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THE METABOLISM OF *SINAPIS ALBA* SEEDS IN WATER UNDER ANAEROBIC CONDITIONS*

A. W. MISSEN and A. T. WILSON

Chemistry Department, Victoria University of Wellington, New Zealand

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Abstract—Tritiated water has been used to study the metabolism of *Sinapis alba* (White mustard) seeds in water under anaerobic conditions. Although some metabolism does take place under these conditions it does not lead to the formation of a seedling. Moreover it has been found that the active metabolism of *S. alba* seeds for 24 hr, under anaerobic conditions, does not lead to their death, since on restoration to aerobic conditions they do germinate, albeit much more slowly. Significant differences exist in the pattern of labelling of *S. alba* seeds under anaerobic conditions, from that under aerobic conditions. Thus, large amounts of tritium accumulate in lactic acid under anaerobic conditions; on transferring the seeds to an oxygen atmosphere the tritium in the lactic acid greatly decreases. It appears that the anaerobic metabolism leads to the accumulation of lactic acid which contributes to the inhibition of the metabolism leading to germination. This was supported by experiments which showed that lactic acid inhibited the germination of *S. alba* seeds. The possible role of lactic acid in the metabolism of the seeds is discussed.

INTRODUCTION

IN A previous paper,[1] Spedding and Wilson described the use of tritiated water (THO) as a medium for the germination of *Sinapis alba* seeds, in order to elucidate the metabolites involved in the early stages of their germination. The principle of the method[2] is that the only way a compound can become non-exchangeably tritiated under these conditions is by a biochemical (or chemical) reaction. Thus any compound which becomes labelled must have been involved in some metabolism. Under some circumstances the presence of an unlabelled compound can indicate that this compound was not being metabolized.

Most seeds will not germinate if placed in water under anaerobic conditions. They will, however, remain in a state whereby they are still able to germinate if later placed in an aerobic environment. The aim of this work was to study what differences in metabolism were taking place under anaerobic conditions and how this metabolism was controlled so that it did not lead to the death of the seed. This may be of interest in understanding the longevity of seeds with impervious seed coats. It is also of interest in that any differences found would lead to a greater understanding of the metabolism occurring during the seed's normal germination.

RESULTS

Unidentified Metabolites

An unidentified metabolite previously designated[1] compound "M", was found to become labelled strongly under anaerobic conditions. On subjecting the compound to electrophoresis over a range of pH values, it was found to have no basic ionizable groups and to

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

[1] D. J. SPEDDING and A. T. WILSON, *Phytochem.* **7**, 897 (1968).
[2] A. T. WILSON, *J. New Zealand Inst. Chem.* **28**, 87 (1964).

have properties suggesting it to be an organic acid. A plot of its absolute mobility against pH gave a curve very similar to that obtained for lactic acid. Compound "M" also co-chromatographed exactly with lactic acid in the six neutral, acidic and basic solvent systems used. When some tritiated compound "M" was mixed with lactic acid and the benzylthiuronium derivative formed, the derivative was found to recrystallize to constant specific activity, confirming compound "M" to be lactic acid.

The compound tentatively suggested to be fructose by Spedding and Wilson[1] was shown by co-chromatography in six solvents and by electrophoresis in a borate buffer to probably be sucrose.

Comparison of Metabolites Labelled under Hydrogen and Under Air

Sinapis alba seeds were allowed to imbibe tritiated water under air and under hydrogen, for varying time intervals, ranging from 1 to 24 hr. As Table 1 indicates, the chief differences between compounds labelled under hydrogen and those labelled in air were that, under hydrogen: no glutamine was labelled, there were no lipids labelled, only very small amounts of labelled sugar phosphates were observed, glutamic acid was rarely observed, the amount of labelled sucrose was greatly decreased and the amount of labelled lactic acid was greatly increased, relative to other labelled compounds.

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

Compound(s) labelled	Time and extracting solvent									
	1 hr		3 hr		6 hr		12 hr		24 hr	
	EtOH	H_2O	EtOH	H_2O	EtOH	H_2O	EtOH	H_2O	EtOH	H_2O
(a) Under air*										
γ-Aminobutyric acid	+	+	++	+	+	+	t	+	++	++
Alanine	+	+	+++	++	++	+	+	++	++	++
Glutamic acid		+		++		++		++		++
Aspartic acid		+		++		+		++		+
Lactic acid			+	t	+	+	+	++	+++	+++
Sucrose	+		++		+++	++	++	++	++	+
Malic acid		+		++		++		++		+
Glutamine						++	t	++	+	++
Sugar phosphates						+		+		
Lipids					+		+		+	
(b) Under hydrogen										
γ-Aminobutyric acid	+	+	++	++	++	+++	+	+++	+	++
Alanine	+	+	++	++	++	+++	+	+++	+	++
Glutamic acid		+								t
Aspartic acid		+		+		++				
Lactic acid			t		+	+	+++	+++	+++	+++
Sucrose					t			+	+	+
Malic acid				t		+		+		+
Glutamine										
Sugar phosphates								t		
Lipids										

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

* The relatively large amounts of lactic labelled in seeds germinating in air for 12 hr and 24 hr, could be partly due to a buildup in CO_2 concentration around the seeds. Under the closed system employed, this might lead to anomalous results. In an O_2 atmosphere this phenomenon does not occur.

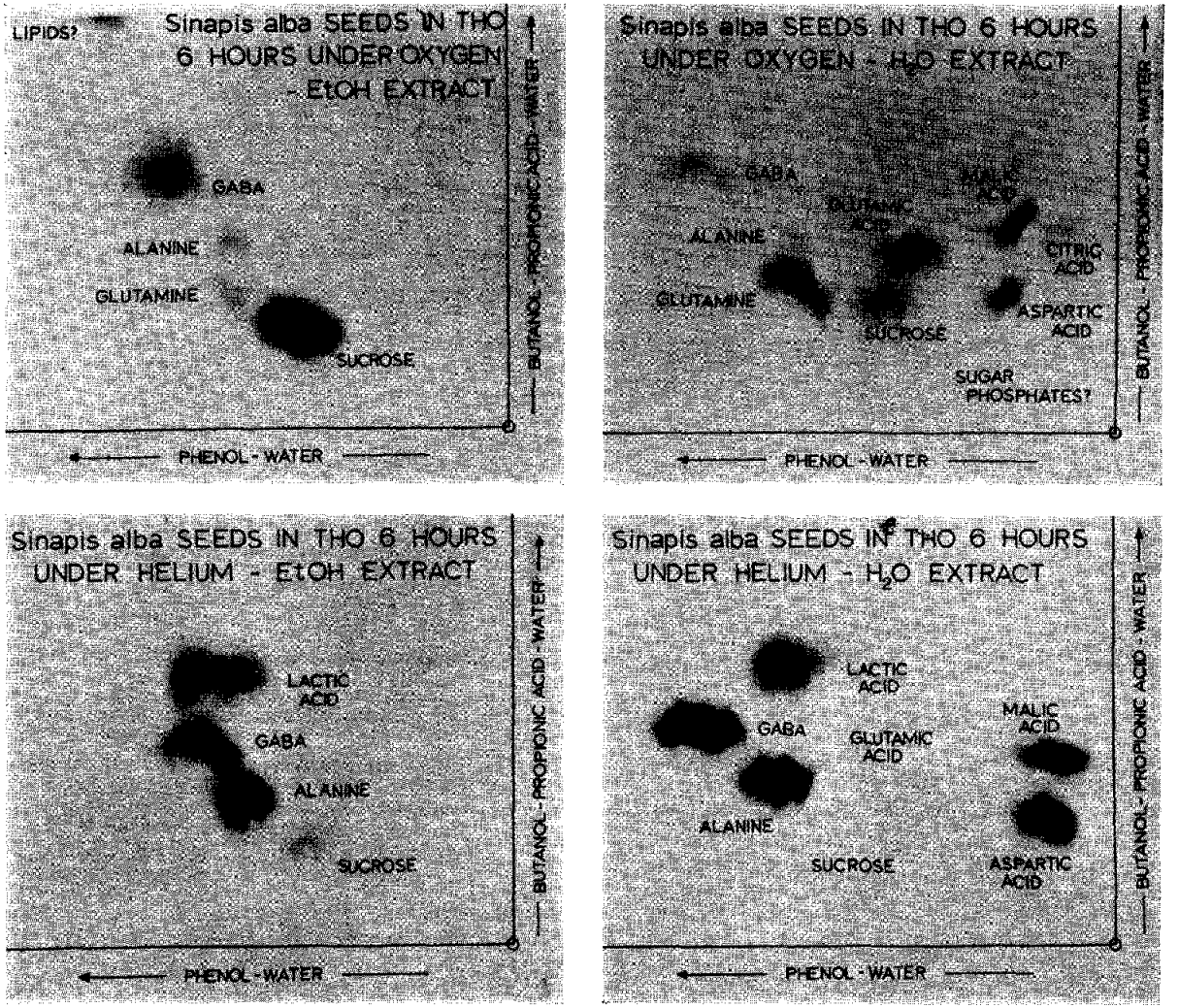

FIG. 1. COMPARISON OF LABELLING UNDER OXYGEN AND UNDER HELIUM.

The compounds tritiated under air were lactic acid, γ-aminobutyric acid (GABA), alanine, glutamic acid, aspartic acid, malic acid, sucrose, glutamine, sugar phosphates and lipids. After 6 hr imbibition of THO, under air sucrose labelling increased relative to the other compounds, while under hydrogen lactic acid labelling increased relative to the other compounds. When seeds with part of the testa removed were allowed to imbibe THO for 3 hr under air, the pattern of labelling corresponded closely to that found for seeds with whole coats, in the same conditions, except for the fact that no lactic acid was labelled.

Metabolites Labelled Under Helium

To ensure that hydrogen was in fact providing an inert and anaerobic environment, *S. alba* seeds were allowed to imbibe tritiated water under helium for 6 hr. The helium would be expected to be chemically inert with respect to the seeds. The pattern of labelling obtained (Fig. 1) was very similar to that obtained for hydrogen, verifying the validity of using hydrogen for an anaerobic atmosphere.

Metabolites Labelled Under Oxygen

Seeds were allowed to imbibe tritiated water under oxygen for 6 hr, using the same apparatus as that used for hydrogen. The most noticeable feature (Fig. 1) was that no labelled lactic acid was observed, while sucrose was very strongly labelled. Other features were the strong labelling of lipids and the weak labelling in citric acid.

When seeds which had imbibed tritiated water under hydrogen for 12 hr were placed in a pure oxygen atmosphere and allowed to imbibe tritiated water for a further 12 hr, the label in lactic acid greatly decreased and the pattern of labelling obtained was very similar to that obtained for 6 hr under oxygen except that a slight trace of lactic acid was observed.

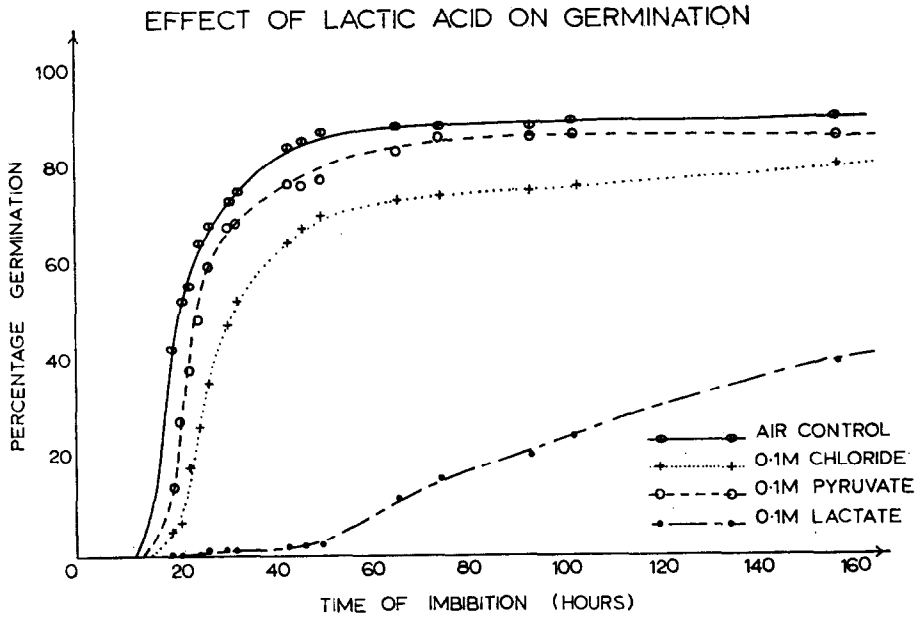

FIG. 2. THE COMPARATIVE RATE OF GERMINATION OF *Sinapis alba* SEEDS IN 0·1 M LACTATE.

Germination Studies

When *S. alba* seeds were allowed to imbibe water under a hydrogen atmosphere, none had germinated after 700 hr. Next, the seeds were allowed to imbibe water under hydrogen for 24 hr, after first flushing for 7 days. When they were introduced into air their rate of germination was found to be greatly reduced from that of the control (>96 per cent), with only 29 per cent germinated after 50 hr and 70 per cent after 100 hr.

Thus, it is apparent that the seeds do not germinate under hydrogen, and that, on reverting to normal conditions, their germination is inhibited in some way. Since lactic acid had been found to be labelled in significant quantities, on imbibition under anaerobic conditions, experiments were done to see if it exerted any unusual effect on the germination of the seeds. *S. alba* seeds were allowed to germinate in 0·1 M solutions of sodium lactate, pyruvate, and chloride at pH 7 alongside a control in distilled water. It was found (Fig. 2) that in the sodium chloride and pyruvate solutions, the germination rate was slightly less than that of the control. However, in the sodium lactate solution the seeds germinated very slowly and after 240 hr only 48 per cent of the seeds had germinated. It was also found that seeds germinate more rapidly in oxygen than in air.

DISCUSSION

Important Factors in Germination

From the results obtained it is apparent that while *Sinapis alba* seeds under hydrogen in the presence of water carry out some metabolism, this metabolism does not lead to their germination. Furthermore this metabolism does not lead to their death, since seeds after imbibing water under hydrogen for 24 hr and then being introduced into air, still germinate, although at a slower rate. Tests with lactate showed that this also had an appreciable effect on the germination of *S. alba* seeds. The effect of 0·1 M lactate was much greater than that of 0·1 M chloride or 0·1 M pyruvate solutions, indicating that the effect could not be due to the ionic strength of the solution but rather must be specific to lactate. Since lactic acid has been shown to become labelled in seeds during the imbibition of THO under hydrogen and under helium, it is possible that at sufficient concentration it inhibits the germination of the seeds. While it is acknowledged that growth is inhibited in the absence of oxygen by the lack of ATP and cycle derivatives, lactate accumulation could be more than just a symptom of this. Lactate itself might affect the concentration of cycle derivatives by affecting utilization of certain amino acids.

The inhibition is slowly reversible, as shown in the experiment in which seeds were allowed to imbibe tritiated water for 12 hr under hydrogen and then 12 hr under oxygen. The presence of a detectable amount of labelled lactic acid, from that experiment, could indicate that the rate of conversion of lactic acid (to pyruvic acid presumably) is slow.

While under hydrogen there is a great deal of lactic acid labelled and a negligible quantity of labelled sucrose, under oxygen the situation is reversed. The absence of labelled lactic acid from the germination for 6 hr in oxygen could be correlated with the fact that seeds germinate more rapidly in oxygen than in air. Furthermore, lactic acid was found to be absent after 3 hr germination in air, of seeds with cut coats in contrast to seeds with whole coats. Therefore it seems apparent that *S. alba* seeds are under partially anaerobic conditions during germination in air, probably because of the relatively impermeable seed coat.

The effect of lactic acid on germination might be more specifically explained by its effect on the enzyme systems involved in the metabolism of glutamic and aspartic acids. Under

anaerobic conditions, after 12 hr, these amino acids have been found to be present, but not appreciably labelled. Under aerobic conditions they are strongly labelled. It is possible that the reactions leading to the labelling of glutamic acid and aspartic acid are inhibited by the lactic acid accumulated under anaerobic conditions. The inhibition of the metabolism of these compounds might well upset the metabolic processes leading to germination.

Metabolism during Germination

In an earlier paper, Spedding and Wilson[1] postulated reactions whereby many of the compounds, particularly the amino acids, could have become labelled. Their arguments will not be recapitulated in this paper. However, in view of the present identification of lactic acid, it would be worthwhile to consider the place it might have in the seed's metabolism during germination. The relatively large amounts of labelled lactic acid formed in germinating *S. alba* seeds, particularly when under anaerobic conditions, suggest that it is being formed by glycolysis. This pathway has been suggested to occur in other germinating seeds.[3,4] The lactic acid can become labelled with tritium, through the keto-enol tautomerism of pyruvic acid, forming labelled pyruvic acid, which can then become converted to lactic acid labelled in the methyl position.

In this work, the amount of tritium incorporated into sucrose as compared to that into other compounds varies very greatly between anaerobic and aerobic conditions. While sucrose could acquire label due to the reversibility of invertase, the build up of sucrose under aerobic conditions suggests that it may actually be synthesized during germination, although clearly more quantitative work would have to be carried out to establish this point. Sucrose would be useful to the young plant for the translocation of food reserves.

Gould and Rees[5] found that *S. alba* seeds contain the carbohydrates sucrose (2·3%), stachyose (1·8%), and raffinose (0·15%). Furthermore, they found that within the first day of germination the stachyose was being broken down within the seed, with the galactose formed being utilized rapidly and virtually completely. It is suggested that the galactose might be converted to UDP-glucose and glucose-1-P by the pathways shown to be operating in soya beans.[6] The UDP-glucose could be utilized in the formation, under aerobic conditions particularly, of labelled sucrose. The glucose-1-P could pass into the glycolytic pathway. Further work using C^{14}-galactose and C^{14}-sucrose as tracers could clarify this.

Under aerobic conditions the pyruvic acid formed in glycolysis could be passed via acetyl-CoA into the tricarboxylic acid cycle, resulting in the release of energy for synthetic purposes. When the seed is placed in water under anaerobic conditions the pyruvic acid is converted to lactic acid. On restoration to aerobic conditions this "stored" lactic acid could then become available for further metabolism. The accumulated lactic acid could also possibly, as mentioned earlier, contribute to the inhibition of the seed's metabolism.

EXPERIMENTAL

Germination of Seeds

The *Sinapis alba* (White mustard) seeds used in this work were found to give greater than 90 per cent germination.

For aerobic imbibition, five seeds were placed in a 10 ml conical centrifuge tube. THO (5 c/ml; supplied

[3] A. M. MAYER and A. POLJAKOFF-MAYBER, *The Germination of Seeds*, Pergamon Press, Oxford (1963).
[4] M. D. HATCH and J. F. TURNER, *Biochem. J.* **69**, 495 (1958).
[5] S. E. B. GOULD and D. A. REES, *J. Sci. Food Agri.* **16**, 702 (1968).
[6] J. H. PAZUR, M. SHADAKSHARASWAMY and G. E. MEIDELL, *Arch. Biochem. Biophys.* **99**, 78 (1962).

by the Radiochemical Centre, Amersham), sufficient to wet the seeds for the period of imbibition, was added to the seeds, which were left at room temperature for the specified time.

For anaerobic imbibition an apparatus was used consisting basically of a glass tube sealed at one end, and with a small side-arm near its open end, on which fitted a glass joint containing inlet and outlet tubes. Five seeds were rolled into the side-arm, and 2–3 drops of THO were placed in the bottom of the tube. The top piece was sealed on and the apparatus connected to the flow from a H_2 (or helium) cylinder, with the THO immersed in liquid air. After flushing thoroughly with H_2 (160 hr) the apparatus was removed from the liquid air and the THO allowed to thaw. The seeds were then tipped into it and allowed to imbibe at room temperature under a gentle stream of H_2 (or He).

The same procedure was adopted for O_2, except that THO was immersed in a mixture of dry-ice and acetone In each case, germination was halted by adding absolute ethanol followed immediately by grinding the imbibed seeds in an all-glass Potter–Elvehjem tissue homogenizer.

Extraction of Imbibed Seeds

After grinding the seeds with absolute alcohol, the crushed seeds were centrifuged down to give the supernatant alcohol extract. The process of grinding and centrifugation was then repeated twice more with small portions of water, which gave the water extract. These extracts were kept separate and evaporated to dryness under reduced pressure.

Chromatography of Extracts

The extracts were chromatographed in two dimensions on Whatman No. 4 chromatography paper. The 18 × 12-cm chromatograms were run first in phenol:water and then in butanol:propionic acid:water.[7] Tritium-labelled compounds were detected by scintillation autography.[8]

Identification of Labelled Metabolites

Tritium-labelled compounds were identified by reference to previous assignments and by co-chromatography in the solvents described above.

Other solvents were also used in the co-chromatography of labelled lactic acid and sucrose. These were lutidine:collidine:water; butanol:acetic acid:water;[9] butanol:pyridine:water (1:1:1); ethanol:880 NH_4OH:water (18:1:1); *iso*-propanol:water (4:1); and propanol:ethyl acetate:water (7:1:7), the latter two being used only for the co-chromatography of labelled sucrose. Amino acids were detected with ninhydrin spray; organic acids were detected with a mercurochrome spray (0·1% in 95% ethanol) or with a sulfanilamide reagent;[10] and sugars were detected by spraying with analine hydrogen phthalate.[11]

Lipids were detected by cutting from the chromatogram of the ethanol extract, 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 counted in a liquid scintillation counter. Counts in excess of three standard deviations of counting above a reference piece, cut from the same position in the chromatogram of a water extract, were presumed to indicate the presence of labelled lipids.

Those metabolites subjected to electrophoresis were run on Whatman No. 1 paper (43 × 2 cm) at a potential of 300 V for 2 hr. Glucose was normally used as a marker for electroendosmosis, except when 0·05 M borate was used as the electrolyte for carbohydrates.

Germination Studies

In these experiments, the rate of germination of seeds subjected to certain conditions was compared with that of a control. One hundred *S. alba* seeds were placed on 11-cm diam. Whatman No. 1 filter paper in a covered Petri dish, and allowed to imbibe water under specified conditions. A control, with distilled water, was always run simultaneously.

[7] A. T. WILSON and M. CALVIN, *J. Am. Chem. Soc.* 77, 5950 (1955).
[8] A. T. WILSON, *Biochim. Biophys. Acta* **40**, 522 (1960).
[9] F. C. STEWARD, R. M. ZACHARIUS and J. K. POLLARD, *Ann. Acad. Sci. Fennicae*, Ser. A, II, **60**, 321 (1955).
[10] G. C. SCHMIDT, C. FISCHER and J. M. MCOWEN, *J. Pharm. Sci.* **52**, 468 (1963).
[11] S. M. PARTRIDGE, *Nature* **164**, 443 (1949).