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# AN EXPERIMENTAL STUDY OF METABOLISM DURING ANHYDROBIOSIS

A thesis
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MURRAY VICKERS

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## ABSTRACT

Certain organisms can survive desiccation. Examples are seeds, pollen and spores of plants, lichens, the eggs of many insects, many rotifers and tardigrades and certain nematodes. The term *anhydrobiosis* has been used to describe the state of such organisms when desiccated. In this thesis anhydrobiosis is defined as 'the maintenance of the living condition at low water activity', using the word *activity* in the thermodynamic sense. The term *resting metabolism* is proposed to describe any metabolism occurring during anhydrobiosis. The literature on anhydrobiosis and resting metabolism is reviewed.

The aim of this work has been to study anhydrobiosis. This has required the development of an acceptable technique to study resting metabolism and the evaluation of the technique by the study of a selection of anhydrobiotic organisms.

The experimental difficulties associated with studying resting metabolism are discussed. Clearly the addition of any solvent to an anhydrobiotic organism will disturb anhydrobiosis, and hence classical biochemical techniques cannot be used. The requirements of a technique that would not disturb the organism are defined.

A novel technique for studying the resting metabolism of any anhydrobiotic system is described and results from experiments on the resting metabolism of various seeds, pollen and spores are given. It is shown that complex metabolism (involving amino acids, organic acids, sugars and sugar phosphates) occurs above 64% relative humidity. However, these results could be complicated by active fungal growth

that may occur above 70% relative humidity. Consequently the experimental work concentrates on metabolism occurring at 64% and four other lower relative humidities to a minimum of 15%.

This new technique is a development of a method previously used to study the germination metabolism of seeds. The essential principle of the technique is the exposure of the organism to tritiated water vapour. This labels the water and other components containing exchangeable hydrogen in the organism. Since many biochemical reactions proceed with the incorporation of a non-exchangeable hydrogen atom, the detection of a tritium label in a compound indicates that it must have been involved in a chemical or biochemical reaction. If, however, a metabolite is found not to be tritium labelled then this can be evidence that certain metabolic pathways involving this particular metabolic pool are not operating.

An exposure chamber is described in which biological specimens can be exposed to tritiated water vapour at constant relative humidity. After several days exposure to tritiated water vapour the specimen is extracted and the metabolites that have incorporated tritium are detected and identified. This technique has the advantage of detecting *in vivo* metabolism and does not alter the resting state of the organism.

The exposure of plant propagules to tritiated water vapour is shown not to destroy their viability. This was established for samples of *Pinus ponderosa* pollen, *Allium cepa* and *Sinapis alba* seeds, and the spores of *Cyathea dealbata*. In all cases germination proceeds but at a slower rate than with unexposed propagules. Control experiments show that the tritium activity detected is due to metabolic processes and not due to non-physiological chemical activity.

The incorporation of tritium into metabolites by the resting propagules is slight. An investigation into the chromatographic and scintillation autographic techniques which would allow maximum

sensitivity of detection is described. Thin layer electrophoresis followed by chromatography in a second direction is found to be particularly suited to this work. An attempt to develop low temperature liquid scintillation autography is discussed. It is contended that this shows promise of very great sensitivity.

Results obtained by the technique show that between 45% and 64% relative humidity the resting metabolism of all propagules studied involves mainly amino acid metabolism with varying amounts of organic acid metabolism. It is suggested that amino acids become labelled by low level transaminations and/or deaminations occurring within the dry propagule. Also the tritium labelling of citric, malic, succinic and possibly fumaric acids may indicate the operation of at least parts of the tricarboxylic acid cycle in the dry propagules at relative humidities of 45% and above.

The extent of tritium labelling at 34% is much reduced. In the cases of *Pinus ponderosa* pollen and *Allium cepa* seeds no tritium labelling that can be detected by scintillation autography occurs at all. Other propagules studied at this low relative humidity label only one compound except in the case of *Phormium tenax* pollen which incorporates traces of tritium into two amino acids. No propagule will incorporate tritium detectable by scintillation autography at 15% relative humidity.

Scintillation counting of lipids extracted from the propagules shows that small amounts of tritium are incorporated into these compounds by all propagules at all relative humidities. Evidence is adduced to suggest that this incorporation is due to non-metabolic processes.

Tritium labelling of water soluble macromolecules is negligible. This suggests that there is little synthesis of such compounds in

resting propagules at 64% relative humidity and below.

The solid residue left after extraction is always found to be tritium labelled. Further examination suggests that the label in the solid residue is acquired by non-metabolic means.

Scintillation counting of extracts containing small molecule water soluble metabolites implies that these contain varying amounts of semi-labile tritium. It is contended that this is lost during chromatography and that chromatography is a very effective means of decontaminating the extracts of semi-labile tritium. Results from further experiments support this contention.

Sucrose is a carbohydrate often found to be tritium labelled in experiments with germinating propagules in tritiated water. Attempts to detect tritium labelling of sucrose in *Pinus ponderosa* pollen after exposure to tritiated water vapour are described but only traces of label are found. This does not unequivocally eliminate the possibility of carbohydrate metabolism in the resting pollen but does indicate that it is less comprehensive than in germination.

The techniques developed by the author have since been used by other workers to study the resting metabolism of *Pithomyces chartarum* spores and of two examples of lichens in some detail. These results, together with the author's work on eleven species of pollen, four species of seed, one species of fern spore and one of fungal spore, show a striking similarity in the resting metabolism of diverse anhydrobiotic systems.

It is concluded as a result of this work that there is considerable metabolic activity at surprisingly low relative humidities (that is, low water activities). It is proposed that resting metabolism involves a branch of biochemistry dealing with metabolism at low water activity and consequently solutions of high concentration. Presumably only certain types of metabolic activity are possible in the very concentrated

solutions existing in an anhydrobiotic system. It appears that this metabolic activity involves amino acids and organic acids. This hypothesis would predict that the reactions taking place in all anhydrobiotic systems are similar and this proved to be the case for all organisms studied. It will be interesting to determine the nature of resting metabolism of animal anhydrobiotic systems, but time did not permit such studies.

Experiments on extracts from *Pinus ponderosa* pollen show that tritium labelling does occur at 34% relative humidity but to such a small extent that it could not be detected by scintillation autography. It is suggested that even at this low water activity enzymes in the pollen have not been rendered inoperative but that labelling has been greatly attenuated by the extremely high viscosity of the intracellular fluid. This could mean that diffusion of substrates and products becomes rate limiting. It is suggested that a glass-like state of extremely high viscosity may exist within the cell at 34% relative humidity and below.

Preliminary experiments indicate that there may be a critical relative humidity between 64% and 75% where the comparatively simple metabolism of intermediate relative humidities become much more complicated. It may be significant that it is in this region of relative humidity that most biochemical substances deliquesce.

It is suggested that some enzymes will operate in much more concentrated solution than others. As relative humidity increases more enzymes become active. The implications this may have for the nature of enzymes in anhydrobiotic systems is considered.

Suggestions are made for the application of the newly developed technique to various other biological problems.

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| | |
|---|---|
| ala | alanine |
| asp | aspartic acid |
| glu | glutamic acid |
| gaba | 4-aminobutyric acid |
| gly | glycine |
| lac | lactic acid |
| mal | malic acid |
| suc | succinic acid |
| sucr | sucrose |
| TLC | thin layer chromatography |
| TLE | thin layer electrophoresis |
| MCW | methanol, chloroform, water extraction solvent. |

# CHAPTER 1 INTRODUCTION

## 1-1 Occurrence of Organisms that can Survive Desiccation

### 1-1.1 Description

Since the beginning of civilization mankind has relied upon the ability of seeds to survive desiccation. In fact the occurrence of civilization itself was probably totally dependent upon the implicit recognition of this remarkable faculty of seeds, in allowing the cultivation of crop plants.

The ability of seeds to survive desiccation is shared with other types of organism. Pollen (Stanley and Linskens, 1972), and the spores of ferns and fungi (Smith, 1955) are, like seeds (Roberts, 1972), specialized plant structures developed to survive a period of desiccation during which time the structure is propagated. Hence they are referred to collectively as plant propagules (Scagel *et al.*, 1969). In the case of lichens the entire organism can survive long periods of desiccation (Hale, 1974). In the animal kingdom it is found that many adult rotifers and tardigrades (microscopic aquatic organisms) and certain nematodes can survive desiccation, as can the eggs of many insects (Borradaile *et al.*, 1963).

### 1-1.2 A Necessary Adaptation

This capacity to survive desiccation is an integral part of the adaptive features of the above diverse range of organisms and is usually essential for the survival of each organism in its habitat. Plant propagules usually have to remain dormant during a period of transportation, and often remain dormant until suitable environmental conditions pertain. For example, many types of seed remain dormant in the ground

throughout winter and only germinate in the more favourable conditions of spring (Crocker and Barton, 1957). Lichens are believed to have a very important role in colonizing barren terrestrial environments (Hale, 1974). Many lichens live in very exposed areas and are capable of experiencing long periods of desiccation. They have obviously evolved very effective mechanisms for surviving these and yet readily resume their growth when water becomes available.

Rotifers and tardigrades usually inhabit freshwater pools that are susceptible to drying out during periods of drought. These organisms have to survive these periods until rainwater replenishes the pools (Borradaile *et al.*, 1973). This adaptation is shared by certain nematodes (*ibid.*) living in environments prone to desiccation.

The ability of many insect eggs to remain dormant during periods of low relative humidity is of considerable importance. In most cases this has proved harmful to mankind as, for example, in the spoilage of certain foodstuffs during storage and in the continued survival of certain pests such as borer, fleas and cockroaches in the domestic environment.

### 1-1.3 Question of the Status of these Organisms

Despite the obvious commercial importance to man, the question of the biological status of these organisms has received remarkably little attention. Many early biologists regarded these desiccated organisms as being in an abiotic state waiting to be revived or resuscitated by an increase in water content. This is not particularly remarkable when one considers the great difference between the conditions existing in the desiccated organism and those in a normally metabolising organism. Some biologists even had difficulty in considering the desiccated organism as a living one. The term *anabiosis* has been widely used to describe what Preyer (1891) refers to as a state of 'viable lifelessness' such as existing in dried organisms that survive

desiccation. More recently the term *anhydrobiosis*, initially introduced by Giard (1894), has gained general acceptance to describe this state. In the next section this phenomenon is discussed in some detail.

## 1-2 Anhydrobiosis

### 1-2.1 Definition

The term *anhydrobiosis* is now used to describe the biological status of dried organisms that survive desiccation. The origin of this term is discussed below. Anhydrobiosis has been defined as 'latent life induced by the removal of water from the living system' (Crowe, 1971). In the author's opinion this definition is not precise enough as these organisms, while they may not contain liquid water, do contain loosely bound water. For instance, wheat seeds stored at 50% relative humidity contain approximately 12% moisture at 25 C (Greer, 1953). Consequently it is suggested a more precise definition would be 'maintenance of the living condition at low water activity'. (The term activity, unless qualified, is used in this thesis in the thermodynamic sense.)

It is suggested that the term 'latent life' is a rather abstract one for a practical biological definition. The term 'water activity' refers to a measurable quantity which reflects the degree of desiccation of the organism. In other words the desiccated *anhydrobiote* may be regarded as containing very concentrated solutions, i.e., the activity of the water (its chemical potential) is considerably lower than in the dilute solutions encountered in 'normal' biological systems. For most non-anhydrobiotic organisms it is essential that the water content is kept relatively high and these organisms have evolved many diverse systems to achieve this.

In the case of most animals we can regard the situation pertaining in blood as typical. Here the water activity is very nearly that of

pure water. In terrestrial plants there may be greater fluctuations but it is unlikely, even during periods of extreme water stress, that the water activity in a plant departs much from that of pure water. The situation in desiccated anhydrobiotes is radically different. This difference must in part explain the lack of research on the matter. Most classical biochemical techniques rely on dilute solutions of metabolites and cell extracts. The lack of techniques available must have discouraged research on anhydrobiotes as they have such a low water content. The question of water activity in dry anhydrobiotes is discussed later in Section 2-6.

1-2.2 Nomenclature

The term anhydrobiosis was introduced by Giard (1894) to describe the biological processes occurring within an organism during desiccation. More recently Keilin (1959) proposed the term *cryptobiosis* to cover any condition where the metabolism of an organism is greatly reduced or even suspended entirely by the external conditions. Such conditions include as well as desiccation, low temperatures (cryobiosis), low oxygen concentrations (anoxybiosis) and high salt concentrations (osmobiosis). It can be seen that the last condition of high salt concentration will usually occur internally as a consequence of anhydrobiosis.

Other terms have been used which include the condition of anhydrobiosis. These are *anabiosis* (Preyer, 1891), *abiosis* (Schmidt, 1948) and *hypobiosis* (Monterosso, 1934). All these terms have been used in the past with varying degrees of precision and may well appear in future publications. Because of the unclear status of the anhydrobiotic organisms a researcher into this field must be aware of the associated confusion of the nomenclature. Hopefully this confusion will have been largely dissipated since the publication in 1973 of *Anhydrobiosis* (Crowe and Clegg, 1973).

This thesis sets out to develop an experimental technique to study what biochemical processes are occurring in a dry anhydrobiotic organism and to determine how this is affected by relative humidity. The term 'resting metabolism' is proposed to describe any metabolic activity occurring during any crytobiotic state, including anhydrobiosis.

1-2.3 General

This thesis is concerned with anhydrobiosis in plant propagules i.e., seeds, pollens and spores. More particularly it is an attempt to elucidate with a novel technique, the basic metabolic activities of desiccated plant propagules. While this may be of immediate interest with regard to longevity of propagules and the cause of their ultimate loss of viability, there is also the more general issue of metabolic processes at low water activity to be considered.

In the following discussion anhydrobiosis in seeds will be reviewed and discussed. Also anhydrobiosis in organisms such as rotifers and tardigrades not studied by the techniques described in this thesis, but nevertheless relevant, will be considered.

The metabolic status of a dried seed was not questioned for a long time. It is only recently that researchers have considered the question of what happens in a seed during dry storage. The question has been asked of animal anhydrobiotes (e.g., rotifers, tardigrades, etc.) ever since they were first discovered by Leeuwenhoek in 1702.

So remarkable was the discovery of these animals which could survive desiccation for long periods that there was initial disbelief in their very existence. However the fact that higher plants produced seed which could perform the same feat was accepted without reserve. This is understandable in that human familiarity with seeds dates back to before the beginning of civilization.

The discovery of animal anhydrobiotes had to await the invention of the microscope. Once this had happened the question of biological

processes in the desiccated state became the source of much debate. But for a long time, while controversy raged about animal anhydrobiosis, the question of anhydrobiosis in seeds remained untouched. There now follows a brief account (largely of Keilin, 1959) of the controversy surrounding animal anhydrobiotes both for its own intrinsic interest and because it raises questions equally applicable to any anhydrobiotes including plant propagules.

1-2.4 Historical

The study of anhydrobiosis, it has been suggested (Keilin, 1959), began when Leeuwenhoek used his primitive microscope to demonstrate the revival of desiccated rotifers. This work, described by Leeuwenhoek in a letter entitled 'On certain animicules found in the sediment of gutters of roofs of houses' (1702), failed to attract the notice of his contemporaries and it was not until forty years later that his work was followed up. J.T. Needham (1743) and later H. Baker (1753) studied the revival of desiccated microscopic eelworms.

The phenomenon of the revival of desiccated organisms was so extraordinary that the observations of Needham and Baker were treated with skepticism. Spallanzani (1769), one of the foremost biologists of the time, proposed that what were being studied were not organisms but dried vegetable fibres which swelled and twisted when soaked in water.

This rejection of the observations of Needham and Baker, for which Spallanzani had the support of the majority of the scientific thinkers of the time, illustrates the difficulty early biologists had in accepting the existence of animals that could survive desiccation. It is interesting to note that no one drew an analogy with seeds.

Later Spallanzani (1803) reversed his earlier views and studied the phenomenon extensively. In a paper entitled 'Observations and experiments on some singular animals that can be killed and revived' he expounded the view that these animals upon desiccation suffer death and

can be later resurrected by the addition of water. This rather extravagant viewpoint could be seen as an over-correction of his first opinions of the phenomenon.

Spallanzani further demonstrated that desiccated organisms proved much more resistant to high and low temperatures than did non-desiccated organisms. This study was further advanced by Doyére (1842) and others.

Ehrenberg (1838) took issue with the prevailing view of 'death and resurrection' and claimed instead that the organisms in question merely had their metabolism greatly reduced by desiccation. However, he further claimed that rotifer still feed and reproduce while in a desiccated condition, in spite of careful experiments by others, results of which contradicted this view.

Between 1858 and 1860 a new debate developed in France about the very existence of desiccation-resistant animals. Pouchet rejected the idea as it threw into question his views on spontaneous generation. The Biological Society of France appointed a commission whose task was to resolve the matter. The report of the commission prepared by Broca (1860) concluded that the existence of anhydrobiotic animals was proved beyond doubt. This important result was somewhat overshadowed at the time by the Darwin-Wallace theory of evolution in England and the continuing debate on spontaneous generation in France — though the last issue, as already indicated, was intimately linked with the resuscitation of desiccated organisms.

So by 1860 the existence of desiccation-resistant organisms was unequivocally accepted by the scientific world, though the issue of what happened to the organisms during desiccation and rehydration had not even begun to be resolved. It has only been recently, with the availability of modern techniques, that these problems have been studied in earnest.

## 1-2.5 The Origin of Life

It has recently been suggested that anhydrobiosis is implicated in the origin of life (Hinton, 1968). According to this hypothesis primeval organisms originally developed in environments such as shore line rock pools. This type of environment would be prone to desiccation. This would also allow a concentration of molecules, a process presumably favourable to cell formation. Any primeval cells formed in such a process may well then have to survive a period of complete desiccation. So it is suggested that the ability to survive desiccation was a fundamental requirement of the first life forms. As more complicated organisms developed so too did the ability to invade the marine environment where desiccation was not a problem. So the ability to survive desiccation was lost by most organisms. When organisms began to colonize the land, new adaptations dealt with the problem of the non-aquatic environment by preventing the loss of water.

This theory relies on the assumption that life originated in isolated marine rock pools, or something similar. This idea does not enjoy much support at the moment (Bernal, 1967). In particular it is objected that such a scenario would lead to many origins in many different pools, giving rise to many fundamentally different forms and that the process could still be observed today. In answer to this it can be said that once a biological entity was created and started to reproduce it would immediately alter the environment to make another origin much less likely. Further, even if a number of different origins occurred in the past then we can simply say what resulted from this profusion of primeval organisms was determined by natural selection. As a third point it may simply be that not all that many possibilities exist as far as fundamentally different life forms are concerned. Also no theory on the origin of life has particularly compelling arguments in its favour, so Hinton's proposal that anhydrobiosis was involved in the

origin of life is still worthy of consideration.

## 1-3 Longevity of Seeds and Other Anhydrobiotic Organisms

### 1-3.1 Reported Claims

It appears that among anhydrobiotes, seeds are the best adapted to surviving long periods of dry storage. However, it is found that some seeds are very much better adapted than others to survive such storage. In fact, seeds can be roughly divided into three categories on this basis: (i) macrobiotic seeds — which remain viable under suitable storage conditions for 15 years or more, (ii) mesobiotic seeds — which can be stored for 3 to 15 years, (iii) microbiotic seeds — which lose viability after less than three years storage (Ewart, 1908). Common examples of microbiotic seeds are *Alium cepa* L (onion), *Pastinace sativa* (parsnip), *Lactuca sativa* L (lettuce), *Capsicum frutescens* (pepper), *Brassica oleracea* (cauliflower). Mesobiotic seeds include most cereal crops, e.g., *Triticum vulgare* (wheat), *Hordeum vulgare* (barley), *Sinapis spp* (mustard), *Secale cereale* (rye) and *Zea mays* (maize). Macrobiotic seeds include most legumes and a small group of seeds from other families (Barton, 1961).

Many claims have been made about seeds of supposed great antiquity germinating readily. Some of these claims have been recently questioned. What now follows is a brief review of literature on this subject.

The oldest seeds still capable of germinating and for which there is documentary proof of age are those of a *Nelumbium* species (Ramsbottom, 1942). These were collected in 1705 and germinated in 1942 after storage in the British Museum herbarium. Specimens of *Albizza julibrissin* seeds collected and stored at the same institution from 1793 were still capable of germinating in 1940 (*ibid.*).

In 1923 Ogha claimed to have found lotus seeds with an estimated age of over 400 years that could still germinate. Later Chaney (1951) proposed geological evidence indicated the seeds were many thousands of

years old. Libby (cited in Godwin and Willis, 1964) carried out $^{14}C$ dating procedures on the seeds and concluded their age was 1040 ± 210 years B.P. Libby also carbon dated the remains of a prehistoric boat associated with the seeds and found them to be 3052 ± 200 years B.P.

The interest of Godwin and Willis (1964) in the question of seed longevity led them to obtaining their own samples of the seeds and boat remains to carry out an independent assessment of their age by carbon dating. This gave the interesting results of 100 ± 60 years B.P. for the seeds and 3195 ± 110 years B.P. for the boat remains. Thus while affirming the later result of Libby's, Godwin and Willis contradicted the earlier more important result, that of the age of the seeds. The authors of the latter work could see no obvious explanation for the discrepancy between their results and those of Libby. However they point out that the high germination percentage reported by Ogha would be more in keeping with relatively young seeds rather than seeds a thousand years old.

Later Godwin (1968) published another paper which called into question a number of recent claims regarding allegedly very old seeds that were still viable. In it he discussed the dangers of assuming the seeds to be as old as artifacts that are found in close proximity. This is illustrated by the seeds found by Ogha. In both carbon dating experiments the seeds and boat remains found nearby have quite different ages. Another example is that of some seeds of *Lupinus arcticus* found in conjunction with a rodent skull (Porsild, Harington and Mulligan, 1967). The skull was carbon dated at approximately 10,000 years B.P. However it cannot be assumed that the seeds are the same age, as they may have been deposited at a much later time than the associated remains.

The claim by Sivori, Nakayana and Cigliano (1968) that they had discovered some viable 600 year old seeds of *Canna compacta* in an

Argentinian tomb, was also disputed by Godwin. However, Lerman and Cigliano (1971) published a reply to Godwin carefully elaborating their evidence and arguments and these appear very convincing. This means that 600 years is the maximum authenticated limit for the survival of seeds or, for that matter, any anhydrobiote.

Pollen is generally much shorter lived than seeds. Some, such as *Pinus* species, can be stored for over three years before losing their ability to protrude a germ tube upon wetting (Malloy, 1965). Other pollen, such as *Zea mays*, reportedly lose their viability in a few days (Stanley and Linskens, 1972). It has been suggested that certain pollen may not be anhydrobiotic at all but is 'biological material in transit'. (Wilson, 1973). It may be that work on resting metabolism of pollen may throw some light on this issue.

Fern and fungal spore appear to be much more long-lived than pollen. There have been reports of spore surviving 20 years of dry storage (Zobh, 1943) though the subject has not been thoroughly investigated.

Lichen can survive for many years when desiccated (Hale, 1974). Rotifers, tardigrades and nematodes have been reported as surviving for decades in a desiccated state (Crowe, 1971).

1-3.2 Factors Affecting Longevity

The conditions of storage have a marked effect on the longevity of anhydrobiotic organisms. In seeds it is generally conceded that temperature and moisture content are the two most important factors (Barton, 1961; Roberts, 1972). Most seeds favour low temperature and low relative humidity. Seed storage laboratories usually maintain a temperature of 4C and a relative humidity of 30% (Roberts, 1972). These conditions would probably be favourable for most other anhydrobiotes as well. There are, of course, exceptions. For example, the seeds of some aquatic plants, such as the river maple, actually lose viability on drying (Jones, 1920). Such seeds could not be regarded as anhydrobiotic.

Some work has been done to find the effect of gaseous composition of the storage atmosphere. In some cases oxygen was found to be detrimental (Sayre, 1940), but in others no marked effect was noticed (e.g., Simpson, 1953).

Light has been found to have little effect on longevity of seed (Barton, 1961), but a beneficial effect has been claimed by Jensen (1941).

Longevity is most likely a characteristic that is in part genetically determined. Weiss and Wentz (1937) have shown that some genotypes of *Zea mays* have greater longevity than others indicating that it is an inheritable trait.

The nature of the seed coat can contribute to long viable seed storage. This is achieved by excluding water, oxygen and microflora (Barton, 1961).

Microflora can effect longevity of a seed but usually only when relative humidity rises above 70%. This subject has been reviewed (Christensen and Lopez, 1963). The main significance of this is that in the experimental work described in this thesis, relative humidities were kept below those known to allow fungal growth.

Outside the laboratory there are several situations that give rise to conditions favourable to long periods of seed storage. One obvious condition is in areas of permafrost, where the seed may have been stored at low temperatures for long periods. The absence of liquid water and the low relative humidities often found in such regions would also be favourable conditions for lengthy seed storage. Another situation is that found in tombs where the relative humidity is low and temperature fluctuations are slight. Much excitement was generated by the discovery of cereal grains in various ancient tombs, including the Egyptian pyramids, but early claims of their viability have been refuted (Barton, 1961). This has not prevented unscrupulous individuals

from selling modern wheat as 'mummy wheat' at the entrance of a pyramid. In fact the seeds of cereals are quite short-lived even under ideal conditions (*ibid.*).

It is likely that the longevity of most other anhydrobiotes would be affected in a similar way in that temperature and moisture content would be the major factors with other factors playing a minor role.

1-3.3 Cause of Death in Anhydrobiotes

Why do dried anhydrobiotic organisms die? This question is far from resolved. Most of the literature associated with it deals with seeds. A number of theories have been proposed to explain the loss of viability in seeds. These have been reviewed by Roberts (1972) and may be roughly divided into two classes: stochastic theories and accumulation/depletion theories. Stochastic theories encompass those that hold random events, such as mutations, responsible for the ageing and eventual death of a seed. The frequency of these random events is dependent on such factors as temperature, moisture and oxygen tension. Accumulation/depletion theories propose that ageing and death in the seed is a consequence of the accumulation or depletion of certain compounds. The second group of theories are of particular interest in this thesis as they could be explored by the technique developed to study resting metabolism.

Viability theories may be divided another way into those that propose extrinsic factors are responsible for ageing and death and those that propose intrinsic factors for these processes. Extrinsic factors generally recognised are background radiation and storage fungi. The three important intrinsic factors are accumulation of toxic metabolites, denaturation of macromolecules and depletion of essential metabolites. Each of these may be subdivided further and Roberts (1972) provides a very succinct classification of viability theories. This is reproduced in Figure 1.1, p.14. No one of these theories has

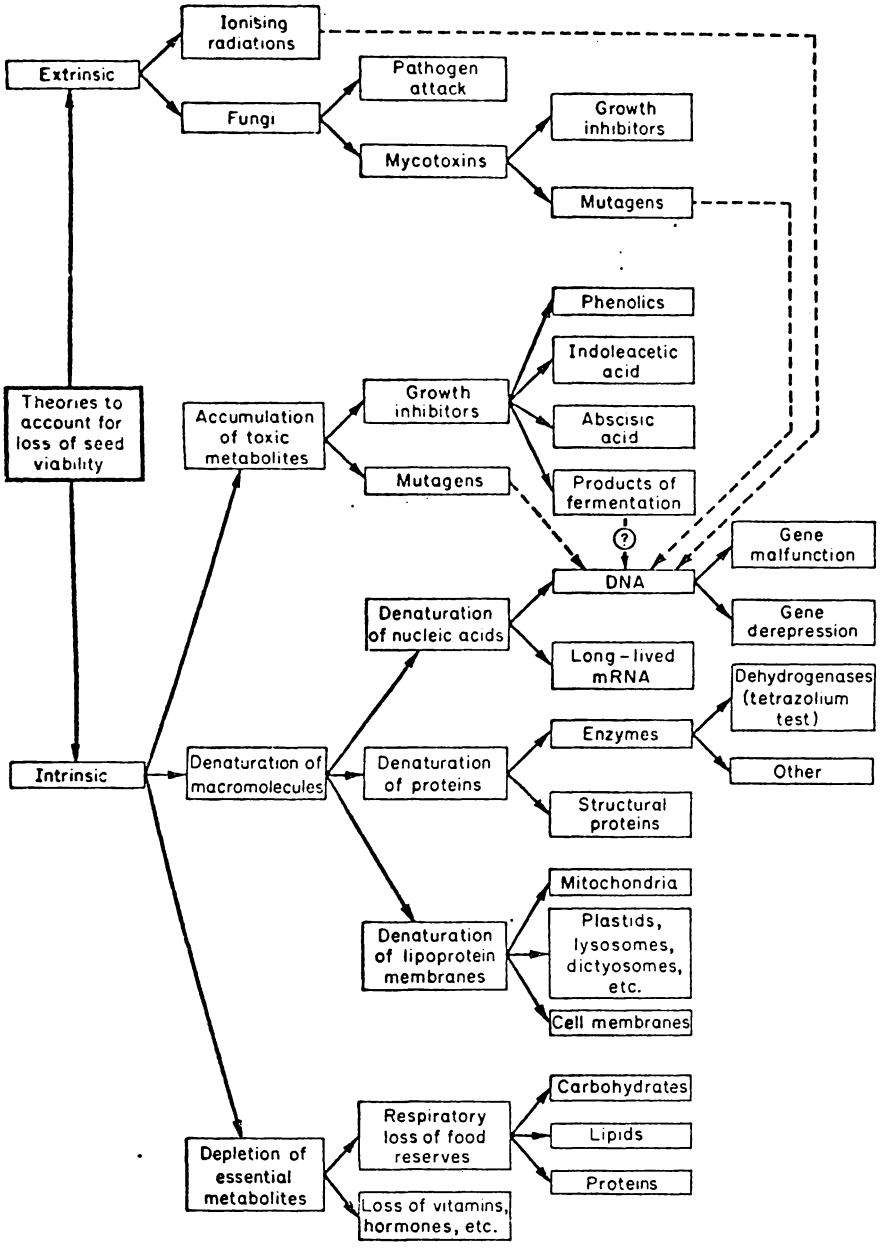



FIGURE 1.1 A classification of viability theories.

achieved any particular pre-eminence over the others, although background radiation and storage fungi are not regarded as primary causes for loss of viability.

The accumulation of lactic acid has been suggested (Wyttenbach, 1955) as a cause of loss of viability in three species of seed. However, Roberts (1972) adduces evidence to show this to be unlikely. A number of other substances have been suggested, such as fatty acids, indole acetic acid and abscisic acid whose accumulation causes loss of viability. This is an interesting field of research but more work needs to be done to show unambiguously the inhibiting effect of these substances on germination of seeds.

Research into the effects of endogenous mutagens is proceeding and it appears that some seeds definitely do produce such substances (D'Amato and Hoffman-Ostenhof, 1956). However, it has been shown that this is not the main cause for loss of viability in research on other seeds (Abdulla and Roberts, 1968).

It is now considered unlikely that loss of viability in seeds is due to depletion of food reserves (Barton, 1961). Old non-viable seeds do not show a great difference in overall seed reserves compared with fresh viable seeds. However it may be that the amount of some crucial organic molecule (e.g., thiamine or ascorbic acid) may decrease with time until there is not enough to facilitate germination.

The well-known thermodynamic instability of proteins suggests that the denaturation of proteins, particularly enzymes, may be responsible for the loss of viability. Experimental results so far have not related loss of viability with loss of enzyme activity. It is possible though that only one particular crucial enzyme be denatured to prevent germination and this has to be identified in each seed.

Certain evidence suggests that the denaturation of nucleic acids is responsible for the loss of viability of seeds (Greer and Zamenhof, 1962).

Once again, however, there is evidence from other studies that is contrary to this hypothesis (Barker, Bray and Detlefsen, 1971).

A relatively new explanation is that the denaturation of the lipoprotein cell membranes is responsible for loss of viability in seed (Berjak, 1968). This idea is being investigated with electron microscopy and may prove quite tenable.

Roberts concludes his review by emphasising that we must keep an open mind when considering the causes of death in seeds. It seems to the author of this thesis, in view of the mass of contradictory results, that the problem may be a multifactored one. For different types of seed different factors could be dominant in ageing and death. Even in the same type of seed the mechanism may be different under different storage conditions.

It is hoped that a study of resting metabolism using the various techniques developed in this thesis may allow a greater understanding of ageing and death in dried anhydrobiotes. Such a study may support or deny present theories, or perhaps provide evidence for a new explanation.

# CHAPTER 2 RESTING METABOLISM

## 2-1 General

Any anhydrobiotic organism goes through a non-desiccated stage of development. For example, a seed begins to develop from a fertilized ovule. At this stage it is probable that the immature seed has the same moisture content and a similar metabolism to that of the parent plant. As the seed matures it is transformed by desiccation to an organism of very little or perhaps no metabolism. This condition can be maintained for many months or even years, then by the addition of water under favourable conditions it can become once more an actively metabolising organism.

The metabolic changes occurring during these three stages have not been studied extensively. Presumably during the first stage the seed's 'metabolic factory' is shut down in some way until upon desiccation resting metabolism commences. When the third phase, germination, begins the 'metabolic factory' of the seed is started up again and eventually is returned to normal.

The work described in this thesis is an attempt to discover the nature of any metabolism occurring during the second phase. The technique used to study resting metabolism was a development of a technique used to study germination metabolism.

An interesting question is: 'What happens to the metabolism of a seed at it ripens and desiccates?' Three possibilities that can be considered are (i) the metabolism slows gradually until at desiccation it has been reduced to many orders of magnitude or ceases entirely, (ii) metabolic pathways 'shut down' one at a time until at desiccation

there is a very simple metabolism or none at all, (iii) the process of desiccation is associated with the operation of special metabolic pathways peculiar to anhydrobiotes. Eventually the metabolism of the organism reaches a very low level or ceases entirely.

Recently Crowe and Madin (1975a) have shown that the anhydrobiotic nematode *Aphelenchus avenae* has to be prepared for anhydrobiosis by slow water loss at high relative humidity prior to more complete dehydration. If active animals are transferred immediately to an atmosphere of relative humidity below 97% they die. This implies that some metabolic or structural changes must occur during the initial period of slow water loss if the animals are to survive desiccation. In a subsequent publication the same authors (1975b) showed that glycerol and trehalose accumulated during the initial slow drying phase. This accumulation was strongly correlated with survival of subsequent desiccation, and may well be the main adaptive feature that allows these organisms to survive desiccation.

The function of trehalose and glycerol may be to replace water associated with macromolecules and membrane systems and stabilize these structures at low water activities. It is not clear if seeds are similarly prepared for desiccation or if other adaptations have been developed. However, many pollens contain high carbohydrate concentrations and these may serve to protect macromolecules and membranes of the pollen from the vicissitudes of desiccation.

Another question, and in fact the principal one asked in this thesis is: 'What metabolism, if any, occurs in the desiccated anhydrobiotic organism?' As has been implied above there are two possibilities; (i) that there is no metabolism at all, (ii) that there is a metabolism but this must necessarily be of a very low level. It is obvious that something is happening in the organism because after a certain period it loses its viability.

Whether this is evidence of metabolism is a moot point and is discussed in greater detail below.

The question of germination metabolism has been more studied than that of ripening or resting metabolism. Two possibilities that could be considered regarding germination metabolism are (i) all metabolic pathways begin to operate at the onset of germination, increasing in intensity with time; (ii) metabolic pathways begin to operate consecutively so that the initial metabolism is simple and becomes more complex with time. The work of Spedding and Wilson (1968) indicates that the latter is the case in germinating seeds. This may indicate that if resting metabolism occurs at all it will be simple in nature.

## 2-2 Does the Dry Anhydrobiote have any Metabolism?

### 2-2.1 General

In the previous section the question was asked 'Does the anhydrobiotic organism have any metabolism?' There now follows a discussion of the theoretical aspects of this issue.

In spite of mankind's intimate and necessary association with seeds, botanists have only recently considered that the metabolic status of seeds is a significant issue. Indeed, many early botanists seemed to regard the seed as an abiotic organisation which did not qualify as an organism until germination was complete.

While the question of metabolism in the dry seed, until quite recently remained largely unasked, there has been considerable debate on the metabolic status of dried anhydrobiotic animals. Two general hypotheses as mentioned above have been developed on this matter, one that there is a total cessation of metabolism following desiccation, the other that metabolism is reduced to a very low but finite level. (Obviously any metabolism that does occur must be very slight as the dry organism has no input of food or nutrients.) So the question arises: 'Are anhydrobiotes merely quantitatively different from

normally metabolising organisms or is there a qualitative difference between them?' However, until quite recently this question was not studied experimentally because of the lack of sensitive techniques for measuring very low metabolic rates.

2-2.2 Prevailing Ideas

In 1973 Clegg discussed this question in broad outline in a paper entitled 'Do cryptobiotes have a metabolism?' — this confined itself to dried organisms containing < 5% water. It is obvious that changes do occur in these organisms during storage because they lose the ability to germinate or be resuscitated after a period of time. However, as Crowe points out, although these changes could be regarded as physiological they should not necessarily be regarded as evidence of metabolism. Clegg (1973) proceeds to define metabolism as 'enzymatically catalysed chemical reactions integrated into pathways that are regulated in terms of rate and direction and that contribute to the maintenance of the cell in which they are located'.

The last phrase could be questioned although the objection is not serious. It seems possible that enzymatically catalysed reactions may occur in a cell which are not necessary for the maintenance of the cell. In fact it has been shown (Vickers, 1971) that *Sinapis alba* seeds produce considerable amounts of 4-aminobutyric acid during early germination by the decarboxylation of glutamic acid. However, it appeared that the 4-aminobutyric acid was not utilized by the seed and it was suggested its formation was the result of some sort of metabolic accident — neither harmful nor beneficial to the germinating seed. The existence of such processes seem to have been little considered. However, if evolution is regarded as a continuing process initiated by random mutations, then such a possibility does not seem unreasonable. Furthermore if these processes did not waste so much of the cell's metabolic energy as to threaten its survival and one accepts the

Mendelian view of inheritance, then it would be reasonable to expect the metabolic capability for these processes would be transmitted from generation to generation.

It could be argued in the case of the germinating *Sinapis alba* seeds that the 4-aminobutyric acid is later utilized by the developing seedling, but even this is no guarantee that it is actually beneficial to the cell. 4-aminobutyrate can be converted, via succinic semi-aldehyde, to succinate and thence oxidized in the tricarboxylic acid cycle to provide NADH and ATP. But the same could be said for the glutamic acid from whence the 4-aminobutyrate came. So the process would be just an unnecessarily complicated way of oxidizing glutamic acid. Consequently a reaction (or series of reactions), even though it may be integrated into other important metabolic pathways, may have no overall beneficial effects on the maintenance of the cell. It would seem rather arbitrary to exclude this from what is regarded as the cell's metabolism, but Clegg's definition would require this.

Considering Clegg's question: 'Do dried cryptobiotes have a metabolism?' again it would seem there are three possible situations: (1) Cryptobiotes have no enzyme catalysed reactions whatsoever and the only reactions are entirely non-physiological. (2) They have some enzymes still operational at low water activities which catalyse isolated reactions. (3) They have integrated pathways still active (e.g., TCA cycle) but operate at extremely low levels. Whilst it would be reasonable to regard situations (1) and (2) as those where metabolism could be said to be non-existent, it would not be reasonable to claim this in situation (3) even though the operation of the pathways was not beneficial and perhaps even harmful in the long term to the maintenance of the organism.

It could be argued that Clegg has defined his terms such that

there can be only one answer to his question. As it is generally accepted that dried cryptobiotes deteriorate with time and with the crucial phrase in Clegg's definition '... contribute to the maintenance of the cell ...' then one must conclude that there is no metabolism in dried cryptobiotes in terms of his definition. This author would like to include in a definition of metabolism, enzyme catalysed reactions integrated into pathways even though they may not contribute to the maintenance of the cell in which they are located.

Clegg proceeds to discuss his question from a physio-chemical point of view to try to demonstrate the impossibility of any operational enzymes at low water activity. He cites results obtained by workers studying the hydration of proteins and ribosomes as well as some more direct evidence.

Fuller and Brey (1968) found that water adsorbed by anhydrous egg albumen was immobile until 0.075 g of water per 1 g of protein had been adsorbed. 0.15 g of water per 1.0 g of protein had to be adsorbed before there was any water that could be regarded as loosely bound. In a similar study Bull and Breese (1968) showed that albumen required 0.3 g $H_2O$ per 1 g protein to saturate fully all the polar residues and, further, that nine other proteins behaved similarly. In the same paper the dehydration of albumen solutions is discussed. At 0.7 g water per 1 g protein the solution became a glass and at 0.2 g water per 1 g protein the glass became very brittle. If the proteins in a dried cryptobiote are in the form of a brittle glass it is difficult to imagine how any enzyme catalysed reactions could occur to an appreciable extent when one considers such problems as the extremely slow rates of diffusion which would affect the rates of all reactions.

In 'Do dried cryptobiotes have a metabolism?' Clegg proposed that a cryptobiote with a water content of 5% would not have enough water to hydrate completely all its protein (estimated at 35% of its dry weight)

let alone all the other structures and compounds in its cells.

Skujins and McLaren (1967) have adduced more direct evidence about enzyme activity at low water activities *in vitro*. In a series of experiments, well-mixed samples of urease and $^{14}C$ urea were held at different relative humidities and the enzyme activity and hydration levels of urease and urea were measured. It was found that no activity could be detected below a urease hydration of 8%, and above this the enzyme activity followed the adsorption isotherm of the enzyme and not its substrate. Clegg fails to point out that these results are interesing as they indicate a much lower hydration level for useful catalysis than did the results discussed in the previous paragraph.

Clegg also reviews the hydration of nucleic acids and considers this as important as that of proteins. Low hydration levels of ribosomes causes them to collapse into irregular shapes and presumably lose the ability to function normally. It has certainly been shown that ribosomes can be completely desiccated and yet completely regain activity upon rehydration (Christman and Goldstein, 1971; Leary *et al.*, 1969). However Clegg considers normal ribosome function extremely unlikely with the amounts of water present in the dried cryptobiote.

Unfortunately all of the work on proteins and nucleic acids under conditions of desiccation has been carried out on macromolecules from non-cryptobiotic organisms. This leaves open the question of cryptobiotes having proteins and nucleic acids that are especially resistant to desiccation. Consequently it is possible much of the previous discussion may not be directly applicable to dried cryptobiotes — a line of reasoning suggested by Clegg.

At the conclusion of 'Do dried cryptobiotes have a metabolism?' Clegg answers his question with a 'no'. He finally turns to what he describes as the most compelling argument, which has been referred to before, i.e., whatever is happening in the dried cryptobiote it is

deleterious and therefore cannot be metabolic in terms of Clegg's own definition of metabolism. The logic of this argument is indeed compelling, but it is the definition of metabolism which needs further consideration.

Taken overall Clegg's conclusion that cryptobiotes containing less than 5% water have no metabolism at all seems the most reasonable, even if one were to exclude the most stringent requirement of Clegg's definition of metabolism. However, between 5% water content and that of an organism's liquid water there is a whole range of water content where the answer to Clegg's question is much less certain. A seed stored at common room temperatures and humidities, for instance, has a water content of about 12% (e.g., see Roberts, 1972, p.430). What evidence is available so far indicates that such seeds definitely do have a metabolism, although not strictly in terms of Clegg's definition as it is not apparently beneficial to the seed. The next section discusses this evidence and the techniques used to acquire it.

## 2-3 Previous Work on Resting Metabolism

### 2-3.1 General

Very little work has been carried out on dried organisms pertaining directly to their metabolic activities. This is largely because of the lack of suitably sensitive techniques. If dried organisms have any metabolism at all it would presumably be very slight. In fact it is proposed that there may be three possibilities: (1) that there is no metabolism during anhydrobiosis, (2) that the normal metabolic activity of the organism has been greatly reduced by desiccation, (3) that there is a type of metabolism peculiar to anhydrobiosis. In an editorial comment in *Anhydrobiosis*, Crowe and Clegg (1973) claim that any other situation than (1) would render the term cryptobiosis ('secret' biosis) suspect. Such semantic argument is of no great concern to the biochemist. The claim of Crowe and Clegg that any situation other than

no metabolism would remove uniqueness from the condition seems exaggerated when one considers all aspects of anhydrobiosis. The condition has much that is peculiar to it even if a total cessation of metabolism does not occur.

A question that arises is how to tell metabolism from what Clegg describes as 'adventitious chemistry'? Obviously reactions that are not enzymatically catalysed could not be regarded as metabolic but even a reaction which is enzymatically catalysed, if occurring in isolation, could be excluded from metabolism as discussed earlier.

2-3.2 Ways of Studying Resting Metabolism

Measurement of $O_2$ and $CO_2$ exchange by manometry as an indication of metabolism is fraught with difficulties. The rate of exchange in dried anhydrobiotes must be very low if the organisms are not going to metabolise away their food reserves in a short time. Manometry is not a very sensitive technique. Interpretation of results is also difficult as gas exchange is such a rough yardstick of metabolism. Low level gas exchange could easily be a result of non-metabolic activities in the seed.

The use of a radiochemical method is far more sensitive as Crowe and Clegg (1973) point out. The problem of this technique lies in introducing the radioactive tracer into the dried cryptobiote, although, as discussed later, some progress has been made with $^{14}C$ labelled compounds. The experimental work described in this thesis is an attempt to develop a new method for this type of study.

2-3.3 Manometric Studies

Carbon dioxide production by dried grain of *Zea mays* has been studied by Bartholomew and Loomis (1967). Gas chromatographic analysis was carried out using both live and dead sterile *Zea mays* grain. A range of moisture content from 1.4% to 12.6% was studied. Time intervals ranged up to 100 days. The study gave results which defied

rational analysis and the authors concluded that $CO_2$ production was not a dependable measure of respiration in dry seeds. They further claimed that oxygen uptake was also not a suitable indicator of metabolism in maize grain.

In two papers Pigon and Weglarska (1953, 1955) describe measuring the $O_2$ uptake of dried *Macrobiotus spp.* (a type of tardigrade). The second paper describes careful measurements of oxygen uptake by the organisms over a range of relative humidities from 25% to 96%. Below 48% relative humidity the $O_2$ consumption was at an extremely low but constant level. Above this humidity the $O_2$ uptake increased gradually but remained very low until about 95% relative humidity when the uptake rate increased rapidly.

All of these papers indicate the equivocal nature of the results obtained from manometric studies and it is not surprising that very little work has been carried out on anhydrobiosis using this technique.

2-3.4 Radiochemical Studies

In a study using a technique very closely related to the technique developed in this laboratory, freshly harvested seeds of *Avena fatua* (wild oats) were exposed to $^{14}C$ ethanol. Wild oat seeds are dormant when freshly harvested and acquire the ability to germinate only after several months of dry storage. The object of the above mentioned study by Chen (1972) was to try to discover some of the metabolic changes that occur during the time between harvesting and acquiring the ability to germinate. As Chen points out, an important aspect of his technique was that the moisture content of the seed is not affected by the introduction of the tracer. Seeds with a moisture content of about 10% were exposed to $^{14}C$ ethanol — it is implied but not clearly stated that this was as a vapour. No mention of viability was made. Exposure intervals were for one week followed by extraction

with 80% ethanol. The insoluble residue was subjected to various hydrolyses and the hydrolysate analysed. The ethanol soluble fraction contained most of the activity (95%), which proved to be mostly sugars and amino acids and only traces of organic acids. The hydrolysates of the insoluble residue contained labelled amino acids — this the author presumed was from proteins synthesis in the presence of $^{14}C$ tracer during dry storage. Due objection to this conclusion is that the initial extraction with 80% ethanol is a technique proven to be inefficient in extraction of metabolites supposedly soluble in this solvent (Beileski and Turner, 1966). However, the fact that a pronase hydrolysis showed a higher activity in amino acids than an acid hydrolysis does give a little more credence to Chen's conclusions.

Finally, Chen describes a pathway by which proteins could become labelled in his experiments: ethanol → acetaldehyde → acetate → α keto acids → amino acids → proteins.

This paper is a very significant one in spite of its uncertainties for, as indicated earlier in this chapter, there is a considerable body of opinion that holds anhydrobiotes are incapable of any metabolism and yet here we are given evidence of protein synthesis in a dried anhydrobiote!

Another interesting study which holds some promise in the field of experimental approaches to resting metabolism is one by Meyer and Mayer (1971). In this study the authors attempt the permeation of dry seeds with chemicals using a solvent of anhydrous dichloromethane. The work demonstrated that treatment with the solvent for periods of 44 hours did not affect subsequent germination of the seeds. Furthermore, they elegantly demonstrated the penetration of chemical substance by the technique by introducing coumarin into the dry seeds. This substance is an inhibitor of germination and the treated seeds failed to germinate. However, if the seeds were soaked in an excess of water

after this treatment, they were able to germinate. This was interpreted as meaning the coumarin had been successfully introduced and yet could be removed again by soaking in water.

The above technique would seem very promising, using radiochemical tracers, for studying metabolism of dry seeds and also the very earliest stages of germination. However, in view of the obviously very low rate of metabolism in dry seeds, certain problems would be encountered. Very high initial activities would have to be introducted to obtain detectable quantities of subsequent metabolites. This would require very high standards of radiochemical purity of the introduced compound. This criterion is not always easily attainable in the author's experience (Vickers, 1971). Also, attempting to find traces of activity in various metabolites in the presence of one with a very high activity is often difficult in that normal isolating and detection procedures become overloaded.

The technique of introducing radiochemicals into seed was undertaken prior to the work of Meyer and Mayer, although these authors do not refer to it. Wilson and Harris (1968) introduced $^{32}P$ sodium phosphate into crested wheatgrass seeds in an attempt to determine the type of phosphorylation reactions occurring at low water activities. In these experiments $Na\ H_2{}^{32}PO_4$ was dissolved in absolute ethanol and the seeds imbibed this solution. The ethanol was then quickly evaporated and the seeds allowed to equilibrate to various water potentials (relative humidities) and left for several days. This treatment was shown not to reduce significantly the germination percentage of the seeds. The results showed that phosphorylation occur at 52% relative humidity and above but did not occur at 15% relative humidity.

Although this paper deals with only one aspect of resting metabolism (phosphorylations) it is nevertheless important as it uses a technique that may have wider application for the study of resting metabolism in

anhydrobiotic organisms.

In 1965 Mann pioneered a possible technique for studying resting metabolism using radiochemicals. It was this technique which was developed during the experimental work described in this thesis. The principle of Mann's technique was to expose the specimen to tritiated water vapour at constant relative humidity. The details of this technique are described in the next chapter. The results obtained by Mann indicated that chemical or biochemical activity occurred in *Pinus radiata* pollen stored at four relative humidities (62%, 52%, 43%, 15%).

Mann's results showed that at 43% relative humidity a number of metabolites were tritium labelled by the pollen. Not all the labelled compounds were identified but the results showed the promise of the technique. Preliminary experiments by Mann with *Sinapis alba* seeds and *Pithomyces chartarum* spore also showed tritium labelling even at 15% relative humidity after several months storage in tritiated water vapour.

It was these results of Mann's that inspired this author to refine the technique of exposing propagules to tritiated water vapour at constant relative humidity. The shortcoming of Mann's techniques and the changes made by the author of this thesis are discussed in detail in the next chapter.

## 2-4 The Aim of this Thesis

The primary aim of this thesis was to develop and evaluate a technique for studying resting metabolism. It was then intended to study the effect of relative humidity on resting metabolism and to compare results from a wide range of organisms to get an overall view of the subject.

In order to study the metabolism occurring in a dried organism any technique used must not interfere with the dehydrated state of the organism. This is a very difficult requirement to fulfill and explains

why little research has been undertaken in this field.

Most biochemical techniques that have been devised for studying metabolism involve the use of aqueous media — this approach, of course, would be an anathema to studying resting metabolism in anhydrobiotes. The technique developed by the author of this thesis is one which circumvents many of the problems encountered by other techniques. It involves storing the dried organism in an atmosphere containing tritiated water vapour at constant relative humidity. The details of this technique are described in the next chapter.

2-5 Scope of this Thesis

The organisms chosen to study were restricted to plant propagules. Initially it was hoped to find in what compounds most of the tritium was incorporated at constant relative humidity. A range of relative humidities were chosen between 15% and 64% with the intention of finding how tritium incorporation varied with relative humidity.

Most of the work involved various types of pollens and seeds from widely different plant types. It was hoped to get a wide range of qualitative results to find if there were any remarkable differences in tritium incorporation between different species.

As the work progressed new questions arose and attempts were made to deal with some of these. This included using more sensitive techniques to detect tritium incorporation; (i) at low relative humidities, (ii) in lipids and lipid-like compounds, (iii) in macromolecules.

These latter techniques were applied only to a few organisms because time did not permit of their application to all other organisms studied. In this regard the two organisms studied most were *Sinapis alba* seeds and *Pinus ponderosa* pollen.

Careful tests on radiosensitivity were carried out to ensure the exposure to tritiated water vapour did not kill the organisms studied.

Also a number of subsidiary experiments, such as the water content of the organisms at different relative humidities were undertaken.

## 2-6 Water Relations in Dried Organisms

### 2-6.1 Introduction

An apparently dry seed (or other anhydrobiotic organism) will usually contain an appreciable amount of water. Most seeds, when stored under ambient room conditions, will contain in the order of 10% water. The precise amount will depend on the type of seed, the temperature and relative humidity of storage. Hence for a given seed stored at a constant temperature the water content of the seed will be determined by the relative humidity. The relationship between relative humidity and water content seems to vary considerably between different species of seed. Figure 2.1 shows the relationship between water content and relative humidity for various seeds stored at 25 C (data from Roberts, 1972; Hubbard, Earle and Senti, 1957).

Another interesting feature of seed water content is that some water is irreversibly lost during initial drying to a very low relative humidity. Consequently when seeds are equilibrated with a given relative humidity after storage at a higher relative humidity, they will always have a higher water content than the same species of seed equilibrated with the given relative humidity after storage at a lower relative humidity. This is illustrated in Figure 2.2 for *Oryzasativa* seeds (Roberts, 1972).

In chemical terms the water content (often called moisture content) of the seed is not a very meaningful quantity. If the availability of water dictates the chemical or biochemical behaviour of the seed it would seem reasonable to assume that the chemical activity (or chemical potential) of the water is the critical quantity. The activity of the water in the seed is determined by the partial pressure of the water vapour in the atmosphere above the seed.

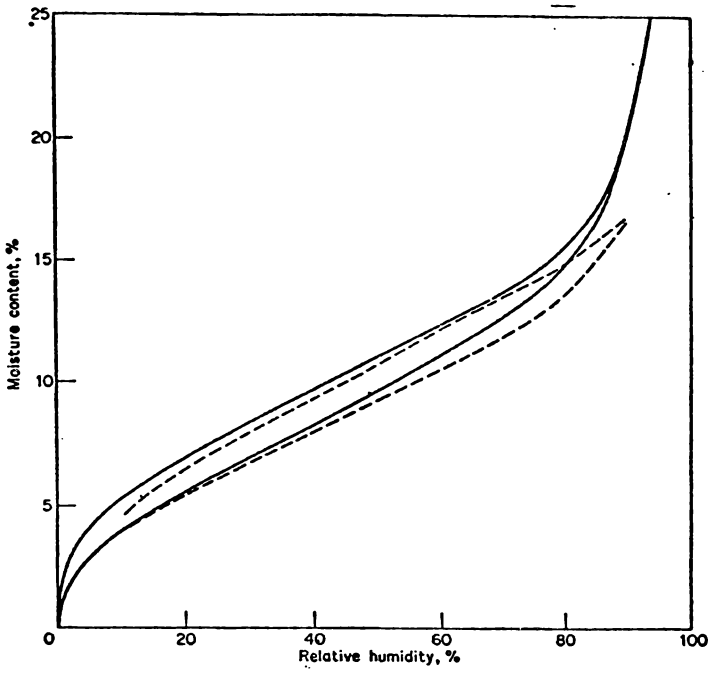



FIGURE 2.1 Absorption/desorption relationship for wheat (solid line) and rice (dotted line). In each case upper curve represents desorption and lower curve absorption.

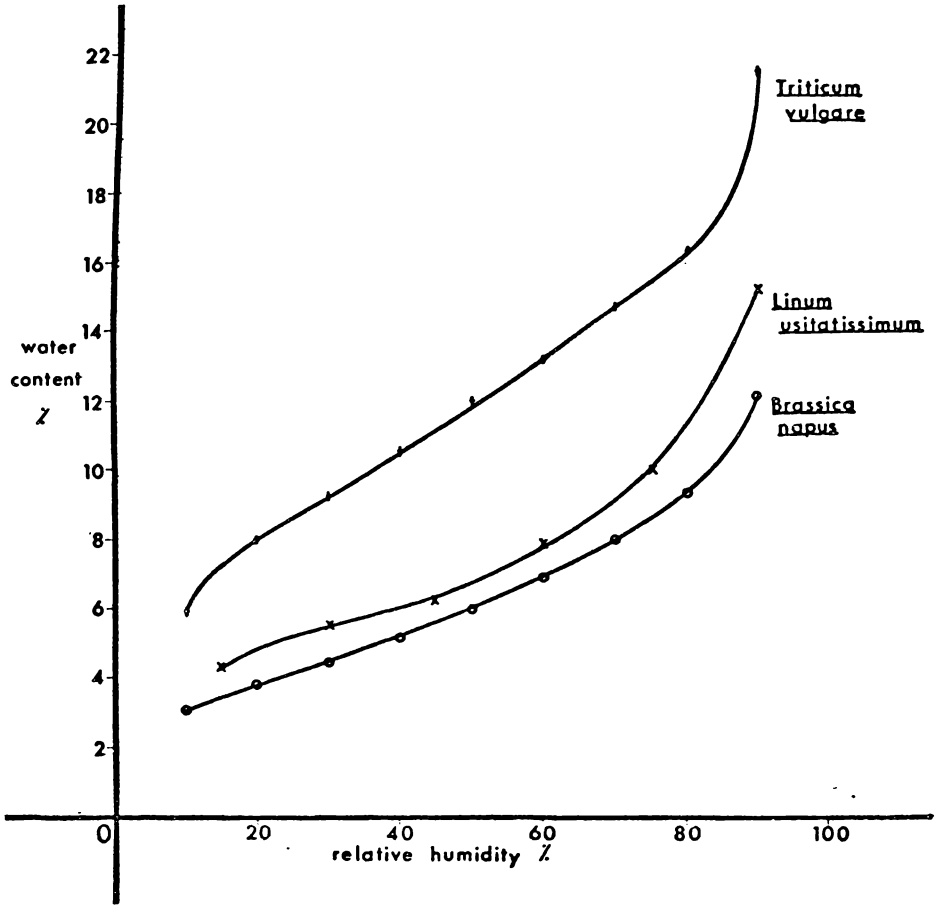



FIGURE 2.2 Relationship of water content and relative humidity for three species of seed.

$$\text{activity} = \frac{P}{Po} \qquad \text{(Morris, 1974)}$$

P = partial pressure of water vapour at specified temperature

Po = saturated water vapour pressure at same temperature

But relative humidity % could be written as

$$RH = \frac{P}{Po} \times 100$$

It follows that activity = $\frac{RH}{100}$

The relative humidity, then, is a direct measure of the water activity in the storage atmosphere and hence the water activity in the seeds. This explains why the moisture content of a seed varies with relative humidity of storage. However, the chemical constituents of the seed will also affect the water content. As the chemical constituents of various seeds differ markedly, so does the moisture content of different types of seed in equilibrium with a particular relative humidity. Hence oil-bearing seeds have a lower water content than, for example, cereal grains.

Many references dealing with anhydrobiosis provide only the water content of the dried organisms and not the relative humidity of storage. The meaning of published values for water content is further confused by the arbitrary manner with which it is determined. Consequently it is not always possible to accurately infer the water activity in a given organism from the water content.

2-6.2 Methods for Determining Water Content

The International Seed Testing Association (1966) has adopted standard procedures for measuring the water content of seeds. Three methods are suggested:

(1) Heating a five gram sample to 130 ± 3 C for 60 minutes in non-corrosive metal dishes.

(2) Heating a five gram sample to 105 ± 2 C for 16 hours.

(3) Distilling a sample of seed with toluene and collecting

the water present in the distillate and measuring its volume.

The first two techniques are proximate and, in fact, are measuring the content of the sample that is readily volatile. Figures obtained by such techniques may be of value to some researchers, but this author thinks that they are of limited value in trying to understand the processes occurring in anhydrobiosis.

Another objection to the oven drying method is that the International Seed Testing Association suggests that all but the smallest of seeds be ground in a mill to facilitate water loss during heating. However, there is a danger that water would be exchanged with the atmosphere during the milling process, particularly as the mill heats up considerably when in operation.

Another problem with oven drying methods to determine water content is that the samples do not achieve a constant weight under the conditions previously specified. In the experience of this author the time taken for this, even at 130°C is considerable. In general, seeds will undergo an initial rapid loss of weight followed by a slow loss of weight for a period of weeks. This may be explained as an initial rapid evaporation of loosely-bound water and other volatile compounds. The slow loss of weight following could be attributed to three things; (1) slow evaporation of strongly-bound water, (2) slow evaporation of slightly volatile compounds (e.g., some lipid substances), (3) slow production of volatile compounds from the thermolytic lesions of non-volatile compounds in the seeds.

The above discussion indicates the problems of attaching much meaning to the quantity often referred to as water or moisture content.

The toluene distillation method could give a reasonable measure of actual water content, but it will not give very accurate results as it is a volumetric technique.

At the present time the main value of water contents of anhydrobiotic organisms is to allow comparisons of research carried out in different laboratories. If it proves necessary to know the absolute amount of water in such organisms this may best be carried out by some chemical technique that is more specific for measuring water. One possibility would be the Karl Fischer titration a variation (Peters and Jungnickel, 1955) of which has been used by Crowe and Madin (1975a) to determine water content of nematodes. Another possibility, and one investigated by this author, is to dry the seeds over phosphorus pentoxide. This would remove even strongly bonded water, as $P_2O_5$ can remove water from such hygroscopic compounds as LiCl. The disadvantages of this technique are that $P_2O_5$ would also remove volatile bases (but it is most unlikely these would be present in seeds considering the weakly acidic nature of plant tissue) and that it is possible that $P_2O_5$ may be absorbed by the seeds. This could be easily checked by determining if the phosphate content of the seeds increased appreciably during the drying process.

Some preliminary experiments by this author have shown that seeds and pollen stored over $P_2O_5$ at room temperature do eventually attain constant weight although the process is slow (about 20 days for pollen and three months for intact barley seed). However, further storage at 50 C under vacuum over $P_2O_5$ does not lead to further weight loss. This implies that some equilibrium state has been achieved.

Although the water content of a number of organisms studied in this thesis were determined they have been given no prominence in view of their doubtful value.

2-6.3 Physical Nature of Water Associated with Anhydrobiotic Organisms

Very little research has been carried out on the biophysical characteristics of water associated with dried anhydrobiotes. It will be necessary to have an understanding of this topic to explain the

the chemistry and biochemistry of anhydrobiosis. The few studies undertaken so far indicate the matter is far from simple.

One area of research associated with the subject is the nature of the hydration of macromolecules. It would be reasonable to expect much of the water associated with macromolecules such as proteins, nucleic acids and carbohydrates. The hydration characteristics of even one such macromolecular substance appears to be a complex process. In general, there appears to be two ways in which water associates with macromolecules: that where the water binds chemically by a weakly exothermic process (12.5 - 25 $kJ.mol^{-1}$ water) and that where the water is loosely adsorbed involving no energy release (Jirgensen, 1962). However, there is no sharp boundary between these two processes of water adsorption. This may be illustrated by the water vapour adsorption isotherm for tobacco mosaic virus at 25 C (Figure 2.3).

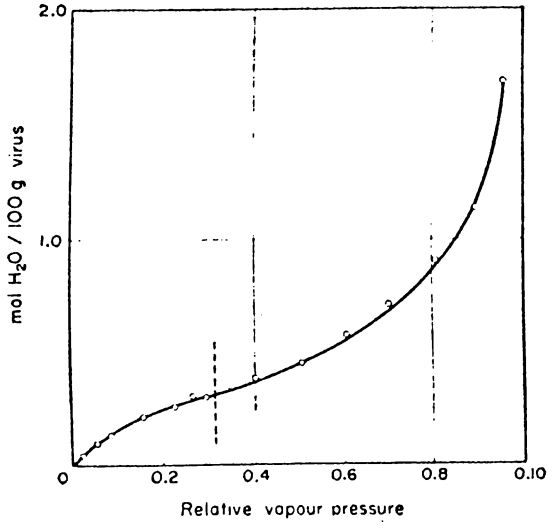



FIGURE 2.3 Water vapour adsorption isotherm for tobacco mosaic virus (Katchman, Cutler and McLaren, 1950)

The sorption isotherm shown does not display any sharp discontinuity which could correspond with the end of chemical and the beginning of loose association although the authors of it propose an arbitrary point (dotted line on figure). It appears that for many proteins 0.05 - 0.2 g of water is 'chemically' bound for each gram of protein (Jirgensen, 1962).

Koga, Echigo and Nunamura (1966) have studied the physical properties of cell water in partially dried *Saccharomyces cerevisiae* This study, involving NMR, enthalpy and dielectric measurements, suggests that there are four states of cell water in the organism. These correspond to regions of the adsorption isotherm described as: localized water region (strongly adsorbed), mobile adsorption region, gel region and solution region. The first two regions, or the first region by itself, could correspond with the 'chemically' bound water discussed above. It remains to be seen what significance this classification has for the study of resting metabolism. It would be interesting to find if there were any abrupt changes in metabolism associated with these physical changes in the state of cell water in anhydrobiotes in general.

The work of Koga *et al.* suggests that the most strongly bound water represents only 4% of the total mass of *Saccharomyces cerevisiae* cells. This would correspond with a low water activity. If metabolism were to occur in the organism above this level of hydration, one may find metabolism at surprisingly low relative humidities (or water activities).

It is obvious that a considerable amount of data has yet to be obtained before even the outlines of resting metabolism can be constructed. However, once this has been done, it is most likely that some correlation will be found between the change of resting metabolism with water activity and the physical state of the water in the organism under study.

2-6.4 A New Branch of Biochemistry?

The results discussed in Section 2-3 indicate that metabolism can be detected at water activities appreciably less than one. If this is so, it suggests the development of a branch of biochemistry dealing with metabolism in solutions of high concentration, i.e., solutions of low

water activity. It has been suggested above that the nature of macromolecular constituents of anhydrobiotes may be different from those encountered in non-anhydrobiotic organisms. It may be necessary to revise our ideas on macromolecular structure and function when dealing with organisms that can survive desiccation. At the very least caution must be used when transferring classical biochemical concepts to the biochemistry of anhydrobiotic organisms.

# CHAPTER 3 DEVELOPMENT OF A NOVEL TECHNIQUE FOR STUDYING RESTING METABOLISM

## 3-1 Discussion

### 3-1.1 Introduction

The primary goal of the work described in this thesis and a considerable amount of the effort expended was to develop and evaluate a successful technique to study resting metabolism. The requirements of suitable techniques are outlined in the previous chapter.

The almost complete lack of knowledge on the subject of resting metabolism implies the need for a new experimental approach. This chapter discusses the general characteristics, then describes the experimental details of the technique developed. This technique was developed from the use of tritiated water to study germination metabolism (Spedding and Wilson, 1968). It is obvious that the extent of metabolism in the dried organism must be very slight. Consequently, analytical techniques have to be very sensitive to detect any such metabolism. Chapter 4 describes routine analytical procedures. Also described are the attempts of the author to obtain maximum sensitivity from these procedures.

### 3-1.2 Rationale for the Technique

In Chapter 2 the experimental problems of studying resting metabolism were discussed (Section 2-3). The technique developed by the author is believed to overcome many of the problems that beset other techniques.

In principle the technique involves equilibrating the specimen with a certain relative humidity then exposing it to tritiated water vapour

at the same relative humidity. After several days the propagule is extracted and the extracts analysed chromatographically. Tritium labelled metabolites are detected by scintillation autography and identified by co-chromatography (see Chapter 4). Enumerated below are the advantages of the technique:

1. It is a technique capable of detecting *in vivo* metabolism in a resting propagule during dry storage.
2. The technique uses water as the radioactive tracer; this has the advantage of being one of the most mobile of cell constituents, increasing the likelihood of the radio isotope reaching the reaction centres. On the other hand, $^{14}C$ labelled molecules, even if they are introduced into the propagule may not be able to diffuse to the centres of reaction.
3. The technique proved to be non-lethal to propagules tested for radiosensitivity to tritiated water vapour over the time intervals used.
4. The technique is simple, requiring only a few special pieces of glassware. The tritiated water is relatively cheap and easily obtainable.
5. The technique has high sensitivity.

3-1.3 Principles of the Technique

The theory of using tritiated water to detect *in vivo* metabolism has been outlined by Wilson (1964). Briefly, if a compound is found after repeated washing to be $^{3}H$-labelled then it must have been involved in some chemical or biochemical reaction. If however a compound is found not to be $^{3}H$-labelled then this can be evidence that certain metabolic pathways involving this compound are not operating.

The technique has three problems which must be considered when

interpreting results: (i) The isotope effect between $^{3}H$ and $^{1}H$ can be very large, therefore the results may not completely correspond to normal metabolism. (ii) Common to all radiochemical techniques is the problem of radiation damage. (iii) Important biochemical reactions can occur in the presence of tritiated water without the incorporation of tritium. This can be considered when arriving at hypotheses based on the presence or absence of a tritium labelled compound.

These questions are discussed more fully at the end of this chapter under the title 'Interpretation of Results'.

## 3-2 Experimental Procedure

### 3-2.1 Storage of Propagules — The Humidistats

A series of 'humidistats' were set up to equilibrate the propagules to various relative humidities. Each of these was a standard eight inch desiccator having a saturated salt solution in the bottom in place of a desiccant, and stored in an incubator at 24 C. Different salts provided different relative humidities.

| Salt | % Relative Humidity (24 C) |
|---|---|
| $NH_4NO_3$ | 62 |
| $Mg(CH_3COO)_2$ | 64 |
| $NaNO_2$ | 64 |
| $Mg(NO_3)_2 \cdot 6H\ O$ | 54 |
| $K_2CO_3 \cdot 2H_2O$ | 45 |
| $MgCl_2 \cdot 6H_2O$ | 34 |
| $LiC\ell \cdot H_2O$ | 15 |

These values were taken from two references, *Chemical Rubber Company Handbook* (1975) and *International Critical Tables* (1933). It was found necessary, because of a series of confusing results and some contradictory references, to check experimentally the reported relative humidity provided by each salt. This was done by measuring the vapour pressure above the appropriate saturated salt solutions at 24 C with a mercury manometer. The calculated values were in good agreement with the values given in the table.

The choosing of a salt was dictated by two factors. First, that it be, if possible, non-toxic (sodium nitrite proved to be an exception as discussed later, though its use was eventually discarded). Second, that it provided a relative humidity below that which fungal and bacterial spore germination can occur. Almost all propagules could be expected to be contaminated with saprophytic and parasitic fungi and

bacteria (Christensen and Lopez, 1963). Fortunately these all appear to have a minimum relative humidity requirement for germination. For almost all, this requirement is above 70%, though for one fungus it is as low as 65% (Snow, Chrichton and Wright, 1943).

Several species of propagule were stored for four years at 65% relative humidity and showed no sign of mould growth. However, slight growth was observed on some onion seeds stored at 75% relative humidity. This was taken to indicate that any mould spore present were not germinating at the highest relative humidity commonly studied.

Samples of a propagule were transferred to various humidistats and weighed daily until they reached a constant weight. At this point it was assumed that the sample had achieved an equilibrium moisture content (see Section 2-6 for discussion of this) corresponding to the relative humidity pertaining in the humidistat.

Exposure of the propagule to tritiated water vapour was commenced as soon as possible after the propagule had reached its equilibrium moisture content.

The humidistats were not always stored at 24C. For practical reasons humidistats were often left on the laboratory bench for long periods and suffered the usual fluctuations in laboratory temperatures. In experiments to find the changes of resting metabolism with time the propagules would have been stored under variable temperature conditions. However, the humidistats were always returned to 24C for several days before samples were removed for exposure to tritiated water vapour.

### 3-2.2 Exposure of the Propagules to Tritiated Water Vapour at Constant Relative Humidity — The Manifolds

The first important task in the experimental work was to design a suitable apparatus for carrying out the exposure of the propagules to tritiated water vapour. This involved a considerable amount of careful thought, taking various factors into account. The apparatus that was finally constructed was called a 'manifold' for reasons that will be

obvious. Below is a discussion on an earlier apparatus used to study resting metabolism with tritiated water vapour, followed by a discussion on the factors that influenced the development of the manifolds.

In 1965 Mann carried out some preliminary studies on exposing plant propagules to tritiated water vapour. The apparatus used was referred to as a 'pentapus' (see Figure 3.1). The central 'leg' contained a saturated solution of the appropriate salt in tritiated water. The outer legs contained dried propagules. After a certain exposure time one outer leg was removed by melting the glass at the restriction and drawing the leg away. This left the remainder of the pentapus sealed and the removed specimen could be dealt with separately. This entailed opening the sealed tube in a fume cupboard in the high level radiochemistry laboratory, extracting the sample and analysing the extracts for tritium labelled metabolites. The remaining specimens from the original pentapus could be dealt with later as required.

The author initially used this technique but felt that it had the following disadvantages:

(1) The propagules were not apparently equilibrated to the relative humidity at which they were exposed to tritiated water vapour. It was felt that this was a possible source of ambiguity as some metabolic changes may be associated with the dehydration or rehydration of the sample as the moisture content adjusts to the equilibrium value corresponding with the relative humidity being studied. (No mention is made in Mann's publication of previous treatment of the propagules but it is likely they were stored over silica gel, relative humidity = 18%, prior to placement in the pentapus.) Consequently the metabolism detected by Mann in his preliminary experiments could be a combination of resting metabolism at the humidity being studied, plus any metabolic changes occurring in the initial stages before the propagule had reached an equilibrium moisture content.

FIGURE 3.1

A Pentapus: The central 'leg' contains a saturated salt solution in tritiated water. The outer 'legs' contain botanical specimens.

(2) The author felt that the total volume of air in the pentapus was too small to ensure that gross changes in the gaseous composition did not occur during the course of the exposure. The apparatus designed by the author had a volume of approximately one litre. The work of Bartholomew and Loomis would indicate the carbon dioxide production by grain of *Zea mays* under conditions typical of those used in the author's experiments would be less than one μ mole. This amount would not appreciably alter the total fraction of atmospheric carbon dioxide (equivalent to approximately 15 μ mole) initially present in the manifold. Changes in oxygen concentration are less of a problem as the initial amount of oxygen is 1500 times as great. An experiment was also devised to ensure that no changes occurred in the conditions used in the type of experiments undertaken in this thesis. Two manifolds were set up each connected to a mercury manometer. Both contained four samples of propagule and had a salt solution to maintain a relative humidity of 64%. One manifold also contained a piece of filter paper soaked in NaOH solution that had been previously equilibrated to 64% relative humidity. A third manifold was set up as a thermobarometer. After three weeks storage at 24 C no change was detectable in either manometer. This indicated that the changes in oxygen and carbon dioxide partial pressures were less than that detectable by the mercury manometer, that is to say, less than 1 mm of mercury. As the partial pressure of the least abundant gas associated with metabolism, carbon dioxide is about 2.3 mm of mercury, we can state that no gross changes of the gases in the manifold have occurred.

However, it was recognised that localised changes in gas composition could occur unless precautions were taken. Consequently, the manifolds were designed to facilitate the diffusion of gases. This was achieved by making passageways as short as practicable.

(3) The other disadvantage of the pentapus was in removing samples

for analysis. First, it was a delicate operation removing a leg of the pentapus, incurring an appreciable health hazard, particularly as 0.5 Curie of activity was involved. Furthermore, the glass blowing necessitated heating part of the apparatus to red heat and it was uncertain how this affected the remaining samples. The procedure may have given rise to gross fluctuations in the relative humidity and temperature of the atmosphere in the pentapus. So the manifolds were designed with easily removable vessels attached to standard ground glass joints.

(4) A problem recognised by Mann, but not dealt with experimentally, was that of radiosensitivity of the propagules to tritium. Experiments described in this thesis give results that indicate Mann's experiments involved exposure time lethal to the propagules. Exposure times used in this thesis were below lethal levels, as was demonstrated by the fact that those tested still germinated.

Specification of the Manifolds:

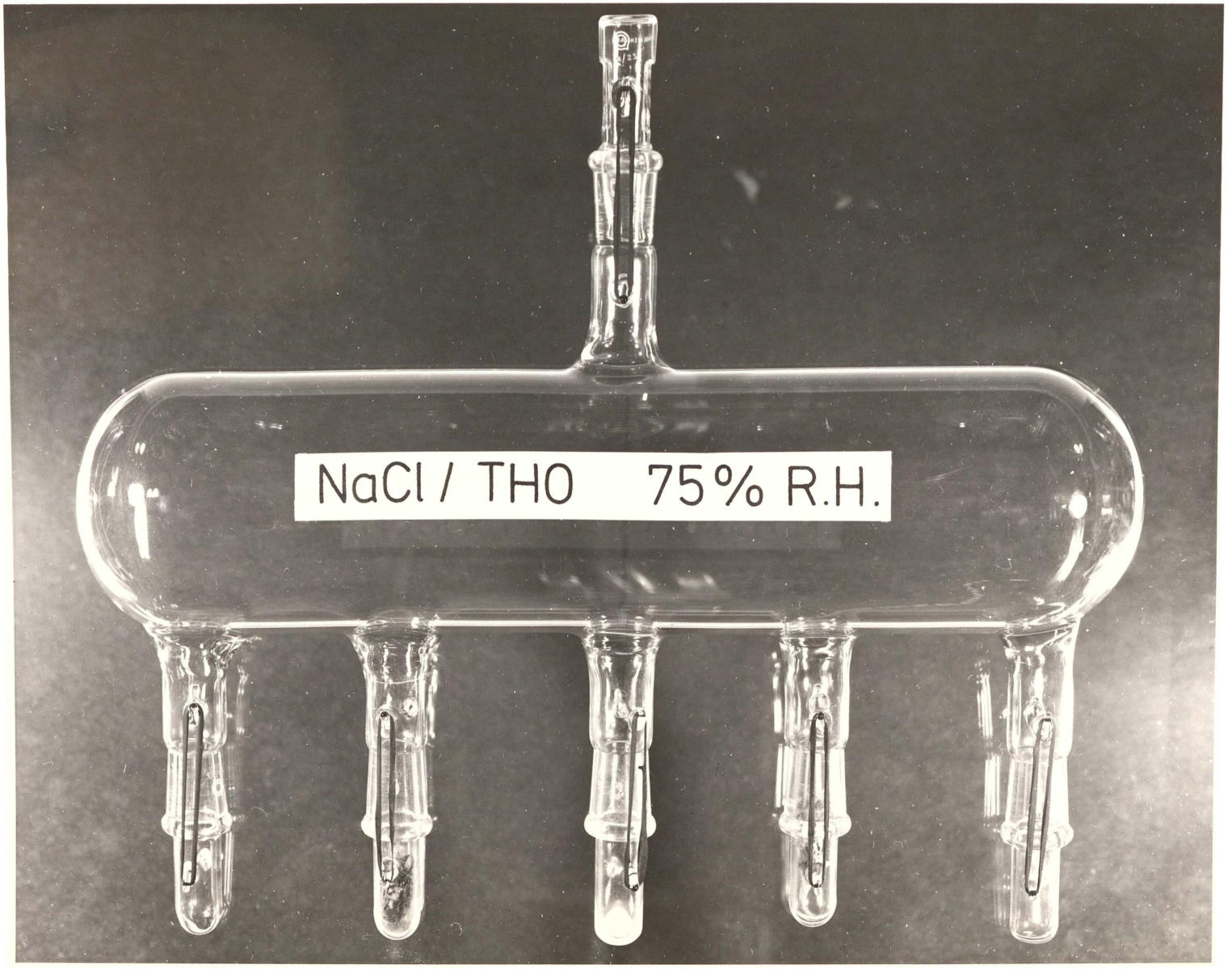
The manifolds were designed in consultation with the university glassblower, Mr R. Barbour. They consisted essentially of a glass cylinder 70 mm diameter and 300 mm length with five B14 female ground glass joints arrayed longitudinally (see Figure 3.2). Some examples had a tap at one end connected to another B14 joint. Small vessels having a B14 male joint, containing botanical specimens could be plugged into the ground glass joints of the manifold. Each vessel was 10 mm diameter and 30 mm long. The joints were greased with apeizon M grease to prevent escape of tritiated water. Both the manifolds and the vessels had small glass hooks on them so that the vessels could be held on firmly with rubber bands.

Preparation of the Manifolds:

The constant relative humidity in the manifolds was maintained by a saturated salts solution of the appropriate salt in tritiated water (5 Curies.$m\ell^{-1}$).

FIGURE 3.2

The Exposure Manifold. The central vessel contains a saturated salt solution in tritiated water, providing a constant relative humidity throughout the vessel. The other vessels contain botanical specimens.


NaCl / THO 75% R.H.

A problem arose over the possible dilution of the tritium by protium ($^1H$) present in the salt. Consequently, anhydrous salts were used wherever possible. All salts that required dehydration were ground finely and left over phosphorus pentoxide for several days in a desiccator that had been evacuated with a rotary oil pump. Heating was not employed as many of the salts used decomposed on heating (e.g., $MgCl_2 \cdot 6H_2O$ decomposes to MgCl(OH) on heating.

There are three problems associated with choosing a suitable salt for the tritiated water vapour manifolds:

(1) Some salts could not be dehydrated by the technique used. However, the amount of water left was insufficient to decrease greatly the specific activity of the tritium. For example, magnesium chloride hexahydrate was converted to a dihydrate on drying, but this diluted the tritium by only 20%.

(2) One salt chosen, ammonium nitrate, and used for eighteen months gave very confusing results. The total amount of labelled compound that appeared on chromatograms was much less than expected. The problem was not clearly defined until a scintillation counter was installed at this university, and it was found that tritium incorporation from these experiments was consistently lower than expected. Analysis of scintillation counting of extracts showed the specific activity of the tritiated water in manifold with saturated ammonium nitrate solutions was much lower than in other manifolds. Finally, it was realised that the protons of the ammonium ions ($NH_4^+$) were diluting the radioactive trace by a factor of three times!

A third problem was encountered in trying to find a substitute for ammonium nitrate.

(3) The first salt used to replace ammonium nitrate was sodium nitrite as it gave a similar relative humidity. As mentioned previously, care had to be taken not to choose toxic salts. It was

considered that nitrites, although toxic to animals are not particularly toxic to plants. A humidistat was set up containing saturated sodium nitrite and a trace of sodium hydroxide to inhibit formation of oxides of nitrogen. No such compounds were detected after three weeks so a tritiated water vapour manifold was set up using sodium nitrite. However, after a few weeks a distinct brownish tinge could be seen in the atmosphere of the manifold. It was supposed that the decay of tritium had given rise to oxides of nitrogen — in particular brown dinitrogen tetroxide. It was decided the presence of this gas was not conducive to experimental consistency so the use of this manifold was discontinued and results obtained from it were discarded.

Finally magnesium acetate was chosen as a salt to produce a relative humidity of 64% in place of ammonium nitrate.

A slight excess of the salt required to produce a saturated solution with 100 µℓ of tritiated water was weighed into a manifold vessel. A Pasteur pipette, which had been roughly calibrated to 100 µℓ, was used to transfer this amount of tritiated water into the vessel. The pipette was immediately discarded into a large volume of water to dilute the traces of radioactivity remaining. The vessel was promptly plugged into a manifold, which had all openings sealed with empty vessels to prevent the escape of the tritiated water vapour. All vessels were firmly secured with rubber bands. The vessel containing the salt and tritiated water was warmed to about 40 C to ensure the complete saturation of the solution. Then the manifold was placed in an incubator at 24 C and left for at least one day to ensure that vapour equilibrium had been attained. The solution was examined for excess salt to check for saturation.

Introducing Specimens to the Manifold:

Preparation of the vessels containing propagules varied slightly according to the type of propagule used. Any specimen put into a

vessel was weighed to enable later calculation of tritium uptake per unit mass. After the weighing procedure the vessel containing the specimen was returned to the storage humidistat to re-equilibrate it in case of any surface exchange of water vapour in the laboratory atmosphere. After this the vessel was rapidly plugged into a manifold — an opening was made available immediately beforehand to prevent upsetting the atmosphere of the manifold. The manifold was returned to the incubator and left until an extraction was to be carried out.

The introduction of the propagule into the manifold did cause dilution of the tritiated water present due to the moisture associated with the botanical material. Because of this, certain procedures were adopted to minimise inconsistencies attributable to this dilution. For example, if a short and long exposure of the same type of propagule was intended, both samples were introduced simultaneously. Consequently both samples were exposed to the same specific activity tritiated water vapour.

When the tritium in the saturated salt solution became seriously diluted after several experiments, fresh solutions were prepared.

When a series of experiments were completed, air was drawn through the manifold for a few minutes by attaching the top opening to a vacuum pump and removing a stopper from one of the bottom openings. This ensured that changes in the gaseous composition of the manifold atmosphere did not accumulate over a series of experiments.

## 3-3 Development of Techniques for the Extraction of Metabolites from Plant Propagules

### 3-3.1 Discussion

It was recognised that efficient extraction procedures would be very important in order to detect the expected very low rate of resting metabolism. Consequently, a number of different extraction procedures were experimented with until a very efficient one was adopted, as described below. This section deals with the problems involved with extraction of metabolites from plant propagules. The next section describes the technique finally adopted.

Wilson and his colleagues have used tritiated water to study the metabolism of plant propagules in this laboratory and at Victoria University of Wellington since 1963. The original extraction technique involved two extractions with absolute ethanol, or methanol, followed by two extractions with water. The ethanol extract contained small molecule alcohol soluble metabolites and the water extract contained some less soluble small molecule metabolites and a certain amount of soluble macromolecules. This technique had the following disadvantages:

(1) Some substances were partially extracted by alcohol and partially by water (e.g., glutamic acid, sucrose). This did often help in tentative identification of the metabolite but made it difficult to estimate even roughly the comparative intensity of labelling of various compounds. This was particularly so as the fraction extracted by the alcohol varied greatly with the degree of hydration of the alcohol.

(2) The ethanol extract contained lipids which interfered with the chromatography of the more polar compounds. This restricted the loading of chromatograms and gave rise to poor resolution.

(3) The water extract contained macromolecules that interfered with chromatography of small molecule metabolites.

(4) Biochemical changes could occur during the initial stages of the extraction procedure as it often took several minutes to grind some of the larger propagules used. One suggested solution to this problem is to put biological material into boiling methanol to 'kill the organism instantly'. Whilst the reasoning behind this procedure can be appreciated, on further thought it makes little sense biochemically. During the time taken for the methanol and heat to penetrate the biological material (and seeds are particularly stable to heat) major changes could occur, facilitated especially by the rising temperature. With tritiated water there is also a considerable health problem as the escaping alcohol vapour would be tritiated (from exchange with tritiated water).

In experiments with resting metabolism it was recognised that only small amounts of radioactivity may be incorporated by the propagules, so it would be desirable to have extracts which would allow heavy loadings on the chromatograms. Also, as the rate of metabolism, if any, would be very low it was obviously desirable to minimize any non-metabolic changes during extraction no matter how slight. An extraction technique of Bieleski and Turner (1966) was investigated and found to be perfectly suited for the experimental requirements of this thesis.

Bieleski and his co-workers have done a great deal to advance the cause of the extraction and analysis of metabolites from plant material. Several of his elegant techniques have been used by the author in the work described in this thesis.

3-3.2 Description of the Extraction Procedure Finally Adopted

MCW Extraction: Solvent methanol:chloroform:water 12:5:3.

A 3 mℓ aliquot of this solvent was cooled to -30 C in an all glass tissue grinder, then the botanical sample was placed in it. Seeds were macerated by a hand powered grinder, pollen and spore

with a motor-driven grinder. Spore required powdered glass <400 mesh to achieve efficient maceration. The suspension was centrifuged and the supernatent transferred to a centrifuge tube. The residue was resuspended in another 3 mℓ of MCW solvent at 0 C and centrifuged again. The supernatents were combined to give the 'MCW extract'.

Water Extraction:

The residue was then resuspended twice in 2 mℓ aliquots of distilled water and the supernatents combined to give the water extract.

Fractionation of MCW Extract:

Meanwhile the MCW extract had 1.5 mℓ chloroform and 2.2 mℓ of water added, and decanted four times to ensure thorough mixing. This was then centrifuged, resulting in two layers. The top layer was principally methanol and water. It contained water soluble, small molecule metabolites. It is referred to in this thesis as the 'methanol extract'. This was carefully drawn off with a Pasteur pipette and transferred to a pear-shaped flask. Five mℓ of water were added to this to prevent bumping during subsequent freeze-drying. The lower layer was principally chloroform and methanol. It contained lipids and lipid-like materials. It is referred to in this thesis as the 'chloroform extract'. This was drawn off with another Pasteur pipette into another pear-shaped flask. Care was taken not to contaminate the two layers with each other.

All three extracts (methanol, chloroform and water extracts) were then freeze-dried to remove volatile radioactivity. Care had to be taken with the chloroform and methanol extracts not to allow them to heat up too quickly or violent boiling ejected material from the flask. The residues left after freeze-drying were then subjected to various forms of chromatographic analyses described in the next chapter.

The Solid Residue:

The residue left after the extraction procedure was first washed three times in 10 mℓ of distilled water to remove any traces of volatile

tritium. The washed residue was then resuspended in a small quantity of water (one to three mℓ), and a drop of this suspension was placed on a piece of chromatography paper and left for several days to dry. This was then tested for tritium activity by scintillation autoradiography.

3-3.3 Advantages of the Extraction Technique

Bieleski (1964) began a paper on the problem of halting enzyme action when extracting plant tissue with the sentence 'The composition of a tissue extract should reflect the state of the tissue at the moment of sampling'. To achieve this it is obvious that enzyme inactivation must occur at the beginning of the extraction process. The common practice of extracting tissues with absolute or 80% ethanol, or methanol, at room temperature will not inactivate all enzymes at the moment of sampling. At the very least enzymes will remain intact until the maceration process is complete, when the alcohol will denature most of the enzymes. Even so, it is known certain enzymes, such as phosphatase,can survive and operate in cold alcoholic solution (Ullrich and Calvin, 1962; Ullrich, 1963). One answer would be to kill the organism by transfering it to boiling methanol. As mentioned previously, while this procedure may lead to death of the tissue, it may actually facilitate chemical or even biochemical activity during the brief period taken for the solvent to penetrate the tissue. Killing the tissue may not necessarily lead to enzyme inactivation. Phosphatase action and certain hydrolytic reactions have been implicated in post mortem changes of bulky tissue killed by boiling alcohol (Bieleski, 1963).

As an alternative to boiling Bieleski (1964) suggested transferring the tissue to a solvent well below room temperature. This makes much more sense chemically as all reaction will be greatly retarded whether the tissue has been killed or not. While at this low temperature the tissue may be macerated thus destroying enzymic organisation and allowing the solvent to penetrate cellular barriers and denature enzymes as much

as possible. Also the dissolution of substrates throughout the bulk of the extraction solvent will greatly reduce their concentration, further militating against effective catalysis by any still active enzymes.

Bieleski (1964) adduces evidence to show that effective phosphatase activity was much less during extraction at -25 C than at 70 C. However, even at -25 C phosphatase activity was still measurable and the destruction of the enzyme was very slow.

Phosphatases, however, appear to be particularly robust enzymes and many enzymes denature rapidly under the conditions of extraction used by the author. Bieleski (1963) used a solvent of methanol-chloroform-formic acid. The formic acid helped inactivate phosphatases and also assisted in extracting certain slightly soluble phosphate compounds. This solvent was used initially along with MCW (methanol-chloroform-water) but as no further tritium labelled compounds were extracted compared with MCW, its use was discontinued.

The MCW solvent had two advantages irrespective of low temperature use. The first was that it was much more efficient at extracting amino acids, and probably most other metabolites. Bieleski and Turner (1966) have shown that two extractions with MCW will extract as much amino acid as five extractions with 80% ethanol. This may be due to the presence of chloroform in the solvent which assists in rupturing lipid membranes in the cells. The second advantage was the separation of the MCW extract in two phases. A lower chloroform phase containing lipids and the upper methanol phase containing small molecule, water soluble metabolites.

Consequently each class of compounds could be chromatographed without interference from the other. This lead to better resolution of compounds by chromatography.

## 3-4 The Question of Controls

One of the questions that may be asked of the results reported in this thesis is: Does the detection of tritium labelled metabolites truly indicate metabolism? The question of what constitutes metabolism is discussed earlier in this thesis (Chapter 2). However, it could be that the tritium labelling of compounds is a result of non-enzymic chemical processes. This could be seen as a criticism of the validity of the results obtained. In fact the results would still be valid, in that they would imply changes in the dry organism with time which may still have significance with respect to other aspects of anhydrobiosis.

It would obviously be desirable to distinguish 'chemical' and 'biochemical' processes in anhydrobiosis. As a consequence some consideration was given to the appropriateness of biochemical controls. The most obvious one was to use autoclaved organisms. Such controls were indeed used with *Pinus ponderosa* pollen and *Sinapis alba* seeds. The sample of biological material was autoclaved for fifteen minutes at 121 C in a standard autoclave. The autoclaved sample was transferred to a humidistat and left to achieve an equilibrium water content. The sample was then transferred to a tritiated water vapour exposure manifold of appropriate relative humidity and left to expose for several days. The exposed sample was then treated in the same manner as other organisms. Results from *Pinus ponderosa* pollen and *Sinapis alba* seeds (discussed later) showed no incorporation of tritium except for one faintly labelled compound in *Pinus ponderosa* pollen. The compound had unusual chromatographic qualities and did not correspond to any of the metabolites found in live pollen.

However, this author does not regard autoclaved organisms as a completely valid control. It is possible that some unstable compounds, involved in non-metabolic processes when the live organisms are exposed to tritiated water vapour, are destroyed by autoclaving. Hence

non-metabolic reactions that may be occurring in live seeds would not be detected. Consequently another control was devised that may have more chemical justification. This involved extracting a sample of the biological material with the extraction solvent normally used, methanol: chloroform:water, 12:5:3 (MCW — see above). This extract was freeze-dried, resulting in a dry mixture of the metabolites normally extracted by the extraction procedure. The dried extract, referred to as 'MCW control', remaining in the freeze drying flask was transferred to a humidistat and left to equilibrate (*ca.* three days). The flask was then attached to a tritiated water vapour exposure manifold of appropriate relative humidity and left for a period of days. The flask was then washed out with MCW and the resulting solution treated as any other MCW extract to give a methanol fraction and a chloroform fraction.

It is suggested that this represents a more strictly chemical control because, (i) the extration with MCW is unlikely to cause any gross changes to the metabolites normally extracted by this solvent, (ii) it is very unlikely that any enzymes would be extracted by, and remain operative after the extraction procedure. However, it must be conceded that the metabolites extracted are no longer organised in the way they were in the original organism and this may well affect their 'chemical' as well as their 'biochemical' behaviour. Nevertheless, a number of control experiments of this type were carried out. The results from these were used in conjunction with those obtained from autoclaved controls.

Results from the MCW control showed no tritium incorporation in small molecule water soluble metabolites.

## 3-5 Use of Tritium as a Biological Tracer

### 3-5.1 Isotope Effect

In chemical reactions isotopic fractionation may be due to difference in equilibrium constants or in reaction rates (Duncan and Cook, 1968). This effect becomes more pronounced as the mass difference between the isotope increases. The mass difference between protium ($^1H$) and tritium ($^3H$) is greater than for any other isotopes. Theoretically then, the potential degree of fractionation of $^1H$ and $^3H$ could be large. However, in practice, this does not appear to be the case.

Fractionations of hydrogen isotopes in major chemical pathways of plants and animals have been reviewed by Brunner (1973). Values for H/T fractionations have been reported to be in the order of 9% between carbohydrates and lipids (Smith and Epstein, 1970). However, fractionations were found absent in transpirational processes (Zimmerman, Ehhalt and Munnich, 1967). MacFarlane (1976) compared isotope ratios in various parts of plant tissue and found differences in the order of only 20%.

The above studies indicate that isotope effects even for hydrogen isotopes in biological systems is very slight. Furthermore, as only 0.11% of the hydrogen atoms were $^3H$ atoms in the tritiated water used, it is most unlikely that isotopic fractionation would affect metabolic pathways in the organisms studied.

It would also appear that although qualitative results could not be obtained because of varying fractionations between tritium and protium, tritium labelling can be used to infer qualitatively the path of protium in living systems.

### 3-5.2 Radiation Damage

In the technique for studying resting metabolism described earlier in this chapter biological specimens were exposed for several days to tritiated water vapour of high specific activity. The possibility of

major radiation damage is appreciable. However, it is difficult to assess accurately the radiation dose received by the specimen during the exposure period. Mann (1965) estimates the maximum dose received by *Sinapis alba* seeds imbibed in liquid THO at 5 $Ci.m\ell^{-1}$ to be $3 \times 10^5$ rads. Ballie (1965) has shown that radiation doses of more than $2 \times 10^6$ rads were lethal to *Sinapis alba* seeds. However, in exposure to tritiated water vapour some days would elapse before the specific activity of the tritium in the seeds reached 5 $Ci.m\ell^{-1}$. This would imply that *Sinapis alba* seeds could survive exposure times of at least six days.

Consequently it was decided to carry out simple germination tests of various propagules after exposure to tritiated water vapour. The most extensive tests were carried out on *Pinus ponderosa* pollen because a milligram of pollen would contain a statistically significant number of organisms. The scale of the manifold exposure apparatus was not such as to allow a large number of experiments with seeds. Nevertheless, a number of isolated tests were carried out on seeds.

In a series of experiments on *Pinus ponderosa* pollen at 64% and 34% relative humidity. A sample (*ca.* 30 mg) of pollen was exposed to tritiated water vapour at the appropriate relative humidities. After 1, 2, 5 and 10 days exposure a small portion (*ca.* 1 mg) of the sample was taken and put into a sealed test-tube with 0.1 mℓ distilled water. Controls were run with each germination test. After 24, 48 and 72 hours an aliquot of the suspended pollen was transferred to a small Hirsch funnel and thoroughly washed with distilled water to remove exchangeable volatile radioactivity. The pollen was resuspended in a very small volume of water and transferred to a microscope slide where it was observed under 40× and 100× magnification.

Control samples began germinating (> 30%) after 24 hours, and > 98% germination had occurred after 48 hours imbibition with water. After

two days exposure to THO vapour the germination rate of the pollen was obviously affected. After 24 hours less than 2% of the pollen had protruded germ tubes. After 48 hours roughly 50% of the pollen had germinated and after 72 hours germination was above 95%. The germ tube produced by the pollen exposed to THO vapour were shorter and less branched than those of the control.

After 5 days exposure to THO vapour germination was in the order of only 50% after 72 hours. Beyond this time fungal growth inhibited further study. After 10 days exposure to tritiated water vapour none of the pollen had germinated after 72 hours, although considerable swelling, as initially occurs with control samples, did take place.

The above results applied to both relative humidities studied, although a slightly more marked effect was noticed at 64% than at 34%. This suggests the radiation damage is largely determined by the specific activity of the tritium in the pollen rather than the total activity.

It was decided as a result of the above experiments to expose the pollen to THO vapour no longer than five days. It should be mentioned that the minimum amount of radiation required to prevent germination is not likely to do so by altering metabolic patterns in the pollen. Critical radiation damage would first occur at higher levels of cellular organisation than that of the metabolic pathways. Consequently it was felt by this author that if an exposure time was chosen that still allowed some of the pollen to germinate it was not likely that basic metabolism would have been grossly affected. Hence the resting metabolism detected by the technique could be assumed to be at least qualitatively similar to that occurring in pollen that was not exposed to THO vapour.

Simple tests were carried out on small samples of *Sinapis alba* and *Alium cepa* seeds which showed that some germination of these seeds was possible after exposure to THO vapour for times commonly used in resting

metabolism experiments. It should be noted, as with pollen, that the germination percentage and germination rate was lower with exposed seed than with controls.

It can be seen from the above discussion that radiation damage of speciments exposed to THO vapour cannot be said to be insignificant. While the overall metabolic pattern is unlikely to have been grossly affected if the propagule can still germinate, albeit more slowly than usual, it is possible that one or more labelled metabolites detected may be a consequence only of radiation damage. As a result a degree of caution may be advisable when interpreting results. If one particular compound were to appear labelled in all resting metabolism experiments then this could be the result of some radiochemical process. This can be dealt with to some extent by controls for the tritiated water vapour exposure experiments.

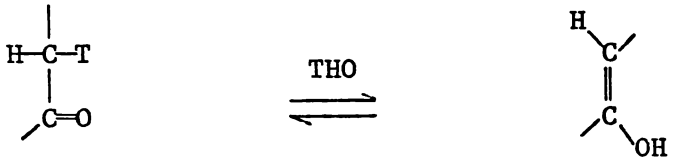
The question of radiation damage by endogenous tritium is an interesting one in itself. Most studies on radiation damage centre on radiation from an external source. This gives rise to ionisations within the irradiated organism which disrupts chemical processes occurring. If a radioisotope, such as tritium, is incorporated into an organism then not only is there the damage done by the radiation consequent upon a disintegrating tritium nucleus, but also one would expect disruption of the molecule of which the tritium atom may be part as ${}^{3}_{1}H$ is transmutted to ${}^{3}_{2}He$. Although in many metabolites this effect may be slight due to the low concentrations and relatively long half life of ${}^{3}H$, in some important molecules (e.g., DNA) this effect could be a crucial one. It is suggested that the technique of exposing tissues to tritiated water vapour described earlier in this chapter may well have application in the study of various aspects of radiosensitivity of biological material.

3-5.3 Interpretation of Results

When an organism metabolises in the presence of tritiated water, tritium is constantly being incorporated and eliminated. Three categories of incorporated tritium can be distinguished.

1. Labile tritium: Tritium ions will exchange readily with protons in such groups as —OH, —COOH, —SH, —$NH_2$. This tritium labelling will be just as easily reversed by back exchange during subsequent extraction and chromatography. Hence such tritium labelling will not be detected by the techniques described in this thesis.

2. Semi-labile tritium: Some reactions may give rise to tritium labelled molecules by slow exchange processes such as keto-enol tautomerism:

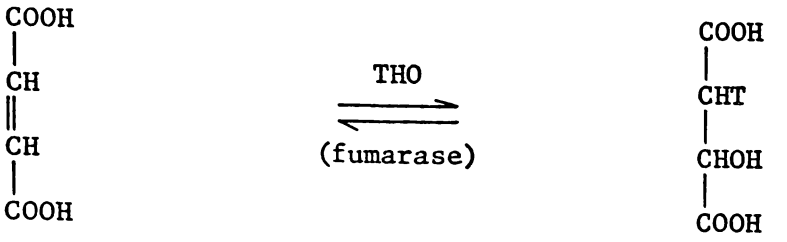



keto form enol form

Compounds labelled by such a process would probably lose the label again by slow back exchange with extraction and chromatography solvents. (Later in this thesis evidence is adduced to suggest that semi-labile tritium is present in extracts after extraction, but this is efficiently removed during chromatography.)

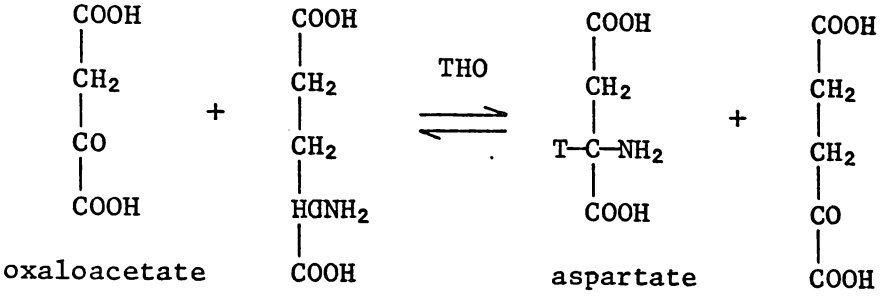
3. Non-labile tritium: Biochemical reactions, excluding those described in 2, occurring in tritiated water that give rise to the formation of C—T bonds will produce compounds with non-labile tritium atoms. Such tritium labelled compounds will be detected after chromatography as radioactive. Four examples of such reactions are given below:

1. Hydration of fumarate



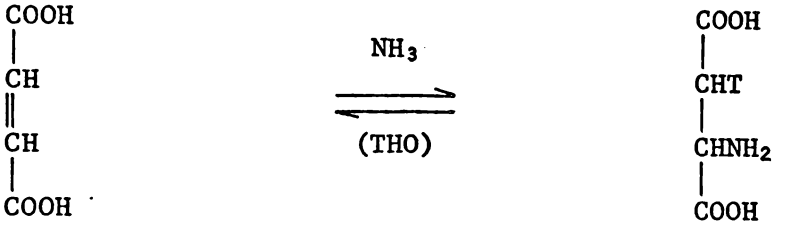

fumarate malate

2. Transamination of oxaloacetate



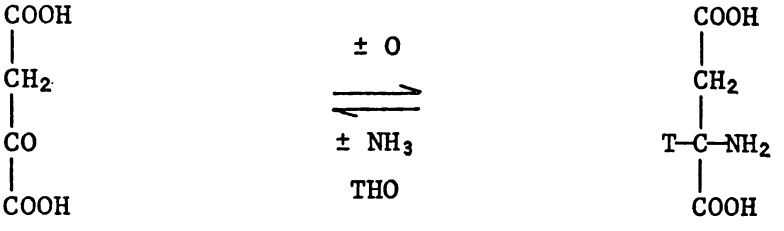

oxaloacetate glutamate aspartate 2-oxoglutarate

3. Amination of fumarate




fumarate aspartate

4. Reductive amination of oxaloacetate




oxaloacetate aspartate

One of the difficulties with using tritiated water in biological systems is in deciding what particular reaction is responsible for the tritium labelling of a compound. If aspartic acid were found to be labelled in an experiment then it would have acquired this label from

any of the last three reactions or a number of others. Further experimentation would be required to determine which reaction was responsible for the tritium labelling. In this sense the use of $^{14}C$ labelled compounds in biological systems gives rise to results that are more readily interpreted.

If labelled aspartate were detected in an experiment one obvious step would be to find where the tritium label was located on the molecule. It will be noted that in reaction 3, the tritium is located at $C_2$ and in reaction 4, it is located on $C_3$.

The possibilities for tritium labelling of compounds by an organism, metabolising in the presence of tritiated water, are extensive. Mann (1965) has surveyed all the enzyme catalysed reactions known at the time of the survey, and attempted to predict which of these would give rise to tritium labelled metabolites if they occurred in the presence of tritiated water. As Mann points out, this cannot be done with a high degree of confidence, especially if the mechanism of the reaction is unknown. For instance, stereo-specificity requirement may lead to no incorporation of tritium where one might otherwise expect it.

If aspartate were not found to be labelled by a biological system metabolising in the presence of tritiated water then it is possible to exclude the possibility of certain reactions involving aspartate such as the three given above. However, it is not correct to assume that no reactions involving aspartate are occurring, as some reactions may proceed without the incorporation of tritium.

Another problem is that if no labelled aspartate is detected it may be that it is just below the level of detection. It does not follow that a low level of labelling of a compound implies a low level of metabolism of that compound. If the compound has a very rapid turnover and small metabolic pool size, the amount of tritium labelled compound at any instant may be small. This dilemma may be resolved by resorting to very

sensitive techniques of detection. It is fortunate that scintillation counting can detect even just a few tritium disintegrations, so that if a compound is purified it should be possible to say whether it has a low level of radioactivity or none at all.

To summarise then, it is advisable to be cautious about making definite conclusions from results of experiment involving tritiated water. Such experiment, however, will often allow the development of hypotheses which may be tested with further experimentation.

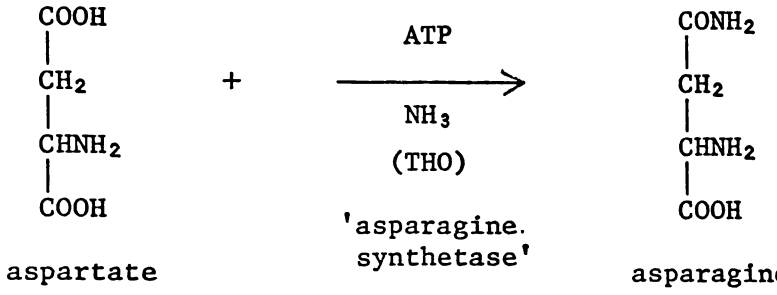
## 3-6 Potential of Studies on the Rate and Total Amount of Tritium Activity

Once an organism has been stored for some time at constant water activity (i.e., relative humidity) then it would be reasonable to expect that a steady state condition exists. This contrasts with the situation in, for instance, a germinating system. The existence of a steady state condition could allow the determination of various parameters associated with resting metabolism.

Exposing the organism to tritiated water vapour should allow in principle an *in vivo* measure of enzyme activity. For instance, studying the rate of incorporation of tritium into a metabolite may give information about the relative importance of different reactions. It must be stressed here that in such studies isotope effects would have to be taken into consideration.

Information about the specific activity of a labelled metabolite once the steady state has been reached and the time taken for this, may suggest something about the organisation of various metabolites in the organism. For instance, if a compound extracted from the organism rapidly achieves a relatively low steady state specific (tritium) activity, this could imply that only a small fraction of the extracted compound is involved in metabolism. Further, changes in specific activity with relative humidity may indicate something about the organisation of a metabolite in an organism. For instance, a sharp decrease in specific activity of a metabolite below a particular relative humidity may indicate that only a small pool of the total amount of metabolite present is being metabolised at the lower relative humidities. It is quite possible that at very low relative humidities, when viscosities were particularly high, only the metabolite immediately adjacent to the enzymes responsible for its metabolism will be affected.

Tritium can be incorporated into a metabolite directly by *de novo* formation of C—T bonds or indirectly by its formation from a metabolite already containing a tritium atom. For instance, asparagine can be formed from aspartate but no tritium would be expected to be incorporated if the reaction occurred in the presence of tritiated water.




aspartate asparagine

However, if the aspartate were already tritium labelled then tritium labelled asparagine would result. By studying the nature of the increase of tritium activity in the two compounds with time, it may be possible to show whether or not the labelled asparagine has been formed from aspartic acid.

It is possible that many more questions could be answered by such studies as those suggested above. However, it must be emphasised once again that any quantitative studies would have to take into account the effects of isotopic fractionation. This may necessitate carrying out reactions *in vitro* to determine experimentally the degree of isotopic fractionation in any particular reaction.

The work described in this thesis did not include any quantitative study on the rate of labelling of metabolites, but was confined to qualitative studies aimed at finding which metabolites are involved in resting metabolism. The above discussion was to show another obvious use of the technique developed, which would allow the discovery of information otherwise unobtainable by other techniques.

# CHAPTER 4 ANALYTICAL METHODS

## 4-1 Introduction

It has been suggested before that if resting metabolism occurs at all its extent will be very slight. It can be assumed then that even with the high specific activity tritiated water used in the technique described in Chapter 3, the amount of tritium incorporation will be small. Consequently the author had to explore many avenues for increasing the sensitivity of tritium detection. To this end, considerable effort was put into refining chromatographic and scintillation autographic techniques. This chapter describes the standard techniques adopted after the investigation of several possible variations. There are allusions to some of the techniques investigated though these are not discussed exhaustively.

Another important characteristic required of the chromatographic procedures was that they were capable of resolving a wide range of metabolites and that there is enough information about each compound to enable a tentative identification of each metabolite that has a reasonable probability of being correct. This last characteristic was achieved in part by the use of more than one chromatographic technique for a particular extract.

## 4-2 Chromatographic Analysis of Extracts

### 4-2.1 Methanol Extract

This extract, obtained in the manner described in Chapter 3, was by far the most important of the three extracts as it contained most of the compounds that had incorporated non-exchangeable tritium. The methanol extract contained most of the water soluble, small molecule metabolites, principally amino amino acids, sugars, organic acids and most sugar phosphates. In all cases this extract was redissolved in 100 µℓ of 10% isopropanol in distilled water.

Paper Chromatography:

In previous work with tritiated water on the germination of plant propagules, two-dimensional paper chromatography was used to analyse extracts containing small molecule water soluble metabolites. The author experimented with a wide range of solvent combinations but eventually adopted the pair used by previous workers, i.e., phenol, water, followed by butanol, propionic acid, water.

For each extract two paper chromatograms (150 cm × 150 cm Whatman # 4) were loaded, one with a single application of 5 µℓ of redissolved extract and the other with four applications of 5 µℓ with thorough drying of the chromatograms following each application. When the origins were finally dry the chromatograms were developed by descending chromatography (see, for example, Bassham and Calvin, 1957). At no time were the extracts or chromatograms exposed to high temperature, to avoid any thermal decomposition.

Solvents:

First direction: Phenol, water. 100 g redistilled phenol mixed with 29 g of distilled water. This was prepared in bulk and stored at 4 C in the dark.

Second direction: Butanol, propionic acid, water. 6:3:4, v:v:v mixed immediately before use.

After each development chromatograms were dried overnight in a high

speed fume cupboard.

Thin Layer Chromatography:

A survey of literature on chromatographic and scintillation autographic techniques indicates that thin layer chromatography allows greater sensitivity of detection as well as greater resolution than paper chromatography. Randerath (1970) contends that tritium on thin layer chromatograms can be detected with a sensitivity of fifty times that of paper chromatograms. In the author's opinion this claim is rather optimistic; however, the much greater resolution of thin layers gave chromatograms that concentrated the activity of a compound into a much smaller area. This had the effect of increasing the overall sensitivity of detection.

It was realised that the total amount of tritium incorporated into metabolites would be small, particularly at low relative humidities and a number of thin layer techniques developed by Bieleski and Turner (1966) and Turner (1966), which would allow detection of even faintly labelled compounds, were investigated. The thin layers employed by Bieleski consisted of a mixture of silica gel and cellulose. Such layers were superior to those containing only one of the two ingredients. For example, silica gel layers form compact spots with amino acids but have low RF values. Cellulose layers have a wide range of RF values but spots show pronounced 'tailing'. Mixed layers form compact spots over a large range of RF values with amino acids.

These mixed layers were used to analyse extracts by electrophoresis in one direction followed by chromatography in a second direction and this proved very successful for separating and detecting tritium labelled amino acids. The mixed layers were also used for two-dimensional chromatography. A variety of solvent combinations were experimented with but the sensitivity of tritium detection seemed much lower than with electrophoresis followed by chromatography. This was

rather an enigma, and although a large number of two-dimensional thin layer chromatograms were produced, their results were of little value.

Mixed Layer Preparation:

4 g silica gel H (Merck), 10 g cellulose MN300 (Maschery Nagel) and 80 mℓ of water for 250 μm layers;

7 g silica gel H (Merck), 17.5 g cellulose MN300 (Maschery Nagel) and 130 mℓ of water for 500 μm layers.

The materials were mixed in a high-speed blender and spread on thoroughly cleaned 200 × 200 mm plates with a Quickfit spreader. The plates were left to air dry for a few hours. They were then left in an incubator overnight at 40-50°C. The plates were used directly for electrophoresis (see below) but were washed if used for chromatography. This washing was to prevent serious 'tailing' of organic acids and began with an initial development with 1% oxalic acid (sometimes incorporated in the original slurry). This was followed by development with distilled water, then left overnight to develop with a B.A.W. solvent (see below). This treatment was shown to lead to much better resolution of organic acids.

Thin Layer Electrophoresis for Amino Acids:

A 25 mm wide origin band was lightly pencilled onto the layer 25 mm in from the side and 65 mm from one end (see Figure 4.1). Two 10 μℓ of the sample were applied to this origin band with thorough drying (by a cold air stream) between applications.

The electrophoresis buffer was applied by dipping the plate in a perspex tank containing buffer. The buffer was allowed to almost reach the origin and care was taken to ensure the plate was vertical in the tank. The plate was then quickly blotted with filter paper to remove excess buffer, and the other end immersed in the tank until the two buffer fronts met. Care was taken not to swamp the origin at any time with buffer.

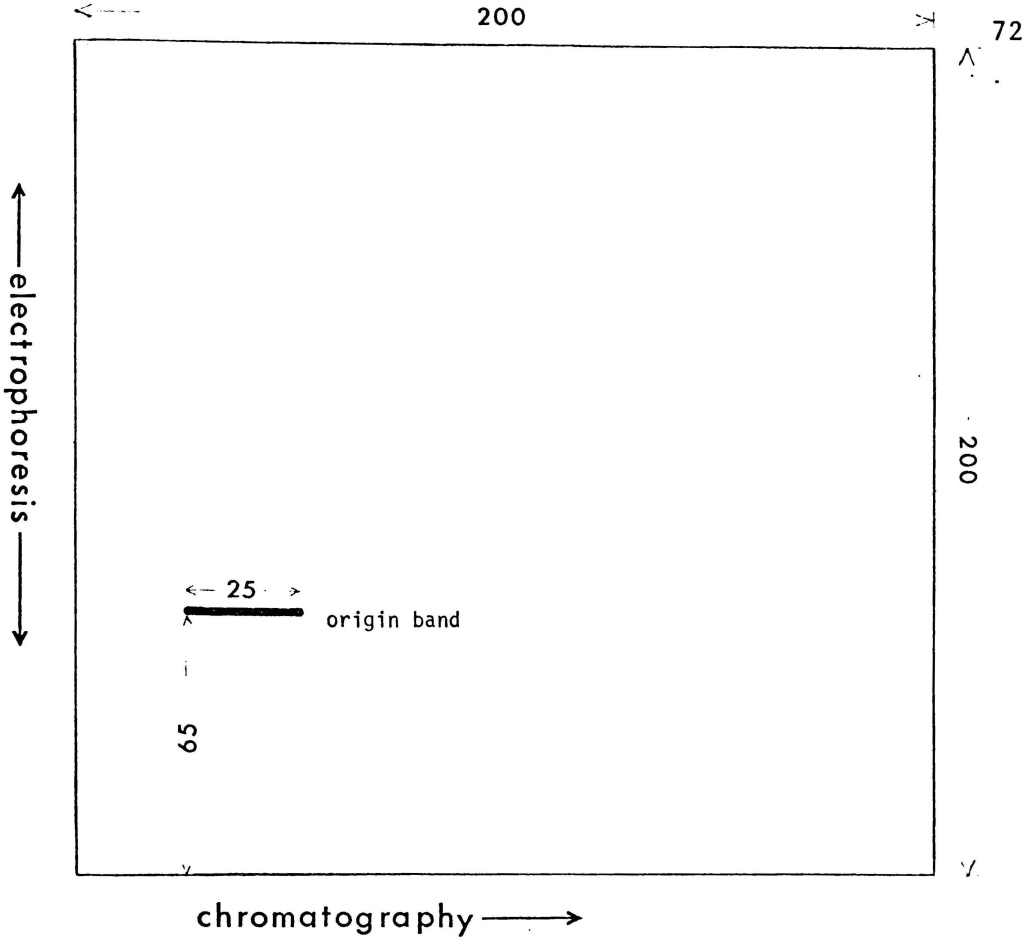



figure 4.1 : Thin layer plate for combined electrophoresis and chromatography

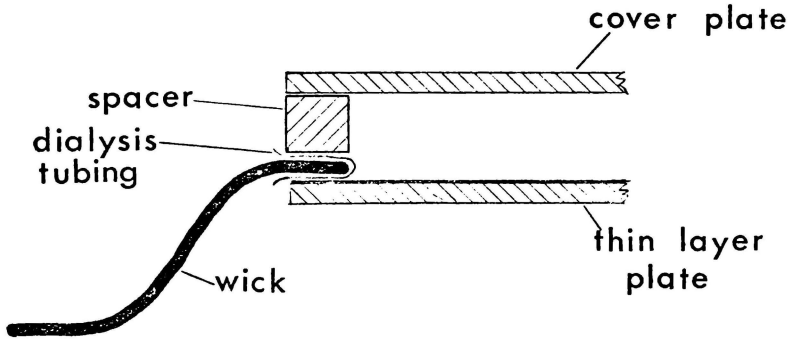



figure 4.2 : Detail of attachment of electrophoresis wick to thin layer plate

Electrophoresis buffer: pH 1.9

42 mℓ formic acid

142 mℓ acetic acid

Distilled water to 2.5 ℓ.

It was necessary to work quickly with the plate at this point to prevent evaporation. Two wet wicks had been previously prepared, each consisting of a strip of Whatman 3MM chromatography paper 80 × 200 mm, with one side sandwiched between a piece of dialysis tubing slit along one side. The dialysis tubing prevented syphoning of buffer onto the plate. These wicks were placed at opposite ends of the plate (top and bottom of Figure 4.1). Two perspex rods 200 mm long and 5 mm square cross-section were placed on the wicks and a 200 × 200 mm glass plate placed on top to prevent the wicks falling off (see Figure 4.2).

The electrophoresis tank had been prepared to receive this assembly. This tank had two buffer compartments each with a platinum electrode and 500 mℓ of buffer into which the wicks could reach. The tank was then filled with Varsol (a dry cleaning solvent supplied by the Atlantic Oil Company) previously colled to about 5°C.

The assembly was thoroughly immersed in the coolant in the tank, with the origin towards the anode, and the wicks pushed down into the buffer. The lid of the tank was closed and the depression filled with ice to keep the temperature of the coolant below 12 C. Above this temperature diffusion of compounds was liable to become unacceptably fast. The potential across the apparatus was adjusted to 1000 V which gave rise to currents of about 20 mA for 250 μm layers and 50 mA for 500 μm layers. After 20 minutes the current was switched off, the assembly removed from the tank and dismantled. The thin layer plate was stood vertically for three minutes on a piece of filter paper to allow most of the coolant to run off. The plate was then placed horizontally in a drying cabinet at 40°C for one hour to dry. All compounds on the

plate would now be in bands parallel to the origin. Compounds having an overall positive charge at pH 1.9, mainly amino acids, would migrate to the cathode and those such as phosphate esters, having a negative charge, would migrate to the anode. Sugars and organic acids having no charge would be on the origin. Electrophoretic drift usually carried the origin a few millimetres towards the cathode. Where sugars and organic acids were to be subject to separate analysis, the area of the layer containing them was cut out and this was extracted with 10% isopropanol and the resulting solution freeze dried.

The remaining bands on the plate were concentrated into spots. This was achieved by immersing the plate's righthand side (Figure 4.1) downward in a tank of water. The level of the water almost reached the bands and water was allowed to soak up the layer until it reached the other side of the bands. Surplus water was swabbed off the layer and a current of cold air was used to dry the plate. The compounds left on the plate were present as spots on the lefthand side (Figure 4.1) of the plate.

The plate was then developed from left to right (Figure 4.1) with two chromatography solvents.

Chromatography Solvents:

First: Butanone, pyridine, water, acetic acid (70:15:15:2);

Second: Propan-1-ol, water, propyl acetate, pyridine, acetic acid (120:60:20:4:1).

After development in the first solvent the plate was dried for one hour at 40°C. Both developments were carried out in an 'S' tank to maintain solvent vapour equilibrium.

Two-dimensional Thin Layer Chromatography:

A washed plate had an 25 mm origin band lightly pencilled 25 mm in from one side and 25 mm from the bottom. Ten µℓ of sample was applied to this origin and dried in a stream of cold air. A whole

range of solvents were investigated but finally the pair used by Turner was chosen.

First solvent: Phenol, water, 100:29 (as for paper);

Second solvent: Butanol, acetic acid, water, 12:3:5 (Smith, 1969).

After development in the first solvent (5-6 hours) the plate was dried thoroughly (overnight at 40°C).

The bands then present on the chromatogram were then concentrated into spots by allowing 1% acetic acid to soak across the plate at right angles to the original direction of development. After drying the process was repeated, otherwise 'tailing' of acidic compounds occurred. The dried chromatogram was then developed in the second dimension by the second solvent. 'S' tanks were used in both developments.

4-2.2 Chloroform Extract

This contained all the neutral and polar lipids extracted from the propagule. These two classes of compound require different techniques for analysis. A number of reference works were consulted, principally *Lipid Chromatographic Analysis* (Marinetti,1969) and *Thin Layer Chromatography* (Randerath, 1966), also a technique of Bieleski (1965) for dealing with polar lipids was investigated. What was required was a simple technique that separated the major groups of lipid or phospholipid to find what type of compound, if any, had incorporated a tritium label. The author finally developed two chromatographic systems for analysing the chloroform extract. One separated neutral lipids and the other separated phospholipids.

Layers: 50 × 100 mm strips of commercially prepared silica gel coated aluminium foil (Merck, Cat. No. 5562).

Application of Samples: Each extract was dissolved in 100 µl of chloroform methanol, 1:1. Loading tests with extracts from each propagule were carried out. According to these 1 to 5 µl of extract were applied with a volupette to the origin of the

chromatogram.

Chambers: Glass vessels with lids, of appropriate dimensions, lined with filter paper to provide solvent vapour saturation, were used.

Solvents for Neutral Lipids: Redistilled chloroform (developing time 15-20 mins). Although this is not a recognised solvent, in the author's opinion it is better than many others published. It has properties similar to dry ether (a commonly used solvent) but has several advantages:

1. It does not absorb water to the same extent, assisting consistency.
2. It is not quite so polar as ether and gives better separation at high RF values.
3. It always runs with a straight solvent front, which cannot be said for ether.
4. Peroxides which form in ether and are known to decompose lipids do not form in chloroform.

Solvent for Polar Lipids: Chloroform, methanol, water, 25:4:1 (development time 20-25 min). This is a standard solvent of known characteristics (Randerath, 1966) which gives good separation of polar lipids.

4-2.3 Water Extract

The main concern with the water extract was to find if any significant amounts of activity could be found in any macromolecule dissolved in this fraction. It was of course recognised that a great many types of macromolecules would be denatured during the initial methanol, chloroform, water (12:5:3) extraction. So this work should be regarded as merely exploratory.

As the water extract could well contain traces of small molecular weight compounds not extracted by MCW solvent, these had to be removed

before trying to detect tritium activity in macromolecular compounds. This was achieved by dissolving the extract in 0.1 µℓ of water and transferring 5 µℓ of the solution to the origin of a paper chromatogram. This was then developed in one dimension with butanol, propionic acid, water.

Any macromolecular compounds present in the water extract will have remained on the origin.

4-2.4 Solid Residue

The solid residue left after each extraction was resuspended in 1 mℓ of water and a drop of this suspension was transferred to a piece of chromatography paper. This was left to dry and exchange away volatile tritium in a high speed fume cupboard for one week. This was then tested for tritium activity by scintillation autography, see Section 4-2.

## 4-3 Detection of Tritium Labelled Compounds

The beta-emissions of tritium are too weak to allow tritium detection by radio autography. Earlier workers in this laboratory used the technique of liquid scintillation autography developed by Wilson (1960) to detect tritium activity on chromatograms. This technique is the photographic equivalent of scintillation counting. It involves exposing X-ray film to the chromatogram whilst they are immersed in a scintillating solution of 1,4 diphenyl benzene in toluene. Randerath (1970) has published a variation upon this technique that allows the exposures to be carried out in the dry state. Although this did not give any increase in sensitivity, it did reduce the health risk incurred by the previously described method. With the wet exposure method the experimentalist was exposed to toluene vapour as well as skin contact with toluene.

In Randerath's technique a chromatogram is dipped in 5% 2,5 diphenyl oxazole in ether and left to dry. This was then exposed to a sheet of X-ray film for 24 hours or more. The sensitivity was greatly increased by lowering the temperature to -80 C with dry ice.

In practice each chromatogram of methanol or water extract had three $^{14}C$ marker spots applied to the corners to allow alignment of the chromatogram with the film after development. The chromatogram was then dipped in 5% 2,5 diphenyl oxazole and dried in a high speed fume hood for 5 minutes. The chromatograms were left in the dark for a few hours to allow any phosphorescence to decay and then placed between two sheets of paper with a 200 × 200 mm sheet of X-ray film. The orange paper that surrounded each sheet of X-ray film proved opaque to the scintillated light. A stack of chromatograms, each in contact with a sheet of X-ray film, was placed into a black plastic bag which was placed in another black plastic bag. This was put into a heat insulating expanded polystyrene box then covered with dry ice and the

box was put into a deep freeze at -20 C. The dry ice took about 7 days to sublime. After this time the polythene bag containing the chromatograms was removed from the box and allowed to warm up to room temperature. The stack was dismantled and each sheet of film numbered with pencil in the top righthand corner, and the chromatograms stacked in order. The films were developed in a Kodak X-ray film developer and fixed with May and Baker Rapid Amfix with hardener. The films were washed in running water for 15 minutes then dried. Each film was then aligned with the markers and the position of tritium labelled compounds was indicated by dark areas. By pressing hard on the film with a pencil an outline of the radioactive areas could be made on the chromatogram.

A very similar procedure was adopted for lipid chromatograms except that scintillator impregnating solution was 5% 2,5 diphenyl oxazol in petroleum ether toluene, 4:1. This was used because ether would have dissolved some of the lipids from the chromatogram.

## Investigation into Increasing the Sensitivity of Tritium Detection

Randerath (1970) has shown that the sensitivity of tritium detection by dry scintillation autography (described above) was increased twenty-fold by lowering the temperature from 20°C to -80°C. This increase was attributed to a change in characteristics of the X-ray film as temperature decreased. The sensitivity of the cold dry technique was about the same as Wilson's (1960) liquid scintillation autography, i.e., approximately 10 n $Ci.cm^{-2}.week^{-1}$. It was reasoned then that lowering the temperature of the liquid technique might yield a significantly more sensitive technique.

The scintillant used in Wilson's technique 1,4 diphenyl benzene was only sparingly soluble in toluene at room temperature and crystallised out when the temperature was lowered. Consequently, the

liquid scintillant used was toluene containing 3 $g.\ell^{-1}$ 2,5 diphenyl oxazole. When an exposure was carried out using this procedure at -80 C the scintillation autograph obtained had a blotchy appearance and showed a decreased sensitivity. It was then found that the toluene became cloudy when cooled to -80 C, this would disperse the scintillated light and reduce the exposure of the film. The cloudyness was believed to be caused by water dissolved in the toluene. Various attempts were made to dry the toluene but only phosphorus pentoxide was capable of preventing the cloudyness at -80 C. Unfortunately the phosphorus pentoxide reacted with the scintillant. As water was absorbed very rapidly from the atmosphere, no way could be seen to render the toluene transparent at -80 C.

It was then decided to add 10% ethoxyethanol to the scintillant. This was found to provide a transparent solution at -80 C. Unfortunately when an exposure was carried out using this scintillant the film showed serious fogging and this was attributed to a chemically reductive effect of ethoxyethanol on the silver halide of the film emulsion. Methanol and acetone were both tried in place of ethoxyethanol but the fogging was worse in the case of methanol and no better in the case of acetone. At this point it was decided to give up this line of investigation and persevere with the dry technique to continue the study of anhydrobiosis.

This line of research may still be worth following. It is suggested that very pure solvent could be tried to prevent fogging the film, or finding a suitable desiccant to add to the scintillant. It might also help to handle solutions at very low relative humidity so that moisture was not absorbed from the atmosphere.

Despite a considerable amount of work no practical technique was found that was superior to that of either Wilson or Randerath. It is the author's opinion, however, that techniques of scintillation autography are well worth further study. It seems most likely than an

increase of an order of magnitude of tritium detection will be possible once the problems mentioned above have been solved.

## 4-4 Identification of Tritium Labelled Metabolites

### 4-4.1 Tentative Identification

Radioactive compounds detected by scintillation autography were at first identified by reference to standard maps of the various systems used. The use of thin layer chromatography assisted greatly in this initial tentative identification because of much higher resolution than that of paper chromatography. Also the existence of at least two chromatograms produced by different techniques provided much more evidence for the initial tentative identification. At this stage the thin layer plate obtained by electrophoresis followed by chromatography was sprayed with ninhydrin to identify the positions of amino acids. Any radioactive areas were carefully compared with ninhydrin positive areas. If a radioactive area was found to coincide in size and shape with an amino acid area (as detected by ninhydrin) it was inferred that this amino acid had probably incorporated tritium. This was further checked by co-chromatography.

Co-chromatography:

Once tentative identities had been assigned to radioactive areas, these areas were cut out of chromatograms and subject to co-chromatography. This was carried out principally with areas from paper chromatograms.

A section of a radioactive area was cut from the chromatogram. This was attached with one corner vertically downward to one end of a small chromatography paper wick. The other end of the wick was placed into a trough containing 10% isopropanol in distilled water. Eventually a drop of liquid formed at the bottom corner of the strip. This was presumed to contain the radioactive compound eluted from the strip. The drop was drawn off with a capillary and this transferred to the origin of another chromatogram. This process was repeated with a second drop formed on the corner of the strip to ensure that all the

compound had been transferred to the new chromatogram.

The strip was retained to check for radioactivity later. The new chromatogram had some of the authentic compound that was believed to be identical to the radioactive compound added to the origin, then it was developed with two solvents different from those used initially.

Both the new chromatogram and the strip cut from the original chromatogram were then subjected to scintillation autography. If the strip showed no activity then it was assumed it had been all successfully transferred to the chromatogram. No problems were ever encountered in this regard.

The chromatogram was then sprayed with, or dipped into, a suitable detecting agent and the radioactive area compared in position and shape with that of the authentic compound detected by the detecting agent. If the two areas corresponded the initial identification was confirmed. If not, the relative position of the two may allow a new tentative identification of the compound.

Solvents used for co-chromatography:

(a) For amino acids:

1. Butanol:pyridine:water, 1:1:1 v:v:v;
2. Butanol:acetic acid:water, 12:3:5 v:v:v (Smith, 1969).

(b) For organic acids and sugars:

1. Phenol:ethanol:ammonia (phenol solvent previously described: absolute ethanol; 0.88g.m$\ell^{-1}$ ammonia, 150:40:10);
2. Butanol:acetic acid:water, 12:3:5 v:v:v;
3. Butan-1-ol: propanone:diethylamine:water, 70:70:14:35 (Smith, 1969);
4. Propan-1-ol:Formic acid:water, 65:22:13 (Cook and Bieleski, 1969).

In some cases non-amino acid compounds were co-chromatographed from

thin layer plates. To achieve this a Pasteur pipette had a small piece of tissue lodged in the constriction. The narrow end was attached to a vacuum pump. The radioactive area on the chromatogram was scraped off the plate and sucked into the Pasteur pipette. The pipette then had one mℓ of 10% isopropanol passed through it to dissolve the radioactive compounds. The layer material was retained by the tissue. The solution was freeze-dried in a pear-shaped flask and redissolved in a minimum of solvent. This solution was transferred to the origin of another chromatogram which had had some of the authenticated compound added. The procedure then followed was the same as before.

## 4-5 Quantitative Work and Detection of Low Level Tritium Activity

In the later stages of this thesis scintillation counting of extracts and individual compounds was undertaken. This had to await the arrival of a scintillation counter at this laboratory. The machine used was a Packard Tricarb Model 3330.

Samples were diluted preferably to give a count rate between $10^4$ and $10^5$ c.p.m. Each scintillation vial contained 10 mℓ of scintillation fluid. This was miscible with up to 0.6 mℓ of aqueous solution.

Scintillation Fluid:

3 g.$\ell^{-1}$ 2,5 diphenyl oxazole in toluene:ethoxy ethanol, 10:6.

Samples were counted for up to 10 minutes or until the counting error fell below 1%. Blanks were run with each lot of samples and had a count rate in the order of 50 ± 5 c.p.m. Standards containing 10 and 100 n Ci $^3$H-U-uridine were included with each lot of samples. For any particular type of extract (e.g., chloroform extract *Sinapis alba* seeds) a duplicate of one was prepared and 'spiked' with standard $^3$H-U-uridine, to determine any quenching effect. This was never found to be greater than the error in the finally calculated activity.

Results have generally been calculated as total activity in the original extract. Errors have been estimated and have been attributed largely to the volumetric procedures adopted and not to the counting itself. This is discussed further in Chapter 8.

# CHAPTER 5 RESULTS FROM EXPERIMENTS WITH SEEDS

## 5-1 Introduction

Once the experimental methods described in Chapters 3 and 4 had been developed it was decided to study a variety of plant propagules using these techniques. Examples were studied as they became available and various aspects of experimental technique were improved as the work proceeded.

This chapter describes preliminary experiments on seeds from *Zea mays*. These showed the potential of the technique but further work on this species was not contemplated for reasons described below. Also in this chapter are results from all the other types of seed studied.

The order in which the results are presented do not represent the chronological order of study. After the results of the preliminary study on *Zea mays* seeds, results from *Sinapis alba* (mustard) seeds are given, as this species was the seed most exhaustively studied. Later results from *Pastinaca sativa* (parsnip) seeds, *Allium cepa* and *Hordeum vulgare* seeds are presented to provide a comparison of a selection of seed types. During the time when the resting metabolism of seeds was being studied experiments on pollen and spore were also undertaken. These are presented in Chapters 6 and 7.

## 5-2 Preliminary Experiments with *Zea mays* Seeds

### 5-2.1 Comments

The construction of the exposure manifolds described in Chapter 3 was completed at a time when the Waikato crops of *Zea mays* (maize) had ripened. Consequently it was decided to carry out experiments at two

relative humidities to find if any resting metabolism could be detected in the *Zea mays* seeds.

Samples of *Zea mays* seeds were stored at 86% and 64% relative humidity. Once equilibrium moisture content had been attained, one healthy-looking seed was put into a manifold vessel and exposed to tritiated water vapour at the approximate relative humidity. One seed was exposed for five days and another for twenty days at each relative humidity before extraction. Only the methanol extract was analysed, and this by two-dimensional paper chromatography.

## 5-2.2 Results

TABLE 5.1 Compounds tritium labelled by *Zea mays* seeds exposed to tritiated water vapour.

| Compound | Relative Humidity 86% | | Relative Humidity 64% | |
|---|---|---|---|---|
| | 5 days | 20 days | 5 days | 20 days |
| alanine | + + | + + + | - | + |
| glutamate | + | + + | + | + + |
| aspartate | + | + | - | + |
| sucrose | - | + | - | - |
| gaba | - | + | - | - |
| succinate ? | - | + | - | - |
| lactate ? | - | + | - | - |
| malate ? | - | + | - | - |
| citrate ? | - | t | - | - |
| unknown | - | t | - | - |

Number of + indicate intensity of label.

t indicates trace of label.

? signifies identification of compound is not positive.

- indicates no label.

5-2.3 Discussion of Results

It is immediately obvious that the technique is successful in detecting metabolism in the dry seeds. Ten radioactive compounds can be distinguished from the experiment at 86% relative humidity where the seeds were exposed to tritiated water vapour for 20 days. Even at 64% relative humidity one compound is detected as radioactive after only five days exposure to tritiated water vapour.

Amino acids are the first class of compounds to become labelled and are the most prominently labelled compounds in all cases. In fact non-amino acid compounds are found to be labelled only after 20 days exposure to tritiated water vapour at the higher relative humidity.

Another noticeable feature of the results is that alanine is the most heavily labelled compound at 86% relative humidity but glutamate is the most heavily labelled compound at 64% relative humidity.

The problem with the results obtained at the higher relative humidity is that they may represent fungal metabolism, a problem discussed previously (3-2.1). Consequently only a few experiments were carried out at relative humidities above 64% in later work.

Certain difficulties were encountered in using *Zea mays* seeds. Because of these difficulties work on *Zea mays* was discontinued. The difficulties included:

(1) The seeds were large and difficult to macerate, particularly after storage at low relative humidity.

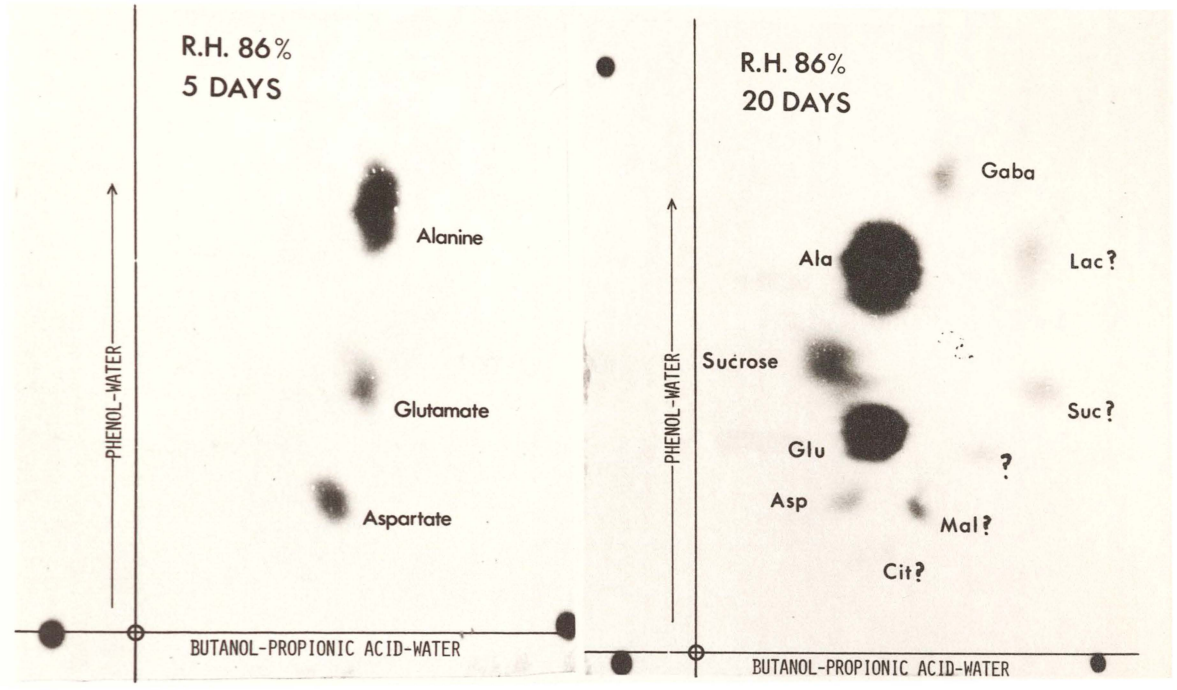
(2) The large amount of water associated with each seed (because of its size) quickly diluted the activity of the tritium in the exposure manifold.

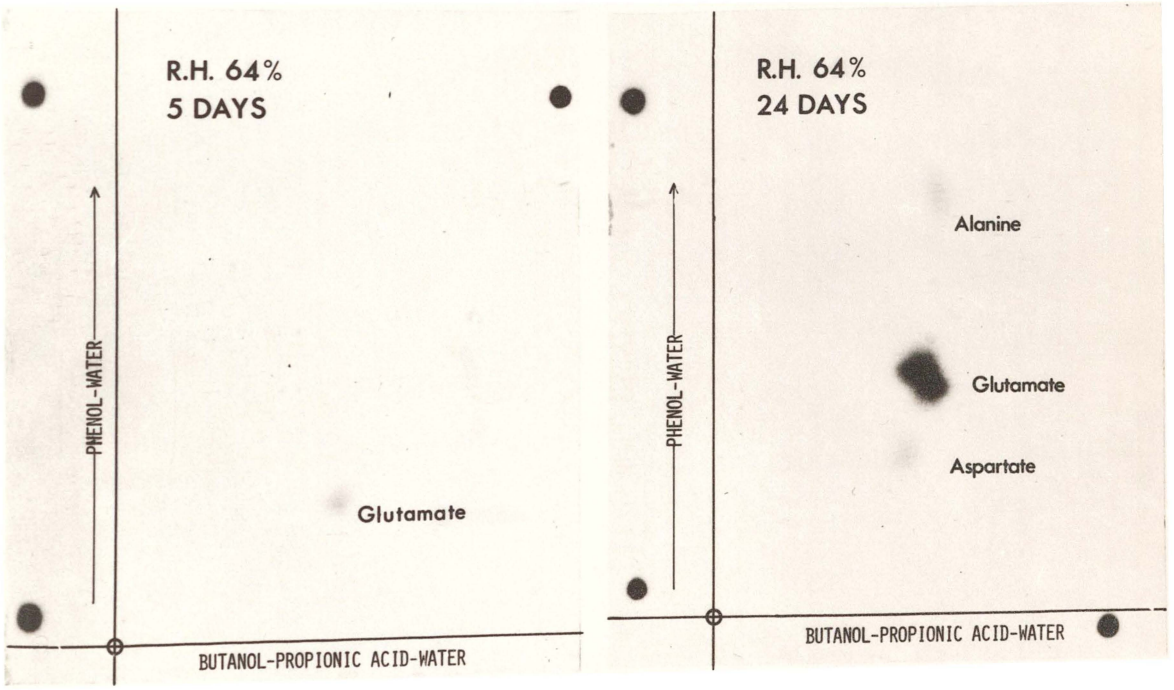
(3) Only one seed could be used for each extraction which could allow an appreciable probability of choosing a non-viable seed (= 0.02), in spite of high overall germination percentage. The choice of a much smaller seed would allow the extraction of, for example, five seeds. If

FIGURE 5.1

Scintillation autographs of paper chromatograms from experiments with *Zea mays* seeds for relative humidities (RH) and exposure durations, to tritiated water vapour, indicated.

(Unlabelled spots at periphery of chromatograms are locating marks.)


R.H. 86%
5 DAYS
Alanine
Glutamate
Aspartate
PHENOL-WATER
BUTANOL-PROPIONIC ACID-WATER
R.H. 86%
20 DAYS
Gaba
Ala
Lac?
Sucrose
Suc?
Glu
?
Asp
Mal?
Cit?
PHENOL-WATER
BUTANOL-PROPIONIC ACID-WATER


R.H. 64%
5 DAYS
Glutamate
PHENOL-WATER
BUTANOL-PROPIONIC ACID-WATER
R.H. 64%
24 DAYS
Alanine
Glutamate
Aspartate
PHENOL-WATER
BUTANOL-PROPIONIC ACID-WATER

the germination percentage was 98% the probability of selecting five non-viable seeds is $0.02^5 = 3.2 \times 10^{-9}$.

(4) No activity could be detected below 64% relative humidity.

The rest of this chapter describes experiments with seeds that did not have the above disadvantages.

## 5-3 Results from *Sinapis alba* seeds

### 5-3.1 Comments

This seed was chosen as an example of a mesobiotic seed, i.e., one with moderately good keeping properties. It is also an example of a dicotyledon. When freshly harvested these seeds have a very high germination percentate (> 98%) and germinate rapidly. This makes them useful experimental seeds as their viability can be easily tested. Another reason for this choice was that the germinating metabolism of these seeds has been studied extensively with liquid tritiated water (Spedding, 1963; Mann, 1965; Missen, 1968; Irwin, 1969; Reynolds, 1970).

Plants of *Sinapis alba* were grown by the author and fresh seeds harvested from them. These seeds were found to have > 98% germination in 36 hours at 20 C. Germination was tested by spreading one hundred seeds onto seed test paper (Whatman) moistened with tap water. Any obviously deformed seeds were rejected. Samples of seeds were equilibrated to various relative humidities as described in Section 3-2.1.

Ten seeds were exposed to tritiated water vapour at 54% relative humidity for twelve days. After this treatment eight out of the ten seeds germinated after 48 hours at 20 C.

## 5-3.2 Results

TABLE 5.2 Tritium labelled compounds from *Sinapis alba* seeds exposed to tritiated water for 12 days.

| Compound | Relative Humidity | | | | | |
|---|---|---|---|---|---|---|
| | RH = 75% | 64% | 54% | 45% | 34% | 15% |
| glutamate | + + + | + + | + | t | - | - |
| aspartate | + + + | + | t | t | - | - |
| alanine | + + + | + | t | t | - | - |
| gaba | + + | - | - | - | - | - |
| unknown C | + | - | - | - | - | - |
| asparagine | + | - | - | - | - | - |
| glycine | t | - | - | - | - | - |
| sucrose ? | + | - | - | - | - | - |
| unknown A | + | - | - | - | - | - |
| unknown $P_1$ | + | - | - | - | - | - |
| lactate | t | t | t | t | - | - |
| malate | t | t | - | - | - | - |
| unknown B | t | t | - | - | - | - |
| sugar phosphates | t | - | - | - | - | - |
| unknown S | - | t | + | + | t | - |

Number of + indicate intensity of label.
t indicates trace of label.
? signifies identification of compound is not positive.
- indicates no label.

## 5-3.3 Discussion of Results

1. Most of the tritium label was incorporated in the amino acids glutamate, aspartate and alanine. At the highest relative humidity the amino acids asparagine, serine, 4-amino butyrate and glycine were also labelled. It would appear that amino acids are most important

compounds in the resting metabolism of *Sinapis alba* seeds.

2. There are two obvious decreases in the extent of tritium labelling: (i) between 75% and 64% relative humidity, (ii) between 45% and 34% relative humidity. This implies that the extent of resting metabolism is reduced sharply at these two points.

3. Traces of malic acid at 75% and 64% relative humidity indicate that this compound is involved in some sort of metabolism at higher relative humidities. Lactic acid, another organic acid is still labelled even at 45% relative humidity.

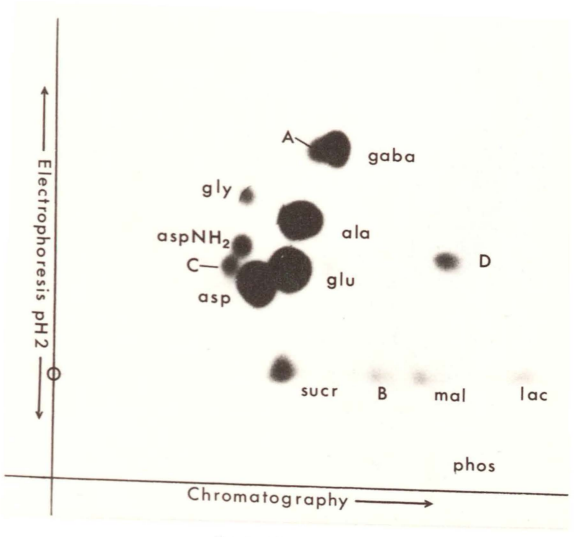
4. A compound, thought to be sucrose, was found labelled at 75% relative humidity only. This suggests the operation of a metabolic pathway at the highest relative humidity that does not operate at lower relative humidities. The same reasoning could be applied to unknown A and P.

5. A compound appearing to be a sugar phosphate, or other phosphate type compound, is labelled at 75% relative humidity but at no lower relative humidities. This may imply that phosphate metabolism ceases between 75% and 64% relative humidity.

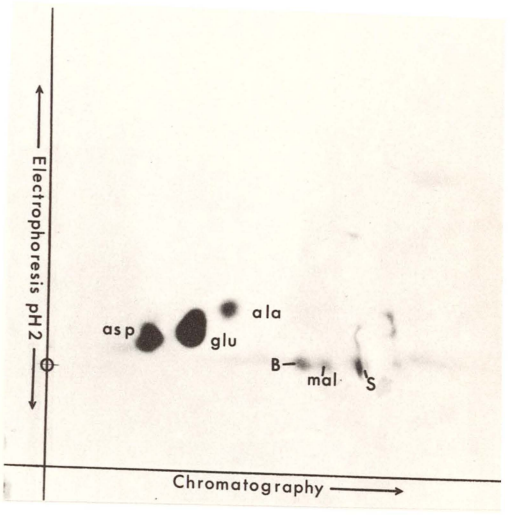
6. Unknown S was unusual as it was not detected labelled at 75% but was detected at lower relative humidities. The label in this compound may signify some type of resting metabolism, peculiar to the seeds, between 64% and 34% relative humidity. Unknown S behaved chromatographically like a neutral compound and could possibly be a 5 carbon sugar (e.g., ribose).

7. No labelled lipids were detected by scintillation autography at any relative humidity. This suggests that lipids are not important in the resting metabolism of *Sinapis alba* seeds.

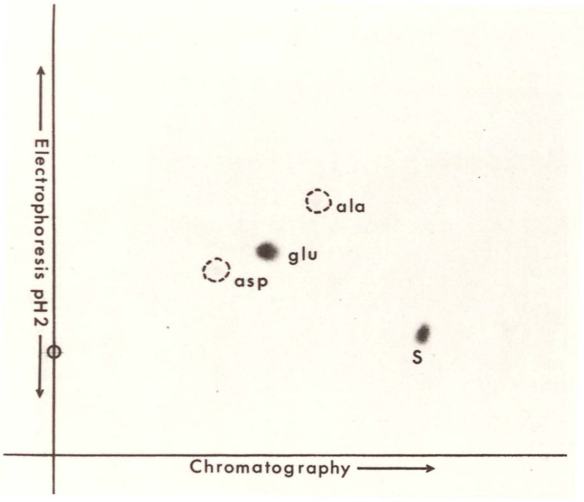
8. No labelled macromolecules were found at any relative humidity indicating that these are probably not being synthesised during resting metabolism by *Sinapis alba* seeds.



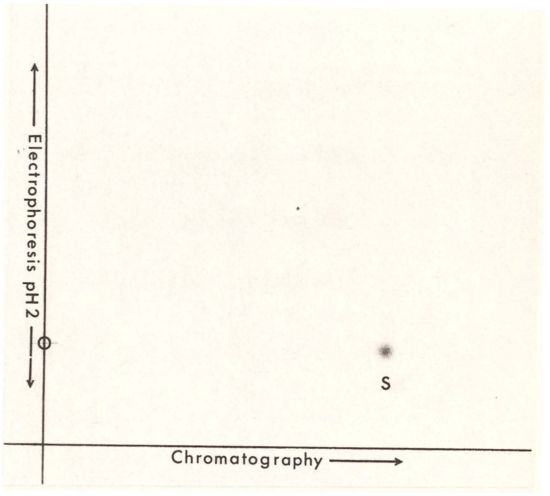

RH 75%



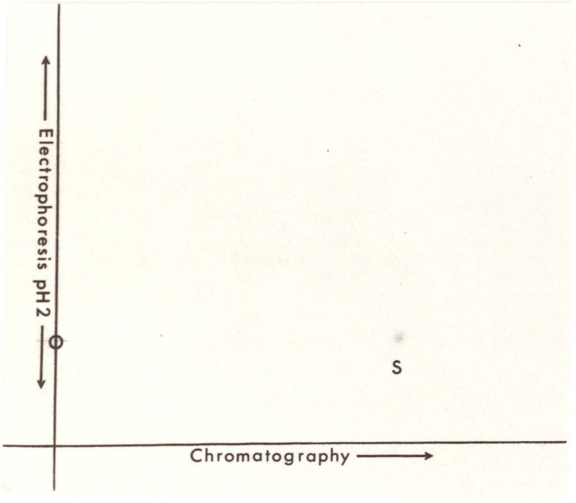

RH 64%



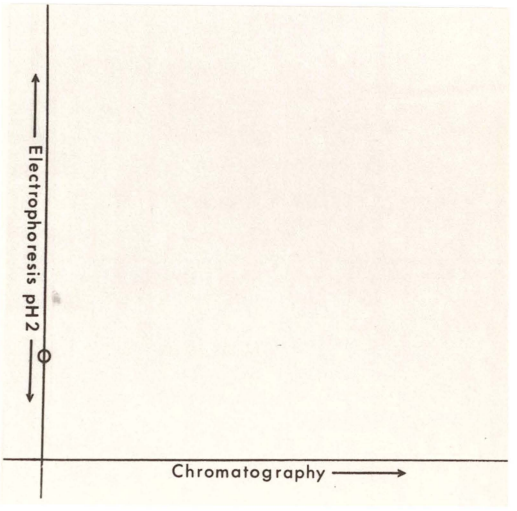

RH 54%




RH 45%



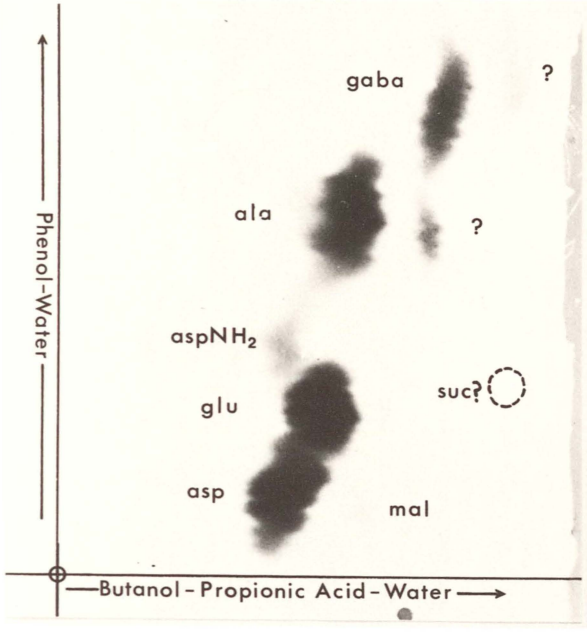

RH 34%



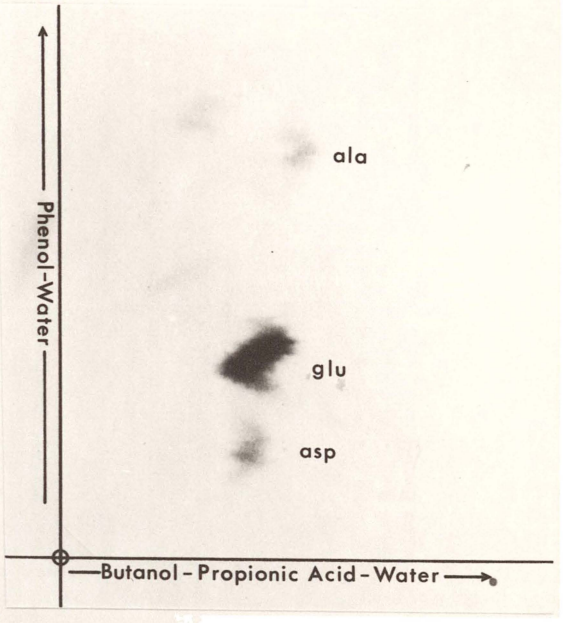

RH 15%

FIGURE 5.3

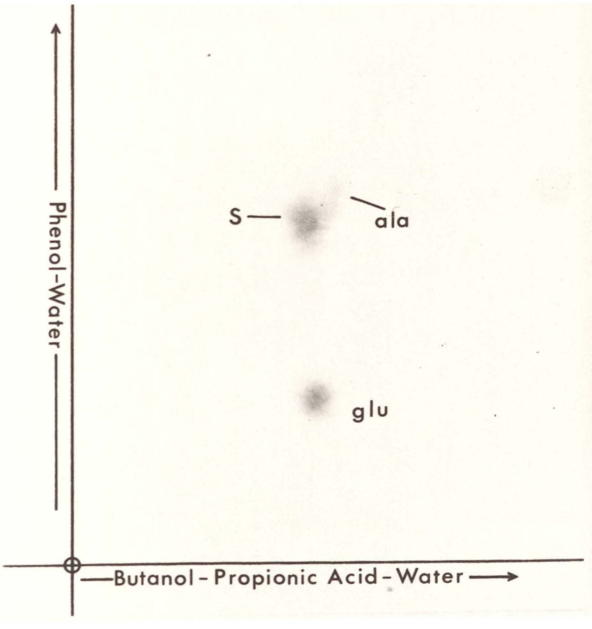
Scintillation autographs of paper chromatograms from a series of experiments with *Sinapis alba* seeds exposed to tritiated water vapour for 12 days at relative humidities (RH) indicated.
(Unlabelled spots at periphery of chromatograms are locating marks.)



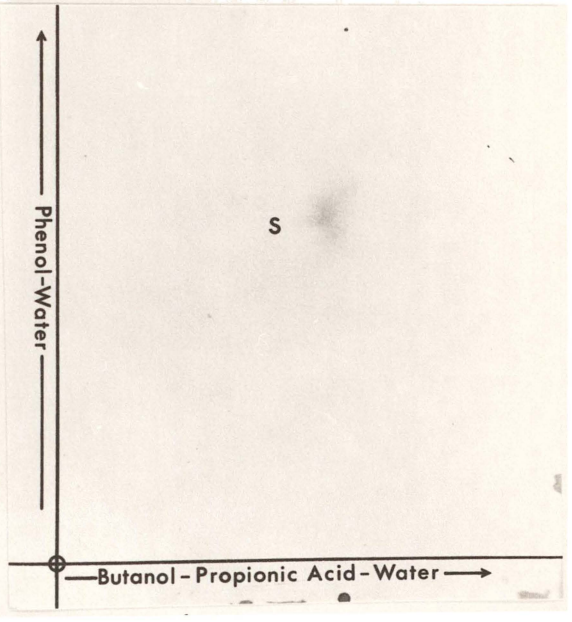

RH 75%




RH 64%




RH 54%




RH 45%

### 5-3.4 Controls

Control experiments for *Sinapis alba* seeds were carried out using autoclaved seeds exposed to tritiated water vapour at 64% relative humidity for twelve days. Also MCW control experiments (see 3-4) were carried out at 64% and 34% relative humidity (twelve days exposure to tritiated water vapour). No compound was detected as tritium labelled on paper chromatograms from these experiments by scintillation autography.

Any tritium labelling of the controls is obviously below the level of detection of scintillation autography with paper chromatograms. This does not completely rule out tritium labelling and later experiments suggest that labelling does occur in the controls but it is much less than in the live seeds

## 5-4 Results from *Pastinaca sativa* seeds

### 5-4.1 Comments

*Pastinaca sativa* (parsnip) seeds were chosen as a representative of microbiotic (short keeping) (Myers, 1935) dicotyledonous seeds.

Material: Fresh seeds were collected from parsnip plants that grew near the author's home in the Waikato. Great care was taken to ensure that the seeds were not exposed to any conditions likely to be harmful to them. In spite of this they had a low germination percentage (about 10%). It is assumed, therefore, that most of the seeds were exhibiting some form of dormancy.

This was of particular interest as the metabolic aspects of dormancy have been as yet little studied. It was hoped that the results from parsnip seeds would help to give some understanding of this topic.

## 5-4.2 Results

TABLE 5.3 Tritium labelled compounds detected by scintillation autography from extracts of parsnip seeds exposed to tritiated water vapour for 12 days.

| Compound | RH = 64% | RH = 54% | RH = 34% |
|---|---|---|---|
| glutamate | + + + | + + | - |
| aspartate | + + | + | - |
| alanine | + | + | - |
| gaba | + | - | - |
| asparagine | ? t | ? t | - |
| malate | + | t | - |
| citrate | t | - | - |
| unknown O | t | + + | + |
| unknown Y | t | t | - |
| unknown Z | t | - | - |
| lipid | - | - | - |
| 'macromolecules' | - | - | - |
| sugar phosphates | - | - | - |
| unknown SP | + | t | - |
| solid residue | + | + | + |

Number of + indicate intensity of label.
t indicates trace of label
- indicates no label.

## 5-4.3 Discussion of Results

1. Most of the tritium label is in amino acids, principally glutamic acid, aspartic acid and alanine. Also there are trace amounts of label in 4-aminobutyric acid, asparagine and unknown Y, which also appears to be an amino acid (see below).

2. At higher relative humidity the pattern of tritium labelling is

generally similar to that of *Sinapis alba* seeds in that amino acids contain most of the label with organic acids containing a small portion of the overall labelling.

3. Unknown O is a tritium labelled compound of particular interest. It appears at 54% relative humidity quite heavily labelled and is easily detectable at 34% relative humidity. However, it is not found labelled at the highest relative humidity of 64%. This suggests that a metabolic process occurs at 54% which does not occur at 64% relative humidity. The chromatographic properties of O suggest that it is an organic acid, possibly fumaric or succinic acid. As unknown O was not found in *Sinapis alba* seed experiments it was at first thought that it may be related to the dormancy of the *Pastinaca sativa* seeds. However, a tritium labelled compound chromatographically similar to O was found in non-dormant barley seeds in later studies.

4. No tritium labelled lipids or soluble macromolecular compounds were detected by dry scintillation autography.

5. No sugar phosphates were detected as tritium labelled but one compound which did not move under electrophoresis and yet formed a compact spot with RF = 0.70 in the chromatography solvent was recognised as being a neutral compound possibly a sugar (unknown SP). This compound was not identified. It could be identical to unknown S from *Sinapis alba* seeds.

6. Unknown Y was ninhydrin positive and moved to a position roughly corresponding to tyrosine. However, it was not successfully co-chromatographed. Tyrosine has not been found labelled in experiments with seeds and THO before.

7. Unknown Z was not ninhydrin positive and did not move near to a position occupied by any compound on the standard map and was not even tentatively identified. The electrophoretic properties imply that this compound acts as a weak base at pH2 and moves to a position between

FIGURE 5.4

Scintillation autographs of chromatograms obtained from experiments with *Pastinaca sativa* seeds exposed to tritiated water vapour for 12 days.

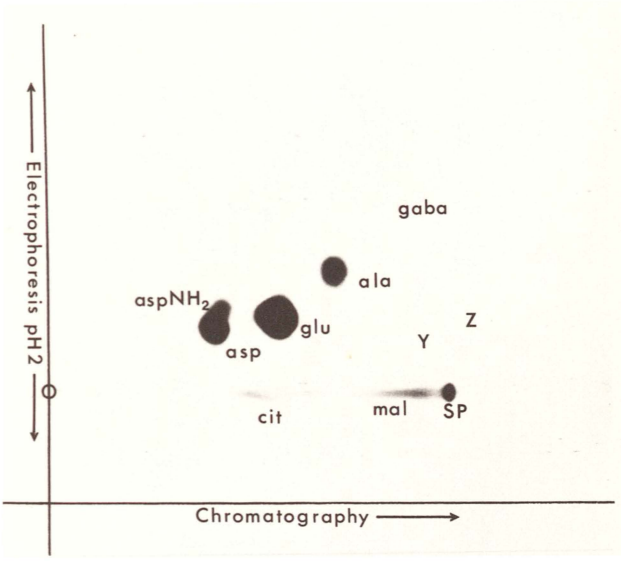
(a) TLE/TLC plate from 64% relative humidity.

(b) Paper chromatogram from 64% relative humidity (exposed to X-ray film for 5 times the duration of (c) and (d).

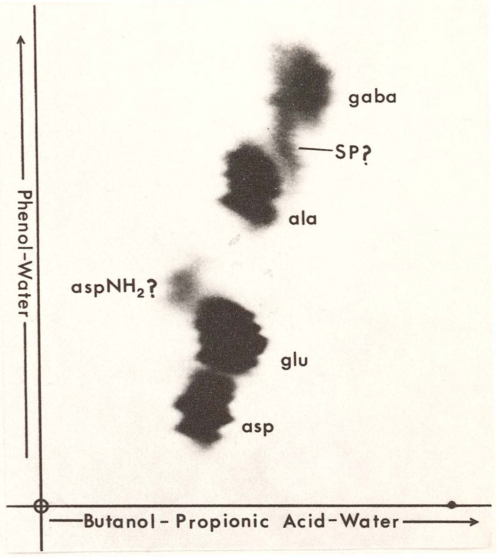
(c) Paper chromatogram from 54% relative humidity.

(d) Paper chromatogram from 34% relative humidity.

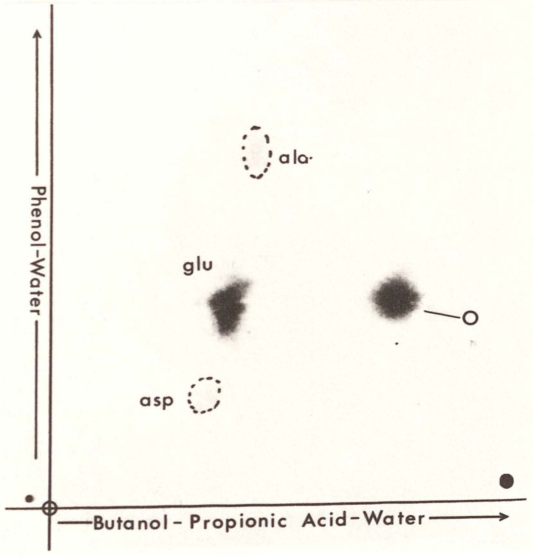
(Unlabelled spots at periphery of chromatograms are locating marks.)



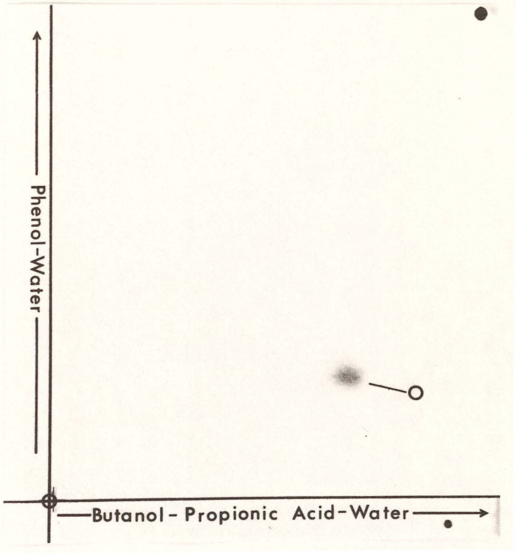

a RH 64%




b RH 64%




c RH 54%




d RH 34%

glutamate and alanine. Its chromatographic properties imply that it behaves as a rather non-polar substance in the solvents used (RF = 0.71).

## 5-5 Results from *Allium cepa* (onion) Seeds

### 5-5.1 Comments

Seeds of *Allium cepa* were chosen to represent microbiotic (poor keeping) (Pitchards, 1933) monocotyledonous seeds.

Fresh seeds (Spanish Brown variety, Coopers) were obtained from G.E. Clarke Ltd. These seeds had a germination percentage of about 90% after six days.

Samples of ten seeds were exposed to tritiated water vapour at various relative humidities and checked for germination. At 64% relative humidity eight out of ten germinated in six days. At 75% relative humidity four out of ten seeds germinated in six days. At 54% eight out of ten germinated in six days.

## 5-5.2 Results

TABLE 5.4 Tritium labelled compounds from *Allium cepa* seeds exposed to tritiated water vapour.

| | 10 days exposure | | 20 days exposure | |
|---|---|---|---|---|
| Compound | RH = 75% | RH = 64% | RH = 54% | RH = 34% |
| glutamate | + + | + | + | - |
| alanine | + + | t | t | - |
| aspartate | + | t | - | - |
| gaba | + | t | - | - |
| serine | ? t | - | - | - |
| lactate | ? | - | - | - |
| unknown V | + | - | - | - |
| sugar phosphates | ? t | - | - | - |
| sugars | - | - | - | - |
| lipids | - | - | - | - |
| macromolecules | t | - | - | - |
| solid residue | + | + | + | + |

Number of + indicate intensity of label.
t indicates trace of label.
? indicates identity not established.
- indicates no label.

## 5-5.3 Discussion of Results

1. Amino acids are the main class of compound to be labelled by *Allium cepa* seeds, just as they were for the three previous types of seed studied. In onion seeds the amino acids labelled included glutamic acid, alanine, aspartic acid, 4-aminobutyric acid and possibly serine.

2. At 64% relative humidity and below, amino acids were the only type of compound found to be labelled.

3. The overall incorporation of tritium by onion seeds was obviously much less than by most other propagules studied. This may indicate that the overall metabolism in dry onion seeds is less than in other propagules. This is evidence suggesting that the poor keeping qualities of onion seeds is not due to their 'burning up' food reserves — one of the suggested reasons for loss of viability in seeds.

4. Unknown V occupied a chromatographic position similar to that of unknown Z from parsnip seeds. It was not identified.

5. Traces of a labelled compound were found occupying a position that would be normally occupied by sugar phosphates on a chromatogram of the methanol extract from onion seeds at 75% relative humidity. This suggests that sugar phosphate metabolism occurs at this high relative humidity.

6. A trace of labelled 'macromolecules' was found to be labelled at 75% relative humidity. This may indicate that such compounds are being synthesised at this RH.

7. No labelled sugars could be detected at any relative humidity, suggesting that these compounds are not important in the resting metabolism of onion seeds.

## 5-6 Results from *Hordeum vulgare* (barley) Seeds

### 5-6.1 Comments

*Hordeum vulgare* seeds were chosen to represent mesobiotic (Barton, 1961) monocotyledonous seeds.

Barley plants were grown by the author in a glasshouse and freshly harvested seeds were placed into humidistats. These fresh seeds had a germination percentage of over 98%.

## 5-6.2 Results

TABLE 5.5 Tritium labelled compounds from *Hordeum vulgare* seeds exposed to tritiated water vapour for 17 days.

| Compound | Relative Humidity | | | |
|---|---|---|---|---|
| | 64% | 54% | 45% | 34% |
| glutamate | + + | + | + | - |
| alanine | + + | + | t | - |
| fumarate ? | + + | + | + | + |
| succinate ? | + | - | - | - |
| lactate | + | + | t | - |
| asparagine | + | + | - | - |
| aspartate | + | t | - | - |
| malate | + | + | - | - |
| sugars | - | - | - | - |
| phosphates | - | - | - | - |
| lipids | - | - | - | - |
| macromolecules | - | - | - | - |
| solid residue | + | + | + | - |

Number of + indicate intensity of label.
t indicates trace of label.
? signifies identification of compound not positive.
- indicates no label.

## 5-6.3 Discussion of Results

1. Much of the tritium label was incorporated in amino acids. The amino acids glutamate, alanine, asparagine and aspartate were found labelled at 64% and 54% relative humidity. Glutamate and alanine were found labelled at 45% relative humidity.

2. One of the most intensely labelled compounds appeared to be an

organic acid, possibly fumarate or succinate. This compound travelled slightly ahead of glutamate in phenol water solvent and slightly ahead of lactate in butanol, propionic acid, water solvent. It was not successfully co-chromatographed. This compound appeared labelled at all relative humidities and was the only compound labelled at 34%. It appears to be chromatographically similar to unknown 0 in *Pastinaca sativa* seeds. It is interesting to note that this compound was not labelled at 64% by the parsnip seeds but was at lower relative humidities, but it was labelled by barley seeds at all relative humidities studied.

3. Lactic acid was found labelled at the three higher relative humidities. Malic acid is tritium labelled at 64% and 54% relative humidity.

4. No tritium activity was found in sugars, sugar phosphates, lipids or soluble macromolecules. This suggests these compounds are not important in the resting metabolism of barley seed and relative humidities of 64% and below.

5. Traces of label were found in four amino acid-like compounds. One of these could have been glycine. Another moved with glutamate under electrophoresis and with alanine in the chromatography solvent. The other two occupied positions in an area occupied by common non-polar amino acids, e.g., leucine, iso-leucine, phenyl alanine, valine and tyrosine.

FIGURE 5.5

A comparison of chromatographic techniques. Scintillation autographs obtained from extracts of *Hordeum vulgare* seeds exposed to tritiated water vapour at 64% relative humidity for 20 days.

(a) TLE/TLC.

(b) Two-dimensional TLC.

(c) Two dimensional paper chromatography.

NOTE: The area labelled suc? in (c) is probably fumarate.

(Unlabelled spots at the periphery of chromatograms are locating marks.)

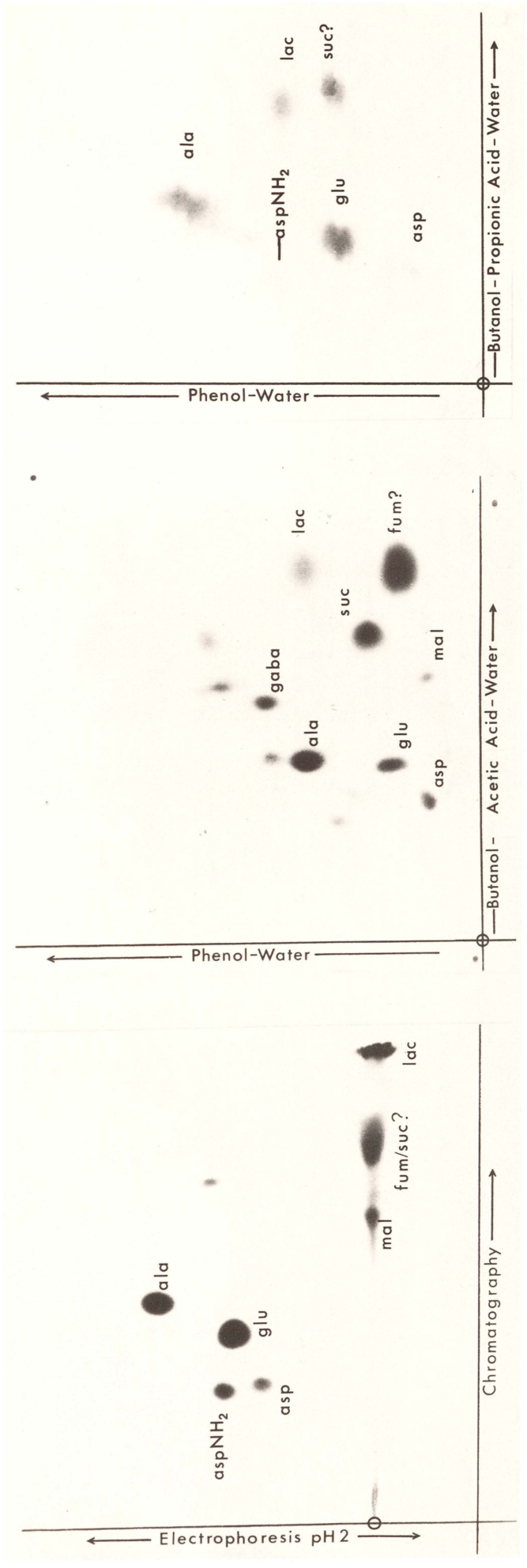

Electrophoresis pH 2
Chromatography
aspNH2
asp
glu
ala
mal
fum/suc?
lac
a
Phenol-Water
Butanol- Acetic Acid-Water
gaba
ala
glu
asp
suc
mal
fum?
lac
b
Phenol-Water
Butanol-Propionic Acid-Water
ala
aspNH2
glu
asp
lac
suc?
c

FIGURE 5.6

Effect of long exposure of chromatograms to X-ray film.

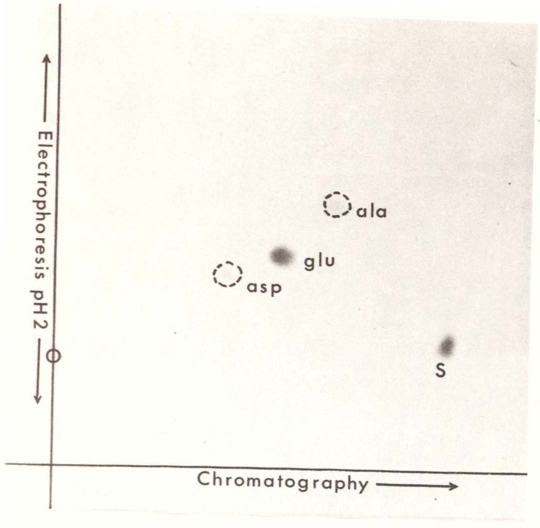
(a) Scintillation autograph from TLE/TLC plate of extract from *Sinapis alba* seeds exposed to tritiated water vapour at 54% relative humidity for 12 days. X-ray film exposed for 2 weeks.

(b) The same TLE/TLC plate as (a) exposed to X-ray film for 4 months.

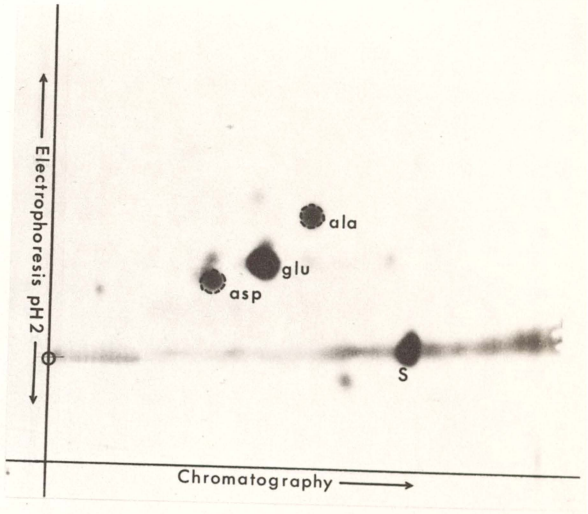
(c) Scintillation autograph from TLE/TLC plate of extract from *Hordeum vulgare* seeds exposed to tritiated water vapour at 54% relative humidity for 20 days. X-ray film exposure, 2 weeks.

(d) Same TLE/TLC plate as (c) exposed to X-ray film for 4 months.

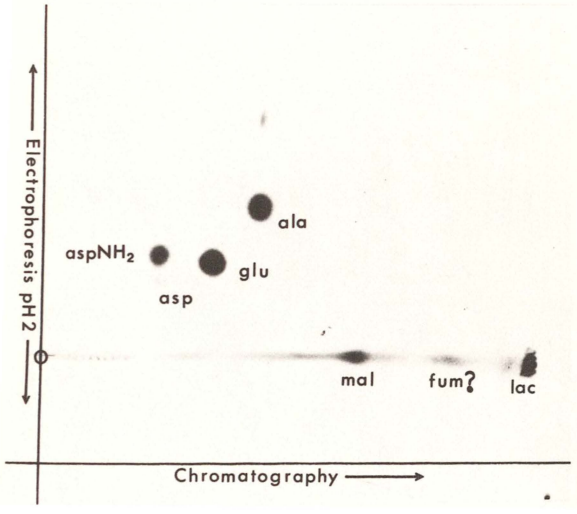
Comments: The long exposures show tritium labelled compounds not detected in the short exposure for both chromatograms. This is more striking in the chromatogram from *Hordeum vulgare* seeds, in that the newly detected compounds are quite clearly labelled. Of particular note is the compound at the top right corner of (d) which has characteristics similar to unknown P from *Pinus ponderosa*. Also in (d) are the two compounds at the lower left corner having characteristics of phosphate type compounds. Such compounds have not been found labelled at a relative humidity as low as 54% in any other propagule studied.


Electrophoresis pH 2
ala
glu
asp
S
Chromatography

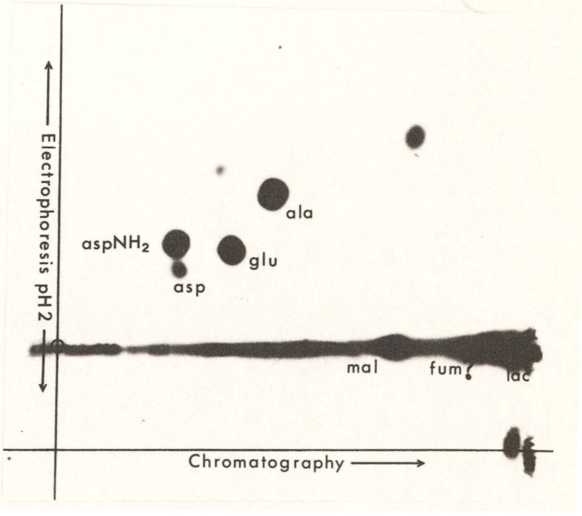
a


Electrophoresis pH 2
ala
glu
asp
S
Chromatography

b


Electrophoresis pH 2
ala
aspNH2
glu
asp
mal
fum?
lac
Chromatography

c


Electrophoresis pH 2
ala
aspNH2
glu
asp
mal
fum?
lac
Chromatography

d

# CHAPTER 6 RESULTS FROM EXPERIMENTS WITH POLLEN

## 6-1. Introduction

At the end of Chapter 1 it was mentioned that pollens are generally much shorter lived than seeds. If this loss of viability was due to a high rate of resting metabolism, depleting important metabolites, then it would be reasonable to assume that resting metabolism would be relatively easy to detect in pollen. It was decided, for this reason, that pollen might be a useful propagule to study. Another reason was that fresh pollen was available at the time when preliminary experiments showed the success of the technique.

The most important pollen studied was that of *Pinus ponderosa*. This was chosen because it was abundantly available and could be germinated readily.

Various other pollens were collected as they became available. A variety was chosen to represent a wide range of pollen types including wind propagated and insect propagated pollen from gymnosperms and angiosperms, and among the latter group, pollen from monocotyledons and dicotyledons.

## 6-2 *Pinus ponderosa* Pollen

### 6-2.1 Comments

Male cones of *Pinus ponderosa* were collected at noon on the first fine day after they began shedding pollen. The cones were immediately taken to the laboratory and laid out on sheets of newsprint. Pollen was shaken from the cones each day, collected and transferred to constant relative humidity containers.

It was thought that allowing the pollen to dry thoroughly at a low relative humidity and then returning it to a higher relative humidity for equilibration may introduce uncertainties. It was thought possible that irreversible changes may occur as the water content of the pollen decreases. So to keep the situation as simple as possible the pollen was transferred rapidly to the humidistats, especially for samples at higher relative humidities. To this end the first sample of pollen collected was put into the humidistat of highest relative humidity. This pollen was also the first to be exposed to tritiated water as it lost its viability more rapidly than pollen stored at lower relative humidity.

Samples of pollen were stored in large flat vessels to assist rapid equilibration. The vessels were weighed daily until they attained constant weight.

Approximately 30 mg of pollen were weighed into a manifold vessel and stored in the humidistat until required.

The pollen was tested for viability and radiosensitivity. Fresh pollen germinated about 100% in distilled water after 48 hours. After five days exposure to tritiated water vapour at 64% relative humidity the rate of germination of the pollen had diminished considerably as had the percentage germination. After 72 hours about 90% of the pollen had germinated, and no more germinated after this.

## 6-2.2 Results

TABLE 6.1 Tritium labelled compounds from *Pinus ponderosa* pollen exposed to tritiated water vapour for 5 days.

| Compound | Relative Humidity | | | | |
|---|---|---|---|---|---|
| | 64% | 54% | 45% | 34% | 15% |
| glutamate | + + + | + + | + | - | - |
| aspartate | + + | + | t | - | - |
| alanine | t | t | t | - | - |
| 4-amino-butyrate | + + | t | t | - | - |
| malic acid | + | t | t | - | - |
| unknown P | t | t | t | - | - |
| unknown Q | t | - | - | - | - |
| unknown R | t | - | - | - | - |
| lipids | - | - | - | - | - |
| macromolecules | - | - | - | - | - |

Number of + indicates intensity of label.

t indicates trace of label.

- indicates no label.

## 6-2.3 Discussion of Results

1. At 45% relative humidity and above, metabolic activity can be easily detected in the pollen.

2. The activity detected by the incorporation of tritium involves mainly amino acids, in particular glutamate, aspartate, 4-amino-butyrate and alanine. Malic acid was the only non-amino acid compound to be labelled to any extent.

3. At 34% and 15% no metabolic activity could be detected by the technique.

4. The overall labelling pattern is similar to that of seeds, particularly with regard to points 1 and 2 above.

5. The intensity of label in 4-amino-butyrate diminishes much more abruptly than the other compounds between 64% and 54% relative humidity. This may indicate that the reactions responsible for the labelling of this compound are more sensitive to water activity than reactions responsible for labelling the other compounds.

6. Unknown P has unusual chromatographic qualities. It acts as a weak base at pH 2 and moved to a position between ala and gaba under electrophoresis and is a very non-polar compound in the chromatography solvent (RF = 0.77). It was not detected by ninhydrin. The possibility has to be considered that this compound may be an as yet unidentified biochemical.

7. Unknowns Q and R (RF = 0.81 and 0.80 respectively) both appeared to be amino acids but were not successfully co-chromatographed. From the map of Bieleski and Turner (1966), they could be two of the five amino acids valine tyrosine, phenyl alanine, leucine or isoleucine.

8. As with seeds, no labelled lipids, sugars or macromolecules could be detected at 64% relative humidity and below.

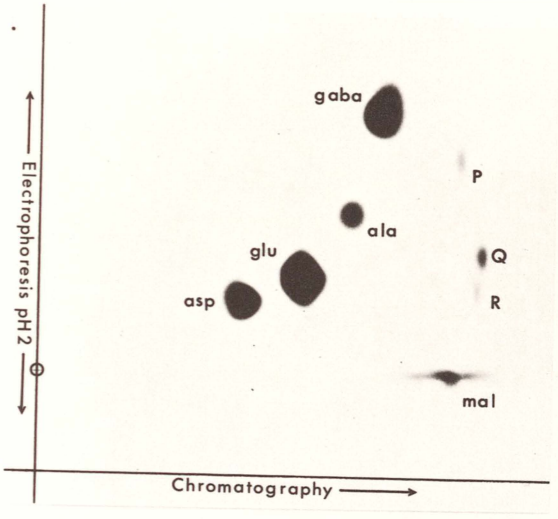
### 6-2.4 Control Experiments

Control experiments were carried out using autoclaved *Pinus ponderosa* pollen and MCW controls (see 3-4) at 64% and 34% relative humidity. Only a trace of tritium activity was found from autoclaved pollen at 64% relative humidity. This was incorporated in a compound with unusual chromatographic qualities — very high RF in phenol, water ($\approx$ 0.9) and an intermediate RF in butanol, propionic acid, water (= 0.65). This compound was not identified, nor was it detected in experiments with live pollen.

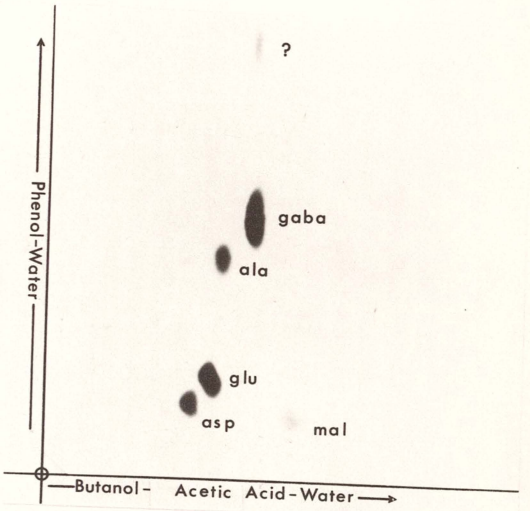
These results imply that all of the tritium incorporation found in

FIGURE 6.2

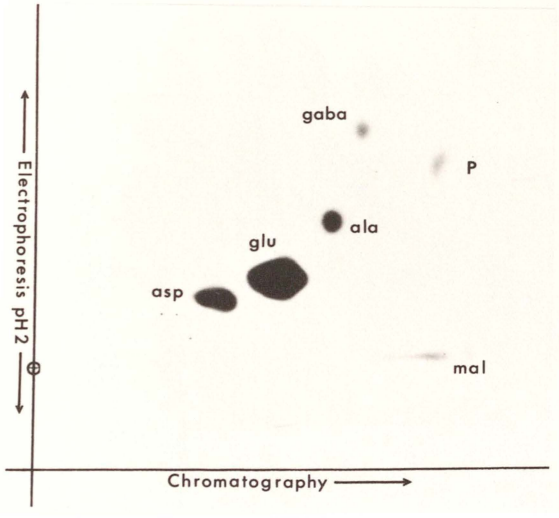
Scintillation autographs from paper chromatograms of *Pinus ponderosa* pollen extracts exposed to tritiated water vapour for 5 days at relative humidities (RH) indicated.
(Unlabelled spots at periphery of chromatogram are locating marks.)



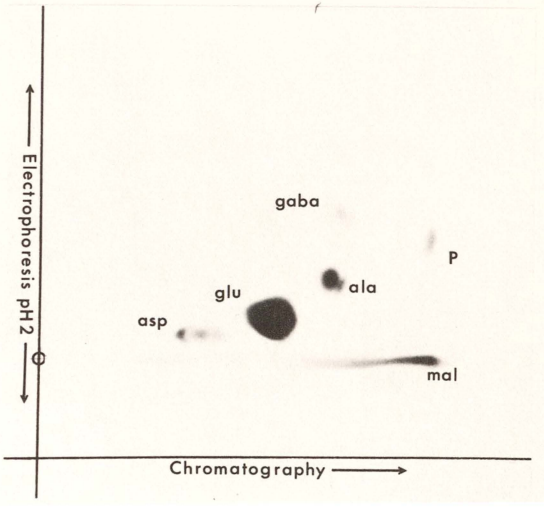

a RH 64%




b RH 64%




c RH 54%




d RH 45%

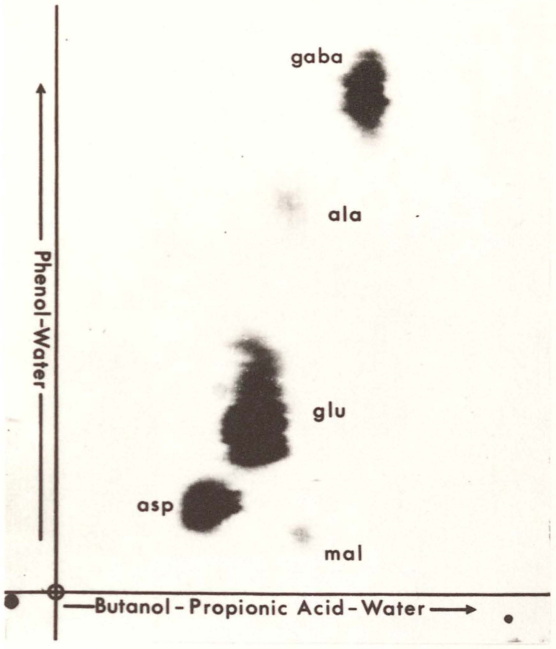
FIGURE 6.1

Scintillation autographs from extracts of *Pinus ponderosa* pollen exposed to tritiated water vapour for 5 days and relative humidities (RH) indicated.

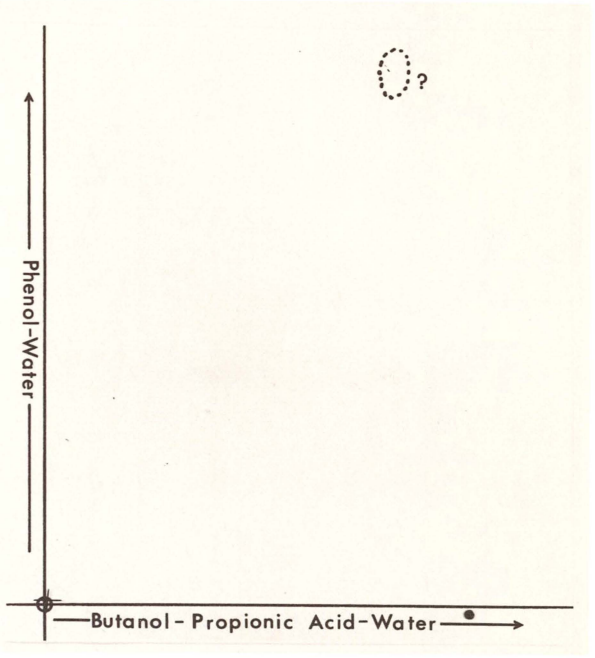
(a), (c), (d) TLE/TLC plates.

(b) Two-dimensional TLC plate.

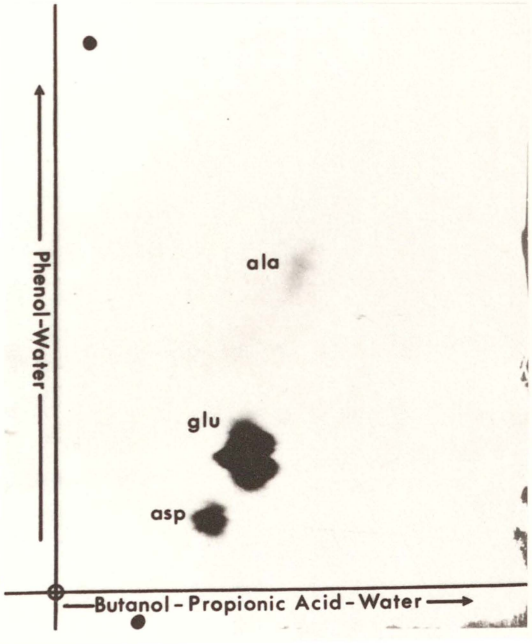
(Unlabelled spots at periphery of chromatograms are locating marks.)



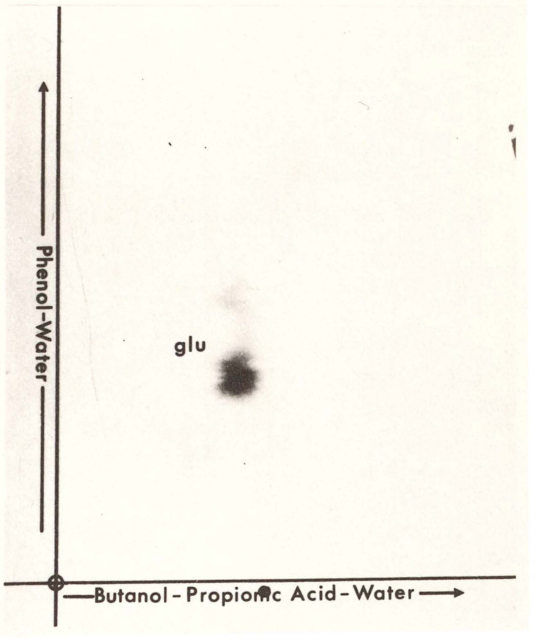

RH 64% live pollen




RH 64% autoclaved pollen




RH 54% live




RH 45% live

the live *Pinus ponderosa* pollen was due to metabolic and not chemical processes.

### 6-2.5 Experiments with Disrupted Cells and Water Extract of *Pinus ponderosa* Pollen

Two experiments were devised to find the importance of cellular organisation in the resting metabolism of pollen. The first of these involved rapidly macerating the pollen in distilled water at 0 C. The entire resulting suspension was transferred to a pear-shaped flask and immediately freeze-dried. (No complete cells could be detected by microscopic examination of a portion of the suspension.) The flask containing the freeze-dried disrupted cells was transferred to a humidistat at 64% relative humidity and left for several days to equilibrate. The equilibrated disrupted cells were then exposed to tritiated water vapour at 64% relative humidity for five days. The flask was removed from the tritiation manifold and 3 mℓ of MCW solvent at -30 C was added. A stirring rod was used to suspend the solid present in the flask and the suspension was transferred to an all-glass tissue grinder. The subsequent extraction was the same as that described in Section 3-3.

In another experiment a sample of pollen was macerated in distilled water at 0 C and the resulting suspension centrifuged. This water extract would contain water soluble metabolites and macromolecules from the pollen. The supernatant was transferred to a pear-shaped flask and freeze-dried. The flask was then transferred to 64% relative humidity for several days to equilibrate. It was then attached to a tritiated water vapour manifold and exposed for five days. The contents of the flask were then extracted in the usual way. Results for the two experiments just described are given below with those from live pollen.

TABLE 6.2 Results from disrupted cells and water extract of *Pinus ponderosa* pollen.

| Compound | Exposure: 5 days at 64% RH | | |
|---|---|---|---|
| | Live Pollen | Disrupted Cells | Water Extract |
| Glutamate | + + + | + + | + |
| 4-amino-butyrate | + + | + + | - |
| aspartate | + + | + | - |
| alanine | t | - | - |
| malate | + | - | - |

Number of + indicates intensity of label.

t indicates trace of label.

- not detected as labelled.

The results show that the disrupted pollen cells give a labelling pattern remarkably similar to intact live pollen cells. The overall intensity of labelling is less in the disrupted pollen and it is possible that alanine and malate are just below the level of detection. These two compounds are labelled only slightly by live pollen. It would appear that the resting metabolism of the disrupted pollen cells is not very different from live pollen cells. This suggests that cellular organisation is not crucial to the operation of resting metabolism.

The water extract showed a considerably reduced metabolism compared with live pollen. This suggests that some of the mechanisms responsible for resting metabolism are dependent on insoluble structures of the pollen. However, it appears that the enzyme or enzymes responsible for labelling glutamate are soluble.

### 6-2.6 Experiments with Pollen Stored for an Appreciable Time

Some pollen that had been stored for 12 months at 4 C 18% relative humidity was transferred to a humidistat at 64% relative humidity at 24 C. This pollen was still viable. After equilibration at the higher relative humidity a sample of this pollen was exposed to tritiated water vapour at 64% relative humidity for five days. This was extracted and the extracts analysed as described previously.

The tritium labelling pattern obtained by scintillation autography was identical to that obtained from fresh pollen equilibrated to 64% relative humidity. This suggests that the resting metabolism in the stored viable pollen, once it is returned to a higher relative humidity, is the same as the fresh pollen. Consequently, it can be said that irreversible changes in respect to resting metabolism do not occur when *Pinus ponderosa* pollen is stored for long periods at very low relative humidity then returned to an intermediate relative humidity.

In another experiment some pollen, which had completely lost viability, stored for 12 months at 64% relative humidity and 24 C was exposed to tritiated water vapour. (*Pinus ponderosa* pollen loses viability after about three months under the above storage conditions.) The scintillation autographs obtained showed only traces of labelling in glutamic acid. It appears that drastic reduction of resting metabolism has occurred in the old, non-viable pollen stored at 64% relative humidity at 24 C.

## 6-3 Experiments with Other Pollens

### 6-3.1 Comments

While a comprehensive study of *Pinus ponderosa* pollen was being undertaken various other types of pollens were collected and exposed to tritiated water vapour to compare the tritium labelling patterns of different pollens.

Wind propagated pollens were collected in a manner similar to that described for *Pinus ponderosa*. Insect propagated pollen was brushed off the flower with a small artist's brush, into a glass container. All samples were immediately transferred to a constant relative humidity chamber. In all cases approximately 30 mg of pollen was exposed to tritiated water vapour.

Pollen collected early in the experimental work was analysed by paper chromatography, whereas pollen collected later was analysed by thin layer chromatography and paper chromatography. As tritium detection on thin layer chromatograms proved to be considerably more sensitive than on paper chromatograms, the results from the earlier pollen are not strictly comparable with those from the later pollen. Consequently the results are presented separately.

As a comparison results from *Pinus ponderosa* are included at the head of each list.

6-3.2 Results

(i) From paper chromatography:

TABLE 6.3 Tritium labelled compounds detected from various pollen species after exposure to tritiated water vapour for 5 days.

| Pollen Species/ Relative Humidity | Compound | | | | | |
|---|---|---|---|---|---|---|
| | glu | asp | ala | gaba | other | |
| *Pinus ponderosa* 64% | + + + | + | t | + + | mal | - |
| *Pinus radiata* 64% | + + + | + | t | + + | - | - |
| *Betula alba* 64% | + | + | + + | + | gly? | lac? |
| *Podocapus dacrydioides* 64% | + + | + | + | t | - | - |
| *Camellia sp.* 64% | + + | + | + | - | - | - |
| *Salex discolor* 64% | + + + | + | t | t | gly? | - |
| *Salex discolor* 54% | + + | t | - | - | - | - |
| *Salex discolor* 45% | + | - | - | - | - | - |
| *Salex discolor* 34% | - | - | - | - | - | - |
| *Salex discolor* 15% | - | - | - | - | - | - |

Number of + indicates intensity of label.

t indicates trace of label.

- indicates no label.

? indicates uncertainty of identification.

FIGURE 6.3

Scintillation autographs of paper chromatograms of various pollens exposed to tritiated water vapour at 64% relative humidity for 5 days.

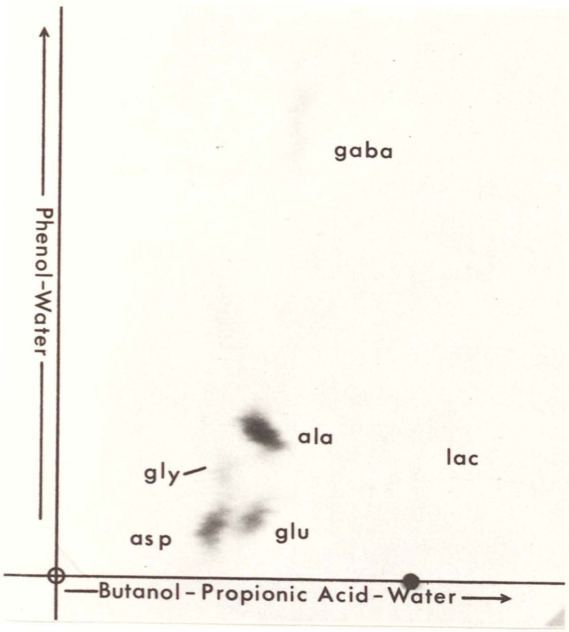
(a) *Betula alba.*

(b) *Camellia sp.*

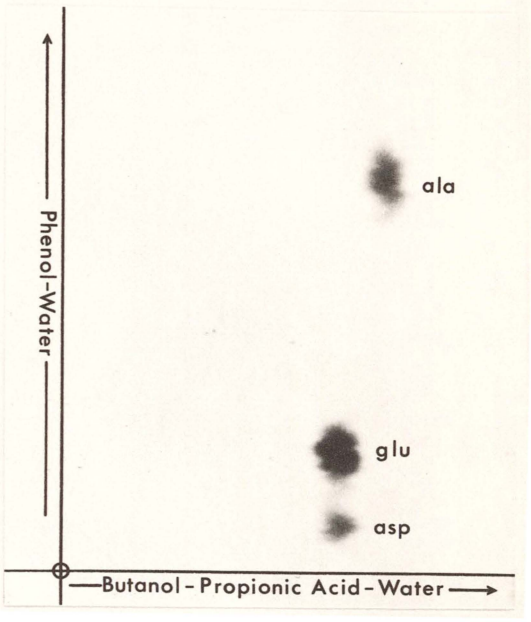
(c) *Podocarpus dacrydioides.*

(d) *Salex discolor.*

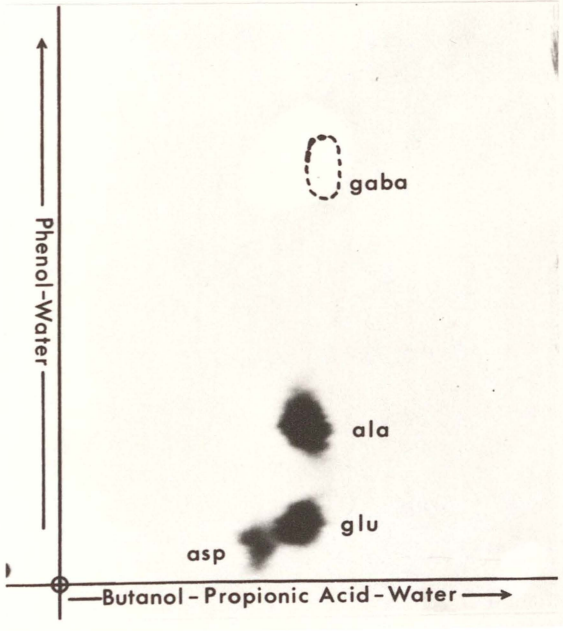
(Unlabelled spots at periphery of chromatogram are locating marks.)


gaba
Phenol-Water
ala
lac
gly
asp
glu
Butanol - Propionic Acid - Water

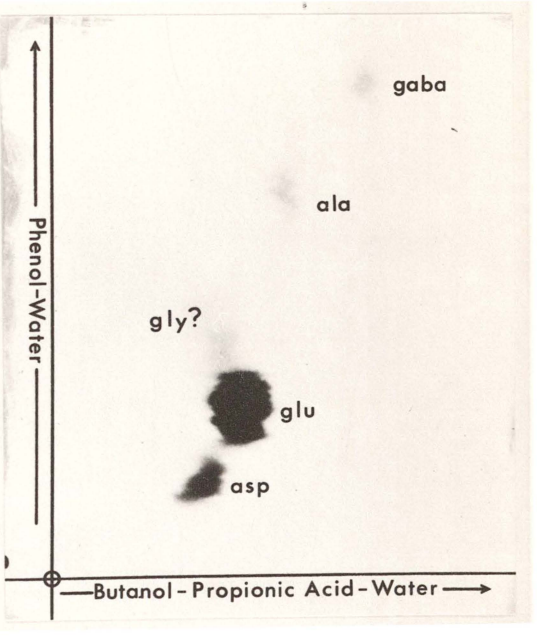
a


Phenol-Water
ala
glu
asp
Butanol - Propionic Acid - Water

b


gaba
Phenol-Water
ala
glu
asp
Butanol - Propionic Acid - Water

c


gaba
ala
gly?
Phenol-Water
glu
asp
Butanol - Propionic Acid - Water

d

(ii) From paper and thin layer chromatography:

TABLE 6.4 Tritium labelled compounds detected from various pollen species after exposure to tritiated water vapour for 5 days.

| Pollen Species/ Relative Humidity | Compound | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | glu | asp | ala | 4-ab | aspNH$_2$ | gly | ser | mal | X |
| *Pinus ponderosa* 64% | + + + | + | t | + + | - | - | - | + | 3 |
| *Magnolia sp.* 64% | + + + | + + | + + | + | + | t? | + | t | 4 |
| *Cystiscis scoparins* 45% | + + | t | + + | t | + | t? | t | - | - |
| *Cupressus macrocapa* 64% | + + | t | - | + | - | - | - | t? | 2 |
| *Arum macalutum* 54% | + + | t | + | - | - | t? | - | - | 3 |
| *Phormium tenax* 64% | + + + | + | + | t | + + | - | - | - | 3 |
| *Phormium tenax* 54% | + + | + | + | - | + | t? | - | - | - |
| *Phormium tenax* 45% | + | t | t | - | + | - | - | - | - |
| *Phormium tenax* 34% | t | - | - | - | t | - | - | - | - |
| *Phormium tenax* 12% | - | - | - | - | - | - | - | - | - |

Column X: number of unidentified tritium labelled compounds.

Number of + indicates intensity of label.

t indicates trace of label.

- indicates no label.

? indicates uncertainty of identity.

FIGURE 6.4

Scintillation autographs of TLE/TLC plates of extracts of various pollens exposed to tritiated water vapour for 5 days at 64% relative humidity.

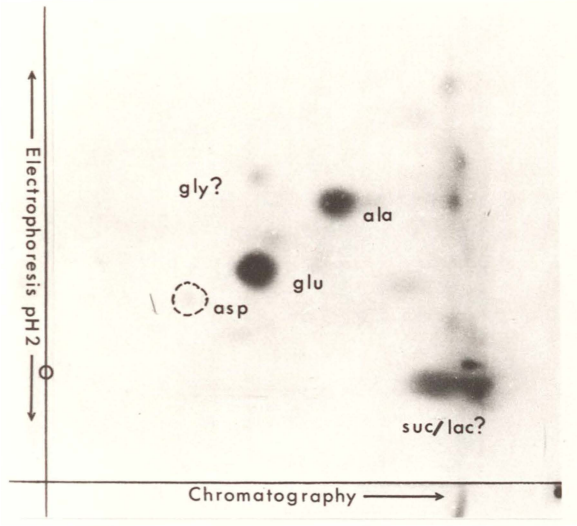
(a) *Arum maculatum*.

(b) *Cupressus macrocapa*.

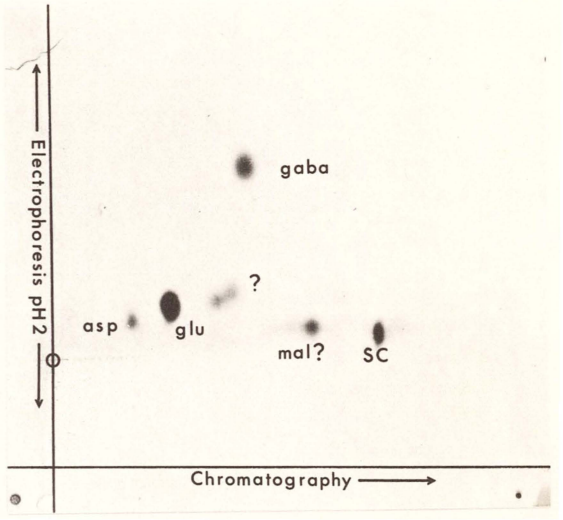
(c) *Phormium tenax*.

(d) *Magnolia sp*.

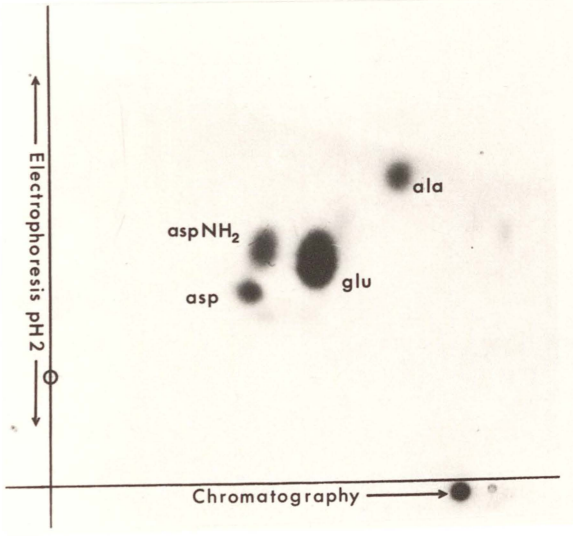
(Unlabelled spots at periphery of chromatograms are locating marks.)


Electrophoresis pH2
gly?
ala
glu
asp
suc/lac?
Chromatography

a


Electrophoresis pH2
gaba
?
asp
glu
mal?
SC
Chromatography

b


Electrophoresis pH2
ala
aspNH2
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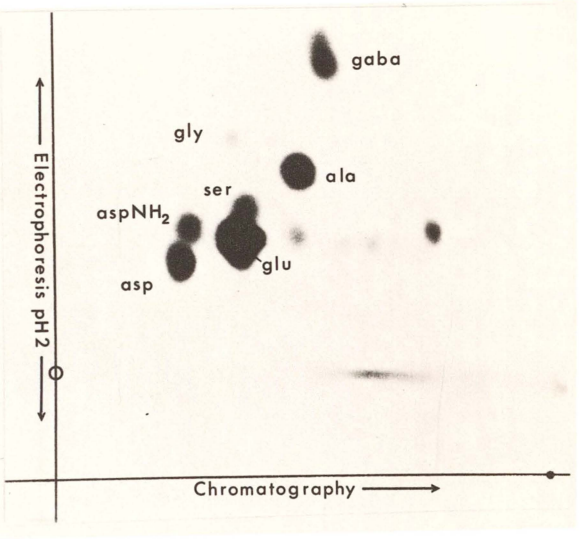
Chromatography

c


gaba
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Electrophoresis pH2
Chromatography

d

(iii) Lipids and Macromolecules

In no experiment with different pollen species were any labelled lipids or macromolecules detected by the method outlined in Chapter 4.

6-3.3 Discussion of Results

1. As with *Pinus ponderosa* pollen tritium activity in all the pollen species tested was confined largely to amino acids.

2. The particular amino acids labelled and the relative intensity of labelling varies considerably from one species to another.

3. Some species of pollen showed considerably more activity than others.

4. The species that showed great diversity and high intensity of label, compared with *Pinus ponderosa* pollen, were all insect pollenated species. These were the pollen of *Cystiscus scoparins*, *Magnolia sp.* and *Phormium tenax*. It would be interesting to propose that this higher rate of metabolism contributes to the brief longevity of insect propagated pollen (Stanley and Linskens, 1972) compared with wind propagated pollen. It could be suggested that the higher rate of metabolism cause the pollen to deplete its reserves quickly and thus lose viability in a shorter time. However, this hypothesis could not obviously be general as the insect propagated pollens, *Arum macalutum*, *Camellia sp.* and *Salex discolor* show no more tritium labelling than does the *Pinus ponderosa* pollen.

6-4 Summary

All the pollen studied showed tritium labelling at higher relative humidities. No labelling in *Pinus ponderosa* pollen was detected at 34% relative humidity or below. Traces of activity were found in *Phormium tenax* pollen at 34% relative humidity but not at 15%. Controls of *Pinus ponderosa* pollen showed no tritium labelling.

Virtually all the tritium detected was incorporated into amino acids and only traces were incorporated into organic acids. In this respect amino acids appear more important in the resting metabolism of pollen than of seeds.

No labelling of lipids or macromolecules was detected in resting metabolism experiments with pollen. In all cases the solid residue left after extraction was labelled.

# CHAPTER 7 RESULTS FROM EXPERIMENTS WITH FERN AND FUNGAL SPORE

## 7-1 Introduction

Once the work on various seeds and pollens was completed it was decided to carry out some resting metabolism experiments on an example of each of the other two important plant propagules, fern spore and fungal spore. It was hoped to find whether or not resting metabolism was similar in all four types of propagule.

## 7-2 *Cyathea dealbata* (silver fern) Spore

### 7-2.1 Comments

*Cyathea dealbata*, a common tree fern, was chosen to represent fern spore. A fertile frond was cut from the plant and laid out on paper on a laboratory bench. After one day a large quantity of spore had been deposited on the paper. This was sieved to remove debris and samples were stored at 64%, 54% and 34% relative humidity. The spore could be germinated easily by sprinkling it on the surface of a nutrient solution in a crystallising dish which was then covered. The dish was left in diffused sunlight on a laboratory shelf. The green gametophytes were easily discernible after twenty days.

Nutrient solution (Heyes, 1974):

0.15 g $MgSO_4 \cdot 7H_2O$

0.12 g $KNO_3$

1.44 g $Ca(NO_3)_2 \cdot 4H_2O$

0.25 g $KH_2PO_4$

17 mg $FeCl_3 \cdot 6H_2O$

2 mg Nystatin (Sigma Chemical Co., U.S.A.)

dissolved in one litre of distilled water.

Samples of this spore were exposed to tritiated water vapour at 64% and 34% relative humidity for ten days. These were then tested for germination. After twenty days both samples showed green gametophyte though not as profuse as in the control. No attempt was made to determine the germination percentage because of radiochemical hazards in laboriously examining the dishes containing the germinating spore.

7-2.2 Results

TABLE 7.1 Tritium labelled compounds from *Cyathea dealbata* spore after 10 days exposure to tritiated water vapour.

| Compound | Relative Humidity | | |
|---|---|---|---|
| | 64% | 54% | 34% |
| glutamate | + | - | - |
| citrate | + | + | - |
| malate | t? | t? | - |
| unknown E | + | t | - |
| sugars | - | - | - |
| sugar phosphates | - | - | - |
| lipids | - | - | - |
| macromolecules | - | - | - |

+ labelled.

t faintly labelled.

- not labelled

? identity uncertain.

7-2.3 Discussion of Results

1. The most striking results compared with those from other propagules is that amino acids are not the main class of compound to incorporate most of the tritium label. In fact at 54% relative humidity, while labelled citric acid and unknown E were detected, no labelled amino acids were found.

2. The next most obvious result is that the overall incorporation of tritium is less diverse than in most other propagules studied.

3. Unknown E was not successfully co-chromatographed. It did not form a compact spot on thin layer chromatography and was therefore probably an organic acid. It is possible that it was in fact 'tailed' citric acid although it appeared separate.

4. No labelled sugars, sugar phosphates, lipids or macromolecules were detected at any relative humidity suggesting that these compounds were not involved in the resting metabolism of *Cyathea dealbata* spore.

## 7.3 Experiments with *Scleroderma bovista*

### 7-3.1 Comments

A giant puffball (fruiting body of *Scleroderma bovista*) was collected from a Waikato farm paddock. This was cut open and the spore removed from it. Samples of the spore were transferred to a humidistat at 64% and 34% relative humidities. Several techniques were tried to germinate the spore but these were unsuccessful. It was presumed the spore were in the dormant stage.

## 7-3.2 Results

TABLE 7.2 Tritium labelled compounds from *Scleroderma bovista* spore exposed to tritiated water vapour for 10 days.

| Compound | Relative Humidity | |
|---|---|---|
| | 64% | 34% |
| alanine | t | - |
| aspartate | t | - |
| 4-aminobutyrate | t | - |
| asparagine | t | - |
| glutamate | t | - |
| malate | t | t |
| organic acids | - | - |
| sugar phosphates | - | - |
| lipids | - | - |
| macromolecules | - | - |
| unknown G | t | t |
| unknown H | + | t |

+ labelled.

t faintly labelled.

- not labelled.

## 7-3.3 Discussion of Results

1. Amino acids are the main class of compounds detected as labelled. This indicates that amino acid metabolism is the most significant in the resting metabolism of *Scleroderma bovista* spore. In this sense the fungal spore are essentially similar to most of the seeds and pollen studied.

2. Only trace amounts of tritium labelled compounds could be

detected at 34% relative humidity. This indicates that the resting metabolism of the spore was at a very low level at this relative humidity. This result is essentially similar to those obtained from other propagules studied at low relative humidity.

3. Glutamate, a compound prominently tritium labelled in most other propagules studied is only faintly labelled by *Scleroderma bovista* spore. The principal compound labelled by the spore is 4-aminobutyrate, and the amino acids alanine and aspartate are labelled to a small extent.

4. Unknown G appears to be very similar to unknown P from *Pinus ponderosa* experiments. It is not ninhydrin positive, moves to a position between alanine and 4-aminobutyrate under electrophoresis and has a high $R\vec{F}$ (= 0.71) in the chromatography solvent. Unknown H, which forms a compact spot in chromatography (RF = 0.65) yet does not move under electrophoresis is probably a sugar.

## 7.4 Summary

The results of this chapter indicate that the resting metabolism of *Cyathea dealbata* and *Scleroderma bovista* spore is similar in kind to that of the seeds and pollen studied. In both species of spore amino and organic acids are tritium labelled at higher relative humidities but it appears that organic acid metabolism is relatively more important in the fern spore (*Cyathea*) than in the other propagules studied.

In neither type of spore were labelled lipids or macromolecules detected, but the solid residue left after extraction of the spore was left labelled in all experiments.

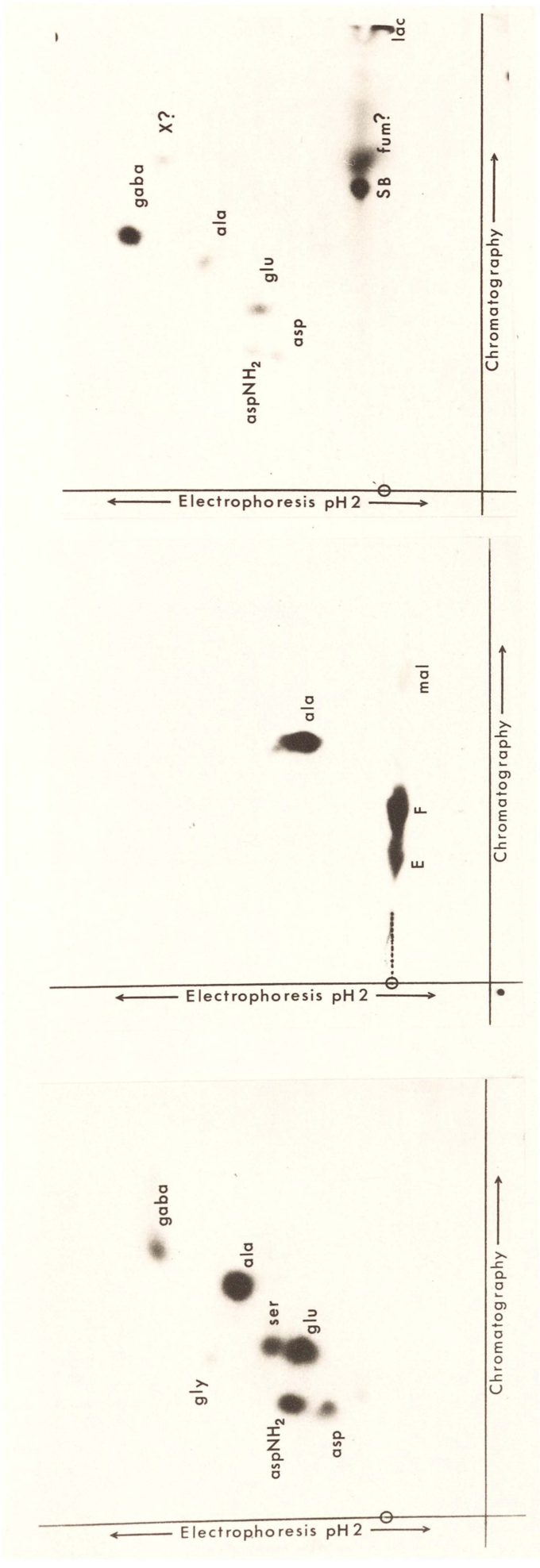
FIGURE 7.1

(a) Scintillation autograph from TLE/TLC plate of extract from *Cystiscus scoparins* pollen exposed to tritiated water vapour for 5 days at 45% relative humidity.

(b) Scintillation autograph from TLE/TLC plate of extract from *Cyathea dealbata* spore exposed to tritiated water vapour for 10 days at 64% relative humidity.

(c) Scintillation autograph from TLE/TLC plate of extract from *Scleroderma bovista* spore exposed to tritiated water vapour for 10 days at 64% relative humidity.

(Unlabelled spots at periphery of chromatogram are locating marks.)


gaba
ala
ser
glu
gly
aspNH2
asp
Electrophoresis pH 2
Chromatography
a
ala
mal
E
F
Electrophoresis pH 2
Chromatography
b
gaba
X?
ala
glu
asp
aspNH2
SB
fum?
lac
Electrophoresis pH 2
Chromatography
c

# CHAPTER 8 QUANTITATIVE EXPERIMENTS AND THE DETECTION OF LOW LEVEL TRITIUM ACTIVITY

## 8-1 Introduction

The work described so far has been a wide-ranging study of resting metabolism of various species and types of plant propagules. It appears that the nature of resting metabolism is essentially the same in all the organisms studied. The same has been found to be true of two species of lichen by Cowan (1977) and facial eczema spore by Teh (1977) using the technique developed by this author. In all the examples studied significant metabolism can be detected down to 45% relative humidity, but at 34% very little metabolism can be detected by scintillation autography. At 15% relative humidity no metabolism could be detected. This chapter describes an attempt to find more information about this sudden change in resting metabolism with relative humidity.

It has been suggested in Chapter 2 that metabolism occurring during anhydrobiosis is that which occurs in highly concentrated solution. The type of metabolism that has been indicated by the results just described in Chapters 5, 6 and 7 appears to be broadly independent of species and type of propagule. The relative humidity at which all activity stopped did vary from species to species.

An interesting question that arises is why tritium labelling ceases at a particular relative humidity. Two possibilities can be considered: (i) the enzymes responsible for the reactions that label the compounds detected may become inoperative because of changes in their tertiary structure due to the changing water relations in the

organism; (ii) the viscosity of the medium may increase ultimately to a glass-like state and the rate at which the substrates and products diffuse toward and away from the active sites of enzymes may become a limiting factor. It has been suggested that the cellular components present in the dried anhydrobiote are in a glass-like state (Clegg, 1973). As the viscosity of a glassy (solid state) solution is many orders of magnitude greater than that of a liquid solution (Trevena, 1975) this could explain the sudden decrease of metabolism between 30-45% relative humidity.

An experimental investigation was instigated to try to decide which of the above alternatives explained the lack of tritium labelled compounds from organisms exposed to tritiated water vapour at very low relative humidities. This involved using a scintillation counter to measure the total amount of radioactivity incorporated in the freeze dried extracts from organisms exposed to tritiated water vapour at various relative humidities. The advantage of scintillation autography is that it allows both detection and identification of tritium labelled compounds. Scintillation counting does not allow this easy detection and identification of tritium labelled compounds but it does allow quantitative results and is at least a thousand times more sensitive than scintillation autography. Consequently tritium activity that remained undetected by scintillation autography may be easily detected by scintillation counting.

If no tritium activity was detected at low relative humidities it would indicate that no metabolism was occurring, supporting the concept that enzymes are rendered inoperative by low water activities. If low level tritium labelling was detected it would imply that the rates of reactions have been greatly reduced, supporting the idea that the viscosity of the cellular medium has increased so much as to become a limiting factor.

It was also considered that a number of other intriguing questions could be pursued by scintillation counting. These questions included:

(1) What was the overall uptake of tritium by the organism and how was this affected by relative humidity and duration exposure?

(2) How much of this tritium was incorporated into non-volatile compounds and how was this affected by relative humidity and duration of exposure?

(3) Was there any tritium labelling in the lipid fractions and water extracts that was below the level of detection by scintillation autography, and if there was, how did this compare in amount with the labelling in the methanol fraction?

(4) Did certain compounds of interest in germination (e.g., sucrose) have no tritium label or did they not have sufficient to be detected by scintillation autography?

In order to answer some of the above questions a routine was adopted of taking aliquots of solutions at various stages of the experimental procedure. This included: (a) an aliquot from the initial combined MCW extract. This would provide a measure of the total tritium uptake by the organism. It was supposed that all the exchangeable tritium in the organism would rapidly exchange with protium in the solvent. Also this extract contained virtually all the soluble compounds containing non-exchangeable tritium. Preliminary experiments showed that taking an aliquot from the initial suspension after maceration (thus including tritium incorporated in the solid residue) showed slightly less activity than an aliquot taken from the supernatant. It was assumed that the solid material included in the former sample decreased the counting efficiency. Hence all subsequent aliquots were taken from the supernatant. (b) aliquots from each of the freeze-dried extracts after they were re-dissolved. This, it was hoped, would allow the determination of the amount of tritium incorporated

into non-volatile compounds in each extract.

## 8-2 Experimental

The experimental procedure adopted was to take 5 μℓ from each combined MCW extract, and dilute this to 5 mℓ with 10% isopropanol. Then 5 μℓ of this solution was transferred to a scintillation vial and counted as described in Section 4-4.

The freeze-dried methanol extracts were redissolved in 0.1 mℓ of 10% isopropanol and 5 μℓ of this was diluted to 5 mℓ. 0.1 mℓ of this solution was transferred to a scintillation vial.

A similar procedure was adopted for the other two types of extract except that after redissolving in 0.1 mℓ of the appropriate solvent, 5 μℓ were transferred directly to a scintillation vial.

## 8-3 Precision of Results

It should be stressed that the results obtained from scintillation counting were not envisaged as being highly precise. Various factors were not conducive to precision, such as:

(1) the possible inefficiency of extraction;

(2) the difficulties in accurately transferring organic solvents of low surface tension (e.g., MCW extraction solvent, chloroform methanol 1:1 for redissolving lipids);

(3) the volumetric errors introduced by micro-scale handling.

All these factors were accentuated by considerations of radiochemical safety (particularly in handling volatile radioactive solvents) which militated against best volumetric procedures being adopted.

The errors given in the results below are estimated from the statistical uncertainty of counting and the errors claimed by the manufacturers of the micro-volumetric apparatus used.

It should be realised that the questions posed at the beginning

of this chapter do not require more than semi-quantitative results. In some cases only the order of magnitude of tritium labelling was required, and in others simply whether or not tritium label was present.

## 8-4 Problems Encountered with Scintillation Counting of Extracts

### 8-4.1 Initial Results

Initial results obtained from scintillation counting of extracts were rather puzzling in that they did not reflect the trend of results obtained by scintillation autography. This applied mainly to the methanol fraction. An example of this is given in the following table of results from parsnip seeds, though similar results were found with extracts from other propagules.

TABLE 8.1 Tritium activity in freeze-dried methanol fraction* from *Pastinaca sativa* seeds exposed to tritiated water vapour for 12 days.

| Relative Humidity % | Activity of Fraction $nCi \cdot mg^{-1}$ |
|---|---|
| 64 | 145 ± 9 |
| 54 | 47 ± 3 |
| 45 | 93 ± 5 |
| 34 | 104 ± 6 |
| 15 | 38 ± 3 |

*This fraction contains most of the water soluble, small molecule metabolites.

It would appear from these results that there is more tritium labelling at 45% and 34% than at 54% relative humidity. This is contrary to the results obtained by scintillation autography.

After a number of such anomolous results had been obtained from

barley and *Sinapis alba* seeds it was suspected that some of the tritium detected by scintillation counting was labile (or semi-labile), and that this labile tritium was lost during subsequent chromatography in the procedure for scintillation autography.

### 8-4.2 Results from Re-freeze Drying Extracts

To test the possibility that the extracts contained labile tritium, they were dissolved in 5 mℓ of solvent (10% propan-2-ol in water) and freeze dried again. This, it was hoped, would allow the exchange of any labile tritium remaining in the extract and this would be carried over with the solvent during evaporation. This was tested with extracts from parsnip seeds at 64, 54 and 34% relative humidity.

TABLE 8.2 Effect of second freeze drying on methanol extracts from parsnip seeds.

| Relative Humidity % | Initial Freeze Drying $nCi \cdot mg^{-1}$ | Second Freeze Drying $nCi \cdot mg^{-1}$ |
|---|---|---|
| 64 | 145 ± 9 | 104 ± 9 |
| 54 | 45 ± 3 | 45 ± 4 |
| 34 | 104 ± 6 | 17.5 ± 1.6 |

The above results clearly support the hypothesis that the once freeze-dried extracts contain labile tritium. They also indicate that the amount of labile tritium in each extract is extremely variable. The presence of some labile tritium can be explained by the presence of exchangeable tritium in many of the metabolites present in the extract. It is also possible that some semi-labile tritium does not have sufficient time to back exchange with the extraction solvent during the extraction process. Both of these factors could be responsible for the presence of labile tritium in the freeze-dried

extracts and the latter could give rise to variable amounts of labile tritium depending on the time between the beginning of extraction and the freezing of the extracts prior to freeze drying.

### 8-4.3 Attempts to find the Nature of the Labile Tritium in Methanol Extracts

In order to investigate the labile tritium in methanol extracts it was decided to take samples from a methanol extract and expose them to various conditions to find how these affected the subsequent tritium activity detected by scintillation counting.

Some parsnip seeds that had been exposed to tritiated water vapour for three months at 64% relative humidity were extracted as described previously. The freeze-dried methanol extract was dissolved in 5 mℓ of 10% propan-2-ol and a 5 μℓ aliquot was taken for a scintillation vial. The remaining extract was divided into five 1 mℓ samples and treated in the following way:

(1) Freeze dried.

(2) Heated to 50 C for one hour then freeze dried.

(3) Acidified to pH 2 with 0.1 mol $\ell^{-1}$ HCl. (a) Half of this solution was freeze dried; (b) half was heated to 50 C for one hour then freeze dried.

(4) Brought to pH 11.5 with 0.1 mol $\ell^{-1}$ NaOH then freeze dried.

(5) Left for 10 days at 4 C then freeze dried.

The object of processes (2), (3) and (4) was to accelerate any slow exchange reaction so as to leave only non-exchangeable tritium in the extract. If conditions had been too extreme (i.e., strong acid or alkali) the breakdown of compounds (e.g., glutamine) containing non-exchangeable tritium may have occurred.

For the samples (1) to (4) the freeze-dried extracts were dissolved in 1 mℓ of water, a 5 μℓ sample taken for scintillation counting and the process for each flask, described above, was repeated. This was

followed by the same procedure a third time.

TABLE 8.3 Results from experiments to remove semi-labile tritium from extracts of *Pastinaca sativa* seeds.

| Process Used | Initial Activity $nCi \cdot mg^{-1}$ | First Treatment $nCi \cdot mg^{-1}$ | Second Treatment $nCi \cdot mg^{-1}$ | Third Treatment $nCi \cdot mg^{-1}$ |
|---|---|---|---|---|
| 1 | 236 ± 12 | 256 ± 23 | 193 ± 17 | 193 ± 17 |
| 2 | " | 219 ± 19 | 176 ± 17 | 160 ± 14 |
| 3 (a) | " | 181 ± 16 | 160 ± 14 | 149 ± 13 |
| 3 (b) | " | 192 ± 17 | 158 ± 14 | 150 ± 14 |
| 4 | " | 198 ± 18 | 159 ± 14 | 143 ± 13 |
| 5 | " | 81 ± 7 | - | - |

8-4.4 Discussion

The results show that the only treatment which had a marked effect on activity was that of leaving the extract in 10% isopropanol for ten days at 4 C. It would be unwise to place too much significance on this result because of the problem of experimental artefacts. For example, some micro-organism may have been active in spite of the isopropanol, or tritiated compounds may have esterified with the isopropanol and evaporated during subsequent freeze drying. However, if these possibilities could be excluded the conclusion would have to be that slow exchange processes do take place. It would be interesting to prepare another extract in the same manner as this one and follow the reduction of activity with time. It could be determined if the extract continues to lose activity until it all disappears, or if the activity reaches a limiting value. However, time did not permit this study. The other general aspect of the results is the overall decrease in activity of all treatments with repetition. (A decrease of 0.5%

would be expected because of the 5 μℓ removed for counting.) The large errors do not allow too much emphasis to be placed on this observation, but two possibilities may be considered:

(1) Slow exchange reactions are occurring to remove semi-labile tritium from the non-volatile compounds.

(2) Some of the non-volatile compounds are physically carried over with the evaporating solvent in the freeze-drying process. This possibility could be responsible for a small loss of non-exchangeable tritium by any extract during freeze drying. However, the freeze drying samples were usually inspected during the process to ensure that melting and consequent 'bumping' did not occur.

It is apparent that there may be more to the significance of results obtained by scintillation autography. Other experiments indicated an initial large decrease in activity of some extracts upon the first of repeated freeze dryings. This was followed by small decreases (< 10%) upon subsequent freeze dryings.

A number of interesting questions have been raised by the above results which would warrant further study. However, as considerable time had been devoted to this section of the work, it was decided to adopt a standard procedure for scintillation counting of extracts and continue the study of resting metabolism largely on the basis of results from scintillation autography.

The procedure adopted was to dissolve each extract in 1 mℓ of the appropriate solvent and freeze dry it again. This was repeated, then the extract dissolved in 0.1 mℓ of solvent and 5 μℓ taken for scintillation counting and the remainder used for chromatography.

A set of results using this modified procedure was obtained for *Pinus ponderosa* pollen and *Sinapis alba* seeds, and these follow.

## 8-5 Scintillation Counting of Extracts from *Sinapis alba* Seeds

### 8-5.1 Results

A considerable number of results were amassed from scintillation counting of extracts from seeds. Because the activity detected in the extracts may not represent non-exchangeable tritium as discussed above, it was decided not to publish all of these results but only a typical example. There follows a discussion which would generally apply to other examples. Table 8.4 shows results from a series of experiments with *Sinapis alba* seeds at various relative humidities. Activities are calculated as tritium activity per mg of seed.

### 8-5.2 Discussion of Results

Total tritium uptake: This would be expected to vary according to (a) the specific activity and amount of the tritiated water, and (b) the amount of exchangeable hydrogen in the sample. It should be pointed out that much of the exchangeable hydrogen in the sample is not as $H_2O$, so the uptake of tritium would not be expected to be necessarily directly proportional to the moisture content of the seed. Nevertheless if (a) remained constant it would be reasonable to expect that the total tritium uptake to decrease with relative humidity. However, the specific activity of the tritiated water could have been reduced by residual water in the salts used to maintain constant relative humidity. Also the amount of tritiated water was not carefully measured because of handling difficulties associated with 5 Ci $m\ell^{-1}$ tritiated water. All that can be said of the values in the table is that the total tritium uptake is of the same order of magnitude at each relative humidity.

Methanol Extract: The incorporation of tritium into non-volatile compounds in the methanol extract at 75% relative humidity is nearly seven times that incorporated at 64% relative humidity. This is reflected in the much more intense labelling pattern detected by

TABLE 8.4 Tritium activity in extracts of *Sinapis alba* seeds exposed to tritiated water vapour for 12 days.

| % Relative Humidity | Status of Sample | MCW Extract(a) Total $^3H$ Uptake $mCi \cdot mg^{-1}$ | Methanol Extract(b) $\mu Ci \cdot mg^{-1}$ | Chloroform Extract(Lipid) $nCi \cdot mg^{-1}$ | Water Extract macromolecules $nCi \cdot mg^{-1}$ |
|---|---|---|---|---|---|
| 75 | live seed | 1.77 ± 0.29 | 6.57 ± 0.61 | 28.6 ± 2.5 | 280 ± 25 |
| 64 | live seed | - | 1.02 ± 0.09 | 12.8 ± 1.0 | 259 ± 2 |
| 64 | autoclaved | - | 1.20 ± 0.12 | 10.9 ± 0.9 | 13 ± 1 |
| 64 | MCW control (c) | - | 0.67 ± 0.07 | 77 ± 7 | - |
| 54 | live seed | 1.57 ± 0.26 | 0.46 ± 0.04 | 16.0 ± 1.4 | 174 ± 14 |
| 45 | live seed | 1.91 ± 0.30 | 0.40 ± 0.04 | 14.9 ± 1.3 | 140 ± 12 |
| 34 | live seed | 1.29 ± 0.20 | 0.17 ± 0.015 | 1.4 ± 0.1 | 37 ± 3 |
| 34 | MCW control (c) | - | 0.23 ± 0.02 | 33.4 ± 3 | - |
| 15 | live seed | 1.66 ± 0.26 | 0.15 ± 0.013 | 1.1 ± 0.1 | 34 ± 3 |

(a) MCW - methanol:chloroform:water (12:5:3); the initial extraction solvent.

(b) Methanol extract: this contains most of the small-molecule, water-soluble metabolites.

(c) See Section 3-4 for explanation of this control.

scintillation autography at the higher relative humidity. At lower relative humidities the difference in tritium incorporation as detected by scintillation counting is not as great as would be expected from the results of scintillation autography. It may be that even after three freeze dryings the extracts still contain semi-labile tritium which is lost during chromatography in the scintillation autographic technique. This could be tested by eluting all the material from a chromatogram of an extract from each relative humidity and count the total activity present after chromatography (see Section 8-5.3).

Another possibility is that there are at 34% and 12% relative humidity a large number of compounds each with a very small amount of activity and none of which have enough radioactivity to be detected by scintillation autography, but when this is measured in total it represents a considerable amount of activity. At higher relative humidities, perhaps, the extra activity is present in only a few compounds allowing these to be detected by scintillation autography. This possibility would have to be investigated experimentally. One way would be to cut the entire chromatogram into small squares and determine the activity in each square by scintillation autography.

The autoclaved seeds and MCW control showed surprisingly high tritium activity in the methanol fraction. The fraction from the autoclaved seeds had slightly more activity than that from the live seeds. Even the MCW control showed more than half as much activity as the live seeds. These results are in direct contrast with those obtained by scintillation autography, where neither of these controls showed any detectable tritium activity. These results are rather intriguing and indicate the need for more research on the nature of the tritium label in non-volatile compounds left after freeze drying. Once again, the possibility of compounds containing semi-labile

tritium contaminating the freeze-dried extract exists. If this is so, then this semi-labile tritium is presumably exchanged during chromatography. This possibility could be checked as suggested above by eluting the material from the appropriate chromatogram and measuring its activity.

It should be remembered that the organisms have been exposed to tritiated water vapour for relatively long periods (12 days) during which time slow exchange reactions could occur which may not normally be observed in other experiments such as those studying germination with liquid tritiated water where maximum exposure times are in the order of hours.

Chloroform Extract: From examining the results it is easy to understand why no labelled lipids were detected, as the amount of tritium incorporated into the chloroform extract was always much less than in the corresponding methanol extract. This suggests that the metabolism of lipids in dry seeds, if it occurs at all, is of much less significance than that of small molecule water soluble metabolites.

It should be noted that repeated freeze drying seems to have no significant effect upon labelling in the chloroform extract. This suggests that the activity detected was a reasonable measure of the non-labile tritium incorporated into lipids. If this is so, then the high levels of labelling in the controls indicates that the labelling of lipids in the live seeds is due to non-metabolic processes. (The lipids of the MCW control at 64% relative humidity have six times as much tritium activity as those from live seeds.)

Water Extracts: These show remarkably high tritium activities at higher relative humidities, although no activity could be detected by scintillation autography. It is interesting that autoclaved seed showed very little activity in the water extract. It is possible that extracts from the live seeds contained considerable amounts of semi-

labile tritium that was lost during chromatography. Hence the high activity detected by scintillation counting may be a consequence of non-metabolic activity.

8-5.3 Activity in Methanol Extract After Chromatography

A chromatogram from each relative humidity was taken after scintillation autography and the entire chromatogram was eluted with water. This was achieved by attaching the chromatogram to a wick placed in a trough of distilled water (as in two-dimensional descending chromatography). The chromatogram had been cut at an angle across the bottom to cause the eluate to accumulate in one corner and then be collected in a beaker. When about 4 mℓ of water had eluted from the chromatogram the eluate was made up to 5 mℓ with water and an aliquot taken for counting. Further eluate was collected but it was always found to contain less than 1% of the tritium eluted off initially. The chromatogram was dried and re-exposed to film, but no activity was detected.

The activity present in the initial eluate was calculated as total activity in extract per mg of seed. These results are presented below and compared with those of the methanol fraction prior to chromatography.

TABLE 8.5 Tritium activity of methanol extracts eluted from chromatograms.

| Sample | $^3H$ Activity of Eluate $nCi \cdot mg^{-1}$ | $^3H$ Activity of Extract Prior to Chromatography $nCi \cdot mg^{-1}$ |
|---|---|---|
| 75% live | 1650 ± 160 | 6570 ± 610 |
| 64% live | 330 ± 40 | 1020 ± 100 |
| 64% autoclaved | 70 ± 7 | 1200 ± 120 |
| 64% MCW control | 50 ± 6 | 670 ± 70 |
| 54% live | 250 ± 30 | 460 ± 40 |
| 45% live | 120 ± 10 | 400 ± 40 |
| 34% live | 50 ± 6 | 170 ± 20 |
| 34% MCW control | 7 ± 0.8 | 230 ± 20 |
| 15% | 4 ± 0.5 | 150 ± 20 |

These results clearly show that in all extracts most of the tritium label found in the methanol extract does not survive chromatography. The results of tritium labelling detected in the eluate from extracts of live seeds compare well with those of scintillation autography. It would appear that the tritium labelling at 34% is almost half that at 45% relative humidity. Chromatography of extracts from 34% probably contain tritium labelled compounds just below the limit of detection. However, there is a very marked drop in tritium labelling at 15% relative humidity. Presumably resting metabolism in *Sinapis alba* seeds undergoes a considerable decrease between 34% and 15% relative humidity.

The controls at 64% relative humidity show much less tritium activity in the eluate than the live seeds. This contrasts with the results obtained from the methanol extract prior to chromatography, but compares favourably with the results from scintillation autography.

The control at 34% relative humidity shows that the tritium activity in the live seeds even at this low relative humidity is probably due to physiological (not merely chemical) processes.

It appears, then, that the chromatographic processes used have afforded a simple means of decontaminating the methanol extract from relatively large amounts of semi-labile tritium activity.

### 8-5.4 Summary

The results from this section of work indicate that scintillation counting of extracts was more complicated than it first appeared. No simple procedure would yield unequivocal results. This suggests an interesting line of radiochemical research to discover the sources of semi-labile tritium in methanol extracts from resting metabolism experiments. It is probably significant that the experiments were of such a duration (several days) as to allow tritium labelling to slow exchange reactions not normally observed. Chromatography was shown to be an effective means of decontaminating the methanol extract of the semi-labile tritium.

Labelling in lipids was considerably less than that of the corresponding methanol extracts, though most of the labelling measured was thought to be non-labile. It was likely that the water extract contained semi-labile tritium. It was suggested that the labelling in both the chloroform and water extracts was due to non-metabolic processes.

## 8-6 Effect of Relative Humidity on Tritium Labelling in *Pinus ponderosa* Pollen Exposed to Tritiated Water Vapour

### 8-6.1 Results

As with seeds a large number of results were obtained from scintillation counting of extracts from pollen. The following table

is representative of these results and illustrates the effect of relative humidity of tritium activity in the various extracts from *Pinus ponderosa* pollen.

TABLE 8.6 Tritium activity in extracts of *Pinus ponderosa* pollen exposed to tritiated water vapour for 5 days.

| Relative Humidity % | MCW Extract Total Uptake $mCi \cdot mg^{-1}$ | Methanol Extract $nCi \cdot mg^{-1}$ | Chloroform Extract(Lipids) $nCi \cdot mg^{-1}$ |
|---|---|---|---|
| 64 | 3.20 ± 0.26 | 1080 ± 90 | 20 ± 1 |
| 64 autoclaved | - | 157 ± 12 | 13.2 ± 0.6 |
| 64 MCW control | - | 126 ± 10 | 42 ± 2 |
| 54 | 2.11 ± 0.17 | 470 ± 40 | 12.5 ± 0.6 |
| 45 | 1.68 ± 0.13 | 280 ± 22 | 10.0 ± 0.5 |
| 34 | 0.91 ± 0.09 | 151 ± 12 | 6.9 ± 0.3 |
| 15 | 1.16 ± 0.10 | 172 ± 6 | 5.0 ± 0.3 |

8-6.2 Discussion

Total Tritium Uptake: The results show a general decrease with relative humidity except that the figure for 15% is higher than the figure for 34%. This is not too surprising as the salt solution to provide 34% relative humidity (magnesium chloride solution) was made with a dihydrate. The water of hydration of this salt would have diluted specific activity of the tritiated water. It is interesting that apart from this anomaly the trend is more as would be expected than in the case of *Sinapis alba* seeds. One explanation is that the free water in pollen represents a greater proportion of the exchangeable hydrogen than in seeds. This is not a very significant feature of the work and no further study was initiated. However, it is worth noting that the total tritium uptake per milligram of

tissue by the pollen was of the same order of magnitude as that of the mustard seeds.

Methanol Extract: The results clearly show, as expected, a decrease in tritium incorporation with relative humidity. However, the activities at 34% and 15% relative humidity are higher than would be expected from the results of scintillation autography. The explanation for this may be the same as for the extracts from *Sinapis alba* seeds. Either the extracts contain semi-labile tritium which is lost during chromatography, or there are a large number of compounds each containing a small amount of label. To discover if either of these proposals are tenable it would be necessary to conduct further experiments. Section 8-8 describes a series of experiments designed to give further information about tritium labelling at low relative humidities.

The results from control experiments show much less tritium activity in the methanol extract than with the live pollen. This is in general agreement with results from scintillation autography. However, the tritium activity measured in the controls is still quite appreciable. It is possible that at least some, or perhaps most, of this activity is semi-labile.

Chloroform Extract: The tritium activity of the chloroform extracts was very much less than that of the corresponding methanol extracts. This may explain why no tritium activity was detected in the chloroform extracts by scintillation autography.

The tritium activity in the chloroform extracts from control experiments is relatively high. In fact, in the MCW control it is twice as high as in the live pollen. This suggests that the labelling found in lipids from the pollen is incorporated by non-metabolic means.

### 8-6.3 Summary

The total tritium uptake by *Pinus ponderosa* pollen exposed to tritiated water vapour showed some correlation with relative humidity and was of the same order of magnitude as the total tritium uptake in *Sinapis alba* seeds.

The tritium activity found in methanol extracts decreased with relative humidity but was unexpectedly high at 34% and 15% relative humidity. There was appreciable labelling in the controls although this may be semi-labile.

Tritium labelling was detected by scintillation counting in lipids from *Pinus ponderosa* pollen, but this may well have been labelled by non-metabolic means.

## 8-7 Effect of Duration of Exposure on Tritium Labelling by *Pinus ponderosa* Pollen Exposed to Tritiated Water Vapour

### 8-7.1 Results

During the course of the research results were accumulated from *Pinus ponderosa* pollen exposed to tritiated water vapour for various time intervals at relative humidities of 64, 45 and 34%. These are produced in Table 8.7 and their significance discussed.

### 8-7.2 Discussion of Results

It must be pointed out that a more comprehensive set of results would have been very desirable. However, as it was a very time-consuming procedure to obtain these results, and as each extraction involved the ingestion of small amounts of volatile tritium by the experimenters in spite of all precautions, it was decided not to obtain a wider set of results.

The activity measured in the MCW extracts indicates that tritium uptake by the pollen is rapid. At 64% and 34% relative humidity the

TABLE 8.7 Tritium uptake and labelling by *Pinus ponderosa* pollen for various time intervals.

| Exposure Days (RH%) | MCW Extract $^3H$ Uptake $mCi \cdot mg^{-1}$ | Methanol Extract $nCi \cdot mg^{-1}$ | Chloroform Extract (Lipids) $nCi \cdot mg^{-1}$ |
|---|---|---|---|
| 1 (64) | 2.31 ± 0.21 | 240 ± 19 | 2.5 ± 0.2 |
| 2 (64) | 3.20 ± 0.29 | 570 ± 46 | 6.7 ± 0.5 |
| 5 (64) | 3.42 ± 0.31 | 1080 ± 86 | 20.0 ± 1.6 |
| 11 (64) | 4.30 ± 0.39 | 1640 ± 131 | 55 ± 4 |
| 2 (45) | 1.33 ± 0.12 | 37.6 ± 3.0 | 3.3 ± 0.2 |
| 5 (45) | 0.98 ± 0.09 | 115 ± 9 | 9.8 ± 0.8 |
| 10 (45) | 2.08 ± 0.19 | 264 ± 21 | 10.4 ± 0.8 |
| 20 (45) | 1.41 ± 0.13 | 405 ± 32 | 22.6 ± 1.8 |
| 1 (34) | 0.68 ± 0.06 | 7.2 ± 0.6 | 0.7 ± 0.1 |
| 2 (34) | 1.00 ± 0.09 | 58 ± 5 | 1.6 ± 0.2 |
| 5 (34) | 0.91 ± 0.08 | 150 ± 12 | 7.5 ± 0.6 |

tritium uptake after two days exposure to THO vapour is within experimental error the same as at five days. At 45% relative humidity the activity detected after five days exposure to THO vapour is actually less than at two days. This is probably due to an experimental anomaly. Even after a one day exposure to tritiated water vapour the $^{3}H$ uptake is not much less than at two days at 64% relative humidity.

It would appear that the initial incorporation of tritium into non-volatile compounds in the methanol extract occurs almost linearly at 64% relative humidity, followed by reducing rate of incorporation as time increases. At 45 and 34% relative humidity, however, it appears that the initial rate of incorporation of tritium into metabolites is slow and then increases with time, then presumably decreases again. It may be that tritiated water rapidly gets to the centres of metabolism responsible for tritium labelling at 64% relative humidity, but at lower relative humidities there is a time lag. The reason for this is not readily apparent, but it is possible that the process responsible for tritium labelling at 34% relative humidity exhibits a time lag. This process may also occur at 64% relative humidity but it is overshadowed by other processes that occur at the higher relative humidity. The situation at 45% relative humidity could represent an intermediate phase. In other words, there may be two (or more) separate processes responsible for the tritium labelling that was found. These may be situated in different sites in the pollen cells, or it may be that the intracellular organisation of the pollen changes with relative humidity.

The rate of lipid labelling appears to increase with time at 34% and 64% relative humidity. It is suggested that lipid is distributed throughout the pollen cell and as the tritiated water diffused in the

cell more lipid becomes available to be labelled. Thus the rate of tritium labelling could increase.

## 8-8 Attempt to Discover the Nature of Tritium Labelling at Low Relative Humidity

### 8-8.1 Introduction

The results from Sections 8-5, 8-6 and 8-7 have given rise to a number of interesting questions. Each one would require considerable experimental work to answer. Consequently an arbitrary choice had to be made as to which direction would be taken to proceed. It was decided to examine possible tritium labelling at all relative humidities in those compounds found to be labelled on paper chromatograms at 64% relative humidity in *Pinus ponderosa* pollen. This was principally designed to find if label was present, just below the level of detection by scintillation autography, in trace amount, or not at all. This is described below.

### 8-8.2 $^{3}H$ Activity of Selected Areas from Chromatograms of *Pinus ponderosa* Pollen Methanol Extracts

Because of the rather inconclusive results obtained from scintillation counting of whole extracts it was decided to cut various areas from paper chromatograms corresponding with compounds found to be detected by scintillation autography. Extracts from *Pinus ponderosa* pollen exposed to tritiated water vapour for five days at five different relative humidities were analysed by paper chromatography. Radioactive areas detected by scintillation autography were found. Each chromatogram was dipped in 0.2% ninhydrin in acetone and warmed to 40 C. As soon as ninhydrin positive spots appeared they were marked and cut out, then placed into a scintillation vial containing 0.5 mℓ of water. The areas where malic acid and sucrose would be expected to be found were also cut out and placed into separate scintillation vials containing

0.5 mℓ of water. These two areas were located with respect to known amino acids. This procedure was not entirely satisfactory in view of the variable nature of chromatography. However, the areas cut out were large, with the hope that at least negative results would be reasonably conclusive. If no tritium labelling was found in the 'sucrose area' then it can be said with reasonable confidence that this compound does not incorporate tritium. If labelling was found then it can only be said that sucrose may incorporate tritium because the 'sucrose area' on the chromatogram will almost certainly contain compounds other than sucrose.

Each vial now had 10 mℓ of scintillation fluid added (Section 4-4) and counted for tritium activity. The efficiency of the entire procedure was checked with some $^{3}H$ labelled 4-aminobutyric acid on chromatography paper which was dipped in ninhydrin and warmed until a purple colouration was observed. The efficiency, compared with putting the same amount of $^{3}H$ labelled 4-aminobutyric acid straight into a scintillation vial, was excellent (92%).

In the results below the activity of each compound is calculated for the entire extract.

TABLE 8.8 $^{3}H$ activity in compounds cut from chromatograms from experiments with *Pinus ponderosa* pollen exposed to THO for 5 days.

| RH % | alanine nCi | aspartic acid nCi | 4-amino butyric nCi | glutamic acid nCi | "malic acid" area | "sucrose" area |
|---|---|---|---|---|---|---|
| 64 | 147±17 | 712±85 | 672±81 | 2180±260 | 96±11 | 27±3 |
| 54 | 216±26 | 318±38 | 47±6 | 1730±210 | 37±4 | 21±3 |
| 45 | 82±10 | 53±6 | 35±4 | 318±38 | 24±3 | 25±3 |
| 34 | 8.0±1.6 | 3.0±0.8 | 4.4±0.9 | 2.0±0.4 | - | 9.8±1.7 |
| 15 | 4.7±1.0 | 1.2±0.4 | 6.5±1.2 | 1.1±0.3 | 1.2±0.4 | 10.0±1.8 |

8-8.3 Discussion of Results

Each compound is considered individually then a general discussion follows.

Alanine: The most significant feature about the activity of this compound is the ten-fold drop between 45% and 34% relative humidity. The activity at 64% is surprisingly lower than that at 54%. This may be the consequence of an experimental artefact.

Aspartic acid: The activity of this compound drops with relative humidity showing a very sharp decrease between 45% and 34%.

4-aminobutyric acid: The activity of this compound decreases more than ten-fold between 64% and 54% relative humidity. A further considerable drop is observed between 45% and 34%.

Glutamic acid: At the three higher relative humidities this is clearly the most intensely labelled compound having three times as much activity as the next most intensely labelled compound at any particular relative humidity. This is consistent with the results from scintillation autography. At 34% and 15% relative humidity, however, the tritium activity in glutamic acid is almost negligible, showing a drop of over one hundred times.

Malic acid area: This area shows a very sharp decrease in tritium activity below 45% relative humidity. In fact, at 34% relative humidity the count rate was the same as the background (48 c.p.m.).

Sucrose area: It is interesting that appreciable amounts of this compound were found at all humidities, although sucrose was never detected by scintillation autography. This may have been due to a low specific activity of tritium labelled sucrose, i.e., there was a high concentration of sucrose on the chromatogram. It is known that sucrose is a major compound of *Pinus* pollens (Helmers and Machlis, 1956). The lack of a sharp drop in activity with decreasing relative humidity may indicate non-metabolic labelling of the sucrose area. Further

experimental evidence is required to resolve this question.

General discussion: The results are consistent with those from scintillation autography showing a sharp drop in tritium activity between 45% and 34% relative humidity. This indicates that the processes responsible for tritium labelling of all the compounds, except those in the 'sucrose' area are very dependent upon relative humidity and hence the water activity in the pollen.

The relationship of these results to thos obtaining by counting an aliquot of the entire methanol extract is interesting. At all relative humidities the total activity of the six compounds measured is at least several times less than that of the activity of the original methanol extract. While there may be traces of activity on areas of the chromatogram that was not measured, it is unlikely that these will greatly affect this conclusion. This is further evidence that the original extract still contains semi-labile tritium which is removed during chromatography.

It is, of course, possible that the chromatograms themselves contain semi-labile tritium. To check this an 18-month old chromatogram was re-exposed to X-ray film but no discernible change could be observed in the exposure compared with an exposure made just after the preparation of the chromatogram.

## 8-9 Conclusions

The scintillation counting of extracts did not yield the simple results expected initially. However, a number of intriguing results were obtained. Although not all of the questions asked at the beginning of this chapter can be unequivocally answered, the results that were obtained posed a number of new and interesting questions — a common consequence of research.

As the main thrust of the research undertaken by this author

involved using scintillation autography to study the resting metabolism of a variety of organisms, the experiments with scintillation counting had to be limited eventually. The questions that result from this work must be left for subsequent researchers.

The results from the two organisms studied indicate that scintillation counting of freeze-dried extracts does not give a reliable indication of the non-exchangeable tritium incorporated. Further, it appears that semi-labile tritium is very difficult to remove even by repeated freeze dryings. It became clear that paper chromatography was an effective way of removing this semi-labile tritium from mixtures of biological compounds. This capability may be a consequence of the chemical characteristics of the chromatography solvent, or the surface characteristics of the paper fibres, or both.

It appears that in the case of *Pinus ponderosa* pollen the tritium labelling at 34% relative humidity is greatly reduced from that at 45% relative humidity. However, it did not cease entirely. It is suggested that the sharp decrease in labelling is due to a change in the properties of intracellular medium surrounding the enzyme. This greatly reduces the rate of diffusion of the substrates and products, and this becomes a rate-limiting step. If this is so it indicates that the operation of enzymes in the anhydrobiotic organism is extremely resistant to desiccation. More experimental data would be required to validate this hypothesis and this is discussed in 'Suggestions for Further Work' (Chapter 11).

The results from this section show that there are appreciable amounts of tritium activity in the chloroform and water extract even though this was not detected by scintillation autography. It is suggested that this label may be due largely to non-metabolic processes. Some further work was carried out on these extracts and this is described in the next chapter.

# CHAPTER 9 FURTHER EXPERIMENTS ON THE WATER EXTRACT, CHLOROFORM EXTRACT AND SOLID RESIDUE

## 9-1 Nature of Tritium Labelling in the Water Extract

### 9-1.1 Discussion

Although scintillation autography showed no tritium labelled macromolecules from any of the water extracts from resting metabolism experiments, scintillation counting showed considerable activity in most water extracts. It was decided to determine if any of this tritium label was incorporated into macromolecular compounds using a more sensitive technique. It is possible that the activity detected by scintillation counting may be residual amounts of tritium labelled small molecule metabolites, that were not extracted by MCW solvent. In fact, in some experiments such compounds were detected by scintillation autography (these moved from the origin of the one-dimensional chromatogram, see 4-1.3).

Gel filtration columns such as Sephadex provide a convenient way of separating substances on the basis of relative molecular mass (Mr). Sephadex G-25 allows compounds with Mr > 5000 to pass through a column without retention and it is commonly used to separate macromolecular compounds from small molecule metabolites. If a sample of a water extract was passed through such a column the macromolecular compounds will be eluted unhindered, but metabolites of the sort encountered in the methanol extract (small molecule) will be strongly retained. If fractions of the eluate are measured for tritium activity it will be possible to tell with a high degree of confidence whether macromolecules in the water extract are labelled or not.

9-1.2 Experimental

A Sephadex G-25 column was prepared as follows: 1 g of Sephadex G-25 was left to soak in pH 7 phosphate buffer (0.05 mol $\ell^{-1}$) for 24 hours. This was transferred into a 10 × 150 mm column. Molecules with Mr > 5000 were eluted from the column with the void volume of 3 mℓ; this was checked with blue dextran (Mr ≈ $2 \times 10^5$). Small molecules (Mr < ≈ 300) were eluted after another 3 mℓ of buffer had passed through the column; this was checked with bromophenol blue.

The water extract was dissolved in 1 mℓ of pH 7 phosphate buffer (0.05 mol $\ell^{-1}$) and filtered through a small piece of tissue paper lodged in a Pasteur pipette to remove particles of insoluble material often present. 100 μℓ of the filtered solution was placed on the top of the column and this was eluted with pH 7 phosphate buffer (0.05 mol $\ell^{-1}$). 0.5 mℓ fractions were collected in scintillation vials and 10 mℓ of scintillation fluid added to each vial. The vials were then counted for tritium activity.

9-1.3 Results and Conclusions

Experiments of the type just described were carried out on water extracts from *Sinapis alba, Hordeum vulgare* and *Pastinaca sativa* seeds and *Pinus ponderosa* pollen from 64% and 45% relative humidities. All samples showed a peak of activity in about the sixth mℓ, but no sample showed any peak near 3 mℓ.

It can be said with reasonable certainty that no tritium labelled macromolecules are present in any of the water extracts. The activity found in the water extracts appears to be present in small molecule metabolites.

## 9-2 Distribution of Tritium Labelling in Lipids

### 9-2.1 Discussion

Scintillation counting has shown that chloroform extracts contain easily detectable tritium activity (Chapter 8). It was decided to investigate this further to find how this tritium activity was distributed in the lipids present in the extract.

### 9-2.2 Experimental

A small sheet (50 × 100 mm) of aluminium-backed silica gel (Merck Cat. No.5562) was cut out. 5 µℓ of the redissolved chloroform extract was placed 15 mm from one long edge and 10 mm from one end. Another 5 µℓ was placed similarly 15 mm from the other long edge, and 10 mm from the same end. Another chromatogram was prepared in the same way. One of these was developed with redistilled chloroform and the other in chloroform, methanol, water (CMW) (see Section 4-1.2). After development and drying the chromatogram was cut in half (longitudinally) and one half sprayed with 10% phosphomolybdic acid in ethanol then heated to detect lipids.

For each area corresponding with a compound detected on the sprayed half the adjacent area on the unsprayed half was cut out and put into a scintillation vial. The RF value of each area was recorded and the count rate measured.

The efficiency of counting from the thin layer material was checked by transferring 5 µℓ of redissolved chloroform extract to a small square of thin layer material and putting this in a scintillation vial. The count rate of this was compared with that of a 5 µℓ aliquot transferred directly to a scintillation vial.

### 9-2.3 Results

Results from *Sinapis alba*, *Hordeum vulgare* and *Pastinaca sativa* seeds and *Pinus ponderosa* pollen were obtained. They were generally

similar and an example is given below.

TABLE 9.1 $^{3}H$ activity in lipids from *Pastinaca sativa* seeds exposed to tritiated water vapour for 12 days.
(a) Neutral lipids (chromatography solvent chloroform).

| Sample | Count rate |
|---|---|
| RF = 0.53 | 613 ± 25 |
| RF = 0.24 | 723 ± 27 |
| RF = 0.10 | 1160 ± 34 |
| RF = 0 | 3861 ± 59 |
| Total | 6360 ± 140 |
| 5 µℓ aliquot | 7455 ± 86 |
| 5 µℓ of TLC material | 5304 ± 73 |

(b) Polar lipids (chromatography solvent CMW).

| Sample | Count rate c.p.m. |
|---|---|
| RF = 0.89 | 1539 ± 39 |
| RF = 0.78 | 1049 ± 33 |
| RF = 0.69 | 749 ± 27 |
| RF = 0.51-0.63 | 886 ± 30 |
| RF = 0.38-0.51 | 801 ± 28 |
| RF = 0.34 | 634 ± 25 |
| Total | 5660 ± 180 |
| 5 µℓ aliquot | 7455 ± 86 |
| 5 µℓ on TLC material | 5304 ± 73 |

9-2.4 Discussion of Results

1. All of the tritium label present in the sample appears to be confined into phosphomolybdate positive areas.
2. Most of the tritium label appears to reside in polar lipids.
3. It is likely that the area corresponding with RF = 0.89 on CMW chromatograms corresponds with the two compounds having the highest RF values in chloroform.
4. The area RF = 0.78 from CMW chromatogram probably corresponds with RF = 0.1 on the chloroform chromatogram.
5. Visual inspection of the phosphomolybdate sprayed chromatograms indicated that the intensity of tritium label roughly correlated with spot size and intensity.

9-2.5 Conclusions

All the lipids isolated by chromatography of the chloroform extract were labelled to some extent. Furthermore, the degree of labelling of each lipid appeared to be roughly correlated with the concentration of the lipid. It seems likely, especially from earlier results, that all lipids are labelled by non-metabolic processes in dry plant propagules exposed to tritiated water vapour. It is suggested that this may involve the slow hydration of double bonds by tritiated water. Certainly plant lipids tend to have fatty acid residues with double bonds and this indicates the above suggestion is at least feasible.

9-3 Nature of Tritium Labelling in the Solid Residue

9-3.1 Discussion

In all experiments the solid residue left after extraction was tritium labelled. It is possible that this was acquired by exchange processes that were not reversible during extraction. It has been

found (Lang and Mason, 1960) that tritium exchanged by cellulose with tritiated water vapour cannot all be reversibly back exchanged during repeated wetting and drying. Lang and Mason suggest that parts of the cellulose molecule that are accessible for $^{3}H$ exchange originally became inaccessible during the subsequent wetting and drying. This is thought to be due to conformational changes in the cellulose molecule.

It is possible that tritium labelling in the solid residue was caused by a process similar to that just described. During the initial exposure tritium exchanges with protium on various accessible sites on cellulose or other macromolecules present in the organism. When the extraction of the organism begins conformational changes in the macromolecules makes these sites inaccessible for back exchange with the solvent. Even when the solid residue is left to dry for several days prior to scintillation autography the results of Lang and Mason suggest that different areas may become accessible from those that were originally accessible for exchange. Consequently some tritium labelling may become non-exchangeably incorporated into the solid residue by non-metabolic processes. The experiments described below are an attempt to test this suggestion.

### 9-3.2 Experimental

The following is a description of the treatment of solid residue from *Sinapis alba* seeds exposed to tritiated water vapour for 12 days at 64% relative humidity.

The solid residue was suspended in 5 mℓ of water and the suspension centrifuged. The supernatant was referred to as 'first water wash'. 5 μℓ of this was transferred to a scintillation vial for counting. This process was repeated twice to give the second and third water washes. The supernatants were combined and freeze dried. It was hoped that this process would remove any trace of soluble tritium activity.

However, the solid residue remaining was still heavily labelled.

The solid was resuspended in 10 mℓ 0.1 mol $ℓ^{-1}$ HCl. This was centrifuged and 5 μℓ of the supernatant taken for counting. The solid was resuspended in the supernatant and boiled gently for one hour. The resulting suspension was centrifuged and 5 μℓ of the supernatant was counted. The rest of the supernatant was freeze dried.

The residue was resuspended in 5 mℓ of 6 mol $ℓ^{-1}$ HCl and boiled for 10 hours. The suspension was centrifuged and 5 μℓ of the supernatant was neutralised with NaOH and counted. The remaining supernatant was freeze dried and the solid residue remaining checked for activity by scintillation autography.

### 9-3.3 Results

TABLE 9.2 Treatment of solid residue from *Sinapis alba* seeds exposed to tritiated water vapour for 12 days at 64% relative humidity.

| | Tritium Activity $nCi \cdot mg^{-1}$ |
|---|---|
| First water wash | 18,000 ± 1000 |
| Second water wash | 320 ± 20 |
| Third water wash | 6.6 ± 0.5 |
| Non-volatile residue from supernatants of above | 8.3 ± 0.6 |
| 0.1 mol $ℓ^{-1}$ HCl wash | 550 ± 30 |
| 0.1 mol $ℓ^{-1}$ HCl hydrolysate | 1300 ± 100 |
| Non-volatile residue from hydrolysate | 6.3 ± 0.5 |
| 6 mol $ℓ^{-1}$ HCl hydrolysate | 93 ± 7 |
| Non-volatile residue from 6 mol $ℓ^{-1}$ HCl hydrolysate | 1.0 ± 0.2 |

9-3.4 Discussion of Results

The results indicate that the three water washes extract a large amount of tritium but virtually all of this is volatile. It is likely that the tritium detected in the water washes was removed from the residue by back exchange. The minute amount of activity in the water washes that was non-volatile (8.3 ± 0.6 nCi mg $^{1}$) was probably traces of tritium labelled small molecule. metabolites not extracted by previous extraction procedures.

The dilute acid wash extracted a surprising amount of activity. Although subsequent dilute acid hydrolysis removed even more tritium activity from the solid residue, most of this was volatile. This suggests that the dilute acid washing and hydrolysis removed tritium by back exchange.

The concentrated acid hydrolysis removed a relatively small amount of activity from the remaining solid residue and virtually all of this was volatile. Again this suggests the removal of tritium by back exchange and its incorporation into the solvent (i.e., exchange occurs with exchangeable protons in the solvent).

Solid residues from other propagules were treated with variations on the treatment described above. In all cases acid hydrolysis removed tritium labelling from the residue and this label appeared to become incorporated in the solvent suggesting that the labelling was removed by exchange processes. In other words, the acid hydrolyses did not extract tritium labelled sub-units from macromolecules (e.g., amino acids) which would have led to tritium labelling in the non-volatile portion of the acid hydrolysates.

9-3.5 Conclusion

The results from studies on the tritium labelled solid residue left after extraction indicate that this labelling is not a consequence

of metabolic activity but rather of exchange processes which are not entirely reversible during the extraction procedure.

# CHAPTER 10 CONCLUSIONS

## 10-1 Success of Technique

A technique has been successfully developed for studying the metabolism of dried organisms stored at constant relative humidity. This technique was used for the *in vivo* study of the metabolic activity occurring in the seeds of *Sinapis alba*, *Pastinaca sativa*, *Allium cepa* and *Hordeum vulgare*, the pollen of *Pinus ponderosa* and ten other species of pollen, and the spore of *Cyathea dealbata* and *Scleroderma bovista*. Several of these organisms were studied at different relative humidities and in all cases some metabolic activity was found for at least one relative humidity.

## 10-2 Surprising Amount of Metabolic Activity in Dry Organisms

The most striking result from this work was the demonstration of metabolic activity in dried plant propagules. Many biologists do not expect any metabolism in a dried organism such as a seed. Even the suggestion that such an organism may have a metabolism will elicit surprise. However, the work in this thesis has provided results that clearly indicate the occurrence of at least some metabolism in all the dried organisms studied.

This is perhaps the most important conclusion of this thesis — that resting metabolism does indeed occur in dried organisms.

## 10-3 Effect of Relative Humidity in Tritium Incorporation

The amount of tritium labelling and its complexity decreased with decreasing relative humidity but labelling was still easily detectable

at a relative humidity as low as 45%. In some organisms traces of activity were found at 34% relative humidity. At 15% relative humidity no organism showed any tritium labelling as detected by scintillation autography. It can be inferred from this that no appreciable metabolic activity was occurring in the organisms studied at 15% relative humidity, but resting metabolism was definitely occurring at 45% and above. Even at 34% slight metabolic activity was detected for some organisms.

It is suggested that there is a particular relative humidity for each organism at which all enzymes lose sufficient of their bound water to temporarily lose their ability to act as catalysts. This relative humidity varies from organism to organism, but is in the region of 20 to 40% relative humidity.

Above 64% relative humidity tritium incorporation was much more extensive. The number of compounds labelled and the intensity of label increased greatly. This implies a much more extensive, and intensive metabolism in the organisms at 75% relative humidity and above.

### 10-3.1 Biochemistry of Low Water Activity Systems

Metabolic activity detected in the anhydrobiotic systems studied occurred at relative humidities corresponding to solutions of very high concentration, that is, low water activity. This suggested that the metabolism occurring during anhydrobiosis should be viewed as a branch of biochemistry dealing with enzyme activity in highly concentrated solutions of low water activity.

Some enzyme systems appear to be more affected by desiccation than others. The role of bound water in maintaining tertiary structure of enzymes is well known. It appears that enzymes in anhydrobiotes have a varying dependence on bound water. Some of those involved in metabolism of amino acids, and to a smaller extent some of the TCA cycle intermediates, appear less dependent on water for their operation

than others. This suggests that the enzymes and other macromolecular structures of anhydrobiotic organisms have structural characteristics that allow them to operate in solutions of high concentration and low water activity.

At relative humidities above 64% the implied metabolism becomes much more complex and more like that of a germinating system. It is suggested this may be related to the fact that above this relative humidity most biochemical substances go into solution (deliquesce). It is further suggested that solutions in the anhydrobiotes under these conditions are virtually fluid and many enzymes have tertiary structures which allow them to operate as catalysts.

At intermediate and low relative humidities it is likely that solutions within the anhydrobiote are in a glass-like state. The low rate of metabolism may be determined by the very low rate of diffusion of metabolites through such a system.

Scintillation counting of individual compounds from *Pinus ponderosa* pollen indicates that traces of tritium are incorporated at 34% and 15% relative humidity although this could not be detected by scintillation autography. It is suggested that between 45% and 34% relative humidity the viscosity of the intracellular media in the pollen rises sharply and the rate of diffusion of metabolites is greatly reduced so that at 34% relative humidity only substrate in the immediate vicinity of an enzyme is metabolised. This effectively limits the metabolic pool size of the substrate leading to a much reduced tritium incorporation.

## 10-4 Nature of Compounds Detected as Tritium Labelled in Resting Metabolism

(a) Amino Acids and Organic Acids:

In virtually all experiments where tritium incorporation was detected by scintillation autography amino acids had incorporated

tritium. For many of the pollen species studied, amino acids were the only compounds found to be labelled. It is suggested that amino acids are labelled by low level transaminations and/or deaminations occurring in the dry pollen. Glutamate was found to be labelled in most experiments and it is interesting to note that glutamate transaminase and glutamate dehydrogenase (1.4.1.2) are regarded as particularly important enzymes. Further, both these enzymes are believed to be virtually ubiquitous in living organisms (Bonner and Varner, 1965; Kretovich, 1966). It is suggested that the enzyme or enzyme system responsible for the labelling of glutamate is particularly resistant to desiccation.

The two amino acids alanine and aspartate were often found to be tritium labelled by the anhydrobiotes studied. It is suggested that these are also labelled by low level transaminations and/or deaminations. These reactions appear to still function even at 45% relative humidity.

In several species the compound 4-aminobutyrate (gaba) was found tritium labelled at 64% relative humidity but not at 54%. It is suggested that the enzyme(s) responsible for the incorporation of tritium into 4-aminobutyrate is less resistant to desiccation than those responsible for labelling glutamate, aspartate and alanine.

It is significant that the four amino acids mentioned above are important in the early stages of germination in seeds (Haber and Tolbert, 1959; Spedding, 1963), pollen (Malloy, 1965), fern spore (Lever, 1965) and fungal spore (Edwards, 1963). It is suggested that the enzyme systems responsible for metabolising these compounds are not only present in the dry propagule but actually operating at low levels even at relative humidities as low as 45%.

Three other amino acids occasionally found labelled were serine, asparagine and glycine.

Organic acids were often found tritium labelled by the organisms studied, though this applied to seed species more than pollen species. The most common ones found labelled were malate and lactate, although citrate, succinate and possibly fumarate were occasionally found labelled. Generally the labelling in organic acids was less comprehensive or intense than in amino acids. Organic acid metabolism has been implicated in the earlier stages of germination (Haber and Tolbert, 1959; Spedding, 1963), and it appears that the TCA cycle is operating in germinating seeds. It is suggested that at least parts of the cycle is operating at a low level in dry seeds and other dry propagules.

(b) Sugars:

Tritium labelled sugars were not generally found in experiments on resting metabolism of dry propagules. It is suggested that carbohydrate metabolism is not important in resting metabolism. A possible exception to this was the detection of a tritium labelled compound with the characteristics of a sugar or other neutral compound in *Sinapis alba* and *Pastinaca sativa* seeds and *Scleroderma bovista* spore. In each case the compound may have been identical, moving to a position under chromatography similar to that of ribose.

(c) Sugar Phosphates:

The absence of appreciable amounts of tritium labelled sugar phosphates indicate that the metabolism of dried organisms at intermediate relative humidities is less comprehensive than that of germinating organisms.

(d) Lipids:

Tritium labelling of lipids was not detected by scintillation autography in experiments on resting metabolism of dry organisms. It is proposed, consequently, that lipid metabolism is not significant in the resting metabolism of the organisms studied.

Scintillation counting of lipid extracts indicated some incorporation of tritium although this was not detected by scintillation autography. However, controls showed tritium incorporation at least as great and often much greater than that of the live organism. Scintillation counting of individual lipids separated by chromatography indicated that the tritium label was distributed among all the lipids detected. It is suggested that the labelling of the lipids is due to non-physiological processes. Lipids are known to be relative unstable compounds (Gurr and James, 1971). Some process such as the slow addition of water across double bonds in unsaturated lipid moieties may account for the tritium incorporation observed.

(e) Macromolecular Compounds:

The absence of label in macromolecular compounds in the water extract is taken as tentative evidence that the synthesis of such compounds did not occur in the anhydrobiotic state.

(f) Solid Residue:

The solid residue left after extraction was heavily tritium labelled in most experiments. It is proposed, however, as a result of further experiments on the labelled solid residue, that virtually all the tritium was incorporated by non-metabolic exchange reactions.

## 10-5 Similarity in Resting Metabolism in Diverse Organisms

Cowan (1977) and Teh (1977) have used the techniques developed in this thesis to study two examples of lichen (Cowan) and *Pithomyces chartarum* spore (Teh) in some detail. Their results, and the ones obtained by this author for a range of plant propagules, indicate that resting metabolism is basically similar for a diverse range of organisms. It is suggested that anhydrobiotic organisms have certain enzymic systems in readiness for the return of higher water activities.

In all anhydrobiotic organisms these systems are similar involving deaminases and/or transaminases and TCA cycle enzymes. These systems in different organisms are similar in that their operation is particularly resistant to desiccation.

This conclusion adds to that of Spedding and Wilson (1968) who propose that the TCA cycle provided energy for metabolic processes (e.g., enzyme synthesis) in the early stages of germination of seeds. Transaminase and deaminase activity immediately following initial imbibition provided the intermediates required for the TCA cycle. Only later does carbohydrate, phosphate ester and lipid metabolism become significant.

The results of this thesis imply that amino acid metabolism and at least some reactions associated with the TCA cycle, are occurring in many plant propagules even before imbibition when they are stored at as low a relative humidity as 45%.

## 10-6 Anomalous Results from Scintillation Counting — The Effectiveness of Chromatography for Decontaminating Methanol Extracts

Scintillation counting of methanol extracts to determine the incorporation of non-volatile, non-exchangeable tritium did not give reliable results. It was found that these results did not correlate with those obtained by scintillation autography.

It is proposed that freeze-dried extracts contain varying amounts of semi-labile tritium. It is further suggested that chromatographic procedures used in this thesis are effective in removing this semi-labile tritium. This is supported by results obtained by eluting extracts from entire chromatograms and counting the eluate.

It is suggested that the relatively long duration of exposure to tritiated water may allow the incorporation of tritium by slow exchange reaction not normally of significance in, for example, germination

experiments where their duration is of the order of minutes or hours.

## 10-7 Unidentified Compounds that may be Peculiar to Resting Metabolism — 'New' Biochemicals Were Found Tritium Labelled

The compounds S (from *Sinapis alba*), SP (from *Pastenaca sativa*) and SB (from *Scleroderma bovista*) may be identical. In each case the unknown has chromatographic properties similar to a five-carbon sugar, possibly ribose.

Compound P, found tritium labelled in *Pinus ponderosa* pollen, had unusual chromatographic properties. Under electrophoresis at pH 2 it moved with amino acids towards the cathode, but it did not react with ninhydrin. It was more mobile and therefore basic than most amino acids and yet its chromatographic properties were that of a relatively non-polar compound.

Several other tritium labelled compounds were detected but not conclusively identified. It is suggested that some of these compounds might be previously undiscovered biochemicals and that some may be peculiar to resting metabolism.

## 10-8 'Sucrose Area' from *Pinus ponderosa* Pollen

Scintillation counting of the 'sucrose area' from chromatograms of *Pinus ponderosa* pollen extracts showed only traces of tritium activity. Even this could not be positively ascribed to sucrose. It is concluded that sugar metabolism is not as complex as in the germinating pollen and may, in fact, be absent.

# CHAPTER 11 SUGGESTIONS FOR FURTHER WORK

1. A number of compounds were found to be tritium labelled but not identified in this thesis. It would be interesting to discover the identity of these compounds. These include: the neutral compound(s) from *Sinapis alba* and *Pastinaca sativa* seeds and *Scleroderma bovista* spore; unknown 'O' from *Pastinaca sativa* seeds, possibly the same unknown that was found in *Hordeum vulgare* seeds; unknown 'P' from *Pinus ponderosa* pollen which did not appear to be a common metabolite. As has been suggested before any of these may be an as yet unidentified biochemical.

2. There is obvious scope for extending the use of the technique. This could include the study of more examples of fern and fungal spore. Perhaps more significant would be the study of animal anhydrobiotes — rotifer tardigrades, anhydrobiotic nematodes and insect eggs. This could confirm the hypothesis that resting metabolism is similar in all anhydrobiotic organisms.

3. An obvious development would be to change the conditions of storage. Resting metabolism could be studied at different temperatures, under anaerobic conditions and perhaps variations of gaseous atmosphere (e.g., under 100% carbon dioxide, 100% oxygen, etc.).

4. The changes of resting metabolism with time could be an interesting study. For example, the tritium labelling pattern of seeds freshly harvested and equilibrated at 64% relative humidity could be compared with that of seeds stored for six months at 64% relative humidity though still viable. This would provide information about

the changes occurring in the seed as it approaches death.

5. There could be value in studying resting metabolism at higher relative humidities, although problems of active fungal growth would have to be dealt with. This problem might be overcome to some extent by treating organisms with a suitable fungicide.

6. It could be valuable to carry out more studies similar to that of Skujins and McLaren (1967) involving *in vitro* purified enzyme assays at relative humidities below 100%. The activity of enzymes from anhydrobiotes could be compared with those of enzymes from non-anhydrobiotic sources. Similarly, a comparison could be made of the physical properties, such as behaviour toward water absorption, for enzymes from the two types of organism.

7. Another line of research could involve finding more specific information about reactions occurring in resting metabolism. This might answer such questions as: Do amino acids become labelled by deaminations or transaminations, or by some other processes? This may involve extracting specific enzymes from the organisms studied.

8. There are still many questions to be answered about scintillation counting of extracts. These include: discovering the nature of the semi-labile tritium in the methanol extracts, the mechanism by which this is lost during chromatography, and whether or not this semi-labile tritium can be removed by the storage of the extract in 10% isopropanol solution at 0-4 C for long periods.

9. Further studies of tritium labelling that occurs at low relative humidity could be useful. The scintillation counting of individual compounds, such as was carried out for *Pinus ponderosa* pollen, could be carried out for other species. This could allow the discovery of how tritium incorporation is distributed at various relative humidities.

10. The relative humidities at which large changes in the tritium labelling pattern occurs could be determined and related to various properties of the organism dependent on water activity. These properties would include the status of intracellular media, and the properties of such cellular components as macromolecules, membrane ribosomes, etc.

11. A more thorough study of the possible labelling in macromolecules could be undertaken. This could involve exposing an organism to tritiated water vapour then extracting it with distilled water or a buffer solution. This would circumvent the undesirable effects on macromolecules of the MCW extraction.

12. A number of specific problems associated with certain anhydrobiotes could be studied with the techniques developed in this thesis. For instance, the seeds of wild oats (*Avena fatua*) seem to require a period of months of after-ripening subsequent to harvest before they can germinate. The resting metabolism of the ripening seeds could be compared with that of fully ripened seeds. This may give some insight into what occurs during the ripening period.

13. Further development of the scintillation autographic techniques, as discussed in Chapter 4, could be undertaken. The development of low-temperature liquid scintillation autography promises a considerable increase in the sensitivity of detection of tritium compared with scintillation autographic techniques at present available.

14. The interesting question of the radiosensitivity of plant propagules to tritiated water vapour has been considered briefly in Chapter 3. A more intensive study of this question may be warranted. The technique developed for studying resting metabolism would allow a study of the effects of endogenous radio isotopes on dried organisms.

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