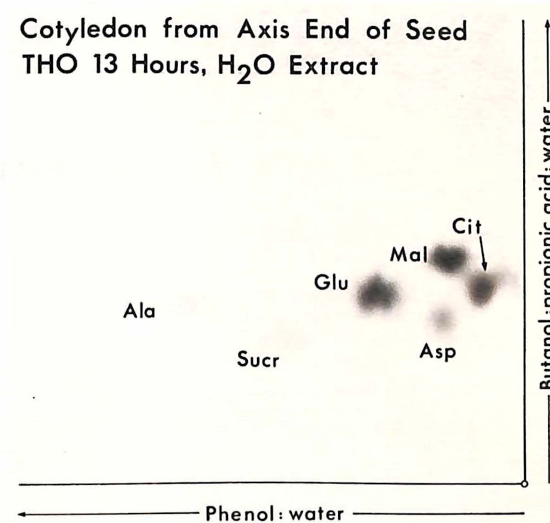












might have had a very low specific activity, so that even though there was some metabolism only insignificant amounts of labelled compounds were formed.

Visual observation of the axis during the experiment indicated that some water had been absorbed, but that the axis was not as hydrated as an excised axis would be after 10-30 minutes of imbibition. The visual observations did not enable the time to be determined any more precisely than this. It is probable therefore that the absence of labelling in the axis was due to a combination of the two effects mentioned above.

Although the patterns of labelling in the pieces of cotyledon can be compared to those in the experiments with excised cotyledons, there are some differences. There was no labelled citrate in the piece of cotyledon from the axis end and only a faint trace of radioactivity that could be citrate in the piece of cotyledon from the middle of the seed. As well as this, both these pieces of cotyledon had traces of radioactivity corresponding to lactate/succinate. In the excised cotyledon at short imbibition times, no labelled lactate/succinate was formed, and citrate was strongly labelled. These differences may indicate the existence of a very low oxygen tension near the axis end of the intact seed. This situation could be expected if water was entering, as it appears to be, from the non-axis end of the seed. As the water moved through the seed its dissolved oxygen would be used for respiratory processes

and the closer to the axis end the cotyledon tissue was, the more anaerobic its metabolism would become. The faintly labelled lactate/succinate spot is therefore probably lactate. Lactate was strongly labelled in the piece of cotyledon from the non-axis end where the water was entering. However, in that case the Krebs cycle acids were also well labelled, indicating that the oxygen tension was much higher there than in other parts of the seed.

The labelling in the pieces of cotyledon from the 13 hour experiment was much more uniform. It was similar to that in excised cotyledons at 3-6 hours, although there were differences in the relative intensities of labelling of some compounds. The labelling in the axis was similar to that of an excised axis at three hours. In contrast to the situation in the six hour, intact seed experiment, only a small amount of labelled lactate was formed in the 13 hour experiment. This indicates that the metabolism in the 13 hour experiment was less anaerobic than in the six hour one. This is probably associated with the observation that the seed in the 13 hour experiment appeared to absorb water more uniformly over its surface. Presumably if water was absorbed more uniformly so too was oxygen.

Four statements can be made on the basis of these results:

1. The labelling in parts of intact seeds can be compared to the labelling in excised parts at shorter times. There were some differences between the two, but these were mainly in the relative intensities of labelling and not in the actual patterns of labelled spots.

2. The results definitely show that it is impossible to assign a time beforehand, at which different parts of an intact seed will start absorbing water.

3. The assumptions made about the effect on metabolism of an intact seed-coat are substantially correct. If information on the sequence of changes during imbibition is to be meaningful, experiments must first be performed on parts of excised seeds.

4. Experiments on excised parts reveal a sequence of changes that indicates the metabolic development of the imbibing seed. The timing of the sequence varies in different parts of intact seeds owing to a number of factors associated with the movement of water into those seeds. Tritiated water could therefore be used to study the water movement into intact seeds by comparing the patterns of labelling from different parts of intact seeds to each other and to the patterns from excised parts.

2-3.2 IMBIBITION OF ATTACHED AXIS AND COTYLEDON

2-3.20 INTRODUCTION

The second step in dissecting a *Phaseolus vulgaris* seed is the excision of the axis from the cotyledon. This could cause differences in the metabolism of the two parts for a number of reasons.

1. Some plant hormone such as gibberellic acid might be transported from the axis to the cotyledon during the first 6 hours of imbibition. Imbibing the axis and cotyledon separately would prevent this transport.

2. Some translocation from the cotyledon to the axis could be occurring in the first six hours. If this were the case, removal of the axis might cause a build-up of some labelled metabolite in the cotyledon. In the presence of the axis this metabolite might be translocated away.

3. The injury caused by excision might produce changes in the metabolism.

It was not expected that the metabolism would be any different, as all the evidence suggests that gibberellic acid is not involved in seed metabolism till much later, translocation from the cotyledon does not appear to occur till after 24 hours, and any injury during excision would probably only have a small local effect. To discover if this was in fact the case, an axis still attached to a small piece of cotyledon was imbibed for 6 hours in

tritiated water. While there was some injury to the seed in doing this it was not as great as in the experiments where the axis was separated completely from the cotyledons.

2-3.21 EXPERIMENTAL AND RESULTS

One axis attached to about one sixth of a cotyledon, was imbibed in 0.05 ml of tritiated water in the usual way. After six hours the axis and attached cotyledon were rinsed twice with water and then separated from each other. They were then extracted and treated separately in the usual way.

The results of this experiment are given in Table 2.3. For comparison the results are also given for the experiment in which axes and cotyledons were imbibed separately for 6 hours.

2-3.22 DISCUSSION

From the results it is clear that axes and pieces of cotyledon imbibed separately have a similar metabolism to those imbibed together. In both cases the same compounds were labelled and sucrose was more heavily labelled in the axis than in the cotyledon. The intensity of the labelled spots was weaker for the attached axis than for the excised ones. Some of this difference is attributed to the fact that only one axis attached to a cotyledon was imbibed whereas two axes were used in the experiments when axes were imbibed separately.

Table 2.3. Compounds labelled in the axis and cotyledon of *Phaseolus vulgaris* when these imbibed tritiated water for 6 hours while joined together. The compounds labelled in the parts imbibed separately are given for comparison.

<table>
<tr><th>Compounds labelled</th><th>JA</th><th>SA</th><th>JC</th><th>SC</th></tr>
<tr><td>4-Aminobutyrate</td><td>t</td><td>+</td><td>++</td><td>++</td></tr>
<tr><td>Aspartate</td><td>++</td><td>++</td><td>++</td><td>++</td></tr>
<tr><td>Glutamate</td><td>++</td><td>+++</td><td>++</td><td>++</td></tr>
<tr><td>Alanine</td><td>+</td><td>++</td><td>+</td><td>t</td></tr>
<tr><td>Citrate</td><td>+</td><td>+++</td><td>+</td><td>++</td></tr>
<tr><td>Malate</td><td>++</td><td>+++</td><td>+</td><td>++</td></tr>
<tr><td>Succinate</td><td rowspan="2">t</td><td rowspan="2">t</td><td rowspan="2">t</td><td>+</td></tr>
<tr><td>Lactate</td><td>t</td></tr>
<tr><td>Sucrose</td><td>+++</td><td>+++</td><td>++</td><td>++</td></tr>
<tr><td>Glutamine</td><td>t</td><td>++</td><td>t</td><td>t</td></tr>
<tr><td>Lipids ?</td><td>+++</td><td>+++</td><td>+++</td><td>++</td></tr>
<tr><td>Sugar phosphates ?</td><td>+</td><td>+++</td><td>+</td><td>+</td></tr>
</table>

Key: +, ++, +++, = increasing relative intensity;
t = trace. JA = joined axis

SA = separated axis

JC = joined cotyledon

SC = separated cotyledon.

Separation of the axis from the cotyledons causes little change in the metabolism of the two parts, at least in the first six hours. It is possible that changes may begin to occur after this time. In this respect it is interesting to note the results of Young et al.[108] They found that excision had no effect on the development of mitochondrial respiration in cotyledons of *Pisum sativum* during the first two days of imbibition, but after this time the differences between excised and non-excised cotyledons became very marked.

2-3.3 CONCLUSION

These experiments show that the metabolism in excised parts develops at a much faster rate than in parts of intact seeds, and that the differences in metabolism of excised parts, as compared to parts in intact seeds, are due mainly to the absence of the seed-coat and not the separation of the axis from the cotyledons. It appears that the faster development of metabolism in the excised parts is due to the faster water absorption that results from removal of the seed-coat. In the presence of the seed-coat there were wide variations in water absorption from one part of the seed to another and from one seed to another. These variations were such that it is not possible to state the order in which metabolic changes occur in the intact seed, except by reference to experiments on its excised parts. However, the assumption that the

metabolic development of excised parts can be compared to the metabolic development of parts in intact seeds appears to be valid.

The experiments on intact seeds described in Section 2-3.1 were difficult to perform and by themselves provided little information about the orderin which metabolism developed during imbibition. However, when performed in conjunction with experiments on excised parts, experiments on intact seeds have a valuable potential for the study of water movement into seeds during imbibition.

2-4 INVESTIGATION OF SUCROSE LABELLING

2-4.0 INTRODUCTION

Mention was made in Section 2-2.1 of the somewhat conflicting information about the role of sucrose. This conflict is elaborated in more detail in the following sections.

In the e*periments on *Phaseolus vulgaris*, sucrose was labelled in both cotyledon and axis at two hours. This means that at least some sucrose was being formed at that time from labelled intermediates. Whether or not the intermediates concerned (fructose, fructose-6-P and UDPG) would be labelled during sucrose synthesis in tritiated water is discussed in section 2-4.1.

There appear to be four possibilities for the origin of the intermediates of the sucrose that is

synthesized during the early germination of *Phaseolus vulgaris*.

1. From glucose: Duperon showed that this is occurring during the first four hours of imbibition, but since there is little free glucose in the seed, [69] this is probably only a minor source of sucrose.

2. From raffinose: The first step in raffinose metabolism is the splitting of the molecule into sucrose and galactose by α-galactosidase (3.2.1.22). The sucrose that is formed directly cannot become labelled if this reaction is occurring in tritiated water. The galactose formed can be metabolised to UDPG. It can therefore enter the pool of intermediates that form sucrose. Since these intermediates can form labelled sucrose in tritiated water (see Section 2-4.1), raffinose breakdown could be responsible for some of the labelled sucrose formed. There is good evidence for believing that raffinose is being broken down in at least the axis of *Phaseolus vulgaris* (see Section 2-1.4), so it is probable that some of the labelled sucrose came from this source.

3. From gluconeogenesis: while it would seem strange if this occurred in a starchy seed during early germination the possibility cannot be ruled out. Thomas and Rees [33] found that both glycolysis and its reverse, gluconeogenesis, were occurring at the same

time in seeds of *Curcurbita pepo* during the first six hours of germination.

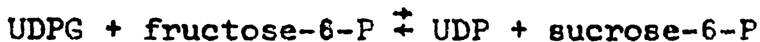
4. From starch: in the imbibing seeds of the closely related *Vicia fabia* [91] and *Pisum sativum* [92], glucose-1-P is formed from starch by the action of phosphorylase (2.4.1.1). This glucose-1-P is eventually metabolised to sucrose. As explained in Section 2-4.1, sucrose would be expected to be labelled if it were being formed in this way from starch in the presence of tritiated water. It is likely that this pathway accounts for the sucrose formed in the cotyledon, as this contains large amounts of starch, but little raffinose or glucose. The results of Oota et al [2] on the closely related *Vigna sesquipedalis* seed suggest that sucrose might also be formed from starch in the axis.

Of the four possibilites outlined above the last two have least evidence to support them. Experiments were therefore carried out to find evidence for or against their existence. Sections 2-4.2 and 2-4.3 report the results of experiments that were done to see if the suggested starch-sugar interconversion in the axis of *Vigna sesquipedalis* occurred also in the *Phaseolus vulgaris* axis. Section 2-4.4 records what happened when ^{14}C-alanine was fed to both the axis and the cotyledon to find out if any gluconeogenesis was occurring.

2-4.1 HYDROLYSIS OF SUCROSE WITH INVERTASE

2.4.10 INTRODUCTION

In the experiments on *Phaseolus vulgaris*(Section 2-2) sucrose was labelled in both the axis and cotyledon from two hoursimbibition onwards. This means that at least some sucrose was being formed at that time from labelled intermediates. There are two well established pathways that are capable of forming sucrose. The first pathway involves the reactions:


UDPG + fructose-6-P $\rightleftarrows$ UDP + sucrose-6-P

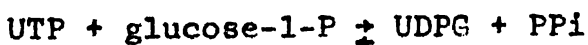
Sucrose-6-P $\rightleftarrows$ sucrose + Pi

The enzymes responsible are sucrose-6-P synthetase (2.4.1.14) and a phosphatase. The second pathway involves only one reaction:

UDPG + fructose $\rightleftarrows$ UDP + sucrose

The enzyme is sucrose synthetase (2.4.1.13). UDPG is involved in both pathways and is connected to the glycolytic sequence through the following three reversible reactions.

UTP + glucose-1-P $\rightleftarrows$ UDPG + PPi


Glucose-6-P $\rightleftarrows$ glucose-1-P

Fructose-6-P $\rightleftarrows$ glucose-6-P

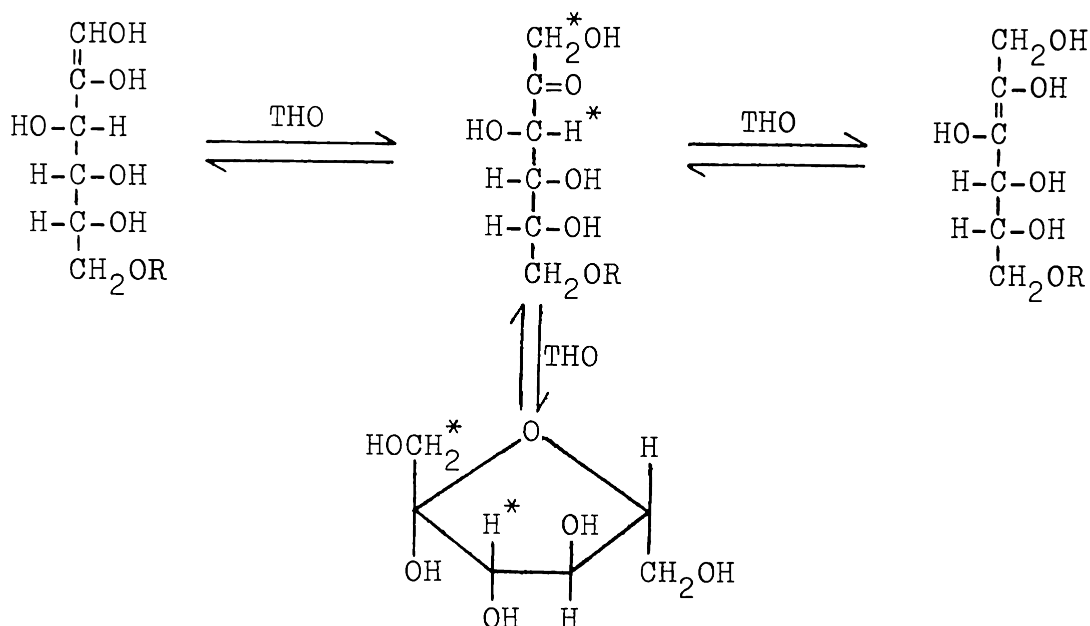
The three enzymes involved are respectively UDPG-pyrophosphorylase (2.7.7.9), phosphoglucomutase (2.7.5.1) and glucosephosphate isomerase (5.3.1.9).

Labelled sucrose would be formed if any of its precursors had a tritium atom bonded directly to a carbon atom. Fructose and fructose-6-P could possibly become labelled in tritiated water by keto-enol tautomerism. UDPG and glucose-1-P cannot become labelled in this way as their respective groups attached to C-1 prevent tautomerisation. However, glucose-6-P could possibly become labelled by keto-enol tautomerism in tritiated water. This label could be passed on to glucose-1-P in the reaction catalysed by phosphoglucomutase (2.7.5.1),and then to UDPG in the reaction catalysed by UDPG-pyrophosphorylase (2.7.7.9).

The possible equilibria through which glucose, fructose, and their C-6 phosphates could become labelled are shown in Fig. 2.3. Only a small percentage of each of these molecules is present in the straight chain form. However, the ring forms, with which these straight chain forms are in equilibrium, do not have carbonyl groups and cannot therefore participate in keto-enol tautomerism.

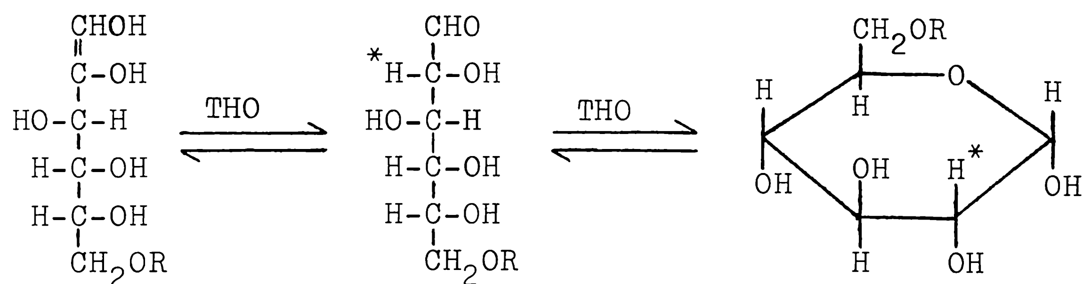
Any compounds labelled by exchange with tritiated water in the way shown in Fig. 2.3, would be expected to lose their label as easily as they had gained it. So compounds labelled in this way would be expected to

FIGURE 2.3



R=P for fructose-6-P

R=H for fructose



R=P for glucose-6-P; R=H for glucose

Possible equilibria through which glucose, fructose and their phosphates could become labelled in tritiated water by keto-enol tautomerism. P represents a phosphate group; an asterisk denotes a position into which tritium could be incorporated; THO represents tritiated water.

exchange their label away again during elution and chromatography with aqueous solvents. However, in the sucrose molecule, the fructose and glucose moieties are locked in their ring configurations and no exchange of label can occur. So if sucrose is formed from precursors that have become labelled in this way, the sucrose will be non-exchangeably labelled even though its precursors are exchangeably labelled. On hydrolysis of sucrose by invertase (3.2.1.26), and chromatography of the glucose and fructose formed, the label should be lost again, as glucose and fructose can then exist in straight chain forms. This was the situation that Missen [18] claimed to have found in *Sinapis alba.* He used a sample of labelled sucrose obtained from an experiment in which *Sinapis alba* seeds were imbibed in tritiated water. He hydrolysed the sucrose with invertase and chromatographed the hydrolysate in two dimensions with the usual phenol:water and butanol:propionic acid:water solvents. He radioautographed the chromatograms and reported that there was not even a trace of radioactivity corresponding to glucose or fructose.

Labelled sucrose, formed in the axis and cotyledon of *Phaseolus vulgaris* when these were imbibed in tritiated water, was treated with invertase to see if it was labelled in the same way as that in *Sinapis alba.*

2-4.11 EXPERIMENTAL AND RESULTS

Samples of sucrose from the six hour tritiated water experiments on the axis and cotyledon were hydrolysed with invertase as outlined in Section 1-4.8. The hydrolysate was chromatographed in one dimension alongside reference sugars using the solvent propanol: ethyl acetate:water 7:1:2. The chromatogram was then radioautographed in the usual way.

The label was not lost during this process. One radioactive spot was formed and this corresponded to glucose and fructose. The solvent used does not separate these two spots completely but it does separate sucrose from them quite conclusively. To see if the spot was indeed glucose and fructose it was eluted in the usual way with 95% ethanol and co-chromatographed in two dimensions with both glucose and fructose. The solvents used were phenol:water 100:29 and ethyl acetate:pyridine:water 12:5:4. A prior investigation had shown that glucose and fructose were completely separated by this pair of solvents under the conditions used. It was found that both glucose and fructose were labelled although the labelling was very weak.

2-4.12 DISCUSSION

This result is the opposite to that found by Missen for labelled sucrose from *Sinapis alba*. The simplest explanation is that the precursors of sucrose

in *Sinapis alba* are exchangeably labelled whereas those in *Phaseolus vulgaris* are not. As pointed out by Mann,[23] glucose and fructose phosphates could possibly become non-exchangeably labelled if they were being formed by reverse of the first few steps of glycolysis, notably the reactions catalysed by the enzymes aldolase (4.1.2.13) and triosephosphate isomerase (5.3.1.1). The results therefore could mean that some reversal of glycolysis occurs in *Phaseolus vulgaris* but not in *Sinapis alba*.

However, there is another explanation. The labelling of the glucose and fructose spots after co-chromatography in phenol:water and ethyl acetate:pyridine: water was very weak. Yet glucose and fructose were quite strongly labelled on the previous one dimensional chromatogram where the solvent was propanol:ethyl acetate:water. For some reason most of the activity was lost in transferring and co-chromatographing the glucose and fructose on the two dimensional chromatogram. Missen chromatographed his sucrose hydrolysate in two dimensions but did not develop a preliminary one dimensional chromatogram. If most of the label in his experiment had also been lost during the two dimensional co-chromatography, he might have been unable to detect any radioactivity corresponding to glucose and fructose even though they were labelled after hydrolysis.

Certainly he would have found none on the two dimensional chromatograms of the sucrose hydrolysate from *Phaseolus vulgaris*, as he radioautographed his chromatograms for only a week. This would not have been long enough to detect the faint labelling that was present.

It is possible therefore that sucrose from both seeds is labelled in the same way and that the different results obtained are caused by differences in experimental technique. To test this possibility a further experiment was done.

2-4.13 SECOND INVERTASE EXPERIMENT.

A sample of labelled sucrose was obtained, by courtesy of M. Vickers, from an experiment in which *Sinapis alba* seeds had been imbibed in tritiated water. A further sample of sucrose was obtained from a *Phaseolus vulgaris* experiment. The two samples were hydrolysed with invertase in the same way as before. Half the hydrolysate from each sample was chromatographed with reference sugars in one dimension; the solvent used was butanol:propionic acid:water 15:7:10. The other half of each hydrolysate was also chromatographed with reference sugars in one dimension; the solvent used this time was propanol: ethyl acetate:water 14:2:4. The chromatograms were then

radioautographed in the usual way.

In all cases a strongly labelled spot corresponding to glucose and fructose was found.

2-4.14 FURTHER DISCUSSION

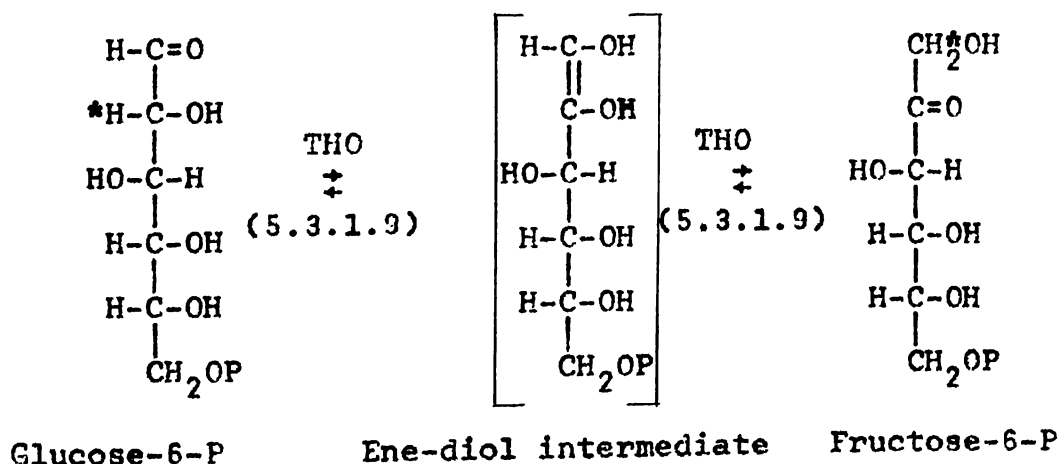
These results indicate that the labelling in the sucrose from the two seeds is much more similar than at first thought. Apparently the label in glucose and fructose is not exchanged away during the hydrolysis of sucrose or the one dimensional chromatography in the above solvents.

There remains the problem of why the label is lost during two dimensional chromatography. A simple explanation would be that the label is only exchanged off slowly and two dimensional chromatography gives it more time to do this. This explanation is unsatisfactory as since the label exchanges on easily it should exchange off just as easily. It appears that the explanation is considerably more complicated.

The assumption that the precursors of sucrose synthesis become labelled in tritiated water by non-enzymic keto-enol tautomerism may not in fact be true. The evidence suggests that the equilibria between glucose, fructose and their ene-diol intermediates can only occur at extremes of pH or at high temperatures,[109, 110] both conditions that are not present in

germinating seeds. It appears that near neutral pH and at room temperature these equilibria are insignificant.[111]

It is more probable that the label in sucrose was originally incorporated into its precursors in the reaction catalysed by glucosephosphate isomerase (5.3.1.9). Topper[42] showed that glucose-6-P could become labelled at C-2 when allowed to react with this enzyme in deuterated water. If this also happened in tritiated water, and the labelled glucose-6-P was then used for sucrose synthesis, the sucrose formed would be labelled. Fructose-6-P would also be expected to become labelled in the presence of this enzyme; so in the sucrose formed, both the glucose and fructose moieties could be labelled. The reaction catalysed by glucosephosphate isomerase (5.3.1.9) and the suggested labelling are shown below. An asterisk denotes the positions into which tritium would be incorporated.



The ene-diol intermediate is the suggested intermediate for the interconversion. It is bracketed to show that it occurs only as part of an enzyme complex. The straight chain forms of the two sugar phosphates will be in equilibrium with their ring forms as shown in Fig. 2.3.

The difference between this form of labelling and that discounted earlier is that this is due to the action of an enzyme whereas the other was not. In favour of this enzymic way of labelling glucose and fructose phosphates is the fact that (2-deutero) glucose-6-P in the absence of the enzyme glucose phosphate isomerase (5.3.1.9) does not exchange away its label in non-deuterated water.[42] If the non-enzymic mechanism was working the label would be lost by exchange.

There still remains the problem of why most of the label was lost during two dimensional chromatography. There are two possible explanations:

1. There was always some loss of the label during co-chromatography due to incomplete elution and diffusion of the compounds during chromatography. However, this is unlikely to have accounted for the consistent loss of label that occurred in all cases.

2. The organic co-chromatography solvents used might catalyse exchange of the label. There is evidence to suggest that anhydrous pyridine can

catalyse the keto-enol tautomerism of glucose and fructose that is required if the label incorporated by the glucosephosphate isomerase reaction is to be exchanged away. Phenol has also been suggested as a possible catalyst.[109] Phenol:water was used in the two dimensional chromatography of both Missen's work and of the work reported here. It was used because it gives a better separation of glucose and fructose on paper chromatograms than any other well known solvent (p. 313, ref. 20). If phenol also catalysed keto-enol tautomerism of glucose and fructose they would lose their label during chromatography. That this could be happening is supported by the fact that the label in glucose and fructose was not lost when they were chromatographed in one dimension using butanol:propionic acid:water as the solvent, but it was almost completely lost when they were chromatographed in two dimensions using phenol:water as the first solvent followed by butanol:propionic acid:water.

2-4.15 CONCLUSION

The evidence suggests that sucrose from both *Sinapis alba* and *Phaseolus vulgaris* was labelled in the same positions. It is suggested that its precursors became labelled in the reaction catalysed by glucosephosphate isomerase (5.3.1.9). This would cause fructose to be labelled at C-1 and glucose at C-2. However, the

possibility cannot be excluded that the glucose and fructose moieties of sucrose became labelled by being formed from reversal of the first steps of glycolysis.

2-4.2 DISSECTION OF AXES AFTER IMBIBITION IN TRITIATED WATER

2-4.20 INTRODUCTION

Oota et al [2] measured the total amount of starch in both the hypocotyl and plumule of the *Vigna sesquipedalis* axis at day intervals from the start of imbibition. The level of starch dropped in the hypocotyl and rose in the plumule even during the first day of imbibition. Since starch cannot be translocated as such, they concluded that it was being converted to sugar in the hypocotyl, and that this sugar was then being translocated to the plumule where it was being converted back to starch.

If the same process occurs in the closely related *Phaseolus vulgaris* seed, and if the sugar being translocated is sucrose, then labelled sucrose would be formed when the axis was being imbibed in tritiated water, and the plumule could be regarded as a sucrose sink. If the axis was imbibed for some hours in tritiated water and then dissected into hypocotyl and plumule most of the labelled sucrose might be found in the plumule. The experiment below was done to test this possibility.

2-4.21 EXPERIMENTAL AND RESULTS

Two axes were imbibed in the usual way for two hours.

They were rinsed as usual and dissected into hypocotyl and plumule. The hypocotyls and plumules were extracted separately. The extracts were chromatographed and radio-autographed in the usual way.

The compounds labelled are given in Table 2.4, in which the labelling in both ethanol and water extracts has been combined. The radioautograms of the ethanol extracts are shown in Plate 2.4. The radioautograms of the ethanol extracts are shown because most of the sucrose was present in those extracts.

2-4.22 DISCUSSION

The plumule is smaller than the hypocotyl and this was reflected in the less intense labelling of the compounds in this part of the axis. The labelling of sucrose in the plumule was somewhat less intense than in the hypocotyl, in line with this general trend. These results give no support to the suggestion that sucrose was being translocated from the hypocotyl to the plumule. However, the possibility that this was occurring to some extent but that most of the sucrose remained in the hypocotyl could not be excluded.

2-4.3 SEPARATE IMBIBITION OF HYPOCOTYLS AND PLUMULES

2-4.30 INTRODUCTION

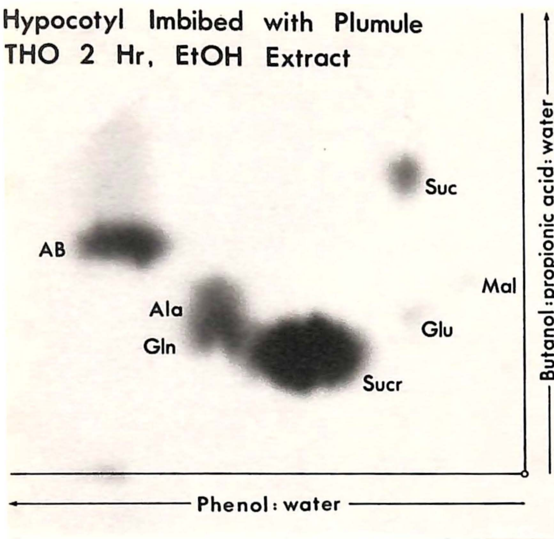
Although large amounts of labelled sucrose were found in both the hypocotyl and plumule in experiment 2-4.2, it is possible that this was being formed only in the hypocotyl and that only some of it was being translocated to the plumule. To test this possibility, plumules and hypocotyls

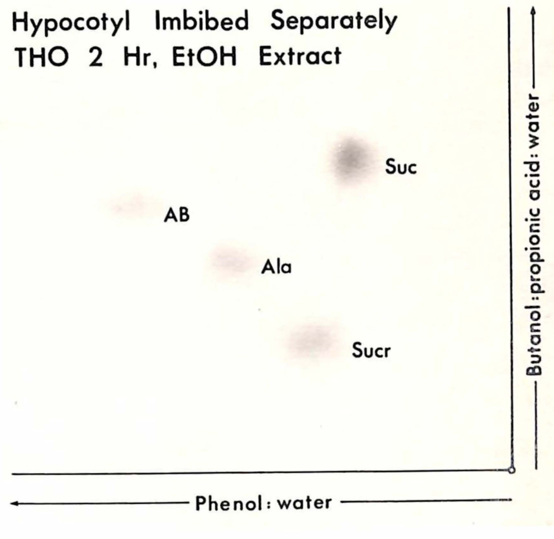
Table 2.4 Compounds labelled in the hypocotyl and plumule when axes of *Phaseolus vulgaris* were dissected into these two parts after imbibing tritiated water for two hours.

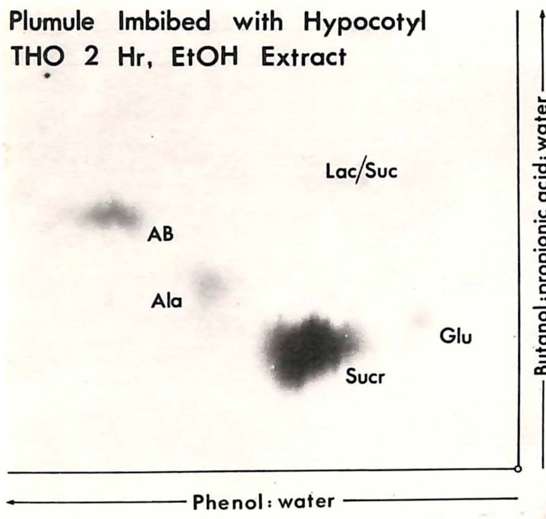
Compounds labelled	Hypocotyl	Plumule
4-Aminobutyrate	++	+
Aspartate	+	t
Glutamate	+	t
Alanine	+	t
Citrate	+	+
Malate	++	+
Succinate	+	t
Lactate		
Sucrose	+++	++
Glutamine	t	
Lipids ?	++	++
Sugar phosphates ?	++	+

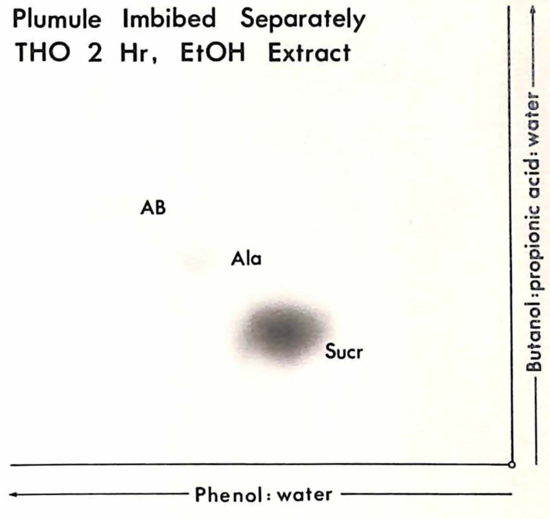
Key : +, ++, +++ = increasing relative intensity;
t = trace

PLATE 2.4









were imbibed separately in tritiated water. If the hypocotyl was the only part of the axis producing sucrose, no labelled sucrose would have been found in plumules imbibed in tritiated water by themselves.

2-4.31 EXPERIMENTAL AND RESULTS

In this experiment six plumules and two hypocotyls were used. Because the plumules are smaller and lighter more of them were used so that the amount of material in both cases was approximately equal. The six plumules were imbibed together for two hours in tritiated water. They were then rinsed and extracted and the extracts were treated in the usual way. The same process was repeated for the two hypocotyls.

The results are given in Table 2.5. In both cases the labelling in the three extracts has been combined for presentation. The radioautograms of the ethanol extracts are shown in Plate 2.4.

2-4.32 DISCUSSION

These results are similar to those reported inSection 2-4.2. The same labelled compounds, including sucrose, were formed in both parts of the axis. Sucrose was more heavily labelled in the plumule than in the hypocotyl in this experiment, whereas the reverse was true for the experiment described in Section 2-4.2 However, this difference can be attributed to six plumules being used in this experiment compared to only two plumules being used in that experiment.

Table 2.5 Compounds labelled when hypocotyls and plumules of *Phaseolus vulgaris* were imbibed separately for two hours in tritiated water.

<table>
<tr><th>Compounds labelled</th><th>Hypocotyl</th><th>Plumule</th></tr>
<tr><td>4-Aminobutyrate</td><td>+</td><td>t</td></tr>
<tr><td>Aspartate</td><td>+</td><td>+</td></tr>
<tr><td>Glutamate</td><td>+</td><td>+</td></tr>
<tr><td>Alanine</td><td>++</td><td>++</td></tr>
<tr><td>Citrate</td><td>+</td><td>+</td></tr>
<tr><td>Malate</td><td>+</td><td>+</td></tr>
<tr><td>Succinate</td><td>+</td><td rowspan="2">t</td></tr>
<tr><td>Lactate</td><td>t</td></tr>
<tr><td>Sucrose</td><td>+</td><td>++</td></tr>
<tr><td>Lipids ?</td><td>++</td><td>++</td></tr>
<tr><td>Sugar phosphates ?</td><td>+</td><td>+</td></tr>
</table>

Key: +, ++ = increasing relative intensity

t = trace

Succinate was more intensely labelled in the hypocotyl than in the plumule in both experiments. This may indicate that the hypocotyl has a larger succinate pool than the plumule. Because these experiments were basically qualitative in nature it is not possible to attach any significance to the small difference between the hypocotyl and plumule in the intensity of labelling of the other compounds.

On the basis of the results in this and the preceding experiment it appears unlikely that sucrose is formed in the imbibing axis of *Phaseolus vulgaris* in the way intimated by Oota [2] for the *Vigna sesquipedalis* axis. The experiments described in these sections also show that the hypocotyl and plumule have very similar metabolism after two hours of imbibition.

2-4.4 EXPERIMENT WITH ^{14}C-ALANINE

2-4.40 INTRODUCTION

Thomas and Rees fed ^{14}C labelled acetate [112] and glucose [33] to imbibing seeds and seedlings of *Cucurbita pepo* to find if and when glycolysis and gluconeogensis were occurring. They found that both pathways were operating before and after germination, although the activity after six hours of imbibition, the shortest time for which they recorded results, was only slight. They suggested that the two pathways must be operating in different parts of the seed for this to occur. A similar situation is also

thought to exist in the case of Castor oil.[5] Both these seeds contain large amounts of stored lipids, whereas the *Phaseolus vulgaris* seed does not. Even so there is lipid metabolism in the *Phaseolus vulgaris* seed during the first six hours of imbibition (Section 2-2.1), and thus gluconeogenesis must be considered as a possibility.

To determine if any gluconeogenesis was occurring in *Phaseolus vulgaris* seeds during the first six hours of imbibition, ^{14}C-alanine was fed to axes and pieces of cotyledon. This compound was used because a preliminary experiment had shown that it was easily absorbed and metabolised by both axis and cotyledon to Krebs cycle intermediates. It is these intermediates, supplied by either the glyoxylate or Krebs cycles that are used in gluconeogenesis.

2-4.41 EXPERIMENTAL AND RESULTS

Universally labelled ^{14}C-alanine was purified as described in Section 1-4.9. Two axes were imbibed for six hours in about 0.05 ml of water containing approximately 0.5 μCi of ^{14}C-alanine. The usual methods of extraction and chromatography were then employed. The chromatograms were exposed to film directly, since the β particles emitted by ^{14}C are energetic enough to penetrate the emulsion of the film. Two pieces of cotyledon were treated similarly. The results are given in Table 2.6, where the labelling in the three extracts has, as usual, been combined for presentation.

Table 2.6 Compounds labelled with ^{14}C when the axis and cotyledon of *Phaseolus vulgaris* imbibed a solution of ^{14}C-alanine for six hours.

Compounds labelled	Axis	Cotyledon
4-Aminobutyrate	t	t
Aspartate	+	+
Glutamate	+	+
Citrate	+	+
Malate	+	+
Succinate Lactate	t	t
Glutamine	t	
Lipids ?	+	t

Key: + = positive identification;

t = trace

2-4.42 DISCUSSION

In the case of the axis there was only a trace of radioactivity that could have been sucrose, on the basis of its R_f value in both solvents and this trace was on the ethanol extract chromatogram . In the case of the cotyledon there was no such trace. Compounds described as having a trace of radioactivity have too little activity to be identified by co-chromatography. Attaching a name to such compounds therefore has a somewhat dubious validity. In the case of the experiments with tritiated water, naming trace amounts of activity is more acceptable in that, in a series of experiments, the compound in question is becoming more heavily labelled with time. The compound is identified in a later experiment and this identification is extrapolated back to the stage at which there was only a trace of radioactivity. This argument does not apply however in the case of this ^{14}C-alanine experiment. The naming of a trace of radioactivity as sucrose, on the basis of it having a similar R_f value in two chromatography solvents, can only be regarded as tentative. The further treatment of this trace of radioactivity to determine if it was sucrose is described in Section 2-4.43.

The labelling in aspartate, glutamate, citrate and malate was strong enough for these to be positively identified by co=chromatography. The fact that these were labelled gives much support to the interpretations put on the results of the tritiated water experiments.

Thus it can be stated with much more certainty that the Krebs cycle is operating and that transaminations are occurring during early imbibition. The greater certainty comes from the fact that the labelling of two compounds in a ^{14}C tracer experiment shows that there must be a pathway connecting them; whereas the labelling of the same two compounds in a tritiated water experiment shows that they are both involved in some metabolism but not necessarily that they are connected. The results of this experiment are in accord with those of the ^{14}C tracer experiments of Duperon (reported in Section 2-1.4).

2-4.43 INVESTIGATION OF POSSIBLE SUCROSE LABELLING

The faint trace of radioactivity corresponding to sucrose, in the ethanol extract chromatogram of the axis, was eluted onto the origin of another piece of chromatography paper. It was developed in one dimension alongside known samples of sucrose, glucose and fructose. The solvent used was ethyl acetate:propanol:water 1:7:2 . Under the conditions used, this solvent took two hours to reach the bottom of the paper and was then over-run for three hours because the sugars are slow moving. After removing the strip of paper containing the suspected sucrose spot the chromatogram was sprayed with the usual sugar sprays to identify the position of the sugars. The strip containing the suspected sucrose spot was cut into six pieces each about 2.5 cm long. The strip was cut in such a way that, by comparison to the known reference

sugars on the chromatogram, it could be stated that any sucrose in the strip would be found in one particular piece and any glucose or fructose would be found in two other pieces; although these last two sugars were not completely separated from each other in this solvent.

Each piece was placed in a vial containing the scintillation mixture described in Section 1-3, and counted in a scintillation counter for 20 minutes. The pieces corresponding to glucose, fructose and sucrose all had counts within two standard deviations of the background count (12.5 ± 1.6 counts/min to two standard deviations). The only piece of the strip that had any activity was the piece that included the origin and even there the activity was very low.

2-4.44 CONCLUSION

The trace of radioactivity from the ethanol extract of the axis, that had been tentatively assigned the name sucrose was not sucrose or any other sugar. Gluconeogenesis from Krebs cycle intermediates proceeds through malate. Malate was labelled in this experiment but there was no labelled sugar formed. It therefore appears that gluconeogenesis by reverse of glycolysis, was not occurring in *Phaseolus vulgaris* seeds during the first six hours of imbibition.

2-4.5 CONCLUSION

These experiments show that the tritiated sucrose, formed in the seeds of both *Phaseolus vulgaris* and *Sinapis alba* was labelled in both its fructose and glucose moieties. The evidence indicates that gluconeogenesis was not occurring in *Phaseolus vulgaris* seeds during the first six hours of imbibition. In the absence of gluconeogenesis, and since the label in glucose and fructose appeared to be partially lost during chromatography in phenol:water, it is somewhat speculatively suggested that the label was introduced into the precursors of sucrose in the reaction catalysed by glucosephosphate isomerase (5.3.1.9). It seems most likely that these precursors of sucrose were themselves formed from hexoses and from the breakdown of raffinose but the possibility that they were also formed from starch breakdown cannot be eliminated.

CHAPTER 3

EXPERIMENTS ON BARLEY SEEDS

3-1 INTRODUCTION

3-1.0 GENERAL

The seed of barley *(Hordeum vulgare)*, variety Carlsberg was the second seed studied for this thesis. Barley is a member of the tribe Hordeae, of the sub-family Festucoideae, of the family Gramineae also known as the Poaceae or grass family (p. 201-204, ref. 17). This family belongs to the monocotyledon class of the angiosperms.

The most interesting characteristic of the seeds of this family is their large starchy endosperm. The embryo is small and is found at the base of the seed.

The barley seed was selected for study because:

1. It is typical of a class of seeds whose germination is extremely important in agriculture. Because of this importance, seed germination has been better studied in this family than in any other.

2. It is used for malting. The first part of this process is similar to the early stages of germination and this has been responsible for stimulating some research on this seed.

3. It is a seed which stores its energy as starch and its storage organ is the endosperm.

4. It was big enough to dissect, yet had a small

enough embryo for several of these to be imbibed together in a small amount of tritiated water.

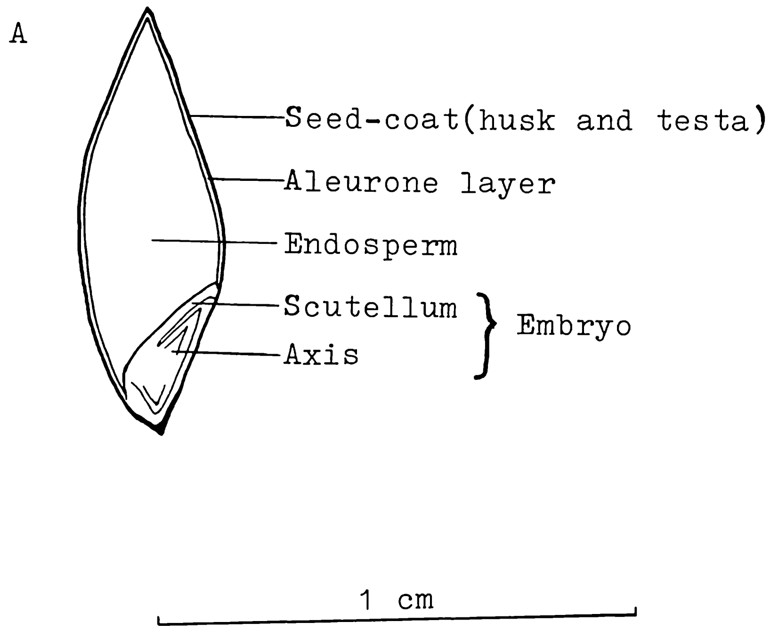
5. It had a high germination percentage (above 95%).

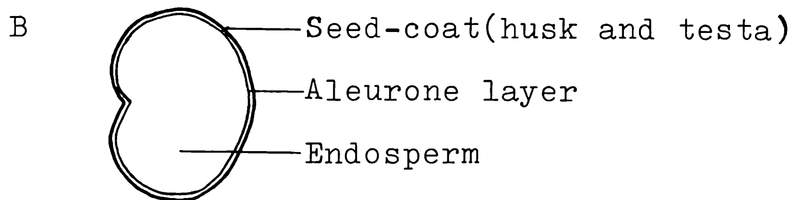
3-1.1 DESCRIPTION AND STORAGE

In the variety of barley used, the seeds were approximately 0.9 cm long and 0.4 cm in diameter. The average seed weighed 0.06 g and the embryo approximately 0.003 g. The husk which was yellow in colour was firmly attached to the underlying testa. As shown in fig. 3.1 most of a barley seed is composed of the endosperm which, in the resting state, is very hard. The embryo is divided into the axis and the scutellum. The scutellum, which is situated between the axis and the endosperm, is regarded as the single cotyledon.[113] This has the function of absorbing nutrients from the embryo, and during germination is the site of intense metabolic activity.[114] Most of the endosperm apparently consists of stored reserves, mainly starch but with some protein, and contains no clearly differentiated cells.[115] However, there is a thin layer of tissue on the surface of the endosperm, beneath the testa, that consists of organised, differentiated cells. This is called the aleurone layer.

The seeds were stored at room temperature and humidity. Under these conditions their water content varied from 9.5 - 10.5%. The germination percentage

FIGURE 3.1





Barley(*Hordeum vulgare*) seed shown in (A) longitudinal and (B) transverse sections.

dropped slowly over the twelve month period in which the experiments were performed but it was still above 90% at the end of this time. At room temperature the seeds began germinating (radicle protrusion) after 18 hours of imbibition and after 24 hours approximately 80% had germinated.

3-1.2 COMPOSITION

The composition of the whole seed given below has been compiled from a number of sources. Where these analyses overlap there are the normal small differences that are expected when the seeds being compared are those of differing varieties and come from differing localities. The composition on a dry weight basis is: starch, 63 - 66%; [115, 116] protein, 10 - 16%; [7, 8] inert husk, 12 - 13%; [115] lipids, 1.7 - 2.5%; [7, 116] sucrose, 0.8 - 2.0%; [115, 117] raffinose, 0 .3 - 0.6%; [116, 117] fructosans, 0.35 - 1.2%; [116, 117] and ash, 2.9 - 3.3%.[7] As well as this the seeds contain small quantities of glucose, fructose and maltose (0.26%),[117] and even smaller quantities of citrate (0.02%) and malate (0.01%).[118]

There have been some analyses done on the embryo and endosperm. These show that the two organs have markedly different compositions. The embryo contains large amounts of lipids and sugars, but no starch. [116] It has 14.1 - 15.4% lipids on a dry weight basis, [116] and this is about one third of the total lipids of the

seed. By comparison, the endosperm contains only 1.2 - 1.5% lipids. Fatty acids comprise more than 85% of the total lipids of the seed. This fatty acid fraction is almost entirely esterified and is composed mainly of linoleic (52%), oleic (28%) and palmitic (11.5%) acids.

The embryo also contains large quantities of the free sugars of the seed. The values given by MacLeod [116, 119] for the dry weight sugar composition of the embryo are: sucrose 11 - 14%; raffinose 9 - 10%; easily hydrolysable polysaccarides, 9%; and cellulose, 3%. James [120] also measured sugar levels, but unfortunately he did not give the percentage composition of sugars relative to the dry weight. He found that the embryo contained almost three times as much sucrose as raffinose.

The endosperm contains less than half of the sugar fraction in the seed and this comprises less than 1% of its dry weight. [119] This is mainly sucrose and there is no raffinose.

3-1.3 METABOLISM

The metabolism of the seeds of the Gramineae (the grass family) and especially of barley has been better studied during early germination than the metabolism of seeds from any other family. This is because of the universal importance of the seeds from this family as a

food source. Even so, the information that has been obtained so far, is sometimes vague and contradictory about the overall changes that are occurring, and is far less adequate in describing the metabolism in the different parts of the seeds.

Brown [29] measured respiration in barley embryos and showed that while this was low after two hours it rose steadily from then on. This rise occurred much faster in excised embryos than in attached ones. After six hours imbibition, he measured respiration in the endosperm and found that it was very low, only 5% of that in the embryo.

Abdul-Baki [121] studied the incorporation of ^{14}C-leucine into protein and ^{14}C-glucose into various compounds during the first 12 hours of imbibition. He compared this to oxygen consumption. During the first two hours oxygen uptake of the seed was low but then it began to increase rapidly. Incorporation and metabolism of leucine and glucose was also at a low level initially but by seven hours this had begun to increase and was still doing so at 13 hours. After ten hours of imbibition the label from ^{14}C-glucose was being incorporated into hemicelluloses, starch, protein and other sugars (there was no attempt at identification), but most of it appeared as $^{14}CO_2$. Isolated embryos incorporated approximately equal amounts of the label into protein and polysaccharide fractions, but endosperms transferred

very little of the label to protein and much more of the label to polysacharides. The incorporation of radio-activity by the endosperm appeared to be greater than that of the embryo but because of the experimentaltechn-ique used it is impossible to compare the two on a weight basis.

James [120] studied changes in carbohydrates of the barley embryo during imbibition. He found that the embryo contained three times as much sucrose as raffinose, these two sugars accounting for 95% of the sugars in the embryo. During the first day there was a large decrease in the levels of both sugars and an increase in the hemicellulose fraction. These changes occurred in both attached and excised embryos, the decrease in the level of sucrose being greater for excised embryos. This is in accord with the findings of Brown [29] and Abdul-Baki [121] that excised embryos have a more active metabol-ism than attached ones. MacLeod [119] also studied the metabolism of raffinose and sucrose. The embryo of the variety she used contained 11% sucrose and 9% raffinose on a dry weight basis. She reported that the embryo was beginning to metabolise its sucrose after six hours of imbibition and its raffinose after 14 hours. The metabolism of the two sugars was not the same in aerobic conditions as it was in anaerobic conditions: sucrose but not raffinose, was metabolised in anaerobic conditions, but in aerobic conditions the

situation was reversed. Under aerobic conditions, the amount of sucrose actually increased with time from six hours onwards and the decrease in raffinose was not sufficient to account for this. Nearly half the sucrose of the seed is in the endosperm and this sucrose began to disappear about the same time as the increase occurred in the embryo. MacLeod suggested that this sucrose was being translocated to the embryo. There is no other evidence for such early translocation, and in view of the fact that the endosperm has an active respiration within ten hours, [121] and that under aerobic conditions there appears to be a reduction in the lipid reserves of the embryo during the first day, [114, 116] it is more likely that the increase in sucrose in the embryo came from lipid breakdown.

As well as sucrose and raffinose and small quantities of other sugars barley seeds also contain some low molecular weight polysaccharides. [117, 119] The role of these in germination has as yet been only superficially studied, but it is known that their overall composition changes and their overall concentration increases in the first day of imbibition. [119]

Indications of the pathways through which carbohydrates are metabolised during early germination, are provided by the identification of enzymes from the

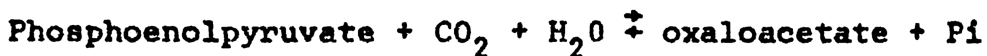
resting seed. A water extraction is often involved. Since this is not usually of more than two hours duration, it can be fairly claimed that the enzymes that are extracted were present in the resting seed. On the basis of the decrease in sugar levels and the increase in respiration that is reported above, it is apparent that the enzymes of a number of pathways become active in the first few hours of imbibition. The embryos of wheat(*Triticum* species) have proved to be a convenient source for the extraction of many enzymes. All the enzymes mentioned below have been isolated from this material. It is very likely that these enzymes are also present in barley embryos, as both barley and wheat belong to the same tribe (p. 204, ref. 17) and the seeds have a similar composition (p. 19, ref. 8).

The enzymes for both sucrose synthesizing pathways have been found.[122, 123, 124] One pathway involves the enzyme sucrose synthetase (2.4.1.13). The reaction is freely reversible and is thought to be more important in sucrose breakdown than synthesis.[125] The second pathway involves the enzymes sucrose-6-P synthetase (2.4.1.14) and a phosphatase. These reactions are outlined in Section 2-4.1.

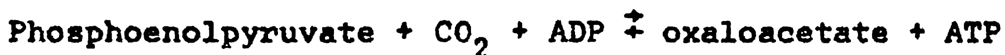
Some of the enzymes of glycolysis,[126, 127] the pentose phosphate pathway,[126, 128] and the Krebs cycle[126]

have been detected. Not all the enzymes of these pathways have as yet been identified in wheat embryos and in any case the identification of all the enzymes of a pathway is not sufficient proof that the pathway is operating. However that some of the enzymes of these pathways are present, suggests that it is these pathways that are responsible for the breakdown of sugars that is known to occur during early germination.

Two important enzymes of intermediary metabolism have also been found in wheat embryos.[129] These are phosphoenolpyruvate carboxylase (4.1.1.31) and phosphoenolpyruvate carboxykinase (4.1.1.e). The two reactions catalysed are respectively:



Phosphoenolpyruvate + CO_2 + H_2O $\rightleftarrows$ oxaloacetate + Pi



Phosphoenolpyruvate + CO_2 + ADP $\rightleftarrows$ oxaloacetate + ATP

These reactions are important because they connect the glycolytic sequence to the Krebs cycle and could be involved either in replenishing Krebs cycle intermediates or in supplying phosphoenolpyruvate for reversed glycolysis.

The situation with regard to lipid metabolism in barley seeds during early germination is somewhat confused. MacLeod and White [116] measured the changes in total lipids of seeds being steeped in water, conditions which approach anaerobiosis. Although more than 80% of the lipid fraction of the seeds was triglycerides, and lipase (3.1.1.3) was

present in the seeds,[130] they found that there was usually little change in the lipid levels over a period of days. However, in a few experiments the lipid levels decreased substantially. They also cite papers where the results were even less consistent. One of the causes of these inconsistent results could be the inefficient extraction of lipids. MacLeod and White were convinced that the quantities of lipids extracted depended not only on the extraction technique but also on the state of the seeds, but they were unable to find a completely reliable extraction method.

Conversely, excised embryos imbibed aerobically always metabolised over 80% of their lipids in two days. This was not due just to the aerobic conditions, as seeds that had been steeped for several days showed only a small drop in lipid levels when subsequently transferred to aerobic conditions. Presumably in this latter case the seeds had access to the carbohydrate reserves of the endosperm and so did not need to use their lipids for respiration.

A number of other papers have also dealt with lipid metabolism in the seeds of both barley[114, 131] and wheat.[132, 133] Wheat is closely related to barley and the seeds of both contain about the same quantities of lipids (p. 19, ref. 8). The somewhat conflicting results in these papers may be due to the

different conditions under which the seeds were germinated. While MacLeod and White [116] showed that the amount of lipid metabolism depends on whether or not the seeds are imbibed aerobically these other papers do not take account of this and give only a vague idea as to how aerobic the conditions used were. However, it appears that lipids can be metabolised in the embryo in the first day of imbibition, and that at least some of the metabolised lipids are triglycerides.[133] Lipids can also be metabolised in the endosperm in the first day, and this metabolism is controlled to some extent by hormones released by the embryo. [132] There is also a suggestion that the scutellum (the part of the embryo that comes between the axis and the endosperm, and can be considered as the cotyledon) is converting lipids to starch after one day of imbibition and that the glyoxylate cycle is involved. [114] One of the distinctive enzymes of this cycle, isocitrate lyase (4.1.3.1), is present in the scutellum of the barley embryo at that time and the cycle has been shown to be responsible for a fat to starch conversion in the scutellum of maize [134] which belongs to the same family as barley A lipid to sucrose conversion therefore appears to be a reasonable explanation for the increase in sucrose that MacLeod [119] found when embryos were imbibed aerobically.

James[120] showed that after one day of imbibition, excised embryos had metabolised most of their stored sugars. On the evidence available from the papers cited above, it is apparent that while lipids, notably triglycerides, can be metabolised in the first day of imbibition, they are not metabolised to the same extent as the sugars are. It therefore appears that part of the function of the lipids in the embryo is to act as a reserve respiratory substrate which can be utilised when sugar reserves are becoming exhausted.

Amino acid metabolism has not been directly studied in barley during early germination but Inatomi and Slaughter[135] tabulated the changes in free amino acids in the embryo under aerobic and anaerobic conditions at 12 hour intervals. Initially, glutamate and alanine were present in greatest concentration, followed by 4-aminobutyrate, proline and glycine. These all increased in concentration under aerobic conditions until after 36 hours 4-aminobutyrate was present in higher concentration than any other amino acid. The other interesting point is that, apart from aspartate, the levels of all the amino acids showed an increase in the first 12 hours. While these increases could have been due to protein hydrolysis, the decrease in aspartate can only be taken as an indication that it was involved in some sort of

intermediary metabolism. The enzyme aspartate aminotransferase (2.6.1.1) has been found in embryos of resting wheat seeds. [136] If it is also present in the closely related barley seed its action could account for the observed decrease in the level of aspartate.

The increase in the level of 4-aminobutyrate that was observed must also have been due to the action of some enzyme other than a hydrolase as 4-aminobutyrate does not occur in proteins. Inatomi and Slaughter [135] assayed for glutamate decarboxylase (4.1.1.15), the enzyme that catalyses production of 4-aminobutyrate from glutamate. They found that it was present in the embryo of both the seedling and the resting seed, but was never present in the endosperm at any stage. A similar enzyme distribution was also found in wheat. [105] Inatomi and Slaughter suggested that this enzyme was responsible for the increase of 4-aminobutyrate that they observed.

The amount of 4-aminobutyrate also increased under anaerobic conditions. Most of the other amino acids increased slightly but glutamate decreased. Glutamate is probably involved in a number of reactions but the production of 4-aminobutyrate must account for some of the observed decrease.

Other papers [118, 137, 138] have reported on the amino acids in barley seeds and seedlings but they only

recorded the changes at two day intervals, so they give no information about the early metabolism. However, they do highlight the importance of glutamate and to a lesser extent proline as storage amino acids. The concentration of 4-aminobutyrate was measured in only one of these three papers. Jones and Pierce [138] found much lower levels than those found by Inatomi and Slaughter. [135] Since Jones and Pierce air-dried their seedlings for 24 hours before analysis their results are of dubious value. However, both papers agree that 4-aminobutyrate is not present in the endosperm.

There has been a certain amount of interest in investigating protein synthesis. Abdul-Baki [121] suggested that it was occurring in barley embryos during the first two hours of imbibition on the basis of the incorporation of ^{14}C-leucine into the so-called protein fraction. However, the incorporation was very low at this stage and from some of his other results it appears that it might have been due merely to contamination of his protein fraction by free leucine. A number of other papers have claimed that such early protein synthesis occurs in the seeds of species of wheat, [139] rye [140] and oat.[141] These are all closely related to barley. The conclusionsin these papers are also based on the incorporation of ^{14}C-leucine but they depend as well on analyses of microautoradiographs

and hybridisation experiments with RNA. It is claimed that resting seeds contain a functional protein synthesizing mechanism that can be activated, at least in isolated systems, as early as the first hour of imbibition. It is also claimed that this system is present in both the embryo [140] and the aleurone cells of the endosperm.[141] While the evidence put forward for protein synthesis occurring after one hour of imbibition is not wholly convincing, and while many of the more detailed assertions in these papers are tentative, [142] there appears to be no doubt that protein synthesis is occurring, if not after one hour, then at least after the first fewhours of imbibition.

From the results of this summary it is clear that even in the first 12 hours of imbibition the barley embryo has an active respiration and a metabolism involving amino acids, lipids and carbohydrates. There is evidence for some metabolism in the endosperm and this appears to be simpler than that in the embryo, but there has been very little study of this organ in the first 12 hours of imbibition. This is not altogether surprising as there is no significant change in the dry weight of the endosperm until after emergence of the embryo has occurred. [131] As well, only a very small part of it, the outside aleurone layer appears to be composed of active, clearly differentiated cells. [113] The embryo

contains large amounts of simple storage compounds: sucrose, raffinose and triglycerides account for at least one third of its dry weight (see Section 3-1.2), whereas in the endosperm these three substances account for less than 2% of the dry weight. Instead, the endosperm has large quantities of stored starch and protein. The enzymes that hydrolyse these are not produced till near the end of the first day of imbibition, so during most of the first day the endosperm has only small amounts of readily usable respiratory substrates. The breakdown of these reserves in the endosperm has been extensively studied. It is controlled by substances, especially gibberellins, which are released from the embryo.

On the basis of the work of MacLeod et al [143] and Varner et al [144] it appears that gibberellins are being made in the embryo and transported to the endosperm after 12 hours of imbibition. By 22 hours α-amylase (3.2.1.1) and other hydrolytic enzymes are being produced in the aleurone layer in response to these gibberellins. This aspect of germination which could be better described as seedling development has been reviewed by MacLeod. [115] It is summarised below because it shows the function of the endosperm in relation to the embryo.

The first hydrolytic enzyme produced in response to the gibberellins is endo-β-glucanase (3.2.1.4). This cleaves the large molecules of β-glucan, the hemiculluloses that form the cell walls of the starchy endosperm. The second enzyme produced is α-amylase (3.2.1.1), which breaks down the starch into smaller units, and the third is a peptidase which attacks proteins. The enzyme β-amylase (3.2.1.2), which is present in the resting seed and which is activated but not synthesized in the presence of gibberellins, degrades these smaller polysaccharide units to maltose. The immediate fate of this maltose has not as yet been fully investigated in barley. However, the way in which it is utilised is indicated by the results of the experiments of Edelman et al [145] and these are supported by the results of studies on the seedlings of species related to barley such as oat [146, 147] and rice. [148, 149] The maltose that is formed is hydrolysed in the endosperm to glucose. Maltase (3.2.1.20) the enzyme responsible, is apparently another enzyme that is also synthesized in response to gibberellins. [146] The glucose formed is transported to the scutellum where it is absorbed and converted to sucrose. All the required enzymes have been found in barley seedlings. [145] This sucrose, and the amino acids that are absorbed from the endosperm, are then translocated to the growing axis.

More recent studies have shown that the control of the hydrolytic enzymes involves much more that just a gibberellin mediated synthesis. Other hormones such as indolyl acetic acid [150] and abscisic acid [151] have been shown to increase enzyme production. The production of the hydrolytic enzymes is also under osmotic regulation. [152] As well as this, there is some controversy over whether or not a large number of other compounds, including a number of amino acids, can also cause the same response as gibberellins. [153, 154]

3-1.4 IMBIBITION

Imbibition in barley was studied by Brown. [29] In an attempt to get reproducible results he imbibed excised embryos and whole seeds on muslin, floating on a water surface. He was well aware that the conditions of water availability had to be kept constant if he was to get consistent results. He decided not to imbibe the seeds in solid media such as sand, because of non-uniform contact between the sand and the medium, and he decided not to immerse the seed entirely in water as this would restrict oxygen availability.

It is unfortunate that Brown's insight into the problems of imbibition has been overlooked or ignored in more recent work. Some workers preferred to completely immerse the seed for hours [119, 135] or days [138] before

placing it under quite different conditions, others placed the seed in moist sand [120, 131] or vermiculite, [118] others placed the seed on moist filter paper, [116] and sometimes it is not clear under what conditions the seed was imbibed. [121] Sometimes the outside husk was removed manually before imbibition, [137] sometimes it was removed by soaking the seed for several hours in 50% H_2SO_4, [114, 135] and sometimes no mention was made of either procedure being used. [152] The effects of these various treatments are largely unknown but it is certain that they cause some changes, [29] possibly very large changes, on the timing of events during imbibition. Some of the treatments, by decreasing the amount of oxygen available to the seed, would cause its metabolism to become anaerobic to a greater or less extent. [131]

Brown [29] studied the differences in imbibition between excised and attached embryos. He found that excised embryos had a greater rate of water uptake and respiration than attached ones. He attributed this to the low permeability of the seed-coat membranes of the intact seed to both oxygen and water. Other authors have also shown that excised embryos have a higher rate of respiration than thosein intact seeds. [116, 120]

Brown [29] measured the water uptake of the attached and excised embryos at 22°C from 2-12 hours of imbibition. He did not excise embryos from seeds before imbibition, but excised them after the seeds had been imbibed for two hours. These embryos imbibed water very rapidly over the next two hours (i.e. from 2-4 hours). This increase levelled off from 4-8 hours and from 8-12 hours the water content showed another sharp increase. Clearly the barley embryo has a three phase imbibition in common with other seeds. The water absorption of attached embryos increased from 2-6 hours but not as rapidly as that of excised ones. It then levelled out and changed little in the next six hours. The water content of the attached embryos in the levelled-off second phase was 50-55% and that of the excised embryos, 60-65%.

Brown compared water absorption of the embryo to that of the endosperm in the intact seed. After two hours the percentages of water in the embryo and endosperm were respectively 29% and 20% and after six hours 52% and 23%. These results are generally similar to those of MacLeod and Palmer. [150] They found that the embryo had a water content of 40% after one hour and 62% after 17 hours. This compared to an endosperm water content of 34% after 16 hours. These are the only figures they quote. Their results are only broadly

comparable to those of Brown because they dehusked their seeds whereas Brown did not, they imbibed them in a different way, and they also used a different variety of barley. However, it is clear that the endosperm absorbs water at a slower rate than the embryo and that eventually the embryo has about twice the water content of the endosperm.

With the variety used by MacLeod and Palmer [150] and under their conditions (25°C), germination as defined by the coleorhiza (a cap-like piece of tissue that covers the rootlet) rupturing the testa, occurred after approximately 17 hours of imbibition. Degradation of endosperm reserves began to occur after 22 hours of imbibition. There was no significant change in the endosperm dry weight till after that time. Between one and two days after the start of imbibition, breakdown of the starch in the endosperm increased rapidly and so did the respiration rate. [131] This reached a peak after six days and by 12 days the carbohydrate reserves of the endosperm were exhausted.

3-2 IMBIBITION OF PARTS OF SEEDS IN TRITIATED WATER

3-2.0 EXPERIMENTAL

The barley seed was the most difficult to dissect of the four seedsstudied for this thesis. As in the case of *Phaseolus vulgaris* the seed contains large amounts of starch and only small amounts of lipids. This

makes the endosperm very hard. As well as this, there is no clear dividing line in the resting seed between the endosperm and embryo. For this reason all the excised embryos may have had small amounts of endosperm tissue attached to them. The pieces of endosperm used for the experiments were taken from the middle of the seed and there was no possibility of them being contaminated with any embryo tissue. The pieces of endosperm were dissected so that the outer aleurone layer and testa were still attached, as well as some of the husk. The husk was attached firmly to the underlying testa and while it could have been scraped away this would have damaged or removed parts of the aleurone layer as well. Three embryos or three pieces of endosperm were imbibed in each experiment. The experiments were carried out as described in Section 1-4.

During the experiments at three minutes the embryo and endosperm did not imbibe enough water to become soft. For this reason grinding took longer than for the experiments at longer times. The time of three minutes can therefore only be regarded as approximate. The ethanol extract and water rinse were combined in the three and 15 minute experiments with the endosperm, and in the three minute experiment with the axis, to

ensure that there was enough radioactivity on the chromatograms to be detected.

3-2.1 RESULTS AND DISCUSSION

The results are given in Table 3.1 where the labelling in the three extracts has been combined for presentation. Some of the radioautograms are shown in Plate 3.1.

From the results it appears that the embryo and endosperm have different metabolism at all four times. Relatively few compounds were labelled in the endosperm and these were never as strongly labelled as the same ones in the embryo.

Embryo metabolism

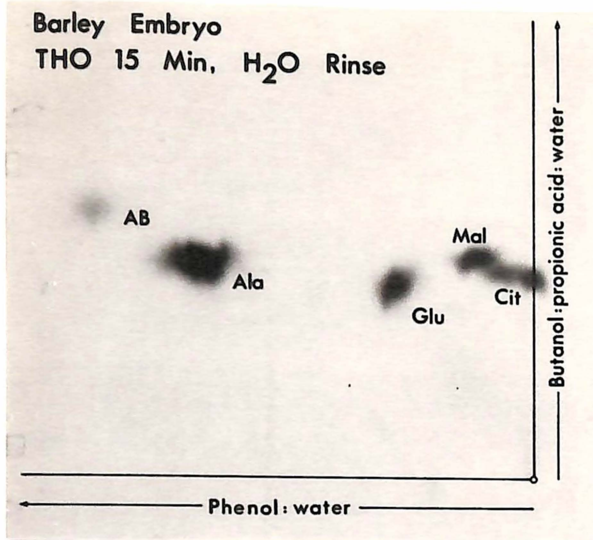
After three minutes imbibitionthere were four faintly labelled compounds in the embryo extracts. The positions of these corresponded to the amino acids alanine, aspartate, glutamate and 4-aminobutyrate. These are the same four compounds that were labelled in the *Phaseolus vulgaris* axis after five minutes and in the *Sinapis alba* seed after ten minutes. After 15 minutes, alanine and glutamate were more strongly labelled and labelled citrate and malate had appeared, as well as a trace of radioactivity that could have been either succinate or lactate. This is similar to

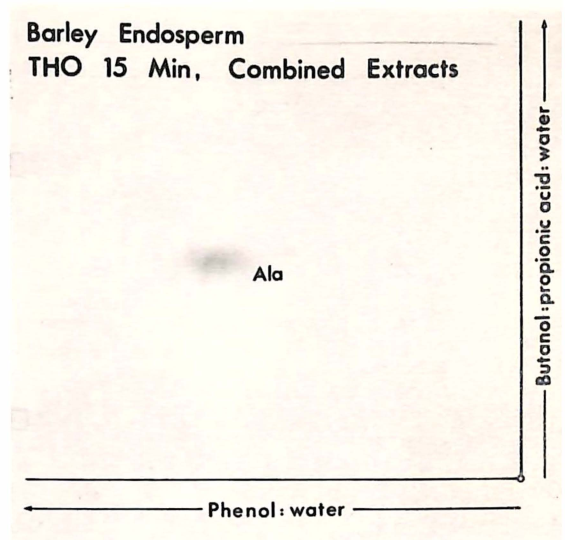
Table 3.1 Compounds labelled when barley embryos and pieces of endosperm imbibed tritiated water.

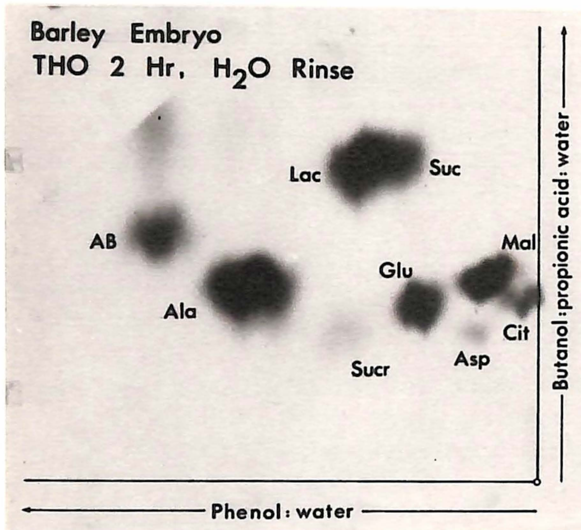
Compounds labelled	Time (min)			
	3	15	120	360
(a) Embryo				
4-Aminobutyrate	t	t	+	++
Aspartate	t	t	t	+
Glutamate	t	+	++	+
Alanine	t	++	++	+++
Citrate		+	+	+
Malate		+	++	++
Succinate			+	++
Lactate		t	+++	++
Sucrose			+	+
Glutamine			+	+
Lipids ?		t	+	+++
Sugar phosphates ?		t	t	+
(b) Endosperm				
4-Aminobutyrate	t	t		
Aspartate				
Glutamate			t	+
Alanine	t	+	+	+
Citrate				
Malate			+	++
Succinate			t	+
Lactate				
Sucrose				
Glutamine				
Lipids ?			t	++
Sugar phosphates ?				

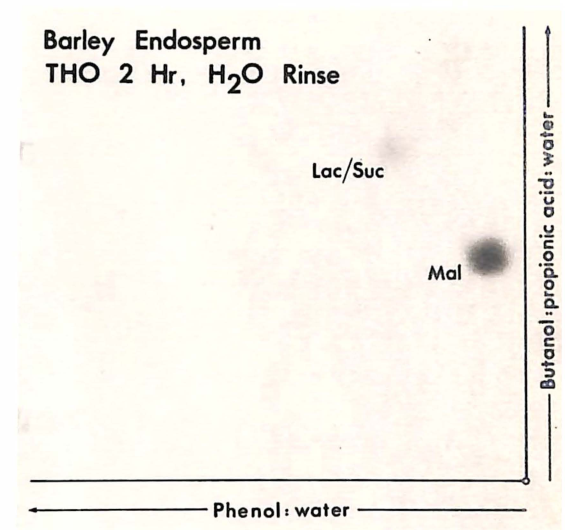
Key: +, ++, +++ = increasing relative intensity
t = trace

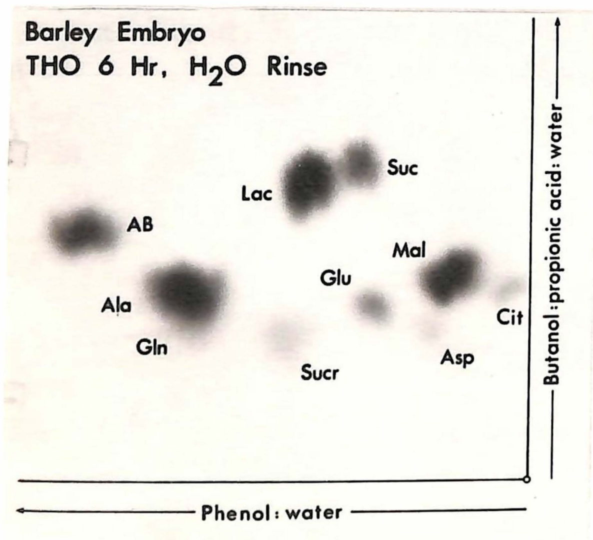
PLATE 3.1

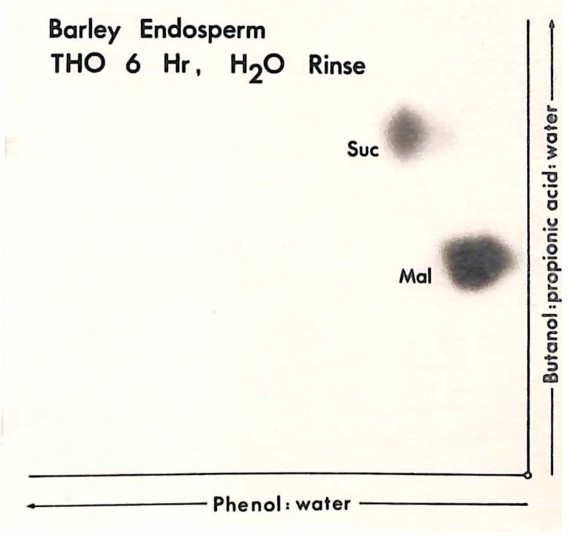












the situation at 30 minutes in both the *Phaseolus vulgaris* axis and the *Sinapis alba* seed. After two hours, labelled succinate, lactate, sucrose and glutamine could be identified and the compounds that had been labelled at 15 minutes were now more strongly labelled. This is similar to the situation in the *Phaseolus vulgaris* axis and cotyledon at two hours. The labelling of compounds in the barley embryo at six hours was very similar to that at two hours except that the lipid fraction had become much more strongly labelled. The order in which compounds were labelled in the barley embryo was more similar to that in the *Phaseolus vulgaris* seed, particularly the axis, than to that in the *Sinapis alba* seed although the same compounds were eventually labelled in all three seeds. Because of the similarity in labelling between the three seeds the reasoning behind the suggested reactions for the labelling in *Phaseolus vulgaris* and *Sinapis alba* also applies therefore to the barley embryo.

Thus alanine, aspartate and glutamate probably became labelled in deamination and transamination reactions. Spedding and Wilson [13] suggested that because 2-oxo acids are unstable they are not stored in the resting seed, but must be produced by deamination and transamination of amino acids soon after the start of imbibition. This definitely seems to be the case for

the seed of wheat which is closely related to barley. Krupka and Towers [155] analysed wheat seeds for 2-oxo acids. They found that these were not present in the resting seed, but that pyruvate, oxaloacetate and 2-oxoglutarate had all appeared after one day of imbibition. In a further experiment using ^{14}C labelled amino acids they showed that these 2-oxo acids were all produced *in vivo* from their corresponding amino acids. As well as this, glutamate dehydrogenase (1.4.1.2) [105] aspartate aminotransferase (2.6.1.1) and alanine aminotransferase (2.6.1.2) [136] have all been found in embryos of resting wheat seeds. If these three enzymes were present in the closely related barley seed, this would account for the labelled aspartate, glutamate and alanine that were formed in that seed when it was imbibing tritiated water.

It is probable that the labelled 4-aminobutyrate was formed in the reaction catalysed by the enzyme glutamate decarboxylase (4.1.1.15). This enzyme has been found in the embryos of both resting and imbibing barley seeds, [135] and would be expected to form labelled 4-aminobutyrate in the presence of tritiated water. The metabolism of 4-aminobutyrate is discussed further in Section 6-3.

Labelled glutamine was probably formed by the action of glutamine synthetase (6.3.1.2) on labelled glutamate.

The Krebs cycle acids, citrate and malate, were labelled after 15 minutes. Aspartate, glutamate and alanine, the amino acids whose corresponding 2-oxo acids form part of the Krebs cycle, were labelled from three minutes onwards. This indicates that these 2-oxo acids were also present from this time onwards. There is some doubt as to whether succinate was labelled at 15 minutes, but it was certainly labelled after two hours. It therefore appears that the Krebs cycle was beginning to operate in the barley embryo after 15 minutes.

As in the *Phaseolus vulgaris* experiments, the formation of lactate in imbibing barley embryos indicates that their metabolism is partially anaerobic. This is not surprising as excised embryos are known to have a high respiration rate.[29] In these experiments they were totally immersed and this would restrict the amount of oxygen available.

Sucrose was not labelled as well in barley embryos as it was in *Phaseolus vulgaris* seeds but it became labelled at the same time, two hours. Its labelling was investigated further and these experiments are described in Section 3-4.

The compounds assumed to be sugar phosphates on the basis of their R_f values in two dimensional chromatography were never strongly labelled at any time and neither was sucrose. It may be that, as suggested in the case of *Phaseolus vulgaris*, the labelled sugar phosphates formed were intermediates of sucrose synthesis.

In the six hour experiment the lipid fraction was strongly labelled in the embryo. Lipids comprise 15% of the embryo.[116] Breakdown of these can occur in the first day of imbibition[133] but it is unlikely that this would lead to labelled lipids being formed. It seems probable that, as in *Pisum sativum*,[106] lipids were being synthesized during early germination to form the precursors of membranes that would be needed when the embryo began its rapid growth phase. Some support for this suggestion comes from the discovery of the enzyme acetyl-CoA carboxylase (6.4.1.2) in resting wheat embryos.[156] Since wheat is closely related to barley this enzyme is probably also present in the barley embryo. This enzyme catalyses the first step in fatty acid synthesis, the carboxylation of acetyl-CoA to form malonyl-CoA.

Endosperm metabolism

The endosperm never produced labelled aspartate, citrate, lactate, sucrose, glutamine or sugar phosphates. There was only a trace of radioactivity in the three and

15 minute experiments that might have been 4-aminobutyrate, but it was never labelled intensely enough to be positively identified. Since 4-aminobutyrate and glutamate decarboxylase (4.1.1.15) were not found in the barley endosperm by Inatomi and Slaughter, [135] it appears unlikely that this trace was 4-aminobutyrate.

The only compounds definitely labelled in the endosperm were glutamate, alanine, malate and succinate. The glutamate and alanine could have become labelled by transamination. Krupka and Towers [155] showed that 2-oxo acids were present in the endosperm of the imbibing wheat seed, although their concentration was much less than that in the embryo.

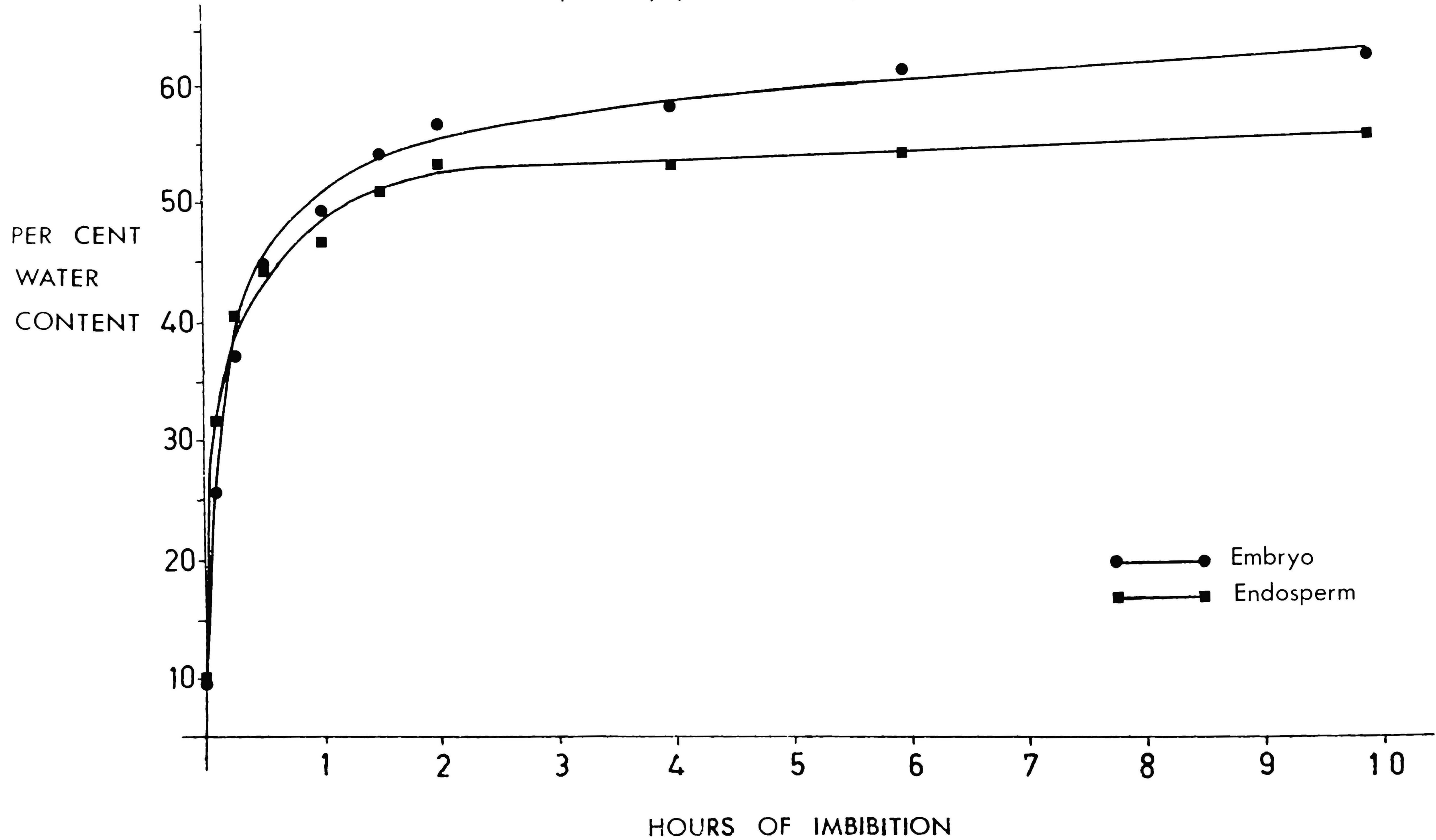
Malate and succinate, but not citrate, were labelled in the endosperm. All three acids would be expected to be labelled if either the Krebs cycle or glyoxylate cycle were operating. Either these cycles were not operating or were operating at such a low level that insufficient citrate was being formed to be detected. Further experiments were carried out to test these two possibilities and these are described in Section 3-3.

Comparison between embryo and endosperm

The changes in water content during imbibition of excised embryos and pieces of endosperm are shown in Fig. 3.2. In both cases there was an initial rapid absorption of water that levelled off after two hours.

FIGURE 3.2

Water absorption by parts of barley seeds



In the first 15 minutes the endosperm and embryo absorbed water at about the same rate but from then onwards the water content of the endosperm was always less than that of the embryo. After six hours imbibition the endosperm had a water content of about 55% compared to that of 60% in the embryo.

MacLeod and Palmer [150] measured water absorption in the intact seed. They found that after 17 hours of imbibition the water content of the endosperm was less than two thirds that of the embryo. They suggested that the low water content of the endosperm was the reason for its limited metabolism. The absorption curve for the excised endosperm given in Fig. 3.2 shows that this is not a likely explanation. The excised endosperm absorbed almost as much water as the embryo; yet, on the basis of the compounds that were labelled in tritiated water, its metabolism was still much simpler. A possible cause of this apparently simpler metabolism is the very small number of active cells in the endosperm. This is supported by the results of the experiments reported in Section 3-3 and the subject is further discussed in that section.

3-2.2 CONCLUSION

During the first six hours of imbibition the metabolic development of the barley embryo, as revealed by the patterns of labelled compounds formed in tritiated water,

was very similar to that of the *Phaseolus vulgaris* axis. In both cases the same compounds were labelled and they became labelled at similar times. The same compounds were also labelled in the *Sinapis alba* seed and the *Phaseolus vulgaris* cotyledon, and there was also some similarity between the order of labelling in these two tissues and that of the barley embryo. Since barley is not closely related to either *Phaseolus vulgaris* or *Sinapis alba*, it is suggested that the embryonic parts of many seeds may have a similar metabolic development during the first hours of imbibition.

The barley endosperm apparently had a much simpler metabolism than the embryo during the first six hours of imbibition. This did not appear to be due to its slower water absorption.

3-3 FURTHER EXPERIMENTS ON ENDOSPERM

3-3.0 INTRODUCTION

The results of the experiments reported in Section 3-2 indicated that the metabolism of the endosperm is much simpler than that of the embryo. Two possible explanations for this are suggested below:

1. The endosperm is believed to contain few living cells. The metabolism might appear simpler than it actually was because there were too few cells to produce a detectable amount of some labelled compounds.

2. The endosperm may have an inherently simpler metabolism either because it lacks gibberellic acid from the embryo to activate its metabolic pathways, or for some other reason.

It was realised that the true explanation might involve a combination of these two possibilities.

Three experiments were carried out to investigate these possibilities and these are reported in the sections that follow.

3-3.1 METABOLISM OF ENDOSPERM WITHOUT ALEURONE LAYER

3-3.10 INTRODUCTION

The endosperm consists predominantly of storage material which is believed to contain no living cells.[115] Only the aleurone layer around the outside of the endosperm is composed of clearly differentiated cells, and this comprises only a very small fraction of the endosperm. An experiment was performed to establish whether or not the metabolism of the endosperm (as revealed by labelled compounds being formed) was due entirely to the aleurone layer.

3-3.11 RESULTS AND DISCUSSION

The experiment in which pieces of endosperm were imbibed for six hours in tritiated water was repeated using pieces of endosperm that had had their aleurone layers completely removed. No labelled compounds were

formed by this central endosperm tissue even in trace amounts. Clearly, the metabolism of the endosperm, at least in the first six hours, is due entirely to the cells of the aleurone layer.

This experiment is important because it shows that although approximately equal weights of embryo and endosperm tissue were used in the four imbibition experiments (Section 3-2), the number of active endosperm cells present would have been much smaller than the number of active embryo cells. Since only a small amount of active endosperm tissue was present the metabolism could have appeared to be simpler than it really was, because some compounds might not have been strongly enough labelled to be detected.

3-3.2 ENDOSPERM IN TRITIATED WATER FOR 12 HOURS

3-3.20 INTRODUCTION

The previous experiment showed that only a small part of the endosperm contained active cells, and it was suggested that the metabolism might appear to be simpler than it really was because of this. However, it is also possible that the endosperm actually does have a much simpler metabolism than the embryo.

In this experiment pieces of endosperm were imbibed in tritiated water for 12 hours. If the metabolism was only apparently simpler, this longer time might enable a great enough accumulation of labelled compounds for these

to be detected. If the metabolism of the endosperm developed at a slower rate than that of the embryo this longer time might enable more compounds to be labelled due to more pathways being activated. If on the other hand there were no more compounds labelled at 12 hours than at six hours this would support the alternative possibility: that the endosperm really does have a much simpler metabolism than the embryo.

3-3.21 RESULTS AND DISCUSSION

Three pieces of endosperm were imbibed for 12 hours in tritiated water. They were extracted and the labelled compounds identified in the usual manner. The results are given in Table 3.2 and, as usual, the labelling of the three extracts has been combined for presentation. The results of the endosperm experiment at six hours are given in the same table for comparison. The radioautogram of the water rinse of the 12 hour experiment is shown in Plate 3.2.

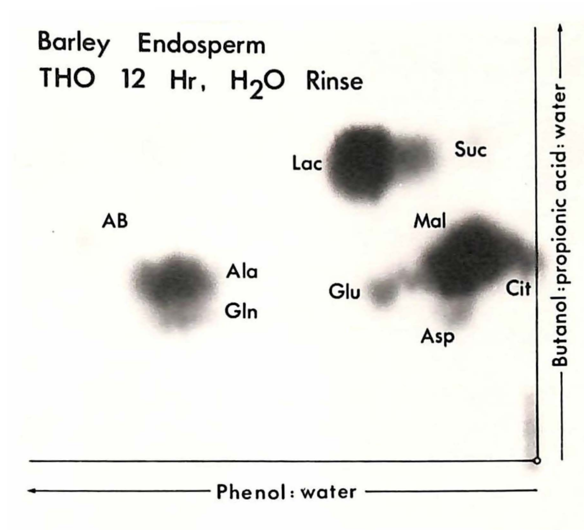
A number of labelled compounds that had not been detected after six hours were present after 12 hours. Lactate and glutamine, which had not been previously labelled, were now strongly enough labelled to be identified by co-chromatography. Trace amounts of activity appeared corresponding to citrate, aspartate and 4-aminobutyrate.

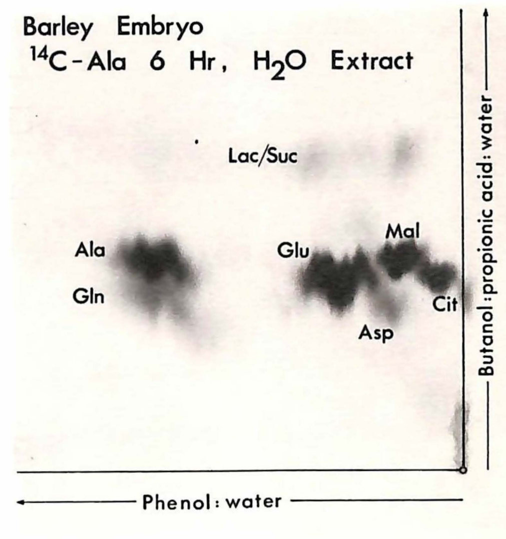
Table 3.2 Compounds labelled when pieces of endosperm imbibed tritiated water for six and 12 hours.

Compounds labelled	6 hr.	12 hr.
4-aminobutyrate		t
Aspartate		t
Glutamate	+	+
Alanine	+	+
Citrate		t
Malate	++	++
Succinate	+	+
Lactate		++
Glutamine		+
Lipids ?	++	++

Key: +, ++, +++ = increasing relative intensity
t = trace.

PLATE 3.2





Although citrate and aspartate were only labelled in trace amounts their presence was an indication that the Krebs cycle had begun to operate.

Whether the trace amount of activity corresponding to 4-aminobutyrate was in fact this compound could not be determined. However, it is interesting to note that neither 4-aminobutyrate nor glutamate decarboxylase (4.1.1.15) was found in the barley endosperm by Inatomi and Slaughter.[135]

The appearance of labelled glutamine was an indication of the presence of glutamine synthetase (6.3.1.2) and a source of ammonia.

The labelling of lactate after 12 hours was probably due to the increased rate of respiration of the endosperm at that time. At six hours the respiration rate is known to be low;[29] some of the labelled intermediates which would be expected to be present if the Krebs cycle were operating were not found even in trace amounts, and no labelled lactate could be detected. After 12 hours however, the compounds expected to be labelled if the Krebs cycle were operating, were all present, and lactate was strongly labelled. Apparently, after 12 hours the endosperm could not obtain enough oxygen to sustain its respiration completely and its metabolism therefore became partially anaerobic.

Sucrose was the only compound labelled in the embryo at six hours that was definitely not labelled in the endosperm at 12 hours. This is consistent with the fact that during seedling growth the endosperm translocates its carbohydrate reserves to the embryo as glucose and not sucrose (Section 3-1.3). The glucose is converted to sucrose in the scutellum, which is part of the embryo.

This experiment showed that the endosperm was capable of more complex metabolism than that apparent at six hours, but, in that no labelled sucrose was formed, its metabolism was still simpler than that of the embryo.

3-3.3 EFFECT OF GIBBERELLIC ACID ON ENDOSPERM

3-3.30 INTRODUCTION

Gibberellic acid has been shown in numerous papers to be important in activating protein synthesis in the aleurone layer (some of these papers were reviewed in Section 3-1.3). Gibberellic acid is apparently not transported to the endosperm till after 12 hours of imbibition.[115] There is therefore the possibility that its absence is one reason for the limited metabolism of the endosperm in the first 12 hours. Accordingly, experiments were performed to find out whether or not gibberellic acid had any effect on the endosperm metabolism (as revealed by the compounds labelled in the

presence of tritiated water).

3-3.31 EXPERIMENTAL AND RESULTS

Three pieces of endosperm with attached aleurone layers were imbibed in 0.05 ml of tritiated water which contained approximately 0.05 mg of gibberellic acid (giving a concentration of 1 mg/ml). Apart from the addition of gibberellic acid the normal experimental technique was employed. Two experiments were performed, with imbibition times of six and 12 hours respectively.

In both cases the patterns of labelling were identical to those of the experiments in which gibberellic acid was not added. Neither did the gibberellic acid have any marked effect on the intensity of labelling. Thus, as far as could be determined by the labelling of compounds in tritiated water, gibberellic acid did not have any effect on the metabolism of the endosperm in the first 12 hours of imbibition.

3-3.4 CONCLUSION

The metabolism of the endosperm developed at a slower rate than that of the embryo, but after 12 hours it was almost as complex as that of the embryo at six hours. It was shown that the endosperm contained only a small percentage of active cells and this probably accounted for its apparently slower development. Only a small fraction of the sample of endosperm tissue used for each experiment would have been metabolically active. Identifiable amounts of labelled compounds

would therefore have taken longer to be formed than if most of the tissue sample were metabolically active as it was in the embryo. However, in one respect the metabolism of the endosperm did appear to be qualitatively different from that of the embryo. Although labelled sucrose was formed in the embryo from two hours onwards, it was not formed in the endosperm even after 12 hours. This accords with the fact that the carbohydrate reserves of the endosperm are known to be transported to the embryo as glucose and not sucrose.

Gibberellic acid appeared to have no effect on the development of endosperm metabolism in the first 12 hours of imbibition.

3-4 INVESTIGATION OF SUCROSE LABELLING

3-4.0 INTRODUCTION

In the experiments reported in Section 3-2, labelled sucrose was found in the embryo from two hours of imbibition onwards, but was never present in the endosperm. The embryo contains large amounts of sucrose which are known to be degraded from six hours of imbibition onwards. If sucrose were being degraded by the reverse of one of the pathways shown in Section 2-4.10, labelled sucrose might be formed if the reactions were in equilibrium from the reaction forming sucrose to the glucosephosphate isomerase (5.3.1.9)

reaction inclusive. Alternatively, it could become labelled if it were being synthesized from this reaction in a compartment separate from the one in which degradation occurs. As shown in Section 2-4.14 , if sucrose were being labelled in this way, the label would be found in both the glucose and fructose moieties.

An experiment was performed in which tritium-labelled sucrose from an embryo experiment was treated with invertase (3.2.1.26). This was done to establish whether or not the label was retained on hydrolysis, and if it was, to find out whether both the glucose and fructose moieties were labelled. On the basis of the reasoning in Section 2-4.14, it was expected that on hydrolysis of labelled sucrose both glucose and fructose would be found to be labelled. However, it was clearly advisable to check that this was indeed so.

The intermediates involved in sucrose synthesis, glucose-6-P and fructose or fructose-6-P, could be formed from the metabolism of other carbohydrates and these appear to be the most likely source. They could also be formed from reversal of glycolysis, possibly utilising intermediates from lipid breakdown. In Section 3-1.1 it was suggested that this was occurring in intact seeds to explain the rise in sucrose observed by MacLeod.[119] To find out if sucrose was being formed

by reversed glycolysis in excised embryos during the first six hours of imbibition, an experiment was performed with ^{14}C-alanine. The results of this are reported in Section 3-4.2.

3-4.1 HYDROLYSIS WITH INVERTASE

A sample of tritiated sucrose formed by the embryo was treated with invertase in the way described in Section 1-4.8; the label was not lost during hydrolysis and the subsequent one dimensional chromatography with the solvent propanol:ethyl acetate:water 7:1:2. When the labelled spot on this chromatogram was transferred to a two dimensional chromatogram and developed with the solvents phenol:water 100:29 and ethyl acetate: pyridine:water 12:5:4, glucose and fructose were both found to be labelled, although the labelling was very weak, as in the case of the *Phaseolus vulgaris* experiment (Section 2-4.1).

The label in sucrose was therefore retained on hydrolysis and was present in both the glucose and fructose moieties. Whether or not they became labelled in the reaction catalysed by glucosephosphate isomerase (5.3.1.9) is uncertain, but as already explained, a study of the reactions leading to sucrose showed that this is the last step at which either glucose or fructose would be expected to become labelled.

3-4.2 EXPERIMENT WITH ^{14}C-ALANINE

Although one aim of this experiment was to establish whether or not reversed glycolysis was occurring it was also done to gain additional information about the general metabolism.

Three barley embryos were imbibed for six hours in 0.05 ml of water containing 1 µCi of ^{14}C-alanine. The experiment was carried out in the same way as that described for *Phaseolus vulgaris* in Section 2-4.41.

The results are given in Table 3.3. The radio-autogram of the water extract is shown in Plate 3.2. The unnamed traces of activity to the right of the lactate/succinate spot were not identified.

No labelled sucrose was found. If reversed glycolysis had been occurring from the level of the Krebs cycle, malate would have been an intermediate. Malate was labelled in this experiment but there was no labelled sucrose. However, malate was not strongly labelled, so any sucrose formed from it might only be weakly labelled. To make absolutely sure that there was no labelled sucrose, the area of the chromatogram to which it would have run was eluted onto the origin of another chromatogram. This chromatogram was developed in one dimension with reference sugars and

Table 3.3 Compounds labelled when barley embryos were imbibed for six hours in a solution of ^{14}C-alanine.

Compounds labelled	
4-Aminobutyrate	t
Aspartate	+
Glutamate	++
Citrate	+
Malate	+
Succinate	t
Lactate	
Glutamine	+
Lipids ?	+

Key : +, ++ = increasing relative intensity
t = trace

the area to which any labelled sucrose would have run was cut out and counted for ^{14}C activity in a scintillation counter. The procedure has been described more fully in Section 2-4.43.

No activity corresponding to sucrose could be detected. This indicates that during the first six hours of imbibition, sucrose was not being formed in excised embryos through reversed glycolysis from the Krebs cycle.

The labelling of the other compounds in this experiment was similar to that in the corresponding *Phaseolus vulgaris* experiment (Section 2-4.4). In both cases the compounds labelled in these experiments support the interpretations of the results from the tritiated water experiments, in which it was suggested that the Krebs cycle was operating and transaminations were occurring.

3-4.3 CONCLUSION

Sucrose appeared to be labelled in the same way in barley embryos as in *Phaseolus vulgaris* and *Sinapis alba* seeds. Although some suggestions have been made about the formation of sucrose in barley, these are somewhat speculative and no direct information was provided by either of the two experiments.

The labelled compounds formed in the ^{14}C-alanine experiment support the interpretations made from the results of the tritiated water experiments. The fact that the same compounds were also labelled when ^{14}C-alanine was fed to embryonic axes of *Phaseolus vulgaris* supports the suggestion made earlier that the embryonic parts of many seeds have a similar metabolic development during the first hours of imbibition.

CHAPTER 4

EXPERIMENTS ON CASTOR OIL SEEDS

4-1 INTRODUCTION

4-1.0 GENERAL

The castor oil (*Ricinus communis*) seed was the third seed investigated in this thesis. Castor oil is a member of the large family Euphorbiaceae which belongs to the dicotyledon class of the angiosperms (p. 338, ref. 17).

In the case of castor oil, more difficulty was experienced in obtaining a sample of seeds with a high germination percentage, than in the case of either *Phaseolus vulgaris* or barley. Two different samples of locally collected seeds and samples of seeds from both England and U.S.A., either failed to germinate or had a very low germination percentage. It was only after using a different germination procedure (Section 1-4.0) that one of the samples of seeds was found to be satisfactory.

The castor oil seed was selected for study because:

1. It is a seed whose energy reserves are mainly lipids and whose storage organ is the endosperm.

2. It was big enough to dissect yet had a small enough embryonic axis for several of these to be imbibed together in a small amount of tritiated water.

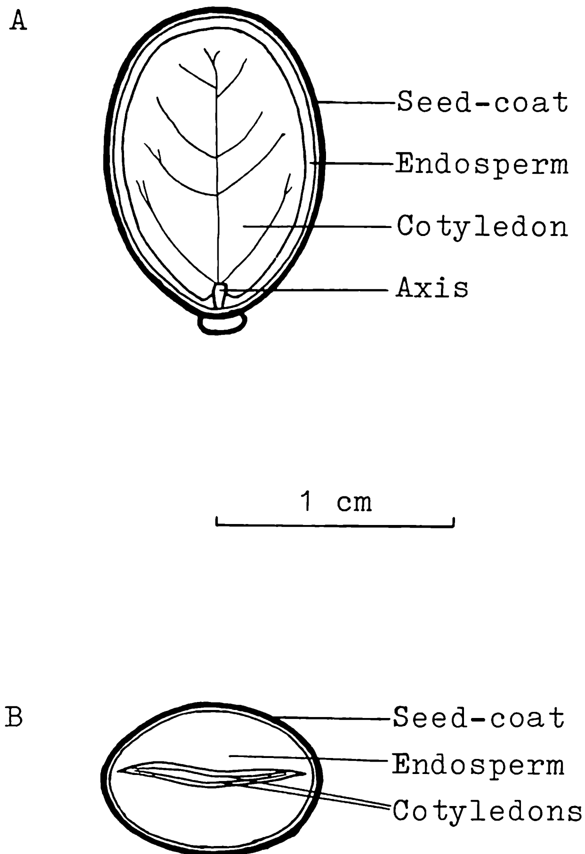
3. It had a high germination percentage (above 90%).

4-1.1 DESCRIPTION AND STORAGE

The sample of castor oil seeds used were described by the supplier as being mixed varieties. The average seed was 1.4 cm long and 0.9 cm in diameter. The smooth seed-coat had a mottled light and dark brown colour. It was very hard and thick but was not firmly attached to the underlying endosperm. As shown in Fig. 4.1, most of a castor oil seed is composed of the endosperm. This has a high lipid content and is much softer than the starchy barley endosperm. The two cotyledons are thin and filamentous with a large surface area, and are attached at their base to the small embryonic axis. It was found that although the cotyledons are not physically attached to the endosperm they are in such intimate contact that it was impossible to separate them intact from the endosperm. The average seed used weighed 0.4 g and the average embryonic axis 0.002 g.

The seeds were stored over silica gel at room temperature. Seeds stored in this way maintained greater than 90% germination over the period in which the experiments were performed. Before attempting to germinate the seeds, the coats were first removed as described in Section 1-4.0. When seeds treated in this way were imbibed they began to germinate (radicle protrusion) after five days and reached their maximum germination percentage after seven days. The germination

FIGURE 4.1



Castor oil(<u>Ricinus communis</u>) seed shown in (A) longitudinal and (B) transverse sections.

is epigeal; as well as absorbing nutrients from the endosperm the cotyledons also carry out photosynthesis.

4-1.2 COMPOSITION

Castor oil seeds contain large quantities of lipids and little carbohydrate. The analysis quoted by Mayer and Poljakoff-Mayber (p. 19, ref. 1) for the air-dried seed is: lipids, 64%; proteins, 18%; starch and sugars, 0%. However, Duperon [157] found that sugar comprised 1% of the fresh weight of the castor oil seed. This sugar was entirely sucrose. This is supported by the results of Yamada [158] who found that about 1% of the endosperm consisted of non-reducing sugar. Yamada [159] also analysed the total fatty acids in the endosperm. Ricinoleic acid comprised almost 90% of the total, and most of the remainder was made up of oleic and linoleic acids.

4-1.3 METABOLISM

Although most studies on the metabolism of germinating castor oil seeds have been concerned with the seedling stage, there have been at least two papers that have reported information about changes during the first day of imbibition.

Hawker [160] measured the activities of a number of enzymes in the endosperm. His first measurement of activity was made after soaking the seeds overnight (presumably after 15-18 hours of imbibition). He found that sucrose synthetase (2.4.1.13), sucrose phosphate synthetase (2.4.1.14), and sucrose phosphatase were all present at that time, but that there was only a very low invertase (3.2.1.26) activity. He also measured the activity of isocitrate lyase (4.1.3.1), one of the enzymes of the glyoxylate cycle. After the overnight soaking he found no activity and after a further day of imbibition the activity was extremely low. It was not till two days after the overnight soaking that the activity increased rapidly. It appears therefore that sucrose can be synthesized during the first day of imbibition but that the glyoxylate cycle does not begin to operate till after two days of imbibition.

Yamada [158] measured the changes in both embryo and endosperm of the lipid and sugar fractions during germination. The sugar level in the endosperm was low for the resting seed and dropped by more than half during the first and second days of imbibition. It then rose steeply to a peak at six days. Yamada did not measure the sugar content of the embryo till after three days of imbibition. It was low at this stage but it then rose quickly to a peak at eight days. Unfortunately, from the

data presented it is not possible to calculate what the sugar (or lipid) content was as a percentage of either the dry or fresh weight of the embryo. Yamada reported that the level of lipids in the endosperm decreased only slightly in the first day of imbibition. The level dropped more quickly during the second day and after seven days only 10% of the original amount remained. Both the axis and cotyledon contained some lipids in the resting seed, but after three days of imbibition these had all been consumed. A neutral lipase (3.1.1.3) was found in the axis after eight hours of imbibition and it reached a peak at 24 hours. [161] In the endosperm there was no activity of this enzyme till 36-48 hours after the start of imbibition. It thus appears that the lipids in the embryo were metabolised earlier than those in the endosperm. Presumably the lipids in the embryo act as a respiratory substrate before translocation from the endosperm begins. Because of the insufficient information it is not possible to say whether sugars or lipids are the important respiratory substrates in the embryo during the first day of imbibition. However, there is some evidence to suggest that sugars are more important than lipids in the endosperm during the first day of imbibition. As with other seeds it is not known at what stage the electron transport system becomes

operative, but cytochrome oxidase (1.9.3.1) has been found in the endosperm of the resting seed. [162]

A large number of papers have been published by the Beevers school about the metabolism of the castor oil seedling. These papers reveal much detail about the function of the cotyledon and the endosperm in supplying nutrients to the growing axis. Those papers that best show the inter-relationship of these organs are reviewed below. It is emphasised that they appertain only to the seedling stage and provide no direct information about metabolism of the seed during the first day of imbibition.

Yamada [158] showed that the castor oil endosperm contains large quantities of lipids which are rapidly broken down during seedling growth, converted to sugar, and then translocated to the axis. The Beevers school has shown that the central pathway of this interconversion is the glyoxylate cycle. [163] Surveys of the two enzymes of this pathway that are not present in the Krebs cycle, isocitrate lyase (4.1.3.1) [164] and malate synthase (4.1.3.2), [165] showed that their presence is restricted to those plant materials, such as the castor oil endosperm, which are actively degrading storage lipids.

Tracer experiments were carried out on the castor oil endosperm with ^{14}C labelled acetate [166] and pyruvate. [167] Both compounds were rapidly metabolised through the

glyoxylate cycle to malate. Malate was converted to phosphoenolpyruvate and this in turn was converted, by reverse of glycolysis, eventually to sucrose. The sucrose was formed directly and free hexoses only arose secondarily.

It is interesting to note at this point that although the overall lipid level decreases with time in the castor oil seedling, it has been shown that some fatty acids, especially C_{18} acids are being synthesised.[168]

A study of the levels of some glycolytic intermediates under both aerobic and anaerobic conditions indicated that glycolysis was proceeding in both directions in the endosperm of the young castor oil seedling.[5] However, the majority of the flow was in the reversed direction, towards sucrose synthesis, and only about 10% was in the forward direction. The authors suggested that these two flows were occurring in different intracellular compartments.

Although most isocitrate in the endosperm is metabolised through the glyoxylate cycle, the Krebs cycle also appears to be active.[169, 170] There is competition for isocitrate between isocitrate lyase (4.1.3.1) of the glyoxylate cycle and isocitrate dehydrogenase (1.1.1.42) of the Krebs cycle. The two papers on the subject reached conflicting conclusions as to why the

glyoxylate cycle was so dominant in vivo. Tanner and Beevers [169] suggested that it was largely because the activity of isocitrate lyase (4.1.3.1) was about three times greater than that of isocitrate dehydrogenase (1.1.1.42). However, Marcus and Velasco [170] found that the activities of the two enzymes were about the same and from additional information concluded that the dominance of the glyoxylate cycle was due to compartmentation phenomena.

Tracer experiments have been performed on castor oil seedlings using ^{14}C labelled sugars [171, 172] and amino acids. [173] These showed that sucrose is absorbed from the endosperm by the cotyledons and translocated down these to the axis. A study with ^{14}C labelled glucose and fructose indicated that these sugars are not involved at any stage in this translocation. [172] This is in contrast to the situation in seeds of the monocotyledons where it is glucose that is absorbed from the endosperm by the scutellum (the single cotyledon).[145, 148] The experiments with ^{14}C labelled amino acids showed that the amino acids closely related to the Krebs cycle such as alanine, glutamate, and aspartate are translocated to the cotyledons in the form of glutamine and sucrose.[173] However, with amino acids not closely connected to the Krebs cycle, such as valine, there is no interconversion and the amino acids are translocated as such.

4-2 IMBIBITION OF PARTS OF SEEDS IN TRITIATED WATER

4-2.0 EXPERIMENTAL

Although the castor oil seed-coats were very hard they were not attached to the underlying endosperm, and it was found that they could be cracked and removed with little or no damage to the rest of the seed. Because it was largely composed of lipids, the rest of the seed was soft and it was easy to excise the axis and to cut out small pieces of endosperm. Care was taken to ensure that none of the pieces of endosperm used in the experiments had any pieces of cotyledon attached. It was found to be impossible to remove the cotyledons intact, but small pieces of cotyledon without any attached endosperm could be obtained and these were used for two experiments. Three axes, three pieces of endosperm, or three pieces of cotyledon were imbibed in each experiment. The experiments were carried out as described in Section 1-4.

4-2.1 RESULTS AND DISCUSSION

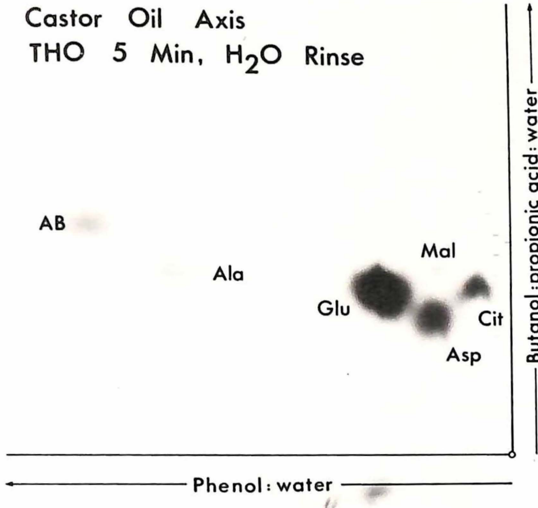
The results are given in Table 4.1 where the labelling in the three extracts has been combined as usual for presentation. The radioautograms of the water rinses are shown in Plate 4.1 and 4.2. On the basis of the patterns of labelling it is clear that the three parts of the castor oil seed that were investigated had different metabolism during the first six hours of imbibition.

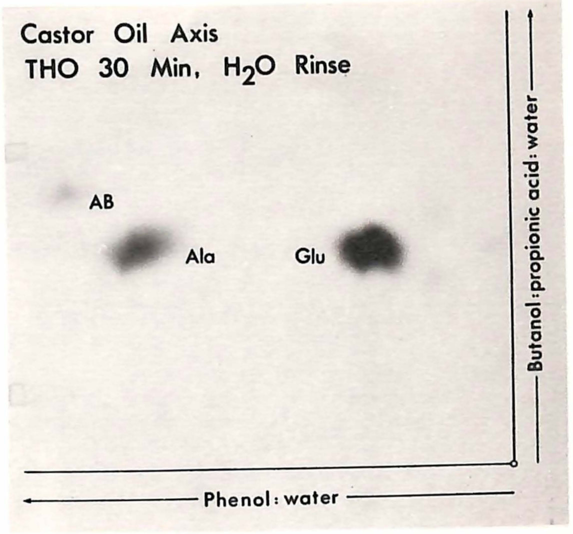
Table 4.1 Compounds labelled when parts of castor oil seeds imbibed tritiated water.

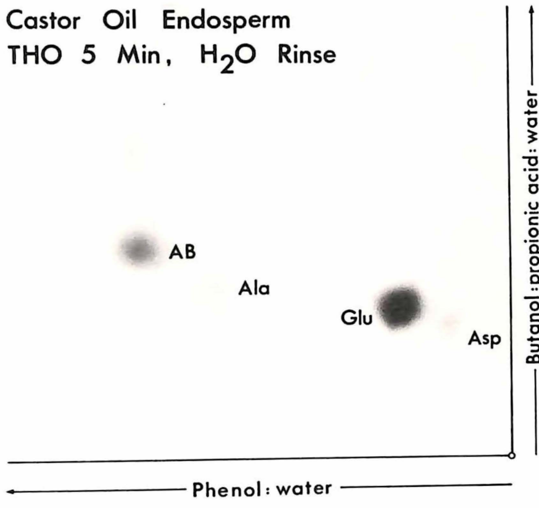
Compounds labelled	Time (min) 5	30	120	360	30	360
	(a) Axis				(b) Cotyledon	
4-Aminobutyrate	t	t	t	+		t
Aspartate	+	t	+	+		t
Glutamate	++	++	++	++	t	+
Alanine	t	+	++	++		t
Citrate	+	++	+	+	t	+
Malate	t	++	+	+		+
Succinate				+		
Lactate		t	t	+		
Sucrose			++	+++		+++
Glutamine			+	+		+
Lipids ?		t	+	++		+
Sugar phosphates ?			+	+		+
	(c) Endosperm					
4-Aminobutyrate	+	t	+	++		
Aspartate	t		+	+		
Glutamate	+	+	++	++		
Alanine	t	t	++	++		
Citrate	t		+	+		
Malate		t	+	++		
Succinate				+		
Lactate			t			
Sucrose			+	+++		
Glutamine				+		
Lipids ?						
Sugar phosphates ?			t	+		

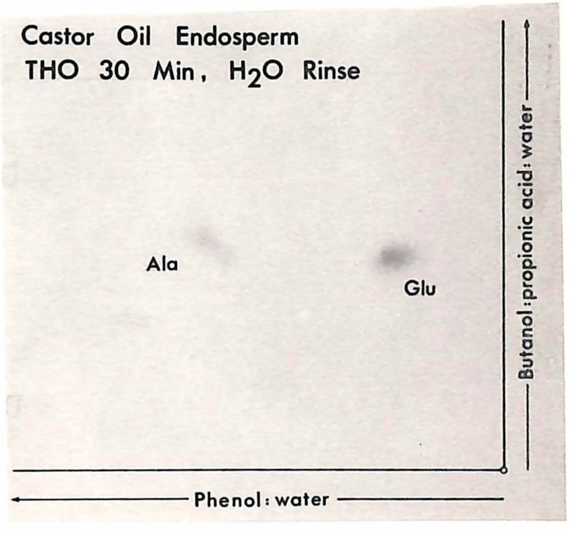
Key: +, ++, +++ = increasing relative intensity
t = trace.

PLATE 4.1









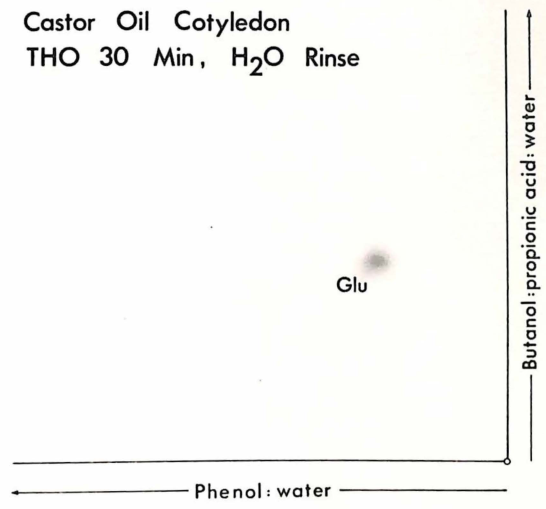
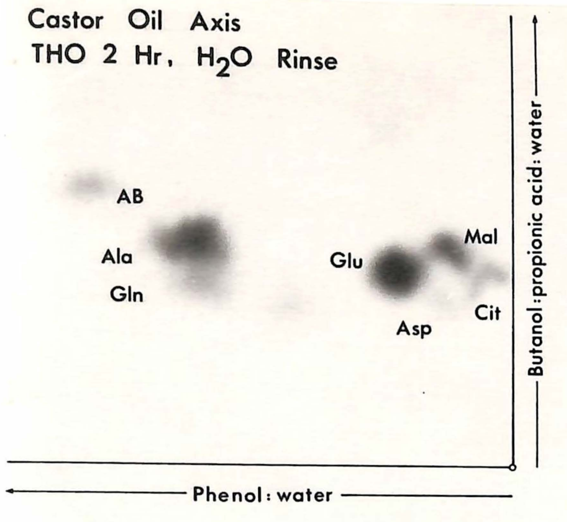
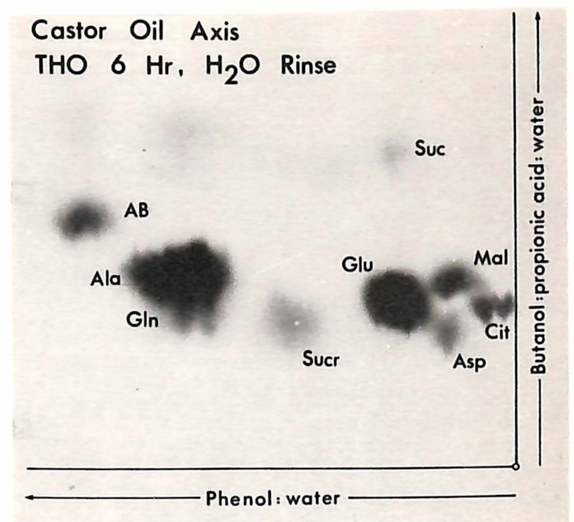
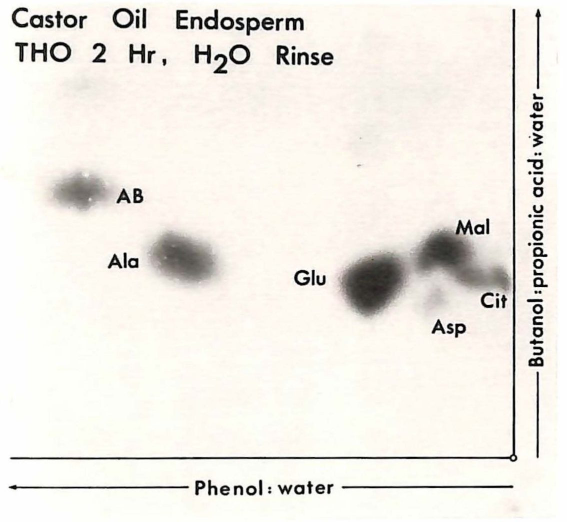
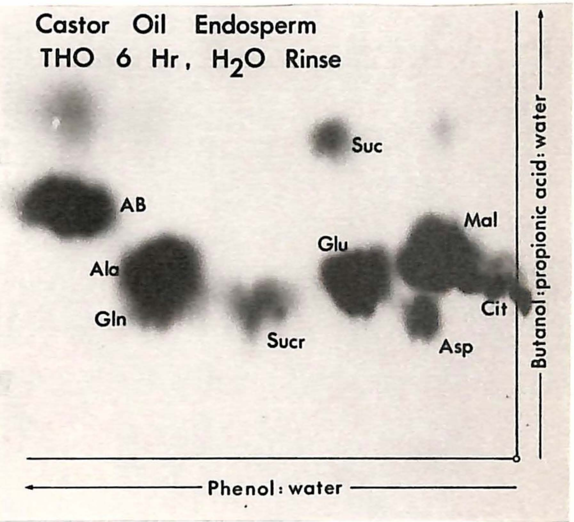


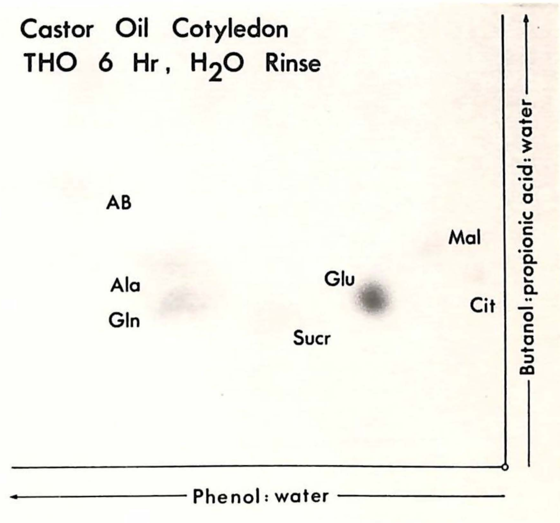
PLATE 4.2











Axis metabolism

After five minutes the axis appeared to have the most active metabolism. Six labelled compounds were present: 4-aminobutyrate, aspartate, glutamate, alanine, citrate and malate. The four amino acids are the same ones that were labelled in the barley embryo, the *Phaseolus vulgaris* axis and cotyledon, and the *Sinapis alba* seed [13] during the first few minutes of imbibition. It is suggested, as it was for the other three seeds, that glutamate, aspartate and alanine probably became labelled by deamination and transamination reactions, and that labelled 4-aminobutyrate was formed in the reaction catalysed by glutamate decarboxylase (4.1.1.15).

A trace of activity that could have been succinate was present in the castor oil axis after 30 minutes. Labelled citrate and malate were also present after 30 minutes, as well as the three amino acids whose corresponding 2-oxo acids are members of, or are closely related to the Krebs cycle. It is suggested on the basis of the labelling in these compounds that the Krebs cycle began to operate at that time. Some of the same labelled compounds would also be expected to be present if the glyoxylate cycle were operating. However, this possibility could be excluded as it has been shown that the glyoxylate cycle does not begin to operate in the

castor oil seed until two days after the start of imbibition.[160, 164] Even then, it apparently operates only in the endosperm and not in the axis.[165] It is interesting to note that 30 minutes was also the time at which the Krebs cycle apparently began to operate in the barley embryo, the *Phaseolus vulgaris* axis and the *Sinapis alba* seed.

After two hours sucrose, sugar phosphates and glutamine had become labelled in the castor oil axis and by six hours sucrose was strongly labelled. Using the method described in Section 2-4.11 it was found that both the fructose and glucose moieties of sucrose were labelled, as they were in the labelled sucrose formed in the barley, *Sinapis alba,* and *Phaseolus vulgaris* seeds. It is suggested that sucrose was formed in a similar way in all these seeds. The labelled sugar phosphates may have been those that were intermediates of sucrose synthesis. The labelled glutamine that was formed at two and six hours was probably formed from the relatively large amount of labelled glutamate that was present, by the action of glutamine synthetase (6.3.1.2).

There was a trace of labelling in the lipid fraction after 30 minutes, and it was strongly labelled after six hours. It has been shown that C_{18} fatty acids are synthesized in the young castor oil seedling.[168] The labelling in the lipid fraction may indicate that this synthesis was occurring in the castor oil axis

even in the first hours of imbibition.

As was the case with the compounds labelled at earlier times, the compounds labelled in the castor oil axis after six hours were the same as those labelled after six hours in the barley embryo and the *Phaseolus vulgaris* axis.

Endosperm metabolism

The first compounds labelled in the endosperm were amino acids. Glutamate and 4-aminobutyrate were the only two compounds strongly enough labelled in the first 30 minutes to be positively identified, but there were also traces of alanine and aspartate. These labelled amino acids were probably formed in the same reactions as the labelled amino acids in the castor oil axis and in the other seeds studied. After two hours, as well as the four amino acids, malate and citrate were labelled and there was a trace of activity corresponding to lactate/succinate. It is likely, therefore, that the Krebs cycle was starting to operate at that time. While some of the same compounds would also have become labelled by the action of the glyoxylate cycle, this is not a possibility at this time, as was previously indicated.

After six hours sucrose was strongly labelled in the endosperm. As in the case of the sucrose formed in the other seeds studied,both the glucose and fructose moieties

were labelled. During seedling growth sucrose is translocated from the endosperm to the axis.[173] It therefore appears that after six hours the endosperm was already producing sucrose for that purpose. The patterns of labelling in the castor oil axis and endosperm were similar after six hours. However, in the endosperm there was no activity in the lipid fraction and no labelled lactate was present, whereas both these were formed by the axis. It is interesting to note that of the parts of seeds so far investigated in this thesis the castor oil endosperm was the only one that contained large quantities of lipids and it was the only one that did not produce labelled lipids in the first six hours of imbibition. It was suggested in Section 1-5.4 that labelled lipids are formed in seeds that are imbibing tritiated water, during lipid synthesis rather than lipid breakdown. That the endosperm did not form labelled lipids supports this suggestion, but it is stressed that it is by no means conclusive evidence.

Cotyledon metabolism

Two experiments were performed on pieces of cotyledon. After 30 minutes there were only two traces of activity, one corresponding to glutamate and the other to citrate. After six hours more compounds were labelled, but apart

from sucrose the labelling was weak. At six hours there was not even a trace of activity corresponding to succinate, so it appears that the Krebs cycle was not operating to any significant extent. Sucrose was strongly labelled in the cotyledon at six hours and glutamine was as well labelled as in the axis and endosperm. These two compounds are known to be involved at the seedling stage, where they appear to be quantitatively the most important compounds in the translocation of nutrients from the endosperm through the cotyledons to the axis. [173] The reason for the cotyledon producing these labelled compounds in such disproportionately great amounts during imbibition is not clear, since, even in the seedling stage, the cotyledon is thought to act only as an active transporter of these compounds and not as a producer. Measurements of enzyme activities indicated that the cotyledon has a complex metabolism during the second day of imbibition, involving at least glycolysis and an electron transport system. [174] As well, it carries out photosynthesis during seedling growth. It is surprising therefore that the experiments reported here indicated that the cotyledon had such a simple metabolism during the first hours of imbibition. It has been claimed that the cotyledon in the resting castor oil seed contains very low levels of stored reserves. [174] It is possible that it has only a low metabolic activity

until it is supplied with nutrients from the endosperm. This could explain why the metabolism of the castor oil cotyledon showed little similarity to that of the *Phaseolus vulgaris* cotyledon. The cotyledons in both seeds translocate nutrients to the axis and also develop a photosynthetic system, but only the *Phaseolus vulgaris* cotyledon acts as storage tissue. The development of metabolism in the *Phaseolus vulgaris* cotyledon depends on the utilisation of its own reserves. This could be expected to occur sooner than the translocation of nutrients from the castor oil endosperm to the castor oil cotyledon.

A comparison of the water absorption by the three parts of the castor oil seed that were used in the imbibition experiments, is shown in Fig. 4.2. The water absorption of the cotyledon was similar to that of the endosperm, while that of the axis was considerably greater. It is clear from Fig. 4.2 that the absorption of water could not be determined as accurately for the cotyledon as for the endosperm and axis. This was because the cotyledon was thin and filamentous and began to dry out rapidly as soon as the surface water was removed.

The axis had the greatest water absorption of the three parts, and it formed more labelled compounds than the other parts in the first two hours of imbibition.

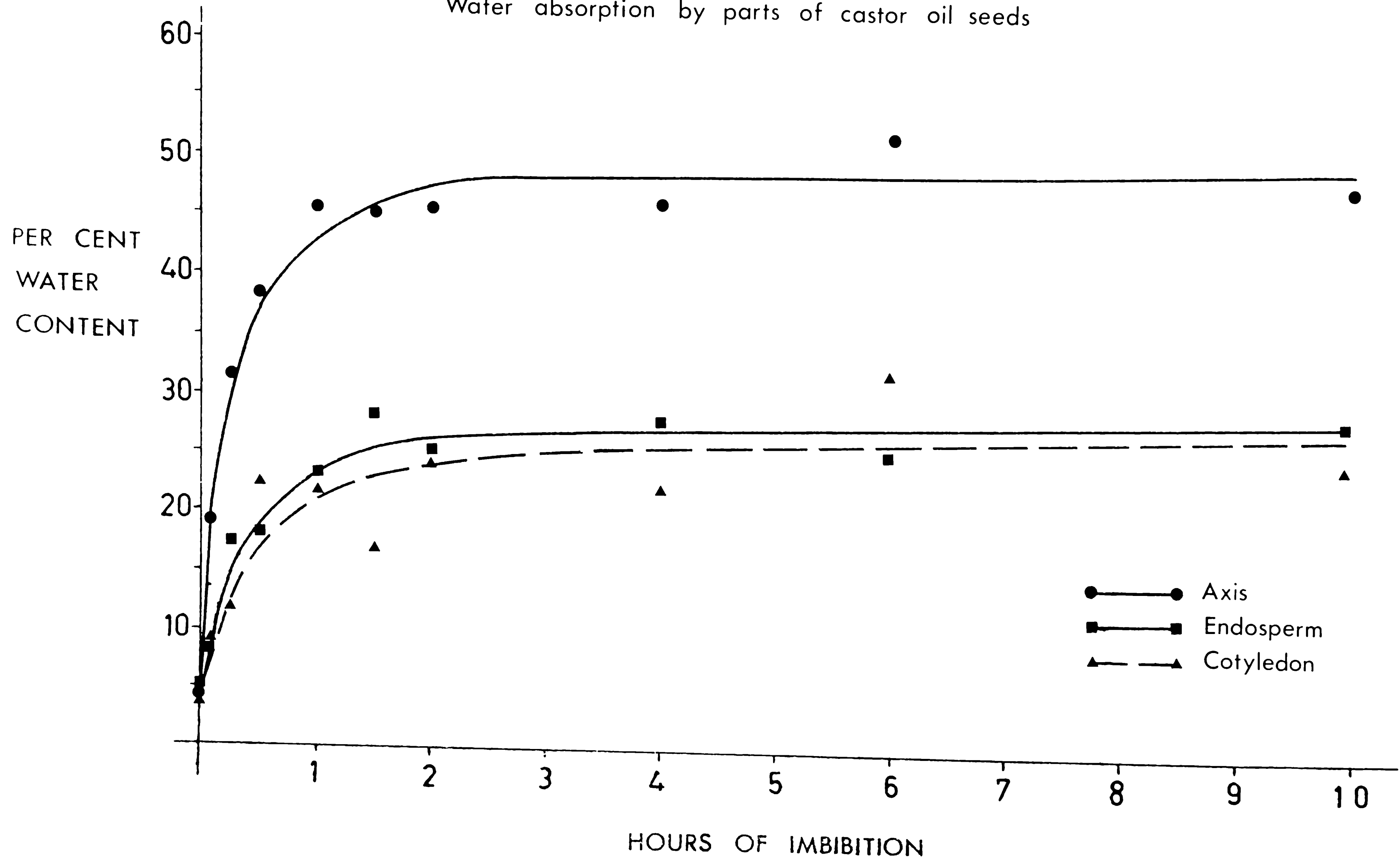


FIGURE 4.2
Water absorption by parts of castor oil seeds
PER CENT
WATER
CONTENT
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
HOURS OF IMBIBITION
Axis
Endosperm
Cotyledon

There may be some connection therefore between activation of metabolism and water absorption in that period. The water content was almost constant from two hours onwards but the metabolism of the endosperm continued to become more complex. At six hours the axis and endosperm had similar metabolism and yet a different water content, while the cotyledon and endosperm had a similar water content but different metabolism. It appears, therefore, that the water content is not a determining factor in the metabolism of the three parts after six hours of imbibition.

4-2.2 CONCLUSION

The metabolism of the castor oil axis during the first six hours of imbibition was similar to that of the *Phaseolus vulgaris* axis and the barley embryo. This supports the hypothesis made earlier that the embryos of many seeds have a similar metabolic development during early imbibition.

There was little similarity between the metabolism of the castor oil endosperm and that of the barley endosperm. Instead the metabolism of the castor oil endosperm more closely resembled that of the barley embryo than the barley endosperm. That the barley endosperm contains few active cells probably largely accounts for this difference. Clearly, although both endosperms serve similar functions in their respective seeds, this does not lead to their having similar metabolism during

early imbibition.

The castor oil cotyledon had much less metabolism than either the endosperm or axis. It also had much less metabolism than the *Phaseolus vulgaris* cotyledon. This may indicate that it has only negligible food reserves in its own tissues.

Although the embryos and the axes of different seeds have very similar metabolism it appears that the organs that store and translocate nutrients to these axes have different metabolism in different seeds.

A comparison of the metabolism of the three parts of the castor oil seed to each other and to the rates at which they absorbed water, showed that although the water content and metabolism of the parts might have been associated in the first two hours of imbibition, this was not the case after six hours.

CHAPTER 5

EXPERIMENTS ON *PINUS RADIATA* SEEDS

5-1 INTRODUCTION

5-1.0 GENERAL

The *Pinus radiata* seed was the fourth and last seed investigated in this thesis. *Pinus radiata* is a member of the family Pinaceae of the order Coniferales.[175] This is one of the orders of the gymnosperms.

Seed bearing plants are categorised into angiosperms and gymnosperms, the most notable difference between them being in their seeds. The seeds of angiosperms are enclosed by an ovary whereas the seedsof gymnosperms are not. Angiosperm seeds contain an endosperm (when it is present) that is formed during fertilisation by combination of nuclei from both the male (pollen) and female (ovule) gametophyte tissues. Gymnosperm seeds also contain a storage organ which is often called the endosperm, but this is composed only of female gametophyte tissue. This storage organ is referred to in this thesis as the female gametophyte. The difference between angiosperm and gymnosperm seeds cannot at present be correlated with any survival value (p. 35, ref. 16).

The *Pinus radiata* seed was selected for study because:

1. It was the only gymnosperm seed easily available with a reasonable germination percentage (70%).

2. It was big enough to dissect yet had a small

enough embryo for several of these to be imbibed together in a small amount of tritiated water.

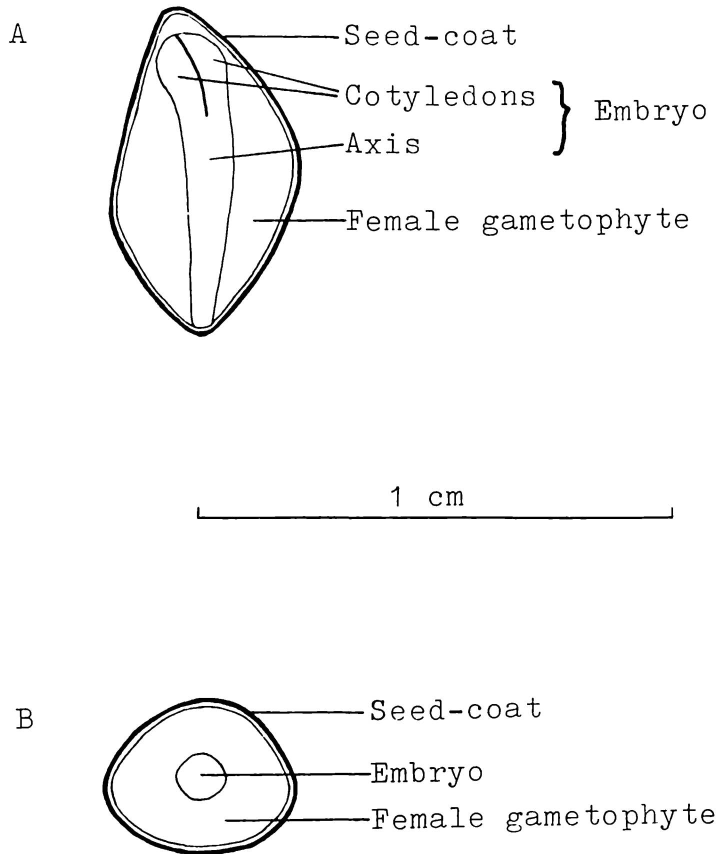
3. It is a typical example of a gymnosperm seed. Its storage organ is the female gametophyte and its energy reserves are mainly lipids.

5-1.1 DESCRIPTION AND STORAGE

The *Pinus radiata* seeds used were approximately 0.7 cm long and 0.4 cm in diameter. The seed-coat was thick, hard and varied from rough to smooth. The seeds varied in colour from pale brown to almost black. As shown in Fig. 5.1, the *Pinus radiata* embryo is surrounded by the female gametophyte and extends almost the full length of the seed. The number of cotyledons is variable (p. 11, ref. 176) but in the sample of seeds obtained was usually 6-8. The cotyledons make up the bulb-shaped end of the embryo. The embryo is in very close contact with the female gametophyte tissue but is not directly attached to it. The average seed weighed approximately 0.03 g and the embryo approximately 0.0025 g.

The seeds were stored over silica gel at 4°C. When stored in this way the germination percentage slowly dropped over the period of nine months in which the experiments were done, from 75% to 68%. The seeds were germinated at room temperature as described in Section 1.4.0. They began to germinate (radicle protrusion) after

FIGURE 5.1



Pinus radiata seed shown in (A) longitudinal and (B) transverse sections.

one week, but did not achieve 70% germination till after 3-4 weeks.

The germination is epigeal and the cotyledons begin photosynthesising while still absorbing the female gametophyte reserves.

5-1.2 COMPOSITION

There appears to be no report in the literature of the composition of the *Pinus radiata* seed. However, the compositions of the seeds of a number of other species of the *Pinus* genus have been analysed, and a histochemical study of the *Pinus radiata* seed, indicates that its composition is similar. [177] The ranges of these analyses are: carbohydrates, 3.7 - 17.9%; proteins, 14.8 - 35.2%; lipids, 31.7 - 78.9%. [7, 11] Although the ranges of measurements are wide, it is clear that *Pinus* seeds contain large quantities of lipids and relatively small amounts of carbohydrates.

Durzan and Chalupa [178] measured the amounts of free amino acids and sugars in the embryo and female gametophyte of the *Pinus banksiana* seed. This species has been placed in the same small group of the genus as *Pinus radiata.* [179] On a dry weight basis, sugars comprised 3.1 - 4.7% of the female gametophyte and 7.9 - 12.6% of the embryo. Approximately half of this sugar was stachyose and there were smaller amounts of raffinose and sucrose.

These sugars have also been found in the seeds of other *Pinus* species.[180] In *Pinus banksiana* seeds the total free amino acids comprised 0.05 - 0.1% of the female gametophyte and 0.4 - 0.7% of the embryo on a dry weight basis.[178] All the protein amino acids were present in the free form as well as citrulline, ornithine, and small amounts of 4-aminobutyrate. Arginine was present in largest amount and accounted for almost 30% of the total.

Goo and Negisi[181] reported that both the embryo and the female gametophyte of the *Pinus thunbergii* seed contained large amounts of lipids. On a dry weight basis, 59.6% of the embryo and 45.2% of the female gametophyte was composed of lipids. Firenzuoli et al[182] separated the lipid fractions from seeds of five different *Pinus* species. They found that in all five cases most of the lipid fraction was composed of triglycerides and that the remainder consisted of small amounts of phospholipids, steroids and free fatty acids.

Hatano[183] reported that small amounts of the 2-oxo acids pyruvate and oxaloacetate were present in the resting *Pinus thunbergii* seed.

5-1.3 METABOLISM

The seeds of *Pinus* species are often used as examples of gymnosperm seeds and a number of papers have studied aspects of their metabolism during the first day of imbibition.

Nyman [184] assayed *Pinus silvestris* seeds for enzymes that catalyse the dehydrogenation of glyceraldehyde-3-phosphate. This is one of the reactions of glycolysis. He found that both an NAD(1.2.1.12) and an NADP (1.2.1.13) dependent enzyme were present in the embryo and female gametophyte of both the resting and imbibing seeds.

Stanley [185] and Stanley and Conn [186] studied mitochondrial preparations from *Pinus lambertiana* seeds. They studied the effect on respiration of adding Krebs cycle acids, and they also carried out ^{14}C tracer experiments. They found that after six hours of imbibition, the Krebs cycle was active in the mitochondrial fraction isolated from both the embryo and the female gametophyte. There was some Krebs cycle activity in the mitochondrial fraction isolated from the female gametophyte of the resting seed, but this was ten times less than that at six hours of imbibition. They made no mention of whether or not there was any Krebs cycle activity in the embryo of the resting seed.

Firenzuoli et al [187] assayed resting seeds of *Pinus pinea* for a number of enzymes that are characteristic of different pathways. They found that glucose-6-phosphate dehydrogenase (1.1.1.49)(pentose phosphate pathway), aldolase (4.1.2.b) (glycolysis), isocitrate dehydrogenase (1.1.1.42) (Krebs cycle), and malate synthase (4.1.3.2)

(glyoxylate cycle) were all present but that there was no isocitrate lyase (4.1.3.1) (glyoxylate cycle) activity. However, this last enzyme was present after six days of imbibition. It therefore appears that, as in the castor oil seed, [160, 164] the glyoxylate cycle does not operate in *Pinus* seeds during early imbibition.

Bartels [188] assayed *Pinus nigra* seeds for malate dehydrogenase (1.1.1.37), aspartate aminotransferase (2.6.1.1), alanine aminotransferase (2.6.1.2), and five of the enzymes of glycolysis. He found that all these enzymes were present in both the embryo and the female gametophyte of the resting and imbibing seed.

Riding and Gifford, [177] using a histochemical technique, found that succinate dehydrogenase (1.3.99.1) was present in the embryo of the resting *Pinus radiata* seed.

Durzan et al [41] used tritiated water to investigate the amino acid metabolism of *Pinus banksiana* seeds. After the seeds had imbibed tritiated water for 12 hours they found that proline, glutamic-γ-semialdehyde, glutamate and alanine were labelled. This showed that these compounds must have been involved in some metabolism at that time. Their next identification of labelled compounds was carried out after four days of imbibition, by which time all the protein amino acids were labelled, as well as some other amino acids, including 4-aminobutyrate. At both times most of the label was present

in so-called "neutral fractions" which they did not analyse. They also measured the free amino acid content of the embryo and the female gametophyte in the resting seed and after 24 hours of imbibition. There was initially a large amount of arginine in both organs, but this had decreased substantially after 24 hours of imbibition. The levels of 4-amino-butyrate, ornithine, aspartate, proline and alanine had also decreased in both organs. Clearly, these amino acids must have been involved in some metabolism for these decreases to have occurred. While there was a large rise in the levels of glutamine and glutamate during the 24 hours, there was no indication as to how much of these increases resulted from protein hydrolysis and how much was at the expense of the other amino acids.

It is clear from this review of the six preceding papers that both the embryo and the female gametophyte of *Pinus* seeds have an active metabolism during the first day of imbibition involving at least the glycolytic sequence, the Krebs cycle, and some amino acid metabolism.

The metabolism of *Pinus* seeds after the first day of imbibition has also been studied. To show the basic similarities between the germination of angiosperm and gymnosperm seeds several of these papers are outlined below.

Although lipids are quantitatively the most important respiratory substrate in *Pinus* seeds, small amounts of sugars are also present. Stachyose, raffinose and sucrose have been found in the resting seeds of *Pinus banksiana* [5] and *Pinus thunbergii.* [180] The enzyme α-galactosidase (3.2.1.22) has also been found in the resting *Pinus thunbergii* seed. [180] After four days of imbibition the two galactose containing sugars disappeared but this was not sufficient to account for the increase in the level of sucrose that occurred. This raised the possibility that a lipid to sugar conversion might be occurring in imbibing *Pinus* seeds in the same way as in castor oil seeds. The glyoxylate cycle, the central pathway of lipid to sugar conversion in castor oil seedlings, has been found to be active in the seedlings of four *Pinus* species. [182]

As with angiosperm seeds, it has been shown in *Pinus* seeds that there is translocation of nutrients from the storage organ to the embryo during seedling growth. Goo and Negisi [181] studied the overall changes of constituents during germination of *Pinus thunbergii* seeds. They found that there was no increase in the dry weight of the embryo till after two days of imbibition and that this increase was only slight till after four days. The dry weight of the embryo then began to increase more rapidly and there was a corresponding decrease in the dry weight

of the female gametophyte. Starch appeared in the embryo and the sugar and protein levels increased, while in the female gametophyte there was a continuous decrease in the lipid and protein levels. The lipid level in the embryo also decreased, but this was a much smaller decrease than that of the female gametophyte.

5-2 IMBIBITION OF PARTS OF SEEDS IN TRITIATED WATER

5-2.0 EXPERIMENTAL

As is the case with the castor oil seed, the *Pinus radiata* seed contains large amounts of lipids and once the hard seed-coat was removed the rest of the seed was soft. Because the embryo was soft and relatively large care had to be taken to avoid damaging it during excision. For each experiment three embryos or three pieces of female gametophyte, each of approximately the same size as the embryo were used. In the case of the experiments at five and 30 minutes, the water rinses and ethanol extracts were chromatographed together.

5-2.1 RESULTS AND DISCUSSION

The results of the experiments are given in Table 5.1 where the labelling in the three extracts has been combined for presentation. Some of the radioautograms are shown in Plates 5.1 and 5.2.

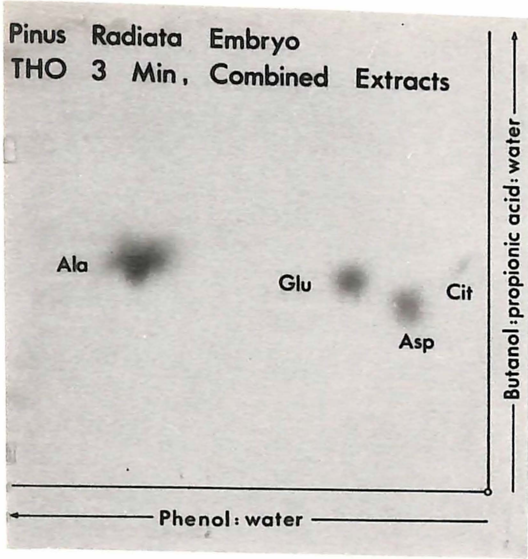
The same labelled compounds were formed in the embryo as in the female gametophyte, at three out of four times studied. After five minutes of imbibition, labelled

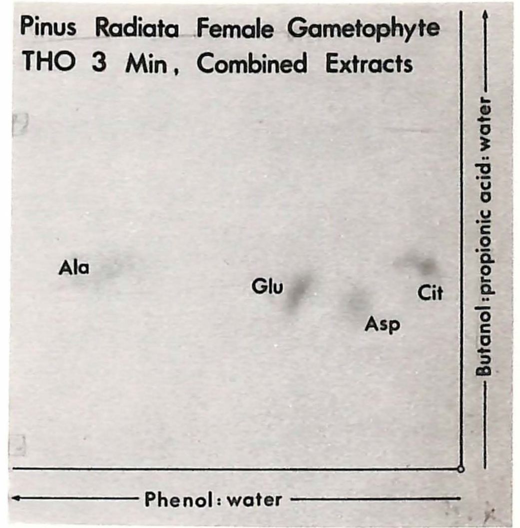
Table 5.1 Compounds labelled when *Pinus radiata* embryos and pieces of female gametophyte imbibed tritiated water.

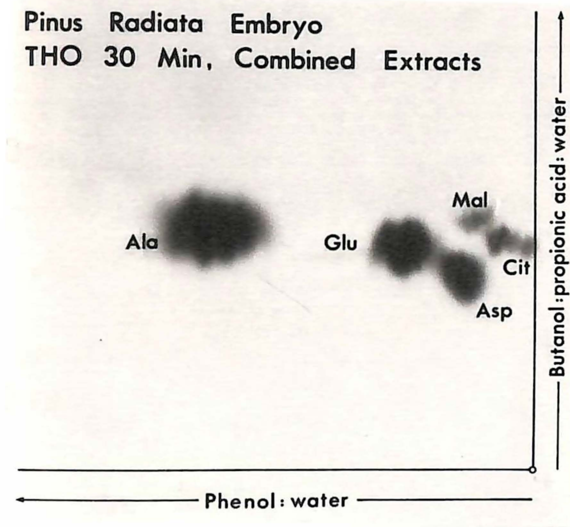
Compounds labelled	Time (min) 5	30	120	360
(a) Embryo				
4-Aminobutyrate			t	t
Aspartate	t	+	+	+
Glutamate	t	++	++	++
Alanine	+	++	++	++
Citrate	t	+	+	++
Malate		+	+	++
Succinate			t	t
Lactate				
Sucrose			+	++
Glutamine			+	+
Lipids ?			t	++
Sugar phosphates ?			++	++
(b) Female gametophyte				
4-Aminobutyrate			t	t
Aspartate	t	t	+	+
Glutamine	t	+	+	+
Alanine	t	+	++	++
Citrate	t	+	+	+
Malate		t	+	+
Succinate				t
Lactate				
Sucrose			t	t
Glutamine			+	+
Lipids ?				t
Sugar phosphates ?			+	t

Key: +, ++ = increasing relative intensity
t = trace

PLATE 5.1







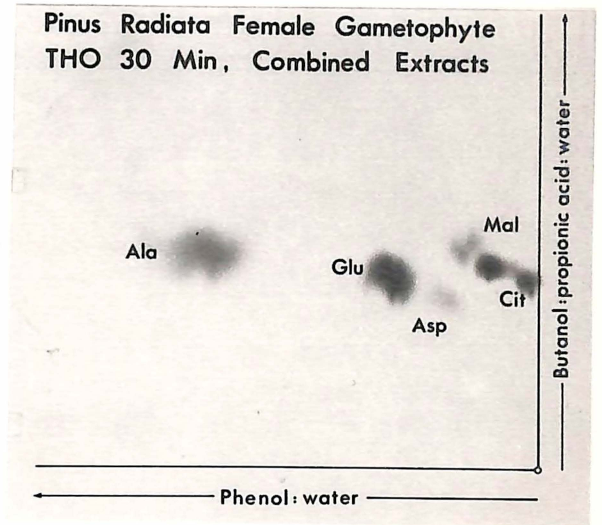
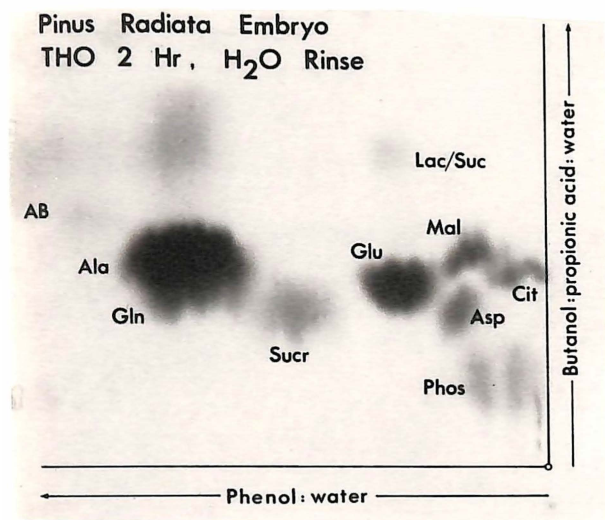
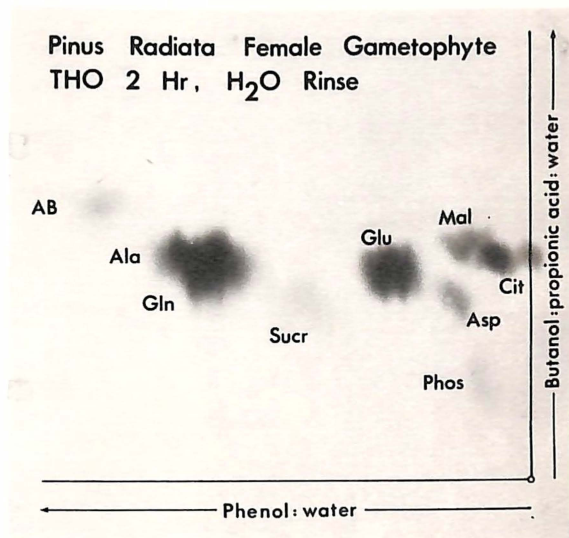
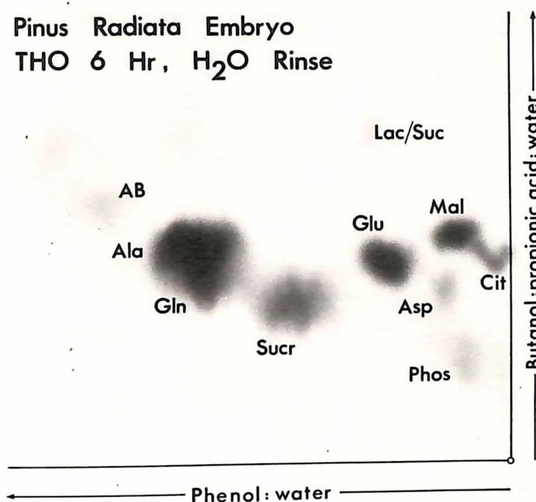
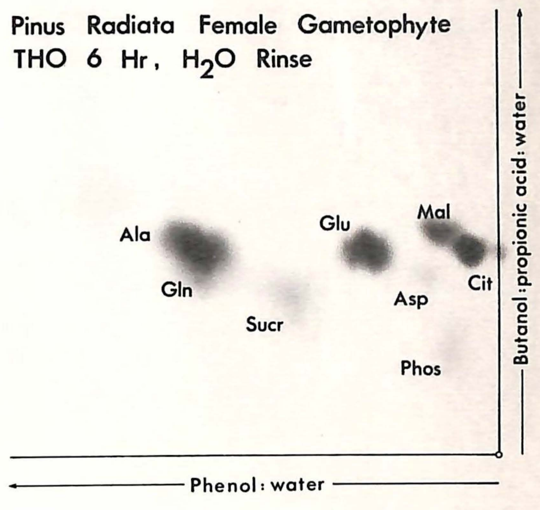


PLATE 5.2









aspartate, glutamate, alanine and citrate were found in both organs, although only the alanine in the embryo had more than a trace of activity. After 30 minutes these four compounds plus malate were labelled in both organs but the labelling was stronger in the embryo. After two hours, sucrose, glutamine and sugar phosphates were labelled in both organs and there were also traces of activity in the embryo corresponding to lactate/succinate and lipids. It was only in the experiments at this time that there was some qualitative difference between the embryo and the female gametophyte in the labelled compounds that were formed. After six hours, traces of activity corresponding to lactate/succinate and lipids were present in the female gametophyte. So at that time, the same labelled compounds were present in the embryo and the female gametophyte. It is not possible to state definitely that labelled 4-aminobutyrate was formed in any of these experiments. Traces of activity that could have been this compound were present in the embryo and female gametophyte after two and six hours, but there was not enough activity to identify them by co-chromatography.

Using the method described in Section 2-4.11 it was shown that the label in the sucrose formed by the *Pinus radiata* embryo was present in both the glucose and fructose moieties. This is the same result as that obtained for the sucrose formed in the seed studied in the

previous chapters and suggests that the sucrose in all these seeds was formed in similar reactions. The other labelled compounds that were formed in the *Pinus radiata* seed are the same as those formed in the other three seeds studied, and a similar reasoning to that advanced in previous chapters can be applied to the suggested reactions of their formation. Thus, glutamine was probably formed by the action of glutamine synthetase (6.3.1.2) on labelled glutamate. Aspartate, glutamate and alanine were probably labelled by deamination and transamination reactions. Transaminases that would cause these three amino acids to become labelled in tritiated water have been found in the embryo and female gametophyte of the resting seed of one *Pinus* species.[188] The corresponding 2-oxo acids of these amino acids are members of, or closely related, to the Krebs cycle. In both the embryo and female gametophyte these amino acids and citrate were labelled from five minutes onwards, and malate was labelled from 30 minutes onwards. It is suggested that the Krebs cycle was operating in both these organs during the first hours of imbibition, and that possibly it began about the time the first trace of labelled succinate was formed: at two hours in the embryo and at six hours in the female gametophyte. The three Krebs cycle acids might also have become labelled by the operation of the glyoxylate cycle. However, the studies of other workers support the contention that in

Pinus seeds during early imbibition there is an active Krebs cycle but not an active glyoxylate cycle. The results of these studies are summarised below.

1. It has been reported that Krebs cycle enzymes are present in the resting seeds of various *Pinus* species.[177, 187, 188] In particular, succinate dehydrogenase (1.3.99.1) was found in the embryo of the resting *Pinus radiata* seed.[177]

2. An active Krebs cycle was found in mitochondrial preparations isolated from both resting and imbibing seeds of one *Pinus* species.[185, 186]

3. In a study of one *Pinus* species it was found that isocitrate lyase (4.1.3.1) one of the key enzymes of the glyoxylate cycle, was not present in the resting seed.[187]

There were some similarities between the labelling in the *Pinus radiata* embryo and that in the embryos or axes of castor oil, barley and *Phaseolus vulgaris*. In all four cases the three amino acids aspartate, glutamate and alanine were labelled at the earliest times examined (three or five minutes), citrate and malate were labelled by 30 minutes, and sucrose and glutamine were labelled after two hours. The lipid fraction took longer to become labelled in the *Pinus radiata* embryo than in the other three embryos or axes but after six hours the intensity of labelling was similar to that in the castor oil axis after six hours. If, as suggested previously,

this labelling occurred during lipid synthesis, this may indicate that both organs had a similar requirement for lipid synthesis at that stage of imbibition.

There are some notable differences between the labelling in the *Pinus radiata* embryo and that in the embryos or axes of the other three species studied. In the *Pinus radiata* embryo there was not even a trace of activity corresponding to succinate until after two hours, whereas in the other three embryos or axes there was such a trace of activity after 15 or 30 minutes. This may be an indication that the Krebs cycle began to operate at a later stage in the *Pinus radiata* embryo than in the other three embryos or axes. 4-Aminobutyrate was not labelled in the *Pinus radiata* embryo, except possibly in trace amounts, and even that not until after 30 minutes; in the other three embry~~ or axes it was one of the first compounds to become labelled. Of the other three embryos or axes studied, it was in the castor oil axis that the labelling of 4-aminobutyrate was most similar to that in the *Pinus radiata* embryo. In the castor oil axis, 4-aminobutyrate was only labelled in trace amounts until after six hours, at which time the labelling was just sufficiently intense for it to be positively identified by co-chromatography.

The female gametophyte has a similar function to the castor oil endosperm and both organs contain large amounts of lipids. However, there were many differences between their patterns of labelling in the first six hours. 4-Aminobutyrate was never labelled in more than trace amounts in the female gametophyte, whereas it was relatively well labelled in the castor oil endosperm. Labelled succinate did not appear in the female gametophyte until after six hours, whereas it was present in the castor oil endosperm after two hours. Sucrose was only labelled in trace amounts in the female gametophyte after six hours, whereas it was strongly labelled in the castor oil endosperm. The two organs were more similar however, in regard to the labelling of the lipid fraction. The lipid fraction was not labelled in the castor oil endosperm at any of the four times studied and there was only a trace of activity in this fraction in the female gametophyte after six hours. The absence of labelling in the lipid fractions of these two organs may indicate, on the basis of the discussion in Section 1-5.4, that little or no lipid synthesis is occurring. This might be expected as both organs are storage tissues and both already contain large amounts of lipids. There are still greater differences in the patterns of labelling between the female gametophyte and the other two storage organs studied. In general, compounds became labelled earlier

and more intensely in the *Phaseolus vulgaris* cotyledon than in the female gametophyte and later and less intensely in the barley endosperm.

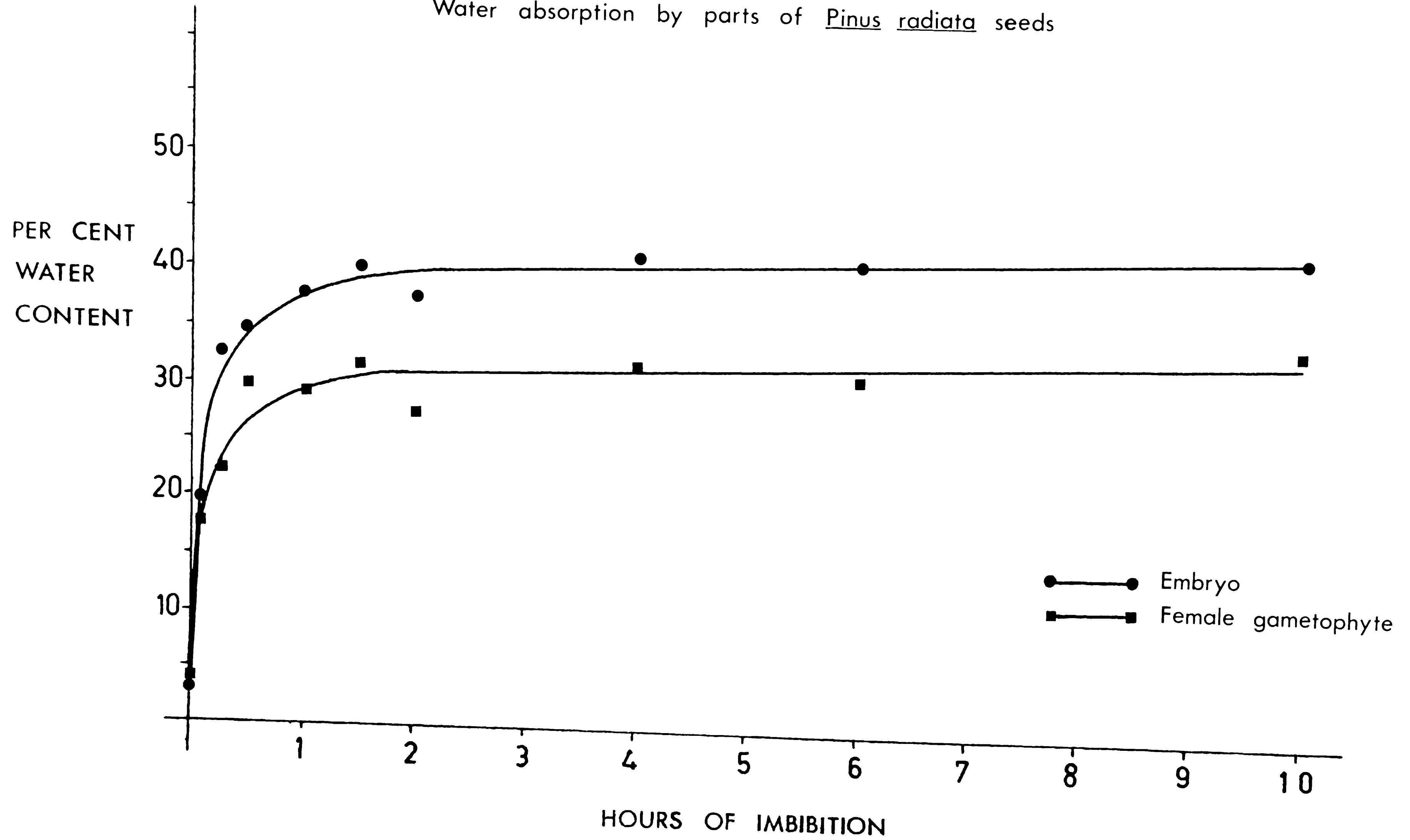
The water absorption curves of the embryo and female gametophyte of *Pinus radiata* are shown in Fig. 5.2. The water content rose steeply for both organs in the first 30 minutes. It then began to level out and, as for the other seedsstudied, the embryo achieved a higher water content than the storage organ (in this case the female gametophyte). While the water absorption of the embryo was slightly faster than that of the female gametophyte, there was only a small difference between the water contents of the two organs after 30 minutes. It is possible that during the first 30 minutes the similarity of water absorption in the two organs may have accounted for their similar metabolic development at that time. However, there was scant correlation between the water content and metabolism after six hours, as at that time the water contents of the two organs were different and yet they had the same patterns of labelling.

5-2.2 CONCLUSION

It was previously hypothesised that the embryos of all seeds have a similar metabolic development during early imbibition. The patterns of labelling in the *Pinus radiata* embryo support this hypothesis to some extent. Amino acids

FIGURE 5.2

Water absorption by parts of *Pinus radiata* seeds



were labelled in the first minutes of imbibition, followed by the Krebs cycle acids, and then sucrose and glutamine. This same development also occurred in the embryos or axes of the other three seeds studied, seeds that are widely separated from evolutionary and embryological points of view. However, there were some differences between the patterns of labelling in the *Pinus radiata* embryo and those in the embryos or axes of the other three seeds studied. Succinate and 4-aminobutyrate were not labelled as early in the *Pinus radiata* embryo as in the other embryos or axes, and they were only labelled in trace amounts after six hours. By contrast these compounds were relatively well labelled in the other embryos or axes. These differences may arise because *Pinus radiata* is a gymnosperm and is further removed from the other three seeds on evolutionary grounds than they are from each other. Alternatively, these differences may reflect the fact that the germination of the *Pinus radiata* seed is a much slower process than the germination of the seeds of the other three species studied.

The female gametophyte had almost the same metabolic development as the embryo. After six hours of imbibition both organs had the same patterns of labelling but different water contents. Water absorption could not therefore be directly related to metabolic development in

the seed at that time. The metabolic development of the female gametophyte was different in many respects from that of the other three storage tissues studied. This supports the conclusion made in Section 4-2.2, that although the embryos and axes of seeds have a similar metabolic development during imbibition, the organs that store and translocate nutrients to these embryos and axes have a different metabolic development in different seeds.

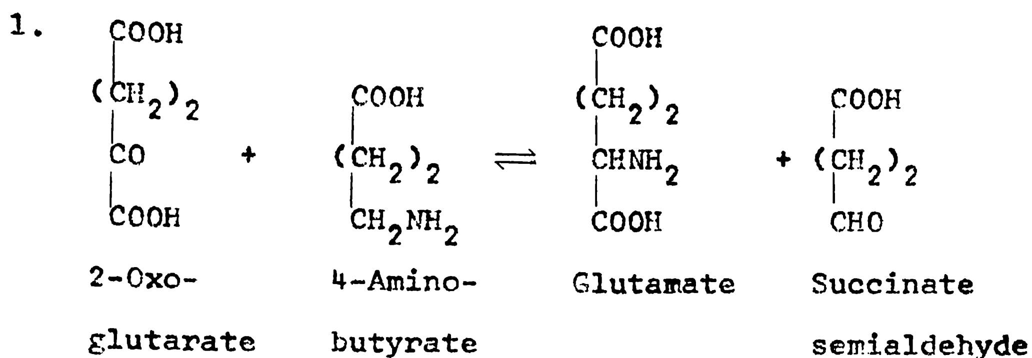
CHAPTER 6

INVESTIGATION OF 4-AMINOBUTYRATE METABOLISM

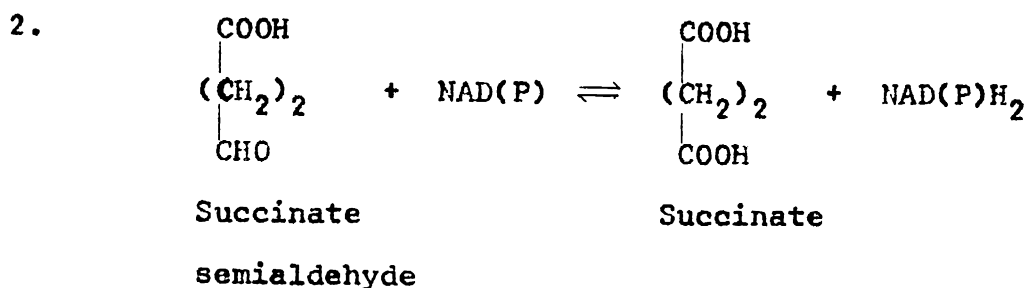
6-1 PRESENT STATE OF KNOWLEDGE OF 4-AMINOBUTYRATE METABOLISM IN PLANTS

The amino acid 4-aminobutyrate is widely distributed in nature and a large number of reactions are known in which it is involved. Apart from its role in the 4-aminobutyrate bypass, which will be discussed below, it has been shown that it can be formed from the breakdown of proline,[189] ornithine,[190] pyrrolidine, spermidine and putrescine,[191, 192] and that it can be metabolised by transamidination to 4-guanidinobutyrate,[193] by methylation to 4-butyrobetaine,[194] by oxidation to 3-hydroxy-4-aminobutyrate,[195] by transamination and reduction to 4-hydroxybutyrate,[196, 197] and by a complex series of reactions to crotonyl-CoA.[198] Biological systems from plants were studied in only two of the papers cited above.[190, 197] All of these reactions may occur in plants, but with the present state of knowledge this is uncertain. The only well established reactions of 4-aminobutyrate in plants are those of the pathway that has been described as the 4-aminobutyrate bypass.[199] The three reactions of this pathway are catalysed by (1) 4-aminobutyrate aminotransferase (2.6.1.c), (2) succinate semialdehyde dehydrogenase (1.2.1.b), and (3) glutamate decarboxylase (4.1.1.15); the equations are given as follows:

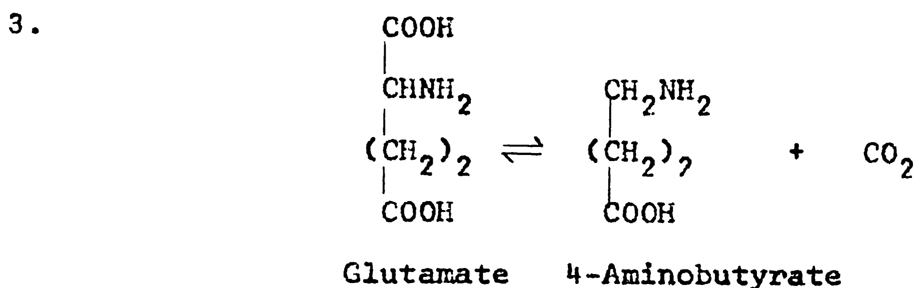
1.



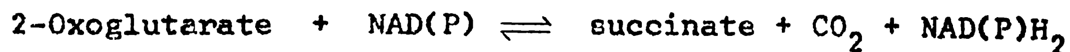
2.



3.



The sum of these three reactions is:

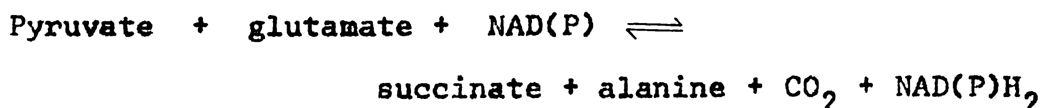


2-Oxoglutarate + NAD(P) $\rightleftharpoons$ succinate + CO_2 + NAD(P)H_2

The pathway is described as a bypass because the sum of the three reactions provides an alternative route to that in the Krebs cycle for converting 2-oxoglutarate to succinate. In mammalian brain tissue, in which this pathway has been intensively studied, there is a net flow from

2-oxoglutarate to succinate, and the pathway appears to be irreversible.[200, 201] However, in one micro-organism, 4-aminobutyrate was found to be formed from succinate by the reverse of reactions (1) and (2) above.[202] Most of the conclusions reached about the functioning of this pathway in plants have been influenced by the results obtained from either micro-organisms or brain tissue: partly because the advances in the understanding of this pathway were first made using non-plant extracts, and also because of the difficulty of isolating succinate semi-aldehyde dehydrogenase (1.2.1.b) from plant sources.

The first enzyme of the pathway, 4-aminobutyrate aminotransferase (2.6.1.c) has been found in mature plant tissues[203, 204] and in seedlings,[204, 205] but does not yet appear to have been identified in resting seeds. Preparations of this enzyme from plants were able to use either 2-oxoglutarate or pyruvate as the amino group acceptor,[204, 205, 206] and in some preparations pyruvate was the preferred substrate.[204, 205] If pyruvate was used as a substrate *in vivo* the sum of the three reactions would become:



$$\text{Pyruvate} + \text{glutamate} + \text{NAD(P)} \rightleftharpoons \text{succinate} + \text{alanine} + CO_2 + \text{NAD(P)}H_2$$

While this would no longer be a bypass of the Krebs cycle, it would be a useful pathway of intermediary metabolism. The bypass could be restored by the three enzymes of the pathway acting in combination with the enzyme, alanine aminotransferase (2.6.1.2), which catalyses the reaction:

2-Oxoglutarate + alanine $\rightleftharpoons$ glutamate + pyruvate

The second reaction of the 4-aminobutyrate bypass is that catalysed by succinate semialdehyde dehydrogenase (1.2.1.b). Until recently, the presence of this enzyme in plants had been inferred from ^{14}C tracer experiments and from comparison with the situation in other organisms. However, the enzyme was recently isolated from the cotyledons of radish (*Raphanus sativus*) seedlings. [206]

The third enzyme of the pathway, glutamate decarboxylase (4.1.1.15) has been found in many plant materials including resting seeds. [102, 103, 105]

There is at present some controversy over whether the 4-aminobutyrate bypass in plants is reversible. One school of thought asserts that the pathway is not reversible and can only proceed in the direction 2-oxoglutarate to succinate. [204, 205, 207] This stand is supported by the results of ^{14}C tracer experiments, and it is claimed that both glutamate decarboxylase (4.1.1.15) and succinate semialdehyde dehydrogenase (1.2.1.b) catalyse (what is described as) essentially irreversible reactions. The other school of thought asserts that the pathway is reversible and that in some cases the net flow through the pathway is in the direction succinate to 2-oxoglutarate. [197, 208, 209] This stand is also supported by the results of ^{14}C tracer experiments; in addition it has been shown that the glutamate decarboxylase (4.1.1.15) from some

plant sources catalyses a weakly reversible reaction,[210, 211] and it has been claimed that a reversible succinate semi-aldehyde dehydrogenase (1.2.1.b) is present in micro-organisms.[202] Many of the results on which these assertions are based are fragmentary. At present it appears that the complete pathway in plants has been intensively studied in only one case.[204] Partly because of the results of this study, but also because of the somewhat doubtful nature of the experimental support for the second school of thought, it now appears to be more commonly believed [135, 212] that the bypass in plants is irreversible and proceeds in the direction 2-oxoglutarate to succinate. The situation can be summarised by saying that while there is some doubt about whether the complete bypass is present in all plants and while there is some doubt about whether the pathway is reversible, there is little doubt that much, if not most, of the 4-aminobutyrate in plants is formed by one or more of the reactions of this pathway.

The experiments that established this pathway in plants were performed on seedlings and mature tissues. There is only a small amount of information available with regard to this pathway in seeds during the first day of imbibition, and this is summarised below. It has been shown that the enzyme glutamate decarboxylase (4.1.1.15) is present in resting seeds [102, 103] and that it begins

to operate at an early stage of imbibition. [105]
When *Sinapis alba* seeds were imbibed in tritiated water, one of the first compounds to become labelled was 4-aminobutyrate. [13, 14] Vickers [104] found that ^{14}C-glutamate was metabolised only to 4-aminobutyrate in powders ground from resting *Sinapis alba* seeds. It has been shown that 4-aminobutyrate is present in many resting seeds, [41, 71, 135] and that its concentration increases in barley seeds during the first day of imbibition. [135] The only definite conclusion that can be drawn from the above evidence, is that some 4-aminobutyrate is formed in seeds during early imbibition by the action of the enzyme glutamate decarboxylase (4.1.1.15)

6-2 4-AMINOBUTYRATE METABOLISM IN *PHASEOLUS VULGARIS* SEEDS

6-2.0 INTRODUCTION

When seeds of *Latuca sativa* or *Sinapis alba* were imbibed in tritiated water 4-aminobutyrate was found to be labelled. This has also been found to be so in the case of *Phaseolus vulgaris*(Section 2-2.1). In all three cases the labelling occurred in the first 30 minutes of imbibition. However, in experiments in which ^{14}C labelled compounds were fed to seeds of *Latuca sativa* [213] and *Phaseolus vulgaris* [69, 74, 76] during the first hours of imbibition, no mention was made of labelled 4-aminobutyrate being formed. Glutamate is widely believed to be the immediate precursor of 4-aminobutyrate and this was labelled in those

experiments. However, Vickers[104] showed that powders ground from resting *Sinapis alba* seeds produced labelled 4-aminobutyrate when soaked in solutions of ^{14}C-glutamate. This may mean that the labelled glutamate formed in the ^{14}C experiments on whole seeds of *Latuca sativa* and *Phaseolus vulgaris*, was produced in a compartment separate from that in which 4-aminobutyrate was formed. Alternatively, since ^{14}C-glutamate was never actually fed in those experiments there may not have been enough formed to be metabolised to a significant amount of 4-aminobutyrate.

As stated in Section 6-1 it is widely (but not universally) accepted that in plants 4-aminobutyrate is formed from glutamate and metabolised through succinate semialdehyde to succinate. If this pathway operates in *Phaseolus vulgaris* seeds during early germination, ^{14}C labelled Krebs cycle acids, especially succinate, should be formed if the seeds are imbibed in a solution of ^{14}C-4-aminobutyrate.

Experiments were therefore performed in which axes and pieces of cotyledon from *Phaseolus vulgaris* seeds were imbibed in solutions of ^{14}C-glutamate to find whether this was the precursor of 4-aminobutyrate, and in solutions of ^{14}C-4-aminobutyrate to determine to which compounds this was metabolised.

6-2.1 EXPERIMENTS WITH ^{14}C-GLUTAMATE

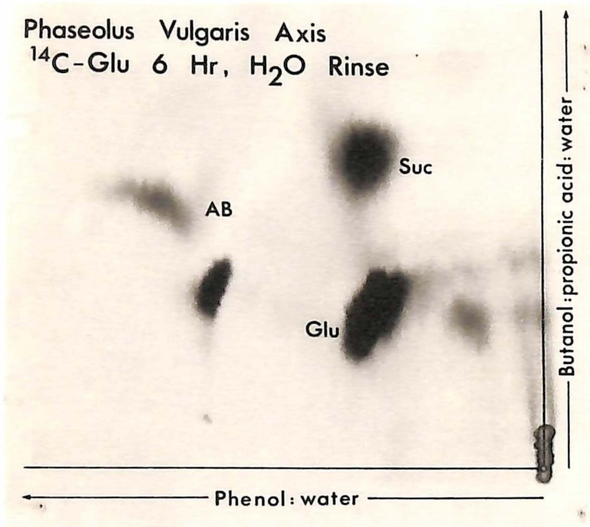
6-2.10 EXPERIMENTAL AND RESULTS

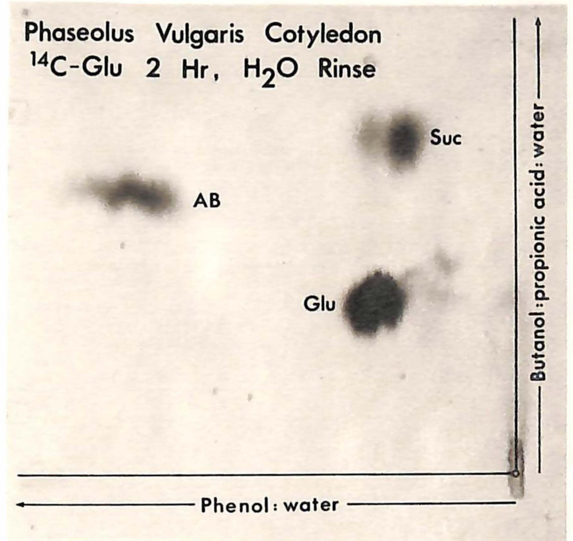
Universally labelled ^{14}C-glutamate of high specific activity (260 mCi/mmole) was purified as described in Section 1-4.9. Two axes were imbibed in approximately 0.05 ml of solution containing about 0.5 µCi of ^{14}C-glutamate. After two hours of imbibition at room temperature the axes were rinsed twice with distilled water and then extracted in the usual way. The extracts were chromatographed in the usual way and then exposed to film directly. Two pieces of cotyledon were treated similarly.

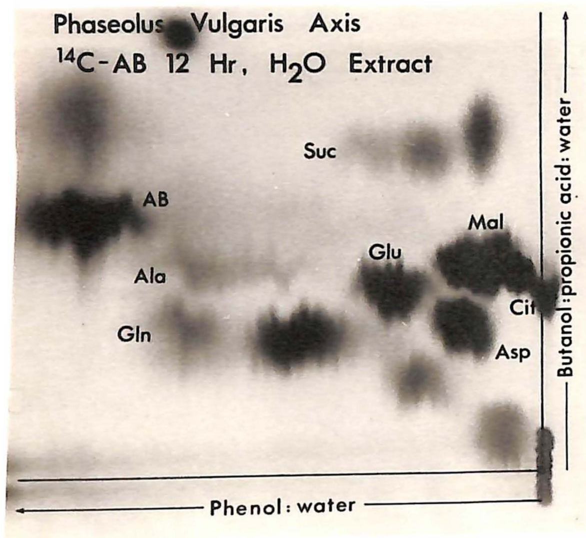
The only chromatogram that had more than traces of radioactivity, apart from glutamate, was the water rinse of the cotyledon. The radioautogram of this is shown in Plate 6.1. Apart from glutamate, 4-aminobutyrate and succinate were the only compounds strongly enough labelled to be identified.

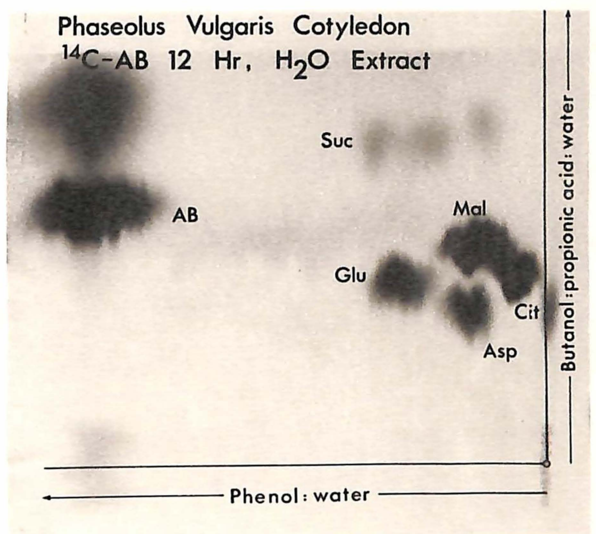
The labelling in the two hour axis experiment was so weak that a further experiment with an imbibition time of six hours was performed. This experiment was performed in the same way as before except for the longer imbibition time. The only well labelled chromatogram obtained in this experiment was that of the water rinse. The radioautogram of this is shown in Plate 6.1. The compound which had a lower R_f in both solvents than 4-aminobutyrate (and which can be seen

PLATE 6.1









below and to the right of 4-aminobutyrate on Plate 6.1) was co-chromatographed and found not to be alanine. Some other compounds in Plate 6.1 have not been named as they were too faint to be identified by co-chromatography.

6-2.11 DISCUSSION

These experiments show that both the axis and cotyledon metabolise glutamate to 4-aminobutyrate. Under the conditions of these experiments it appears to be the major compound formed. This is a similar result to that found for *Sinapis alba* by Vickers. [104]

Faintly labelled compounds were present on the chromatograms of all the extracts. While these were not identified, the patterns of labelling were similar to those found in the experiments with tritiated water. If these faintly labelled compounds were Krebs cycle acids or the amino acids associated with the Krebs cycle they could have been formed either by further metabolism of 4-aminobutyrate or by glutamate being metabolised through 2-oxoglutarate.

6-2.2 <u>EXPERIMENTS WITH (C-1) ^{14}C-4-AMINOBUTYRATE</u>

6-2.20 INTRODUCTION

The preceding experiments show that 4-aminobutyrate was formed from glutamate. The next step was to add ^{14}C-4-aminobutyrate to find its products of metabolism. The ^{14}C-4-aminobutyrate used was labelled in the C-1 position and had a specific activity of 1-5 mCi/mmole.

6-2.21 EXPERIMENTS AT ONE AND TWO HOURS.

Two axes were imbibed for one hour in about 0.05 ml of water containing approximately 1 μ Ci of ^{14}C-4-amino-butyrate. The experiment was performed in the usual way. Two pieces of cotyledon were treated similarly. Apart from 4-aminobutyrate and a diffuse spot which had an R_f value greater than 4-aminobutyrate in the butanol:propionic acid:water solvent, no other labelled compounds were formed. This diffuse spot was found in nearly all the ^{14}C-4-aminobutyrate experiments and its formation is discussed in Section 6-4.

The experiment was repeated with an imbibition time of two hours. The result was the same. Apart from 4-aminobutyrate and its associated diffuse spot there were no labelled compounds.

6-2.22 EXPERIMENT AT 12 HOURS

Two pieces of cotyledon and two axes were imbibed for 12 hours in the same manner as before. The water extracts of both the axis and cotyledon had traces of radioactivity corresponding to malate and citrate. However, the amount of radioactivity was so small that they could only just be perceived on the radioautograms. They could not therefore be identified with certainty.

6-2.3 EXPERIMENTS WITH UNIVERSALLY LABELLED 4-AMINOBUTYRATE

6-2.30 EXPERIMENTAL AND RESULTS

In an attempt to improve the sensitivity of the

previous experiments, analogous experiments were performed using universally labelled ^{14}C-4-aminobutyrate of high specific activity (204 mCi/mmole). Two axes were placed in 0.1 ml of water containing approximately 4 μCi of ^{14}C-4-aminobutyrate. They were left to imbibe for 12 hours at room temperature before rinsing, extraction, chromatography and radioautography in the usual way. Two pieces of cotyledon were treated similarly.

The radioautograms of the water extracts of both axis and cotyledon are shown in Plate 6.1. It must be stressed that the chromatograms were exposed to film for the very long time of two months. The amount of label transferred from 4-aminobutyrate was very small, but appears to be greater because of the long film exposure and because most of the 4-aminobutyrate was in the other two extracts. The labelling of compounds apart from 4-aminobutyrate and its breakdown spots was even weaker in the ethanol extracts and water rinses. However, so much ^{14}C-4- aminobutyrate was used in this experiment and the films were exposed for so long, that the label in 4-aminobutyrate and its breakdown spots blacked out half the radioautograms of the ethanol extracts and water rinses.

The labelled compounds named in Plate 6.1 were identified from either the axis or cotyledon chromatograms, except for the spots labelled succinate, alanine and glutamine. The identity of these was inferred from their

positions on the chromatograms as they were too weakly labelled to be identified by co-chromatography. The radioactive compound below and to the left of glutamate on the axis radioautogram had an R_f value similar to sucrose in both chromatography solvents, but was found not to be a sugar by the method used in Section 2-4.43. The small heavily labelled spot on the title of the axis radio-autogram is that of a marker strip. The trace amounts of activity for which a probable identity could not be advanced have been left unnamed.

6-2.31 DISCUSSION

The label in ^{14}C-4-aminobutyrate was transferred to a very small extent to a number of other compounds. The compounds labelled were the ones that would be expected if this label was entering the Krebs cycle. Thus citrate, malate, aspartate, glutamate and possibly succinate, alanine and glutamine were labelled. Since glutamate was moderately labelled and since there was only a trace of activity corresponding to succinate, it appears that 4-amino-butyrate was being metabolised to the Krebs cycle via glutamate and not succinate. Although, as explained in Section 1-6, it is widely accepted that the 4-aminobutyrate bypass proceeds only in the direction 2- oxoglutarate to succinate, it is believed by some authorities that the net flow through the pathway can be in the reverse direction. These present results suggest that in *Phaseolus vulgaris* seeds during early germination 4-aminobutyrate can only be metabolised by reversal of the reaction catalysed by

glutamate decarboxylase (4.1.1.15).

6-2.4 CONCLUSION

In Section 2-2 it was shown that 4-aminobutyrate was one of the first compounds to be labelled at the start of imbibition. This suggested that it might be important in early metabolism. The ^{14}C tracer experiments showed that it was formed in large amounts from glutamate, and they indicated that it could only be metabolised to other compounds via glutamate. Four statements can be made on the basis of this information.

1. The enzyme glutamate decarboxylase (4.1.1.15) is active in *Phaseolus vulgaris* seeds at an early stage of imbibition.

2. The reason for the formation of 4-aminobutyrate at such an early stage of germination remains unknown. The 4-aminobutyrate bypass is apparently responsible for the formation and removal of 4-aminobutyrate in seedlings (Section 6-1), but the complete pathway does not appear to be operating in the imbibing seeds of *Phaseolus vulgaris*. It is possible that a relatively large amount of 4-aminobutyrate was produced during the first 12 hours of imbibition because one or both of the enzymes of the bypass that catalyses its removal had not been formed at that stage.

3. In the case of *Phaseolus vulgaris* it seems likely that a pathway metabolising 4-aminobutyrate becomes active at some stage between 12 hours and seven days after the start of imbibition. The lower limit of 12 hours is based on the experiments described in the previous sections, and the upper limit of seven days was obtained from the results of Jones and Boulter.[71] These authors reported that 4-aminobutyrate was present in *Phaseolus vulgaris* seeds during the first day of imbibition but was not present in seven day old seedlings.

4. The activity of glutamate decarboxylase (4.1.1.15) in imbibing *Phaseolus vulgaris* seeds appears, from the ^{14}C-glutamate experiments, to be sufficient to account for the labelled 4-aminobutyrate that was formed in the tritiated water experiments reported in Section 2-2. It is therefore suggested that most, and possibly all, of the 4-aminobutyrate that was labelled with tritium in those experiments, was labelled in the reaction catalysed by this enzyme.

6-3 4-AMINOBUTYRATE METABOLISM IN BARLEY SEEDS

6-3.0 INTRODUCTION

Inatomi and Slaughter[135] carried out tracer experiments with ^{14}C-glutamate and ^{14}C-4-aminobutyrate on three day old barley seedlings. They concluded that glutamate was being metabolised through 4-aminobutyrate to succinate.

Their results are not tabulated and appear to be inconclusive in some respects. Their results clearly showed however, that 4-aminobutyrate was formed from glutamate. Not only was this indicated by their tracer experiments, but they also found that the enzyme which catalyses the reaction, glutamate decarboxylase (4.1.1.15), was present in the embryo of both the seedling and the resting seed. However, their results did not unequivocally support their further conclusion that 4-aminobutyrate was then metabolised to succinate. Nevertheless, bearing in mind the discussion in Section 6-1, this conclusion must still be regarded as the most likely possibility.

In the tritiated water experiments with the barley embryo (Section 3-2) there were trace amounts of label in 4-aminobutyrate after three and 15 minutes and it was more strongly labelled at two and six hours. It is evident from the results of Inatomi and Slaughter [135] that, as for *Phaseolus vulgaris* (Section 6-2.1) and *Sinapis alba* [104], this 4-aminobutyrate was formed from glutamate in the reaction catalysed by glutamate decarboxylase (4.1.1.15). In order to see whether this 4-aminobutyrate was further metabolised to succinate, tracer experiments were performed with ^{14}C-4-aminobutyrate.

6-3.1 <u>EXPERIMENTS AT SIX AND 12 HOURS</u>

6-3.10 EXPERIMENTAL

Three embryos were imbibed for six hours at room temperature in 0.05 ml of water containing 5 μCi of

universally labelled ^{14}C-4-aminobutyrate. The embryos were then extracted as usual and the extracts freeze-dried, chromatographed and radioautographed in the usual way. A second experiment was performed with an imbibition time of 12 hours.

6-3.11 RESULTS AND DISCUSSION

Because a relatively large amount of radioactive 4-aminobutyrate was used it gave an intensely labelled spot on the radioautograms, especially those of the water rinses. Associated with the 4-aminobutyrate spot were the usual spots which had R_f values greater and less than it in the butanol:propionic acid:water solvent. These spots appeared to be formed from non-metabolic breakdown of 4-aminobutyrate and this is discussed in Section 6-4. Apart from these spots there were no labelled compounds formed in either experiment.

These results indicate that in the first 12 hours of imbibition either no 4-aminobutyrate was being metabolised to succinate, or too little 4-aminobutyrate was being metabolised for any radioactive succinate to be detected by the radioautography method.

6-3.2 EXPERIMENTS AT SIX and 12 HOURS WITH TRITIUM MARKER

6-3.20 INTRODUCTION

The experiments reported in the previous section showed that if any 4-aminobutyrate was being metabolised to succinate the amount concerned was too small for any labelled succinate

to be detected by radioautography of the chromatograms. With this in mind, methods were sought of adapting the experiments so that radioactive compounds, particularly succinate, could be detected with greater sensitivity. Liquid scintillation counting is a very sensitive method of detecting low energy β particles like those emitted during radioactive decay of ^{14}C. A technique was developed in which the experiments described in Section 6-3.1 were adapted to the use of a scintillation counter. As far as the author is aware this technique has not previously been utilised.

In this technique, pieces of chromatography paper labelled with known tritiated compounds are counted for ^{14}C activity in a scintillation counter. Although both ^{3}H and ^{14}C emit β particles, the maximum energy of the ^{3}H β particle (0.018 MeV) is so much less than that of ^{14}C (0.155 MeV) that very low activities of ^{14}C can be detected by a scintillation counter in the presence of very high activities of ^{3}H. Scintillation counting of ^{14}C activity on pieces of chromatography paper has been shown to be independent of the orientation of the paper in the vial, even in the case when the sample does not dissolve in the scintillation mixture. [214] The method is qualitatively more sensitive than radioautography of chromatograms. Moses [215] stated that if, on a paper chromato-

gram, a ^{14}C radioactive spot is emitting 5000 β particles/ min/cm^2 from each surface of the paper, then it can be just detected after an exposure to film of one day. While exposure to film for longer periods of time would enable lower limits of activity to be detected, the method would still not be as sensitive as scintillation counting, which can detect emissions of only a few β particles/min above background.

Imbibition experiments with tritiated water were performed on various parts of four seeds in this thesis. In these experiments succinate was most heavily labelled in the barley embryo. This indicated that the succinate pool was probably larger in the barley embryo than in the other seed parts studied. If any succinate were being formed from 4-aminobutyrate then, in the presence of ^{14}C-4-aminobutyrate, it should be easier to detect the labelled succinate formed if there were a large succinate pool, since the larger the pool the greater the activity that would remain in it at any one time. It was partly for this reason that greater attempts were made to detect 4-aminobutyrate metabolism in the barley embryo than in the other seed parts studied. However, quite apart from this, the fact that when the barley embryo was imbibed in tritiated water a relatively large amount of labelled succinate was formed, was made use of in the experiments described below. These experiments depended on the fact that succinate

labelled with tritium and succinate labelled with ^{14}C had the same R_f values in paper chromatography. The same situation applied in the case of other compounds. If the extract from a ^{14}C-4-aminobutyrate tracer experiment were chromatographed with ^{3}H-succinate, any ^{14}C-succinate present would be found in the same spot on the chromatogram as the ^{3}H-succinate. If enough ^{3}H-succinate were used the position of the succinate spot could be easily identified after a few days of scintillation radioautography. The area of the chromatogram containing this succinate spot could then be excised, placed in a scintillation vial and counted for ^{14}C activity in a liquid scintillation counter. ^{3}H-succinate and other tritiated compounds were present in extracts from barley embryos that had been imbibed in tritiated water for six hours. These extracts were therefore used to supply the tritiated compounds needed as markers in the technique used in these experiments.

6-3.21 EXPERIMENTAL

The first steps of the experiments reported in Section 6-3.10 were repeated. Three embryos were imbibed for six hours at room temperature in 0.05 ml of water containing 5 µCi of universally labelled ^{14}C-4-aminobutyrate. They were then extracted and the extracts freeze-dried in the usual way. This sequence was repeated, except with an imbibition time of 12 hours, for three more embryos. The freeze-dried extracts were dissolved in the corresponding

extracts from an experiment in which embryos had been imbibed in tritiated water for six hours. Each combination of extracts was chromatographed in the usual solvents and the chromatograms were radioautographed in a scintillation bath. The normal pattern of labelling for the six hour tritiated water experiment (Table 3.1) was obtained, with intensely labelled areas superimposed on it, corresponding to 4-aminobutyrate and its breakdown products. The areas of the chromatograms corresponding to succinate, glutamate and the other tritiated spots were excised and suspended in scintillation vials containing the scintillation mixture described in Section 1-3. Each vial was counted for ^{14}C activity in a scintillation counter.

6-3.22 RESULTS AND DISCUSSION

It was found that apart from 4-aminobutyrate and its breakdown spots, none of the tritiated areas of the chromatograms contained any significant ^{14}C activity.

Clearly, no 4-aminobutyrate was being metabolised to succinate, glutamate or any of the other tritiated marker compounds in the first 12 hours of imbibition. This situation is different from that in the *Phaseolus vulgaris* axis where there was a small amount of 4-aminobutyrate metabolism.

6-3.3 CONCLUSION

It was shown in the two preceeding sections that 4-aminobutyrate is not metabolised to other compounds by the barley embryo during the first 12 hours of imbibition.

This is different from the situation in the *Phaseolus vulgaris* axis.

Inatomi and Slaughter [135] found that there was a continuous rise in the level of 4-aminobutyrate in the barley embryo until after 36 hours of imbibition, when it began to fall. The results of the experiments reported here indicate that this rise occurs because at that time there is no pathway to metabolism away the 4-aminobutyrate that is being formed. Inatomi and Slaughter also showed that 4-aminobutyrate was being metabolised three days after the start of imbibition. It is most likely therefore that the pathway metabolising 4-aminobutyrate in the barley seed begins to operate at the stage when the level of 4-aminobutyrate begins to fall, 36-48 hours after the start of imbibition.

A technique was developed for detecting qualitatively small amounts of particular ^{14}C labelled compounds on paper chromatograms, compounds that are too weakly labelled to be detected by radioautography. The technique relies on using, as a marker compound, the tritiated analogue of the ^{14}C labelled compound being sought.

6-4 BREAKDOWN OF 4-AMINOBUTYRATE DURING CHROMATOGRAPHY

The ^{14}C-4-aminobutyrate used in the experiments reported in Section 6-2 and 6-3 was purified before use by one dimensional chromatography with the solvent butanol: propionic acid:water 15:7:10. Small amounts of radioactive impurities were separated from it in this way. The strip

containing the ^{14}C-4-aminobutyrate was then eluted from the chromatogram and used for tracer experiments. In those experiments the extracts were chromatographed in two dimensions with phenol:water 100:29, followed by butanol:propionic acid:water 15:7:10. On the radioautograms of those chromatograms, there were large diffuse spots with R_f values both greater and less than that of 4-aminobutyrate in the solvent butanol:propionic acid:water, and with similar R_f values to 4-aminobutyrate in the phenol:water solvent. These compounds were not formed metabolically as they were produced even when purified samples of ^{14}C-4-aminobutyrate were chromatographed in the same solvent system. However, when purified samples of ^{14}C-4-aminobutyrate were chromatographed in two dimensions with pyridine:butanol:water 1:1:1 followed by butanol:propionic acid:water, these large diffuse radioactive spots were no longer formed (there were some traces of radioactivity from the 4-aminobutyrate spot but these were of much lower intensity than those being discussed). Since the diffuse spots were formed when the solvent pair phenol:water/butanol:propionic acid:water was used but not when the pair pyridine:butanol:water/butanol:propionic acid:water was used, they can only be due to the phenol:water solvent. While there is no evidence to indicate the type of reaction occurring between phenol and 4-aminobutyrate, the diffuse spots are referred to as breakdown spots for simplicity.

Phenol:water also caused a variable amount of breakdown of tritiated 4-aminobutyrate. When samples of tritiated 4-aminobutyrate were eluted from extract chromatograms and re-chromatographed in the same solvent pair (phenol:water/butanol:propionic acid:water) and then radioautographed, there was a diffuse spot which had a high R_f value in both solvents. The activities of the excised areas of the chromatograms were measured in a liquid scintillation counter and it was found that this diffuse spot always contained less than 20% of the activity of the 4-aminobutyrate spot. Much of the 4-aminobutyrate was extracted in the ethanol extracts during the imbibition experiments with tritiated water. In the chromatograms of these extracts the compounds which had R_f values of 0.3 - 1.0 in both solvents were presumed to be lipids. Most of the breakdown spot from tritiated 4-aminobutyrate would also have been designated as lipids. However, in the tritiated water experiments, labelled 4-aminobutyrate was also present in the water rinse extracts. When a comparison was made in both water rinse and ethanol extract chromatograms, of the activity of the 4- aminobutyrate spot compared to that of the areas that contained compounds with high R_f values in both solvents, it was apparent that this breakdown spot accounted for less than half the lipid count in all cases. Obviously any detailed investigation of lipid labelling would have to use a different method for separating lipids from 4-aminobutyrate.

An attempt was made to use the pyridine:butanol: water solvent instead of phenol:water for the chromatography of extracts from both ^{14}C and tritiated water experiments. However, this solvent coupled with butanol:propionic acid: water did not completely separate the labelled compounds formed. No other solvent pair that was investigated gave satisfactory separation. Since the ^{14}C-4-aminobutyrate was not metabolised to any extent, and since the lipid fraction was not being investigated, there was no necessity to use a different technique such as thin layer chromatography. Accordingly, paper chromatography with the solvent pair phenol:water/butanol:propionic acid:water continued to be used for the investigation of all extracts.

SUMMARY

Previous workers had shown that the tritiated water technique could be used to study the metabolism of seeds during the first minutes and hours of imbibition. These studies had been confined almost entirely to the intact seed of *Sinapis alba*, although it had also been shown that the same labelled compounds were formed in the intact seed of *Latuca sativa*. Both species belong to the dicotyledon class of the angiosperms. It had been planned for this present work to use the tritiated water technique to study the metabolism of seeds from species that are widely separated from an evolutionary point of view, and to combine this with a study of metabolism in different parts of four

seeds that were representative of the main types of seeds among the seed bearing plants. Both these aims have been accomplished by studying the metabolism of parts of four seeds that are widely separated on embryological and evolutionary criteria. Some ^{14}C tracer experiments were performed on two of these seeds, and these provided additional information about the metabolism of some compounds.

The four species chosen and the types of seeds represented were: *Phaseolus vulgaris*, a dicotyledon whose storage organs are the cotyledons and whose main storage material is starch; barley (*Hordeum vulgare*), a monocotyledon whose storage organ is the endosperm and whose main storage material is starch; castor oil (*Ricinus communis*), a dicotyledon whose storage organ is the endosperm and whose main storage materials are lipids; and *Pinus radiata*, a gymnosperm seed whose storage organ is the female gametophyte and whose main storage materials are lipids. In the case of all four seeds, experiments were performed at various times on samples of two or three embryos or axes (embryos minus their cotyledons; axes were used in the case of *Phaseolus vulgaris* and castor oil because their cotyledons are relatively large), and on excised pieces of the storage organ. In the case of the castor oil seed experiments were also performed on pieces of cotyledon.

The most notable result of these experiments is the similarity in the compounds labelled and the times at which they became labelled. This similarity is particularly marked in the experiments performed on the four embryos or axes. In all four cases the three amino acids, aspartate, glutamate and alanine were labelled in at least trace amounts, during the first five minutes of imbibition; citrate and malate were labelled during the first 30 minutes; and sucrose and glutamine were labelled after two hours. Other workers found that a similar metabolic development occurred in intact *Sinapis alba* seeds, but the timing of changes was different from that of the four embryos or axes discussed here. This similarity in labelling among embryos or axes from four very different seeds is a good indication that the embryos and axes of all seeds have a common metabolic development during imbibition. This development begins with amino acid metabolism, and this is joined by metabolism of Krebs cycle acids, and eventually glutamine and sucrose metabolism. The three amino acids, glutamate, aspartate and alanine, are all closely related to the Krebs cycle through their corresponding 2-oxo acids. The enzymes (transaminases and deaminases) that interconvert these amino and 2-oxo acids are present in resting seeds, and would lead to labelled amino acids (but not labelled 2-oxo acids) being formed in the presence of tritiated water. It is suggested that the three amino acids became labelled by the action of transaminases (acting in concert with a

deaminase), and that the presence of these labelled amino acids in seeds during early imbibition was an indication that their corresponding 2-oxo acids were present.

It is suggested that the presence of labelled Krebs cycle acids was due to the action of Krebs cycle enzymes. As well as citrate and malate, there was at least a trace of activity that (on the bases of its R_f values during chromatography) could have been succinate in all four embryos or axes during the first two hours of imbibition. It is concluded from the labelling of these acids, together with the labelling of the three amino acids whose corresponding 2-oxo acids are members of, or closely related to, the Krebs cycle, that the Krebs cycle was beginning to operate in all four embryos or axes during the first two hours of imbibition. This conclusion accords with the results of ^{14}C tracer experiments performed both in this present work and by other workers. The modified Krebs cycle, known as the glyoxylate cycle, which is the other pathway that might also have accounted for the labelling of most of the same compounds as the Krebs cycle, could be excluded as a possibility on the basis of the results of other workers.

The labelling of sucrose indicated that sugar metabolism was occurring during the first two hours of imbibition. It was shown that both the glucose and fructose moieties of sucrose were labelled and a mechanism is proposed whereby this labelling could have occurred.

A number of other experiments were performed on parts of *Phaseolus vulgaris* and barley seeds to further investigate the formation of sucrose. The results of these experiments indicated that sucrose was not being formed in one part of the axis so that it could be transported to another part, and that it was not being formed from reversedglycolysis. It is concluded that it was probably being formed from starch, although the possibility that it was being formed from other sugars cannot be excluded. No satisfactory reason can be advanced to explain why it should be formed in the embryo at that stage. In performing this investigation of sucrose metabolism, it was found that the same labelled compounds were formed in both parts (the plumule and the hypocotyl) of the *Phaseolus vulgaris* axis when they imbibed tritiated water. It thus appears that the same metabolic development occurs throughout the axis.

It is suggested that the labelled glutamine was formed from labelled glutamate, mainly because of the presence of the latter in all cases in which labelled glutamine was formed.

As well as the compounds that have been mentioned above, 4-aminobutyrate, lactate, and groups of compounds designated as lipids and sugar phosphates were labelled in many cases. One of the first compounds to be labelled in the embryos or axes of three of the four seeds studied was 4-aminobutyrate, but in the case of the *Pinus radiata* embryo it was only labelled after two hours of imbibition and was never strongly enough labelled to be identified by co-chromatography.

Experiments were performed on *Phaseolus vulgaris* and barley embryos with ^{14}C tracer compounds to gain information about the metabolic pathways by which this compound was formed and removed. These experiments indicated that it was formed from glutamate but that it was not readily metabolised to other compounds. In the *Phaseolus vulgaris* axis and cotyledon the small amount that was metabolised was apparently metabolised through glutamate, while in the barley embryo (as in the *Sinapis alba* seed studied by another worker) there was apparently no pathway by which it could be removed. Both these results are at variance with the results obtained by other workers on seedlings of these and other species. It is therefore concluded that 4-aminobutyrate is formed in relatively large amounts in seeds during early imbibition because at that stage no pathway for its removal is operating. Since such a pathway (the 4-aminobutyrate bypass) is present at the seedling stage, this pathway must develop at a late stage of germination or during seedling growth.

Labelled lactate was formed mainly in the experiments on the *Phaseolus vulgaris* axis and cotyledon, and the barley embryo; and the intensity with which it was labelled varied greatly from one experiment to another. It is suggested that the amount of labelled lactate formed in a particular tissue sample reflected the degree of anaerobic metabolism, but that even in the case where

lactate was strongly labelled the metabolism was still largely aerobic. These suggestions are consistent with the results of other workers who showed that the amount of water in which a seed is imbibed is a critical factor in determining the degree of anaerobiosis in the seed, and that the degree of anaerobiosis is associated with the amount of lactate formed.

The compounds designated as lipids and sugar phosphates were never separated into their individual constituents or identified by co-chromatography, but the intensities of labelling in these groups of compounds were tabulated for completeness. The lipid fraction was labelled in all four embryos or axes during the first two hours of imbibition. It is suggested that the intensity of labelling in this fraction was an indication of the amount of lipid synthesis occurring. It is noted that the intensity of labelling in the sugar phosphates usually varied with the intensity of labelling in sucrose, indicating that the labelled sugar phosphates were possibly intermediates of sucrose synthesis. Further investigations of germinating seeds using the tritiated water technique, could be profitably directed towards finding methods to separate and identify the individual consitituents of these two fractions.

In the storage organs of the four seeds studied, there were no other labelled compounds formed than those found in the embryos and axes. The metabolic development of the

storage organs was, to a greater or lesser extent, slower than that of their corresponding embryos or axes, and the storage organs did not exhibit as similar a metabolic development among themselves as the embryos and axes did. This may, in the first 1-2 hours of imbibition, have been due to their slower water absorption. In each of the four seeds, the storage organ absorbed water at a slower rate than its corresponding embryo or axis, and its metabolism also developed more slowly. However, the metabolic development after 1-2 hours did not appear to be associated with the water content of the seed part, as the water content of all parts of the seeds reached a relatively constant level while the metabolism of the storage organs continued to become more complex. The metabolic development observed for each storage organ in relation to both its corresponding embryo or axis and also to the storage organs of other seeds is summarised below.

The storage organs in the *Phaseolus vulgaris* seed are the cotyledons, which are part of the embryo. While the metabolic development of the cotyledon was slower than that of the axis it followed a very similar pattern, and after six hours the same compounds were labelled in both the axis and cotyledon.

The storage organ in the barley seed is the endosperm, and the metabolic development of this was much slower than that of the embryo. It was shown that the metabolism of the endosperm was due entirely to a surface layer of cells

the aleurone layer. After 12 hours, the metabolism of the endosperm had become similar to that in the embryo between 15-120 minutes. It is concluded that this apparently very slow metabolic development of the endosperm was at least in part caused by the presence of only very small amounts of actively metabolising aleurone tissue in the endosperm samples. Labelled sucrose was never formed in the endosperm, although it was found in all the other seed tissues investigated. This is consistent with the fact that although sucrose is the sugar most commonly translocated in plants, glucose and not sucrose is the sugar translocated from the endosperm to the embryo in monocotyledon seeds.

The storage organ of the castor oil seed is the endosperm. Its metabolism developed at a slower rate than that of the castor oil axis but after six hours the same compounds, apart from the lipid fraction, were labelled in both parts. The metabolic development of the castor oil endosperm was much more similar to that of the *Phaseolus vulgaris* cotyledon than to that of the barley endosperm. However, from an embryological point of view the two endosperms have similar origins, and the cotyledon is formed in a totally different way. It is clear, therefore, that the metabolic development of the storage organs is not determined by their embryological origin. The metabolism of the castor oil cotyledon was different from that of the castor oil axis and endosperm. The development of metabolism was slower in the cotyledon and varied in several respects from that of the other two tissues. The patterns of

labelling of the castor oil cotyledon were also different from those of the *Phaseolus vulgaris* cotyledon. Since the cotyledons of these two seeds have a similar embryological origin, their differences in metabolism must result from the differences in their morphology and function.

The storage organ of the *Pinus radiata* seed is the female gametophyte. This had a very similar metabolism to that of the *Pinus radiata* embryo, and only at one of the four times studied was there any difference in the patterns of labelling of the two organs. The lipid fraction and 4-aminobutyrate were only labelled in trace amounts in the female gametophyte, but apart from that its metabolism after six hours was similar to that in the *Phaseolus vulgaris* cotyledon and the castor oil endosperm. These three storage organs have different embryological origins; two of them contain mainly lipid reserves, and the other mainly starch reserves; and one of them eventually carries out photosynthesis at the seedling stage, whereas the other two do not. Despite the differences in embryology and function of these two storage organs, there were many similarities in their metabolic development. There were also many similarities between their metabolism and that of the embryos and axes. Of the seed parts studied in this thesis, those which presented the most different patterns of labelling were the barley endosperm and castor oil cotyledon. Even for these organs the

differences could be fairly ascribed to their slower metabolic development, as after 12 and six hours respectively, their patterns of labelling had much in common with those of the other organs studied. Thus, despite the many differences in metabolic development of organs from the same and different seeds, there are many general similarities in the metabolic development of organs from all seeds.

In this thesis the tritiated water technique was used to study the metabolism of excised parts of seeds. To justify the use of excised parts, experiments were also performed, in the case of *Phaseolus vulgaris,* on whole seeds and on an axis attached to a piece of cotyledon. These experiments showed that although the sequence of changes is similar in both excised parts and parts of intact seeds, the changes occur at a slower rate in parts of intact seeds. It is therefore concluded that the times shown for when various compounds first become labelled in excised parts are somewhat shorter than those in parts of intact germinating seeds. These experiments also showed that most of the faster metabolic development of the excised parts, as compared to parts in the intact seed, was due to the absence of the seed-coat and not the separation of the axis from the cotyledon. However, the most interesting result of the experiments with intact seeds was the variation in water absorption over the surface of the seed that was observed. In one experiment the tritiated water was not absorbed uniformly, while in another experiment it apparently was. In the case where the water was absorbed non-uniformly,

the route by which the water entered could be deduced by a comparison of the patterns of labelling formed by different parts of the seed. It is therefore suggested that, when performed in conjunction with experiments on excised parts, experiments on intact seeds have a valuable potential for the study of water movement into seeds during imbibition.

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