LIPID PEROXIDATION AND AGEING

IN SEEDS OF <u>GLYCINE</u> <u>MAX</u>.

by

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OF SEEDS

SOME HAVE MORE VITALITY THAN OTHERS

AS TO KEEPING.

(Theophrastus, c. 372 - 287 B.C.)

PREFACE

The work described in this thesis was carried out in the Department of Biology, University of Natal, Durban, from June 1987 to July 1990, under the supervision of Dr. M. T. Smith and Dr. G. K. Campbell.

These studies represent the original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it is duly acknowledged in the text.

ACKNOWLEDGMENTS

Dr. M. T. Smith. This thesis is as much the fruit of his labours as it is mine.

Dr. G. K. Campbell. For his assistance and many hours of helpful discussion, all of which contributed in no small way to the outcome of this thesis.

I would like to express my sincere gratitude to the staff, both academic and technical, and fellow students of the Department of Biology, in which I have been a student for the last ten years.

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My parents and family, for their encouragement and support.

My Father, without whose conscious and unconscious guidance this work would not have been possible.

ABSTRACT

Six different lots of soya beans (<u>Glycine</u> <u>max</u> (L.) Merr.) were examined. Seed hydroperoxide levels were highly correlated with viability, but not with moisture contents. It was proposed that moisture contents may exert a similar antioxidant effect at intermediate levels as has been observed in dry foods.

Seeds treated with ferrous sulphate were significantly (5% level) invigorated. Furthermore, this treatment was observed to give rise to a reduction in the peroxide value of soya bean axes over the first hour of imbibition, an increase in 2,3,5-triphenyl-tetrazolium chloride reduction and protein synthesis, and a decline in electrolyte leakage. It was proposed that this was due to the antioxidant activity of the ferrous iron, leading to an attenuation of free-radical induced autoxidation.

Ferrous sulphate treated seeds produced more aldehydes than untreated seeds. This result suggested that aldehydes may not be responsible for declining seed vigour.

Hexanal, pentanal and butanal production from heated dry seeds was significantly correlated with seed

germination, CVG and hydroperoxide levels. The thermal

breakdown of the hydroperoxides was postulated to be

the source of these compounds.

A GC technique was developed using model systems of

oxidized methyl oleate, linoleate, linolenate and soya

bean bulk oil. The analysis of seed lipid oxidation

products revealed marked differences in the proportions

of the products compared to bulk and monolayer oxida-

tion. The selective production of the 13-hydroperoxide

implicated enzymatic or metalloprotein involvement.

The implications of the results of this study with

regard to the present theories of seed ageing were

discussed.

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ERRATA

The words argument(s) and occurred (occurring, occurrence) have been consistently misspelt.

SECTION I

INTRODUCTION

CHAPTER 1

1. SEED AGEING - CONCEPTS AND DEFINITIONS

Plant seeds constitute some of the longest lived
assemblages of cells that do not undergo cell division.
Despite the exceptional longevity exhibited, all seeds
gradually lose vigour and ultimately viability.

1.1 Definitions

In this study, a viable seed will be defined as
one that germinates under conditions appropriate for
the species. Germination entails the emergence and
development from the seed embryo of those essential
structures which, for the kind of seed in question, are
indicative of the ability to produce a normal plant
under favourable conditions (McDonald, 1980). Vigour is
notoriously difficult to define adequately, and no
consensus has yet been reached. Numerous tests to
establish seed vigour have been devised. Most of the
tests are based on one of the following criteria:
germination and seedling growth charactaristics,
germination under stress conditions before germination
under favourable conditions, physical parameters,
biochemical charactaristics, and degree of mechanical
damage (Steiner et al., 1989). The definition put

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forward by the International Seed Testing Association in 1977 will be adopted here. Seed vigour is the sum total of those properties of the seed which determines the potential level of activity and performance of the seed or seed lot during germination and seedling emergence. It may be broadly seen as a impaired capacity to germinate.

Seeds can broadly be divided into two groups; recalcitrant and orthodox. Recalcitrant seeds are difficult to define, but can possibly be described as seeds which cannot undergo dehydration on maturation. Orthodox seeds, on the other hand, upon the completion of maturation, enter into a period characterized by low moisture content and metabolic quiescence. It is during this period of time, i.e. between maturation and germination, that seeds will age.

1.2 Factors effecting seed vigour

It is generally agreed that the loss of seed vigour is due to the gradual, random accumulation of damage by the seed tissue, leading to an 'error catastrophe' (Osborne, 1980). This process begins immediately following maturation, the rate of vigour loss being dependent on the rate and site of damage. This is thought to be determined largely by the

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conditions experienced by the seeds preceeding and following maturation; that is, the history of the seed during development and the temperature, moisture content, and the availability of oxygen during storage (Osborne, 1980).

All seeds do not age at the same rate and longevity may differ markedly amongst genera. For example, Canna seeds are reported to have survived ca. 600 years and Nelumbo for ca. 237 years. Hard coated legumes such as Cassia, Albizzia and Mimosa are also very long lived, in marked contrast to other legumes such a soya beans (Glycine max (L.) Merr.) (Osborne, 1980), which under optimal conditions will not maintain their original vigour for longer than two years (Priestley, 1986). Even amongst seeds of the same species, some seeds appear to be genetically superior to others, in that they show a more vigorous growth and higher yields. This may even be apparent between seeds produced by the same parent plant at the same time (Bewley and Black, 1982). Differences in morphology also influence vigour, larger seeds from a particular seed lot often being more vigorous than smaller ones (Bewley and Black, 1982).

1.3 Vigour and storage

Seed history following harvesting and subsequent to storage is complex and often impossible to establish. Two major factors that influence vigour during this period are the physical handling of the seed and the effects of fauna (e.g. rodents, insects) and flora (e.g. fungi). The means of harvesting the seed as well as post-harvest handling can damage seed tissue by bruising or fracturing it. This depends very much on the type of seed (Moore, 1972), legumes being particularly susceptible to this kind of damage (Dickson, 1980).

Microflora, particularly fungi, can influence seed vigour under humid storage conditons (Bewley and Black, 1982). A causal relationship between infection and vigour has not been convincingly demonstrated. Nevertheless, that infection can accelerate loss of vigour is generally accepted (Roberts, 1972).

The conditions of storage, namely temperature and relative humidity, are the main factors thought to be responsible for the loss of seed vigour during dry storage (Bewley and Black, 1982). It has been estimated that storage life may be doubled for each 1% reduction in seed moisture content or for every 5°C reduction in

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storage temperature (Harrington, 1973).

It should nevertheless be noted that this is a generalization, and that there are exceptions. For example, at very low moisture contents (less than 4-5%), certain species of seed is often damaged (Bewley and Black, 1982). Similarly, seeds stored under conditions that permit very high moisture contents without the onset of germination, the rate of the loss of vigour may be greatly reduced. For example, thermodormant lettuce (Lactuca sativa L.) seeds stored fully imbibed display a marked resistance to vigour loss compared to unimbibed seeds (Villiers, 1974).

Exceptions are also evident with regard to temperature. Low temperatures are generally conducive to longevity in orthodox seeds. However, some seeds, particularly those of tropical origin, exhibit a sensitivity to temperatures lower than 10-12°C (Lyons, 1973). High temperatures, predictably, lead to the rapid loss of viability. The technique of rapid or accelerated ageing bears mention here. It has become common practice in seed ageing research to use the simultaneous application of high temperature and relative humidity (commonly 40°C and 100% relative humidity) to obtain, in a relatively short space of time, seeds of different vigour. Implicit in this

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method, however, is the assumption that the effects generated by the above conditions correspond to those that take place under 'normal' conditions (Villiers, 1980). This assumption is being increasingly questioned for two main reasons. Firstly, the use of the technique has lead to the accumulation of a great deal of contradictory information. Secondly, evidence for the activity of enzymes at such high relative humidity is accumulating (Priestley, 1986).

The role of oxygen in the loss of seed vigour is equivocal. For example, Ohlrogge and Kernan (1982) have shown that in soya bean and safflower (Carthamus tinctorius L.) high levels of oxygen, moisture or temperature each acted independently to cause losses in germination, but when applied together, these factors acted synergistically. However, soya bean seeds aged under high temperature (44°C) and humidity (100%) showed no oxygen dependence in seed death. This has lead to the view that although oxygen may act synergistically with either temperature or moisture content, it does not appear to be of primary importance (Osborne, 1980) and its role in the loss of seed vigour remains uncertain (Priestley, 1986).

1.4 Biochemical changes and vigour

In an attempt to account for the loss of vigour observed during dry storage, a number of biochemical changes have been presented as possible causes of the loss of seed vigour. These have been divided into four categories: 1) a decline in metabolic activity and its manifestations (e.g. reduced respiration, slower seedling growth, and lower germination); 2) an increase in the activity of certain enzymes (e.g. phytases, proteases, phosphatases and lipases); 3) a decrease in the activity of other, chiefly respiratory enzymes (e.g. dehydrogenases, cytochrome oxidases, glutamic acid decarboxylase, catalase, peroxidase) and 4) an increase in membrane permeability resulting in the leakage of sugars, amino acids and inorganic solutes from the seed (Abdul-Baki, 1969).

Biochemical tests for vigour may therefore be divided into three groups: they may show unsound areas of seeds which are faulty; they may use the response of an essential enzyme as an index of general vitality; or they may monitor a more complex process vital for the seed in the early stages of germination (Heydecker, 1972).

Before going on to consider theories of seed

ageing, two processes often referred to in the literature need to be briefly addressed. These are free-radical theory and autoxidation.

1.5 Free radical theory

The superoxide radical can be produced directly by a number of enzymes (e.g. peroxidases and dehydrogenases) in both plants and animals (Halliwell and Gutteridge, 1985). Under normal circumstances, no toxicity results due primarily to the action of superoxide dismutase (SOD), which is responsible for converting the superoxide into hydrogen peroxide and oxygen (1).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \ \ldots\ldots (1).$$
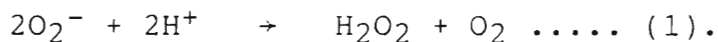
The hydrogen peroxide produced by SOD is broken down by the action of enzymes such as catalase and glutathione peroxidase. Under certain conditions the hydrogen peroxide may be broken down by a Haber-Weiss type reaction to yield the hydroxyl radical (2).

$$H_2O_2 + O_2^- \rightarrow \ \cdot OH + OH^- + O_2 \ \ldots (2).$$
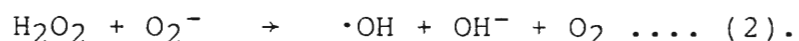
The second-order rate constants of the classical Haber-Weiss reaction are virtually zero in aqueous

solution, making the occurence of the above reaction in vivo very unlikely. This has led to the proposition that the reaction may be catalysed in vivo by traces of transition metals, particularly iron. It is proposed that the superoxide reduces any traces of ferric iron present (3) which in turn reduces hydrogen peroxide by a Fenton type reaction (4) to produce the hydroxyl radical.

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \ldots (3).$$
$$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+} \ldots (4).$$

$$\text{Net:} \quad O_2^- + H_2O_2 \rightarrow O_2 + \cdot OH + OH^- \ldots (5)$$
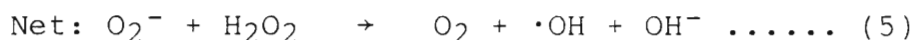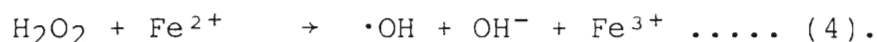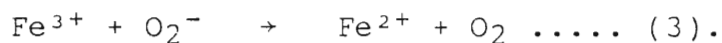
The hydroxyl radical is extremely reactive and can react with any organic molecule in its immediate surroundings. There is general consensus concerning the above mechanisms, the major areas of research in this field being the determination of the source of the superoxide in vivo, and the mechanisms by which the hydroxyl radical leads to the onset of toxicity (Figure 1.1)

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Production of

superoxide radicals

$\downarrow$

SOD

$\downarrow$

H_2O_2 $\rightarrow$ catalases,

$\downarrow$ peroxidases

iron catalysed $\downarrow$

Haber-Weiss H_2O

reaction

$\downarrow$

$\cdot OH$

$\downarrow$

damage to

macromolecules
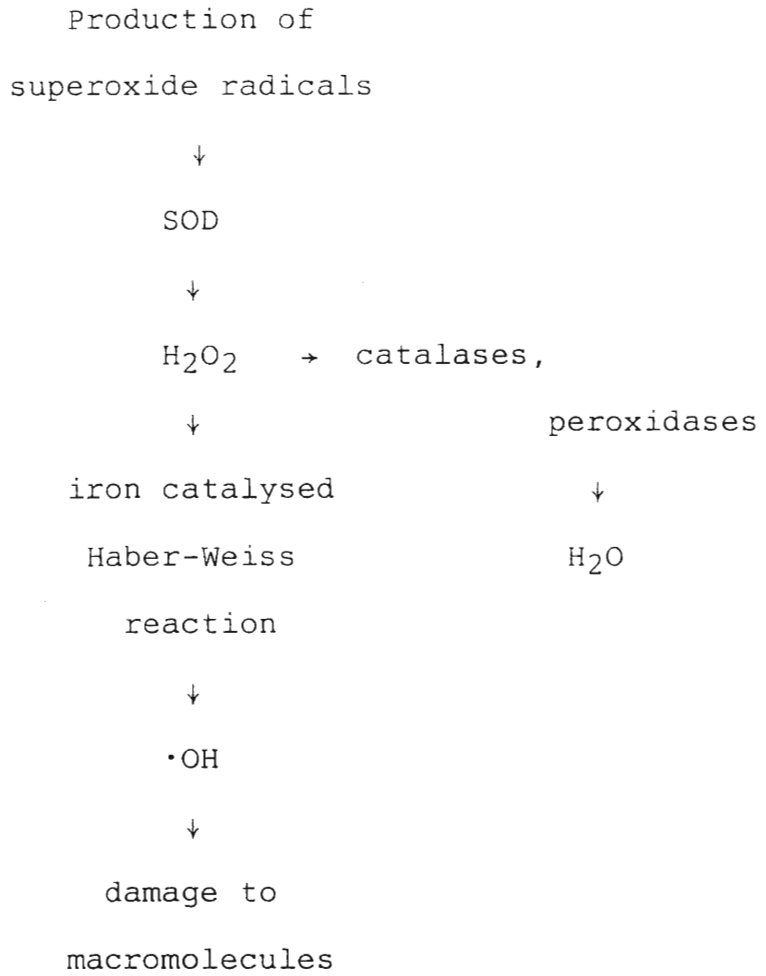
Figure 1.1 The superoxide theory of oxygen toxicity. (Halliwell and Gutteridge, 1985).

1.6 Autoxidation

Lipid peroxidation, or autoxidation, has been suggested to be the cause of membrane damage in seeds (Koostra and Harrington, 1969). The possible effects of peroxidation on mitochondrial membranes in vitro has been extensively investigated by Vladimirov et al. (1980). Lipid peroxidation increases membrane rigidity and negative surface charge, leading to loss of respiratory control, an increase in membrane permeability to calcium and a loss of ATPase activity. It was further shown to bring about an increase in membrane permeability to H^+ (or OH^-) ions and was responsible for the breakdown in membrane dielectric stability. Predictably, the uncoupling of oxidative phosphorylation was also shown to be a direct result.

In bulk oil, autoxidation of polyunsaturated fatty acids is thought to proceed via a free-radical chain reaction. It is initiated by hydrogen abstraction by a free radical and the addition of molecular oxygen to produce a peroxy radical (6,7). This radical in turn abstracts hydrogen from an adjacent molecule, usually another fatty acid, resulting in the formation of a lipid hydroperoxide and a further free radical, thus perpetuating the chain reaction (8). The reaction continues until terminated, usually by an antioxidant.

The hydroperoxide is relatively stable and will tend to accumulate (Frankel, 1982).

$$-CH=CH-CH_2-CH=CH- + R\cdot \rightarrow -CH\underline{=}CH\underline{=}CH\underline{=}CH\underline{=}CH- + RH \quad ....(6)$$

$$-CH\underline{=}CH\underline{=}CH\underline{=}CH\underline{=}CH- + O_2 \rightarrow -CH-CH=CH-CH=CH- \quad .... (7)$$
$$| $$
$$OO\cdot$$

$$-CH-CH=CH-CH=CH- + H\cdot \rightarrow -CH-CH=CH-CH=CH- \quad .... (8)$$
$$| \qquad\qquad\qquad\qquad\qquad |$$
$$OO\cdot \qquad\qquad\qquad\qquad\quad OOH$$
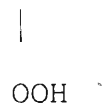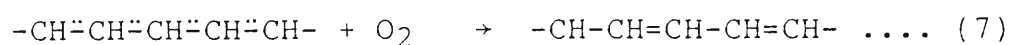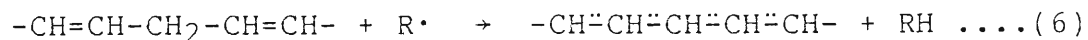
Autoxidation of linoleate (after Halliwell and Gutteridge, 1985).

In plants, three fatty acids usually predominate, namely oleic (18:1), linoleic (18:2) and linolenic acid (18:3). Autoxidation of these oils usually produces a complex mixture of allylic hydroperoxides (Frankel, 1982). Autoxidation of monolayers of these oils appears to be different inasmuch as higher proportions of epoxy compounds are produced (Logani and Davies, 1979).

Hydroperoxides are apparently relatively benign (Vladimirov et al., 1980). The breakdown of

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hydroperoxides, however, has been shown to yield a large number of products including short chain aldehydes and alkoxy radicals (Frankel, 1982). These would be potentially damaging if produced _in vivo_ (Esterbauer, 1982). Not only are they implicated in changes in membrane fluidity, surface charge and permeability (Vladimirov et al., 1980), but they have been shown to react with polypeptides (Gardner, 1979) and may also be involved in polynucleotide damage (Inouye, 1984).

Hydroperoxide breakdown is thought to be catalysed by presence of reduced iron (Frankel, 1982) by a similar mechanism to that given for hydrogen peroxide breakdown (3-5). Figure 1.2 gives a schematic representation of the major steps involved. The origin and nature of the free radical attack implicated remains uncertain and is a major area of research in this field.

1.7 Lipoxygenase

No discussion of lipid peroxidation would be complete without a consideration of the activity of lipoxygenase. Lipoxygenase (linoleate: oxygen oxidoreductase; EC 1.13.1.13) is found in a wide variety of plants, particularly the legumes. It is

POLYUNSATURATED

FATTY ACID POOL

↓

free radical

attack

↓

LIPID HYDROPEROXIDE

POOL

↓

hydroperoxide breakdown

(heat, Fe^{2+})

↓

organic free radicals

short chain aldehydes

↓

damage to

macromolecules
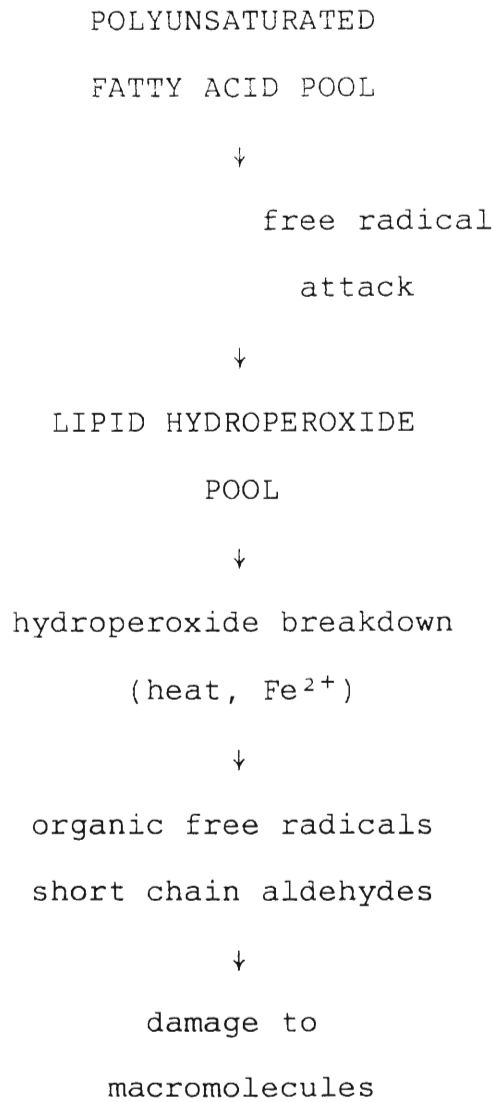
Figure 1.2 Free radical induced lipid peroxidation - the fate of the hydroperoxide.

highly specific for the oxidation of fatty acids which contain a cis,cis penta-1,4-diene unit. The naturally occuring fatty acids, linoleic, linolenic and arachidonic acids, which contain one or more such unit, are therefore potential substrates for LOX. Oleate, having a single double bond, is not a substrate. However, owing to the stereospecificity of LOX, a cis,cis system with a hydrogen atom of L-configuration at the ω-8 postion is essential. In most cases, the hydroperoxy group is introduced in the ω-6 position, except for a very small amount of ω-10 hydroperoxy acid formed with linoleate.

Autoxidation, in contrast, produces a 50:50 mixture of 13-hydroperoxy and 9-hydroperoxy acids. The discovery of non-heame iron in soya LOX has lead to the development of the mechanism given in reactions 9-12 (Gardner, 1980).

$$E-Fe^{+++} + LH \rightarrow E-Fe^{++}L\cdot + H^+ \quad \dots \, (9)$$

$$E-Fe^{++}L\cdot + O_2 \rightarrow E-Fe^{++}LOO\cdot \quad \dots \, (10)$$

$$E-Fe^{++}LOO\cdot \rightarrow E-Fe^{+++}LOO^- \quad \dots \, (11)$$

$$E-Fe^{+++}LOO^- \rightarrow E-Fe^{+++} + LOO^- \quad \dots \, (12)$$
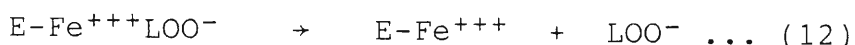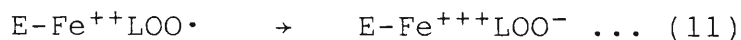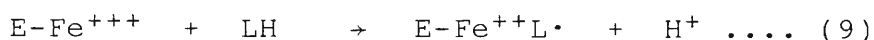
1.8 Vigour theories

"The vigour of a seed is at its highest at maturation. From then on it deteriorates inexorably, continuously and irreversibly." (Heydecker, 1972). As deterioration proceeds, individual members of the total population succumb at different rates. If the number of seed deaths were plotted against time, a 'viability curve' is obtained which approximates a negatively sigmoidal shape.

As Heydecker (1972) has observed; "Loss of germination is clearly an important indication of the loss of vigour but it is, equally clearly, the last relevant indication, the final catastrophe. Many important detrimental changes take place before the seeds lose their ability to germinate."

A broadly accepted, factually based theory to account for seed ageing in orthodox seeds in general has yet to be established (Anderson and Baker, 1983). With hindsight, the many theories put forward to date have been at best hypotheses, but more frequently the speculative nature of these theories has been considerable. Two main lines of approach have been adopted. The first views seed ageing as the successive occurance of sequentially related events leading

finally to the failure to germinate. An example of this approach is the sequence of deterioration given by Delouche (1969).

1) Degradation of cellular membranes and subsequent loss of permeability;

2) Impairment of energy-yielding and biosynthetic mechanisms, and consequently

3) Reduced respiration and biosynthesis;

4) Slower germination and slower heterotrophic seedling growth;

5) Reduced storage potential;

6) Slower growth and development of the autotrophic plant

7) Less uniformity in growth and development amongst plants in the population;

8) Increased susceptibility to environmental stresses (including micro-organisms);

9) Reduced stand-producing potential;

10) Increased percentage of morphologically abnormal seedlings, and,

11) Loss of germinability.

(from Heydecker, 1972)

The second view with regard to seed ageing is that it constitutes a complex of phenomena, primarily

unrelated, any one of which can lead to the loss of germinability. This view is represented by the "classification of viability theories" given by Roberts (1972). Factors proposed to contribute to the loss of vigour include extrinsic factors such as ionising radiation and storage fungi, and intrinsic factors such as the accumulation of growth inhibitors, the accumulation of mutagens, the metabolic depletion of essential reserves, the denaturation of proteins (including enzymes), the denaturation of lipoprotein cell membranes and the denaturation of nucleic acids.

The development of seed ageing research has till recently followed the latter approach. Two hypotheses adopting the former view have recently appeared. The first has been put forward by Berjak et al. (1986) in which it is proposed that "free-radical mediated, deteriorative events might be concentrated at localized intracellular sites, in particular in the mitochondria and in the milieu of the chromatin." A key concept of this hypothesis is "an elevated water content of these organelles compared with other subcellular sites," the rate and location of damage being "a function of the concentration of macromolecular structures vulnerable to free radical attack, occuring within such confined locations which might have relatively higher water contents." The nature or origins of the free radicals

implicated is not addressed. As indirect evidence for
free radical involvement in the seeds investigated,
these workers have shown that the storage of aged seed
in a static, negatively charged field, has been shown
to minimise damage and extend viability (Pammenter et
al., 1974). A similar, though very much smaller,
invigoration was reported on treatment with sodium
thiosulphate and mercaptoethanol (Barnes and Berjak,
1978).

A similar hypothesis has been put forward by
Wilson and McDonald (1986b). They envisage a "steady-
state free-radical flux during ageing concurrent with
accumulation of both co-oxidant injury and oxygenated
fatty acids and their enzymatic breakdown upon
hydration resulting in further seed damage due to free
radical formation and toxic secondary product
production." The paper in which this hypothesis
appeared constitutes essentially a review of work to
date, the authors using work from a variety of
disciplines to support their arguement. However,
evidence beyond broad correlation is lacking, resulting
in the somewhat speculative nature of their hypothesis.
Nevertheless, this paper has contributed greatly to the
clarification of a number of issues in seed ageing
research. Two of these bear mention. "First, the use of
accelerated ageing as a rapid method of mimicking

natural ageing and second, a failure to distinguish

between causes and the potential effects of ageing"

(Wilson and McDonald, 1986b).

These sentiments are shared by the author of the

definitive treatment of seed ageing research to date

(Priestley, 1986). With regard to the use of

accelerated ageing, he states, "It is difficult to

reconcile so many apparently contradictory reports

in seeds during accelerated ageing, save to note

that enzymatic effects - both anabolic and catabolic -

cannot be discounted at high levels of water sorption.

Differences among species or ageing conditions may well

infuence the equilibrium between degradation and

synthesis."

With reference to the second aspect, Priestley

states further, "A wide range of studies have described

potentially debilitating changes in seeds that can

arise as a consequence of ageing, but it often remains

difficult to assess their significance. Investigators

of seed deterioration have repeatedly looked for

physiological or biochemical deficiencies at the

subcellular level in attempts to associate these

changes with loss of vigour and viability; often such

accounts reveal an implicit assumption that the

alteration observed is closely linked to the mechanism

of seed deterioration, and even that it may represent the primary lesion responsible for ageing. Nevertheless, arguments based solely on association of phenomena are often inconclusive when one attempts to unravel a complicated web of cellular interactions; with most studies of this nature we are left no wiser as to whether the effect observed is a cause of a correlate of seed ageing."

The importance of lipid peroxidation in seed ageing remains a contentious issue (Bewley, 1986; Priestley, 1986). Previous work by the author has shown that seed peroxide value was highly correlated with germination in both cabbage (Brassica oleracea L.) and soya beans (Hailstones and Smith, 1988). In this study, it was attempted to obtain further evidence for the involvement of lipid peroxidation. Two main directions of study were employed. The first was a more detailed investigation of the products of lipid peroxidation in order to obtain some information on the causes and mechanisms of lipid peroxidation in the seed itself. The second involved an exploratory study using the invigorating effect of ferrous iron (Hailstones and Smith, 1989b) to obtain information on the possible consequences of lipid peroxidation upon imbibition. In the light of the significant amount of work done on soya bean, this species was chosen for this study.

CHAPTER 2

2. BIOCHEMICAL CHANGES ASSOCIATED WITH SEED AGEING

In this section no attempt will be made to provide an exhaustive review of work done in this field, as much of it is beyond the scope of the present dissertation. A thorough treatment of the entire field, with an exhaustive bibliography, has recently been compiled by Priestley (1986).

2.1 Ultrastructure

A seed is not an amorphous conglomerate of cells, but exhibits marked structural and ultrastructural diversity. The most obvious is the division between cotyledonary and other embryonic structures such as plumule, radicle and endosperm. Even within these structures, ultrastructural diversity is evident. This needs to be borne in mind when considering the changes taking place in the seed as a whole. For this reason, a brief consideration of ultrastructure will follow.

The ultrastructure of dry soya beans and the changes associated with imbibition has been investigated by Webster and Leopold (1977) and Chabot

and Leopold (1982). In the cotyledons, Webster and Leopold (1977) presented evidence suggesting that in dry tissue the plasma membrane was disrupted and unorganised, and that it was structurally altered during imbibition to become an effective barrier. The membranes of organelles appeared usually continuous and well defined. A similar situation was observed for radicle tissue (Chabot and Leopold, 1982). In general, two classes of membrane were observed; "1) membranes of the plasmalemma and protein bodies with closely associated vesicles and lipid bodies, and often meandering or infolded; and 2) the membranes of nuclei, chloroplasts (plastids) and mitochondria which had no associated vesicles and show no meandering or vesiculation, are relatively smooth and lie in contact with the cytoplasm."

Support for the above ultrastructural arrangement is provided by work on maize (Zea mays L.) by Berjak and Villiers (1970, 1972a, 1972b, 1972c, 1972d) and Berjak et al. (1986). These workers reported a similar association of vesicles with the plasmalemma. Their failure to mention a similar situation for the protein bodies may be related to the fact that this work was limited to the root cap. Vesicles, possibly lipid bodies, associated with both the plasmalemma and the protein bodies has also been observed in lettuce

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(Lactuca sativa L.) (Smith, 1978, 1983, 1989). This evidence would suggest that the observed association of lipid vesicles with subcellular structures that must undergo extensive expansion may accurately reflect the situation in dry seeds generally, given the complexities associated with such work on dry material.

Ultrastructural events occuring during ageing has not been done on soya bean. However, Abu-Shakra and Ching (1967) have examined isolated mitochondrial pellets from soya bean axes. Mitochondria from aged material had dilated cristae, a coagulated matrix and some did not have an intact outer membrane. The general disorganization of subcellular membrane systems as a correlate of ageing has been confirmed in both rapidly and slow aged maize (Berjak and Villiers, 1970, 1972a, 1972b, 1972c, 1972d; Berjak et al., 1986) and in lettuce aged under various conditions (Smith, 1989). Changes in the nuclear material were also reported in both maize and lettuce.

Although ultrastructural work is invaluable in the formulation of seed ageing theory, it has a number of limitations. Firstly, it is impossible from the ultrastructural evidence alone to establish the biochemical basis of the lesions observed. Secondly, it is equally impossible to determine whether the lesions

observed are a cause or consequence of seed ageing, and whether they have occcured subsequent to or preceeding imbibition. It may be argued that since the lesions became apparent before any observable vigour loss (Berjak et al., 1986), they possibly constitute a fundamental cause of the loss of vigour. However, biochemical evidence for at least one aspect of ageing, namely lipid peroxidation, has indicated that seeds of both cabbage and soya bean can undergo significant oxidation without any significant decline in vigour (Hailstones and Smith, 1988). As mentioned before, arguements based purely on correlation are often inconclusive.

Biochemical changes associated with seed ageing may be grouped into five main catagories.

1) Cell membranes and permeability and changes in the chemistry of lipids;
2) Changes in the structure and chemistry of proteins, including enzymes;
3) Respiration;
4) Chromosomal abberations and the deterioration of DNA, and
5) RNA and protein synthesis.

Clearly, in the complex web of interaction, changes in

one aspect will effect another. For reasons of convenience, however, they will be dealt with successively.

2.2 Chromosomal abberations and deterioration of DNA

2.2.1 Chromosomal damage

That plants grown from aged seed show a high incidence of morphological abnormality has long been recognized. The first clear evidence that changes in the genetic material were associated with seed ageing came from cytological observations of mitotic cells. Navashin (1933a) demonstrated that plants grown from aged _Crepis_ _tectorum_ L. seeds had a higher incidence of chromosomal abberations. These observations have since been confirmed in a number of seed types, the _locus classicus_ being the work of Roberts _et_ _al_. (1967, Abdalla and Roberts, 1968) on barley (_Hordeum_ _vulgare_ L.), broad beans (_Vicia_ _faba_ L.) and peas (_Pisum_ _sativum_ L.). Work of this nature on soya bean has not come to the writer's attention. In general, it may be said that storage conditions that lead to seed deterioration also promote chromosomal abberations (Priestley, 1986). Although generally true, it should be noted that under certain, usually extreme storage conditions, the correlation between the frequency of chromosomal abberations and germinability breaks down.

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This has been observed to be the case for broad beans (Roberts et al., 1967) and lettuce (Harrison, 1966).

Roberts (1972) has stated that despite a good correlation between the loss of viability and nuclear damage, "the theory is not without its difficulties if one asks the question: what aspect of nuclear damage leads to an inability of the seed to germinate?" The visible damage itself is unlikely to be a causal factor in seed deterioration, as most abberent genomes will be rapidly excluded from the total population. Although chromosome breakage may itself be unimportant, the gene mutations accompanying it presumably are. However, in the light of the observed breakdown in correlation under certain conditions, it cannot be said with any certainty that declining vigour and the loss of viability is the result of the accumulation of mutations. In support of such a position, Roos (1982) has argued that age-induced lesions of the type visualized in mitotic figures are unlikely to threaten the genetic integrity of stored germ plasm.

2.2.2 DNA integrity and synthesis

Changes in the integrity of DNA has also been reported. For example, DNA isolated from aged rye (Secale cereale L.) was greatly fragmented, as evidenced by a decreasing amount of spoolable DNA, and

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the separation of total DNA on a sucrose gradient or polyacrylamide gel revealed an increasing amount of randomly sized fragments of low molecular weight (Cheah and Osborne, 1978; Osborne et al., 1980/81).

There is also evidence to suggest that ^{3}H-thymidine is incorportated into double stranded DNA as part of a postulated repair function during the first hour of imbibition, and that this 'unscheduled' DNA synthesis is more intense in partially deteriorated seeds (Osborne, 1982a, 1982b, 1983). Similarly, scheduled DNA synthesis prior to mitosis is a relatively late event in germination, but is reduced and delayed in older material (Sen and Osborne, 1977). Evidence therefore suggests that impairment of DNA integrity and synthesis is clearly a correlate of declining rye seed vigour. Similar changes in DNA synthesis has been reported in wheat (Triticum eastivum L.) using rapid ageing techniques (Dell'Aquila et al., 1980, Dell'Aquila and Margiotta, 1986). Presumably this may apply to other seeds as well.

The sources of such impaired integrity and synthesis and the chromosomal defects are unknown. Cheah and Osborne (1967) have postulated that endodeoxyribonuclease activity, triggered by age-induced deficiencies of the enzyme's inhibitor, may be

responsible for the fragmentation they observed.
Natural background radiation has been generally
discounted, as it is insufficient in magnitude to
account for the changes observed (Priestley, 1986).
Automutagenis, that is, the production of toxic or
mutagenic compounds as a result of physiological or
biochemical activity within the seed has long been
implicated (James, 1961). A recently proposed
hypothesis has suggested that the breakdown products of
lipid oxidation may be responsible for declining seed
vigour (Wilson and McDonald, 1986b). However, although
such products have been implicated in damage to the DNA
(Inouye, 1984), direct evidence for such interactions
within the seed itself is lacking.

2.3 RNA and protein synthesis.

2.3.1 Protein synthesis

Protein synthesis, which is usually evident within
the first hour of imbibition, is impaired in
deteriorated material, and soya beans are no exception
(Anderson, 1977; Gidrol et al., 1988). The declining
rate of protein synthesis may be related in part to the
levels of available energy, particularly ATP and GTP,
but it is clear that ageing involves severe deficien-
cies in the cellular mechanisms responsible for the
translation of mRNA into protein (Priestley, 1986).

2.3.2 Ribonucleic acid

The messenger RNA responsible for protein synthesis during imbibition may be of two sorts. Some 'long-lived' or 'presumptive' mRNA survives desiccation within the seed and is capable of being translated soon after rehydration. In adddition, newly transcribed mRNA is produced within a few hours of imbibition. Lesions in either of these mRNAs would predictably effect protein synthesis (Priestley, 1986).

There is evidence to suggest that long-lived mRNA may be lost to some extent during storage. For example, in rye seeds, Osborne (1983) has shown that the amount of retrievable polyadenylated RNA from unimbibed embryos was greatly reduced in aged material, and specific template activity was also reduced by ageing. However, it is possible that much of the stored mRNA is not essential for germination, as it is subject to extensive degradation during imbibition of wheat. Indeed, it appears that the degradation of this RNA may be in fact delayed or impaired in deteriorated seeds (Smith and Bray, 1982, 1984; Smith et al., 1986; Bray and Smith, 1985). The significance of declining levels of long-lived mRNA during ageing is therefore still uncertain (Priestley, 1986).

Although it is generally suggested that declining synthesis of 'new' mRNA may be a correlate of seed deterioration, it is still not clear at what level this lesion manifests itself. That is, is it a reduced level of available energy, deficiencies in transcription or deficiencies in translation. Ribosomes may suffer some degree of degradation during ageing. For example, in rye embryos the 18S and 28S rRNA subunits may suffer loss of integrity (Roberts and Osborne, 1973a), which has been attributed to ribonuclease activity. Despite this, the sedimentation characteristics of ribosomes from non-viable rye embryos suggest that all major components are still present (Osborne et al., 1977).

2.3.3 Lesions associated with the soluble factors.

At least one major age induced lesion is associated with the soluble factors that mediate peptide bond formation at the ribosome. The capacity for GTP-dependent binding of amino acyl-tRNA to ribosomes by elongation factor 1 is impaired in deteriorated rye (Roberts and Osborne, 1973a), pea (Bray and Chow, 1976a) and wheat (Dell'Aquila et al., 1976). In aged pea, the deterioration of elongation factor 2 and phenylalanyl-tRNA synthetase have been reported (Bray and Chow, 1976a).

The evidence in this field has been succinctly

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summarized by Priestley (1986). "The evidence currently available clearly indicates that each of the principle elements of the protein synthesizing system in aged seeds may suffer from deficiencies: long-lived mRNA is lost or less efficient, newly synthesized messenger may be absent or incoherent, ribosome structure and effectiveness are altered to a certain degree, and some of the soluble factors responsible for peptide formation are incapacitated. There is, in addition, some question of a sufficient and continuing supply of the nucleoside triphosphates required by the synthetic apparatus. Several or all of these factors probably serve to reduce the overall ability of aged seeds to synthesize protein."

2.4 Changes to the proteins, including enzymes

In seeds, as in most organisms, proteins have both a structural and functional role. It is the latter role that is of particular interest in seed ageing research, and may be divided into two main groups; storage reserves and enzymes.

2.4.1 Storage reserves

As early as 1915, Crocker and Groves demonstrated that both seed ageing and protein denaturation in vitro were similarly influenced by temperature and moisture

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content. Solubility properties have also been found to change during long term storage, and such effects have been noted in soya bean (Echigo, 1965; Saio et al., 1980). Most investigators, however, have not attempted to relate these changes with declining seed vigour, and the reasons for such changes remain obscure (Priestley, 1986).

Proteolysis has not been widely linked to seed ageing (Priestley, 1986), although Ovcharov and Genkel (1973) have reported declining levels of protein in the embryos and endosperm of deteriorated maize. However, such changes were observed in wheat only at high moisture levels, seeds having 10-11% moisture content showing no such changes (Krishtofovich, 1974). The possible involvement of fungi at such high moisture levels needs to be taken cognisance of (Priestley, 1986).

2.4.2 Enzymes

Attempts to correlate changes in enzyme activity with seed ageing are not new to the field, the work of MacLeod (1952) on barley being an oft sited example. Proteinase, β-amylase, phosphatase and catalase activity were observed to decline slowly, and was still evident after loss of viability. Peroxidase activity declined more rapidly, in close correlation with seed

vigour.

Some enzymes are absent or inactive in newly hydrated seeds, and become detectable only after a few hours of imbibition. The rate at which these new enzymes appear is frequently retarded in aged material (Priestley, 1986). For example, Rao and Wagle (1983) suggested that lower levels of isocitrate lyase in aged soya bean was due to developmental deficiencies in de novo protein synthesis.

Many studies have shown that enzyme activity is reduced in deteriorated seed of many different species, including amylases (Saxena and Maheshwari, 1980) and lipase (Luczyńska, 1973) in soya bean. However, it is not always clear whether these shortcomings are due to degradation of latent enzymes in the stored seed or arise less directly from a failure of proper development (Priestley, 1986).

Some studies have shown an increasing activity of certain enzymes. Rao and Wagle (1981) have reported an increased β-amylase activity in rapidly aged soya beans. Proteinase activity has been observed to increase in wheat (Shvetsova and Sosedov, 1958) and Sorghum bicolor L. (Perl et al., 1978). Grange et al.

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(1980) found that endopeptidase activity in kidney bean (Phaseolus vulgaris L.) declined in the early stages of ageing but increased thereafter. Zelenskii and Zelenskaya (1983) reported a similar pattern for proteinase in rapidly aged soya beans. It should be stressed that reports of increasing activity during deterioration almost always come from studies employing rapid ageing techniques (Priestley, 1986).

2.4.3 The tetrazolium test

Related to the above discussion is the wide use of tests for respiratory dehydrogenases, in particular, triphenyltetrazolium chloride (Lakon, 1942). The continued use of this technique as a convenient and valid assessment of seed vigour attests for its usefulness and broad applicability. However, the exact physiological basis for the test is uncertain. Much of the reducing ability is probably associated with the respiratory chain, but cannot be confidently regarded as exclusively so (Priestley, 1986). Other factors may also be invloved, such as the level of reducing substances and competition between hydrogen acceptors in the cell (Smith, 1952), as well as considerations of penetrability (Priestley, 1986). Its ability to provide a valid index of vigour is, however, undisputed.

In conclusion, the weight of evidence clearly

indicates that enzymes may suffer deterioration as a consequence of ageing and that others fail to develop normally on germination (Priestley, 1986). However, given the enormous complexities of enzyme activity, any speculation with regard to the causes of these effects and questions about whether they constitute cause or effect of reduced seed vigour would be premature at this stage.

2.5 Respiration

It has long been suspected that declining seed vigour would be reflected as metabolic deficiencies, and in this vein, respiration and related aspects of seed metabolism have received considerable attention. Unfortunately, the complexity of the mechanisms involved, together with the use of rapid ageing, has detracted from the significance of these studies, and no definite conclusions have been forthcoming (Wilson and McDonald, 1986b; Priestley, 1986).

2.5.1 Respiration and vigour

Low rates of oxygen uptake have been consistently reported during imbibition of aged seeds of maize (Woodstock and Feeley, 1965), barley (Abdul-Baki, 1969) and soya bean (Woodstock et al., 1984). In soya bean, this may be evident in the isolated axes and cotyledons

separately (Wahab and Burris, 1971; Parrish and

Leopold, 1977). Also in soya bean, Woodstock and

Taylorson (1981a) have interpreted an increase in

ethanol and acetaldehyde during early imbibition of

rapdily and slow aged seeds as due to an imbalance

between glycolysis and the tricarboxylic acid cycle. A

similar situation has been observed in rapidly aged pea

(Gorecki et al., 1985). An increased respiratory

quotient (the molar ratio of carbon dioxide produced to

oxygen consumed) has also been observed in deteriorated

seeds of maize (Woodstock and Grabe, 1967), barley

(Anderson, 1970) and soya bean (Byrd and Delouche,

1971; Woodstock et al., 1984).

2.5.2 Mitochondria

Impaired respiration has been usually ascribed to

impaired mitochondrial function. One of the earliest

observations in support of this was the work of Abu-

Shakra and Ching (1967) on mitochondria isolated from

the axes of deteriorated and 'fresh' soya bean. Axes

from aged material exhibited a reduced amount of

inorganic phosphate esterified into ATP per unit oxygen

consumption (ADP:O ratio), and the application of an

exogenous respiratory uncoupler, dinitrophenol,

indicated that the mitochondria from aged material were

endogenously uncoupled. More recently, Ferguson et al.

(1990a) has reported a decrease in the rates of state 3

respiration and in the respiratory control ratios of mitochondria from rapidly aged soya bean.

Recent attempts to unravel the complexities of respiration at the early stages of germination have met with little success, due mainly to the uncertainties associated with the experimental protocol and the question of the involvement of lipoxygenase and the alternate (cyanide resistant) pathway (Siedow and Girvin, 1980; Leopold and Musgrave, 1980; Parrish and Leopold, 1977, 1978a, 1978b). Attempts to correlate the involvement of the alternate pathway with declining vigour have proved inconclusive. A more thorough attempt to resolve these difficulties has been provided by Puntarulo et al. (1987).

2.5.3 Availability of energy

The availablity of energy, principally as ATP, has also received some attention (Ching, 1982). Levels of ATP and energy charge, effectively the ratio of high energy phosphate species (ATP, ADP) to low energy phosphate species (AMP), tend to be very low in dry seeds, but increase substantially on imbibition (Priestley, 1986). A number of attempts have been made to correlate ATP levels with vigour in seeds of crimson clover (Trifolium incarnatum L.) (Ching, 1973), lettuce (Ching and Danielson, 1972), barley (Van Onckelen et

al., 1974) and rapidly aged soya bean (Anderson, 1977).
In general, a negative correlation between ATP content
and age has been observed, although Buchvarov and
Alekhina (1984) have reported an increased level of ATP
in rapidly aged soya bean.

Caution needs be applied when considering such
data, however. Perl (1986) has drawn attention to
"three pionts that should be emphasized in order to
come to proper conclusions" with regard to ATP levels
and seed vigour. Firstly, a consideration of data that
indicated the presence of a correlation between seed
vigour and ATP accumulation in seeds at their early
stages of imbibition revealed that the positive
correlation may be attributed to the variation in the
size of the seeds used, and not the amount of ATP
accumulated per se. The correlation between seed vigour
and ATP accumulation at the early stage of germination
was examined in over a dozen species and in 90% of them
no such correlation was found.

Secondly, some ATP requiring activities in the
early stage of germination, (protein and RNA synthesis
and membrane-protein phosphorylation) utilize large
amounts of ATP compared with the amounts of ATP
accumulated. The accumulated ATP consists of at most 5%
of the total ATP synthesized. Over 95% of the produced

ATP is utilized concomitantly with its synthesis. Thus if the accumulated ATP increases or decreases even by 100%, its significance and value for evaluation of the rate of ATP synthesis is negligible.

Thirdly, the fact that ATP requiring systems start to act immediately after imbibition, at the same time as the ATP synthesizing system, points to the conclusion that the accumulated ATP is a result of the balance between processes of synthesis and utilization. Hence, large amounts of ATP accumulated may be a result of either active ATP synthesis, reflecting high vigour seeds, or of impaired ATP utilization indicative of low vigour seeds. Similar conflicting conclusions can be drawn from seeds with low levels of accumulated ATP. The data so far published in the literature indicate that ATP accumulation cannot serve as a measure of seed quality (Perl, 1986).

Although the above may represent an extreme and somewhat theoretical view, (indeed, papers by the above author attempting to correlate ATP levels and synthesis with seed vigour continue to appear: Perl and Kretschmer, 1988), it does highlight the fact that a more critical approach needs to be adopted. As the above sentiments may be leveled at other aspects of respiration, such as oxygen consumption and carbon

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dioxide production (Wilson and McDonald, 1986b; Perl, 1986), such caution may be broadly applicable to this field.

2.6 Cell membranes and permeability

2.6.1 Permeability

One of the most consistent observations correlated with seed vigour has been an increased 'leakiness' on the part of deteriorated material. A considerable amount of work has been done in an attempt to establish the cause of this phenomenon. This has led to the accumulation of a large body of literature on the subject.

Attempts to measure this increased leakiness has involved a number of procedures, including examining the ability of cells to plasmolyze (Doroshenko, 1937), the measurement of the elastic modulus of cotyledonary tissue (Parrish et al., 1982) and the use of vital dyes such as Evan's blue (Schoette and Leopold, 1984). The most commonly used approach, however, is the measurement of the electrical conductivity of the steep water. A great number of studies on numerous seed types have reported an increased conductivity with declining vigour, the effect being most graphically demonstrated by the work of McDonald and Wilson (1980) on rapidly

aged soya bean. Despite some concern expressed over the "physiological relevance of the leakage phenomenon" (Priestley, 1986), it is generally agreed that it represents some form of altered membrane integrity. The biochemical basis for this phenomenon has become the subject of extensive investigation.

2.6.2 Membranes and damage of the seed lipid

Of the two major components of membranes, namely lipids and proteins, lipids have received the most attention, although incorrectly so in the opinion of some (Bewley, 1986).

When considering seed lipid, it is necessary to distinguish between true membrane lipid (polar or phospholipid), and storage lipid, which in most cases occurs in vastly greater amounts. A decrease in total lipid content during storage has been reported in some seeds. This was presumably due predominately to losses in the storage lipid. For example, rapidly aged soya beans lost 15% of the oil fraction over six months (Nakayama et al., 1981). It has been suggested that this may be due to slow metabolism by the seed or seed microflora under relatively humid storage conditions (Priestley, 1986).

Changes in the lipids of the membranes has

received much greater attention. A marked decline in the level of phospholipids has been reported in rapidly aged cucumber (Cucumis sativus L.; Koostra and Harrington, 1969), peanut (Arachis hypogea L.; Pearce and Abdel Samad, 1980), pea (Powell and Matthews, 1981b), soya bean (Priestley and Leopold, 1979; Nakayama et al., 1981), tomato (Lycopersicon esculentum Mill.; Francis and Coolbear, 1984) and wheat (Petruzelli and Toranto, 1984). In general, no such decline has been observed in slow aged material, the notable exception being a study on peanuts (Pearce and Abdel Samad, 1980). This once again serves to highlight the differences in responses to the two techniques.

Two suggestions have been offered to account for the chemical alteration of seed lipids: lipid peroxidation and the action of lipases. Phospholipase D is present in dry seeds (Quarles and Dawson, 1969), and minor accumulations of phosphatidic acid has been reported in aged soya bean (Priestley and Leopold, 1979, Nakayama et al., 1981). An increase in lysophopholipids has also been reported (Nakayama et al., 1981). The existence of phospholipase A in plants is questionable, however (Galliard, 1980), and the identity of such lipolytic acivity is therefore uncertain (Priestley, 1986).

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2.6.3 Lipid peroxidation

Evidence for the involvement of lipid peroxidation in seed ageing, principally as a decline in the level of polyunsaturated fatty acids, is equivocal. The data obtained from rapid ageing studies is fraught with contradiction. Some workers have reported no change (Priestley and Leopold, 1979; Pearce and Abdel Samad, 1980), while others have reported a slight decline (Harman and Mattick, 1976, Stewart and Bewley, 1980; Ferguson et al., 1990b).

This contrasts markedly with work done on slow aged material, in which a decline in polyunsaturated fatty acids has been consistently reported in a variety of seeds (Spencer et al., 1973; Flood and Sinclair, 1981; Priestley and Leopold, 1983; Hailstones and Smith, 1988), the notable exception again being the work of Pearce and Abdel Samad (1980) on peanut.

An increase in lipid oxidation products, primarily hydroperoxides, has also been commonly reported. Spencer et al. (1973) reported an increase in hydroxy and epoxy products in Cichorium intybus L. and Crepis spp. Radrupal and Basu (1982) demonstrated a significant negative correlation between a product of lipid peroxidation (malondialdehyde) and rapidly aged wheat and mustard (Brassica juncea, Cross) seed vigour,

although no such relationship was observed in rapidly aged soya bean using similar techniques to assess peroxidation (Stewart and Bewley, 1980). Seed peroxide value was highly correlated with germination and vigour in slow aged cabbage and soya bean (Hailstones and Smith, 1989a), although Pearce and Abdel Samad (1980) were unable to detect hydroperoxides in aged peanut. This contrasts with the work of Mathur et al. (1956), in which a negative correlation between peroxide value and peanut seed vigour was observed.

Attempts to relate seed vigour to endogenous antioxidant levels such as tocopherols have proved inconclusive (Fielding and Goldsworthy, 1980; Priestley et al., 1980). A similar result has been obtained in studies attempting to modulate lipid peroxidation with exogenous antioxidants (Priestley, 1986). One other aspect that has received substantial attention is the involvement of free radicals.

2.6.4 Free radicals and seed vigour

Electron spin resonance (ESR) spectroscopy and low level chemiluminescence analysis have been increasingly employed in the investigation of free-radical based processes involved in seed deterioration. Despite difficulties in interpretation and uncertainties inherent in the application of the techniques to seeds,

ESR has been used in a number of studies. Priestley et
al. (1980) examined cotyledonary material from slow and
rapidly aged soya beans, and found no appreciable
change in free radical signal. This was supported by
the work of Buchvarov and Gantcheff (1984). However,
these workers reported that the free radical signal in
axes of both rapidly and slowly aged soya increased
markedly. Subsequently, Priestley et al. (1985b) has
observed an increased free radical signal in rapidly
aged soya bean axes, but not in axes from slow aged
material. No evidence for a connection between the free
radical levels and lipid peroxidation was obtained,
however, and the identity of the free radicals remains
uncertain (Priestley, 1986).

Kiyashko (1981) and Likhatchev et al. (1984) have
reported an increase in low-level chemiluminescence
from aged soya bean. In contrast, Perelberg et al.
(1981) has observed a decline in the level from soya
beans during long term storage, although an increase
was detected in rapidly aged seed. According to
Buchvarov et al. (1983), the level of chemiluminescence
was greater from aged soya beans during the first
minutes of hydration. In general, the evidence
accumulated is inconclusive, and the involvement of
enzymes, particularly under high humidity conditions
and during imbibition cannot be excluded (Priestley,

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1986). As was pointed out above, there is no direct evidence to link this phenomenon with lipid peroxidation.

To conclude, "it is still difficult to define the role of lipid peroxidation in seed ageing, and a satisfactory understanding of the importance of free radicals in deterioration is even more elusive. Almost any generalization that may be made can be controverted by at least one significant observation" (Priestley, 1986). Nevertheless, from the weight of evidence it is clear that lipid peroxidation does constitute an important aspect of seed deterioration.

2.7 Resumé

In a recent review of the literature pertaining to this field, Wilson and McDonald (1986b) have put forward a lipid peroxidation model of seed ageing, in which they envisage that seed lipids are subject to a slow oxidation during storage. It was proposed that these oxidation products are broken down on imbibition, leading to further free radical production and other related products which result in extensive cellular damage, hence the idea of "incipient death".

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This model constitutes the most recent attempt to provide a unifying hypothesis for seed ageing in which all the different aspects discussed above are incorporated. Thus it is postulated that the breakdown of the oxidation products "may damage the seed further by forming toxic secondary products which inhibit respiration, protein synthesis, DNA synthesis and denature protein." The mechanisms of lipid peroxidation are ascribed variously to a "free-radical flux", autoxidation, or the action of lipoxygenase. Key concepts are the enzymatic breakdown of the hydroperoxides and the consequent production of aldehydes. In the following study, it will be attempted to establish to what extent this model is a true reflection of ageing in soya bean seeds.

SECTION II

CHAPTER 3

3.1 THE EVALUATION OF SEED VIGOUR IN RELATION TO LIPID PEROXIDATION.

3.1.1 INTRODUCTION

Evidence for the involvement of lipid peroxidation in seed ageing has proved inconclusive. In a recent review, Priestley (1986) has stated that, "it is still difficult to define the role of lipid peroxidation in seed ageing." Earlier work on the relationship between lipid peroxidation and ageing in soya bean seeds has provided contradictory results (Stewart and Bewley, 1980; Priestley and Leopold, 1979), leading to controversy over the role, if any, of lipid peroxidation in seed ageing (Bewley and Black, 1982; Priestley, 1986).

In a recent paper (Hailstones and Smith, 1988), evidence was presented for a decline in polyunsaturated fatty acids concomitant with decreasing seed vigour in soya bean and cabbage seeds. In support of this, Priestley and Leopold (1983) have reported a similar decline in polyunsaturated fatty acids in slow aged soya bean. Spencer et al. (1973) have reported an increase in the level of oxygenated acids in the oils of Cichorium intybus L., Crepis thomsonii Babc. and C.

vesicaria L. seeds with storage, while more recently,
Radrupal and Basu (1982) demonstrated a highly
significant negative correlation between lipid
peroxidation and germination in the seeds of wheat
(Triticum aestivum L.) and mustard (Brassica juncea
Cross). Futhermore, a significant correlation was
obtained between germination percentage and
hydroperoxide levels in the cabbage (r = -0.99) and
soya bean (r = -0.89) seeds (Hailstones and Smith,
1988). The weight of evidence, therefore, implicates
lipid peroxidation in seed ageing, although proof of a
causal role has not yet been obtained (Priestley,
1986).

As noted previously, the role of relative
humidity, and hence moisture content, in seed ageing is
well documented. Harrington (1973) has estimated that
seed storage life may be doubled for every 1% reduction
in seed moisture content. However, work completed three
years previously on cabbage and soya bean seeds
(Hailstones and Smith, 1988) revealed a poor
correlation between seed moisture content and
germination percentages. In contrast to this, a marked
positive correlation between moisture content and seed
peroxide levels was observed in the cabbage seeds. In
view of the marked correlations obtained between
germination percentages and peroxide value in both seed

types, it was suggested that increasing moisture contents may lead to increasing levels of peroxidation, thereby causing a decline in seed vigour. However, data from soya bean seeds investigated at the same time did not support this proposal. A thorough evaluation of the soya bean seeds was therefore undertaken in order to obtain further information on the possible causes of lipid peroxidation in soya bean seeds.

The use of the coefficent of velocity of germination (CVG) as a simple measure of the 'speed' of germination, and hence seed vigour, has gained wide acceptance in seed testing laboratories. According to the coefficient developed by Kotowski (1926), if all seeds germinate on the first day, that lot will have a CVG of 100. Declining seed vigour will be reflected in a declining CVG. However, Brown and Mayer (1988), who evaluated single-value indices of germination, state that the method of Kotowski cannot be recommended as a means of summarizing germination. In this study, the ability of the method to reflect seed germination was evaluated.

3.1.2 MATERIALS AND METHODS

Seed Material.

Soya beans (<u>Glycine</u> <u>max</u> (L.) Merr., cvs Ibis, Impala, Pioneer and Hartebees) were obtained from the Summergrain Research Center, Potchefstroom, and stored in air-tight containers at 5°C. All seed lots were harvested in 1984, with the exception of Hartebees, which was harvested in 1983. Freshly harvested lots of were obtained again in 1988.

Lipid extraction.

Lipids were extracted using the solvent system of Khor and Chan (1985). Two grams of ground seed material were extracted with 10 mℓ of methylene chloride/methanol (2:1,v/v) containing 0.1 g.ℓ^{-1} butylated hydroxytoluene for 15 minutes and centrifuged in a bench top centrifuge at 1500$\underline{g}$ for 5 minutes at room temperature. The solvent was decanted and washed with a quarter volume of 2M NaCl and recentrifuged for 5 minutes at 1500$\underline{g}$ and room temperature. The lower phase was aspirated, dried with anhydrous NaSO$_4$ and then evaporated at 35°C under nitrogen.

Hydroperoxide determination.

Peroxide values were determined using the method of Stine <u>et</u> <u>al</u>. (1954). 0.02 mℓ of lipid were dissolved in

0.5 mℓ methylene chloride/ methanol (2/1,v/v). 0.02 mℓ
of 0.01 M ferrous chloride were added to 5 mℓ of
methylene chloride/methanol, followed by 0.02 mℓ of
lipid solution and 0.02 mℓ of 3M potassium thiocyanate.
The reagents were mixed and the absorbance measured
against a blank of the reagents at 505 nm.
Determinations were done in replicates of four. Using a
standard curve of Fe^{3+} concentration against
absorbance, absorbance values were converted to
micrograms of Fe^{3+} per 10 mℓ of solvent. Peroxide
values, as milli-equivalents $O_2.kg^{-1}$ fat, were then
calculated as: peroxide value = conc. Fe^{3+} per 10 mℓ/ g
of fat used x 55.84.*

Germination tests.

Two replicates of 50 seeds each of each seed batch were
germinated in 14 cm Petri dishes. To prevent damage on
imbibition, seeds were germinated in 25 mℓ of 20% PEG
8000 (ψ = -0.51 MPa) instead of on filter paper. Seeds
were kept in the dark at 24°C. Percentage germination
was monitored daily for four days. From the data, the

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* Due to the sensitivity of this technique, it is
crucial that all glassware be thoroughly clean,
that reagents be made up fresh immediately before
use, and that extracted lipid be processed
immediately following extraction.

coefficient of the velocity of germination (CVG) was
calculated using a modification of the formula of
Kotowski (1926):

$$CVG = \Sigma n \times \%G / \Sigma(Dn)$$

where n is the number of seeds germinated on day D
after sowing. %G is the final germination percentage.

Moisture content.

Two replicates of two grams of whole seeds were dried
in a convection oven at 130°C for 24 hours. Moisture
content was expressed as percentage wet weight.

Statistical Methods.

The significance of difference was determined using
Student's t-test. The Poisson product moment
correlation coefficient was used to calculate the
correlation coefficients of the data. The significance
of the correlation was read off from tables of
significance levels for the Poisson correlation
coefficient (Garvin, 1986).

3.1.3 RESULTS

Both germination percentages and seed CVG was highly correlated with peroxide value ($r = -0.81$ and $r = -0.90$ respectively, Table 3.1.1). This was consistant with the results obtained in 1985 (Table 3.1.2).

In contrast, seed percentage germination bore no apparent relationship to the duration of storage. Comparison with seed lots obtained in 1988 confirmed this observation (Table 3.1.3). Similarly, no evidence for the genetic superiority of one cultivar over another was obtained (Table 3.1.3).

Table 3.1.3. Germination percentages of soya bean seeds obtained in 1985 and 1988, and tested in 1988.

Cultivar	Germination percentage	
	1985	1988
Ibis	85%	27%
Impala	94-78%	70%
Pioneer	62%	71%
Hartebees	42%	43%

Table 3.1.1. Mean percentage germination, coefficient of velocity of germination (CVG) and peroxide value of soya bean seeds examined in 1988.

Cultivar	CVG		Germ.	Peroxide value
	$\%.d^{-1}$		$\%$	$m.eq.O_2.kg^{-1}$
Ibis	41.50a	34.98a§	85a	0.000
Impala 4028	37.13b	35.52a	94b	4.920a
Impala 4023	36.58b	28.52b	78a	8.707a
Impala 4031	35.23b	27.14b	78a	14.371b
Pioneer	33.13b	20.24c	62c	20.815c
Hartebees	32.70b	13.04d	42d	18.850c

Correlation with				
germination	0.74	0.98**		
Peroxide value		-0.90*	-0.81*	

* significant at the 5% level using Students t-test.

** significant at the 1% level.

§ corrected for %G. Means in the column followed by the same letter are not significantly different.

Table 3.1.2. Percentage germination, coefficient of velocity of germination (CVG) and peroxide value of soya bean seeds examined in 1985. (After Hailstones, 1987; Lipid peroxidation and ageing in seeds of cabbage and soya bean. M.Sc. Thesis).

Cultivar	CVG $\%.d^{-1}$	Germ. $\%$	Peroxide value $m.eq.O_2.kg^{-1}$
Ibis	54.92a[§]	97a	8.00a
Impala 4028	53.89a	99a	7.15a
Impala 4023	55.07a	98a	11.25a
Pioneer	51.12a	95b	12.98a
Hartebees	42.11a	91b	15.14a
Impala 4031	45.28a	91b	20.11a
Correlation with Peroxide value	-0.80	-0.89*	

* significant at the 5% level using Students t-test.

§ corrected for %G. Means in the column followed by the same letter are not significantly different.

Moisture content was poorly correlated with germination, CVG and hydroperoxide levels. (Figure 3.1.1). Peroxide levels were greatest towards the lower and upper limits of the range of moisture contents obtained. Seed lots of intermediate moisture contents showed a possible trend of declining and then increasing peroxide values as seed moisture contents increased. This result was again consistent with the data obtained in 1985 (Figure 3.1.2).

The results obtained in this study for the evaluation of the method of Kotowski (1926) revealed that CVG did not accurately reflect seed germination, giving a correlation of only 0.75 (Table 3.1.1). Seeds of significantly different germination percentages gave CVGs that were not statistically different. The method described by Kotowski assumes that a given seed lot will attain 100% germination given sufficient time (Brown and Mayer, 1988). If the calculated value is multiplied by the final percentage germination and not 100, then results are obtained that correspond very well with the actual germination data. By this method, a correlation coefficient of 0.98 was obtained (Table 3.1.1).

A consideration of some hypothetical data will further demonstrate the piont. Seed lots A, B, C and D
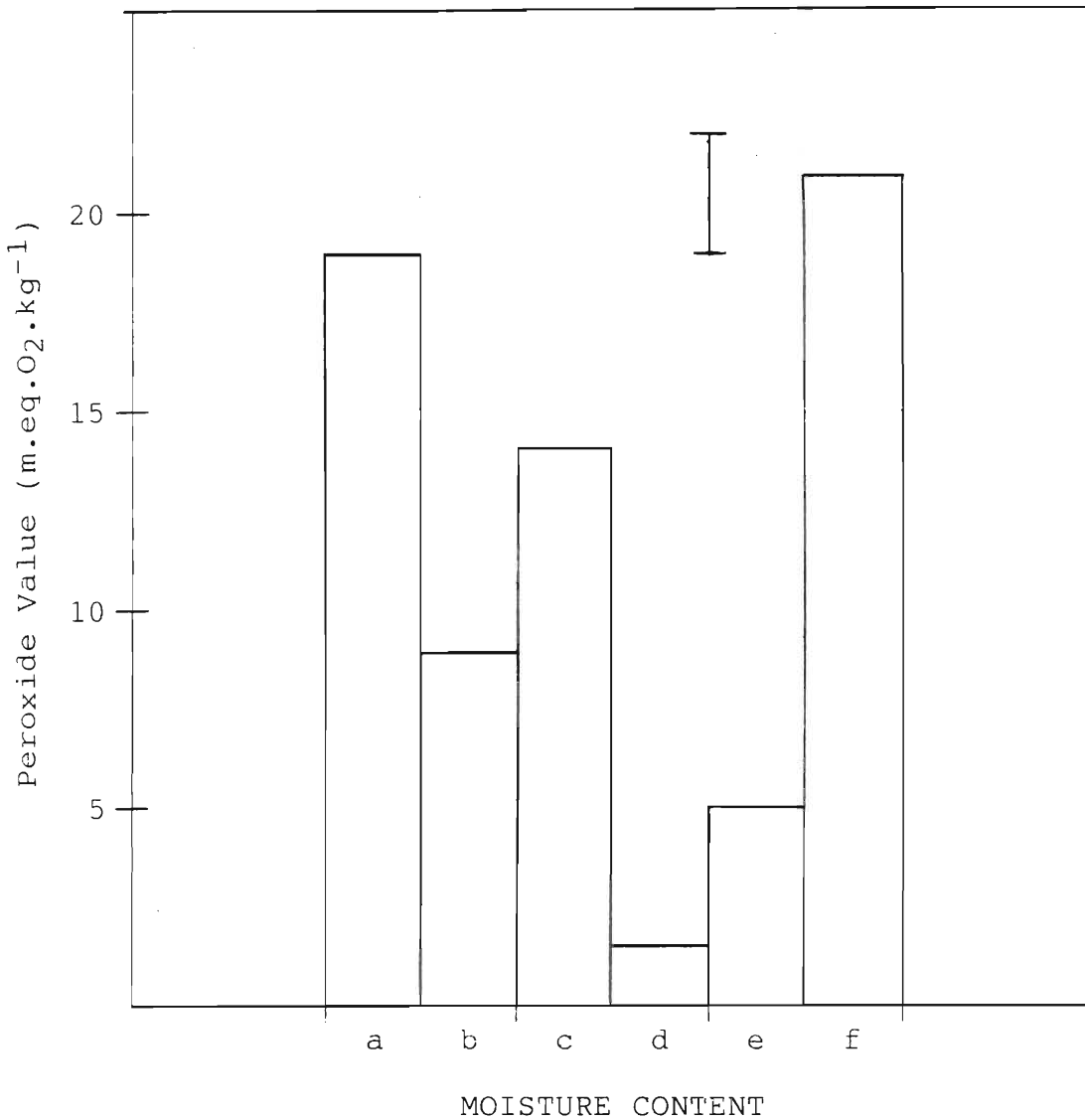
Fig 3.1.1 Relationship between moisture content
and peroxide value (1988). Vertical bar represents
LSD (5%).

a - Hartebees   5.8%
b - Impala 4023   8.4%
c - Impala 4031   9.9%
d - Ibis   10.2%
e - Impala 4028   10.3%
f - Pioneer   11.5%
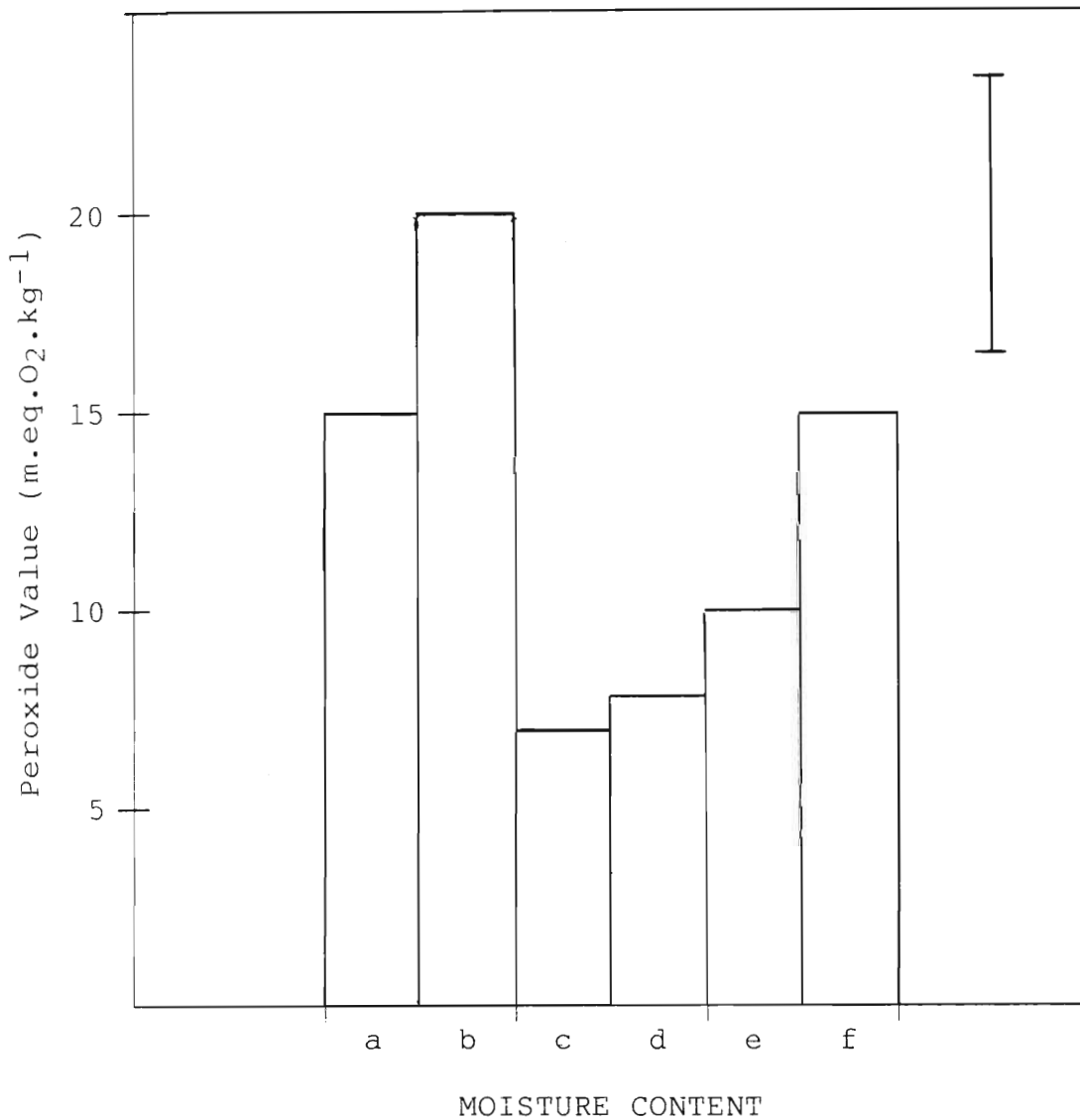
Fig 3.1.2 Relationship between moisture content
and peroxide value (1985). Vertical bar represents
LSD (5%).

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a - Hartebees      5.1%
b - Impala 4031    7.9%
c - Impala 4028    8.3%
d - Ibis           8.6%
e - Impala 4023    8.9%
f - Pioneer        9.7%
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(adapted from Hailstones, 1987. Lipid peroxida-
tion and ageing in seeds of cabbage and soya bean.
M.Sc. thesis)

.have the following germination response over 5 days.

	Day1	Day2	Day3	Day4	Day5
Lot A	20	50	80	90	100
Lot B	15	37.5	60	67.5	75
Lot C	10	25	40	45	50
Lot D	5	12.5	20	22.5	25

If we assume further that no further increase in percentage germination was observed after 5 days, the CVG for all of the lots will work out at 38.46. If the values are calculated with the altered formula, then the CVGs will then work out at 38.46, 28.85, 19.23 and 9.62 respectively. When a linear regression was applied to the data, a correlation of 0.9999 with percentage germination was obtained, as apposed to an r of zero for the original data.

3.1.4 DISCUSSION

Although seed germination percentages and CVG were highly correlated with peroxide levels, seed moisture contents and peroxide values gave somewhat variable trends. Previous work on cabbage seeds (Hailstones and Smith, 1988) indicated that seed peroxide levels consistently increased with increasing moisture content. In this and previous work (Hailstones and Smith, 1988), peroxide levels were observed to be lowest at intermediate moisture contents and to increase as moisture content increased or decreased. These data may suggest that in soya bean seeds the rate of lipid peroxidation may also be accelerated at low moisture contents, in addition to the observed increase in the rates of oxidation at higher moisture contents.

The relationship between the rate of lipid peroxidation and moisture contents in foods has been extensively investigated (Karel, 1980). Evidence indicates that water acts as an antioxidant at intermediate levels by decreasing the catalytic activity of metal catalysts, by promoting quenching of free radicals and by promoting non-enzymatic browning which causes the production of antioxidants. At high water levels, the rate of oxidation increases. This is thought to be due to the increased the mobilization of

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reactants and catalysts. Similarly, at very low water contents, the rate of oxidation is again increased (Karel, 1980).

It has been shown that seeds exists in dynamic equilibrium with the moisture content of the atmosphere (Priestley, 1986). If one measures seed moisture contents as a function of relative humidity at constant temperature, a seed sorption isotherm results. This has a negatively sigmoidal shape, permitting the definition of three distinct zones of hydration at the points of inflection (Priestley, 1986). In soya bean, the point of inflection between zones I and II, and zones II and III occur at a moisture content of approximately 5% and 10% respectively (Vertucci and Leopold, 1984). The moisture contents of the seed lots examined in this study all fall within zone II, with the exception of Pioneer, which fell in zone III. Furthermore, of the lots within zone II, Hartebees, with a moisture content of 5.8%, would be placed very near the boundary between zones I and II.

These zones correspond with the three zones of oxidative activity in foods mentioned above, and suggest that the rates of lipid peroxidation may be predicted from the seed isotherms. From the results of this study, it is tempting to suggest that moisture

contents may play a similar role in soya bean seeds. The intermediate moisture contents of zone II may retard lipid peroxidation, possibly by similar mechanisms operative in dry foods, although natural antioxidants (tocopherols) and inhibitors of lipid peroxidation might also be important.

The relationship obtained between moisture content and peroxide value has suggested another important consideration. Wilson and McDonald (1986b), in their lipid peroxidation model for seed ageing state that a steady-state free-radical flux will lead to a constant increase in hydroperoxide levels, in proportion to the age of the seeds. Clearly, this was not the case for the soya beans investigated. The results of this study may therefore suggest a more dynamic relationship between seed peroxide value and moisture contents in soya beans, and add further support for the determinant role that moisture contents have in seed ageing in general.

Evidence clearly suggests that even below the species level seed longevity is genetically determined, and soya beans are no exception (Priestley, 1986). However, the results of this study suggest that the fundamental processes of ageing were the same in all seed cultivars examined. The differences in germination percentages were readily attributable to factors such

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as seed moisture contents and the extent of oxidation of the lipid, rather than to cultivar differences. Such a view is supported by the observation that recently harvested seeds of these cultivars has equivalent or greatly reduced germination percentages. For this reason, the seed lots used in this study were compared only on the basis of empirically determined data and not on the basis of possible genetic differences. Cultivar names were, however, retained for convenience of reference.

Brown and Mayer (1988) have stated that the index of seed vigour proposed by Kotowski could not be recommended as a way of summarizing germination. However, from the results obtained in this study, it would appear that the formula given by Kotowski (1926) could be altered to read CVG = $\Sigma n / \Sigma(Dn)$ x %G. Furthermore, the highly significant correlation obtained between CVG and germination data argues in favour of the continued use of Kotowski's CVG, corrected for %G, as a biologically valid and useful means of summarizing germination.

CHAPTER 4

4.1 THE EFFECT OF FERROUS IONS ON SOYA BEAN SEED GERMINATION.*

4.1.1 INTRODUCTION

The free radical theory of ageing assumes that there is a single basic cause of ageing, modified by genetic and environmental factors, and postulates that free radical reactions are involved in ageing (Harman, 1981). The possible involvement of free radical reactions in seed ageing has received considerable attention over the last decade. However, evidence for a causal role of free radical reactions in seed ageing has proved inconclusive, and has engendered much debate as to the role, if any, of such reactions in declining seed vigour (Bewley, 1986; Priestley, 1986).

Berjak et al. (1986) have postulated that ageing in maize (Zea mays L.) caryopses was due to free radical mediated, deteriorative events that might be concentrated at localized intracellular sites, in particular in the mitochondria and in the milieu of the chromatin. The concept of free radical involvement in seed ageing has been applied to seed ageing in general by Wilson and McDonald (1986b). These workers have

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* - appendix 2

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postulated that lipid peroxidation, a free radical chain reaction, may constitute a common underlying mechanism in seed ageing. They propose that "a steady-state free-radical flux during ageing concurrent with accumulation of both co-oxidant injury and oxygenated fatty acids and their enzymatic degradation upon hydration" will lead to "further seed damage due to free radical formation and toxic secondary product production."

The treatment of seeds with antioxidants in order to retard free radical mediated oxidation and thus prolong shelf-life has been a concept of long standing. As early as 1961, Kaloyeras et al. showed that the vigour of onion (Allium cepa L.) and okra (Abelmoschus esculentus L.) seeds stored at room temperature could be improved by pretreatment with α-tocopherol or starch phosphate. More recent attempts have met with limited success however, problems with the toxicity of the antioxidants or the solvents used to apply them often presenting insurmountable obstacles (Wilson and McDonald, 1986b).

Most of such experiments to date have investigated the ability of antioxidants to increase storage life or to protect seeds under various ageing treatments (Wilson and McDonald, 1986b). In contrast to this,

Pammenter et al. (1974; Berjak, 1978) have shown that
the provision of a static electric charge during and
after rapid ageing improved the vigour of maize seeds.
The decrease in viability of maize seeds during dry
storage is associated with abnormalities in cellular
membranes and lysis of cellular components. It was
proposed that this was due to free radical damage of
macromolecules. Embryonic root tips from cathodically
protected seeds revealed none of the abnormalities
evident in the controls. There was little evidence of
abberation in mitochondrial profile, and most of these
organelles had well-defined, normal inner membrane
development. The above treatment also produced vigorous
cellular activity. Cells of experimental embryos
"presented a very dynamic picture with respect to lipid
utilization and possible de novo membrane synthesis."
It was proposed that the provision of electrons by the
static charge quenched any free radicals produced as a
consequence of ageing, thus preventing the accumulation
of free-radical induced damage.

Previous work (Hailstones, 1987, M.Sc. thesis;
Hailstones and Smith, 1989b) has shown that cabbage
(Brassica oleracea L.) seeds imbibed in a solution of
ferrous ion in high concentration were markedly
invigorated, giving significantly higher germination
percentages than controls. It was proposed that the

reducing property of the ferrous ions had provided a chemical source of electrons, thus quenching any free radicals that may be produced during early imbibition. Ferrous ion has been reported to act as an antioxidant at high concentration (Pokorný, 1987). In this study, the ability of ferrous ions to invigorate soya bean seeds was investigated.

4.1.2 MATERIALS AND METHODS

Seed material, germination tests and the statistical treatment of data were the same as described previously (chapter 3.1, pg. 55).

Chemical treatment

Seeds were germinated as described (pg. 56) in solutions of ferrous sulphate ($FeSO_4$) of different molarities.

4.1.3 RESULTS

Seeds of low (cv. Pioneer and Hartebees) and intermediate germination percentages imbibed in a solution of 0.04M $FeSO_4$ were markedly invigorated. In Hartebees, this treatment resulted in a significant increase in germination percentage (Figure 4.1.1) and CVG (Table 4.1.1). Treatment of cv. Impala 4023 resulted

Table 4.1.1 The effect of ferrous ions on soya bean seed germination.

| | Coefficient of the velocity of germination | | | |
cultivar	control	0.04M	0.005M	LSD(5%)
cv. Pioneer	17.38	30.38**	11.52*	5.30
cv. Hartebees	19.30	26.60*	–	6.20
cv. Impala 4023	22.84	35.32**	–	7.16

* significant at the 5% level

** significant at the 1% level

– not determined.

in a significant increase in germination (Figure 4.1.2) and a highly significant increase in CVG (Table 4.1.1). The same treatment gave a very highly significant increase in germination (Figure 4.1.3) and a highly

significant increase in CVG in cv. Pioneer (Table 4.1.1) In contrast to this, the treatment of this seed lot with a 0.005 M solution resulted in a significant decline in both germination (Figure 4.1.3) and CVG (Table 4.1.1).
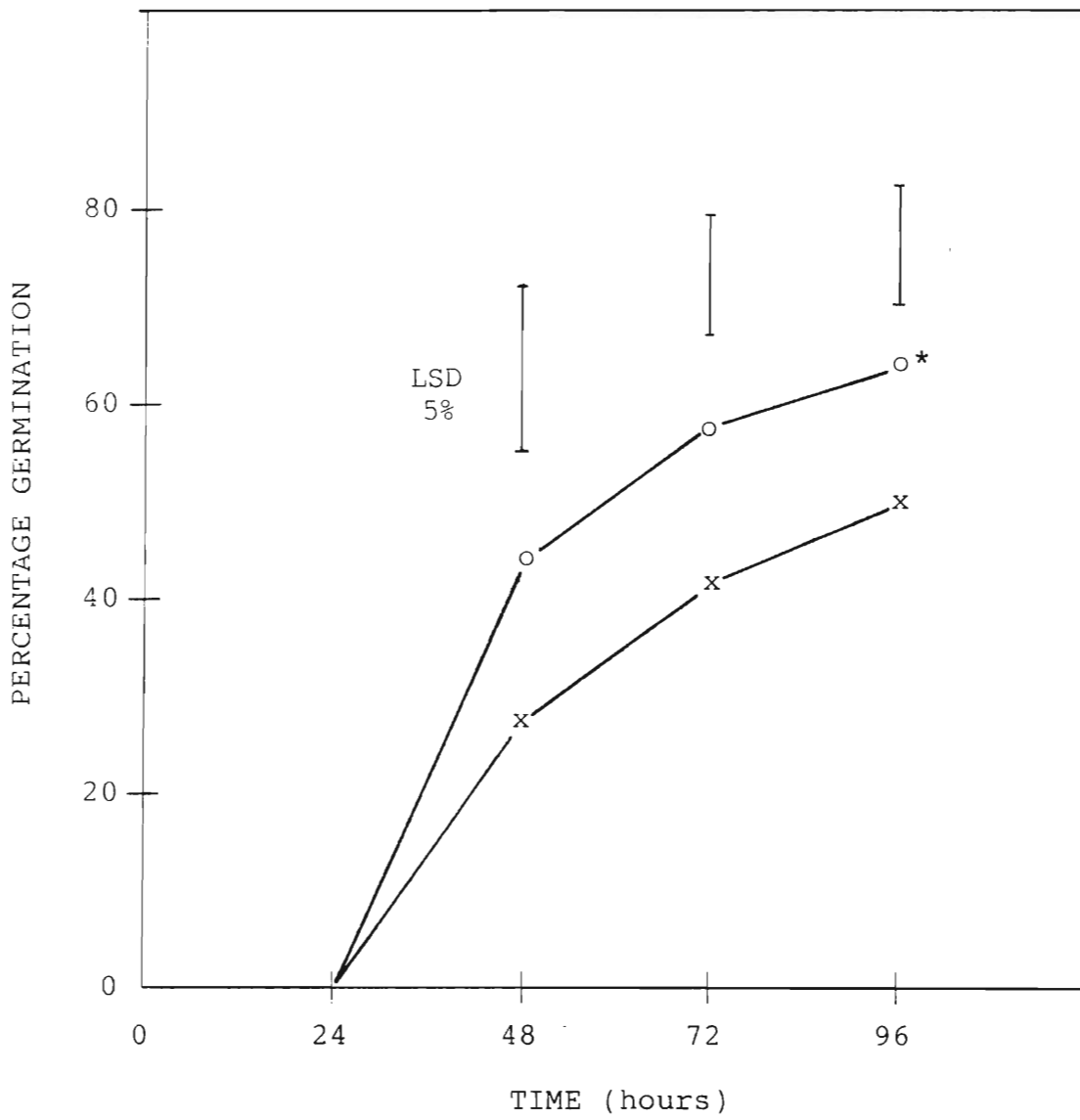
Fig 4.1.1 Germination of soya bean cv. Hartebees
treated with a ferrous sulphate solution. x control;
o 0.04M. * - sig. at the 5% level.
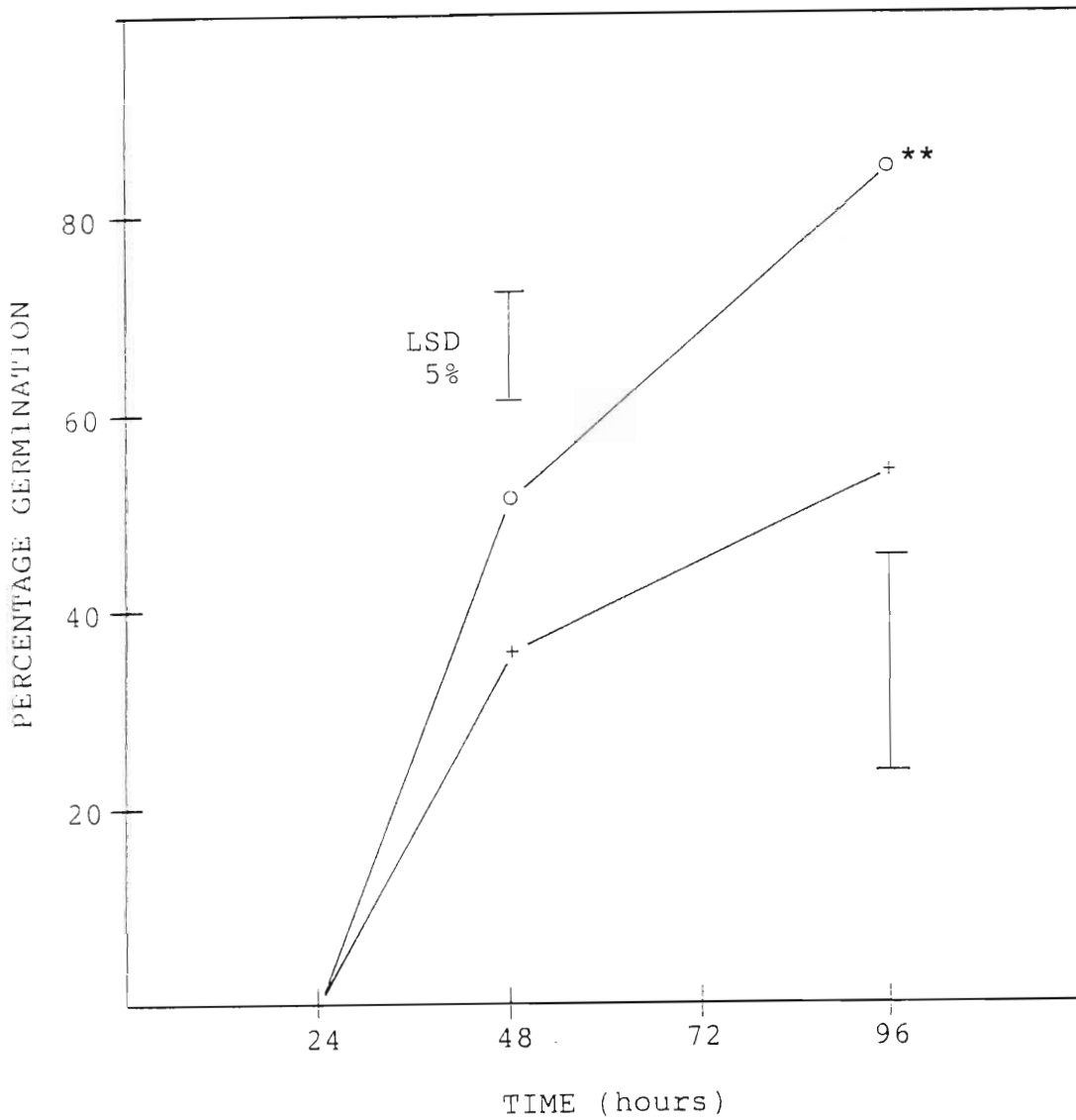
Fig 4.1.2. Germination of soya bean cv. Impala
4023 treated with a ferrous sulphate solution.
+ - control; o - 0.04 M $FeSO_4$. ** - significant
at the 1% level.
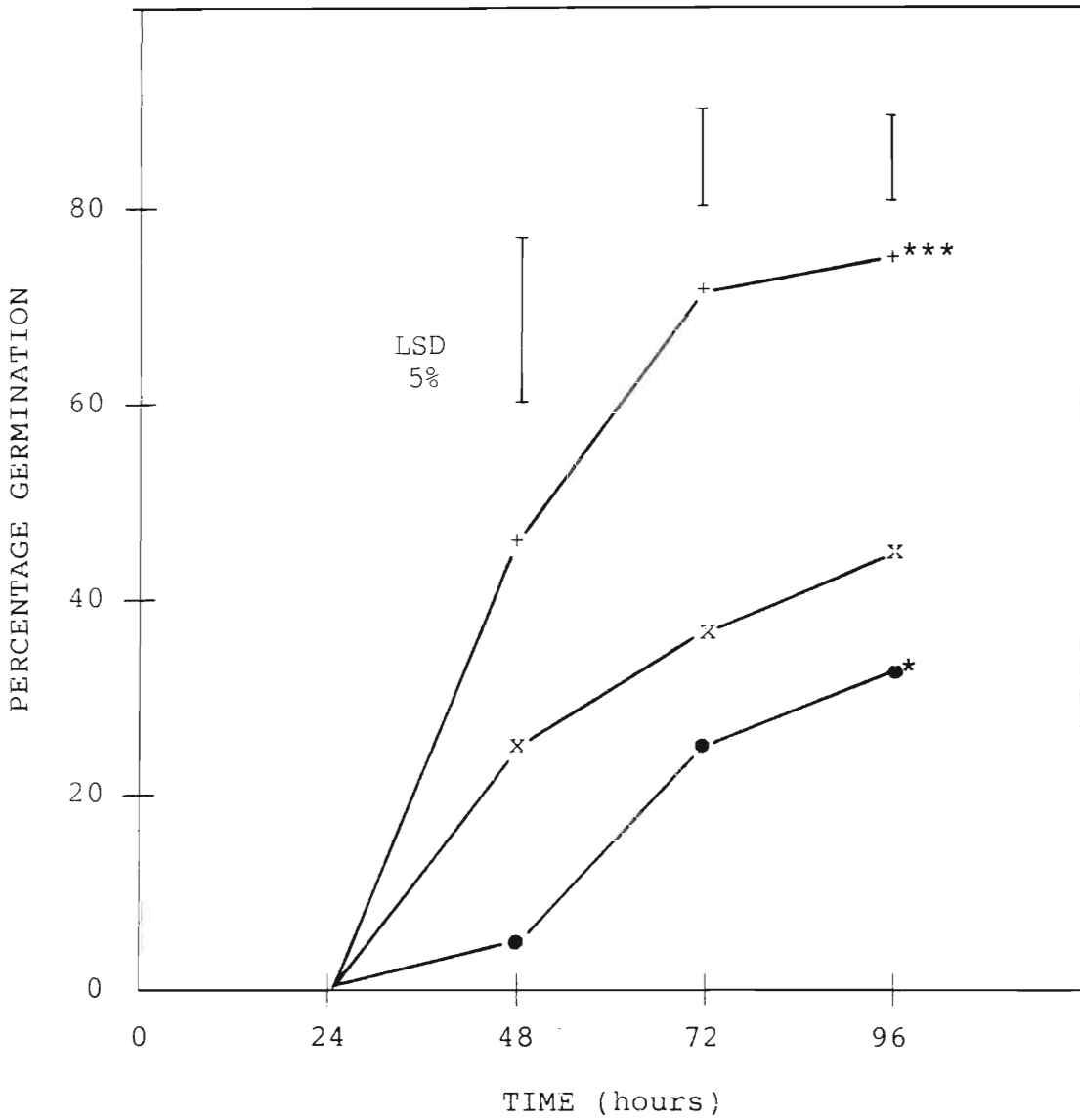
Fig 4.1.3 Germination of soya bean cv. Pioneer
treated with a ferrous sulphate solution. x control;
● 0.005M; + 0.04M. * - sig. at the 5% level, *** -
sig. at the 0.1% level.

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4.1.4 DISCUSSION

Results indicated that in all instances a high
concentration of ferrous ions produced a marked
invigoration of the soya bean seeds examined. These
results suggest that events during early imbibition are
important with respect to the determination of vigour
in aged soya bean seeds. Berjak (1978) has suggested
that the membrane damage visible in aged maize embryos
occurs as a result of free radical induced peroxidation
when intracellular water content is raised during
imbibition. With respect to age induced lesions to the
DNA of the type visualized in mitotic figures, it is
uncertain whether the damage occurs during storage or
upon imbibition (Roos, 1982). Wilson and McDonald
(1986b) have stated that "the phenomenon of incipient
death during germination of aged seeds suggests the
presence of a destructive element which becomes active
only after imbibition." They propose that the
destructive element may be oxygenated fatty acids which
are enzymatically broken down on imbibition. This
reaction may damage the seed further by producing an
increase in free radicals and by forming toxic
secondary products. The results obtained in this study
therefore add support to the above idea of incipient
damage on imbibition.

The reason for the invigoration is proposed to be the antioxidant activity of high concentrations of ferrous ions. It has already been mentioned that the pro-oxidant activity of heavy metals can revert to antioxidant activity at high concentration (Pokorný, 1987). The marked reduction in the germination percentage of seed imbibed in a 0.005M solution of $FeSO_4$ support the above proposition, as it suggests that a high concentration of the ferrous ion is necessary to obtain the invigoration. The toxicity of the low concentration of ferrous ion observed may possibly be due to its pro-oxidant activity.

It is proposed that any free-radicals produced on imbibition may therefore be quenched by a reaction similar to that given below (1).

$$R\cdot + Fe^{++} \rightarrow R^- + Fe^{+++} \ldots (1)$$
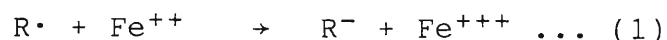
Any further damage due to free radical reactions is therefore prevented, resulting in an improvement in germination. The results of this study therefore indirectly support the occurence of free radical-mediated reactions during early imbibition.

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4.2 THE EFFECT OF FERROUS IONS ON THE EXTENT OF LIPID PEROXIDATION DURING EARLY IMBIBITION.

4.2.1 INTRODUCTION

With regard to seed ageing, it is not clear whether the critical damage occurs during storage or on imbibition. Villiers (1974) has argued that the dry state of seeds prevents the activity of macromolecular turnover and repair, and damage is therefore accumulated during storage. In support of this, seeds of lettuce (Lactuca sativa L.) and ash (Fraxinus americana L.) kept fully imbibed but unable to germinate allowed a high germination capacity to be maintained for long periods, together with a very low incidence of chromosomal abberations. It was proposed that "if the loss of viability in seeds is caused by the inactivity of repair systems, damage to macromolecules and organelles can only be repaired when seeds are imbibed." In support of such repair activity, Berjak and Villiers (1972) have demonstrated an increase in RNA and protein synthesis in rapidly aged maize, while ultrastructural investigation revealed that the damage to the mitochondria and plastid membranes appeared to be reversible during early germination. Osborne (1982a) has demonstrated that the incorporation of ^{3}H-thymidine into DNA as part of a postulated repair function was

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more intense in aged material. It has therefore been argued that if on imbibition the accumulated level of damage should exceed the seeds ability to repair it, the seed will fail to germinate (Villiers, 1974; Priestley, 1986)

Alternatively, damage accumulated during storage may reflect an incipient form of damage which will be manifest only upon imbibition (Wilson and McDonald, 1986b). They suggest that on imbibition, certain toxic substances (aldehydes and free radicals) are released or produced which lead to reduced vigour and eventually failure to germinate. This group sites the invigorating effects of hydration-dehydration treatments as indirect evidence for such a proposal. They argue that the very short time necessary to invigorate seeds by this method does not support the idea of repair. Rather, they suggest that the brief hydration of the seeds allows for the removal of the proposed toxic substances (Wilson and McDonald, 1986b). Berjak (1978) has argued that the absence of evidence for lipid peroxidation in maize seeds investigated suggested that "in dry maize seed it is possible that only free radical generation takes place, and that membrane damage occurs upon imbibition."

In support of the latter arguement, work on

cabbage seeds and soya bean axes has shown that in aged seeds a marked increase in lipid peroxidation occured during early imbibition, which was very much less or absent in seeds of high viability (Hailstones and Smith, 1989b). In the previous section (4.1), the invigorating effect of ferrous ions was demonstrated. In this study, the possible effects of ferrous ions on lipid peroxidation in soya bean axes during early imbibition was investigated.

4.2.2 MATERIALS AND METHODS

Seed material, germination tests, chemical treatment, lipid extraction, hydroperoxide determination and statistical methods were the same as described previously (chapter 3.1, pg. 55).

The determination of peroxide value on imbibition.
Forty isolated soya bean axes were imbibed in 9 cm Petri dishes moistened with 5 mℓ of $FeSO_4$ solution or distilled water. At $\frac{1}{2}$ hour and 1 hour, the seed lipid was extracted and the peroxide value determined as described (pg. 55).

4.2.3 RESULTS

In a previous study, the peroxide value in axes
from seeds of high viability (cv. Ibis), showed no
increase during the first hour of imbibition. Axes from
seeds of low vigour, however, gave a significant
increase in peroxide value at $\frac{1}{2}$ hour, followed by a
significant decline at 1 hour (Figure 4.2.1).

In soya bean axes from seeds of low viability (cv.
Pioneer), treatment with a 0.04 M solution resulted in
a very highly significant attenuation of the peroxide
value at $\frac{1}{2}$ hour (Figure 4.2.2). In order to establish
the lower limit of invigoration, axes from this seed
lot were treated with a 0.01 M solution of $FeSO_4$. No
change in the levels of hydroperoxides was observed.
Treatment of axes from a lot of intermediate viability,
cv. Impala 4023, did, however, resulted in a very
highly significant reduction in peroxide value at $\frac{1}{2}$
hour (Figure 4.2.3).
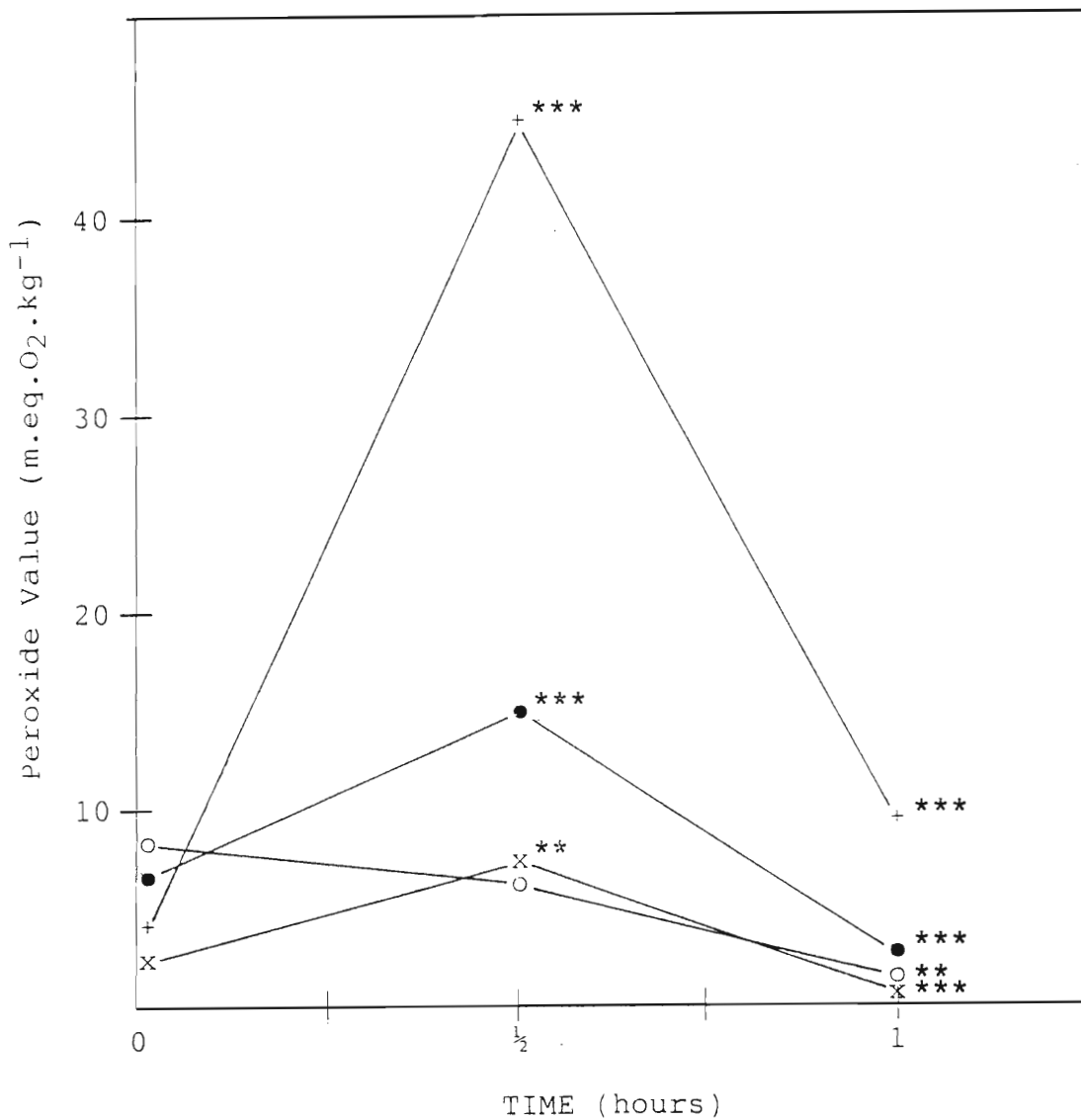
Fig 4.2.1. Changes in peroxide value in soya bean axes over the first hour of imbibition. o - Ibis; x - Impala 4028; + - Impala 4023; ● - Impala 4031. ** - significant at the 1% level. ***- significant at the 0.1% level.

(After Hailstones, 1987. Lipid peroxidation and ageing in seeds of cabbage and soya bean. M.Sc. thesis)
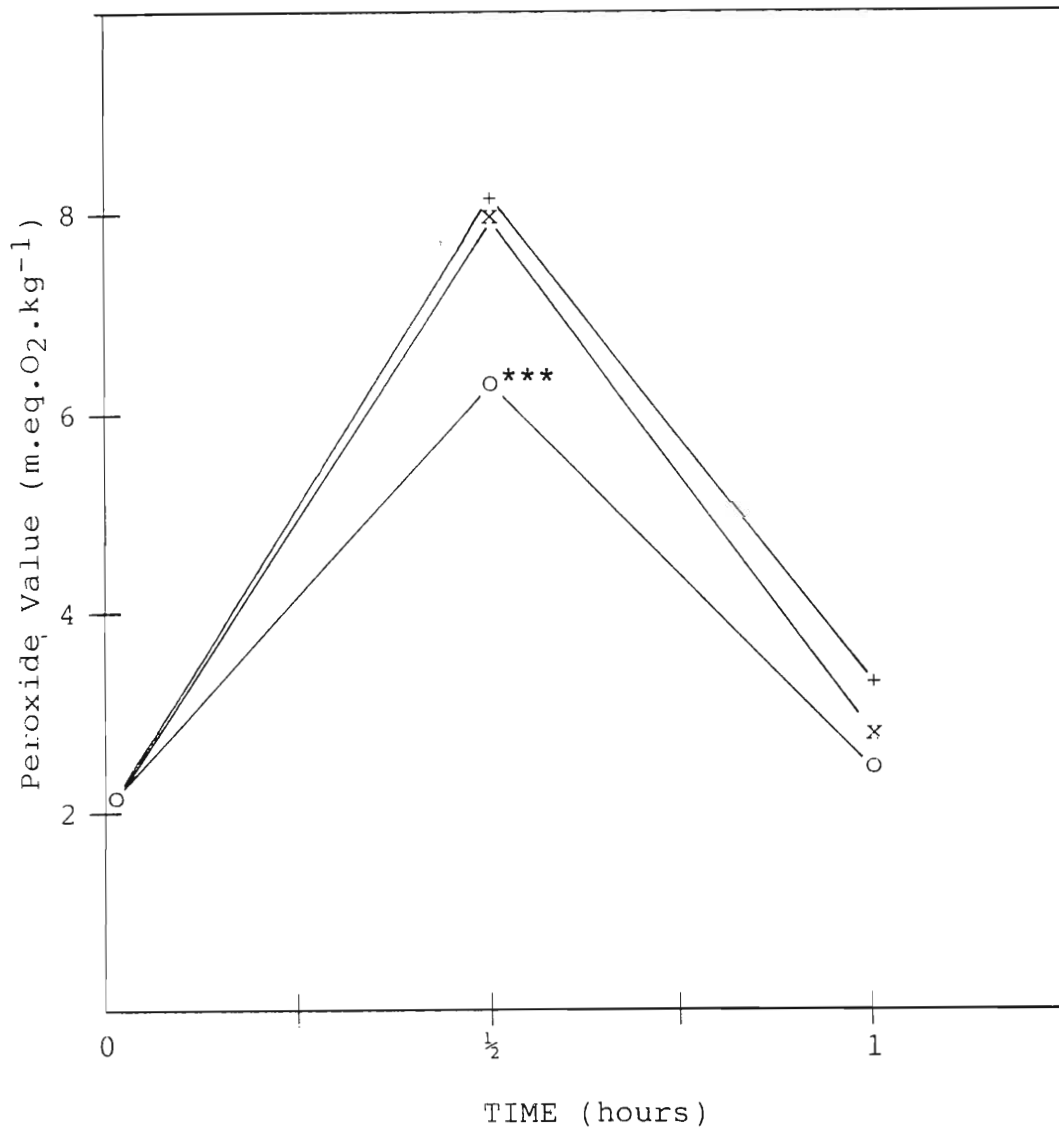
Fig 4.2.2. Changes in peroxide value over the first hour of imbibition in soya bean (cv. Pioneer) axes. x - water; + - 0.01 M $FeSO_4$; o - 0.04 M $FeSO_4$. *** - significant at the 0.1% level.
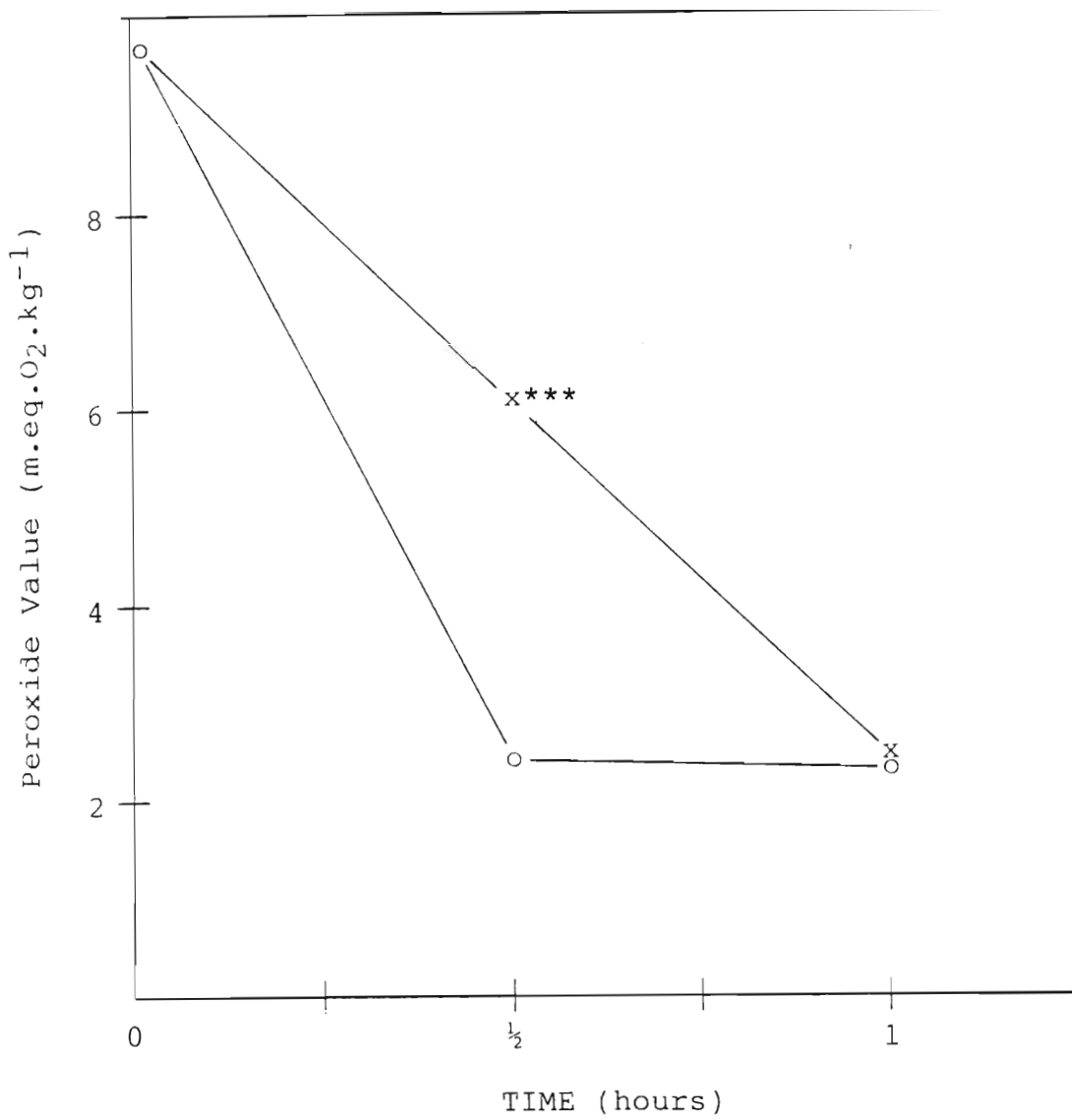
Fig 4.2.3. Changes in peroxide value over the first hour of imbibition in soya bean (cv. Impala 4023) axes. x - water; o - 0.01 M FeSO$_4$. *** - significant at the 0.1% level.

4.2.4 DISCUSSION

The results of previous work on soya bean axes (Hailstones and Smith, 1989b) has shown that in seeds of high viability, any accumulated peroxides are broken down by the activity of possible 'repair' processes. Results suggest further that the breakdown of the hydroperoxides occurs immediately upon imbibition. In seed axes from intermediate and low viability lots, however, a short term increase in peroxide value was observed, suggesting that seeds of low viability are subject to increased lipid peroxidation immediately following imbibition. The continued operation of the mechanisms of hydroperoxide breakdown was suggested by the observed decline in peroxide value at 1 hour.

Stewart and Bewley (1980) have demonstrated an increase in malondialdehyde, a product of lipid peroxidation, during early imbibition in rapidly aged soya bean axes. However, in this instance, the effects of this increase in lipid peroxidation with respect to seed viability were difficult to evaluate, as the seeds had lost all viability after only two days of ageing. The results of this study, however, suggested that the increase in lipid peroxidation observed in both soya axes and cabbage seeds (Hailstones and Smith, 1989b) may be related to seed viability. The marked reduction

in the level of hydroperoxides in the axes treated with ferrous ions may indicate that the invigorating effect demonstrated in the previous section was due to the proposed antioxidant activity of the ions inhibiting lipid peroxidation during early imbibition.

However, in addition to the antioxidant activity suggested previously, the results obtained from the treatment of the axes from cv. Impala suggest that the ferrous iron may also be catalysing the breakdown of the hydroperoxides directly (Frankel, 1982).

As mentioned in the previous section, these reactions could be beneficial to the seed, as it would inhibit lipid peroxidation and prevent the production of toxic secondary products such as aldehydes and alkoxy free radicals (Esterbauer, 1982; Wilson and McDonald, 1986b). The results obtained in this study therefore provide further evidence for a role of lipid peroxidation and free radicals in soya bean seed ageing.

The following working model is proposed. In seeds of high viability, any damage that may have occured during storage is repaired (Berjak and Villiers, 1972; Villiers, 1974). These repair processes are activated immediately on imbibition. Wilson and McDonald (1986b)

have proposed the activity of hydroperoxide lyase.
Alternatively, natural antioxidants such as α-
tocopherol and the classical systems of repair
involving enzymes (e.g. peroxidases) may be involved
(Halliwell and Gutteridge, 1985). If, however, the
level of damage accumulated during storage exceeds a
critical level, delaying the activation of repair
mechanisms or rendering the seed repair processes
ineffective, the situation will be exacerbated by a
increase in lipid peroxidation, leading to increased
levels of damage. The increase in lipid peroxidation
may be due to the breakdown of the hydroperoxides
present, thus leading to further free radical damage
and the production of toxic secondary products (Wilson
and McDonald, 1986b). Extensive damage resulting from
these reactions may severely impair cellular function,
leading to a decline in seed viability.

In their review, Wilson and McDonald (1986b)
state that one of the most promising approaches to
the investigation of lipid peroxidation in seed
ageing is the application of exogenous factors to
modulate in vivo peroxidation. The method
described here may possibly represent such a technique.
By using ferrous iron solutions to modulate in vivo
peroxidation on imbibition, the role of lipid
peroxidation in many of the classical events associated

with a loss of vigour could be evaluated. However, it is possible that the ferrous ions are giving rise to other effects in addition to the postulated dismutating and antioxidant ability. Further work is therefore necessary to establish the exact biochemical mechanism of the invigoration observed.

4.3 THE EFFECT OF FERROUS IONS ON SOME PHYSICAL AND BIOCHEMICAL ASPECTS OF SEED AGEING.

4.3.1 INTRODUCTION

Many seed ageing studies to date have been concerned with attempting to associate various physiological and biochemical changes within the seeds with the loss of seed viability. Priestley (1986), in a recently published review of seed ageing, states that "often such accounts reveal an implicit assumption that the alteration observed is closely linked to the mechanism of seed deterioration, and even that it may represent the primary lesion responsible for ageing." Speculation as to what may constitute the "primary lesion responsible for ageing" has led to much debate in the literature (Bewley, 1986; Priestley, 1986). This is largely due to the fact that the question of causality is not easily addressed because of the correlative and circumstantial nature of the evidence (Bewley, 1986).

Bewley (1986) has stated that evidence for lipid peroxidation in ageing seeds is tenuous. In contrast to this, Priestley (1986) has observed that seed lipids do undergo peroxidative degradation in long-term dry storage, but stresses the contradictory nature of the evidence. He nevertheless concedes that seeds will not

retain viability if their lipids are subjected to extensive peroxidation. He concludes by saying that "the challenge is to understand how the intergrated system becomes subject to disarray and to trace the overall effect of alterations to nucleic acids, membrane integrity, respiratory activity and repair functions."

The tetrazolium test developed by Lakon (1942) is now well established as a standard test for seed vigour. It is widely accepted that the test is indicative of the activity of enzymes of the dehydrogenase group. Living tissue stains red, dead tissue not at all, and tissue in which the normal biochemical activity has in some way been impaired somewhere in between. Experience has revealed that the interpretation of seed staining patterns are rarely as simple as this. For example, the presence of micro-organisms can be misleading, as tissue will stain red due to the microbial respiratory activity (Lakon, 1949). Furthermore, the ability of seed tissue to reduce the salt is dependent not only on the enzyme activity, but also on the level of reducing sustances and the competition between hydrogen acceptors within the cell (Smith, 1952). Nevertheless, it is generally agreed that the tetrazolium test does provide a general index of the metabolic ability or activity of a given

seed tissue.

Some of the earliest biochemical studies on seed ageing were concerned with the correlation of DNA and protein synthesis with seed vigour (Woodstock, 1973). More recently, Dell'Aquila and Margiotta (1986) have reported that ageing in wheat (Triticum durum Desf.) seeds was accompanied by a progressive decline in the rate of DNA synthesis as measured by 6-^{3}H-thymidine incorporation into embryonic tissue. In a similar study, Dell'Aquila and Taranto (1986) showed that in wheat the rates 6-^{3}H-thymidine and 4,5-^{3}H leucine uptake were markedly less in deteriorated material. Incorporation, expressed as percentage uptake, was little different for leucine, but greatly different for thymidine. Similar effects have been demonstrated for protein synthesis in aged seeds. For example, Gidrol et al. (1988) has reported a decrease in protein synthesis measured as ^{35}S-methionine incorporation after 12 hours imbibition in aged soya bean and pea (Pisum sativum L.) seeds. Evidence therefore suggests a general decline in anabolic activity in aged seeds.

One of the most frequently cited phenomenon associated with declining seed vigour is an increased leakage of various substances from the seeds as ageing progresses. This may be readily monitored by

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determining the conductivity of the steep water of such seed lots. Frequently, an increased level of leakage is observed. This was one of the earliest observed events associated with seed ageing, and led to the suggestion that seed membrane integrity was somehow impaired with age (Bewley, 1986; Priestley, 1986).

In the previous sections, the invigoration of ferrous ions on soya bean seeds was demonstrated. It was proposed that lipid peroxidation may represent a causal factor in seed ageing due to the production of toxic free-radicals and aldehydes. If the proposed central role for lipid peroxidation is correct, then an overall improvement in cellular metabolic activity might be expected following ferrous ion treatment. In this study therefore, the effect of ferrous ions on certain physical and biochemical aspects of imbibition was investigated.

4.3.2 MATERIALS AND METHODS

Seed material, germination tests, chemical treatment, lipid extraction, hydroperoxide determination and statistical methods were the same as described before (cf. chapter 3.1, pg. 55).

Conductivity determination

Four replicates of 10 seeds were germinated in 9 cm Petri dishes containing 15 mℓ of osmoticum as described above (pg. 56). Two mℓ of 200 g.ℓ^{-1} chloramphenicol was included to prevent any bacterial growth. A control for both the treated and untreated seeds was prepared as decribed, without the seeds. After 24 hours imbibition, the conductivity of the steep water was measured.

Protein and DNA synthesis

Protein and DNA synthesis was assayed by the method of pulse-labelling and determined as radioactive incorporation into TCA (trichloroacetate)- insoluble material after the method of Dell'Aquila and Taranto (1986). After 24 hours imbibition, four replicates of 10 seed axes were dissected and placed in 0.30 mℓ labelling medium in 10 mℓ plastic vials. The labelling medium consisted of 0.274 mℓ of 2% sucrose (DNA) or distilled water, 0.02 mℓ of 200 mg.ℓ^{-1} chloramphenicol and either 6 $\mu\ell$ of L(4,5-^{3}H) leucine (specific activity

112 Ci.mmol^{-1}; radioactive concentration 1mCi.mℓ^{-1}) or

6 $\mu\ell$ of (6-^{3}H) thymidine (specific activity 23 Ci.mmol^{-1}; radioactive concentration 1mCi.mℓ^{-1}).

After pulse labelling for 24 hours for DNA and one hour for protein synthesis, the axes were rinsed several times with distilled water containing 100 fold excess of either unlabelled leucine or thymidine prior to homogenization in 2 mℓ of 80% ethanol containing 100 fold excess of the two compounds. For uptake determination, 0.2 mℓ of homogenate were transferred directly to scintillation vials together with 3 mℓ of commercial solubilizing cocktail and counted in a Beckman scintillation counter.

For protein synthesis determination, after centrifugation of the homogenate at approximately 2000$\underline{g}$ in a bench top centrifuge for 5 minutes, the resulting pellet was resuspended in 1 mℓ of 10% TCA, heated at 90°C for 5 minutes to discharge aminoacyl-tRNA and then cooled on ice. The total suspension was filtered through a glass filter and washed sequentially with 5% TCA and 80% ethanol. The filter was dried and counted in a 5% (w/v) PPO-toluene scintillation cocktail. For DNA synthesis determination, after the centrifugation of the homogenate as before, the resulting pellet was solubilized in 1N NaOH, boiled for three minutes to

hydrolyse proteins, cooled on ice and then precipitated
with 2 mℓ of 10% TCA. Filtration and counting were
carried out as described above.

Tetrazolium staining

Four replicates of twenty soya bean seeds were imbibed
in 9 cm Petri dishes lined with two sheets of Whatman
No. 1 filter paper and moistened with 10 mℓ of 20% PEG
8000. Another four replicates were germinated in a
0.04M ferrous sulphate solution. After 24 hours, the
seed coats were removed and the seeds placed in 0.5%
tetrazolium solution for 2 hours. The seeds were then
rinsed and the formazan extracted for 24 hours in the
dark with 10 mℓ acetone. The extract was cleared by
centrifugation at 1500g in a bench-top centrifuge and
the absorbance read at 490 nm against an acetone blank.
The tetrazolium reducing ability of ferrous sulphate
was determined by adding the two solutions directly to
each other.

4.3.3 RESULTS

Tetrazolium Staining.

As may be predicted from the redox potential of the salts (approximately $-0.08V$ as opposed to $+0.74V$ for Fe^{++}) no tetrazolium reducing ability was observed for the 0.04 M ferrous sulphate solution used. In both seed lots investigated, a marked increase in tetrazolium reducing ability was observed in the treated seeds. In cv Pioneer, a significant increase was observed over the control, while in cv. Hartebees, a highly significant increase was observed (Table 4.3.1).

Table 4.3.1. The effect of ferrous ions on the tetrazolium reducing ability of soya bean seed.

Cultivar	Absorbance (490 nm)	
	Water	0.04 M $FeSO_4$
Pioneer	0.514	0.579*
Hartebees	0.708	0.962**

* significantly different at the 5% level

** significantly different at the 1% level

DNA and Protein Synthesis.

No significant increase in either uptake or incorporation by the treated seeds was observed for either DNA or protein synthesis. The percentage of labelled thymidine incorporated in the axes from treated seeds was not significantly different from that in the controls (Table 4.3.2).

Table 4.3.2. The effect of ferrous ions on DNA synthesis (cpm $(6\text{-}^3\text{H})$ thymidine.10 axes^{-1}) in axes from cv. Hartebees seeds.

	Water	0.04 M FeSO$_4$
uptake ($\times 10^4$)	23.65±12.76	26.50±3.22
incorporation ($\times 10^3$)	15.53±10.78	14.20±5.12
% incorporation	6.4±1.87	5.3±1.73

In contrast to this result, the percentage of labelled leucine incorporated was significantly greater in the treated seeds (Table 4.3.3). In both DNA and protein

synthesis, the variability associated with uptake and incorporation of label was markedly reduced by the treatment (cf. Tables 4.3.2 and 4.3.3).

Table 4.3.3. The effect of ferrous ions on protein synthesis (cpm L(4,5-^{3}H) leucine.10 axes^{-1}) in axes from cv. Hartebees seeds.

	Water	0.04 M FeSO$_4$
uptake (x10^4)	3.20±0.99	3.38±0.21
incorporation (x10^2)	2.30±0.73	2.98±0.41
% incorporation	0.72±0.03	0.88±0.12*

* - significantly different at the 5% level

The conductivity of the steep water of the ferrous sulphate treated seeds showed a significant decline in cv Hartebees and a highly significant decline in cv Pioneer (Table 4.3.4).

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Table 4.3.4. The effect of ferrous ions on the conductivity of the steep water ($mS \times 10^{-2}$).

Cultivar	water	0.04 M $FeSO_4$
Hartebees	24.35	7.75*
Pioneer	17.98	8.81**

* - significant at the 5% level

** - significant at the 1% level

4.3.4 DISCUSSION

Treatment with ferrous sulphate had no effect on
DNA synthesis over the time period investigated (24 -
48 hours). Evidence indicates that in most vigorous
seeds, scheduled DNA synthesis prior to mitosis is a
relatively late event in germination, and is
considerably reduced and further delayed in aged
material (Priestley, 1986). This has been demonstrated
in aged rye (Sen and Osborne, 1977). A delayed onset of
cell division and a low rate of DNA synthesis has also
been reported in aged wheat embryos (Dell'Aquila et
al., 1980; Dell'Aquila and Margiotta, 1986;
Dell'Aquila and Taranto, 1986). However, the results
of this study suggest that the invigoration of the soya
bean seeds by ferrous ions was not associated with an
improvement in DNA synthesizing ability.

In contrast to this, protein synthesizing activity
was significantly greater in the treated seeds. In a
recent paper, Gidrol et al. (1988) have demonstrated a
decrease in in vivo protein synthesis in aged pea and
soya bean seed axes after 12 hours imbibition, the
observed effect being greater in the soya bean seeds.
They also demonstrated a lower level of adenylate
energy charge in the soya beans after 12 hours
imbibition. They suggested that the observed difference

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in protein synthesis could be due to a greater reduction of the energy supply in soya bean axes. This arguement is supported by the results obtained for tetrazolium reducing ability in this present study. The above authors postulate a possible role of deterioration of the mitochondrial membrane as a consequence of seed ageing, leading to an inefficient oxidative phosphorylation and thus resulting in a restriction of the energy supply. In support of this, Gidrol (1989) has demonstrated a greater increase in superoxide dismutase activity in aged soya beans during early germination. This was proposed to be due to the ageing-induced release of the superoxide radical from mitochondria. Damage of the mitochondrial membrane as an underlying phenomenon in seed ageing has also been postulated by Woodstock (1976) and Berjak et al. (1989).

Bewley (1986) has observed that the status of the plasmamembrane as it passes almost instantaneously from a dry to a fully hydrated state is a subject of continuing interest in seed ageing research. It is envisaged that this membrane may undergo a tempory disorganization related to the instantaneous rehydration of the membrane. Since the integrity of the plasmalemma and associated membranes is an integral requirement for normal cellular function, "it is perhaps not surprising to find that workers have

surmised that viability loss can be effected trough perturbations of membrane systems" (Bewley 1986). However, he states further that there is as yet little incontrovertible evidence that membrane deterioration is a primary event in the course of viability loss.

The results obtained in this study indicate that the invigorating effect of ferrous ions was associated with a marked reduction in the leakage of electrolytes from the soya bean seeds. Furthermore, the work descibed in the previous sections may suggest that this improved membrane integrity was due to the antioxidant activity of the ferrous ions inhibiting the increase in lipid peroxidation associated with early imbibition in aged seeds. The results obtained here therefore provide evidence for a possible link between lipid peroxidation and membrane integrity.

Work to date on the role of membrane integrity in the loss of seed vigour is fraught with contradictions (Bewley, 1986). This is only compounded by the fact that the use of rapid ageing techniques long held to be equivalent to slow ageing are being increasingly questioned (Wilson and McDonald, 1986b). Nevertheless, evidence indicates that in soya bean seeds aged in the long term the polar fraction (membrane lipid) does show signs of peroxidative damage, as evidenced by a decline

in polyunsaturated fatty acids (Priestley and Leopold, 1983; Hailstones and Smith, 1988). These accumulated hydroperoxides may on imbibition break down, giving rise to a free-radical chain reaction leading to the increase in peroxide value observed (Hailstones and Smith, 1989b). It is proposed that this would be limited largely to the lipid milieu, with cell membranes being a major site of attack. Dismutation of the hydroperoxides so formed, possibly by metallo-proteins, would lead to the production of organic free-radicals (Halliwell and Gutteridge, 1985), which may diffuse from the sites of production, causing widespread damage to the cellular tissue.

In conclusion, the present results indicate that a general improvement in biochemical activity was observed on treatment with ferrous sulphate, and support the hypothesis proposed above. It is proposed that the ferrous sulphate may prevent the potentially damaging free-radical reactions during early imbibition, thus allowing the re-establishment of functional membrane integrity and the onset of the normal processes of germination, including respiration and protein synthesis. An improved membrane integrity would also explain the marked reduction in the variabilty observed in the uptake of both leucine and thymidine. The disruption of membrane integrity due to the breakdown

of lipid hydroperoxides and the resulting production of alkoxy free-radicals may therefore constitute an underlying event leading to the onset of age related phenomena associated with declining seed vigour in soya bean. Furthermore, the fact that cabbage seeds were similarly invigorated by this treatment (Hailstones and Smith, 1989b) suggests that the lipid peroxidation model of seed ageing proposed by Wilson and McDonald (1986b) may be essentially correct.

CHAPTER 5

5.1 THE EFFECT OF FERROUS IONS ON THE EVOLUTION OF VOLATILE ALDEHYDES DURING EARLY SOYA BEAN SEED GERMINATION.

5.1.1 INTRODUCTION

The phenomenon of the evolution of volatile carbonyl compounds from imbibing seeds is well documented, and a number of studies have revealed a clear relationship between seed age and the amount of volatile products formed. Harman et al. (1978) has reported that aged pea (Pisum sativum L.) seeds produced up to 24 times more volatile carbonyl compounds than unaged seed. In another study (Harman et al., 1982), volatile aldehyde production was up to 30 times higher in aged than in unaged pea seeds and 3 to 7 times higher in poor than in high-quality soya bean seeds. More recently, Wilson amd McDonald (1986a) have shown that aldehydes captured by passive collection during the first and second day of germination were highly correlated with field emergence.

A number of workers have attempted to characterize the volatiles evolved from hydrated seeds. Heydanek and McGorrin (1981a, 1981b) reported that major amounts of C4-C6 alcohols, hexanal, 1-octen-3-ol, nonanal and 3,5-octadien-2-one were derived from hydrated oat (Avena

sativa L.) groats. In studies more relevent to seed ageing, both Woodstock and Taylorson (1981) and Gorecki et al. (1985) reported increasing amounts of ethanol and acetaldehyde from aged soya bean and pea seeds.

The source of these volatile compounds is still a matter of debate. Woodstock and Taylorson (1981) and Gorecki et al (1985) suggest that an imbalance between tricarboxylic and glycolytic activities, present even in vigorous seeds, may become more pronounced in aged seeds, leading to the accumulation of ethanol and acetaldehyde. Alternatively, Wilson and McDonald (1986b) have postulated that the breakdown of lipid hydroperoxides may also give rise to volatile aldehydes on imbibition.

The effects of these volatiles are also subject to investigation. Harman et al (1978) has shown that volatiles from aged germinating pea seeds stimulated fungal spore germination much more than unaged seeds. It was proposed that the evolution of such compounds may have a direct bearing on fungal infection of aged seeds in the soil. It has also been proposed that aldehydes may also effect seed vigour more directly due to their cytotoxicity (Wilson and McDonald, 1986b). The potential cytotoxic effects of aldehydes are well documented (Esterbauer, 1982, Esterbauer et al., 1988).

Wilson and McDonald (1986a) have therefore proposed that oxygenated fatty acids accumulated during storage may be broken down upon enzymatic activation of the seed to yield cytotoxic aldehydes, thereby causing extensive damage to the germinating seed (Wilson and McDonald, 1986b).

A marked invigoration and an attenuation of lipid peroxidation during early imbibition has been demonstrated in soya bean seeds on treatment with a solution of ferrous ions (Chapter 4). The effect of ferrous ions on volatile aldehyde production over the first 24 hours of imbibition was therefore monitored in order to establish if a relationship between the observed invigoration and the level of volatile aldehydes existed.

5.1.2 MATERIALS AND METHODS

Seed Material, germination tests, lipid extraction, hydroperoxide determination and statistical methods were the same as described above (chapter 3.1, pg. 55).

Aldehyde assay

Four replicates of 25 seeds were germinated in glass jars on eight sheets of paper towel. Seeds were moistened with 25 ml of water or 0.04 M ferrous sulphate containing two ml of 200 g.l^{-1} chloramphenicol. Four plastic vials containing five ml 0.2% (w/v) 3-methyl-2-benzothiazolone hydrazone (MBTH) solution were included in the vessel which was sealed with PTFE tape. The experiment was carried out at 5°C in the dark for 24 hours.

Colourimetric analysis

One ml of MBTH solution from each vial was added to 2.5 ml of 0.23% $FeCl_3$ and incubated for five minutes at room temperature. Acetone (6.5 ml) was then added and the absorbance measured at 635 nm. A standard curve was prepared using formaldehyde, and aldehyde production was expressed as nl of aldehyde.ml^{-1} per 25 seeds (Wilson and McDonald, 1986a).

5.1.3 RESULTS

A highly significant (1% level) increase in
volatile aldehyde production was observed from the
ferrous sulphate treated seed of both intermediate (cv.
Impala) and low viability (cv. Pioneer; Table 5.1.1).
Previous work has shown that these two lots differed
significantly in germination percentage and peroxide
value (Table 3.1.1, pg. 59). In this study, volatile
evolution from untreated Impala seeds was significantly
different from Pioneer at the 5% level.

Table 5.1.1. The effect of ferrous ions on volatile aldehyde
production (nℓ.mℓ^{-1} per 25 seeds) during the first 24 hours of
inbibition. (%G, germination percentage; PV, peroxide value).

Cultivar	%G[1]	PV[1]	Control[1]	Experiment
Impala	78	8.71	0.37	3.18**
Pioneer	63	20.82	1.09	5.09**

** significant at the 1% level

1 significantly different at the 5% level

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5.1.4 DISCUSSION

The weight of evidence indicates that volatile aldehyde production during early germination is positively correlated with seed viability and field emergence (Wilson and McDonald, 1986b). In accordance with this, the amount of aldehyde produced from the untreated seeds in this study was significantly greater in the less vigorous seed lot. However, the significantly greater levels of aldehydes produced from the treated seeds did not support a role for adehydes in the reduction of soya bean seed vigour. This result may therefore suggest that the implication of aldehydes in general as the possible toxic products giving rise to reduced seed viability may be incorrect (Wilson and McDonald, 1986b).

The identity of the volatile compounds was not established in this present study. The identity of the compounds would, however, be important in establishing the origin of the volatiles. An imbalance in the processes of respiration would produce relatively short chain compounds. The occurrance of short chain compounds, particularly ethanol and acetaldehyde, has been consistently reported from imbibing seeds of soya bean (Woodstock and Taylorson, 1981), pea (Gorecki et al. (1985) and cotton (Gossypium hursitum L.; Nelson,

1987). In contrast to this, lipid peroxidation would give rise to longer chain compounds. Heydanek and McGorrin (1981a, 1981b) have reported the production of predominately C4-C6 alcohols, hexanal, 1-octen-3-ol, nonanal and 3,5-octadien-2-one from hydrated oat groats, although it is questionable whether the method used (seeds were immersed in water at 55°C) allowed normal germination. Smith (1986, Ph.D. thesis) has observed the production of volatile alcohols (butanol, pentanol, hexanol) and aldehydes (pentanal, hexanal) from heated, imbibed lettuce (Lactuca sativa L.) seeds. Furthermore, a possible positive correlation between the amounts of volatiles produced and seed viability was demonstrated. In this instance, it was proposed that lipid hydroperoxides were the source of the compounds identified (Smith, 1986).

The results obtained in this study have demonstrated an increase in lipid peroxidation during early imbibition of aged seeds (section 4.2, pg. 82) and a possible relationship between this increase in oxidation and seed metabolism (section 4.3, pg. 94). This result would support both of the origins of volatile compounds proposed above, i.e. lipid peroxidation and metabolic imbalance. It is therefore proposed that the increase in lipid peroxidation in aged soya bean could give rise to the long chain volatile compounds

(Smith, 1986; Wilson and McDonald, 1986a), while the concomitant free-radical damage associated with peroxidation could lead to further disruption of membranes, thus leading to the proposed imbalance between tricarboxylic and glycolytic activity and the release of shorter chain compounds (Woodstock and Taylorson, 1981; Gorecki et al., 1985).

The possible toxicity of volatile carbonyl compounds has been speculated upon at length (Wilson and McDonald, 1986b). However, although their ability to cause a wide range of damage is not disputed (Esterbauer, 1982; Esterbauer et al., 1988), evidence for a causal link between volatile aldehydes and seed viability is tenuous. In animal systems, extensive research has revealed that 4-hydroxyalkenals, particularly 4-hydroxynonenal and 4-hydroxyhexenal, are the major products implicated in the toxic effects observed following free radical-induced lipid peroxidation (Esterbauer, et al., 1988). The characterization of the volatile aldehydes produced during early germination would therefore be important in establishing the possible mechanisms by which cytotoxicity occurs in seeds.

Evidence for the production of 4-hydroxy alkenals in seeds is, however, lacking. Such compounds were not

detected in volatiles derived from hydrated oat groats
(Heydanek and McGorrin, 1981a, 1981b) or aged lettuce
seeds (Smith, 1986). Furthermore, enzymatic breakdown
of the hydroperoxide, often implicated in the produc-
tion of volatile compounds (Heydanek and McGorrin,
1981a, Wilson and McDonald, 1986b), may not produce such
oxidation products. For example, Galliard et al. (1976)
have reported almost exclusive production of hexanal
from the 13-hydroxy linoleate and trans-2-nonenal and
cis-3-nonenal from the 9-hydroxy linoleate in cucumber
(Cucumis sativus L.) fruit. The analysis of oxidation
products of soya bean seed lipids revealed that the 13-
hydroperoxide predominated, the 9-hydroperoxide being
found in relatively small amounts (cf. Chapter 6).
Hexanal may therefore be predicted to be the major
product of enzymatic hydroperoxide breakdown in soya
beans. Hexanal has been reported to constitute the
major component of the volatiles evolved on imbibition
of rancid oat groats (Heydanek and McGorrin, 1981b). In
contrast to this, hexanol was observed to predominate
on imbibition of aged lettuce seeds (Smith, 1986). It
may therefore be surmised that other aldehydic
compounds than those identified in animal systems might
be responsible for the proposed toxicity in seeds.

Furthermore, the uncertainty with regard to the
identity of the cytotoxic compounds is compounded by

the observation that the response of different seeds to different volatile compounds is inconsistent. French and Leather (1981), who examined the effects of a wide range of volatile aldehydes and alcohols, including hexanal, nonanal and nonanol, on weed seed germination, reported a variable response. Some seeds exhibited a marked sensitivity to most of the compounds tested, others only to some, and yet others exhibited an improved germination response to certain of the compounds, including hexanal, nonanal and nonanol.

In conclusion, further work is necessary in order to establish a causal relationship between the volatile compounds produced from seeds on imbibition and seed viability. The results obtained in this study indicate that the invigoration of soya beans by ferrous ions was not associated with a reduction in the amount of volatile aldehydes produced.

5.2 THE EVOLUTION OF VOLATILE ALDEHYDES ON HEATING IN RELATION TO SEED VIABILITY IN SOYA BEAN.*

5.2.1 INTRODUCTION

The need to provide an objective assessment of the rancidity of vegetable oils led to the development of a number of tests based on the measurement of volatile lipid oxidation products. Scholz and Ptak (1966) demonstrated a simple gas chromatographic procedure for measuring the degree of rancidity of cottonseed (Gossypium hursitum L.) oil by monitoring headspace pentane levels. They reported a moderate correlation between the pentane levels and the rancidity score as determined by the organoleptic test. In a similar investigation, Evans et al. (1969) reported a significant correlation between the amount of thermally derived pentane and the peroxide value of soya bean and cottonseed oils. Both the above authors proposed that autoxidation products were the source of these volatiles. That lipid hydroperoxides can give rise to such volatile compounds has been firmly established. Frankel (1982) and Esterbauer (1982) have reported the production of predominately alkanal and related compounds from the thermal decomposition of oxidised

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oleic, linoleic and linolenic acids.

The application of this approach to monitoring
seed ageing (Fielding and Goldsworthy, 1982) has
demonstrated that thermally derived volatiles from dry
wheat (Triticum aestivum L.) seeds were related to seed
viability. Lipid oxidation products were postulated to
be the source of the volatiles. A positive correlation
between the germination percentage and the amount of
volatiles derived on heating dry seeds has also been
obtained for cabbage (Brassica oleracea L.), soya beans
and lettuce (Lactuca sativa L.; Hailstones, 1987;
Smith, 1986). The marked correlation between seed
peroxide value and the amount of volatiles produced
indicated that lipid hydroperoxides may have been the
source of these compounds.

Heydanek and McGorrin (1981a, 1981b) have shown the
production of primarily $C_{10}H_{16}$ monoterpenes,
alkylbenzenes and hexanal from heated dry oats (Avena
sativa L.). Smith (1986) has demonstrated the evolution
of pentane, ethane-thiol, alcohols (butanol, pentanol,
hexanol) and aldehydes (pentanal and hexanal) from
heated lettuce seeds. The objective of this study,
therefore, was to determine the origin and identity of
the volatiles produced on heating soya bean seeds and
to evaluate the relationship of these compounds to seed

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viability and hydroperoxide levels.

5.2.2 MATERIALS AND METHODS

Seed material was the same as described previously
(chapter 3.1, pg. 55).

Evolution of volatiles.

Two grams of ground seed material were placed in 15 mℓ
serum vials and sealed. The sealed vials were heated at
130°C for one hour and left to stand overnight. Vials
were reheated at 130°C for 20 minutes immediately
before sampling. Four replicates of 0.05 mℓ samples
from each seed lot were injected directly onto the GC
column using gas-tight syringes. Aldehydes were
identified by injecting 0.05 mℓ samples of gas from the
headspace of standards of hexanal, pentanal and butanal.

Gas liquid chromatography.

A Varian 3300 gas chromatograph fitted with a flame
ionisation detector and a 15 metre non-polar (DB1)
megabore capillary column was used. Injector and
detector temperatures were 250°C. Column temperature
was programmed at 40°C for three minutes, ramped to
46°C at 3°C.minute^{-1}, held for 30 seconds, and then
ramped to 80°C to drive off any late eluting compounds.
Carrier gas was helium at 6 mℓ.minute^{-1}.

Lipid extraction, hydroperoxide and moisture content

determination, germination tests and statistical

methods were the same as described previously (chapter

3.1, pg. 55).

5.2.3 RESULTS

A representative gas chromatogram of the volatiles evolved on heating is given in Figure 5.2.1. On average, 25 peaks were resolved. An initial investigation showed that none of the major peaks bore any relationship to germination (data not shown). A number of smaller peaks, however, did show a relationship to germination. Three of these could be identified using standards as butanal, pentanal and hexanal, and the rest of the study was limited to the investigation of these compounds.

In order to determine the origin of the volatiles, seed powder that had been extracted of its oil was heated and sampled as above. A comparison of the chromatograms indicated that most of the volatiles were derived from the oil, including the three aldehydes investigated (Figure 5.2.2).

Percentage germination and CVG were highly correlated with peroxide value (r = -0.81 and -0.90 respectively, Table 3.1.1, pg. 59). The amount of volatile aldehyde produced on heating was poorly correlated with CVG, germination and peroxide value for all three aldehydes (Table 5.2.1). If expressed as a percentage of the total volatiles produced, however,
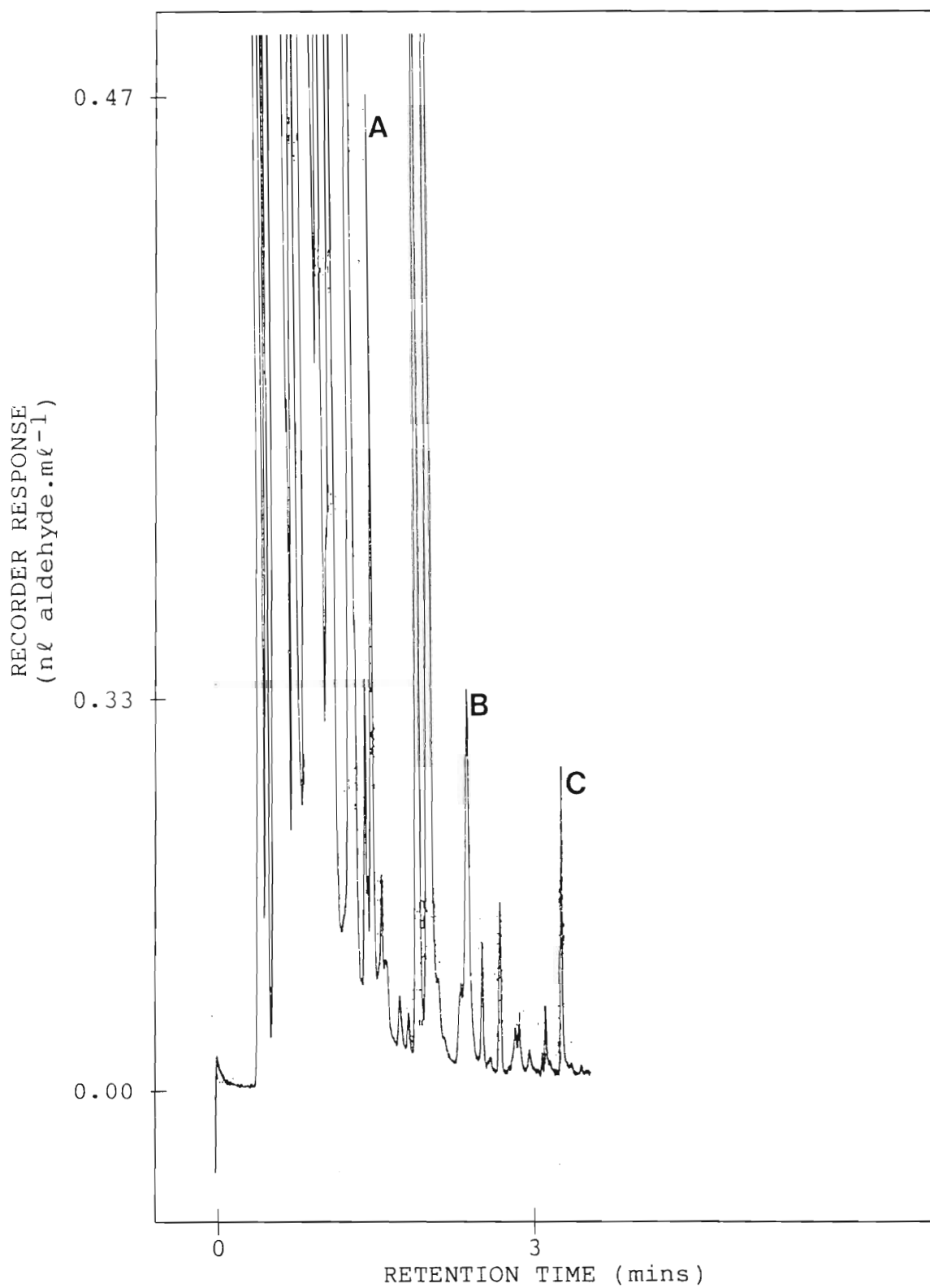
Fig 5.2.1 A typical gas chromatogram of volatile compounds evolved on heating soya bean powder. Cultivar Pioneer. A, butanal; B, pentanal; C, hexanal.

Fig 5.2.2 A gas chromatogram showing volatiles
derived from heated seed powder depleted of lipid.

the amounts of hexanal, pentanal and butanal were all highly correlated with CVG, germination and peroxide value (Table 5.2.2). Hexanal showed a highly significant relationship ($r = -0.94$) with germination and seed CVG ($r = -0.96$) and a significant relationship with peroxide levels ($r = 0.85$). Pentanal was significantly correlated with both seed CVG and hydroperoxide levels, giving -0.86 and 0.81 respectively (table 5.2.2). Butanal was highly correlated with CVG ($r = -0.94$), peroxide value ($r = 0.89$) and germination ($r = -0.87$).

Table 5.2.1. Amount ($n\ell.m\ell^{-1}$ of headspace) of butanal, pentanal and hexanal derived on heating six cultivars of soya bean.

Cultivar	Butanal	Pentanal	Hexanal
Ibis	0.223a	0.135a	0.121a
Impala 4028	0.236a	0.166a	0.138a
Impala 4023	0.159a	0.120b	0.154a
Impala 4031	0.214a	0.131a	0.131a
Pioneer	0.375b	0.236a	0.297b
Hartebees	0.235a	0.160a	0.190c
Correlation with			
germination	-0.34	-0.35	-0.58
peroxide value	0.55	0.57	0.76
CVG	-0.36	-0.40	-0.63

Table 5.2.2. Percentage area of butanal, pentanal and hexanal
derived on heating six cultivars of soya bean.

Cultivar	Percentage Area		
	Butanal	Pentanal	Hexanal
Ibis	0.377a	0.220a	0.202a
Impala 4028	0.539b	0.406b	0.349b
Impala 4023	0.902b	0.663b	0.477b
Impala 4031	0.950b	0.541b	0.384b
Pioneer	0.977b	0.605b	0.778c
Hartebees	1.320b	0.805b	1.040c
Correlation with			
germination	-0.87*	-0.78	-0.94**
peroxide value	0.89*	0.81*	0.85*
CVG	-0.94**	-0.86*	-0.96**

* significant at the 5% level using Student t-test

** significant at the 1% level using Student t-test

5.2.4 DISCUSSION

The correlations obtained between peroxide value and the relative area percentages of aldehydes, and the results from the chromatographic analysis of seed tissue depleted of lipid indicated that the volatile aldehydes were derived from the thermal breakdown of lipid hydroperoxides.

The occurance of hexanal as a product of lipid oxidation is well documented. Hoffmann (1962) identified hexanal as one of the cleavage products from oxidized soya bean oil. Frankel (1982) reported the evolution of hexanal as a major product from heated oxidized linoleate hydroperoxides and methyl esters. With respect to hexanal production from seeds, Heydanek and McGorrin (1981a, 1981b) reported the evolution of hexanal from oat (Avena sativa L.) groats. Wilson and McDonald (1986b) have stated that hexanal appears to be ubiquitous in aged seeds, and suggested that its measurement may be useful as an index of lipid peroxidation.

The 13-hydroperoxide of linoleate is considered to be the source of hexanal from heated oxidized linoleate (Frankel, 1982). Results from the analysis of lipid oxidation products indicated that the 13-hydroperoxide

was the major product of lipid oxidation in soya bean seeds (following section, pg. 169). Hexanal is thought to be formed by homolytic cleavage of the 13-hydroperoxide, followed by unimolecular decomposition of the alkoxy radical formed, to yield hexanal (Frankel, 1982, Figure 5.2.3).

$$CH_3(CH_2)_3CH_2-CH-CH=CH-R'$$
$$|$$
$$OOH$$

$$\downarrow$$

$$CH_3(CH_2)_3CH_2-CH-CH=CH-R' + \cdot OH$$
$$|$$
$$O\cdot$$

$$\downarrow$$

$$CH_3(CH_2)_4CHO + R'$$
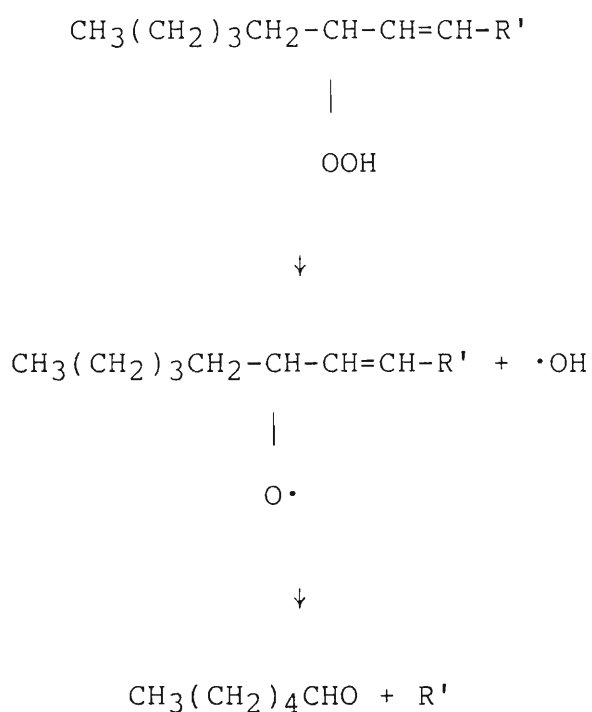
Figure 5.2.3. Hexanal formation from the 13-OOH of linoleate.

Hexanal was however produced in relatively small amounts, being less than either pentanal or butanal. Frankel (1982) has noted that at relatively low temperatures, the cleavage of the carbon-carbon bond between the peroxide group and the double bond is

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favoured, while at high temperatures, the cleavage of

the carbon-carbon bond on the other side of the

peroxide group is favoured (Figure 5.2.4). It is

possible that the conditions used in this study

favoured the latter reaction, thus resulting in the

relatively low levels of hexanal produced. This

arguement is supported by the significant levels of

pentanal detected.

$$\downarrow \quad \downarrow$$

$$CH_3(CH_2)_3-CH-CH=CH-R$$

$$|$$

$$OOH$$
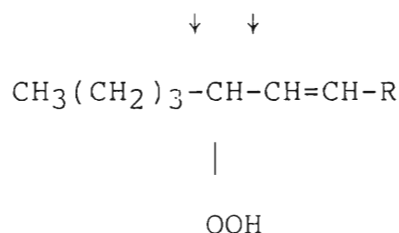
Figure 5.2.4. Different positions of peroxide cleavage.

In contrast to hexanal, pentanal production is not

well documented in the literature. Hoffmann (1962), who

identified pentanal in the volatile fraction from

oxidized soya bean oil, suggested that it may be formed

from a 14-hydroperoxy octadecadienoate. Frankel (1982)

has reported small amounts of pentanal associated with

the thermal breakdown of oleate methyl esters, but not

from oleate hydroperoxides. Pentanal was also observed

in volatiles from linoleate hydroperoxides, but not

linolenate methyl esters. From a consideration of the

proposed mechanisms of secondary breakdown (Frankel,

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1982), a possible source of pentanal may be the cleavage of the alkoxy radical formed from 13-hydroperoxy octadecadienoate (Figure 5.2.3) to yield a pentane radical. This may then undergo the proposed steps of peroxidation and hydroperoxide breakdown to produce pentanal (Figure 5.2.5).

$$CH_3(CH_2)_3-CH_2\cdot + O_2 + RH$$

$$\downarrow$$

$$CH_3(CH_2)_3-CH_2$$
$$|$$
$$OOH$$

$$\downarrow$$

$$CH_3(CH_2)_3CHO + \cdot OH + \cdot H$$
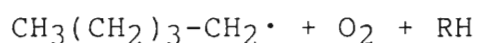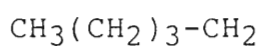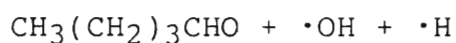
Figure 5.2.5. Pentanal formation from the 13-hydroperoxy linoleate.

The production of butanal from oxidized linolenate hydroperoxide and linoleate methyl ester on heating has been reported by Frankel (1982). Small amounts of butanal have also been observed in volatiles derived

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from the thermal breakdown of trilinolein, but none was detected from linoleate hydroperoxides or methyl esters. One possible mechanism for the formation of butanal is the secondary breakdown of 3-hexenal by peroxy attack at the double bond (Figure 5.2.6).

$$CH_3-CH_2-CH=CH-CH_2-CHO + O_2 + 2RH$$

$$\downarrow$$

$$CH_3-CH_2-CH_2-CH-CH_2-CHO$$
$$|$$
$$OOH$$

$$\downarrow$$

$$CH_3(CH_2)_2CHO + \cdot CH_2CHO$$
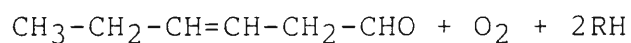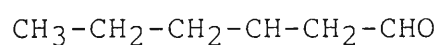
Figure 5.2.6. Butanal formation from 3-hexenal.

However, 3-hexenal is thought to be derived from the 12 and 13-hydroperoxy octadecatrienoate, relatively minor components of oxidized linolenate (Frankel, 1980). 3-hexenal itself was reported to constitute only 1.4% of the total volatile products formed from the

heated linolenate hydroperoxides (Frankel, 1982). An alternative source of butanal may therefore exist.

The source of the volatiles in this study was not pure oils, but seed tissue, and a multiple complex of cross-reactions would be expected. Although the lipid appears to be the major source of the volatiles detected, the involvement of other cellular components cannot be excluded. Speculation as to mechanisms involved must therefore be viewed as tentative.

It has been proposed (Wilson and McDonald, 1986a) that the measurement of thermally derived volatile compounds may be used as an index of seed vigour. Results obtained here support such a proposal. However, such a technique would require careful standardization of all parameters, including the quantity of seed tissue used, the details of the temperature regime, the duration of treatment, the volumes of the headspace and sampling methods. Clearly, further work would be required before the technique could be generally recommended as a convenient means of measuring seed vigour.

In conclusion, the results of this study indicated that only certain volatile compounds evolved on heating were correlated with seed viablity. The significant relationship obtained between seed hydroperoxide levels

and the evolution of the volatile aldehydes identified, and the fact that seed material depleted of lipid did not produce these compounds, suggested that the lipid hydroperoxides were the source of these aldehydes.

CHAPTER 6

6.1 ANALYSIS OF THE AUTOXIDATION OF OLEATE, LINOLEATE AND LINOLENATE BY GAS CHROMATOGRAPHY.

6.1.1 INTRODUCTION

Hydroperoxides are thermally labile, making them impossible to chromatograph successfully (Frankel, 1980). Reduction of the hydroperoxides, usually with NaBH$_4$, provides a stabler product, but complete separation of the different hydroxy products has proved difficult (Frankel et al., 1977b), and certain hydroxy compounds are reported to break down at the temperatures used for GC analysis (Freedman, 1967). Derivatization of the hydroxy products and hydrogenation of the lipid has been used by a number of workers in an attempt to overcome these problems (Freedman, 1967; Frankel et al., 1977a). Hydrogenation of unsaturated lipid removes all double donds, thus producing saturated (stearate) derivatives from oleate, linoleate and linolenate. Gas chromatography-mass spectroscopy (GC-MS) analysis of hydrogenated and silylated autoxidized lipid has revealed that the main components of oxidized oleate were 9, 10-epoxystearate, 8-, 9-, 10-, and 11-hydroxystearates and small amounts of di-hydroxystearates (Frankel et al., 1977a). Oxidized linoleate produced predominately 9- and 13-ketostearates, and 9- and 13-hydroxystearates (Frankel

et al., 1977b). Autoxidation of linolenate resulted in a mixture of 9-, 12-, 13-, and 16-hydroxystearates (Frankel et al., 1977c).

Further analysis using the combined techniques of of GC-MS and high pressure liquid chromatography (HPLC), together with computer summation, has been used to accurately determine the relative proportions of the hydroxy products (Frankel et al., 1977b; Neff et al., 1978). This work revealed that the isomeric composition of oleate, linoleate and linolenate remained remarkably constant at different levels and temperatures of autoxidation (Frankel, 1980). Oxidized oleate showed a small but consistently higher amount of the 8- and 11-isomers than the 9- and 10-isomers (Frankel et al., 1977a). Oxidized linoleate produced equal amounts of 9- and 13-hydroperoxides (Frankel et al., 1977b). Oxidized linolenate formed the 9- and 16-isomers in significantly higher proportions than the 12- and 13-isomers (Frankel et al., 1977c).

In this investigation, the ability of a megabore capillary column to resolve products of lipid peroxidation was investigated. Autoxidized oleate, linoleate and linolenate were examined for the relative proportions of oxidation products. The ability of the column to resolve the different hydroxy products was compared

with recent studies in the literature, with the

ultimate aim of resolving the oxidation products from

seed lipids.

6.1.2 MATERIALS AND METHODS

Seeds of salsify (Tragopogon porrifolius),
Dimorphotheca sp. and Helichrysum sp. were obtained
from local seed merchants (Collingwoods). The oil of
Ricinus communis L. (castor oil) was obtained
commercially.

Autoxidation

Pure standards of oleic, linoleic and linolenic
acids were obtained from the Sigma Chemical Company.
Autoxidations were done with 0.05 ml of pure standard
in 50 ml of methylene chloride/ methanol (2/1,v/v),
stirred under O_2 at 40°C.

$NaBH_4$ reduction

0.02 ml of 0.5 M aqueous $NaBH_4$ was added to 2 ml
of autoxidized lipid solution and left to stand for 10
minutes. Half a millilitre of water was then added and
the vial shaken to obtain separation of the two phases.
Excess $NaBH_4$ was detroyed with dilute HCl. One micro-
litre of the lower phase was then injected into the GC.

Silylation

Silylation of the lipid was carried out using
trimethylsilyl-imidazole (TMSI). An aliquot of the
reduced lipid solution was dried down under N_2,

ensuring that all methanol was evaporated. Two hundred microlitres of pyridine and 0.2 mℓ of TMSI were added and the vial heated at 100°C. The course of silylation was monitored by GC. One microlitre of the pyridine-TMSI mixture was injected directly onto the column.

Hydrogenation

Hydrogenation of the lipid was carried out under a slight positive pressure of hydrogen in 95% ethanol with NiO_2 as catalyst. The course of hydrogenation was monitored by GC.

Lipid extraction

Lipid extraction was carried out as described previously (cf. chapter 3.1, pg. 55).

Esterification

Esterification was carried out using the organic base-catalysed technique of Metcalfe and Wang (1981). An aliquot of lipid was dissolved in 2 mℓ of methylene chloride/methanol (2/1,v/v). Half a millilitre of 0.1 M tetramethylammonium hydroxide (TMAH) was added and left to stand for five minutes. Half a millilitre of water was then added and the vial shaken to separate the phases. One microlitre of the lower phase was then analysed by GC.

Chromatographic conditions

A Varian 3300 gas chromatograph fitted with a flame ionization detector and a 15 metre DB 1 megabore capillary column was used. Injector and detector temperatures were 250°C. Column temperature was programmed from 155°C to 230°C at $4°C.minute^{-1}$. Carrier gas was helium at 10 mℓ.minute^{-1}.

Thin layer chromatography

TLC was carried out on hand-spread glass plates. Silica gel G 60 (0.01 - 0.04 mm) containing binding agent ($CaSO_4$) was spread to a thickness of 0.250 mm. The plates were developed in petroleum ether:diethyl ether:acetic acid (70:30:1). Spots were vizualized with 1% 2',7'- dichlorofluorescein in methanol and viewed under UV light. These were examined by GC by redissolving in diethyl ether and shaking with an equal volume of water. The ether layer was then concentrated and one microlitre injected into the GC.

6.1.3 RESULTS AND DISCUSSION

By comparison with the results of work on autoxidized oleate, linoleate and linolenate in the literature using columns of equivalent properties (OV 101, OV 1), most peaks obtained could be tentatively identified. Further information with respect to peak identity was obtained by the analysis of the oil from seeds reported to contain epoxy or hydroxy compounds. By this method, preliminary identification of most of the compounds could be made (Figure 6.1.1) Following convention in the literature, the autoxidized lipid was referred to as reduced, oxidized lipid (Frankel, 1980).

It was found that neither silylation nor hydrogenation provided any improvement in the resolution of the compounds obtained. Complete hydrogenation could also not be obtained without extensive changes in the proportions of the oxidation products.

Autoxidized oleate

The oxidation products of reduced, oxidized oleate eluted as two peaks (peaks III and IV, Figure 6.1.1). By comparison with the results of autoxidation of oleate in the literature (Frankel et al., 1977a), the two peaks were tentatively identified as epoxy stearate and hydroxy oleate respectively. Frankel et al (1977a)
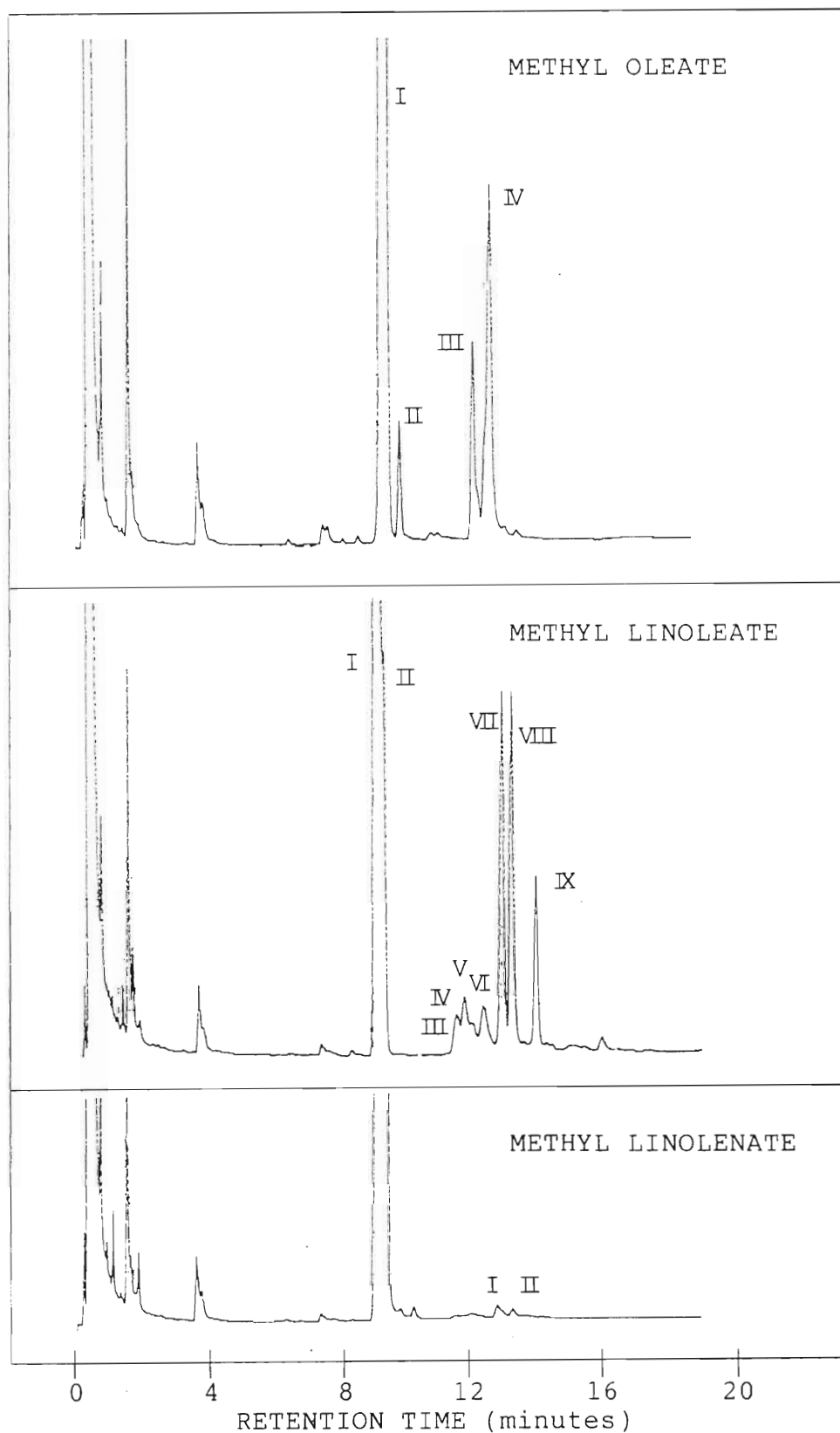
Fig 6.1.1 Gas chromatograms of $NaBH_4$ reduced,
oxidized methyl oleate, linoleate and linolenate.
Methyl oleate: I, methyl oleate; II, methyl stearate;
III, epoxy stearate; IV, hydroxy oleate. Methyl lin-
oleate: I,II, methyl linoleate; III,IV,V, epoxy oleate;
VI, unknown; VII, VIII, IX, monohydroxy linoleate.
Methyl linolenate: I,II, monohydroxy linolenate.

has reported the production of 9,10-epoxy stearate as the major epoxide in oxidized oleate. Peak III was therefore tentatively identified as 9,10-epoxy stearate (cis-9,10-epoxy-octadecanoic acid).

The analysis of castor oil revealed that ricinoleate (12-hydroxy-cis-9-octadecenoic acid) coeluted with peak IV (Figure 6.1.2). This suggested that this peak consisted of hydroxy monoene products. Oxidized oleate has been shown to yield a mixture of 8-, 9-, 10- and 11-hydroxy-cis and trans-octadecenoic acids (Frankel et al., 1977a), eluting as a single, unresolved peak. This peak was therefore identified as consisting of 8-, 9-, 10-, and 11-hydroxy hydroxy oleate (Table 6.1.1).

Autoxidized linoleate

GC of reduced, oxidized linoleate yielded a number of distinct peaks (Figure 6.1.1). By comparison with work in the literature (Frankel et al., 1977b; Neff et al., 1978), peaks III to IV were tentatively identified as consisting of epoxy products, and peaks VII to IX as hydroxy compounds. Peak VI was not detected in further samples of autoxidized linoleate and may have been artifactual.

Helichrysum seed oil has been reported to contain
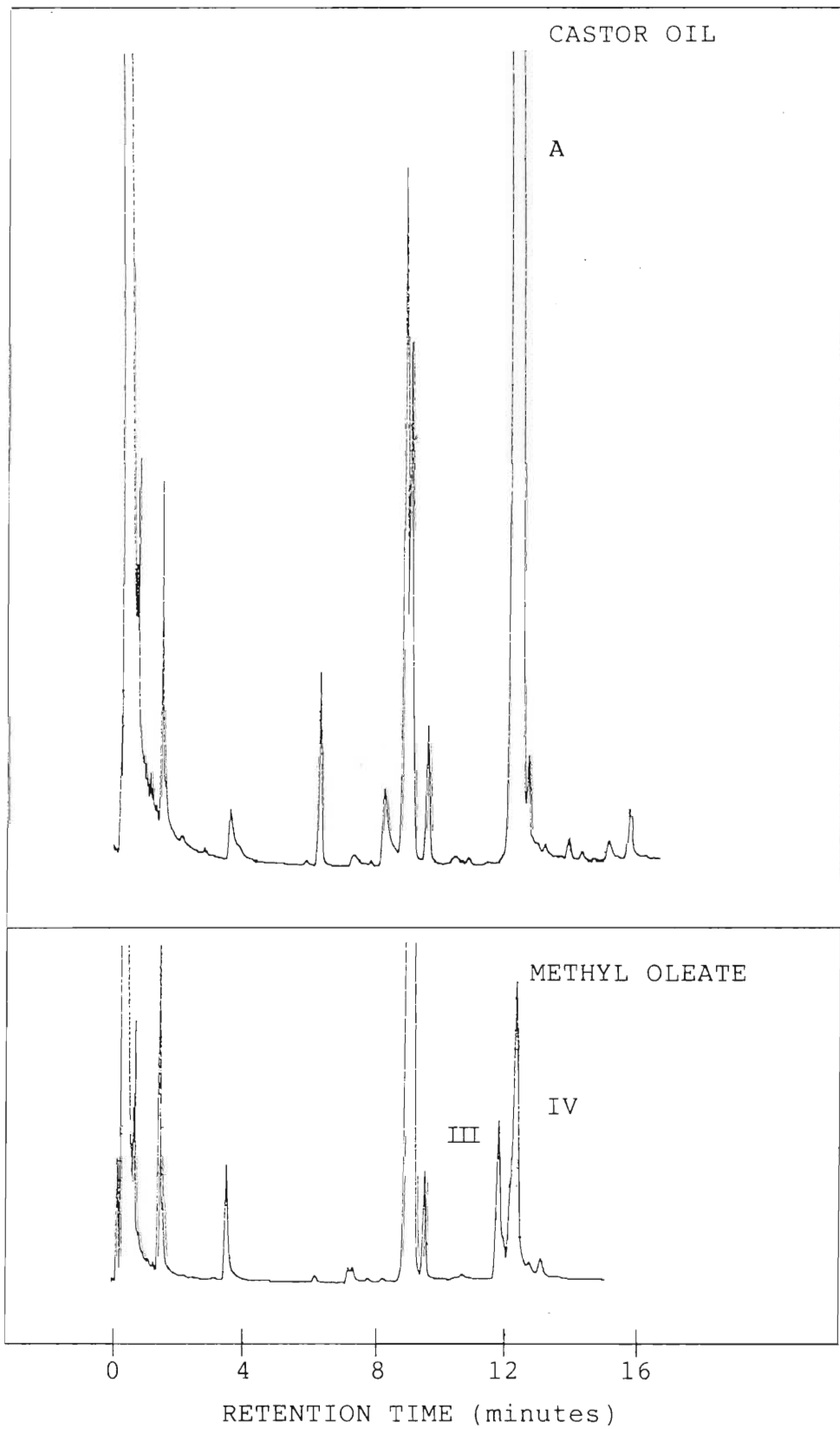
Fig 6.1.2 Gas chromatograms of castor oil and methyl oleate. A, ricinoleate. III, epoxy stearate; IV, monohydroxy oleate.

coronaric acid (cis-9,10-epoxy-cis-12-octadecenoic

acid; Powell et al., 1965). The analysis of this oil

under the same chromatographic conditions indicated

that the first group of partially resolved peaks (III

to V) was constituted of epoxy monoenes, confirming the

initial tentative identification. By this method, the

major peak of this group, peak IV, was tentatively

identified as 9,10-epoxy oleate.

Table 6.1.1. The results of GC analysis of autoxidized oleate.

Component	Percent of total oil
I octadecenoic acid	84.56%
II octadecanoic acid	1.10%
III cis-9,10-epoxy octadecanoate*	2.58%
IV hydroxy monoenes	6.12%
8-hydroxy-cis and trans-9-octadecenoate	
9-hydroxy-cis and trans-10-octadecenoate	
10-hydroxy-cis and trans-8-octadecenoate	
11-hydroxy-cis and trans-9-octadecenoate	

* - identification tentative

Work on autoxidized linoleate has demonstrated
that the 9- and 13-hydroperoxides are present in equal
amounts (Frankel et al., 1977b; Neff et al., 1978). The
similar proportions of peaks VII and VIII obtained in
this study therefore indicated that these two peaks
were the 9- and 13-hydroxy derivatives of the 9- and
13-hydroperoxides. Tragopogon porrifolius oil has been
shown to contain both 9-hydroxy-trans-10-cis-12- and
13-hydroxy-cis-9-trans-11-octadecadienoic acid
(Chisholm and Hopkins, 1960). Furthermore, 13-hydroxy
linoleate has been reported to be less polar than 9-
hydroxy linoleate (Morris et al., 1960b). By the
combination of TLC (Figure 6.1.3) and GC analysis of
the oil from Tragopogon seeds, peaks VII to IX were
tentatively identified as 13-hydroxy cis, trans
linoleate and 9-hydroxy trans, cis linoleate respec-
tively (Table 6.1.2).

Neff et al. (1978) using an equivalent column has
shown that GC of silyl esters of reduced, oxidized
linoleate gave complete separation of cis, trans from
trans, trans isomers of the monohydroxy component. The
silylation of autoxidized linoleate in this study
altered the elution characteristics of peaks VII, VIII
and IX, the first two compounds eluting as a single
peak, and peak IX as two separate peaks. This result
indicated that peak IX may have consisted of two
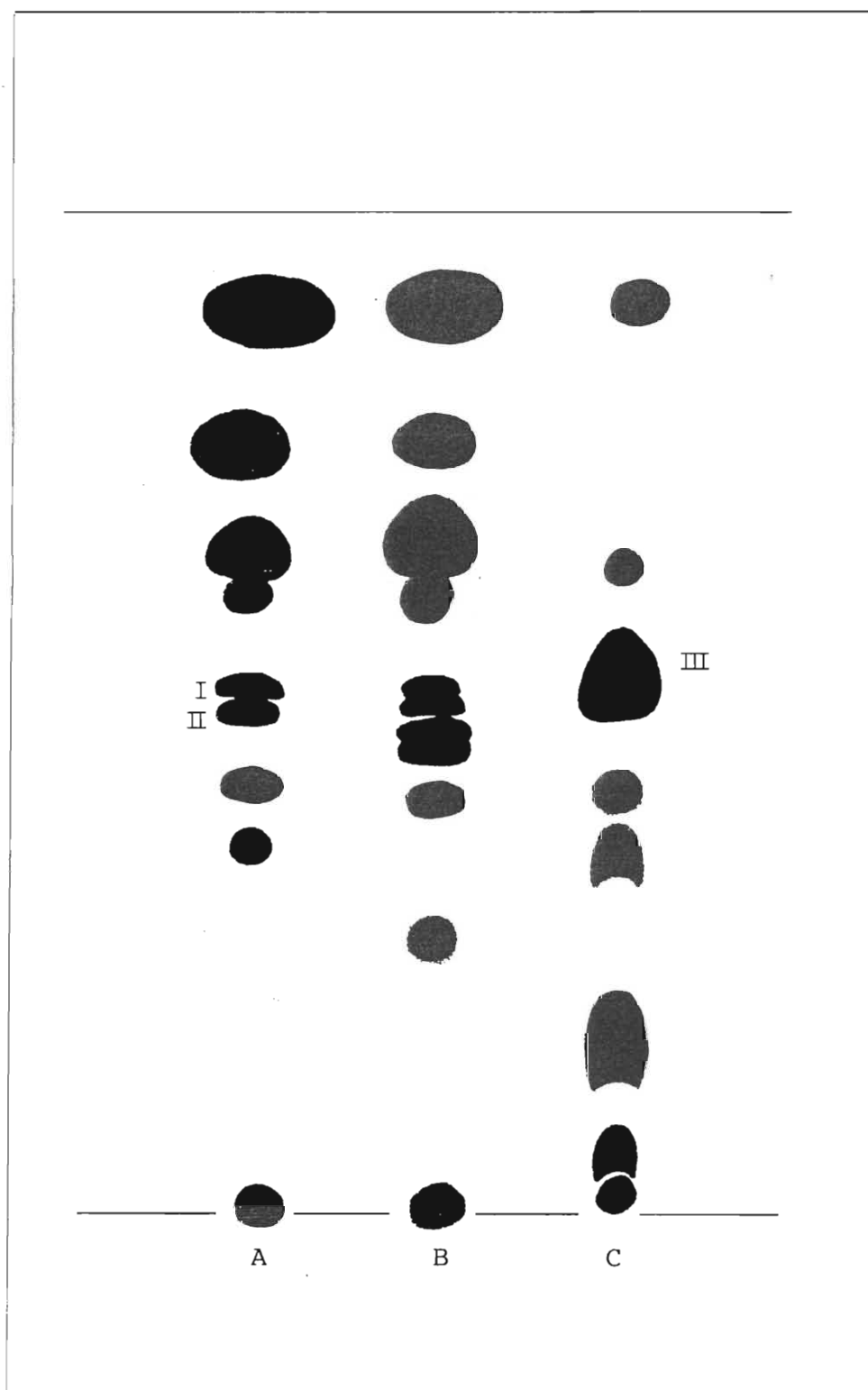
Fig 6.1.3 Thin layer chromatographic separation
of Tragopogon (A), Helichrysum (B) and castor oil(C)
methyl esters. I, 13-hydroxy linoleate; II, 9-
hydroxy linoleate; III, ricinoleate.

closely related compounds (Figure 6.1.4). Peak IX was therefore tentatively identified as monohydroxy <u>trans</u>, <u>trans</u> dienes, or more specifically, 13-hydroxy-<u>trans</u>-9-<u>trans</u>-11-octadecadienoic acid and 9-hydroxy-<u>trans</u>-10-<u>trans</u>-12-octadecadienoic acid (Table 6.1.2).

Table 6.1.2. Results of GC analysis of autoxidized linoleate.

Component	Percent of total oil
I <u>cis</u>, <u>cis</u>-linoleate	67.69%
II <u>cis</u>, <u>trans</u>-linoleate	4.63%
III epoxy-oleate*	1.04%
IV 9,10-epoxy-<u>cis</u>-oleate*	2.12%
V epoxy-oleate*	0.21%
VI unknown	1.55%
VII 13-hydroxy-<u>cis</u>, <u>trans</u>-linoleate	6.66%
VIII 9-hydroxy-<u>trans</u>, <u>cis</u>-linoleate	6.55%
IX 9- and 13-hydroxy-<u>trans</u>, <u>trans</u>-linoleate	3.05%

* identification tentative
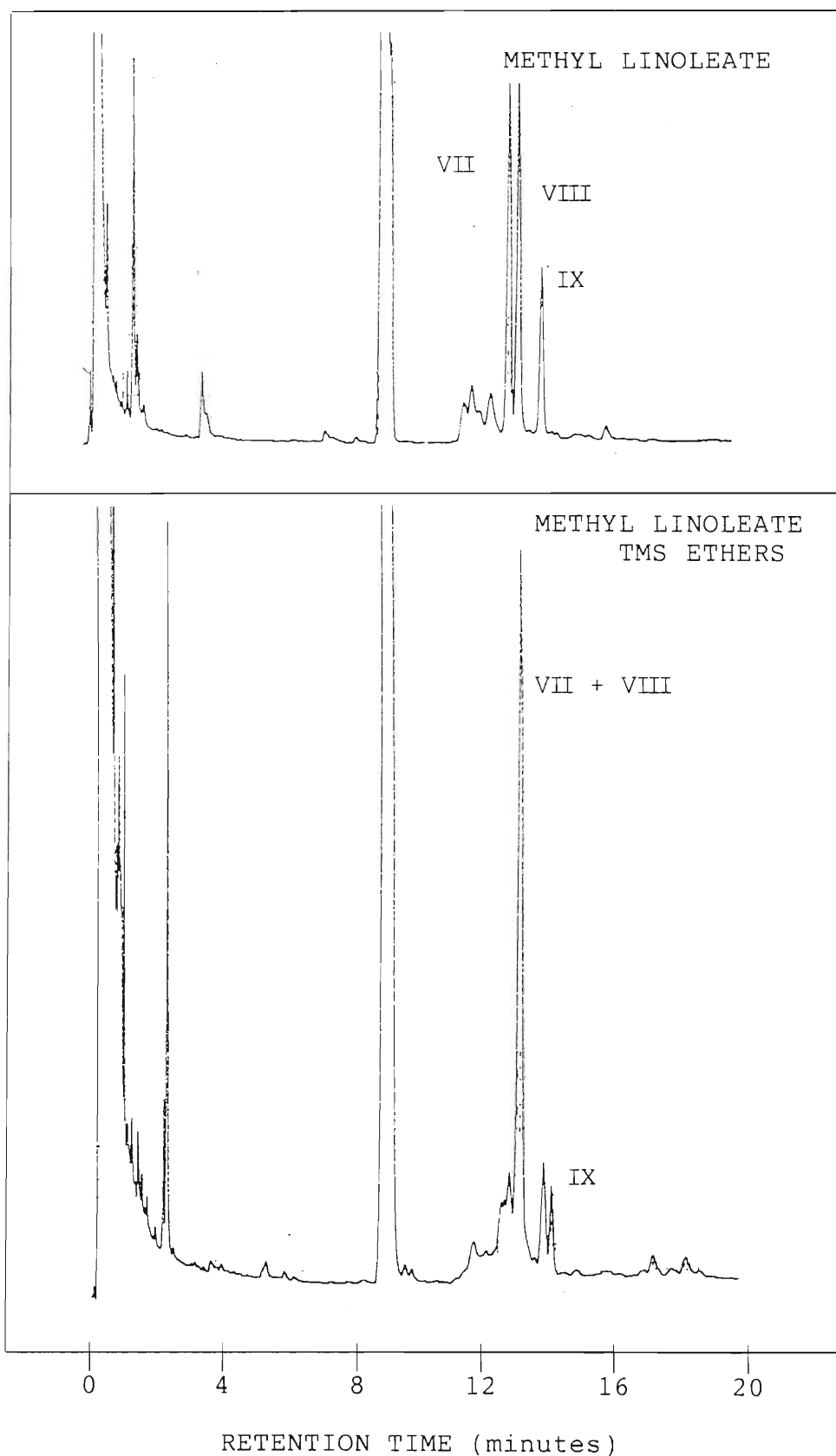
Fig 6.1.4 Gas chromatograms of reduced, oxidized
methyl linoleate showing the effects of silylation.
VII,VIII, cis,trans hydroxy linoleate; IX, trans,trans
hydroxy linoleate.

Following the initial investigation described above, standards of the cis and trans isomers of 9- and 13-hydroxy linoleate were made available by Dr. W. E. Neff*. This allowed positive confirmation of peak identities. Comparison with these standards confirmed the identities given above for peaks VII, VIII and IX.

Autoxidized linolenate

The analysis of reduced, oxidized linolenate revealed two main peaks co-eluting with the monohydroxy compounds (Figure 6.1.1). Frankel et al. (1977c) has reported a mixture of 9-, 12-, 13- and 16-hydroxy linolenate from autoxidized linolenate. Consistently higher amounts of 9- and 16-hydroperoxides were found, particularly at higher peroxide values (Frankel et al., 1977c).

From a consideration of the behaviour of the hydroxy products of linolenate given in the literature (Frankel et al., 1977c), it was possible to tentatively identify the peaks obtained. The first peak may represent 9-hydroxy-trans-10, cis-12, cis-15-octadeca-trienoic acid. The 12-hydroxy-cis-9, trans-13, cis-15-

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octadecatienoic acid and 13-hydroxy-cis-9,trans-11,cis-15-octadecatrienoic acid might be expected to co-elute together, and possibly with the 9-hydroxide. The distinct shoulder on peak I (Figure 6.1.1) may represent either the 12- and 13-hydroxide or the trans component of this group. A consideration of the literature would support the former, as relatively small amounts of trans, trans compounds are present in autoxidized linolenate (Frankel et al., 1977c). The final peak may therefore represent the 16-hydroxy-cis-9,cis-12,trans-14-octadecatrienoic acid (Table 6.1.3).

Table 6.1.3. A summary of the results of oxidized linolenate.

Component	Percent of total oil
I 12-,13- and 9 hydroxy linolenate*	1.07%
II 16-hydroxy linolenate*	0.61%

*identification tentative

By comparison with autoxidized linoleate, it was evident that the hydroxy products of linolenate will co-elute with those of linoleate (Figure 6.1.1). If the

tentative identification given above is correct, then

the 9-, 13-, and 12-hydroxides will have eluted with

the 13-hydroxy-cis, trans-linoleate and the 16-

hydroxide with the 9-hydroxy-trans, cis-linoleate.

A number of workers have reported the lability of

certain hydroxy compounds on GC analysis. For example,

dimorphecolic acid (9-hydroxy-trans-10, trans-12-

octadecadienoic acid) has been reported to dehydrate

during GC analysis to give conjugated trienes

(Freedman, 1967). Similarly, α-hydroxy monoenes have

been shown to be altered to conjugated dienes (Morris

et al., 1960a).

Morris et al. (1960b) found that methyl

dimorphecolate decomposed in the flash heater and not

on the GC column. Freedman (1967) tried to prevent this

by injecting straight onto the column, but found

extensive dehydration still occured at temperatures as

low as 200°C. Binder et al. (1964) reported complete

decomposition of methyl dimorphecolate at 250°C, but no

decomposition at 200°C.

In order to determine if any decomposition was

occuring in this study, the oil of Dimorphotheca seeds

was examined under the same chromatographic conditions

used for the analysis of the autoxidized oils. No

evidence for the breakdown of methyl dimorphecolate was obtained. This result indicated that no alteration of hydroxy compounds had occured in this study.

Linolenate has been shown to be particularly susceptible to oxidation due to the fact that it contains three methylene-interrupted double bonds. For this reason, relatively greater amounts of hydroperoxides would be expected in oxidized linolenate compared with oxidized oleate and linoleate. This contrasts with the results obtained in this study, as only relatively small quantities of linolenate hydroperoxides were detected. However, Frankel (1980) has noted that the hydroperoxides of linolenate are more labile than those of oleate and linoleate, and therefore do not accumulate as rapidly as in the less unsaturated lipids.

In conclusion, the analysis of autoxidized lipid with the use of a megabore capillary column provided comparable resolution of hydroxy and epoxy compounds to that obtained in the literature. Separation was apparently achieved on the basis of both the position of the oxygen containing group and <u>cis</u>, <u>trans</u> isomerism. Results, therefore, suggested that megabore GC may provide a simple yet effective tool for the monitoring of lipid peroxidation.

6.2 ANALYSIS OF AUTOXIDIZED SOYA BEAN OIL LIPIDS BY GAS CHROMATOGRAPHY.

6.2.1 INTRODUCTION

Soya bean oil contains approximately 10% palmitic, 3% stearic, 20% oleic, 60% linoleic and 7% linolenic acid. Frankel et al. (1977c) examined the relative proportion of hydroxy products in different mixtures of autoxidized oleate, linoleate and linolenate. GC-MS analysis of a 1:1:1 mixture of oleate, linoleate and linolenate showed that linolenate was the main source of hydroperoxides at low peroxide value (PV), but that at higher PV, linoleate products predominated. It was suggested that this was due to the hydroperoxides of linolenate being less stable relative to linoleate, and thus do not accumulate as much as linoleate hydroperoxides during autoxidation (Frankel, 1980). Both the 12- and 16-hydroperoxides, primarily products of linolenate, declined with increasing PV. The 9- and 13- hydroperoxides predominated at all PVs, their relative proportions increasing with increasing oxidation.

In a mixture of fatty acids more closely approximating soya bean oil, GC-MS analysis revealed an unexpectedly high proportion of 12-hydroperoxides at low

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peroxide value (Frankel and Neff, 1979). Photosensitized oxidation was suggested as a likely source of this compound. The relative proportions of the other compounds were comparable with those obtained previously (Frankel et al., 1977c), the 9- and 13-hydroperoxides still predominating, particularly at high peroxide values.

In the previous section (6.1), the application of megabore capillary gas chromatography to the analysis of autoxidized oleate, linoleate and linolenate was described. Results indicated that the column used was capable of resolving most of the hydroxy compounds. In this study, the ability of the column to resolve the autoxidation products of soya bean oil was investigated.

6.2.2 MATERIALS AND METHODS

Autoxidation

Soya bean oil was obtained commercially.
Autoxidation was done with one mℓ of oil in 50mℓ
of methylene chloride/ methanol (2/1,v/v), stirred
under O_2 at 40°C.

The method for lipid extraction and hydroperoxide
determination, and statistical analysis was the same as
described in chapter 3.1 (pg. 55), and that for
reduction and esterification of the lipid, and the
procedure for TLC was the same as described in chapter
6.1. (pg 143).

Chromatographic conditions

A Varian 3300 GC fitted with an FID and 15 m DB1
megabore capillary column was used. Detector and
injector temperatures were 250°C. Column temperature
was 180°C. Carrier gas was helium at 3 mℓ.minute^{-1}.

6.2.3 RESULTS AND DISCUSSION

Gas chromatographic analysis of reduced oxidized soya bean oil (PV 504) revealed complete separation of major hydroxy compounds. The epoxy compounds eluted separately (Figure 6.2.1.). As might be expected, $NaBH_4$ reduced, autoxidized soya bean oil gave a very similar chromatographic profile to linoleate (Figure 6.1.1, pg. 147). From the results of the previous investigation of oxidized oleate, linoleate and linolenate, a number of peaks were readily identifiable (Table 6.2.1).

By using a combination of TLC (Figure 6.2.2) and GC, peaks I, II and VIII were shown to all elute with the neutral lipid fraction. As this consisted of normal, unoxidized lipid compounds, it seemed improbable that these peaks were products of oxidation.

The analysis of Helichrysum oil as before (section 6.1, pg 148) indicated that peak III coeluted with coronarate. Peak III was therefore tentatively identified as cis-9,10-epoxy-cis-12-octadecenoate. Mead et al. (1980) has reported the occurance of both the 9,10- and 12,13-epoxides in autoxidized linoleate monolayers. It is possible therefore that peak III may also have contained the cis-12,13-epoxy-cis-9-octadecenoic
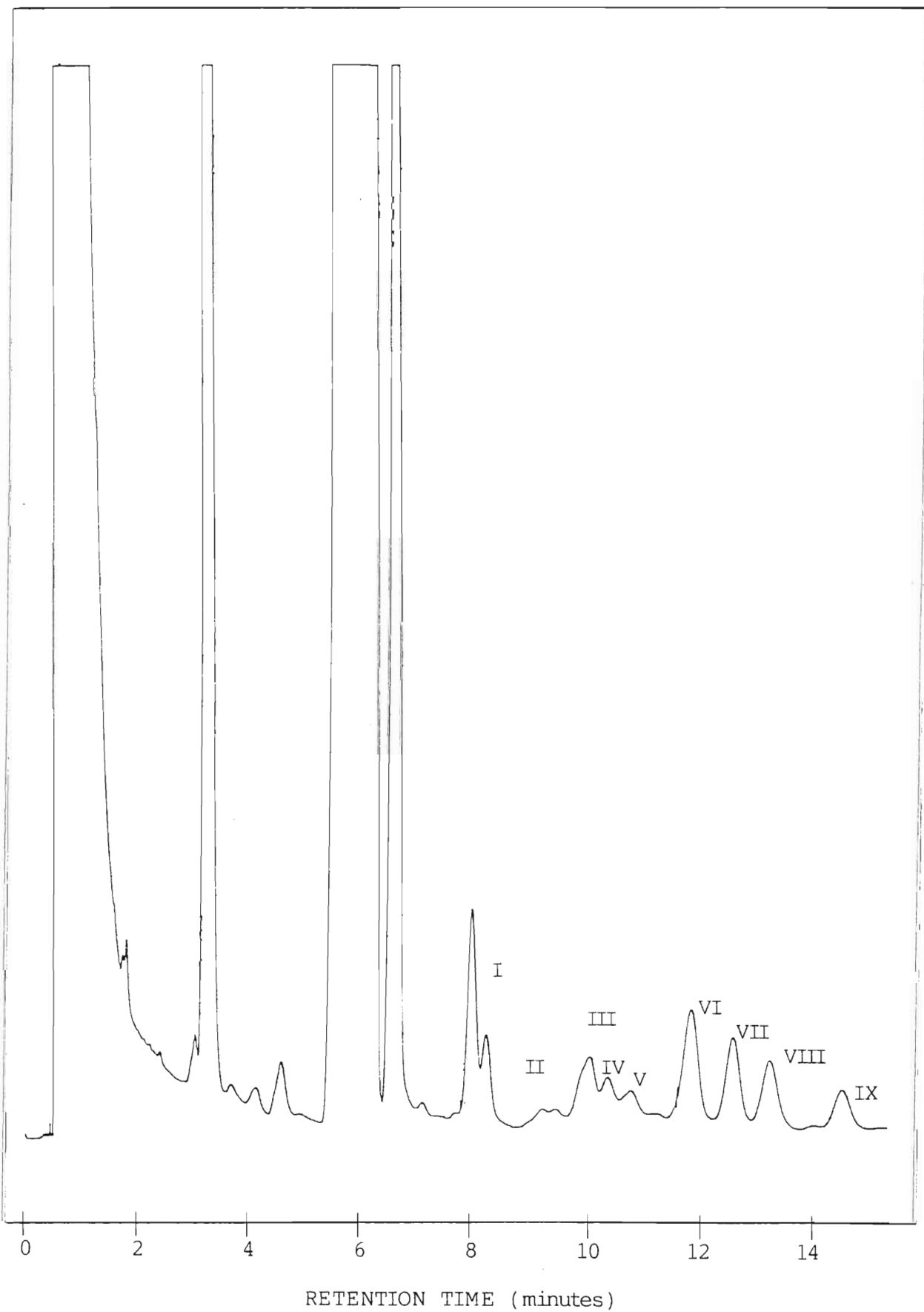
Fig 6.2.1 Gas chromatogram of reduced, oxidized soya bean oil.

acid. In addition, peak III could also have contained the epoxy products of oleate autoxidation. Further work is therefore necessary in order to positively identify the compounds of peak III.

Peaks IV and V were tentatively identified as monohydroxy monoenes. TLC-GC analysis of the soya bean methyl esters revealed that peaks IV and V were fractionated, peak IV eluting with the 13-hydroxide and peak V with the 9-hydroxide (Figure 6.2.2). This result supported the initial identification given above. The initial study (6.1) indicated that the DB 1 column permitted the resolution of cis and trans isomers. This result was consistent with similar work in the literature (Neff et al., 1978). This may suggest that peaks IV and V consisted of partially resolved cis and trans hydroxy monoenes. From the relative proportions obtained in this study, this would indicate that there was a marginally higher proportion of cis than trans hydroxy monoenes in the autoxidized soya bean oil. In support of this, Garwood et al. (1977) have shown a slightly higher proportion of cis to trans isomers in oleate oxidized at low temperature. As the oil used in this study had undergone autoxidation at low temperature, peaks IV and V were tentatively identified as partially resolved cis and trans-hydroxy oleate.
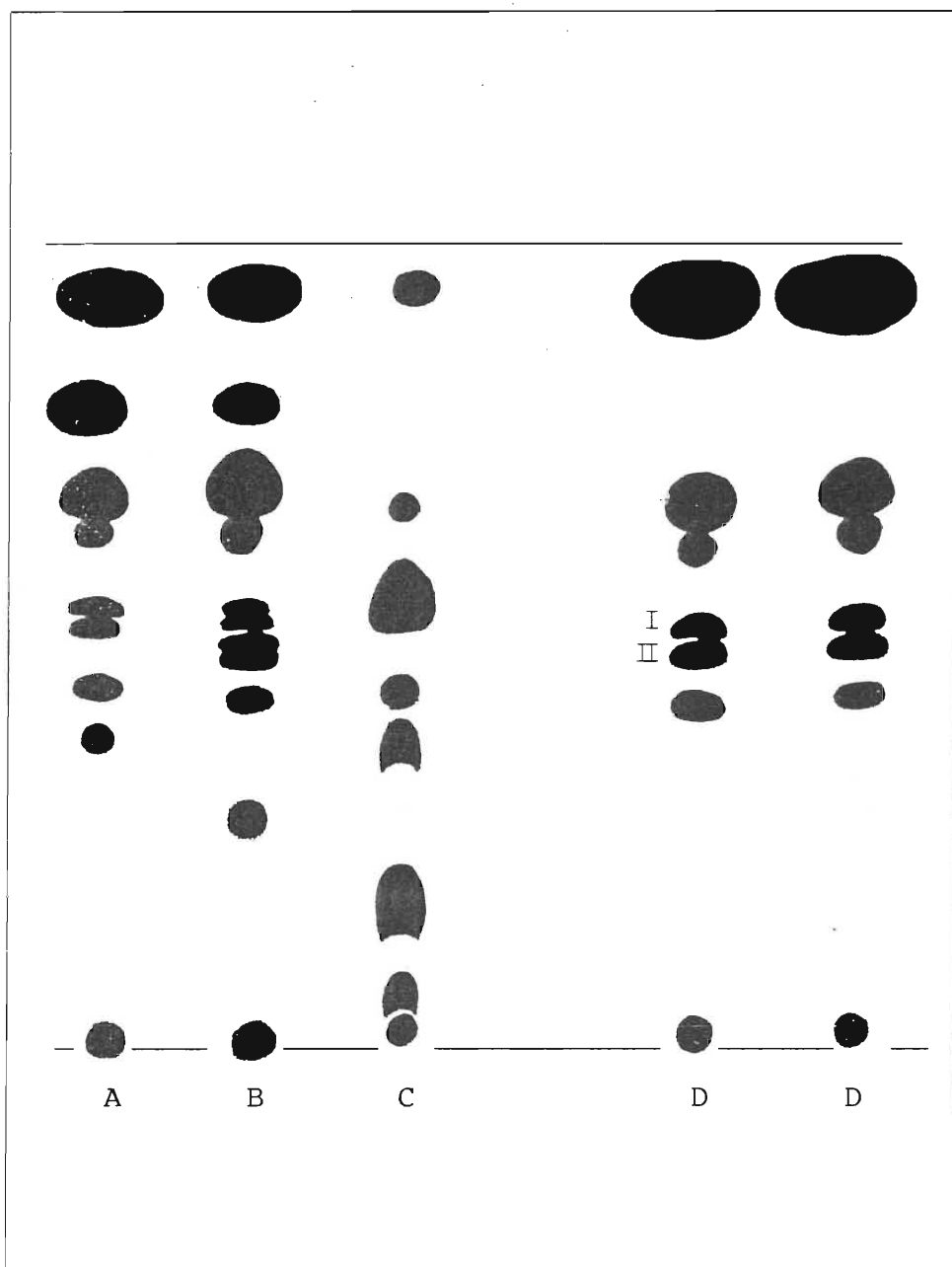
Fig 6.2.2 Thin layer chromatographic separation
of Tragopogon (A), Helichrysum (B), castor oil (C)
and soybean (D) methyl esters. I, 13-hydroxy lin-
oleate; II, 9-hydroxy linoleate.

From the results of the previous study, peaks VI, VII and IX were identified as monohydroxy diene compounds. This was confirmed by TLC-GC analysis of hydroxy products from <u>Tragopogon</u> seeds and by comparison with standards of linoleate hydroperoxides. Peaks VI and VII were therefore identified as 13-hydroxy-<u>cis</u>-9, <u>trans</u>-11-octadecadienoate and 9-hydroxy-<u>trans</u>-10, <u>cis</u>-12-octadecadienoate respectively, and peak IX as 9-hydroxy-<u>trans</u>-10, <u>trans</u>-12-octadeca-dienoate and 13-hydroxy-<u>trans</u>-9, <u>trans</u>-11-octadeca-dienoate (Table 6.2.1).

Work on autoxidized mixtures of oleate, linoleate and linolenate and soya bean oil has revealed that the 9-hydroperoxide of linoleate predominates, particularly at low peroxide values. In contrast to this, the relative proportions obtained in this study indicated that the 13-hydroperoxide predominated, the ratio of the 9- to the 13-hydroperoxide being approximately 43:57. Chi-square analysis revealed that this ratio was significantly different (p = 0.05) from that obtained for a 1:1 mixture of linoleate and linolenate (9OH:13OH, 54:46) and for a 1:1:1 mixture of oleate, linoleate and linolenate (9OH:13OH, 55:45). It was not, however, significantly different from the proportions obtained from soya bean oil (9OH:13OH, 53:47) and oxidized linoleate (9OH:13OH, 50:50) (Frankel and Neff,

1979, Frankel, 1980).

The higher proportions of the 13-hydroperoxide obtained in this study may have been due to the coelution of the products of linolenate autoxidation (cf. Figure 6.1.1, pg 147). The relative contribution of linolenate oxidation products could not be estimated from the GC analysis of soya bean oil in this study. However, linolenate constitutes only approximately 7% of soya bean oil. Furthermore, Frankel (1980) has observed that the hydroperoxides of linolenate are more labile than those of linoleate, and do not accumulate as rapidly. This suggests that the contribution of linolenate autoxidation products may have been negligible.

In conclusion, the results of this study showed that autoxidized soya bean oil could be analysed by megabore capillary GC for the relative proportions of hydroperoxides present.

6.3 ANALYSIS OF AUTOXIDIZED SOYA BEAN SEED LIPIDS BY GAS CHROMATOGRAPHY.

6.3.1 INTRODUCTION

The autoxidation of bulk oil leads to the production of complex mixtures of allylic hydroperoxides and epoxides. Research into the mechanisms involved has revealed that these products are produced in remarkably constant proportions (Frankel, 1980). For example, autoxidation of linoleic acid gives two main products, the 13- and 9-hydroperoxides, in a 1:1 ratio. Similarly, autoxidation of oleate gives the 8-, 9-, 10- and 11-hydroperoxides in the proportions of 27:23:23:27 respectively, and that of linolenate yields the 13-, 11-, 12-, and 16-hydroperoxides in the ratio 13:11:12:46 respectively (Frankel, 1980).

The autoxidation of lipid monolayers, by contrast, yields predominately epoxides. Although hydroperoxides are present, they seem to form as intermediate products and do not accumulate, but instead oxidize other lipid in the monolayer to epoxides. This oxidation is stereospecific yielding cis epoxides from cis precursors and trans epoxides from trans precursors (Mead et al., 1980).

The only thorough analysis of oxidation of seed lipid in relation to the duration of storage is that of Spencer et al. (1973). These wokers monitored the oxidation of lipid in seeds of Cichorium intybus L., Crepis thomsonii Babc. and Crepis vesicaria L. over 5 years at 5°C and then 18 months at a variety of conditions. An increase in conjugation was observed in all three seed lots over the period of storage, the levels increasing markedly on storage at room temperature. Oxygenated acids in C. intybus increased from 1% initially to 3% after five years storage at 5°C and 17% after 18 months storage at room temperature. The corresponding levels in C. thomsonii were 2, 6 and 18% respectively. The levels of conjugated hydroxy acids at these three stages for C. vesicaria was 0.3, 2 and 9% respectively. The major products formed during storage were hydroxy acids with conjugated unsaturation and 9,10-epoxy acids. The almost exclusive production of the 9,10-epoxide was interpreted as being indicative of enzymatic involvement.

The involvement of enzymes, in particular lipoxygenases, in the oxidation of seed lipid during storage has been suggested by a number of workers (Wilson and McDonald, 1986b; Priestley, 1986). This view has gained some support from the observation of the anomalous role of oxygen in seed ageing (Ohlrogge

and Kernan, 1982; Priestley et al., 1985); a direct relationship between oxygen levels and seed ageing has not been conclusively established.

The autoxidation of lipid by lipoxygenase is both regiospecific, giving rise to specific positional products, and stereospecific, producing an optically active product. For example, the peroxidation of linoleate by soya lipoxygenase gives the 13L-hydroperoxide as the predominate product, the 9D-hydroperoxide being produced in relatively small amounts (Gardner, 1980).

In this study, megabore capillary GC analysis of autoxidized lipids developed in the previous sections (6.1, 6.2) was used to investigate the oxidation of lipid from aged soya bean seeds.

6.3.2 MATERIALS AND METHODS.

Seed material, lipid extraction and peroxide value determination were the same as described in chapter 3.1 (pg. 55). The reduction and esterification of lipid and chromatographic conditions were the same as described previously (chapter 6.1, pg. 143).

Column chromatography

The fractionation of the lipid was carried out using a modification of the column chromatographic technique of Vioque et al. (1961). A 20 x 1cm 'Biorad Econo-column' was packed with silica gel G (0.2 - 0.5 mm, 35 - 70 mesh) in petroleum ether. Lipid (5 mℓ) was eluted with petroleum ether containing successively increasing proportions of diethyl ether. Fractions of 50 mℓ each were collected, the elution of compounds being monitored by GLC, and fractions containing similar compounds were combined. Fractions 1 and 2 of 3% and 5% diethyl ether respectively consisted of normal non-oxygenated lipid. Fractions 3 and 4 (8 and 10% diethyl ether) consisted of predominately epoxy compounds. Fractions 5 and 6 (12% diethyl ether) contained the monohydroxy oxidation products. Compounds remaining on the column were then eluted with 100% diethyl ether. Fractions 5 and 6 were combined and dried down under nitrogen at 70°C. Further fractionation of the hydroxy

components was achieved by modulating the water content of the silica gel. The above procedure was repeated using silica gel containing 20%, 10% and 5% water respectively.

Polarimetry

The purified 13-hydroxide sample was examined for optical activity, using diethyl ether as solvent.

6.3.3 RESULTS AND DISCUSSION

GC analysis of the lipid from four cultivars of
soya bean seeds revealed no qualitative differences
between the oxidation compounds detected in the seed
oils and those obtained in bulk soya bean oil (Figure
6.3.1, cf. Figure 6.2.1, pg. 164). In contrast to this,
marked quantitative differences were observed between
bulk oil and the seed lipid from all four cultivars.
The proportions of oxidation products were, however,
similar in each of the four cultivars (Table 6.3.1).

Results demonstrated that epoxy compounds
constituted from 0.33% to 0.58% of total lipid. A
similar result was obtained in soya bean bulk oil
(Table 6.3.1). Proportionally, a much greater level of
epoxides was detected in the seed oils than in bulk
oil. In the seeds the epoxides constituted 33% to 40%
of oxygenated products identified in contrast to 15% in
bulk oil.

The autoxidation of membranes has been postulated
to be a causal factor during ageing in dry seeds.
Biomembranes contain a high proportion of unsaturated
lipids and are often closely associated with enzymes
and other proteins, making the initiation of free-
radical reactions both facile and damaging (Mead et

174
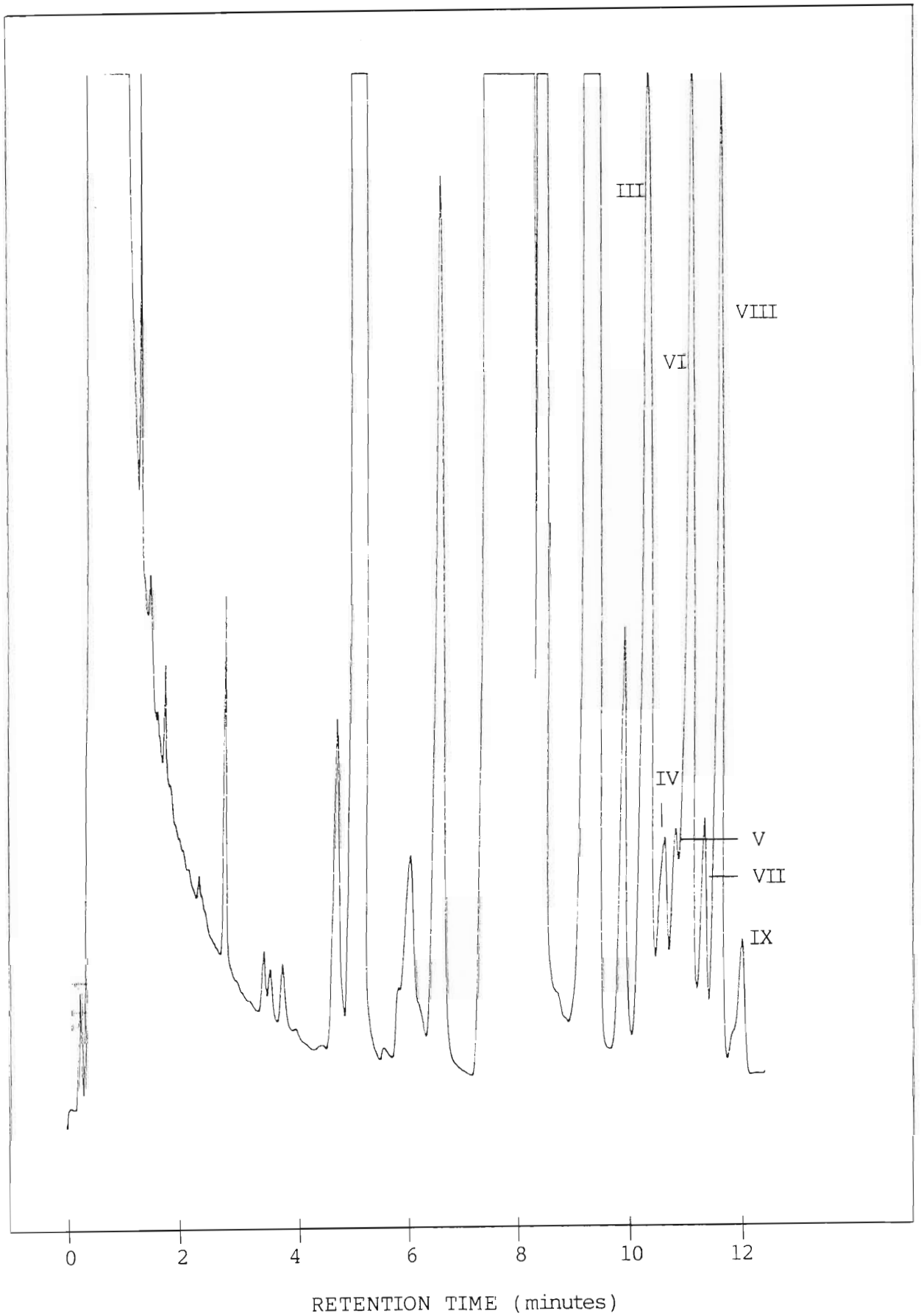
Fig 6.3.1 Gas chromatogram of reduced oil from soya bean
seed cv. Hartebees.

al., 1980). In an attempt to establish the possible mechanisms involved in the oxidation of membranes, Mead et al. (1980) have used model oxidation systems to investigate the autoxidation of lipid monolayers. Results indicate that epoxides are formed as the major product of monolayer oxidation (Mead et al., 1980). It was proposed that these epoxides were formed by the oxidation of adjacent lipid molecules by a peroxy radical (Logani and Davies, 1980). In contrast to bulk oxidation, hydroperoxides did not accumulate, but formed as intermediate products. Instead, the hydroperoxides oxidized other olefins in the monolayer to epoxides, or rearranged to form hydroxy-epoxy compounds (Mead et al., 1980). The above findings contrast with the results of this study, hydroperoxides being present in equivalent amounts to the epoxides (Table 6.3.1).

Such a result suggested that oxidation of soya bean seed lipid had proceeded by a mechanism different to that observed in monolayer oxidation, and may indicate that the autoxidation of lipid in aged seeds may occur predominately in non-membrane lipid. This result is supported by the observation that the polyunsaturated fatty acids from the polar lipid fraction in aged soya bean seeds showed only a very slight decline, while marked changes were observed in

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the total lipid (Hailstones and Smith, 1988; Priestley
and Leopold, 1983). From the above, it may be surmised
that the oxidation of soya bean oil may occur predomi-
nately in the storage or bulk lipid phase.

Spencer et al. (1973) have demonstrated the
accumulation of predominately hydroxy and epoxy
compounds in aged Cichorium intybus L., Crepis
thomsonii Babc. and Crepis vescicaria L. seeds. These
workers also reported the almost exclusive production
of the 9,10-epoxide. It was proposed that lipoxygenase
activity may have been responsible for the selective
formation of the epoxides. They postulate the existance
of a per-epoxide intermediate which may then give rise
to either a hydroperoxide or an epoxide. The equivalent
proportions of epoxy to hydroxy components observed in
this study may support the above mechanism (Table
6.3.1), and might implicate the possible involvement of
lipoxygenases in the oxidation of seed lipids.

From the results of the previous work (chapter 6.1
and 6.2), peaks IV and V were identified as hydroxy
monoenes. As oleate is not a substrate for lipoxyge-
nase, peaks IV and V were possibly derived from
classical free radical-mediated autoxidation. However,
results obtained from all four cultivars revealed the
selective production of the 13-cis, trans-hydroper-

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oxide. In all seeds examined the proportion of the 13-
to the 9-hydroxy peak was greater than 80:20 (Table
6.3.1). This result strongly implicates the involvement
of lipoxygenase.

However, Gardner (1980) has advocated caution in
the implication of lipoxygenase activity purely on the
basis of the relative proportions of the products.
Firstly, autoxidation may occur during lipoxygenation.
Furthermore, rearrangement (isomerisation) of the
hydroperoxides occurs very readily. The presence of a
trans, trans component in the oxidation products from
the seed oils (peak IX, Figure 6.3.1) would support the
occurance of the latter process.

Secondly, it has been observed that oxidations
catalysed by metalloproteins may exhibit marked regio-
selectivity. Chan et al. (1978b) have demonstrated that
in such reactions the 13-carbon of linoleate was
selectively oxidized, giving ratios of 13-hydroper-
oxides to 9-hydroperoxides as high as 70:30. The
identification of specific stereoisomers is therefore
necessary for proof of enzymatic activity. No optical
activity was, however, observed in a partially purified
13-hydroxy fraction from cultivar Hartebees.

However, Hamberg (1971) has shown that non-

specific oxidations can play a role in the formation of hydroperoxides by lipoxygenase. Furthermore, the greater selectivity observed in this study than that reported by Chan et al. (1978b) for metalloproteins may be used to argue in support of lipoxygenase involvement. In addition, lipoxygenase contains nonhaem iron (Gardner, 1980) and therefore would be a 'metalloprotein'.

In a recent study using model oxidation systems, Priestley et al. (1985) have shown that, with the exception of polar lipids of embryonic axes, seed lipids were highly resistant to oxidative degradation provided seed structure was maintained intact. These workers suggested that the degradative changes identified in the lipids of soya beans stored for several years probably resulted from enzymatic or metabolic activities in the stored seed. Brockmann and Acker (1977) have shown that lipoxygenase may give rise to slow lipid peroxidation of model systems at moisture contents very much lower than those usually encountered in dry seeds. The weight of evidence therefore supports the involvement of enzymes (metalloproteins), and in particular lipoxygenase, in the oxidation of seed lipid during ageing.

In conclusion, the results of this study indicate

that the oxidation of seed lipid occured by a mechanism fundamentally different to that of free radical-mediated lipid peroxidation or the autoxidation of monolayers. The production of equivalent amounts of epoxy and hydroxy compounds did not support the autoxidation of membranes during ageing, suggesting that oxidation may be limited to the bulk lipid phase. The selective production of the 13-hydroperoxide indicated the involvement of enzymes (Spencer et al., 1973) or 'metalloproteins' (Chan et al., 1978b). In both cases, lipoxygenase involvement is implicated. The failure to detect optical activity may only be surmised upon. Further work is therefore necessary in order to obtain conclusive proof of lipoxygenase involvement in the oxidation of lipid in aged seeds.

A detailed investigation to positively identify all components involved and to analyze the stereochemistry of the different products would be necessary to resolve this question. However, as lipoxygenases from different species have different selectivities (Gardner, 1980), a simple examination of oxidation products from different seed types may provide an additional line of investigation in order to establish the activity, if any, of this enzyme.

SECTION III

CHAPTER 7

OVERVIEW AND SYNTHESIS

7.1 THE ROLE OF FREE RADICALS IN SOYA BEAN SEED AGEING - A RE-EVALUATION.

7.1.1 FREE RADICALS AND AGEING.

The onset of free-radical chain-reactions is due to the production of the superoxide anion, O_2^- (Halliwell and Gutteridge, 1985), the quinone-semiquinone system being often cited as a possible source (Borg et al., 1978). Other possible sources include enzymes, particularly various oxidases and dehydrogenases, and even direct reduction by ferrous iron, although this is usually regarded as a product rather than a source of the superoxide (Halliwell and Gutteridge, 1985). Free radical reactions are an integral part of normal cellular function such as respiration and photosynthesis, and under physiological conditions no toxicity is observed. This is due to the activity of superoxide dismutase (SOD), catalases and other protective mechanisms, including ascorbate peroxidase and glutathione peroxidase (Halliwell and Gutteridge, 1985). In the event of the absence or failure of such protective mechanisms, the development of cytotoxicity due to the production of the superoxide, and ultimately H_2O_2 and $OH\cdot$, would ensue.

The coupling of lipid peroxidation to these

reactions is accommodated via an initiation reaction by a free radical, usually the OH·. Alternatively, the superoxide radical may reduce molecular iron from the ferric to the ferrous form. This in turn may then catalyse the breakdown of lipid hydroperoxides, thus giving rise to a free radical chain reaction (Halliwell and Gutteridge, 1985). For this reason, lipid hydroperoxides are regarded as one of the main possible sources of cytotoxic products following free radical-mediated lipid peroxidation (Halliwell and Gutteridge, 1985; Esterbauer et al., 1988). Considerable evidence for the role of lipid-peroxidation products in many disease-related effects, as well as its role in irradiated tissues and in vitro peroxidation systems, has accumulated (Halliwell and Gutteridge, 1985).

7.1.2 FREE RADICALS AND SEED AGEING.

The direct application of free radical theory to seeds does pose a number of theoretical problems, the major one of which is the dry state in which seeds persist for protracted periods of time. Many of the classical reactions involving the formation of the superoxide, hydroxy radical and hydrogen peroxide in fully hydrated systems may not be readily implicated in dry seeds.

The state of water in dry seeds.

The state of water in dry seeds is a subject of
continuing interest in seed ageing research. The
moisture content of dry seeds depends very much on the
prevailing storage conditions, but is generally of the
order of less than 15% (Bewley, 1986). Water in seeds
has been described as 'bound' or 'free'. Bound water
may be defined as that which occurs in the vicinity of
large molecules, and whose structural and dynamic
properties therefore differ from that of 'free' water
in the same system (Priestley, 1986). Evidence suggests
that dry seeds possess little or no 'free' water
(Priestley, 1986). For example, ^{1}H-NMR studies of soya
bean cotyledons have demonstrated that 'free' water was
absent below 11% moisture contents (Seewaldt et al.,
1981).

The effects of the dry state on the mobility of
molecular components of seeds has been investigated
using nuclear magnetic resonance (NMR) techniques. ^{13}C-
NMR analysis of soya beans has revealed that the only
freely mobile components in unimbibed seeds were the
triacylglycerols (Schaefer and Stejskal, 1975; Anderson
et al., 1977; Colnago and Seidl, 1983), neutral lipids
that are apparently as undisturbed by the absence of
water as by its presence (Priestley, 1986). In contrast
to this, proteins and carbohydrates are held relatively

rigid (Priestley, 1986). Similar studies on soya by ^{31}P-NMR indicated that compounds such as sugar phosphates and nucleotides are similarly motionless at moisture levels lower than 8% (Priestley and de Kruiff, 1982).

Metabolic activity in dry seeds.

The effects of moisture content on the relative mobility of cellular components is likely to have a profound influence on the rates of reactions (Priestley, 1986). Karel (1980) has demonstrated that in oxidative reactions occuring in dry foods, water acts as an antioxidant at intermediate moisture contents. At higher moisture contents, however, water acts as a pro-oxidant. It is proposed that at this level water plastisizes the matrix, making it permeable to various reactants and catalysts rendered non-reactive at intermediate moisture levels (Karel, 1980). It is interesting in this respect to note that results obtained in this study suggest that moisture contents may exert a similar influence on the rates of lipid oxidation in dry seeds. It would be interesting to determine to what extent the proposed mechanisms operative in dry foods govern the oxidation of seed lipids.

Enzymes would be similarly effected by seed

moisture contents. Priestley (1986) has noted that, with regard to enzymatic activity in dry seeds, activities normally associated with the hydrated states of cells are negligible or non-existant in sorption zone I (below 5% moisture content in soya bean), are very low in sorption zone II (5-10%), and become increasingly active with the onset of zone III (above 10%). Respiratory activity, commonly implicated in free radical production, is neglible to non-existant below zone III in soya beans (Vertucci and Leopold, 1984).

In contrast to this, investigations using dry model systems has indicated that lipolytic activities may be expressed at extremely low moisture levels, the rate limiting factor being the mobility of the substrate (Priestley, 1986). Acker and Wiese (1972) have shown that the activity of oat seed lipase in dehydrated model systems was directly dependent on the fluidity of the triacylglycerol substrate. In a similar study with soya bean lipoxygenase, Brockmann and Acker (1977) have observed slow enzymic oxidation at moisture levels below 5%. The degradation of amphipathic lipids by phospholipases B and D in barley, by contrast, was seen to occur only at higher moisture levels (Acker and Kaiser, 1959), an observation of singular importance with respect to the oft reported decline in phopholipids during rapid ageing.

Free radicals and seed ageing.

Priestley (1986) has stated that evidence for the involvement of free radicals in the ageing of dry material is equivocal. In support of this, the results of the GC analysis of autoxidized seed lipids described in this study did not support the involvement of the classical mechanisms of free radical-mediated autoxidation of polyunsaturated lipid. This contrasts with the present state of seed ageing theory which envisages a steady-state free-radical flux during storage giving rise to a slow, constant attack of lipid and other macromolecules (Wilson and McDonald, 1986b). Molecular oxygen is known to be important in many free-radical generating and propagating reactions (Halliwell and Gutteridge, 1985). However, evidence for a causal role of oxygen in seed ageing is equivocal (Abdalla and Roberts, 1968; Ohlrogge and Kernan, 1982). Priestley et al. (1985a) have stated that evidence currently available suggests that the degradative changes previously identified in the lipids of soya beans stored for several years (Priestley and Leopold, 1983) was probably due to enzymatic or metabolic activities in the stored seeds. The results obtained in this study support such a proposal. The selective production of the 13-hydroperoxide in aged soya bean seeds suggested the involvement of enzymes. Enzymatic oxidation in stored seeds has also been proposed by Spencer et al.

(1973). Alternatively, the catalytic activity of metalloproteins may be involved (Chan et al., 1978b).

In conclusion, the available evidence suggests that seed lipid mobility and the activity of certain lipid enzymes may be little effected by desiccation. Furthermore, from the observation that certain lipid enzymes (lipases, lipoxygenases) may be localized in close proximity to their substrate (Gardner, 1980), it may be surmised that the activity of such enzymes during storage must be regarded as highly probable. The results of this study indicate that oxidation of soya bean seed lipid had occured via a mechanism fundamentally different to free-radical mediated autoxidation of bulk oil. The selectivity evident in the oxidation of the seed lipids would be more consonant with enzymatic or metalloprotein catalytic mechanisms than those associated with bulk oxidation.

7.1.3 MEMBRANES AND SEED AGEING.

Membranes are comprised of a lipid bilayer containing both intrinsic and extrinsic proteins. This bilayer acts as a barrier to the general diffusion of materials into and out of the cell, and it provides the appropriate milieu in which proteins and transmembrane carriers function. Due to the specific structural and

physical properties of phospholipids, the main lipid

component of membranes, the spontaneous formation of

a bilayer results upon contact with bulk water (Bewley,

1986).

Membrane proteins.

Membrane proteins confer characteristic

biochemical properties to a membrane system (Bewley,

1986). Intrinsic proteins are associated with the

hydrophobic interior of the membrane, whereas extrinsic

ones are localized on the membrane-water interface.

Intrinsic proteins can constitute as much as 80% of the

surface area in mitochondria (Bewley, 1986), and are

generally strongly influenced by the fluidity of the

membrane system. Extrinsic proteins, by contrast, are

only superficially associated with the membrane

surface, and are readily released following osmotic

shock (Bewley, 1986).

The state of membranes in dry seeds.

The state of membranes in the dry seeds has been

the subject of some controversy. Simon (1974) and Simon

and Mills (1983) have postulated that membranes may

undergo a major phase transition on desiccation, as

there is insufficient water to maintain the bilamellar

structure. However, the available evidence indicates

that although the membrane may suffer perturbations on

drying (Bewley, 1986), there is no compelling evidence to suggest that the characteristic bilayer organization is lost (Priestley, 1986). Ultrastructural studies on dry soya beans (Chabot and Leopold, 1982) have shown that the membranes of organelles were well defined and intact. The plasmamembrane was often greatly convoluted and withdrawn from the cell wall in places, but no evidence for a major alteration of the basic membrane structure was obtained.

In contrast to this, ultrastructural evidence indicates that an increasing amount of proteins was imbedded in the plasmamembrane of dry soya beans as moisture content increased (Chabot and Leopold, 1983). A similar result has been obtained by Sato and Ashahi (1975) in pea mitochondria. Apparently, when tissue moisture content is low, some membrane proteins detach themselves from the membrane and become dispersed in the cytoplasm (Sato and Asahi, 1975). During imbibition, these proteins return to their former position in the membrane (Sato and Ashahi, 1975). In conclusion, the evidence suggests that membranes may be little altered by drying, whereas membrane proteins are strongly influenced by it. Evidently, membrane proteins are more dependent on interactions with water for structure and function than membrane lipid itself.

Membranes and free radical attack.

Membranes have long been implicated as a primary site of oxidative damage during seed ageing. However, evidence may indicate that in soya bean seeds, the oxidation of membranes may be of minor importance during storage. Firstly, evidence from the analysis of polyunsaturated fatty acids has indicated that the polar fraction suffers only a small degree of oxidation during ageing (Priestley and Leopold, 1983; Hailstones and Smith, 1988). In contrast, the total lipid fraction showed marked changes in the levels of the poly-unsaturates. Secondly, evidence from the analysis of the oxidation of lipid in seeds obtained in this study indicates that oxidation had occured by a different mechanism to that reported for the oxidation of lipid monolayers (Mead et al., 1980). This result suggests that membranes were not the principal site of oxidative activity during storage.

Soya bean seed ageing - a working hypothesis.

Chabot and Leopold (1983) have shown that in dry soya bean seeds, the membranes of the plasmalemma and protein bodies was closely associated with vesicles and lipid bodies within an electron dense matrix (Figure 7.1.1). They have postulated that these lipid bodies may represent lipid that would be incorporated into the

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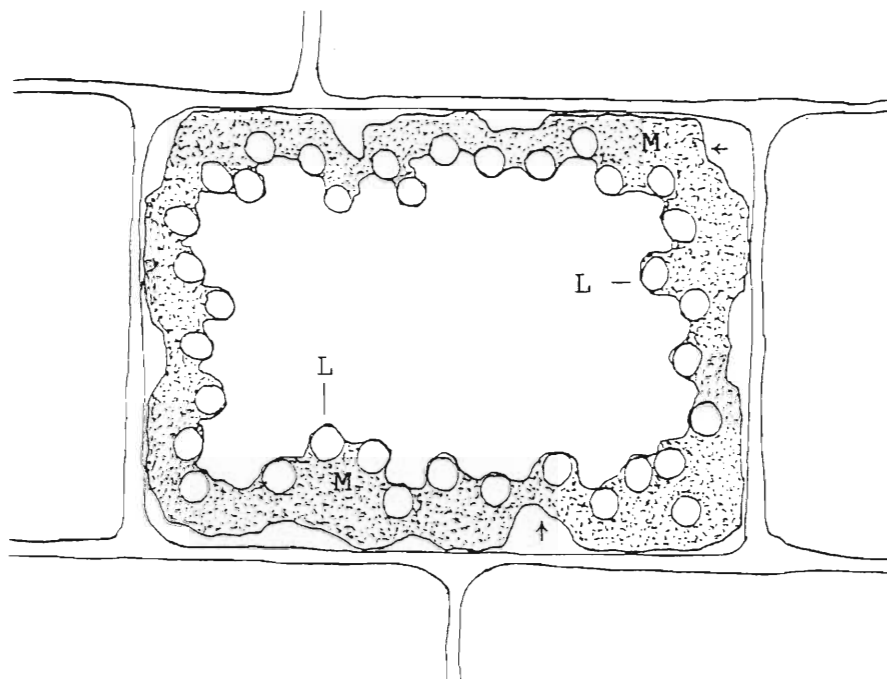
Lipid bodies - L

Electron-dense matrix - M

Plasmamembrane - arrows

A schematic representation of the ultrastructure
of dry soya bean tissue showing the relationship
between the plasmamembrane, lipid bodies and
electron-dense matrix. Adapted from Chabot and
Leopold, 1982.

Figure 7.1.1

membrane upon hydration. On the basis of the results of this study, it is therefore hypothesized that oxidation of lipid in dry storage may be associated primarily with these lipid bodies. Upon hydration, the breakdown of the oxygenated lipid accumulated during storage may give rise to free radical chain reactions within the immediate vicinity of these lipid bodies. The close proximity of other cellular components (electron dense matrix) and of the plasmamembrane would mean that extensive free radical mediated reactions associated with such lipid bodies could lead to widespread damage to both the plasmamembrane and associated macromole-cules. This would be of particular importance with respect to the possible re-incorporation of lipid and proteins into the membrane upon hydration. Such damage would be manifest initally as perturbations of plasma-membrane integrity, leading ultimately to further changes in cellular metabolism (Figure 7.1.2).

7.1.4 IMBIBITIONAL EVENTS IN RELATION TO AGEING.

The oxidative activities in soya bean embryonic axes during early germination has recently been investigated in some depth (Puntarulo et al., 1987). These workers demonstrated that cyanide-sensitive oxygen uptake accounted for the main part of oxygen uptake during the early stages of imbibition. An

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$$LH \xrightarrow{\quad} LOOH \xrightarrow{\quad} LO\cdot$$

with E-Fe above and E-Fe below on the left transition, and M^{n+1} above and M^n below on the right transition.
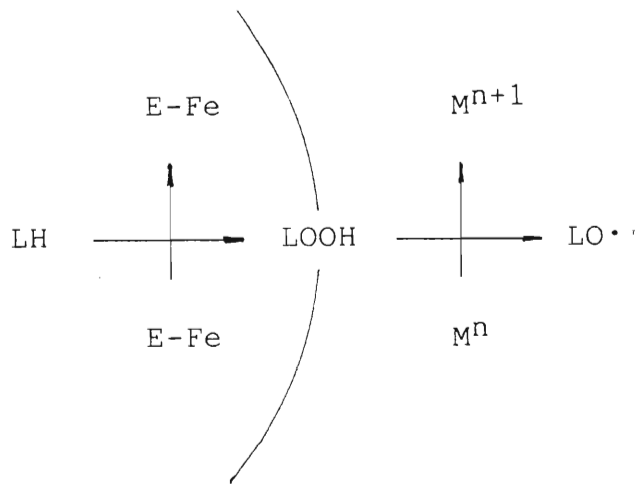
Soya bean seed ageing - a working model.

Enzymatic or metalloprotein (E-Fe) oxidation
of seed lipid, confined predominately to the
lipid bodies during storage, leads to the
accumulation of lipid hydroperoxides (LOOH)
which break down on imbibition due to transition
metal catalysis (M^n), producing lipid (alkoxy)
free radicals (LO·) which in turn catalyse
free radical-mediated lipid peroxidation and
derived reactions, causing extensive disruption
of the processes associated with the recon-
stitution of functional membrane integrity
during early germination.

Fig. 7.1.2.

increase in the cytochrome pathway and in cytochrome oxidase activity without a change in alternate pathway activity was interpreted as indicative of the fact that mitochondria may be immature on imbibition and gain respiratory competency only after several hours of imbibition. Alternate (cyanide resistant) oxygen consumption accounted for only 18% of total oxygen uptake initially, and even declined subsequently to 15%. Lipoxygenase activity was responsible for a significant proportion of oxygen uptake (15-35%). In contrast to this, the production of the superoxide anion and H_2O_2 was responsible for less than 4% of total oxygen uptake.

Free radicals and germination.

Further research by these workers (Puntarulo et al., 1988) revealed that the mitochondria were the primary site of H_2O_2 production, accounting for 0.9 to 1.5% of total oxygen uptake. Catalase was shown to be the major enzyme involved in the removal of the H_2O_2 produced. Gidrol (1989) has shown that in deteriorated (rapidly aged) soya bean axes there was a dramatic increase in SOD activity during early imbibition. Catalase, ascorbate peroxidase and glutathione peroxidase activity were severely reduced or absent in aged material. It was proposed that the increased SOD activity was indicative of an increased level of H_2O_2

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production, and that this may have resulted in the reduction of seed vigour. Such a conclusion contrasts with the results of Puntarulo et al. (1988), who demonstrated that treatment of seeds with aminotria-zole, an irreversable inhibitor of catalase, did not effect axes growth, despite a marked increase in H_2O_2 production (100 mM over against an estimated steady-state level of 5 micromolar). On the basis of the limited evidence available, it may be concluded that although the production of the superoxide and hydroxyl radicals has been shown to be a possible correlate of ageing, a causal link between such radical production and seed vigour had not been convincingly demonstrated.

Lipid peroxidation and germination.

An alternative source of cytotoxicity may be lipid peroxidation. The presence of small amounts of lipid hydroperoxides in seeds is amply attested to in the literature (Priestley, 1986). Increasing levels of hydroperoxides in seeds during storage has been demonstrated in both cabbage and soya bean (Hailstones and Smith, 1988). Breakdown of such lipid hydroperoxides could lead to the initiation of a free-radical chain-reaction. This is supported by the results obtained in this study with respect to changes in peroxide value during early imbibition of axes from aged soya bean seeds and from the invigoration of soya

bean seeds with ferrous ions. The involvement of free
radical mediated damage during early germination has
also be postulated to be a causal factor in the
viability of maize (Berjak et al., 1986) and seeds in
general (Wilson and McDonald, 1986b). The exact
mechanisms involved are still to be conclusively
established, but it seems probable that reducing
substances such as ferrous iron may catalyse such a
reaction in vivo (Halliwell and Gutteridge, 1985).
Alternatively, enzymatic breakdown of the hydroper-
oxides may be involved, giving rise to toxic secondary
products (Wilson and McDonald, 1986b).

Cytotoxic breakdown products.

The identities of the cytotoxic products have not
as yet been established. As alluded to above, the role
of the classical free-radical products, particularly
the hydroxyl radical, is uncertain. It may therefore be
surmised that the breakdown products of lipid
hydroperoxides may be of greater importance. Many of
the products of hydroperoxide breakdown are volatile.
It has been proposed that these compounds, in
particular the aldehydes, may be responsible for the
development of cytotoxic effects (Wilson and McDonald,
1986b). A specific group of aldehydes, the 2-hydroxy
alkenals, have been implicated in the development of
cytotoxicity in animal systems (Esterbauer et al.,

1988). However, although aldehydes have been shown to be highly correlated with seed viability (Woodstock and Taylorson, 1981; Gorecki et al., 1985), the role of aldehyde production in seed vigour is uncertain. Furthermore, results obtained in this study revealed that a reduction in the level of aldehydes was not associated with the invigorating effect of ferrous ions. This result may indicate that aldehydes, at least in general, may not be implicated in the reduction in soya bean seed vigour. The alkoxy radicals, primary products of hydroperoxide breakdown, would therefore seem to be the most probable agent of cytotoxicity. In support of this, evidence obtained from invigoration experiments described in this study suggested that free radical reactions during early imbibition may be causally related to seed vigour.

7.1.5 RÉSUMÉ

It is proposed that soya bean seed lipids are subject to a slow rate of oxidation during long term dry storage. Of particular importance in this regard is the possible involvement of enzymes (lipoxygenase) or metalloproteins (1), and the possible role of protein sulphydral catalysis of lipid oxidation by the provision of the sulphydral radical and reducing species such as ferrous iron (2) (Gardner, 1980).

$$E\text{-}Fe^{+++} + LH \quad \rightarrow \quad E\text{-}Fe^{+++} + LOOH \ldots (1)$$

$$RSH + Fe^{+++} \quad \rightarrow \quad RS\cdot + Fe^{++} \ldots (2)$$
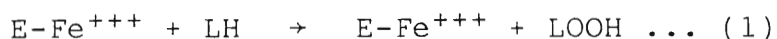
These reactions will lead to a gradual accumulation of lipid hydroperoxides (LOOH) and other co-oxidative injury. On imbibition, the accumulated hydroperoxides are broken down by the co-ordinated action of enzymes such as lipases and lyases (Vick and Zimmerman, 1976) together with peroxidases, rapidly removing any hydroperoxides present (Hailstones and Smith, 1989b). If, however, the accumulated level of hydroperoxides should exceed the ability of the cell to remove them, dismutation, possibly catalysed by transition metals, will lead to the initiation of autoxidation within the lipid milieu. Extensive damage would then ensue, particularly within the lipoprotein domain of the membranes. Furthermore, the release of any alkoxy radical products may extend the level of damage beyond the immediate confines of the membranes. Disruption of membrane integrity may, however, be the single major event in the reduction of seed vigour, as this would have extensive consequences within the cell (Bewley, 1986; Priestley, 1986).

A secondary free-radical mechanism may be induced by general membrane dysfunction, leading to the release

of the superoxide from flavin-linked dehydrogenases, the hydroquinone-semiquinone-quinol system and cytochrome P450. This would result in the formation of H_2O_2, and ultimately the hydroxyl radical. Alternatively, as flavin-linked dehydrogenases cannot reduce O_2 directly, the failure of membrane integrity may lead to the release of the contents of certain microsomes (glyoxysomes), releasing flavin-linked oxidases into the cytoplasm. These are known to reduce O_2 directly to give the superoxide (Lehninger, 1979).

7.1.6 CONCLUSION

The work presented here supports a major role of lipid peroxidation and derived reactions in soya bean seed ageing. Lipid oxidation during storage appears to proceed via a mechanism fundamentally different to that of either bulk or monolayer oxidation. Furthermore, the selective production of the 13-hydroperoxide implies that the oxidative processes are subject to particular mechanistic constraints, implicating the possible involvement of enzymes (lipoxygenase) or metallo-proteins.

The events of early germination were observed to be crucial to seed vigour. In particular, an increase in hydroperoxides and their subsequent dismutation may

give rise to extensive free-radical mediated damage to the plasmamembrane and other subcellular membrane systems. Results suggest that the postulated disruption of membrane integrity may be of fundamental importance during early germination, and have a direct bearing on the establishment of normal cellular function, thus giving rise to the observed decline in vigour of soya bean seeds.

These results do not support the idea of a steady-state free-radical flux during storage or a causal role of aldehydes in the decline in seed vigour. They do, however, support the idea of incipient damage proposed by Wilson and McDonald (1986b). The results are consistent with the reported declines in respiration, and protein synthesis in aged seeds, and indicate a possible link between lipid peroxidation and cell metabolism in general. It was hypothesized that lipid peroxidation, via its effect on membrane integrity and damage to macromolecules other than lipid, could give rise to most, if not all, of the phenomena shown to be associated with declining seed vigour. Lipid peroxidation may therefore constitute the single major cause leading to the decline in soya bean seed vigour.

SECTION IV

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APPENDIX I

Thermally-derived volatile aldehydes in relation to seed viability in soybean seeds

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Summary

The standard germination test is sometimes inadequate in predicting the field performance of soybean seeds and other tests have been developed to assess vigour. Thermally-derived volatiles aldehydres released from dry soybeans (*Glycine max*) were examined for their ability to measure seed vigour. Volatiles identified by gas chromatography as hexanal. pentanal and butanal. when expressed as a percentage of the total volatiles produced. were significantly correlated with seed germination. seed vigour and hydroperoxide levels in the six batches of soybeans investigated. Evidence is presented that the thermal breakdown of lipid hydroperoxides is the source of these volatiles and that lipid peroxidation may be an important determinant in seed ageing.

Résumé

Aldéhydes volatils produits par chauffage. en relation avec la viabilité des graines de soja

Le test standard de germination est parfois insuffisant pour prédire les performances au champ des graines de soja. C'est pourquoi d'autres tests ont été développés pour évaluer la vigueur. On a recherché si les aldéhydes volatils émis par chauffage des graines sèches de soja (*Glycine max*) pouvaient permettre de mesurer la vigueur. Les composés volatils identifiés par chromatographie en phase gazeuse comme étant l'hexanal. le pentanal et le butanal. exprimés en pourcentage des produits volatils totaux émis. étaient significativement corrélés à la germination des graines. à leur vigueur et au taux d'hydroperoxyde chez les six lots de soja étudiés. Il est apporté des preuves que la dégradation thermique des hydroperoxydes lipidiques est la source de ces composés volatils et que la peroxydation des lipides pourrait être un phénomène essentiel dans le vieillissement des graines.

Zusammenfassung

Thermisch entwickelte flüchtige Aldehyde in Beziehung zur Lebensfähigkeit von Samen bei Sojabohnensaatgut

Die Standardkeimfähigkeitsbestimmung ist manchmal für die Vorhersage des Feldaufgangs von Sojabohnensamen unzureichend und andere Prüfmethoden zur Bestimmung der Triebkraft wurden erstellt. Thermisch entwickelte. von trockenen Sojabohnen abgegebene flüchtige Aldehyde wurden auf ihre Brauchbarkeit zur Bestimmung der Triebkraft hin überprüft. Mittels Gaschromatographie wurden die flüchtigen

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Stoffe als Hexanal, Pentanal und Butanal identifiziert. Wurden diese als Prozentsatz der insgesamt flüchtigen Stoffe dargestellt, waren sie bei den sechs untersuchten Sojabohnenproben signifikant mit der Keimfähigkeit, der Samentriebkraft und dem Wasserperoxidgehalt korreliert. Es wird der Nachweis geführt, daß die thermische Zerstörung von Wasserperoxiden der Lipide die Ursache für die flüchtigen Stoffe ist, und daß die Lipidperoxidation bei der Samenalterung einen wichtigen Faktor darstellt.

Introduction

The need to provide an objective assessment of rancidity in vegetable oils has led to the development of tests based on the measurement of volatile lipid oxidation products. Scholz and Ptak (1966) reported a simple and reliable gas chromatographic procedure for measuring the degree of rancidity of cottonseed (*Gossypium hirsutum* L.) oils by monitoring headspace pentane levels.

The application of this approach to seed technology has been a more recent development. Heydanek and McGorrin (1981) characterised volatiles distilled from dry oat (*Avena fatua* L.) groats by gas chromatography-mass spectroscopy as primarily $C_{10}H_{16}$ monoterpenes, alkylbenzenes and hexanal. The suggestions that volatiles, derived from the heating of air-dried seeds, may provide a very sensitive indicator of seed age and potential vigour was first suggested by Fielding and Goldsworthy (1982), who reported that thermally-derived volatiles from dry wheat (*Triticum aestivum* L.) seeds were related to seed vigour. Lipid oxidation products were postulated to be the source of the volatiles, although the identity of the compounds was not determined.

Preliminary studies in our laboratories have shown correlations between the hydroperoxide levels and the amount of volatiles derived on heating air-dry seeds of cabbage (*Brassica oleracea* L.), soybean (*Glycine max* (L.) Merr.) and lettuce (*Lactuca sativa* L.). The objective of this study was to determine the origin and identity of the volatiles produced on heating soybean seeds and to evaluate the relationship between these compounds and seed vigour and hydroperoxide levels.

Materials and methods

Seed material
Soybean seeds (cultivars 'Ibis', 'Impala', 'Pioneer' and 'Hartebees') were obtained from the Summergrain Research Center, Potchefstroom, and stored in airtight containers or over silica gel at $5\,^\circ C$.

Evolution of volatiles
Two grams of ground seed material were placed in 15 ml serum vials and sealed. The sealed vials were then heated at 130 C for one hour and left to stand overnight. Vials were reheated at 130 C for 20 minutes immediately before sampling. Four replicates of 50μl samples from each seed lot were removed from the vials and injected into the gas chromatograph using gas-tight syringes. Aldehydes were identified by injecting 50 μl samples of gas from the headspace of standards of hexanal, pentanal and butanal.

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Gas liquid chromatography
A Varian 3300 gas chromatograph fitted with a flame ionisation detector and a 15 m non-polar (DB1) megabore column was used. Injector and detector temperatures were 250 °C. Column temperature was held at 40 °C for three minutes. ramped to 46 °C at 3 °C/minutes^{-1}. held for 30 seconds and then ramped to 80 °C to drive off any late-eluting compounds. Carrier gas was helium at 6 ml/minutes^{-1}.

Lipid extraction
Lipids were extracted using the solvent system of Khor and Chan (1985). Two grams of ground seed material were extracted with 10 ml of methylene chloride/methanol (2:1. v/v) containing butylated hydroxytoluene (0.1 g l^{-1}) for 15 minutes and centrifuged in a bench top centrifuge at 1500 g for five minutes at room temperature. The solvent was decanted and washed with a quarter volume of aqueous sodium chloride (10 g l^{-1}) and recentrifuged as before. The lower phase was aspirated. dried with anhydrous sodium sulphate and the lipid recovered by evaporation at 35 °C under nitrogen. '

Hydroperoxide determination
Peroxide values were determined using the method of Stine. Harland, Coulter and Jenness (1954). Twenty μl of lipid were dissolved in 0.5 ml methylene chloride/methanol (2/1. v/v). Twenty μl of 0.01 M ferrous chloride were added to 5 ml of the methylene chloride/methanol. followed by 20 μl of the lipid solution and 20 μl of 3M potassium thiocyanate. The reagents were mixed and the absorbance measured against a blank of the reagents at 505 nm. Determinations were done in replicates of four. Using a standard curve of Fe^{3+} concentration against absorbance. absorbance values were converted to micrograms of Fe^{3+} per 10 ml of solvent. Peroxide values. as milli-equivalents O_2 kg^{-1} fat. were then calculated as follows: Peroxide value = concentration Fe^{3+} per 10 ml g^{-1} of fat used × 55.84.

Germination tests
Two replicates of 50 seeds from each seed lot were germinated in 14 cm petri dishes. To prevent damage on imbibition, seeds were germinated in 25 ml of 20% PEG 8000 (Ψ = −0.51 MPa) instead of on filter paper. Seeds were kept in the dark at 24 °C. Percentage germination was monitored daily for four days. From the data. the coefficient of the velocity of germination (CVG) was used to measure seed vigour according to the formula:

$$CVG = \Sigma n \times 100/\Sigma (Dn)$$

where n is the number of seeds germinated on day D after sowing (Kotowski. 1926).

Moisture content
Two replicates of 2 g of whole seed were dried in a convection oven at 130 C for 24 hours. Moisture content was expressed as percentage wet weight.

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Table 1. Mean percentage germination. seed vigour (coefficient of velocity of germination-CVG). peroxide value and moisture content of six cultivars of soybean seeds.

Cultivar	CVG (percentage day⁻¹)	Germina- tion	Peroxide value (m eq. O_2kg^{-1})	Percentage moisture content
Ibis	41.50a[1]	85a	0.000	10.2a
Impala 4028	37.13b	94b	4.92a	10.3a
Impala 4023	36.58b	78a	8.71a	8.4b
Impala 4031	35.23b	78a	14.37b	9.9a
Pioneer	33.13b	62c	20.81c	11.5c
Hartebees	32.70b	42d	18.85c	5.8d
Correlation with moisture content	0.32	0.61	−0.22	
Correlation with peroxide value	−0.96**	−0.80		

[1] means in the column followed by the same letter are not significantly different.
** Significant at the 1% level using Students t-test.

Statistical methods

The significance of difference was determined using the Student's t-test. The Poisson product moment correlation coefficient was used to calculate the correlation coefficients of the data. The significance of the correlation was read off from tables of significance levels for the Poisson correlation coefficient (Garvin. 1986).

Results

Seed viability and lipid hydroperoxides

Both the percentage germination after four days. and seed vigour were highly correlated with peroxide value (r = 0.80 and −0.96. respectively: table 1). In contrast to this. moisture content was poorly correlated with percentage germination. seed vigour and hydroperoxide levels. A closer consideration of the data. however. did reveal a distinct. non-linear relationship between peroxide levels and moisture content. Seed lots having very low or very high moisture contents both had high peroxide values ('Pioneer' and 'Hartebees'. table 1). Seed lots of intermediate moisture contents showed declining and then increasing peroxide values as seed moisture contents increased. This is supported by comparison with the data obtained for the same seed lots three years previously (figure 1). At that time. a significant correlation between peroxide value and percentage germination was obtained (r = 0.88). whereas moisture content was poorly correlated to both of these parameters (r = −0.25 and −0.43. respectively). In general. over the subsequent three years percentage germination declined in all lots except 'Impala 4028' and '4031'. while moisture content increased in all seed lots except 'Impala 4023'. In all seed lots showing a small increase in moisture content. namely 'Impala 4028'. '4031' and 'Ibis'. peroxide values declined. In 'Pioneer' and 'Hartebees'. however. peroxide value increased markedly. As noted above. 'Pioneer' has a particularly high moisture content at 11.5%. while 'Hartebees' has a very

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low moisture content at 5.8%.

Seed vigour and volatiles

A representative gas chromatograph of the volatiles evolved on heating ground seed material is given in figure 2A. On average. 25 peaks were resolved. An initial investigation showed that none of the major peaks bore any relationship to germination (data not shown). However a number of smaller peaks were seen to show a relationship to germination. Three of these were identified as butanal. pentanal and hexanal by co-chromatography with standards. Consequently the rest of the study was limited to an investigation of these compounds.

While the amounts of volatile aldehydes produced by heated seeds were poorly correlated with seed vigour. germination and peroxide value for all three aldehydes (table 2), when expressed as a percentage of the total volatiles produced hexanal. pentanal and butanal were all highly correlated with vigour, germination and peroxide value (table 3). Hexanal showed a highly significant relationship ($r = 0.94$) with germination and a significant relationship with both peroxide levels ($r = 0.85$) and seed vigour ($r = 0.86$). Pentanal was significantly correlated with both seed vigour and hydroperoxide levels ($r = 0.88$ and 0.81. respectively: table 3). Butanal was highly correlated with seed vigour ($r = 0.91$). peroxide value ($r = 0.89$) and germination ($r = 0.87$).
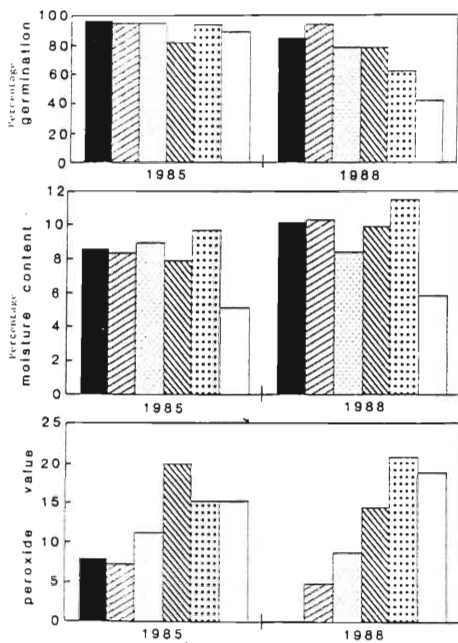

Figure 1. Comparison between the percentage germination. moisture content and peroxide value of the same six lots of soybean seeds in 1985 and 1988.
Key to cultivars: ■ Ibis: ▨ Impala 4028: ☐ Impala 4023: ▧ Impala 4031: ▦ Pioneer: ☐ Hartebees.
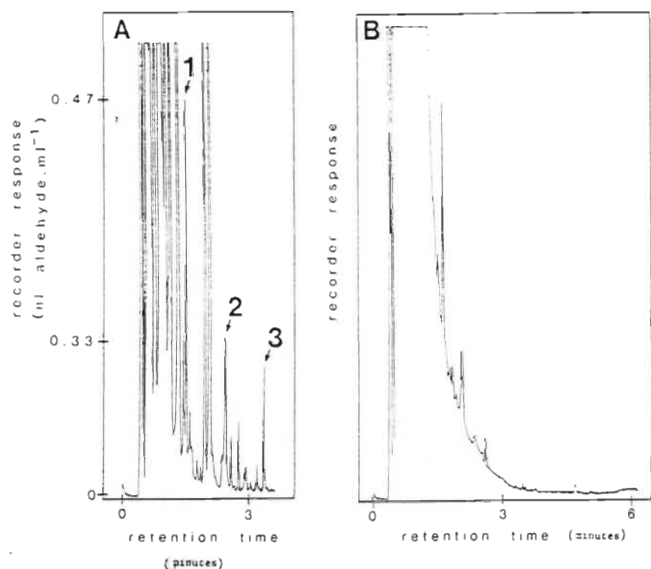
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Figure 2. Gas chromatographic separation of volatiles derived from heating:
(A) ground soybean (cultivar Pioneer) showing peaks co-chromatographing with butanal (1). pentanal (2) and hexanal (3)
(B) soybean powder depleted of lipid.

In order to determine the origin of the volatiles. seed powder freed of oil by solvent extraction was heated and the headspace sampled as above. A comparison of the chromatographs clearly indicated that most of the volatiles were derived from the oil. including the three aldehydes investigated (figure 2B).

Discussion

Spencer. Earle. Wolff and Tallent (1973) have reported an increase in the level of oxygenated acids in the oils of *Cichorium intybus* L.. *Crepis thomsonii* Babc. and *C. vesicaria* L. seeds with storage. while more recently. Radrupal and Basu (1982) demonstrated a highly significant negative correlation between lipid peroxidation and germination in the seeds of wheat (*Triticum aestivum* L.) and mustard (*Brassica juncea* Cross). These results. together with the highly significant correlation of peroxide value and seed vigour in this study. add further support for a role of lipid peroxidation in seed ageing.

In spite of much contradictory data to date. we have additionally obtained evidence for a decline in polyunsaturated fatty acids with decreasing vigour in soybean and cabbage seeds. Furthermore. a very significant correlation was obtained between germination and hydroperoxide levels (r = −0.99) in the cabbage seeds. For these seeds the marked relationship between moisture content and peroxide levels was interpreted

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Table 2. Amount (nl ml⁻¹ of headspace) of butanal, pentanal and hexanal derived on heating six cultivars of soybean seeds.

Cultivar	Butanal	Pentanal	Hexanal
Ibis	0.22a[1]	0.135a	0.121a
Impala 4028	0.24a	0.16a	0.13a
Impala 4023	0.16a	0.12b	0.15a
Impala 4031	0.21a	0.13a	0.13a
Pioneer	0.37b	0.24a	0.30b
Hartebees	0.23a	0.16a	0.19c
Correlation with germination	−0.34	−0.35	−0.58
Correlation with peroxide value	0.55	0.57	0.76
Correlation with seed vigour	−0.44	−0.52	−0.68

[1] Means followed by the same letter are not significantly different.

Table 3. Percentage area of butanal, pentanal and hexanal derived on heating six cultivars of soya bean.

Cultivar	Percentage Area		
	Butanal	Pentanal	Hexanal
Ibis	0.38a[1]	0.22a	0.20a
Impala 4028	0.54b	0.41b	0.35b
Impala 4023	0.90b	0.66b	0.48b
Impala 4031	0.95b	0.54b	0.38b
Pioneer	0.98b	0.60b	0.78c
Hartebees	1.32b	0.80b	0.04c
Correlation with germination	−0.87*	−0.78	−0.94**
Correlation with peroxide value	0.89*	0.81*	0.85*
Correlation with seed vigour	−0.91*	−0.88*	−0.86*

[1] Values in the column followed by the same letter are not significantly different.
 * significant at the 5% level using Student t-test.
** significant at the 1% level using Student t-test.

as indicating that increasing moisture contents may lead to increasing levels of peroxidation. On the other hand no support for this proposal was evident from an examination of the results obtained from the same seed lots of soybean seeds, either in 1985, or in the present study (figure 1).

Wilson and McDonald (1986) have suggested a lipid peroxidation model for seed ageing in which a steady-state free radical flux leads to a constant increase in hydroperoxide levels, in proportion to the age of the seeds. Our results suggest that while this might be so for cabbage seeds, it was not the case for soybean seeds. It may be necessary to invoke a more dynamic relationship between the level of peroxidation and storage conditions.

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If the rate of hydroperoxide formation should exceed the rate of breakdown (as may occur at high moisture content) or if such breakdown is reduced. or should not occur (as may take place at low temperatures). then hydroperoxides will accumulate. If factors within the seed should favour either an increased rate of breakdown (as may occur at high moisture content and temperatures). or a decreased rate of formation (as may take place at low temperatures). seed hydroperoxide levels will decline.
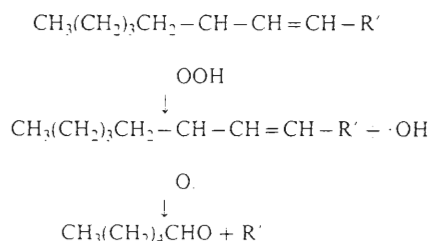
Furthermore. since peroxidation is autocatalytic. prematurational effects and early storage history may predispose the seed to later deterioration once conditions arise which accelerate peroxidation. The controlled deterioration tests which have been developed (Powell and Matthews. 1981) may be an empirical approach for bringing out such latent defects.

The present study has provided evidence that the volatile aldehydes observed were derived from the thermal breakdown of lipid hydroperoxides. That lipid hydroperoxides can produce such volatiles has been firmly established. Esterbauer (1982) and Frankel (1982) have both reported the production of predominantly alkanal and related compounds from oxidised oleic. linoleic and linolenic acids.

Hoffman (1962) has identified hexanal as one of the cleavage products from oxidised soybean oil and Frankel (1982) reported the evolution of hexanal from heated oxidised linoleate.

In seeds. Heydanek and McGorrin (1981) reported the evolution of hexanal from oats (*Avena sativa* L.) groats. while Wilson and McDonald (1986) have suggested that hexanal appears to be ubiquitous in aged seeds. and its measurement may be useful as an index of lipid peroxidation. We have recently shown a relationship between seed hydroperoxide levels and the amount of butanal. pentanal and hexanal released from heated lettuce (*Lactuca sativa* L.) seeds.

The 13-hydroperoxide of linoleate is considered to be the source of hexanal from heated oxidised linoleate (Frankel. 1982). It is thought to be formed by homolytic cleavage of the hydroperoxide. followed by unimolecular decomposition of the alkoxy radical formed. to yield hexanal:

$$CH_3(CH_2)_3CH_2 - CH - CH = CH - R'$$

$$\begin{array}{c} OOH \\ | \\ CH_3(CH_2)_3CH_2 - CH - CH = CH - R' - \cdot OH \end{array}$$

$$\begin{array}{c} O \\ | \\ CH_3(CH_2)_4CHO + R' \end{array}$$

Hexanal formation from the 13-hydroperoxide of linoleate. R' denotes carboxylic end of the fatty acid.

In contrast to hexanal. pentanal production is not well documented in the literature. Hoffmann (1962) identified pentanal in the volatile fraction from oxidised soybean oil and. on theoretical grounds. suggested that it may be formed from a 14-hydroperoxy octadecadienoate. Although 14 hydroperoxy fatty acids have not been noted in

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model oxidation studies of lipids (Frankel. 1980). we have detected pentanal in the headspace of heated. solvent extracted lipids of lettuce seeds. The production of butanal as a product of free radical attack on 3-hexenal. itself a minor breakdown product of linolenate oxidation. has been suggested (Frankel. 1982). A possible mechanism is illustrated below:

$$CH_3-CH_2-CH=CH-CH_2-CHO + O_2 + 2RH$$

$$CH_3-CH_2-CH_2-\overset{\cdot}{C}H-CH_2-CHO$$

$$OOH$$

$$CH_3(CH_2)_2CHO + \cdot CH_2CHO$$
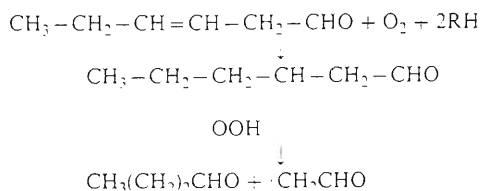
Proposed mechanism for the formation of butanal from 3-hexenal. RH denotes molecule with abstractable hydrogen. such as an unsaturated fatty acid.

The significant correlations obtained between volatile aldehydes. germination percentage and seed vigour indicate a definite relationship between lipid peroxidation and seed vigour: furthermore volatile headspace sampling of heated seeds may offer the seed technologist a technique of considerable diagnostic potential.

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APPENDIX II

Increased lipid peroxidation during the imbibition of deteriorated seed and the invigorating effect of ferrous ions

In the seeds of both soyabean (*Glycine max* L.) and cabbage (*Brassica oleracea* L.), a change was noted in lipid peroxide values over the first hour of imbibition. Peroxides declined rapidly in seeds of high viability, whereas in seeds of lower viability a marked increase in lipid peroxidation was observed 30 minutes after imbibition. A significant invigoration of seeds was observed following treatment with aqueous solutions of ferrous sulphate or ferrous chloride, possibly as a consequence of the quenching of free-radical reactions. The results are discussed in relation to current theories of seed ageing, the role of antioxidants and the invigoration of seeds by hydration-dehydration treatments.

It has been proposed that damage to membranes, enzymes and the genome during the ageing of seeds in dry storage may be the result of free-radical peroxidation reactions initiated by lipid autoxidation.[1-3] With regard to the decline in seed viability, it is not clear whether the critical damage occurs during storage or on imbibition. Thus it is possible that damage accumulates in proportion to the duration and conditions of storage. On imbibition, repair processes may be activated, rectifying any damage that would have occurred. The greater the amount of damage, the longer the time required to effect repair, leading to an increased lag period before the onset of germination, as is typically observed in deteriorating seeds. If, however, the damage should exceed the repair capability of the seed, it will fail to germinate.[2] Alternatively, it is possible that the toxic products of lipid peroxidation which are released or formed on imbibition lead to reduced viability and the eventual failure to germinate. It is argued that the invigorating effects obtained by the hydration-dehydration treatment of aged seeds are too short for metabolic repair to take place and that any beneficial effects of such treatments are likely to be the result of the removal of toxic substances.[3] The provision of a static electric charge during and after rapid ageing was shown to improve the viability of maize (*Zea mays* L.) seeds.[4] This treatment also increased the percentage germination of seeds not subjected to ageing. It was suggested that this was due to the provision of electrons by the static charge, which quenched any free radicals produced, and thus inhibited lipid peroxidation.

In this study we report on lipid peroxidation during early imbibition in seeds of soyabean and cabbage, since this would be helpful in clarifying whether lipid peroxidation itself, or its products, are implicated in seed deterioration. In addition, we investigated the effect of ferrous ions, as a chemical source of electrons, and hence a means of quenching free-radical reactions, on the germination of seeds.

Materials and methods

Seed material. Cabbage seeds (*Brassica oleracea* L.) were obtained from Mayford Seeds in sealed tins during 1985-86 and stored hermetically under ambient laboratory conditions (20 – 24°C). With the exception of cabbage seeds of cv. Savoy Perfection lot B, which were specifically provided by the supplier in response to a request for low viability seed, all seeds were from currently marketed stocks.

Soyabean seeds (*Glycine max* L.) were obtained from the Summergrain Research Centre, Potchefstroom, and stored in hermetically sealed containers at 5°C.

With the exception of cv. Duiker, which were purchased two years previously, all soyabean seeds were drawn from the 1985 stocks used for cultivar evaluation trials. The relationship between moisture contents, peroxide values, fatty acid levels and seed viability have been previously reported.[5]

Germination tests. In the case of cabbage, four replicates of

100 seeds were germinated in 9-cm Petri dishes lined with two sheets of Whatman No. 1 filter paper and moistened with 5 ml water. Percentage radicle emergence was recorded over four days. For soya, four replicates of 50 seeds were germinated in 14-cm Petri dishes. To prevent imbibitional damage, the seeds were moistened with 20 ml of 20% PEG 8000 (ψ = −0.51 MPa). Percentage radicle emergence was recorded over four days. The coefficient of the velocity of germination was calculated using the formula

$$CVG = \Sigma n / \Sigma (Dn) \times \%G,$$

where n is the number of seeds germinated on day D after commencement of imbibition.[6]

Determination of peroxide value on imbibition. One gram of cabbage seeds and 40 isolated soybean axes were imbibed in 9-cm Petri dishes as described above. At 0.5 hour and 1 hour, the seed tissue was extracted as described below.

Lipid extraction. To extract seed lipids, samples were ground in a mortar and pestle with 10 ml methylene chloride:methanol (2:1 v/v) containing 0.01% butylated hydroxy toluene.[7] After centrifugation at 1500 × g in a benchtop centrifuge, the solvent was washed with a quarter volume of 1% NaCl, recentrifuged, and the lower phase dried under nitrogen at 35°C.

Hydroperoxide determination. Peroxide values were determined by the ferrous thiocyanate method.[8] Twenty microlitres of 0.021 M ferrous chloride was added to 5 ml methylene chloride:methanol, together with an aliquot of lipid and 20 μl of 3 M potassium thiocyanate. The reagents were mixed and the absorbance measured against a blank of the reagents at 505 nm. Using a standard curve of Fe^{3+} concentration against absorbance, absorbance values were converted to micrograms of Fe^{3+} per 10 ml solvent. Peroxide value, as milliequivalents O_2 per kg fat, were then calculated as follows: Peroxide value = conc. Fe^{3+} per 10 ml per gram of fat × 55.84.

Chemical treatment. Seeds were germinated as above in solutions of ferrous chloride and ferrous sulphate of different molarities.

Statistical methods. Significant differences were determined using the Student's *t*-test.

Results

Cabbage seeds of high viability showed a decline in peroxide value over the first hour of imbibition (Table 1). In seeds of intermediate viability (73% germination), no change was observed at 0.5 h, and the peroxide value fell significantly after 1 h. Seeds of low viability showed a significant increase in peroxide levels at 0.5 h, followed by a significant decline at 1 h. A similar trend

Table 1. Changes in lipid peroxides in seeds of cabbage and soyabean over the first hour of imbibition.

	Percentage germination	Peroxide value		
		0 h	0.5 h	1 h
Cabbage				
cv. Golden Acre	100	10.4	8.5	7
cv. Glory of Enkhuizen	98	11.0	10.3	9.8
cv. Savoy Perfection Lot A	73	14.7	15.1	9.8*
cv. Savoy Perfection Lot B	39	16.8	25.1*	16.2*
Soya				
cv. Ibis	97	5.2	6.4	1.5
cv. Impala Lot A	95	5.2	7.5	1.7
cv. Impala Lot B	95	10.7	43.0**	12.8**
cv. Duiker	82	59.0	64.3	26.1*

* Significantly different from control at the 5% level.
** Significantly different from control at the 1% level.

was observed for soyabean axes. Seeds of high viability showed no clear change at 0.5 h, but peroxide values had declined by 1 h (Table 1). The peroxide value of seeds of low viability rose significantly at 0.5 h, then fell significantly at 1 h.

A comparison between seed lots A and B of soyabean (cv. Impala), 95% of which germinated, revealed marked changes over time. An initial twofold difference between peroxide levels in the dry state was increased to sixfold at 0.5 h. This contrasts with a greater than fivefold difference between lot B and cv. Duiker in the dry state and a less than twofold difference at 0.5 h after imbibition.

Treatment of cabbage seeds of low viability (cv. Savoy Perfection) with concentrations of 0.01 M and 0.04 M ferrous chloride did not greatly affect the pattern of invigoration, although there were changes in the levels of significance when compared to the control.

On the other hand, 0.02 M ferrous chloride increased both the coefficient of velocity of germination and the percentage germination after four days (Table 2). Treatment of cabbage seeds with a 0.02 M or 0.04 M solution of ferrous sulphate also resulted in a significant increase in germination after four days, and a highly significant (at the 0.1% level) increase in CVG. These same trends were observed with soyabean seeds treated with 0.04 M ferrous sulphate (Table 3). In one experiment, a significant decline in both germination and CVG was observed when seeds of cv. Pioneer were exposed to a 0.005 M solution of ferrous sulphate.

Discussion

These observations might suggest that one possible reason for the invigoration of aged wheat seeds[9] and the reduction in membrane damage of maize seeds,[10] when given hydration-dehydration treatments, is a reduction in the lipid hydroperoxide status of tissues. Lettuce seeds of differing viability have been shown to undergo transient changes in lipid peroxidation, the levels being maximal between two and four hours after imbibition and thereafter declining to values seen in dry seeds by 15 hours post-imbibition (Smith, unpublished).

A progressive decline has been observed in the levels of malondialdehyde, a putative end-product of lipid peroxidation, during the first four hours of imbibition in both aged and unaged soyabean seeds.[11]

Such transient changes in hydroperoxide levels may be part of the process of restitution taking place during imbibition, and also of the proposed cellular repair process.[2] We would further like to suggest that the transient increase in hydroperoxide levels may be due to the decomposition of the hydroperoxides, since their dismutation, possibly by metallo-proteins, could lead to the production of free radicals that would enhance lipid autoxidation if not quenched.[3]

The results of this study support the idea of incipient damage on imbibition, rather than just a build-up in dry storage. The invigorating effect of ferrous salts is consistent with such a proposal, and may be explained by the fact that the pro-oxidant

Table 3. The influence of ferrous sulphate on the germination of two cultivars of soyabean seeds.

| | | Percentage germination | | | |
| | | cv. Pioneer | | cv. Hartebees | |
Days of imbibition	Control	Molarity Fe^{2+} 0.005	0.04	Control	Molarity Fe^{2+} 0.04
1	0	0	0	0	0
2	25	4	46 (17.09)	28	44 (16.78)
3	37	25	71 (10.06)	42	58 (13.71)
4	45	33	75 (8.44)	50	64 (13.32)
CVG	17.4	11.5	30.4 (5.30)	19.3	26.6 (6.20)

Values in parenthesis denote least significant difference at $P = 0.05$

activity of heavy metals can revert to antioxidant activity at high concentration.[12] In such a case, any free radicals produced may be quenched by the reaction:

$$R^{\cdot} + Fe^{2+} \rightarrow R^{-} + Fe^{3+}.$$

Any further damage is therefore prevented, resulting in improved germination. The observation that a 0.005 M solution of ferrous sulphate reduced seed viability (Table 3) may presumably be explained by iron acting as a pro-oxidant. Support for a possible quenching action of ferrous ions has recently been forthcoming from the observed reduction in lipid peroxides when seeds of cabbage and soyabean were imbibed in ferrous salts (manuscript in preparation).

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Table 2. The influence of various molarities of ferrous ion on the germination of cabbage seeds, cv. Savoy Perfection.

| | | Percentage germination | | | | |
| | | $FeCl_2$ molarity | | | $FeSO_4$ molarity | |
Days of imbibition	Control	0.01	0.02	0.04	0.02	0.04
1	0	5	0	5	7	6 (3.00)
2	7	22	25	17	28	25 (5.10)
3	13	32	34	37	34	36 (9.54)
4	16	38	36	41	40	41 (8.89)
CVG	5.7	15.2	15.0	15.8	17.5	17.5 (3.761)

Values in parenthesis denote least significant difference at $P = 0.05$