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STUDIES OF THE EARLY REACTIONS IN THE GERMINATION OF *SINAPIS ALBA* SEEDS*

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Abstract—Tritiated water has been used as the aqueous medium for the germination of *Sinapis alba* (Mustard) seeds. ^{3}H-labelled compounds formed during the germination processes have been separated and identified to give information on the biochemistry of reactions taking place during early germination. Since ^{3}H is incorporated into aspartic acid, glutamic acid, γ-aminobutyric acid and alanine in the first 20 min of germination it is concluded that each of these compounds is involved in biochemical reactions during the early steps in germination. Citric acid, malic acid and succinic acid do not incorporate tritium until after 30 min. No tritium could be detected in the lipid fraction until after the third hour of germination. The results indicate that amino acid metabolism is important in the early stages of germination. It is proposed that the seed stores unstable α-oxo-acids as the corresponding amino acids until required for germination. Deamination and transamination produce the α-oxo-acids for the Krebs cycle. This is later supplied with acetate from the degradation of lipid reserves.

INTRODUCTION

THE mechanism by which a seed can remain dormant for many years, yet rapidly turn into a vigorously growing organism on the addition of water has fascinated man for many years. The problem of storing a viable biochemical system, consisting as it does of unstable chemical substances, would appear to present very considerable chemical problems. The initial steps of germination are thus of considerable interest as they may lead to an understanding of the mechanism that enables seeds to break the period of suspended animation and produce a young growing seedling.

An excellent review of seed germination has been compiled by Koller *et al.*,[1] who noted that the observed metabolic changes in seeds may either be the result of germination or its cause. These authors warn that ambiguous results may be obtained in studying isolated parts of seeds because of the undoubted interrelationship of the metabolism in these seed parts.

The next section will describe a novel approach to the study of germination. Seeds were germinated in tritiated water (^{3}HHO) and the metabolites which incorporated tritium (^{3}H) were identified so that it was possible to elucidate which compounds were involved in metabolism during the early steps of germination. It was also possible to show that some metabolites, although present, did not incorporate ^{3}H from ^{3}HHO in short times and therefore were not involved in active metabolism.

This study was carried out *in vivo* on the whole seed at much shorter times than had previously been possible. This has eliminated the ambiguity of experiments on separate seed parts and has allowed the early metabolic processes of germination to be studied.

* The work described in this paper constitutes a part of the thesis of D. J. Spedding, submitted in partial fulfilment of the requirements for the M.Sc. degree at Victoria University of Wellington, New Zealand.

[1] D. KOLLER, A. M. MAYER, A. POLJAKOFF-MAYBER and S. KLEIN, *Ann. Rev. Plant Physiol.* 13, 437 (1962).

RESULTS AND DISCUSSION

The results of a large number of experiments are given in Table 1. Some chromatograms are shown in Fig. 1.

TABLE 1. COMPOUNDS LABELLED WITH TRITIUM WHEN *Sinapis alba* SEEDS ARE GERMINATED IN TRITIATED WATER FOR VARIOUS TIME INTERVALS

	Time (min)								
	5	10	15	30	60	90	120	180	1440
γ-Aminobutyric acid	+	+	+	+	+	+	+	+	+
Aspartic acid	+	+	+	+	+	+	+	+	+
Glutamic acid		+	+	+	+	+	+	+	+
Alanine		+	+	+	+	+	+	+	+
Malic acid			+	+	+	+	+	+	+
Citric acid			+	+	+	+	+	+	+
Succinic acid				+	+	+	+	+	+
Fructose?				+	+	+	+	+	+
Lipid								+	+

Other compounds present on chromatogram but not identified.

The theory of using tritium-labelled water to detect metabolism *in vivo* is described in detail in an earlier paper.[2] In the present investigation it would be difficult to study the metabolism taking place in the first few minutes of the germination of a seed using conventional biochemical techniques. The results of this present work can be interpreted thus:

Since tritium is incorporated into aspartic acid, glutamic acid, γ-aminobutyric acid and alanine during the first 15 min of germination, it is concluded that each of these compounds is involved in some biochemical reaction which would lead to the incorporation of tritium into some non-exchangeable position in the molecule. It is perhaps unexpected that γ-aminobutyric acid appears to be involved in active metabolism during the early stages of the germination of this seed. The remaining amino acids are closely related to the α-oxo-acids of the Krebs cycle. As is well known, α-oxo-acids are chemically unstable with respect to decarboxylation, thus it is an attractive hypothesis to postulate that the seed stores the α-oxo-acids of the Krebs cycle as amino acids, which are transaminated or deaminated on germination to provide the unstable Krebs cycle intermediates. The metabolites present which do not become labelled also provide important information about the metabolism during the early steps of germination. In this case malic acid is present during the first 15 min but does not become labelled, showing that malic acid is not involved in Krebs cycle metabolism until after 30 min. Similarly, fat metabolism would be expected to lead to labelling of the lipid pool. Since labelled lipids are not detected until the third hour of germination, it is concluded that β-oxidation of fat reserves is not important in the very early steps of germination.

Having discussed the general conclusions of the work, it is now proposed to discuss the labelling in detail:

[2] A. T. WILSON, *J. New Zealand Inst. Chem.* **28**, 87 (1964).

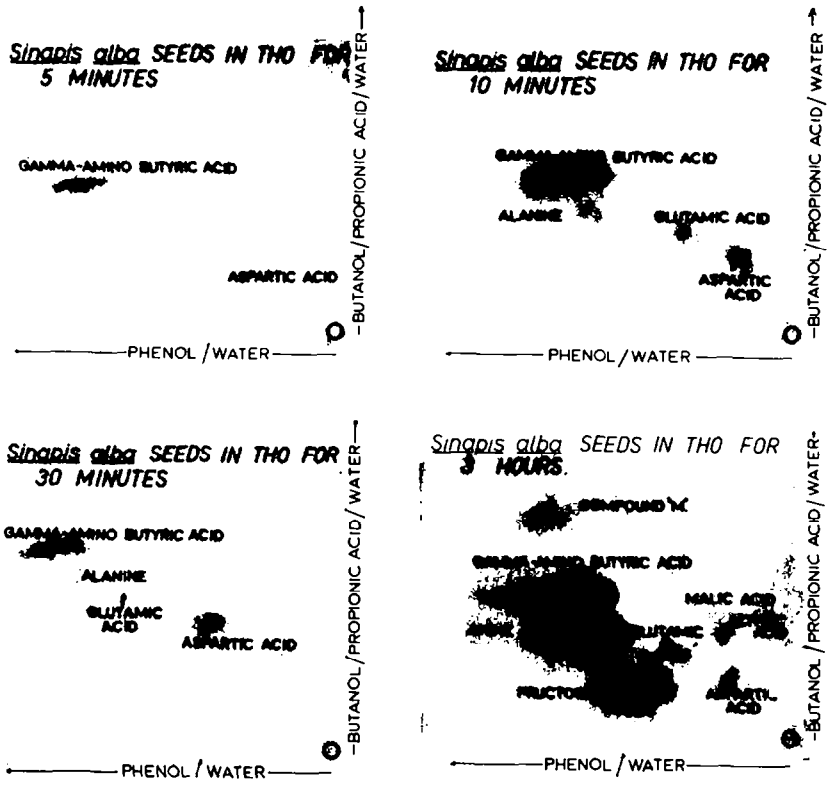

FIG. 1. AUTORADIOGRAPHS OF ^{3}H-LABELLED COMPOUNDS PRODUCED DURING THE GERMINATION OF *Sinapis alba* SEEDS IN ^{3}HHO. COMPOUND 'M' HAS NOT BEEN IDENTIFIED.

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Amino Acid Metabolism

(*a*) Transamination: The amino acids involved in a transamination in the presence of ^{3}HHO would be expected to have a ^{3}H atom stably bound at the α-carbon atom.[3]

(i) γ-Aminobutyric acid is most likely to become ^{3}H-labelled in the germinating seed by transamination with α-oxoglutaric acid. Transamination of γ-aminobutyric acid with aspartic acid and alanine are also possible.[4] The presence of γ-aminobutyric acid and its transaminases has been shown in a number of seed species.[5–9]

(ii) Glutamic acid, alanine and aspartic acid and their transaminases have all been found in resting seeds.[10–14]

(*b*) Glutamate decarboxylation would directly yield ^{3}H-labelled γ-aminobutyric acid in the presence of ^{3}HHO. In this reaction glutamic acid would also be expected to become labelled at the α-carbon atom. Glutamate decarboxylase has been implicated in the germination of *Cucurbita pepo* seeds[15] and wheat germ.[16] Haber and Tolbert[17] were unable to detect ^{14}C-labelled γ-aminobutyric acid in extracts from lettuce seeds germinated in ^{14}C-bicarbonate solution. However, the present authors[18] have detected this amino acid labelled with ^{3}H after the germination of another variety of lettuce seed in ^{3}HHO. The germination of these latter seeds gave a similar pattern of ^{3}H-labelled compounds as *Sinapis alba* seeds. This indicates that either γ-aminobutyric acid is associated with glutamic acid only by transamination, which would give a ^{3}H label but no ^{14}C label to γ-aminobutyric acid, or that the association is through glutamic decarboxylation, in which case the carboxyl group removed contained all the ^{14}C of glutamic acid.

(*c*) The reverse of the amino acid dehydrogenase reactions can lead to ^{3}H-labelled amino acid in the presence of ^{3}HHO. Glutamic acid dehydrogenases have been found in a number of seeds.[19–21]

(*d*) It is probable that during the early germination processes of *S. alba* seeds, transaminations involving glutamic acid, aspartic acid, γ-aminobutyric acid, and alanine take place in order that the α-oxo-acids (α-oxo-glutaric acid, succinic semi-aldehyde, oxalacetic acid and pyruvic acid) may be obtained for the several transformations in which they could be involved.

This postulate poses a problem—where do the original keto acid(s) come from to initiate the

[3] A. S. Konikova, M. G. Kritsman and R. V. Teis, *Biokhimiya* **7**, 86 (1942).
[4] R. O. D. Dixon and L. Fowden, *Ann. Botany (London)* **25**, 513 (1961).
[5] T. Suzuki, T. Hasegawa, A. Maekawa and Y. Sahashi, *Bull. Agr. Chem. Soc. Japan* **22**, 341 (1958).
[6] P. O. Larsen, *Acta Chem. Scand.* **16**, 1511 (1962).
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[14] N. B. Das and P. K. Sen Gupta, *Trans. Bose Inst. (Calcutta)* **15**, 95 (1942).
[15] K. Okunuki and M. Inagaki, *Proc. Japan Acad.* **27**, 658 (1951).
[16] M. Rohrlich, *Getreide Mehl* **7**, 89 (1957); seen in *Chem. Abstr.* **53**, 20593 (1959).
[17] A. H. Haber and N. E. Tolbert, *Plant Physiol.* **34**, 376 (1959).
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[19] D. D. Davies, *J. Exptl Botany* **7**, 203 (1956).
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[21] M. Damodaran and K. R. Nair, *Biochem. J.* **32**, 1064 (1938).

transaminations? It is suggested that these arise from the deamination of amino acids which, being chemically more stable than α-oxo-acids, are more likely to be stored by the seeds in the resting stage prior to germination. Deamination by amino oxidases would not lead to ^{3}H-labelled products in the presence of ^{3}HHO but this does not eliminate the possiblity of this reaction proceeding in the early phases of germination.

Organic Acid Metabolism

(*a*) Krebs cycle reactions in the presence of ^{3}HHO would lead to citric, isocitric, succinic and malic acids labelled in a stable position with ^{3}H. However, provided the ^{3}H atom remains in a stable position, it should be possible to pass the ^{3}H label to all the intermediates of the cycle. As the seed in the early phases of germination must obtain energy for metabolic processes, and as ^{3}H-labelled citric, malic and succinic acids are present in extracts from seeds germinated for short times in ^{3}HHO, it would seem that the Krebs cycle is in operation in the early phases of *S. alba* germination. Krebs cycle enzymes have been detected in many seeds.[19, 22–25]

(*b*) Glyoxylate cycle reactions would be expected to produce the same ^{3}H-labelling pattern as the Krebs cycle reactions. The advantage to the germinating seed of having the glyoxylate cycle in operation would be that the succinate formed could act as a precursor to many cell constituents.

Lipid Metabolism

Largely because of the extensive lipid reserves of many seeds, lipid metabolism has been regarded as being very important in seed germination. *S. alba* seeds do not show detectable lipid metabolism until 3 hr after the initial imbibition of water.

The amount of radiation to which each *S. alba* seed was exposed was estimated to be approximately 3×10^4 r.e.p. for a 1 hr experiment. The radiation damage at this level would probably be at the genetic rather than physiological level. Baillie[26] has found that *S. alba* seeds need doses of above 2×10^6 r.e.p. before the percentage germination falls.

CONCLUSIONS

Haber and Tolbert[17] germinated lettuce seeds in ^{14}C-bicarbonate and suggested that the ^{14}C was fixed primarily by carboxylations into organic acids and the Krebs cycle and transaminations were functioning during the early phases of germination. A similar pattern would seem to be true for the early germination of *Sinapis alba* seeds. However, due to the fact that ^{3}H can be incorporated into amino acids by *S. alba* seeds in as little as 5 min after the addition of ^{3}HHO it is possible to suggest the sequence in which these metabolic processes come into operation. (Haber and Tolbert[17] were unable to detect ^{14}C-labelled compounds until after 20 min germination of lettuce seeds.) The present authors have found that lettuce seeds follow the same pattern of ^{3}H-labelled compounds during their germination as do *S. alba* seeds.[18]

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[25] T. E. HUMPHREYS, *Plant Physiol.* **30**, 46 (1955).
[26] W. J. H. BAILLIE, Personal communication.

The evidence presented above indicates that very early in germination the seeds of *S. alba* carry out amino acid metabolism, probably deaminations followed by transaminations, to produce the oxo-acid intermediates of the Krebs cycle. These oxo-acids are chemically unstable, hence it is reasonable to expect that their storage in the seed would present difficulties.

Metabolism involving some of the Krebs cycle organic acids joins the initial amino acid metabolism after approximately 30 min, probably indicating that the Krebs or the glyoxylate cycle is in operation to produce the energy needed for the rapid changes that will occur in the embryo.

The functioning energy-producing cycle is probably supplied later with acetyl-COA from the degradation of the lipid reserves (3 hr after the initial imbibition of water).

EXPERIMENTAL

Germination of Seeds

The *Sinapis alba* (Mustard) seeds used in this work were found to have greater than 90 per cent germination. Five seeds, each with approximately one-eighth of its seed coat removed to facilitate the introduction of water to the internal tissues of the seed, were placed in a 10 ml conical centrifuge tube. A surface film of ^{3}HHO (5 C/ml; supplied by the Radiochemical Centre, Amersham, England) was added to the seeds which were left at room temperature ($20 \pm 2°$) for the required time interval. Germination was halted by adding absolute alcohol followed immediately by grinding the imbibed seeds in an all-glass Potter–Elvehjem tissue homogenizer.

Extraction of Imbibed Seeds

The absolute alcohol added to halt germination provided the first alcohol extract after the grinding process. A second alcohol extract was obtained by adding a further aliquot of absolute alcohol to the crushed seeds in the homogenizer and grinding once again. These two extracts were combined to give the alcohol extract. A water extract was obtained similarly by combining two small deionized water extracts of the crushed seeds. The solid material was centrifuged down from these extracts and the supernatants were combined and evaporated to dryness under reduced pressure.

Chromatography of Extracts

The extracts were chromatographed in two dimensions on Whatman No. 4 chromatography paper that had previously been washed with 0·5% oxalic acid solution.[27] The 15-cm square chromatograms were run first in phenol:water solvent and then in butanol:propionic acid:water as described by Calvin *et al.*[28] ^{3}H-labelled compounds were detected by scintillation autography.[29]

Identification of ^{3}H-Labelled Compounds

^{3}H-Labelled compounds were identified by co-chromatography in four different solvents. The phenol:water and butanol:propionic acid:water solvents described above, and n-butanol:pyridine:water (1:1:1) and ethanol:880 ammonium hydroxide:water (80:4:16).

Amino acids were detected with ninhydrin spray (supplied as an aerosol by Sigma Chemical Co., St. Louis, U.S.A.); carboxylic acids were detected with a spray of bromocresol green (0·04 g in 95 ml ethanol and 5 ml water); and sugars were detected with a spray of analine hydrogen phthalate.[30]

Lipids were detected by cutting from the chromatogram the corner diagonally opposite the origin where compounds of R_f 0·9–1 in both solvents appear. In the solvents used these were presumed to be lipids. The piece cut from the chromatogram was extracted with toluene and counted in a liquid scintillation counter. Counts in excess of three standard deviations of counting above background were presumed to indicate the presence of ^{3}H-labelled lipids.

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