The involvement of short-chain fatty acids in the control of thermodormancy and germination in *Lactuca sativa* L. cv. 'Grand Rapids'

A Thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

by

Robert Russell Cameron Stewart

September 1978

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Abbreviations

A	-	the hyperfine splitting constant.
Axx	-	the hyperfine splitting constant with the magnetic field oriented along the primary x - axis = 600 µtesla.
Ayy	-	the hyperfine splitting constant with the magnetic field oriented along the primary y - axis = 600 µtesla.
Azz	-	the hyperfine splitting constant with the magnetic field oriented along the primary z - axis = 3.2 mtesla.
$A_{\parallel}$	-	the hyperfine splitting constant with the magnetic field oriented parallel to the membrane normal.
$A_{\perp}$	-	the hyperfine splitting constant with the magnetic field oriented perpendicular to the membrane normal.
ABA	-	abscisic acid.
Bq	-	becquerel.
°C	-	degrees Celsius (0°C = 273.15K).
CCCP	-	carbonyl cyanide m-chlorophenylhydrazone.
cv.	-	cultivar.
E.P.R.	-	electron paramagnetic resonance.
FID	-	flame ionisation detector.
FR	-	Far red light (wavelength about 730 nm).
g	-	gram.
g	-	a measure of the absolute magnetic field position of an E.P.R. spectrum.
GA	-	gibberellin.
GA_{4+7}	-	a mixture of gibberellin A_4 and gibberellin A_7.
GC-MS	-	gas chromatography-mass spectrometry.
G.L.C.	-	gas-liquid chromatography.
G_{50}	-	that dose which reduces germination to 50% of the corresponding control.

h - hour.

h - Planck's constant.

Ho - applied magnetic field.

Hz - hertz - cycles per second.

$H_{\parallel}$ - magnetic field parallel to membrane normal.

$H_{\perp}$ - magnetic field perpendicular to membrane normal.

IAA - indole-3-yl-acetic acid.

J - joule.

lettuce seed - botanically fruits.

m - metre.

M.E.S. - 2(N-morpholino)ethanesulfonic acid.

M.I.M. - multiple ion monitoring.

mol - mole.

$NADH+H^{+}$ - reduced nicotinamide adenine di-nucleotide.

P - phytochrome.

Pfr - phytochrome, far-red absorbing form.

P.L. - percentage leakage

$P.L._{17}$ - that dose which brings about a P.L. value of 17.

Pr - phytochrome, red absorbing form.

RL - red light (wavelength about 660 nm).

S - the order parameter.

W - watt.

μ - the magnetic moment.

μ_z - the component of μ along the direction of Ho.

ν - frequency.

5-doxyl stearate - the 4',4'dimethyl-oxazolidone-N-oxyl derivative of 5-ketostearic acid.

16-doxyl stearate - the 4',4'dimethyl-oxazolidone-N-oxyl derivative of 16-ketostearic acid.

Acknowledgements

I should like to thank:

My supervisor, Dr A.M.M. Berrie, for his assistance and guidance during the period of this study.

Professor M.B. Wilkins, for providing facilities in his department.

Dr R.J. Cogdell, for an introduction to the preparation and use of liposomes.

Dr A. L. Porte, of the Chemistry Department, for his advice, use of E.P.R. spectrometer and for useful discussion of the results thereof.

Dr V. B. Math, for mass spectrographs.

Ms. L. Hazard, for typing this thesis (in record time).

Mr T.N. Tait, for the reproduction of the photograph herein, and for many other occasions.

My parents, for the sacrifices they made throughout my education.

Mary, who was always there.

S U M M A R Y

Short-chain fatty acids (chain length 6-9) were found to be inhibitory to lettuce seed germination. This inhibition was affected by the imbibition temperature in a particular manner. When the G_{50} dose of each short-chain fatty acid was calculated, the G_{50} dose being that dose which reduced germination to 50% of the corresponding control, and plotted against temperature they were found to be linearly related. As the imbibition temperature increased, the dose required to reduce germination to 50% of the control was reduced. Nonanoic acid was the most effective in reducing lettuce seed germination of the fatty acids tested, while hexanoic acid was the least so. All four lines were found to extrapolate to zero dose around 36.5°C. This temperature corresponds to that which has been reported as being a thermal block to germination (Dunlap and Morgan, 1976).

When the gradients of the G_{50} dose against temperature lines were plotted against chain length, these were also found to be linearly related. It is thus possible to predict the G_{50} dose for a short-chain fatty acid (of chain length 6-9) at any temperature in the range 12-28°C from the formula:

$$G_{50} = \frac{T - 36.5}{-2.32C + 10.61} \quad \text{mol m}^{-3}$$

where T = temperature in °C

C = chain length of the fatty acid.

The G_{50} was also affected by the phytochrome state. A high level of Pfr increased the G_{50} dose and a high level of Pr reduced it. High levels of GA_{4+7} (100 mmol m^{-3}) also

increased the dose required, while 10 mmol m^{-3} GA_{4+7} and kinetin (10 and 100 mmol m^{-3}) had no effect upon the G_{50} dose.

Liposomes were prepared so that they contained KCl within the vesicles and NaCl in the surrounding medium. When used along with the specific potassium ionophore, valinomycin, these liposomes may be utilised to determine the proton permeability of the liposome bilayer. This was performed at a range of temperatures for liposomes made from lecithin only. By intrapolation, it was estimated that at 36°C, these control liposomes would have a PL value (a measure of the proton permeability) of 17.

When short-chain fatty acids were incorporated into the liposomes, these were found to increase the proton permeability of the bilayer. The PL_{17} dose of each short-chain fatty acid was calculated at each of 20, 25, 30 and 35°C (the PL_{17} dose is that dose which brings about a PL value of 17 at the temperature; it is derived in an analogous manner to the G_{50} dose). When the PL_{17} doses for short-chain fatty acids (C_6-C_9) were plotted against temperature, these were found to be linearly related, also the four lines extrapolated to zero in the region 35-38°C.

Rothman (1973) has shown that when a membrane undergoes the thermotropic phase transition and the "stearoyl" residues become more fluid, only the distal portions of the chains increase their mobility. The first 6-9 carbon atoms nearest the glycerol backbone are held tightly in the all-trans configuration, even in the liquid-crystal phase. Since there is a similarity between this non-cooperative chain length and the active chain lengths in this study, it was hypothesised that the short-chain fatty acids were becoming intercalated between the lipid molecules and would therefore increase the mobility of the distal portions of the

lipid chains.

The incorporation of nonanoic acid into lecithin bilayers increased the mobility of a 16-doxyl stearic acid analogue, but did not increase the mobility of a 5-doxyl stearic acid analogue. It would thus appear that short-chain fatty acids can become incorporated into a lipid bilayer and increase its fluidity.

There is evidence to suggest that short-chain fatty acids occur in lettuce seeds. However the endogenous levels could not be correlated with the physiological state of the seed.

The hypothesis is presented that, even if short-chain fatty acids do not play a regulatory role *in vivo*, the control of thermodormancy may lie at the level of membrane fluidity, possibly acting *via* membrane-associated enzymes.

PUBLICATION:

The effect of temperature on the short-chain fatty acid induced inhibition of lettuce seed germination. by R. Stewart and A. Berrie, Plant Physiology, in press.

INTRODUCTION

Seed germination has long been studied by plant physiologists and while there is available a wealth of literature on the subject, there is no concrete evidence for the involvement of any of the known plant growth regulators in the control of germination. There are several reviews of seed germination available (e.g. Taylorson and Hendricks, 1977; Mayer and Poljakoff-Mayber, 1975; Mayer and Shain, 1974) and the reader is referred to these. Lettuce seed germination in particular has been the subject of considerable research and I will review some of the data available because of the direct relevance to the topic of this thesis.

Before proceeding, it would be useful to define germination and dormancy. Germination, in lay-man's terms, is taken to mean the onset of growth resulting in the production of a seedling. Seed physiologists, however, generally consider germination to be those changes which take place in a seed after imbibition but prior to the onset of growth (Evenari, 1949). These changes are reversible (Berrie and Drennan, 1971) while the subsequent growth is not. Having stated this, we nevertheless score germination as having taken place when the radicle protrudes from the covering. Obviously any process which stops the early post-germination growth might be scored as nil germination. However, the speed and ease with which germination can be scored in this manner makes possible the trial of many more treatments than would be logistically feasible if using a more time consuming biochemical score system e.g. polysome formation.

The dormant condition is considered to exist when the environmental conditions appear to be suitable for germination, but it does not take place.

Lettuce seeds (c.v. Grand Rapids) exhibit a type of dormancy which is neither universal nor unique. This is manifest in germination being maximal at temperatures up to 25°C, but above this critical temperature germination is reduced and approaches zero at 30°C (e.g. Berrie, 1966). This type of dormancy is called thermodormancy.

Above the critical temperature germination is promoted by electromagnetic radiation of wavelength around 660 nm (red light). Since the seeds respond positively to light they are termed positively photoblastic. This promotion of germination by red light may be reversed on irradiation with wavelength around 730 nm (far red light) given immediately after the red light (Borthwicks et al., 1952). These red/far red cycles may be repeated many times, germination being dependant on the irradiation given last. This is a classic manifestation of a phytochrome mediated response (Hendricks, 1960).

PHYTOCHROME AND LETTUCE SEED GERMINATION

Phytochrome is a chromoprotein and it exists in two photoconvertible states designated Pr (red absorbing form) and Pfr. (far red absorbing form). Thus,

$$Pr \underset{\text{Far red}}{\overset{\text{Red light}}{\rightleftharpoons}} Pfr.$$

There are several intermediates for both pathways, differing in the forward and reverse reactions (Smith, 1975). It has been suggested that Pfr is the active form leading to a promotive event. While this is generally accepted, it is equally valid to suggest that Pr is active interfering with the promotive event. There are several theories as to the action of phytochrome; these include gene de-repression (Mohr, 1966), enzyme activation (Tezuka and Yamamoto, 1974), and phytochrome-induced changes in membrane properties (Hendricks and Borthwicks, 1967).

There is considerable evidence to suggest that phytochrome is associated with membranes. However, the exact role of phytochrome in lettuce seed germination is unclear. Most of the work regarding phytochrome effects has centred on an alteration of endogenous hormone levels after a light treatment. The most commonly studied growth regulator is GA. While it is attractive to suggest that since GA promotes germination, a red light treatment may lead to an increase in the GA content (c.f. Reid et al., 1968), there is little evidence to suggest that this is so. Kohler (1962) and Slabnik (1977) have both reported increased GA-like activity after a red light treatment. However, there is more substantial evidence against a red-light induced increase in GA levels, e.g. Vidaver and Hsiao (1973) reported that red light induced an increase in germination after 2 days dark storage, while GA had no effect: Negbi (1968) showed that continuous far-red irradiation stopped germination despite the presence of GA: Bewley et al (1968) showed that red light and GA interact synergistically, not additively as would be expected if red light were causing GA synthesis. It would appear, therefore, that red light treatment does not lead to an increase in GA content of lettuce seeds as it does in other systems, e.g. Reid et al (1968).

To return to the promotion of lettuce seed germination, it has been noted that there is considerable variation within one batch of seeds as regards germination in the temperature range 25 - 28°C. It is thought that this is due to variability in the amount of Pfr present in the dry seed. This level is fixed during the drying phase of seed maturation and is affected by the degree of shading of the mother plant. Leaf canopies absorb relatively more red light than far red, and will thus alter the RL/FR ratio resulting in an alteration of the photo-stationary state. It has been shown that alterations in the environment of the mother plant can profoundly alter the development of the seed

(e.g. Koller, 1962; Shropshire, 1973; Eenink, 1977). Thus a hostile maternal environment may deepen the dormancy of the progeny. It is intuitively obvious why this should be ecologically advantageous, i.e. an environment hostile to the mother plant, due to shading, water stress etc., is unlikely to be suitable for seedling development, and a resulting dormancy may allow time for the climatic conditions to become more favourable.

GERMINATION AND GROWTH REGULATORS

Much work has been performed and a large amount of data is available on the subject of the effects of plant growth regulators on the germination of lettuce seeds. However, this has taken our understanding of the natural control mechanism little further forward. The effects of the various growth regulators have been reviewed (Mayer and Shain, 1974). It would be useful, however, to mention briefly the effects of the major growth regulators.

GA promotes the germination of lettuce seeds (Khan et al., 1957; Ikuma and Thimann, 1960). This has been shown on many occasions, but all attempts to show an increase in GA content correlated with the breaking of dormancy have failed.

There has been little study of the involvement of auxins in the control of thermodormancy. Robertson et al (1976) reported that IAA inhibited lettuce seed germination and that this inhibition could be relieved by application of GA's and cytokinins. However, they could find no correlation between IAA levels and the control of thermodormancy.

Exogenous applications of cytokinins have been reported to promote lettuce seed germination (e.g. Rao et al, 1976). Baryilae and Mayer (1968) claimed that they isolated cytokinins from germinating seeds, but could find none in non-germinating seeds. In contrast, Van Staden (1973) isolated unidentified cytokinins from non-germinating seeds

and claimed that a stimulus for germination (GA or red light) caused interconversion of these cytokinins. Khan has proposed a hypothesis that germination is controlled by an interplay of phytochrome, cytokinins and a natural inhibitor (probably ABA) (Khan, 1967(a), 1967(b), 1968), culminating in a proposal that cytokinins have a "permissive" role, only relieving an inhibitory block, and that GA is necessary for the germination process itself. It has been counter-claimed that cytokinin induced germination is abnormal and is an artefact due to swelling of the cotyledons, leading to rupture of the pericarp. As stated earlier, attempts to follow endogenous levels of cytokinins have yielded conflicting results. It must therefore be concluded that we cannot, at present, propose any definite role for cytokinins in the control of thermodormancy.

ABA will reduce the germination of lettuce seeds (Khan, 1968) and this reduction has been reported to be relieved by GA, red light and/or cytokinins, depending on the author. Sankhla and Sankhla (1968) and Khan (1968) reported that GA was ineffective in relieving ABA-induced dormancy, but cytokinins were. However, Robertson (1976) and Bewley and Fountain (1972) reported that GA was effective. A similarly unclear picture is apparent when endogenous ABA levels are followed: Braun and Khan (1976) reported that ABA levels dropped more quickly in germination inducing conditions, while Berrie and Robertson (1977) could find no such relationship. ABA thus cannot be ascribed a regulatory role in the control of lettuce seed germination.

Thus, our knowledge of the involvement of the major growth substances (ABA, IAA, GA, and cytokinins) in the control of lettuce seed germination is unclear. In summary, exogenous GA and cytokinins tend to promote germination, while exogenous ABA tends to reduce it. However, there is no strong evidence for the involvement of any of these compounds in the endogenous control of germination. To some

extent, this problem is a microcosm reflecting the confusion which exists in hormone physiology in general. The statement made by Hillman (1970) on apical dominance is equally valid as regards seed germination: "Thus, before ascribing a regulatory role to any of the four growth substances in this phenomenon, it is necessary to consider their concentration and method of application, especially in view of the importance of environmental effects, which may affect the endogenous hormonal status of the plant." It should be remembered that an alteration in the gross level of a hormone is only one way in which it can lead to an alteration of response. The rate of turnover may vary, while the level remains constant, also an environmental (or other) factor may alter the susceptibility of the tissue to the hormone, thus altering the response. If the turnover of these plant growth regulators were followed, or if the effect of differing environments on the response were studied, a clearer picture may arise.

WHY SHORT-CHAIN FATTY ACIDS?

In 1953, Bennet-Clarke and Kefford developed a technique for separating constituents of a crude plant extract. They used partition purification followed by paper chromatography, and found three regions of growth regulating activity on the chromatograms:

(i) auxin

(ii) promoter α

(iii) inhibitor β

While promoter α has been largely overlooked, the inhibitor β complex has been the subject of extensive research. Many growth regulating compounds have been identified in this complex (Gross, 1975).

Before the isolation of inhibitor β, there had been reports of growth inhibiting substances of plant origin, e.g. Hemberg (1949), who suggested that growth inhibitors, rather than auxin, may be

responsible for the onset of the rest period in potatoes. These reports, however, lacked the purification of the inhibitory substance achieved by Bennet-Clarke and Kefford.

Phillips and Wareing (1958) found seasonal changes in the growth substance content of shoots. They found higher levels of inhibitor β in winter and the lowest levels in May and June, when the plants were actively growing. The same workers (1959) showed an increase in inhibitor levels on transfer of sycamore shoots into dormancy-inducing conditions. In 1963, Dürffling found cyclical changes of inhibitor β levels in Acer similar to those in sycamore, reported by Phillips and Wareing (1958).

Eagles and Wareing (1963, 1964) found an inhibitor in an extract from Betula: when this was re-applied to Betula seedlings, apical growth was arrested. They also found increased levels of the inhibitor in plants in short days.

By 1965, there had been accumulated a large amount of evidence for the occurrence of the growth regulating complex, inhibitor β, and for its involvement in resting state development. When, in the same year, ABA was identified and subsequently found to be present in the inhibitor β complex, most of the work centred on identifying the active component ceased, as many workers assumed that this was ABA. Certain anomalies were reported, but these tended to be overlooked, e.g. Holst (1971) noted that inhibitor β was more active than ABA.

While this work was going on, Berrie and co-workers (unpublished data) were attempting to identify another inhibitory fraction of plant origin. This "oat inhibitor", from wild oat, was found to contain a mixture of short-chain fatty acids (Berrie et al., 1975). These fatty acids, of chain length 6-14, were found to have chromatographic properties similar to those of inhibitor β. When re-applied to lettuce seeds, these short-chain fatty acids caused a reduction in germination,

even after a red light treatment.

Working independantly, Reid (pers. comm.) found a reduction in carboxymethylcellulase production as the result of an application of an inhibitor β extract from fenugreek seeds. He could find no ABA in this extract, but found short-chain fatty acids. He also found that short-chain fatty acids reduced the GA-induced amylolysis in embryo-less barley half-seeds (Reid and Buller, 1976).

Ando and Tsukamoto (1974) had previously identified capric, lauric and myristic acids in an isolate of dormant iris bulbs. They found that these were inhibitory to growth as tested by the Avena straight growth test, but they did not show these acids to be part of the inhibitor β complex. Similarly, Le Poidevin (1965) had shown short-chain fatty to be inhibitory to the germination of mustard seeds, but he did not show that these occur endogenously.

These short-chain fatty acids were known to be inhibitors of the growth of organisms. They are uncouplers of oxidative phosphorylation in fungi and bacteria (Hochster and Quastel, 1963). They may also be the active component of the anti-bacterial chlorellin, isolated from Chlorella (Pratt et al., 1944). Spoehr (1949) identified Chlorellin as a mixture of the photo-oxidation products of unsaturated long-chain fatty acids. Stumpf (1965) showed how the oxidation of unsaturated C_{18} fatty acids will give rise to a mixture of short-chain fatty acids and dicarboxylic acids. It has been reported that the algal anti-bacterials are formed on the death of the mature algal cell (Proctor, 1957), a precursor being released which is subsequently photo-oxidised in the medium.

Vegis (1956) has stated that the oxidation of NADH + H^+ by molecular oxygen proceeds very slowly in embryonal cells. A restricted oxygen supply will slow this even more. He later suggested (1966) that the restriction of the availability of oxygen will favour the

formation of fatty acids, as this formation can act as an acceptor of hydrogens from NADH + H^+ which do not go to molecular oxygen (Lynnen, 1954). Thus the build up of ethanol or acetaldehyde may be avoided. He also suggested that a build up of fats leads to a loss of protoplasmic contact with the cell wall, leading to the isolation of single protoplasts. The permeability to water and solutes is thus greatly reduced. This could explain why seeds with lipid reserves survive immersion better than those with starch.

Short-chain fatty acids are thus known to have physiological effects on a range of organisms and could possibly explain the development of the resting state, as described by Vegis (1966). Their uncoupling activity could be predicted on the basis of the requirements for uncoupler activity: (i) uncouplers are usually weak organic acids; (ii) they possess the ability to distribute the charge when in the ionised form. The negative charge of the anion would be distributed over the whole of the carboxyl, according to the theory of resonance, thus satisfying the second requirement. However, all the physiological effects are not explicable on the basis of their activity as uncouplers, so there must be some other mode of action, which is not yet known.

SOME CHEMICAL PROPERTIES OF FATTY ACIDS

There is available a considerable amount of information on the chemistry of fatty acids. An excellent review was written by Markley (1960). It would be useful to mention a few of the properties. Figure 1(a) shows the melting points of the fatty acids with chain length 6-15. From this it can be seen that they fall into two homologous series, one with an odd number of carbon atoms in the chain and the other with an even number. This alternation is also seen in other physical properties. Muller (1923) suggested that this is due

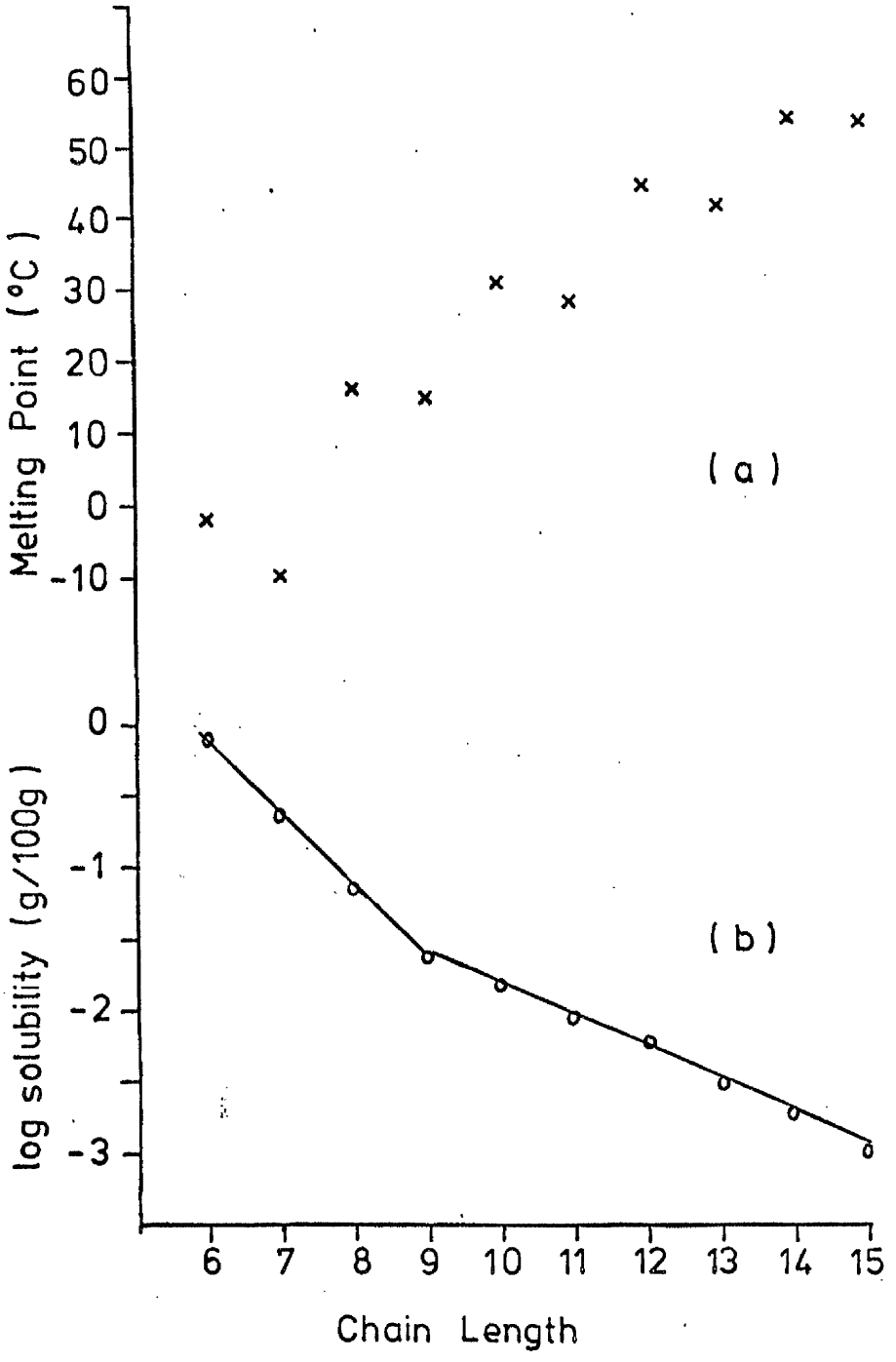


Figure 1: (a) The melting point of short-chain fatty acids. Note the alternating series.

(b) The solubility of short-chain fatty acids in water at 20°C. Note the two straight lines, intercepting at nonanoic acid.

to the arrangement of the zig-zag carbon skeletons in the crystal. If there is an even number of carbon atoms the functional end groups would be parallel. If there is an odd number of carbon atoms, the functional groups would not be parallel. The repeating unit in the crystal would thus be one molecule if an even-numbered chain, and two if an odd-numbered chain. However, Malkin (1931) attributed the alternation in properties to the tilt of the chains in the crystal. He argued that Müller's hypothesis implied that this alternation should apply in all long-chain compounds, while the alkanes, alcohols and ketones exhibit no such alternation. Malkin also pointed out that all non-alternating series possess vertical chains, whereas alternating series possess tilted chains. He demonstrated how Müller's theory could be extended to show that the alternation of properties will only occur if the zig-zag chains are tilted. It is now generally accepted that the alternation of properties is due to the angle of tilt. The alternation of properties is also seen in biological systems: Don and Wilmer (pers. comm.) have shown such an alternation in the inhibition of stomatal opening in Commelina.

Figure 1(b) shows a plot of the $\log_{10}$ of the solubility of the fatty acids in water against chain length. The fatty acids are much more soluble than the corresponding alkanes, due to the presence of the polar carboxyl group. It is evident from figure 1(b) that, as the hydrocarbon chain increases, the ability of the carboxyl group to bring about solubility of the acid in water becomes decreasingly effective. This is not a simple relationship. As figure 1(b) shows there is a break in the linear relationship at chain length nine. It is believed that this is due to the fact that chain length nine represents a transition point below which the polar carboxyl group exerts the greater influence over the molecule's properties and above which the hydrophobicity of the alkane chain predominates.

The fatty acids are weak acids. The dissociation constants (pK) vary little over the range of chain lengths 4-9 (4.66 - 4.91), while those for the acids of longer chain length have not been determined, due to their very slight solubility. Although all these acids are weakly acidic, they are stronger than carbonic acid (pK = 6.29), thus the fatty acids will be liberated from their alkali salts by mineral acids, and they in turn will liberate carbonic acid from its salts.

The fatty acids of chain length 4-16 are freely soluble in several organic solvents e.g. diethyl ether, dichloromethane, chloroform, toluene. In contrast the salts of these acids, which are comparatively soluble in water, are much less soluble in the less polar organic solvents. These solubility properties form the basis of the purification procedure which will be detailed later.

GERMINATION BIOASSAYS

INTRODUCTION

As has been stated previously, short-chain fatty acids have been reported to reduce lettuce seed germination (Berrie et al., 1975). Initially, it was intended to confirm this work.

MATERIALS AND METHODS

Fruits of Lactuca sativa L. cv. Grand Rapids were obtained from the Page Seed Company, Greene, New York and stored at -15°C prior to use. All the bioassay work was performed using seeds of the same batch.

A range of short-chain fatty acids was obtained from British Drug Houses Ltd and the acids were used without further purification.

The fatty acids were applied in solution in redistilled diethyl ether to a 4.25 cm diameter Whatman's No 3 filter paper lining the base of a 5 cm glass petri dish. The concentration of fatty acid in ether was appropriate to give, on evaporation of the ether, an amount that would result in the desired nominal molar concentration when 1.5 cm^3 of distilled water was added to the dish. Control (zero concentration) dishes were treated with diethyl ether. Fifty lettuce seeds were dispensed into each petri dish. Normally, there were four replicate petri dishes per treatment, these being sealed in a light-tight container and incubated at a range of temperatures, each being held to $\pm$ 1°C.

Initially, the petri dishes were arranged such that the replicate petri dishes were held in different light-tight containers, each container having four different treatments in it. This led to very variable results, which can be attributed to cross-contamination

between dishes by vapour because of the volatile nature of the fatty acids. For this reason in subsequent work the four replicate dishes were kept together in the same light-tight container.

GA_{4+7} and kinetin were prepared in aqueous solution and when these were tested, this solution was substituted for the imbibition water.

Red and far-red irradiation was carried out using a 12 V 100 W tungsten halogen lamp in a light-tight housing. The emitted radiation was filtered through 20 cm of water and monochromatic light was generated by means of interference filters made by Barr and Stroud, Anniesland, Glasgow. The red light was produced using a filter with peak transmission at 656 nm and band width 10 nm. This gave an intensity of 475 $\mu W\ cm^{-2}$. Normally, the seeds were exposed to this intensity for 5 minutes giving a dose of 142.5 $mJ\ cm^{-2}$. The far red was produced by using a filter with peak transmission at 730 nm and band width 10 nm. This gave an intensity of 1.72 $mW\ cm^{-2}$. The seeds were exposed to this for 10 minutes, giving a dose of 1.03 $J\ cm^{-2}$.

Unless otherwise stated, the seeds were given a red light treatment 2 h after imbibition.

A seed was considered germinated when the radicle protruded.

Standard error was calculated from the formula:

$$S.E. = \frac{\sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}}}{\sqrt{n}}$$

Correlation coefficients were calculated from the formula:

$$r = \frac{\Sigma (x - \bar{x})(y - \bar{y})}{\sqrt{[\Sigma(x - \bar{x})^2][\Sigma(y - \bar{y})^2]}}$$

The regression (least squares) line was calculated from the formula:

$$\hat{y} = b.x + (\bar{y} - b.\bar{x})$$

where $$\bar{x} = \frac{\Sigma x_i}{n}$$

$$\bar{y} = \frac{\Sigma y_i}{n}$$

and $$b = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

RESULTS

Preliminary experiments at 20°C showed that nonanoic (C_9) acid was ineffective in reducing lettuce seed germination at concentrations less than 500 mmol m^{-3}. However, germination was completely inhibited at 5 mol m^{-3}. It was therefore decided to define more precisely this threshold region, by using a narrow concentration range. The fatty acid solutions were prepared using a dilution factor of 1.77. (This was chosen as it is $\sqrt[4]{10}$, and will thus allow a narrow concentration range, while maintaining the logarithmic scaling.) A range of concentrations from 10 mol m^{-3} to 559 mmol m^{-3} was therefore prepared. The germination was scored after 24, 48, 72 h, except for the 12°C test which was scored after 48, 72, 96 and 120 h.

The full data for the effect of nonanoic acid on lettuce seed germination at 12, 20, 24, 26 and 28°C are shown in Tables 1-5. When these are compared it can be seen that temperature does have an effect on the fatty acid induced reduction of germination. For example, at 12°C, there is still substantial germination in 3.16 mol m^{-3} nonanoic acid, but at 28°C, germination is zero in 1.77 mol m^{-3}. To allow easy comparison of a number of these tables, a value called the G_{50} dose was derived. The G_{50} dose is that dose which reduces

Table 1: The effect of nonanoic acid on lettuce seed germination at 12°C. Mean and standard error of number germinated per fifty seeds.

Conc./mol m^{-3}	Time after imbibition (h) 48	72	96	120
0	27.25 ±1.31	41.00 ±2.35	46.75 ±1.11	47.50 ±0.96
0.316	24.00 ±4.88	36.50 ±4.41	47.75 ±1.03	48.50 ±1.19
0.559	25.50 ±2.90	37.75 ±2.14	44.50 ±0.96	47.00 ±0.82
1.00	8.75 ±1.60	22.25 ±1.80	29.75 ±0.25	37.75 ±0.63
1.77	5.75 ±1.11	22.75 ±2.72	28.50 ±1.85	36.25 ±2.50
3.16	0.75 ±0.48	4.50 ±0.96	7.50 ±2.33	10.25 ±3.09
5.59	0 -	0.25 ±0.25	0.50 ±0.29	1.25 ±0.95
10.00	0 -	0 -	0 -	0 -

Table 2: The effect of nonanoic acid on lettuce seed germination at 20°C. Mean and standard error of number germinated per fifty seeds.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	31.75 ±1.50	42.00 ±0.71	45.75 ±1.32
0.316	19.50 ±2.79	38.00 ±2.45	45.25 ±1.03
0.559	27.75 ±0.25	39.50 ±1.66	46.00 ±0.71
1.00	28.50 ±2.96	38.25 ±2.25	46.00 ±0.71
1.77	2.50 ±0.87	11.25 ±0.95	15.50 ±2.10
3.16	0.25 ±0.25	0.25 ±0.25	1.00 ±0.58
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

Table 3: The effect of nonanoic acid on lettuce seed germination at 24°C. Mean and standard error of number germinated per fifty seeds.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	24.75 ±1.38	36.25 ±1.60	36.25 ±1.60
0.316	24.25 ±1.11	34.45 ±2.29	37.50 ±1.45
0.559	20.50 ±4.67	28.50 ±1.00	34.50 ±2.79
1.00	10.00 ±2.52	20.75 ±1.55	30.00 ±3.14
1.77	1.00 ±0.71	2.00 ±0.91	3.75 ±1.32
3.16	0 -	0 -	0.75 ±0.48
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

Table 4: The effect of nonanoic acid on lettuce seed germination at 26°C. Mean and standard error of number germinated per fifty seeds.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	24.00 ±3.08	34.25 ±3.04	35.25 ±3.48
0.316	21.75 ±2.56	29.75 ±3.66	30.00 ±3.16
0.559	18.25 ±2.25	30.25 ±1.85	31.00 ±2.24
1.00	11.00 ±2.49	18.75 ±3.43	22.50 ±3.89
1.77	0.75 ±0.48	4.50 ±2.18	5.50 ±2.26
3.16	0 -	0 -	0.25 ±0.25
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

Table 5: The effect of nonanoic acid on lettuce seed germination at 28°C. Mean and standard error of number germinated per fifty seeds.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	9.75 ±2.14	10.75 ±3.86	11.25 ±3.17
0.316	6.25 ±1.38	7.25 ±1.93	7.25 ±1.93
0.559	7.75 ±3.12	9.50 ±3.75	9.75 ±4.17
1.00	2.25 ±1.03	5.25 ±2.29	5.25 ±2.29
1.77	0 -	0 -	0 -
3.16	0 -	0 -	0 -
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

germination to 50% of that of the corresponding control; i.e. from Table 2, the G_{50} dose is that dose which reduces germination to 22.88 per fifty. To arrive at the estimate of the G_{50} dose, it was necessary to derive an equation, as visual estimates from the dose-response curves were not sufficiently accurate. To allow this, the dose-response curve is considered to be linear in the threshold region, and can thus be assigned an equation of the form,

$$y = mx + c,$$

where m is the gradient

and c is a constant.

This could be rewritten,

$$N = md + c$$

where N is number germinated

and d is the dose.

Solving two of these equations simultaneously and intrapolating for N_{50} $(= \frac{N \text{ control}}{2})$ leads to the equation:

$$G_{50} = \frac{N_{50} - (N_1 - \frac{N_1 - N_2}{d_1 - d_2} . d_1)}{\frac{N_1 - N_2}{d_1 - d_2}}$$

From Table 2, this yields:

$$G_{50} = \frac{22.88 - (46 - \frac{46 - 15.5}{1 - 1.77} .1)}{\frac{46 - 15.5}{1 - 1.77}} \text{ mol m}^{-3}$$

$$= 1.58 \text{ mol m}^{-3}$$

This G_{50} value was calculated for the range of temperatures and was found to be related to temperature in a particular way. When the calculated G_{50} dose was plotted against temperature a linear relationship was obtained. It was decided to calculate the G_{50} value at the same range of temperatures for the other short-chain fatty acids.

The accumulated germination data for hexanoic, heptanoic and octanoic acids are contained in Appendix I. When the G_{50} values for these acids were calculated they were found to be linearly related to temperature, as were those of nonanoic acid. A plot of these G_{50} values, along with the calculated linear regression lines are shown in Figure 2.

It is also evident, from Figure 2, that the calculated regression lines converge at zero concentration at approximately 36.5°C.

When the gradients of these four lines are plotted against chain length, it is noted that these are also linearly related. This is shown in Figure 3. While care must be taken when using a correlation coefficient of only four points, it would appear to be significant when a value as high as -0.964 is obtained. It is therefore possible to predict the G_{50} dose for any of these four fatty acids at various temperatures, simply by substituting the values in the following equation:

$$G_{50} \text{ dose} = \frac{T - 36.5}{-2.32.C + 10.61} \text{ mol m}^{-3}$$

where C is the number of carbon atoms in the molecule

and T is the temperature in degrees Celsius.

Subsequent tests showed that fatty acids with chain lengths greater than nine do not fall into this pattern. They are less effective than nonanoic acid.

It was then decided to investigate the possibility of an interaction between fatty acids and germination effectors. Nonanoic acid was used to investigate interactions, as it is the most effective of the acids tested. The first to be looked at was light-treatment. This was repeated on three occasions. The data from one are presented in Table 6. From Table 6, it can be seen that red light increases the dose of nonanoic acid required to reduce germination by 50%, while far red reduces it. These effects are consistent and reproducible.

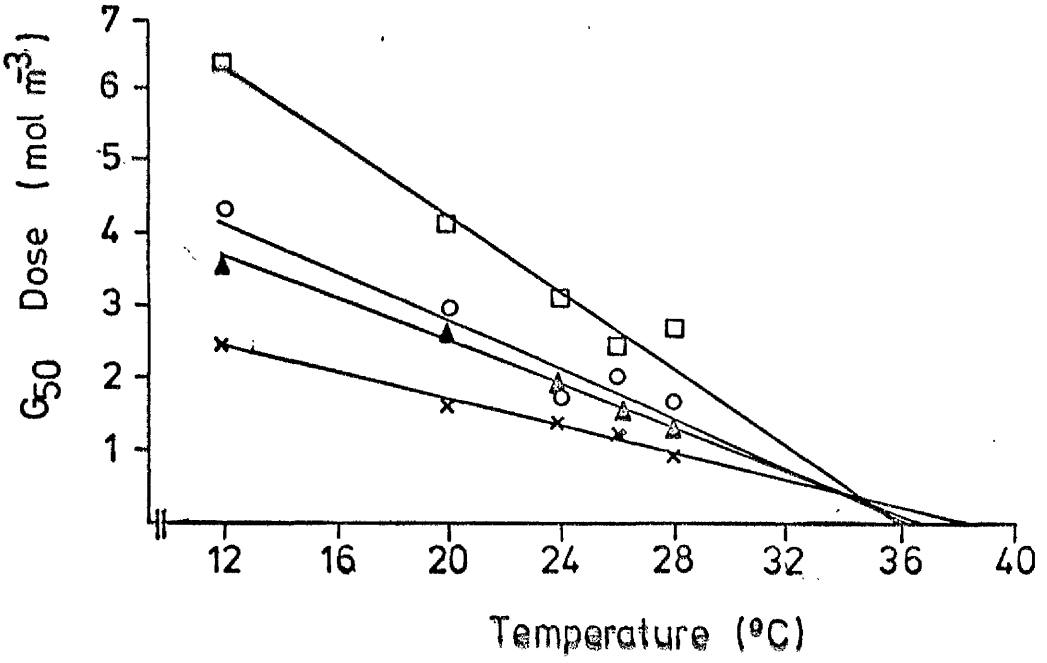


Figure 2: A plot of G_{50} dose against temperature for four short-chain fatty acids. The lines are the calculated regression lines. The correlation coefficients are shown in the table below, as is the key to the symbols.

		r
□	Hexanoic acid	-0.979
o	Heptanoic acid	-0.971
▲	Octanoic acid	-0.990
×	Nonanoic acid	-0.993

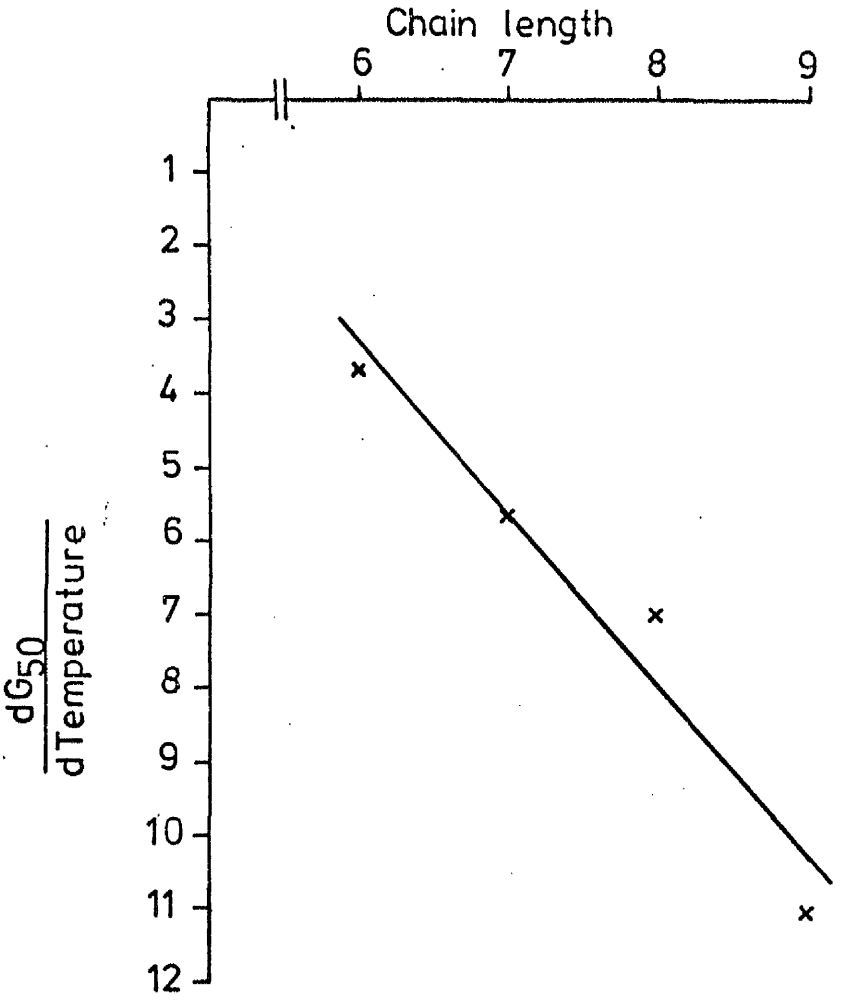


Figure 3: A plot of the gradients of the G_{50} curves against chain lengths. This regression yielded a correlation coefficient of -0.964.

Table 6: The effect of nonanoic acid and light treatment on the germination of lettuce seed at 20°C after 24 h. The table shows the complete data for one trial; the mean number germinated per fifty (and standard error), the calculated G_{50} dose, and the mean and standard error of the calculated G_{50} dose from three such trials.

nonanoic acid conc/mmol m^{-3}	Light treatment		
	Dark	Red light	Far red
0	8.75 (1.11)	28.75 (3.50)	2.25 (0.48)
316	8.75 (1.11)	31.75 (1.44)	2.25 (0.75)
559	5.50 (0.50)	22.00 (1.69)	1.00 (0.71)
1000	0.50 (0.29)	6.00 (0.91)	0.25 (0.25)
Calculated G_{50} (mmol m^{-3})	658	769	535
Mean G_{50} dose (mmol m^{-3})	664	780	535
S.E.	35	17	22

Since nonanoic acid and light treatment interact, it is possible that the fatty acid induced reduction of germination could be relieved by an increased red irradiation. To check this, the dose-response curves for germination against light dose with and without nonanoic acid present were determined. The dose-response curves for red irradiation, with and without 1 mol m^{-3} nonanoic acid are shown in Figure 4. It is clear that no such a relief is possible: the curve for the seeds with fatty acid plateaus at the same light dose as does that without. A similar test was performed with far-red irradiation. A similar result was obtained in that the response to far-red light saturated at the same dose whether or not fatty acid was present. This is shown in Figure 5. There was an additional problem in this case. Since the level of germination in dark-imbibed seeds in 500 mmol m^{-3} nonanoic acid is about 9%, when these seeds are also given a far-red light treatment, percentage germination becomes so low, that natural variation becomes relatively large.

Gibberellin promotes lettuce seed germination in darkness. GA_{4+7} was applied at 10 and 100 mmol m^{-3} to lettuce seeds with a range of nonanoic acid concentrations. The results of this are shown in Table 7. From this, it can be seen that 10 mmol m^{-3} GA_{4+7} promotes germination, but has no effect on the G_{50} dose of nonanoic acid. 100 mmol m^{-3} GA_{4+7}, however, both promotes seed germination and increases the G_{50} dose. This was repeated on three occasions and was found to be reproducible.

Kinetin has been reported to promote the germination of lettuce seeds in darkness. 10 mmol m^{-3} and 100 mmol m^{-3} kinetin was co-applied to lettuce seeds along with a range of concentrations of nonanoic acid. The results of this are shown in Table 8. This shows that while kinetin promoted the germination of lettuce seed, it had no effect on the G_{50} dose. It would therefore appear that kinetin and nonanoic acid do not

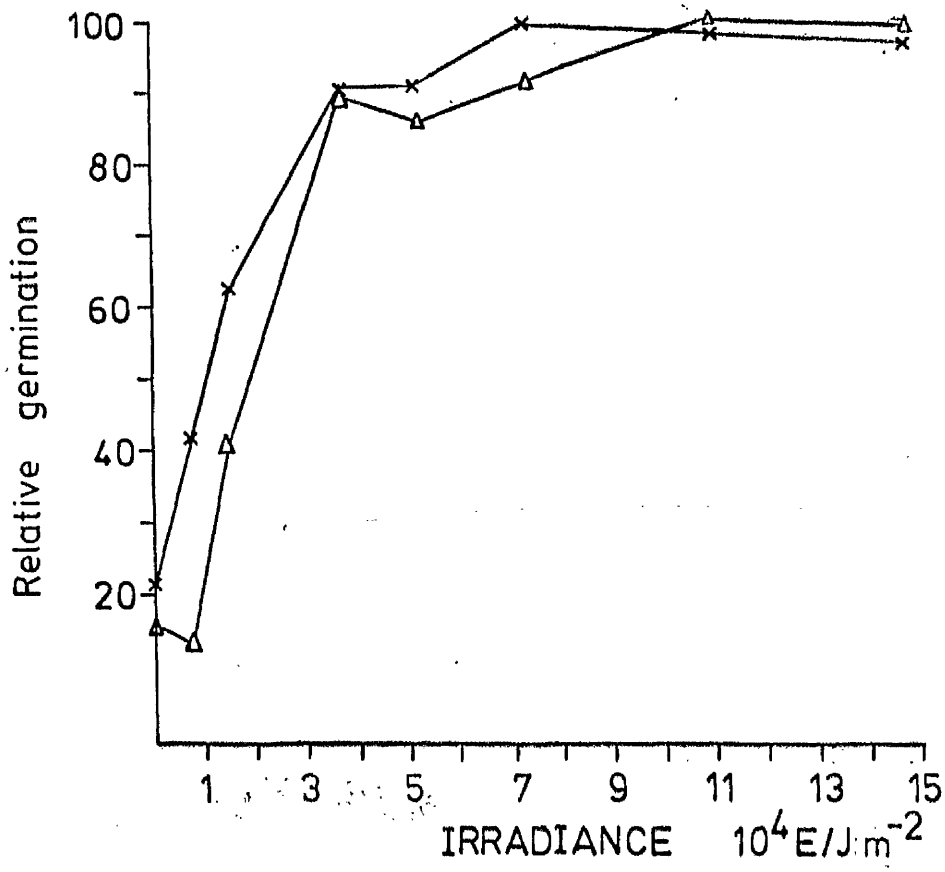


Figure 4: The dose-response curves for lettuce seed germination against dose of "red light" with and without nonanoic acid.

x Control

Δ 1 mol m^{-3} nonanoic acid

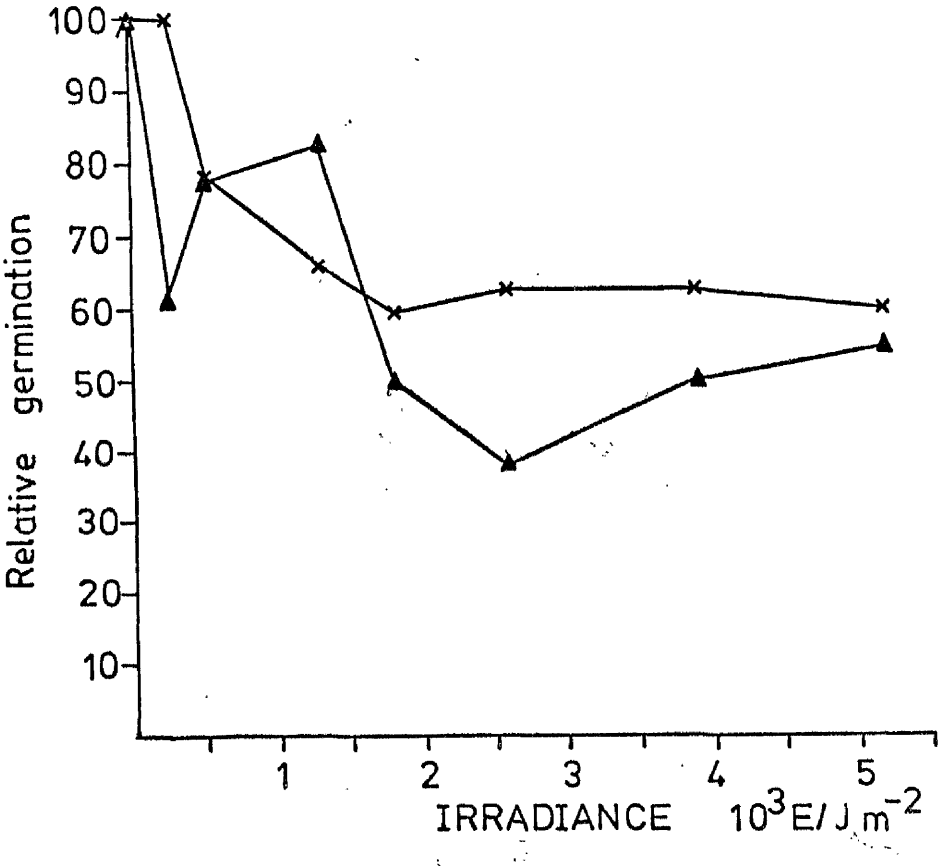


Figure 5: The dose-response curves for lettuce seed germination against dose of "far-red light" with and without nonanoic acid.

x Control

Δ 500 mmol m^{-3} nonanoic acid

Table 7: The effect of GA_{4+7} and nonanoic acid on the dark germination of lettuce seeds at 20°C after 24 h. The table shows the complete data for one trial; the mean number germinated per fifty (and standard error), the calculated G_{50} dose, and the mean and standard error of the calculated G_{50} dose from three such trials.

Nonanoic acid conc/mmol m^{-3}	Conc/mmol m^{-3} GA_{4+7}		
	0	10	100
0	8.25 (1.32)	39.00 (0.71)	39.75 (1.32)
559	5.25 (0.25)	22.00 (1.29)	34.75 (1.99)
1000	2.00 (0.41)	9.00 (0.91)	20.75 (2.50)
1777	0.75 (0.75)	0.75 (0.48)	4.75 (1.03)
Calculated G_{50} (mmol m^{-3})	771	644	1040
Mean G_{50} dose (mmol m^{-3})	709	684	1120
S.E.	31	26	88

Table 8: The effect of kinetin and nonanoic acid on the dark germination of lettuce seeds at 20°C after 24 h. The table shows the complete data for one trial; the mean number germinated per fifty (and standard error), the calculated G_{50} dose, and the mean and standard error of the calculated G_{50} dose from three such trials.

Nonanoic acid conc/mmol m^{-3}	Kinetin conc/mmol m^{-3}		
	0	10	100
0	11.00 (1.35)	14.25 (1.25)	17.50 (1.94)
559	11.25 (1.03)	12.75 (0.75)	11.50 (0.96)
1 000	2.50 (0.87)	7.50 (1.04)	8.00 (1.36)
1 777	0.50 (0.50)	1.50 (0.65)	1.50 (0.65)
Calculated G_{50} (mmol m^{-3})	848	1040	906
Mean G_{50} dose (mmol m^{-3})	744	760	718
S.E.	68	140	91

interact.

Lettuce seed germination is thus reduced by fatty acids, and that reduction may be modified by the photo- or chemi-environment of the seed.

DISCUSSION

There are several examples of interactions between short-chain fatty acids and environmental factors included in the results. It would be useful to consider those factors which affect germination and compare this with their effect on the G_{50} dose.

	Effect on germination	Effect on G_{50} dose
Temperature	germination $\alpha \frac{1}{\text{Temperature}}$	G_{50} dose $\alpha \frac{1}{\text{Temperature}}$
Red light	promotes germination	increases G_{50} dose
Far red light	reduces germination	decreases G_{50} dose
Gibberellin	promotes germination	increases G_{50} dose
Kinetin	promotes germination	no effect on G_{50} dose

Thus, there is a parallelism between the effects of the first four stimuli on germination and on the G_{50} dose. Kinetin is an exception, since it appears to have no effect on the G_{50} dose.

Fatty acids could, possibly, be included in the list above as it has been reported that the presence of short-chain fatty acid, as well as reducing germination, enhance the reduction by another fatty acid in a synergistic manner (Berrie, et al., 1975). Fatty acids thus interact with each other.

There are three ways in which one can consider evaluating the role of an endogenous component in controlling a process:

1. Changes in Levels

One expects the total level of the growth regulator to change

according to the physiological state.

2. Rates of Turnover

It is necessary to follow the metabolism of a growth regulator; if only the total endogenous level is followed, this will give no guide whatsoever to the turnover. The total level may remain constant, while rates of synthesis and of breakdown both increase.

3. Changes in Susceptibility

Physical (or chemical) parameters may alter the susceptibility of the system to the same dose - e.g. if the level of the growth regulator is constant, the tissue may vary in its response to this constant amount depending on, for example, temperature.

The results of the bioassay studies reported could be interpreted in terms of a "susceptibility" system as described in (3).

It is also interesting to note that the stimuli which alter the G_{50} dose have all been ascribed membrane effects.

1. Temperature

Temperature is well known to affect the state of membranes. The most profound effect is that on the thermotropic phase transition. This is an alteration in the degree of order of the hydrocarbon chains of the lipid bilayer from a highly ordered crystalline state to a less ordered liquid-crystalline state (e.g. Overath and Trauble, 1973). This sharp thermal phase transition is seldom found in natural lipids, rather there occur, what are termed, phase separations: there co-exist regions of crystallinity and fluidity. At elevated temperatures the percentage of the lipid present in the crystalline state is reduced.

The calculated regression lines in Figure 2, are seen to extrapolate to zero at 36.5°C. This appears to be significant, since there are many reports of lettuce seeds being promoted to germinate at up to 35°C, but not above. Negm et al (1972) reported that ethylene and

carbon dioxide together promoted germination up to 35°C. Braun and Khan (1975) also succeeded in promoting germination at 35°C. Similarly, Dunlap and Morgan (1976) reported that ethylene and either GA or red light together would promote germination above 32°C, but not at 36°C, which they termed a thermal block to germination. However, there is then a problem of identifying this thermal block to germination. Hendricks and Taylorson (1976) reported a marked increase in the permeability of the plasmalemma of lettuce seeds in the 35-40°C range. It is known that small molecule permeability of membranes is proportional to the degree of fluidity (e.g. Bangham, 1968), and since Hendricks and Taylorson used measurement of the conductivity of the bathing medium as a measure of the permeability of the plasmalemma, it is possible that this 36°C block is due to a rapid increase in the degree of fluidity of the membrane at this temperature. Such an increase in fluidity could increase small molecule permeability to such an extent as to overcome active transport processes and to allow solutes to flow along the concentration gradient. Similarly, if the seed membrane became freely permeable to water, excess water may enter along the osmotic gradient between the cytosol and the medium. This would lead to severe damage to the seed. In fact, it was found that when seeds were incubated at 38°C for 72 h, they were damaged and exhibited a characteristic syndrome. This consisted of an abnormal swelling of the endosperm to such an extent that the testa was ruptured. Such a syndrome was first reported by Ikuma and Thimann (1964). This syndrome could also be induced at a much lower temperature by overdosing with short-chain fatty acid. Plate 1 shows this syndrome for seeds incubated at 38°C; at 20°C with 10 mol m^{-3} nonanoic acid, c.f. normal thermo-dormant seeds, incubated at 30°C.

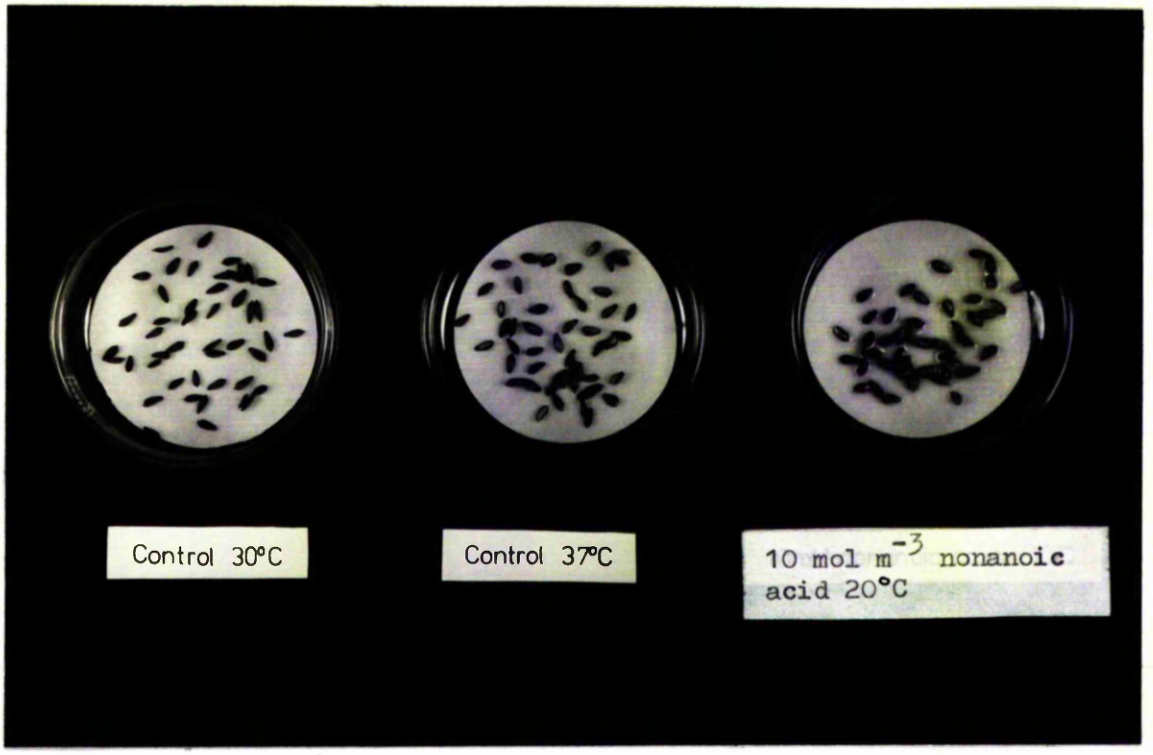


Plate 1: A characteristic syndrome in which the endosperm swells to such an extent that the testa ruptures. Such a syndrome may be induced by imbibition at high temperatures or in the presence of high concentrations of short-chain fatty acids.

2. Phytochrome

There is a considerable amount of evidence that phytochrome acts on membranes (for review, see Marmé, 1977). Marmé (1974) showed *in vitro* phytochrome binding. He suggested that Pfr induces conformational changes in the membrane, altering its functional properties. However, Pratt and Marmé (1976) have shown that *in vivo* and *in vitro* phytochrome binding are different and suggest that *in vitro* binding may be an artefact. They also reported that phytochrome is released from the pelletable fraction, after a far red irradiation, with a half-life of 25 minutes at 25°C and 100 minutes at 13°C and 3°C. It is possible that this change in half-life between 25°C and 13°C, but not between 13°C and 3°C is due to the membrane phase transition. More recently, Hendricks and Taylorson (1978) have shown that for Pfr to be effective in promoting germination of *Amaranthus retroflexus* B. seeds, the membrane must be in a crystalline state. They claim that Pfr is ineffective if the membrane is fluid.

Wiesenseel and Smeibidl (1973) showed that the plasmolysis of *Mougeotia* is controlled by phytochrome: red light increases the permeability. Marmé (1977) summarises the major phytochrome effects on membranes to be:

1. Membrane permeability
2. control of the activity of membrane bound enzymes:
   (a) modulate enzyme activities which are capable of controlling the active translocation of ions and molecules into and out of the cell: (b) modulate metabolism of membrane-bound molecules: (c) control the activity of membrane-bound enzymes, which catalyse the metabolism of soluble molecules.

Thus, there is considerable evidence for the involvement of phytochrome in membrane controlled processes.

3. Gibberellin

GAs have been reported to affect membranes. Wood and Paleg (1974) and Wood et al (1974) reported that GA increased membrane fluidity, however their results are far from unequivocal. The major criticism of this work is that the amount of gibberellin incorporated is much above that required to bring about physiological responses. It should be noted from Table 7, that while 10 mol m^{-3} GA_{4+7} promotes the germination of lettuce seeds, it has no effect on the G_{50} dose. 100 mol m^{-3} GA_{4+7} does not promote germination any more than does 10 mol m^{-3}, but it does alter the G_{50} dose. This suggests that the GA is acting in two different ways: at low concentrations it promotes germination: at higher concentrations this promotion is saturated, but the large GA dose acts in some other manner. It is possible that this abnormal, high dose effect of GA is due to its incorporation into membranes. The levels of GA may then approach those used by Wood and Paleg

In summary, germination of lettuce seed is reduced by application of short-chain fatty acids, and this reduction is modulated by temperature, light treatment and gibberellin.

LIPOSOME PERMEABILITY

INTRODUCTION

In the previous section it was shown that short-chain fatty acids reduce lettuce seed germination in a particular way.

Because of the amphipathic nature of the short-chain fatty acids, and since the factors which affected the G_{50} dose, i.e. temperature, large doses of GA, and phytochrome, have all been ascribed membrane-affecting properties it was believed that the short-chain fatty acids may be acting on membranes. However, it is obviously desirable to obtain a guide to the fundamental action of the short-chain fatty acid. To this end, it was decided to use an artificial membrane system, liposomes.

Liposomes can be produced simply by shaking phosphatidylcholine in water. However such vesicles are multi-lamellar, non-uniform, and tend to precipitate on standing (Mizamoto and Stoeckenius, 1972). More stable homogeneous liposome preparations can be obtained by ultrasonication, which promotes formation of uniform mono-bilayer vesicles (Bangham et al., 1974). Liposomes have been shown to be completely sealed, and thus, they constitute a simple model system for permeability studies of lipid bilayers, particularly because of their very high surface area to volume ratio.

Since most liposome dispersions act as perfect osmometers, measurements of bulk water permeability can be derived from the rate of swelling or shrinking when an osmotic gradient is applied across the membrane. Bittman and Blau (1972) measured the initial volume changes in multi-lamellar liposomes after establishing concentration gradients of potassium chloride across the bilayers. They showed that the initial rate of permeability to water increased

with the introduction of increasing numbers of unsaturated bonds into the constituent phospholipid molecules, indicating that the rate of diffusion is a function of the fluidity of the hydrocarbon chains. The action of cholesterol on water permeability was predictable on the basis of its effect on the mobility of the hydrocarbon chains (Sackmann and Trauble, 1973), i.e. cholesterol increases permeability below the transition temperature, but reduces it above.

Biological membranes and lipid bilayers have rather similar permeability properties as regards water and non-polar solvents. There are, however, marked differences as regards their permeabilities to hydrophilic solutes, particularly inorganic cations: the permeability of lipid bilayers to these being generally much smaller. It has been suggested that this difference is due to the presence in a natural membrane of certain proteins, which may be directly concerned in this process. Since inorganic ions are highly hydrated, the energy required for their release from an aqueous environment and transfer to the hydrophobic region of the membrane provides a major barrier to transport via hydrophobic pathways. The presence of an anionic charge on the surface of a bilayer assists the permeation of cations across phospholipid bilayers, and the effect of these cannot be overlooked. There is also a considerable amount of evidence to show that cholesterol has an effect on cation permeability similar to its effect on water permeability (Papahadjopoulos et al., 1971). It is generally accepted that hydrocarbon chain mobility is an important parameter in the passive diffusion of hydrophilic solutes across lipid bilayers (Schreier-Muccillo et al., 1976).

It has been shown then that the water and small molecule permeabilities of a number of phospholipid and phospholipid-cholesterol systems have been found to approximate to those of natural membranes.

MATERIALS AND METHODS

Chemicals

Lecithin (egg) (M.W. about 800) was obtained from British Drug Houses Ltd, and was used without further purification. Since this is a natural lipid, it is likely that it is heterogeneous. In fact 38 molecular species have been identified in egg phosphatidylcholine (Kuksis and Marai, 1967).

2(N-morpholino) ethanesulphonic acid (M.E.S.) was obtained from British Drug Houses Ltd.

The specific potassium ionophore, valinomycin and the uncoupler, carbonyl cyanide m-chlorophenylhydrazone (C.C.C.P.) were obtained from Calbiochem, La Jolla, California 92037. Valinomycin was made to 90 mmol m^{-3} in ethanol and the CCCP was made to 490 mmol m^{-3} in ethanol.

Liposome Generations

250 mg (312 μmoles) of lecithin were dissolved in approximately 10 cm^3 of chloroform in a round-bottom flask. The chloroform was removed by rotary evaporation at 35°C. This left a thin film of lecithin on the inside of the flask. 5 cm^3 of 500 mol m^{-3} KCl and 500 mmol m^{-3} M.E.S. (pH 6.5) solution were added to the flask, which was vigorously shaken by hand. The resulting suspension was transferred to a 20 cm^3 vial, surrounded by ice, and sonicated for 5 x 30 seconds, with 1 minute rests between. The suspension was loaded onto a short column of Sephadex G_{25} coarse, swollen and eluted in 500 mol m^{-3} Na Cl (pH 6.5). This results in lecithin vesicles, or liposomes, with NaCl outside and KCl inside the vesicles.

12 cm^3 of 500 mol m^{-3} NaCl (pre-incubated to required

temperature) were placed in a cuvette with a water jacket. (See Figure 6) Water was circulated through the jacket by means of a Baird and Tatlock, Circon 2, circulating water pump and heater. This allowed the temperature of the cuvette to be held constant. 0.5 cm^3 of the liposome suspension was also added to the cuvette, and the pH was adjusted to 6.5 using 10 mol m^{-3} NaOH and 10 mol m^{-3} HCl.

Changes in the pH of the bathing medium were recorded using an Electronic Instruments Limited pH meter (Model 7020), coupled from its jack-plug output to a Servoscribe Chart recorder.

After the addition of the liposome suspension and the correction of the pH to 6.5, 10 minutes equilibration were allowed, during which the drift of pH was noted. Valinomycin was then added and any deflection in the response noted. A few minutes later the uncoupler CCCP was added and the deflection noted. So that correction could be made for different buffering capacities of the medium, 1 mm^{-3} 10 mol m^{-3} NaOH was then added as a standard and the deflection noted. The theory behind this is that the valinomycin will pump potassium ions out of the vesicles, but this will be resisted by the immediate establishment of a voltage difference between the inside of the vesicles and the medium, so few potassium ions will be moved. If, however, the membrane is "leaky", protons will flow into the vesicles and allow a greater amount of potassium ions to be extruded, resulting in a net acidification inside the vesicles and a net alkalisation in the bathing medium. (Only the change in the external medium is important since the M.E.S. buffer inside the vesicles will compensate and maintain the pH.)

The CCCP is an uncoupler, i.e. a proton carrier, and will allow the free flow of protons regardless of the permeability of the membrane to protons. This is summarised in Figure 7.

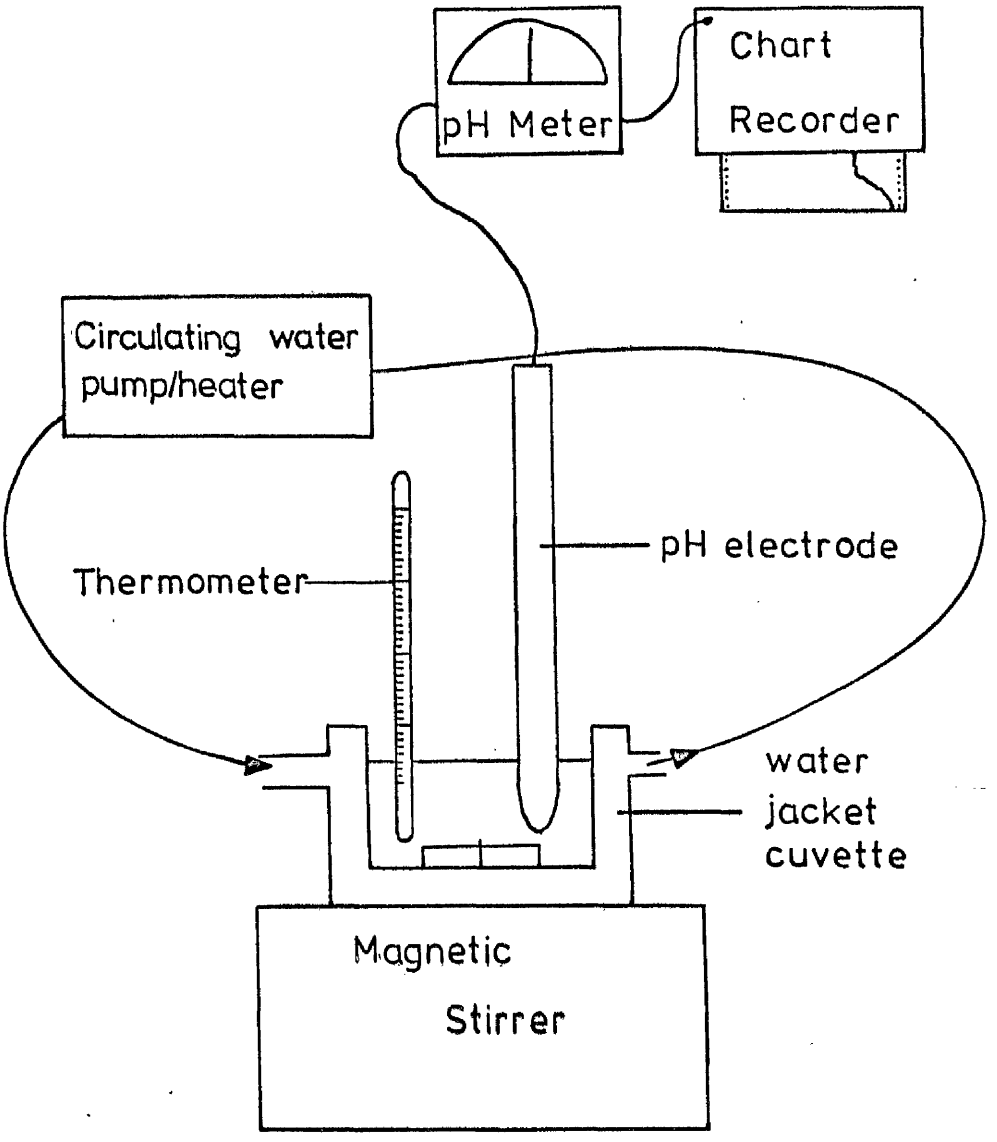


Figure 6: The isothermal cuvette system used for the liposome permeability studies. The pH meter and chart recorder allowed measurement of the small pH changes in the bathing medium.

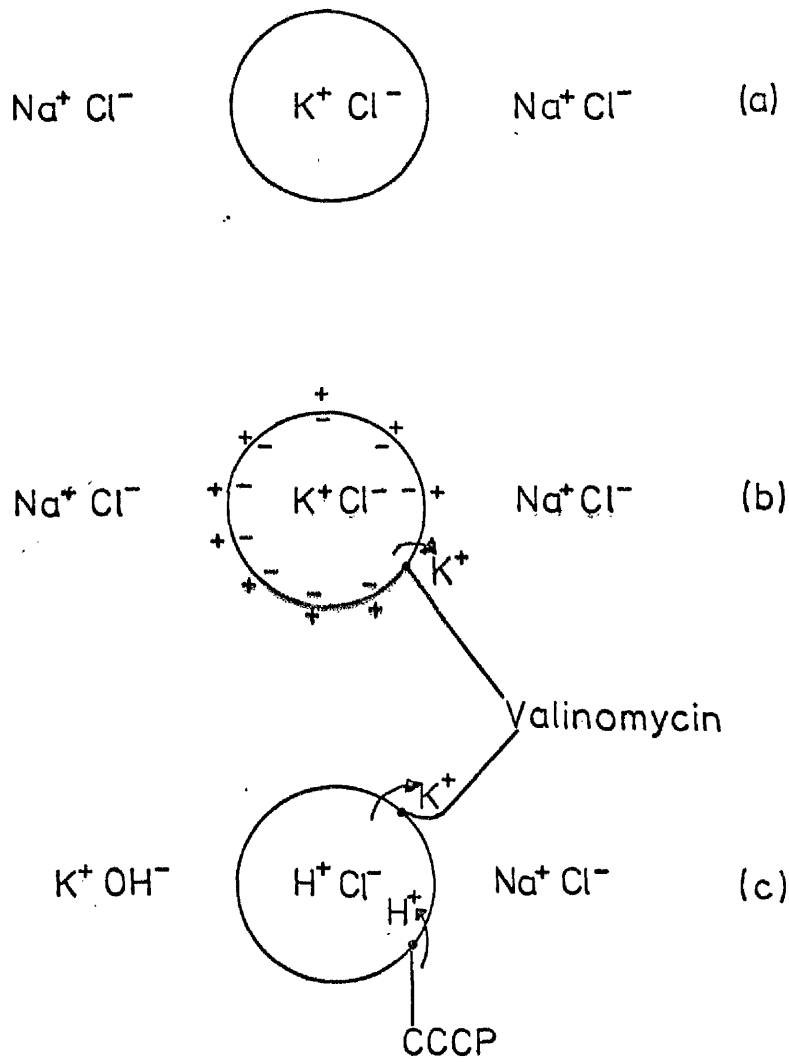


Figure 7: A diagrammatic representation of a liposome.

(a) After passing down the Sephadex G_{25} column: Na Cl in the medium, KCl inside the vesicles.

(b) After addition of the valinomycin: some potassium ions are extruded, leading to the establishment of a potential difference across the vesicle bilayer.

(c) After addition of the CCCP: this allows protons to enter the vesicle along the potential gradient, re-establishing electrical neutrality and allowing the valinomycin to extrude potassium ions to completion. This results in a net alkalisation of the external medium.

Short-chain fatty acids were incorporated into the liposomes by adding to the lecithin-chloroform solution the required amount of 10 mol m^{-3} fatty acid in chloroform solution, then taking to dryness as above.

Since the lecithin has a molecular weight of about 800, the 250 mg will contain about 312 μmoles. Short-chain fatty acids were added to this in the range 25 to 200 μmoles, which correspond to a mole to mole ratio of from 0.081:1 to 0.64:1 respectively, short-chain fatty acid to lecithin (or 0.04 to 0.32 fatty acid to stearoyl residue).

There was a limiting factor to the amount of short-chain fatty acid which could be incorporated since liposomes would not form when more than 100 μmoles of nonanoic was incorporated. However, liposomes would form with 200 μmoles of hexanoic acid, but not with more than this amount. This is presumably due to the hydrotropic action of the short-chain fatty acids.

Statistics

Mean, standard error, linear regression and correlation coefficient were calculated as detailed in section I.

Two way analyses of variance were performed using the following formulae:

If there are r rows and k columns:

$$SSR = \left(\sum_{j=1}^{r} \frac{T_j^2}{k} \right) - \frac{T_{..}^2}{rk}$$

$$SSC = \left(\sum_{i=1}^{k} \frac{T_i^2}{r} \right) - \frac{T_{..}^2}{rk}$$

$$SST = \sum \sum x_{ij}^2$$

$$SSE = SST - SSR - SSC$$

$$MSR = \frac{SSR}{r-1}$$

$$MSC = \frac{SSC}{k-1}$$

$$MSE = \frac{SSE}{(r-1)(k-1)}$$

$$F_C = \frac{MSC}{MSE}$$

$$F_R = \frac{MSR}{MSE}$$

Where SSR is the among rows sum of squares; SSC is the among columns sum of squares; SST is the total sum of squares; SSE is the within mean square; MSR is the mean square of rows; MSC is the mean square of columns; MSE is the within mean square.

RESULTS

Initially, it was difficult to decide how to interpret the charts produced by the Servoscribe coupled to the pH meter. An example of one is shown in Figure 8. The major problem was that in some cases an addition resulted in a net alkalisation, as does that of CCCP in Figure 8, but in other cases, it resulted merely in a reduction in the acidification drift, as with the valinomycin in Figure 8. From this trace, it can be seen that the drift is constant, i.e. the lines are parallel before and after any addition. The traces can therefore be projected, as shown by the dotted lines in Figure 8, to find the position of the trace if no addition had taken place. It can therefore be considered that a measure of the effect would be the displacement of the trace. It was therefore decided to measure the perpendicular distance between the lines (denoted by the arrows in Figure 8), and use this as our parameter to assess

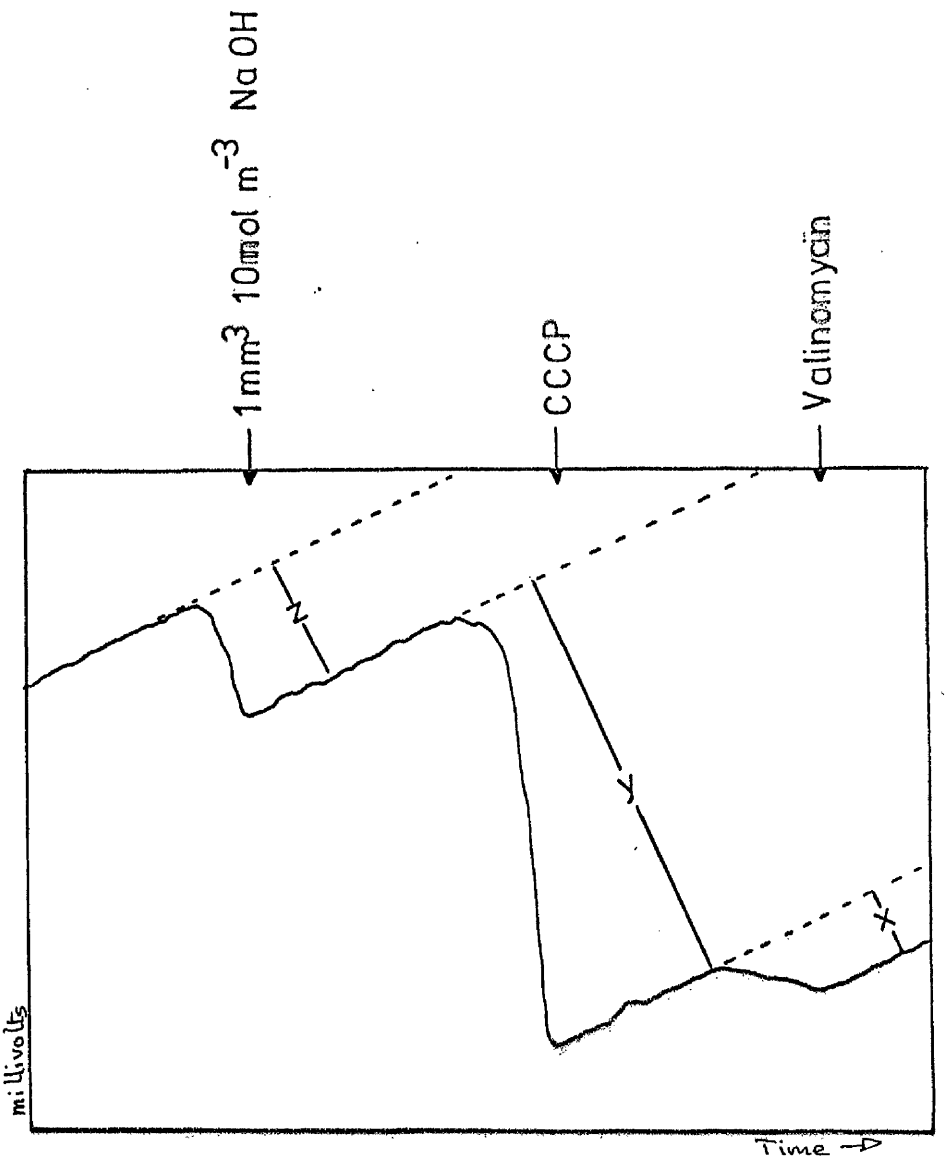


Figure 8: An example of a trace produced by the pH meter-chart recorder. The perpendicular displacements due to valinomycin and CCCP (x and y respectively) were measured and divided by that due to the NaOH standard, this gave the values V and C respectively.

alkalisation. The displacements caused by the valinomycin (x) and the CCCP (y) were then divided by the displacement caused by the NaOH standard (z) to arrive at values V and C respectively, which were in nanomoles. In order to compensate for variations in the preparation of different batches of liposomes a value, called the percentage leakage, was calculated thus:

$$\text{Percentage leakage} = \frac{V}{V + C} \times 100$$

Thus, if the membrane of the vesicle is impermeable to protons, then C will be much larger than V and thus the percentage leakage will be small.

The calculated percentage leakage values for control liposomes, i.e. liposomes without short-chain fatty acids added, at a range of temperatures are shown in Table 9. From Table 9, it can be seen that as the temperature increases, the percentage leakage value increases, i.e. the liposome membrane becomes more permeable to protons.

When nonanoic acid was incorporated into the lecithin liposomes, it was found that 25 to 100 μmoles in the 250 mg of lecithin caused the plot of percentage leakage against temperature to be shifted to the left: i.e. nonanoic acid reduced the temperature required to bring about a certain percentage leakage value. The amount of thermal shift depended on the amount of nonanoic acid incorporated into the liposomes: i.e. the more nonanoic acid, the lower the temperature required. The percentage leakage values for liposomes containing nonanoic acid in the range 25 to 100 μmoles per 250 mg of lecithin are shown in Table 10.

To allow easy comparison between the various percentage leakage values, it was decided to calculate, from the data in Table 10,

Table 9: The mean percentage leakage values and standard errors for control liposomes (i.e. without added short-chain fatty acid) at a range of temperatures. Analysis of variance showed that the temperature effect was significant at better than the 1% level, while the difference between replicates was not. Linear regression of the data yielded the equation:

$$y = 0.75\,x - 9.85$$

with $r = 0.973$.

T/°C	Percentage leakage
20	4.66 ± 1.71
25	8.20 ± 1.84
30	12.67 ± 1.20
35	14.61 ± 1.13
40	18.62 ± 4.64
45	26.25 ± 2.80

Table 10: The mean percentage leakage values and standard errors for liposomes containing various amounts of nonanoic acid per 250 mg (312 μmoles) of lecithin at a range of temperatures. Two way analysis of variance (up to 35°C) showed that the both temperature and the nonanoic acid effects were significant at 1% level. The PL_{17} values (derived by intrapolation) are also shown. These are linearly related to temperature with r = -0.956.

	Amount of nonanoic acid (in μmoles)				
T/°C	0	25	50	100	PL_{17}
20	4.66 ± 1.71	6.79 ± 1.21	8.51 ± 2.97	27.16 ± 4.26	72.8
25	8.20 ± 1.84	8.96 ± 1.15	22.51 ± 3.51	24.00 ± 5.33	39.8
30	12.67 ± 1.20	21.90 ± 3.98	26.24 ± 3.59	22.31 ± 6.91	11.7
35	14.61 ± 1.13	22.03 ± 2.67	33.83 ± 5.25	29.20 ± 3.23	8.0
40	18.62 ± 4.64	22.66 ± 2.67	-	39.07 ± 1.92	-

the amount of nonanoic acid required to bring about a percentage leakage value of 17%, since this is the percentage leakage value for control liposomes at 36°C. This value was denoted the PL_{17} dose. While it could be argued that to choose a PL value of 17 for comparison is artificial, this is admitted. Any other PL value could have been adopted, however the significance of the 17 value lies in its relationship with 36°C for control liposomes. It should be remembered that 36°C appears to be an important physiological temperature to the seed, since it is a thermal block to germination and the cluster point of the G_{50} curves shown in Figure 2.

The PL_{17} doses at a range of temperatures from 20 to 35°C were calculated for nonanoic acid from the percentage leakage values in Table 10, and for hexanoic, heptanoic and octanoic acids from the data in Tables 11, 12, and 13 respectively.

A plot of these calculated PL_{17} doses against temperature is shown in Figure 9. From this it can be seen that there is a linear relationship between the PL_{17} dose and temperature. (It proved impossible to gain an estimate of the PL_{17} dose for hexanoic acid at 20°C, since liposomes would not form when 400 μmoles of hexanoic acid were added to the 250 mg of lecithin.)

DISCUSSION

There is an obvious similarity between the pattern in Figure 9 and that in Figure 2. This would suggest that the short-chain fatty acid is affecting the membrane *in vivo*. It would be worthwhile at this point to say a little about what is known of membrane structure.

The "normal" membrane consists of lipid, proteins and

Table 11: The mean percentage leakage values and standard errors for liposomes containing various amounts of octanoic acid per 250 mg (312 μmoles) of lecithin at a range of temperatures. Two way analysis of variance showed that the temperature effect was significant at 1% level and the octanoic acid at the 5% level. The PL_{17} values (derived by intrapolation) are also shown. These are linearly related to temperature with r = -0.989.

	Amount of octanoic acid (in μmoles)				
T/°C	0	25	50	100	PL_{17}
20	4.66 ± 1.71	3.68 ± 1.21	7.04 ± 1.87	15.86 ± 2.94	106.0
25	8.20 ± 1.84	11.58 ± 2.16	15.39 ± 1.75	22.53 ± 3.42	61.2
30	12.67 ± 1.20	15.49 ± 2.86	17.64 ± 3.18	25.69 ± 5.13	42.4
35	14.61 ± 1.13	39.90 ± 3.81	19.20 ± 2.10	26.75 ± 2.71	2.4
40	18.62 ± 4.64	42.66 ± 3.92	24.41 ± 4.12	39.93 ± 3.10	-

Table 12: The mean percentage leakage values and standard errors for liposomes containing various amounts of heptanoic acid per 250 mg (312 μmoles) of lecithin at a range of temperatures. Two way analysis of variance (up to 100 μmoles and 35°C) showed that the temperature effect was significant at 0.5% level, while the heptanoic acid effect was significant at the 5% level. The PL_{17} values (derived by intrapolation) are also shown. These are linearly related to temperature with r = -0.997.

	Amount of heptanoic acid (in μmoles)					
T/°C	0	25	50	100	200	PL_{17}
20	4.66 ± 1.71	6.38 ± 2.13	4.32 ± 1.41	3.63 ± 0.74	21.73 ± 3.06	173.9
25	8.20 ± 1.84	7.45 ± 0.96	8.82 ± 0.74	16.30 ± 3.12	18.78 ± 3.01	128
30	12.67 ± 1.20	12.88 ± 2.10	11.61 ± 4.17	19.09 ± 3.13	-	86
35	14.61 ± 1.13	16.76 ± 2.86	25.11 ± 3.87	33.30 ± 4.21	-	27.8

Table 13: The mean percentage leakage values and standard errors for liposomes containing various amounts of hexanoic acid per 250 mg (312 μmoles) of lecithin at a range of temperatures. Two way analysis of variance (up to 30°C and 100 μmoles) showed that the temperature effect was significant at the 1% level, while the effect of the hexanoic acid was insignificant. The PL_{17} values (derived by intrapolation) are also shown. These are linearly related to temperature with r = -0.994.

	Amount of hexanoic acid (in μmoles)					
T/°C	0	25	50	100	200	PL_{17}
20	4.66 ± 1.71	4.74 ± 1.32	5.16 ± 0.59	3.72 ± 1.72	7.65 ± 1.12	-
25	8.20 ± 1.84	6.81 ± 1.32	12.26 ± 1.27	11.33 ± 1.98	19.48 ± 1.84	169.6
30	12.67 ± 1.20	10.13 ± 2.84	9.74 ± 2.15	17.49 ± 2.08	-	96.8
35	14.61 ± 1.13	15.91 ± 2.55	17.15 ± 2.42	-	-	46.9

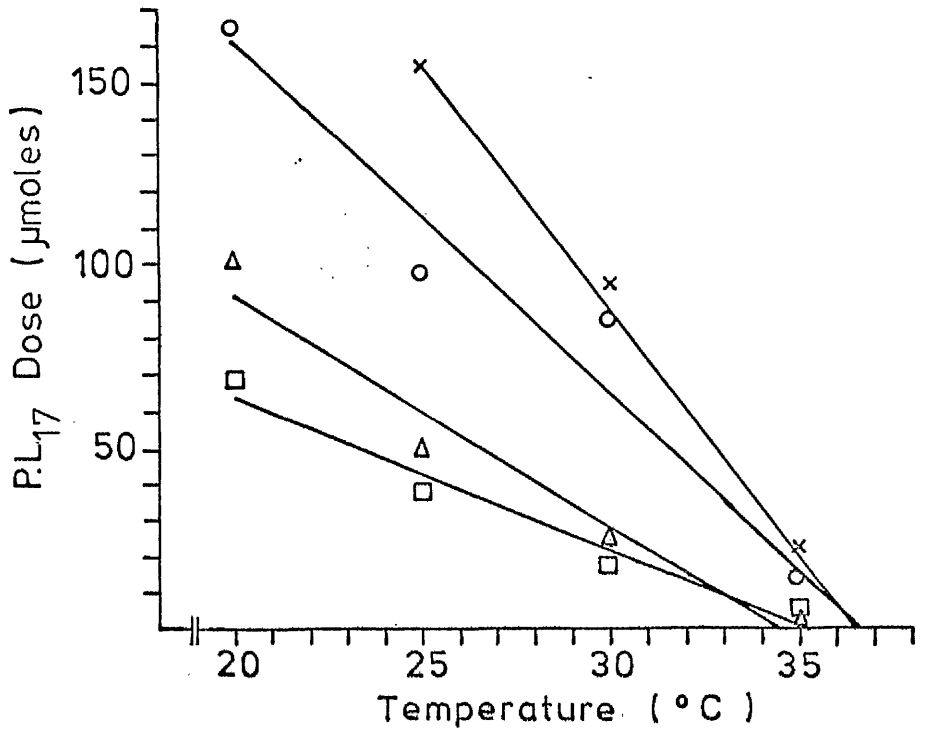


Figure 9: A plot of the calculated dose required to bring about a percentage leakage value of 17 for the four short-chain fatty acids at 20-35°C.

	Acid	r
×	Hexanoic	-0.984
o	Heptanoic	-0.965
Δ	Octanoic	-0.986
□	Nonanoic	-0.999

glycoproteins. In 1935, Danielli and Davson proposed a unit membrane model, in which a lipid bilayer is sandwiched between two layers of protein. However, this model was found to be incompatible with later evidence on several points. Singer and Nicholson (1972) proposed a fluid mosaic model of membrane structure. The basis of this model is a bilayer of lipid intercalated to various extents by proteins. The lipids and integral proteins are thus disposed in a mosaic arrangement. They suggest that biological membranes are quasi-fluid structures in which both lipids and integral proteins are able to perform translational movements within the overall bilayer. This fluidity implies that the main components, i.e. lipids, proteins and oligosaccharides, are held in place by non-covalent interactions. This is shown by the fact that components of the membrane can be dispersed by solvents, detergents or denaturing agents which do not involve the breaking of truly chemical bonds.

Lipids and many of the intrinsic proteins are amphipathic molecules, i.e. they have hydrophobic and hydrophilic groups on one molecule. These amphipathic molecules are arranged in the thermodynamically favourable form of a bilayer, in which the polar groups are directed towards the water phase and the non-polar groups are inside the bilayer. It is possible that a protein molecule, or a group thereof may pass across the entire membrane. Such traversing proteins could be in contact with the aqueous solvent on both sides of the membrane. However, current knowledge simply does not permit the formulation of any specific membrane model and it is considered unlikely that there is one unique structure common to all membranes.

Phase transitions have been observed, by physical methods, in the membranes of micro-organisms (Reineat and Steim, 1970;

Overath and Trauble, 1973). These changes correlate well with the thermotropic phase transitions typical of phospholipid systems (Overath and Trauble, 1973). The phase transition is a change in the degree of the lipid-water organisation as from partial order (gel) towards disorder, or as an extreme from crystalline (highly organised) to a liquid-crystalline (mesomorphic) state. The gel phase only superficially resembles the gel encountered in cross-linked polymers: the fatty acid chain of the phospholipids may undergo restricted rotation about their long axis, but not about carbon to carbon bonds (Steim, 1972). In the liquid-crystal state the hydrophilic head groups remain tightly packed, but the fatty acyl residues are more fluid and disordered than in the gel. This increase in disorder is much less than that produced by melting n-alkanes, but increases with distance along the chain from the polar head (Levine et al., 1972). The phase transition is accompanied by a decrease in the width of the bilayer, and an increase in its surface area (Trauble and Haynes, 1971).

The temperature at which the phase transitions occur is dependant on the head group of the lipid and the length and degree of saturation of the fatty acyl chains (Chapman, 1975). Heterogeneity of lipid species, which always occurs in nature, can complicate the process. In this case a single sharp transition is not seen: rather, at intermediate temperatures there is segregation of the phospholipid components of a mixture into domains of crystalline and liquid-crystalline structure (Verkleij et al., 1972; Ververgaert, 1973) termed phase separations. The presence of sterols further complicates the process (Phillips, 1972). Below the transition sterols interrupt the packing of the fatty acyl chains, thus increasing fluidity, while in the liquid-crystal phase, the sterols restrict the mobility of the fatty acyl

chains, thus reducing the fluidity. In this way, sterols act as "fluidity buffers".

Rothman (1973) calculated the entropy of the transition for lipids with various fatty acyl chain lengths. He found that the change in entropy was linearly related to chain length and extrapolation of this relationship suggested that the change in entropy would be zero with chain length 6-9. The exact value was dependant on the degree of saturation of the phospholipid. This value is believed to correspond to the number of carbon atoms near the glycerol backbone which do not take part in the transition, i.e. they are held in the all-trans configuration even in the liquid-crystal phase. This is shown in Figure 10(b). This means that the motion of any individual "stearoyl" residue, in the liquid crystal phase will describe a cone, with long axis oriented parallel to the membrane normal, and with its apex at the sixth to ninth carbon from glycerol backbone. This is represented in Figure 11.

There are three major possible modes of action of the short-chain fatty acid, acting on the membrane.

Firstly, it has been suggested that the short-chain fatty acids dimerise, by hydrogen bonding between two adjacent carboxyl groups, in a non-polar solvent (Markley, 1960). It is obvious why this would be thermodynamically favourable, as such a dimerisation would tend to cancel out the polarity of the carboxyl group. A short-chain fatty acid dimer, thus formed, would then approximate to the chain lengths commonly encountered in a membrane. However, it seems unlikely that such a dimerisation would occur in a membrane, as even the diminished polarity of a pair of hydrogen-bonded carboxyl groups would not be expected to associate with the non-polar hydrocarbon core of the bilayer: also in such an

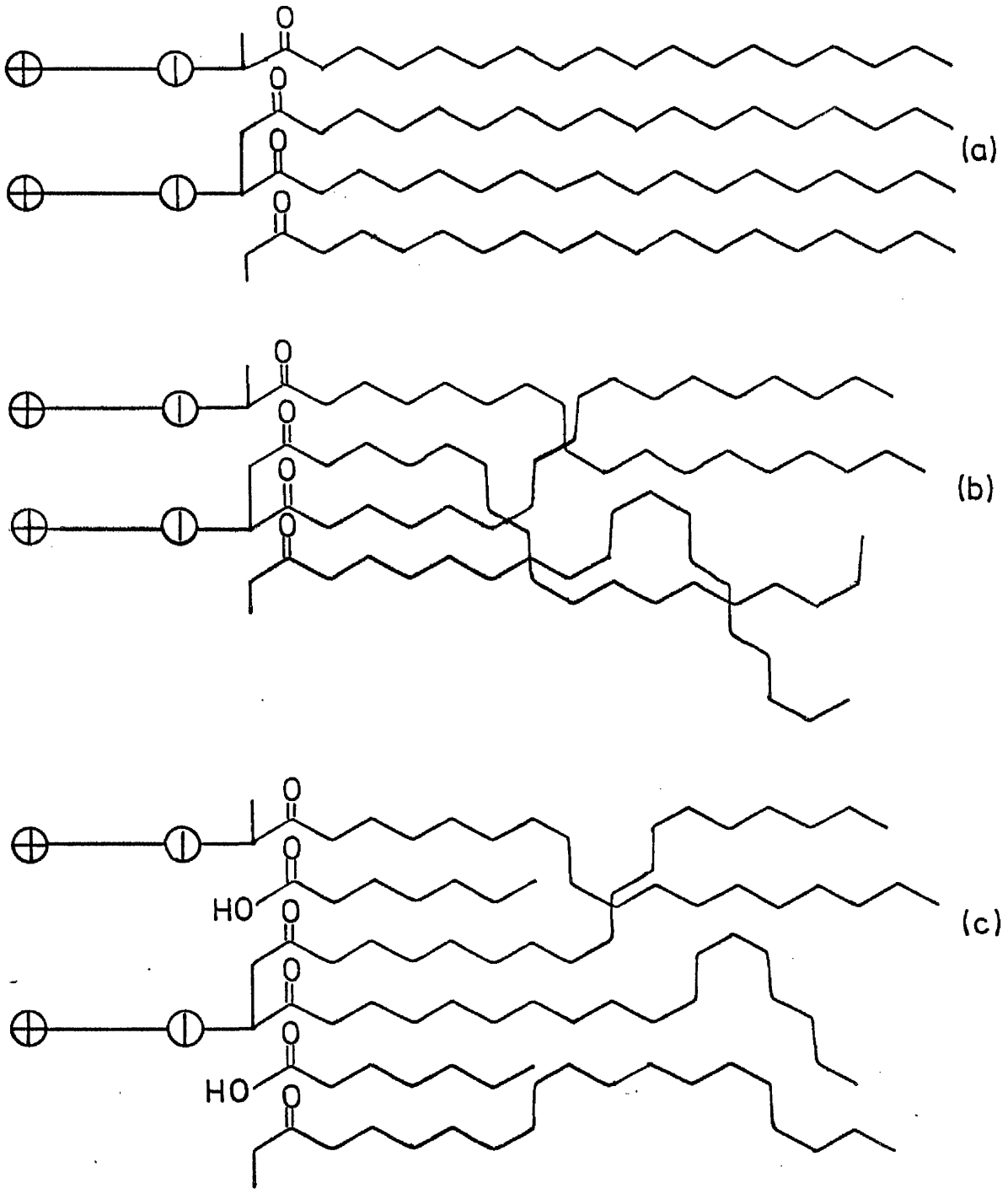


Figure 10: A diagram of half of a lipid bilayer.

(a) In the quasi-crystalline state, all carbon-carbon bonds are in the trans configuration.

(b) In the liquid phase, gauche configuration are present in the "stearoyl" residues: only the 6-9 carbon nearest the glycerol backbone are held in the trans configuration.

(c) The hypothetical manner in which a short-chain fatty acid (in this case hexanoic acid) may be incorporated into a bilayer, and will reduce the interactions between the "stearoyl" residues.

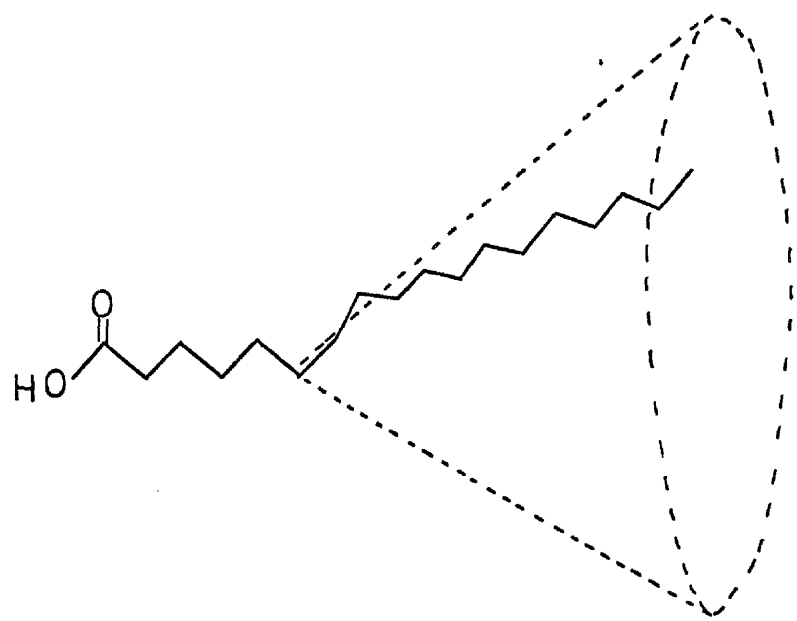


Figure 11: The mobility of a single "stearoyl" residue in the liquid phase. The first 6-9 carbons, nearest the glycerol backbone, are rigidly held in the all-trans configuration. Beyond this limit, there is a proportional increase in the mobility along the chain. Thus, the locus of permitted movements forms a cone with apex at the 6-9 carbon.

orientation one acyl end of the dimer would be proximal to the polar head groups of the bilayer. Such a juxtaposition of polar and non-polar groups would not be considered to be thermodynamically favourable.

Secondly, it is interesting to note that the all-trans chain length of Rothman (1973) is similar to the active chain lengths in these studies. This led to the development of an hypothesis that when a short-chain fatty acid is incorporated into a lipid bilayer, the fatty acid becomes intercalated between the lipid molecules, with the carboxyl group oriented towards the aqueous phase, between the lipid head groups. The short-chain fatty acid would thus reduce the interaction between the distal regions of the neighbouring "stearoyl" residues. The incorporation of the short-chain fatty acid would thus mimic the effect of elevated temperature, as shown in Figure 10(c).

Thirdly, it has been reported that the short-chain fatty acids act as uncouplers of oxidative phosphorylation in bacteria and fungi (Hochter and Quastel, 1963). It is possible that this uncoupler activity is the source of the apparent increase in permeability of the liposomes, since proton permeability alone was followed. However, if the short-chain fatty acids were merely acting as proton carriers, such a clear interaction with temperature, as shown in Figure 9, would not be expected. Similarly, if the short-chain fatty acid were inhibiting seed germination, simply because it is an uncoupler, an interaction with temperature and phytochrome would not be expected.

In summary, the short-chain fatty acids appear to affect membranes, increasing the permeability. Care must be excercised, however, as at least part of this apparent effect may be due to the fatty acid acting as an uncoupler.

L I P I D B I L A Y E R F L U I D I T Y

INTRODUCTION

It was shown in the previous section that short-chain fatty acids appear to affect liposome permeability. It was suggested that this action may be due to the short-chain fatty acid artificially fluidising the membrane, however this can only be considered an hypothesis. It is obviously necessary to try to substantiate this hypothesis. The method of choice to follow any changes in membrane fluidity would be to use "spin-labelled" stearic acid analogues. The spin-labelled compound contains a long-lived nitroxide radical which, by virtue of its unpaired electron, exhibits paramagnetism.

When a paramagnetic species is placed in a magnetic field, it can adopt one of two orientations relative to the magnetic field: a high energy configuration in which the net magnetic moment of the spin-label (designated μ_z) is oriented parallel to the applied field (Ho) and a low energy configuration in which the net magnetic moment is anti-parallel to Ho. Thus the energy difference between the two orientations is $2\ \mu_z$ Ho (Knowles et al., 1976).

Transitions between the two levels can be induced by electromagnetic radiation of frequency ν, such that

$$h\nu = 2\ \mu_z\ Ho$$

where h = Planck's constant.

This condition is termed resonance, and since before resonance the low energy state will be preferred, resonance may be seen as a net absorption of the applied electromagnetic radiation. For

a given species (i.e. constant μ_z) only one value of ν will cause transitions at a constant field strength Ho. Thus, if ν is fixed and the magnetic field is swept over a range, all paramagnetic species of a single type should undergo transitions at the same Ho value. This was apparently the case when the first Electron Paramagnetic Resonance Spectroscopy (EPR) experiments were performed. However, with the development of more precise instruments, distinctions could be noted. An example of an EPR spectrum for a spin-labelled stearate analogue is shown in Figure 12, which shows the normal trace of an EPR: the first derivative of the absorption (i.e. $\frac{\text{d. absorption}}{\text{d. Ho}}$) rather than the absorption itself.

From Figure 12, it can be seen that the "theoretical single" peak of the nitroxide radicle has become three peaks. This is termed hyperfine splitting. An EPR is defined by two values, the g-value, which shows the position of the centre peak, and the hyperfine constant, A, which is a measure of the splittings. Both these values are shown in Figure 12. This hyperfine splitting arises from the interaction of the paramagnetic electron with the magnetic moments of neighbouring nuclei.

Nitroxide radicles exhibit, what is termed, spectral anisotropy, i.e. the spectrum obtained is dependant on the orientation of the doxyl group relative to the applied magnetic field. The most marked difference is in the hyperfine constants (A) obtained with different orientations. This is shown in Figure 13. It is possible to define three mutually perpendicular axes relative to the doxyl group. The Z-axis lies in the plane containing the P(π) orbital in which the unpaired electron is largely located. If the magnetic field is oriented parallel to the Z-axis, then a hyperfine constant, designated A_{zz}, of 3.2 mtesla is obtained. If the magnetic field is oriented parallel to either of the other

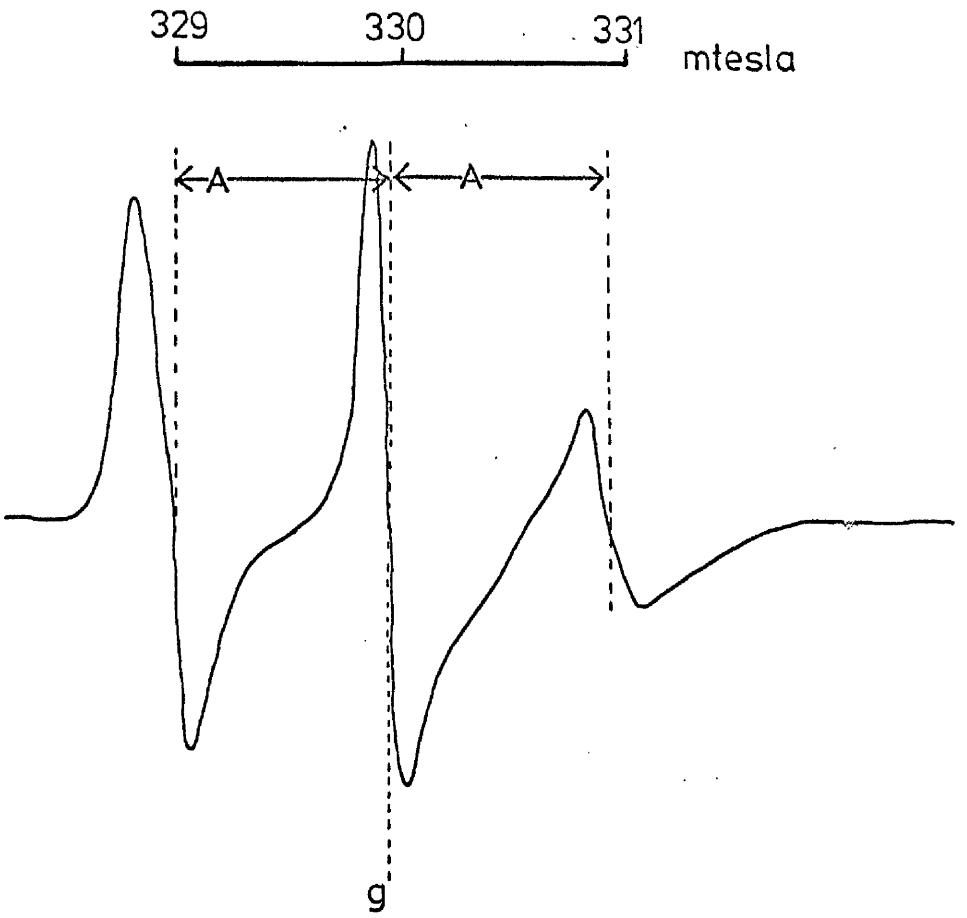


Figure 12: The electron paramagnetic resonance spectrum of an isotropically tumbling nitroxide radical in solution.

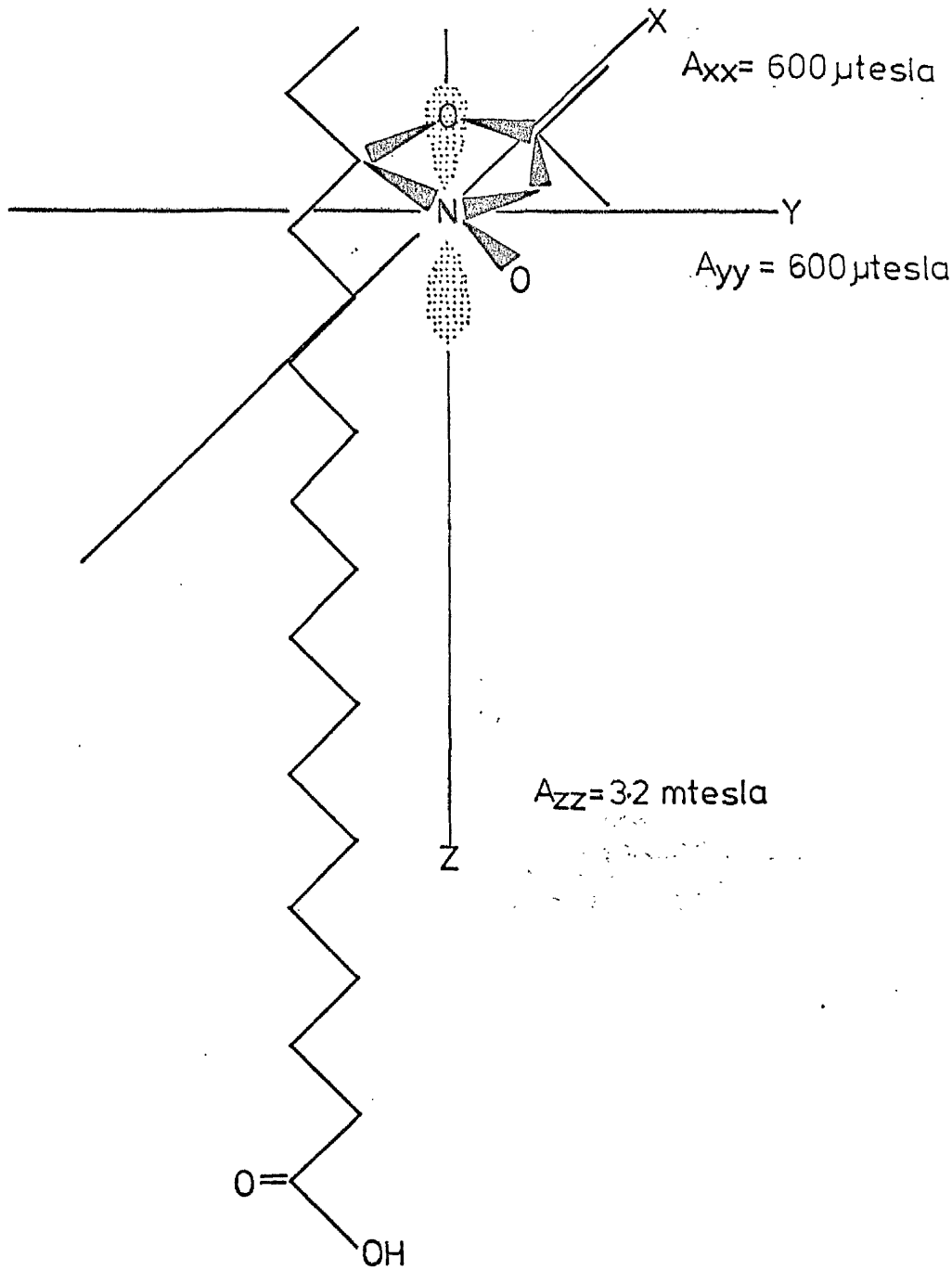


Figure 13: A diagram of 16-doxyl stearate showing the principal axes, and the anisotropy of the hyperfine splitting constant A. Thus, the maximum hyperfine splitting is seen along the Z-axis, i.e. the long axis of the molecule, and the minimum along the x and the y-axes. Also shown is the Pπ orbital, in which the unpaired electron, which gives rise to the paramagnetism, is largely located.

primary axes, i.e. x or y axis, then a hyperfine constant, designated A_{xx} or A_{yy} respectively, of 600 μtesla is obtained (e.g. Griffith and Waggoner, 1969).

If a spin-labelled molecule is moving freely and randomly in solution, it is performing isotropic motion and no anisotropy will be seen, since the spectral anisotropy will be averaged out due to the molecules adopting random orientations relative to the applied magnetic field, and a hyperfine constant intermediate between the 3.2 mtesla and 600 μtesla principal axes maxima and minima would be obtained.

If a spin-labelled stearic acid analogue were held rigidly within a lipid bilayer, as shown in Figure 14(a), then the maximum hyperfine splitting will be obtained with the applied magnetic field in a direction parallel to the membrane normal, designated the $\parallel$ direction, since this is along the principal Z-axis. Similarly, the minimum hyperfine splitting will be obtained with the applied magnetic field perpendicular to the membrane normal, designated the $\perp$ direction. Thus if a spin-labelled stearate is held rigidly then,

$$A_{\parallel} = A_{zz} = 3.2 \text{ mtesla}$$

also,

$$A_{\perp} = A_{xx} = A_{yy} = 600 \text{ μtesla}$$

If the spin-labelled molecule undergoes rapid anisotropic motion of limited amplitude, e.g. motion in a cone (as in Figure 11 and Figure 14(b)), then part, but not all, of the spectral anisotropy will be averaged. Thus $A_{\parallel}$ will be less than A_{zz} and $A_{\perp}$ will be greater than A_{xx}. The spectral anisotropy ($A_{\parallel} - A_{\perp}$) will therefore be less than 2.6 mtesla ($A_{zz} - A_{xx}$). The values $A_{\parallel}$ and $A_{\perp}$ may be derived from a nitroxide spectrum as shown in Figure 15. From these values a measure of the averaging may be

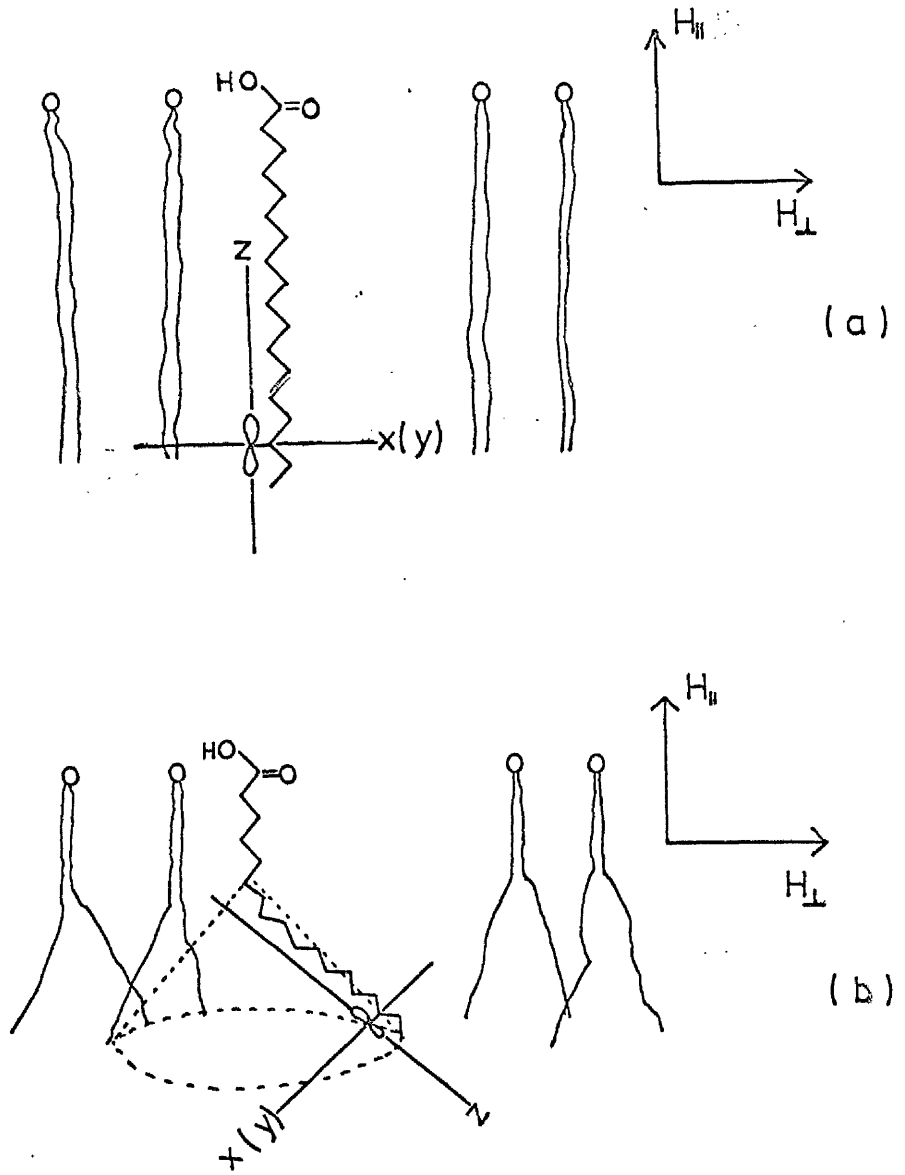


Figure 14: The effect of the phase of the membrane lipids on the spectral anisotropy.

(a) If the lipids are in the crystalline phase, the doxyl group is held such that the z-axis is parallel to the membrane normal. Thus if the magnetic field is oriented parallel to the membrane normal, $H_{\parallel}$, then a hyperfine splitting constant of 3.2 mtesla is seen. If the field is at right angles to the membrane normal, $H_{\perp}$, a hyperfine splitting of 600 μtesla is seen. Thus $A_{\parallel}$ and $A_{\perp}$ are equivalent to the principal axes values A_{zz} and A_{xx} respectively.

(b) If the lipids are in the liquid phase, then some of the spectral anisotropy will be averaged out. Thus $A_{\parallel}$ will be less than A_{zz} and $A_{\perp}$ will be greater than A_{xx}.

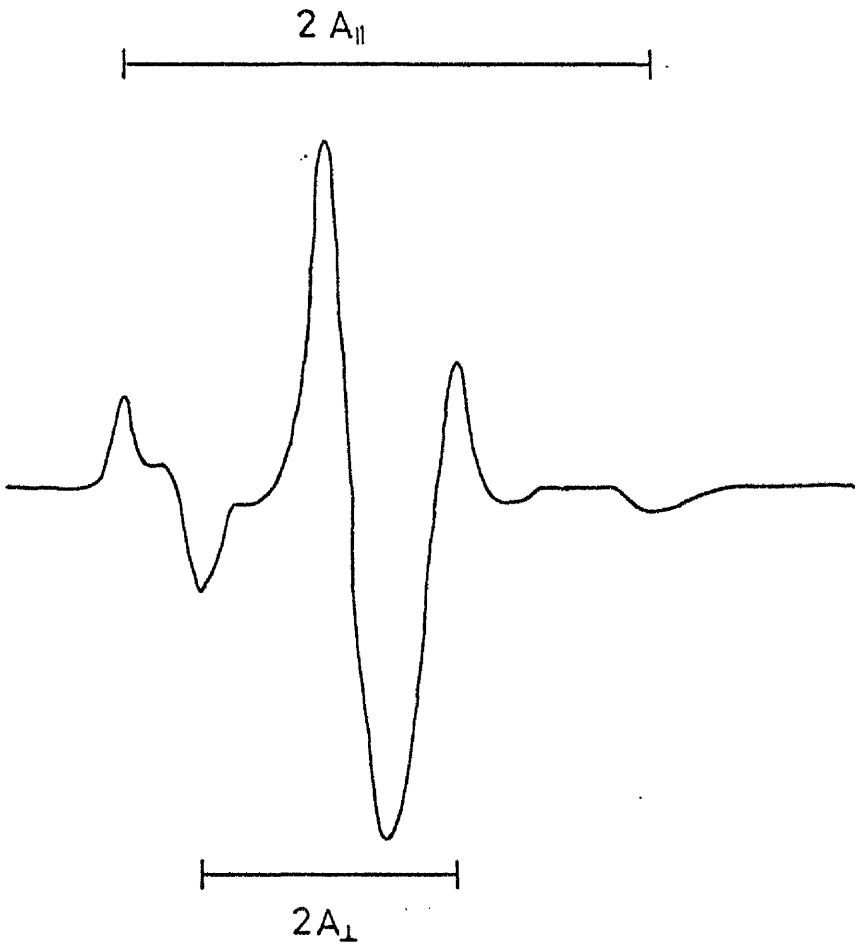


Figure 15: A theoretical nitroxide spectrum of a dispersion of liposomes containing spin-labelled analogues in water. This shows how $A_{||}$ and $A_{\perp}$ values may be extracted from a spectrum. (from Knowles *et al.*, 1976)

obtained by calculating the order parameter, S, such that

$$S = \frac{A_{||} - A_{\perp}}{A_{zz} - A_{xx}}$$

S is simply the ratio of the observed spectral anisotropy to the 2.6 mtesla theoretical maximum of the completely rigid principal axes values. Thus, S = 1 implies total rigidity and S = 0 implies completely isotropic motion.

In this way spin-labelled stearic acid analogues are very useful to show changes in the degree of fluidity of a bilayer.

In the previous section it was hypothesised that the incorporation of a short-chain fatty acid into a lipid bilayer would increase the fluidity of the core of the bilayer. The best method for testing this hypothesis is to use two doxyl stearate analogues: one in which the spin-label is in the 5-position, and one in which the spin-label is in the 16-position. In the crystalline phase, both of these spin-labels should be relatively restricted. This is shown in Figure 16. According to Rothman (1973) in the liquid-crystal phase, a 16-doxyl stearate spin-label should show comparatively free motion, while a label in the 5-position should not. This is shown in Figure 17. It should be remembered that in the liquid-crystal phase, as in Figure 17, the "stearoyl" residue undergo rapid molecular motion. Each gauche configuration is short-lived, but there is an average of two per chain at any instant. It should also be remembered that any individual "stearoyl" chain undergoes motion in a cone, as shown in Figure 11, and therefore the diagram in Figure 17 should be looked on as a two-dimensional representation of a three-dimensional system.

If our hypothesis is correct, then the incorporation of a

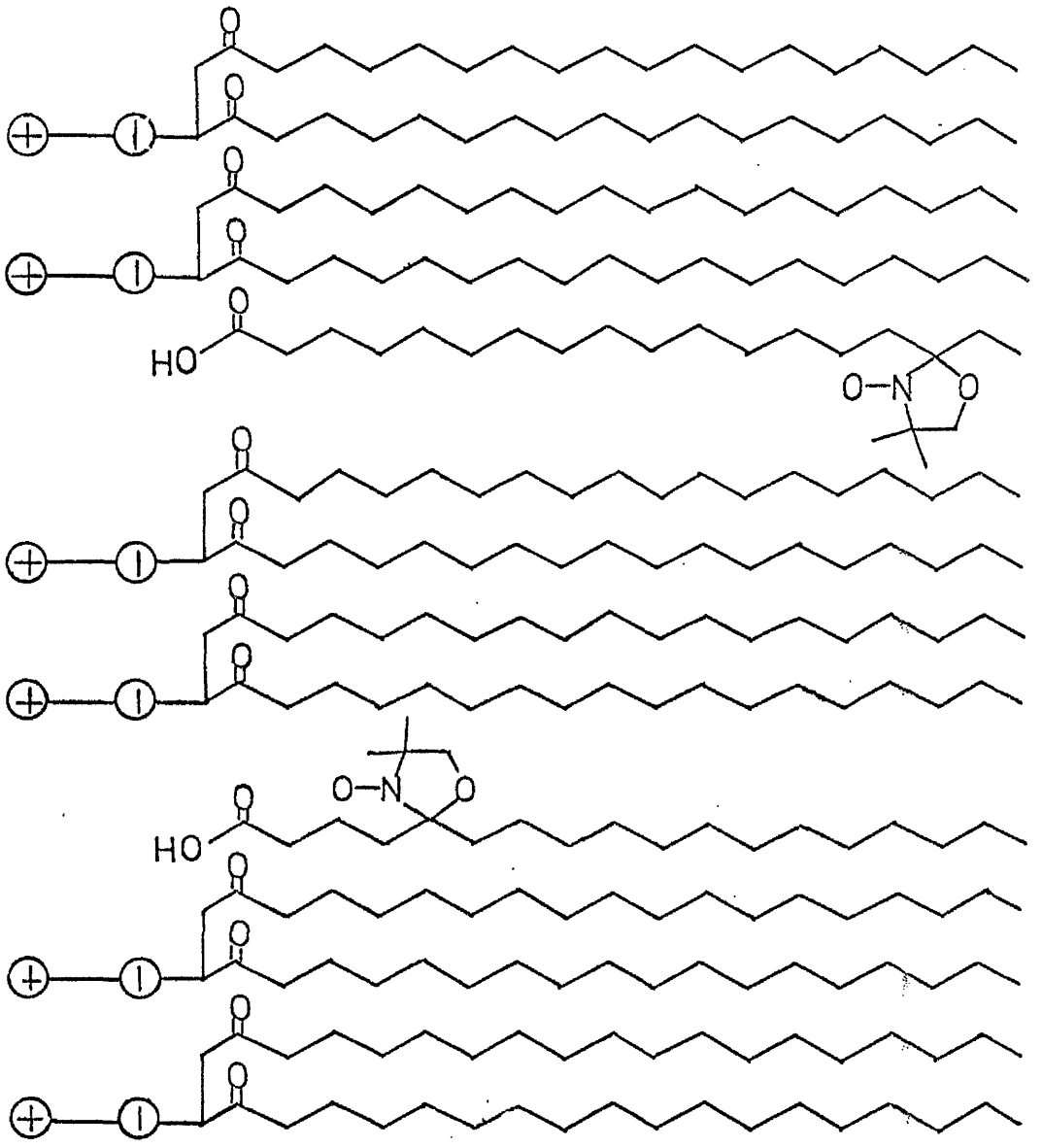


Figure 16: A lipid bilayer, containing spin-labelled stearate analogues, in the crystalline phase. Thus, both the 5-doxyl and 16-doxyl stearate analogues would give spectra indicative of relatively restricted motion. (Obviously, both 5-doxyl and 16-doxyl stearate analogues would not be incorporated into the bilayer at the same time.)

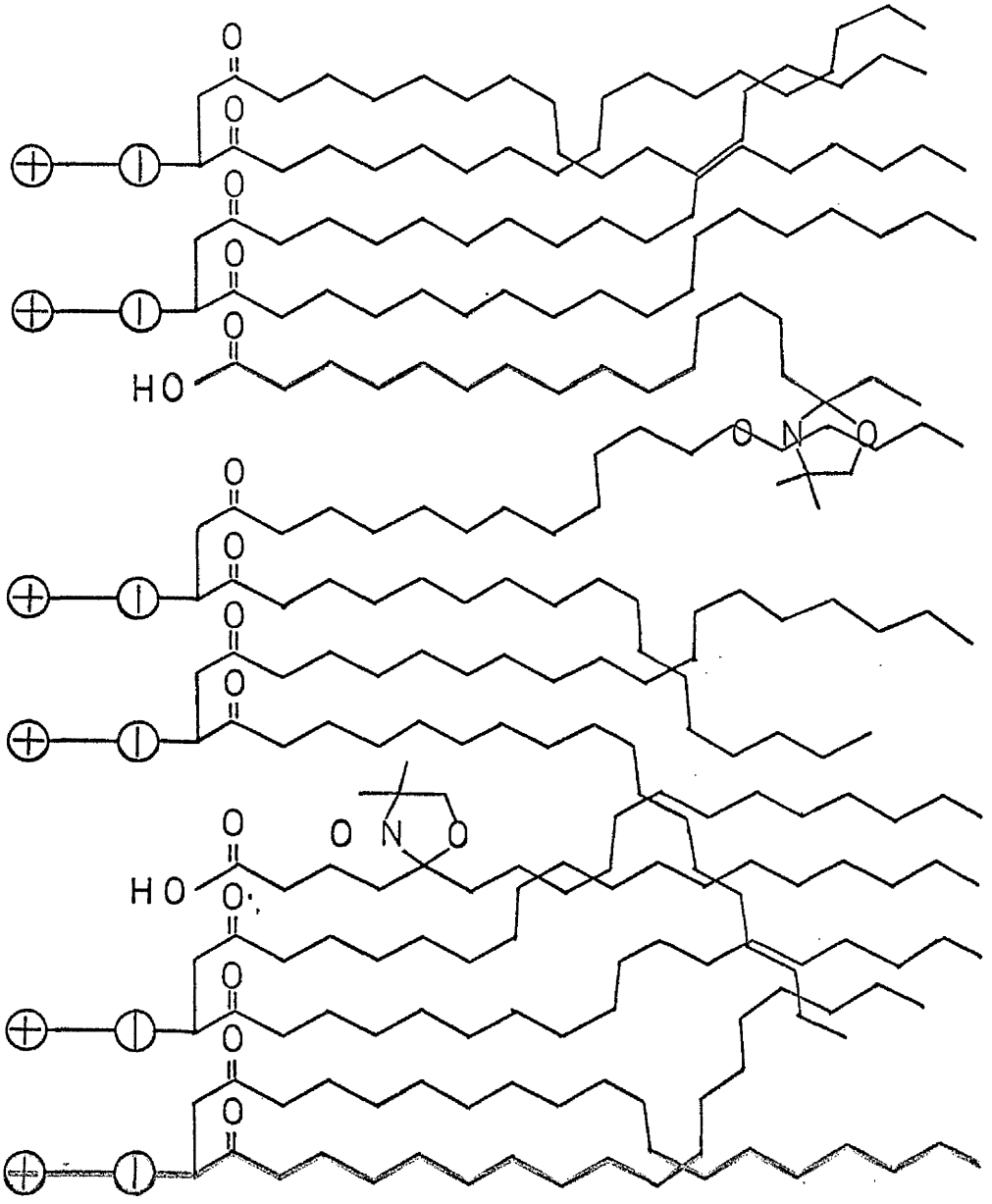


Figure 17: A lipid bilayer, containing spin-labelled stearate analogues, in the liquid phase. The 16-doxyl stearate will give a spectrum indicative of relatively free mobility, while that of the 5-doxyl will be indicative of restricted mobility.

short-chain fatty acid into a bilayer should increase the mobility of a spin-label at the 16-position, but not at the 5-position. This is diagrammed in Figure 18.

MATERIALS AND METHODS

5-Doxyl stearate and 16-doxyl stearate (the $4^1,4^1$-dimethyl-oxazolidine-N-oxyl derivative of 5 and 16 keto-stearic acid respectively) were obtained from Synvar Associates, 3221 Porter Drive, Palo Alta, California, 94304.

Lecithin (egg) was obtained from British Drug Houses. The lecithin was dissolved in Analar chloroform at 59 mg/cm^3, and the doxyl stearate was added to give a mole ratio of 200:1 (lecithin:doxyl stearate). 20 mm^3/cm^3 of distilled water was added, so that on removal of the chloroform the lecithin had a water content of approximately 25%.

Before use, the above solution was shaken to ensure dispersion, as there was a tendency for the hydrated lipid to form a layer on the glass round the solvent level. The solution was streaked onto one side of a Spectrosil quartz probe, and onto one side of a Spectrosil quartz "coverslip". The chloroform was blown off under wet air and the coverslip was placed over the probe, with coated faces together, and sealed in position with "Bostik" clear adhesive.

Spectra were recorded on a Newport Instruments electron resonance spectrometer with a fixed micro-wave frequency of 927 MHz, care being taken to ensure that the sample was not saturated with the micro-waves. The magnetic field was swept from 325 mtesla to 340 mtesla, with markers at 330 and 340 mtesla from a proton magnetometer.

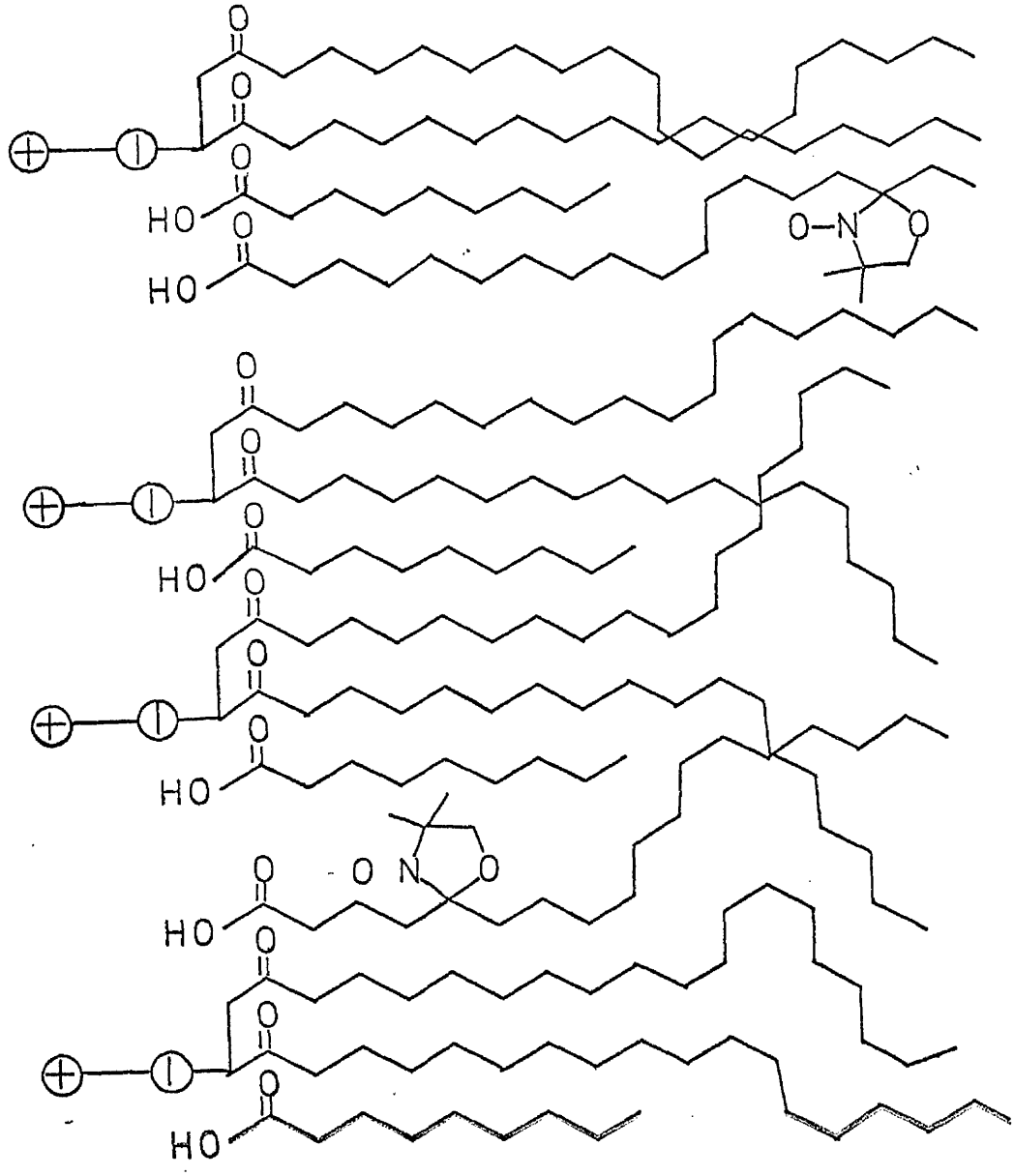


Figure 18: A lipid bilayer, containing spin-labelled stearate analogues, into which nonanoic acid has become incorporated. The nonanoic acid would reduce the interaction between the distal ends of the "stearoyl" chains. Thus 16-doxyl stearate will give a spectrum indicative of relatively free mobility, while that of 5-doxyl stearate will be indicative of restricted mobility.

Temperature was varied by passing heated (or cooled) nitrogen over the probe *in situ*, between the magnetics of the spectrometer. The temperature was checked by means of a copper:constantin thermocouple located close to the probe, with reference at the temperature of melting ice.

RESULTS

The first spectra to be recorded were those for lecithin bilayers including 16-doxyl stearate. These are shown, for a range of temperatures, in Figure 19. At least two spectra were recorded at each temperature, and reversibility of the temperature-induced changes was checked. From Figure 19, it can be seen that the spin-label is relatively mobile, however the high-field peak (i.e. the peak at approximately 332 mtesla) is seen to be split into two sub-peaks. It can also be seen that the ratio of the intensity of these peaks changes with temperature, the higher field sub-peak decreasing in intensity as the temperature increases, until it disappears completely at 50°C. These spectra are interpreted on the basis that the lower field sub-peak corresponds to the spin-label being in a region of fluidity, while the higher field sub-peak corresponds to the spin-label being located in a region of crystallinity: as the temperature is raised the amount of lipid present in crystalline "domains" is reduced. It should be remembered that the bilayer is made from egg lecithin, i.e. a natural phospho-lipid, thus the lipid is heterogeneous and would thus be expected to exhibit phase separations over a wide temperature range, rather than a single sharp phase transition.

When nonanoic acid was incorporated into the lecithin bilayers (at 10% mole to mole with the lecithin), it was found to increase

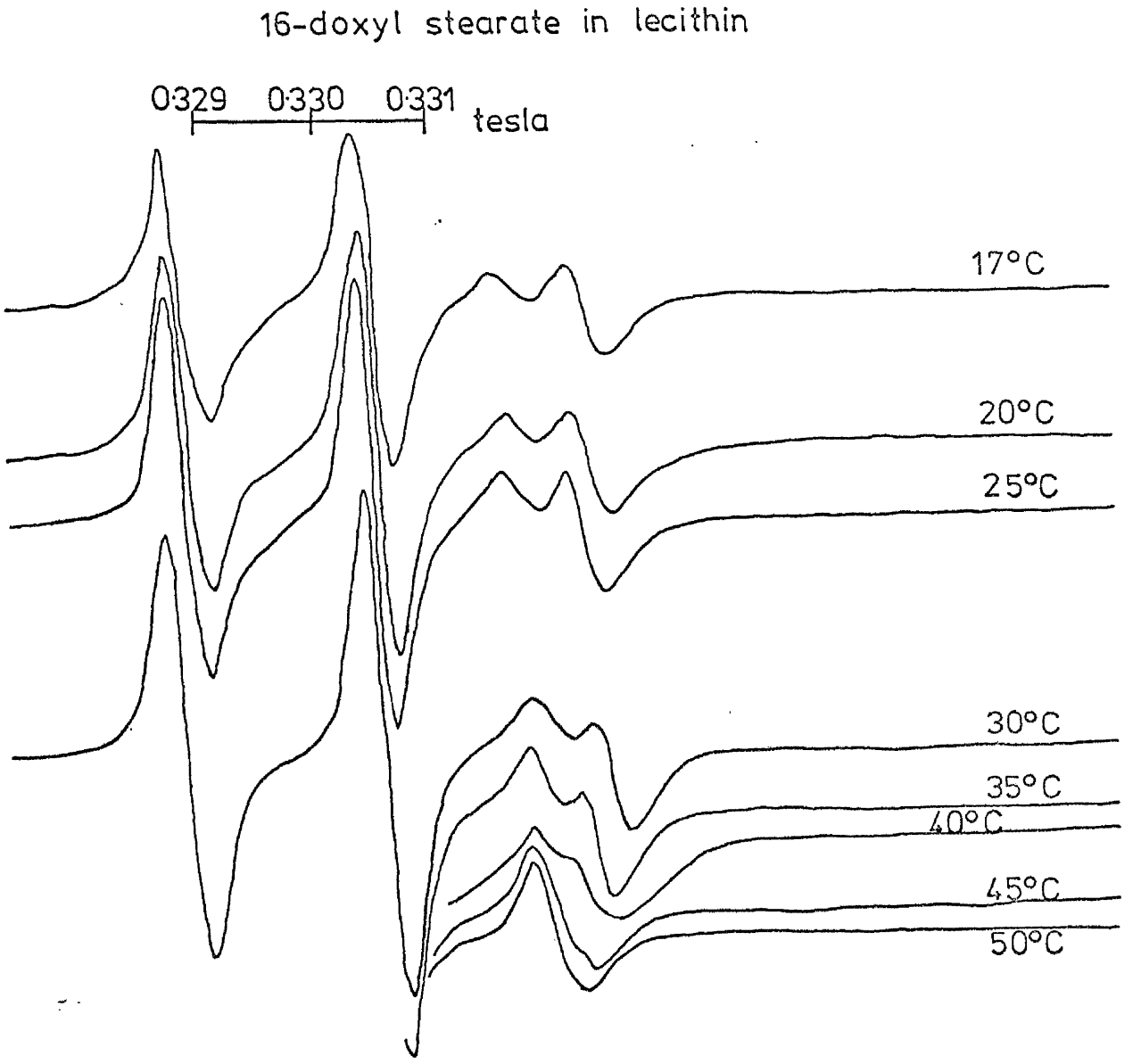


Figure 19: The electron paramagnetic resonance spectra of 16-doxyl stearate in lecithin bilayers. The high-field peak is seen to be split into two sub-peaks, the ratio of which changes with temperature. It would appear that the higher field sub-peak corresponds to a region of crystallinity, which reduces with increasing temperature.

the mobility of the 16-doxyl stearate spin label. This is shown in Figure 20. When the peaks in Figure 20 are compared with those in Figure 19, it is evident that there is considerable sharpening of the peaks on incorporation of nonanoic acid. It can also be seen that the high-field peak is no longer split: the sub-peak corresponding to a region of crystallinity is no longer seen, even at 20°C. The incorporation of nonanoic acid into a bilayer thus appears to substitute for temperature in increasing fluidity.

When spectra were recorded from 5-doxyl stearate in the lecithin, the spectra of an immobile spin-label was noted. This is shown in Figure 21. From this, it can be seen that the high-field peak has become much broadened, compared with the spectra from the 16-doxyl stearate. It should be remembered that this trace is the first derivative of absorption, so the plateau at zero gradient, followed by a negative gradient seen as the high-field peak is representative of absorption over a wide range. It can also be seen from Figure 21 that the low-field peak has become split into two sub-peaks, and the ratio of these two peaks changes with temperature, as did those of the high field peak of the 16-doxyl stearate in Figure 19.

When bilayers were made up containing both 5-doxyl stearic acid and nonanoic acid (See Figure 22), there was little difference between the spectra recorded from those shown in Figure 21. There was some sharpening of the mid-field peak, but there was not as profound an alteration as was noted for the 16-doxyl stearate.

The hyperfine splitting constants ($A_{\parallel}$ and $A_{\perp}$) were measured from the spectra as shown in Figure 15. From these, the order parameter, S, was calculated as detailed previously. A plot of

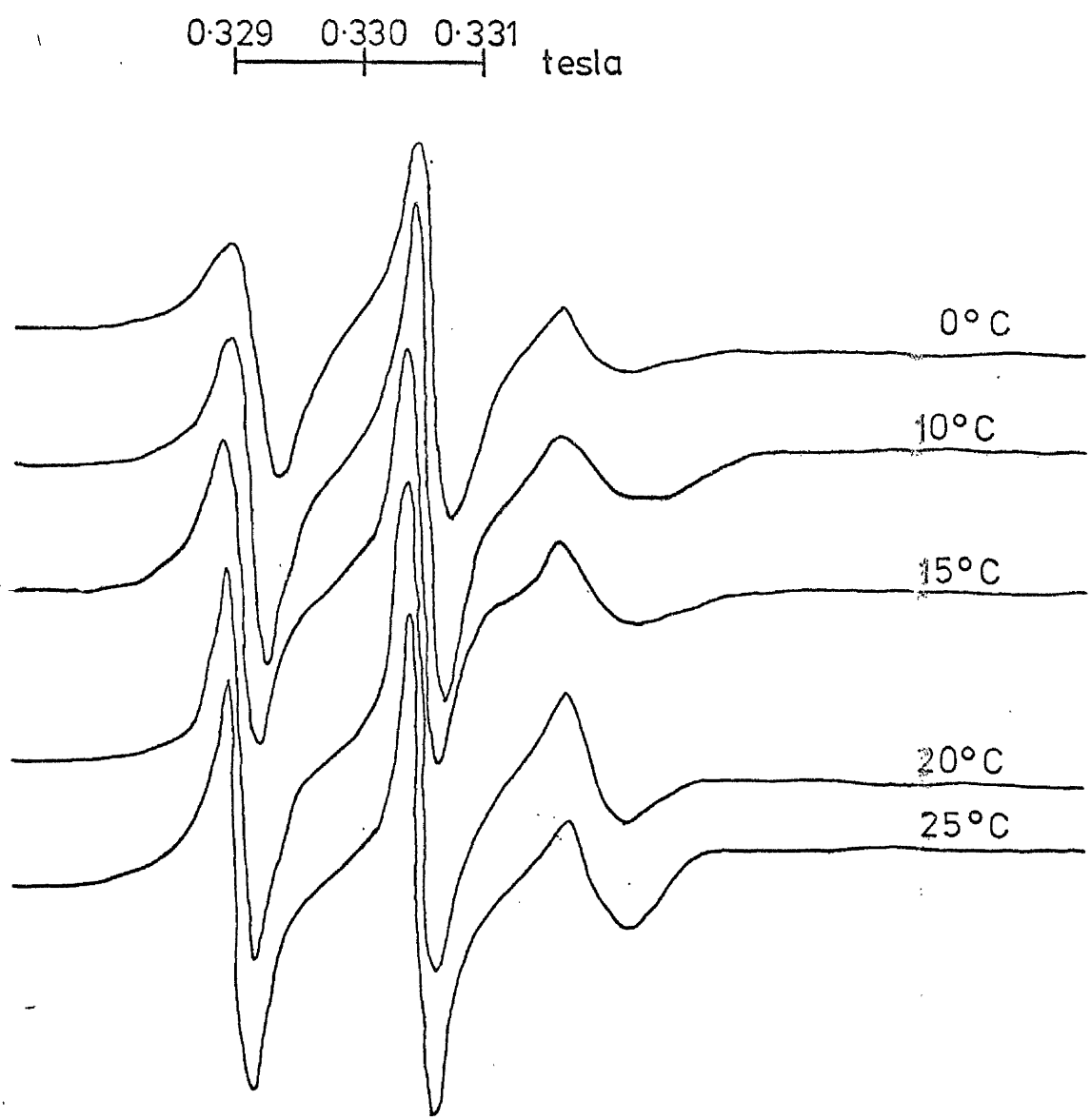


Figure 20: The electron paramagnetic resonance spectra of 16-doxyl stearate in lecithin bilayers containing nonanoic acid (10:1, mole to mole, lecithin to nonanoic acid). The high-field peak is no longer split (cf. Figure 19), that sub-peak which corresponded to the region of crystallinity is no longer seen.

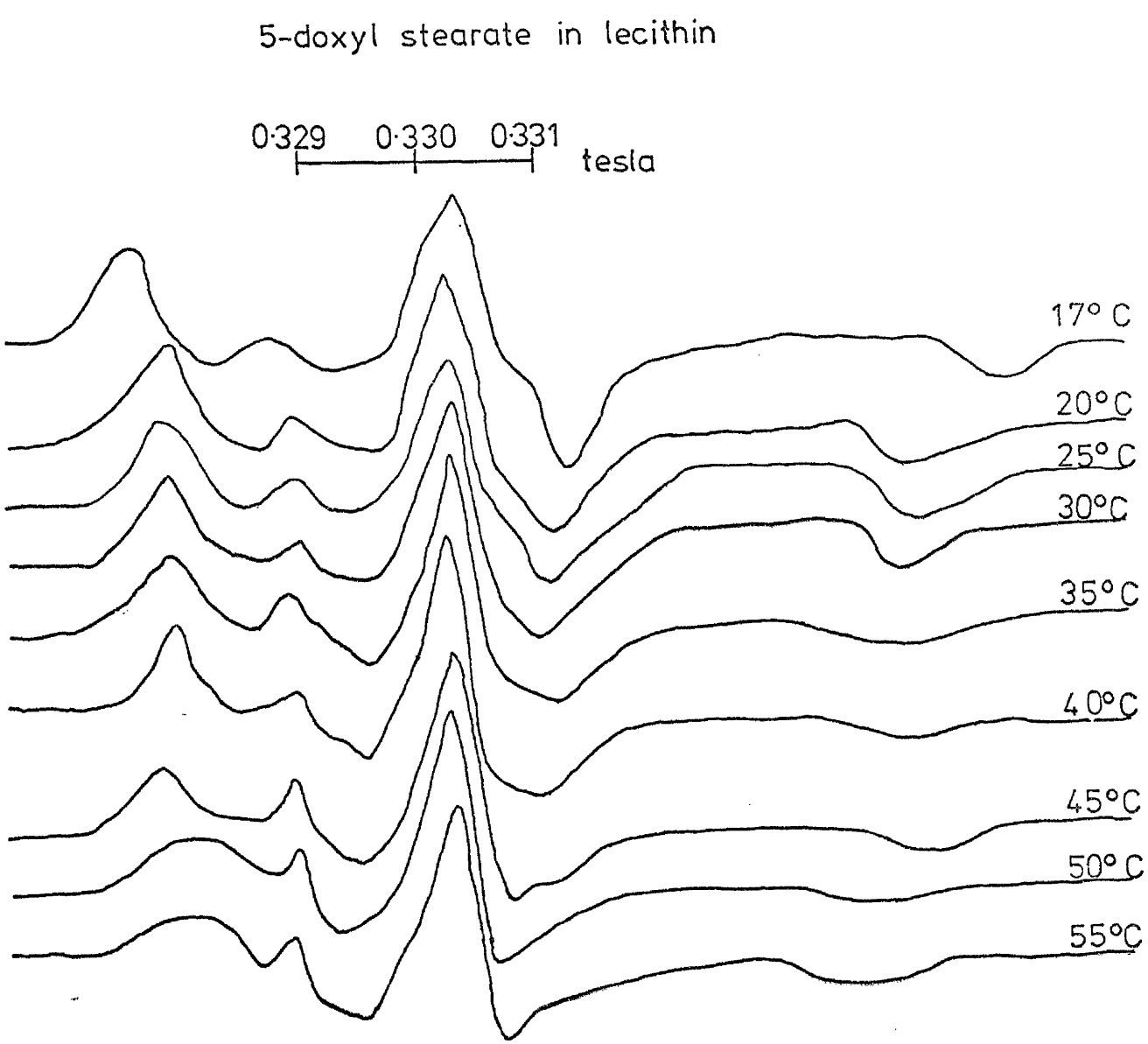


Figure 21: The electron paramagnetic resonance spectra of 5-doxyl stearate in lecithin. These spectra are indicative of restricted mobility. In these the low-field peak is split in two: the ratio of these changes with temperature. This is interpreted as the lower of the sub-peaks corresponding to a region of crystallinity, which decreases as the temperature increases.

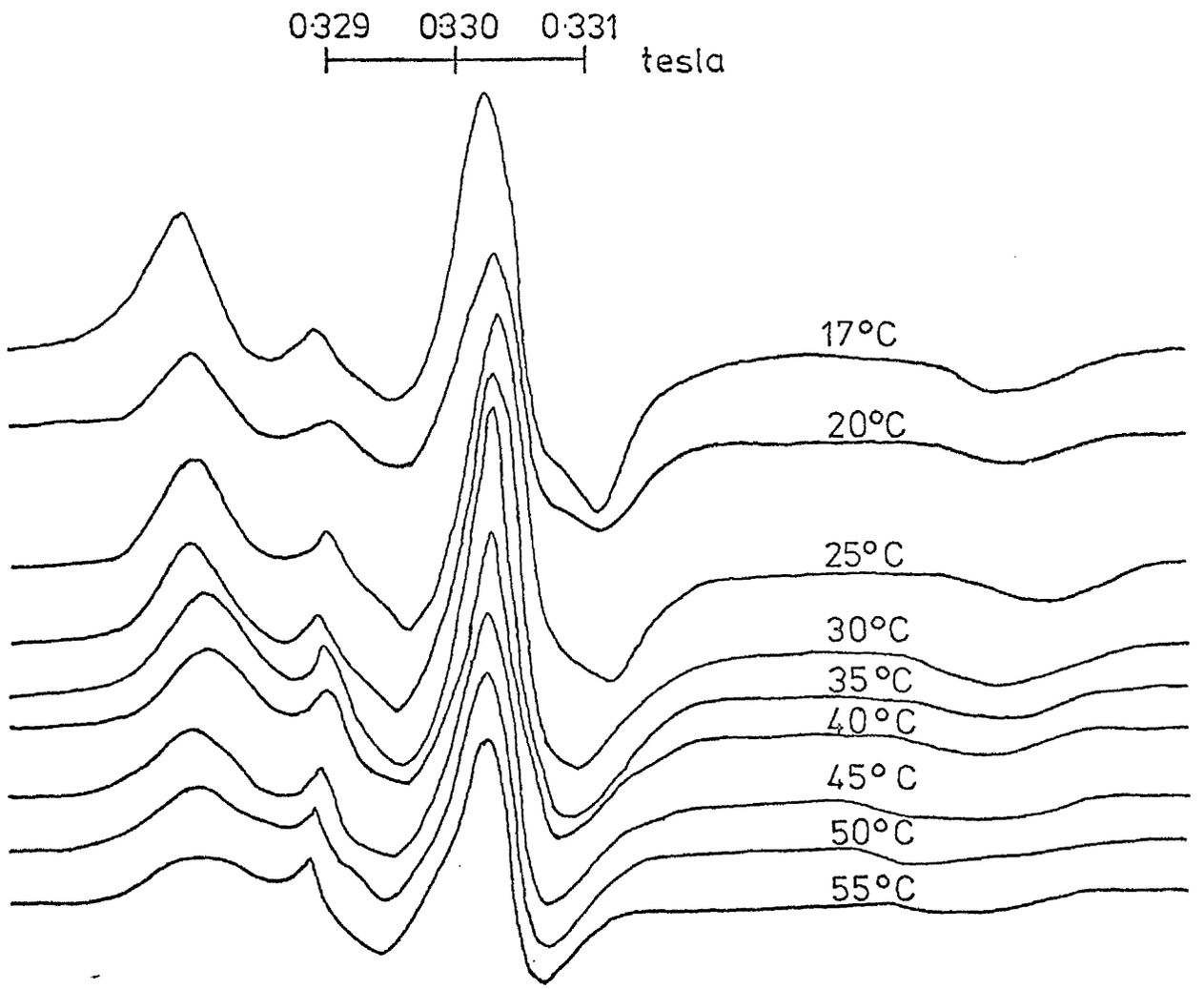


Figure 22: The electron paramagnetic resonance spectra of 5-doxyl stearate in lecithin bilayers with nonanoic acid incorporated (10:1, mole to mole, lecithin to nonanoic acid). While there is some sharpening of the peaks (cf. Figure 21), there is no difference in the splitting of the low field peak into two sub-peaks.

the order parameters for the spectra in Figures 19-22 and for other (unpublished) spectra are shown in Figure 23. From this it can be seen that the S-values for the 16-doxyl stearate form two distinct populations, depending on whether or not nonanoic acid was incorporated into the bilayer. The order parameters for the 5-doxyl stearate do not form two such distinct populations. From Figure 23, it may also be noted that the order parameter are linearly related to temperature. The linear regression was calculated for each of the four groups of order parameters, the data from this being presented in Table 14. By comparing the gradients of the calculated regression lines (the m values in Table 14), it can be seen that the lines are close to parallel. The effect of the incorporation of the nonanoic acid would thus seem to cause a lowering of the temperature at which a value of S is attained. This effect is superimposed upon the temperature effect on the order parameter and could be regarded as merely shifting this relationship to the left (i.e. a lower temperature).

While this effect on the order parameter is apparent, a certain amount of caution is required in interpreting these results. From the orientation of the E.P.R. probe, it would seem likely that the bilayer would be arranged such that the magnetic field is oriented parallel to the bilayer normal (i.e. parallel direction) and therefore the extraction of $A_{\perp}$ would not be valid. However, the spectra were similar to those published by Knowles et al (1976), for a random dispersion of spin-label containing liposomes, from which they extract the order parameter. It must therefore be considered possible that the bilayers in this study were not as specifically oriented as was previously thought. On the other hand, it is possible that the extraction of $A_{\perp}$ from the data in Figures 19-22 was not valid. Bearing this qualification in mind,

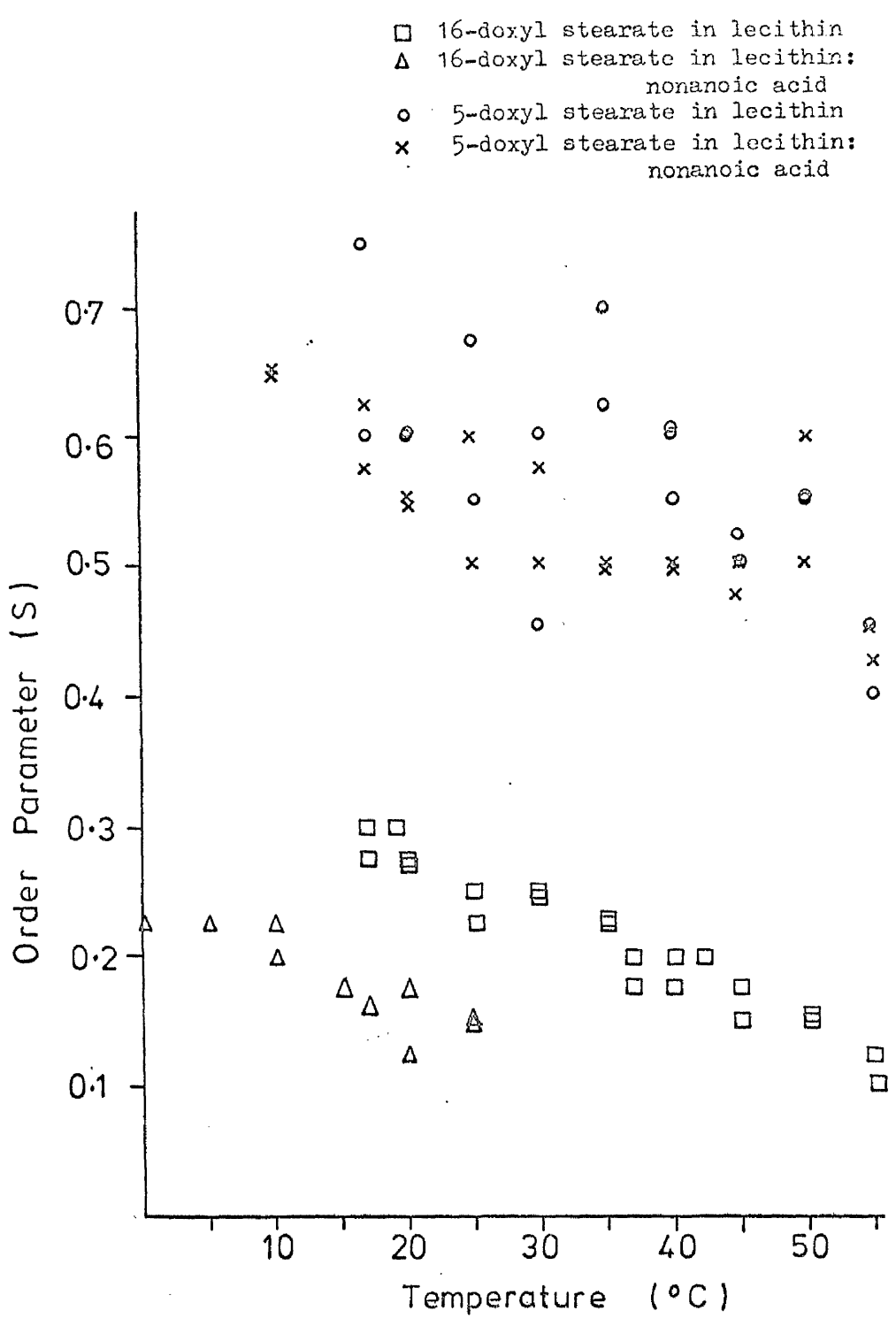


Figure 23: A plot of the order parameters for EPR spectra against temperature. The incorporation of nonanoic acid obviously reduces the order parameter of 16-doxyl stearate, but does not do so for 5-doxyl stearate.

Table 14: The calculated linear regression data and correlation coefficient for the order parameter against temperature as shown in Figure 23.

$y = mx + c$

	n	r	m	c
16-doxyl stearate in lecithin	23	-0.804	-0.0033	0.321
16-doxyl stearate in lecithin: nonanoic acid	11	-0.869	-0.0038	0.236
5-doxyl stearate in lecithin	19	-0.712	-0.0047	0.752
5-doxyl stearate in lecithin: nonanoic acid	20	-0.762	-0.0034	0.647

$\bar{m} = -0.0038 \pm 0.00033$

it nevertheless appears that the incorporation of nonanoic acid may substitute for elevated temperature in increasing the fluidity of the hydrocarbon core of a lipid bilayer.

DISCUSSION

From the data in Figure 19-23, it would appear that the incorporation of a short-chain fatty acid into a lipid bilayer does increase the fluidity of the core, as was suggested in Figure 18. However, care must be excercised in comparing such results to a natural system. Natural membranes have been shown to contain sterols, and their presence has been shown to alter the properties of the membrane (Demel et al., 1968; Sackman and Trauble, 1972). Also natural membranes contain up to 60% protein, so any results obtained from lipid bilayers must be considered to be only a guide to the contribution of the lipid portion to the whole. Often overlooked is the effect of inorganic cations, which have been shown to alter the conformation of the head groups of lecithin bilayers (Brown and Seelig, 1977). Thus a membrane should be looked on a dynamic association of phospholipid, proteins, oligosaccharides and inorganic ions, rather than as a simple lipid bilayer containing proteins.

While realising that this is the case, it is necessary to use as defined a system as possible, and to this end, the use of lipid bilayers appears justified. It should be noted that the incorporation of a stearic acid analogue containing a bulky doxyl will introduce perturbations into the bilayer and reduce the close packing to some extent. However, Levine et al (1972) have shown that the inclusion of spin-labels into a bilayer had little effect on the ^{13}C nuclear magnetic resonance relaxation times of synthetic lecithin, and thus the perturbations must be slight.

There remains the problem of the relevance of increased membrane fluidity to the control of thermodormancy. There are two major ways in which this could occur.

Firstly, as was stated previously, permeability is a function of fluidity. Reeves and Dowben (1970) showed that the permeability of liposomes to water was affected by temperature. Papahadjopoulos et al. (1971) have shown that cation permeability is affected by the degree of fluidity. Demel et al. (1968) showed that increased fluidity increased the permeability of liposomes to glucose. Schreier-Muccillo et al. (1975; 1976) showed that the permeability of lipid bilayer to ascorbate was also a function of the degree of fluidity of the hydrocarbon core. Thus fatty acyl chain fluidity has been shown on many occasions to affect the permeability of the bilayer to a range of molecules. Hendricks and Taylorson (1976) have shown that incubating lettuce seeds at elevated temperatures (35-40^{o}C) results in increased efflux of solutes from the seeds, apparently due to increased permeability of the plasma-lemma. The permeability of the seed membranes thus do appear to be affected by temperature. However, it is unlikely that natural control of thermodormancy lies at the level of changes in the solute permeability status of the membrane, since prolonged exposure to non-germinating temperatures would then be expected to result in loss of viability. Similarly, there is no evidence to suggest changes in water permeability: seeds take up water in non-germination-inducing conditions equally as well as they do in favourable conditions. There is therefore no evidence to suggest that membrane permeability is the key to control of thermodormancy.

The other major way in which the degree of fluidity of a bilayer may affect a process is by an alteration in membrane-

associated enzymes. Lyons and Raison (1970) showed that mitochondria extracted from chilling-sensitive plants showed a break in the Arrhenius plot of respiration rate. Chilling-resistant plants had no such break. This work was extended (Raison et al., 1971) to show that disruption, sonication, hypotonic swelling, and freezing-thawing had no effect on the break in the Arrhenius plot. However when the membranous components were dispersed using detergents, no break was seen. Kumamoto et al. (1971) suggested that these breaks were real and a consequence of the membrane phase-change. Raison (1972) showed that a number of mitochondrial enzymes exhibit breaks and these breaks correlated with the membrane phase change as demonstrated by spin-labels. Not all mitochondrial enzymes exhibited the break, only those which are membrane-associated. He also showed that non-mitochondrial membrane-associated enzymes show the transition-related break also. Similarly, Towers et al. (1972) showed that incorporation of amino acids by free ribosomes gave straight line Arrhenius plots. However rough endoplasmic reticulum showed two linear regions with a break point. This break point correlated with a phase-change in the lipids of the rough endoplasmic reticulum as demonstrated by E.P.R.

There is therefore a large amount of evidence to suggest that the degree of fluidity of a membrane is more than merely a biophysical parameter. It has profound biochemical consequences. Such an alteration of enzyme activation energies may have in itself a profound effect on the physiological status of the tissue.

If the short-chain fatty acids are affecting the membrane in vivo, as was suggested previously, it appears, from the

evidence presented, that such an effect is likely to be on the degree of fluidity. If this is so the short-chain fatty acid may substitute for temperature by increasing the fluidity artificially.

ENDOGENOUS LEVELS OF SHORT-CHAIN FATTY ACIDS

INTRODUCTION

Many growth regulators of plant origin have been ascribed a regulatory role in control of thermodormancy in lettuce seeds. The onset of dormancy could be due to the build-up of inhibitors, and the release from dormancy may be due to a reduction in their levels. Conversely, the induction of dormancy may be due to a reduction in the levels of growth promoters, while the release may be due to an increase in the levels of these.

However there is no definitive evidence to implicate any of the growth regulators in the control of thermodormancy in lettuce, e.g. Braun and Khan (1975) reported that ABA levels decreased more quickly in germination-inducing conditions than in non-germination-inducing conditions, but this decrease did not always correlate with germination. Berrie and Robertson (1976) could find no such correlation and concluded that no clear role for ABA could be established.

Similarly conflicting results have been reported for the levels of the other major growth regulators during dormancy and germination.

In 1975, Berrie et al. identified short-chain fatty acids as an active component of the inhibitor β complex. These acids were shown to be potent inhibitors of lettuce seed germination. While they showed that the short-chain fatty acids were present in a wide range of plant tissues, they did not show that they were an endogenous component of lettuce seeds. The levels of the

short-chain fatty acid present in wild and cultivated oats (Avena fatua and A. sativa respectively) correlated well with the depth of dormancy exhibited by the fruit. However, it is not possible to distinguish whether the presence of the short-chain fatty acid induces the dormant condition or the dormant condition leads to synthesis of the fatty acids, as was suggested by Vegis (1956).

MATERIALS AND METHODS

Fruits of Lactuca sativa L. cv Grand Rapids were obtained from the Page Seed Company, Greene, New York and stored at $-15^{o}C$ prior to use. All the work attempting to follow the endogenous levels of the short-chain fatty acids was performed using the same batch of seeds.

25 g of lettuce seeds were placed in a 26 cm x 18 cm x 10 cm polythene box lined with aluminium foil and were wetted with 40 cm^3 of distilled water. The boxes were then sealed with an air-tight lid and incubated at the prescribed temperature in darkness.

Organic solvents were routinely re-distilled before use.

Extractions

Initially a cold methanolic extraction was used, based on Bennet-Clarke and Kefford (1953). Macerated tissue was extracted in methanol for 24 hours at $4^{o}C$. The methanol was filtered and reduced to the aqueous phase by rotary evaporation under reduced pressure at $35^{o}C$. The pH was adjusted to 2, using 10 kmol m^{-3} H_2SO_4. The acidified aqueous fraction was partitioned three times with diethyl ether. The aqueous phase was discarded, while the

ethereal phases were combined and washed three times with 600 mol m^{-3} aqueous sodium hydrogen carbonate. The aqueous solutions were combined and taken to pH 2 with 10 kmol m^{-3} H_2SO_4. This was partitioned three times with diethyl ether. The ethereal solutions were combined and reduced, *in vacuo*, at low temperature to a small volume which was loaded onto a potassium hydroxide/silica gel column as detailed by Berrie *et al.* (1975). The sample was methylated using diazomethane and the content of fatty acid was assayed by G.L.C., as detailed later.

While the above extraction/purification procedure was successful, it was believed that it could be improved upon. There appeared to be considerable losses associated with the reduction of the methanolic extract to the aqueous phase. The following toluene-based extraction was developed and was used extensively in the studies of the endogenous content of the short-chain fatty acids.

Seeds were placed in a Soxhlet extractor with a Dean and Stark tube included between the Soxhlet and the condenser. This is shown in Figure 24. The seeds were soxhletted for 24 hours and the extracted water was collected in the delivery arm of the Dean and Stark tube. The use of this apparatus thus also allowed estimation of the water content of the seeds.

After extraction, the toluene was allowed to cool and was made to 250 cm^3 with redistilled toluene. The toluene extract was extracted three times with 200 mol m^{-3} aqueous di-sodium carbonate. The toluene was discarded: the water collected in the Dean and Stark tube was added to the di-sodium carbonate solution which was washed once with diethyl ether to remove residual toluene. The ether wash was discarded, and the pH of the aqueous solution was adjusted to 2 using 10 kmol m^{-3} H_2SO_4. The

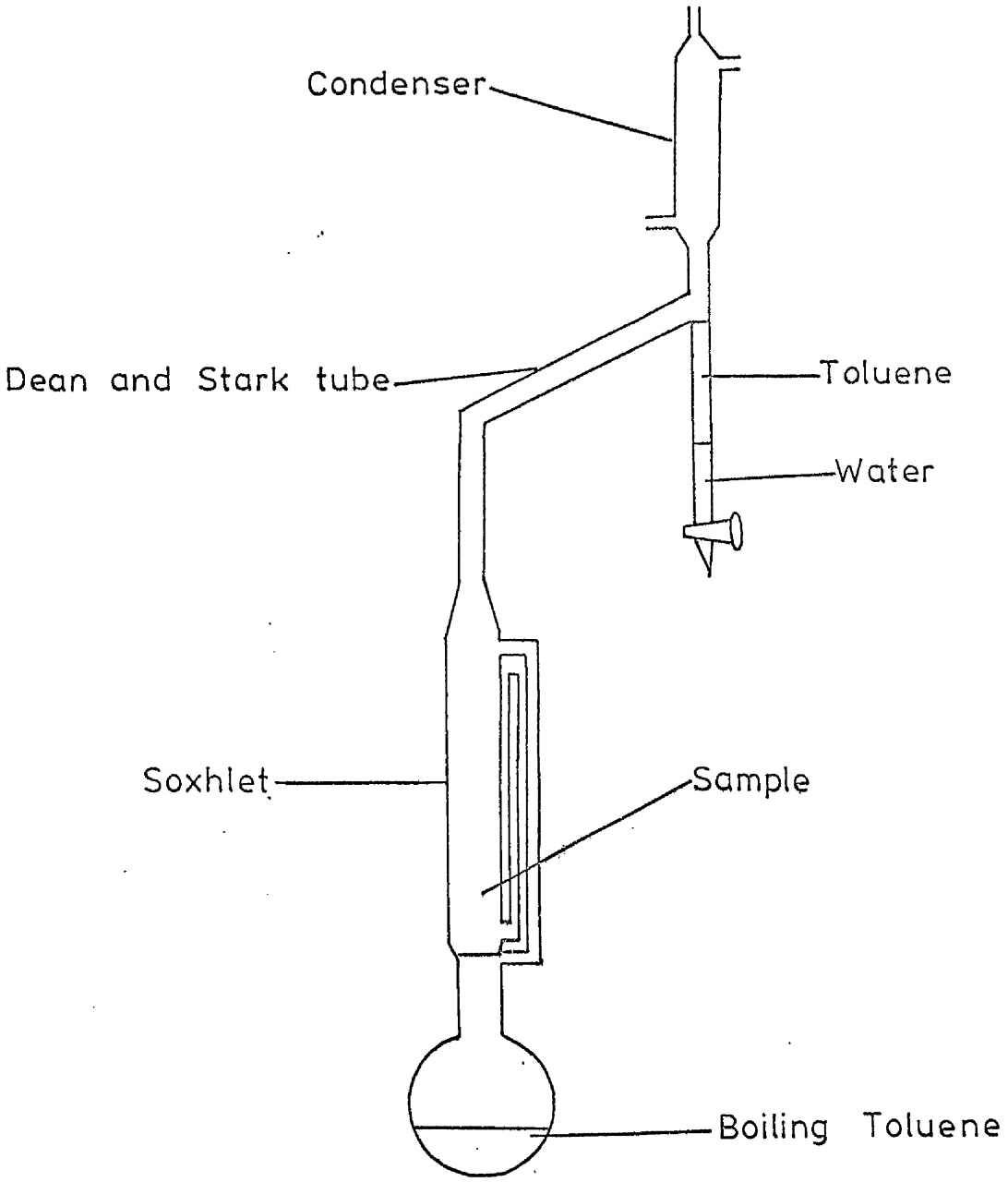


Figure 24: A diagram of the Soxhlet-Dean and Stark tube combination used to extract the fatty acids.

acidified aqueous solution was extracted three times with diethyl ether. Residual water in the ethereal solution was frozen out and the ether was removed by rotary evaporation *in vacuo*. The sample was methylated using diazomethane and the fatty acid content was determined by G.L.C.

Purification Efficiency:

It is obviously desirable to have an estimate of the efficiency of a purification procedure. To test this 1 mg of each of the fatty acids C_6 to C_{12} was added to 250 cm^3 of toluene and extracted using either di-sodium carbonate or sodium hydrogen carbonate as the base. The percentage of the applied fatty acid found in each of the organic solvents is shown in Table 15. From Table 15, it is clear that there is a marked difference in the efficiency of extraction of the range of fatty acids when sodium hydrogen carbonate is used as the base. It is interesting to note that much of the loss is due to the fatty acids entering the ether wash of the base, prior to its acidification. This suggests that the partition coefficients of the various fatty acids between the sodium hydrogen carbonate solution and ether are very different. If this is so, then the extraction published by Berrie *et al.* (1975) could not have been equally efficient for all the fatty acids.

However, when di-sodium carbonate is used as the base there is more equality of the efficiency of extraction, albeit rather less efficient than $NaHCO_3$ for the shorter chain fatty acids.

As a further check of the efficiency, 3 batches of 25 g of lettuce seeds were extracted by Soxhlet in toluene. To each was added 10 kBq of ^{14}C-nonanoic acid, before purification. The radioactive content of each solvent was tested by scintillation counting. The results are shown in Table 16. These suggest

Table 15: The amount of fatty acid remaining in each of the organic extractants after use: expressed as a percentage of that applied.

	SODIUM HYDROGEN CARBONATE			DI-SODIUM CARBONATE		
Percentage in:	Toluene	ether wash of alkali	final ether	Toluene	ether wash of alkali	final ether
Hexanoic acid	0	0	97	0	0	84
Heptanoic acid	0	0	100	0	0	82
Octanoic acid	2	6	92	1	0	82
Nonanoic acid	2	21	77	0	0	80
Decanoic acid	2	54	44	0	1	80
Undecanoic acid	15	70	15	1	2	78
Dodecanoic acid	71	39	2	1	7	80

Table 16: The distribution of radioactivity remaining in each solvent after extraction. 10 kBq of 1-^{14}C nonanoic acid was added to the initial toluene. The numbers in the table are net disintegration per minute.

TOLUENE	21 222	20 592	17 523
ETHER WASH	12 426	13 104	11 340
ACIDIFIED BASE	36 787	49 140	46 575
FINAL ETHER	529 998	499 821	524 772
PERCENTAGE EFFICIENCY	89	86	88

that the extraction of nonanoic acid by the standard procedure is about 87% efficient. This estimate is less than 10% different from that quoted in Table 15 for nonanoic acid.

Synthesis of Diazomethane

Diazomethane (CH_2N_2) was prepared thus:

Nitrosan (Du Pont Chemicals Ltd) was placed in a 100 cm^3 flask with a delivery tube and was covered with a layer of 1,2-ethanediol. Onto this layer 20 cm^3 of diethyl ether is poured and 5 cm^3 of saturated aqueous sodium hydroxide is added. The flask is sealed immediately with a cork stopper wrapped in silver foil and is immersed in warm water. The delivery tube is placed in a small conical flask containing a little ether. The receiver flask is kept in an ice bath. Diazomethane is distilled over with the ether and gives a yellow-coloured ethereal solution in the small conical flask. This diazomethane in ether solution was then added to purified extracts along with 1 cm^3 of methanol as a catalyst. The sample was left for several hours and then was reduced in vacuo to 1 cm^3, prior to analysis by G.L.C.

Gas Chromatography

Gas chromatography was performed using a Perkin Elmer F17 gas chromatograph with a 1/8" outside diameter, 1 metre long stainless steel column, packed with 5% FFAP (Free Fatty Acid Phase) on Chromosorb G: AW DMCS, 80-100 mesh. Chromatography of both the free fatty acids and of the methyl esters was performed. For the free acids, a temperature programme of 160°C to 210°C at 4°C min^{-1} was used. For the methyl esters a programme of 80°C, held for 4 minutes, and then 4°C min^{-1} to 160°C was used.

It was decided to construct a series of calibration curves for

the flame ionisation detector (FID) response to various amounts of the fatty acids with chain lengths 6-12. Before this could be done it was necessary to decide how to estimate the area of each peak. The first method used was to estimate the area of an approximating triangle. This appeared to be highly subjective and led to extreme variability, particularly where tailing of the peaks occurred.

The next method of area estimation to be tested was cutting out the peaks from the chart-recorder trace and weighing them. It must therefore be shown that the paper is uniform and will give a constant weight per unit area. There will also be an error factor due to the cutting out, but for the purposes of this test, these two sources of error will be considered inseparable.

To test the uniformity of the paper, 70 similar size strips of paper were cut out. Fifty of these were selected at random and weighed to 0.01 mg. This test was performed three times with different size strips of paper.

Test 1

The fifty strips had a mean weight of 1.82 mg and a standard deviation of 0.08 mg. Thus, 95% of the strips will be in the range 91-109% of the mean and 99% will be in the range 88-112% of the mean.

Test 2

Mean weight 0.91 mg; standard deviation 0.03 mg. 95% will be in the range 93-107% of the mean and 99% will be in the range 91-109% of the mean.

Test 3

Mean weight 2.84 mg; standard deviation 0.10 mg. 95% will be in the range 95-105% of the mean and 99% will be in the range

92-108% of the mean.

These tests show that errors due to variation in the paper (and the cutting) are not excessive. This method of estimating the area of the peaks was adopted and used to construct a calibration curve.

Seven calibration curves, one for each of the fatty acids C_6-C_{12}, of FID response against dose were plotted. The data for these are shown in Table 17. It can be seen that there is a linear relationship between the FID response and the dose of short-chain fatty acid.

Mass Spectrometry

Mass spectrometry of the fatty acids with chain length C_6 to C_{12} was performed using an AEI MS30 spectrometer interfaced, by means of a membrane separator, to a Pye 104 gas chromatograph. The column used was 3% SP 2250 on Supelcoport 100-120 mesh (2.7 m length), with a flow rate of 40 cm^{-3} min^{-1}. A temperature programme of 120°C for 5 min and then 8°C min^{-1} was employed.

The mass spectrometer was run in two modes: full scan and multiple ion monitoring (MIM).

Full-Scan Mass Spectrometry

The full-scan mass fragmentograms for the methyl esters of the fatty acids C_6 to C_{12} are shown in Figures 25-31. The molecular ion, M^+, is always present in the spectra of unbranched, saturated methyl esters of fatty acids, although the intensity with respect to the base ion, m/e = 74, varies with chain length, being a minimum at chain length 5 and increasing with longer chain lengths. As a result of this, the molecular ion is not seen in the spectra of methyl hexanoate to methyl undecanoate inclusive, as shown in Figures 25-30, but is seen in the spectrum of methyl

Table 17: The data for the calibration curves of the GLC FID response to the seven short-chain fatty acids. The table shows the mean and standard error of the arbitrary response units.

Amount Injected	Hexanoic acid	Heptanoic acid	Octanoic acid	Nonanoic acid	Decanoic acid	Undecanoic acid	Dodecanoic acid
1 µg	1 150 ± 47	1 171 ± 84	1 313 ± 90	1 224 ±102	1 146 ±105	1 130 ± 46	1 111 ± 92
500 ng	647 ± 12	663 ± 31	705 ± 45	670 ± 38	690 ± 41	612 ± 49	644 ± 41
250 ng	246 ± 7	241 ± 7	265 ± 12	261 ± 21	264 ± 12	218 ± 10	221 ± 18
125 ng	110 ± 5	110 ± 4	129 ± 5	127 ± 7	120 ± 5	105 ± 4	123 ± 8
62 ng	62 ± 3	59 ± 2	66 ± 4	65 ± 1	67 ± 3	63 ± 3	67 ± 2

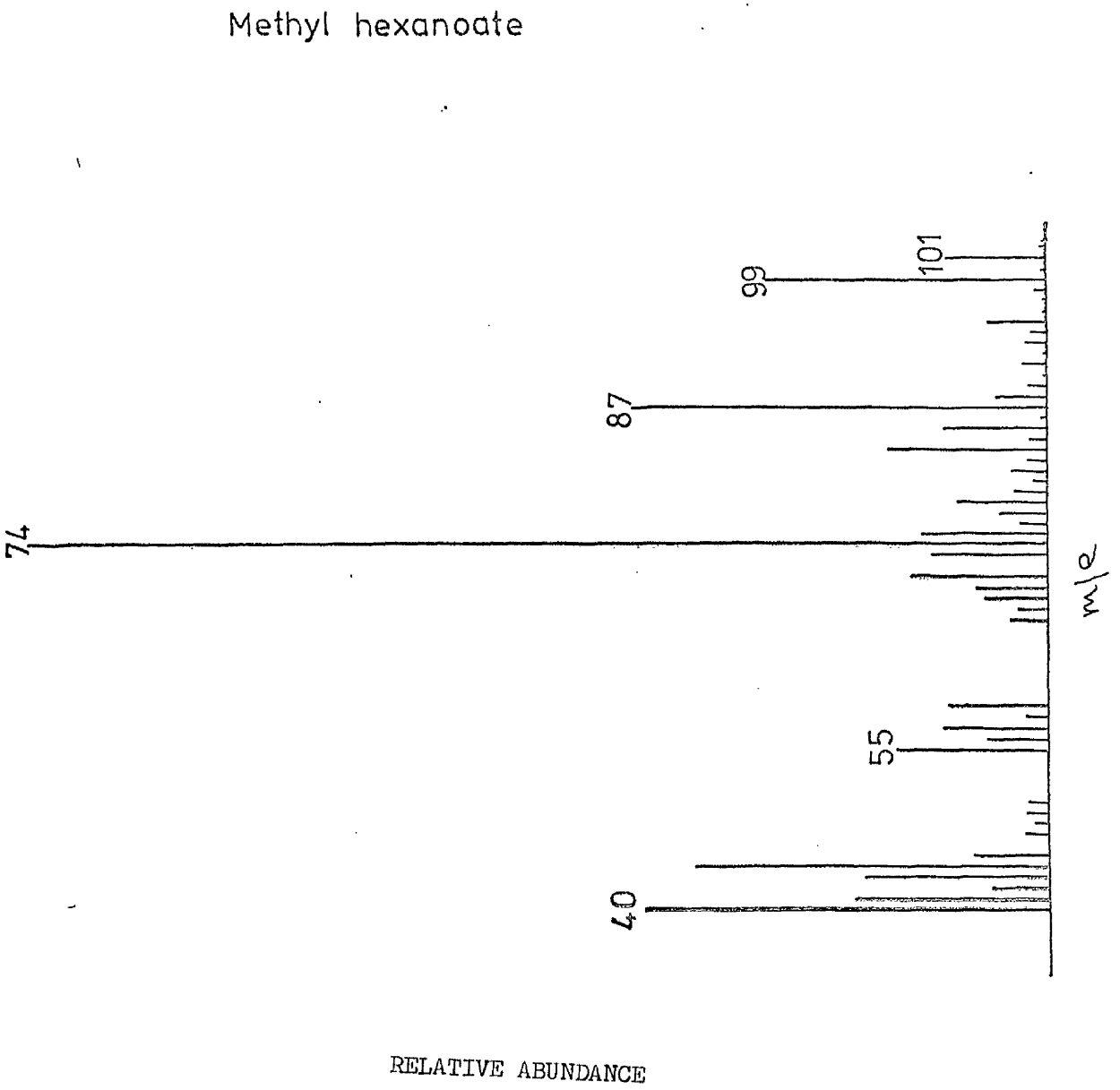
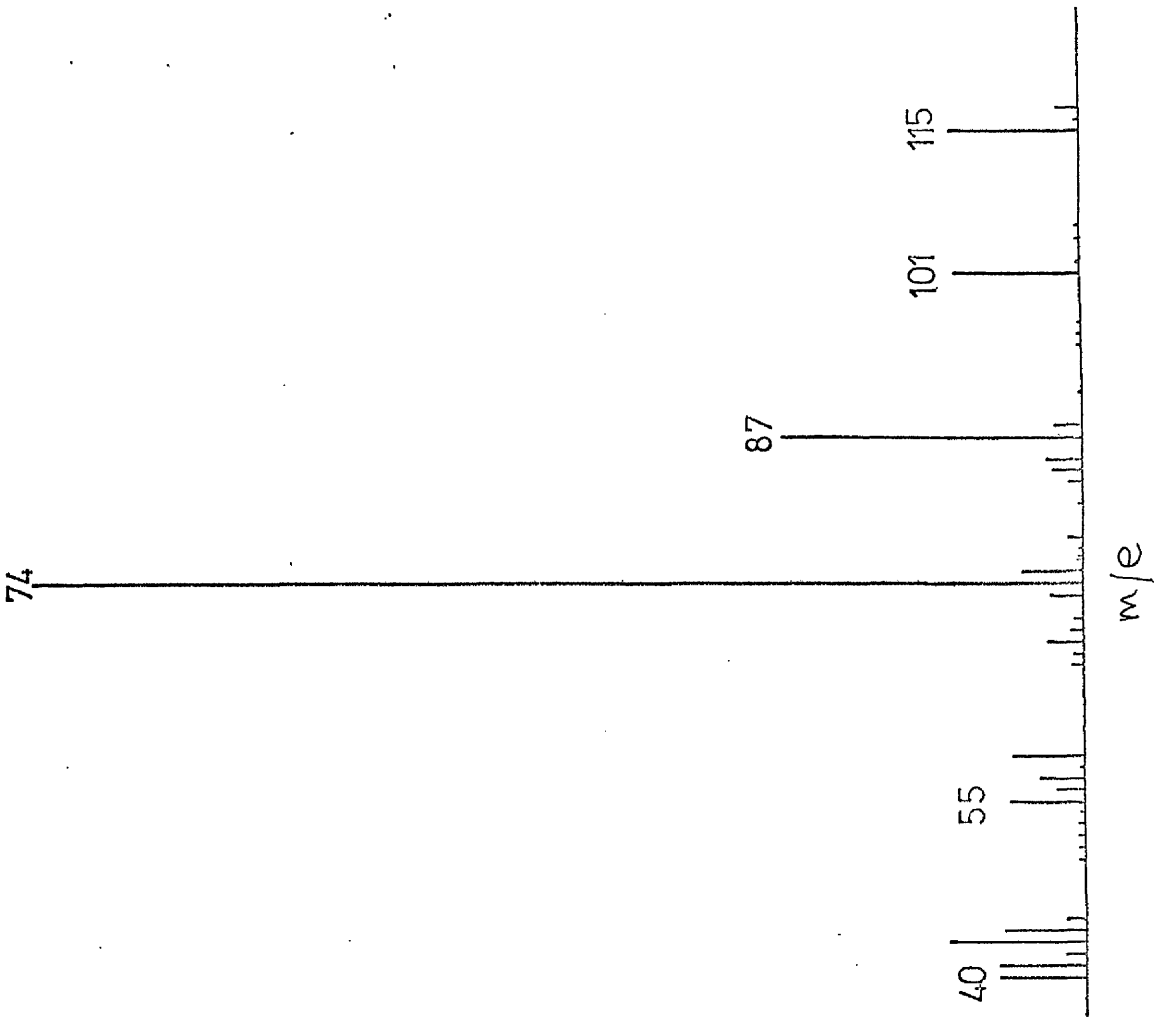


Figure 25: The full scan mass spectrum of methyl hexanoate.

Methyl heptanoate



RELATIVE ABUNDANCE

Figure 26: The full scan mass spectrum of methyl heptanoate.

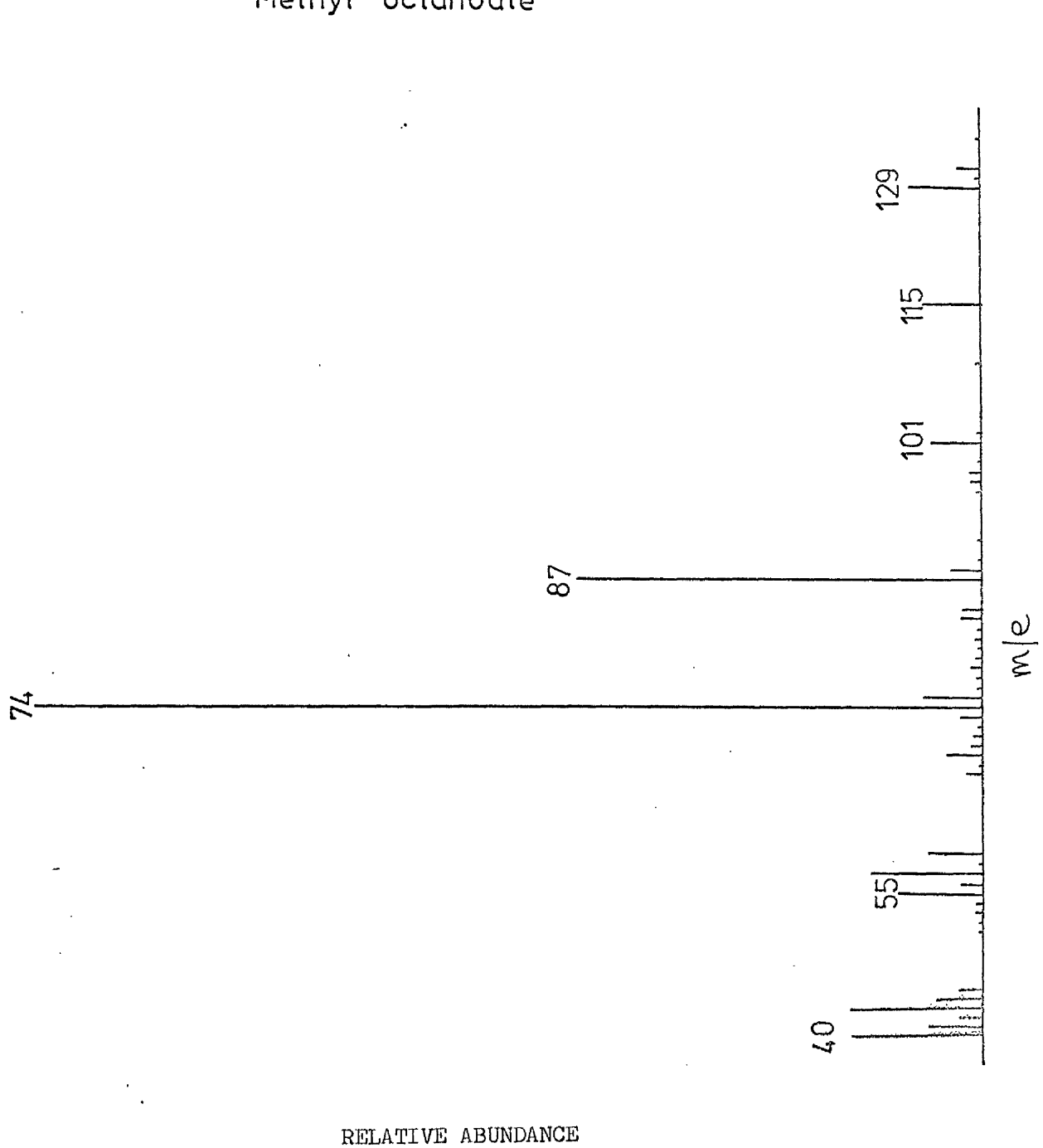


Figure 27: The full scan mass spectrum of methyl octanoate.

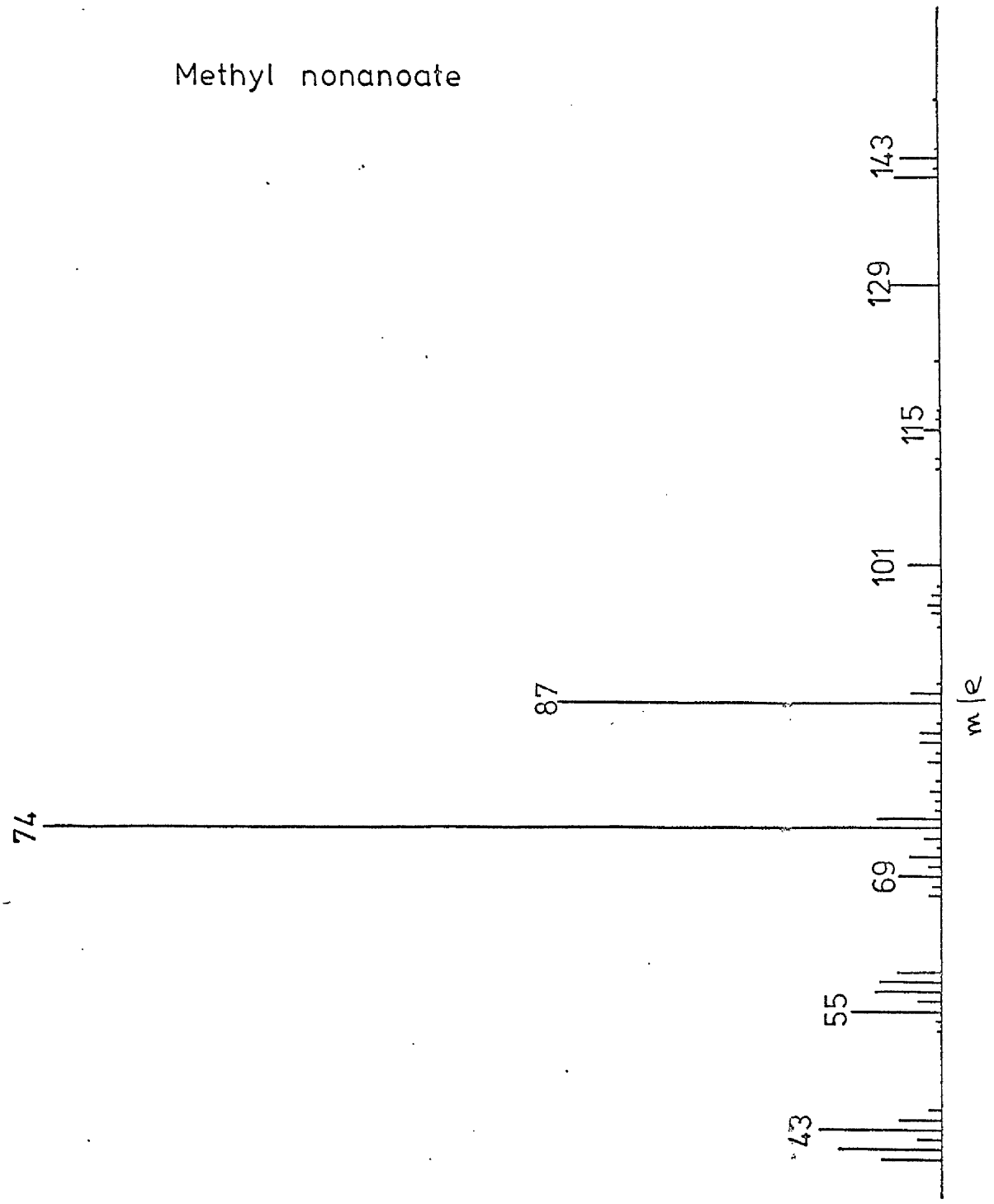


Figure 28: The full scan mass spectrum of methyl nonanoate.

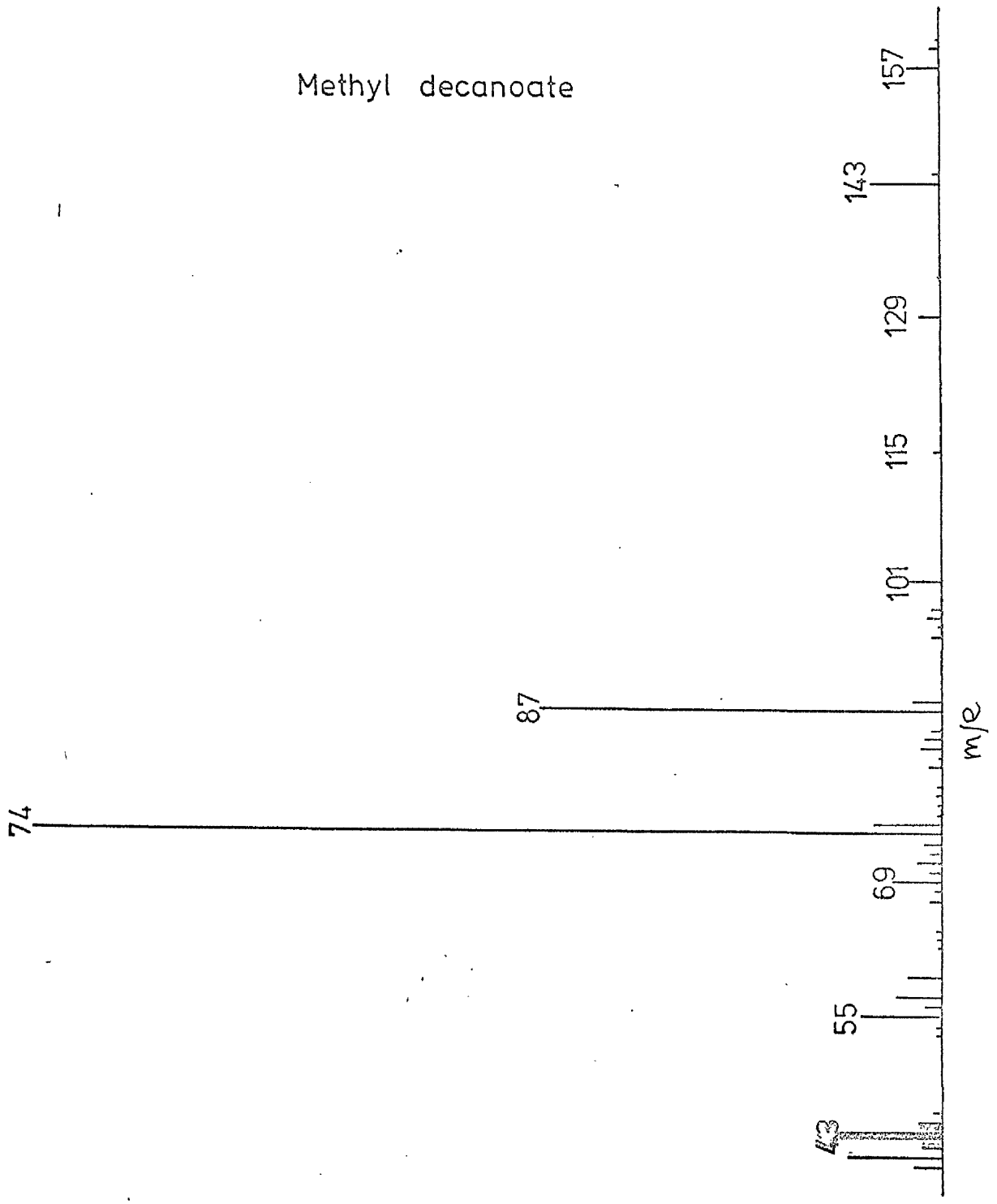


Figure 29: The full scan mass spectrum of methyl decanoate.

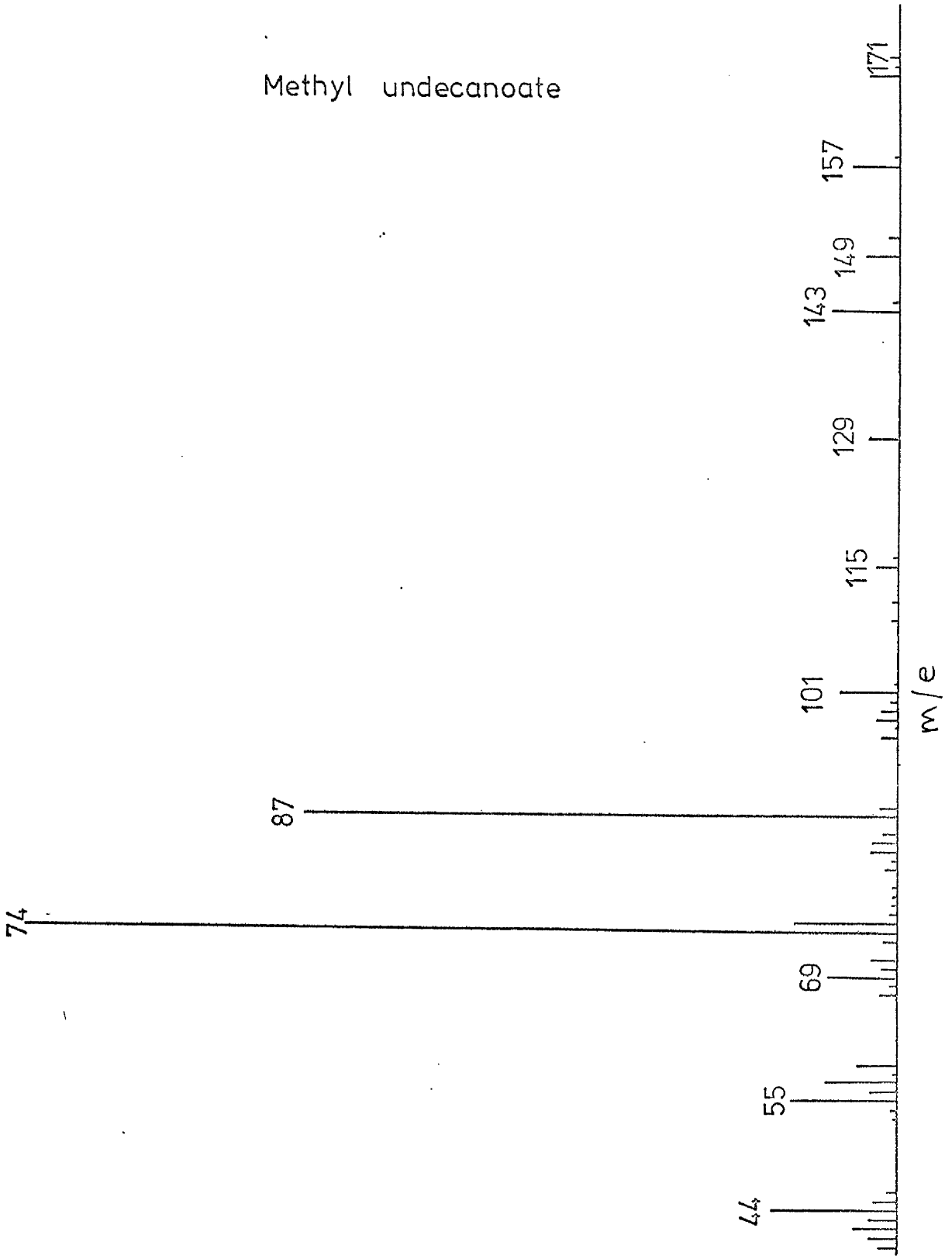


Figure 30: The full scan mass spectrum of methyl undecanoate.

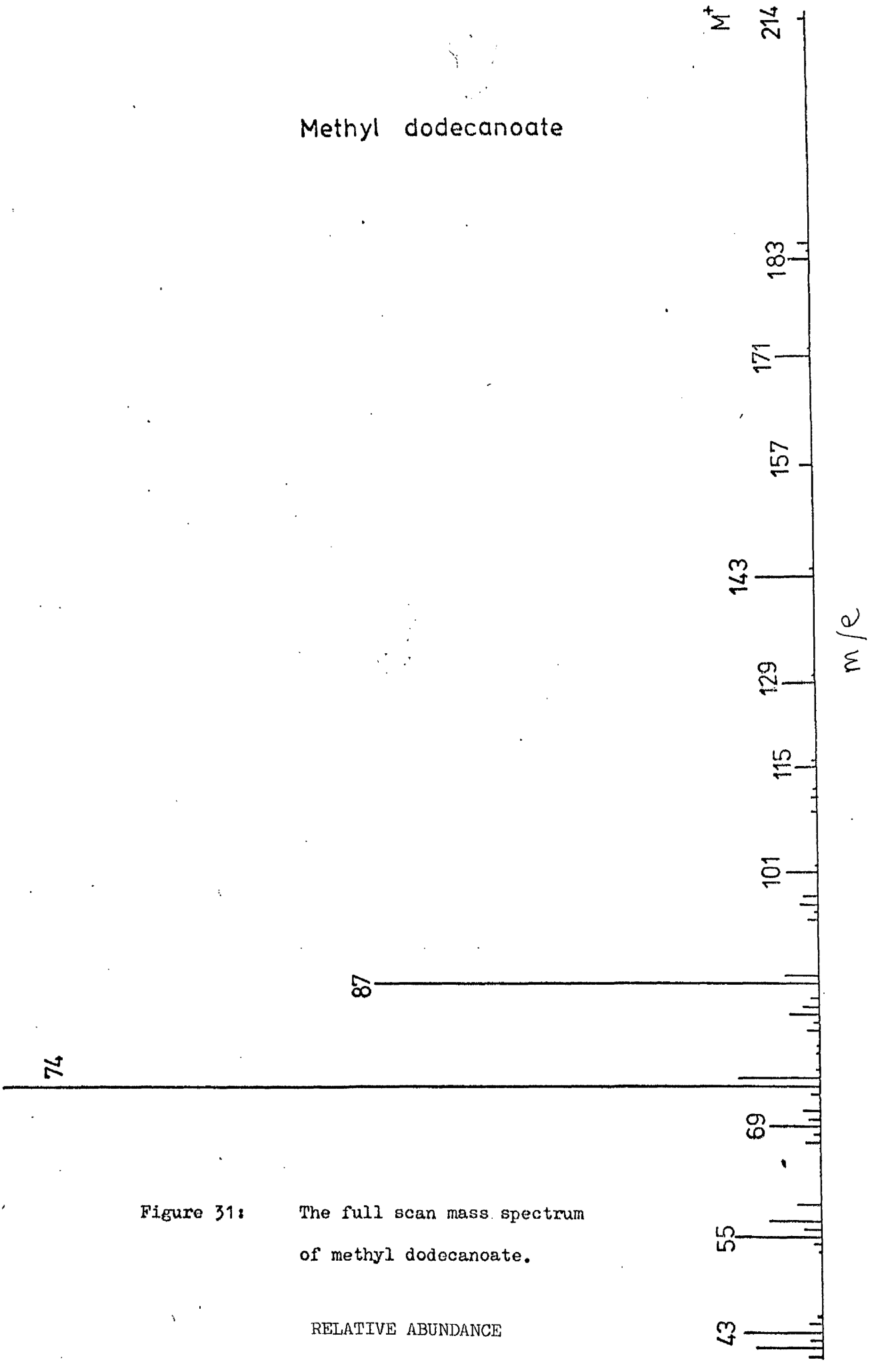


Figure 31: The full scan mass spectrum of methyl dodecanoate.

dodecanoate (Figure 31).

The base peak of the mass spectrum of the methyl ester of a fatty acid is m/e = 74. The mode of formation of this radical ion is known as a McLafferty rearrangement. This rearrangement involves the specific transfer of a γ-hydrogen atom in a six-membered transition state to the carbonyl oxygen atom with subsequent cleavage at the β-carbon atom, as shown in Figure 32.

Also present in the spectrum are strong ions of the form:

$$[(CH_2)_n\ COOCH_3]^+ ,$$

for all values of n up to the molecular ion. The predominant members of this group are n = 2 (m/e = 87), n = 3 (m/e = 101) and n = 6 (m/e = 143).

Also seen are m/e 43 and 57 corresponding to 3 and 4 carbon mono-unsaturated hydrocarbon fragments respectively. The analogous di-unsaturated hydrocarbon fragments are also evident (m/e 41 and 55).

Multiple Ion Monitoring

In the MIM mode, the spectrometer was tuned to m/e 74, 87, 101 and 143 to monitor for the major ions, as detailed previously.

Figure 33 is a copy of the MIM trace for an extract of dry lettuce seeds. This shows that the extract contains methyl pentanoate, hexanoate, heptanoate, octanoate, nonanoate and (off the page) dodecanoate. There is a peak of m/e 74 and 87 where that of methyl decanoate would be expected, but no peak is seen at m/e 101 nor 143. It therefore cannot be stated that methyl decanoate is present.

Care must be exercised, however, in the interpretation of

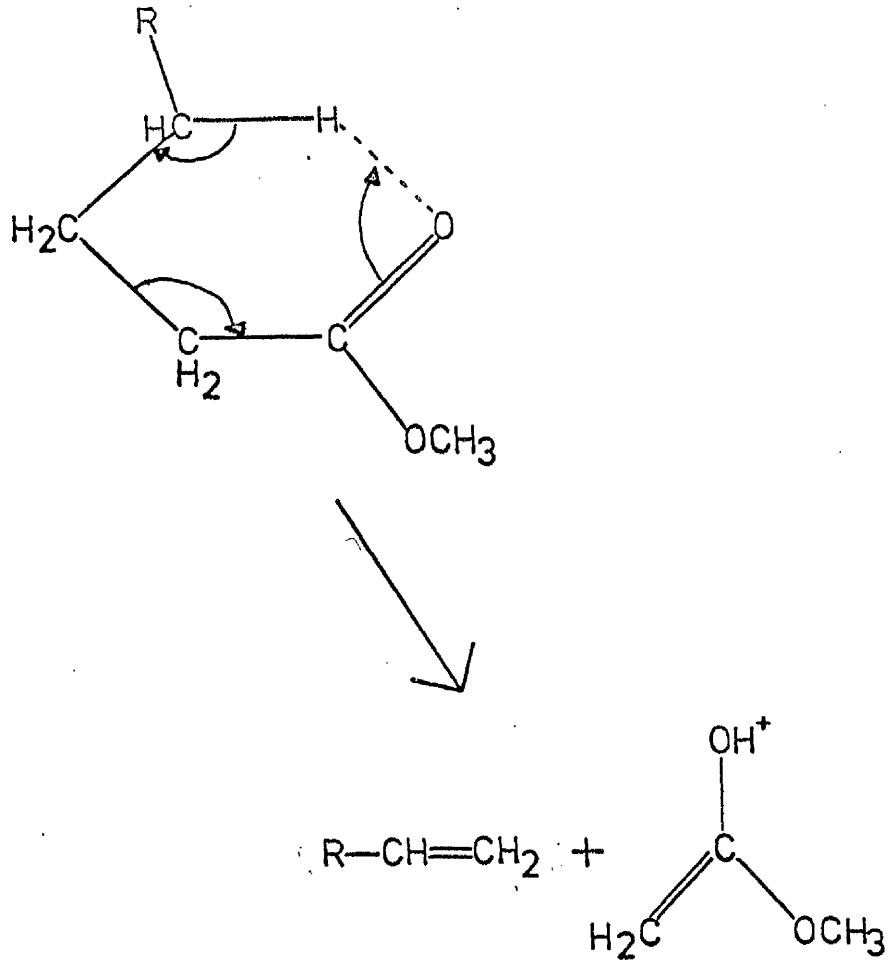


Figure 32: The McLafferty re-arrangement which gives rise to the m/e = 74 base ion of the mass spectrum of methyl esters of fatty acids.

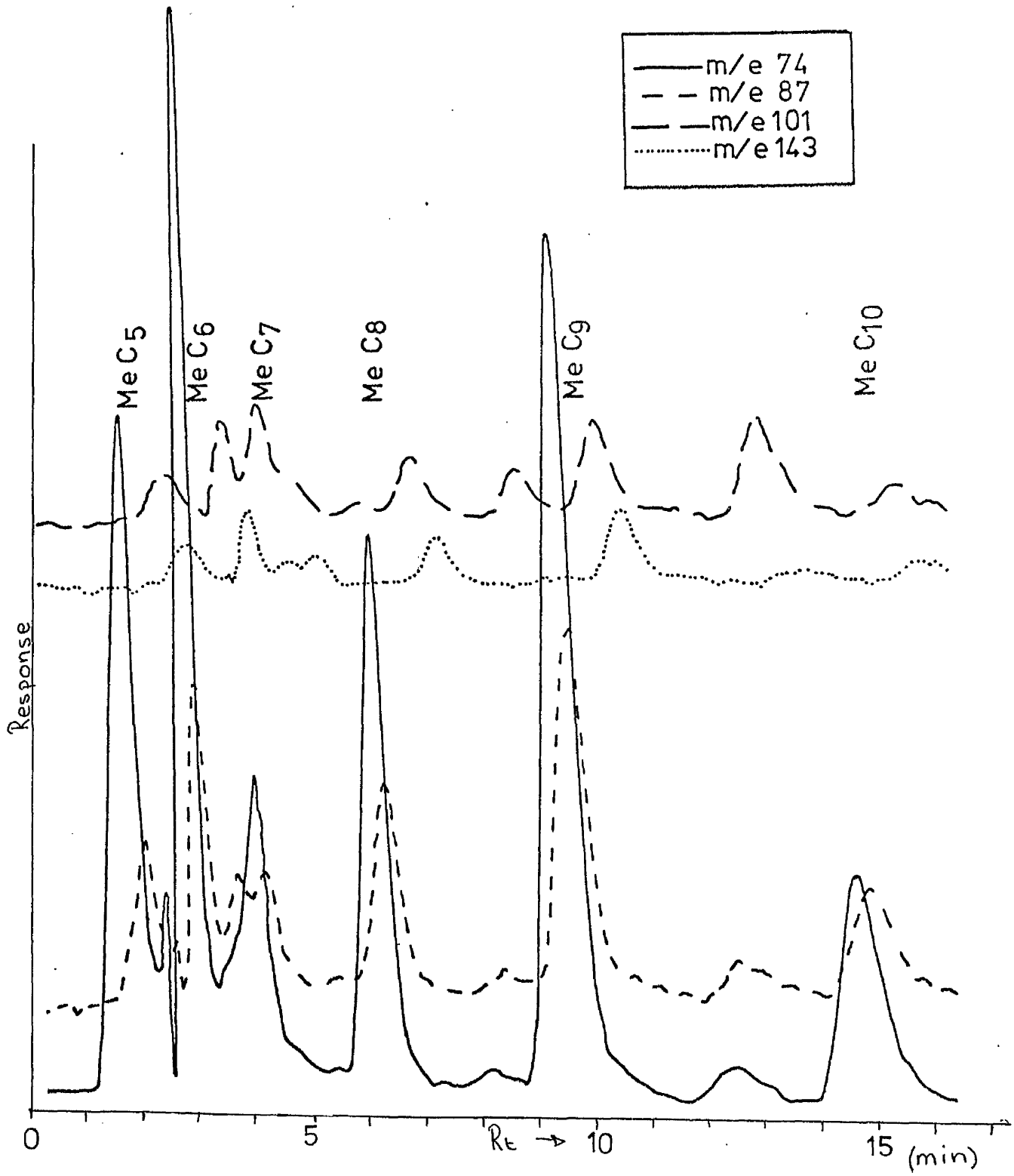


Figure 33: The multiple ion monitoring mass spectrometry traces of an extract of dry lettuce seeds. This suggests that methyl pentanoate, hexanoate, heptanoate, octanoate, nonanoate, and decanoate are all present. However, methyl hexanoate (M.W. 130) has given a response in the m/e 143 channel.

these data. Figure 34 shows part of an MIM trace of standards of methyl fatty acids, chain length 6-12. It may be noted that there is a peak on the m/e 143 channel corresponding to methyl hexanoate, which has a molecular weight of 130. If such artefacts can be seen in standards, the MIM traces of corresponding unknowns cannot be considered useful in the search for an unequivocal identification of the unknown.

RESULTS

Dry lettuce seeds were extracted in toluene by Soxhlet. The resulting toluene extract was treated as detailed previously. The final extract was assayed by GLC as the methyl esters. The resulting GLC trace is shown in Figure 35. By subsequent co-injection with standards, the peaks marked as methyl hexanoate, octanoate and dodecanoate in Figure 35, could not be separated from the standards either on the 5% FFAP nor on a 10% SP1200/1% H_3PO_4 on 80/100 Chromosorb stationary phase. Later GC-MS used in MIM mode identified methyl pentanoate, hexanoate, octanoate, nonanoate and decanoate as present, but with reservations as detailed in the section on mass spectrometry.

Seeds were incubated at 20°C and then surface dried and extracted by Soxhletting in toluene. The estimated content of the short-chain fatty acids is shown in Table 18. From this it is evident, that there is a drop off in the level of hexanoic acid up to 11 hours after imbibition thereafter no clear pattern is apparent. The levels of octanoic and dodecanoic acids are more variable, although there is a clear tendency for the levels to increase with longer imbibition.

Seeds were subsequently incubated at 30°C and dried and

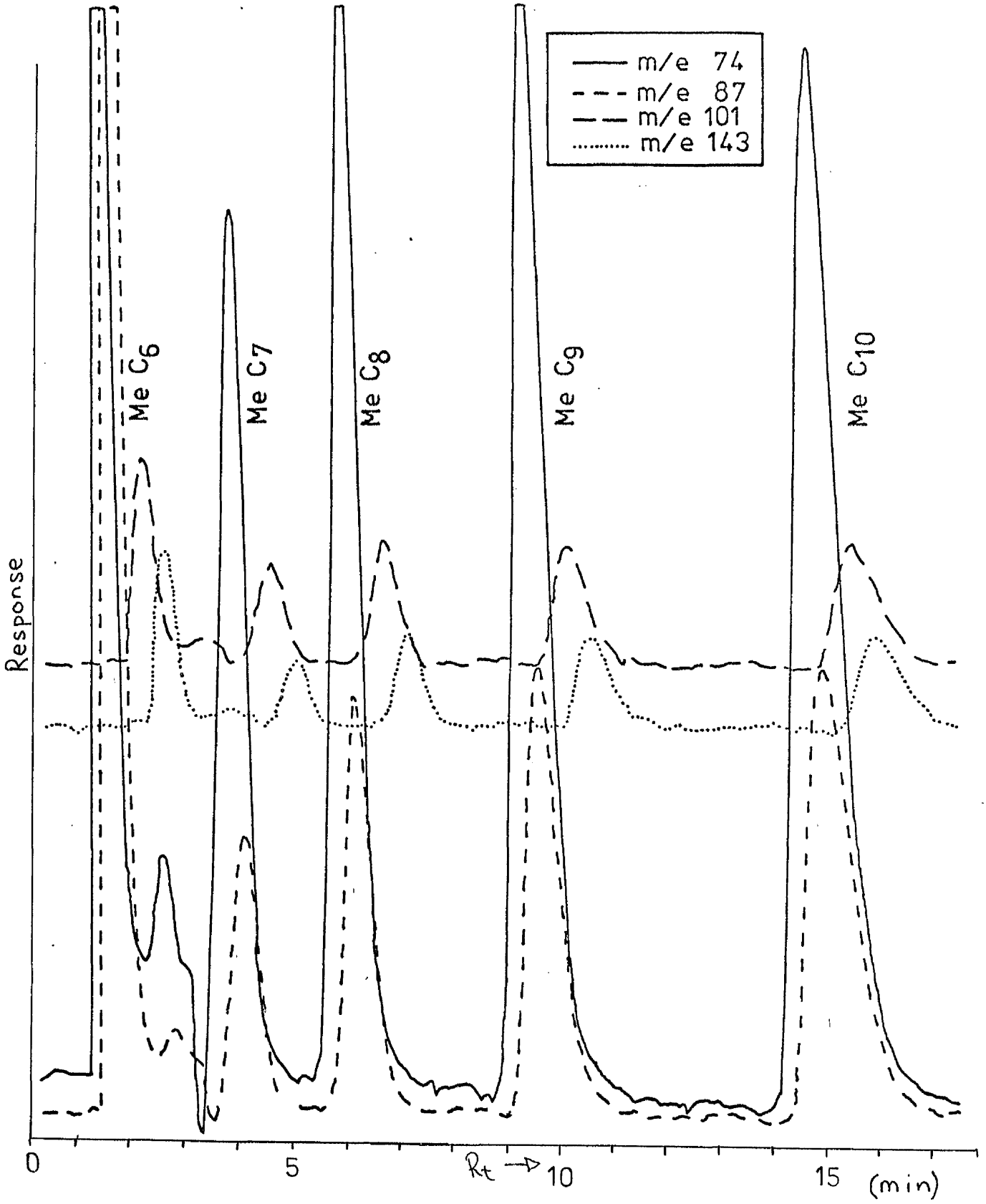


Figure 34: The multiple ion monitoring mass spectrometry traces of standards of short-chain fatty acids C_6 to C_{10}. Note that methyl hexanoate (M.W. 130) has given a response in the m/e 143 channel.

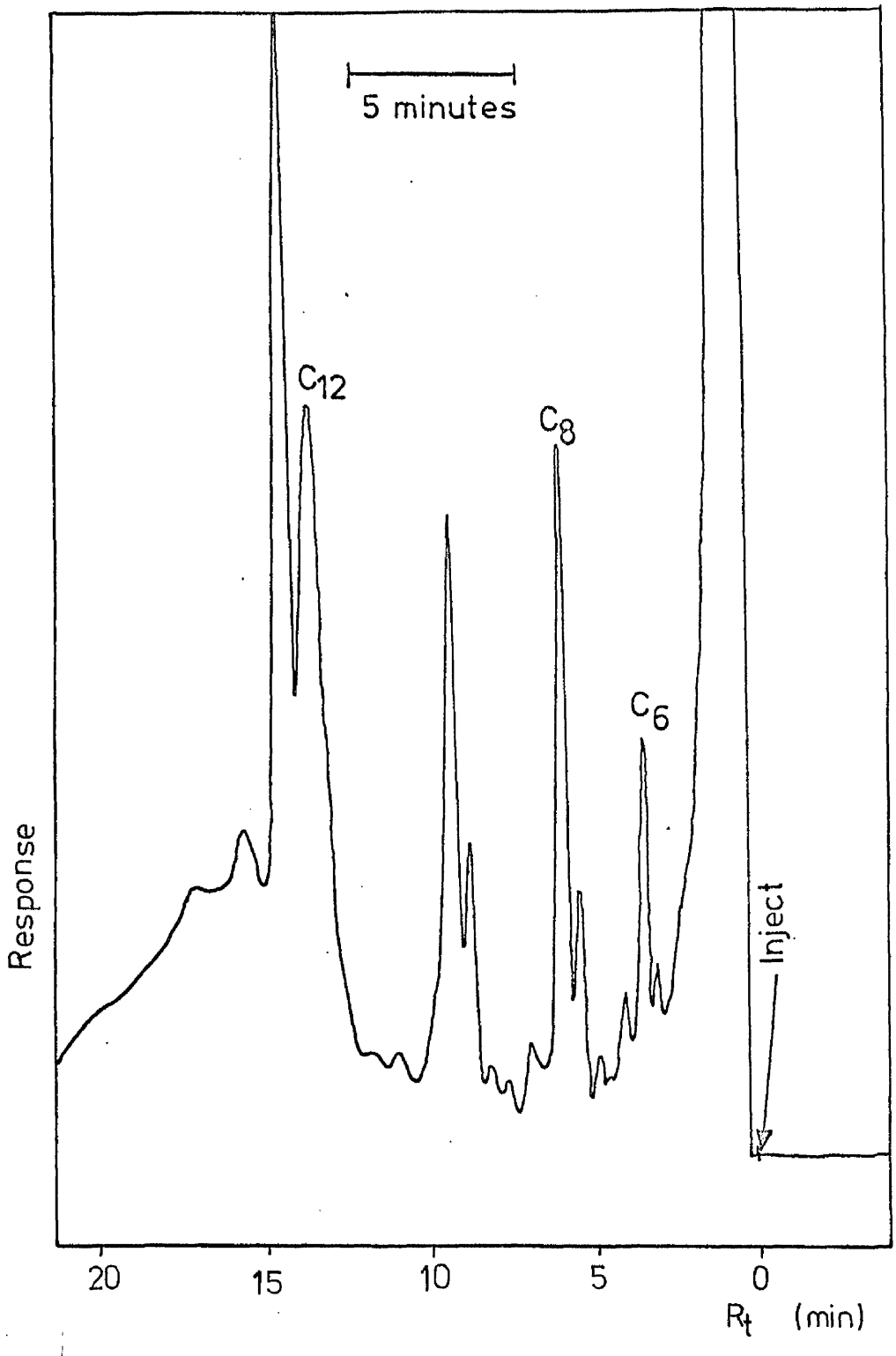


Figure 35: A GLC trace of a methylated extract of dry seeds. The peaks marked as C_6, C_8 and C_{12} co-chromatographed with methyl hexanoate, methyl octanoate, and methyl dodecanoate respectively.

Table 18: The amount of short-chain fatty acids in lettuce seeds at various times after imbibition at 20°C. The values are mg/kg of dry seeds.

Time after Imbibition/h	Hexanoic acid	Octanoic acid	Dodecanoic acid
0	4.23	9.20	7.61
1	8.13	0.53	0.39
3	3.65	0.42	-
5	1.93	0.78	0.64
7	0.75	1.02	0.91
11	0.35	0.88	-
16	0.50	-	6.41
19	0.29	1.66	3.46
24	0.82	2.04	7.17

extracted as previously. A pattern similar to that shown in Table 18 was found. There was no obvious difference between the levels of the short-chain fatty acids during imbibition at 20°C and 30°C.

DISCUSSION

There is no evidence that there are different amounts of short-chain fatty acids in seeds when they are incubated at germination-inducing (20°C) and non-germination -inducing temperatures (30°C). It is doubtful whether there is sufficient evidence even to state unequivocally that short-chain fatty acids are an endogenous component of lettuce seed. This is due to the fact that the extraction procedure employed is of extremely low selectivity. The partition purification procedure would be expected to include all organic acids which would be ionised in the sodium carbonate solution. Similarly there would be an error associated with claiming that co-chromatography with standards in the GLC could be used to establish identity, when it in fact merely fails to show the compounds to be different. Similarly, the response of the FID is unspecific, since it would respond to any organic compound to some degree. Therefore it is not justified to claim that a response to a co-injection which is the sum of the responses to the components of that co-injection can be used as a criterion for identification any more than can simple co-chromatography.

It is often stated that co-chromatography in several different systems is a useful guide to identification. However, this cannot be considered the case with GLC columns, as there is a high degree of correlation between retention times in different

stationary phase systems.

The use of the external standard method for estimating the amount of a compound which would give rise to the response noted, would also cause errors to be included in the estimate. The external standard method is based upon an absolute amount of standard which is injected into the gas chromatograph and is not relative to anything within the sample. Thus the precision of the method is based upon injection techniques, the precision of which has been estimated at 4-6% (Hammarstrand, 1978) for a reasonably skilled operator. Since two injections are required for external standardisation (one for standard, one for sample), the errors would be additive and would reduce precision to the order of 88%. Also the detector response must remain constant, and to this end it is desirable to have standards and sample analysed as near together in time as is possible.

In an analogous manner, there are likely to be errors associated with the use of an external standard for extraction efficiency. It is dubious whether the purification of a pure standard is representative of that of a plant extract. If there are different amounts of the compound of interest in the standard and the plant extract, they may exhibit rather different properties. This is seen in both solvent partition purification and in many forms of chromatography. Errors of this sort may be reduced if it can be shown that a procedure is equally efficient for various amounts of the standard, particularly at the concentrations which would be expected to occur in the extract. However, such a test would not exclude errors due to the compound of interest interacting with contaminants in the plant extract, which may lead to the estimate of the efficiency of the extraction being erroneous.

The use of an internal standard would overcome most of the problems associated with extraction-purification, but would introduce a few of its own. It is obviously desirable that the internal standard has chromatographic properties essentially indistinguishable from those of the compound of interest, yet at the point of assay, they must be distinguishable. Radioisotopically labelled analogues of the compound of interest would appear to be the best internal standard, however there are reports of isotopes being chromatographically separable: also the label should be of as high specific activity as is available, so that as small a number of standard molecules have to be added as is possible, which will in itself reduce the possibility of autoradiolysis.

Since many of the plant growth regulators are organic acids, methylation prior to analysis by GLC is often performed routinely. Diazomethane is often the methylating agent of choice. It is usually assumed that diazomethane will methylate carboxyl acid and possibly alcoholic hydroxyl groups only. However, diazomethane has been used to elongate hydrocarbon chains by insertion of a methylene group within the chain (e.g. Morrison and Boyd, 1966). In this way nonanoic acid could become methyl decanoate, or even longer. In an analogous manner, indole acetic acid could become indole propionate methyl ester. This would obviously lead to erroneous estimation of the endogenous status of these compounds.

Methanol is also often used as a solvent in at least one stage in the extraction of plant growth regulators. However, it has recently been noted in this laboratory that when organic acids were in alcohols, there occurred spontaneous ester formation. This was noted for fatty acids in methanol, indole acetic acid in methanol (Mclaughlin, pers. comm.) and dicarboxylic acids in ethanol (Berrie, pers. comm.). Therefore, if an internal standard

(of an organic acid) were stored in alcoholic solution prior to its addition to the extract, it would be likely to overestimate the loss of the test acid over to the ester. It is likely that a radioactive labelled standard would form the esters more quickly due to the formation of radicals in the alcoholic solution on absorption of the emitted radiation.

The major conclusion which can be drawn from this section of the research is that attempts to identify plant growth regulators and to follow their levels requires a high degree of specialisation. It cannot be adequately covered as a side branch of another line of research. It could also be concluded that much of the published literature on the endogenous growth regulator content of plant could possibly be more critical.

D I S C U S S I O N

Normally, thermodormant lettuce seeds show maximum germination only at low temperatures. In the variety used in this study, Grand Rapids, this is manifest as maximum germination up to 26°C (e.g. Berrie, 1966) above which there is an abrupt thermal cut off, germination approaching zero at 30°C. The presence of a short-chain fatty acid reduces the germination at low temperatures. If the data in Tables 1-5 are re-examined, and plotted as percentage germination against temperature for each dose of nonanoic acid, a pattern as shown in Figure 36 is seen. Thus, the short-chain fatty acid may be considered to be lowering the temperature at which the thermal cut off of germination is taking place. In the three systems studied, one *in vivo* and two *in vitro*, the short-chain fatty acids are doing nothing unique in their effects on germination, liposome permeability, and membrane fluidity. In each case they are merely lowering the temperature at which a thermal event takes place, be it germination cut off, increasing permeability or increasing fluidity.

Contrary to the situation with wild oats (Berrie *et al.*, 1975), there is no evidence to suggest a regulatory role for the short-chain fatty acids *in vivo*. It has not been possible to correlate their endogenous levels with the physiological state of the seed. However, this does not invalidate our argument. Obviously any amphipathic molecule which is present in the medium *ab initio* could be incorporated into the membrane bilayer at levels far in excess of that which would occur in a natural condition. If these short-chain fatty acids were incorporated into the bilayer of seed

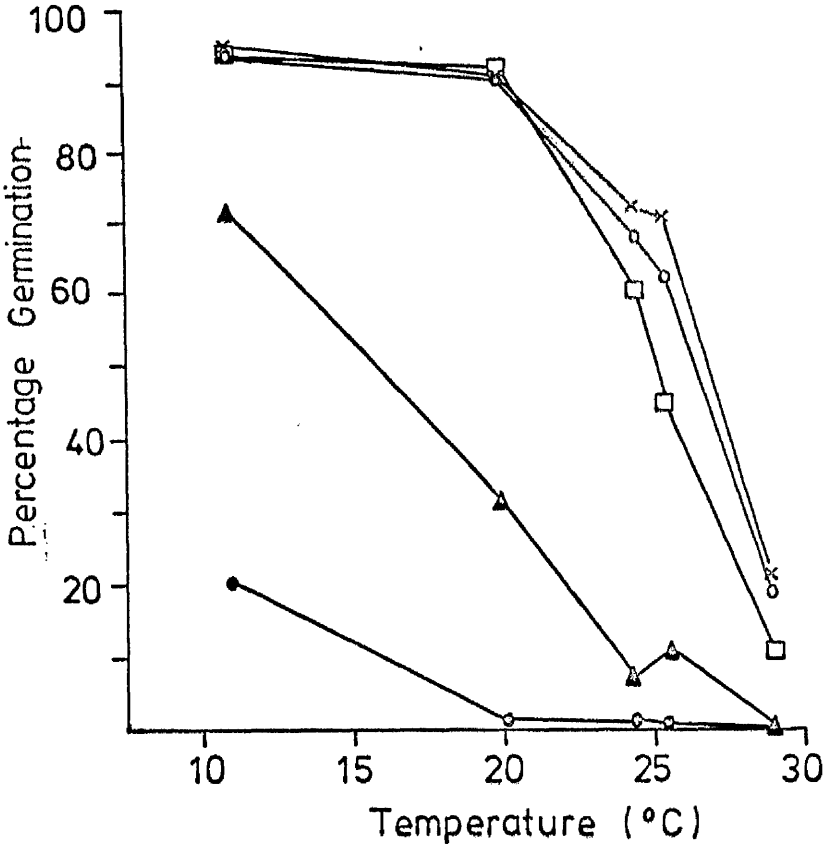


Figure 36: The effect of nonanoic acid on the germination of lettuce seeds at a range of temperatures. The increasing dose of nonanoic acid may be considered to be shifting the thermal cut-off to a lower temperature.

Conc/mol m^{-3}

× 0

o 0.559

□ 1.00

△ 1.77

• 3.16

membranes, they may artificially increase the fluidity of the bilayer, substituting for the elevated temperature normally required for this process. The degree of fluidity of membranes is critical to control of physiological processes. There is a considerable amount of data available which shows the extent to which an organism will alter its membrane lipids to maintain a constant degree of fluidity. De Silva et al (1975) have shown that the pattern of the fatty acid composition of near-isogenic lines of spring and winter wheats were altered in response to the growth temperature. They suggested that these changes were the result of a gene-linked temperature-sensitive mechanism controlling desaturation of fatty acids. At the lower temperatures, there was an increased proportion of di- and tri-unsaturated fatty acids. Thus the membrane would be in a fluid state at a temperature at which a more saturated membrane would be crystalline.

Unfortunately, there are no mutants of higher plants which could be used to test De Silva's hypothesis. However, there are strains of Acholeplasma laidlawii which exhibit a degree of auxotrophy for membrane fatty acids. With these mutants it is possible to alter the fatty acid composition of the bacterium's membrane at will (Silvius et al., 1977). It is thus possible to directly correlate the degree of saturation of the membrane fatty acids with the temperature of the phase transition.

The effect of temperature is not merely at the level of the well known kinetic parameters of enzymes, but it must also be seen as a determinant of the molecular configuration of the membrane. This molecular configuration itself is a determinant of the activation energy of reactions controlled by membrane-associated enzymes. Raison (1972(a); 1972(b)) has shown that the thermotropic membrane transition correlated with a break-point in the

Arrhenius plot for respiratory and other membrane-associated enzyme. Soluble enzymes had continuous, unbroken, Arrhenius plots. It is suggested that the degree of fluidity may alter the depth into a membrane which a protein penetrates. Such an alteration of the depth of penetration may alter the quaternary structure of the protein, and be possibly a "quasi-allosteric" effect, or it may cause active sites to be either exposed to the cytosol or concealed within the bilayer depending on its position.

It was suggested that the 36.5°C may be a temperature at which a thermal event takes place. It should be considered unlikely that such a temperature would represent a point of transition of the bulk membrane lipids, since it is normally the case that the bulk membrane lipids are in a fluid state at normal growth temperatures. However, there is evidence that membrane associated proteins are surrounded by a halo of crystalline lipid which undergoes a thermal transition well above that of the bulk lipid.

Stier and Sackman (1973) used spin labels as substrates of the cytochrome P_{450}-reductase system. The temperature dependence of the reduction kinetics of a fatty-acid spin label revealed an abrupt decrease in activation energy at 32°C, whereas no such break is seen for the reduction of the water soluble spin label, Tempo. At 10°C, there is no reduction of the membrane spin label, but an appreciable reduction of the water soluble spin label, whereas at 35°C, the rates of reaction are equal. Conventional spin labelling reveals that the bulk of the membrane does not undergo a transition at 32°C, and is fluid at 10-12°C. These experiments reveal a functional lipid mosaicism in which a halo of a quasi-crystalline phospholipid is segregated around the cytochrome P_{450}-reductase complex. This halo undergoes a transition

at 32°C, and may have a regulatory function in the hydroxylating reactions of the cytochrome.

It is possible that the 36.5°C thermal event which was suggested previously is the thermotropic phase transition of a lipid halo around a critical enzyme. This enzyme may be inhibitory to germination (cf. Black and Richardson, 1967) or it may be an enzyme which is necessary for the successful completion of germination, and the fluidity alters its properties in such a way as to render this impossible.

Recently Hendricks and Taylorson (1978) have suggested that the action of phytochrome in seeds depends on membrane organisation. They state that at high temperatures the membrane is in such a disorganised state that a high level of Pfr is required to elicit the response. If the short-chain fatty acids are in fact increasing membrane fluidity *in vivo*, then the data shown in Table 6 may be indicative of an effect of phytochrome state on the physiological expression of the physical state of the membrane. Haupt *et al.* (1969) showed that the photoconvertion of Pr to Pfr in *Mougeotia* involved a re-orientation of the chromophore in the plasma-lemma. Such a radicle alteration in the molecular arrangement of the phytochrome molecule is likely to alter at least its immediate environment. However, caution must be excercised as Morré (unpublished data) has recently suggested that in *Mougeotia* a single band of endoplasmic reticulum parallels the plasma membrane, thus the data of Haupt *et al.* could be interpreted in terms of phytochrome being associated with the endoplasmic reticulum.

Hendricks and Taylorson (1978) also suggested that the configuration of the polar head groups of the membrane lipids may be critical for phytochrome action. This is interesting since it is well documented that the maintenance of phytochrome association

with a pelletable fraction requires Mg^{++} to be present in the medium (Pratt and Marmé, 1976). This pelletability of phytochrome is unaffected by monovalent cations, so it is not a simple ionic strength effect. Brown and Seelig (1977) have shown that the presence of trivalent cations alters the orientation of the head groups in lecithin bilayers. Monovalent cations had no effect. However, they did not test divalent cations. It is possible that phytochrome associates with a lipid. Gressel and Quail (1976) have suggested that phytochrome associates with a protein component of the membrane, since treatment of phytochrome-containing membrane fractions with phospholipase C resulted in the release of more than 80% of the choline but no bound phytochrome was released. They interpreted this as indicating that pigment could not bind to the polar head groups. However, it is possible that the attachment of the large phytochrome molecule would make a lipid molecule inaccessible to the phospholipase. If this were the case, the phytochrome could still be associated with the 20% of the lipid which was not hydrolysed by the enzyme.

The hypothesis is presented that the control of thermodormancy lies at the level of membrane fluidity. It must be emphasised, to the point of being repetitious, that a membrane is much more than a physical barrier leading to the compartmentation of the cytosol. It should be seen as an integral, active part of the protoplasmic system, a dynamic association of lipid, protein, saccharides and inorganic ions.

While the use of lipid bilayers allows the development of easily reproducible artificial pseudo-membranes, the role of proteins, which can account for 60% of the membranes dry weight, must not be overlooked.

There is one obvious inconsistency between the results of

the bioassay and liposome studies on the one hand and the spin-label studies on the other. If the short-chain fatty acid were becoming incorporated into the membrane, as suggested in Figure 10(c), then an equimolarity of effectiveness would be expected between the different chain length fatty acids. However, such an equimolarity is not seen (See Figures 2 and 9). This is likely to be due to the difference between the water solubilities of the fatty acids (See Figure 1(b)), giving different partition coefficients between the lipid and aqueous phases. Thus, while the water solubility of hexanoic acid is greatest, there is likely to be the least percentage, of the total applied, in the membrane lipids.

In conclusion, it is our hypothesis that the control of thermodormancy lies at the level of membrane fluidity. The artefactual incorporation of a short-chain fatty acid into a membrane will increase the fluidity of the bilayer, thus mimicking the effect of elevated temperature.

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A P P E N D I X

APPENDIX

Table 1: The effect of octanoic acid on lettuce seed germination at 12°C. Mean and standard error.

Conc./mol m^{-3}	Time after imbibition (h) 48	72	96	120
0	22.50 ±3.67	39.75 ±1.44	46.75 ±1.65	48.00 ±0.91
0.316	19.75 ±3.20	40.75 ±1.44	47.00 ±1.43	49.00 ±0.41
0.559	14.75 ±1.11	38.00 ±2.74	43.50 ±1.85	47.50 ±0.96
1.00	16.75 ±2.33	41.25 ±3.77	45.00 ±2.12	47.75 ±1.65
1.77	15.50 ±1.50	26.75 ±1.03	35.75 ±0.25	43.75 ±0.95
3.16	1.75 ±0.75	16.00 ±4.04	20.75 ±4.01	27.00 ±3.72
5.59	0 -	0.75 ±0.48	3.25 ±1.44	5.50 ±2.22
10.00	0 -	0 -	0 -	0 -

APPENDIX

Table 2: The effect of octanoic acid on lettuce seed germination at 20°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	21.00 ±2.33	35.25 ±2.50	40.25 ±1.89
0.316	19.50 ±1.56	37.50 ±2.54	41.50 ±0.96
0.559	22.00 ±2.38	39.25 ±0.86	43.75 ±1.11
1.00	18.00 ±4.09	32.25 ±2.14	43.75 ±1.38
1.77	8.00 ±2.04	26.75 ±0.95	31.25 ±1.11
3.16	0.50 ±0.50	8.50 ±2.33	12.75 ±2.87
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

APPENDIX

Table 3: The effect of octanoic acid on lettuce seed germination at 24°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	24.25 ±3.28	25.50 ±1.94	30.50 ±2.96
0.316	25.25 ±3.71	29.00 ±2.55	30.75 ±3.43
0.559	20.50 ±1.85	25.75 ±3.09	27.75 ±3.80
1.00	14.25 ±1.93	22.00 ±2.10	23.50 ±1.33
1.77	7.25 ±2.40	14.25 ±3.30	14.50 ±3.12
3.16	1.25 ±0.75	2.50 ±1.33	2.75 ±1.38
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

APPENDIX

Table 4: The effect of octanoic acid on lettuce seed germination at 26°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	28.25 ±2.69	39.50 ±1.19	40.25 ±1.55
0.316	25.50 ±2.26	36.75 ±1.50	38.00 ±1.47
0.559	23.50 ±3.53	36.00 ±3.49	38.25 ±2.96
1.00	20.50 ±2.40	34.00 ±3.83	37.25 ±2.85
1.77	6.25 ±1.55	13.75 ±2.18	17.75 ±3.97
3.16	1.50 ±0.65	3.50 ±1.19	4.75 ±1.60
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

APPENDIX

Table 5: The effect of octanoic acid on lettuce seed germination at 28°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	13.25 ±2.10	15.25 ±1.50	15.75 ±1.65
0.316	11.50 ±1.26	15.25 ±1.11	16.00 ±1.08
0.559	7.00 ±1.42	11.75 ±1.65	15.00 ±1.69
1.00	4.75 ±1.93	9.75 ±2.03	9.75 ±2.03
1.77	1.25 ±0.75	4.75 ±2.50	4.75 ±2.50
3.16	0 -	0.50 ±0.50	0.50 ±0.50
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

APPENDIX

Table 6: The effect of heptanoic acid on lettuce seed germination at 12°C. Mean and standard error.

Conc./mol m^{-3}	Time after imbibition (h) 48	72	96	120
0	28.25 ±2.40	44.25 ±1.50	47.50 ±0.96	47.50 ±0.65
0.559	24.25 ±3.50	42.75 ±1.11	47.00 ±0.41	47.75 ±0.63
1.00	18.00 ±4.02	41.75 ±1.11	47.25 ±1.11	47.25 ±1.11
1.77	16.75 ±3.91	30.50 ±1.71	42.25 ±1.32	46.00 ±1.08
3.16	10.00 ±1.42	26.00 ±1.47	29.00 ±1.08	36.00 ±0.41
5.59	0.25 ±0.25	3.50 ±1.19	4.75 ±1.75	8.75 ±0.39
10.00	0 -	0 -	0 -	0 -

APPENDIX

Table 7: The effect of heptanoic acid on lettuce seed germination at 20°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	27.50 ±1.04	41.75 ±1.44	48.00 ±0.71
0.559	21.75 ±2.93	42.00 ±2.08	46.25 ±1.38
1.00	13.00 ±0.91	37.00 ±1.92	43.75 ±1.65
1.77	4.00 ±1.78	30.25 ±2.78	34.00 ±3.11
3.16	3.00 ±0.91	17.00 ±3.03	21.00 ±2.68
5.59	0.50 ±0.50	0.75 ±0.48	2.25 ±1.32
10.00	0 -	0 -	0 -

APPENDIX

Table 8: The effect of heptanoic acid on lettuce seed germination at 24°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	25.50 ±4.88	34.75 ±3.71	36.50 ±4.85
0.559	21.00 ±2.67	31.25 ±2.20	33.00 ±5.12
1.00	13.50 ±1.54	22.50 ±2.11	35.50 ±2.22
1.77	7.25 ±1.11	16.00 ±3.39	16.50 ±3.12
3.16	1.25 ±0.63	4.75 ±1.38	7.00 ±1.64
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

APPENDIX

Table 9: The effect of heptanoic acid on lettuce seed germination at 26°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	10.00 ±2.86	17.25 ±1.18	15.00 ±2.97
0.559	10.75 ±3.04	13.00 ±3.58	15.50 ±4.74
1.00	7.50 ±2.54	13.25 ±3.40	13.75 ±3.12
1.77	2.50 ±1.72	7.00 ±1.58	8.25 ±1.89
3.16	0.50 ±0.29	1.75 ±0.25	3.25 ±0.48
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

APPENDIX

Table 10: The effect of heptanoic acid on lettuce seed germination at 28°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	5.00 ±3.39	13.00 ±6.18	13.25 ±6.25
0.559	6.50 ±1.26	10.75 ±2.46	11.00 ±2.49
1.00	4.25 ±1.65	8.75 ±4.50	9.75 ±5.12
1.77	0.25 ±0.25	4.50 ±1.04	6.00 ±1.08
3.16	0 -	0 -	0.25 ±0.25
5.59	0 -	0 -	0 -
10.00	0 -	0 -	0 -

APPENDIX

Table 11: The effect of hexanoic acid on lettuce seed germination at 12°C. Mean and standard error.

Conc./mol m^{-3}	Time after imbibition (h)			
	48	72	96	120
0	29.50 ±3.12	41.75 ±0.48	44.25 ±1.44	47.25 ±0.86
0.559	24.50 ±2.06	39.50 ±1.55	45.50 ±1.19	46.25 ±1.03
1.00	26.50 ±1.85	39.25 ±2.25	42.50 ±2.10	43.00 ±2.04
1.77	25.00 ±3.08	36.00 ±0.82	42.25 ±0.48	43.50 ±0.50
3.16	22.50 ±0.65	30.00 ±0.91	38.50 ±0.96	40.50 ±0.50
5.59	10.25 ±1.65	19.00 ±2.49	26.50 ±3.67	29.50 ±3.07
10.00	0 -	0 -	0.25 ±0.25	0.50 ±0.50

APPENDIX

Table 12: The effect of hexanoic acid on lettuce seed germination at 20°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	18.75 ±2.46	32.00 ±2.97	38.75 ±3.30
0.559	21.50 ±1.85	37.50 ±2.21	44.25 ±1.18
1.00	16.25 ±1.93	33.50 ±2.90	40.75 ±3.83
1.77	7.00 ±0.71	23.50 ±2.33	34.00 ±1.47
3.16	3.00 ±0.41	18.25 ±1.03	25.00 ±1.33
5.59	0.25 ±0.25	7.25 ±1.70	9.25 ±0.86
10.00	0 -	0 -	0 -

APPENDIX

Table 13: The effect of hexanoic acid on lettuce seed germination at 24°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	22.00 ±3.58	26.50 ±3.71	31.00 ±2.68
0.559	23.25 ±3.82	24.75 ±3.38	28.00 ±5.12
1.00	22.00 ±1.47	27.75 ±2.06	29.00 ±1.23
1.77	13.75 ±0.86	21.00 ±1.69	23.00 ±1.69
3.16	5.25 ±1.25	12.50 ±3.02	14.75 ±3.33
5.59	0.25 ±0.25	3.00 ±1.47	4.75 ±1.98
10.00	0 -	0 -	0 -

APPENDIX

Table 14: The effect of hexanoic acid in lettuce seed germination at 26°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	16.50 ±3.18	19.75 ±3.91	21.00 ±4.12
0.559	13.25 ±1.70	20.50 ±3.97	20.50 ±3.97
1.00	8.75 ±2.93	14.25 ±1.80	14.75 ±1.98
1.77	3.50 ±1.56	12.00 ±2.12	14.00 ±1.83
3.16	0.75 ±0.25	1.75 ±0.48	6.25 ±1.70
5.59	0 -	0.50 ±0.29	0.50 ±0.29
10.00	0 -	0 -	0 -

APPENDIX

Table 15: The effect of hexanoic acid on lettuce seed germination at 28°C. Mean and standard error.

Conc/mol m^{-3}	Time after imbibition (h) 24	48	72
0	7.50 ±0.29	10.25 ±0.86	10.50 ±1.04
0.559	8.50 ±1.85	10.50 ±2.58	13.00 ±3.08
1.00	5.50 ±1.23	6.50 ±2.18	6.50 ±2.18
1.77	5.25 ±0.65	8.75 ±1.89	10.25 ±2.25
3.16	0.50 ±0.29	2.75 ±0.48	3.00 ±0.71
5.59	0 -	0 -	0.25 ±0.25
10.00	0 -	0 -	0 -