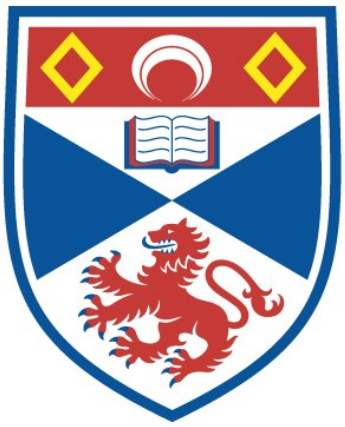
LIPID COMPOSITION AND HABITAT SELECTION IN HIGHER PLANTS

Alistair MacCulloch Hetherington

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



1983

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Lipid Composition and Habitat Selection
in Higher Plants

THESIS ABSTRACT

Lipid analyses of the leaves of Empetrum nigrum subspp. hermaphroditum with an upland distribution in the U.K. and the lowland E. nigrum subspp. nigrum revealed

a) that the lowland subspecies had higher total and neutral lipid levels throughout 1979

b) that total lipid levels remained constant within the leaves of both subspecies throughout the year.

c) that storage lipid (triacylglycerols) contributed 1.4% and 4.5% to the total lipid of subspecies hermaphroditum and nigrum respectively.

This data is inconsistent with the suggestion that the high leaf total lipid levels associated with alpine species represent high levels of storage lipid. Instead it is suggested that the high lipid content of Empetrum leaves may be a reflection of a well developed waxy cuticle.

Iris pseudacorus occupies habitats characterized by poor O_2 availability and is able to tolerate up to two months total anoxia without any loss in viability. By contrast the cultivated Iris germanica var Quechei typically a plant of well drained soils suffers 100% mortality during 8 weeks anoxia. Further the cut primary shoot of I. germanica was observed to be more susceptable to anoxic injury than the remainder of the rhizome. As the biosynthesis of polyunsaturated fatty acids requires the participation of molecular oxygen it was thought profitable to compare what changes occurred in the anoxia tolerant I. pseudacorus and intolerant I. germanica when subject to anoxic stress.

In I. pseudocorus there were a number of lipid modification during anoxia. Glycolipids declined dramatically and although all fatty acids declined it was surprising that saturated acids decreased the most. It was suggested that the decline in glycolipids might reflect mobilization of carbohydrate reserves and/or a replenishment of the fatty acid pool through glycolipid breakdown. The significance of the alterations in membrane fluidity which might be expected to result from alterations in the saturated /unsaturated ratio remain unexplained. By complete contrast, the anoxia intolerant I. germanica although possessing a highly similar lipid profile exhibited no changes in lipid composition in response to anoxia. Therefore membrane dysfunction through lipid component omission is not a major factor in anoxic mortality.

Through production of cytotoxic species such as H_2O_2, $O_2^{\cdot -}$, .OH and $^1O_2$, O_2 may bring about peroxidative damage. On rexposure to air it was found that the highly anoxia sensitive primary shoot tissue of I. germanica produced 38 times more malondialdehyde (M.D.A. - a lipid peroxidation product) than material which was maintained aerobically. I. pseudacorus did not exhibit such differences. Although the overall levels of M.D.A. are higher in I. pseudacorus it may be that the primary shoot tissue contains efficient endogenous secondary protection mechanisms to make good peroxidative damage. However, in the natural environment it is unlikely that the species would ever be exposed to such rapid alterations in O_2 concentrations.

Declaration

I hereby declare that this thesis has been composed by myself, and that it is a record of work which has been done by myself. None of the work has been accepted in any previous application for a degree. Any other sources of information have been acknowledged.

Signed

Alistair Hetherington

Statement

I, Alistair Hetherington, was admitted as a research student of the University of St. Andrews in October 1979 in accordance with Ordinance General No. 12 and the Resolution of the University Court, 1967, No. 1. The thesis was completed in August 1982.

Certificate

I hereby declare that Alistair M. Hetherington has been engaged upon research from October 1979 onwards to prepare the accompanying thesis for the degree of Doctor of Philosophy.

Signed

Prof. R. M. M. Crawford

Dr M. I. S. Hunter

St. Andrews

August 1982

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PREFACE

In the study of physiological factors imposing constraints on habitat selection, and hence limiting ecological range, research at the level of the whole plant has been the most prolific. There are much fewer data attempting to relate ecological limitation to cellular events. There are a number of reasons why this might have arisen, among these are difficulties in the application of physiological/biochemical techniques developed for cereal crops to field species, and the necessity to develop suitable methods for pot cultivation. However, foremost among these is the desire to examine the plant as an integrated unit in relation to its environment. This is of course a very laudable approach, however it does make it very difficult to examine the fundamental basis of adaptation to a particular environment. At the whole plant level one is dealing with a mosiac of effects, which make it difficult to identify the underlying event(s) in adaptation. In relation to tolerance phenonema it may be more appropriate to examine the problem at the cellular level where the temporal spacing between cause and effect is less.

This thesis attempts to relate constraints on habitat selection (effects) to cellular lipid physiology (causes). The two functional aspects of lipids in higher plants under investigation are, (i) their role in energy storage and (ii) as membrane components.

Lipids have long been known to serve as a means of energy storage in seeds where their high energy to bulk ratio makes them ideal reserves. It has also been suggested that they might serve a similar function in the leaves of alpine plants where there are similar constraints on storage volume (5: 11: 12: 13)

From leakage studies and the known fluidizing effect of EtOH on membranes, several workers have suggested the membrane as a target for anoxic/hypoxic damage (for review see 47). Then by implication membrane lipids may be involved in the tolerance of anoxia/hypoxia, and ultimately influence habitat selection. Both areas had recieved some attention prior to this work. However as the question of the possible involvement in energy metabolism still required the rigorous application of lipidological methods, and the interpretation of lipid compositional changes under anoxia was proving difficult, it was considered an appropriate time for a critical rexamination of both the problems.

CHAPTER 1

GENERAL INTRODUCTION

Lipids are a heterogeneous group of substances having in common the property of insolubility in water, but solubility in non-polar solvents such as chloroform, hydrocarbons or alcohols. This definition encompasses a wide range of compounds including long chain hydrocarbons, alcohols, aldehydes, fatty acids and derivatives such as glycerides, wax esters and sulfolipids.

Lipids can be conveniently subdivided into 2 groups on the basis of their solubility in certain solvents. Within the neutral lipid grouping are free fatty acids, sterols, wax esters and hydrocarbons, the pigments carotenoids, xanthophylls and chlorophylls and of great commercial importance, the neutral acylglycerols, in which fatty acids are esterified to the trihydric alcohol glycerol. In seeds fully esterified triacylglycerols are typical seed oils (Fig 1.i.)

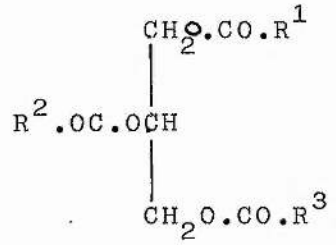
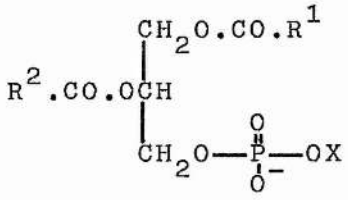


Fig 1.i. A typical triacylglycerol in which R^1, R^2 and R^3 represent fatty acyl side chains which may be nonidentical or identical.

Polar lipids are those which contain a polar group such as the phosphate group of phospholipids, the sulfate group of sulfolipids or the sugar moiety of the glycolipids. Polar lipids have 2 subdivisions, sphingolipids which contain sphingosine or a related amino alcohol are of minor

Fig 1.ii.

The Major Phospholipids(after Harwood, J. L., 1980)



Basic structure

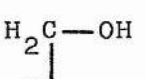
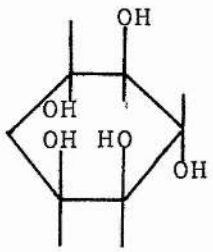
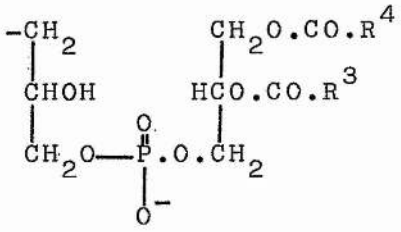
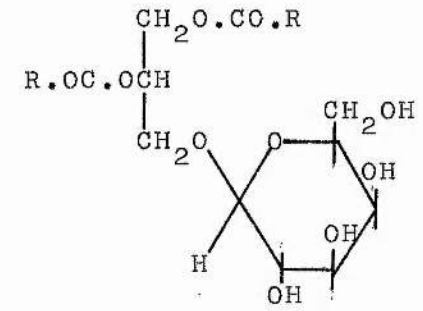
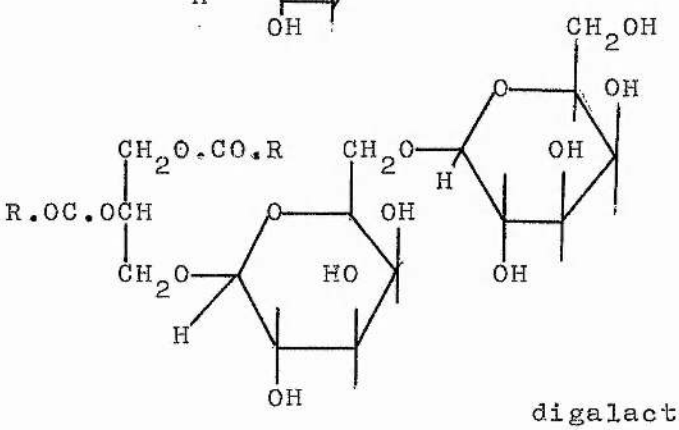
Base moiety	Phospholipid	
X = -H	Phosphatidic acid	(PA)
X = $-CH_2CH_2\overset{+}{N}(CH_3)_3$	Phosphatidylcholine	(PC)
X = $-CH_2CH_2NH_2$	Phosphatidylethanolamine	(PE)
X = 	Phosphatidylserine	(PS)
X =	Phosphatidylglycerol	(PG)
X = 	Phosphatidylinositol	(PI)
X = 	Cardiolipin	(CL)

Fig 1.iii. Structure of the commonly occurring glycolipids.



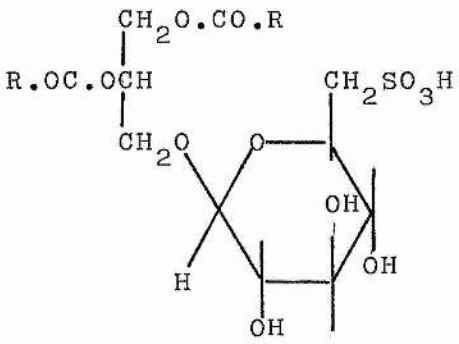
monogalactosyldiglyceride

(MGDG)



digalactosyldiglyceride

(DGDG)



sulfoquinovosyldiglyceride

(SQDG)

importance in plants (1) and glycerolipids which are major structural components; glycerophospholipids are based on phosphoric acid. The structures of those commonly occurring in plant tissues are shown in Fig 1.ii.

PA is generally found in only trace amounts in plant tissue (2), which is commensurate with its role as a biosynthetic intermediate. The most abundant phospholipids of plant (and animal) membranes are PC and PE. Interesintly, PE is not found in chloroplast membranes, where PG is of greatest importance. PI is also a major phospholipid of many plant tissues but PS, although generally distributed, is a minor component. In mammalian membranes it is PS which assumes the major role while PI is found in lower amounts, CL is an interesting phospholipid as it is exclusively localized in mitochondrial membranes, and as such may be used as a marker to study mitochondrial development. The commonly occurring plant glyco and sulfolipids (Fig 1.iii.) can be used in the same manner as they occur in very high amounts in plastid membranes. In photosynthetic tissue the amount of MGDG consistently exceeds the amount of DGDG whereas, the reverse is true in non-photosynthetic tissue (3). SQDG is a ubiquitous constituent of higher plant leaves where it is concentrated in the chloroplast membranes (4).

Lipids can also be subdivided on a functional basis. Structural lipids include all the polar lipids (as membrane components), but also include some of the neutral

Table 1.i. Structures of the Major Fatty Acids.

The shorthand notation for fatty acids is X : Y, where X indicates the carbon chain length while Y indicates the number of double bonds.

COMMON NAME	SYMBOL	STRUCTURE	SYSTEMATIC NAME
Lauric	12 : 0	$CH_3(CH_2)_{10}COOH$	Dodecanoic acid
Myristic	14 : 0	$CH_3(CH_2)_{12}COOH$	Tetradecanoic acid
Palmitic	16 : 0	$CH_3(CH_2)_{14}COOH$	Hexadecanoic acid
Stearic	18 : 0	$CH_3(CH_2)_{16}COOH$	Octadecanoic acid
Oleic	18 : 1	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	Octadecenoic acid
Linoleic	18 : 2	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	Octadecadienoic acid
Linolenic	18 : 3	$CH_3(CH_2CH{=}CH)_3(CH_2)_7COOH$	Octadecatrienoic acid

grouping. Sterols are important membrane components and wax esters and hydrocarbons are found within the cuticle. The most important constituents of the storage lipids are the acylglycerols, and of these, it is the triacylglycerols which are the most abundant.

Although the number of fatty acids detected in plant tissues approaches 300, most species possess only about 10-12 major acids (Table 1.i.). The hydrophobic portion of polar lipids commonly comprise either saturated or unsaturated monocarboxylic acids with an unbranched even numbered carbon chain (Table 1.i.). Of the major saturated acids, lauric, myristic, palmitic and stearic tend to predominate. While the most frequently encountered unsaturated acids are oleic, linoleic and linolenic.

Absolute specificities of fatty acids for single lipids seldom occur in plant tissue (1). Although PG has trans-3-hexadecenoate representing between 15 to 30% of its total acyl moieties (1).

CHAPTER 2

AN ASSESSMENT OF THE ROLE OF STORAGE LIPID IN THE ENERGY METABOLISM OF EMPETRUM SPP.

INTRODUCTION

The ability of alpine species to tolerate extended periods of low temperature has stimulated considerable interest in the underlying mechanisms of such an adaptation. Species adapted to such environments exhibit rapid rates of growth during the short period of favourable weather (5). The contribution of abundant carbohydrate reserves to the energy metabolism of these species has been investigated by a number of workers (6 ; 7 ; 8 ; 9 ; 10). Others (11 ; 5 ; 12 ; 13)suggest that rapid growth rates are supported by the catabolism of storage lipid which represents an adaptive mechanism in species limited in storage volume by their small size.

Energy production from the catabolism of lipids is likely to occur by β -oxidation of fatty acids released by lipase action from neutral acylglycerols. This leads to the formation of acetyl CoA which can either be catabolized by the TCA cycle or, by way of the glyoxalate cycle can be converted to carbohydrate in a manner analagous to the situation in germinating oil seeds. By quantitatively comparing the acylglycerols isolated from an alpine species with those of a closely related species of lowland distribution it was hoped to ascertain whether lipids contribute significantly to the energy metabolism of montane species.

The genus Empetrum was thought to be particularly

suitable for this purpose as Bliss (11) reported that E. eamessii subspp. hermaphroditum had the highest total lipid content of a range of upland species surveyed. In the U.K. Empetrum nigrum L. subspp. nigrum has a low altitude distribution while E. nigrum L. subspp. hermaphroditum (Hagerup)Bocher is restricted to upland sites. Leaves were selected for study as it had been reported (14; 9) that the greatest seasonal lipid fluctuations occurred in these organs.

MATERIALS AND METHODS

Sites

Empetrum nigrum subspp. hermaphroditum was collected from a marked population at an altitude of 800 m from an exposed site in Glen Clova, Tayside, Scotland (NGR NO 259739) on the first Tuesday of every sampling month. E. nigrum subspp. nigrum was sampled from a marked population at Tentsmuir forest, Fife on the east coast of Scotland (NGR NO 500267) at sea level. Collection of material took place on the first Thursday of the sampling month. This proceeded from November 1979 to October 1980 at 3 month intervals.

Preparation of material

Leaves were ground in liquid N_2 and immediately transferred to a freeze drier. The leaf material was dried for 24 hours to 0.03 mmHg and was then ground in an electric mill. Ground material was either immediately

extracted or stored at -18 °C.

Lipid extraction

All solvent mixtures are given as v/v. All solvents were freshly dried and redistilled and 50 mg.l^{-1} butylatedhydroxytoluene was added as antioxidant. Lipids were extracted by modification of the methods of (15) and (16). To 1 g of the freeze dried leaf material 76 ml of a mixture of $CHCl_3$-MeOH-H_2O (1 : 2 : 0.8) was added, shaken for 5 mins and centrifuged (800 g, 10 mins). The supernatant was removed and the pellet resuspended in 20 ml $CHCl_3$. Following recentrifugation the pellet was discarded and the supernatants combined. To the combined extracts 20 ml H_2O was added and the solutions further centrifuged (800 g, 10 mins). The lower lipid rich layer was removed and the supernatant was washed with a further 20 ml $CHCl_3$ followed by centrifugation. The lipid rich lower layers were combined and concentrated under reduced pressure in a rotary evaporator. Lipid extracts were transferred to vials where they were taken to dryness at 45 °C under a stream of N_2. For dry weight determination vials were transferred to a freeze drier and were dried for 24 hours to 0.03 mmHg. The lipid was taken up in 0.5 ml $CHCl_3$-MeOH (1 : 2), the vials were gassed out with N_2 and stored at -18 °C.

Column chromatographic separation

Lipids were fractionated by chromatography on acid washed Florisil (17). Pigments and neutral lipids were

eluted with $CHCl_3$. Polar lipids were eluted with MeOH.

Thin Layer Chromatography (TLC)

Neutral lipids were applied to TLC plates (20 x 20 cm) precoated with 0.25 mm Silica Gel 60 (Merck) following activation of the plates at 110 °C for 60 mins. The plates were developed in paper lined TLC tanks at 4 °C. A number of different solvent systems were employed: Hexane-diethyl ether-acetic acid (90 : 10 : 1) (18); hexane-isopropyl ether-diethyl ether-acetone-acetic acid (85 : 12 : 1 : 4 :1) (19); diethyl ether-benzene-ethanol-acetic acid (40 : 50 : 2 : 0.2)(20); and a 2 step development using hexane-diethyl ether (98 : 2) followed by hexane-diethyl ether-acetic acid (50 : 50 : 1)(21). For preparative separations plates were prepared with 0.5 mm Silica Gel G and developed in the system of (18). Lipids were visualized with either I_2 vapour or Rhodamine 6G. Neutral lipids were identified by comparison with standards.

Analysis of Fatty Acids

Prior to transesterification the acylglycerol fraction isolated by preparative TLC was saponified according to (22). Fatty acid methyl esters (FAMEs) were prepared from the saponified material according to (22) but with 1.5% MeOH-H_2SO_4 as transesterification reagent, in the presence of methyl heptadecanoate internal standard. FAMEs were analysed by Gas Liquid Chromatography (GLC) isothermally at 176 °C and at 225 °C using a (2 m x 4 mm i.d.)

Table 2.i. Leaf total lipid from the leaves of *E. nigrum* subspp. *nigrum* and *hermaphroditum* at 3 month intervals. mg lipid. g^{-1} freeze dried tissue weight. n=5

	Subspp. *nigrum*		Subspp. *hermaphroditum*	
MONTH	x	SE	x	SE
Nov.	166.0	0.89	134.8	1.98
Feb.	167.2	1.02	137.0	3.42
May	170.9	0.64	137.8	1.02
Oct	168.8	1.11	135.5	1.24

Table 2.ii. Polar and neutral lipid class distribution in the leaves of *E. nigrum* subspp. *nigrum* and *hermaphroditum* during May.
mg lipid. g^{-1} freeze dried tissue weight. recovery=85%
n=5

	Subspp. *nigrum*		Subspp. *hermaphroditum*	
FRACTION	x	SE	x	SE
POLAR	24	2.35	21	2.02
NEUTRAL	125	1.21	96	4.38
POLAR/ NEUTRAL	0.192		0.218	

glass column packed with SP - 2330 (Supelco, Inc. Bellefonte, PA, USA) with a N_2 flow rate of 45 ml.min^{-1}. FAMEs were identified by comparison with standards.

RESULTS

During the course of 1979-1980 there was little variation in the leaf total lipid of either lowland or alpine Empetrum subspp. In this period the lowland subspp. nigrum exhibited the higher total lipid content (Table 2.i.). The ratio of polar to neutral lipid was similar (Table 2.ii.). Analysis of the polar lipid fatty acids revealed qualitative similarities. The ratio of saturated to unsaturated fatty acids of the polar fraction, computed during May, is also similar (Table 2.iii.).

Table 2.iii. Per cent fatty acid composition of the polar fraction from the leaves of E. nigrum subspp. nigrum and hermaphroditum during May; n=1

	Fatty Acids (% of Total Acids)							
Subspp.	16:0	16:1	18:0	18:1	18:2	18:3	Others	Sat/ Unsat
nigrum	13.8	0.9	5.8	8.0	28.7	38.8	4	0.26
hermaphroditum	11.7	1.6	7.0	6.6	21.7	47.5	3	0.25

Separation of the neutral fraction of subspp. hermaphroditum by TLC demonstrated that the triacylglycerols were present

Fig 2.i.

Thin layer chromatogram of neutral lipids from E. nigrum subspp. hermaphroditum showing absence of triglycerides component. Loadings A and J = 50 µg; B and I = 40 µg and C and H = 65 µg on Silica Gel 60. Solvent system Hexane-Ether-HAc (90 : 10 : 1). Visualization by Iodine vapour. Identification was by comparison with standards.

Reference Number;

1 = Polar lipid

2 = 1,2 and 1,3 diacylglycerols and pigments

3 = cholesterol and other sterols

4 = unidentified

5 = free fatty acid

6 = unknown

7 = triacylglycerols

8 = unknown

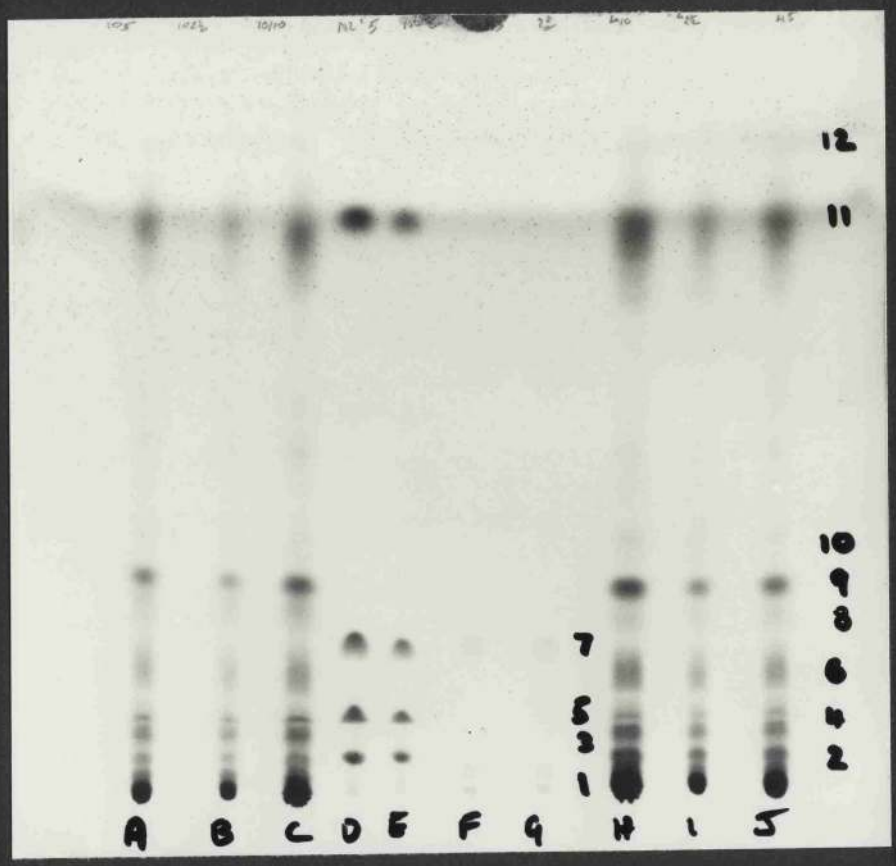
9 = unknown

10 = unknown

11 = wax esters and sterol esters

12 = wax hydrocarbons.

Lanes A, B, C, lipid extracted after (15) and (16), H, I, J, extracted after (23). Lanes D, E, F, G, neutral lipid standards:- DG:FFA:TG:Cholesterol esters.


12
11
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A
B
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D
E
F
G
H
I
J

Table 2.iv. Per cent fatty acid composition of the triacylglycerols fraction isolated from the leaves of E. nigrum subspp. nigrum and hermaphroditum during May. n=1

	Fatty Acids (% of Total Acids)								
Subspp.	12:0	14:0	16:0	18:0	18:1	18:2	18:3	22:0	Others
nigrum	3.3	2.9	18.2	4.8	10.8	23.5	29.3	2.0	6
hermaph-roditum	1.4	3.0	18.3	3.5	4.5	19.2	42.9	1.0	7

Table 2.v. Triacylglycerols content (%) of the total and neutral lipid from leaves of E. nigrum subspp. nigrum and hermaphroditum during May; n=1

Triacylglycerols	Subspp. nigrum	Subspp. hermaphroditum
% of Total Lipid	4.5	1.4
% of Neutral Lipid	5.4	1.7

in very low amounts. Other neutral lipids identified were wax hydrocarbons, wax esters, free fatty acids, sterols, pigments. Many components remained unidentified (Fig 2.i.). The constituent fatty acids from the triacylglycerols fraction are also qualitatively identical, with few quantitative differences (Table 2. iv.) between the 2 subspp. By the use of an internal standard the fatty acid content of the triacylglycerol fraction for May was used to compute the contribution of triacylglycerols to the total neutral lipids. In subspp. hermaphroditum this was found to be lower than in its lowland relatives (Table 2.v.).

DISCUSSION

Lipid analyses of higher plant leaves indicate that up to 7% of higher plant leaf dry matter is present as lipid (24), the lipid content of Empetrum leaves (13-17%) may therefore be regarded as high (Table 2.i.). Such values are consistent with another recent report (25) for other dwarf alpine shrubs. In this study high leaf total lipid levels are not exclusively associated with montane species as E. nigrum subspp. nigrum (lowland) maintains the higher levels throughout the year (Table 2.i.). Seasonal variation in Empetrum leaf lipid is slight irrespective of habitat (Table 2.i.). This situation has also been reported in the dwarf New Zealand evergreen shrubs Celmisia haastii and C. prorepens (14). This contrasts with the marked seasonal variation found in

Loiseleuria procumbens(26; 25) and Celmisia viscosa (14). Apparent lipid variation may result from alterations in other contributors to dry weight known to fluctuate seasonally such as corbohydrates (10). More importantly variation in total lipids may result from alterations in other non-storage lipid components such as, polar lipids (9), pigments or cuticular components. Thus it is invalid without specific quantitation of the acylglycerol component to assign a role in energy metabolims to lipid.

Prior to 1959 it was believed that the triacylglycerols were the major higher plant leaf lipid (27). However, Weenink (28; 29) working with clover was able to demonstrate that the majority of leaf lipid was present in the polar fraction as galactolipid. Fractionation of Empetrum total lipid revealed that the bulk was present in the neutral fraction (Table 2.ii.). This result is not consistent with the suggestion (9) that high leaf lipid levels reflect extensive membrane proliferation. TLC analysis revealed that the amount of triacylglycerols present in the leaf extract was low (Fig 2.i.). This was confirmed by GLC analysis of the constituent fatty acids (Table 2.iv. and v.). The triacylglycerols represented respectively only 4.5% and 1.4% of the total lipid of subspp. nigrum and hermaphroditum. For comparative purposes reports of the triacylglycerols content of higher plants are scarce. However, the results are of the same order as the 2% occurrence in Trifolium repens (30). Even if these results represent a seasonal

minimum then in order for the triacylglycerols to become a major leaf component they would have to increase dramatically. It would be expected that such an increase would be reflected in the seasonal variation in the total lipid, but this was clearly not the case (Table 2.i.). Further, the amount of triacylglycerols in the leaves of the lowland Subspp. was more than 3 times that in Subspp. hermaphroditum.

Examination of Fig 2.i. suggests that a large proportion of the neutral lipid from Subspp. hermaphroditum is made up of wax esters. Together with the wax hydrocarbons these form the lipid components of the leaf cuticle. In Tasmanian eucalyptus it was observed (31), that the possession of glaucous leaves was restricted to high altitude populations. In upland species wax may serve to protect against frost damage and resist pathogen attack (32) and may provide a barrier to near UV (33). In E. nigrum subspp. nigrum the cuticle may provide protection against other factors such as desiccation.

By increasing the ratio of unsaturated to saturated polar lipid fatty acids a species might ensure that a functional membrane was maintained at low temperatures (34). In Empetrum Subspp. no difference in this ratio was found (Table 2.iii.). This may reflect seasonality of sampling, or may be another example of a species which does not respond to low temperatures by increasing the unsaturation of its fatty acids (35; 36).

In conclusion, the data presented in this study for

the genus Empetrum are inconsistent with a role for lipids in the overwintering metabolism of alpine species. Without fractionation of neutral lipid or other estimation of the triacylglycerols Tschager et al (25) reached similar conclusions. It is suggested that the high total lipid values probably reflect the presence of a well developed waxy cuticle. An extension of this approach will be required before it is possible to make general statements about the role of lipids in adaptation to this habitat.

CHAPTER 3

SURVIVAL OF IRIS SPP. UNDER ANOXIA

INTRODUCTION

It is becoming increasingly apparent that certain higher plants have the ability to withstand periods of anoxia. In the germinating seeds of Cicer arietinum it has been suggested that anoxia occurs after imbibition and before rupture of the seed coat, but prolongation of this natural anaerobic period by continued soaking results in death (37).

However it is in relation to flooding that the phenomenon has received the greatest attention. In seeds, rice (38) and barn grass (39) stand out from most other species in their ability to germinate in the absence of O_2, although development is limited to coleoptile extension. The rhizomes of aquatic plants also vary in their tolerance of anoxia with species such as Schoenoplectus lacustris and Scirpus maritimus being able to survive and extend their shoots in the absence of O_2, while the morphologically similar Juncus effusus and J. conglomeratus are killed (40). The ability to survive periods of anoxia with or without shoot extension confers a selective advantage on species which inhabit wetlands or flood prone sites, in that when the supply of O_2 from shoot to root is interupted, either by flooding, silting or winter dieback, the lack of O_2 will not prove immediately fatal to the plants.

The genus Iris contains a number of species which

although morphologically similar, differ in their ecological preferences for wet or dry sites. An example of such contrasting ecological behaviour is found in the morphologically similar species pair Iris pseudacorus L. and I. germanica L. I. pseudacorus occupies habitats characterized by poor O_2 availability such as lakeside muds. Even during midsummer O_2 concentrations within the rhizome are low (2.7% v/v)(41). During the winter following shoot dieback O_2 transport to the rhizome will be interrupted and this might be expected to lead to extensive periods of anoxic stress within the organ. By contrast, the cultivated I. germanica var Quechei probably of Mediterranean origin, is typically a plant of well drained soils. The aim of this work was to compare survival after anoxia of these two morphologically similar species. The effects of both temperature and duration of treatment were investigated as (42) had demonstrated pea survival to be dependent on both factors. It was also hoped to investigate whether there was any differential tissue survival within the rhizomes.

MATERIALS AND METHODS

I. pseudacorus collected locally, and I. germanica supplied by the University Botanic Garden, were planted out for 2 months in sand with supplementary light under 16 hour days. Nutrients were supplied once a week. Immediately before treatment the roots and leaves were

removed and the rhizomes were carefully cleaned and trimmed to between 7 and 8 cm length. They were then transferred to the Anaerobic Workbench (a.w.b.)(Forma Scientific, Ohio, U.S.A.) where they were placed in anaerobic jars (Gas Pak, from Becton, Dickinson & Co., Cockeysville, U.S.A.), containing a Pd catalyst to remove any traces of O_2, under an atmosphere of 85% N_2, 10% H_2, 5% CO_2.

Each jar contained 5 rhizomes which rested on moist filter paper sitting on specially constructed trays. The efficiency of the anaerobic system was monitored using methylene blue indicator. In all the anaerobic treatments this indicator remained completely colourless. After 12 hours the jars were sealed and placed in incubators where they were kept in the dark for the duration of the experiment, at the end of which the rhizomes were planted out in wet sand. Aerobic control rhizomes were treated in exactly the same manner except that the jars remained unsealed.

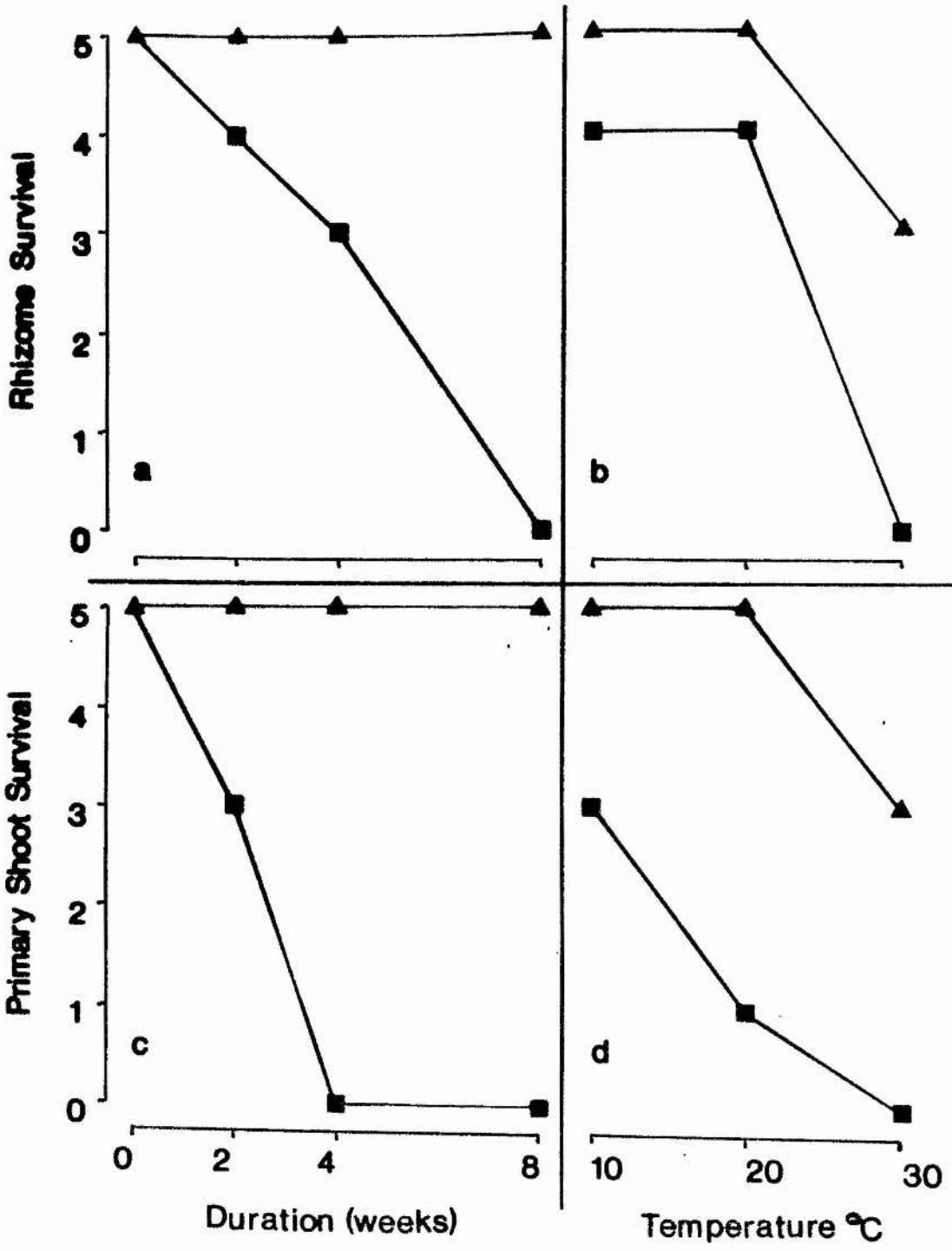
RESULTS

Rhizome survival was defined as the capacity for regrowth from the cut primary shoot or growth from previously dormant shoot buds. Observations were continued up to 2 months after planting out. All control rhizomes of both species grew from the cut shoot. No growth was observed under anoxia. I. pseudacorus withstood prolonged periods of anoxia at 20 ^{o}C without loss of viability

Fig 3.i.

Survival of I. pseudacorus ▲—▲ and I. germanica ■—■ rhizomes and primary roots at 20 °C after different periods of anoxia (a) and (c) and after 2 weeks anoxia at 3 temperatures (b) and (d); 5 rhizomes per treatment.

fig. 3·1


Rhizome Survival
Primary Shoot Survival
0
1
2
3
4
5
a
b
c
d
0
2
4
8
Duration (weeks)
10
20
30
Temperature °C

(Fig 3.i.a) and, even after 2 weeks at 30 °C, 60% of the rhizomes under test recovered (Fig 3.i.b). By contrast, 8 weeks exposure to anoxia at 20 °C (Fig 3.i.a) or 2 weeks at 30 °C (Fig 3.i.b) resulted in 100% mortality of I. germanica. Growth in I. pseudacorus resumed without exception from the cut shoot. In I. germanica the cut primary shoot was observed to be more susceptible to anoxic injury (Fig 3.i.c and 3.i.c) than the remainder of the rhizome. Growth occurred from previously dormant buds following cut primary shoot mortality.

DISCUSSION

The use of shoot growth as an index of viability seems to be validated since all rhizomes in which no growth was observed eventually decayed. The response of I. germanica to anoxia is similar to pea seedlings (42) and graminaceous seed (43). Over the anoxic period mortality increases with both increasing temperature and duration of incubation (Fig 3.i.a and b). However, unlike previous reports the cut primary shoot was more susceptible to anoxic injury than the remainder of the rhizome. This differential tissue survival is surprising in view of the suggestion (44 ;45) that meristematic areas are frequently found to operate a fermentative metabolism. This results from the poor O_2 supply to the closely packed cells in this region. In the I. germanica rhizome if fermentation occurs in the meristematic region it does not appear to confer tolerance. Instead, it is possible

that the high metabolic rate encountered in such regions (45) may contribute to lethality either as a result of a rapid depletion of reserves or to the accumulation of potentially toxic by-products which, due to the tissue structure are slow to diffuse away.

In I. pseudacorus this response was not observed (Fig 3.i.c and d). Perhaps the ability of I. pseudacorus to withstand prolonged exposure to anoxic conditions represents an important adaptation to its natural environment. Following winter shoot dieback the O_2 supply to the underground components will be interrupted. If the rhizome lies buried below the level of the water table, unless there is a lateral flush of the surrounding soil with aerated water then anaerobic conditions will result (46). The ability of I. pseudacorus to tolerate 2 months anoxia may be important in competing with other species which occupy wetland sites which are subject to seasonal fluctuations in the water table.

In the following chapters the contribution of biochemical adaptation to the ability of I. pseudacorus to occupy such habitats will be investigated. In order to best identify these factors a comparative approach employing I. pseudacorus and I. germanica was attempted as the lethal events of anoxic injury are poorly understood.

CHAPTER 4

EFFECTS OF ANOXIA ON THE FATTY ACIDS OF IRIS SPP.

INTRODUCTION

In the previous chapter it was demonstrated that the morphologically similar species pair I. pseudacorus and I. germanica differ in their tolerance of anoxia. It was the object of the work in this and subsequent chapters to investigate the biochemical basis of tolerance. This might be achieved through a better understanding of the nature of anoxic injury.

It is possible to predict certain biochemical modifications which will accompany transfer to an anoxic environment. The causal factors in anoxic mortality may be divided into 2 categories on the basis of whether there is a direct involvement of molecular O_2. Primary mechanisms result from the failure of O_2 requiring reactions for which there is no alternative anoxic pathway. The second grouping includes events associated with the necessity to operate alternative pathways under anoxic conditions such as an inherently inefficient form of energy metabolism. In the following table an attempt has been made to assign biochemical processes sensitive to anoxia to one or other of the above categories.

Research has been concentrated within the "secondary" category (for reviews see [47] ; [48] ; [49]). It is therefore felt appropriate to investigate those reactions falling into the first category.

Possible Molecular Bases for Anoxic Injury

Primary	Secondary
Through the direct involvement of molecular O_2 in a mechanism.	Through the necessity to operate alternative pathways.
(a) Unsaturated fatty acid biosynthesis.	(a) Production of possibly toxic anaerobic end products.
(b) Sterol biosynthesis.	(b) Depletion of reserves.
	(c) Alterations in energy charge affecting other reactions.
	(d) Difficulties in regenerating NAD(P).

It has been demonstrated (50 ;51) that O_2 is essential for maintaining plant membrane integrity, although the biochemical basis for this is not yet understood. In animals the site of anoxic damage to mitochondria has been shown to be the membrane phospholipids (52). Molecular oxygen is required for the biosynthesis of unsaturated fatty acids which are known to be essential to membrane structure and function (53). The possibility therefore exists that in some anoxia-intolerant plant species a factor contributing to cell death may be the inability to synthesize new, or conserve existing, fatty acids. If unsaturated fatty acids are essential to

membrane structure and function then, by implication, tolerant species must either greatly reduce membrane lipid turnover or possess a novel desaturation mechanism.

MATERIALS AND METHODS

Prior to experimentation I. pseudacorus and I. germanica were grown in the greenhouse as previously described (Chapter 3). Before treatment, rhizomes were trimmed to 9 cm in length, washed and roots and leaves removed. They were then placed on moist filter paper in darkened plastic containers. Anaerobic treatments were carried out in the a.w.b. as previously described (Chapter 3). The aerobic controls were placed in an incubator at 20 ^{o}C in an air atmosphere.

Lipid Extraction

After trimming to 8.5 cm to remove any possible microbial contamination from cut surfaces, rhizomes were immediately grated into liquid N_2 to minimize lipid degradation due to endogenous lipase activity and freeze dried for 24 hours. Samples were then milled and immediately before extraction with hexane-iso-PrOH (23) the samples were rehydrated with distilled H_2O (3 ml. g^{-1} tissue dry wt). Lipids were fractionated on acid washed Florisil as described previously (Chapter 2).

Analysis of Fatty Acids

The analysis of fatty acid following transesterification has been described in Chapter 2.

Table 4.i. Total lipid content (mg. g^{-1} dry wt rhizome) of I. pseudacorus and I. germanica rhizomes incubated under aerobic and anaerobic conditions at 20 ± 2 °C for 14 days.

	Aerobic Control				Anaerobic Treatment				Significance
Species	$\bar{x}$	sd	se	n	$\bar{x}$	sd	se	n	(t-test)
I. pseudacorus	11.2	1.4	0.6	5	9.4	1.7	0.6	7	P 0.05
I. germanica	67.5	7.6	3.4	5	71.2	3.1	1.4	5	Non-significant

Table 4.ii. Polar and neutral lipid content (mg. g^{-1} dry wt rhizome) of I. pseudacorus rhizomes incubated under aerobic and anaerobic conditions at 20 ± 2 °C for 14 days.

	Aerobic Control				Anaerobic Treatment				Significance
Lipid Class	$\bar{x}$	sd	se	n	$\bar{x}$	sd	se	n	(t-test)
Polar	4.43	0.96	0.43	5	2.55	1.04	0.39	7	P<0.01
Neutral	6.75	0.87	0.39	5	6.49	1.19	0.39	7	Non-significant

Table 4.iii. Polar and neutral lipid content (mg. g^{-1} dry wt rhizome) of I. germanica rhizomes incubated under aerobic and anaerobic conditions at 20 ± 2 °C for 14 days.

	Aerobic Control				Anaerobic Treatment				Significance
Lipid Class	$\bar{x}$	sd	se	n	$\bar{x}$	sd	se	n	(t-test)
Polar	34.52	5.11	2.28	5	30.74	4.31	1.93	5	Non-significant
Neutral	27.08	12.05	5.39	5	25.18	12.43	5.56	5	Non-significant

Table 4.iv. Saturated/unsaturated fatty acid methyl esters (by wt) from polar lipids of I. pseudacorus and I germanica rhizomes incubated under anaerobic and aerobic conditions at 20 ± 2 °C for 14 days.

	Aerobic Control				Anaerobic Treatment				Significance
Species	$\bar{x}$	sd	se	n	$\bar{x}$	sd	se	n	(t-test)
I. pseudacorus	0.91	0.28	0.14	5	0.46	0.10	0.04	7	$P<0.01$
I. germanica	0.76	0.28	0.20	5	0.77	0.22	0.10	5	Non-significant

Figure 4.i. Neutral lipid fatty acid methyl esters mg/g dry weight rhizomes) of I. pseudacorus incubated under aerobic and anaerobic (Shaded) conditions at 20 ± 2 °C for 14 days. Mean of 5 and 7 replicates for aerobic and anaerobic treatments respectively. Results of students t-test between aerobic and anaerobic treatments; 14:0 Non sig.; 16:0 P>0.02; 18:0 Non sig.; 18:1 Non sig.; 18:2 Non sig.; 18:3 Non sig.; 22:0 Non sig.; Error bars = S.E.M.

fig. 4·1

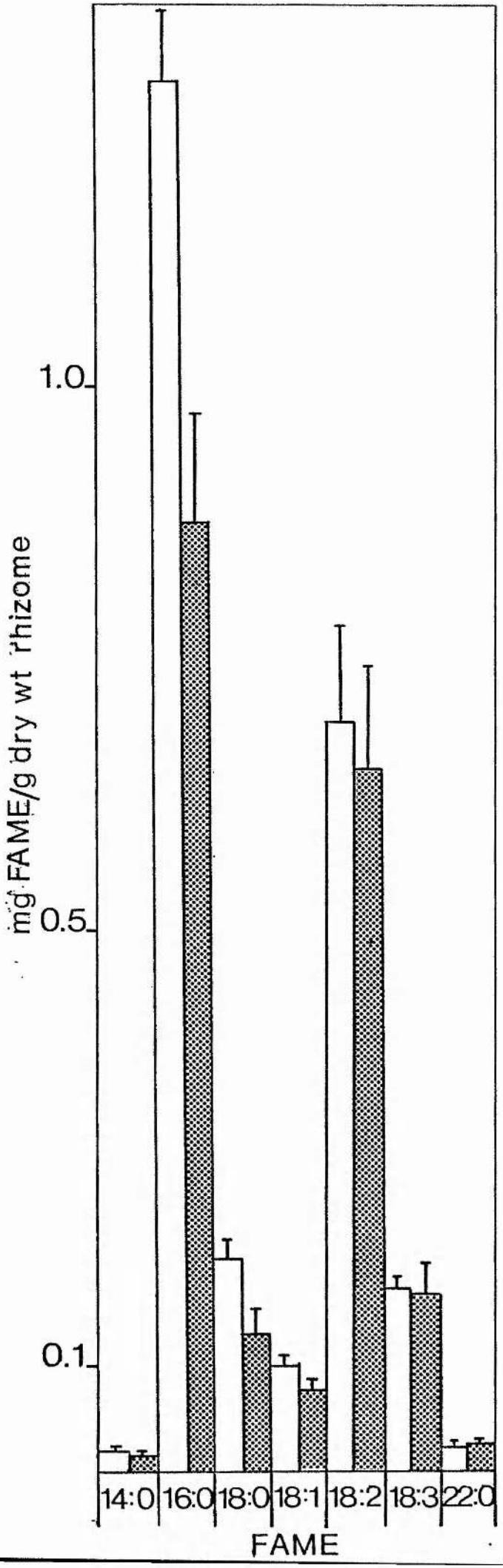

1.0
0.5
0.1
mg FAME/g dry wt rhizome
14:0
16:0
18:0
18:1
18:2
18:3
22:0
FAME

Figure 4.ii. Polar lipid fatty acid methyl esters (mg/g dry weight rhizomes) of I. pseudacorus incubated under aerobic and anaerobic (shaded) conditions at 20 ± 2 °C for 14 days.
Mean of 5 and 7 replicates for aerobic and anaerobic treatments respectively.
Results of students t-test between aerobic and anaerobic treatments; 14:0 $P>0.05$; 16:0 $P>0.01$; 18:0 $P>0.05$; 18:1 $P>0.05$; 18:2 $P>0.05$; 18:3 $P>0.05$; 22:0 $P>0.05$;
Error bars = S.E.M.

fig. 4·2

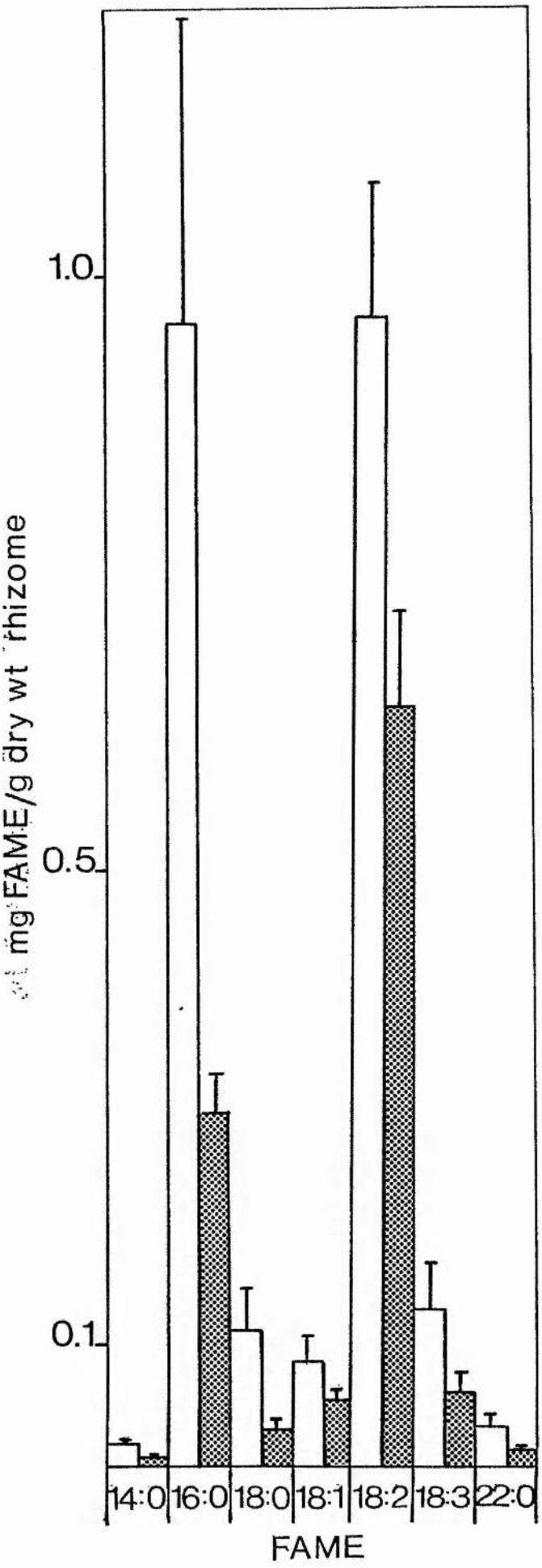

1.0
0.5
0.1
mg FAME/g dry wt rhizome
14:0
16:0
18:0
18:1
18:2
18:3
22:0
FAME

Figure 4.iii. Neutral lipid fatty acid methyl esters (mg/g dry weight rhizomes) of *I. germanica* incubated under aerobic and anaerobic (shaded) conditions for 14 days at 20 ± 2 °C.
Mean of 5 replicates (both treatments).
Results of students t-test between aerobic and anaerobic treatments; all non significant.
Error bars = S.E.M.

fig. 4.3

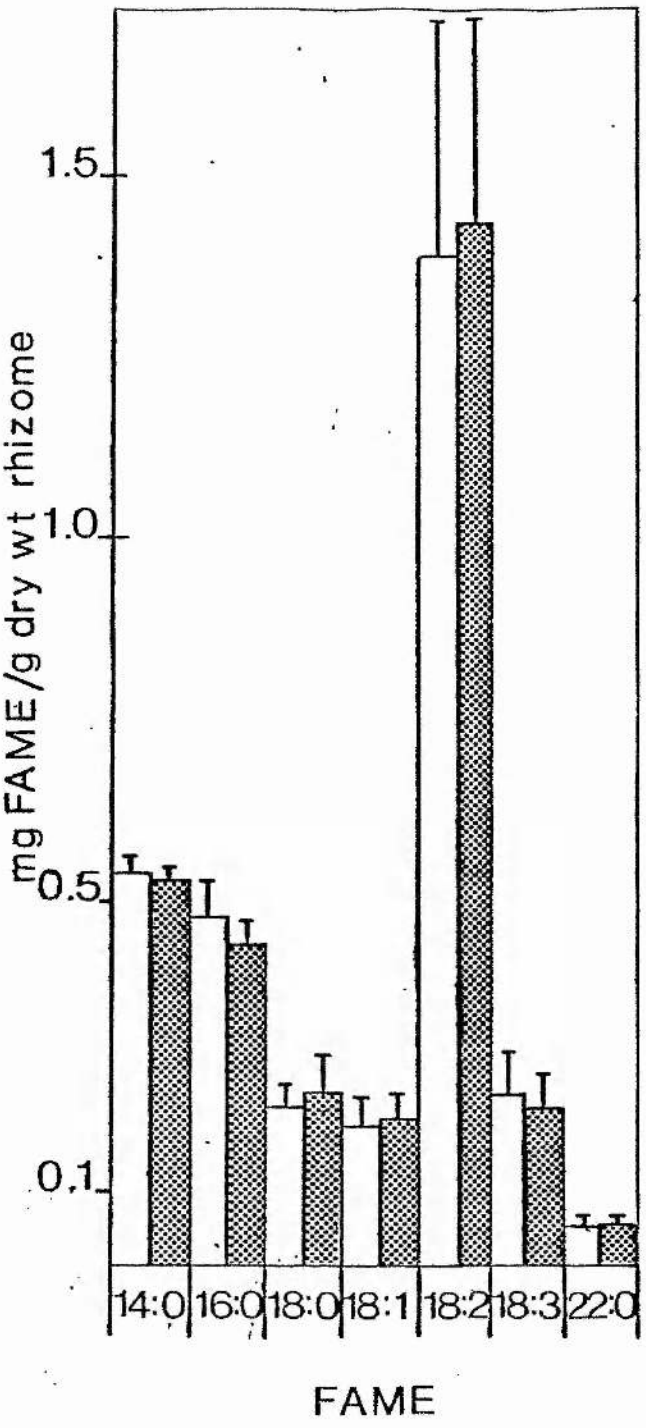
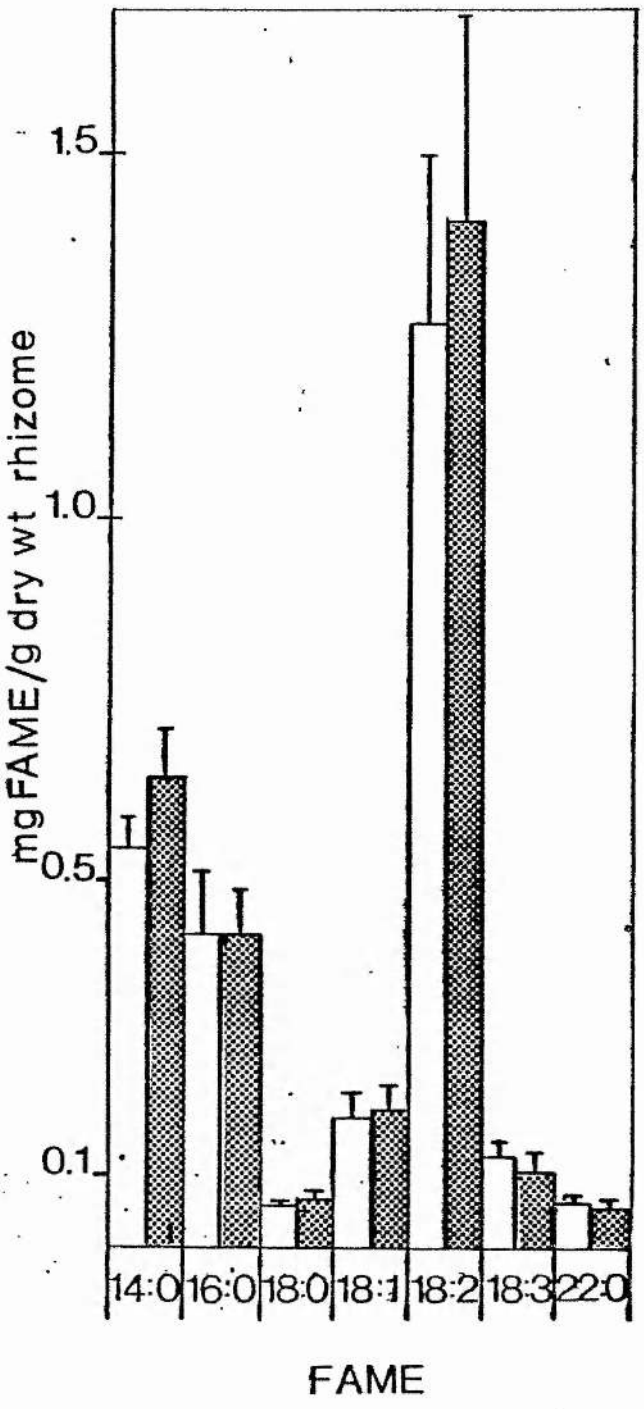

1.5
1.0
0.5
0.1
mg FAME/g dry wt rhizome
14:0
16:0
18:0
18:1
18:2
18:3
22:0
FAME

Figure 4.iv. Polar lipid fatty acid methyl esters (mg/g dry weight rhizomes) of *I. germanica* incubated under aerobic and anaerobic (shaded) conditions at 20 ± 2 °C for 14 days.
Mean of 5 replicates (both treatments).
Results of students t-test between aerobic and anaerobic treatments; all non significant.
Error bars = S.E.M.

fig. 4·4


1.5
1.0
0.5
0.1
mg FAME/g dry wt rhizome
14:0
16:0
18:0
18:1
18:2
18:3
22:0
FAME

RESULTS

Of the 2 species the total lipid content of *I. germanica* was the greater (approx. 6 times)(Table 4.i.). Further, the ratio of polar to neutral lipid in *I. germanica* was higher than in the rhizome of the wetland species (Tables 4.ii. and 4.iii.).

During 14 days anoxia, neither species exhibited any growth. In *I. pseudacorus* rhizomes, total lipid significantly declined (Table 4.i.). This decrease reflects significant reductions in polar lipids, the neutral fraction remaining unaltered (Table 4.ii.). All polar lipid fatty acids decreased significantly. The greatest losses were of the saturated fatty acids chiefly palmitic and stearic (Figs 4.i. and 4.ii.) although there is also a considerable reduction in linoleate in the polar fraction. In contrast to *I. pseudacorus*, the total lipids of *I. germanica* were not significantly decreased (Table 4.i.). Similarly there were no significant losses of either the polar or neutral fractions (Table 4.iii.) or of the individual fatty acids from the above classes (Figs 4.iii. and 4.iv.).

DISCUSSION

Anoxic conditions would undoubtedly inhibit *de novo* synthesis of unsaturated fatty acids by the mechanisms known to operate in higher plants (54), although the biosynthesis of saturated acids could continue. Indeed, through

the use of labelling techniques this has been verified in the rice coleoptile (55). If turnover of the fatty acyl side chains of membrane lipids is occurring, then in the absence of utilization of unsaturated fatty acids from non-membrane lipid, a preferential depletion of unsaturated fatty acids might be expected in response to anoxia.

It is surprising therefore, to observe in I. pseudacorus that under anoxia it is the saturated acids which decrease most markedly. One possible expanation for this result might be the activity under anoxia of an acyl hydrolase specific for the 1 position on polar lipids. It has been shown (56 ; 57) that this position is occupied preferentially in certain phospholipids and glycolipids by a saturated acid. This activity would thus result in the release of free saturated fatty acids, which might then, in the absence of further degradation, appear in the neutral fraction. However, Figure 4.i. shows that there is no compensatory rise in the saturates in the neutral lipids under anoxia. How the saturated acids released under anoxia from the polar fraction might be catabolized in the absence of a mechanism for the regeneration of NAD^+ is unclear. In rat myocytes it has been demonstrated that ischemic damage, (which can occur due to the interruption of blood supply to a cell) is due to the lack of O_2 *per se* rather than some other essential factor or nutrient (58). The resultant membrane dysfunction has been attributed to activation of endogenous

phospholipases either by depleted ATP supply (59) or as a result of increased cell Ca^{2+} (60). It is possible that one of the above factors might be involved in the activation of an acyl hydrolase in Iris. The adaptational advantage of such a mechanism to I. pseudacorus is not clear.

The significant alteration observed in the overall ratio of saturated to unsaturated fatty acids (Table 4.iv.), which might be expected to lead to an increase in membrane fluidity in the absence of other compensatory mechanisms, may have important consequences for membrane function. Such mechanisms are unlikely to include the insertion of sterols, as sterol biosynthesis would probably also be interrupted under anoxia (61). The preferential loss of polar lipids is also probably indicative of changes in membrane composition and is consistent with the loss of phospholipids observed in rice and wheat under anoxic conditions.

It has been suggested that a metabolic adaptation to anoxia does involve the accumulation of lipids (62). Specifically it has been suggested that through synthesis of fatty acids a mechanism would be provided for the re-generation of NAD^+ and for the production of a non-toxic end product of anaerobiosis (62). Interestingly, in support of this hypothesis Vartepetian et al (55) found an increase in neutral lipid labelling under anoxia in the rice coleoptile. Under anoxia there is no increase in the neutral fraction (containing free fatty acids) in

I. pseudacorus (Table 4.ii. and Fig 4.i.). The data for this anoxia tolerant species are therefore inconsistent with the above suggestion. However, a conclusive answer might be obtained by examining the pattern of free fatty acid synthesis under anoxia through the use of labelling techniques.

The situation in I. germanica completely contrasts with the changes in I. pseudacorus. Although the fatty acids of the two species are similar in the aerobic controls, the lack of significant changes in either lipid classes or fatty acid composition raises the question of whether the observed alterations in I. pseudacorus lipids represent an adaptation to anoxia. Vartepetian et al (55) found that the fatty acid composition of anaerobically and aerobically germinated rice coleoptiles was similar. It must be remembered that although rice is flood tolerant, unlike I. pseudacorus its viability under anoxia is severely limited (47). In contrast, others (63) have shown that, as in this work, anaerobiosis results in an increased ratio of unsaturated to saturated acids in both rice and wheat. This change is interpreted as an adaptive mechanism in the former.

In the next chapter the effect of anoxia on the polar lipids was investigated in greater detail. By analysing both phospho and glycolipids it was hoped to determine whether the effects of anoxia were localized to a single lipid component and/or organelle.

CHAPTER 5

EFFECTS OF ANOXIA ON THE POLAR LIPIDS OF IRIS SPP.

INTRODUCTION

In the previous chapter it was demonstrated that a 14 day period of anoxia resulted in losses of polar lipids, particularly their saturated fatty acid components from the anoxia-tolerant species *I. pseudacorus*. By complete contrast, the anoxia intolerant *I. germanica*, exhibited no changes in membrane lipid composition in response to anoxia. In this chapter the effect of anoxia on the individual polar lipid classes was investigated.

In anoxia-intolerant species such as *Carex leporina* mitochondrial disintegration has been observed after 24 hours anoxia (64). In rice coleoptiles germinated under anoxia, mitochondria assume a distinctive structure remaining physiologically active. Transfer to aerobic conditions results in mitochondria forming the typical aerobic configuration (38). It has been suggested that the ability of rice mitochondria to resist anoxia may be due to the presence of reserve lipids within the coleoptile (65). This suggestion must advocate that when there is a deficiency of unsaturated fatty acids in the membrane lipids then transfer of fatty acids from neutral (reserve) lipids such as triglycerides is taking place, This would be a novel system and would still leave membranes deficient in sterols, as the synthetic enzyme squalene oxidase is oxygen dependent (66).

Certain polar lipids are exclusively located in

specific subcellular organelles. Cardiolipin for example is only found in mitochondrial membranes, while the glycolipids mono and digalactosyldiglyceride (MGDG and DGDG) are associated with chloroplastic membranes (3). By investigation of these marker lipids it was hoped to determine whether the lipid changes in Iris pseudacorus subjected to anoxia were a general membrane phenomenon or whether they were localized in any group of subcellular components or to any one organelle.

MATERIALS AND METHODS

Preparation of Iris pseudacorus and I. germanica for experimentation was similar to that described in Chapter 3.

Anoxia/Aerobic treatments:-

These were carried out in work jars at 20 ^{o}C in the manner outlined in Chapter 3.

Lipid Extraction:-

After preparing the rhizome tissue according to the method of Chapter 4, the lipids were extracted from the freeze dried groung material in $CHCl_3$-MeOH-H_2O (2:1:0.8) (16) which has been described previously (Chapter 2).

Thin Layer Chromatography:-

Plates (20 x 20 cm) precoated with silica gel 60 (Merck) were activated at 110 ^{o}C for 60 mins and allowed

Table 5.i.

Phospho and glycolipid content of I. pseudacorus and I. germanic rhizomes incubated under anoxic conditions for 14 days.

	μg P. g^{-1} tissue dry wt				
	Aerobic		Anaerobic		Signif-icance
	$\bar{x}$	se	$\bar{x}$	se	(t-test)
I. pseudacorus	29.36	2.16	26.64	1.06	NS
I. germanica	31.44	2.74	22.08	3.56	NS

	ug Galactose. g^{-1} tissue dry wt				
	Aerobic		Anaerobic		Signif-icance
	$\bar{x}$	se	$\bar{x}$	se	(t-test)
I. pseudacorus	4208	166.57	2656	271.76	$P<0.002$
I. germanica	16200	1014.69	15840	645.10	NS

to cool. The lipid sample (c. 5000 µg total lipid) was applied immediately and the plates were developed in the following systems. First dimension chloroform-methanol-ammonia (25% w/v)-water, (168:88:11:11)(v/v). Chloroform-methanol-glacial acetic acid, (50:27:12)(v/v) was used to develop the second dimension. Further details of this system, together with specific detection reagents employed are described in the Appendix.

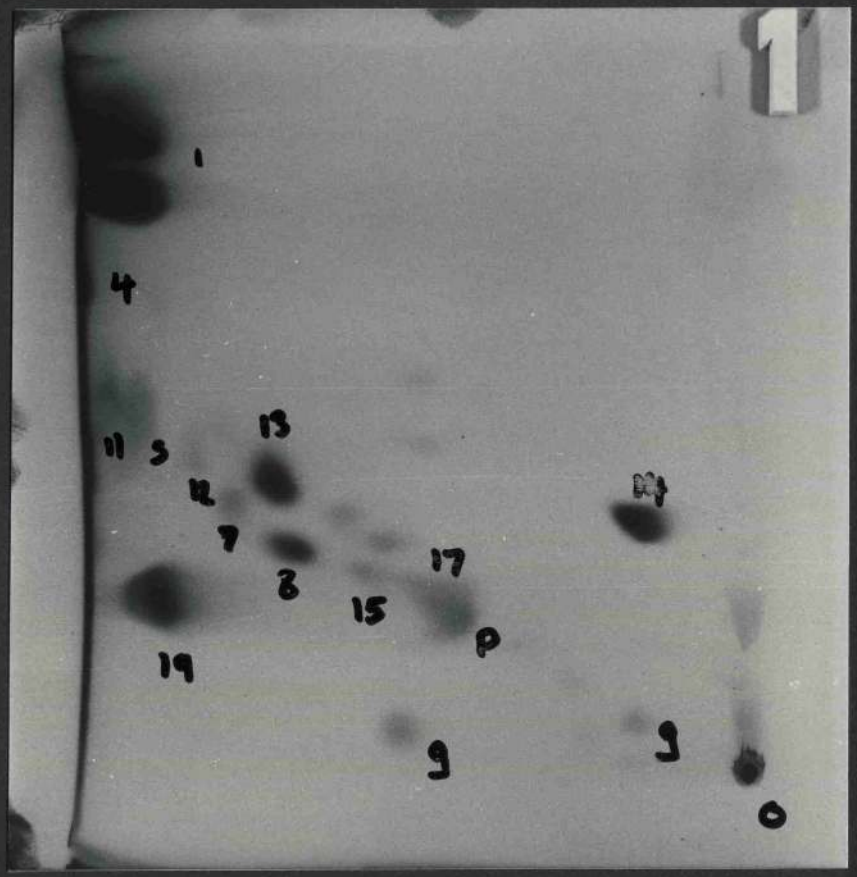
Quantitative assay of phospholipids and glycolipids:-

Phospholipids were assayed according to the method of Rouser et al (67). Glycolipids were assayed according to Roughan and Batt (68).

RESULTS

After 14 days anoxia phospholipid content (expressed as µg P. g^{-1} tissue dry wt) remained unaltered in both Iris species. In I. germanica this also held for glycolipids (expressed as µg galactose. g^{-1} tissue dry wt). However, glycolipids decreased significantly in I. pseudacorus (Table 5.i.)

Figures 5.i. and 5.ii. illustrate a chromatographic separation of Iris polar lipids. Of these the most abundant component is phosphatidic acid. A result of this nature is possibly indicative of phospholipase D action, and the generation of PA as an artefact of extraction should be excluded in future work. However, it was possible to identify the major polar lipids. Both species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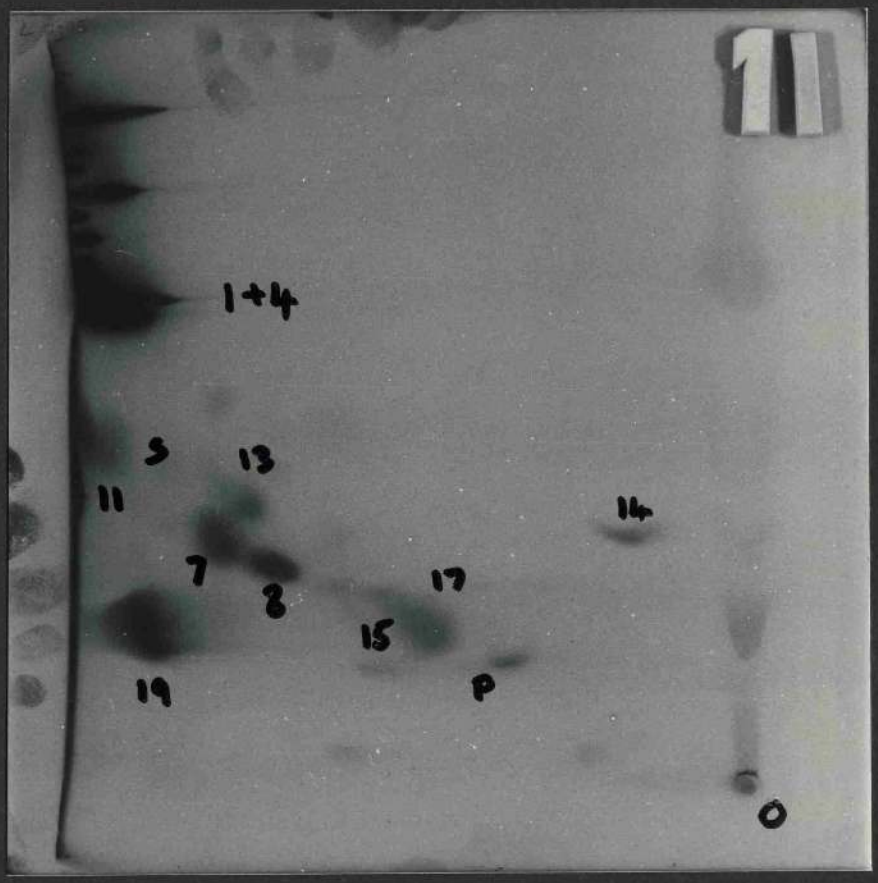
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P
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contained PE, PC, PG, PS, PI, DGDG and SQDG. CL and MGDG were also identified although partially masked by other lipids. Additionally the presence of a number of unidentified glycolipids and sterol compounds was indicated by their differential staining reactions. Both species contained the same major polar lipids.

DISCUSSION

During 14 days anoxia polar lipids declined in I. pseudacorus while being maintained at the same level in I. germanica. This confirms the findings of the previous chapter. Interestingly, the polar lipid losses in I. pseudacorus were restricted to the glycolipid class (Table 5.i.). Others (55; 69) using ^{14}C-acetate report phospholipid losses in both rice and wheat, whilst (55) found no reduction in rice MDGD labelling under anoxia. However it should be noted that in the present work phospholipids were estimated as lipid phosphorus, while in previous reports they have been estimated through acetate incorporation into the constituent fatty acids. If as advocated in the previous chapter a lipid acyl hydrolase specific for the one position on phospholipids is operating, then although total phospholipid (by weight) may decline the amount of lipid phosphorus will not necessarily alter. The apparent discrepancy between these results and those of (55) may therefore be explained.

It is believed that glycolipids are exclusively associated with chloroplastic membranes whilst in non-photosynthetic tissue they are localized in other plastids

such as amyloplasts and chromoplasts (3). It may be that the decrease in glycolipid observed in I. pseudacorus reflects a preferential breakdown of such organelles under anoxia which might be of adaptational significance. Preferential loss of these organelles may represent a mechanism for the conservation of other membranes when the supply of lipids for turnover is limited. Further, a breakdown of starch storing amyloplasts might also involve the mobilization of reserves and therefore contribute to energy metabolism.

Losses of polar lipids under anoxia have an interesting parallel in animal tissue (in which the ratio of phospholipid to glycolipid is much higher). Accelerated phospholipid degradation and its resultant membrane dysfunction have been proposed as the critical alteration that produces irreversible rat liver cell injury and ultimately cell death in ischemia (the interruption of blood supply to a tissue)(60). It has been demonstrated that it is the depletion of O_2 *per se* which causes cell injury (58), and (60) suggests that the loss of phospholipid results from the activation of endogenous membrane bound phospholipases by the increase in cell Ca^{2+} which occurs in ischemia. From work with rat cardiac myocytes it has been suggested (59) that in ischemia or anoxia where ATP levels are very severely depressed the resistance of the membrane to attack by endogenous phospholipases is reduced. They further suggested that this might have resulted from a failure of ATP-dependent protective

mechanisms such as phosphorylation-dephosphorylation of membrane components.

No accumulation of lyso-phospholipids in either species of Iris was found in this work, and there were no obvious changes in proportions of either phospholipid or glycolipid classes in response to anoxia (Figs 5.i. and 5.ii.). However, as is apparent in Figs 5.i. and 5.ii. the major phospholipid component was found to be phosphatidic acid. This is normally a minor phospholipid (2). The presence of large amounts of PA is possibly indicative of the action of phospholipase D (70). This enzyme, confined to the plant kingdom, hydrolyses phospholipids to phosphatidic acid. It is known to be released during tissue disruption and remains active in organic solvents such as chloroform (71). In this study no precautions were taken to prevent its activity. Inactivation of the enzyme is frequently carried out by boiling the tissue in iso-propanol prior to extraction (22).

Although it is highly unlikely that 100% phospholipid degradation will have occurred it is difficult to draw many conclusions from this experiment about the influence of anoxia on phospholipid classes.

CHAPTER 6

POST ANOXIC PEROXIDATION IN IRIS SPP.

INTRODUCTION

Continuing with the investigation into the causal factors of anoxic injury, in this chapter consideration will be given to possible changes occurring on rexposure to oxygen after a period of anoxia. One such hitherto uninvestigated mechanism for cellular damage may be membrane lipid peroxidation. Peroxidative damage underlies a number of membrane pathologies (72) and in plants has been implicated in such processes as leaf senescence (73), wounding (74), drought suscepibility (75) and in ozone damage (76).

Although essential to aerobic life, under certain conditions O_2 may also behave as a cytotoxin. It has been observed that on exposure to pure O_2 higher plants grow badly and develop lesions in both roots and leaves (77). The toxic effects of O_2 result from its participation in certain cellular reactions which generate highly reactive species. These include peroxide (H_2O_2) and the superoxide radical ($O_2^{\cdot -}$) which are not particularly damaging in themselves but can give rise to the hydroxyl radical ($\cdot OH$) and singlet oxygen ($^1O_2$). Hydroxyl radicals are extremely reactive and they will attack and damage almost every cellular molecule (78). One group of molecules particularly sensitive to attack by these species are the polyunsaturated fatty acid components of cell membranes (79). These can also be attacked directly by $^1O_2$ (77).

Within the cell there are a number of endogenous mechanisms to protect against the deleterious effects of O_2. These may be divided into primary and secondary mechanisms on the basis of the timing of the defensive action. Primary mechanisms operate during and prior to lipid peroxidation while secondary mechanisms are associated with post lipid peroxidative events (Fig 6.i.). These mechanisms include the enzymes superoxide dismutase (SOD), catalase and peroxidase. Small molecules such as glutathione, hydroquinones, carotenoids and α-tocopherol are also involved in protective processes (80). Of the protective mechanisms glutathione reductase is induced by oxygen in cotton leaves (81) but unlike animals (82), bacteria (83), blue green bacteria (84), yeast (85) and green algae (86) cotton leaf SOD is not induced by oxygen (81). However, SOD is induceable in other higher plants (87). If these protective mechanisms decline during anoxia it may be that the critical phase of anoxic damage may not be during the anoxic period itself but in the period immediately after rexposure to O_2 when defences may be inadequate.

Interestingly, in rat cardiac tissue (88) and gerbil brain (89) it has been demonstrated that post hypoxic reoxygenation results in lipid peroxidative damage.

The purpose of this investigation was to assess the degree of lipid peroxidation occurring in the 2 Iris species after anoxia. By measuring levels of the lipid peroxide product malondialdehyde (MDA), it would be possible to

Fig 6.i. Scheme showing reactive species in polyunsaturated fatty acid (PUFA) peroxidation and endogenous protection mechanisms.

Reactive Species	Notes		Protective mechanisms
H_2O_2	May be formed through action of eg. urate oxidase, L-amino oxidase and glycollate oxidase	PRIMARY MECHANISMS	catalase; peroxidase
$O_2^{-}\cdot$	May be formed through action of eg. xanthine oxidase and tryptophan dioxygenase		superoxide dismutase (SOD)
$\cdot OH$ $^1O_2$	Formed through reaction of $O_2^{\cdot -}$ and H_2O_2		glutathione dependent protective proteins

peroxidized ///

Fig 6.i. continued on facing page.

Fig 6.i. (Continued from facing page)

Reactive Species	Notes		Protective mechanisms
peroxidized PUFA intermediates	The above species can attack PUFA's. Once the reactions have been initiated the system is autocatalytic.	SECONDARY MECHANISMS	α-tocopherol carotenoids (as free radical scavengers act as chain breakers).
Lipid peroxides			glutathione peroxidase; phospholipase A_2+lyso-phosphatide acyl transferases
Fragmentation products eg. MDA			aldehyde dehydrogenase (animals)

observe if a parallel existed between plants and animals in post anoxia/hypoxic response. The primary shoot was chosen as the region for investigation since it appeared particularly sensitive to anoxia in the intolerant species I. germanica (Chapter 3).

MATERIALS AND METHODS

Preparation of rhizomes for the anaerobic work bench and anaerobic treatments were carried out as previously described (Chapter 3). After 14 days all rhizomes were harvested and treated in the following manner:- a segment of tissue c. 0.75 cm^2 was rapidly excised from the meristematic region of the cut primary shoot. About ⅓ of the tissue was weighed and used in the thiobarbituric acid (TBA) reaction, while the remaining ⅔ was also weighed and immediately ground in liquid N_2 and freeze-dried overnight. The 5 anaerobic and aerobic control rhizomes were harvested on termination of the experiment. The anaerobic rhizomes were harvested and processed inside the anaerobic workbench (with the exception of the liquid N_2 treatment) while a further 5 were treated as for the aerobic controls after 6 hours exposure to laboratory atmosphere.

Thiobarbituric Acid Reaction (TBA)

This was carried out after the methods of (90) and (73) with modifications (91 ; 92). The tissue (c. 550 mg fresh weight) was ground in 5 ml 72% trichloroacetic acid.

After transfer to a boiling tube, 5 ml 0.5% thiobarbituric acid, 0.23 ml $FeCl_3.6H_2O$ (286 mg/100 ml H_2O) and 0.23 ml butylated hydroxytoluene (233 mg/100 ml absolute EtOH)(to prevent further peroxidation) were added. The tube was heated at 95 ^{o}C for exactly 60 minutes, and then rapidly cooled in an ice bath, 4 ml n-butanol were added and the contents vortex mixed. After centrifugation the spectrum of the supernatant was run between 490 and 550 nm versus a reagent blank. The malondialdehyde (MDA) concentration was calculated from OD 535-520 and the molar extinction coefficient for MDA of 1.56×10^5 $M^{-1}cm^{-1}$ (93).

In the anaerobic treatment after grinding in TCA and adding the reagents, the boiling tube was removed from the a.w.b. and the remainder of the reaction proceeded in the laboratory.

Lipid Extraction

Lipids were extracted from the freeze dried tissue by the method previously described (Chapter 4).

RESULTS

The results of the MDA estimations expressed on a dry weight basis are given in Fig 6.i. and Tables 6.i. and 6.ii. The levels of MDA are lower in I. germanica than in I. pseudacorus regardless of treatment. After 14 days anoxia there are no significant differences for I. pseudacorus between the aerobic control, the anoxic

Table 6.i. Malondialdehyde concentrations related to dry weight, fresh weight and total lipid in primary shoots of I. pseudacorus rhizomes subjected to varying degrees of anoxia. Figures in parentheses are S.E. Ratio[a] = Anoxic treatment/aerobic control. Each value represents the mean of 5 replicates except those marked * where n=4.

	n mol MDA.mg^{-1} fresh weight	Ratio[a]	n mol MDA.mg^{-1} dry weight	Ratio[a]	n mol MDA.mg^{-1} lipid	Ratio[a]
Anoxia (14 days)	6.38×10^{-4} (8.00×10^{-5})	0.56	4.95×10^{-3} (7.50×10^{-4})	0.88	6.50×10^{-2}* (1.00×10^{-2})	0.96
Anoxia (+14d) + 6 h air	7.95×10^{-4} (9.50×10^{-5})	0.69	4.90×10^{-3} (2.50×10^{-4})	0.88	8.25×10^{-2} (1.75×10^{-2})	1.22
Aerobic (14 days)	1.15×10^{-3} (1.60×10^{-4})	-	5.58×10^{-3} (6.25×10^{-4})	-	6.75×10^{-2}* (1.25×10^{-2})	-

Table 6.ii. Malondialdehyde concentrations related to dry weight, fresh weight and total lipid in the primary shoots of *I. germanica* rhizomes subjected to varying degrees of anoxia. Figures in parentheses are S.E. Ratio[a] = Anoxic treatment/aerobic control. Each value represents the mean of 5 replicates.

	n mol MDA.mg^{-1} fresh weight	Ratio[a]	n mol MDA.mg^{-1} dry weight	Ratio[a]	n mol MDA.mg^{-1} lipid	Ratio[a]
Anoxia (14 days)	7.70×10^{-5} (1.65×10^{-6})	32.49	5.83×10^{-4} (1.45×10^{-5})	37.6	6.18×10^{-3} (1.33×10^{-3})	27.96
Anoxia (+14d) + 6 h air	3.30×10^{-4} (6.50×10^{-5})	139.24	2.43×10^{-3} (5.95×10^{-4})	156.8	3.50×10^{-2} (7.50×10^{-3})	158.37
Aerobic (14 days)	2.37×10^{-6} (6.63×10^{-7})	-	1.55×10^{-5} (4.38×10^{-6})	-	2.21×10^{-4} (4.45×10^{-5})	-

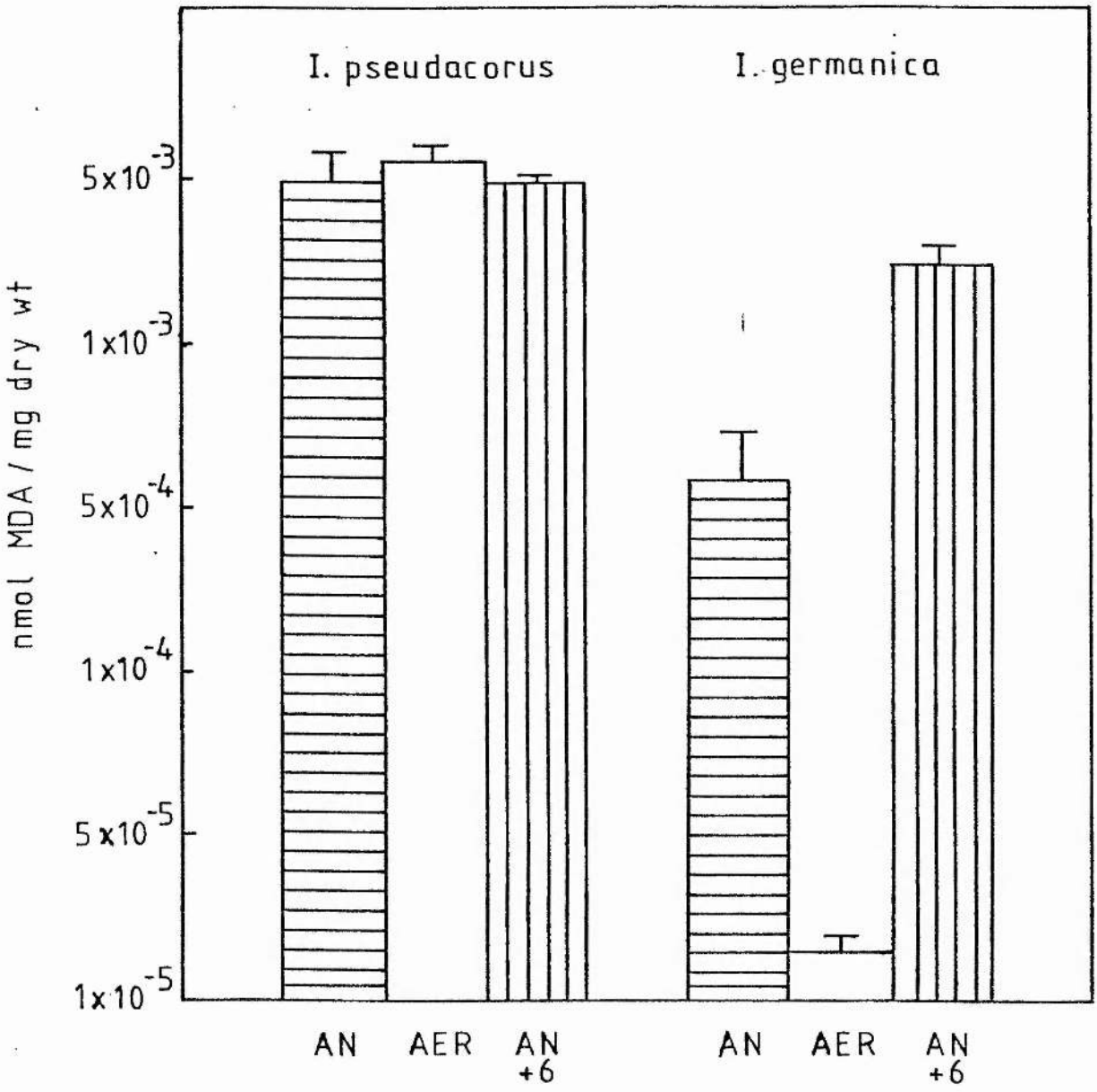
Table 6.iii.

	Anoxia/Anoxia + 6h	Anoxia/Control	Control/Anoxia + 6h
I. pseudacorus	N.S.	N.S.	N.S.
I. germanica	Significant*	Significant*	Significant*

Results of significance test among treatments in Iris peroxidation experiment (MDA expressed on a mg tissue dry weight basis). An initial test of significant variation among treatments means was carried out using a one way analysis of variance; pairwise comparisons were made by utilising the LSD calculated from the residual mean squares. Because the individual treatment variances were not equal in I. germanica a logarithmic transformation of the data was performed. * significant at P 0.002. n=5

Fig 6.ii. Malondialdehyde concentrations expressed on a dry weight basis in primary shoots of rhizomes of Iris pseudacorus and Iris germanica. Rhizomes were subjected to 14 days anoxia (AN), 14 days anoxia followed by 6 h exposure to air (AN + 6) or 14 days in air (AER). Error bars represent S.E.; n=5.

fig. 6.2


I. pseudacorus
I. germanica
nmol MDA / mg dry wt
5x10-3
1x10-3
5x10-4
1x10-4
5x10-5
1x10-5
AN
AER
AN +6
AN
AER
AN +6

control and the anoxia +6 hours exposure to laboratory air treatments (Table 6.iii.). By contrast, there is a significant increase in MDA in I. germanica rhizomes kept under anoxia even without exposure to air but after 6 h in the air levels rose to 150 times those in the air.

These differences are maintained when the results are expressed on a total lipid basis (Table 6.i. + 6.ii.). When expressed on a fresh weight basis the pattern is maintained in I. germanica, however a significant difference results between the aerobic and anoxic treatments in I. pseudacorus.

DISCUSSION

Anoxia brings about a decrease in fresh weight and dry weight in both I. pseudacorus and I. germanica (results not shown), and additionally total lipid in I. pseudacorus (Chapter 4). Similarly, in rice the absolute levels of reducing sugars (94) and proteins (95 ; 96) decrease under anoxia. As MDA production is frequently expressed on the basis of one of the above parameters, it is therefore difficult to make meaningful comparisons between anoxic and aerobic treatments. As it is the polyunsaturated fatty acids attached to polar lipids which undergo peroxidation, then it would be useful to relate MDA production to total (phospho and glyco) lipids. However, it was demonstrated in Chapter 6 that

I. pseudacorus glycolipids decline under anoxia, making the comparison invalid. In this study, by the inclusion of a third experimental treatment (a second control) which was analysed under anoxia, it was possible to differentiate the influence of post anoxic events on MDA production from the effects of anoxia alone. For comparatime purposes results were expressed on a dry weight, fresh weight and total lipid basis. MDA production maintained a similar pattern regardless of the method of expression. Thus, variation in moisture or lipid content could account for the changes in MDA observed.

On a dry weight basis, there were no significant differences between aerobic and anoxic treatments in I. pseudacorus (Fig 6.ii. and Table 6.iii). This was in marked contrast to the 38 fold increase in MDA in the I. germanica primary shoot under anoxia.

An increase in peroxidative end products under anoxia is surprising, but may reflect the existence of traces of O_2 not removed from the rhizome in the a.w.b. transfer chamber. The high level may also reflect MDA production initiated when the leaves were removed from the rhizomes prior to experimentation, which in the aerobic control may be further metabolized. If MDA is neither further metabolized nor undergoes other reactions under anoxia it may remain to react in the TBA test after 14 days. In addition, after 6 hours exposure to laboratory air there was a 150 fold increase in the peroxidative end products relative to the aerobic control. A similar

increase in lipid peroxidation in apical buds of Phaseolus vulgaris stored under N_2 (22 hours), and then incubated in air (0.5 hours) has been reported (97). Of great interest is the increase in MDA between the anaerobic treatments. This is in the region of a 4 fold increase, and may be responsible for the observed bud destruction after anoxia.

Although, the absolute levels of lipid peroxidation appear higher in I. pseudacorus than in I. germanica it is obvious from the survival data (Chapter 3) that the primary shoots of the former species suffer no ill effects. In I. germanica during anoxia there may be failure to maintain both primary and secondary peroxidative defence mechanisms (Fig 6.i.). This would account for the inverse correlation between increased MDA production and decreased primary shoot viability after anoxia. Although the absolute amounts of MDA are always greater in I. pseudacorus, there is no increase in MDA production between treatments, this may be due to the maintenance of effective (ie. at the aerobic level) primary and seondary protective mechanisms. The primary defense systems guard against any increase in peroxidation after anoxia, while the maintenance of secondary systems ensures that damage can be withstood (Fig 6.i.). It is suggested that in I. germanica it is the increase in peroxidation which is responsible for damage,* and that this increase is due to the failure of primary protection mechanisms under anoxia, and an inability to resist/repair peroxidative damage due to

* or vice versa

inadequate secondary mechanisms. It is interesting to note that in I. pseudacorus membrane lipid changes accompany anoxia (Chapter 4) and although post anoxic exposure to laboratory air was limited it may be indicative of such repair mechanisms.

In this work a correlation has been observed between primary shoot mortality (Chapter 3) and increased MDA production in I. germanica, which was not present in I. pseudacorus. In order to determine whether there was any causal basis to these observations additional experimentation would have to be carried out. This might take the form of monitoring the activity of defensive enzymes such as SOD, treatment with antiperoxidative agents such as EDW (76) and removal of rhizomes into differing O_2 atmospheres.

CHAPTER 7

GENERAL DISCUSSION

The object of this research was to investigate possible metabolic adaptations to anoxia. In order to achieve this a better understanding of the causal events in anoxic injury was required. In Chapter 4 a number of mechanisms were put forward to account for anoxic mortality. These were divided into primary and secondary causes on the basis of whether there was direct participation of molecular O_2 in the reaction. The emphasis in this work was on the former category, with particular interest in the maintenance of membrane integrity under anoxic conditions.

Two weeks anoxia at 20 ^{o}C results in 20% of I. germanica rhizomes perishing (Fig. 3.i.). If membrane dysfunction through lipid component omission was a major factor in lethality, an alteration in lipid composition would be expected. As evidenced in Chapters 4 and 5 this was not the case. However, this does not rule out primary lipid participation in primary shoot mortality (which appeared much more sensitive to anoxia (Fig. 3.i.)), as in the whole rhizome lipid assay compositional changes restricted to the primary shoot may have been concealed.

In the anoxia tolerant species I. pseudacorus there were a number of lipid modifications during anoxia. Glycolipids declined dramatically (Table 5.) and although all fatty acids declined it was surprisingly the

saturates which decreased the most (Chapter 4). It was suggested (Chapter 5) that the decline in glycolipids might reflect mobilization of carbohydrate reserves and/or replenishment of the fatty acid pool through glycolipid breakdown. The significance of the alterations in membrane fluidity which might be expected to result from alterations in the saturated/unsaturated ratio remain unexplained.

For more conclusive results it would be necessary to investigate the dynamic aspects of anoxic lipid adaptation. Although one would predict that synthesis of saturated acids could continue, labelling techniques would provide valuable information on the rates of synthesis and possibly turnover of membrane components. These additional data might aid in interpreting the significance of the alterations in the ratio of saturated to unsaturated fatty acids in I. pseudacorus. In I. germanica where there were no apparent changes in lipid composition such data would indicate whether anoxia induces a quiescent state in the rhizome or whether lipid metabolism still continues.

However, it must be stressed that an isotopic approach would not suffice on its own. Such a study might not have revealed the massive glycolipid decline in I. pseudacorus, and without gross membrane lipid compositional data a statement on membrane fluidity is impossible. In future isotopes could profitably be employed to investigate the "extension growth" observed

in a number of species under anoxia (42). Such "growth" whether due to cell expansion or cell division would seem likely to involve the production of new membrane and its lipid composition and metabolism would be of interest.

In summary, although there are lipid alterations in the tolerant I. pseudacorus (which may be of adaptational significance) the lack of lipid modifications in the sensitive I. germanica are not consistent with a primary role for lipids in lethality under anoxic conditions. However, it should be borne in mind that lipid compositional data for the whole rhizome might nevertheless mask significant localized lipid changes either at the anatomical or subcellular level.

It has always been implied that the lethal event(s) occurred under anoxia, and that transfer to an oxygen atmosphere would alleviate the problem by reactivating aerobic metabolism. However as indicated in this work (Chapter 6) the act of transfer itself can result in the generation of cytotoxic molecules, through peroxidative reactions.

The highly anoxia sensitive primary shoot tissue of I. germanica produced significantly increased amounts of malondialdehyde (MDA), a lipid peroxidation by-product after rexposure to air, while the tolerant I. pseudacorus did not show this increase. Although the overall levels of MDA are higher in I. pseudacorus it may be that primary shoot tissue contains efficient endogenous secondary protection mechanisms to make good peroxidative

damage, enabling the tissue to cope with higher levels of peroxidation. Also the site of peroxidative damage may be important, if this is localized to one specific membrane in I. germanica then its effects might be more devastating than a general membrane peroxidation in I. pseudacorus. It would be of interest to determine whether there is a decline in I. germanica protective mechanisms during anoxia, and whether peroxidative damage could be avoided by introduction of the rhizome into atmospheres of gradually increasing O_2 concentration. In I. pseudacorus the adaptational significance of tolerance to high levels of peroxidation is unclear. In the natural environment it is unlikely that the species would ever be exposed to rapid alterations in O_2 concentration. Rather, the transfer from anaerobiosis to aerobiosis will take place gradually as muds dry out, the water table recedes or new shoots break the surface.

However, if peroxidative damage underlies anoxic pathology it may have a number of important commercial applications. In the agricultural industry where large amounts of produce are stored in bulk, it is conceivable that the central portion of a stored mass may be subject to anoxia. If this is indeed the case, when the produce is moved the rapid inrush of O_2 could lead to peroxidative damage. It would therefore be of interest to determine which crops (especially vegetable) contained adequate protection mechanism and which were peroxidation susceptable. Applied research into induction of protection mechanisms,

storage at different O_2 concentrations or into methods of ensuring continued crop aeration might be of commercial interest.

It is now possible to amend the table of anoxic toxicity mechanisms presented in Chapter 4. Of the primary mechanisms it would seem that lipid omissions are not directly responsible for mortality in Iris, however it may be that membrane lipid adaptation is important to continued tolerance. A third category must be added to the scheme, which is post anoxic peroxidative damage. Further, the differential tissue survival observed in this work underlines the importance of taking into consideration tissue type before embarking on physiological experiments. If differing tissue metabolism contributes to the differential tissue survival then by implication care must be taken in selecting tissue of a similar phenology.

APPENDIX

For the investigation of the effects of anoxia on the membrane lipids of *Iris* species a high resolution TLC system capable of resolving the major phospholipid classes was required. As published systems (98 ; 99) frequently displayed overlap of certain phospholipid classes as well as glycolipids, it seemed appropriate to extend the TLC system currently being developed in Dr Hunter's laboratory to the analysis of plant polar lipid species.

Plates (20 x 20 cm) precoated with silica gel 60 (Merck) were activated at 110 ^{o}C for 60 minutes, and allowed to cool and the lipid (*c.* 5,000 µg Total Lipid) sample applied as a spot.

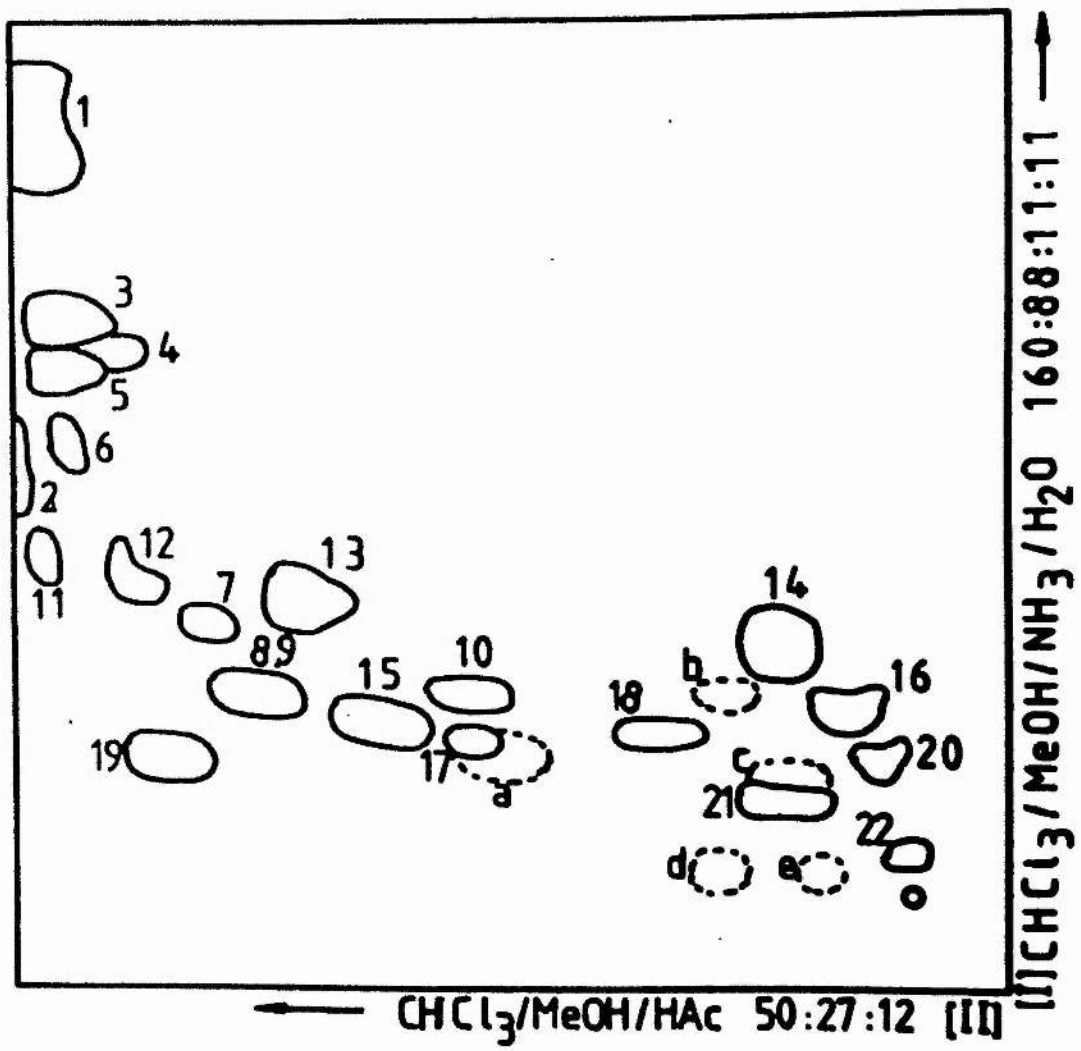
Tanks containing solvent were lined with chromatography paper and allowed to equilibriate at 4 ^{o}C for at least 1 hour before use. Low temperature was felt to be desirable since it improves resolution by minimizing diffusion of spots and autoxidation of polyunsaturated fatty acids. Plates were developed in the first dimension using chloroform-methanol-ammonia (25% w/v)-water (160:88:11:11) (v/v) to within 0.5 cm of the top (120 mins), removed from the tank and dried using a hair drier (15 mins), complete removal of the solvent being essential. Chloroform-methanol-glacial acetic acid (50:27:12)(v/v) was used to develop the second dimension for the same distance (180 mins) and the plates removed and dried as above. During chromatography, the tanks were sealed with parcel

tape. All solvents were dried and redistilled before use and butylatedhydroxytoluene, 50 $mg.l^{-1}$ added as antioxidant.

Iodine vapour was used as a general detection reagent, and molybdate reagent (100) for phospholipids. For glycolipids, anthrone (101) proved to be most useful. After spraying with 0.2% anthrone in conc. H_2SO_4 the plates were heated on a hot plate at 70 ^{o}C for 20 mins. The glycosyldiglycerides gave green/blue spots within 10 mins, while the violet spot characteristic of sulfoquinovosyldiglyceride (SQDG) took up to 15 mins for full colour development. Phospholipids appeared as brownish-grey spots. For analytical purposes the best results were only obtained with freshly prepared samples, this may be due to oxidation in older samples altering the chromophore to produce intermediate colours. Individual spots were tentatively indentified from their differential staining reactions, by comparison with authentic standards and with the work of others in Dr Hunter's laboratory.

A composite map showing the positions of some 25 polar lipids is shown in Fig A1. In addition to separating most naturally occurring phospholipids, several glycolipids were also resolved. Fig A2 and Fig 5.i illustrate separations of leaf (broccoli) and rhizome (I. pseudacorus) polar lipids. On these chromatograms spots which could not be positively identified due to either the lack of availability of authentic standards or lack of specific stain have been tentatively identified. Fig A3 shows a separation of lipid standards in this system, it should

fig. A·1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a
18
b
14
16
c
20
21
22
d
e
CHCl3/MeOH/HAc 50:27:12 [II]
[I]CHCl3/MeOH/NH3/H2O 160:88:11:11

Fig A1.

Map of polar lipids after two-dimensional TLC with $CHCl_3$-MeOH-NH_3 (25% w/v)-H_2O 160:88:11:11 (v/v)(1st dimension) and $CHCl_3$-MeOH-HAc, 50:27:12 (w/v) (2nd dimension) at 4 ^{o} on precoated silica gel 60 (Merck) plates. Spots outlined with solid lines denote authentic standards; dotted lines signify tentatively identified naturally occurring components.

Phospholipids: 11 = cardiolipin (CL); 12 = phosphatidyl glycerol (PG); 13 = phosphatidyl ethanolamine (PE); 14 = phosphatidyl choline (PC); 15 = phosphatidyl serine (PS); 16 = sphingomyelin (SM); 17 = phosphatidyl inositol (PI); 18 = lysophosphatidyl ethanolamine (LPE); 19 = phosphatidic acid (PA); 20 = lysophosphatidyl choline (LPC); 21 = lysophosphatidyl serine (LPS); 22 = diphosphoinositide (DPI).

Glycosyl Diglycerides: 4 = monogalactosyl diglyceride (MGDG); 7 = sulphoquinovosyl diglyceride (SQDG); 8 = digalactosyl diglyceride (DGDG).

Glycosphingolipids: 3 = monoglucosyl ceramide (GcC); 5 = mongalactosyl ceramide (Type II - non-hydroxy-fatty acids) (GaCII); 6 = monogalactosyl ceramide (Type I - hydroxy-fatty acids) (GaCI); 9 = lactosyl ceramide (LC); 10 = psychosine (Ps); a = triglycosyl ceramide (C_3); c = tetraglycosyl ceramide (C_4); b, d, e = unidentified neutral glycosphingolipids.

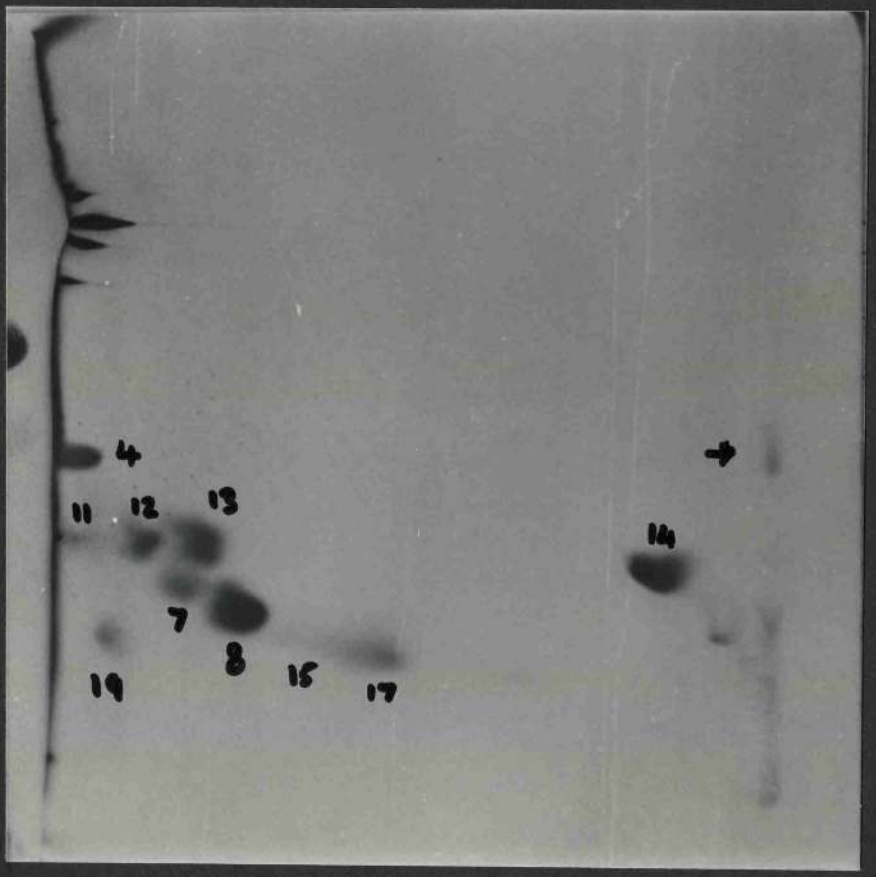
Others: 1 = neutral lipids (eg. mono-, di-, tri-glyceride, cholesterol, carotenoids); 2 = rhodamine; 0 = origin.

Fig A2.

Thin-layer chromatogram of total lipids from broccoli leaves. Conditions, labelling and staining as in previous figures. Loading 20 µg lipid P.
Note glycolipid breakdown products (arrowed).

Fig A3.

Thin-layer chromatogram of standard phospholipids.
Conditions and labelling of spots as in Fig A1.
Detection I_2 vapour.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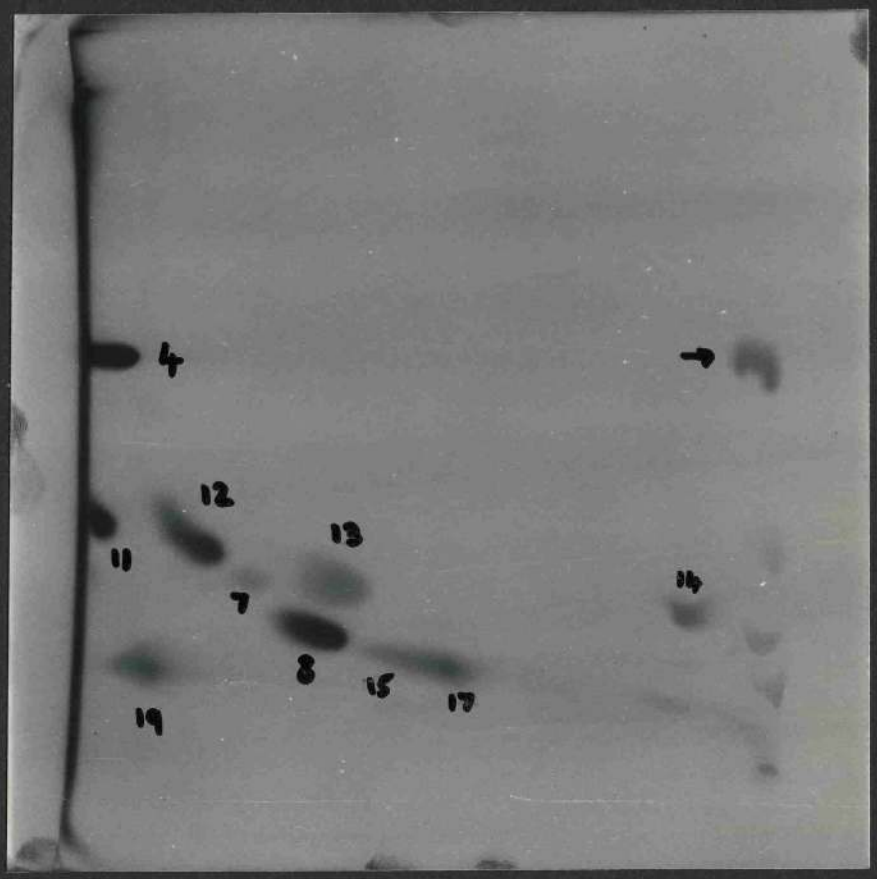
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be noted that incomplete resolution of standard phosphatidyl serine (bovine brain) and phosphatidyl inositol (yeast) was achieved, yet separation was always satisfactory in plant tissue extracts (Figs A2 and 5.i.) and human samples (pers comm.). This may be due to fatty acid compositional differences. This TLC system would thus appear to be applicable to the separation at low temperature of a wide range of polar lipids from diverse cell types.

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