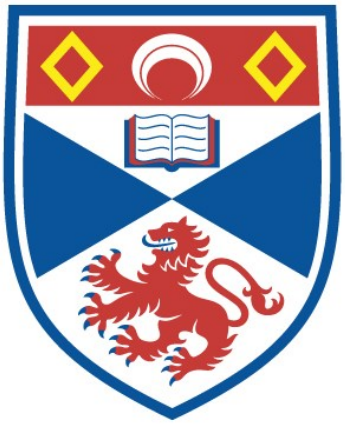
THE ENZYMIC CONTROL OF FLOODING TOLERANCE IN HIGHER PLANTS

Martin McManmon

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A thesis submitted for the degree of Doctor of Philosophy, University of St. Andrews, October 1969.

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CAREER

I entered the University of Manchester in October 1963, and graduated in July 1966 with a First-class Honours degree in Botany.

In October 1966 I matriculated at the University of St. Andrews under Ordinance General No. 12, and later as a candidate for the degree of Ph.D. under Resolution of the University Court, 1967, No.1.

After three years as a research student I left St. Andrews, in September 1969, to take up an appoint--ment with the British Antarctic Survey, at the University of Aberdeen.

DECLARATION

I hereby declare that the following thesis is based upon work done by me, that the thesis is my own composition, and that it has not been previously presented for a higher degree.

The research work was carried out in the Department of Botany, University of St. Andrews, under the supervision of Dr. R.M.M.Crawford.

CERTIFICATE

I hereby certify that Martin McMammon has been engaged upon research work for a minimum of nine terms under my supervision, that he has fulfilled the conditions of Ordinance No. 12, and that he is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

ACKNOWLEDGEMENTS

I wish to express my appreciation to my supervisor, Dr. R.M.M.Crawford, for his great patience and good nature over the past three years, and similarly to other staff and students in the Botany Department.

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The work was carried out during the tenure of an S.R.C. Research Studentship.

CONTENTS

INTRODUCTION

Although waterlogging is probably less of a problem than drought in world agriculture, nevertheless some soils, notably in Australia and some tropical areas, are subjected to regular flooding during the wettest months. Considerable research has been devoted to the effect of this periodic flooding on pasture crops in these areas, and to the general agricultural importance of waterlogging (e.g. Marshall and Millington, 1967; Humphries, 1962; Pearsall, 1950). Waterlogging can also present problems on a smaller scale in most of the temperate agricultural regions of the world. Fortunately, in some tropical and semi-tropical areas, rice has evolved as a crop eminently suited to almost permanently flooded soils ("paddy" soils). With the growth of rice as a major grain crop, large areas have actually been flooded for this purpose, and the chemical and biological properties of these flooded soils have received much attention.

Apart from agricultural considerations, many members of the British flora habitually grow in permanently water-logged soils, and still more are able to survive periodic flooding without apparent damage. Others, however, exhibit adverse effects varying from a slight reduction in growth

to rapid death of the whole plant. Elucidation of the mechanisms whereby flood-tolerant plants are able to continue active growth when at least partially submerged in water may assist in controlling the damage to crops caused by such flooding. Information on the nature of flooding damage, and its avoidance by plants, may also make possible the breeding of strains resistant to such damage, and even indicate new types of crops which may be grown in these conditions.

As Williams and Barber (1961) point out, any damage caused to plants by waterlogging at the roots is unlikely to be due to an excess of water itself, but to the reduction of oxygen available to the root system. Water can contain at saturation only 1/30 of the oxygen found in the atmosphere, and this figure is further reduced by the low diffusion coefficient of the gas in water and by the respiratory activity of micro-organisms. In this context it may be pointed out that the movement of ground water is at least as important as the actual level of the water table. Stagnant flood-water is much more likely to cause damage (other than mechanical) to plants than flowing water (Webster, 1962; Armstrong and Boatman, 1967). This is due to the low oxygen content of stagnant water, and to the magnification of other damaging factors discussed below.

In fact the picture is rather more complicated than a simple lack of oxygen. Russell (1961) states that aeration of the soil (or its lack) affects plant roots through three factors: in oxygen content, in carbon dioxide content, and in the content of anaerobic decomposition products. To these may be added two more - the modification of the available nutrient status, and the levels of toxic products of the plant's own metabolism. Although the relative importance of all these features has not been fully evaluated, the oxygen level in root tissues is probably the single most important factor affecting submerged roots. Before this is dealt with, three of the other factors will be briefly discussed.

Carbon dioxide at high concentrations has a generally depressing effect on plant metabolism, resulting in wilting and cessation of growth (Vlamis and Davis, 1944). Chang and Loomis (1945) found that carbon dioxide inhibited both water and mineral absorption, and Webster (1962) found that under conditions of experimental flooding carbon dioxide accumulated in soil water to a level equivalent to 55% in air, sufficient to severely affect the growth of Molinea caerulea. These effects are related neither to the pH nor to the oxygen content, but appear to be due to a toxic effect of the carbon dioxide itself.

Products of anaerobic decomposition are as varied as the organisms producing them; they include hydrogen, hydrogen sulphide, methane, ethylene, and butyric acid. The unpleasant smell of a waterlogged soil is due to these products. Their effects of plant growth are very varied. Hydrogen sulphide (Russell, 1961) and ethylene (Smith and Russell, 1969) are extremely toxic, the latter at concentrations as low as 1 p.p.m. Methane was found by Vlamis and Davis (1944) to inhibit the growth of barley but enhance that of rice. Butyric acid was cited by Vamos (1957) as a cause of root damage in flooded rice-fields.

The reducing conditions prevailing in waterlogged soils cause a general reduction in the availability of several important nutrients, notably phosphorus and nitrogen. The uptake of potassium by the plant is reduced, and potassium may even be excreted if high carbon dioxide concentrations are also present (Chang and Loomis, 1945). Mn^{++++} and Fe^{+++} ions may be reduced to the Mn^{++} and Fe^{++} forms, and may then form insoluble sulphides (Mandal, 1962). In any case, the toxic Fe^{++} (ferrous) ion increases markedly. Jefferey (1961) suggests that the state of reduction of a waterlogged soil should be measured in terms of the ratio of ferrous to ferric iron available. The most immediate effect of this general reduction of available nutrients,

together with the adverse effects on salt uptake, is chlorosis of the leaves. This will be described in more detail in Part I.

The features of waterlogged soil so far noted can hardly be modified directly by the plant. The levels of oxygen within the root, and possibly in its immediate environment, can however be modified. When a soil is flooded, it has been found that all dissolved oxygen can disappear within ten hours (Scott and Evans, 1955). Despite this, the roots of many plants which are permanently submerged have been found to contain oxygen in sufficient quantities to allow at least some aerobic respiration (Vallance and Coult, 1951; Coult, 1964; Armstrong, 1964). It is well known that many (but not all) plants whose roots are habitually submerged contain extensive aerenchymatous tissue continuous throughout the roots and shoots, and it was assumed that this formed an efficient pathway for the diffusion of oxygen to the roots. Williams and Barber(1961) have questioned this assumption, however, and claim that aerenchyma may not give a more efficient diffusion pathway than the normal air-spaces of a mesophyte root. They suggest that aerenchyma is more likely to be a means of reducing the amount of living tissue per unit volume, while maintaining adequate mechanical strength with a honeycomb structure.

In addition, there have been numerous instances of non-aerenchymatous tissues allowing marked gaseous diffusion (Brown, 1947; Heide, Boer-Bolt and Van Raalte, 1963; Greenwood, 1967). The plants used by these authors (mainly agricultural and horticultural species) would certainly suffer damage by quite short periods of flooding (Van t'Wandt and Hagen, 1957) despite any diffusion of oxygen down to and out of the roots. Laing, (1940), in studies of a wide range of water plants, stressed the wide diversity of structure of underwater rhizomes, and stated that resistance to anaerobic conditions is not due to diffusion of oxygen through the aerenchyma; on occasions the internal atmosphere of these plants contained little or no oxygen.

The diffusion of oxygen through air-spaces therefore does not seem adequate to explain the difference in flooding tolerance between higher plant species, and it is likely that the vast majority of species, whatever their morphology, will suffer some degree of anaerobiosis at the roots during periods of flooding. Whether or not this damages the plant will depend on the form which anaerobic metabolism will take in a particular species.

Although there are descriptions of the effects of flooding upon the composition of plant communities (Rutter,

1955; Webster, 1961), and upon the performance of individual plants (e.g. Ahlgren and Hansen, 1957), little attention has been given to the metabolic changes which precede these visible effects. There have been even fewer comparisons of the metabolic responses of flood-tolerant and flood-intolerant species, since the studies of Taylor (1942) on respiration, fermentation, and growth in wheat and rice. More detailed and up-to-date investigations of anaerobic metabolism have come from Boulter, Coult and Henshaw(1963) and Effer and Ranson(1967), but each used only one species (Iris pseudacorus and Fagopyrum esculentum respectively) and made no comparisons with other species.

Crawford (1966) made an initial study elucidating the metabolic differences between 'helophytes' and 'non-helophytes' (these terms were used in a purely experimental sense, according to the plants' reactions to artificial flooding). Species of the genus Senecio which were intolerant of high water-table conditions showed increases in their rates of glycolysis after being flooded for one month. Senecio species tolerant of experimental flooding showed no such change. It was suggested that the excessive accumulation of a toxic product such as ethanol, the end-product of glycolysis, could account for some of the damage suffered by the non-helophytes under conditions of high

water-table; plants tolerant of flooding avoided this damage by some form of metabolic control system. In a later paper (1967a) this observation was extended to a wider range of species, and an increase in alcohol dehydrogenase activity was demonstrated, but only in those plants intolerant of flooding. This latter observation is of course a possible explanation of the acceleration of glycolysis previously noted. It is also, incidentally, an additional explanation for the well-known Pasteur effect, or the acceleration of glycolysis under anaerobic conditions, previously explained only on the basis of an alteration of the ADP/ATP balance in anaerobiosis (Beevers, 1961). While this may be true in the short-term, alcohol dehydrogenase induction may contribute to the effect under persistent anaerobiosis.

The behaviour of a metabolic system depends to a great extent (but not entirely) upon the presence and properties of the enzymes which catalyse the reactions involved. Also, variations in metabolism which are genetic in origin (as are the differences between halophytes and non-halophytes) are most likely to be controlled through enzyme (i.e. protein) synthesis, as this process, of all the metabolic activities of an organism, is most directly under genetic control. The control mechanisms may there-

fore be logically sought in the appropriate enzyme systems. Alcohol dehydrogenase provides the first example of this, in that its induction in non-helophytes by flooding, and its lack of induction in helophytes, provides a partial explanation of the acceleration of glycolysis on flooding of non-helophytes, but not of helophytes. The explanation remains only partial, and many questions remain unanswered regarding the nature of the inductive process, the identity of the inductive stimulus, and the properties of the induced enzyme. Also, no alternative pathway has been suggested to enable helophytes to avoid ethanol accumulation.

The aims of the present study can therefore be presented as follows:-

1. To clarify the inductive process of alcohol dehydrogenase, identify the inductive stimulus, and study the induced enzyme.

2. To investigate the differences between helophytes and non-helophytes with regard to alcohol dehydrogenase induction.

3. To investigate similarly other related enzymes, selecting them as appear appropriate to the areas of metabolism as they come under study (a simplified plan of the areas of metabolism investigated, with the enzymes studied, is presented in Figure 3).

4. In addition, some assays of co-enzyme levels were performed, as it became apparent during the work that they were of considerable importance in controlling enzyme-catalysed reactions in these metabolic systems.

5. To combine these results and formulate a metabolic control system whereby the homeostatic properties of helophytes in flooded conditions can be explained in terms of their enzyme systems.

x x x

Arrangement of the Thesis

The methods used, results obtained, and conclusions reached are presented separately for each enzyme and co-enzyme studied. Relevant points are discussed as they arise in each section, including their relation to the general thesis, therefore no final discussion is included. The summary is, however, expanded to cover briefly all the major points which have arisen in the separate sections.

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PART I

THE PLANT MATERIALS

PART I THE PLANT MATERIALS

A total of nineteen species was used in these experiments, from nine families. Thirteen species were dicotyledons, and the rest monocotyledons, giving considerable taxonomic diversity. At the same time, however, a preponderance of the genus Senecio will be noticed; while this is mainly due to their use in previous work on this topic, it also serves to give an often useful comparison of the behaviour of closely-related species under the experimental conditions described.

All of the species used are members of the British flora with the exception of Pisum sativum L. and Vicia faba L., and were obtained entirely from natural sites, by collection of the plants or seeds.

Experimental Species

1. Caltha palustris L. Ranunculaceae. The two British subspecies and various forms were not distinguished. This species is normally found in marshes, fens, and ditches, and is therefore a natural helophyte. Whole plants were collected from the Nature Conservancy's reserve at Morton Lochs, near Tayport, Fife, and from Loch of Lowes, near Dunkeld, Perthshire, before being trimmed and planted in sand culture.

2. Ranunculus flammula L. Ranunculaceae. The various subspecies were not distinguished. Also a natural helophyte, specimens of whole plants were collected from various wet areas in Fife and Perthshire, then trimmed and planted in sand culture.

3. Pisum sativum L. var. 'Meteor'. Leguminosae. Seeds were obtained from a local commercial source, and germinated and grown in sand culture in the glasshouse as required.

4. Vicia faba L. var. 'Green Windsor'. Leguminosae. As for Pisum

5. Myosotis scorpioides L. Boraginaceae. This species was found in pools of standing water at Morton Lochs. Whole plants were collected, trimmed, and planted in sand culture. This is another natural helophyte, as is the next species.

6. Mentha aquatica L. Labiatae. Portions of plants were collected from slowly-running water in a drainage ditch on the Nature Conservancy's reserve at Tentsmuir, Fife, from standing water at Morton Lochs, and from marshy ground near Lindores Loch, Fife. Short cuttings were made and rooted in sand culture. Variations in form between specimens from the various sources disappeared in cultivation, and no hybrid forms were encountered. Some material developed severe rust infection in cultivation and was discarded on

the grounds that the metabolism may have been affected.

7. Senecio jacobea L. Compositae. This is a weed of waste land and poor pasture; it is not normally found in wet habitats. Whole plants and seeds were collected from sand-dunes near St. Andrews. The plants were trimmed and grown in sand culture, and the seeds gave generally good germination and grew on rapidly given the same treatment.

8. Senecio aquaticus L. Compositae. A natural helophyte, this species is commonly found in marshes and wet meadows. Seeds were collected from the island of Wyre, Orkney, and whole plants from Crag Lough, near Hexham, Northumberland.

9. Senecio squalidus L. Compositae. This is an introduced species generally found on drier waste ground, embankments, etc. Seed was collected from plants growing on spoil-heaps near Barnsley, Yorkshire, and germinated in sand in the glasshouse.

10. Senecio sylvaticus L. Compositae. Normally a plant of dry sandy soils among open vegetation; seeds were collected from the same site as the previous species.

11. Senecio viscosus L. Compositae. A species normally found on waste ground, railway embankments, etc., generally on well-drained soils. Seeds were obtained from the same source as S. squalidus and S. sylvaticus, and germinated similarly.

12. Senecio vulgaris L. Dune race (Crawford, 1966). Compositae. The dune race is distinguished as a physiological race of S. vulgaris because it differs from the garden race in its response to experimental flooding. It is not to be confused with the variety radiatus Koch., which is also often found on dunes. Whole plants were collected from sand-dunes at Tentsmuir, Fife, and further propagation was by seed collected in the greenhouse. The garden race of S. vulgaris was not used in these experiments.

13. Hieracium pilosella L. Compositae. This is commonly found on dry grassy banks, walls, etc. Whole plants were collected from dunes at Tentsmuir and near St. Andrews. They were grown in sand culture and propagated by stolons.

14. Juncus effusus L. Juncaceae. Like most Juncus species a natural helophyte, this species is very common in wet pastures and bogs on acid soils. Plants were collected from Tentsmuir and from Lindores Loch, Fife. They were divided and planted in sand.

15. Iris pseudacorus L. Iridaceae. Another natural helophyte, this is found in marshes and wet ground generally. Plants were collected from Lindores Loch, Fife, and Loch Clunie, Perthshire. The rhizomes and leaves were trimmed, and small portions planted in sand.

16. Carex arenaria L. Cyperaceae. Commonly found in sandy places by the sea, this species often colonises blow-outs in fixed dunes, but may persist in the wetter conditions of dune slacks. Whole plants and rhizomes were collected at Tayport, Fife, and planted in sand.

17. Glyceria maxima (Hartm.) Holmberg. Gramineae. A grass usually found in standing or slowly-moving water and therefore a helophyte. Plants were collected from Lindores Loch, Fife and trimmed and grown in sand culture.

18. Ammophila arenaria (L.) Link. Gramineae. This species is common on most sand dunes as a stabiliser, and is often planted for this purpose. Seeds and young plants were collected from Tentsmuir, and germinated or grown in sand culture.

19. Phalaris arundinacea. L. Gramineae. This grass, a natural helophyte common in a wide range of wet habitats, was collected from Lindores Loch, Fife, and propagated by rhizomes in sand culture.

x x x

Once collected, the plants were grown in the glass-house as described, and re-propagated, often several times, by cuttings (Mentha aquatica), seeds (most Senecio species), or by trimming and planting suitably-sized plants or parts of plants, depending on the growth-form.

All material was grown in sand culture, and watered with Hoagland's solution (Thomas, Ranson, and Richardson, 1956) weekly, keeping the sand moist with distilled water at other times. Material selected for experimental flooding was grown in 7-litre polythene buckets filled to within two inches of the top with sand. In early experiments the water-table was visibly kept at the desired level by means of a fine-mesh zinc inspection tube in the centre of each bucket, but later free drainage was allowed through a small hole in the bottom of each bucket; this was easily plugged when experimental flooding was commenced. Material destined for other experimental treatments was grown in 3-inch deep polythene trays with drainage holes, or in individual 4-inch diameter plastic pots. All plant material received the same treatment until used for experimental purposes.

After growth in sand culture, all the species used were classified as helophytes or non-helophytes according to their reaction to experimental flooding (Crawford, 1966). One bucket of plants was flooded with Hoagland's solution, and the water level kept above the sand surface by the addition of distilled water as necessary, and Hoagland's solution weekly. Another bucket was flooded with Hoagland's solution which was allowed to drain freely from the base; the sand was kept moist by addition of Hoagland's solution

weekly and distilled water as necessary.

There was never enough plant material available to allow replicate sampling methods to be used before and during the experimental period, to obtain fresh and dry weight data. In order to determine the effect of flooding on growth, therefore, specimens were chosen which were closely comparable in size (this was more feasible when the plants were raised from seed), and growth determined in three ways:

1. By measurements appropriate to the growth-form of the plant (growth of a marked leaf, plant height, total shoot growth, etc.) before and after the experimental period.

2. By cropping the aerial portions of the plants after the experimental period, and recording fresh weights and

3. dry weights, after drying at 100°C in an oven.

Table I shows the effects on one month's flooding upon all the species used, in terms of method 1. The figures in the third column are arrived at by calculating the percentage increase of the measured parameter during the experimental period, and expressing the growth of the flooded plants as a percentage deviation from that of the unflooded.

Tables 2 and 3 give the results of measurements by

Table 1. Effect of Flooding upon Growth

Measurements made over one month's experimental flooding

Species	Measurement made	Effect of Flooding
Caltha palustris	Leaf length x breadth[x]	21% increase
Ranunculus flammula	" " "	Not available[xx]
Pisum sativum	Plant height	30% decrease
Vicia faba	" "	15% decrease
Myosotis scorpioides	Total shoot growth	17% decrease
Mentha aquatica	Leaf length x breadth[x]	8% increase
Senecio jacobea	" " "	37% decrease
S. aquaticus	" " "	9% increase
S. squalidus	Plant height	28% decrease
S. sylvaticus	Number of leaves[x]	Not available
S. viscosus	Leaf length x breadth	Not available
S. vulgaris (dune)	Plant height	52% decrease
Hieracium pilosella	Leaf length x breadth	21% increase
Juncus effusus	None valid	---
Iris pseudacorus	Leaf length	28% increase
Carex arenaria	Number of leaves[x]	11% increase
Glyceria maxima	Leaf length	31% decrease
Ammophila arenaria	" "	51% increase
Phalaris arundinacea	" "	48% increase

[x] The youngest visible leaf was measured and marked with waterproof ink at the beginning of the experimental period.

[xx] Measured leaves matured and died before the end of the experimental period.

Table 2. Effect of Flooding upon Growth

Fresh weight of aerial portions after one month's flooding

Species	Average Fresh Weight per Plant		
	Unflooded	Flooded	Ratio Unfl.:Fl.
Caltha palustris	1.4g	1.4g	1:1.00
Ranunculus flammula	0.5g	1.4g	1:2.80
Pisum sativum	6.1g	2.0g	1:0.33
Vicia faba	19.1g	18.9g	1:0.99
Myosotis scorpioides	4.5g	5.7g	1:1.27
Mentha aquatica	1.3g	1.8g	1:1.38
Senecio jacobea	23.0g	17.3g	1:0.75
S. aquaticus	3.9g	6.4g	1:1.64
S. squalidus	4.8g	3.4g	1:0.71
S. sylvaticus	14.6g	7.9g	1:0.54
S. viscosus	5.0g	4.5g	1:0.90
S. vulgaris (dune)	4.4g	4.0g	1:0.91
Hieracium pilosella	5.7g	6.6g	1:1.16
Juncus effusus	2.5g	3.7g	1:1.48
Iris pseudacorus	8.6g	12.4g	1:1.44
Carex arenaria	0.7g	2.2g	1:3.14
Glyceria maxima	3.9g	3.5g	1:0.90
Ammophila arenaria	1.1g	0.9g	1:0.82
Phalaris arundinacea	1.3g	2.5g	1:1.92

Table 3. Effect of Flooding upon Growth

Dry weight of aerial portions after one month's flooding

Species	Average dry weight per plant		
	Unflooded	Flooded	Ratio Unfl.:Fl.
Caltha palustris	0.19g	0.27g	1:1.42
Ranunculus flammula	0.08g	0.15g	1:1.88
Pisum sativum	0.91g	0.30g	1:0.33
Vicia faba	1.74g	1.73g	1:0.99
Myosotis scorpioides	0.57g	0.58g	1:1.02
Mentha aquatica	0.20g	0.23g	1:1.15
Senecio jacobea	2.20g	2.20g	1:1.00
S. aquaticus	0.62g	0.86g	1:1.39
S. squalidus	0.50g	0.41g	1:0.82
S. sylvaticus	1.22g	0.84g	1:0.69
S. viscosus	0.66g	0.62g	1:0.94
S. vulgaris (dune)	0.60g	0.25g	1:0.42
Hieracium pilosella	0.70g	0.81g	1:1.16
Juncus effusus	0.68g	0.85g	1:1.25
Iris pseudacorus	1.12g	1.45g	1:1.29
Carex arenaria	0.19g	0.48g	1:2.51
Glyceria maxima	0.56g	0.46g	1:0.82
Ammophila arenaria	0.32g	0.30g	1:0.94
Phalaris arundinacea	0.35g	0.45g	1:1.29

methods 2 and 3.

As well as these measurable effects of experimental flooding, certain visible symptoms of flooding damage were observed. These are listed below for the nineteen species used. Growth parameters used in Table 1 are omitted.

The Visible Effects of Experimental Flooding

Caltha palustris - leaves deeper green.

Ranunculus flammula - plants larger, deeper green; leaves more numerous.

Pisum sativum - leaves paler; chlorosis between the main veins; flowers fewer; seeds fewer and smaller; blackening of the root-tips.

Vicia faba - reduction of lateral root growth; blackening of root-tips.

Myosotis scorpioides - no difference visible.

Mentha aquatica - no differences visible.

Senecio jacobea - chlorosis and distortion of the leaves; root growth reduced and distorted.

Senecio aquaticus - no differences visible.

Senecio squalidus - narrowing of leaf-segments; increased anthocyanin production; reduction of root growth.

Senecio sylvaticus - general chlorosis; wilting; reduction of root and shoot growth (Plate I).

Senecio viscosus - general chlorosis; browning of roots

PLATE I

Senecio sylvaticus

Unflooded

Flooded

one month

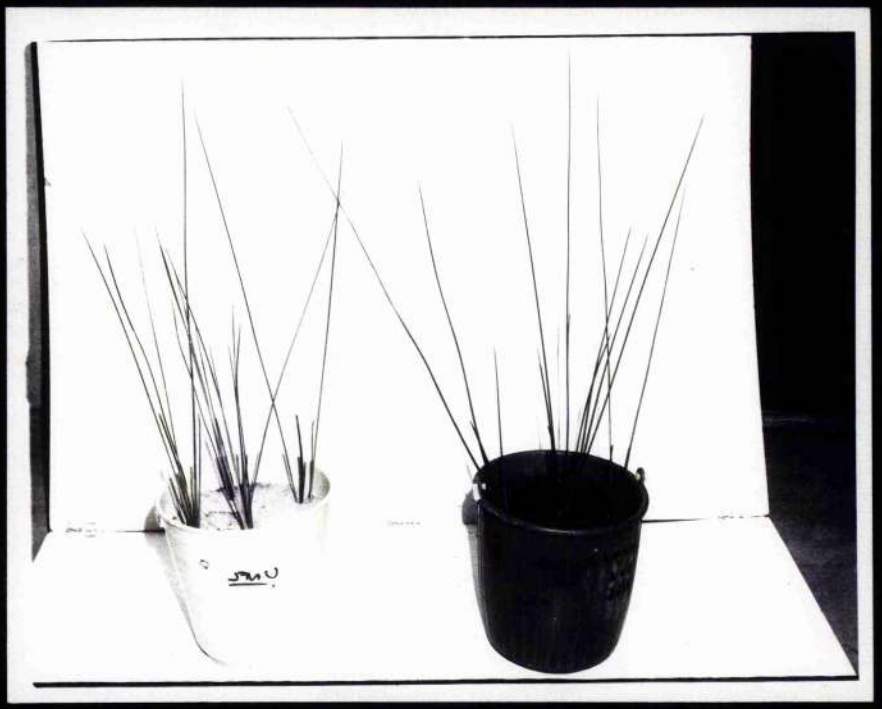
PLATE II

Juncus effusus

Unflooded

Flooded

one month



and reduction of root growth.

Senecio vulgaris (dune) - narrowing of leaf-segments, general chlorosis; browning of roots and reduction of root growth.

Hieracium pilosella - general chlorosis; reduction of stolon production; browning of roots and reduction of root growth.

Juncus effusus - no differences visible (Plate II).

Iris pseudacorus - no differences visible.

Carex arenaria - leaves larger, deeper green.

Glyceria maxima - no differences visible.

Ammophila arenaria - narrowing and chlorosis of leaves; death of older leaves; browning of the root system and reduction of root growth.

Phalaris arundinacea - no differences visible.

x x x

Most of the nineteen species could be classified without trouble as helophytes or non-helophytes on the basis of all measurable and visible effects of flooding. The various criteria used proved less satisfactory for the remainder. In these cases the figures for growth and fresh and dry weight were strongly contradicted by the visible evidence, e.g. Glyceria maxima apparently had its growth reduced by flooding, but appeared perfectly healthy and

showed none of the visible symptoms of flooding damage, while Hieracium pilosella, whose growth on all counts was increased on flooding, nevertheless displayed marked leaf chlorosis and browning of the roots, as well as delayed flowering after the experimental period. These 'doubtful' species were grown for more extended periods (up to three months) in flooded conditions to find which would survive. They were then classified accordingly.

As a result of these observations upon the effects of flooding for one month and longer, all the species were classifies as experimental helophytes or non-helophytes, the former being those whose growth was unaffected or stimulated by flooding, and the latter those whose growth was reduced or which were otherwise adversely affected by flooding. Table 4 gives this classification. The species are not listed in any particular order within the classes, as not only are these results not considered significant enough to give a gradation of flooding tolerance or intolerance, but the effects of flooding are so varied and complex (as will be seen later) that such a grading would be a gross oversimplification.

It must be emphasised that this is a purely experimental classification, and may or may not coincide with the distribution of the species in the field, where many factors

Table 4. Classification of the Experimental Species as Helophytes and Non-Helophytes

Helophytes	Non-Helophytes
Phalaris arundinacea	Senecio jacobea
Glyceria maxima	Senecio squalidus
Senecio aquaticus	Senecio sylvaticus
Ranunculus flammula	Senecio viscosus
Mentha aquatica	Senecio vulgaris (dune)
Caltha palustris	Ammophila arenaria
Juncus effusus	Hieracium pilosella
Myosotis scorpioides	Pisum sativum
Carex arenaria	Vicia faba
Iris pseudacorus	

other than flooding tolerance are operating. Also, the conditions in a waterlogged bucket are not nearly so severe (in terms of dissolved oxygen and accumulation of sulphides and ferrous iron) as those in a similarly waterlogged sandy soil in the field (Crawford, 1966), therefore only those species which are very susceptible to flooding will appear as non-helophytes under experimental conditions.

In fact, most of the species chosen for experiments would be classified ecologically in the same way as they have been experimentally (with the possible exception of Carex arenaria, although this can persist in periodically-flooded dune slacks), but many of the species considered and rejected for experimental purposes did not conform to this pattern. Notably, most grasses appeared as experimental helophytes, but would certainly not survive prolonged flooding in the field.

As may be surmised from the rather oddly-assorted list of non-helophytes, some difficulty was experienced in building up a collection with suitable experimental properties. For these experiments, the properties sought were sufficiently rapid growth, ease of propagation, and a convenient growth-form.

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PART II

ALCOHOL DEHYDROGENASE

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Introduction

Alcohol dehydrogenase (ADH) has been one of the more widely-studied of the pyridine nucleotide-linked dehydrogenases. Some of the reasons are its early purification and crystallisation (Negelein and Wulff first crystallised the enzyme in 1937), its independence of any co-factors other than the nucleotides, and its unique position at the end of a major metabolic chain. Sund and Theorell (1963) give an excellent review of the properties of the enzyme; since then Harris (1964) has elucidated the amino-acid sequence around the active sites, and several workers have discovered isoenzymes similar to those well-known for lactate and other dehydrogenases (Koen and Shaw, 1966; Blair and Vallee, 1966; Pikkarainen and Räihä, 1969).

Despite the very wide occurrence of ADH in many living organisms, only the yeast and the liver (Bonnichsen and Wassén, 1948) enzymes have been crystallised and can be studied in the pure state. Berger and Avery (1943) included ADH in a study of the dehydrogenases of Avena coleoptiles. Stafford and Vennesland (1953) reported the occurrence of the wheat-germ enzyme and partially purified it. Subsequent reports of the enzyme in higher plants have been numerous, and it may be concluded that its occurrence is general

throughout the plant kingdom.

A brief summary of the more important properties of alcohol dehydrogenase is given in Table 5. The Michaelis Constants given are only approximate, and vary from author to author, and also with pH.

The reaction usually catalysed in vivo by ADH is:

$$CH_3CHO + NADH^+ H \rightleftharpoons CH_3CH_2OH + NAD^+$$

Substrate specificity, especially of the liver enzyme, is very wide, and a variety of higher alcohols, aldehydes and some aromatic ketones can act as substrates. Most preparations of the liver enzyme have so far given highest activity with the higher aliphatic alcohols. The yeast ADH has a rather narrower specificity, and gives highest activity with ethanol.

Induction of alcohol dehydrogenase Inductive enzymes in higher plants were first reported by Tolbert and Cohen (1953), who studied glycollic acid oxidase. Indole acetic oxidase (Galston and Dalberg, 1954) and nitrate reductase were further additions to the list. This latter enzyme has received much attention regarding the influence of environment on induction and stability (Hageman and Flesher, 1960a; Afridi and Hewitt, 1964 and 1965; Beevers, Schrader, Flesher, and Hageman, 1965). Afridi and Hewitt found that induction by nitrate and light was prevented by an anaerobic environment, and concluded that anaerobiosis was inhibiting the

Table 5. Some Properties of ADH

	Liver Enzyme	Yeast Enzyme
Molecular weight	84,000	150,000
No. of active sites	2	4
Zinc atoms bound	2	4
Km acetaldehyde	2.7×10^{-4} M*	1.1×10^{-4} M**
Km ethanol	2.1×10^{-4} M*	2.4×10^{-2} M**

Km = Michaelis Constant

* Theorell, Nygaard and Bonnischen, 1955

** Hayes and Velick, 1954

protein synthesis necessary for induction. This is in marked contrast to the case of alcohol dehydrogenase. This enzyme was first reported as inducible in higher plants by App and Meiss (1958), who studied germinating rice, although the induction of ADH activity in rat kidney by feeding the rats with ethanol had been described in 1938 by Leloir and Muntz; this must have been one of the earliest records of adaptive enzyme formation in any species.

App and Meiss found that aeration of rice seedlings germinated under water reduced the ADH activity; when aeration stopped, the activity rose again. Added ethanol caused a rise in activity, but acetaldehyde either had no effect or else depressed activity. Hageman and Flesher (1960b) found increased activity in corn seedlings when they were placed in an anaerobic environment, but said that acetaldehyde was the inductive agent and that ethanol had no effect. The results of App and Meiss and those of Hageman and Flesher are thus in disagreement, but, as the latter point out, the apparent contradiction may be explained by the great differences in ecology and metabolism between the two species. Kollöffel (1968) performed similar experiments on germinating peas, and found both acetaldehyde and ethanol to be inductive agents, the former at one-tenth the concentration of the latter.

Crawford (1967a) followed the changes in root ADH activity consequent upon experimental flooding of a wide range of plants which he classified experimentally as 'helophytes' or 'non-helophytes' according to the affect of flooding upon growth. He found that the plants tolerant of experimental flooding showed less than a threefold increase in root ADH activity, while the non-helophytes exhibited 14- to 70-fold increases. This marked ADH induction was adduced to explain the previously-recorded acceleration of glycolysis upon flooding of non-helophytes, but not helophytes (Crawford, 1966). Crawford's papers neither attempt to explain the lack of induction in helophytes, nor identify the inductive agent. The paper of Crawford and McManmon (1968) extends the work in these directions, but as most of the results in that paper are incorporated in the present thesis, they will be discussed as they arise.

x x x

(i) Flooding Experiments (including extraction and protein assay methods)

All of the experimental species were grown for one month under high and low water table ('flooded' and 'unflooded' conditions as already described in Part I. The roots were then washed free of sand, first in tap water, then in distilled water, blotted damp-dry with paper tissues, and quickly weighed before being homogenised in a chilled mortar with 2-10 times (volume to weight) their own amount of Tris-HCl buffer, 0.1M, pH 8.0, kept at 4°C. A small amount of acid-washed sand was added to aid homogenisation.

In earlier experiments the homogenate was then passed through a double layer of muslin, but this stage was omitted, apparently without deleterious effects, when the amount of root material was limited. The homogenates (at least two different plant replicates per treatment) were centrifuged at 12,000 g for 20 minutes at 4°C, and the supernatant retained for enzyme assay; in some cases this crude extract was stored deep-frozen until assayed, without apparent reduction in activity, but normally assays were carried out immediately.

For convenience of presentation and reference, all the spectrophotometric enzyme assay systems used, together with the principles involved and other relevant information, are

presented together as an appendix (Appendix A).

All enzyme activities, unless otherwise stated, are expressed as International Units (Bergmeyer, 1963), and the figures are, throughout every experiment, the average of two independent (different plant) replicates. Protein was estimated by the method of Murphy and Kies (1960), which utilises the difference in extinction between 215nm and 225nm in very dilute protein solutions. The method of Warburg and Christian (1941) gave comparable results, but the former was preferred because of the simplicity of calculation. Only in one case was this method not used (See Table 11), because of unknown interfering substances; then the biuret method (Robinson and Hogden, 1940) was used, with bovine serum albumin as a reference.

x x x

Table 6 gives the results, in terms of ADH activity, of flooding the experimental species for one month. The species are divided into helophytes and non-helophytes, as described in Part I.

The data confirms that of Crawford (1967a) in terms of the differences in induction of root ADH activity in helophytes and non-helophytes, and thus supports his hypothesis that one of the mechanisms by which helophytes withstood flooding is by avoidance of the increase in

Table 6. Alcohol Dehydrogenase activity in roots of plants grown under flooded and unflooded conditions for one month

Species	ADH activity (International Units/mg protein)		
HELOPHYTES	Unflooded	Flooded	Ratio Unfl.:Fl.
Phalaris arundinacea	0.244	0.091	1: 0.37
Glyceria maxima	0.143	0.109	1: 0.76
Senecio aquaticus	0.088	0.102	1: 1.16
Ranunculus flammula	0.126	0.123	1: 0.98
Mentha aquatica	0.057	0.057	1: 1.00
Caltha palustris	0.350	0.350	1: 1.00
Juncus effusus	0.078	0.073	1: 0.94
Myosotis palustris	0.140	0.130	1: 0.93
Carex aronaria	0.031	0.042	1: 1.35
Iris pseudacorus	0.066	0.035	1: 0.53
NON-HELOPHYTES			
Senecio jacobea	0.030	0.136	1: 4.54
S. squalidus	0.030	0.073	1: 2.44
S. vulgaris (dune)	0.016	0.081	1: 5.06
S. viscosus	0.100	0.510	1: 5.10
S. sylvaticus	0.027	0.102	1: 3.78
Ammophila aronaria	0.015	0.042	1: 2.80
Hieracium pilosella	0.055	0.102	1: 1.86
Pisum sativum	0.014	0.218	1:15.6
Vicia faba	0.013	0.076	1: 5.85

glycolosis and accumulation of ethanol consequent upon induction of root ADH. The figures of Table 6, however, show a degree of induction far less than that found by Crawford. This is undoubtedly due at least in part to the modification of the assay system used by him to one using 200 μmoles of acetaldehyde per experimental cuvette instead of 40 μmoles (see Appendix A). It is considered that this modified assay system gives a more accurate picture of the maximum ADH activity in the extract; this is discussed more fully and put on a quantitative basis in a later section dealing with the kinetics of the induced enzyme.

x x x

Short-term induction by flooding An induction period of one month is very long compared to other studies on enzyme induction, even in higher plants. Hageman and Flesher (1960b) demonstrated induction in two to three days, and Kollöffel (1968) used a similar period.

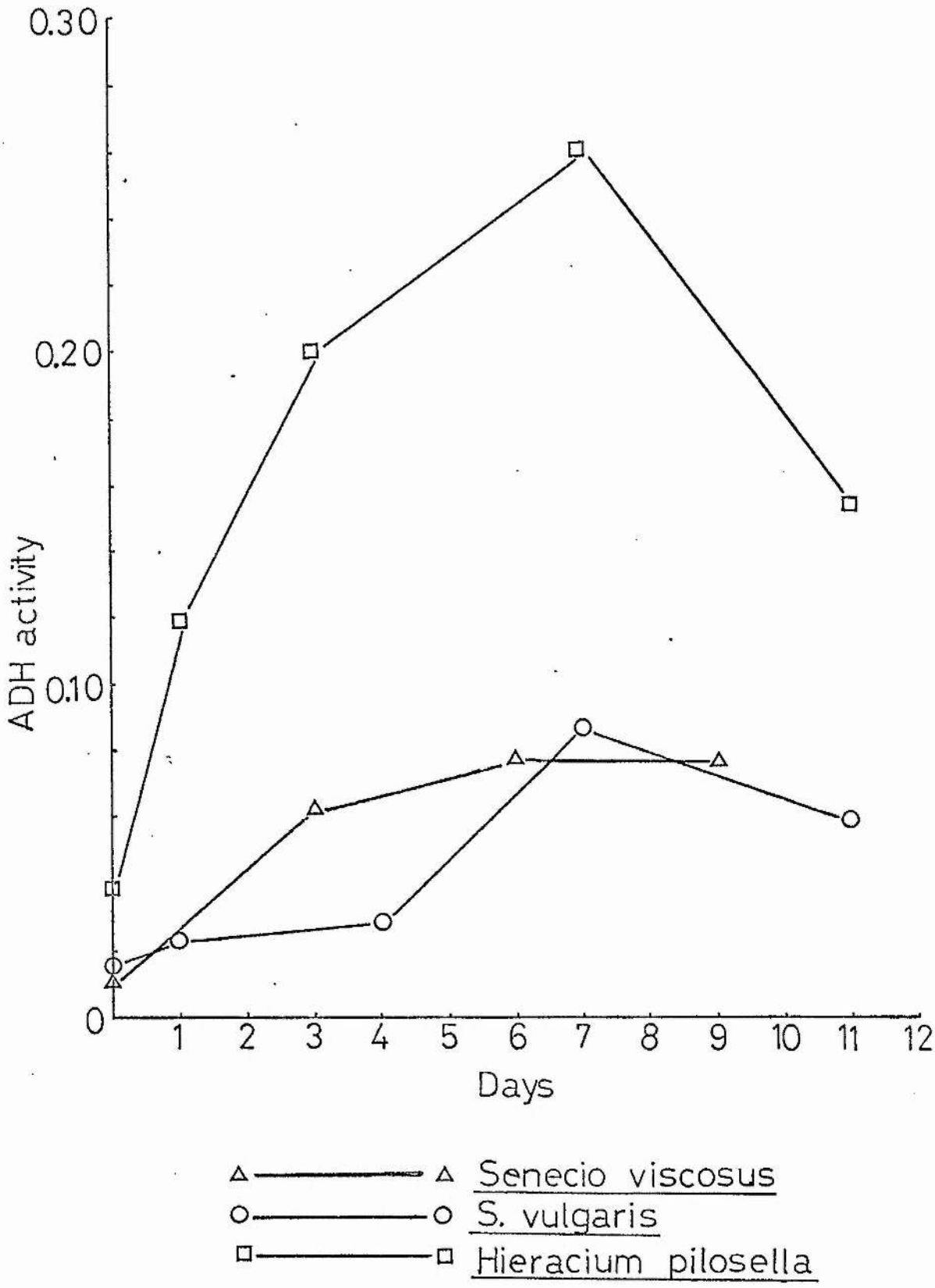
Three non-helophytes were flooded as previously described for periods of from one to eleven days, and the root ADH activity assayed. The results are expressed graphically in Figure 1. The starting point (0 days) is in each case the value for the unflooded control.

It is apparent that not only is ADH induction

FIGURE 1.

Development of ADH activity in three non-helophytes by flooding for a period of days.

Enzyme activities are expressed as International Units per milligram protein.


0.30
0.20
0.10
0
ADH activity
1
2
3
4
5
6
7
8
9
10
11
12
Days
Senecio viscosus
S. vulgaris
Hieracium pilosella

demonstrable over this shorter time-scale, but that it may even exceed that apparent over a month. There is some indication in Figure 1 that induction tails off after about a week, and activity may fall if flooding persists. This seems to be particularly true of Hieracium pilosella. Table 7 gives the results of another experiment, conducted over a period of one to two weeks, in which the ADH activity induced in the roots of H. pilosella during the first week is apparently lost during the second week; the root ADH of Senecio jacobea retains its induced activity. The two helophytes included in this experiment show a steady fall in activity throughout the period.

Discrepancies in the magnitude of induction of ADH in a given species from experiment to experiment were characteristic of these investigations, and caution must be exercised when comparing one experiment with another, especially if they are performed on different batches of plant material at different times of the year. The induction phenomenon seems to be least marked during the winter, and most in evidence during spring and early summer. Most of the results in Table 6 were obtained between November and March, while those of Figure 1 were obtained during April and May.

x x x

Table 7. Root ADH activity of four species grown under flooded and unflooded conditions for one and two weeks

Enzyme activities expressed as International Units per mg. protein.

Species	ADH activity		
HELOPHYTES	Unflooded	Flooded 1 week	Flooded 2 weeks
Myosotis scorpioides	0.068	0.063	0.022
Mentha aquatica	0.159	0.083	0.049
NON-HELOPHYTES			
Senecio jacobea	0.040	0.138	0.162
Hieracium pilosella	0.029	0.052	0.034

(ii) Water Culture Experiments

In an attempt to achieve greater control over the root environment, and as a prelude to later experiments using enzyme substrates for induction purposes, an experiment was performed in which two helophytes and two non-helophytes were grown in aerated and non-aerated water culture for periods of one to nine days.

Plants were raised in sand culture as previously described in Part I, and transferred to water culture in Hoagland's solution on reaching a suitable size. The containers used were one-pint milk bottles painted matt black on the outside and closed by rubber stoppers with three holes, for the plant, air inlet, and air outlet respectively. Anhydrous lanolin (Adeps lanae, B.P.) was used to provide an airtight seal between plant and stopper. Continuous aeration was provided to the aerated cultures by 'Hy-Flo' aquarium pumps. Non-aerated cultures were gassed with nitrogen for one hour, then the air inlets and outlets were sealed. On removal from the water culture the plant roots were washed thoroughly with distilled water, blotted, weighed, and homogenised for assay as previously described.

The results of this experiment are depicted in Figures 2 and 3. For comparison, in the case of one of the non-helophytes, Senecio viscosus, the induction of root ADH

FIGURE 2.

ADH activities in the roots of two non-helophytes grown for different periods in aerated and non-aerated water culture.

Enzyme activities are expressed as International Units per milligram protein, x100

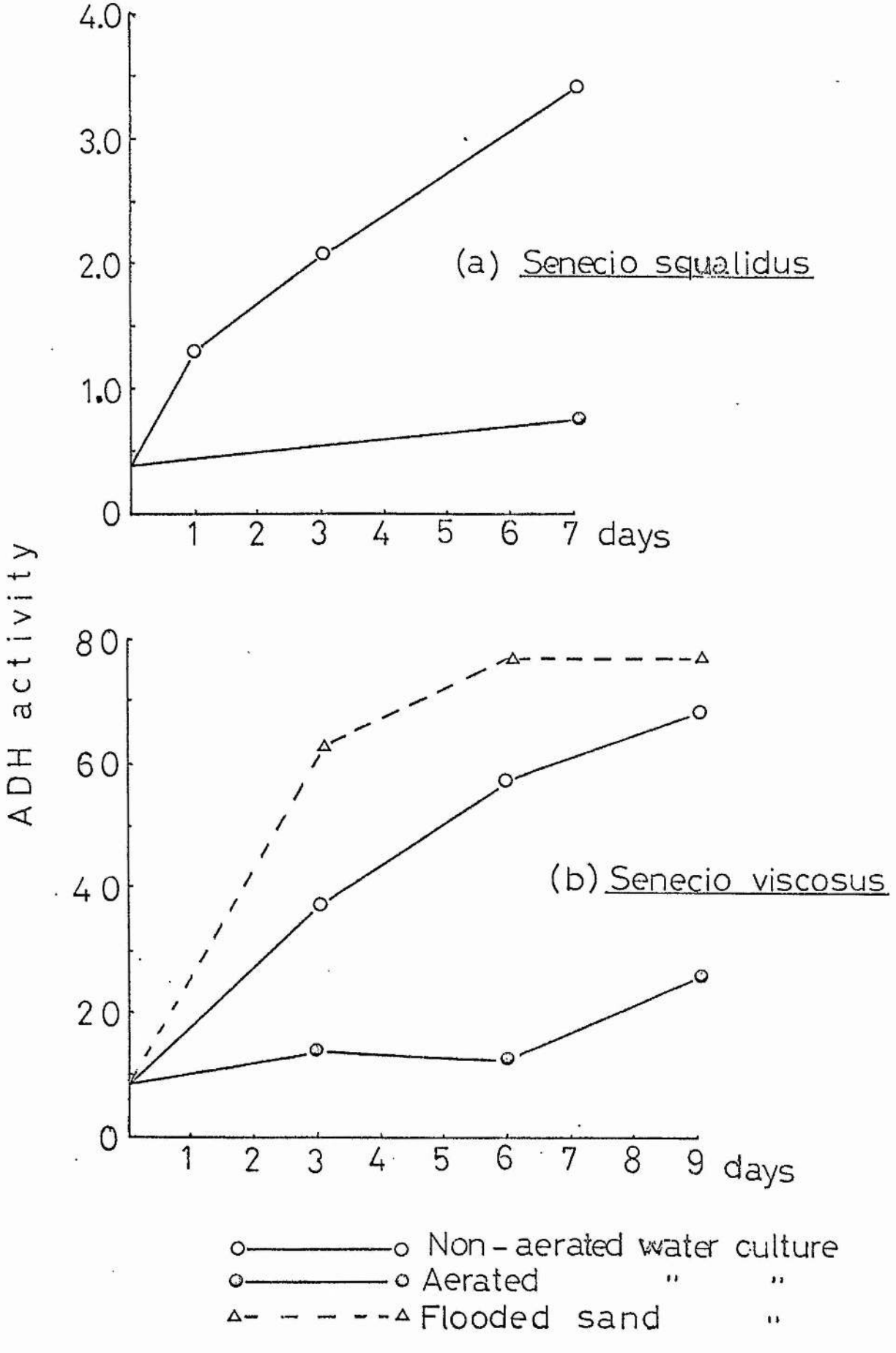
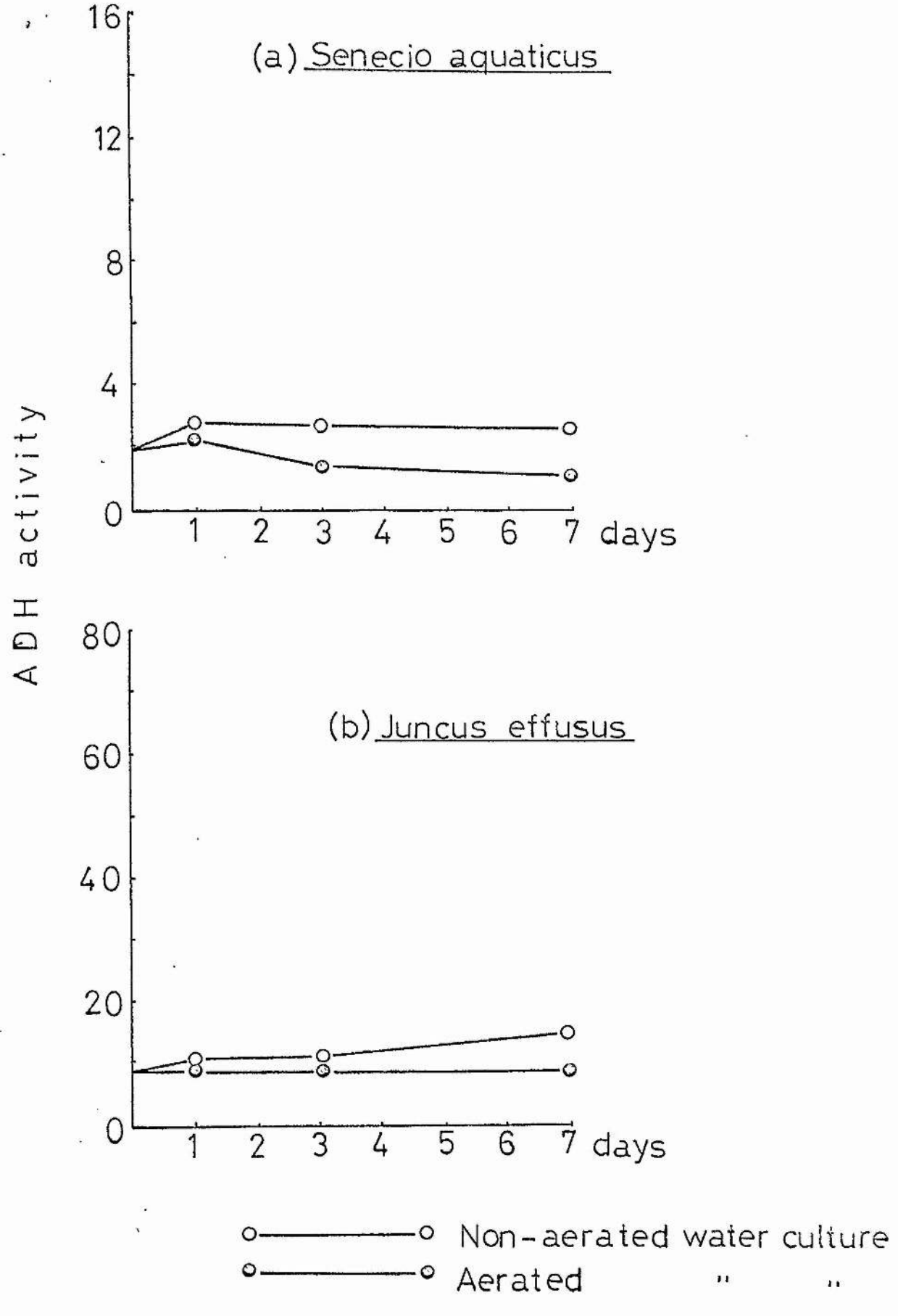

4.0
3.0
2.0
1.0
0
1 2 3 4 5 6 7 days
(a) Senecio squalidus
ADH activity
80
60
40
20
0
1 2 3 4 5 6 7 8 9 days
(b) Senecio viscosus
Non-aerated water culture
Aerated " "
Flooded sand "

FIGURE 3.

ADH activities in the roots of two helophytes grown for different periods in aerated and non-aerated water culture.

Enzyme activities are expressed as International Units per milligram protein, x 100


(a) Senecio aquaticus
16
12
8
4
0
1 2 3 4 5 6 7 days
ADH activity
(b) Juncus effusus
80
60
40
20
0
1 2 3 4 5 6 7 days
Non-aerated water culture
Aerated " "

in sand culture, previously given in Figure 1, is re-plotted here.

The induction of root ADH in non-helophytes by non-aerated water culture is similar to that in flooded sand culture, although possibly less rapid. However, as has already been stated, quantitative comparisons of the results of different experiments should be made only with caution. The main point to emerge from this experiment is that aerated and non-aerated water cultures are comparable, for experimental purposes, to unflooded and flooded sand cultures; the degree of induction of ADH is similar, and the differences between helophytes and non-helophytes are similarly apparent. Thus a clearer basis is given to the interpretation of later, more elaborate, water culture experiments in 'helophyte' and 'non-helophyte' terms; these are described in subsequent sections.

A subsidiary experiment involving aerated and non-aerated water culture demonstrates the reversible nature of the induction of ADH in non-helophyte roots. Plants of the dune race of Senecio vulgaris, previously grown for one month in flooded and unflooded sand culture, were transferred to aerated water culture. In some of the cultures, aeration was stopped after seven days. The results are presented in Table 8. The high ADH activity induced on flooding is lost on transfer to aerated water culture, but

Table 8. Reversibility of Induction: ADH activity in the roots of Senecio vulgaris(dune race) after various treatments

Enzyme activities are expressed as International Units per mg. protein.

	Original Sand Culture	
	Flooded	Unflooded
Further Water Culture		
None	0.0518	0.0018
Aerated 7 days	0.0082	0.0027
Aerated 7 days, then non-aerated 5 days	0.0573	0.0882

is regained on cessation of aeration. Induction on cessation of aeration is similarly displayed by the plants not previously grown in flooded sand culture. This reversibility of induction has also been found in rice (App and Meiss, 1958) and germinating peas (Hollöffel, 1968). If induction is a result of de novo enzyme synthesis, then this reversibility is attributed to normal protein turnover combined with cessation of induced enzyme sythesis consequent upon removal of the inductive agent or environment.

x x x

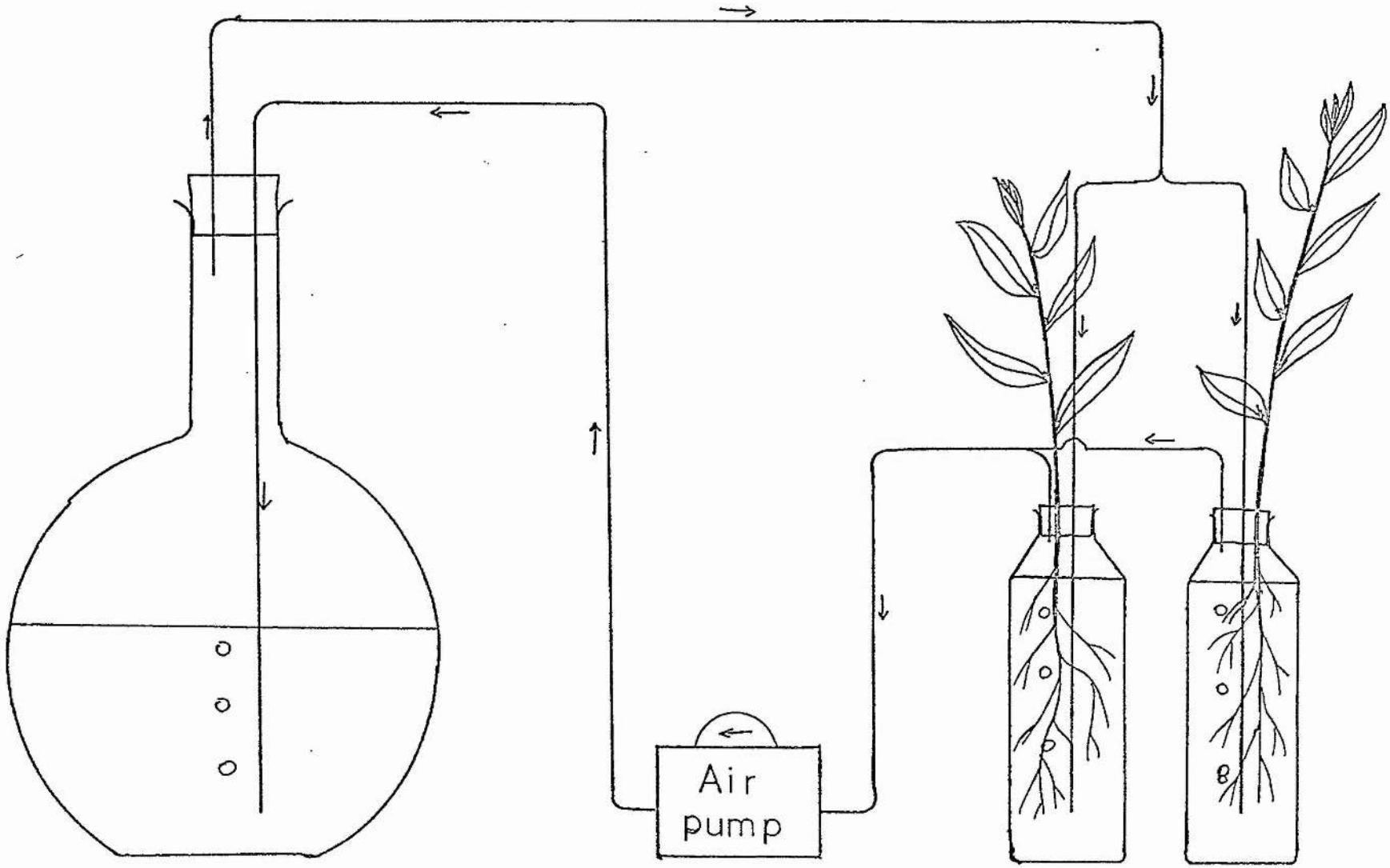
(iii) The Inductive Stimulus

The inductive agent, if any, for ADH has been in dispute. Enzyme induction is most commonly by substrates, and either acetaldehyde (Hageman and Flesher, 1960b), or ethanol (App and Meiss, 1958), or both (Kolloffel, 1968) have been suggested. Hageman and Flesher found that pyruvate also induced ADH (in corn seedlings), and suggested that this was decarboxylated to acetaldehyde, the actual inductive stimulus. Other alcohols and aldehydes do not appear to have been tested. The present experiments are also confined to these three substances, mainly because the demonstration of ADH induction by more exotic compounds would be mainly of theoretical interest, whereas pyruvate, acetaldehyde, and ethanol are all likely to be present and increasing in conditions of anaerobiosis and accelerated glycolysis.

The volatile nature of acetaldehyde and ethanol made it necessary to devise a closed cycle of aeration whereby aerobic conditions could be maintained in the culture solution while preventing the loss by volatilization of the added substrate. The system is outlined in Figure 4. Black-painted milk bottles were again used as culture vessels. Air from the culture solution containing the ethanol or acetaldehyde was bubbled through a five-litre flask containing 2.5 litres of a solution of similar strength

FIGURE 4.

Closed aeration cycle employed in induction experiments using volatile substances.


Air
pump
Reservoir solution
Replicate water cultures

in distilled water, before being returned to the culture solution. This system served three purposes:

1. Aerobic conditions were maintained in the culture solution. (Tests for dissolved oxygen using a Mackelreth oxygen meter before and after an experiment showed no significant reduction of the oxygen content of the solutions.)

2. Volatile metabolic products which may have affected the inductive process were removed by dilution into the reservoir solution.

3. The concentration of ethanol or acetaldehyde in the culture solution was maintained, as the gas phase was in continuous circulation and equilibrium with the liquid phases of culture solution and reservoir.

Kollöffel (1968) has devised a similar system for his studies on germinating peas.

After 60 hours, the plants were removed from the culture solutions, and the roots washed very thoroughly, first in running tap water then in several changes of distilled water, to remove traces of ethanol or acetaldehyde which, as substrates of ADH, would have affected the enzyme assay. They were then blotted, weighed, and homogenised for assay as previously described.

Acetaldehyde Concentrations from 10^{-5}M to 5×10^{-2}M were used, and their effects upon root ADH activity in seven species are shown in Figures 5, 6, 7. Because of the marked falls in protein extracted per gram fresh weight of

FIGURE 5.

ADH activities in the roots of three helophytes grown in aerated culture solutions containing different levels of acetaldehyde.

Enzyme activities are expressed as International Units per gram fresh weight of roots.

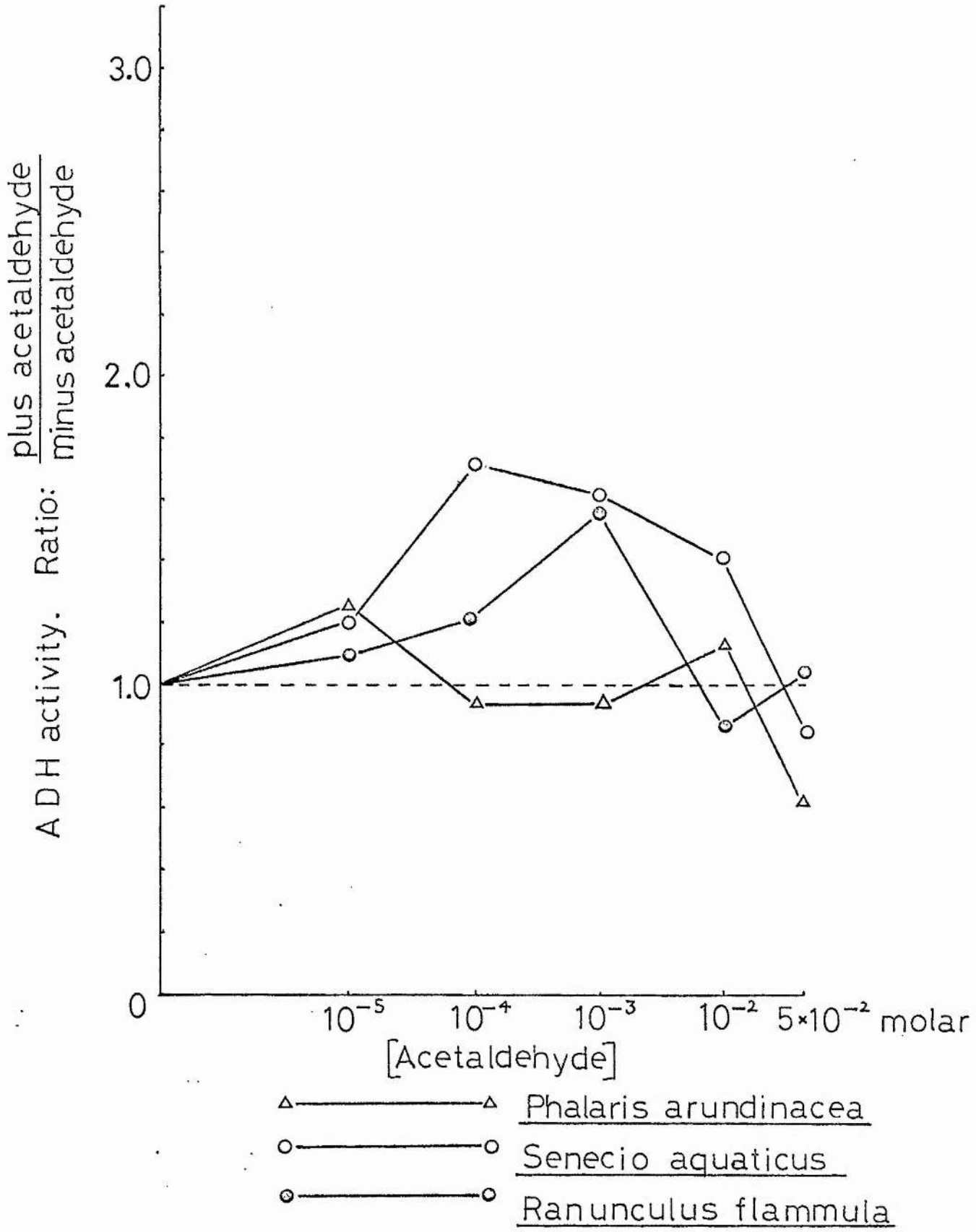

ADH activity. Ratio: plus acetaldehyde / minus acetaldehyde
3.0
2.0
1.0
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
5×10^{-2} molar
[Acetaldehyde]
Phalaris arundinacea
Senecio aquaticus
Ranunculus flammula

FIGURE 6.

ADH activities in the roots of two non-helophytes grown in aerated culture solutions containing different levels of acetaldehyde.

Enzyme activities are expressed as International Units per gram fresh weight of roots.

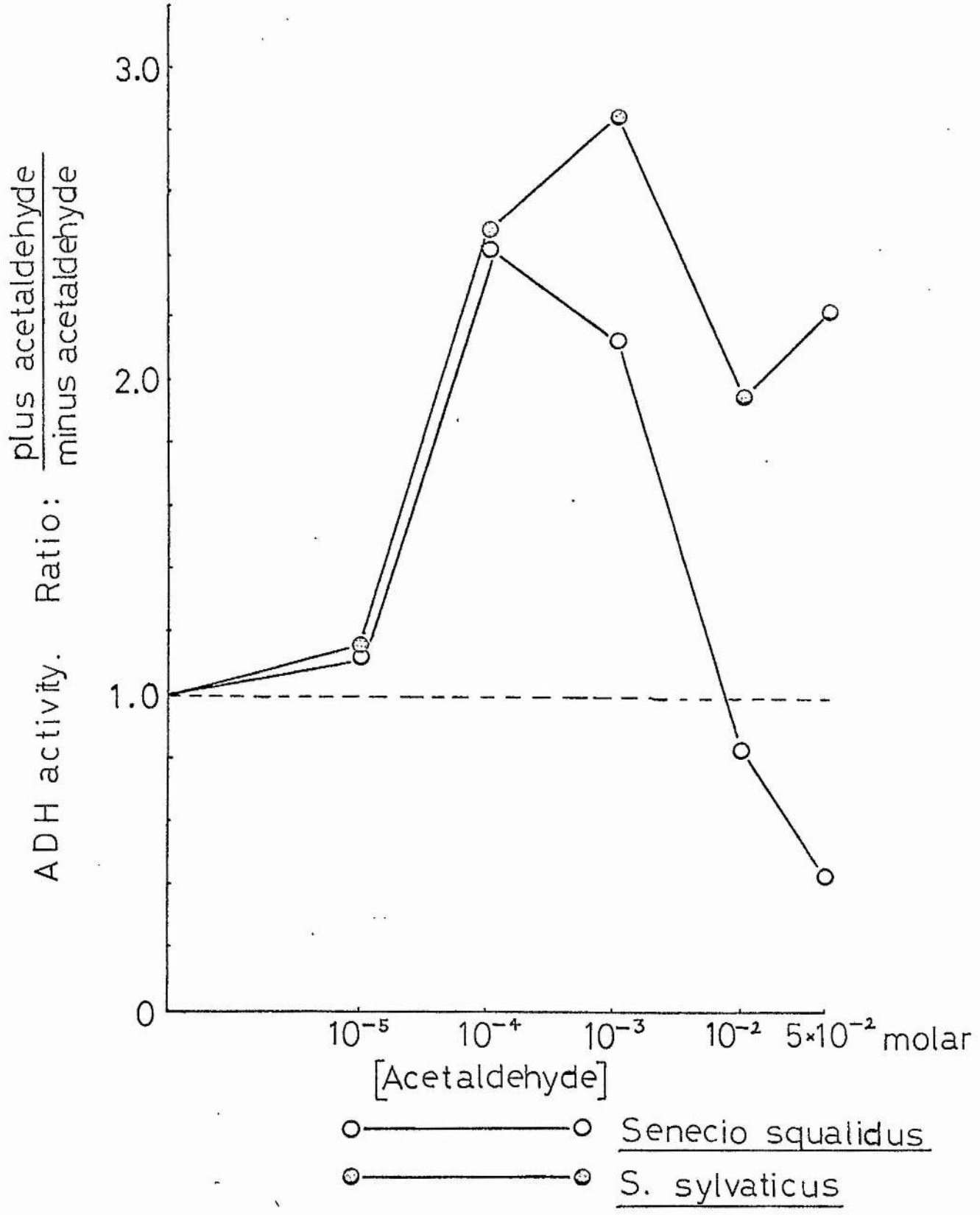
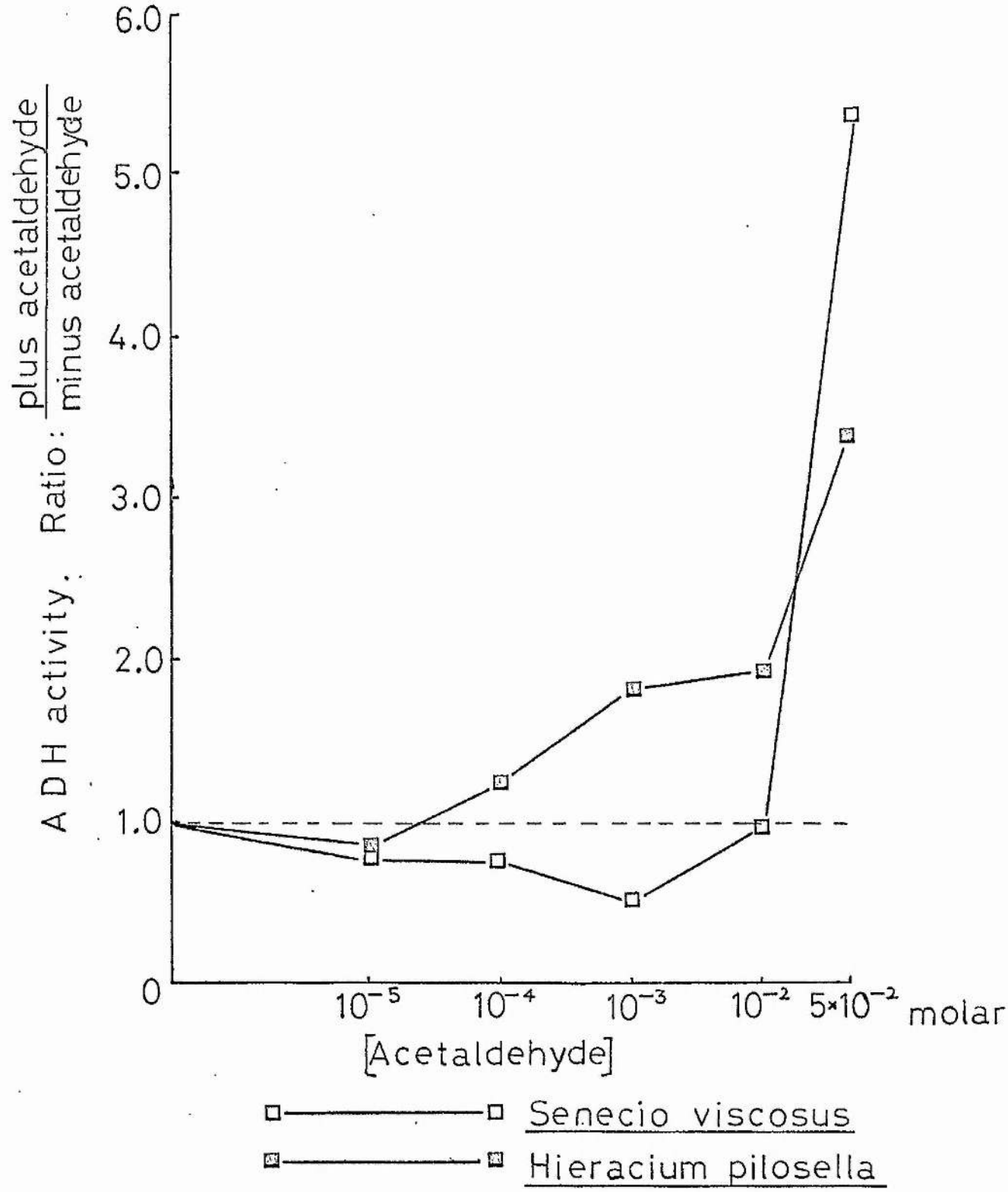

ADH activity. Ratio: plus acetaldehyde / minus acetaldehyde
3.0
2.0
1.0
0
10⁻⁵
10⁻⁴
10⁻³
10⁻²
5×10⁻²
molar
[Acetaldehyde]
Senecio squalidus
S. sylvaticus

FIGURE 7.

ADH activities in the roots of two non-helophytes grown in aerated culture solutions containing different levels of acetaldehyde.

Enzyme activities are expressed as International Units per gram fresh weight of roots.


A D H activity. Ratio: plus acetaldehyde / minus acetaldehyde
6.0
5.0
4.0
3.0
2.0
1.0
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
5×10^{-2}
molar
[Acetaldehyde]
Senecio viscosus
Hieracium pilosella

roots cultured in the high acetaldehyde concentrations (see Table 9), it was felt that a more accurate picture of ADH induction would be given by expressing these results in terms of ADH activity per gram fresh weight instead of per milligram protein. The log scales of the abscissae in Figures 5, 6, 7 should be noted.

The three helophytes used showed little or no ADH induction, while all four non-helophytes showed induction to some degree. These different induction reactions of helophytes and non-helophytes to acetaldehyde are of particular interest when we consider the different induction effects of flooding which have already been established, as it seems to suggest basic differences in the enzyme induction mechanisms of the plants concerned. This, however, does not exclude other causes of differences in behaviour on flooding, such as accumulation of different quantities of acetaldehyde, and the existence of other inductive stimuli.

It is also obvious, from Figures 6 and 7, that the behaviour of the non-helophytes is not at all uniform, and here at least they seem to fall into two categories:

1. ADH is induced up to threefold by acetaldehyde concentrations of 10^{-4}M to 10^{-3}M (Senecio squalidus and S. sylvaticus).

2. ADH is induced up to sixfold by acetaldehyde concentrations of about 5×10^{-2}M (Senecio viscosus and Hieracium pilosella).

Table 9. Protein extracted from the roots of seven species grown in different concentrations of acetaldehyde for 60 hours

(a) NON-HELOPHYTES

(Acetaldehyde)	Senecio squalidus	Senecio sylvaticus	Senecio viscosus	Hieracium pilosella
Nil (control)	34.0	15.5	23.4	33.3
10^{-5}M	28.9	16.2	27.1	33.5
10^{-4}M	30.4	17.3	28.4	35.6
10^{-3}M	31.9	13.9	24.8	34.8
10^{-2}M	19.7	10.7	20.9	26.4
5×10^{-2}M	7.7	7.0	7.6	17.9

(b) HELOPHYTES

(Acetaldehyde)	Phalaris arundinacea	Senecio aquaticus	Ranunculus flammula
Nil (control)	22.4	24.6	14.1
10^{-5}M	30.4	29.9	14.1
10^{-4}M	25.2	26.1	13.8
10^{-3}M	24.2	18.3	14.1
10^{-2}M	17.1	16.3	11.1
5×10^{-2}M	8.2	6.7	4.6

Protein was determined by the method of Murphy and Kies (1960), and is expressed as milligrams per gram fresh weight.

These differences in behaviour are puzzling. There is a considerably greater degree of induction in the present experiments, but otherwise the results for S. viscosus and Hieracium pilosella agree with those of Hageman and Flesher (1960b) and Kollöffel (1968), in that induction did not become maximal until an acetaldehyde concentration of between 0.01M and 0.05M was used. It is unlikely that roots would accumulate such quantities of acetaldehyde naturally, as even in large fruits, when injured, acetaldehyde concentrations seldom exceed 0.005M (Thomas, 1931; Haagen-Smit, Kirchner, Prater, and Deasy, 1945). On the other hand, the acetaldehyde concentrations causing root ADH induction in Senecio squalidus and Senecio sylvaticus could conceivably be accumulated by the roots in flooded conditions, but it has not proved feasible to measure such small quantities of acetaldehyde in the limited root material available. For the same reason any suggestion that differences in permeability of root tissues to acetaldehyde may contribute to the variable behaviour of non-helophytes must remain purely speculative.

The main conclusion to be drawn from these experiments is that acetaldehyde is in fact an inductive stimulus for alcohol dehydrogenase, and that its effectiveness is greater in non-helophytes than in helophytes.

Ethanol Concentrations from 10^{-4}M to molar were used, as

the concentration of ethanol where present in tissues is generally about ten times that of acetaldehyde (Thomas, 1931). The effects upon the root ADH activity of six species (three helophytes, three non-helophytes) are shown in Figures 8 and 9. The results are presented in the same way as those from the acetaldehyde induction experiment.

There is no regular pattern of induction, either with the helophytes or the non-helophytes. In fact the actual forms of the graphs probably have little significance, except for the general fall-off in activity at high ethanol concentrations (earlier in the case of Ammophila), probably a result of the general toxicity of ethanol rather than any specific repressive effect upon ADH. There is a decline in extractable protein per gram fresh weight when the plants are treated with high alcohol concentrations (Table 10). Ability to withstand this ethanol poisoning does not seem to be correlated with the flooding tolerance of the plant. This confirms Crawford's opinion (1966, 1967a) that helophytes avoid the increased rate of glycolysis and accumulation of toxic products consequent upon flooding, rather than exhibiting resistance to poisoning by such products.

Normally in aerobic conditions any ethanol present in roots would be metabolised and thus may even contribute to the growth of the plant (Cossins and Turner, 1962, 1963;

FIGURE 8.

ADH activities in the roots of three non-helophytes grown in aerated culture solutions containing different levels of ethanol.

Enzyme activities are expressed as International Units per gram fresh weight of roots.

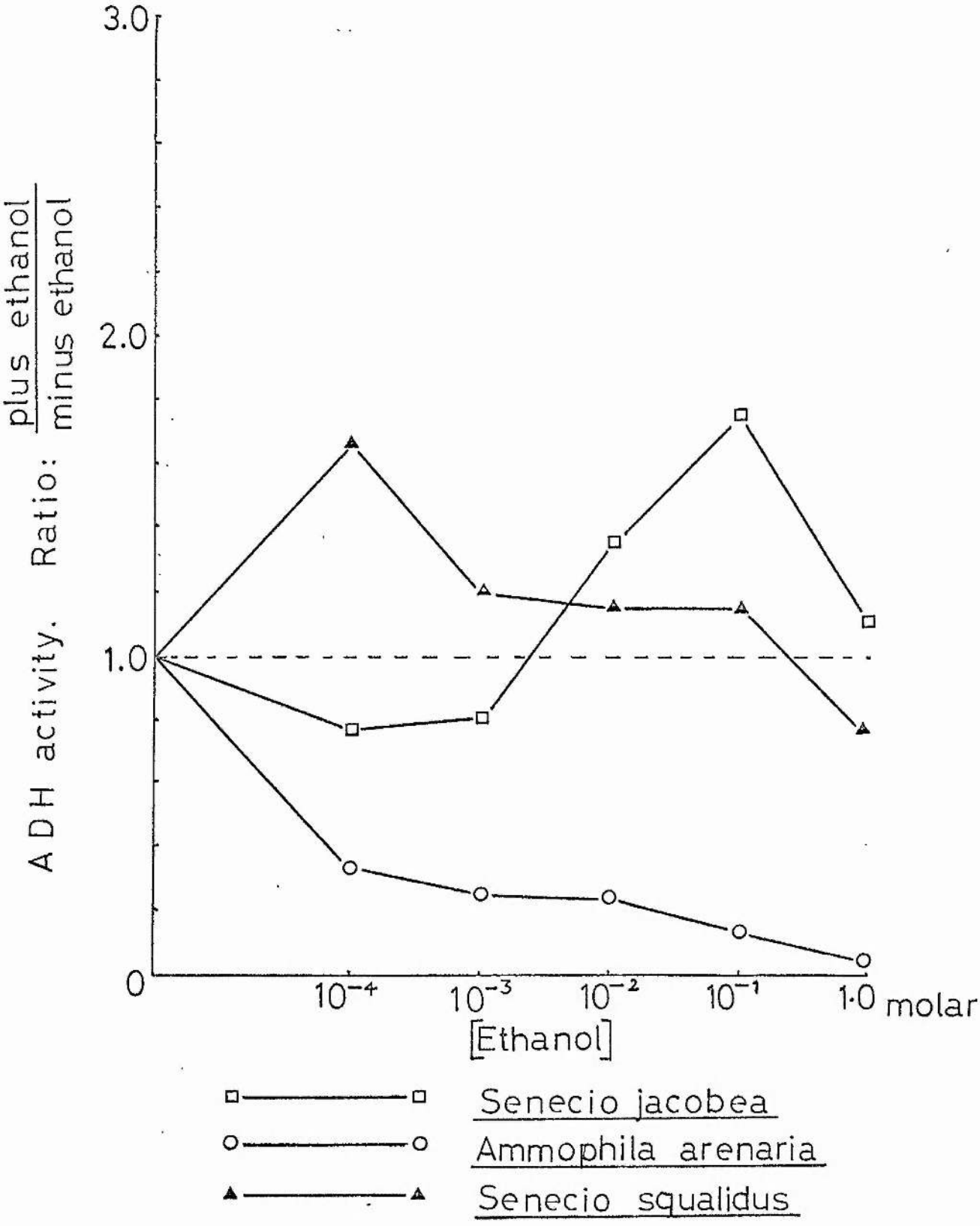

3.0
2.0
1.0
0
ADH activity. Ratio: plus ethanol / minus ethanol
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1·0 molar
[Ethanol]
Senecio jacobea
Ammophila arenaria
Senecio squalidus

FIGURE 9.

ADH activities in the roots of three helophytes grown in aerated culture solutions containing different levels of ethanol.

Enzyme activities are expressed as International Units per gram fresh weight of roots.

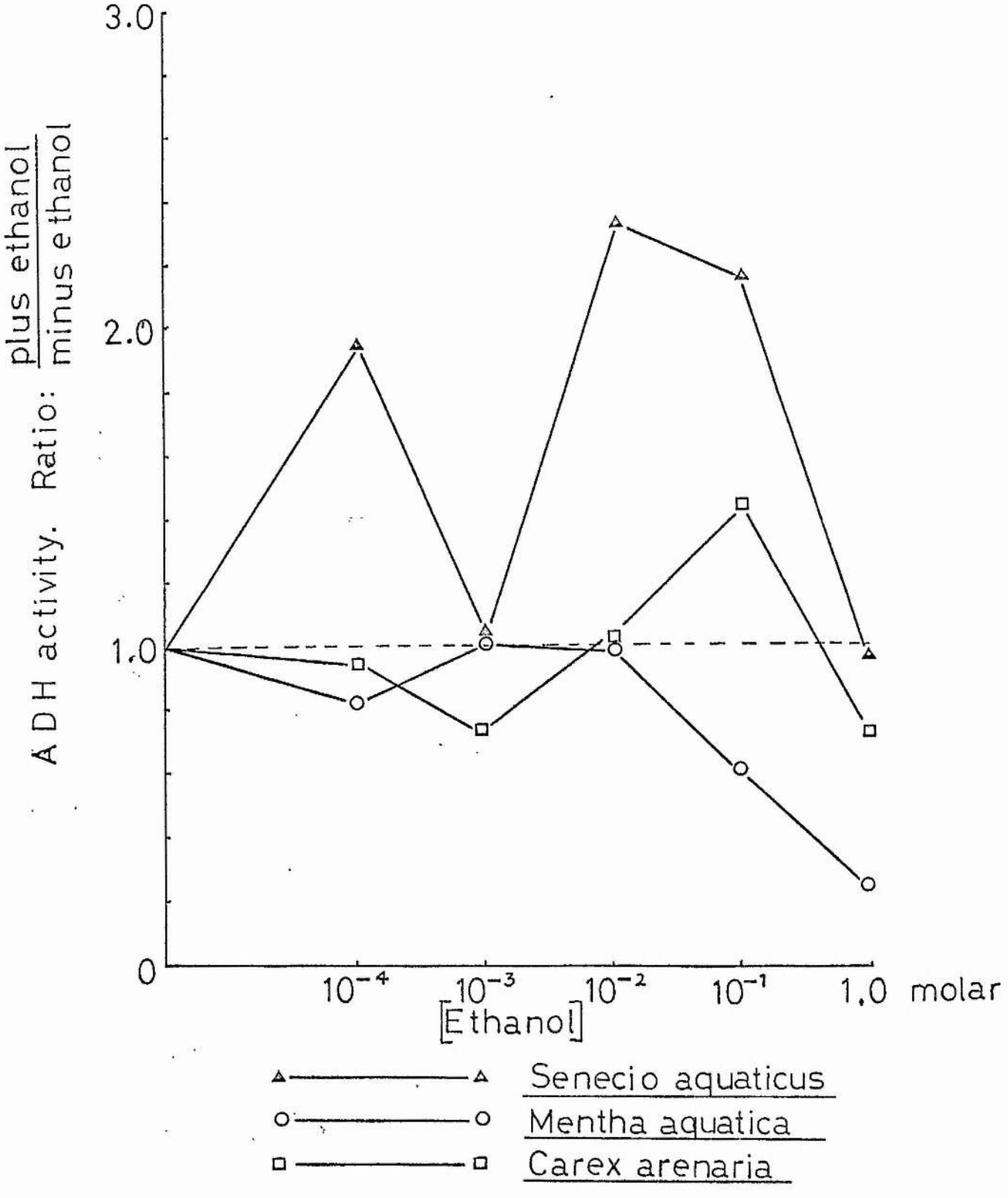

3.0
2.0
1.0
0
ADH activity. Ratio: plus ethanol / minus ethanol
10⁻⁴
10⁻³
10⁻²
10⁻¹
1.0 molar
[Ethanol]
Senecio aquaticus
Mentha aquatica
Carex arenaria

Table 10. Protein extracted from the roots of six species grown in different concentrations of ethanol for 60 hours

(a) NON-HELOPHYTES

(Ethanol)	Senecio jacobea	Ammophila arenaria	Senecio squalidus
Nil (control)	47.7	17.0	34.4
10^{-4}M	47.7	17.3	37.6
10^{-3}M	52.7	21.9	44.4
10^{-2}M	49.9	20.8	35.3
10^{-1}M	44.4	9.4	30.1
Molar	20.5	7.4	17.7

(b) HELOPHYTES

(Ethanol)	Senecio aquaticus	Mentha aquatica	Carex arenaria
Nil (control)	26.0	28.5	20.8
10^{-4}M	31.2	33.9	17.7
10^{-3}M	32.5	30.3	25.9
10^{-2}M	26.5	30.3	18.2
10^{-1}M	22.5	16.2	19.8
Molarr	11.2	11.4	19.9

Protein was estimated by the method of Murphy and Kies (1960) and is expressed as milligramm per gram fresh weight.

Cossins and Beevers, 1963) until it is used up. In the present experiment, although ethanol may be metabolised, it is continually replenished from the reservoir and remains at a constant level, thus simulating in aerobic conditions the accumulation of ethanol in anaerobiosis.

The failure here of ethanol to show any regular inductive effect on ADH agrees with the result of Hageman and Flesher, but not those of App and Meiss or Kollöffel. This may not be particularly significant, as the role and development of ADH in mature plants is probably very different from that in germinating seeds, which were used by all these workers. There are pronounced changes in ADH activity during the germination of pea and corn seeds (Davidson, 1949; Throneberry and Smith, 1955), and such changes may complicate the investigation of the effects of external factors upon ADH activity.

Pyruvate As pyruvate is non-volatile, it was not necessary to adopt the closed cycle of aeration used for ethanol and acetaldehyde. The plants, raised in sand culture, were transferred to water-culture, using Hoagland's solution, in 100 ml Erhlenmeyer flasks covered with aluminium foil to exclude light. Sodium pyruvate was added to the culture solutions to give concentrations of 10^{-4}M, 10^{-3}M, 10^{-2}M, and 5×10^{-2}M pyruvate. A set of control cultures contained

FIGURE 10.

ADH activities in the roots of three helophytes grown in aerated culture solutions containing different levels of pyruvate.

Enzyme activities are expressed as International Units per gram fresh weight of roots.

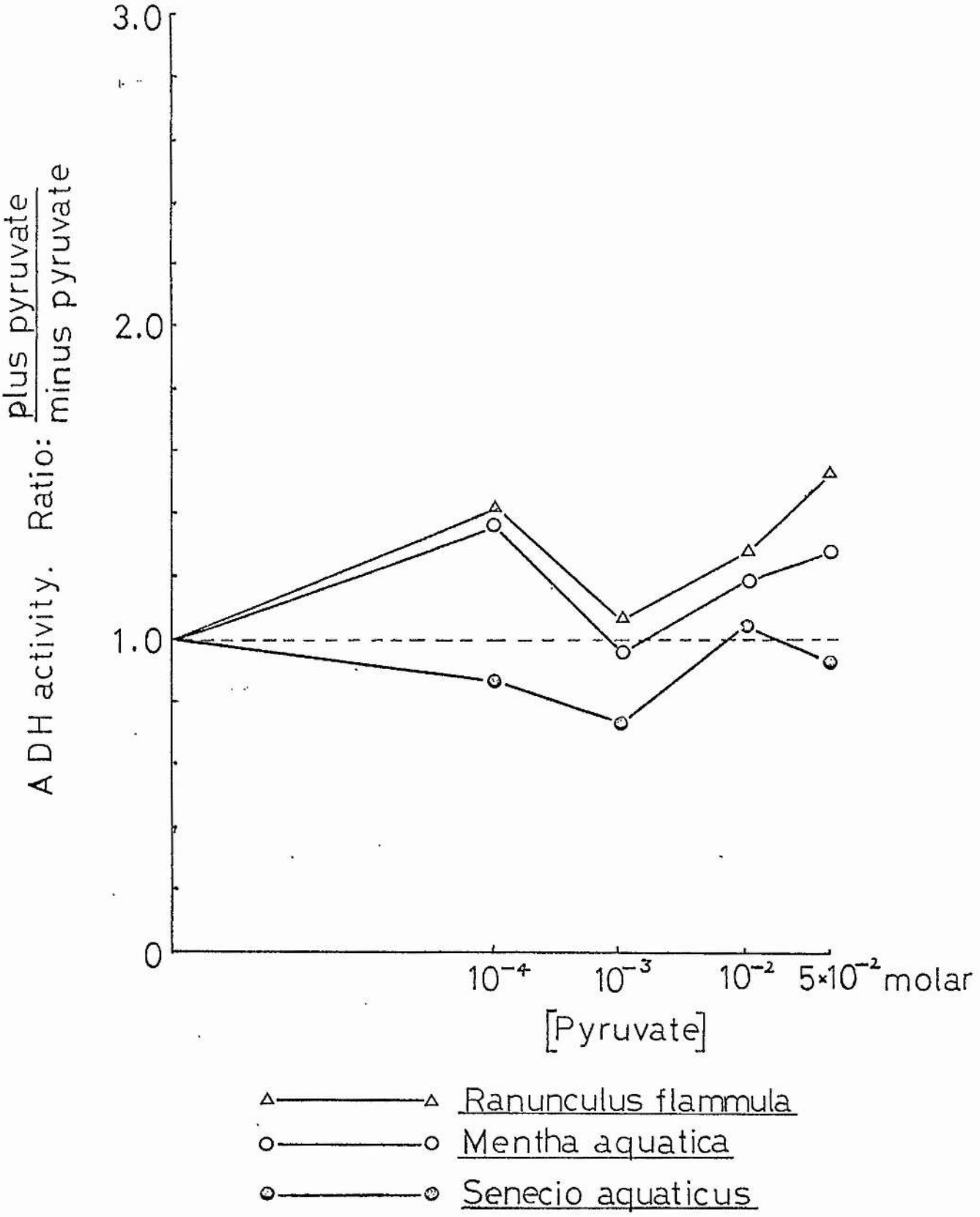
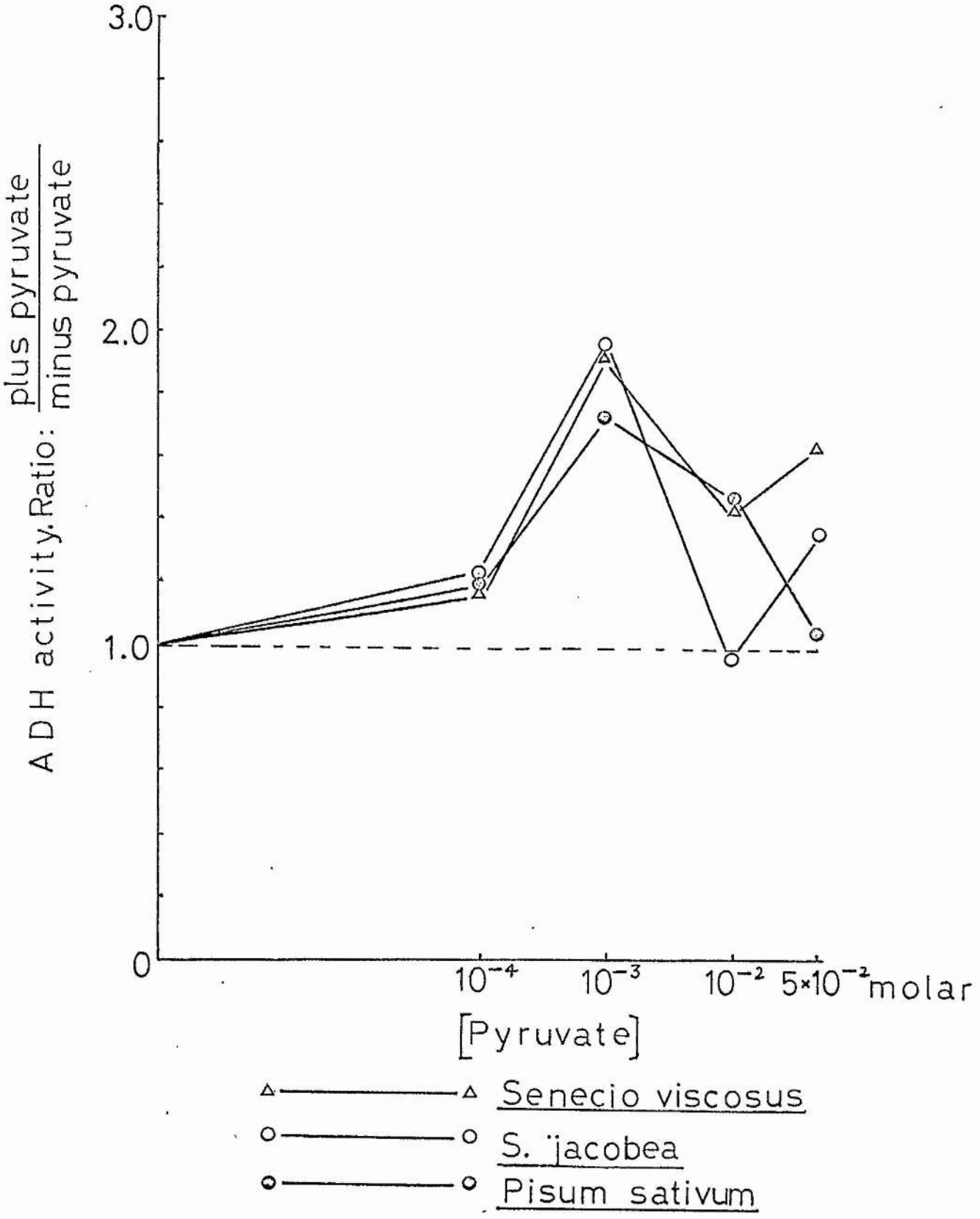

3.0
2.0
1.0
0
ADH activity. Ratio: plus pyruvate / minus pyruvate
10⁻⁴
10⁻³
10⁻²
5×10⁻² molar
[Pyruvate]
Ranunculus flammula
Mentha aquatica
Senecio aquaticus

FIGURE 11.

ADH activities in the roots of three non-helophytes grown in aerated culture solutions containing different levels of pyruvate.

Enzyme activities are expressed as International Units per gram fresh weight of roots.


3.0
2.0
1.0
0
ADH activity. Ratio: plus pyruvate / minus pyruvate
10⁻⁴
10⁻³
10⁻²
5×10⁻² molar
[Pyruvate]
Senecio viscosus
S. jacobea
Pisum sativum

no pyruvate. The solutions were aerated intermittently for 60 hours using an aquarium pump, and the roots were then washed thoroughly in running tap water, then distilled water as previously described, before being homogenised and assayed for ADH activity.

The root ADH activities of three helophytes and three non-helophytes treated in this way are shown in Figures 10 and 11. For ease of comparison, the activities are expressed in the same way as those for acetaldehyde and ethanol, although depression of protein content at high pyruvate concentrations was much less common than at high acetaldehyde or ethanol concentrations. Presumably this is because pyruvate is not toxic in itself, but in certain cases may give rise to the toxic products of anaerobiosis in an aerobic environment, as in this present case. This will be most likely to occur when pyruvate is present in sufficient quantities to saturate the aerobic respiratory pathways (Krebs cycle) and overspill into acetaldehyde and ethanol production. Thus a 'threshold' effect will be present, depending upon the efficiency of operation of the Krebs cycle, and other routes of pyruvate breakdown. High ethanol or acetaldehyde accumulation appears to be toxic to all species (Tables 9 and 10), therefore 'overspill' will cause damage to any plant. Aerobic respiration efficiency

is variable but appears to be unrelated to flooding tolerance (Crawford, 1966). The pyruvate 'threshold' and hence its apparent toxicity, therefore, is also variable but unrelated to flooding tolerance. This is confirmed in Table 11. (Assuming, as previously, that damage is reflected in a reduction of protein content per gram fresh weight of roots.)

Figures 10 and 11 seem to show that there is a definite inductive effect on root ADH by pyruvate at 10^{-3} molar, in non-helophytes but not in helophytes. The forms of the graphs are like those of Figures 5 and 6 (acetaldehyde) but less pronounced. Hageman and Flesher (1960b) suggest that pyruvate is inductive by virtue of its decarboxylation to acetaldehyde, but two facts throw doubt upon a similar interpretation of the present results:

1. Pyruvate at 10^{-3} molar causes induction of Senecio viscosus root ADH, but induction by acetaldehyde is not apparent until concentrations of the order of 5×10^{-2} molar are used.

2. The culture solutions in the present experiment, unlike those of Hageman and Flesher, were actively aerated, thus facilitating the rapid removal of the volatile acetaldehyde. Whether or not this actually occurred is not known.

Whether pyruvate acts as a direct inductive stimulus for ADH therefore remains doubtful. Its inductive effects

Table 11. Protein extracted from the roots of six species grown in different concentrations of pyruvate for 60 hours

(a) NON-HELOPHYTES

(Pyruvate)	Senecio viscosus	Senecio* jacobea	Pisum sativum
Nil (control)	44.5	28.0	18.2
10^{-4}M	44.0	34.2	18.0
10^{-3}M	47.5	37.1	18.7
10^{-2}M	43.0	36.9	9.5
5×10^{-2}M	41.5	42.5	6.5

(b) HELOPHYTES

(Pyruvate)	Ranunculus flammula	Mentha aquatica	Senecio aquaticus
Nil (control)	20.9	32.2	24.1
10^{-4}M	21.0	28.7	44.9
10^{-3}M	17.0	29.0	36.2
10^{-2}M	10.4	20.2	20.2
5×10^{-2}M	7.6	20.2	20.8

Protein was estimated by the method of Murphy and Kies (1960), except for * S. jacobea, where the biuret method was used (Robinson and Hogden, 1940). The protein is expressed as milligrams per gram fresh weight.

in practice, however, are apparent, and the different behaviour of helophytes and non-helophytes in this case gives another pointer to the means whereby helophytes avoid damage caused by accelerated glycolysis on flooding.

x x x

(iv) Kinetic Studies

During the course of routine ADH assays using the assay method of Crawford (1967a), in which 40 µmoles acetaldehyde are added to each experimental cuvette on the assumption (justified on previously published data) that this saturates the enzyme, it was noticed that the enzyme activity seemed to vary with varying amounts of acetaldehyde around this figure, up to the addition of about 150 µmoles acetaldehyde per cuvette. (For details of the assay system, see Appendix A). Extracts of helophyte roots in particular needed high concentrations of acetaldehyde to produce maximum reaction velocity, whereas non-helophyte ADH appeared to be saturated at much lower concentrations, especially after ADH induction by the various means which have been outlined.

It was decided therefore to conduct an investigation into the variation of reaction velocity with acetaldehyde concentration in crude ADH extracts from the roots of various species grown under flooded and unflooded conditions.

Eight species (four helophytes, four non-helophytes) were grown in sand culture for one month in flooded and unflooded conditions as previously described in Part I. Crude extracts were then made of the roots in the normal way, and assayed for ADH activity as outlined in Appendix A.

The variation in reaction velocity with acetaldehyde concentration was then determined using the same assay system but substituting appropriate amounts of M/10 and M/100 acetaldehyde solutions to give final substrate concentrations varying from M/30 to M/5000. Each series of velocity measurements was carried out on two replicate (different plant) extracts.

The reciprocals of the reaction velocity and the substrate concentration were calculated in each case and plotted against each other to give the well-known Lineweaver-Burk plot (Dixon and Webb, 1958), which in uncomplicated cases gives a straight line cutting the base-line at -1/Km (See Figure 12), where Km is the Michaelis constant of the enzyme with respect to a particular substrate. (The Michaelis constant, following the practice of Dixon and Webb, is defined as the substrate concentration giving half maximal velocity.)

Preliminary experiments using a commercial preparation of yeast ADH, obtained from Boehringer (London), and diluted in 10% bovine serum albumin, gave a Km value for ADH with respect to acetaldehyde of 6.3×10^{-4}M. This is rather greater than the value of 1.1×10^{-4}M given by Hayes and Velick (1954), but Nygaard and Theorell (1955) give values of up to 5.5×10^{-4}M. Preliminary experiments were also

FIGURE 12.

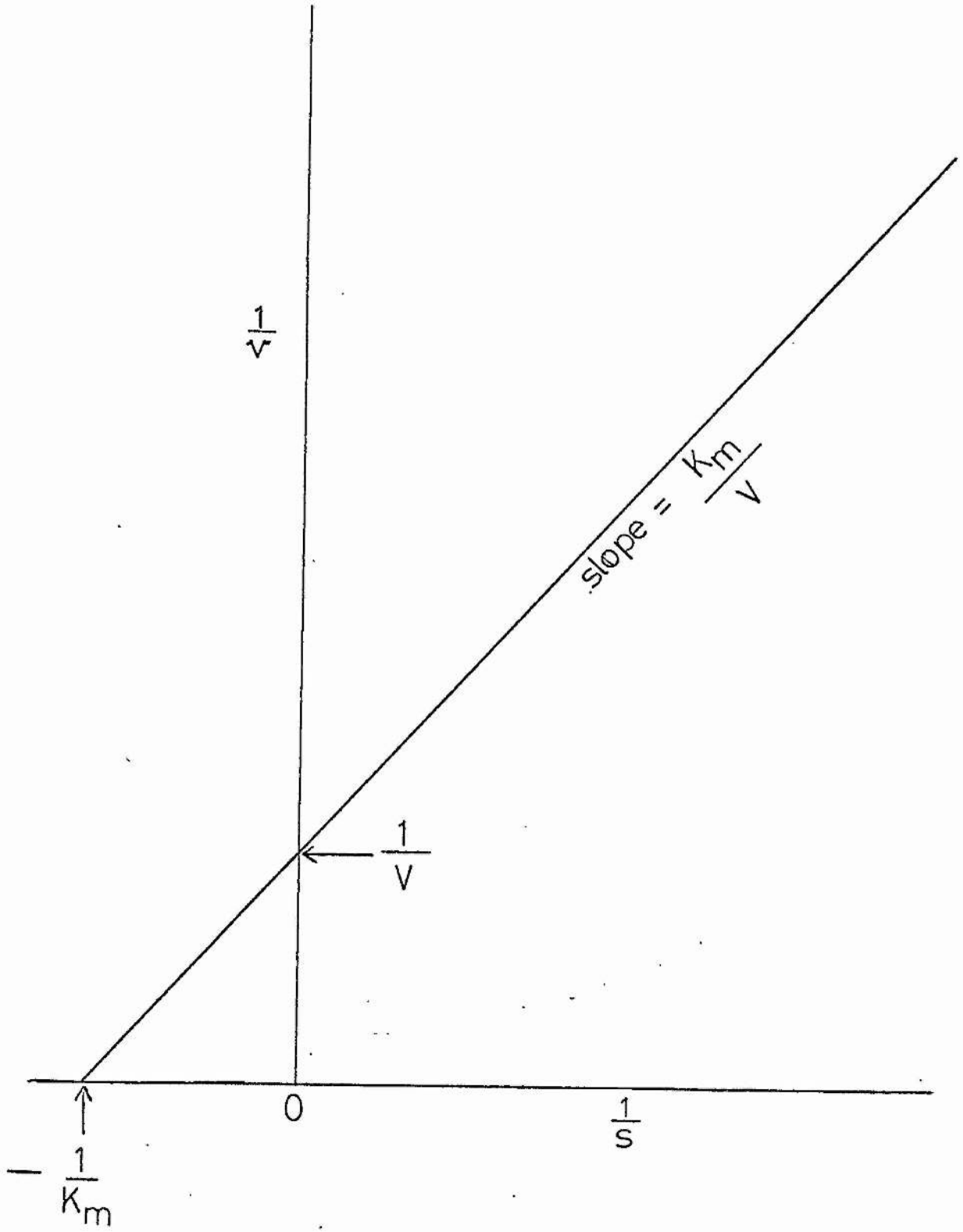
The Lineweaver-Burk Plot.

v = reaction velocity

V = maximum reaction velocity

s = substrate concentration

Km = Michaelis Constant


$\frac{1}{v}$
slope = $\frac{K_m}{V}$
$\frac{1}{V}$
0
$\frac{1}{s}$
$-\frac{1}{K_m}$

performed on pea seedling root extracts, and these gave a Km value of 5.8×10^{-3} M acetaldehyde. The Lineweaver-Burk plots of the yeast and pea root enzymes are presented in Figure 13. Reaction velocities were expressed in terms of the change in extinction at 366nm per minute per unit volume of root extract (See Appendix A), but the actual units are in this case immaterial.

Table 12 gives the apparent Km values for ADH with respect to acetaldehyde using crude extracts of the roots of eight experimental species.

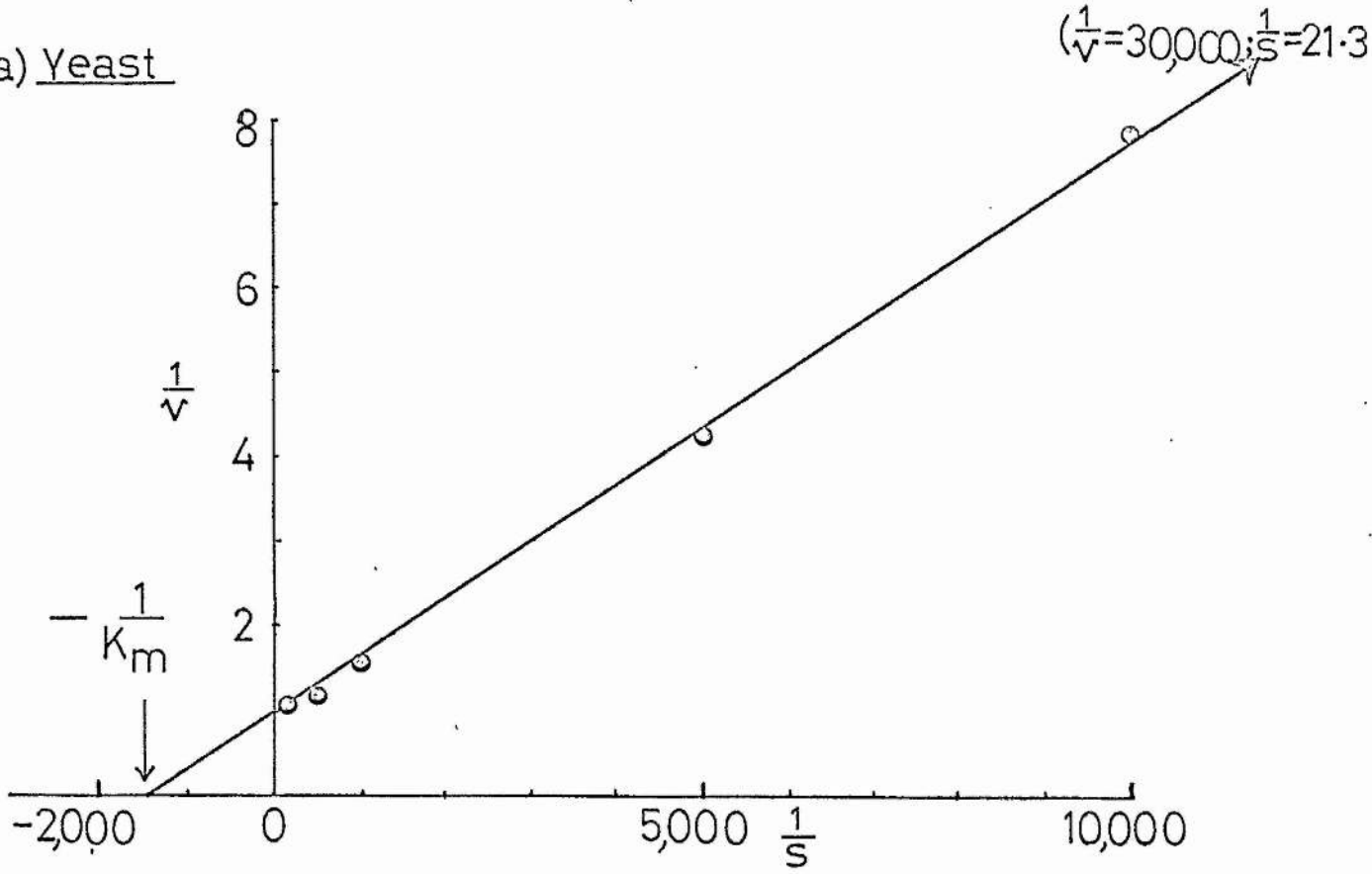
The most striking point about these figures is the marked fall in apparent Km value on flooding of the non-helophytes. While it is recognised that Km values do not give a measure of the affinity of an enzyme for a substrate except in the simplest cases, nevertheless they do indicate how easily an enzyme-catalysed reaction will take place: the smaller the Km, the faster will the reaction proceed at most substrate concentrations.

Caution must be exercised when discussing apparent Km values derived from crude tissue extracts, but it does seem that these results reveal yet another reason for the increased glycolytic rate apparent on the flooding of non-helophytes: if the Km of the last enzyme in the glycolytic chain falls, then equilibria will be shifted throughout the

FIGURE 13.

Lineweaver-Burk plots for ADH with respect to acetaldehyde.

(a) Yeast



(b) Pea seedling root

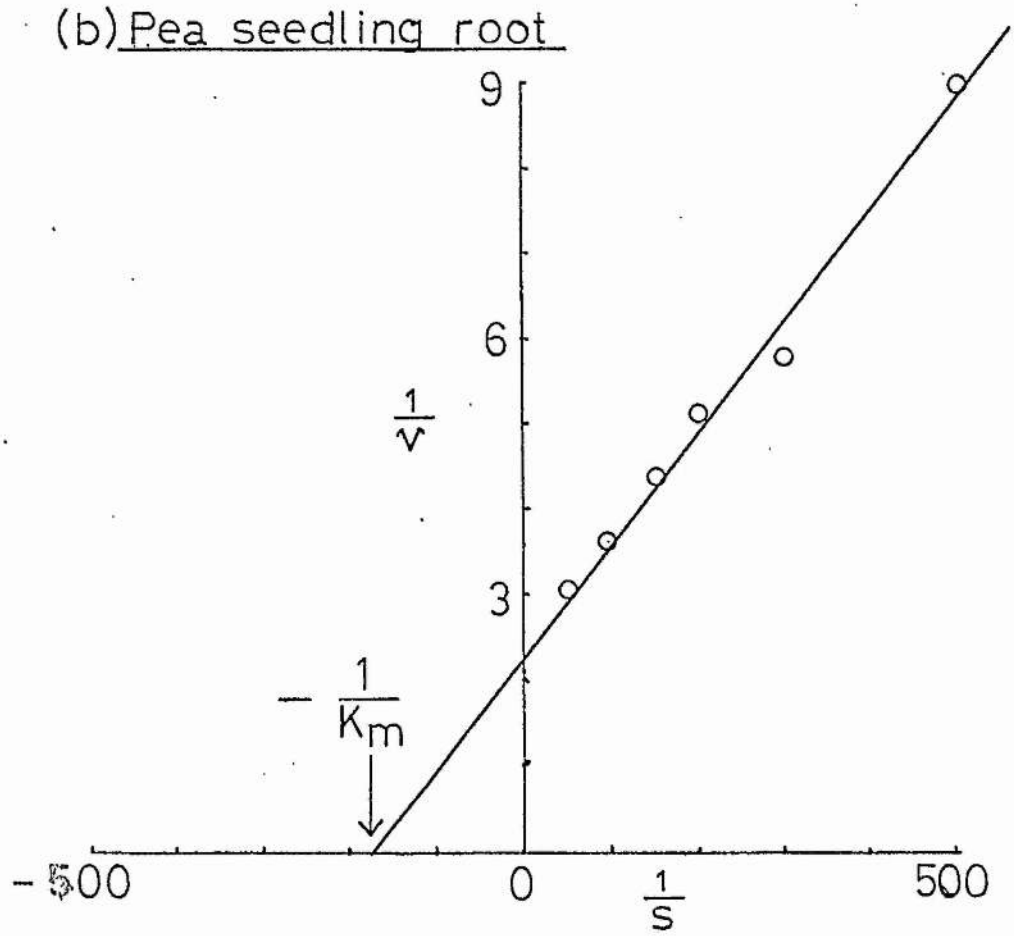


Table 12. Apparent values of Km, with respect to acetaldehyde, of the root ADH of eight species grown under flooded and unflooded conditions for one month.

	Apparent Km values (x 10^{-3}M acetaldehyde)		
HELOPHYTES	Unflooded	Flooded	Unfl./Fl.
Senecio aquaticus	21	16	1.3
Caltha palustris	7.6	18	0.4
Mentha aquatica	c.55	c.55	1.0
Ranunculus flammula	33	26	1.3
NON-HELOPHYTES			
Senecio jacobea	3.5	1.2	2.9
Hieracium pilosella	13.7	3.0	4.6
Senecio viscosus	12.0	1.0	12.0
Pisum sativum	27	1.1	24.5

sequence, and the whole process will be accelerated. Helophytes show no such fall in apparent Km value, and therefore are more resistant to increases in glycolysis on flooding.

There are at least three possible reasons for the change in apparent Km of ADH for acetaldehyde on the flooding of non-helophytes:

1. The appearance on flooding of an enzyme activator. Many enzymes are activated by metallic cations, by anions, or by their substrates. There is some evidence for the activation of yeast ADH by high ethanol concentrations (Sund and Theorell, 1963) which has been explained on the basis of changes in charge distribution on the enzyme surface, but it is obviously impossible to check this possibility without extensive purification.

2. The disappearance on flooding of an enzyme inhibitor otherwise permanently present. This seems inherently unlikely, as it would necessarily have to be present most of the time in nearly every plant species. There have been no reports of such a substance. If it existed, it would be of a competitive type, because of the effect on the apparent Km (Dixon and Webb, 1958).

3. The appearance or increase on flooding of a separate enzyme form with a lower Km for acetaldehyde. One feature

of the kinetic investigations which lent credence to this idea of isoenzyme induction was the form of some of the Lineweaver-Burk plots. Contrary to expectation, these plots were not linear. The form in fact was often reminiscent of the form of graph obtained when two enzymes with different Michaelis constants are acting upon the same substrate (Figure 14). In this graph, the 'apparent' value for $-1/Km$ lies somewhere between the two actual values.

Some of the Lineweaver-Burk plots obtained during the present experiments are presented in Figures 15, 16, 17, and 18. In each case the straight-line extrapolation to the base-line was used to give the results in Table 12. The curved extrapolations should cut the base-line at $-1/K_1$, where K_1 is the higher of the Michaelis constants involved (if indeed only two are involved). The form of the curve is common to both helophytes and non-helophytes, indication that isoenzymes of ADH may be a factor of both.

Such non-linear plots are difficult to interpret, and this explanation is only one of several. Nygaard and Theorell (1955) found similar non-linear plots using ethanol, and invoked a completely different explanation. The speculative nature of such reasoning is not denied, especially when using unpurified enzymes. No further inferences will be drawn here. Nevertheless it was these features

FIGURE 14.

Form of the Lineweaver-Burk plot when two enzymes act upon the same substrate.

(Modified from Dixon and Webb, 1958)

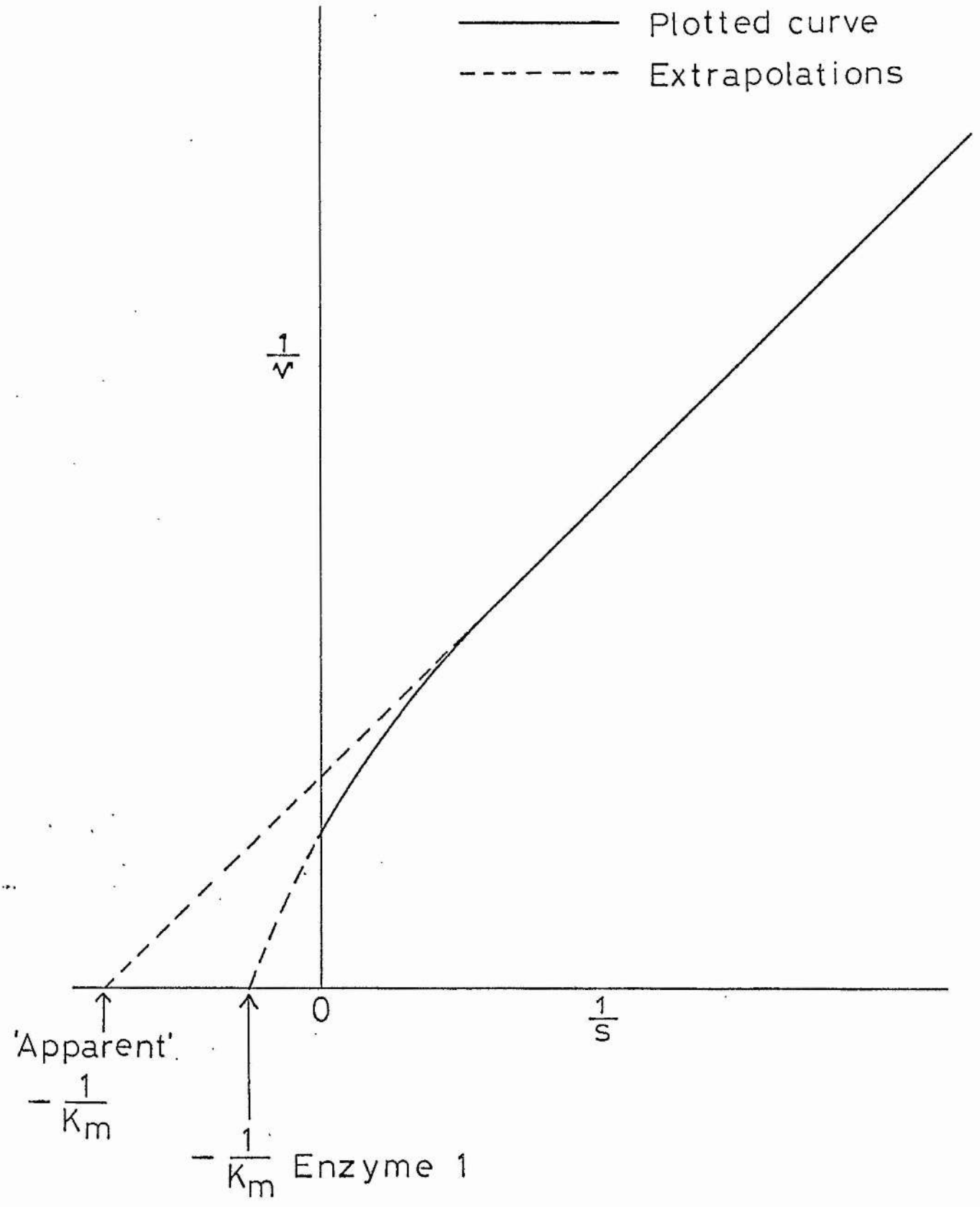

Plotted curve
Extrapolations
1/v
0
1/S
'Apparent'
-1/Km
-1/Km Enzyme 1

FIGURE 15.

Lineweaver-Burk plot of ADH with respect to acetaldehyde, using Ranunculus flammula, flooded one month.

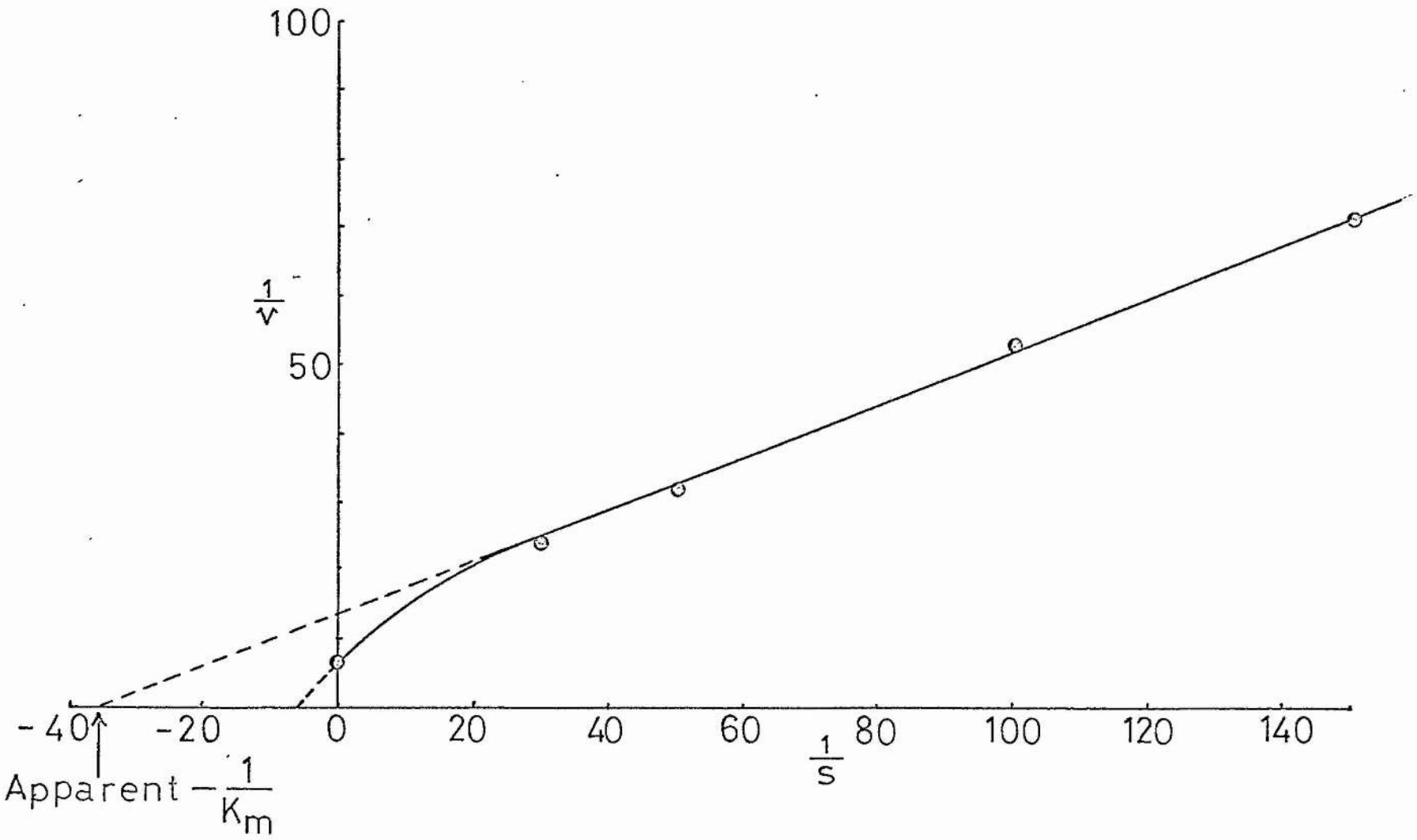

100
1/v
50
-40
-20
0
20
40
60
80
100
120
140
1/s
Apparent −1/Km

FIGURE 16.

Lineweaver-Burk plot of ADH with respect to acetaldehyde, using *Caltha palustris*, flooded one month.

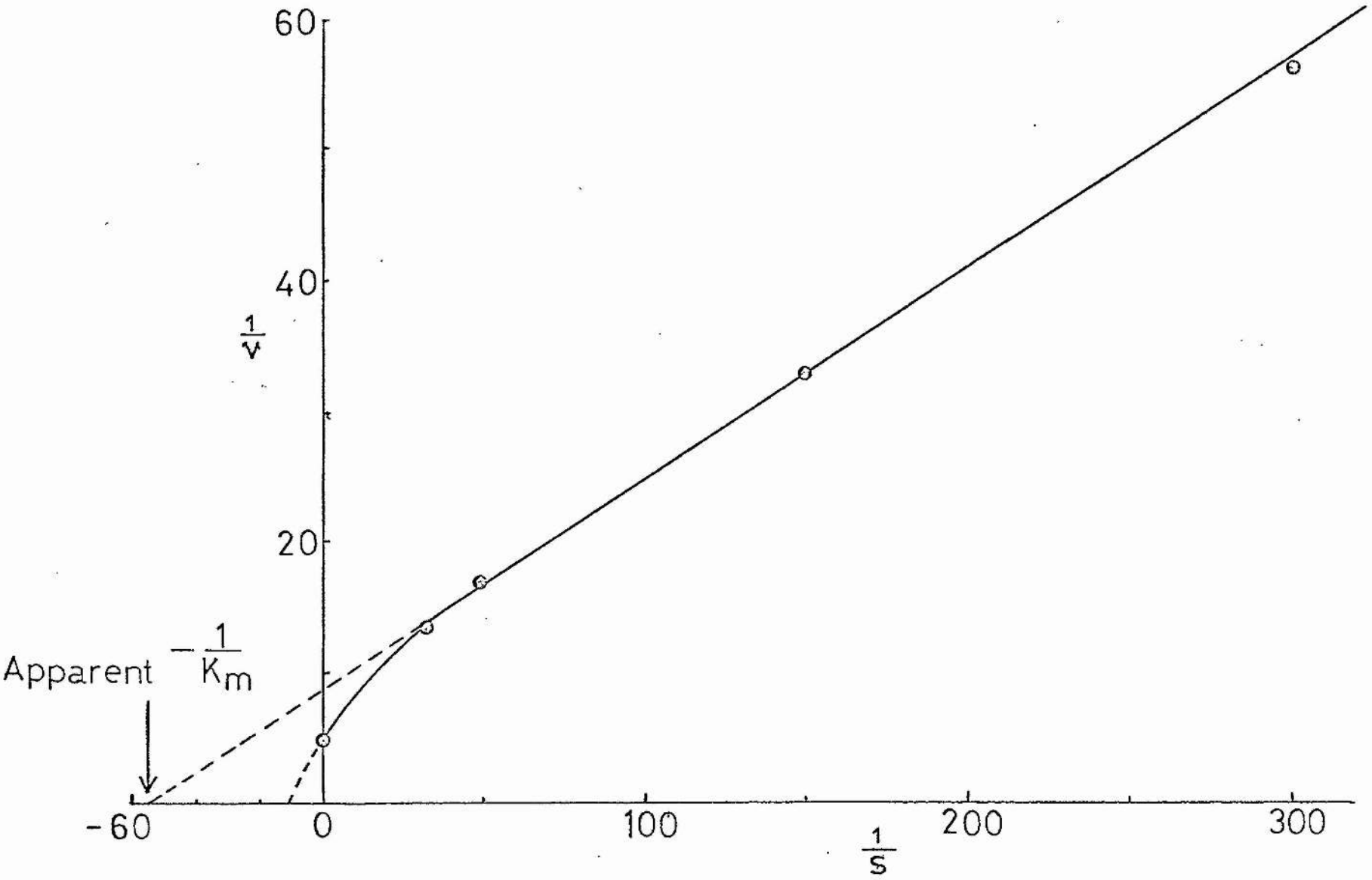

60
40
20
1/V
Apparent −1/Km
-60
0
100
200
300
1/S

FIGURE 17.

Lineweaver-Burk plot of ADH with respect to acetaldehyde, using *Senecio viscosus*, flooded one month.

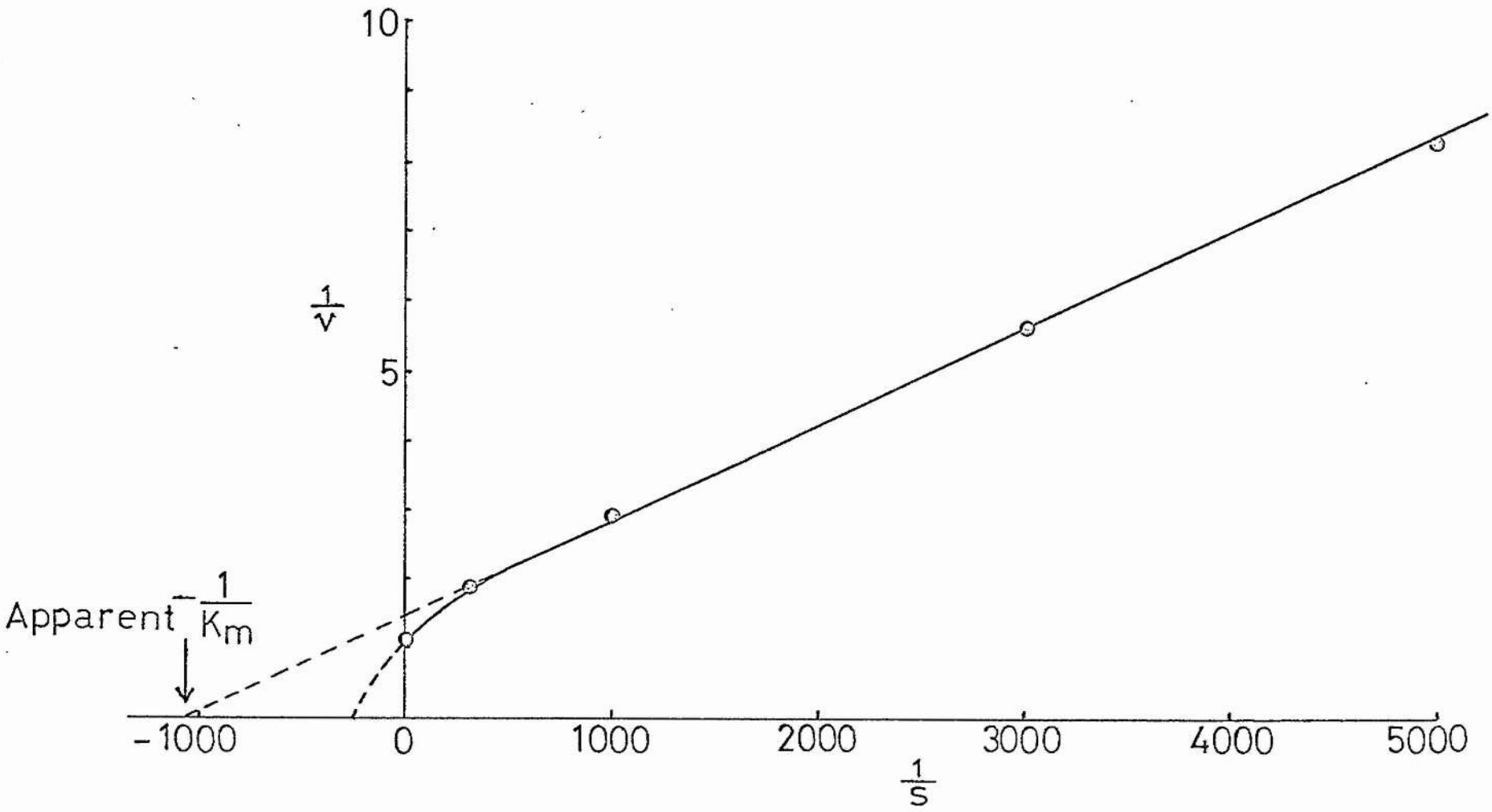
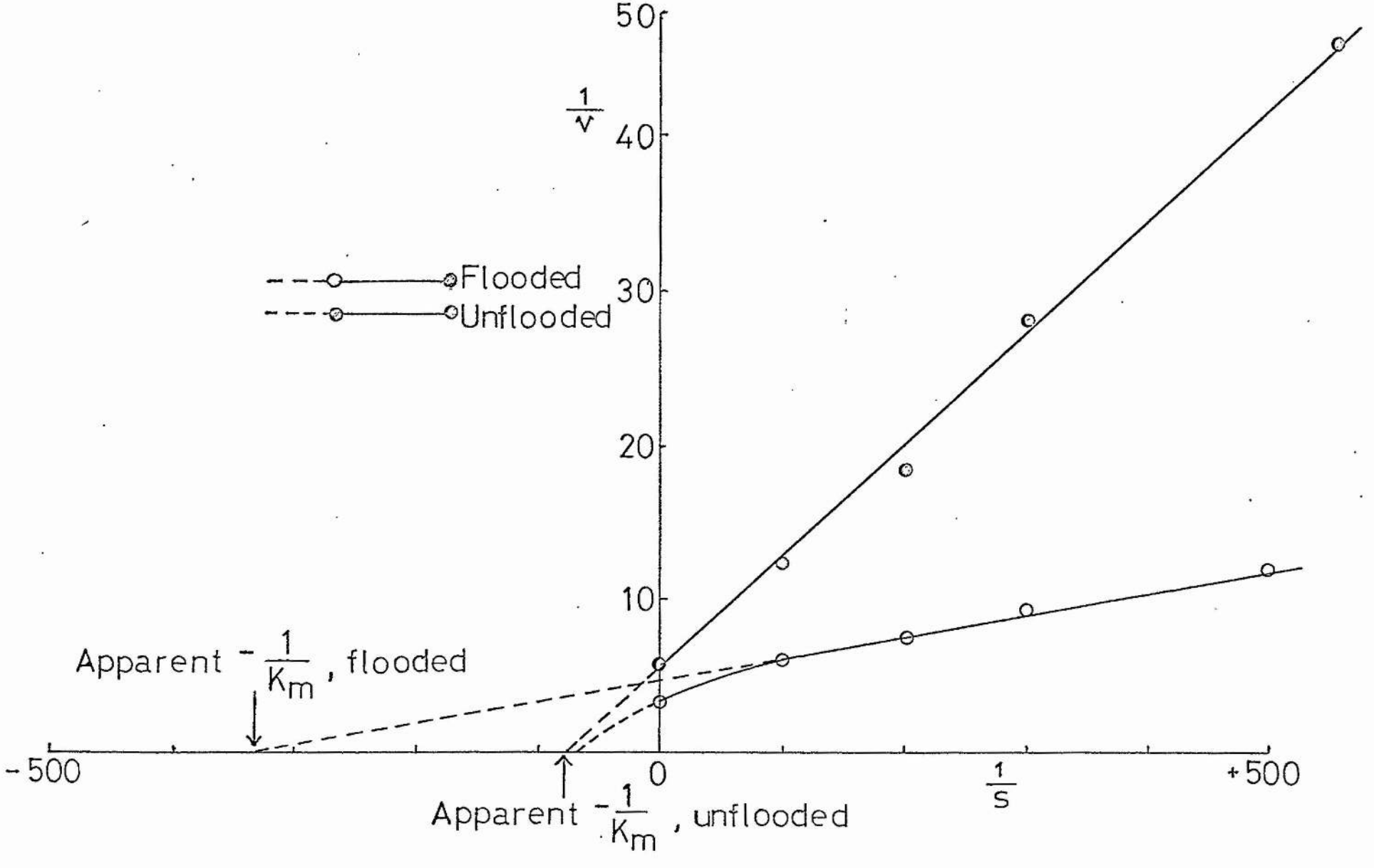

10
5
$\frac{1}{v}$
Apparent $-\frac{1}{Km}$
-1000
0
1000
2000
3000
4000
5000
$\frac{1}{S}$

FIGURE 18.

Lineweaver-Burk plot of ADH with respect to acetaldehyde, using *Hieracium pilosella*, flooded and unflooded.


50
40
30
20
10
1/v
Flooded
Unflooded
Apparent -1/Km, flooded
-500
0
+500
1/s
Apparent -1/Km, unflooded

which prompted the investigation of isoenzyme induction which forms the subject of the next section.

x x x

(v) Isoenzyme Studies

In recent years many dehydrogenases and other enzymes have been found to consist of multiple molecular forms (isoenzymes) in the same tissue. (For reviews of the subject see Wilkinson, 1965; Shannon, 1968. The latter deals only with plant isoenzymes.) Not until even more recently has the existence of ADH isoenzymes become established. Purified human liver ADH has yielded up to three fractions (Wartburg, Bethune, and Vallee, 1964), while unpurified enzyme from the same source gave five bands (separated by gel electrophoresis) appearing successively during foetal and infant development (Pikkarainen and Räihä, 1969). Pietrusko's group (1969), in a detailed study of horse liver ADH, found nine isoenzymes and suggest that they may be composed of different combinations of subunit species bearing active sites not equivalent in substrate-binding (and hence kinetic) properties.

So far, plant ADH has been resolved into isoenzymes only in maize, where it was studied on a genetic basis. Two sets of isoenzymes are distinguished on a basis of electrophoretic mobility and intensity of staining (Scandalios, 1966; Schwartz and Endo, 1966; Schwartz, 1969). It is suggested that the enzyme is a dimer, as hybrid enzymes are found in heterozygotes. Schwartz therefore postulates control by

two ADH genes.

Macko, Honold and Stahmann (1967) subjected a wide variety of enzymes from wheat seedlings to polyacrylamide gel electrophoresis, and only ADH persistently gave a single band in the gels.

Experimental

Six species (three helophytes and three non-helophytes) were grown for periods of up to one month in flooded and unflooded sand culture as described in Part I. The roots were then washed thoroughly in tap water and distilled water, blotted, weighed, and homogenised, using a chilled mortar and pestle, in cold Tris-HCl buffer, 0.1 M, pH 8.2, containing 10 % (weight/volume) sucrose, 0.006 M ascorbic acid and 0.006 M cysteine according to Staples and Stahmann (1964). In order to avoid loss of enzyme activity by the use of concentration procedures, these extracts were made as strong as possible, subject to the efficient separation of the liquid phase by centrifugation. Usually 1 ml of extracting medium was added per gram fresh weight of root tissue. The homogenates were centrifuged at 15,000 g for 20 minutes at 4°C before being subjected to polyacrylamide gel electrophoresis ('disc' electrophoresis) by the method of Davis (1964) as modified by Tombs and Akroyd(1967). In this method the 'sample' and 'spacer' gels of Davis are

omitted, and the sample, made more dense by the addition of sucrose during extraction, is layered directly onto the separating gel. Sample volumes of 20 to 100 µlitres per gel were used, containing 400 to 2000 µgrams total protein, as determined by the method of Murphy and Kies (1960).

Experiments with various strengths of gels showed that a gel containing 7.5 % acrylamide gave the best results with ADH. This was polymerised chemically, using dimethyl-amino-proprionitrile (DMAP) as an accelerator (Tombs and Akroyd, 1967).

Various buffer systems were used; the most satisfactory was found to be the following:

Gel buffer: Tris-HCl, 0.1 M, pH 8.2.

Tank buffer: 28.8 g glycine + 6.0 g Tris, dissolved in 1 litre of distilled water. This gives a solution of pH 8.3.

Addition of bromophenol blue as a marker for the electrophoretic front was rendered unnecessary by the presence of noticeable amount of oxidised polyphenols (despite the presence of ascorbic acid and cysteine in the extraction medium) forming a narrow brown or yellow band migrating with or just behind the electrophoretic front.

Electrophoresis was performed in a refrigerator at approximately 4° C. The current was maintained at 1 mA

per gel tube until the samples had entered the gels, to prevent loss of the samples by convection, and then raised to 3 mA per gel tube. Use of a constant current supply caused heating of the gels as the voltage rose during the course of a separation, therefore a constant voltage supply was used. The current fell to about 1mA per gel tube as separation proceeded. Electrophoresis was continued until the polyphenol bands had progressed to a suitable point in the gels.

The gel rods were then extracted from the tubes and placed in specimen tubes containing the following staining solution, specific for ADH. This is modified from Fine and Costello (1963) by increasing the ethanol concentration tenfold:

Tris-HCl, 0.1 M, pH 8.2	-	25 ml
NAD	-	18 mg
Nitro-blue tetrazolium	-	5 mg
Phenazine methosulphate	-	1 mg
Ethanol	-	0.5 ml

Sites of tetrazolium reduction are indicated in the gels by the deposition of the deep blue insoluble formazan.

At least one of the eight replicate gels obtained from each root extract was placed in this staining solution minus ethanol, to identify any staining not due to alcohol

dehydrogenase activity, such as the 'nothing dehydrogenases' mentioned by Wilkinson (1965) and the reduction of the NBT by the ascorbic acid and cysteine present in the extraction medium (Macko, Honold, and Stahmann, 1967). In fact only the latter phenomenon was observed, and not in every case.

The specimen tubes containing the gels in the staining solution were wrapped in aluminium foil to exclude light and shaken on a flask shaker at room temperature. Other staining procedures recommend a temperature of 37°C, but much of this work has been done with mammalian tissues.

Most of the literature states specifically that staining for dehydrogenases is complete in one to two hours. Only Schwartz and Endo (1966), using maize, found minor isoenzymes appearing after prolonged staining. In the present experiments there was often no staining visible after two hours, and the gels were therefore left in the staining solution for 24 hours, after which time it was assumed that no further staining would occur. Sometimes staining did not become apparent until after ten hours.

On removal from the stain, the gels were washed and stored in distilled water in the dark; the stained bands did not fade when kept for several weeks in these conditions, although the background tended to darken. The results were recorded in three ways:

1. By diagrammatic representation.

2. By recording densitometer. A Joyce-Loebl Chromoscan was used, with a red filter.

3. By photography, from a distance of 8 inches using an Edixa single lens Reflex camera and 'Ilford' Pan F (fine grain) film. The gels, in tubes of water, were subjected to strong diffuse background lighting from an 'Ilford' negative viewer.

Although most subjective, the first method was most satisfactory; visual inspection gave the clearest indication of the number and position of the bands. Finer points disappeared in photographs and densitometric scans. The latter, though giving a quantitatively accurate picture of the degree of staining, do not necessarily reflect the level of enzyme activity. The long periods of staining employed will exaggerate the importance of the lesser isoenzymes.

The results are depicted in Figures 19 to 30 and Plates III to VIII. In each case the gel marked 'A' has been stained with the complete staining mixture for ADH. That marked 'B' has been stained with a mixture omitting the ethanol, and any staining in the latter is due to the extraneous reactions already mentioned. For purposes of comparison, the diagrams and densitometric tracings of the gels are adjusted in scale such that the electrophoretic

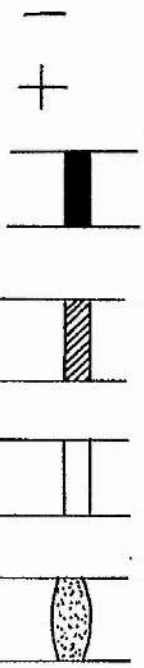
fronts (as identified by the polyphenol bands always immediately behind) are always the same distance from the origin. Because of slight variations in running conditions, this was not always the case in the actual gels. The photographs are all to the same scale.

The results for individual species are discussed separately, in conjunction with the figures and plates.

x x x

Key to Figures 19 to 30

Gel diagrams



— cathode (origin)

\+ anode

strong formazan staining

moderate formazan staining

weak formazan staining

band of oxidised polyphenols

Densitometer tracings

Vertical axis - Optical Density (arbitrary units)

Horizontal axis - length of gel

Senecio sylvaticus (Figures 19 and 20. Plate III)

Non-helophyte.

Before flooding, one band of ADH is visible in the gels, immediately behind the yellowish-brown band of polyphenols, at R.f. 0.98. On flooding this band increases in intensity and two additional ones appear, at R.f.'s 0.86 and 0.69. These two isoenzymes may have been present in the unflooded roots in quantities too small to be detected, or may have been newly synthesised on flooding.

In this case there appeared to be no reduction of the nitro-blue tetrazolium by the ascorbic acid at the electrophoretic front.

x x x

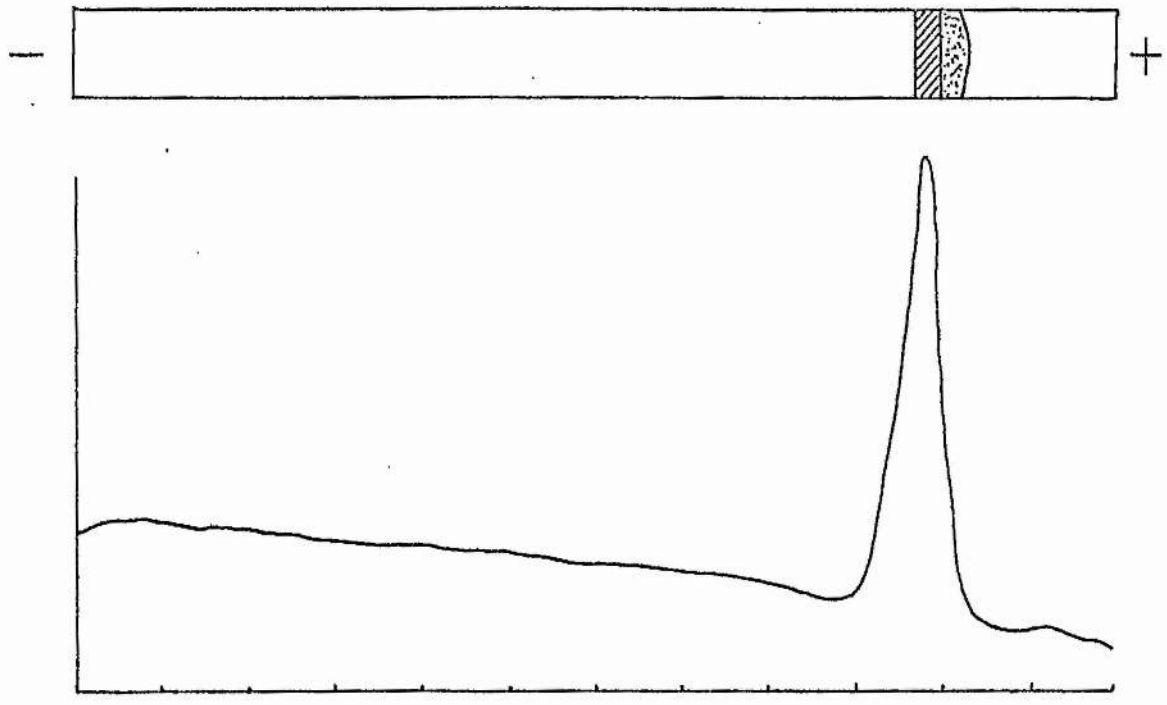
FIGURE 19.

ADH isoenzymes in the roots of Senecio sylvaticus, unflooded.

(A) Full staining mixture.

(B) Staining mixture minus ethanol.

(A)



(B)

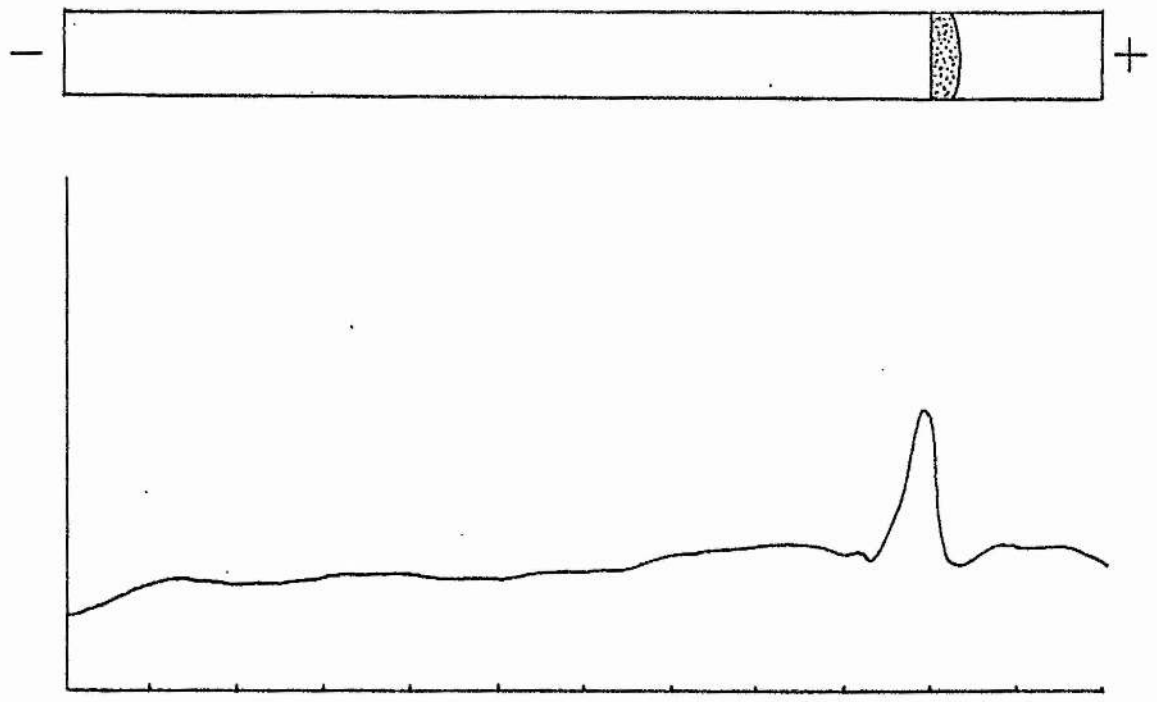
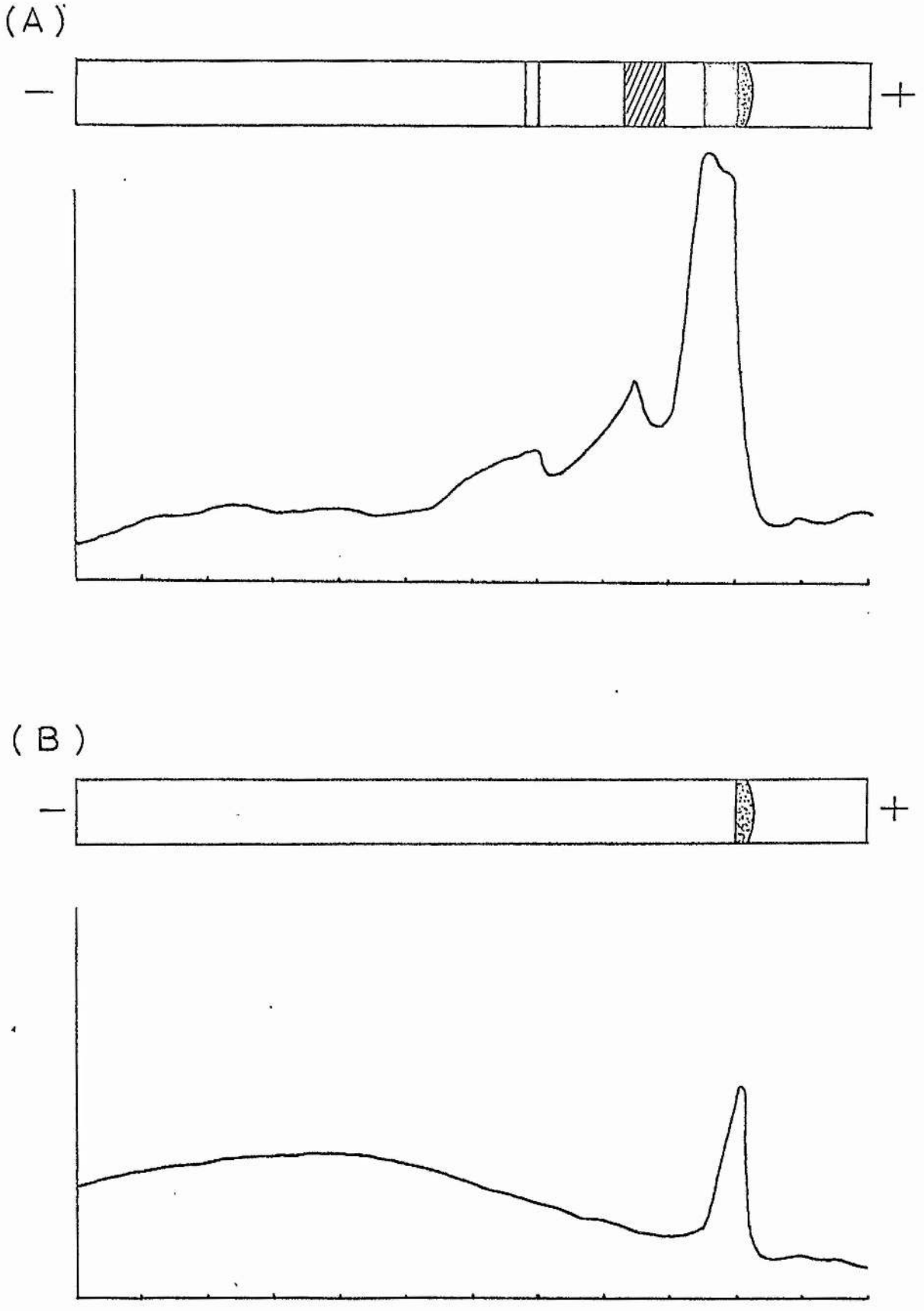


FIGURE 20.

ADH isoenzymes in the roots of *Senecio sylvaticus*, flooded one month.

(A) Full staining mixture.

(B) Staining mixture minus ethanol.


(A)
−
+
(B)
−
+

Hieracium pilosella (Figures 21 and 22. Plate IV)
Non-helophyte.

No bands of ADH were apparent on electrophoresis of extract from unflooded roots. Since a comparable extract showed ADH activity when assayed spectrophotometrically (See Table 6), it can only be concluded that the activity was too small to be detected in the gels, or that inactivation took place during electrophoresis.

In the extract from flooded roots, at least four isoenzymes are apparent, at R.f.'s 0.73, 0.69, 0.63, and 0.59, with a possible fifth appearing very faintly in some of the gels at R.f. 0.53. This fifth band was visible only on inspection and did not appear on densitometric tracings or in photographs.

Hieracium pilosella showed the greatest number of isoenzyme bands of all the species tested.

x x x

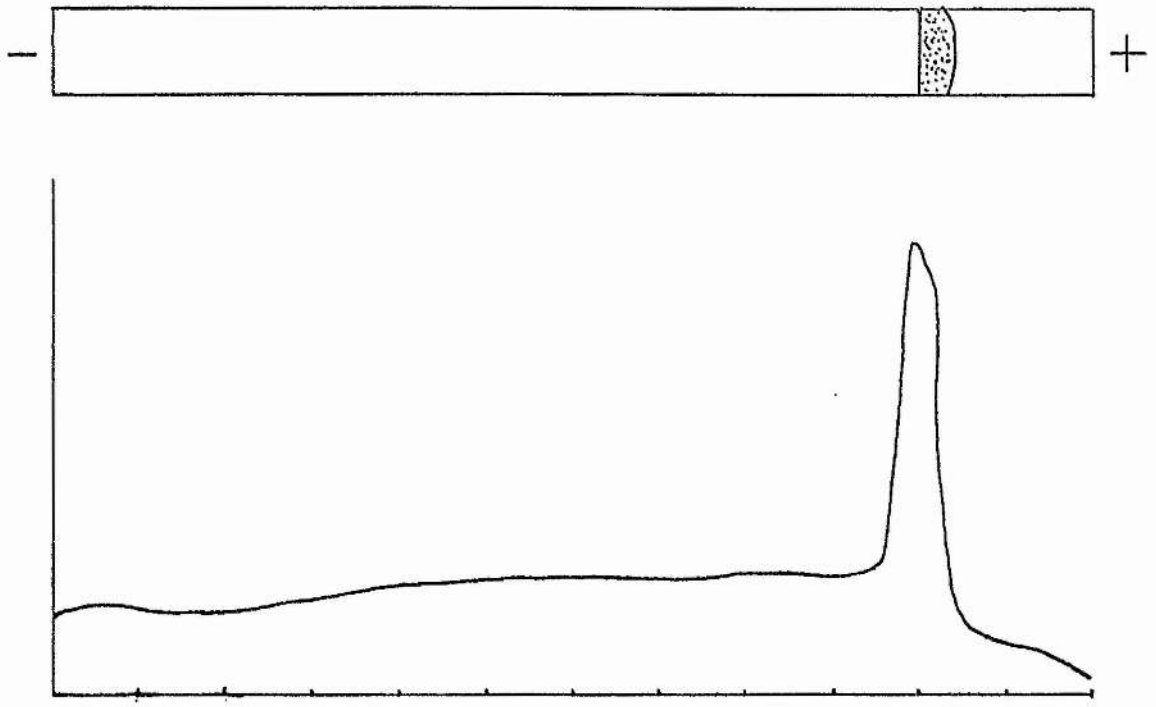
FIGURE 21.

ADH isoenzymes in the roots of Hieracium pilosella, unflooded.

(A) Fullstaining mixture.

(B) Staining mixture minus ethanol.

(A)



(B)

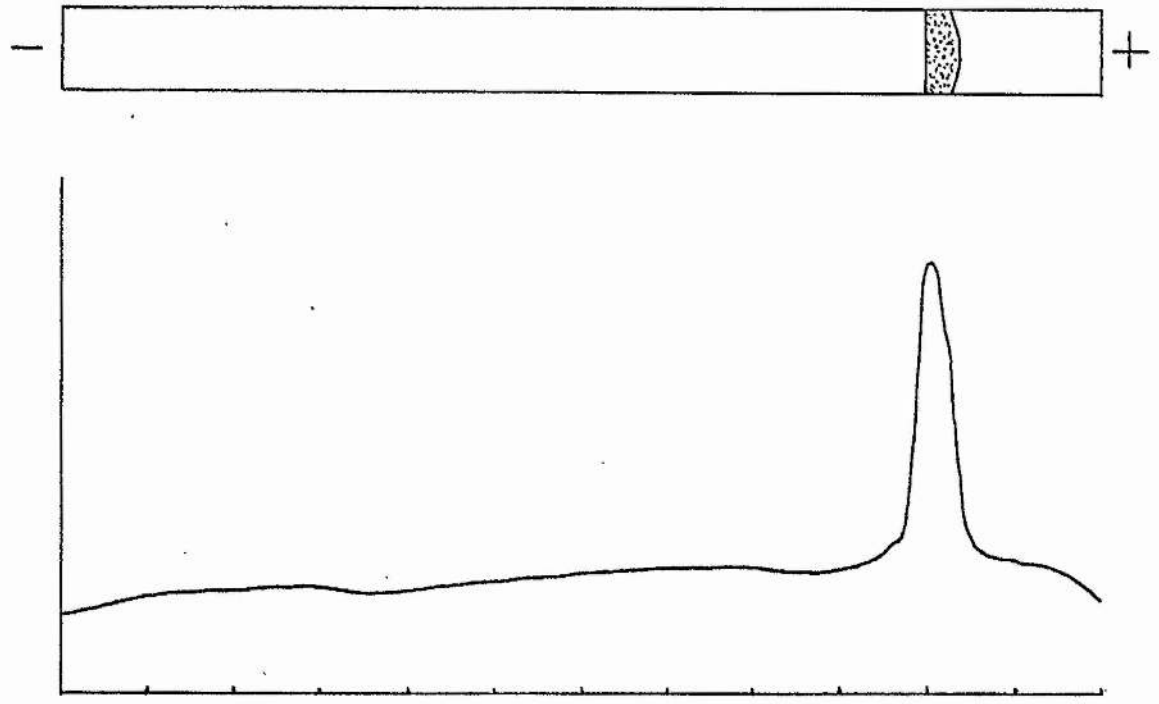


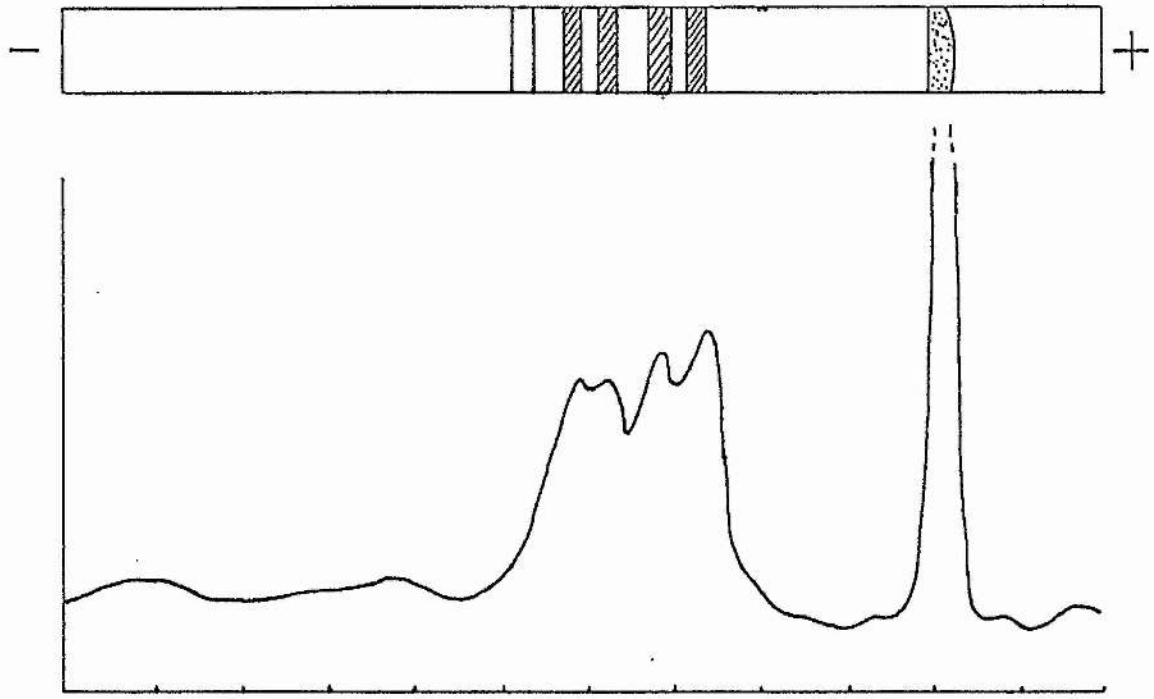
FIGURE 22.

ADH isoenzymes in the roots of *Hieracium pilosella*, flooded one month.

(A) Full staining mixture.

(B) Staining mixture minus ethanol.

(A)



(B)

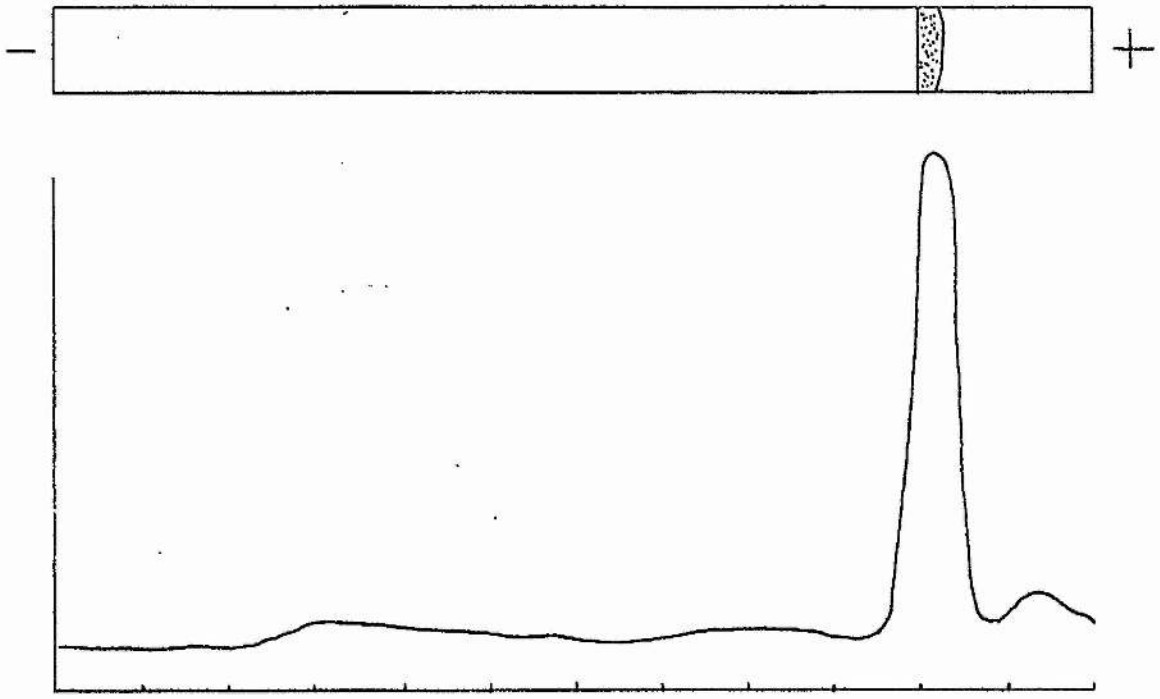


PLATE III

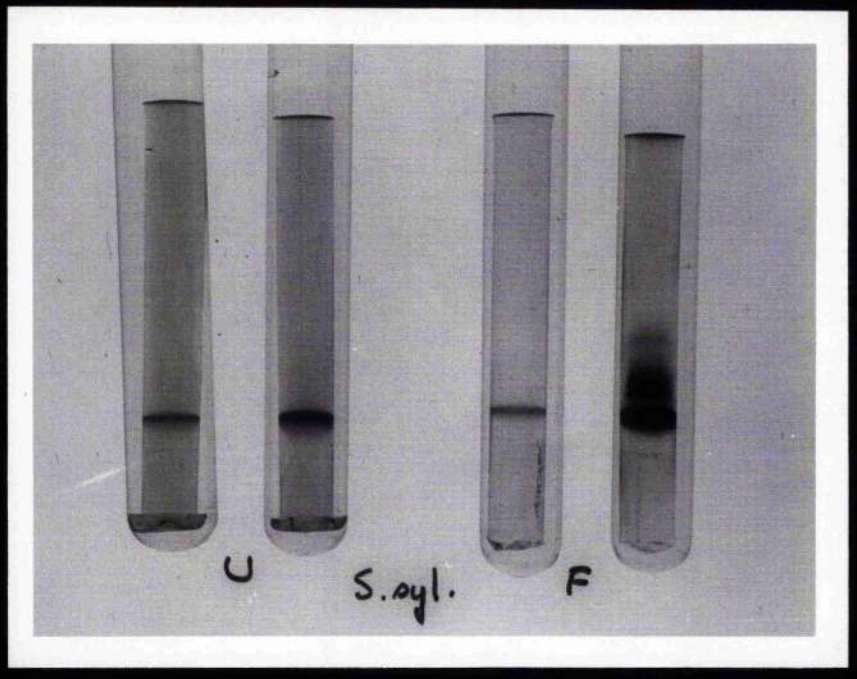
Senecio sylvaticus isoenzymes

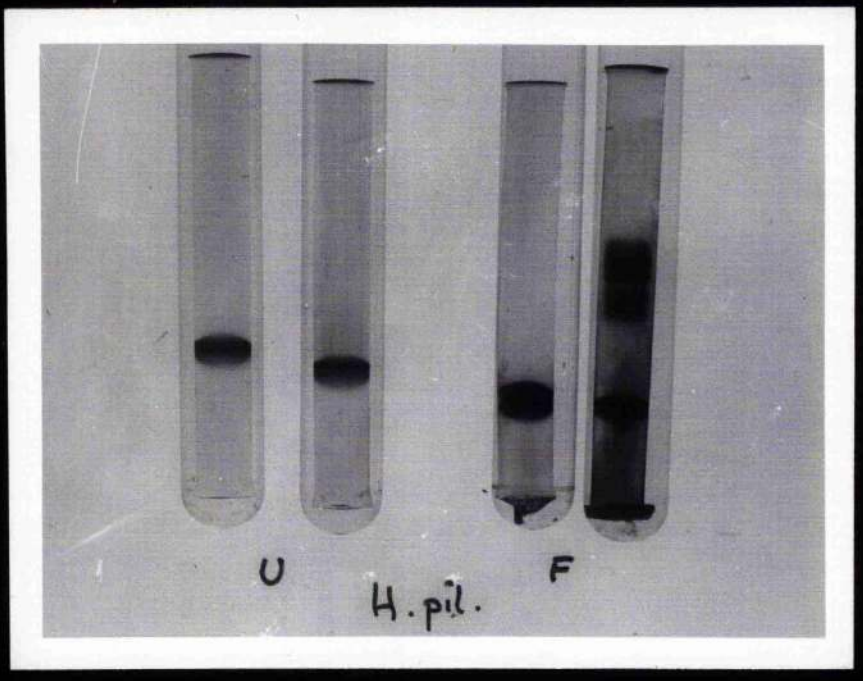
Unflooded		Flooded	
(B)	(A)	(B)	(A)

PLATE IV

Hieracium pilosella isoenzymes

Unflooded		Flooded	
(B)	(A)	(B)	(A)


U
S. ayl.
F


U
F
H. pil.

Senecio vulgaris (dune race) Figures 23 and 24. Plate V) Non-helophyte.

Like Hieracium, Senecio vulgaris showed no stained bands of ADH in gels from extracts of unflooded roots, despite the fact that spectrophotometric assay showed ADH activity. In this case the assay was of an extract made from the same set of roots as was used for electrophoresis.

On flooding, ADH activity increases, as can be expected in a non-helophyte, but there is only a single band of ADH staining, immediately behind the polyphenol band. Any other isoenzymes, if present, are in quantities too small to detect.

x x x

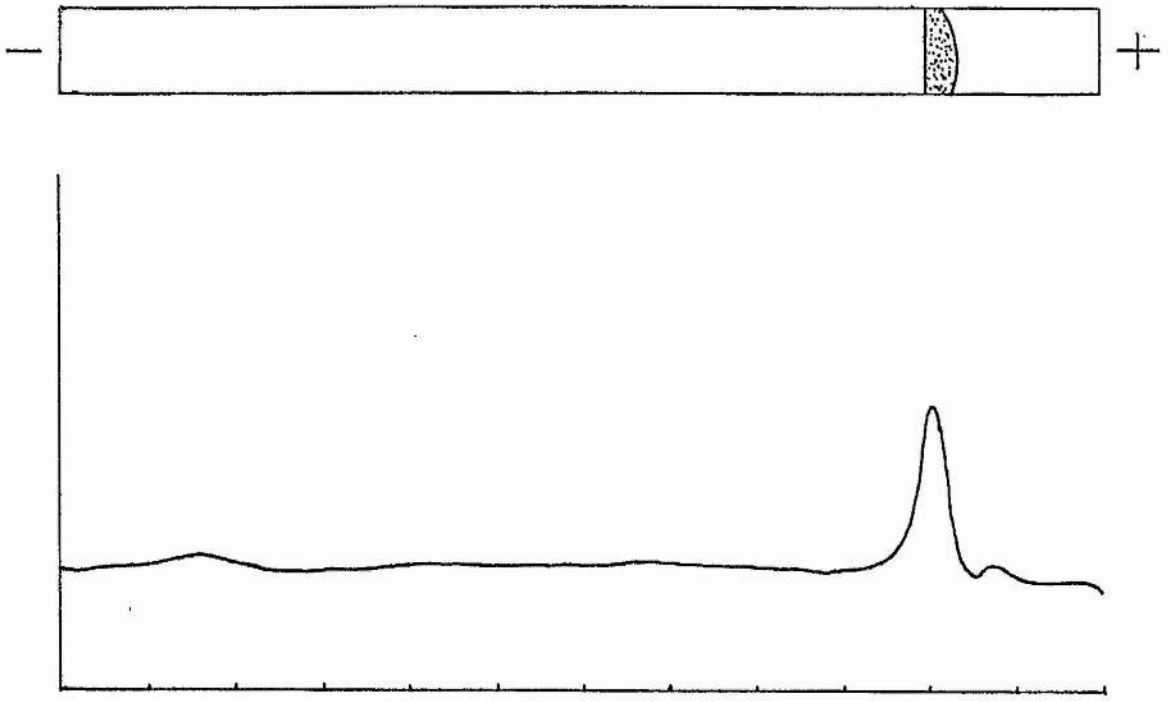
FIGURE 23.

ADH isoenzymes in the roots of Senecio vulgaris, unflooded.

(A) Full staining mixture.

(B) Staining mixture minus ethanol.

(A)



(B)

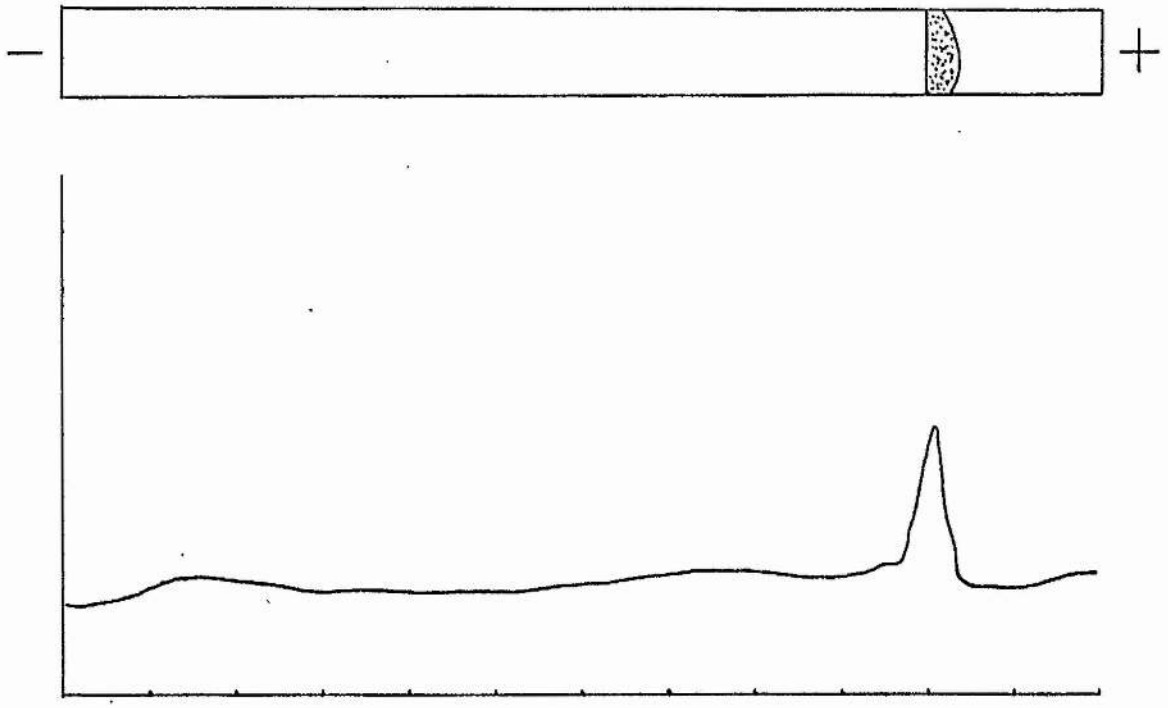


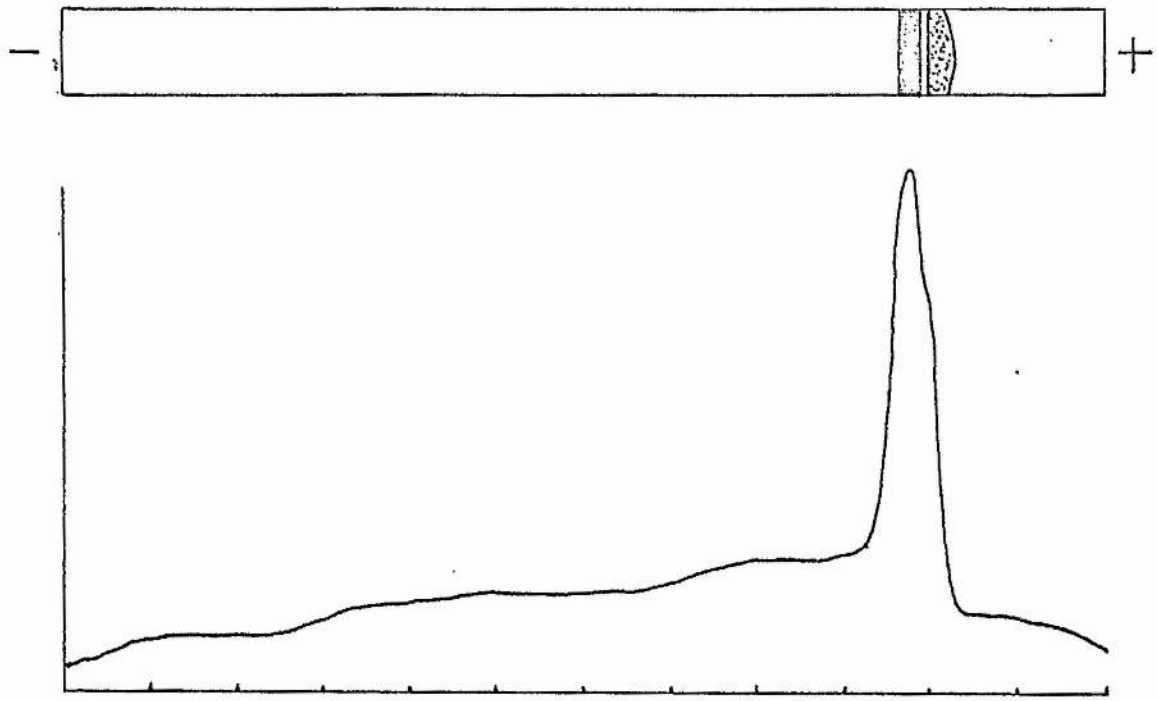
FIGURE 24.

ADH isoenzymes in the roots of Senecio vulgaris, flooded one month.

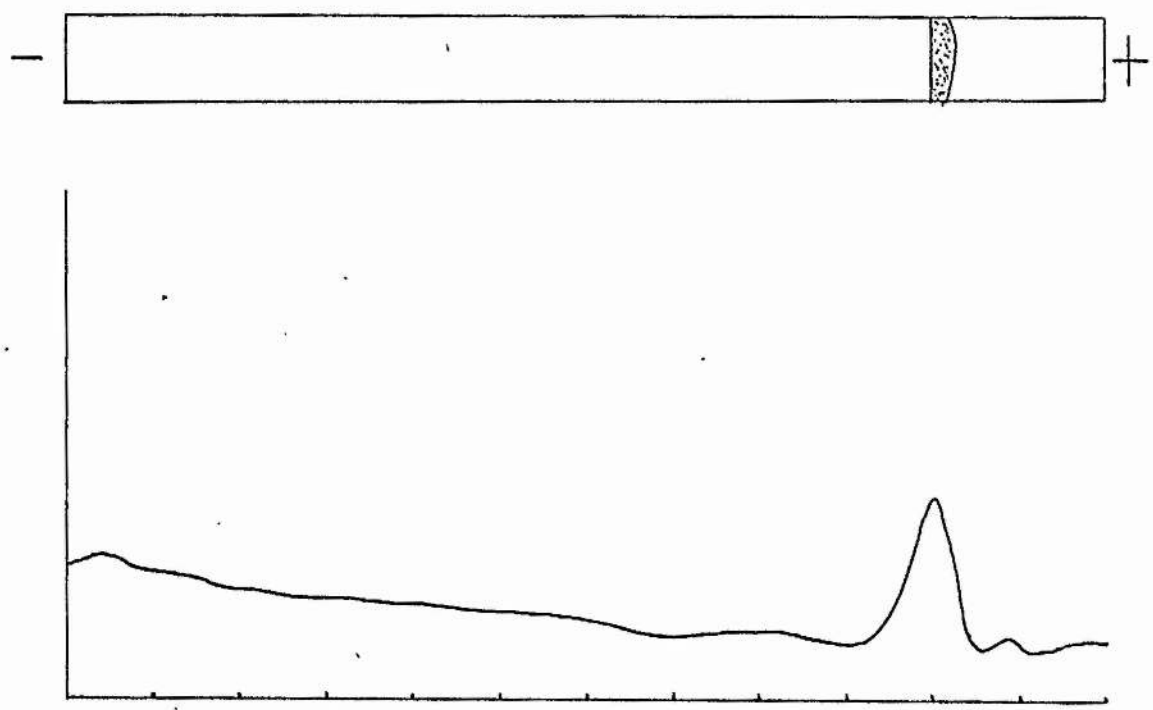
(A) Full staining mixture.

(B) Staining mixture minus ethanol.

(A)



(B)



Carex arenaria (Figures 25 and 26. Plate VI)

Helophyte.

A single band of ADH at R.f. 0.85 appears in gels made from extracts of unflooded roots. On flooding, this appears to become resolved into two rather narrower and fainter bands at the same position. As can be expected, there is no intensification of staining on flooding of this species. It is likely that another isoenzyme very similar to the original increases to detectable levels on flooding, rather than the original becoming separated into enzyme subunits such as those discovered by Pietruszko's group (1969). Any subunits would be likely to have rather different gel electrophoretic mobilities from the original enzyme.

The staining at the electrophoretic front is not due to enzyme activity, as is shown by its presence in gels 'B' of the figures, and Plate VI.

x x x

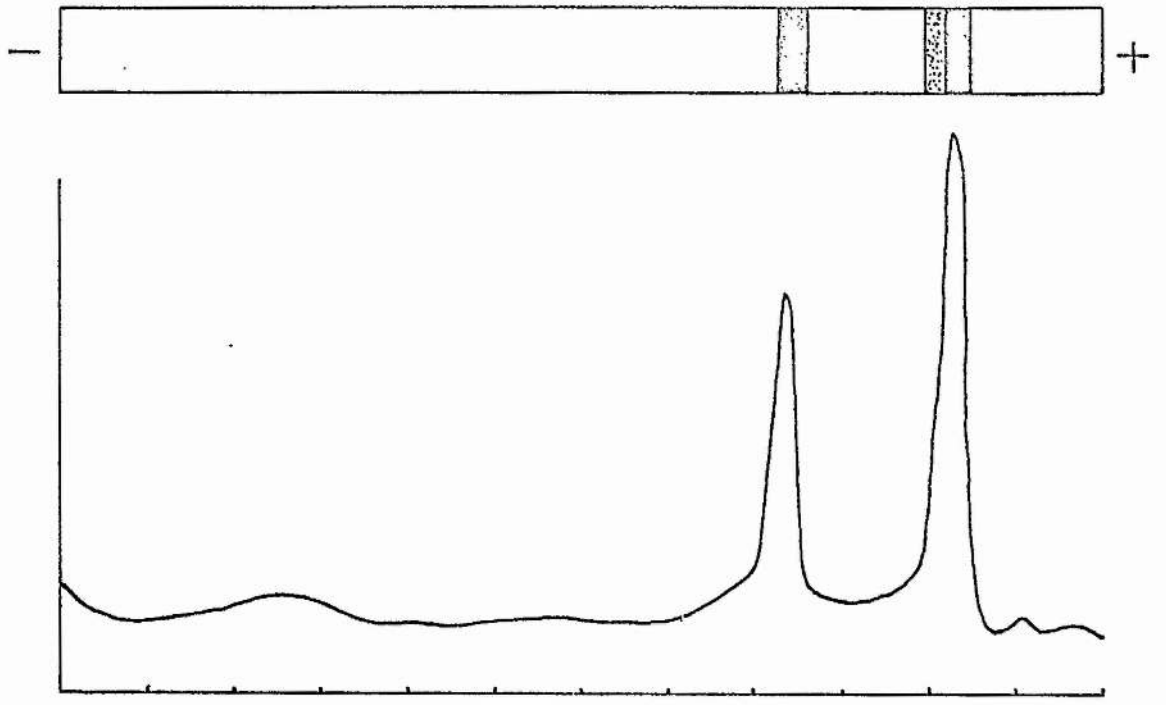
FIGURE 25.

ADH isoenzymes in the roots of *Carex arenaria*, unflooded.

(A) Full staining mixture.

(B) Staining mixture minus ethanol.

(A)



(B)

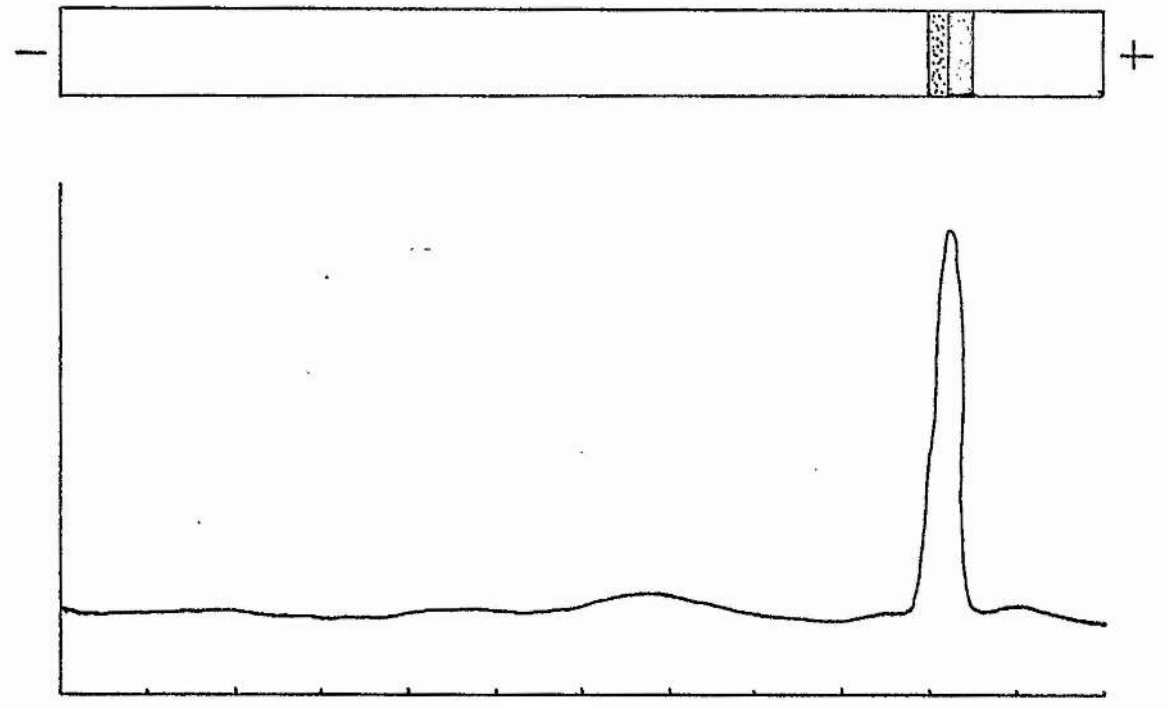


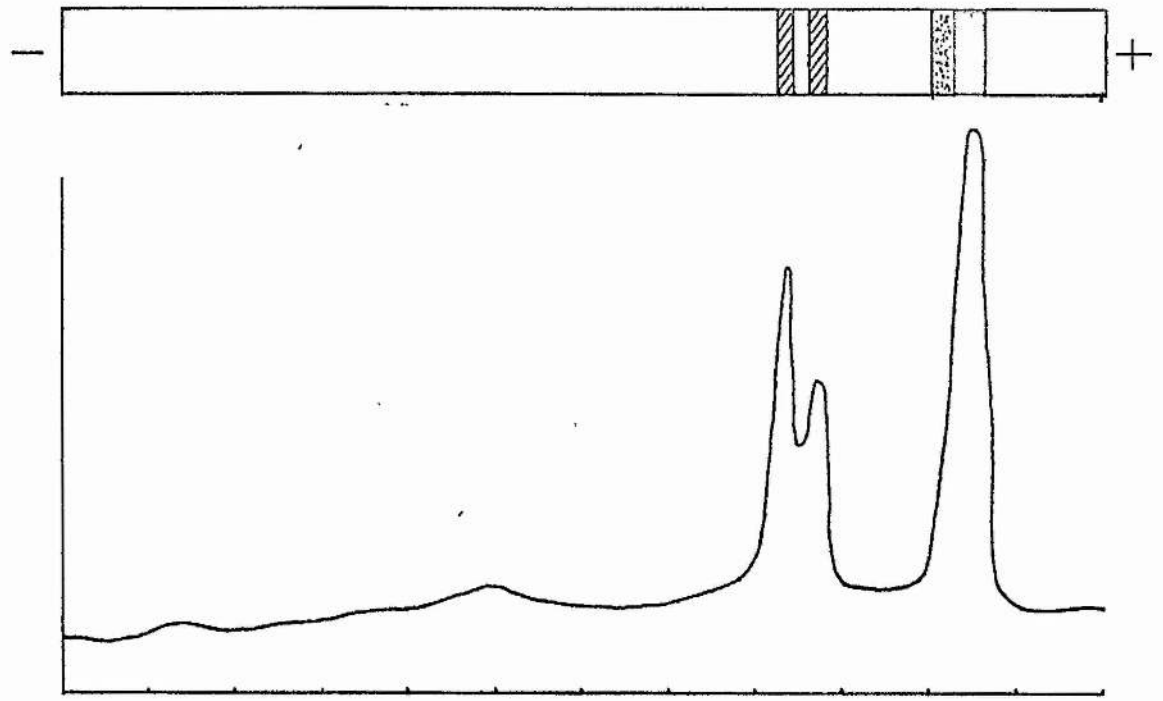
FIGURE 26.

ADH isoenzymes in the roots of *Carex arenaria*, flooded one month.

(A) Full staining mixture.

(B) Staining mixture minus ethanol.

(A)



(B)

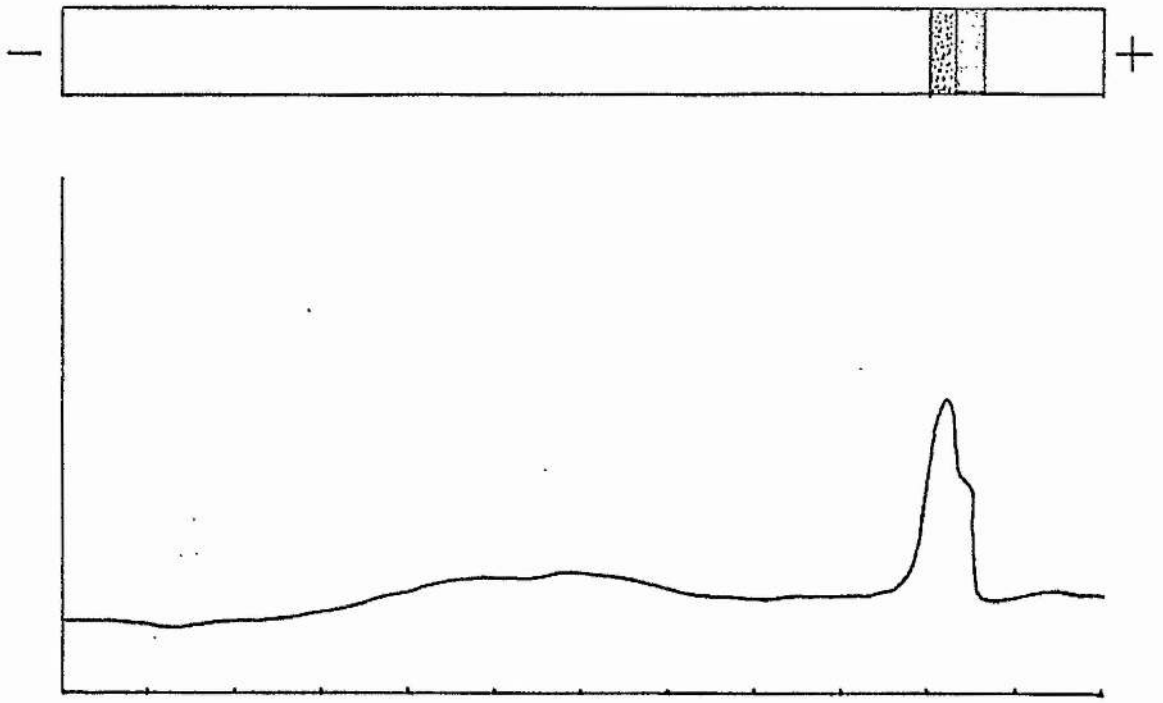


PLATE V

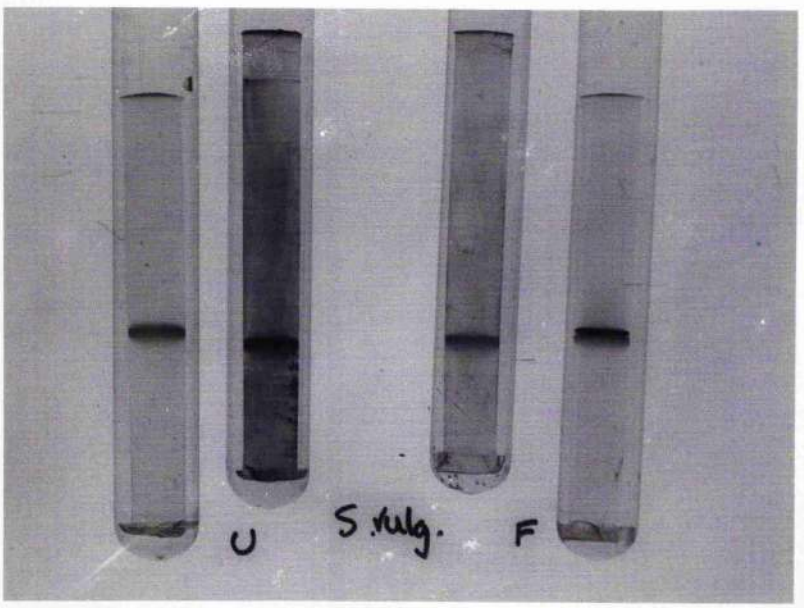
Senecio vulgaris isoenzymes

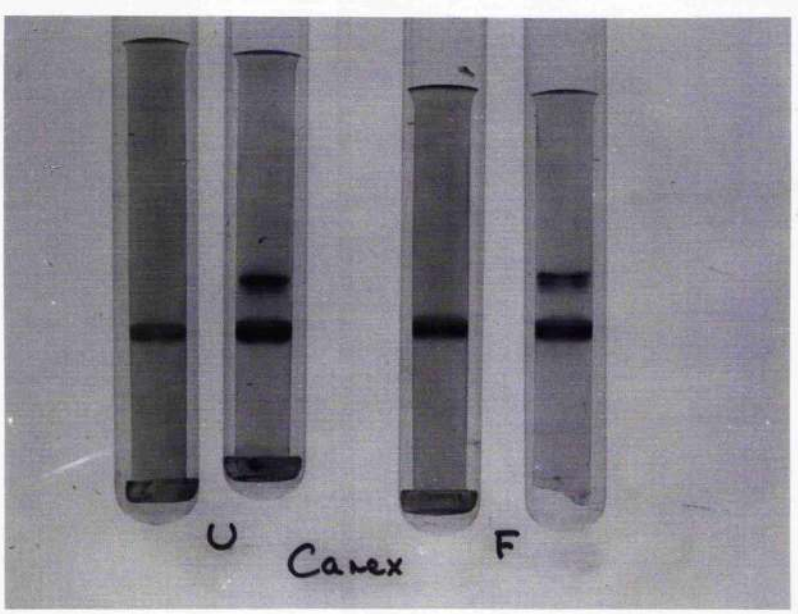
Unflooded		Flooded	
(B)	(A)	(B)	(A)

PLATE VI

Carex arenaria isoenzymes

Unflooded		Flooded	
(B)	(A)	(B)	(A)


U
S. vulg.
F


U
Carex
F

Iris pseudacorus (Figures 27 and 28. Plate VII)
Helophyte.

The isoenzyme pattern for this species is rather difficult to interpret. Extracts of flooded and unflooded roots showed very similar ADH activities when assayed spectrophotometrically (Table 6), but on electrophoresis it was only in the extracts of flooded roots that ADH staining appeared. Staining at the electrophoretic front in Figure 27 appeared also in gel 'B' and was not due to ADH.

Three isoenzymes were detected in extracts made from flooded roots, at R.f.'s 0.98, 0.80. and 0.59 respectively. The last showed by far the strongest staining.

The absence of staining in the gels made from unflooded roots may have been due to loss of activity during electrophoresis.

x x x

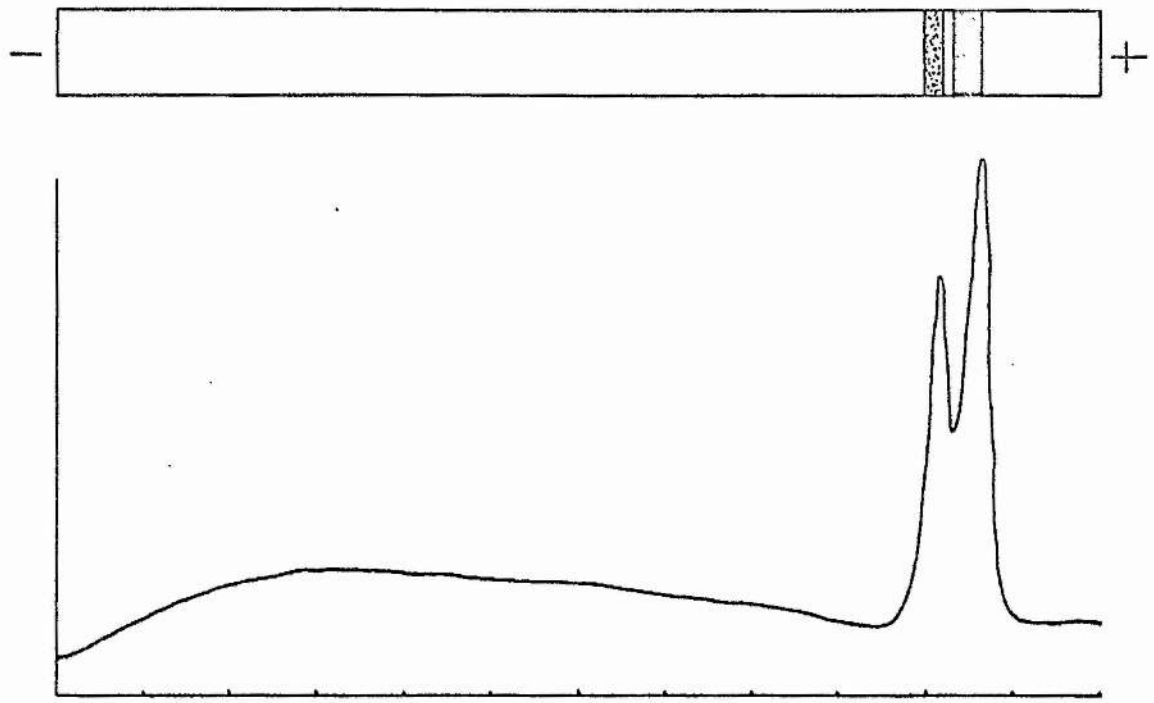
FIGURE 27.

ADH isoenzymes in the roots of *Iris pseudacorus*, unflooded.

(A) Full staining mixture.

(B) Staining mixture minus ethanol.

(A)



(B)

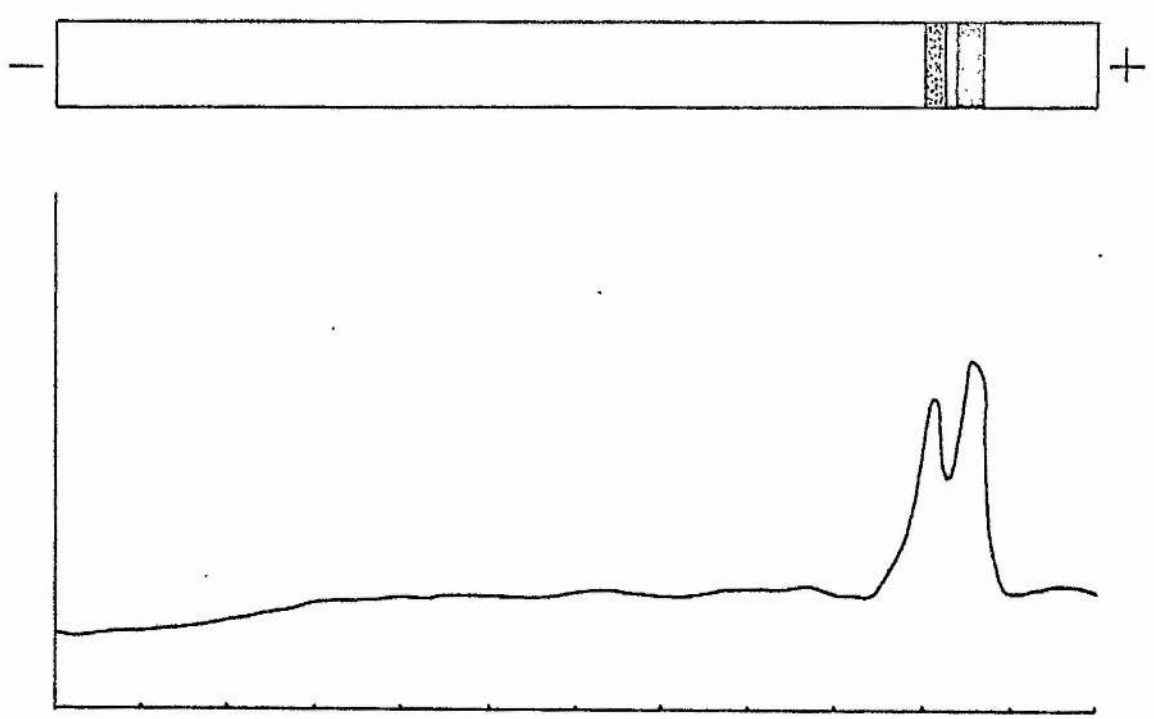


FIGURE 28.

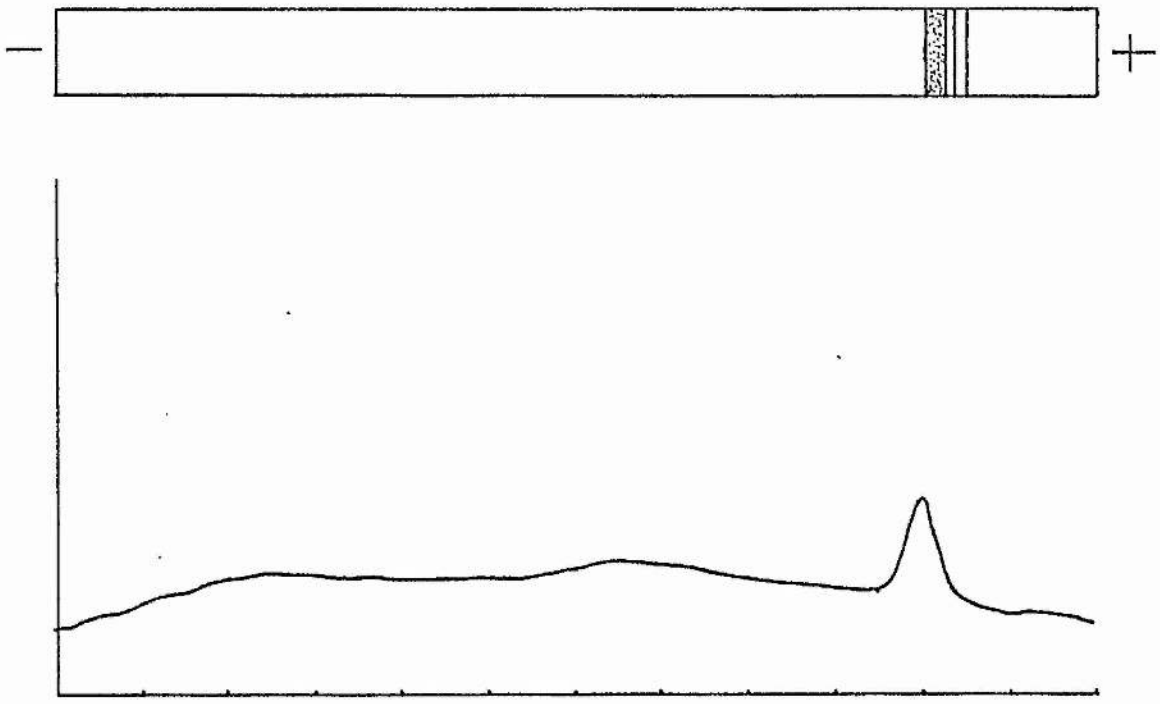
ADH isoenzymes in the roots of *Iris pseudacorus*, flooded one month.

(A) Full staining mixture.

(B) Staining mixture minus ethanol.

(A)

(B)



Mentha aquatica (Figures 29 and 30. Plate VIII)

Helophyte.

Like Iris, staining attributable to ADH activity appeared only in gels made from extracts of flooded roots, although spectrophotometric assays gave similar activities for flooded and unflooded treatments. It must be again concluded that activity was lost during the electrophoretic procedure.

In the flooded treatment, two isoenzymes appeared, at R.f.'s 0.94 and 0.85, the former being the more strongly stained.

x x x

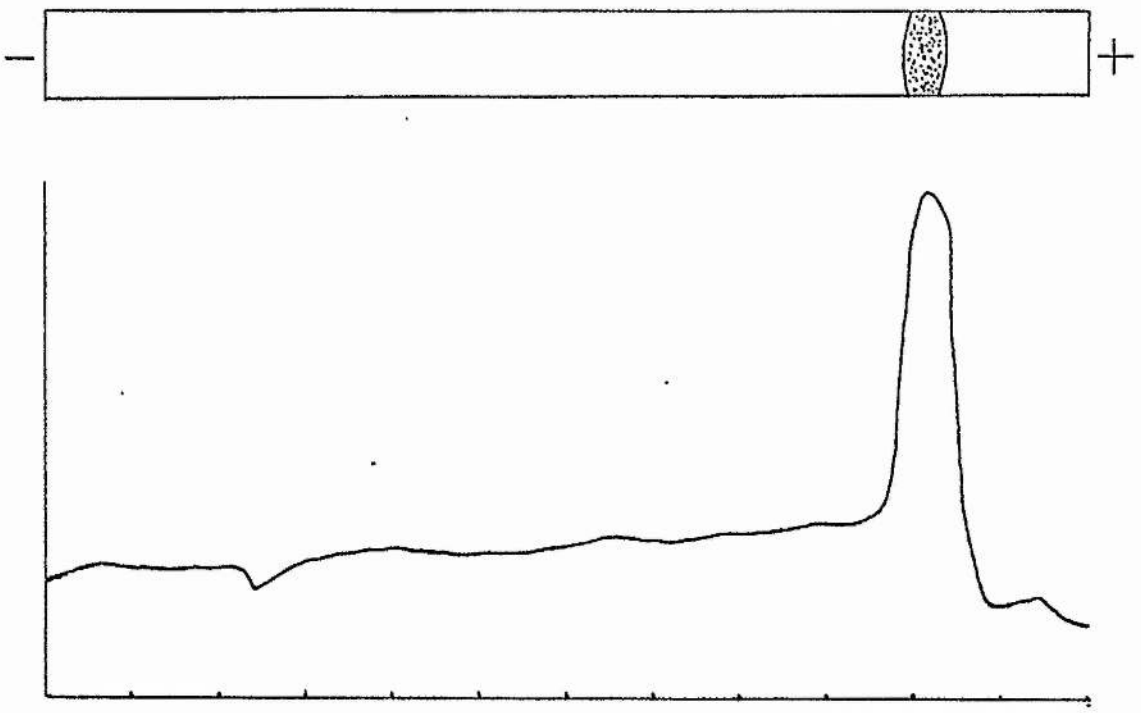
FIGURE 29.

ADH isoenzymes in the roots of Mentha aquatica, unflooded.

(A) Full staining mixture.

(B) Staining mixture minus ethanol.

(A)



(B)

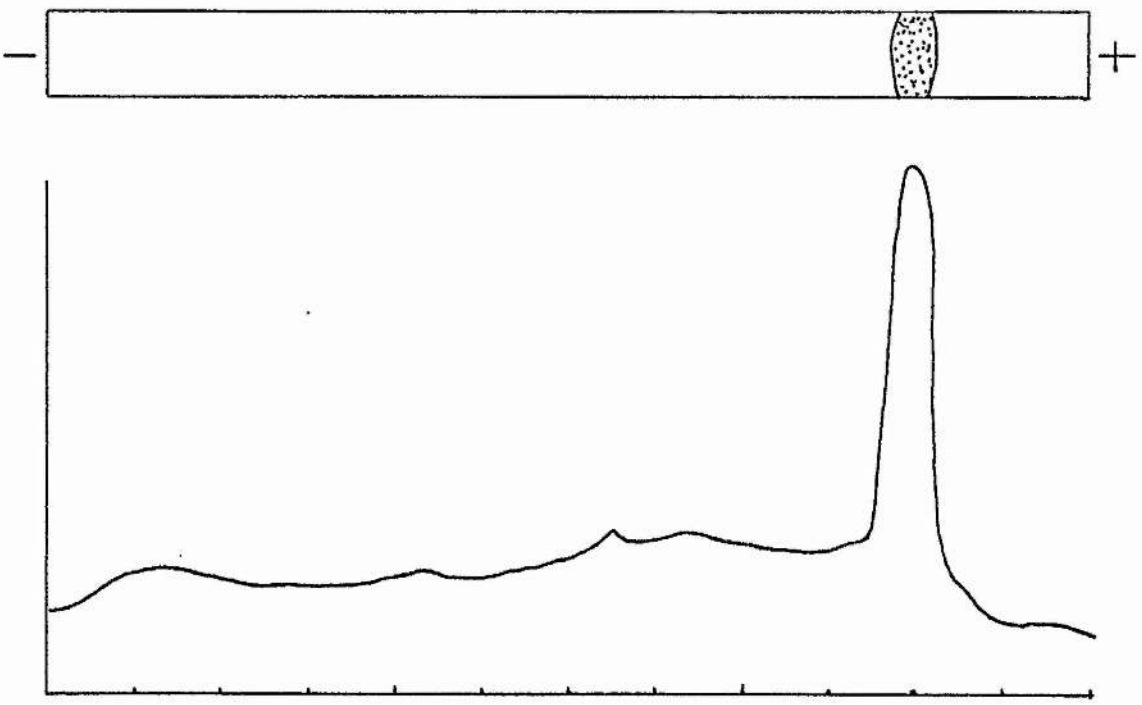


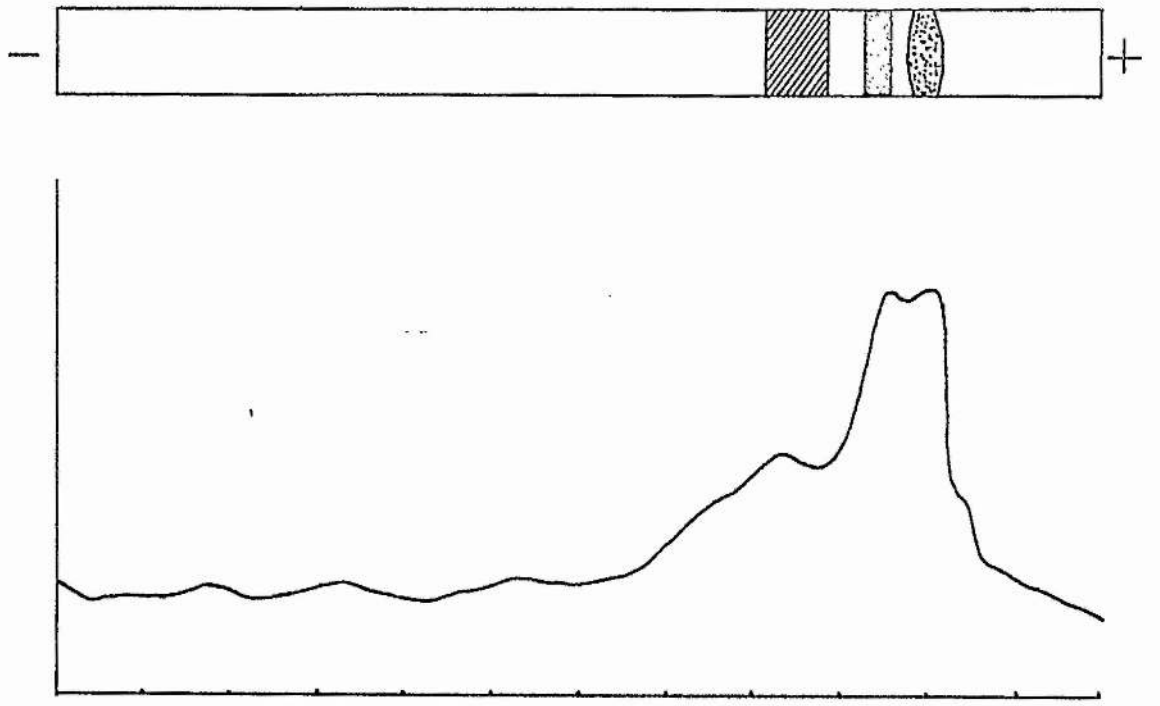
FIGURE 30.

ADH isoenzymes in the roots of *Mentha aquatica*, flooded one month.

(A) Full staining mixture.

(B) Staining mixture minus ethanol.

(A)



(B)

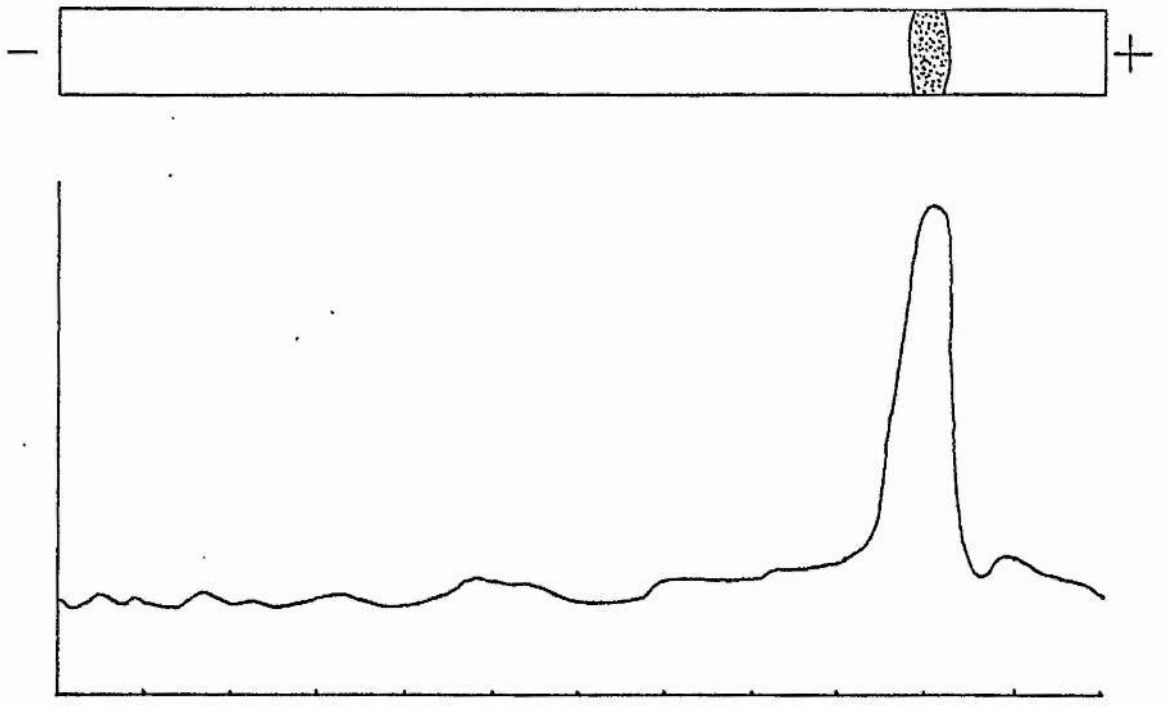


PLATE VII

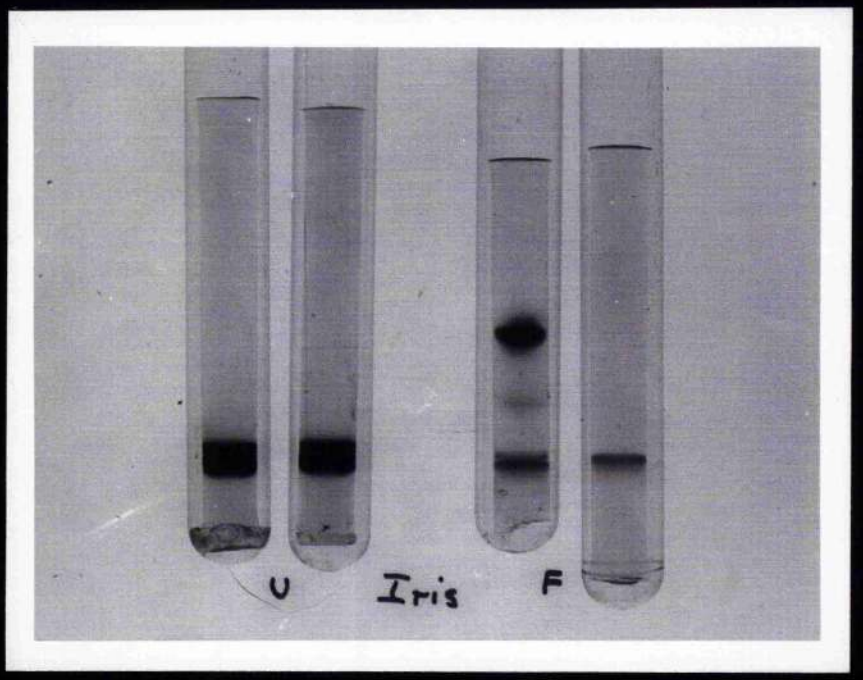
Iris pseudacorus isoenzymes

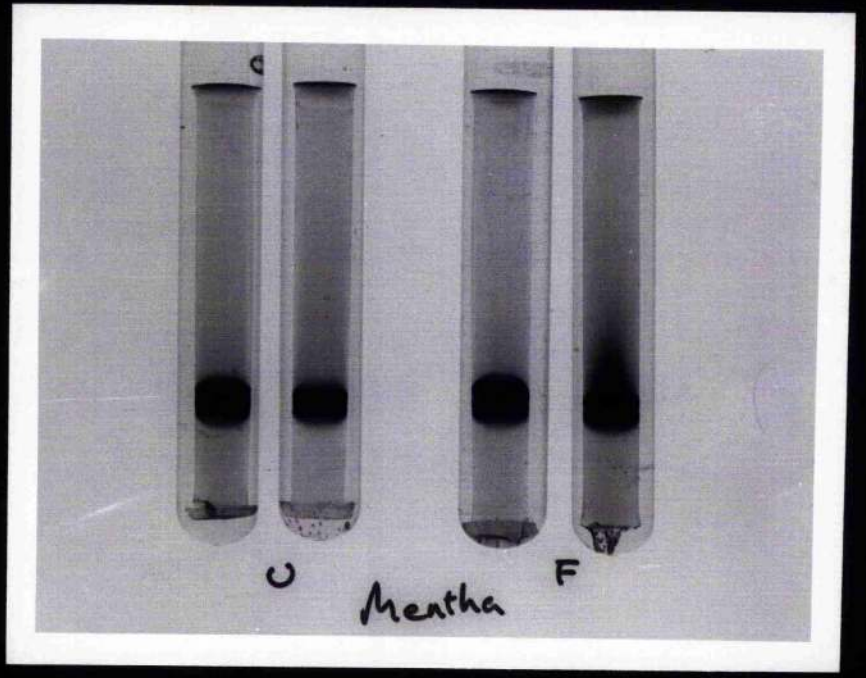
Unflooded		Flooded	
(A)	(B)	(A)	(B)

PLATE VIII

Mentha aquatica isoenzymes

Unflooded		Flooded	
(B)	(A)	(B)	(A)


U
Iris
F


U
Mentha
F

It is evident, therefore, that isoenzymes of ADH, which have so far received little attention in comparison with those of other enzymes, do in fact exist in most of the species studied here, and are affected by the environmental conditions. The present experiments do not give any evidence of the properties of these isoenzymes; they differ in number and electrophoretic mobility from species to species, and although all of the separations described were performed under similar conditions, it is not possible to correlate the pattern in one species with that in another. Isoenzymes of different species showing similar R. f. values are not necessarily identical, as is shown by the frequently very close proximity of bands in the same gel (e.g. Carex arenaria, flooded, and Hieracium pilosella, flooded).

Where additional bands appear on flooding, however, bands present in 'unflooded' gels are still present in 'flooded' gels, and can be identified as such, although possibly changed in intensity (e.g. Senecio sylvaticus, Carex arenaria).

Absence of a particular isoenzyme band from a gel does not necessarily indicate that the isoenzyme is absent from the root, as is shown by the absence of any staining where ADH has been shown by other methods to be present. A certain threshold value of activity is probably necessary

before staining occurs. In some cases the relatively labile enzyme extracts may not survive electrophoresis; this was probably the case with the extracts of unflooded Iris and Mentha roots.

Although the isoenzymes demonstrated here may have similar structural and functional relationships to those of horse liver ADH, as discussed by Pietruszko et al (1969), it is obviously impossible to draw any conclusions until similar work has been done on this same selection of plants.

It is interesting to note that staining at the electrophoretic front, attributed by Macko, Honold and Stahmann (1967) to reduction of the tetrazolium by the ascorbic acid in the extraction medium, occurs only in gels made from extracts of some helophyte roots, and is entirely absent from non-helophyte gels. The purpose of the ascorbic acid and cysteine is to prevent inactivation of the enzyme by oxidation of -SH groups, but another very obvious effect is to some extent to prevent the oxidation of polyphenols beyond the quinonic (colourless) state on homogenisation of the roots, thus giving a lighter-coloured extract. Crawford (1967b) notes that polyphenol oxidase is present in appreciable amounts only in non-helophytes. If these observations are connected, it may be that the amounts of the reducing agents used are critical, and that active

polyphenol oxidation produces enough quinone to cause complete oxidation of the ascorbic acid (Beevers, 1961), leaving none available to cause formazan staining at the electrophoretic front.

The relationship of the isoenzyme investigations to the other studies of ADH in helophytes and non-helophytes is discussed in the section immediately following.

x x x

Alcohol Dehydrogenase - General Discussion and Conclusions

That the regulation of glycolysis and associated metabolic pathways is important in anaerobiosis is well-established (Crawford, 1966; Effer and Ranson, 1967). The precise role of ADH, and its behaviour, are to be discussed here in the light of the present results.

In general the suggestions of Crawford (1966, 1967a) are confirmed, namely that in non-helophytes (but not in helopytes) the induction of ADH by anoxia on flooding causes an acceleration of glycolysis and damage to the plant. However, the present results show that the behaviour of higher plant root ADH is rather more complex than previously thought. While some aspects may be correlated with the flooding tolerances of the species concerned, the simple induction differences between helophytes and non-helophytes found by Crawford (1967a) form only a part of the picture.

When a plant is flooded, it appears that one or more of three things can happen to the ADH in its roots:

1. Simple induction - the enzyme already present increases in amount or activity (Non-helophytes).

2. The apparent Michaelis constant of the enzyme with respect to acetaldehyde falls (Non-helophytes).

3. The isoenzyme pattern changes, often with an increase

in the number of the isoenzymes detected. This is not necessarily related to flooding tolerance, but is demonstrated more readily in non-helophytes because of the greater ADH activity present after flooding.

The latter two features, as has already been suggested, may be linked, in that the new isoenzymes may have different Km values from those already present. This is not proven, however, and its converse is certainly not true - appearance of new isoenzymes does not necessarily mean a change in the Km value, as closely-related isoenzymes are quite likely to have similar Km values. This is illustrated by Carex arenaria, where two isoenzyme bands appear close together on flooding in place of one in a similar position from the 'unflooded' extract. There is little increase in apparent ADH activity on flooding of Carex; it is a typical helophyte. Yet it is clear that flooding does induce some enzyme synthesis, as an extra isoenzyme appears. Thus enzyme induction is not necessarily related to the ability of a plant to withstand flooding. It is the degree of induction and perhaps the kinetic properties of the newly-formed enzyme which are important in this respect.

The results of Crawford (1967a) and other workers (Hageman and Flesher, 1960b; Kollöffel, 1968) are quite likely to include both true induction and the reduction

of the apparent Km which has been noted here. The relative importance of these two features seems to vary from species to species, as is shown in Table 13, which shows the reduction of apparent Km and increase in maximum enzyme activity in four species (all of course non-helophytes). The increase in "effective activity" of ADH upon flooding of a species would depend both upon the degree of induction and the reduction of the apparent Km. On this count, of the four species listed, Senecio viscosus and Pisum sativum should be severely affected by flooding, and this is indeed the case. The figures given for Hieracium pilosella are illustrated graphically in Figure 19: the plotted lines cut the vertical axis at 1/V max, and are quite close, therefore actual induction is not great. The straight-line extrapolations giving the apparent -1/Km on the horizontal axis are widely different, indicating that the increase in effective ADH activity, in this case at least, is dependent mainly on reduction of the apparent Km, and less on actual increase of enzyme already present.

The tentative theoretical treatment (Page 58) of the extrapolation of the curves plotted in Figures 15 to 18 receives some support in the case of Hieracium. It was suggested that the curved forms of the Lineweaver-Burk plots resembled the graph-forms obtained when two or more

Table 13. Induction of ADH activity and reduction of apparent Km values in four non-holophytes

	ADH induction (Flooded/Unflooded)	Apparent Km fall (Unflooded/Flooded)
Senecio jacobea	4.54	2.9
Hieracium pilosella	1.86	4.6
Senecio viscosus	3310	12.0
Pisum sativum	15.6	24.5

enzymes with different Michaelis constants act upon the same substrate. The 'flooded' plot has just such a form, and no less than four (possibly five) isoenzymes of ADH were present in an extract made from flooded Hieracium roots (Figure 22, Plate IV). Four isoenzymes, possibly with different Km values, make the curved plot of Figure 18 impossible to interpret in terms of real and apparent Km values, but remain entirely compatible with the downward curved form of the graph. As was generally the case, the ADH activity of the extract of unflooded roots was not great enough to give a staining reaction after electrophoresi so we cannot say if a linear Lineweaver-Burk plot would be reflected in a single major isoenzyme. Also, no other direct comparisons of isoenzyme patterns with graph-forms can be made, as no other species was used for both these investigations, due mainly to the choice of material available.

Whatever the isoenzyme pattern, however, the differences in kinetic and electrophoretic properties from species to species do tend to confirm that the variation of induction of ADH on flooding is indeed linked with variations in the enzyme itself, rather than to different levels of the inductive stimulus (acetaldehyde) being present.

Electrophoresis of plant proteins as an aid to

taxonomy has in recent years received some attention. Thurman, Boulter, Derbyshire and Turner (1967) correlated the isoenzyme patterns of formic and glutamic dehydrogenases in members of the Fabaceae with their taxonomic positions. It is interesting to note that the ADH isoenzyme patterns present and induced in the species used here do not seem to be related to their taxonomic positions. This is not surprising if we assume that the properties of the enzymes are genetically determined, and modified by environment. Genetic and ecological characteristics like flooding tolerance are usually evolved after the major taxonomic differences are established. Also, other features of the ADH reaction to flooding do not seem to be taxonomically correlated, within the genus Senecio at least (degree of induction, reduction of the apparent Km, and the relative importance of these two factors).

The three reactions of root ADH may be all caused by the same stimulus, or may be the results of various aspects of the changing root environment consequent upon flooding. Simple induction (taking this to mean an increase in maximum activity), we have shown, can be attributed at least in part to a specific stimulus - acetaldehyde. (This aspect of the ADH reaction potentially presents a classic feedback situation as the enzyme is induced, glycolysis is accelerated to

produce more acetaldehyde, which induces more enzyme.)
It would be a simple matter to extend experiments with acetaldehyde and other substances to discover if the other two reactions of ADH to flooding also have specific causative agents, and whether these are identical to the substance causing simple induction. Certain other features of the inductive process have been studied - its reversibility, its time-course, but such features have not been investigated with regard to the kinetic and isoenzyme properties. It seems likely that the three aspects are closely connected. If they are not, and their function can be separated experimentally, then this would form an interesting investigation in comparative biochemistry, although outside the scope of the present work.

x x x

PART III

OTHER ENZYMES

PART III OTHER ENZYMES

Introduction

Three major metabolic pathways are responsible for respiration in most tissues - the EMP (glycolysis), TCA (Krebs cycle) and pentose phosphate pathways. Their relative importance varies from tissue to tissue and, more important in the present context, in response to changes in the environment. The classic example is the Pasteur Effect, where anaerobiosis produces a blocking of the TCA cycle (due to oxygen not being available to the terminal oxidase systems) and acceleration of glycolysis, thought to be due mainly to changes in the ATP/ADP balance. (This view has been challenged by Barker, Khan and Solomos (1966), and by Effer and Ranson (1967), who question the validity of the Pasteur Effect.) Such variations in the contribution to respiratory catabolism of these three major metabolic pathways have usually, as in the above example, been ascribed to changes in co-enzyme and other metabolite levels (Davies, 1961). This explanation must necessarily be true at least in part - such a complicated system of dynamic equilibria must be disturbed when the level of a major component such as oxygen is altered. It need not, however, be the only explanation. An additional factor could be changes (or inherent specific differences) in the amount or activity

levels of the catalytic agents, the enzymes,

There do not appear to be any integrated studies of the effects of alterations in the environment upon the levels of activity of the more important enzymes of all three major respiratory pathways in one species or tissue. This is not surprising in view of the battery of extraction and assay procedures which would be necessary. Selected enzymes of these systems have been assayed, as for example in the study by Hageman and Flesher (1960b) of the effect of anaerobiosis of corn seedlings on three enzymes simultaneousl - aldolase, triose phosphate dehydrogenase, and alcohol dehydrogenase. In this case only ADH activity was found to vary. Abarov and Petinov (1964) and Abarov (1965) studied the effect of drought upon selected enzymes of the glycolytic and pentose phosphate pathways of corn. Effer and Ranson's (1967) studies on the effect of anoxia on respiration in buckwheat (Fagopyrum esculentum) assess only by inference the operation of the pathways, by measuring the levels of respiratory intermediates and the rate of carbon loss. It is interesting to note that they find no evidence for the Pasteur Effect as previously conceived.

Enzyme activities in fungi have received rather more attention. Casselton (1966) studied the glycolytic and pentose phosphate pathways of Polyporus brumalis, while

Cochrane and Cochrane (1966) noted changes in anaerobic metabolism and glycolytic enzyme activities during the development of Fusarium solani. Also interesting is Caltrider and Gottleib's (1963) extensive study of respiratory enzymes in the spores of four fungus species, including one saprophyte and three parasites. Although specific differences were found in the levels of many enzymes, they could not be related to the ecology of the fungi. Possibly this is due to a lack of information in the precise environmental conditions of fungal spores. Work on actively-growing material may produce more revealing differences.

Most of the enzymes under discussion have been reported from several species of higher plants grown under various conditions, but collation of such data would be of little use, as the enzyme extraction and assay systems vary considerably, rendering the data useless for comparative purposes except under strictly replicate conditions. There are however examples of the possession of specific enzymes or enzyme systems being related to obvious ecological , morphological or otherfeatures of the plants possessing them. Examples are the secretion of 'digestive' enzymes by organs of certain carnivorous plants, the presence of the enzymes of the glyoxalate cycle in fatty seeds, and the very active preparations made from succulent plants of the enzymes involv

in Crassulacean acid metabolism (Walker, 1957, 1960).

Work on alcohol dehydrogenase (Part II of this thesis) has shown that species differences in the properties and induction behaviour of a universally-occurring enzyme can be shown to follow a general pattern related to an aspect of the ecology of the species concerned. It was decided, therefore, to make limited investigations (in terms of occurrence and activity) of other selected respiratory enzymes in helophytes and non-helophytes, and also to investigate the effects of flooding on enzyme activity, extending the investigations as indicated by the results obtained. It was hoped that the results for all the enzymes could be combined to formulate a more complete picture of the mechanism of flooding tolerance. The enzymes chosen for study were: malic dehydrogenase (MDH), 'malic' enzyme, glucose 6-phosphate dehydrogenase (G6-PDH), lactic dehydrogenase (LDH), phosphoenolpyruvate (PEP) carboxylase and carboxykinase, enolase, pyruvate kinase. Of these enzymes, the first received the most attention, because of the results obtained in preliminary experiments.

x x x

(i) Malic Dehydrogenase (MDH)

The reaction catalysed is:

L-malate + NAD^+ $\rightleftharpoons$ Oxaloacetate + $NADH^+ + H^+$

MDH is of even more universal occurrence than ADH, and has been shown to be active in most living tissues. It was chosen for study because one of its substrates, L-malate, is thought to be widely accumulated in plants under anaerobic conditions (Mazelis and Vennesland, 1957; Boulter, Coult and Henshaw, 1963). Crawford and Tyler (1969) found that malic acid accumulated in helophytes under flooded conditions, but disappeared from non-helophytes, and suggested that malic acid provided a non-toxic alternative to ethanol as a product of anaerobiosis in plants, and one which could be more easily metabolised on subsequent return to aerobic conditions.

The study of MDH is complicated by the fact that there are not only two distinct fractions, located in the mitochondria and cytoplasm respectively, but the presence of a variable number of isoenzymes is well-established in many tissues. (Intracellular localisation is a problem not encountered in the study of alcohol dehydrogenase, although Barker, Khan and Solomos (1966) postulate a glycolytic particle. This, unlike the mitochondrion, must be very easily broken by homogenisation during enzyme extraction.)

As in most previous studies, a simple homogenisation technique was adopted, as for alcohol dehydrogenase, and the resultant extract probably contained a mixture of MDH components. Even if separation of the mitochondrial and cytoplasmic fractions had been attempted, an extensive purification procedure would have been necessary to establish their identity. As the activity of the purified enzyme fraction would be unrelated to its total activity in the root tissue, this was not attempted. The interpretation of the results for MDH must therefore be made more cautiously than when considering those for alcohol dehydrogenase.

x x x

1. Flooding Experiments

The methods used for investigating the effects of experimental flooding upon root MDH activity were essentially the same as for alcohol dehydrogenase activity (Part II, Section (i)). Four species (two helophytes, two non-helophytes) were subjected to experimental flooding for one month, then the roots were homogenised and assayed for MDH activity, using the assay system described in Appendix A. This is identical to the ADH assay system except that 5 μmoles of sodium oxaloacetate replace the acetaldehyde.

Table 14. The effects of one month's flooding upon the levels of MDH activity in the roots of four species

Enzyme activities are expressed as International Units per mg. protein.

	MDH ACTIVITY		
	Unflooded	Flooded	Ratio Unfl:Fl
Hieracium pilosella	0.023	0.053	1:2.3
Senecio viscosus	0.051	0.122	1:2.4
Senecio aquaticus	0.097	0.094	1:0.97
Mentha aquatica	0.011	0.011	1:1.00

* * *

Table 15. The effect of short periods of flooding upon the level of MDH activity in the roots of Senecio viscosus

Enzyme activities are expressed as International Units per mg. protein.

Treatment	MDH activity
Unflooded control	0.035
Flooded 3 days	0.047
Flooded 6 days	0.058
Flooded 9 days	0.070

The results are set out in Table 14.

In addition, Senecio viscosus was flooded for shorter periods (3, 6 and 9 days), then the roots were assayed for MDH activity. A control bucket remained unflooded. The results are shown in Table 15.

The non-helophytes show an increase of about twofold on flooding, while the activity in the two helophytes remains constant. MDH therefore shows a similar reaction to ADH on flooding, at least in the four species examined.

2. Water Culture Experiments.

Simultaneously to the flooding experiments, a series of water culture experiments was carried out, similar to those described earlier in the section on alcohol dehydrogenase, and using the same four species. The plants were grown in aerated and non-aerated culture solutions for up to nine days, and the root extracts subsequently assayed for MDH activity. The results are shown in Figures 31 and 32. The data from Table 15 is plotted onto Figure 31 (b) for purposes of comparison.

Fluctuations in activity in response to environmental conditions do not seem to have been previously

FIGURE 31.

MDH activities in the roots of two non-helophytes grown for different periods in aerated and non-aerated water culture solutions.

Enzyme activities are expressed as International Units per milligram protein, × 100

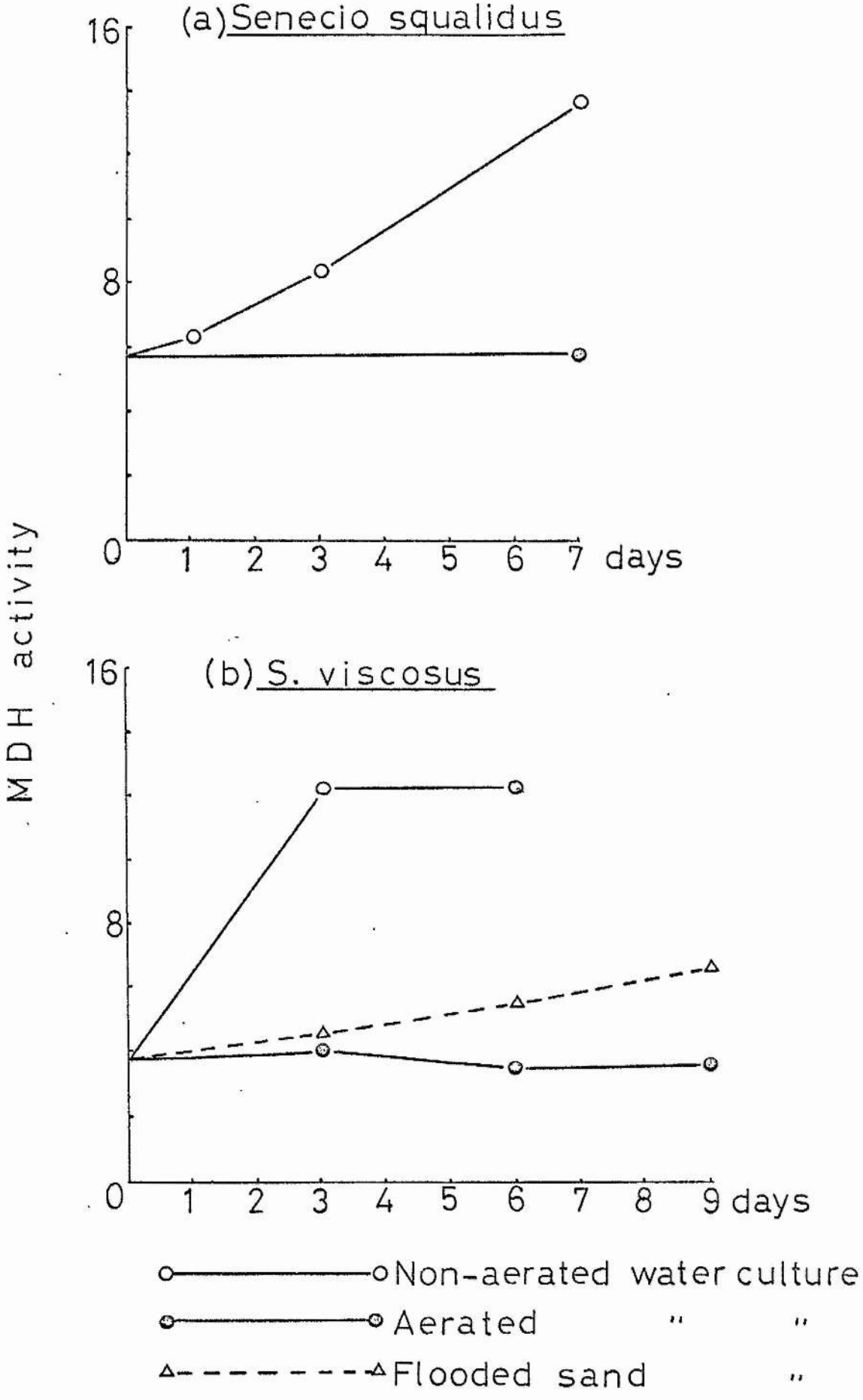
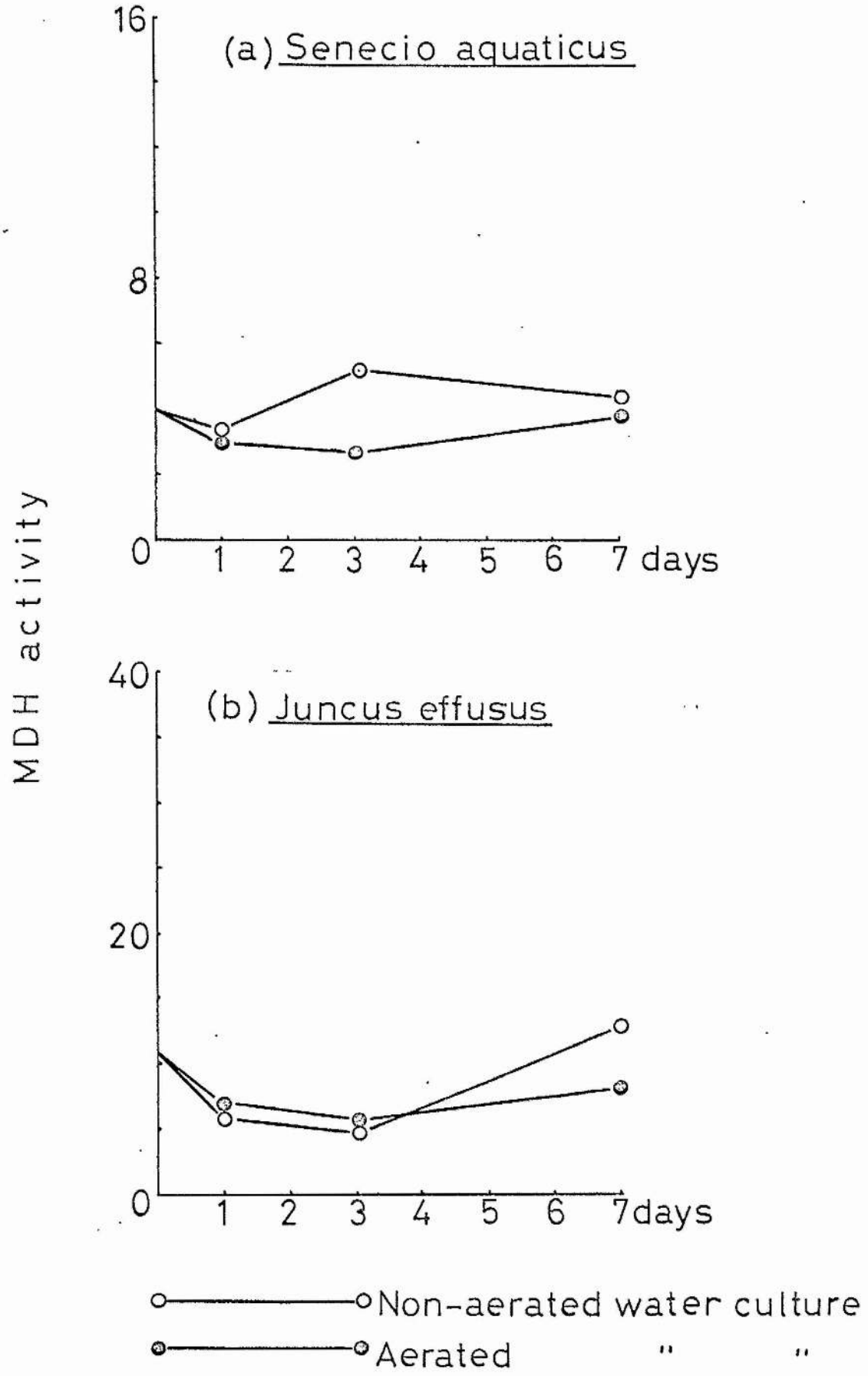

(a) Senecio squalidus
16
8
0
1 2 3 4 5 6 7 days
(b) S. viscosus
16
8
0
1 2 3 4 5 6 7 8 9 days
MDH activity
Non-aerated water culture
Aerated " "
Flooded sand "

FIGURE 32.

MDH activities in the roots of two helophytes grown for different periods in aerated and non-aerated water culture solutions.

Enzyme activities are expressed as International Units per milligram protein, x 100.


(a) Senecio aquaticus
16
8
0
1 2 3 4 5 6 7 days
MDH activity
(b) Juncus effusus
40
20
0
1 2 3 4 5 6 7days
Non-aerated water culture
Aerated " "

reported for this enzyme. The induction of MDH upon flooding (Tables 14 and 15; Figure 31 (b)) is evident to a much lesser degree than in non-aerated water culture, and seems relatively insignificant in Figure 31 (b). The anoxia produced by experimental flooding is much less marked than in non-aerated water culture, where all oxygen is rigidly excluded, except for any which may diffuse through the plants themselves. Flooded sand culture permits the free diffusion of air downwards through an exposed water surface. In fact, Crawford (1966) found that experimental flooding produced a root environment much less anaerobic than that found in flooded conditions in the field. Non-aerated water culture may therefore be comparable to field conditions, and MDH activity may increase to a similar extent.

As with alcohol dehydrogenase, MDH is induced in non-helophytes by non-aerated water culture, but not in holophytes. The significance of this is not clear; at this stage it is tempting to connect it with the marked fall in malic acid content of roots consequent upon the flooding of non-helophytes, but not helophytes (Crawford and Tyler, 1969). Discussion of this point must take account of the compartmentation of malate and MDH in the cell. This problem will be more fully

dealt with later, but it can be pointed out here that if MDH induction and malate loss are in fact connected, then this suggests that malate is lost by conversion to other metabolites via oxaloacetate. The equilibrium constant of 2.3×10^{-5} (Stern, Ochoa and Lynen, 1952) strongly favours the reduction of oxaloacetate to malate, and for the reaction to be reversed a very large NAD/NADH ratio would be necessary. This is unlikely under anaerobic conditions (Yamamoto, 1966 a). It is obvious that the situation is not as clear as the acceleration of glycolysis associated with the induction of alcohol dehydrogenase in non-helophytes.

3. Substrate - induction Experiments.

In order to clarify this situation, an attempt was made to find the inductive stimulus for MDH, using both of the immediate substrates of the enzyme, L-malate and oxaloacetate.

Young plants of two helophyte and two non-helophyte species were raised in sand culture, using Hoagland's solution, and transferred to water-culture in 50 ml glass specimen-jars covered with aluminium foil to exclude light. There were five treatments, each with two replicates:

(i) Control - Hoagland's solution only.

FIGURE 33.

MDH activities in the roots of two non-helophytes grown for three days in water culture containing L-malate and oxaloacetate.

Enzyme activities are expressed as International Units per gram fresh weight root material.

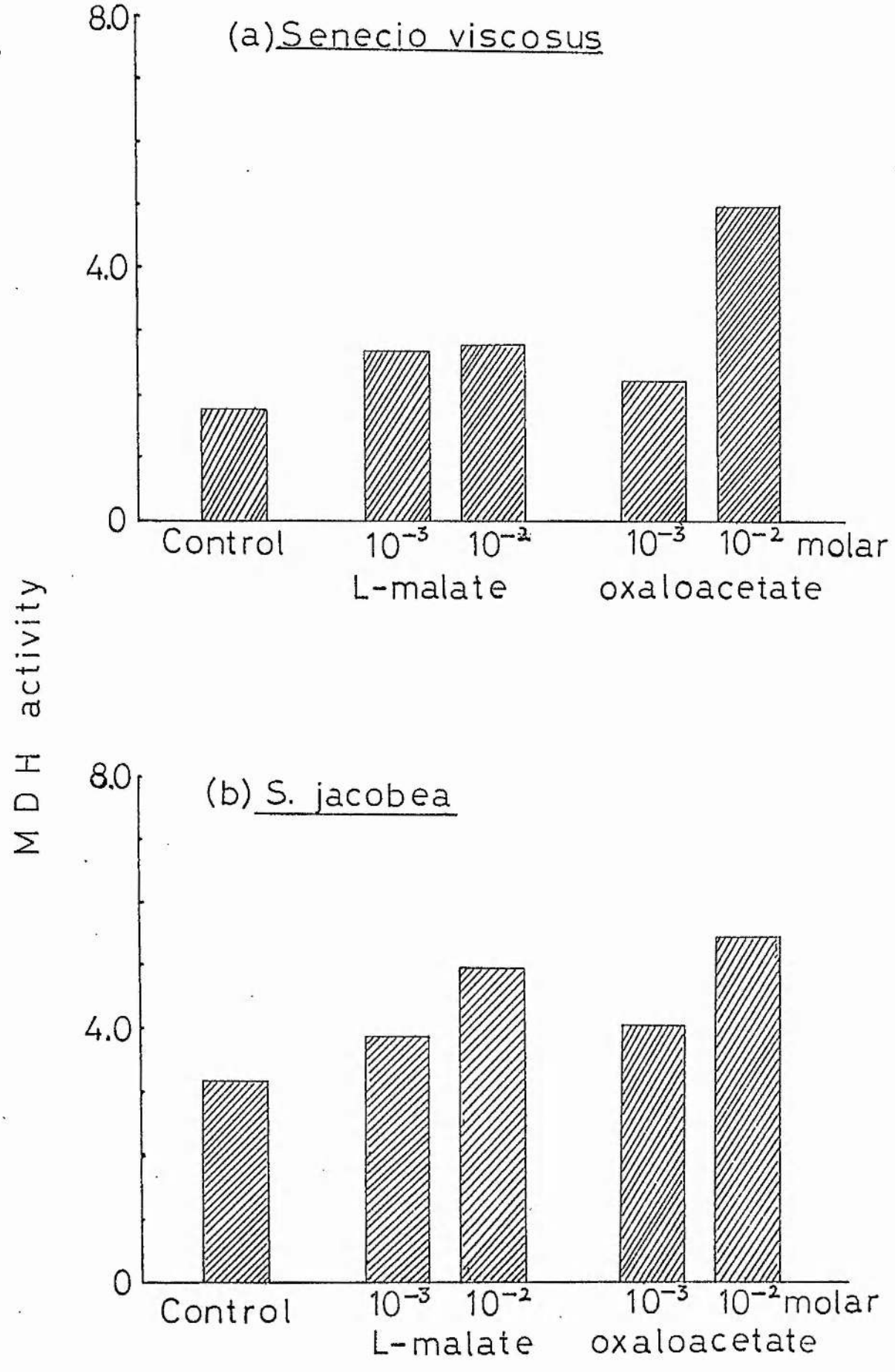
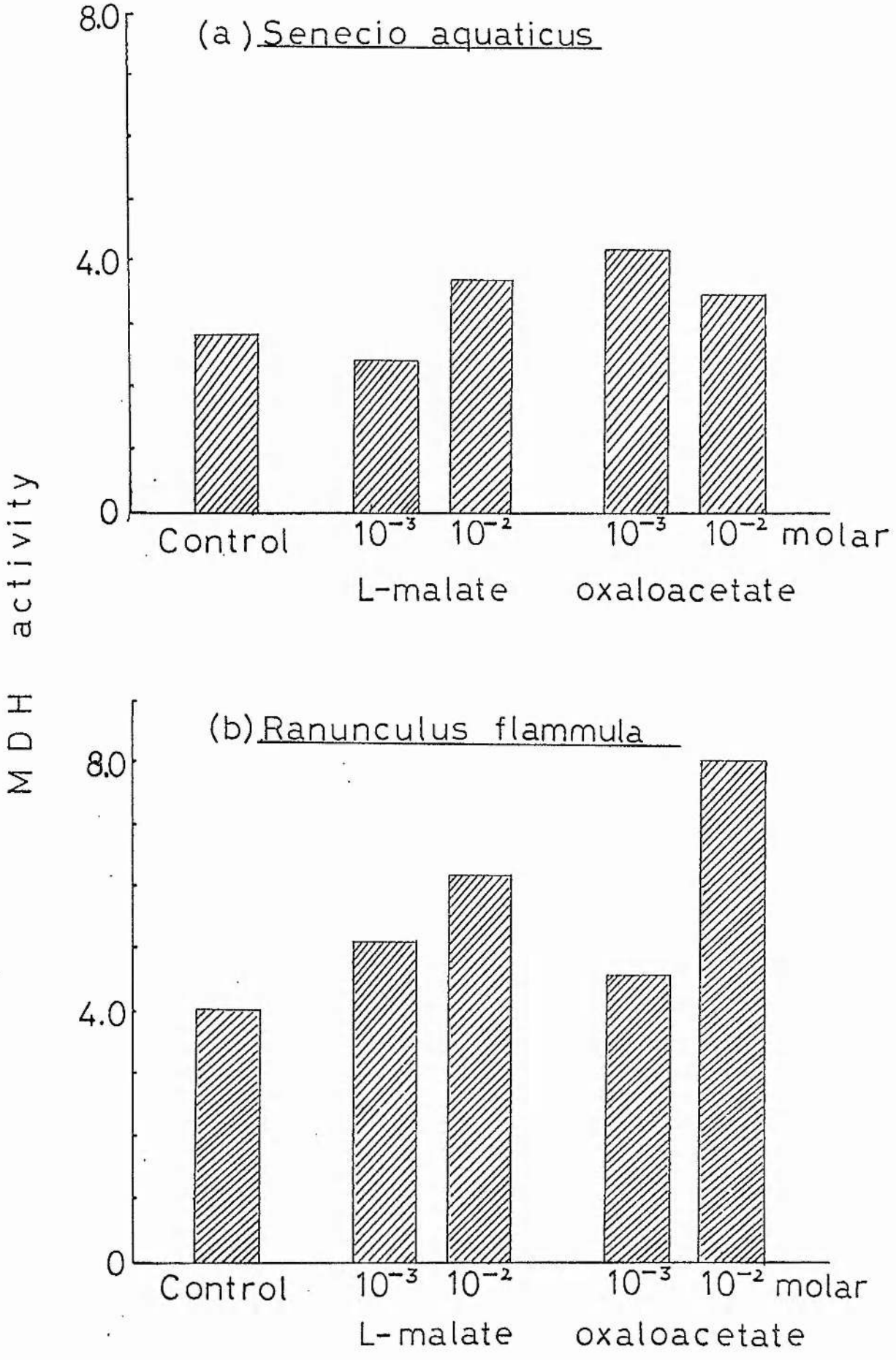

(a) Senecio viscosus
8.0
4.0
0
Control
10^{-3}
10^{-2}
L-malate
10^{-3}
10^{-2} molar
oxaloacetate
M D H activity
(b) S. jacobea
8.0
4.0
0
Control
10^{-3}
10^{-2}
L-malate
10^{-3}
10^{-2} molar
oxaloacetate

FIGURE 34.

MDH activities in the roots of two helophytes grown for three days in water culture containing L-malate and oxaloacetate.

Enzyme activities are expressed as International Units per gram fresh weight root material.


(a) Senecio aquaticus
8.0
4.0
0
MDH activity
Control
10⁻³
10⁻²
10⁻³
10⁻² molar
L-malate
oxaloacetate
(b) Ranunculus flammula
8.0
4.0
0
Control
10⁻³
10⁻²
10⁻³
10⁻² molar
L-malate
oxaloacetate

(ii) Hoagland's solution + 10^{-3} M L-malate

(iii) Hoagland's solution + 10^{-2} M L-malate

(iv) Hoagland's solution + 10^{-3} M oxaloacetate

(v) Hoagland's solution + 10^{-2} M oxaloacetate.

The culture solutions containing the organic acids were neutralised to pH 6.0 using 0.1 N sodium hydroxide. There were no closures on the jars, and free diffusion of air to the culture solutions was permitted. In addition there was intermittent aeration by aquarium pump. These treatments were continued for three days, after which time the roots were removed, washed, and allowed to stand over a period of half-an-hour in several changes of distilled water to remove as much as possible of the malate and oxaloacetate, which otherwise might have interfered with the subsequent enzyme assay. A crude extract was then made as previously described, and assayed for MDH activity. The results are shown in Figures 33 and 34. Because of appreciable variations in the protein contents of the extracts, the enzyme activities are expressed in terms of fresh weight of the root tissue.

There is an increase in MDH activity over the control in almost all cases. Both malate and oxaloacetate therefore seem to stimulate MDH activity when applied in vivo, and in each case this effect

is more noticeable at the higher concentration of applied substrate. There are no apparent differences in the behaviour of different species, and the helophytes react in a similar way to the non-helophytes. This is in marked contrast to the behaviour of the enzyme on growth of the plants in non-aerated water culture (Figures 31 and 32). Also, the degree of induction in the present experiment is much less than in the comparable 3-day non-aerated water culture.

Since the concentrations of malate applied are comparable with, and those of oxaloacetate rather greater than, those found occuring and accumulating naturally (Crawford and Tyler, 1969), it therefore seems doubtful if either of these substances is acting as an inductive agent in the water-culture experiments. This view is reinforced by the lack of difference in behaviour of helophytes and non-helophytes in the present experiments, as compared with the water-culture experiments; the results of Crawford and Tyler (1969) should also be noted: L-malate apparently disappears while MDH activity rises in non-helophytes, and accumulates while MDH activity remains steady in helophytes. These facts make it appear most unlikely that malate is acting as an inductive stimulus for MDH.

A major complication is the presence in the cell of

at least two MDH components (Ting, Sherman and Dugger, 1966), which may have separate functions and behaviour. Malate also occurs in two separate 'pools', one in the mitochondria, produced and used in the Krebs cycle, and one in the cytoplasm apparently produced by carbon dioxide fixation. The two are not easily interchangeable in normal circumstances (Lips and Beevers, 1966). This makes interpretation of metabolic experiments with MDH and malate very difficult. It is likely that any simple explanation on the basis of accumulation of a metabolite and induction of the enzyme would be erroneous. It is not clear from this present study which of the MDH components is induced in anaerobiosis, and neither do Crawford and Tyler's results indicate which of the metabolic pools is concerned with the accumulation and loss of malate on flooding.

The precise role of MDH in the mechanisms of flooding tolerance cannot therefore be deduced from its behaviour as decribed here, as can that of alcohol dehydrogenase, and must remain at this point obscure.

x x x

(ii) "Malic" Enzyme

This is more fully known as NADP - malic dehydrogenase (decarboxylating). Either of two reactions are catalysed, according to pH (Ochoa, 1955):

1. At pH 7.3 malate is oxidised and decarboxylated:

$$\text{L-malate} + \text{NADP} \rightleftharpoons \text{Pyruvate} + \text{NADPH} + \text{CO}_2$$

2. At pH 5.2 oxaloacetate is decarboxylated:

$$\text{Oxaloacetate} \longrightarrow \text{Pyruvate} + \text{CO}_2$$

Mn^{++} ions are necessary for both reactions; other metal ions are less effective.

This enzyme was chosen for study because both reactions catalysed are possible pathways whereby malate may be removed from tissues. Reaction 1 may also act as a route of malate accumulation, being readily reversible, with an equilibrium constant of 19.6 (litres x moles^{-1}) (Hazary, Korey and Ochoa, 1953), favouring malate breakdown to pyruvate under most conditions. This reaction has been most studied.

The enzyme is widely distributed, and was first found in plants by Conn, Vennesland and Kraemer (1949). Only reaction 1 was studied in the experiments described below.

Experimental.

Two series of flooding experiments were carried

out, the first lasting for two weeks and the second for one month. In all, eight species were assayed for "malic" enzyme activity (See Appendix A for the spectrophotometric assay system), after growth for either of these periods under flooded and unflooded conditions as previously described in Part I. In making the extract for enzyme assay, the roots were homogenised in Triethanolamine - HCl - NaOH buffer, 0.1 M, pH 7.5. In earlier experiments Tris - HCl buffer was used, but gave a false assay result due to the high absorption produced at 366 nm when Mn^{++} ions react with Tris. This phenomenon has previously been recorded by De Moss (1955).

Table 16 gives the results of these experiments. The most noticeable point is that "malic" enzyme activity is apparent only in non-helophytes and is entirely absent, or present to a degree too minute to assay, in the helophytes. The activities in the non-helophytes, while very much smaller than those of alcohol and malic dehydrogenases, were quite easily measurable with the techniques and apparatus used, and replicate measurements showed them to be accurate within the prescribed assay conditions. There does not appear to be any significant change in activity on flooding in those species possessing the enzyme.

The absence of "malic" enzyme activity from helophytes is very interesting when we consider the results of Crawford and Tyler, (1969). Any accumulation of malic acid via pyruvate, which would be catalysed by this enzyme, cannot take place in its absence, and other routes must be considered. If the Krebs cycle is still at least partially operative after flooding of helophytes, as would be the case if adequate aeration were provided by diffusion through the plant, then malate would be accumulated by this route. In the absence of an operative Krebs cycle, the only other route of malate accumulation appears to be by the carboxylation of phosphoenolpyruvate to form oxaloacetate, by PEP carboxylase and PEP carboxykinase, which are present in a wide variety of plant tissues. Mazelis and Vennesland (1957) in fact propose that malate is a major product of anaerobiosis in plants, and is formed by this route. Whatever the route of accumulation, the loss of malate via pyruvate is certainly made less likely by the absence of "malic" enzyme from helophytes.

The presence of "malic" enzyme in non-helophytes, however, provides a probable route of the malate loss observed on flooding in these plants by Crawford and Tyler. Decarboxylation of malate by "malic" enzyme is thought to take place in apple fruits during ripening (Dilley, 1966)

Table 16. "Malic" enzyme activities in the roots of plants grown in flooded and unflooded conditions

Enzyme activities are expressed in International Units per mg. protein.

(a) Two Weeks Treatment

	NON-HELOPHYTES		HELOPHYTES	
	Senecio jacobea	Hieracium pilosella	Mentha aquatica	Myosotis scorpioides
Unflooded	0.0013	0.0017	0	0
Flooded	0.0017	0.0016	0	0

(b) One Month Treatment

	NON-HELOPHYTES		HELOPHYTES	
	Senecio viscosus	Pisum sativum	Senecio aquaticus	Glyceria maxima
Unflooded	0.00034	0.0054	0	0
Flooded	0.00023	0.0028	0	0

and in succulents during the day (Walker, 1960). The alternative - oxidation of malate to oxaloacetate - is made less likely by the reducing conditions of anaerobiosis, one result of which is an unsuitably high NADH/NAD ratio (Yamamoto, 1966a). Given the presence and activity of "malic" enzyme in these conditions, then an important factor will be the level of NADP present as a co-enzyme for the reaction. The pyridine nucleotide co-enzymes form the subject of a later section.

In conclusion it is interesting to note that "malic" enzyme is not the first enzyme to be related to flooding tolerance in terms of its distribution rather than its behaviour. Crawford (1967b) records that phenol oxidase activity is also present in non-helophyte root extracts but absent or very low in helophytes, with no change on flooding. Phenol oxidases have been implicated in the metabolism of lignin precursors (Beevers, 1961) such as shikimic acid, which accumulates in subaquatic woody tissues (e.g. rhizomes of Iris pseudacorus and Nuphar luteum) (Boulter, Coult and Henshaw, 1963; Crawford and Tyler, 1969). It is possible that phenol oxidase distribution is related to shikimic acid accumulation, even as "malic" enzyme distribution is linked to malate accumulation. Obviously further investigations on this point are necessary.

Even more interesting is Dilley's (1966) note that apple fruit "malic" enzyme is inactivated by polyphenols, thus providing a possible link between these two enzymes showing very similar distribution but apparently remote function. The exact connection is by no means clear.

x x x

(iii) Phosphoenolpyruvate Carboxylase and PEP Carboxykinase.

These two enzymes both catalyse the carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate. The reactions are:

1. PEP carboxylase

$$PEP + CO_2 \rightleftharpoons \text{Oxaloacetate} + Pi$$

(Pi represents inorganic phosphate)

2. PEP carboxykinase

$$PEP + ADP + CO_2 \longrightarrow \text{Oxaloacetate} + ATP$$

In view of the relationships of malic dehydrogenase behaviour, "malic" enzyme distribution, malate accumulation and loss, and flooding tolerance, all of which have been discussed in the preceding two sections, it is likely that at least one of the two enzymes in this section will have some importance in the regulation of metabolism in conditions of anoxia. PEP carboxylase has already been shown to be involved in malate accumulation in succulent plants (Walker, 1957), and the metabolic diversion from glycolysis to malate accumulation which occurs on flooding of helophytes (Crawford and Tyler, 1969) may be very similar to that found in Crassulacean acid metabolism.

Both enzymes are widely distributed in plants

and are often (though not always) found together. (Mazelis and Vennesland, 1957).

The PEP carboxylase reaction is practically irreversible, and it is generally accepted that the PEP carboxykinase reaction will favour the carboxylation of PEP in most conditions (Vennesland, 1962). Loss of oxaloacetate by decarboxylation would be more likely to take place by a non-enzymic spontaneous process or by the malic dehydrogenase and "malic" enzyme reactions, than by the PEP carboxykinase reaction. An exception is in the synthesis of carbohydrate from organic acids, usually in fatty tissues, where it appears that PEP is indeed formed, with the aid of ATP. The ADP/ATP ratio may therefore be important, and this is dealt with in a later section (Part IV, Section (ii)).

Experimental

Young plants of six species (three helophytes, three non-helophytes) were raised in sand culture and subjected to experimental flooding, with unflooded controls, for one month. The roots were then homogenised and assayed for enzyme activity. Extraction was in Tris-HCl buffer, 0.1 M, pH 7.5. Some of the extracts were stored deep-frozen for several weeks before assay.

Most of the assay systems for these two enzymes involve the incorporation of $C^{14}O_2$ into oxaloacetate in an exchange reaction (Vennesland, 1962; Tchen and Vennesland, 1955). This is not strictly necessary for the irreversible PEP carboxylase reaction, but the PEP carboxykinase assay will not be otherwise accurate except at zero concentrations of oxaloacetate. For the present purposes strict accuracy was not required; the presence and activity of either or both of these enzymes would be enough to establish that the carboxylation of PEP could take place. Accordingly, an assay procedure based on the estimation of oxaloacetate by the malic dehydrogenase reaction was devised. This is detailed in Appendix A. Interference with the assay by other enzymes in the extract (pyruvate kinase, lactic dehydrogenase, pyruvate decarboxylase, alcohol dehydrogenase) certainly occurred, but not to an extent sufficient to invalidate the present assay if the results are only considered on a semi-quantitative basis. Pyruvate kinase activity was probably the greatest source of interference. This is assayed elsewhere (Section (vi)), and is found to be insufficiently high to obscure the activity of the PEP carboxylating enzymes if lactic dehydrogenase is not present in excessive amounts.

PEP carboxykinase activity was found only in Carex arenaria root extracts, and was about one-third that of the PEP carboxylase. Whether this enzyme was absent in the other species or whether the assay system was at fault is not clear. No definite conclusions can be drawn regarding PEP carboxykinase.

The results of the PEP carboxylase assays are shown in Table 17. Lack of root material prevented an assay being made of Carex arenaria (unflooded). Otherwise these results show that all the species tested exhibited a definite PEP carboxylase activity There is no discernible pattern of distribution, and behaviour on flooding is irregular. Apparent differences in activity between the flooded and unflooded treatments are probably not significant. Both helophytes and non-helophytes, therefore, can potentially form oxaloacetate (and therefore malate) by this pathway in both flooded and unflooded conditions. Whether they actually do so will depend on other factors - substrate and co-enzyme levels, activity of alternative pathways, etc. Accumulation of the malate will also depend on loss via other routes, notably the decarboxylation catalysed by "malic" enzyme.

x x x

Table 17. PEP carboxylase activities in the roots of plants grown in flooded and unflooded conditions

Enzyme activities are expressed as International Units per mg. protein.

Species	Unflooded	Flooded
NON-HELOPHYTES		
Ammophila arenaria	0.0036	0.0071
Senecio sylvaticus	0.0014	0.0015
Senecio squalidus	0.0009	0.0010
HELOPHYTES		
Carex arenaria	-	0.0066
Iris pseudacorus	0.0010	0.0019
Mentha aquatica	0.0051	0.0038

(iv) Lactic Dehydrogenase (LDH)

The reaction catalysed is:

Pyruvate + NADH $\rightleftharpoons$ L (+) - lactate + NAD^+

Lactic acid is the normal end-product of glycolysis in animal tissues, and here lactic dehydrogenase occurs universally, being especially abundant in muscle (Schwert and Winer, 1963). A different type of LDH occurs in yeast and is independent of nucleotide co-enzymes (Dixon, 1955).

In higher plants lactate and LDH are much less common. Phillips (1947) found that lactic acid was produced by barley seedlings in anaerobiosis, and lactate has also been found to be important in potato tubers respiring anaerobically (Barker and el Saifi, 1964) and during early germination of peas (Cossins, 1964). Recently, LDH has been demonstrated in Phaseolus vulgaris seedlings and found to show a higher activity when the seedlings were grown under anaerobic conditions. (Sharwin, 1968). If lactic dehydrogenase can be demonstrated in plant roots, then lactate production may be considered, like malate production, as an alternative to alcohol as an end-product of glycolysis.

Experimental

Young plants of four species (two helophytes, two

non-helophytes) were raised in sand culture and flooded for two weeks. A further set of four species were flooded for one month. After this time the roots were homogenised in Tris - HCl buffer, 0.1 M, pH 7.5, and assayed for LDH activity as described in Appendix A. The assay for the nucleotide-dependent type of LDH was used, rather than that for the LDH found in yeast, as the LDH found in pea seedlings by Sherwin was of the former type.

The results are set out in Table 18. LDH activity was detected in most of the species tested, but often in very small amounts. Although there appears to be some induction on flooding of some of the non-helophytes and one helophyte, the activities are really too small for these differences to be considered significant. These figures, together with Crawford and Tyler's (1969) findings that lactic acid was absent, or present only to a very minor degree, in most of the species tested, lead to the conclusion that in more mature plants at least, lactic dehydrogenase is not usually an important enzyme, and plays little part in adaptations to flooding tolerance.

x x x

Table 18. Lactic dehydrogenase activity in the roots of plants grown in flooded and unflooded conditions.

Enzyme activities are expressed in International Units per mg. protein.

(a) Two Weeks Treatment

	NON-HELOPHYTES		HELOPHYTES	
	Senecio jacobea	Hieracium pilosella	Mentha aquatica	Myosotis scorpioides
Unflooded	0.0060	0	0.0010	0.0008
Flooded	0.0134	0.0002	0.0009	0.0011

(b) One Month Treatment

	NON-HELOPHYTES		HELOPHYTES	
	Pisum sativum	Senecio vulgaris	Caltha palustris	Phalaris arundinacea
Unflooded	0	0	0.0028	0.0024
Flooded	0	0.0045	0.0015	0.0058

(v) Glucose 6-phosphate Dehydrogenase (G6 - PDH)

The reaction catalysed is:

Glucose 6-phosphate + NADP $\rightleftharpoons$ 6-phosphogluconate + NADPH

Although glucose 6-phosphate dehydrogenase is not immediately related to the enzymes so far studied, it does catalyse the first step of the pentose phosphate pathway, an alternative to glycolysis and the Krebs cycle as a route of respiration, non-mitochondrial and utilising a different set of pyridine nucleotide co-enzymes. The relative importance of the pentose phosphate pathway in respiration, as indicated by the release of labelled carbon from sugars, has been shown to vary between species (Beevers and Gibbs, 1954), with the age of a tissue (Gibbs and Beevers, 1955), and upon wounding or fungal infection (Daly, Bell and Krupka, 1961). Abarov and Petinov (1964) found an increase in the activity of the pentose phosphate pathway in conditions of soil drought. They measured NADP-dependent dehydrogenase (including G6-PDH) activity. Abarov (1965) found this increase to be due to de novo protein synthesis. Thus adaptive changes in G6-PDH activity may have a part to play in the control of respiration under abnormal conditions.

Experimental

Only one set of experiments was performed, in which four species (two helophytes, two non-helophytes) were grown in sand culture and flooded for one-month periods as previousl;

described. The roots were then homogenised in Tris-HCl buffer, 0.1 M, pH 8.2, and assayed for G6-PDH activity as detailed in Appendix A. The results are set out in Table 19.

Activity was not detected in Mentha root extracts. In the other three species, activity rose to varying degrees on flooding. Whether this is a general phenomenon can only be discovered by extending the experiments to further species. It is conceivable that the pentose phosphate pathway can serve as an alternative to the EMP-TCA pathways under abnormal conditions of various kinds, but the relative importance of the two systems in vivo can only be estimated using isotope techniques.

In preliminary assays of G6-PDH, activity was only detected in about half the species tested. It is not therefore likely to be of general importance in the control of metabolism on flooding.

x x x

Table 19. Glucose 6-phosphate dehydrogenase activity in the roots of four species grown under flooded and unflooded conditions

Enzyme activities are expressed as International Units per mg. protein.

NON-HELOPHYTES	Unflooded	Flooded
Vicia faba	0.0023	0.0205
Senecio sylvaticus	0.0006	0.0012
HELOPHYTES		
Mentha aquatica	0	0
Iris pseudacorus	< 0.0002	0.0042

(vi) Pyruvate Kinase

The reaction catalysed is:

Phosphoenolpyruvate + ADP $\rightleftharpoons$ Pyruvate + ATP

Mg^{++} and K^{+} ions are required for activity.

The conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase is an alternative to the formation of oxaloacetate and malate by the PEP carboxylase reaction (Section (iii)). How the PEP will be metabolised depends on several factors, one of which is the activities of the two enzymes present. Any changes in either enzyme will affect the relative importance of the two pathways.

Experimental

Two sets of four species each were subjected to experimental flooding, for one week and one month respectively, then the roots were homogenised in triethanolamine-HCl-NaOH buffer, 0.1 M, pH 7.5, in which the assay was subsequently performed (Appendix A). The results are shown in Table 20.

Pyruvate kinase is similar to PEP carboxylase in that there is no apparent pattern of distribution or behaviour on flooding. Thus it is unlikely that the actual amounts of these two enzymes play any significant part in the differences in metabolism between helophytes and non-helophytes.

The pyruvate kinase reaction, however, by its requirement for ADP as a co-enzyme, is likely to be affected by the

Table 20. Pyruvate kinase activity in the roots of plants grown under flooded and unflooded conditions

Enzyme activities are expressed in International Units per mg. protein.

(a) One Week Treatment

	NON-HELOPHYTES		HELOPHYTES	
	Senecio jacobea	Hieracium pilosella	Mentha aquatica	Myosotis scorpioides
Unflooded	0.0194	0.0093	0	0.0022
Flooded	0.0032	0.0050	0	0.0017

(b) One Month Treatment

	NON-HELOPHYTES		HELOPHYTES	
	Senecio vulgaris	Vicia faba	Ranunculus flammula	Phalaris arundinacea
Unflooded	0.0040	0.0080	0.0025	0.0830
Flooded	0.0047	0.0380	0.0028	0.0260

ADP/ATP ratio. It is known that this ratio often rises in anaerobiosis, and this fact has been used as a basis for explaining the Pasteur Effect (Beevers, 1961). Anaerobiosis therefore may tend to favour the pyruvate kinase reaction, perhaps at the expense of the PEP carboxylase reaction, which does not require ADP. The adenosine phosphates are treated briefly in Part IV, Section (ii). Obviously further information is needed on the levels of glycolytic intermediates and co-enzymes, such as that given by Effer and Ranson (1967). Until this is available, the data here given for PEP carboxylase and pyruvate kinase activity levels cannot be fully interpreted.

x x x

(vii) Enolase

The reaction catalysed is:

2-phosphoglycerate $\rightleftharpoons$ Phosphoenolpyruvate + H_2O

Mg^{++} ions are required for activity.

An important enzyme in the glycolytic sequence, enolase catalyses the production of phosphoenolpyruvate, a possible point of diversion from the glycolytic pathway to the production of malate.

Experimental

Seven species were tested for enolase activity, using the assay system described in Appendix A. The first group of three (two non-helophytes, one helophyte) were subjected to one week of experimental flooding, and the second group of four species (two non-helophytes, two helophytes) were similarly subjected to one month's flooding. The results are shown in Table 21.

Although there is great variation in enzyme activity in the various extracts, no overall pattern is evident; enolase activity appears to depend neither on the flooding tolerance of the species nor upon the environmental conditions. Activities are generally small; some are extremely small. The accuracy of the assay at these levels is not great, and individual variations are probably not significant. It is improbable that enolase activity is entirely absent from any

Table 21. Enolase activities of root extracts of seven species grown under flooded and unflooded conditions

Enzyme activities are expressed in International Units per mg. protein.

(a) One Week Treatment

	NON-HELOPHYTES		HELOPHYTES
	Senecio jacobea	Hieracium piloselle	Myosotis scorpioides
Unflooded	0.0069	0.0100	<0.0002
Flooded	0.0038	0.0110	0.0040

(b) One Month Treatment

	NON-HELOPHYTES		HELOPHYTES	
	Senecio vulgaris	Vicia faba	Glyceria maxima	Mentha aquatica
Unflooded	0.0052	<0.0002	0.0013	0.0026
Flooded	0.0078	<0.0002	0.0004	0.0028

species, as this would make the glycolytic pathway inoperable, but no special role can be assigned to the enzyme in the metabolic adaptations of flooding tolerance, at least on the data obtained here.

x x x

Experiments on Enzyme Activity Levels - Discussion and Conclusions

With the exception of glucose 6-phosphate dehydrogenase, the seven enzymes studied (counting the PEP carboxylating enzymes as one system) are linked metabolically in that they catalyse an important network of reactions linking the glycolytic and TCA cycle sequences. Further, all these enzymes are theoretically capable of operation in anaerobiosis when normal respiration is slowed or stopped. Figure 35 is a simplified plan of the metabolic pathways involved, and a brief summary of the results of the investigations on the activity of these enzymes is given below.

Alcohol dehydrogenase - present in all species. Activity increases and apparent Km falls only on flooding of non-helophytes. Activity is induced in non-helophytes by acetaldehyde *in vivo*.

Malic dehydrogenase - present in all species. Activity increases in severe anoxia, in non-helophytes only. Activity is induced slightly in all species by L-malate and oxaloacetate *in vivo*.

"Malic" enzyme - present only in non-helophytes. No change in activity on flooding.

PEP carboxylase - present in all species. No regular change on flooding.

FIGURE 35.

Some metabolic pathways involved in flooding tolerance.

ENOL. - enolase

P.K. - pyruvate kinase

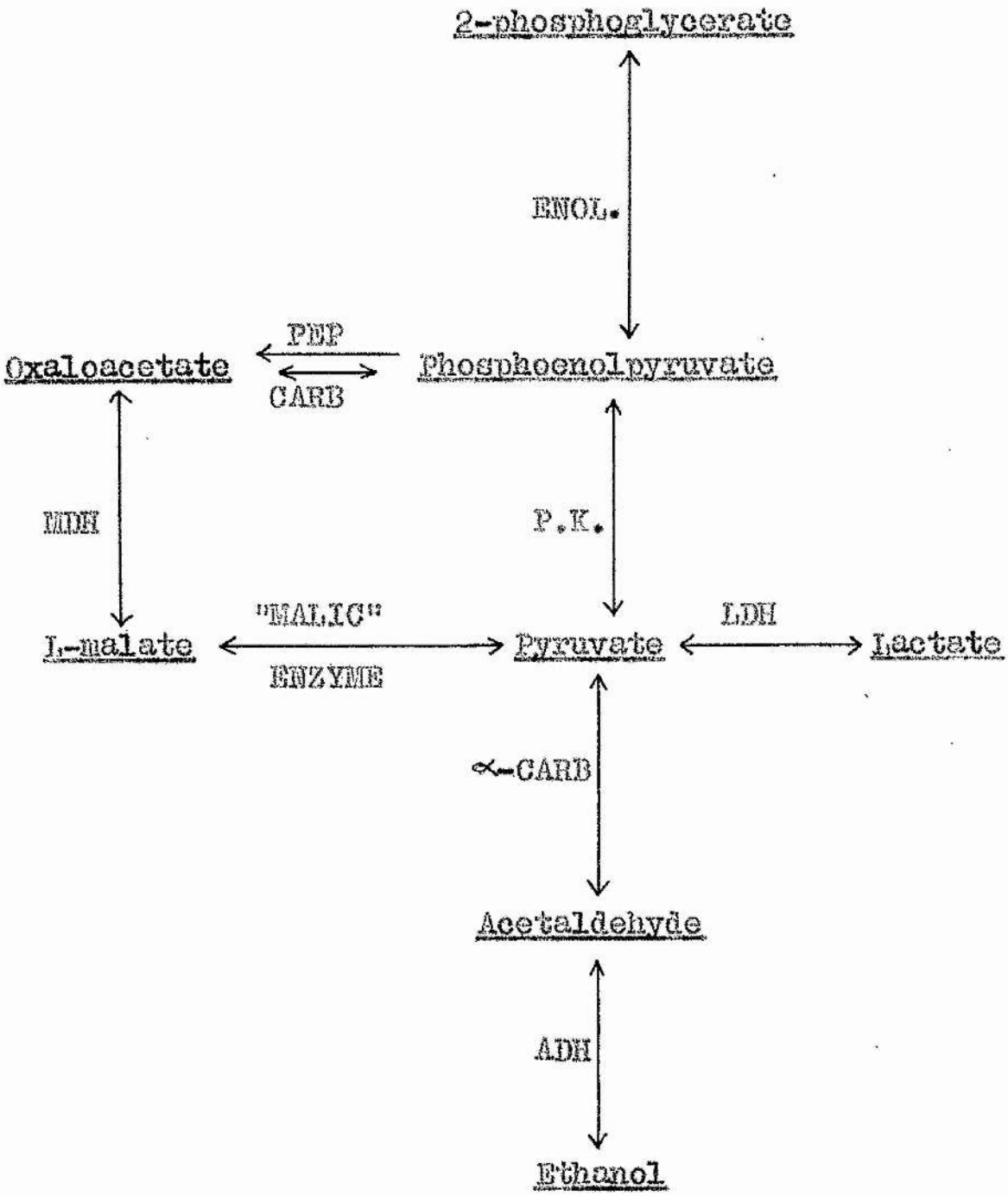
PEP CARB - phosphoenolpyruvate carboxylase and carboxykinase

MDH - malic dehydrogenase

LDH - lactic dehydrogenase

α-CARB - α-carboxylase (not studied)

ADH - alcohol dehydrogenase


2-phosphoglycerate
ENOL.
PEP
Oxaloacetate
CARB
Phosphoenolpyruvate
MDH
P.K.
"MALIC"
L-malate
ENZYME
Pyruvate
LDH
Lactate
∝-CARB
Acetaldehyde
ADH
Ethanol

Lactic dehydrogenase - occurrence irregular.

Pyruvate kinase - present in most species. No significant pattern.

Enolase - present in most species. No significant pattern.

Glucose 6-phosphate dehydrogenase - occurrence irregular. Some rise in activity on flooding, where present.

If these results are considered together with those of Crawford (1966, 1967a) and Crawford and Tyler (1969), concerning the acceleration of glycolysis in, and production of ethanol by, non-helophytes, and accumulation of malate by helophytes in anoxia, then the following scheme of events may be tentatively proposed for this area of metabolism. It is assumed that some degree of anoxia ensues on flooding of both helophytes and non-helophytes (see Introduction, Page 6).

Non-Helophytes

On flooding, normal respiration is blocked and glycolysis proceeds to the production of acetaldehyde and ethanol. Acetaldehyde induces alcohol dehydrogenase activity which, together with a reduction in the apparent Km value, accelerates glycolysis; a positive feedback effect may induce ADH still further. Malate present is decarboxylated by "malic" enzyme to pyruvate and thence to acetaldehyde, contributing further to ethanol production. Oxaloacetate and hence malate

may be formed by the carboxylation of phosphoenolpyruvate, but the malate will not accumulate. Ethanol and acetaldehyde do accumulate, and contribute to the poisoning of metabolism.

Helophytes

On flooding, normal respiration is at least partially blocked, and glycolysis may proceed to the production of acetaldehyde and ethanol, but the former fails to induce ADH, the apparent Km value remains unchanged, and no acceleration of glycolysis ensues. Malate present is not decarboxylated, because malic enzyme is absent. Oxaloacetate and hence malate are produced by the carboxylation of phosphoenolpyruvate, and malate accumulates. This is non-toxic, and may remain without harm to the plant until aerobic conditions are restored.

x x x

Lactate production in anaerobiosis via lactic dehydrogenase probably varies in importance from tissue to tissue, and may occur in both helophytes and non-helophytes. Apparently it is important in young pea (Wager, 1961) and Phaseolus vulgaris (Sherwin, 1968) seedlings. The importance of the pentose phosphate pathway, as indicated by glucose 6-phosphate dehydrogenase activity, also seems variable. It is suggested that the LDH and G 6-P DH reactions are of relatively minor

importance in the mechanisms of flooding tolerance proposed above.

Evidence for the phosphoenolpyruvate → oxaloacetate → malate (→ pyruvate) sequence, catalysed by PEP carboxylase malic dehydrogenase, and "malic" enzyme is provided by Ting and Dugger (1965), who found just such a sequence in corn root tissue. Walker (1962) considers this system to be of general importance in the metabolism of higher plants.

Features which the proposed scheme does not explain are the induction of malic dehydrogenase activity in non-helophytes by severe anoxia, and the similar but smaller induction caused by in vivo application of malate and oxaloacetate in all species. These apparent inductive effects are incompatible on first view with a rise in malate content of helophytes in anoxia, and a fall in non-helophytes (Crawford and Tyler, 1969). A rise in malic dehydrogenase activity in anoxia should tend to increase the production of malate in non-helophytes, the equilibrium of the reaction being highly favourable to this view (Mazelis and Vennesland, 1957). As has already been mentioned, however, simple tissue level data for MDH and malate are likely to be misleading, as both are subject to compartmentation within the tissues. Ting, Sherman and Dugger (1966) found one cytoplasmic and two mitochondrial enzymes, and suggest different functions

for them. Lips and Beevers (1966) similarly distinguish two malate pools, one cytoplasmic and one mitochondrial. These pools seem to be physically separated, so it is not correct to assume that the total malate content of the root should be related to the total MDH activity, even if induction is a real phenomenon here.

The metabolic system of flooding tolerance as proposed so far relies entirely on enzyme distribution and behaviour, and substrate levels. The levels of co-enzymes available for the reactions involved are obviously very important in deciding whether or not any particular reaction will proceed. Especially important is the oxidation-reduction state of the pyridine nucleotides (NAD/NADH and NADP/NADPH ratios), co-enzymes of the dehydrogenases studied, and the degree of phosphorylation of the adenosine phosphates (ADP/ATP ratio). These co-enzymes are the subject of Part IV.

ADDENDUM

Since Parts II and III were written, Glasziou (1969) has published a comprehensive review on the control of enzyme formation and inactivation in plants.

x x x

PART IV

CO-ENZYMES

PART IV CO-ENZYMES

Introduction

All of the enzymes studied, except PEP carboxylase and enolase, depend for their activity upon the pyridine nucleotide co-enzymes NAD and NADP and their reduced forms NADH and NADPH, or upon the adenosine phosphate co-enzymes ATP and ADP. The levels of these co-enzymes available will therefore affect the rate and extent of a particular enzyme-catalysed reaction.

Because of the large number of enzymes associated with these co-factors, many not involved in the areas of metabolism studied here, it would be unrewarding to theorise on possible rates and routes of production, use, and regeneration of the co-enzymes, and their effect on the rates of enzyme reactions studied. A more practical approach is to determine the tissue levels of these metabolites, and attempt to relate any differences observed (between species, environments, experimental treatments) to possible changes in enzyme reaction rates. There are two major problems involved in this approach:

1. The degree of compartmentation of co-enzymes

is only imperfectly known. All of the co-enzymes here studied are known to occur within and without the mitochondria. The degree of permeability of the mitochondria to the co-enzymes in their various forms is in doubt (Burges and Hurst, 1965). The compartmentation of ATP even in glycolysis, on an easily-disintegrated 'glycolytic particle', has been proposed by Barker, Khan and Solomos (1966). Thus simple tissue levels can be misleading, and may be significant only on a semi-quantative basis.

Lack of information on compartmentation makes even more difficult the estimation of the effects of competition for the co-enzymes by other enzyme reactions. As all the enzymes studied exist at least partially in the extramitochondrial soluble phase, it can only be provisionally assumed that the tissue level of a particular co-enzyme is that available to all enzymes equally.

2. The extremely rapid interconversion of various forms of the co-enzymes (oxidation/reduction of the pyridine nucleotides; phosphorylation of the adenosine phosphates) necessitate great care in extraction. Otherwise co-enzyme levels in tissue extracts may bear no relation to those in the living plant. Moss (1963) recommends that tissues be frozen

rapidly, using metal tongs cooled in liquid air, but this may be necessary only with mammalian tissues. Also, the morphology of many plant tissues makes this method impractical. Yamamoto (1963, 1966a) has obtained good results merely be ensuring that the time spent in handling tissues before homogenisation is reduced to the minimum. Some delay is often unavoidable, however, and duplicate experiments involving different handling times are essential if this source of error is to be minimised.

If co-enzyme levels are to be of significance in enzyme reaction rates, then the levels must be rate-limiting for a particular reaction. If they are not, then changes in the amounts of co-enzyme will not affect the rate. Yamamoto (1963) found that both NAD and NADP levels were rate-limiting factors in the metabolism of a variety of plant tissues. Both the pyridine nucleotides and the adenosine phosphates have been frequently quoted as rate-limiting factors in respiration, glycolysis, and other areas of metabolism (Wolstenholme and O'Connor, 1959; Umbarger, 1963). It is therefore assumed that any variation found in the present study will be of significance in affecting the course of metabolism in flooded and unflooded plants,

bearing in mind the considerable sources of error outlined above.

x x x

(i) Pyridine Nucleotides

When a plant is flooded, oxygen is unavailable to the terminal cytochrome oxidase, and NADH produced by the operation of the Krebs cycle can no longer be oxidised through the cytochrome system with the production of ATP and regeneration of NAD. The Krebs cycle activity thus ceases, at least partially, until NAD is again available. Under these conditions, therefore, NADH can be expected to accumulate. Of course, other, extramitochondrial, routes of NAD production are available (the alcohol and malic dehydrogenase reactions, for example), but the ability of pyridine nucleotide co-enzymes to penetrate mitochondria is limited (Burges and Hurst, 1965). Also, as glycolysis proceeds, the extramitochondrial NAD is used again in the triose phosphate dehydrogenase reaction.

This outline is grossly oversimplified and ignores many other dehydrogenase systems which involve this co-enzyme, but it does serve to show that in conditions of anoxia the tendency will be for the NAD/NADH ratio to fall. Less NAD will be available for oxidative energy (ATP) - producing reactions, and the metabolic activity and growth of the plant may decline. A plant which by metabolic or physical means manages to

maintain its NAD/NADH ratio therefore has the advantage, as long as poisonous metabolites such as alcohol are not produced in the process. The accumulation of malate by helophytes (Crawford and Tyler, 1969) can now be seen as a means of maintaining an adequate supply of oxidised co-enzyme in conditions of anoxia.

The position of NADP and its reduced form is less clear. Two of the enzymes studied utilise this co-enzyme (glucose 6-phosphate dehydrogenase and "malic" enzyme). "Malic" enzyme is apparently important in the anaerobic metabolism of non-helophytes, converting malate to pyruvate. If this system is to operate as proposed, then a supply of NADP, the oxidised form of the co-enzyme, is necessary. The source of this is not clear. One possibility is the NAD kinase reaction discussed by Yamamoto (1966b), in which NADP is formed from NAD and ATP. This seems unlikely in view of the reduction in efficiency of this system under anaerobic conditions (NADH is not utilised). In the absence of information on this point, therefore, it was decided to investigate tissue levels of both pyridine nucleotide co-enzymes to see whether or not they behaved similarly.

In any study of the pyridine nucleotide co-enzymes, more stress should be laid upon the ratio of oxidised

and reduced forms than upon absolute tissue levels, as the latter will necessarily vary with the type and condition of the tissue used. The NAD(H)/NADP(H) ratio may also be important (Yamamoto, 1966a).

Experimental

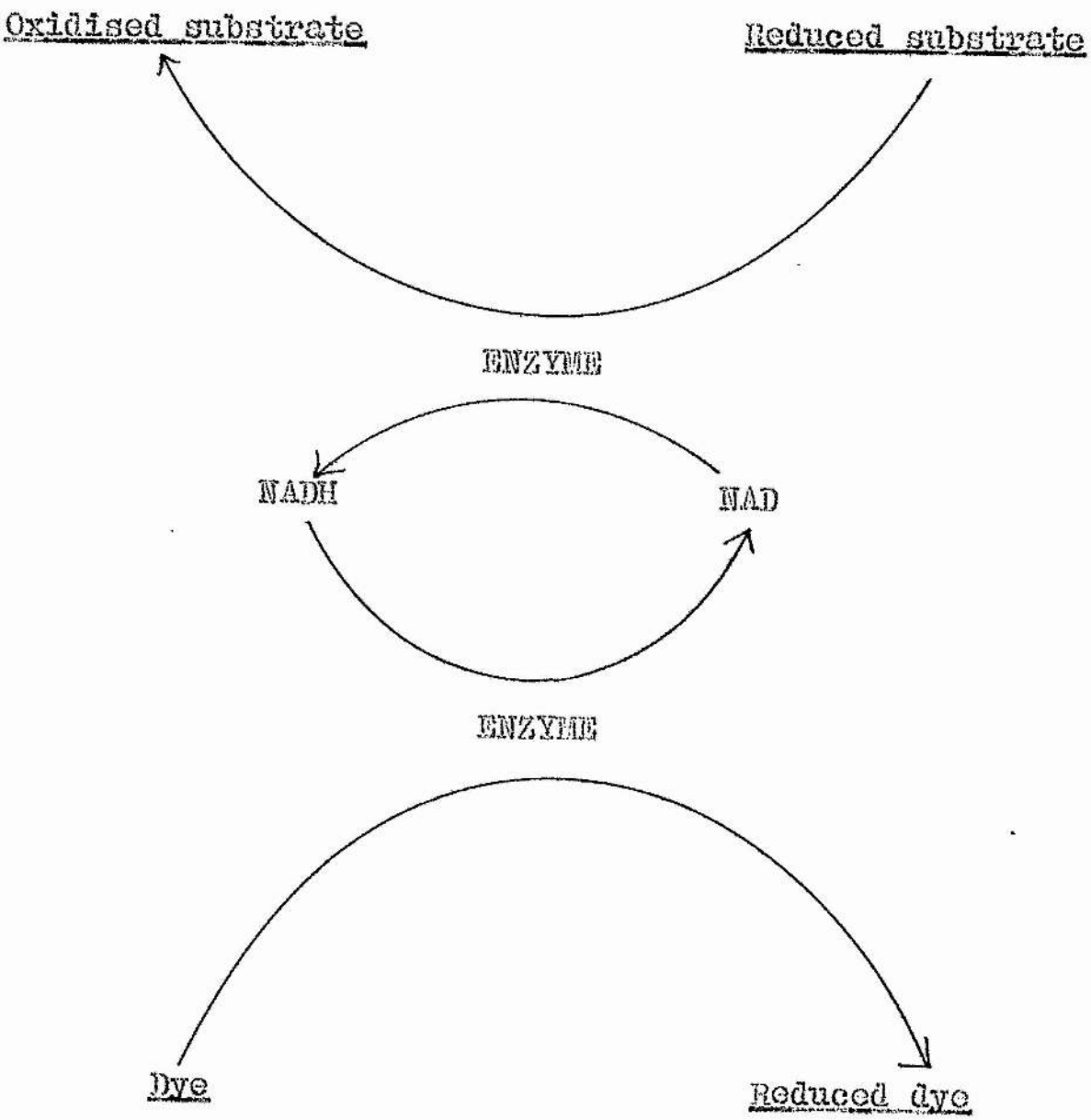
All of the enzyme assays described in Appendix A depend directly or indirectly upon the measurement of the oxidation or reduction of pyridine nucleotide co-enzymes by the change in extinction at 366 mm. The direct spectrophotometric estimation of NAD(H) and NADP(H) is therefore potentially very simple, and this method has been applied utilising a variety of dehydrogenases (e.g. Ciotti and Kaplan, 1957; Klingenberg, 1963). Unfortunately the sensitivity of this method is limited to approximately 10^{-6} moles per gram fresh weight of tissue, even using the shorter wavelength of 340 mm (with a greater extinction coefficient for NADH and NADPH).

Glock and McLean (1955) have developed a much more sensitive method in which metabolic levels of pyridine nucleotides can easily be estimated, down to 10^{-9} moles per gram fresh weight. The pyridine nucleotide is involved in a continuous oxidation-reduction cycle utilising two enzymes and their substrates, one of which is a dye which is continuously reduced, as is

shown in Figure 36. The progress of the reaction is followed spectrophotometrically as the dye is reduced. As the co-enzyme is acting catalytically, this method is very sensitive. Glock and McLean used cytochrome C and cytochrome C reductase, following the reaction at 550 nm. Marré and Bianchetti (1961) and Yamamoto (1963) modified the method to use dichlorphenol indophenol (DCIP) and diaphorase. The latter system was used in the present study. Any suitable NAD- or NADP- dependent dehydrogenase of sufficient purity may be used, together with its reduced substrate, for the other coupled reaction. In the present case the alcohol dehydrogenase and glucose 6-phosphate dehydrogenase reactions were utilised for the NAD(H) and NADP(H) estimations respectively. The complete assay systems are given below. The reaction rates were measured by following the reduction of DCIP at 578 nm on the Eppendorf photometer and recorder described in Appendix A. Standard curves were prepared, using solutions containing from 2×10^{-9} moles to 10^{-7} moles, and a stoichiometric relationship between reaction rate and pyridine nucleotide content was maintained throughout this range. After each assay using a tissue extract, a known amount of pure pyridine

FIGURE 36.

Reactions involved in the pyridine nucleotide assay.


Oxidised substrate
Reduced substrate
ENZYME
NADH
NAD
ENZYME
Dye
Reduced dye

nucleotide was added to the experimental cuvette to discover to what extent the enzymes of the assay system were inhibited by substances in the root extract. Some extracts caused considerable inhibition, and in these cases the results were discarded as invalid.

Assay system for NAD and NADH (Modified from Yamamoto, 1963)

Each experimental cuvette contained (in a total volume of 3.0 ml):

170 umoles Tris-HCl, pH 7.6

0.25 umoles dichlorphenol indophenol

Tissue extract (0.5 or 1.0 ml)

These were mixed and left to stand at room temperature for 30 minutes, to allow endogenous reductive processes to be completed, before adding:

Alcohol dehydrogenase - 0.1 mg protein.

NADH diaphorase - 0.05 mg protein.

The reaction was then initiated by adding:

0.1 ml 95% ethanol.

Assay system for NADP and NADPH (Modified from Marré and Bianchetti, 1961)

Each experimental cuvette contained (in a total volume of 3.0 ml):

170 umoles Tris-HCl, pH 7.6

0.25 umoles dichlophenol

Tissue extract (0.5 or 1.0 ml)

To P83

These were mixed and left for 30 minutes as described in the other system, before adding:

Glucose 6-phosphate dehydrogenase - 0.05 mg protein

NADPH diaphorase - 0.1 mg protein

The reaction was initiated by adding:

0.1 ml of 0.1M glucose 6-phosphate.

The NADPH diaphorase was prepared from spinach chloroplasts by the method of Avron and Jagendorf (1956), up to and including the resolubilization of the cold acetone precipitate. All other enzymes and substrates were obtained commercially.

As will readily be seen, this method of estimation does not distinguish between the oxidised and reduced forms of the co-enzymes, therefore these must be separated during the preparation of the extract. To achieve this, use is made of the lability of the oxidised co-enzyme in hot alkali and the similar lability of the reduced form in hot acid. The extracts were made as follows:

Between one and two grams of root tissue was quickly rinsed, blotted and weighed, then transferred to 5 or 10 mls of 0.1N HCl or NaOH maintained at 90 - 95 ^{o}C in a morter heated over a water-bath. The roots were then ground for two minutes exactly

(the disintegrative effect of the acid and alkali made the addition of sand unnecessary), and the homogenate was poured into a 25 ml beaker in an ice-bath and allowed to cool before being neutralised to pH 7.6 using 0.1 N HCl or NaOH. One ml of Tris-HCl, 0.1 M, pH 7.6 was added to stabilise the pH. The final volume was then recorded, and the extract centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was stored deep-frozen until assayed. The oxidised pyridine nucleotides (NAD and NADP) were estimated in the neutralised acid extracts, and the reduced forms (NADH and NADPH) in the neutralised alkali extracts.

Two replicate (different plant) extracts were prepared from each experimental treatment, and as each replicate necessarily involved a different handling time after removal from the experimental environment (usually flooded or non-flooded sand culture), this acted as a check on any changes in pyridine nucleotide ratios which may have taken place during extraction.

This extraction method of distinguishing the oxidised and reduced nucleotides is very efficient. In experiments using known added amounts of the co-enzymes, the recovery of NAD from the hot acid was between 90% and 95%, while none survived the hot alkali extraction, and vice versa with NADH. Comparable results

were obtained using NADP and NADPH.

Eight species in all were used. Each was subjected to one month's experimental flooding, with unflooded controls, before the roots were extracted and assayed as discribed. The results of the flooding experiments are presented in Tables 22 and 23. In addition, Pisum sativum was subjected to water culture in aerated and non-aerated conditions, as described in Part II, (Page 37), for 8 hours, then the roots assayed for pyridine nucleotide content. Table 24 shows the results of this experiment.

Where a zero figure appears in any of the tissue level figures, this means that pyridine nucleotides were present at less than accurately measurable levels (about 0.4×10^{-9} moles per gram fresh weight could be measured). Oxidised/reduced ratios derived from such figures are very approximate. The absence of detectable NAD in any of the Pisum extracts is difficult to explain, and seems to be a phenomenon of older roots. In a preliminary assay using pea seedling roots, large amounts of NAD (22×10^{-9} moles/gm fresh weight) were detected. This confirms the results of Yamamoto (1963), who used Vigna sesquipedalis roots.

The figures for NAD and NADH are very variable, making their significance doubtful. Nevertheless the

Table 22. Effect of flooding on Pyridine nucleotide root tissue levels - NAD and NADH

Pyridine nucleotide levels are expressed as moles x 10^{-9} per gram fresh weight.

	Treatment	(NAD)	(NADH)	(NAD)/(NADH)
NON-HELOPHYTES				
Senecio jacobea	Unflooded	5.9	1.3	4.5
	Flooded	4.7	16.2	0.3
Pisum sativum	Unflooded	0	1.7	0
	Flooded	0	1.2	0
Hieracium pilosella	Unflooded	0.5	0	>10
	Flooded	3.5	3.3	1.1
HELOPHYTES				
Caltha palustris	Unflooded	2.0	0.8	2.5
	Flooded	0.5	0.4	1.25
Mentha aquatica	Unflooded	13.1	0	>100
	Flooded	4.8	0	>20
Glyceria maxima	Unflooded	0	3.7	0
	Flooded	6.9	1.4	4.9

Extracts made from Vicia faba and Juncus effusus roots caused almost complete inhibition of the assay systems, and the results were discarded.

Table 23. Effect of flooding on pyridine nucleotide root tissue levels - NADP and NADPH.

Pyridine nucleotide levels are expressed as moles x 10^{-9} per gram fresh weight.

	Treatment	(NADP)	(NADPH)	(NADP)/(NADPH)
NON-HELOPHYTES				
Senecio jacobea	Unflooded	7.0	6.7	1.05
	Flooded	10.6	6.3	1.7
Pisum sativum	Unflooded	0	1.2	0
	Flooded	6.4	0	> 40
Hieracium pilosella	Unflooded	0	1.2	0
	Flooded	1.4	1.9	0.74
HELOPHYTES				
Caltha palustris	Unflooded	1.3	2.0	0.65
	Flooded	0.8	1.0	0.8
Glyceria maxima	Unflooded	2.4	4.7	0.51
	Flooded	0	8.3	0

Extracts made from Vicia faba, Juncus effusus, and Mentha aquatica roots caused almost complete inhibition of the assay systems, and the results were discarded.

Table 24. Effect of aerated and non-aerated water culture on pyridine nucleotide root tissue levels in Pisum sativum.

Pyridine nucleotide levels are expressed as moles x 10^{-9} per gram fresh weight.

(a) NAD and NADH

Treatment	(NAD)	(NADH)	(NAD)/(NADH)
8 hrs. aerated	0	0.8	0
8 hrs. non-aerated	0	2.5	0

(b) NADP and NADPH

Treatment	(NADP)	(NADPH)	(NADP)/(NADPH)
8 hrs. aerated	1.5	16.4	0.09
8 hrs. non-aerated	2.8	10.4	0.27

three species showing a rise in NADH levels in anoxia are all non-helophytes (flooded Senecio jacobea and Hieracium, and Pisum in non-aerated water culture). NAD levels in anoxia do not seem to fall as expected in Senecio jacobea and Hieracium. The helophytes show a range of behaviour, and no general conclusions can be drawn here.

The NADP and NADPH results are rather more interesting. It is clear that in all the non-helophytes NADP levels are maintained and even increased in conditions of anoxia, while the reverse is true of the helophytes. The levels of the reduced co-enzyme are rather more variable.

The increase in NADP levels (and the NADP/NADPH ratio) on flooding of non-helophytes is very difficult to explain, contradicting as it does the results of Yamamoto (1966a), whopostulates the formation of NADP from NAD, a process occurring more readily in aerobic conditions, where (oxidised) NAD is freely available. The other main source of NADP has been quoted as fat metabolism (Beevers,1961), which is unlikely in the tissues used here. Stafford and Vennesland (1953) have described an NADP-dependent alcohol dehydrogenase from wheat-germ, and suggest a linkage of the pentose phosphate pathway with NADP-dependent ADH activity. In the course of experiments with ADH in the present work, a large

apparent NADP-dependent ADH activity was detected in Senecio viscosus and Hieracium pilosella, but in vitro tests for the accumulation of NADP by this route (using glucose 6-phosphate and glucose 6-phosphate dehydrogenase) were negative.

Whatever the source of the NADP of non-halophytes in anoxia, it is evident that the oxidised co-enzyme is present in sufficient quantities for the loss of malate by the "malic" enzyme route to take place as suggested (See Part III, Page 98). In fact, Stafford and Vennesland's suggestion (above) could be modified to link malate decarboxylation to NADP-dependent ADH activity if the latter could be shown to be an important feature of non-halophyte metabolism.

x x x

(ii) Adenosine Phosphates

Although two of the enzyme reactions studied require ADP (the PEP carboxykinase and pyruvate kinase reactions), it is difficult to say at what point the levels of this co-enzyme will seriously limit their operation. It has been found that the ADP/ATP ratio can rise in anoxia (Rowan, Seaman, and Turner, 1956), and this fact has been adduced to explain the Pasteur effect, by the acceleration of the phosphoglyceryl kinase and pyruvate kinase reactions which may ensue. In view of the many competing reactions, and problems of compartmentation regarding the adenosine phosphates, such mechanisms must remain unproven at present.

Also important are the levels of ATP available for general cellular work, water and mineral uptake, solute transport, and the various processes of growth. If energy from ATP is not available for these, growth will cease and the plant will be put at a serious disadvantage.

Experimental

Two species (one helophyte, one non-helophyte) were subjected to experimental flooding for one month as described in Part I. The roots were then quickly washed in distilled water, blotted, weighed, and homogenised in a deep-frozen (-20 °C) mortar, using 3 ml of 6% (W/V) ice-cold perchloric acid per gram of root

tissue. The homogenate was then allowed to thaw, and centrifuged at 12,000 g for 20 minutes at 4°C. The supernatant was neutralised to pH 7.4 using 5 x molar potassium carbonate solution. After noting the final volume, the potassium chlorate was allowed to settle, at 4°C, taking approximately ten minutes. The supernatant was decanted and assayed for ADP and ATP.

Adenosine diphosphate was assayed using the pyruvate kinase reaction (Adam, 1963). The pyruvate produced during the reaction was reduced using lactic dehydrogenase and NADH. The oxidation of the latter was followed at 366 nm, and gave a measure of the extent of the reaction. The following are the reactions involved:

Phosphoenolpyruvate + ADP $\rightleftharpoons$ Pyruvate + ATP

Pyruvate + NADH $\rightleftharpoons$ Lactate + NAD

The assay system used was as follows. Each cuvette contained (in a total volume of 3.0 ml):

Triethanolamine-HCl-NaOH buffer, 0.1 M, pH 7.5

Root extract	-	0.2 to 1.0 ml
0.1 M $MgCl_2$	-	0.1 ml
0.1 M KCl	-	0.1 ml
0.1 M PEP	-	0.05 ml
0.1 M NADH	-	0.05 ml
LDH	-	0.01 mg protein.

At this point a reaction occurred due to pyruvate contamination of the phosphoenolpyruvate. When this ceased, there was added

Pyruvate kinase - 0.01 mg protein

The decrease in optical density over the next ten minutes was used to calculate the ADP content of the solution and hence of the root tissue.

Adenosine triphosphate was similarly measured using the hexokinase reaction (Lamprecht and Trautschold, 1963). This was coupled to the glucose 6-phosphate dehydrogenase reaction, and the reaction was again followed by measuring the change in extinction at 366 nm, consequent upon the reduction of NADP. The reactions involved are as follows:

Glucose + ATP $\rightleftharpoons$ Glucose 6-phosphate + ADP

Glucose 6-phosphate + $NADP \rightleftharpoons$ 6-phosphogluconate + NADPH

The assay system used is shown below. Each experimental cuvette contained (in a total volume of 3.0 ml):

Triethanolamine-HCl-NaOH buffer, 0.05 M, pH 7.5

Root extract - 0.2 to 1.0 ml

0.1 M $MgCl_2$ - 0.1 ml

0.1 M NADP - 0.05 ml

Glucose 6-phosphate dehydrogenase - 0.01 mg protein

Any reaction due to glucose 6-phosphate in the root extract was allowed to go to completion, before adding:

0.5 M glucose - 0.4 ml.

Hexokinase - 0.01 mg protein.

The increase in optical density at 366 nm was then measured after 15 minutes, and this figure used to calculate the ATP content of the root tissue.

The results are presented in Table 25. Each figure is the average of two independent (different plant) replicates involving different handling times during extraction; to check on the effect of extraction procedures upon the adenosine phosphate levels in the tissue.

At first sight, these results would seem to be the opposite of those which may be expected.

In the non-halophyte, showing what may be termed a Pasteur effect (acceleration of glycolysis - see Crawford, 1966), the ADP/ATP ratio nevertheless falls on flooding, and the ATP available for cellular metabolism actually increases. In the halophyte, where the acceleration of glycolysis is avoided and growth continues, the ADP/ATP ratio nevertheless rises.

It must be remembered, however, that these figures reflect the situation a full month after flooding is initiated, and the situation may be quite different from that which exists immediately after the roots are deprived of oxygen. The following scheme is suggested:

Table 25. Effect of flooding upon adenosine phosphate levels in root tissues

Adenosine phosphate levels are expressed as umoles per gram fresh weight.

Species	Treatment	(ADP)	(ATP)	(ADP)/(ATP)
Senecio jacobea	Unflooded	0.058	0.023	2.5
(Non-helophyte)	Flooded	0.032	0.108	0.3
Mentha aquatica	Unflooded	0.049	0.069	0.7
(Helophyte)	Flooded	0.125	<0.010	>10

As the metabolism of the non-helophyte is slowly inhibited by the production of ethanol and by the presence of anaerobic decomposition products in the sand, it is unable to utilise the ATP, produced by the accelerated glycolysis, in synthetic events and cellular work. Therefore, after an initial fall, ATP accumulates and ADP levels fall. In the helophyte, on the other hand, although metabolic damage is avoided, normal respiration is still probably curtailed to some extent, and ATP production therefore reduced. Since growth continues as normal, ATP is utilised at the same rate as before flooding, therefore the amount of free ATP falls, and ADP rises.

One further point will be made, namely that normal glycolysis as it occurs in non-helophytes, probably via pyruvate kinase, involves a nett production of ATP, while the glycolysis suggested for helophytes, with its diversion to malate accumulation via PEP carboxylase, involves no nett production of ATP (The PEP carboxykinase reaction does involve ATP production, but this was not found to be important). This may further contribute towards the long-term accumulation of ATP in *Senecio jacobea* roots on flooding, and its disappearance from *Mentha aquatica*.

These conclusions may be rather over-extended, considering that only two species were compared, although they were very typical, in other ways, of their respective groups. Also, other ATP-ADP reactions, such as that catalysed by adenosine triphosphatase, were not considered. Extension of these experiments to further species is necessary before the conclusions are confirmed.

The metabolic scheme proposed in relation to flooding tolerance in Part III (Discussion) can now be extended to include the co-enzymes involved. It is presented in Figures 37 and 38.

x x x

FIGURE 37.

The metabolic scheme proposed for the flooding of non-helophytes

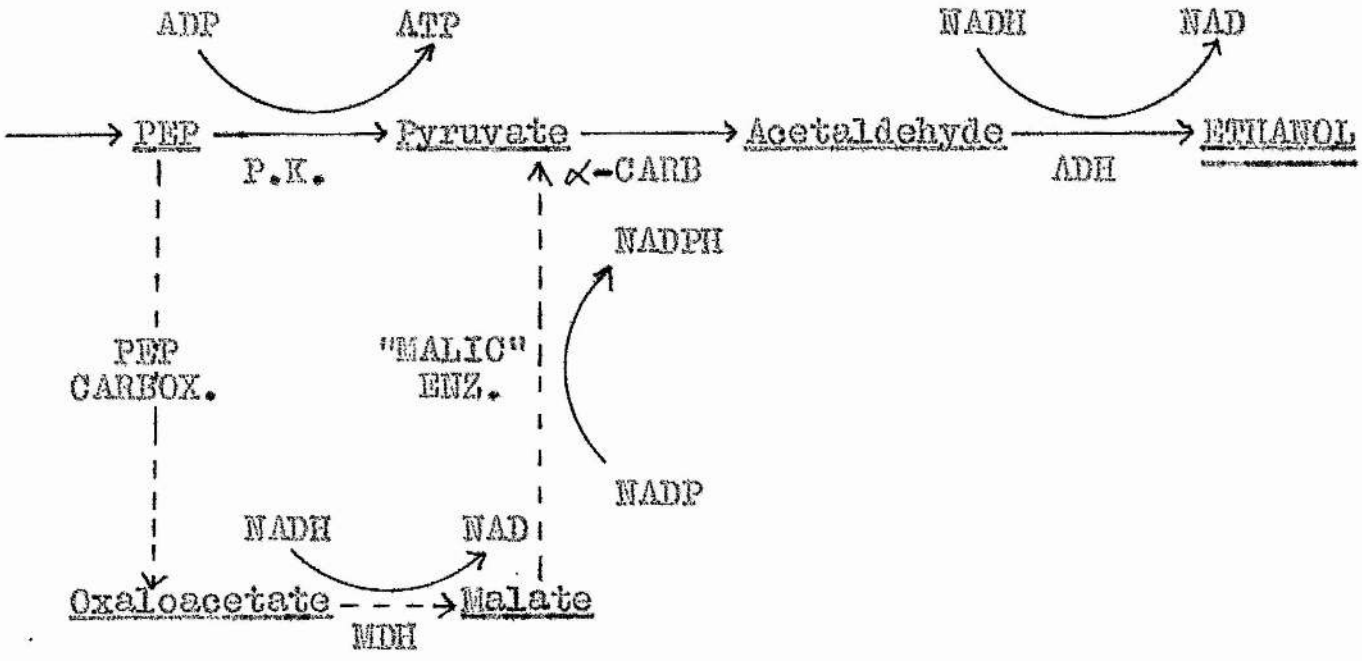
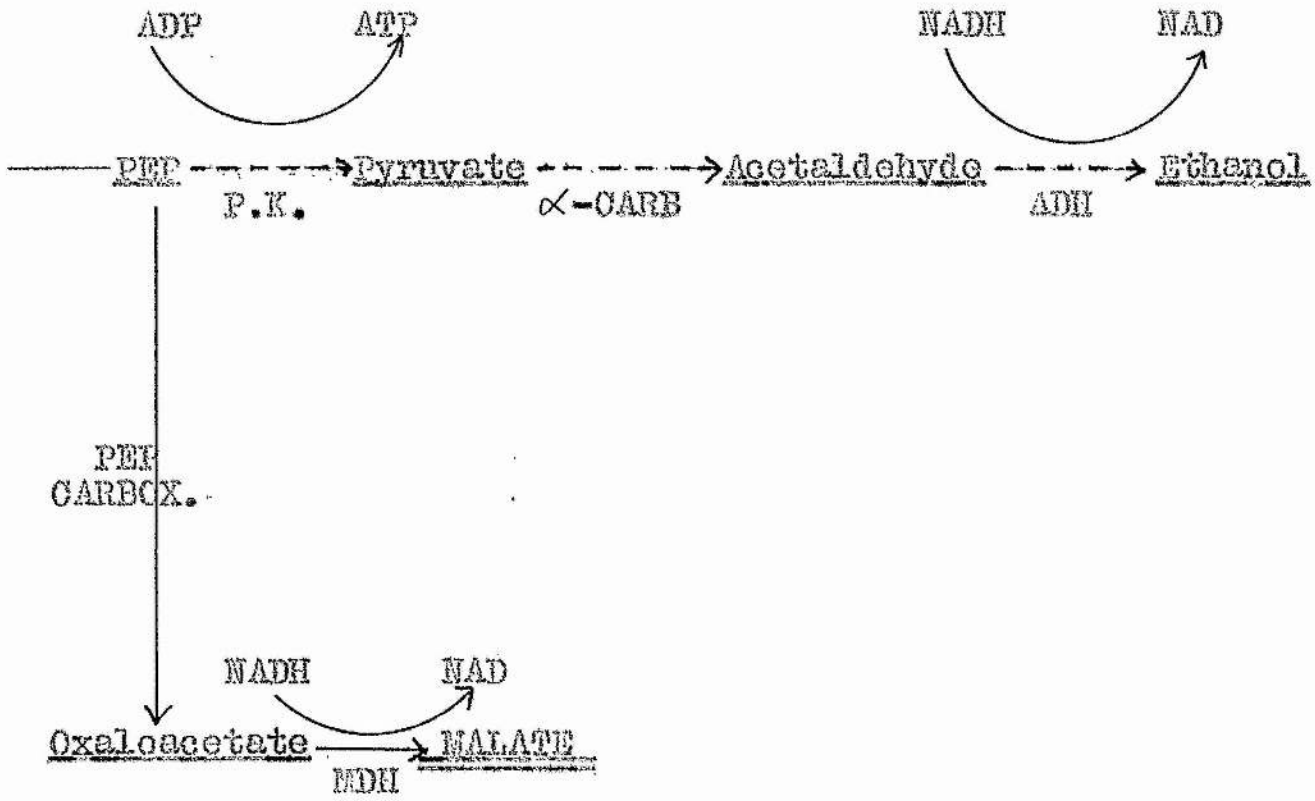

ADP
ATP
NADH
NAD
PEP
P.K.
Pyruvate
Acetaldehyde
ETHANOL
ADH
∝-CARB
NADPH
PEP
CARBOX.
"MALIC"
ENZ.
NADP
NADH
NAD
Oxaloacetate
Malate
MDH

FIGURE 38.

The metabolic scheme of flooding tolerance of helophytes.


ADP
ATP
NADH
NAD
PEP
Pyruvate
Acetaldehyde
Ethanol
P.K.
∝-CARB
ADH
PEP
CARBOX.
NADH
NAD
Oxaloacetate
MALATE
MDH

SUMMARY

(This summary, in the absence of a general discussion, will deal briefly and factually with all the major points which have been raised.)

Introduction. The agricultural importance of flooding is discussed, together with the conditions prevailing in a waterlogged soil, and their effect upon the plant. The physiological significance of aerenchyma is examined, and it is concluded that metabolic modifications are probably necessary for successful growth in waterlogged soils. Previous work on this topic is surveyed.

Part I. Nineteen species were grown in sand culture, and classified as 'helophytes' or 'non-helophytes' on the basis of their responses to experimental flooding for one month and longer. The visible effects of experimental flooding on these species are described, and measurements made of various aspects of growth.

Part II. A brief survey of previous work on alcohol dehydrogenase is made, especially regarding its inductive properties. Experiments are described in which species were grown under conditions of experimental flooding and in aerated and non-aerated water culture. Non-helophytes showed induction of root alcohol dehydrogenase in flooded and non-aerated conditions, while helophytes showed no

such induction. The inductive effect became apparent in 2 to 3 days, and was reversible. Further water culture experiments showed acetaldehyde to be an inductive stimulus for the enzyme; the effect was greater in the non-helophytes. Pyruvate showed a similar but smaller inductive effect, but ethanol caused no induction. It is suggested that this differential effect of acetaldehyde contributes towards the accelerated glycolysis observed on flooding of non-helophytes.

Studies on the kinetics of unpurified alcohol dehydrogenase before and after induction show that the apparent Michaelis constant of the non-helophyte enzyme falls on flooding, while there is no such fall in helophytes. This may contribute further towards the acceleration of glycolysis in non-helophytes on flooding.

The non-linear forms of some of the Lineweaver-Burk plots obtained during the kinetics experiments suggested the possibility of multiple enzyme forms, and polyacrylamide gel electrophoresis revealed variable numbers of alcohol dehydrogenase isoenzymes in most of the species tested, with an indication of changes in the isoenzyme patterns on flooding. It is suggested that newly-synthesised isoenzymes may have different Michaelis constants from those already in the roots. Species differences in the relative importance of enzyme induction

and changes in the apparent Michaelis constant are noted.

Part III. A brief survey is made of previous work on the enzymes of the three major respiratory pathways (glycolysis, the Krebs cycle, the pentose phosphate pathway), and on their responses to environmental conditions. Eight enzymes from this area of metabolism were studied on the basis of their distribution and behaviour in the roots of several experimental species.

Root malic dehydrogenase was induced, in non-helophytes only, by flooding and non-aerated water culture, and to a lesser extent in all species by added malate and oxaloacetate. This is not in agreement with other work on malate accumulation. This and other anomalies may be due to compartmentation of both malate and malic dehydrogenase in the cell. The role of malic dehydrogenase in flooding tolerance remains obscure.

"Malic" enzyme activity was found only in non-helophyte roots. It is suggested that on flooding of non-helophytes, malate is decarboxylated to pyruvate by this route. In helophytes, malate accumulates because the enzyme is absent.

Of the phosphaenolpyruvate carboxylating enzymes, PEP carboxylase was found in all the species tested, providing a possible route of malate accumulation in helophytes. PEP carboxykinase was detected in only one species. Neither enzyme showed any significant distribution between species, or changes in activity on flooding.

Lactic dehydrogenase and glucose 6-phosphate dehydrogenase showed slight increases in some species on flooding, but activities were not thought sufficient to be of general importance in flooding tolerance metabolism.

Pyruvate kinase and enolase activities were detected in small amounts in most species. There was no pattern of distribution or behaviour to suggest any significance in flooding tolerance.

In a general discussion on the enzyme activity results, a scheme of events is proposed for the metabolism of helophytes and non-helophytes on flooding. The more important features are an acceleration of glycolysis due to alcohol dehydrogenase induction and other factors in non-helophytes, together with malate decarboxylation via "malic" enzyme. In helophytes, alcohol dehydrogenase is not induced, but a metabolic diversion via PEP carboxylase leads to malate accumulation; "malic" enzyme is absent. The importance of the other enzymes studied is assessed, and other evidence for the metabolic scheme of flooding tolerance is presented.

Part IV. The significance of the pyridine nucleotides and adenosine phosphates in respiratory metabolism is discussed. Experiments on root tissue assays for these co-enzymes show that sufficiently high NADP levels are maintained in non-helophytes in anoxia to support the

oxidative decarboxylation of malate proposed above. A tentative explanation is offered for the unexpected pattern of AEP and ATP levels after a long-term flooding experiment.

x x x

FIN

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APPENDICES

APPENDIX A SPECTROPHOTOMETRIC ENZYME ASSAY METHODS

Introduction

Many of the enzymes studied were dehydrogenases utilising the pyridine nucleotide co-enzymes nicotinamide adenine dinucleotide (NAD, DPN, Co-enzyme I) or nicotinamide adenine dinucleotide phosphate (NADP, TPN, Co-enzyme II), and their reduced forms NADH and NADPH. Other enzyme reactions studied could be coupled to these dehydrogenase reactions by use of suitable purified enzyme preparations, such that a chain of 2 or 3 reactions was initiated in the assay system, in which the rate-limiting step was the one under study.

Solutions of the reduced forms NADH and NADPH absorb in the ultra-violet, with a peak at 334 nm, while the oxidised forms do not, therefore progress of reactions involving these co-enzymes can be followed by measuring the change in extinction in the assay systems at or near this wavelength. Under optimum conditions, the rate of change in extinction is directly proportional to the speed of the reaction and hence to the concentration of the enzyme, within certain limits.

The apparatus used was an Eppendorf spectrum line photometer with a 366 nm (mercury line) filter, coupled to

a pen recorder through a unit which converted the extinction to a linear scale. The speed of the recorder and sensitivity of the photocell could be varied, and the recorder had a choice of extinction magnification factors. Thus it was possible to detect quite small enzyme activities.

In most cases the limiting factor of sensitivity was the effect of extraneous reactions obscuring the one under study. As most assays were performed on unpurified root extracts, these reactions were always present, but not usually to an excessive extent. The most evident were reactions catalysing the oxidation or destruction of the reduced pyridine nucleotide co-enzymes.

The assays were performed in glass cuvettes of 1 cm light path. After addition of each component, the assay system was mixed using a small glass spatula ('plumper').

Enzyme activity was calculated from the rate of change in extinction (E/unit time) immediately after the addition of the final component of the assay system, except where stated otherwise. At least once in each series of assays, the amount of tissue extract used was varied to test the proportionality of reaction rate to enzyme concentration. Activities were expressed in International Units (Bergmeyer, 1963) per milligram protein or per gram fresh weight of tissue. This was calculated by dividing the change in

extinction (E) per minute by the extinction coefficient of the reduced pyridine nucleotide at 366 nm ($\varepsilon = 3.3/cm^2/\mu mole$).

Assay Systems

Alcohol dehydrogenase (Modified from Crawford, 1967a)

Reaction catalysed:

Acetaldehyde + NADH $\rightleftharpoons$ Ethanol + NAD

Assay system:

200 µmoles acetaldehyde	- 0.05 ml.
2 µmoles NADH	- 0.05 ml.
Root extract	- 0.1 to 1.0 ml.
Tris-HCl buffer, 0.1M, pH 8.2	- to total 3.0 ml.

All components except the acetaldehyde were added, and the rate of change of extinction due to NADH oxidase was subtracted from the rate on addition of the acetaldehyde. This gave the ADH activity. A typical recorder trace is shown in Figure A1. The apparent extinction has been adjusted to a suitable point after the addition of each component.

Malic dehydrogenase (Crawford and McMannon, 1968)

Reaction catalysed:

Oxaloacetate + NADH $\rightleftharpoons$ L-malate + NAD

Assay system:

5 µmoles Na oxaloacetate	- 0.05 ml.
2 µmoles NADH	- 0.05 ml.

FIGURE A.1.

Spectrophotometer trace of alcohol dehydrogenase assay.

Acetaldehyde
NADH
.100
.200
.300
.400
.500
.600
.700
.800
.900
1.000 = T

Root extract - 0.1 to 1.0 ml.

Tris-HCl buffer, 0.1M, pH 8.2 - to total 3.0 ml.

The assay was performed like the ADH assay, substituting 5 µmoles oxaloacetate for the acetaldehyde. The resultant recorder trace was similar to that of the ADH assay. The oxaloacetate was freshly prepared before each series of assays.

"Malic" enzyme (Modified from Ochoa, 1955)

Reaction catalysed:

L-malate + NADP $\rightleftharpoons$ Pyruvate + CO_2 + NADPH

Mn^{++} ions are required for activity. "Malic" enzyme also shows an oxaloacetic decarboxylase activity between pH 4.0 and 6.0. This was not estimated.

Assay system:

10 µmoles Na L-malate	-0.1 ml.
1 µmole NADP	-0.05 ml.
2 µmoles $MnCl_2$	-0.02 ml.
Root extract	-0.5 to 1.0 ml.
Triethanolamine-HCl-NaOH buffer, 0.1M, pH 7.5	-to total 3.0 ml.

The reaction is initiated by addition of the L-malate. The amount of Mn in the assay system is critical; it is essential for the reaction, but an excess produces a rapid increase in extinction due in part to a slight turbidity.

The effect is even more noticeable when Tris-HCl buffer is used (De Moss, 1955), and often obscures the reaction.

A recorder trace of the assay is shown in Figure A2.

Phosphoenolpyruvate carboxylating enzymes

(PEP carboxylase and PEP carboxykinase) (Modified from Vennesland, 1962)

Reactions catalysed:

PEP carboxylase: PEP + CO_2 $\longrightarrow$ Oxaloacetate + Pi

PEP carboxykinase: PEP + ADP + CO_2 $\rightleftharpoons$ Oxaloacetate + ATP

The principle of the assay system lies in the estimation, using the malic dehydrogenase reaction, of the rate of oxaloacetate production. The rate without added ADP is that of the PEP carboxylase-catalysed reaction. The rate increase on addition of ADP should give an estimate of PEP carboxykinase activity. While the following assay system gives a good indication of the rate of the irreversible PEP carboxylase reaction, the PEP carboxykinase assay is probably less reliable. The presence of ADP may allow the alternative pyruvate kinase reaction to operate. Checking on the latter pathway by omission of MDH from the assay system is not possible, because of the large MDH activities present in most extracts.

Assay system:

5 µmoles PEP - 0.05 ml.

FIGURE A.2.

Spectrophotometer trace of "malic" enzyme assay.

L-malate
Mn
Mg Cl2
NADP
.800
.700
.600
.500
.400
.300
.200
.100
0
200
300
400
500
600
700
800
900
1.000 T

10 μmoles $MgCl_2$ - 0.1 ml.

1 μmole NADH - 0.05 ml.

Malic dehydrogenase (pure) - 0.01 mg. protein

Tris-bicarbonate buffer - to total 3.0 ml.

The reaction is initiated by addition of the phosphoenolpyruvate. After it has proceeded long enough for rate measurement of the PEP carboxylase reaction, there is added:

5 μmoles ADP - 0.05 ml.

The increase in the rate should give a measure of PEP carboxykinase activity.

Tris-bicarbonate buffer is prepared by adding 4.2 g sodium bicarbonate per litre of Tris-HCl buffer, 0.1M, pH 7.5.

A recorder trace of the assay is shown in Figure A3.

Lactic dehydrogenase (Modified from Kornberg, 1955)

Reaction catalysed:

Pyruvate + NADH $\rightleftharpoons$ L-lactate + NAD

Assay system:

5 μmoles Na pyruvate - 0.05 ml.

1 μmole NADH - 0.05 ml.

Root extract - 0.5 or 1.0 ml.

Tris-HCl buffer, 0.1M, pH 7.5 - to total 3.0 ml.

The reaction was initiated by addition of the

FIGURE A.3.

Spectrophotometer trace of PEP carboxylase and PEP carboxykinase assay.

ADP
PEP
NADH
$MgCl_2$
MDH
700
600
500
400
300
200
100
0
200
300
400
500
600
700
800
900
1000
T

pyruvate, the assay being carried out like those for ADH and MDH, and giving a similar recorder trace. Triethanolamine-HCl-NaOH buffer gave comparable results to the Tris-HCl.

Glucose-6-phosphate dehydrogenase (Modified from Lohr and Waller, 1963)

Reaction catalysed:

Glucose 6-phosphate + NADP $\rightleftharpoons$ 6-Phosphogluconate + NADPH

Assay system:

2 μmoles G 6-P	- 0.05 ml.
1 μmole NADP	- 0.05 ml.
Root extract	- 0.2 to 1.0 ml.
Tris-HCl buffer, 0.1M, pH 8.2	- to total 3.0 ml.

The reaction is initiated by addition of the glucose 6-phosphate. A recorder trace of the assay is shown in Figure A4.

Enolase (Modified from the 2-phosphoglycerate assay of Czok and Eckert, 1963)

Reaction catalysed:

2-phosphoglycerate $\rightleftharpoons$ Phosphoenolpyruvate + H_2O

Mg^{++} ions are required for activity.

Activity is assayed by coupling the reaction to the lactic dehydrogenase reaction by use of purified pyruvate

FIGURE A.4.

Spectrophotometer trace of glucose 6-phosphate dehydrogenase assay.

1.000
900
800
700
600
500
400
300
200
G 6-P
NADP
0
100
200
300
400
500
600
700
800
900
1.000

kinase and lactic dehydrogenase. The reaction sequence is:

2-PGA $\rightarrow$ PEP $\rightarrow$ Pyruvate $\rightarrow$ Lactate

The rate of oxidation of NADH in the lactic dehydrogenase assay provides a measure of the enolase activity. A possible source of interference is the carboxylation of the phosphoenolpyruvate, which together with the malic dehydrogenase present in the extract, will cause oxidation of the NADH. Omission of the LDH from the system below showed that this interference was not significant.

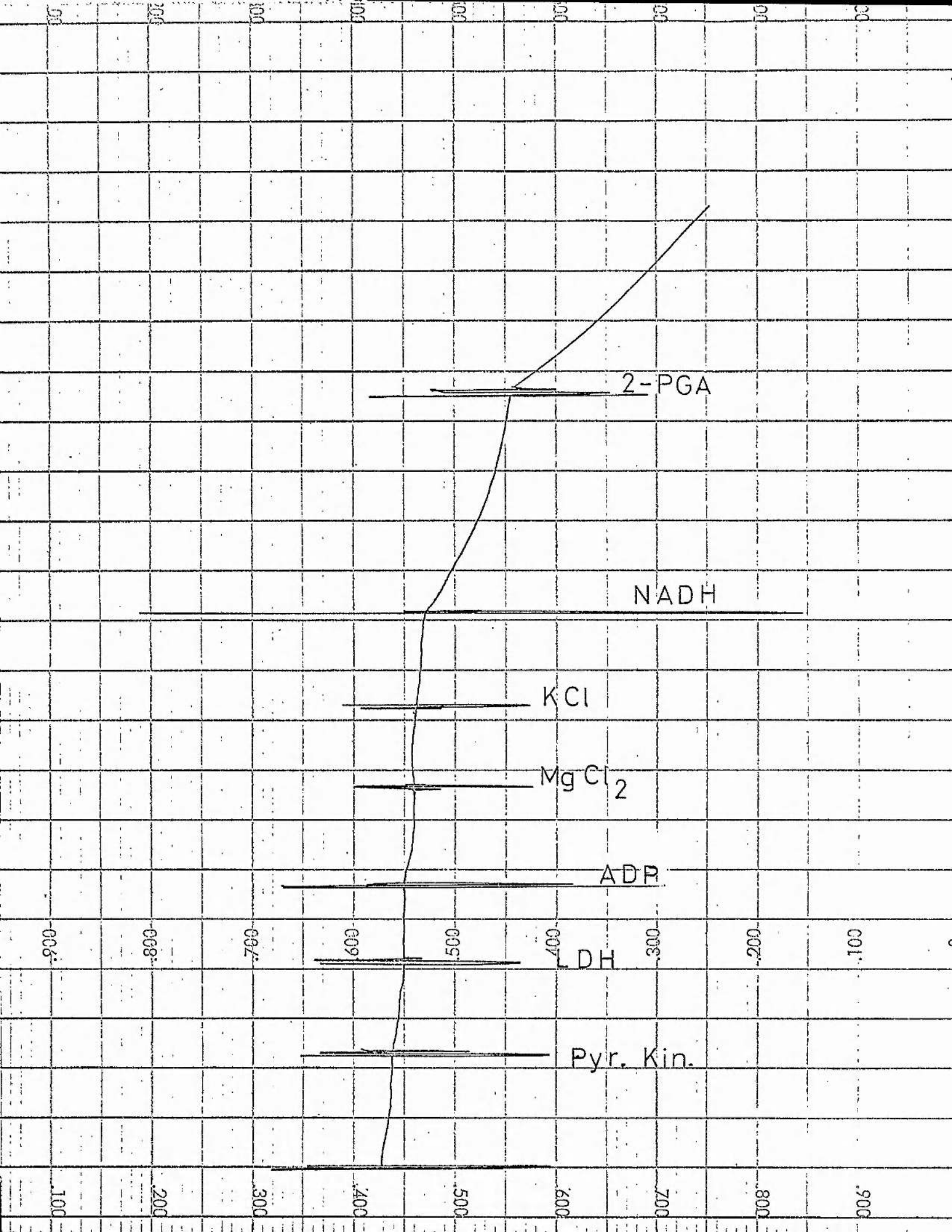
Assay system:

8 µmoles 2-phosphoglycerate	- 0.08 ml.
8 µmoles ADP	- 0.08 ml.
10 µmoles $MgCl_2$	- 0.1 ml.
10 µmoles KCl	- 0.1 ml.
1 µmole NADH	- 0.05 ml.
Pyruvate kinase (pure)	- 0.01 mg protein
Lactic dehydrogenase (pure)	- 0.01 mg protein
Root extract	- 0.5 or 1.0 ml.
Triethanolamine-HCl-NaOH buffer, 0.1M, pH 7.5	- to total 3.0 ml.

The reaction is started by the addition of the 2-phosphoglycerate. A recorder trace of the assay is shown in Figure A5.

FIGURE A.5.

Spectrophotometer trace of enolase assay.


2-PGA
NADH
KCl
$MgCl_2$
ADP
LDH
Pyr. Kin.

Pyruvate kinase (Modified from Bucher and Pfleiderer, 1955)

Reaction catalysed:

Phosphoenolpyruvate + ADP ⇌ Pyruvate + ATP

This reaction is coupled to the lactic dehydrogenase reaction, as in the enolase assay. The assay is performed in a similar way, and sources of interference were also the same, but did not prove to be important.

Assay system:

5 µmoles phosphoenolpyruvate	- 0.05 ml.
1 µmole NADH	- 0.05 ml.
10 µmoles $MgCl_2$	- 0.1 ml.
10 µmoles KCl	- 0.1 ml.
5 µmoles ADP	- 0.05 ml.
Lactic dehydrogenase (pure)	- 0.01 mg protein
Root extract	- 0.5 or 1.0 ml.
Triethanolamine-HCl-NaOH buffer, 0.1M, pH 7.5	- to total 3.0 ml.

The reaction is started by addition of the phosphoenolpyruvate, and the rate is measured after 30 seconds. This allows any pyruvate contaminating the phosphoenolpyruvate to be converted to lactate; subsequent reaction is due to pyruvate produced in the pyruvate kinase reaction. The recorder trace is similar to that obtained in the enolase assay (see Figure A5).

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APPENDIX B ABBREVIATIONS

ADH	-	Alcohol dehydrogenase
ADP	-	Adenosine diphosphate
ATP	-	" " triphosphate
DCIP	-	Dichlorphenol indophenol
DMAP	-	Dimethyl-amino-proprionitrile
DPN	-	Diphosphopyridine nucleotide
EMP	-	Embden - Meyerhof - Parnas pathway
Fl	-	Flooded
G6-PDH	-	Glucose 6-phosphate dehydrogenase
Km	-	Michaelis Constant
LDH	-	Lactic dehydrogenase
MDH	-	Malic dehydrogenase
NAD	-	Nicotinamide adenine dinucleotide
NADH	-	" " " " " " , reduced
NADP	-	Nicotinamide adenine dinucleotide phosphate
NADPH	-	" " " " " " " " reduced
NBT	-	Nitro-blue tetrazolium
PEP	-	Phosphoenolpyruvate
PEP carb	-	Phosphoenolpyruvate carboxylase
P.K.	-	Pyruvate kinase
TCA	-	Tricarboxylic acid (Krebs) cycle
Tris	-	Tris-(hydroxyamino-)methane
Unfl	-	Unflooded