PLANT GROWTH REGULATORS IN THERMODORMANCY AND GERMINATION OF FRUITS OF *LACTUCA SATIVA* L. cv. 'GRAND RAPIDS'

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

by

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Abbreviations

AAA	:	automatic activity analyser
ABA	:	abscisic acid
AES	:	absolute external standard
ATP	:	adenosine triphosphate
BA	:	benzyladenine
c-AMP	:	cyclic 3',5'adenosine monophosphate
Ci	:	Curie
cpm	:	counts per minute
cv	:	cultivar
DEAE	:	diethylaminoethyl
df	:	degrees of freedom
dpm	:	disintegrations per minute
DNA	:	deoxyribonucleic acid
ECD	:	electron capture detector
FID	:	flame ionisation detector
g	:	gram
GA	:	gibberellin
GC-MS	:	gas chromatography - mass spectrometry
GLC	:	gas liquid chromatography
HER	:	high energy reaction
hr	:	hour
IAA	:	indole acetic acid
KN/m^2	:	kilonewtons per metre^2
m	:	metre
M	:	molar concentration
mV	:	millivolts
P.C.I.B.	:	4-chloroindolebutyric acid
PVP	:	polyvinylpyrollidone
Rf	:	relative retention time for TLC
RNA	:	ribonucleic acid
m-RNA	:	messenger ribonucleic acid
t-RNA	:	transfer ribonucleic acid
S.E.	:	standard error
sec	:	second
S.E.V.	:	standard elution volume
TLC	:	thin layer chromatography

Summary

The aim of this research project has been to investigate the hormonal control of germination in light sensitive lettuce seeds. We have used the classic lettuce seed variety, Grand Rapids which, given suitable moisture conditions, will readily germinate below a critical temperature and not above that temperature. This 'thermodormancy' is known to be overcome by red light, mediated through phytochrome, and by a number of exogenously applied chemicals. Little information was available in the literature regarding the endogenous hormonal levels in lettuce seeds. It was decided to examine the involvement of endogenous hormones. Principally we have examined the levels of indole acetic acid and abscisic acid during imbibition at 20°C below the 'critical temperature' and at 30°C above the 'critical temperature'. Abscisic acid and indole acetic acid (hereafter ABA and IAA) have both been identified in fruits of Grand Rapids by combined gas liquid chromatography-mass spectrometry (GCMS). In the mass spectrum (MS) of any chemical the fragment occurring in the largest amounts is known as the most 'abundant ion'. It is possible to 'focus' on one ion instead of taking a full MS and this technique allows the measurement of smaller amounts of material. Using this technique of single ion monitoring the levels of both ABA and IAA have been measured. With ABA the levels have also been measured using an electron capture detector in a gas liquid chromatogram. This detector is several orders of magnitude more sensitive for ABA than the more commonly used flame ionisation detector. Using these techniques we have shown that the levels of both 'free' and 'bound' (released by base hydrolysis) ABA vary during imbibition at 20°C or 30°C. However, it was not possible to correlate these changes with germination behaviour. Furthermore, there were differences between different seed batches. With respect to IAA the data obtained were difficult to interpret. The major problem in working with IAA is that IAA was present in low levels and this necessitated the use of large quantities of seeds for extraction. Changes do take place in the endogenous levels of IAA but as with ABA these could not be correlated with changes in germination.

Initially, it was hoped to look at gibberellin levels in lettuce fruits. It is possible to erect a hypothesis that the control of

photosensitive seeds is under the dual control of promoters and inhibitors and that seeds cannot germinate above the critical temperature because of the presence of inhibitors or the lack of promoters. Control might reside in either reducing the level of inhibitors which would allow the promotive forces present to bring about germination, or in increasing the promotive forces. The latter could entail either the new formation or release of promoters to active sites. Of course, the two are not mutually exclusive. Consequently, we wished to examine the role of gibberellins in lettuce seed germination. Preliminary studies using bioassays indicated that there were major difficulties in studying the levels of gibberellin-like substances during germination. Metabolism of applied radioactive gibberellins might yield meaningful results but in the absence of any information about the gibberellins present in lettuce this is not feasible.

An indirect approach to this problem was carried out using exogenous growth retardants. The theory behind this work is that these growth retardants specifically inhibit gibberellin biosynthesis. If this is the case then applied gibberellins should completely overcome the inhibitory effects of growth retardants, provided saturating levels of gibberellins are added. However, any statistical interaction would be evidence against the proposed mode of action of growth retardants. In fact it was found that such an interaction occurred and that cytokinins and red light both overcame the inhibitory effects of the growth retardants. Consequently, it was not possible to say whether or not red light caused the production of gibberellins.

From this work it also became apparent that lettuce seed germination was promoted by exogenous cytokinins. There is some controversy in the literature about whether cytokinins alone will break thermodormancy or whether the seeds require small amounts of radiant energy in the red region to respond. Furthermore, no comprehensive study of the effect of exogenous cytokinins, abscisic acid, indole acetic acid, gibberellins and red and far-red light on Grand Rapids seeds has been reported. In order to clarify this situation and it was hoped to throw some 'light' on the control of seed germination, this study was undertaken. We have found that cytokinins promote germination in complete darkness at 30°C.

Abscisic acid induced dormancy of lettuce seed can be overcome by red light, by cytokinins and by gibberellins. The fact that gibberellins and red light have been reported as incapable of overcoming ABA would appear to be a function of the concentrations of exogenous chemical added and the time scale of counting germination. In my experiments germination, as assessed by radicle emergence, was counted after 24 hrs and subsequently at 24 hr intervals. This allows analysis in terms of both rate and final germination. Red light was also found to overcome IAA inhibition of germination both at 20°C and 30°C. The inhibition of lettuce seed germination by IAA was lost with time as was ABA inhibition at 20°C. For IAA a 'reverse' escape experiment was conducted where seed was transferred to IAA from water after 1, 2, 3, ----- 24 hrs in water at 20°C. It was found that there was an increasing escape from the inhibitory effect of IAA after 12 hrs imbibition. This was interpreted as showing IAA to have a possible role in germination and not merely radicle growth. A similar experiment with ABA provided more conclusive evidence in favour of ABA inhibiting germination.

Finally, in an attempt to establish what role, if any, either ABA or IAA had in germination, the metabolism of [^{14}C] ABA and [^{3}H] IAA by Grand Rapids lettuce seeds was studied. The results showed that seeds carried out metabolic conversions of ABA but that these changes were not associated with the release from thermodormancy. Changes in labelled IAA indicated that the conversions although requiring the presence of seeds, could not be considered to result from metabolic processes. The results from the IAA metabolism studies do not show a role for IAA.

In summary, we have shown the presence of ABA and IAA for the first time in Grand Rapids lettuce seeds, but have been unable to support the hypothesis that ABA and IAA are controlling compounds in the release or imposition of thermodormancy. If one or both of these compounds imposes dormancy, it can only be the result of its presence and not at a more fundamental level.

SECTION I

GENERAL INTRODUCTION

"The obvious truth is that the moment any matter has passed through the human mind it is finally and for ever spoilt for all purposes of science. It has become a thing incurably mysterious and infinite: this mortal has put on mortality."

G. K. Chesterton

The studies reported in this thesis were aimed at gaining a more complete appreciation of the role of plant growth regulators in thermodormancy and germination in seeds of Lactuca sativa, cultivar (cv) Grand Rapids. For convenience the 'fruits' of lettuce will be referred to as 'seeds' in this thesis. The fruit of lettuce is technically a cypsella, a one seeded fruit with a dry, leathery pericarp.

Studies on the physiology of germination in lettuce seeds started in 1928 when it was shown that certain cultivars of lettuce produce seeds which show a specific temperature requirement for germination. One of the cultivars included in this study was Grand Rapids (Borthwick and Robbins, 1928). Nearly fifty years have passed since this early work, yet the control processes involved in germination are still poorly understood. The literature dealing with the diverse aspects of germination and dormancy is too extensive to be reviewed here and this review will therefore focus its attention on the role of plant growth regulators in the control of germination and dormancy. Germination may be considered as only one of a number of profound morphogenetic events, all of which may be controlled in a similar way. It is intended to make use of literature dealing with other morphogenetic events in an attempt to gain further insight into the control of germination and dormancy. Use will also be made of review articles rather than a compilation of all the available literature.

It is pertinent, before proceeding, to define what is meant by the terms germination and dormancy as used in this study. Following imbibition of water by a whole seed, changes occur in the embryo which result in the onset of cell enlargement and division in the embryonic axis. At some point during the process, the enlarging radicle penetrates the testa or other covering tissue. This first appearance of the radicle is commonly thought of as representing germination itself, but it can also be regarded as being merely the visual evidence of the previous occurrence of germination. If the latter view is adopted this expansion and division should be regarded as post-germinational growth and as such can often be distinguished from germination itself by differences in responses to given physical and chemical environments. The concept of germination subscribed to in this work envisages some controlling activity which occurs prior to,

and results in, the onset of cell enlargement . At present, however, the proximity in time of this germination step to the initial stages of growth makes the study of germination in isolation very difficult.

An adaptive feature of many plant species is the ability of their seeds to remain ungerminated or dormant under conditions which are favourable for growth, and would allow germination of non-dormant seeds of the same species. Dormancy is often manifest as a restriction of the range of conditions permitting germination rather than a total suppression of germination. This is true of Grand Rapids lettuce seeds where germination is temperature sensitive. It is probably not unreasonable to state that the majority of seeds show some form of dormancy, particularly with respect to small seeds and seeds of woody species. Perhaps uniform, rapid germination will be found only in the case of cultivated species where man has exerted a selection pressure. It is important to understand the function of dormancy in seeds. Cohen (1968) has attempted to analyse the optimisation of reproduction of a population, in order to achieve survival of the species and long-term optimal growth. He concluded that uniform germination in wild species is not a desirable character. Baskin and Baskin (1973a) have shown that in Phacelia dubia the ability of seeds to germinate can be correlated with the ability of the seedlings to survive. It is intuitively obvious why dormancy should be essential for the survival of a species. In a cold climate a seedling produced in the Autumn would have little chance of surviving the Winter months to complete its life cycle. The seed must have some means of remaining dormant until the prevailing climatic conditions are suitable for the survival of the young plant.

This immediately raises the question of what factors determine whether a seed is dormant or non-dormant, and to what degree the seed exhibits dormancy. In Grand Rapids lettuce seeds different seed batches show quantitative differences in germination behaviour. Table 1 shows that the level of germination attained at 20°C and 30°C varies amongst seed batches. It can also be seen from this table that little germination occurs at 30°C. However, at this temperature germination is promoted by irradiation at a wavelength of 650 nm in the electromagnetic spectrum, 'red light' (Borthwick et al., 1952). In a seed batch obtained in 1969, a twenty second exposure to red light, after 2 hours imbibition, gave the same response as a two

minute exposure given to a 1972 seed batch (Robertson, unpublished). Different batches of Grand Rapids seeds exhibit quantitatively different responses to treatments given to overcome thermodormancy. Indeed, it would appear that photosensitivity is manifest only in Grand Rapids seeds grown in the United States. The seeds used in this study were purchased from the Page Seed Company, New York, but were grown in the Boise valley in Idaho. This area has low humidity, long day length and a wide diurnal temperature fluctuation during the summer months. In considering what factors may be involved in determining whether a seed is dormant, or to what degree a seed is dormant, we should take into account the contribution of both heredity and environment. Germination ecotypes have been based on differences in germination performance of seeds collected from natural populations. Clearly, before genecological significance can be assigned to these differences the contribution of the environment must be considered (Baskin and Baskin, 1973b).

Before examining the contributions of environment and genetical background it may be worthwhile to consider the difference between a dormant and non-dormant seed. Amen (1968) has proposed the following four developmental phases for seed dormancy:-

1) An inductive phase, characterized by a marked decline in hormone levels.

2) Maintenance phase, constituting an indefinite period of partial metabolic arrest.

3) Trigger phase, representing a period of sensitivity to specific environmental cues.

4) Germination phase, marked by increased hormone and enzyme activity consummating in the resumption of growth of the latent embryonic axis.

It can be proposed that the difference between non-dormant and dormant seeds may be that a trigger phase is not required in non-dormant seeds. In seeds showing some form of dormancy mechanism the requirement for a 'trigger' mechanism to overcome dormancy may be initiated during the inductive phase. The events which take place during the inductive phase could also determine the degree of dormancy found in any one seed crop. The absolute presence or absence of dormancy could depend on the genetic composition of the mother plant.

The inductive phase will here be considered as an unspecified period of time prior to the dispersal of seeds from the mature mother plant. It would be valuable to be able to distinguish between the effect of the environment on the mother plant and on the seed. This ideal cannot often be met, although work by Karssen (1970) indicates that the concept of differential effects may be valid. He demonstrated the existence of two types of dormancy in Chenopodium album, affected by the photoperiod given during the growth of the mother plant. The first disappears after about three months' dry storage and is thought to depend on the level of active phytochrome in the seeds. The second seems to depend on the effect of daylength on photosynthesis and is related to the thickness of the seed coat. A similar situation is found in Chenopodium amaranticolor where the seeds produced under long days possessed unusually thick seed coats, which hindered germination by offering resistance to the radicle emerging (Lona, 1947). Juntilla (1973a) has shown that in Syringa vulgaris and Syringa reflexa, seeds exhibit two types of dormancy, true embryo dormancy and mechanical resistance of the endosperm. Using vegetatively propagated mother plants to eliminate genetic variability, it was shown that the temperature during maturation could affect the mechanical resistance of the endosperm, although not appearing to affect embryo dormancy. Although Karssen interprets his results in terms of an effect on the mother plant, it is conceivable that changes in the thickness or mechanical resistance of restraining layers, are affected by factors affecting the seed rather than the mother plant. There is good evidence that environmental factors, during the latter part of seed maturation, do have an effect on the subsequent germination of the mature seeds, irrespective of whether the effect is on the seed or the mother plant. Koller (1962) has reported that in Grand Rapids lettuce seeds, quantitative changes in the germination responses could be shown in plants harvested under different temperatures or light conditions. The critical factors would appear to be temperature and humidity some 10-30 days preceding harvest (Harrington and Thomson, 1952). As previously mentioned, the lettuce seeds used in this study were grown in an area with wide diurnal temperature fluctuation and low humidity. Temperature during a thirty day period preceding harvest of rose seed populations appeared critical in determining the degree of dormany found (Von Abrams and Hand, 1966). During the later stages of maturation of seeds on the mother plant there

is a rapid loss of water. This occurs in seeds which show subsequent dormancy or not (Mathews, 1973). This loss of water is accompanied by changes at the ultrastructural level. Polysomes disappear and ribosomes are found free in the cytoplasm (Klein and Pollock, 1968). These changes are necessary to prepare the cells to withstand desiccation. The breakdown of polyribosomes, as indicated by an increase in monosomes, is a general result of water stress. Nir et al. (1970) showed that water stress had this effect in maize root tips. These changes appear to be partially reversible during germination (Barker and Rieber, 1967; Sturani et al., 1968). The loss of water is accompanied by an increase in the resistance of the seed to adverse external conditions. During seed maturation there is the formation of large amounts of storage materials, fats, carbohydrates, protein, etc. The process of switching off the seed's metabolism is at present only poorly understood but would appear to be an organised series of events, since not all metabolic activities terminate abruptly and simultaneously (Mayer, 1973). It is well documented that hormone levels in seeds change after the seeds are removed from the mother plant, for example in seeds requiring stratification, but there is also evidence of changes in endogenous hormones during the maturation process (Stoddart, 1965; Curtis and Cantlan, 1968; Thomas et al., 1973). Furthermore, Black and Naylor (1959) found that the application of GA_3 to Avena fatua plants whilst the seeds were maturing resulted in the production of non-dormant seeds. They hypothesised that the GA_3 was transported to the seeds during development, increasing the levels of naturally present GAs. A similar situation was reported by Morgan and Berrie (1970) using the winter wild oat Avena ludoviciana. Furthermore they found that the metabolic inhibitors sodium p-chloromercuribenzoate, sodium iodoacetate and coumarin reduced the development of dormany in ripening seeds of this species. The situation is further complicated by the presence of inhibitors. A new class of 'dormancy inducing'inhibitors, identified as short-chain fatty acids, have recently been found in wild oats (personal communication, A.M.M. Berrie). At present the levels of these fatty acids during maturation are being examined (Don and Buller, personal communication). Black and Naylor (1959) presented no evidence for GA transport to the seed. Treatment of seedlings of Lactuca scariola L. with 100 mg/l GA_3 or 5,000 mg/l C.C.C.

were all shown to result in increases in the level of GA-like compounds in the resulting seed progeny. It was suggested that the effect of daylength on plant performance is mediated by the level of growth regulating substances within the plant and that the behaviour of seed can be modified by the parental environment via the accumulation of different levels of certain growth factors in the seeds (Gutterman et al., 1975). The authors correctly pointed out that an increase in one growth substance in the parent plant can result in the accumulation of a different one in the seeds. We could propose that the endogenous hormone balance of mature seeds will depend on environmental conditions during the final period of seed maturation. A period of drought during this critical period would not only affect the endogenous hormones of the mother plant but could conceivably hinder the movement of these hormones into the seeds.

A second important effect of the environment during the maturation of seeds relates to the endogenous phytochrome status of mature seeds. Many, but not all seeds are light-sensitive, and phytochrome is active in many apparently non-photoblastic seeds. However, the type of seed which is non-photoblastic is often large or belonging to a woody plant species, where the seeds show a dormancy which is overcome by after ripening (Wareing and Saunders, 1971; Chen and Varner, 1973). It is my opinion that the control mechanism in this type of dormancy need be no more subtle than a change in the endogenous hormone balance of mature seeds (which was determined by the environment under which mother plants were matured) in response to subsequent external environmental factors. However, lettuce seeds belong to a group of species with small seeds which often exhibit phytochrome-controlled dormancy. There is considerable evidence that the phytochrome status of mature seeds is affected by the environment under which the mother plants were grown. Koller (1970), studying the action of white light on the germination of Atriplex dimorphostegia, came to the conclusion that the quantitative differences in the photoresponses of 'flat' and 'humped' seeds of this species, resulted from differences in the degree to which their phytochrome system was desensitized by a factor which was susceptible to inactivation by supra-optimal temperature and water stress. During the rapid desiccation encountered in maturing seeds changes may take place in phytochrome, when present. What these changes are remains speculative,

but recent evidence from work carried out with pea epicotyl tissue has shown the presence of a number of intermediates in phytochrome phototransformations (Kendrick and Spruit, 1973a, b and c). It is possible that phytochrome in the dry seed exists as one or more of these intermediates, and that the light conditions at critical times during the maturation process may affect the composition of intermediates present. Furthermore, phytochrome molecules may be present in different cellular compartments, a factor which may be influenced by the external conditions. Kendrick and Spruit (1973d) propose that only phytochrome in certain sites will be active.

Temperature during the period of seed maturation on the mother plant was also found to be critical in determining the subsequent photoresponse of seeds of the species Anagallis arvensis (Grant Lipp and Ballard, 1963). We have previously mentioned the effect of photoperiod on the dormancy of Chenopodium album (Karssen, 1970). Photoperiod has been shown to affect the dormancy of Chenopodium polyspermum (Jacques, 1968), whilst in Diplotaxis harra, seeds showed much higher percentage germination when obtained from plants grown in short day conditions than from long day ones (Evenari, 1965-66). In Arabidopsis thaliana, plants grown under light rich in far-red wavelengths produce light requiring seeds, whilst if the ratio of red/far-red light is high, seeds will germinate in darkness (McCullough, 1969; McCullough and Shropshire, 1970). This effect has been shown to be localised within the seeds and is not an effect on the mother plant. The developing seeds were found to be sensitive to spectral changes throughout embryogenesis until the seeds began to dehydrate, just prior to full maturation. At that point the phytochrome is stabilised in the form photoinduced before dehydration (Hayes and Klein, 1974).

So far we have examined the effect of a number of environmental treatments given to maturing plants, on the subsequent germination response of mature seeds. In some cases genetically identical plants were investigated. It is important to examine the influence of the origin of the mother plant on subsequent germination behaviour. Datta et al.,(1972) have shown that the genetic origin of the mother plant in Aegilops ovata, has an effect on the germination qualities of its offspring. Seeds of Nicotiana tabacum have been reported to require light in order to germinate (Kincaid,1935). According to Kasperbauer (1968) the light requirement varies among lots of the same commercial

variety, from seeds which show no light requirement, to seeds which have an obligate light requirement. He selected lines of initially light-requring lots and carried out a series of self pollinations and reciprocal crosses. Seeds resulting from reciprocal crosses differed in dark germinability from the parents. Both parents were found to contribute towards the light sensitivity of the seed; however, the contribution of the maternal parent was greater than that of the pollen parent. Similar observations have been made in seeds of Cucumis sativa by Suyuki and Takahashi (1969), who present clear evidence for the importance of the maternal parent. Recently, Globerson et al. (1973) isolated two lines of Grand Rapids lettuce seeds which varied greatly in their light requirements, showing that the response of mature seeds to subsequent irradiations was in part of genetic origin. This proposal has been reinforced using three pure lines of Grand Rapids lettuce and studying their F_2 and F_3 progeny. This work indicated that the factors controlling germination could be transmitted in a normal Mendelian manner. The authors showed that the differences in germinability of these lines could not be caused by the seed and fruit coats (Globerson et al., 1974).

To summarise, evidence has been presented that the germination of seeds is dependent on the mother plant from which the seeds were obtained. The genetic composition of the parents, particularly the maternal parent, has been shown to be critical and this genetic potential may be influenced by the external environment in which the parent plants were matured. Of the many environmental factors which could be involved, temperature and radiation seem to be important in determining the quantitative aspect of an inherently dormant population. The period during which maturing seeds appear most susceptible to environmental influence is just before rapid desiccation, prior to the dispersal or fall of the seeds from the mother plant. Exactly how these environmental factors influence the subsequent germination pattern of maturing seeds is uncertain, but there is evidence that the endogenous hormone and phytochrome balance of mature seeds is influenced. I have invoked the 'trigger' suggested in Amen's (1968) theory to explain seed dormancy and how this 'trigger' may operate will be discussed in more detail when dealing with the possible mode of action of plant hormones and phytochrome in overcoming dormancy.

The role of phytochrome in the germination of Grand Rapids lettuce seeds

Mature plants produce seeds which show dormancy, this being of adaptive value in ensuring the continued survival of the species. The first study on the physiology of germination in Grand Rapids lettuce seeds was carried out, as has been mentioned, by Borthwick and Robbins (1928). This study was prompted by the difficulty encountered by Californian lettuce growers, in germinating lettuce seeds at certain times of the year. It was found that in a large number of cultivars of lettuce, including Grand Rapids, germination was inhibited above a certain temperature. The adaptive significance of this is immediately apparent. If the seeds were to germinate at a temperature above the cardinal point for growth of seedlings, then their chances of survival would be slim. This temperature determined dormancy has been well described by Berrie (1966) where it was shown that Grand Rapids lettuce seeds would germinate below a certain 'critical temperature', in this case about 26.5°C (see Fig. 1) but not above this temperature. The seeds can be said to exhibit 'thermodormancy'.

Since the early work of Borthwick and Robbins (1928) a large number of factors has been shown capable of inducing germination in Grand Rapids lettuce seeds, held at a temperature where germination would otherwise not take place. These will be discussed in the relevant sections of this review. The first factor which was shown to influence thermodormancy was white light. Flint and McAlister (1935, 1937), using the cultivar Arlington Fancy, showed that temperature-induced dormancy could be overcome by exposure to white light. The most effective wavelengths in breaking 'thermodormancy' were found to lie in the range 580-700 nm, with a maximum promotion of germination at 670 nm. Maximum inhibition of this promoted germination was found to lie in the bands above 700 nm or at 440-480 nm. Later Borthwick *et al.* (1952, 1954) demonstrated an identical situation in Grand Rapids lettuce seeds with an action spectrum showing maximum promotion at 650 nm and maximum inhibition at 730 nm. Furthermore, at a temperature above the critical temperature, irradiation at 650 nm (red light) had no effect in promoting germination if immediately followed by an exposure to radiation at 730 nm (far-red light). If this cycle was followed by red light then

Figure 1

The effect of temperature on the germination of seeds of Grand Rapids lettuce.
(Data from Berrie, 1966)

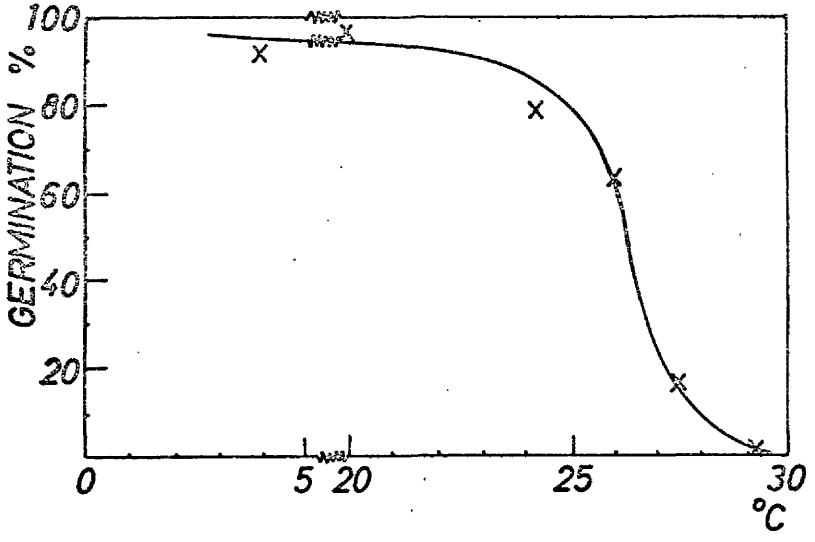


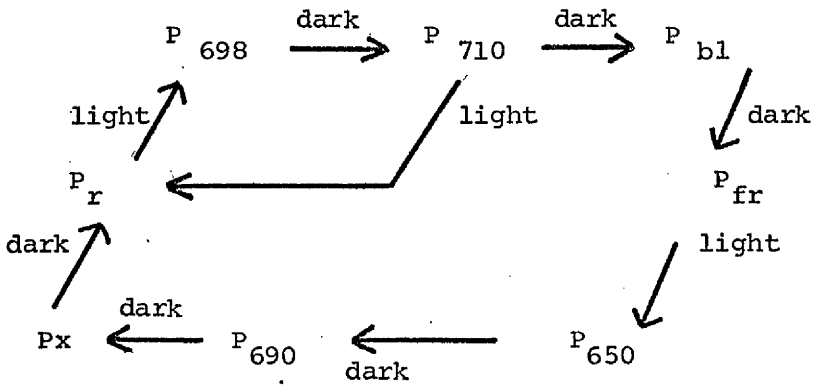
Figure 1. *Germination in darkness of 'Grand Rapids' lettuce seed at different temperatures.*

visible radicle emergence occurred after a specified time; germination was seen to have taken place. Multiple red, far-red reversal could be shown, the germination response being dependent on the last given irradiation.

It is now recognised that this red, far-red reversible photosystem operates through the plant chromoprotein, phytochrome. The term "phytochrome" has been attributed to Warren Butler (Borthwick, 1972) who originally "half jokingly" used the term shortly after the first successful spectrophotometric detection of phytochrome (Butler et al., 1959). Phytochrome in the red-absorbing form is designated P_r whilst phytochrome in the far-red absorbing form is designated P_{fr}. These forms are interconvertible by giving red or far-red light.

$$P_r \underset{730\ nm}{\overset{650\ nm}{\rightleftharpoons}} P_{fr}$$

The process of interconversion involves a large number of intermediates. It is beyond the scope of this review to give these in detail. However, mention of intermediates will be made later. Consequently, I present the most recent thoughts on the intermediates involved in this phototransformation below.



(Kendrick and Spruit, 1973a, b, c and d.)

It has been shown that phytochrome is involved in the control of thermodormancy in Grand Rapids lettuce seeds (Borthwick and Hendricks, 1952). Further work has revealed many of the details on how phytochrome is involved in the control of dormancy. Several lines of investigation have been used. Prior to the naming of

phytochrome and its physical detection, measurement of germination, some time after a particular light treatment, was the only method of showing the involvement of a photomorphogenetic pigment in germination. For reasons which will become apparent, physiological studies are often still the only way of examining phytochrome in seeds. A second approach in studying phytochrome is the use of spectrophotometric assay, using *in vivo* or *in vitro* samples, where phytochrome is extracted and highly purified. As yet no *in vitro* measurements of 'seed' phytochrome have been made. A third approach is the use of immunocytochemical assay using an indirect antibody labelling method employing peroxidase as the ultimate label (Pratt and Coleman, 1971).

In the following discussion we shall describe in more detail the situation with respect to Grand Rapids lettuce seeds, and attempt to interpret these results with reference to other work on seeds and other plant tissue. With Grand Rapids lettuce seeds no direct measurements of phytochrome have been reported: all the available information has been obtained using physiological studies.

Grand Rapids lettuce seeds are positively photoblastic above the critical temperature; that is, they require an exposure to light in order to germinate. Seeds are classified, with respect to light requirements, as positively photoblastic, negatively photoblastic or non-photoblastic. Negatively photoblastic seeds germinate in darkness, their germination being inhibited by exposure to light, whilst non photoblastic seeds appear to be unaffected by the presence or absence of light. The number of studies showing germination to be affected by light, and in later work specifically under phytochrome control is vast; suffice to say that amongst 'small' seeds phytochrome control may be almost ubiquitous (see, for example, Kinzel, refs. cited in Gardner, 1921; Evenari; 1961; Toole, 1961; Rooden *et al.*, 1970). Grand Rapids lettuce seeds have been used to demonstrate a classic phytochrome-mediated germination response. However, all available evidence would suggest that it is in no way unique, and that the principles derived from studying this seed in detail can be applied to other similar seeds.

We have shown that Grand Rapids lettuce seeds respond to red light given to seeds held above the critical temperature. Implicit in this is that the seeds are in water where imbibition can occur. It has been shown that the response of lettuce seeds to irradiation

with red light depends on the water content of the seeds at the time of irradiation (Borthwick et al., 1954; Ikuma and Thimann, 1960). Berrie et al. (1974) have shown that Grand Rapids seeds are capable of responding to red light if the seed moisture content exceeds 6%. It would appear that a slightly higher water content is required for maximum far-red light repression than for red light stimulation of germination (Hsiao and Vidaver, 1971a). After a prolonged period in water lettuce seeds lose their sensitivity to red light (Borthwick et al., 1954; Ikuma and Thimann, 1960), and from physiological studies Hsiao and Vidaver (1973) have interpreted this as being the result of dark reversion of phytochrome in the far-red absorbing form P_{fr}. Although the action of phytochrome is not obvious until visible radicle emergence, some 14 hrs after imbibition, this does not mean that the phytochrome is not immediately active. Bewley et al. (1968) have interpreted a series of experiments as showing P_{fr} to be active within 5 minutes of its formation in Grand Rapids seeds. If the lettuce seeds are given red light and then left for a period of time in darkness before being exposed to far-red light, there comes a time when far-red light is no longer capable of reversing the germination promotion elicited by the red exposure (Borthwick et al., 1954; Ikuma and Thimann, 1960; Black et al., 1967). This 'escape time' varies between seed batches, but is always of several hours duration. The interval between red, far-red irradiations is used for a temperature-dependent reaction which is probably biochemical (Berrie, 1966; A. Kadman-Zahavi, 1960).

If seeds held above the critical temperature are given red light and subsequently dried prior to radicle emergence, then on rehydration the seed can be shown to respond to the red light given before desiccation (Hsiao and Vidaver, 1971b; Loercher, 1974; Berrie et al., 1974). This persistance of a potentiated photoresponse is not unique to lettuce seeds. Kincaid (1935) reported that tobacco seeds retain their capacity to germinate when given light whilst imbibed, and then dried, and a similar situation is reported in seeds of Artemisia monosperma (Koller et al., 1964). Such desiccation of seeds has been shown to do little harm provided it is carried out prior to cell division and enlargment (Berrie and Drennan, 1971). The importance of such a 'potentiated' response is obvious. Vidaver and Hsiao (1972) showed that the effects of red or far-red irradiation on partially hydrated seeds were undiminished

by subsequent dry storage for more than one year before germination. The pigment appears to be extremely stable when dehydrated. Previously it was indicated that rapid dessiccation occurs in the final stages of seed maturation. The form of phytochrome (trapped) during such desiccation would then determine the photoresponse of mature seeds.

When referring to exposures to red or far-red light no mention has yet been made of the duration of these exposures. It is generally accepted that phytochrome control should require only short exposures, of several minutes duration, to red or far-red light. If, after the 'escape period' far-red is given for several hours, a second inhibition of germination can be obtained (Negbi et al., 1968). A requirement for long exposures to far-red is generally considered to indicate the presence of 'high energy reactions' (HER). HER phenomena show action spectra maxima in the blue and far-red regions of the spectrum (Hendricks and Borthwick, 1965). The level of inhibition also depends on the intensity of light, and for this reason the phenomenon was given the name "high energy reaction". Probably the most important question with respect to HER involvement in the control of seed germination is whether or not it operates through phytochrome. For lettuce seeds, an action spectrum for the inhibition of germination has been given by Gwynn and Scheibe (1972) and along with earlier work (Wareing and Black, 1958) indicates that blue wavebands are inhibitory to germination. It should be noted that Evenari et al. (1957a) showed that blue light inhibits the germination of lettuce seeds when given during the first six hours of imbibition, but promotes when given at later times. Can these results be explained in terms of phytochrome as the photoreceptor? Certainly, a model has been put forward (Hartmann, 1966) and extended (Borthwick et al., 1969) to explain HER effects in the far-red region as being solely mediated by phytochrome. This model is supported by experiments, including lettuce seed germination (Hartmann, 1966). The fact that blue wavelengths are inhibitory to lettuce seed germination need not exclude phytochrome as the pigment involved in HER phenomena. It is possible, however, that intermediate pigments may transfer energy to phytochrome (Malcoste et al., 1972b). Furthermore, it has been suggested that the photoreceptor for the HER reactions may be localised in a different part of the seed (Schiebe and Lang, 1969). The site of photoreception in photosensitive lettuce seeds has been shown to be the hypocotyl

(Klein and Preiss, 1958; Ikuma and Thimann, 1958 and 1959). In Cucurbita pepo embryos, Boisard and Malcoste (1970a), using a red light beam of diameter 1 nm, have shown the photosensitivity of the seeds to be localized in the embryonic axis only. These authors propose that phytochrome in the cotyledons is not involved in germination. In a number of seeds in vivo, spectrophotometry has revealed a gradient in phytochrome concentration along the embryonic axis, decreasing from the radicle end towards the cotyledons (Boisard and Malcoste, 1970b; Malcoste et al., 1970). Scheibe and Lang (1969) point out that whilst the P_{fr}/P_r ratio maintained in the radicle end may predominate, the HER reaction may be localised in a different part of the seed. It should be noted that these workers used excised axile portions of seeds and inhibited their germination by bathing them in osmotica. It has recently been indicated that the osmotica used in this work may not be, as thought, wholly inert, and may exert an influence themselves (Berrie et al., 1974). The point still stands that nobody has examined the site of the photoreceptor for the HER! Whilst there is good evidence that phytochrome is the pigment involved in HER phenomena, evidence to the contrary also exists (Rollin, 1966; Negbi and Koller, 1966; Larpert-Gourgand et al., 1974).

The literature dealt with has been concerned with thermodormancy in Grand Rapids lettuce seeds, where P_{fr} must be present in order for the seeds to overcome thermodormancy. We have not considered what is the active form of phytochrome in bringing about germination, nor have we determined whether or not the seed requires phytochrome in the absence of thermodormancy. If we examine the situation found in an apparently photoblastically neutral cultivar of lettuce, Great Lakes, it can be shown that phytochrome is present (Hendricks et al., 1959). Germination of Great Lakes lettuce seeds can be inhibited by exposing the seeds to 24 hrs of light at 400 nm. Red, far-red reversibility could then be displayed which had previously been missing. Obviously phytochrome must be present in the seeds of Great Lakes. It can be shown that in Grand Rapids lettuce seeds, germination can be accelerated (see Tables 36, 45 in this thesis) by irradiating seeds with red light at 20°C, i.e. below the critical temperature. Thus, phytochrome must be present in the seeds even below the critical temperature. This does not prove that the phytochrome present plays a necessary role in germination in the absence of thermodormancy.

However, it can be proposed that under conditions where germination normally takes place there is a balance between the 'promotive' and 'inhibitory' force(s) present. If phytochrome control is primary, as its speed of action would suggest, then it can be proposed that sufficient 'active' phytochrome is present to overcome any inhibitory force(s). When an additional inhibitory force is exerted, viz. temperature, then more 'active' phytochrome is required. The suggestion by Toole (1961) and Roth Bejerano et al. (1966) that the germination of Grand Rapids lettuce seeds, at temperatures where no light requirement is shown, is due to the pre-existence of the 'active' form of phytochrome may be valid.

Further evidence will be presented, when considering spectrophotometric work, to show the presence of phytochrome in seeds as P_{fr}. The 'active' form of phytochrome mentioned is generally accepted as the far-red absorbing form, P_{fr}. The implication is that P_{fr} leads to the production of some factor which brings about germination. Alternatively, P_{fr} could remove an inhibitor of germination. This need not be at the level of synthesis and could well operate by compartmentalising promoters or inhibitors. The same argument can also be assigned to P_r, where the P_r produces an inhibitor or stops a promoter from being active. The removal of P_r by red light would then cause germination. Borthwick (1972) finds this unattractive, arguing that a slight reduction in one form from near saturation is relatively insignificant in comparison to the raising in concentration of the other from near zero to even a few percent. The possibility that either P_r or P_{fr} is the active form should be borne in mind in considering relevant literature.

Many questions remain unanswered from the literature dealing with physiological studies. In an attempt to clarify the position the literature concerned with spectrophotometric measurement will be considered, although phytochrome has not been measured by spectrophotometric assay in Grand Rapids lettuce seeds. In order to interpret the results obtained by spectrophotometry it is necessary to understand both the theory underlying such measurements, and the problems which are met as a result of shortcomings in this physical assay system.

With the discovery of phytochrome photoreversibility it became apparent that changes in optical density of an appropriate sample of

plant material should be produced by alternately irradiating it with red and far-red wavelengths. Using an instrument built by Norris and Butler (1961) the optical density difference of a sample of dark grown maize seedlings at 660 nm and 730 nm, with the pigment predominantly in one form and then in the other, was measured (Butler et al., 1959). The difference between two such measurements is a visible indication of the presence and reversibility of the pigment. This basic principle, on which all spectrophotometric measurements of phytochrome depend, should not be forgotten.

There are two basic sample materials used in spectrophotometric measurements of phytochrome: in vivo measurements, using plant material packed into a cuvette, and in vitro measurements, using extracted and highly purified phytochrome solutions. Neither sample type is ideal. With in vivo spectrophotometry many of the problems which have arisen, have been caused by the fact that samples of plant material contain very little absorbing phytochrome but are highly light scattering. Because real absorption contributes only a few percent to the total attenuation of light in a sample, the use of conventional spectrophotometers is impracticable. To compensate for the low (real) absorption one attempts to measure, not the total optical density but the density difference between a sample and a similar one that has the same scattering properties but no absorption due to the component under study. Two instrument designs have evolved - (a) the double beam and (b) the dual wavelength method. (Figure 2). In (a) comparison is made between two samples that are supposed to be identical, except that one contains the absorbing pigment, while the other does not. In (b) comparison is between two wavelengths of light traversing the same sample in rapid succession. One wavelength is close to an absorption maximum of the substance under study, for phytochrome 730 nm and the other at a wavelength where its absorption coefficient is negligible, 800 nm. By repeating such measurements, at a great number of measuring wavelengths, a difference spectrum of the photoreaction can be obtained. It is beyond the scope of this review to deal with all aspects of phytochrome or even, all the problems associated with the interpretation of in vivo or in vitro measurements of phytochrome. The reason for examining spectrophotometry is in an attempt to interpret some of the results obtained in physiological events. If this is to be meaningful then the problems in making such correlations, indeed if such correlations

Figure 2

Double beam A and dual wavelength B spectrophotometry.

PM : photomultiplier tube

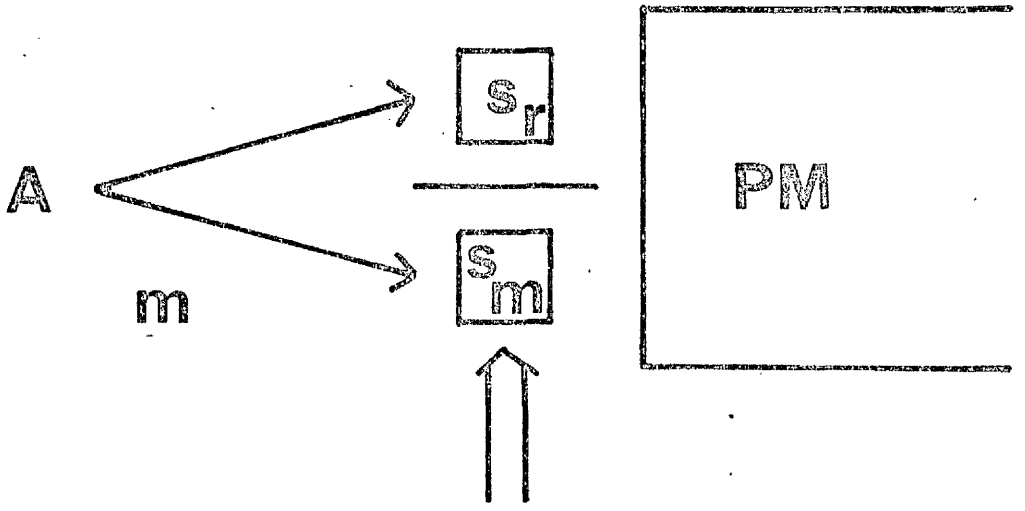
$\Longrightarrow$: actinic light

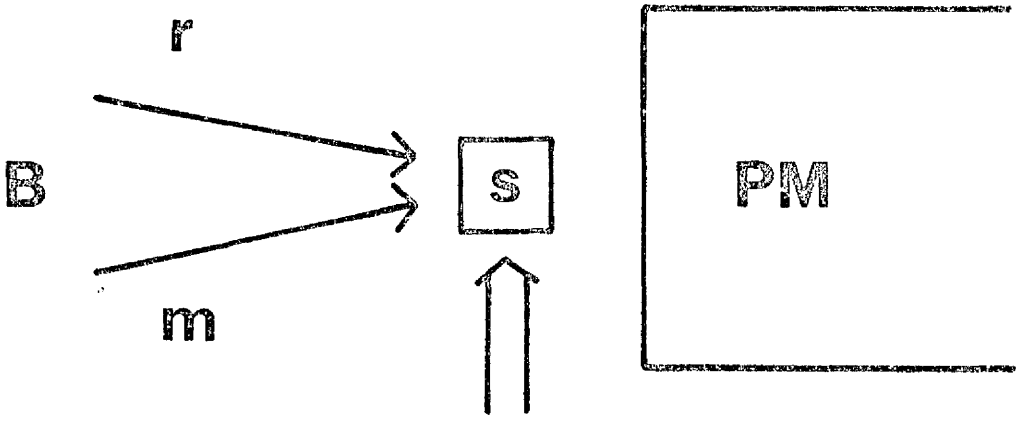
m : measuring wavelength

r : reference wavelength

Sr : reference sample

Sm
&
S : measuring sample


A
m
s_r
s_m
PM


r
B
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PM

are valid, must be considered. Hillman (1972) has gone as far as to say, "in any system examined with rigour, no rational analytically useful relationship has yet been established between a physiological response and the state and content of phytochrome as measured spectrophotometrically." Hillman has called failures in showing a correlation, phytochrome "paradoxes", and has attempted to explain such paradoxes in terms of more than one type of phytochrome, a small active fraction and a bulk fraction, with different physical properties. Can these paradoxes be explained, either in terms of non-homogenous pigment populations or in some other way? Certainly, in vivo measurements of phytochrome have given kinetic curves which deviate from a straight line, which would be the expected for a single type of phytochrome (Boisard et al., 1971; Purves and Briggs, 1968). These studies overlooked one problem of in vivo assay, that is, samples for spectrophotometric measurement are not optically thin. For a reaction, driven by light, the rate should be proportional to the light intensity, provided there is no accumulation of intermediates. In tissue samples, there is a light intensity gradient due to scatter and the actual light intensity affecting the phototransformation is a function of the depth of penetration. For different cross sections of the sample the rate constant of a photochemical reaction will, therefore, have different values. Two groups of workers have presented mathematical models which show phytochrome photoconversion to be first order, assuming the light intensity gradient within the sample to be either exponential (Spruit and Kendrick, 1972), or to approach a gradient predicted by the 'Kubelka-Munk theory of light scatter in turbid materials' (Schmidt et al., 1973). The existence of one population of phytochrome is consistent with the photoreaction being first order with respect to time. A second complicating factor in measuring reaction rates in vivo is that it is assumed that there is no accumulation of intermediates. Kendrick and Spruit (1972a and b) have shown that under certain light conditions, high levels of intermediates are maintained in Amaranthus seedlings. Decay of phytochrome in Amaranthus seedlings deviated from first order rates only under high intensity incandescent light. Pisum epicotyl hooks show no intermediate accumulation and do not deviate from the observed first order phytochrome decay. Evidence from studies on the rate of phytochrome decay is in favour of there being only one type of phytochrome.

A second line of investigation which has been used to indicate

the occurrence of more than one type of phytochrome, is to show deviation of the difference spectra *in vivo* from the shape expected for a single phytochrome. Grill and Spruit (1972) have shown in Pinus species absorption maxima at 656 nm and 710-715 nm and in *Gingko biloba* maxima at 655 nm and 725 nm. It was concluded that the phytochrome of the Gymnospermae differed from that of the Angiospermae and also the Coniferales from the Gingkoales. Spectrophotometric estimation of phytochrome *in vivo* gives information in terms of optical density values. A comparison of optical density values made on different samples is only possible as long as we can be certain that their scattering properties are identical. The effect of scattering is to increase the effective length of the light path in the sample and result in a concominant intensification of absorption bands (Butler and Norris, 1960). Consequently, comparison of 'phytochrome' content of different parts of the same plant or of different samples, must be regarded with caution. Difference spectra may vary considerably in shape, peak position and in the ratio of heights of red to far-red peaks as a result of the presence in the sample tissue of photostable pigments (Spruit and Spruit, 1972; Spruit, 1967). Spruit (1967) concluded that phytochrome in pea leaves was a form of the pigment with abnormal spectroscopic properties. These conclusions are unwarranted in the light of models later proposed (Spruit and Spruit, 1972). It is clear that the study of difference spectra in the search for different forms of phytochrome is of doubtful use if there are other pigments present.

It would seem to be unwarranted to propose the existence of different populations of phytochrome on the basis of *in vivo* spectroscopy. *In vitro* studies of extracted phytochrome do not prove the existence of more than one type of phytochrome, although it should be borne in mind that the evidence is still open to debate (Gardner *et al.*, 1971; Briggs *et al*, 1972). Immuno-cytochemical studies of phytochrome extracted from various plant sources shows no major differences (Pratt, 1973; Rice and Briggs, 1973). If these 'paradoxes' cannot be explained on the basis of different populations, active and bulk phytochrome, are there alternative theories. Recently, Oelze-Karrow and Mohr (1973) have studied 'apparent' synthesis of lipoxygenase in mustard cotyledons, which is suppressed by P_{fr} at a threshold level, in an attempt to show a quantitative correlation between spectrophotometrically detectable P_{fr} and the physiological response. It

has, in previous papers (Oelze-Karrow et al., 1970; Wagner and Mohr, 1966) been suggested that in mustard no 'paradox' exists, but this work has been open to severe criticism. P_{fr} reversion could not be shown (Oelze-Karrow et al., 1970) in Sinapis, in spite of the fact that other workers have demonstrated reversion (Kendrick and Hillman, 1971). Secondly, almost all the Sinapis work has involved prolonged far-red illumination. These criticisms remain unanswered, except in that Mohr appears to have changed his thoughts about the presence of a paradoxical situation! In two recent papers, one dealing with physiological evidence (Oelze-Karrow and Mohr, 1973) and the other with spectrophotometric assay (Schäfer et al., 1973) it was concluded that apparent lipoxygenase synthesis in the COTYLEDONS of the mustard seedlings was controlled by phytochrome (P_{fr}) located in the HOOK. Raven and Spruit studied the induction of rapid (Raven and Spruit, 1972a,b; 1973) chlorophyll accumulation in dark grown pea seedlings. They first showed that phytochrome control was active both in increasing the biosynthetic capacity to form protochlorophyllide, and in initiating the development of a mechanism which protects chlorophyllous pigments from photodestruction (Raven and Spruit, 1972a). Several results which became apparent during studies of photoreversibility (Raven and Spruit, 1972b) were difficult to explain in conventional theories. Far-red irradiation of completely dark grown seedlings gave a relatively high induction of chlorophyll as compared to red. This inductive capacity was progressively lost upon de-etiolation of the tissue. Concomitantly, red, far-red reversibility became more pronounced. The authors have attempted to explain the difference in photoreversibility between dark grown and de-etiolated tissue, to an extremely low P_{fr} requirement for the induction reaction, in addition to transport of phytochrome during the process of de-etiolation (Raven and Spruit, 1973).

Thus it would appear that some of the paradoxes may be explained in terms of phytochrome being active by virtue of its location. In vivo spectrophotometric assays measure an average of the properties of phytochrome in different cellular environments (Kendrick and Spruit, 1973d). The idea of more than one type of phytochrome in a physical or chemical sense appears untenable, but the idea of 'bulk' and 'active' phytochrome, by virtue of localisation may be a useful one in attempting to interpret the data produced by in vivo assay. It must be accepted that there are severe limitations in the in vivo assay of phytochrome.

In using a number of different plant tissues, all of which will have different problems of interpretation, great caution should be exercised in speculating about the correlation with physiological studies.

With this background the literature dealing with *in vivo* spectrophotometric measurement of phytochrome will be considered and an attempt made to interpret what takes place during lettuce seed germination.

In vivo measurements of seed phytochrome have shown its presence in a number of seeds, including one cultivar of lettuce, 'May Queen', and *Nemophila insignis* (Boisard *et al.*, 1968), *Sinapis alba*, *Rhaphanus sativus*, *Phacelia tanacetifolia*, *Nigella damascena* (Malcoste, 1969), a number of species belonging to the family Cucurbitaceae (Malcoste, 1969; Boisard and Malcoste, 1970b; Mancinelli and Tolkowsky, 1968; Spruit and Mancinelli, 1969; Malcoste *et al.*, 1970; Zouaghi *et al.*, 1972), several species of Amaranthus (Kendrick and Frankland, 1969; Kendrick *et al.*, 1969), tomato (Mancinelli *et al.*, 1968), and in a number of Gymnosperms (Tobin and Briggs, 1969; Orlandini and Malcoste, 1972; Orlandini and Bulard, 1972; Grill and Spruit, 1972).

We noted that lettuce seeds were unresponsive to red light until they reach a certain moisture content. In Grand Rapids this moisture content was shown to be 6% (Berrie *et al.*, 1974). As the period of imbibition is increased, in conditions unsuitable for germination, the seeds lose their ability to respond to red light. Is it possible to explain the increase in sensitivity to red irradiation and the subsequent loss of sensitivity from data obtained by measurements of endogenous phytochrome?

Several hypotheses have been offered to explain the increase in sensitivity during imbibition.

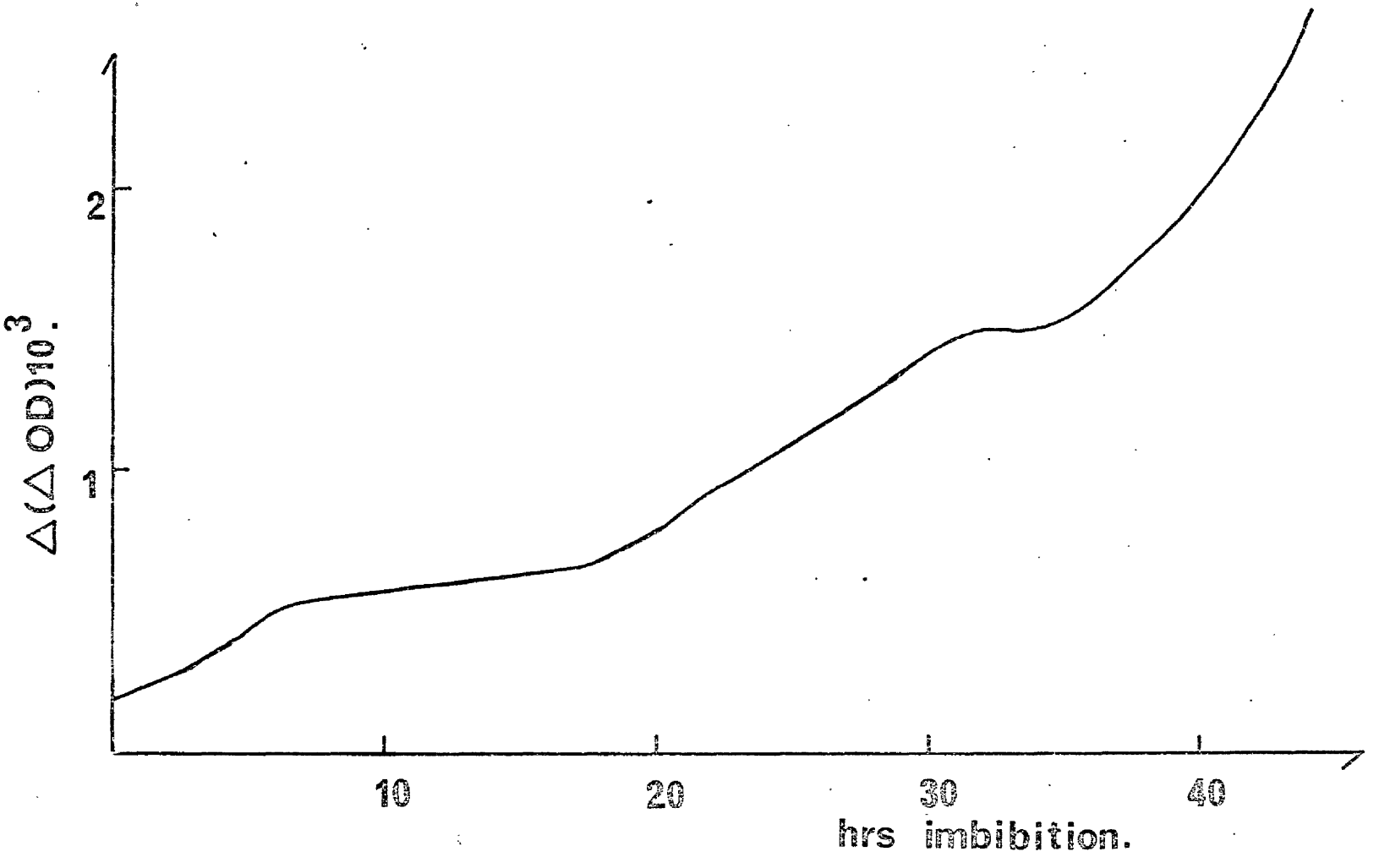
(a) The increase in sensitivity could be due to a gradual activation of P_r from an inactive complex or by synthesis (Negbi and Koller, 1966).

In vivo assay has shown that in several different seeds there is a significant increase in phytochrome content during germination (Boisard, 1969; Boisard *et al.*, 1968; Boisard and Malcoste, 1970b; Mancinelli and Tolkowsky, 1968; Spruit and Mancinelli, 1969; Malcoste *et al.*, 1970; Zouaghi *et al.*, 1972; Kendrick *et al.*, 1969;

Tobin and Briggs, 1969; Orlandini and Malcoste, 1972; Orlandini and Bulard, 1972; Grill and Spruit, 1972). The same time course of appearance of phytochrome has been observed in several seeds, with an initial rapid rehydration phase, a plateau, a second increase, which precedes radicle protrusion, a second plateau and then a rapid increase in detectable phytochrome (see Figure 3). The initial increase in detectable phytochrome, as measured by optical density differences $\Delta(\Delta OD)$, is thought to be a rapid rehydration of pre-existing seed phytochrome. The second increase in phytochrome is unaffected by inhibitors of protein synthesis, cycloheximide or actinomycin D (Zouaghi et al., 1972), which would suggest a release of P_r from an inactive complex. The second 'plateau' is not universal and is absent in lettuce seeds cv 'May Queen' (Boisard, 1969). This may result from the variation in measurements of $\Delta(\Delta$ O.D.) which is larger than in work carried out on a single excised embryo of pumpkin (Malcoste et al., 1970). With lettuce, seeds were packed into the cuvette for measurement of optical density. This means that the measurements obtained were an average of several individuals. The second increase in phytochrome in lettuce seed was observed after 16 hrs whilst radicle protrusion was observed after 24 hrs (Boisard, 1969). The increase was due to the appearance of P_r. The ratio of P_{fr} to P total continuously fell. Following protrusion of the radicle there was a further increase in phytochrome which was found to be sensitive to inhibitors of protein synthesis (Malcoste et al., 1972a). Quail et al. (1973a) found that phytochrome present in Zucchini squash seedlings, hydrated and germinated in D_2O, was heavily labelled with the isotope as determined by isopycnic centrifugation, indicating the protein moiety of phytochrome was synthesised de novo. This de novo synthesised phytochrome has probably no role in germination and has been referred to as 'seedling' phytochrome (Boisard, 1969). Thus, the increase in detectable phytochrome could explain the physiological studies. The seeds would not be able to respond to red light until a certain water content had been attained, as a result of the need for initial hydration of phytochrome molecules. The increase in sensitivity would be due to the increase in phytochrome after the first plateau.

Figure 3

Time course of phytochrome content during the germination of pumpkin seeds (after Malcoste et al., 1970).


Δ(Δ OD)10^3.
2
1
10
20
30
40
hrs imbibition.

(b) A second theory to explain the increase in sensitivity is that a substance or co-factor for P_{fr} is produced Koller *et al.*, 1964). *In vivo* assay cannot rule out this possibility.

(c) The increase in sensitivity could be as a result of a decrease in inhibitor levels in the seeds. This idea has been suggested by Burkart and Sanchez (1969). In *Datura ferox* seeds the response to red light increases with imbibition up to 48 hours. This increase is interpreted as a preparatory phase during which some processes take place which are necessary for the induction of germination by P_{fr}. The seed contains an endogenous inhibitor, the level of which decreases after 24 hours incubation and rapidly falls after 48 hrs. Furthermore, the level of the inhibitor, immediately following harvest, is higher than after several months of dry storage. Typically light sensitive seeds become less light sensitive after storage.

(d) Remobilisation of phytochrome.

These hypotheses to account for increased sensitivity to red light need not be mutually exclusive. They do underline the difficulties in interpreting a physiological event in terms of any one specific hypothesis until other possibilities are excluded.

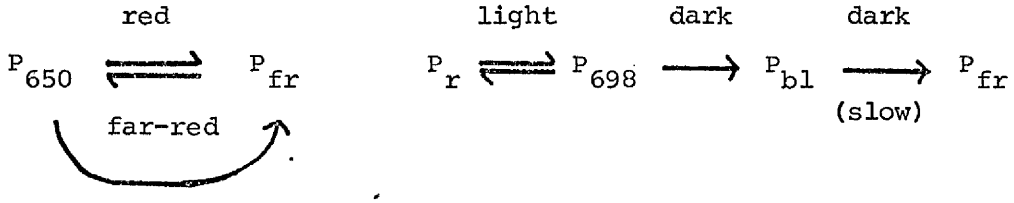
The decrease in sensitivity to red light which takes place when seeds are held, imbibed in conditions unsuitable for germination cannot be explained readily by the *in vivo* assay. However, it has been shown that P_{fr} present in the embryonic axis of seeds of *Cucurbita pepo* begins to 'disappear' after 25 hrs in darkness and after 70-80 hrs cannot be detected (Malcoste *et al.*, 1970a). This may be phytochrome 'destruction'. Phytochrome destruction results in loss of photoreversibility and consequently, detectability. This reaction differs from the dark reversion of P_{fr} to P_r as it is temperature independent (Schäfer and Schmidt, 1974). The mechanism and physiological significance of destruction *in vivo* are unknown.

We have previously mentioned that far-red light, given after the 'escape period' can cause a second inhibition of germination. The exposure to far-red light requires to be of long duration, several hours, and it may be considered to operate through the high energy re-

actions or HER (Negbi et al., 1968). Attempts have been made to explain the HER in terms of phytochrome. Does in vivo assay of phytochrome help in supporting this hypothesis? If HER phenomena are to be explained in terms of phytochrome one of the main properties of HER that requires explanation is that of time dependence. The necessity for continuous or intermittent far-red irradiation over a long period for inhibition indicates that P_{fr} is constantly reappearing during such a period. Mancinelli and Borthwick (1964) and Mancinelli et al.,(1966) assumed that P_{fr} could either be reappearing from rehydration of a reserve pool or by de novo synthesis. However, we have seen that de novo synthesis of 'seedling' phytochrome is in the form of P_r! There is evidence that initially some of the phytochrome present in seeds is in the form of P_{fr}. In dry gourd seeds all the measurable phytochrome was in the P_{fr} form (Zouaghi et al., 1972) whilst an undetermined amount of P_{fr} was found to be present in Amaranthus caudatus (Kendrick et al., 1969) and in lettuce seeds (Boisard, 1969). However, this P_{fr} would be removed by short irradiation with far-red. The reappearance of P_{fr} may be explicable in terms of "inverse" reversion, first suggested by Boisard et al. (1968) for lettuce seeds cv 'May Queen' and in Nemophila insignis, a negatively photoblastic seed. Boisard observed that if the pigment in the seed is transformed to the red absorbing form by far-red irradiation, ten minutes in the dark converts the bulk back to P_{fr}; "inverse reversion". The critical question to be answered is whether this spectrophotometrically measured change in optical density of a sample really represents a change in phytochrome from P_f to P_{fr}. The possibility exists that inverse reversion is merely a hydration phenomenon (Briggs and Rice, 1972). In vivo studies could be confused by the possible existence of low absorbing forms of P_{fr}, due to incomplete hydration, and the degree of light scattering of the sample material. There are now several reports of inverse reversion from in vivo studies, in dry and imbibed seeds, the rate of which is dependent on the species and the degree of hydration (Spruit and Mancinelli, 1969; Malcoste et al., 1970a; Kendrick et al., 1969; Boisard and Malcoste, 1970b). Furthermore, the first in vitro measurement of inverse reversion, with phytochrome extracts from etiolated oats, has lessened the probability of all inverse reversion being merely due to hydration or an artifact of measuring (Kendrick and Spruit, 1973; Spruit, personal communication). This last observation does pose difficult questions as inverse reversion had been

supposed only to occur in the 'seed' phytochrome and not 'seedling' phytochrome. Another question which requires to be answered to understand the role of inverse reversion, is why after a certain duration of far-red exposure, germination does not occur when the seed is placed in darkness. An answer to this question may lie in results cited by Rollin (1972). P_{fr} present in the embryonic axis of seeds of Cucurbita pepo begins to disappear after 25 hrs in darkness and after 70-80 hrs cannot be detected. If the same pattern is assumed to occur in far-red as in darkness, then the duration of far-red in order to obtain a total inhibition of germination depends on the speed of P_{fr} disappearance.

How can we resolve the problem of inverse reversion? Kendrick and Spruit (1974) have presented an explanation of what they think is happening. Seed tissue is in a state of dehydration. On rehydration in vivo studies have shown a rapid increase in detectable phytochrome, some in the form of P_{fr} and some as P_r. We have also indicated that in the maturing seed the spectral quality during the final rapid dehydration phase is critical in determining the photoresponse of the mature seeds. Kendrick and Spruit suggest that under these conditions of dehydration, complete transformation of P_{fr} to P_r and vice versa will be prevented. Thus, the dry seed will contain intermediates of the phototransformations. A scheme showing the proposed pathway of phototransformation is on page 12. There is evidence that on rehydration P_{650} reverts in darkness to P_{fr}. In a partial state of rehydration P_r present will form intermediates which slowly form P_{fr} after red light.



The reaction P_{bl} to P_{fr} is the slowest reaction in the P_r to P_{fr} pathway (Kendrick and Spruit, 1973a). Thus, P_{fr} reappearing in partially hydrated seeds can also be the result of transformation of P_{bl} to P_{fr} in darkness. Under dehydrated conditions P_{fr} can

only be converted to P_{650} by exposure to far-red irradiation, which in darkness will return to P_{fr}, thus explaining the so-called 'inverse reversion'. The occurrence of this reaction *in vitro* is probably associated with the relatively 'dehydrated' state of phytochrome in the presence of high salt and glycerol concentrations. 'Inverse reversion' could only be observed *in vitro* following a red light treatment followed by far-red light. The red light would convert only some of the P_r as far as P_{fr}. Not all the P_{bl} will form P_{fr}. When far-red light is given P_{fr} will be converted only as far as P_{650} on the pathway to P_r. In the dark P_{650} and P_{bl} will then form P_{fr} which could be interpreted as the dark production of P_{fr} from P_r.

Kendrick and Spruit (1974) propose that inverse dark reversion of P_r to P_{fr} does not exist and the term should no longer be used.

The requirement of continuous far-red to inhibit seed germination has led to the postulation that there is continuous production of P_{fr} during imbibition. The dark reversion of P_{650} and P_{bl} to P_{fr} could explain the continuous production of P_{fr}. However, there are unresolved anomalies in the literature. Although a mechanism for P_{fr} production has been shown to exist, *no* increases in P_{fr} can be shown in a time course of phytochrome increase during imbibition. In the work of Negbi *et al* (1968), P_{fr} would be present from the red light treatment; however, at the time of the second far-red treatment the seeds would be fully imbibed and the intermediates P_{650} and P_{bl} would be expected to complete their respective pathways to P_r and P_{fr}. It would be necessary to invoke some other factor. This question remains unresolved.

In summary, Grand Rapids lettuce seeds have been shown to exhibit thermodormancy, this being overcome by red light. Far-red light given immediately after red light stops the promotive effect of red light. The system is under the control of the photomorphogenetic pigment, phytochrome. Further studies have indicated that the seeds can only respond to red light when they attain a certain moisture content. The sensitivity to red light increases during imbibition but eventually decreases. If far-red light is not given for some hours after red light, a point is reached where far-red is unable to reverse the red light potentiated germination. At this time long exposures to far-red light inhibit the expression of germination. Far-red at this time is thought to operate through the high energy reactions (HER) and the evidence for HER acting through phytochrome

is discussed. The questions of what is the active form of phytochrome, and whether or not phytochrome is active in causing germination below the critical temperature for the induction of thermodormancy, are discussed. Having built up a picture of the situation found in Grand Rapids lettuce seeds from physiological studies, spectrophotometric evidence is reviewed in an attempt to interpret these findings. The interpretation of physiological results using a physical assay for phytochrome hinges on being able to correlate changes in optical density with physiological events. This has not always proven possible and, in an attempt to explain the lack of correlation, it has been proposed that two types of phytochrome exist, a small active fraction and a large inactive fraction. Evidence for two different phytochrome populations has come from kinetic studies of phytochrome decay and from difference spectra. However, it is possible to explain the results obtained as showing only one population, if the problems involved in looking at plant samples are taken into consideration. An alternative explanation is that phytochrome is active by virtue of its location. The evidence for this theory is presented. Having considered the difficulties in interpreting the results obtained by in vivo assay, the literature dealing with in vivo measurements of phytochrome in seeds is presented and an attempt made to explain some of the physiological studies on Grand Rapids lettuce seeds, in terms of endogenous phytochrome. Several theories are discussed in relation to the increased sensitivity to red light during imbibition and the involvement of phytochrome in HER. One of the main points which must be explained in relation to HER is the requirement for long exposures to blue or far-red light. It is proposed that P_{fr} must continually reappear or be synthesised. This could be explained in terms of so called 'inverse reversion'. The evidence for this phenomenon is discussed and the most recent theory to explain 'inverse reversion' presented. The idea that 'seed' phytochrome differs in some way from 'seedling' phytochrome is not supported by this recent work. Thus, it has been possible to offer explanations of some of the physiological effects in terms of endogenous phytochrome studies, although a clearer understanding of phytochrome in Grand Rapids seeds will only come when technical difficulties are solved.

The role of growth regulators in the germination of Grand Rapids lettuce seeds

The plant photomorphogenetic pigment, phytochrome, has been shown to be involved in the control of thermodormancy in Grand Rapids lettuce seeds. The fact, there is good evidence that P_{fr} action is required for germination below the critical temperature, may be interpreted as showing P_{fr} to be involved in events leading to germination. Above this critical temperature the action of P_{fr} has been shown to be rapid (Bewley et al., 1968) which would suggest that the action of P_{fr} is the initial, or at least an early, event in the chain of events culminating in germination and radicle emergence. It was previously suggested that there is sufficient P_{fr} in the imbibed seed to cause germination below the critical temperature but at a higher temperature more P_{fr} is required as a result of the increased inhibitory influence. The fact that far-red light can inhibit germination when given to seeds, held below the critical temperature, would seem to rule out the possibility that P_{fr} is not actively involved below the critical temperature (Scheibe and Lang, 1969). Whether P_{fr} or P_r is the 'active' form of phytochrome has been discussed. Regardless of which form is 'active' we can propose that phytochrome overcomes thermodormancy by generating a promotive 'influence' and/or by destroying an inhibitory 'influence'. The word 'influence' is used in preference to 'compound' as the effect may not be on synthesis or destruction but at a physical level. Cohen (1958) has shown that alternating temperature can substitute for light in overcoming thermodormancy in Grand Rapids seeds. It was found that there exists a critical dependence on temperature and no correlation between promotion of germination and the rate or duration of the temperature change. When the thermodynamic values of the reaction were calculated a ΔH value (enthalpy or heat change) of + 70,000 cal/mole and a ΔS value (entropy change) of + 235 cal/deg. mole were obtained. These values are of the same order of magnitude as the respective values for protein denaturation and thermal killing of various living cells. This means the alternating temperatures apparently promote germination by destroying an organised structure or macromolecular arrangement preventing germination in the dark . Evenari (1961) suggests that the structure could be a membrane separating certain reactants or an inactive precursor of an enzyme

which is liberated by the temperature change. These alternatives will be discussed in more detail when 'mode of action' is considered.

Clearly, if this theory is to prove acceptable the presence of inhibitory and/or promotive influences must be shown. Furthermore, these must be shown to vary in a meaningful fashion with respect to the effects of phytochrome in overcoming dormancy. It can be proposed that these 'promoters' or 'inhibitors' are plant hormones. Two approaches have been used in studying the involvement of hormones in the control of seed germination and dormancy, the application of exogenous growth regulators and the extraction of the endogenous growth regulators. It is generally accepted that results obtained exclusively using exogenous studies are insufficient to establish a regulatory role for these substances in the intact organism. Sondheimer et al.,(1968) have stated: "a minimum requirement for such a role would be to show the presence of the hormones in the intact plant and to demonstrate a correlation between hormonal concentration levels and physiological states." This is a minimum requirement and is by no means unequivocal evidence for a regulatory role! With respect to Grand Rapids lettuce seeds a sharp temperature 'cut off' point exists, that is, over a short range of temperature, germination drops from near 100% to below 20% (Berrie, 1966). Any theory erected to explain the control of dormancy must be consistent with the sharp temperature 'cut off' or 'threshold' nature of the phenomenon.

What evidence have we that thermodormancy in lettuce seeds can be overcome by plant hormones? In 1956 Lona first reported that exogenously applied gibberellic acid (GA_3) was capable of inducing germination in the light sensitive seeds of Lactuca scariola. Khan et al. (1957) reported that GA_{1+3} overcame thermodormancy in Grand Rapids lettuce seeds. Since these early reports a number of GAs have been shown to overcome thermodormancy in seeds of Grand Rapids lettuce, including GA_1, GA_2, GA_3, GA_4 (Toole and Cathey, 1961), GA_5, GA_6, GA_7, GA_8, GA_9 (Brian et al., 1962), GA_{4+7} (Ikuma and Thimann, 1963a) and GA_{13} (Bewley et al., 1968). The order of activity reported by Brian et al. (1962) was:

$$A_4 = A_7 > A_3 > A_1 = A_5 > A_2 = A_6 = A_8 = A_9.$$

The germination of a number of photosensitive seeds can be promoted by exogenous gibberellins (Ogawara and Ono, 1957; Rollin,

1959; Fuji et al., 1960; Toole and Cathey, 1961; Evenari, 1961; Bachelard, 1967a; Kollman and Staniforth, 1972; Chancellor and Parker, 1972; Bianco and Pellegrin, 1973; Batanouny, 1974; Bullawa et al., 1975; Palevitch and Thomas, 1974), although this is not ubiquitous (Evenari, 1961). Often a concentration of gibberellin which overcomes dormancy is inhibitory to subsequent radicle growth (Toole and Cathey, 1961). In only one case has far-red light been shown to have any effect on gibberellin-induced germination. Poljakoff-Mayber et al. (1958) reported that far-red could reverse to some degree the promotion of germination brought about by gibberellin. The fact that far-red light cannot overcome exogenous gibberellin has often been used as evidence that red light does not operate via an increase in endogenous gibberellins. This is clearly too facile an argument. If we assume that red light causes an increase in the 'effective' gibberellin content, it is possible that whilst far-red light could inhibit this reaction, the reactions by which gibberellins lead to germination might be unaffected by far-red light. Consequently, the effect of exogenous gibberellin would not be influenced by far-red light. The term 'effective' gibberellin could be interpreted in terms of an increase in the synthesis or a decrease in destruction of gibberellins, or it could involve the release of gibberellins from one part of the seed to another or from some bound, inactive form. These alternatives will be discussed later.

Furthermore, it must be realised that the application of large, indeed massive, doses of exogenous chemicals might lead to unforeseen alterations in the normal pattern of events (Berrie and Robertson, 1973). The question of whether or not red light operates in overcoming thermodormancy through gibberellins can only be answered when the endogenous situation has been examined. A number of workers have shown the presence of 'gibberellin like' compounds in seeds of Grand Rapids (Ikuma and Thimann, 1960; Blumenthal-Goldschmidt and Lang, 1960; Köhler, 1966) and in other lettuce cultivars (Murakami, 1959). Ikuma and Thimann reported that light did not lead to an increase in gibberellin like compounds. Köhler (1966) did report that red light led to an increase in gibberellin like compounds in seeds of Grand Rapids lettuce, but has since retracted this data as it proved unrepeatable (personal communication; Black, 1969). By gibberellin like activity we mean activity

displayed in bioassays reputed to be specific for gibberellins. The gibberellins responsible for such activity in seeds of Grand Rapids lettuce have not been identified. Are we justified in saying that red light does not operate through an effect on gibberellins? Before considering this question in detail, let us examine a second class of promoter found in lettuce seeds and capable of overcoming thermodormancy, the cytokinins.

Miller (1956) originally reported that kinetin was capable of substituting far-red light in breaking thermodormancy in seeds of Grand Rapids lettuce. It was shown that kinetin did not owe its action to increasing the levels of adenine (Haber and Tolbert, 1957). The action of cytokinins in lettuce seed germination has since been an area of unnecessary controversy. Miller (1958) reported that in his original paper of 1956 the levels of promotion which he had reported may have been attributable, in part, to 'stray' light interacting with kinetin. He repeated this work and showed that kinetin did interact with light but also promoted to a lesser degree, in total darkness: e.g. 34% with 5×10^{-5} M kinetin after 24 hrs, 0% in darkness. Evenari (1961) has also reported a small increase in germination brought about by kinetin. Furthermore, kinetin was found to retard the decrease in light sensitivity of the seeds which occurs in water as a function of imbibition time (Leff-Weisy, 1961 cited by Evenari, 1961). Other cytokinins have since been shown to overcome thermodormancy in seeds of Grand Rapids lettuce (this thesis).

As with gibberellins, cytokinins have been found to overcome light sensitive dormancy in some species (Kollman and Staniforth, 1972; Chancellor and Parker, 1972; Reynolds and Thomson, 1973; Palevitch and Thomas, 1974) but in others only in the presence of light (Ogawara and Ono, 1961) or not at all (Bachelard, 1967a; Evenari, 1961). Far-red light has never been shown to be capable of reversing cytokinin promotion of germination. Cytokinin-like activity has been shown to be present in seeds of Grand Rapids lettuce. Barzilae and Mayer (1964) could detect cytokinin-like activity only in seeds which had been stimulated to germinate by red light. Activity was measured in dry seeds and seeds imbibed for 24 hrs. More than one compound was found to be present although these were not identified. More recently, Van Staden (1973a) has presented evidence that red light can bring about a rapid, transient

change in endogenous cytokinins. Against a background of decreasing total cytokinin levels, there is the appearance of butanol-soluble cytokinins (assumed to be nucleotides) at the expense of water-soluble ones (assumed to be nucleosides). Far-red light is capable of partially reversing this effect. If seeds were imbibed for 10 hrs in gibberellic acid and then extracted, similar changes in cytokinins to those brought about by red light were found. The cytokinins present were separated, prior to bioassay, by column chromatography using Sephadex LH20, but were not further identified. This decrease in water soluble cytokinins accompanied by an increase in butanol-soluble cytokinins has been shown in many seeds in response to dormancy breaking stimuli (Van Staden et al., 1972; Van Staden and Wareing, 1972; Webb et al., 1973 ; Brown and Van Staden, 1973; Van Staden and Brown, 1973; Van Staden et al., 1973) although it is not found in all cytokinin-containing seeds (Webb et al., 1973). Although there is good evidence for the type of change found in lettuce seed by Van Staden, the value of the results cited in this paper, with respect to the control of dormancy, must be seriously questioned for a number of reasons. From the germination data published in this paper, it would appear that the batch of seeds used for cytokinin extraction did not respond in classical fashion. Furthermore, Van Staden and Drewes (1974) have since reported that the soybean callus, used for bioassay, may not be specific for compounds generally considered to be cytokinins. We must also question whether or not the purification procedure of Van Staden could lead to the loss of particular cytokinins, resulting in the picture being simplified and misleading. In an attempt to resolve some of the questions relating to the possible involvement of phytochrome in changing endogenous cytokinins, Black et al. (1974) using isolated embryos of Grand Rapids seeds, studied the uptake and formation of cytokinins in response to light or gibberellin treatment. Isolated embryos do not germinate when in contact with solutions of abscisic acid but do so if cytokinin is added (Khan 1971). Presumably, if the excised embryos had an adequate endogenous supply of cytokinins they should be able to germinate in the presence of abscisic acid. This argument was used by Black et al. (1974) to study whether red light or gibberellin causes increases in endogenous cytokinins. They conclude that no increase in cytokinin activity is elicited by light or gibberellin treatments. However, this work cannot rule out transient changes in cytokinins, nor can it determine

whether localised concentrations might be affected, which would not lead to germination when the seed is bathed in high concentrations of abscisic acid. Thus, the question of the role of cytokinins remains unresolved.

It is clear that there are many problems in studying changes in hormone levels in lettuce seeds, and as a result little is known of endogenous hormones in such seeds. The studies which have been carried out serve only to highlight some of these problems, which are often related to the extraction , purification and assay of growth regulating substances. Classical methods involve the extraction of plant materials with organic solvents (or buffers) followed by physico-chemical purification techniques. However, little is known of the efficiency of extraction of the hormones present. It is possible that whole classes of hormones, such as conjugated or other bound forms, will not be extracted. Furthermore, solvent extraction may not remove hormones from tissue or cell compartments. In the absence of any real evidence or knowledge on this, caution should be exercised. If we accept that the hormones present have in fact been extracted there is considerable evidence that hormones with unknown separation properties may be lost during the physico-chemical procedures which follow. For instance, it has been pointed out that a large percentage of the less polar gibberellins such as GA_4, GA_7 and GA_8 migrate into ethyl acetate when partitioned against pH 8.0 buffer although the gibberellins are expected to move into the buffer phase (Crozier et al., 1969a, 1971). Whilst it is possible to keep losses to a minimum where the partition characteristics of compounds are known (Durley and Pharis, 1972), as yet unidentified compounds may be lost. In the past the 'acidic' fraction was often the only one examined for gibberellin activity (Ross and Bradbeer, 1971), other fractions obtained during the partitioning procedure being discarded. There is now considerable evidence for the presence of glucosyl esters and glucosides of gibberellins (Hiraga et al., 1972, 1974a,b; Yokota et al., 1969, 1970, 1971; Schreiber et al., 1967; Yamane et al., 1974; Hemphill Jr. et al., 1973) which are found in neutral and butanol fractions. These esters and glucosides may be the 'bound' or 'conjugated' gibberellins frequently referred to in the literature (Reinhard and Sacher, 1967). A similar situation occurs in the case of cytokinins. A common method for purifying extracts

containing cytokinins is to partition the acidified aqueous extract against ethyl acetate or diethyl ether, so as to remove acidic inhibitors. The water phases are then basified and partitioned against n-butanol. However, it has been shown that losses of 'free' kinetin and isopentenyladenine and their ribosides into the ethyl acetate take place (Hemberg and Westlin, 1973; Hemberg, 1974). Zeatin and its riboside do not move into acidic ethyl acetate (Letham, 1974). As with gibberellins, the presence of glycosyl derivatives of cytokinins has been reported (Letham, 1973; Yoshida and Oritani, 1973). With respect to abscisic acid, a glucoside which is not soluble in acidic ethyl acetate or ether has been demonstrated (Koshimizu et al., 1968; Milborrow, 1970). In the case of indole acetic acid there is a large number of conjugated forms (Schneider and Wightman, 1974). There is good evidence for the conversion of many of these derivatives to active forms (Lang, 1970). Consequently when studying unidentified growth regulatory substances of unknown chemical properties, purification steps may result in the loss of still other physiologically significant compounds. The picture thus obtained would not only be simplified but would be quite misleading.

In a large number of reports on growth-regulatory substances, the hormones contained in a purified extract are partially identified by the use of bioassays. At the very least, bioassays have been used to follow 'activity' during purification steps. Rigorous chemical identification of plant hormones is still the exception, rather than the rule! At best a bioassay will yield information about the overall hormone-like activity of an extract. There are two major problems associated with the use of bioassays: firstly, that one hormone may mask the activity of another; that is, how specific are bioassays. If a chemical separation procedure has failed to separate completely the hormones present, then one hormone may not allow the expression of another. Abscisic acid and other acidic inhibitors will inhibit gibberellin action, in bioassays used to assess gibberellin like activity. We also know that cytokinins can overcome abscisic acid (Sankhla and Sankhla, 1968; Khan, 1968). It is also frequently stated that no cytokinin like activity can be shown in the acidic ethyl acetate phase of a solvent partition system (Van Staden et al., 1972) yet we know that some cytokinins are lost into acidic ethyl acetate (Westlin, 1974). The possibility exists that cytokinin like activity can be masked by the presence of abscisic acid!

Consequently, even if we accept that the bioassay in use is specifically promoted and inhibited by certain hormones, can we tell from the overall hormone like activity whether or not we are looking at actual hormone levels?

A question of more fundamental importance is the ability of bioassays to yield information which is subtle enough to explain the changes in hormones which might be taking place. For example, if we examine changes in gibberellins we may use one of a number of bioassays. The activities of different gibberellins on these bioassays vary widely (Crozier et al., 1969b; Yamane et al., 1973; Hirata et al., 1974c). With respect to cytokinins the activity of ribotides appears to be less than that of free ribosides (Van Staden, 1973b). There are conflicting reports of 'free' cytokinins being less or more active than ribosides (Van Staden,1973b; Black et al., 1974). Hormonal changes taking place in response to some stimulus may go undetected by bioassay as the overall activity, extracted at one moment in time, could be constant regardless of qualitative changes which although not detected by a bioassay, could be highly significant within the system. Furthermore, unless the measurement of hormone activity is continuous there is always the possibility that a transient change might be missed.

It was previously suggested that red light might cause an increase in the 'effective' gibberellin content of lettuce seeds. The term 'effective' could be interpreted in terms of increased synthesis, decreased catabolism, the release of gibberellins either from some 'bound', presumably inactive form, or from a'compartment' of some description. The same argument could be applied to the effect of red light on cytokinins. What evidence is there that red light could act in this way and, if so, why has Grand Rapids lettuce seed not been more thoroughly investigated?

If we turn our attention to the latter, the major problem in examining lettuce seeds is that they are small and hence large quantities are required in order to study hormone changes. Because they are small seeds, it is difficult to separate the seed into its different parts to investigate the possibility that only the radicle or the cotyledons are involved in causing germination. It is possible that the germination of thermodormant lettuce seeds is under the dual control of gibberellins and cytokinins, one class of hormone affecting the radicle and the other, the cotyledons. Ikuma

and Thimann (1963b) believe that cytokinins cause the expansion of the cotyledons which then break through the restraining layers, whilst Haber and Luippold (1960) conclude that cytokinins cause cell enlargement in the radicle. More recently it has been suggested that the endosperm is ruptured by the expanding embryo and a chemical weakening process (Pavlista and Haber, 1970). In Acer pseudoplatanus it has been shown that cotyledon unrolling and expansion is under the control of gibberellins and radicle elongation and geotropic curvature under the control of cytokinins (Pinfield and Stobart, 1972). Thus, there is clear evidence that different parts of the seed can be influenced by different hormones. In examining the 'whole seed' it is possible that any endogenous hormone levels measured could be misleading, as these will represent the sum of all parts.

As a result of the difficulties in working with small seeds, there are few reports which indicate how red light might affect hormone balance. In suggesting possible ways in which red light might operate it is necessary to consider non-seed systems. From the work on Grand Rapids lettuce seeds we observed that no changes in gibberellin activity could be found after red light treatment. However, it is possible that transient changes could have been missed. It is known that red light can induce transient changes in gibberellin like activity in leaves of barley (Reid et al., 1968) or wheat (Beeves et al., 1970). This increase is rapid and appears to be due to release of a 'bound' form and not by de novo synthesis (Loveys and Wareing, 1971). However, this 'release' may be operated against a background of other effects of red light on metabolism (Reid et al., 1972). This release of gibberellin like activity has been shown to be from plastids, either etioplasts or chloroplasts (Cooke and Saunders, 1975; Evans and Smith, 1975; Reid, unpublished), and it is suggested that the organelle exerts a compartmentalisation effect on gibberellins (Railton and Reid, 1974a). Plastids have been shown to be sites of gibberellin synthesis from precursors (Stoddart, 1969) and of gibberellin interconversions (Railton and Reid, 1974b). Rapid transient changes in cytokinin-like activity have also been shown in response to red light (Van Staden and Wareing, 1972; Biddington and Thomas, 1975) although there is no evidence as to how this might be brought about.

Evidence has been presented that these rapid changes, in extractable hormone-like activity, are caused by the release of hormones from a compartmented form. Certainly, the speed of the response would suggest that the increase could not be explained by de novo synthesis. However, it would appear that the release phenomenon is carried out against a background of active metabolism and that this can also be influenced by red light. In studying metabolism, the results obtained from applying radioactively labelled compounds can only be meaningful if the presence of such compounds and their metabolites, as endogenous components of the system, has been shown. Few studies fulfil this criterion. There is evidence that glucosides and glucosyl esters of gibberellins are formed when labelled gibberellins are applied to certain systems (Nadeau and Rappaport, 1972; Nadeau et al., 1972; Yamane et al., 1975). The occurrence of such conjugated gibberellins has raised the question of their role in physiological processes. Evidence reviewed by Lang (1970 and Jones (1973b) and recent work (Browning, 1973; Kopowicz and Panayincki, 1973) have indicated that these glucosides may play a role in controlling the status of free gibberellins within a plant. Conjugation of gibberellins could sequester gibberellins in seeds, thereby regulating the level of free gibberellin. Nadeau et al. (1972) and Stolp et al. (1973) have shown that abscisic acid enhances the conversion of [^{3}H]GA_1 to [^{3}H]GA_1 glucoside, [^{3}H]GA_8 glucoside, [^{3}H]GA_8 and an unidentified [^{3}H]GA conjugate, which has since been shown to be amphoteric (Nadeau and Rappaport, 1974). The stimulation of the formation of these glucosides in abscisic acid treated aleurone layers could not account for the inhibition of gibberellin induced α amylase production in this tissue. A possible explanation of the role of abscisic acid in gibberellin metabolism comes from the work of Musgrave et al. (1972) who examined the fate of [^{3}H]GA_1, [^{3}H]GA_5 and [^{3}H]GA_5 methyl ester applied to isolated aleurone layers of barley and found that the rate of conversion of these gibberellins to more polar metabolites (conjugates) was related to their biological activity in the system. Inactive gibberellins were accumulated to a greater extent than more active ones. It is possible that gibberellin conjugates function to sequester gibberellins when the potential response to gibberellin is blocked by some inhibitor. In seed dormancy such a role for abscisic acid could be implied. Thus,

red light could cause the breakdown of such conjugates releasing biologically active gibberellins. This argument requires that conjugates be shown to be less active in the biological system under examination than free gibberellins. A similar situation is found with cytokinins where there is considerable evidence that glucosides are synthesised (Yoshida and Oritani, 1973; Parker and Letham, 1973, 1974; Gordon et al., 1974; Deleuze et al., 1972; Fox et al., 1973). It is possible that the cytokinin glucosides may be 'active' forms of cytokinin, but this remains open to discussion. Suffice to say that the presence of conjugated forms of gibberellins and cytokinins allows the postulation that rapid increases in extractable activity could be through the destruction or release of these compounds.

It is possible to speculate on the interaction between cytokinins and gibberellins. Railton (1974) has suggested that the rate of turnover of GA_{20} in pea seedlings is enhanced by cytokinin treatment. However, as Bown et al. (1975) point out, in order to determine the rate of turnover, the rate of synthesis, catabolism, possible compartmentation of the sites of synthesis and catabolism and the rates of transport between them, must all be examined before any conclusions regarding rurnover can be reached. Clearly, these criteria are not met by the work of Railton. It is extremely difficult to determine rate of turnover: however, the importance of turnover should not be neglected.

It has been shown that both gibberellins and cytokinins overcome thermodormancy in Grand Rapids lettuce seeds and it has been suggested that phytochrome operates through changing the 'effective' concentration of either or both of these hormone classes. Studies of Grand Rapids seeds have shown the presence of both gibberellin and cytokinin activity, but in neither case could red light be categorically shown to cause a meaningful change in these hormones. The study of endogenous hormones in seeds is beset with a number of problems; only those hormones extracted can be quantified and/or identified; hormones may be lost during the purification procedure resulting in a simplified and false picture, bioassays used to assess hormone activity are not sufficiently specific and cannot reveal the subtlety involved in control. By examining literature concerned with other plant systems, models are presented in an attempt to explain the possible ways in which red light could affect gibberellin and cytokinin levels.

With the present state of knowledge the involvement of phytochrome with gibberellins and cytokinins remains to be resolved.

Gibberellins and cytokinins have been shown to overcome thermo - dormancy. However, these are not the only compounds which will promote germination above the critical temperature. It is not the intention here to deal with all the compounds known to promote germination. However, two compounds have been shown to promote germination which can be considered to be hormones. One of these is ethylene. Ethylene was first reported to break dormancy as early as 1927 (Vacha and Harvey, 1927), and has since been shown to be capable of overcoming thermodormancy in Grand Rapids lettuce seeds (Stewart and Freebairn, 1969; Abeles and Lonski, 1969; Burdett and Vidaver, 1971; Negm et al., 1972; Burdett, 1972) and different types of dormancy in many other species (Egley and Dale, 1970; Esashi and Leopold, 1969; Ketring and Morgan, 1970; Hall and Wareing, 1972). The exact role of ethylene in lettuce seed germination remains unclear. Stewart and Freebairn (1969) concluded that the promotive effect of gibberellic acid resulted from the stimulation of ethylene synthesis. Abeles and Lonski (1969) measured endogenous ethylene production during red light promoted germination and found a doubling of ethylene production. Far-red light was equally promotive of ethylene synthesis. Burdett and Vidaver (1971) have questioned the conclusions reached by Stewart and Freebairn preferring to believe gibberellin promotion of germination to be mediated by more factors than ethylene alone. However, the value of these reports must be seriously questioned following the work of Negm et al. (1972, 1973) who found that carbon dioxide was essential for the ethylene effect on germination. Previously it had been observed that carbon dioxide, which is normally antagonistic to the action of ethylene, does not compete with ethylene in seed germination, indicating that seed germination differs from other ethylene controlled systems (Abeles and Lonski, 1969; Ketring and Morgan, 1972). It has been found that carbon dioxide does inhibit germination during the first few hours of imbibition (Negm et al., 1973). That carbon dioxide was promotive to germination of lettuce seeds has been known since 1936 (Thornton, 1936). The work of Don (1973) would suggest that ethylene is not involved in the endogenous control of Grand Rapids lettuce seed germination. The time course of ethylene production shows that no increase in ethylene occurs

until after radicle elongation has started. It is possible that ethylene production is the result of the radicle penetrating the restraining endosperm layer. However, the possibility cannot be excluded that ethylene is produced prior to radicle emergence but does not leave the seed until this event occurs. We are faced with the problem of cause-effect relations; does ethylene cause germination, or does germination lead to the production of ethylene? This problem is one which occurs in considering the effect of any hormone on the germination of seeds. The problem is more difficult to analyse in lettuce seeds than in many other seeds, because lettuce seeds show visible radicle emergence in under 24 hrs. Lettuce seeds show the first mitosis at 20°C during the 13th hour of imbibition (Evenari et al., 1957b) and soon after, radicle emergence through the seed and fruit coat occurs. In most studies on lettuce seeds, germination is assessed after 24 hrs or 48 hrs. Consequently the time period in which treatments can be made to the seeds is limited to the first 12 hours after imbibition: after this time germination can be thought to have occurred and any subsequent treatments will affect seedling development. Cause-effect relations can only be analysed by critical time course studies.

A fourth regulatory compound, shown to overcome thermodormancy in Grand Rapids seeds is cyclic 3'5' adenosine monophosphate (c-AMP) (Kamisaka and Masuda, 1971). c-AMP was not effective on its own but synergised with gibberellin in overcoming thermodormancy. The presence of c-AMP in lettuce seeds (cv May Queen) has been reported (Narayanan et al., 1970; Pradet et al., 1972). Other evidence that c-AMP may be involved in the control of lettuce seed germination comes from work using the cultivar Spartan Lake. Caffeine and theophylline, inhibitors of phosphodiesterase activity, can promote germination (Hall and Galsky, 1973). Phosphodiesterase breaks down the c-AMP to AMP which is inactive in overcoming thermodormancy. Bianco and Bulard (1974) also showed that c-AMP enhanced the dark, GA_3 induced germination of Grand Rapids lettuce seeds. However, ATP had similar effects although adenosine and adenine were ineffective. The nucleotide was further shown to increase the inhibitory effect of abscisic acid on light induced germination. Despite the reported occurrence of c-AMP in lettuce seeds (Narayanan et al., 1970; Pradet et al., 1972), which is stated to be 'unequivocal', some doubt remains as to whether cAMP does occur in plant tissues. The assays used to

measure c-AMP have been shown to be far from specific and a specific protein kinase assay has failed to show the presence of c-AMP in several plant tissues, previously reported to contain c-AMP (Amrheim, 1974). c-AMP is well known as an animal hormone where it acts as a 'trigger'. It is thought that in the animal the hormone activates the plasma membrane surface by promoting the production, by the cell, of the enzyme (adenyl cyclase) producing c-AMP in the cell; as part of this activation, calcium ions, essential for the enzyme's activity, encounter a more permeable membrane. Thereafter the c-AMP causes proteins in the cell to be phosphorylated by activating protein kinase which phosphorylates RNA polymerase. The RNA polymerase is then thought to stimulate RNA synthesis which then directs protein synthesis (from Steward and Krikorian, 1971). It has been suggested that c-AMP acts in a similar way in plant cells, and that hormones such as gibberellins, owe their activity to promotion of c-AMP synthesis which then acts as the 'trigger'. This view has little experimental backing and c-AMP could not be shown to be produced in response to treatment of barley aleurone by gibberellic acid (Keates, 1973). At present the role of c-AMP, indeed the presence of c-AMP, in plants is dubious. c-AMP represents the dangers in too rigid an attempt to fit concepts derived from animal studies to plant systems. The growth and development of higher animals and plants are very different and there is no reason for supposing that the same type of control mechanisms will operate in both.

So far we have discussed a number of factors which if applied to seeds of Grand Rapids lettuce, above the critical temperature, will overcome thermodormancy. It was proposed that phytochrome acts as a trigger to germination above the critical temperature. The interaction of phytochrome, or at least red light, with other promotive influences has been discussed. However, in the previously mentioned theory of dormancy, put forward by Amen (1968), dormancy is under the dual control of both promoters and inhibitors. We have also suggested that phytochrome may act by increasing the promotive 'influences' present in the dormant seeds or by destroying 'inhibitory' influences. We have not considered the role of inhibitors in dormancy, nor have we offered any explanation of how the seed becomes dormant above the critical temperature. It is to these questions that our attention must now be turned.

Seeds of Grand Rapids lettuce will germinate below the critical temperature, given a suitable aqueous environment, and it was suggested that there was sufficient phytochrome to cause the events leading to the radicle emerging through the restraining layers of the seed. Jones (1926) has described the morphology of the lettuce seed and considers the embryo to be surrounded by three layers.

(a) Pericarp or fruit covering.

(b) Integument; composed of a persistent outer epidermis with thick walls, secondly, remnants of disorganised cells and thirdly, a conspicuous suberized semi-permeable membrane which belongs to the wall of the inner epidermis of the integument, adjacent to the endosperm.

(c) Endosperm: in most parts of the seed consisting of a distinct layer of two cells.

(see Figure 4)

It has been shown that the integrity of the endosperm layer decides the photosensitivity of the seed (Borthwick and Robbins, 1928; Ikuma and Thimann, 1963b; Evenari and Newmann, 1952). The effect of the seed coverings is not as a result of preventing the outward diffusion of gases (Berrie and Hart, 1966), or the inward movement of water or gases (Mayer and Poljakoff-Mayber, 1963). Neither is this characteristic in any way unique to lettuce seeds (Karssen, 1968; Roberts, 1961; Chen and Thimann, 1964; Bachelard, 1967b; Rollin, 1968; Webb and Wareing, 1972; Egley, 1972; Juntilla, 1973b; Lona, 1947; Curtis and Cantlon, 1968). The endosperm provides a mechanical barrier to germination. We have proposed that below the critical temperature the embryo has sufficient growth potential to rupture this mechanical barrier, and that above the critical temperature some stimulus is required to increase the growth potential of the embryo. If the endosperm is punctured, in seeds showing thermodormancy, they will germinate in the absence of any further stimulus, and Speer (1974) has suggested that the integrity of the endosperm could be a natural controlling mechanism. However, we have previously noted that the seed coats cannot explain the quantitative differences in germination behaviour of seeds obtained from different lines of Grand Rapids lettuce (Globerson et al., 1974). In the absence of any evidence for the presence of hydrolytic enzymes which could destroy or weaken the endosperm, it

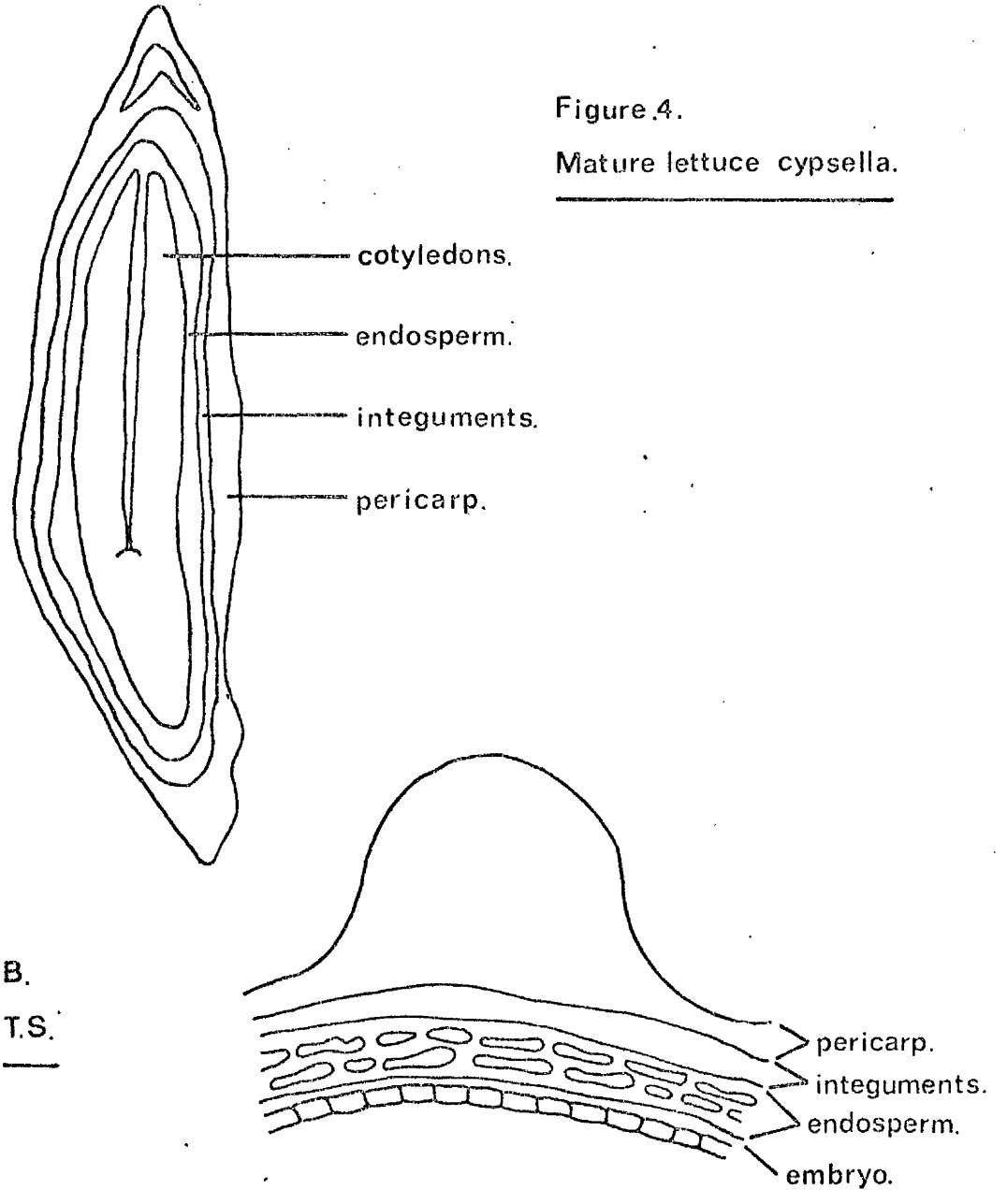


Figure 4.

Mature lettuce cypsella.

(after Jones, 1926.)

seems justified to suggest that control of thermodormancy, whilst requiring an intact endosperm, resides in the embryo.

Seeds of Grand Rapids lettuce will germinate, below the critical temperature, without an additional stimulus to germinate. It seems unlikely, therefore, that there is sufficient inhibitor(s) present to inhibit germination, or at least at sites where they can affect germination processes. Below the critical temperature it can be proposed that there is sufficient 'effective' promoter to overcome inhibitors, if present, or alternatively, the 'effective' inhibitor level is reduced in some way at this temperature but not above the critical temperature. Seeds are known to be rich sources of a wide range of inhibitory material, most of which is not considered to have a control function. It has been suggested that one inhibitor with such a control function could be abscisic acid (Amen, 1968). With respect to studies on seed germination, the involvement of hormones can be examined either by exogenous studies, where known compounds are supplied to seeds, or by investigating the endogenous components of the seeds and their metabolism.

Abscisic acid (ABA) has been shown to inhibit the germination of Grand Rapids lettuce seeds below the critical temperature (Khan, 1968). ABA has also been shown to inhibit the germination of a number of other cultivars of lettuce (Aspinall, et al., 1967; Sankhla and Sankhla, 1968; Reynolds and Thomson, 1973; McWha, 1973). Inhibition of germination, even in continuous contact with an ABA solution, is not permanent, although at high concentrations there is some reduction of the final percentage germination attained (McWha, 1973). In Great Lakes, Aspinall et al. (1967) have shown that ABA inhibition could be overcome by kinetin and to a lesser extent gibberellin A_3 (GA_3), provided the concentration of ABA was not too high. Red light was ineffective in causing germination of seeds held in ABA solution. Khan (1968) working with Grand Rapids seeds, showed that ABA inhibition could be overcome by kinetin but not by GA_3. In a fixed concentration of kinetin, increasing concentrations of GA_3 were shown to cause increased promotion of germination. Bewley and Fountain (1972) found that both kinetin and GA_3 were required to overcome ABA inhibition of whole seeds of Grand Rapids, whilst kinetin alone was effective in isolated embryos. Reynolds and Thomson (1973), using the cultivar Arctic King, showed that kinetin could overcome ABA inhibition but GA_3 or GA_{4+7} were ineffective.

Combinations of kinetin and gibberellin were no more effective than kinetin alone.

Khan (1971) proposed a theory for the control of dormancy, based on his work with lettuce seeds and other systems (Khan, 1967a,b; 1968; Khan and Heit, 1969) where cytokinins are assumed to be 'permissive'. In the presence of inhibitor, which may be ABA, gibberellins are inactive in causing germination unless a cytokinin is present. Khan views the gibberellin as the primary controlling hormone, with cytokinins and ABA, fulfilling permissive and inhibitory roles. Work on Setaria lutescens (Kollman and Staniforth, 1972) supports Khan's theory, and in addition ABA was identified as an endogenous component of this seed. Khan states that the control of seed germination and dormancy by external application of hormones probably reflects the natural control of dormancy and germination (Khan, 1971).

Clearly, seeds of Grand Rapids lettuce have not been fully investigated for the interrelations of cytokinins, gibberellins, abscisic acid and phytochrome.

The abscisic acid used in most exogenous studies is synthetic (RS) - (±) abscisic acid. The naturally occurring enantiomer, (S) - (+) - abscisic acid, has been identified by Oritani and Yamashita (1972) and by Ryback (1972). The synthetic substance is optically inactive, differing from the naturally occurring enantiomer in having ahigher melting point and lower solubility (Milborrow, 1974). While there are physical differences between the natural and synthetic compounds, the question arises as to whether or not their biological properties are the same. The resolution of the stereo isomers (Cornforth et al., 1967) and their subsequent testing by Milborrow (1968) has shown that both the R(-) and S(+) -ABA can be equally active. This depends on the bioassay employed to assay activity. Certain assays rely on their ability to metabolise ABA. It is known that the two forms R(-) and S(+) are not metabolised in the same way (Milborrow, 1970; Walton and Sondheimer, 1971). This point is fully discussed by Milborrow (1974).

As well as stereoisomerism, ABA exhibits geometrical isomerism at the unsaturated bond between carbons 2 and 3 in the side chain. The ABA most commonly isolated from plant tissue is the cis, trans or Z,E isomer, although trans, trans or E,E ABA has been detected in

plant tissues (Milborrow, 1970). In Rosa arvensis only 4% of the total (+) -ABA was present in isolated leaves as the trans, trans isomer. In avocado seeds, which are shielded from sunlight by a thick, pigmented pericarp, no trans, trans isomer was found (Milborrow, 1970). In solvents cis, trans ABA, photoisomerises to a 50:50 equilibrium mixture with the trans, trans isomer (Mousseron-Canet et al., 1960; Cornforth et al., 1968; Lenton et al., 1971). The biological activity of the trans, trans isomer has been reported as equally active to the cis, trans isomer (Addicott and Lyon, 1969), to totally inactive (Cornforth et al., 1965; Milborrow, 1966; Tamura and Nagao, 1969). McWha (1973) assigns the trans, trans isomer 50% the inhibitory activity of the cis, trans isomer in inhibiting germination of seeds of Arctic King or Great Lakes lettuce. This discrepancy may be accounted for in terms of the aforementioned equilibration of trans, trans and cis, trans isomers in light. The growth inhibitory activity of trans, trans ABA, carried out in light, may be attributable to the cis, trans isomer formed by photoisomerisation.

If ABA is to be assigned a control function in seed dormancy the first prerequisite must be the unequivocal identification of ABA in the seed under study. An attempt should then be made to correlate changes in the endogenous hormone with changes in germinability, although the demonstration of such a causal relationship is not itself proof of a controlling function. With respect to lettuce seeds, ABA has been identified in two cultivars, Great Lakes and Arctic King (McWha, 1973) but has not been shown to be present in Grand Rapids. During germination at $20^{o}C$, the endogenous level of ABA in Great Lakes and Arctic King seeds was shown to fall thirty-fold in 24 hrs imbibition. This fall could not be attributed to leaching of ABA. In an attempt to clarify this situation McWha studied the metabolism of 2[^{14}C]-ABA and showed that metabolism did take place. One metabolite was produced which has not yet been identified (McWha, 1973). The presence of ABA as an endogenous component of a number of seeds has been demonstrated (Pieniazek and Grochowska, 1967; Rudnicki and Susyka, 1969; Le Page-Digivry, 1970; Williams et al., 1973) and exogenous ABA shown to inhibit germination in seeds from a large number of species (Milborrow, 1974). When seeds and pericarps are separated it is usually found that the concentration of ABA in the pericarp is greater than that in the seeds (Rudnicki, 1973; Williams et al., 1973) although this need not

always be the case (Diaz and Martin, 1972). Attempts have been made to investigate the role of the ABA contained in the pericarp or the seed in relation to dormancy. Sondheimer et al. (1968) reached the conclusion that only ABA present in the embryo inhibits germination of Fraxinus americana whilst any present in the fruit wall does not affect the seed and is probably associated with regulation of ripening or abscission of the fruit. Williams et al. (1973) reached a similar conclusion for the fruits of Corylus avellana. However, Rudnicki (1973) has postulated that translocation of endogenous ABA, present in the seed coats, to the embryo could play a regulatory role in inhibiting germination. In an attempt to gain some insight into the possible ways in which ABA might control dormancy it is necessary to examine the way in which ABA is degraded during dormancy release.

Few studies have been carried out to examine the metabolism of radioactive ABA by seeds. The most detailed study is by Sondheimer et al. (1974) using embryos from dormant or stratified, non-dormant seeds, or dormant whole seeds of Fraxinus americana. These were imbibed in (S)-2[^{14}C]ABA under a variety of conditions. Both dormant and stratified embryos rapidly metabolize ABA to phaseic acid, 4'-dihydrophaseic acid and an unidentified polar metabolite, apparently derived from 4'-dihydrophaseic acid. Non-dormant embryos had only a slightly greater ability to metabolise ABA. Dormant intact embryos also metabolise (S)-2[^{14}C]ABA during stratification at 5°C or incubation at 25°C. The metabolites formed were similar to those formed by excised embryos although by 12 days a fourth metabolite was observed. Both stratified and non-stratified seeds metabolised ABA equally well. It is difficult to envisage how ABA can be involved in maintaining dormancy in this seed. The metabolic pathway in ash seeds, ABA → phaseic acid → 4'-dihydrophaseic acid, appears to be common to many plant species and tissue types; in embryonic axes of Phaseolus vulgaris (Walton and Sondheimer, 1972; Tinelli et al., 1973); tomato stems (Milborrow, 1970) (where only phaseic acid is formed and not 4'-dihydrophaseic acid). Only (+)-ABA is metabolised to phaseic acid (Sondheimer et al., 1971; Milborrow, 1974).

Phaseic acid was thought to be an artefact of the extraction procedure (Milborrow, 1972) but this does not seem likely as phaseic acid has been identified as an endogenous component of seed of

Phaseolus coccineus (MacMillan and Pryce, 1968), apical tissue of sugar cane (Most, 1971) and cotton fruit (Davis et al., 1972). 4'-dihydrophaseic acid has been identified in dry seed of Phaseolus vulgaris (Walton et al., 1973), endosperm of Echinocystis macrocarpa (Beeley et al., 1975) and apple seeds (Bearder et al., 1975).

A third naturally occurring metabolite of ABA is (+)-abscisyl-β-D-glucopyranoside. This was produced by tomato shoots fed with racemic 2[^{14}C]ABA (Milborrow, 1970) and compared with the same material isolated by Koshimizu et al. (1968) from yellow lupin (Lupinus luteus). The natural glucosyl ester from racemic ABA gave a mixture of (+) and (-) ABA with more of the latter (Milborrow, 1970). (-)-ABA can be conjugated as easily as the natural (+) enantiomer. The activity of the ABA glucoside was equivalent to that of ABA when expressed on a molar basis (Koshimizu et al., 1968). However, the ester is hydrolyzed rapidly by expressed sap from tomato shoots (Milborrow, 1970). Thus its inhibitory activity may be accounted for by the hydrolytic release of ABA. The stability of the ester within the tomato plant, in contrast to its hydrolysis by cell sap, suggests that the conjugate and the esterase molecules are held in different cellular compartments. This glucosyl ester has been shown unequivocally only in the two species discussed. The isolation of the glucoside is extremely difficult as it readily breaks down under acid conditions (Milborrow, personal communication). The presence of the glucosyl ester in other systems is inferred from the release of free ABA by alkaline hydrolysis of methanolic extracts. Seeds of Taxus baccata (Le Page-Digivry, 1973) and Pyrus malus (Bulard et al., 1974) have been shown to contain 'bound' ABA. The glucosyl ester may be a rapid storage product for exogenous ABA when this is applied to tissues (Milborrow, 1974). However, its discovery has led to attempts to implicate 'bound' ABA in the release of dormancy. In Taxus baccata the elimination of dormancy, due to stratification, is associated with a decrease in free ABA with a parallel increase in the amount of bound ABA-like inhibitors (Le Page-Digivry, 1973). During bud dormancy break Lesham et al. (1974) have shown that the level of 'free' ABA decreases whilst there is a parallel increase in 'bound' ABA.

In Grand Rapids lettuce seeds we have noted that the induction of thermodormancy is a threshold phenomenon (Berrie, 1966). Thus, it would appear unlikely that the induction of thermodormancy would involve immediate synthesis of an inhibitor. If an inhibitor is

involved in maintaining dormancy, above the critical temperature, once could envisage this being brought about by the release of ABA from a 'bound' form, assuming 'bound' ABA is biologically inactive, or from some form of compartment. We observed that in tomato Milborrow (1970) showed that the enzymes necessary to degrade ABA glucosyl ester appeared to be separated from the endogenous ABA glucosyl ester. Furthermore Railton et al. (1974) have shown that chloroplasts contain ABA. It is possible to envisage ABA moving from one part of the seed to another. The second critical question is how red light can overcome thermodormancy. We have previously discussed the possibility that red light operates through an effect on promoters. Similar models can be presented for inhibitors, where the promotive influence may remain constant, but the 'effective' inhibitor concentration is reduced. The reduction of effective inhibitor concentration could be envisaged to occur by reversing the effect of temperature in increasing the level of free ABA, in some way returning free ABA to an inactive form. Alternatively the degree of metabolism, via phaseic acid or through other metabolites (McWha, 1973), could be enhanced by red light. None of these possibilities has been examined in seeds of Grand Rapids lettuce.

Although we have discussed these 'alternatives' separately, the theories need not be mutually exclusive. It was proposed that germination was under dual control of promoters and inhibitors. Thus, the induction of thermodormancy might involve both a decrease in effective promoter levels and an increase in effective inhibitor levels. Furthermore, in dealing with more than one compound there is the possibility that a sequential series of events is required. Evidence for such a series of events would be difficult to obtain but the concept should be borne in mind when considering evidence for the involvement of hormones in any physiological event.

There are a few reports which indicate that ABA can affect the endogenous promoters of plant systems. Rudnicki et al. (1972) have shown that the endogenous gibberellins of apple seeds can be related to the level of ABA in the seeds as can the endogenous cytokinins (Rudnicki and Borkowska, 1973). However, zeatin cannot influence the metabolism of radioactive ABA, nor can ABA influence the metabolism of radioactive zeatin in seeds of Fraxinus americana (Tzou et al., 1973; Sondheimer et al., 1974). Whilst it is possible

to speculate on how one hormone might affect the endogenous levels of another, it is difficult to design the relevant experiments to eliminate all other possible explanations, and even more difficult to interpret the results obtained in such experiments!

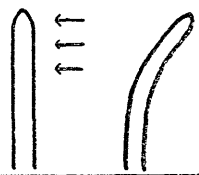
One central problem, not yet considered, is whether ABA is involved in seed germination or in the radicle emergence, required to bring about the visible manifestation of germination. It is difficult to separate the events leading to germination from those required to bring about the visible evidence of germination, radicle growth, which may be considered as seedling development. However, the effect of ABA on these two processes has been separated in one seed, Chenopodium album, as a result of a 'two stage' visible germination (Karssen, 1968). The first stage, which is light sensitive, is the splitting of the outer testa layer and the extending of the radicle from within the seed. The second stage involves protrusion of the radicle through the inner testa and underlying endosperm layer. This second stage is not light sensitive. Using exogenous (RS)-ABA Karssen found that only the second stage was inhibited. Clearly, if germination is not manifest because of a restriction on the 'visible' signs of germination, radicle emergence, then it is possible that we may be studying effects more closely related to seedling development than germination. McWha (1973), using time-lapse photography, studied the rates of radicle extension immediately after emergence through the fruit coats in fruits of the lettuce cultivars Great Lakes and Arctic King. He concluded that the inhibition of radicle elongation brought about by ABA could not explain the effect of ABA in inhibiting germination. However, Bex (1972) has presented evidence that ABA merely inhibits radicle growth and has no effect on the events leading to cell extension. In determining the role of ABA in germination, the question of whether or not ABA affects only radicle elongation, is of paramount importance.

Darwin's conclusion, based on his work with Phalaris, contained in the phrase - "we must therefore conclude that when seedlings are freely exposed to a lateral light some influence is transmitted from the upper to the lower point, causing the latter to bend" (Darwin, 1880, p.474) - may be considered to be the first evidence that the site of perception of a growth stimulus can be remote from the site of action. From this origin the work on the coleoptile of grasses proceeded and the essential steps in its development are summarised in the now classic diagrams of Boysen Jenson (1936). These diagrams (see Fig. 5) show the steps taken in proving that the coleoptile tip produces a chemical substance which is transmitted basipetally and stimulates cellular extension. Boysen Jenson proved that the 'influence' could move through a layer of gelatin, but not through material impervious to water, such as mica. Páal extended these experiments and showed that the growth of a particular organ, the hollow sheath or coleoptile, is controlled by its tip through the action of a diffusible substance. Went showed that this active agent diffused into agar from living oat coleoptile tips and that the agent which had diffused into the agar stimulated cell elongation just as it did when in living tips. If a small block of this agar is placed unilaterally on a decapitated Avena coleoptile the resulting curvature could be shown to be proportional, within limits, to the concentration of the growth hormone present. This enabled the quantification of a plant growth substance for the first time, and is arguably the single most important event in the history of plant physiology, the 'bioassay' was born! The diffusate was called auxin (from the Greek auxein - to increase) following Kögl's suggestion (Steward and Krikorian, 1971) and any substance which elicits a similar response may be said to show 'auxin like activity'. The active substance was shown to be identical with indole-3-acetic acid (IAA) although the first unequivocal evidence for the presence of IAA in oat coleoptiles was not made until 1974 (Bandurski and Schulze, 1974). Over the years IAA has been invoked in one way or another in almost every conceivable biochemical, cell or whole plant physiological response. However, the evidence for any role for IAA in the control of dormancy and germination is sparse.

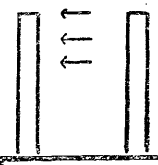
Evenari and Mayer (1954) could not find any promotion of germin-

Figure 5

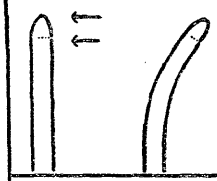
Schematic representation of the early work on hormone control of plant growth (after Boysen-Jenson, 1936).



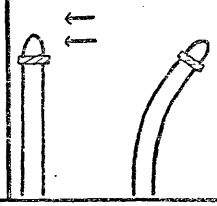
DARWIN, 1880
Light falling from one side upon the tip of a grass coleoptile (Phalaris) causes some influence to be transmitted downward; the coleoptile curves toward the light.



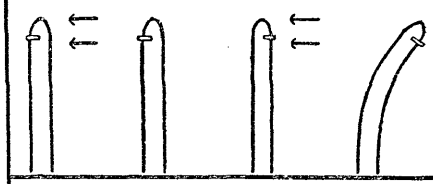
When the coleoptile tip is removed, phototropic response does not occur. Localized sensitivity to light and conduction of a stimulus was observed early in many kinds of plants.



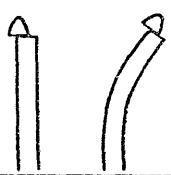
FITTING, 1907
In a room saturated with water vapor, lateral incisions either on one or on both sides of the Avena coleoptile do not prevent its bending toward light from one side.



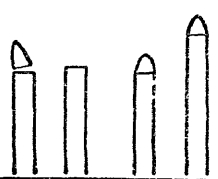
BOYSEN JENSEN, 1910–1911
When an excised coleoptile tip was replaced with gelatin inserted between it and the stump, phototropic curvature resulted as in normal coleoptiles: the tropic stimulus passed over the incision.



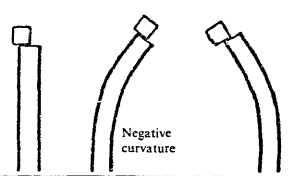
BOYSEN JENSEN, 1910–1911
Insertion of mica plates on the shaded side prevented curvature following unilateral illumination of the tip. When the mica insert was made on the illuminated side, curvature resulted in the usual way. It was concluded that a substance migrates down the back side promoting growth curvature toward light.



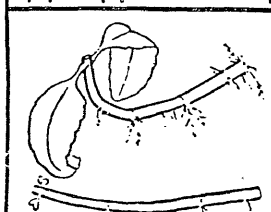
PAÅL, 1918
When an excised tip is replaced on one side of the Avena coleoptile stump, accelerated growth beneath the tip results in curvature.



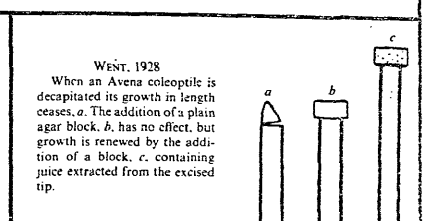
SÖDING, 1925
Decapitation results in diminished growth of the Avena coleoptile, but when the excised tip is replaced, growth in length is renewed.



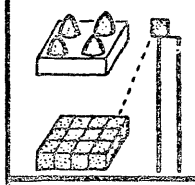
STARK, 1917–1921 SEUBERT, 1925
Expressed sap from Avena coleoptiles was put into agar blocks which were applied unilaterally to coleoptile stumps; curvatures resulted.
Seubert (1925) found by using this method that some substances promote while others inhibit growth, as indicated by negative and positive curvatures.



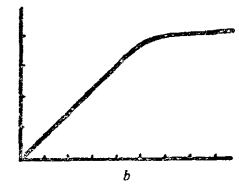
LOEB, 1916
The presence of vigorous leaves on a horizontally placed Bryophyllum stem increases geotropic bending (also the production of roots is stimulated). The action of hormones was suggested as the explanation.

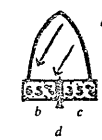


WENT, 1928
When an Avena coleoptile is decapitated its growth in length ceases, *a*. The addition of a plain agar block, *b*, has no effect, but growth is renewed by the addition of a block, *c*, containing juice extracted from the excised tip.



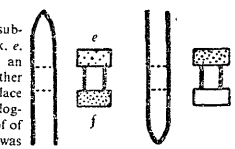
WENT, 1928
Growth hormone is given off from plant tissue (coleoptile tips) into agar. When a small block of this agar is placed unilaterally on a decapitated Avena coleoptile, the resulting curvature, *a*, is proportional, within limits, to the concentration of growth hormone present, *b*.

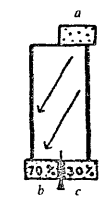




WENT, 1928
When unilateral light falls upon an excised Avena coleoptile tip, *a*, placed in contact with two agar blocks, *b* and *c*, separated by a razor blade, *d*, growth hormone is displaced toward the shaded side; block *b* receives 65 per cent and block *c* 35 per cent of all the recoverable growth hormone given off from the tip.

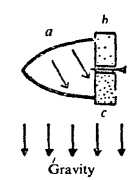
WENT, 1928, AND VAN DER WEIJ, 1932
Transport of growth substance from an agar block, *e*, through a segment from an Avena coleoptile into another agar block, *f*, takes place only toward the morphological base. Qualitative proof of the same phenomenon was given by Beyer (1928).



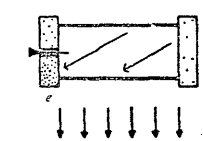


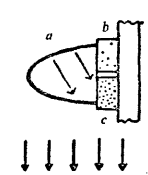
VAN OVERBEEK, 1933
An agar block, *a*, containing growth hormone is placed upon the upper cut surface of a Raphanus hypocotyl segment standing upon two plain agar blocks, *b* and *c*. Exposure to unilateral light causes displacement of growth hormone toward the shaded side; the recoverable portion is present in the two blocks as indicated.

HORMONE EXPLANATION OF PHOTOTROPISM
The growth hormone is displaced by unilateral light into the shaded portion of a hypocotyl, petiole, or similar organ. Its presence in greater concentration promotes growth more rapidly there, and the organ bends toward the light.

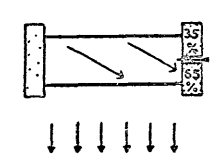


DOLK, 1929
When an excised coleoptile tip, *a*, is placed in a horizontal position in contact with two agar blocks, *b* and *c*, growth hormone is displaced toward the lower side. It accumulates in greater concentration in block *c*.
When a cylindrical segment of an Avena coleoptile is placed horizontally and supplied with growth hormone at the morphological apex, *d*, transport takes place toward the lower side of the morphological base, the hormone accumulating in block *e*.



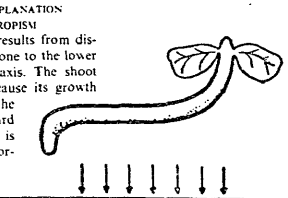


BOYSEN JENSEN, 1933
When an excised root tip of *Vicia Faba*, *a*, is placed horizontally in contact with agar blocks, *b* and *c*, containing 10 per cent glucose, growth hormone is displaced to the lower side and accumulates in the lower block, *c*.



DIJKMAN, 1934
Growth hormone supplied in agar to the cut apex of a segment of Lupinus hypocotyl, placed in a horizontal position, is transported toward the lower side of the morphological base.

HORMONE EXPLANATION OF GEOTROPISM
Tropic bending results from displacement of hormone to the lower side of the plant axis. The shoot curves upward because its growth is promoted, and the root turns downward because its growth is inhibited by the hormone (Cholodny).



ation in imported seeds of Grand Rapids lettuce, held in darkness, over a wide range of concentrations of exogenous IAA. The higher concentrations were found to be inhibitory. When applied in conjunction with red and far-red light, IAA had no significant effect. Seeds of the lettuce cultivar Progress behaved similarly, although higher concentrations of IAA were required to inhibit germination. When the same experiments were carried out with locally grown seeds of Grand Rapids, low concentrations of IAA had a promotive effect in darkness whereas high concentrations inhibited germination (Poljakoff-Mayber, 1953). The anti-auxin P.C.I.B. had no effect on germination. IAA and a number of other compounds, displaying auxin-like activity, were found to be inhibitory to the germination of seeds of Grand Rapids lettuce in light or darkness. Kinetin and GA_3 were shown not to be able to overcome IAA inhibition (Khan and Tolbert, 1966). Sankhla and Sankhla (1968) also showed IAA to be inhibitory to the germination of seeds of two other cultivars of lettuce. There are few reports of the effect of IAA on the germination of other seeds but these few emphasize the point that IAA may promote to a small degree at low concentrations but always inhibits at high concentrations (Vyas and Shrimal, 1973).

As IAA has been shown capable of promoting cell elongation, it might have been proposed that IAA could be involved in the extension of the radicle during seed germination. This seems unlikely in view of the evidence cited above. However, the action of IAA in the extension of oat coleoptile segments is proposed to involve in part the secretion of hydrogen ions. If hydrogen ions were released into the solution, in which seeds were being imbibed, it is possible that the medium could be acidified. This acid bathing solution could then inhibit radicle elongation, stopping the visible manifestation of germination (Rayle, 1973; Reynolds, 1975). There is at present no evidence that IAA solutions become more acid in the presence of seeds. It is also possible that only low concentrations of IAA are required to promote germination as a result of very localised sites of action.

As was the case with all the other hormones considered, two conditions require to be met, in order to satisfy the minimum requirement for indicating a role for IAA in germination; that it is

present in the seeds must be proven, and its endogenous levels must be correlated in some way with dormancy-release and germination. The evidence for both these prerequisites is scant indeed. There is no evidence that auxin like compounds are present in seeds of Grand Rapids lettuce (Blumenthal-Goldschmidt, 1958, 1960), although the possibility that IAA may be present in some bound form, not readily extractable, cannot be ruled out (Blumenthal-Goldschmidt, 1958). Since this early work no reports have been made showing the presence of IAA, 'free' or 'bound' in lettuce seeds. Indeed the only seed, so far shown to contain IAA is sweet corn, Zea mays, which contains 60-80 mgs/kg esterified IAA and 3-4 mgs/kg free IAA (Kopcewicz et al., 1974). Auxin like activity has been shown in seeds of Hyoscyamus muticus (Elkinawy and Hemberg, 1974). Auxin activity was found prior to germination and was promoted by kinetin and diurnal temperature fluctuation, both of which promoted germination. An auxin like compound has also been found in grains of barley, although IAA was shown not to occur (Minchin and Harmey, 1975). It is possible that if only small amounts of IAA are present in lettuce seeds, these levels would have been below the detection limits of the methods in use prior to 1960. Thus, the question as to the presence or absence of IAA in lettuce seeds must remain unresolved.

If we accept that IAA may have a role in the control of dormancy and germination, can we explain how IAA might function in either overcoming or maintaining dormancy? The arguments used for displaying a controlling function for the other hormones can be applied. We can propose that if IAA is promoting germination, this is because of (a) increased synthesis or decreased catabolism, or (b) release from a 'bound form' or from a compartment where it is presumed inactive. If IAA is presumed to inhibit germination, then the opposite argument can be proposed. Release from dormancy could be by decreased synthesis, increased catabolism or removal of 'active' hormone into a bound or compartmented form. In view of the lack of evidence dealing with the role of IAA in seeds, it is difficult to determine which of these alternatives is operating to control the level of endogenous IAA. These alternatives need not be mutually exclusive. A brief survey of the literature dealing with other plant systems does show that the alternatives stated could be operative.

There are numerous forms of 'bound' IAA; glucose (1-(indole-3-acetyl)β-D-glucose), other sugars, aspartic acid and chloro

derivatives of this conjugate, a β-1,4-linked cellulosic glucan, myoinositol, myoinositol arabinosides or myoinositol galactosides (Schneider and Wightman, 1974). If such conjugated forms of IAA are present in seeds, they may be inactive and cleavage may produce free IAA which can either inhibit or promote germination. IAA can also be bound to a particulate fraction in pea seedlings (Jablanovic and Nooden, 1974) or corn coleoptiles (Hertel et al., 1972). Cytokinins can compete for the binding sites, indicating a possible way in which the cytokinin might counteract a possible effect of auxin in seed dormancy. It should be noted that kinetin did not overcome IAA inhibition of lettuce seed germination (Khan and Tolbert, 1966). However, we cannot rule out other cytokinins being effective.

Another interesting report is that of Muir and Chang (1974). These workers showed that red light reduced the growth of oat coleoptiles and synthesis of auxin, the effect being far-red reversible. They interpreted their results as an effect of phytochrome on the cell membrane which delocalizes the tryptophan utilized for auxin synthesis. Furthermore, it is well documented that IAA destroying enzymes, be they IAA oxidase or peroxidases, may be associated with cellular compartments. If IAA and the enzymes involved in catabolism are spatially separated, until a stimulus to germinate has been given, an obvious control mechanism exists for regulation (Schneider and Wightman, 1974).

It was previously suggested, although with little experimental backing, that kinetin and/or red light promote the germination of thermodormant seeds of Grand Rapids lettuce by promoting pectinase and cellulase activity in the seeds (Ikuma and Thimann, 1963b). These enzymes would reduce the mechanical barrier to germination. There is evidence that in pea epicotyls auxin increased cellulases in the cytoplasmic fraction and wall bound cellulases (Ridge and Osborne, 1969; Ferrari, 1972).

As a final 'model' of what the possible involvement of IAA in seed germination might be, it has been shown by Lee (1974) that cytokinin controls the level of IAA oxidase and peroxidase in tobacco callus cells. Other hormones have also been shown to affect the level of IAA destroying enzymes (Lee, 1971a,b).

An increase in IAA destroying enzymes would lead to reduced IAA content, which could release the thermodormant seed to germinate.

Obviously, it is possible to speculate at even greater length on the way in which IAA might act in controlling thermodormancy, either imposing thermodormancy or releasing the seed from dormancy. However, the real need is to look at the endogenous content of IAA in seeds, and whether IAA promotes or inhibits germination, in an attempt to resolve a problem which seems to have attracted little attention from 'auxinologists'.

Seeds of Grand Rapids lettuce will germinate, given suitable moisture, below a critical temperature but not above it. There is a sharp temperature 'cut off' indicating the threshold nature of the phenomenon. This thermodormancy is overcome by the action of phytochrome and it has been proposed that phytochrome acts as a 'trigger' for germination (Amen, 1968). The action of phytochrome is rapid, although the prolonged presence of phytochrome may also be required. It has been suggested that phytochrome may cause germination by altering the 'effective' concentration of (a) growth regulating compound(s) in the seed. The compound(s) would then control the release from dormancy. Whatever the action of phytochrome might be, it can be reversed by far-red light given several hours after red light, and it is assumed that red light leads to certain biochemical events which eventually culminate in the growth of the radicle. In considering how thermodormancy is removed, it must be remembered that phytochrome could act on its own without a secondary 'messenger'. Thus, the possible ways in which phytochrome and/or the secondary messengers act should be considered. It is also important to remember that since germination will take place at a suitable temperature, something must happen when the temperature is raised which inhibits germination. This has already been fully discussed. However, if, for example, gibberellin initiates events which lead to germination, it should be borne in mind that these could take place both below and above the critical temperature. In dealing with the control mechanisms involved, it is probable that the same events occur both in the non-dormant and dormant seeds.

In order to define the possible points at which control may be exerted it is necessary to gain some idea of what happens to the biochemistry of the quiescent seed when it starts to imbibe water. We shall only concern ourselves with the primary events which take place. The biochemistry of seed germination and seedling growth has been the subject of a recent review and need not be dealt with here (Mayer and Shain, 1974).

During seed maturation changes take place which prepare mature seed to withstand desiccation. The process of switching off the seed's metabolism is little understood but appears to be an organised

series of events, since not all metabolic activities terminate abruptly and simultaneously (Mayer, 1973). When the non-dormant, quiescent seed is placed in a suitable environment germination will take place. Reserve materials, stored during maturation will be broken down and utilized as germination and seedling growth proceeds. This breakdown must involve the action of enzymes which are either present in the seed and require to be 'activated', 'released' or synthesized de novo. It has been known for more than a decade that the resting seed of wheat contains a 'preformed' or 'resting' messenger, ribonucleic acid (m-RNA), formed prior to the transcription of DNA after imbibition (Morton and Raison, 1964). The presence of preformed m-RNA has since been shown in cotton seeds (Dure and Waters, 1964; Waters and Dure, 1965), pea seeds (Barker and Rieber, 1967; Sieliwanowicy and Chmielewska, 1973), and rice seeds (Bhat and Padayatty, 1974), and ribonucleic acids having template activity have been found in soybean seeds (Mori et al., 1968). Dry seeds have also been shown to contain ribosomes capable of protein synthesis (Sturani et al., 1968; Marcus and Feeley, 1965; Waters and Dure, 1965; Sieliwanowicy and Chmielewska, 1973; Sasaki and Brown, 1971; Efron et al., 1971; Yamamoto et al., 1974; Reger et al., 1975). The activation of protein synthesis upon imbibition may be due to the formation of polyribosomes from pre-existing ribosomes and m-RNA resulting from a series of reactions which require ATP (Marcus, 1969; Obendorf and Marcus, 1974). In Pisum arvense, Barker and Rieber (1967) suggested that protein synthesis in quiescent seeds is potentially fully operative but is limited by the absence of functional polyribosomes. Studies using electron microscopy revealed the lack of polyribosomes in unimbibed seeds. Polyribosomes have been shown to appear during germination (Berjak and Villiers, 1970; Chapman and Rieber, 1967; Durzan et al., 1971; Marcus and Feeley, 1965; Sasaki and Brown, 1971). An alternative to the formation of active polyribosome formations would be the modification of ribosomal structure which resulted in ribosomes becoming more active in incorporating amino acids. Sturani et al. (1968) provide evidence against this hypothesis.

Pre-formed m-RNA is resistant to desiccation in wheat embryos, whilst newly synthesized m-RNA loses its ability to be transcribed after a cycle of dehydration and rehydration (Chen et al., 1968). It has been suggested that m-RNA in dry embryos is preserved in a

complex form within the ribosomal fraction (Schultz et al., 1972).

Thus, in a number of seeds the sequence of events following imbibition would appear to be a rapid formation of active polyribosomes from pre-existing ribosomes and m-RNA. Although de novo synthesis of ribosomes has been shown to precede polyribosome formation and protein synthesis, in castor bean and broad bean seeds (Marre, 1967; Payne and Boulter, 1968), this does not seem to be general. Furthermore, m-RNA synthesis seems not to be required in the initial stages of germination. Dry seeds of wheat have been shown to contain preserved and protected DNA templates (Chen and Osborne, 1970a) but no replication of the DNA template could be detected during the early period of germination. It is known that DNA synthesis is not a prerequisite of germination in lettuce seeds (Haber et al., 1969). Thus, although continued seedling development obviously requires de novo synthesis of RNAs, DNA, etc. in the context of the early control of seed germination these events may not be important (Frankland et al., 1971).

What is known of the early events of germination in seeds of Grand Rapids lettuce?

Firstly, DNA synthesis is not required for germination. Neither does it seem that de novo synthesis of m-RNA is required. Frankland et al. (1971) showed that the nucleotide base analogue 6-methyl purine inhibited lettuce seed germination but the rate of RNA synthesis in ungerminating and germinating seeds differed only after about 12 hrs at 27°C by which time radicle growth had started. Bewley and Black (1972) reported that there were no major gibberellin induced changes in protein synthesis before visible germination. The picture is complicated by the work of Black and Richardson (1965, 1967) who found that L-threochloramphenicol, actinomycin D and D-chloramphenicol promote the dark germination of lettuce seeds. The suggestion made by these authors was that these substances act by arresting an inhibitory process which requires protein synthesis. Further confusion about the role of nucleic acid and protein synthesis in lettuce seeds has been added by the conflicting results of Mitchell and Villiers (1972) and Fountain and Bewley (1973). Mitchell and Villiers reported that they could not detect polyribosomes in imbibed seeds in darkness and that polyribosomes were formed when a dormancy breaking stimulus of illumination was given. In direct

contradiction of this work the latter authors showed that in ungerminated seeds, polyribosomes build up early in germination and that these polyribosomes are active in protein synthesis. The work of Efron et al. (1971) would tend to support Mitchell and Villiers. They showed that amino acid incorporation increased during the first three hours of imbibition, independent of light and in the absence of synthetic m-RNA. These results infer the presence of both ribosomes and preformed m-RNA. This 'activity' would not lead to germination and illumination is required if incorporation activity is to increase again at 18 hrs, leading to germination.

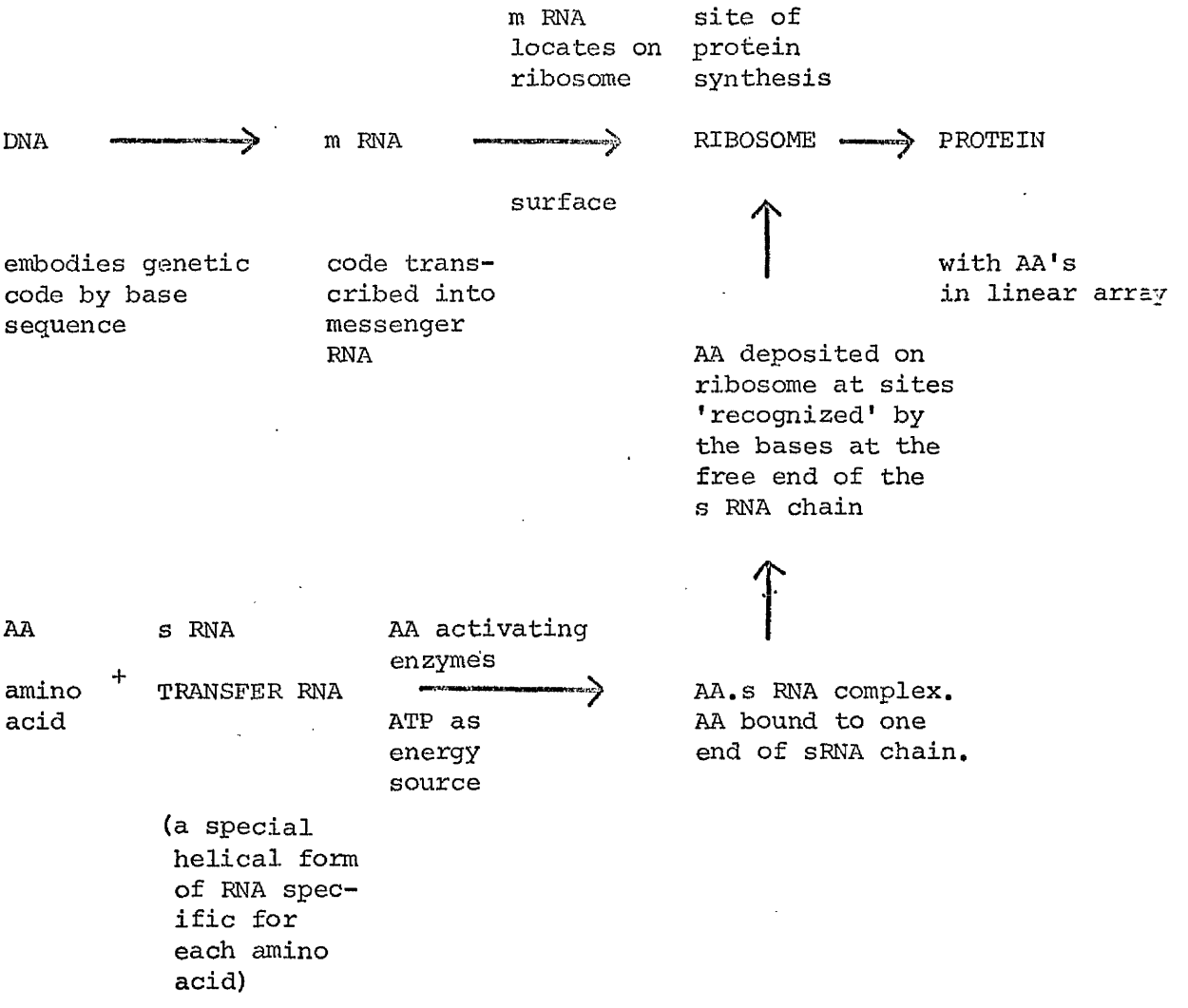
Srivastava and Poulson (1968) and Poulson and Srivastava (1968) have studied the fine structure of the embryo of Lactuca sativa, cv Grand Rapids in the dry seed and during germination. They found that the first cells to be activated were in the hypocotyl just behind the root, after about 12 hrs. In dry embryos ribosomes were common on the cisternae of endoplasmic reticulum as well as in the ground cytoplasm. Polyribosomes were absent and the endoplasmic reticulum was poorly developed. As activation of the embryo took place there was a proliferation of the rough endoplasmic reticulum and aggregates of ribosomes, polyribosomes, could be seen on the profiles of the endoplasmic reticulum and in the ground cytoplasm. These features were especially evident in cells of the root-shoot axis. Dictyosomes (or Golgi apparatus) could also be recognised in cells of the root-shoot axis after 24 hrs. The Golgi apparatus are thought to be involved in lipid metabolism and the production of lipids used in membrane synthesis.

It would appear that in lettuce seeds we have a situation not dissimilar to any otherquiescent seed. Preformed m-RNA and ribosomes exist and the synthesis of m-RNA and DNA are not prerequisites for germination. The difference between quiescent seeds and seeds showing dormancy would appear to lie in their ability to produce polyribosomes, active protein synthesising units! Although there is contradictory evidence in lettuce seeds, it can be proposed that in lettuce, at least insuficient polyribosome formation takes place to cause germination unless some stimulus to germinate is applied. A number of other red light promoted seeds have been shown to require red light treatment if polyribosomes are to be found and subsequent germination take place (Sasaki and Brown, 1971; Yamamoto et al., 1974; Reger et al., 1975).

Thus, whatever red light or various hormones do to promote germination must take place somewhere in the chain of events leading to the production of active polyribosome complexes. The production of polyribosomes itself takes some time which makes it unlikely that this event itself can explain how these promoters effect seed germination. The number of points at which a promoter could control the production of polyribosomes is numerous. Reference to Figure 6 shows that control could reside at any point from the amino acid to the deposition of these on the ribosome, or between the m-RNA and the ribosome. This is a very simplified picture of protein synthesis in plants. Zalik and Jones (1973) have reviewed protein synthesis in plants and it is clear that whilst a general 'outline' may now be accepted as being of universal application, the fine details are only poorly understood. As we have shown that the transcription of m-RNA appears not to be a requirement for the initial steps of seed germination, the evidence for hormones or phytochrome in the control of transcription or DNA synthesis will not be dealt with. It should be understood, however, that the ultimate expression of germination and the further growth of the seedling require these events. Anything which interferes with these events will be seen to influence 'seed germination'.

It has been suggested that phytochrome itself may mediate germination without the aid of a second messenger. Is there any evidence that phytochrome can affect protein synthesis directly? One point where phytochrome could act is indicated from the work of White and Pike (1974). A rapid increase in adenosine-5'-triphosphate (ATP) in etiolated bean buds was shown to be under the control of phytochrome. It has previously been stated that ATP is required for the formation of polyribosomes from pre-existing m-RNA and monosomes (Marcus, 1969; Obendorf and Marcus, 1974). It was thought that phytochrome worked by gene de-repression (Mohr, 1966) a theory still supported by Schopfer (1972). In an attempt to provide evidence for this considerable work was carried out to investigate phytochrome control of *de novo* enzyme synthesis. Whilst it is reasonable to suggest that certain enzymes are indeed synthesised *de novo* in response to phytochrome (the most recent evidence being Acton and Schopfer, 1974; Acton *et al.*, 1974; Attridge, 1974), it would also appear that phytochrome may act by releasing an inactive precursor of the enzyme (Attridge and Smith,

Figure 6. Scheme of events involved in protein synthesis



(from Steward, F.C., 'Plants at Work' Fig. 8-8, page 91)

1973; Attridge et al., 1974). The rapidity of phytochrome action in a large number of responses has led to the idea that it in some way influences membranes. How this might operate and subsequently lead to germination will be discussed at a later stage. We will first consider the evidence relating to the possible mode of action of a secondary messenger.

It is generally accepted that this second messenger will be one of the recognised plant hormones. There is a wealth of literature dealing with the mode of action of plant hormones. We have proposed that control of DNA synthesis and transcription cannot account for the control of dormancy release. It is intended to attempt to examine where control might reside, in the series of events leading to active protein synthesising units, polyribosomes. Evidence using only seed systems is fragmentary and use will be made of non seed systems.

The most comprehensively studied system for the biochemical basis of any plant hormone is the barley or wheat aleurone; the role of gibberellins in this system has been recently reviewed by Jones (1973a). It is not intended to consider all aspects of this system, but to draw a model. Gibberellins have been shown to lead to de novo synthesis of enzymes in the cells of the aleurone layer, these enzymes being secreted into the endosperm where hydrolysis of stored substrates proceeds. The response of isolated aleurone tissue to exogenous gibberellin has a lag period which varies from 4 to 20 hrs after the addition of the hormone. It should be pointed out that α amylase enzyme production is not an event which controls germination (Drennan and Berrie, 1961); however, this fact does not mean that the early results of gibberellin action are not involved in the germination response. What then are the early actions of gibberellin? Evins (1971) and Evins and Varner (1972) have shown that there is both an increase in ribosomes and the formation of polyribosomes in barley aleurone cells. These effects are probably prerequisites for the hormone induction of enzyme synthesis. ABA is known to inhibit α amylase formation (Chrispeels and Varner, 1967) and also ribosome synthesis and the formation of polyribosomes (Evins and Varner, 1972). Another early consequence of gibberellin action is an increase in membrane, particularly endoplasmic reticulum production (Jones, 1969a,b). Evins and Varner (1971)

showed [^{14}C] choline incorporation into a semi-purified fraction, containing endoplasmic reticulum, was increased 4- to 8-fold by gibberellin, with a lag period of about 4 hrs. This parallels the increase in polyribosome formation and is also inhibited by the addition of ABA. That the production of endoplasmic reticulum could be as a result of de novo synthesis is supported by the work of Johnston and Kende (1971). Two enzymes of the cytidine diphosphate choline pathway of lecithin biosynthesis are stimulated within 2 hrs of GA_3 treatment. Cell free extracts from gibberellin treated barley aleurone layers show greater phosphoryl choline glyceride transferase activity than control layers and this activity does not require RNA or protein synthesis. ABA inhibits this gibberellin dependent activation (Ben-Tal and Varner, 1974). Although the evidence in favour of an increase in endoplasmic reticulum via increased synthesis of membrane is strong, Firn and Kende (1974) have failed to show any radical changes in lipid content of aleurone layers when treated with gibberellin, and believe that the accumulation of endoplasmic reticulum in aleurone layers, evidenced during gibberellin treatment, may be an indirect effect. Nevertheless there would appear to be an increase in membrane, whether or not it is caused by increased synthesis of membrane components, and it has been suggested that the synthesis of gibberellin induced hydrolases may depend on the availability of proper membranes for the attachment of polyribosomes that carry hydrolase specific m-RNAs (Tata, 1968). It is known that polyribosomes are the active protein synthesising units (Poulson and Beevers, 1970) but the exact role of membrane bound polyribosomes is unclear. If we accept that the attachment of polyribosomes to membranes may have significance in the regulation of their activity, clearly as yet purely speculative, then it must be important to understand how the polyribosome is 'held' on the membrane. Implicit in the interpretation of much of this type of work is that the formation of polyribosomes is dependent on the availability of m-RNA. It is assumed that polyribosomes are held to membranes by the m-RNA (Chen and Osborne, 1970b). However, Dobberstein et al. (1974) showed that mild ribonuclease digestion released only about 10% of the membrane bound ribosomes. This result makes it unlikely that m-RNA is solely responsible for a specific attachement of ribosomes to membranes.

One of the major problems in any scientific study is the interpretation of results. We have presumed that the action of gibberellin does not involve an effect at the level of transcription, but how easy is it to determine whether an action of a plant hormone is at the level of translation or transcription? Carlson (1972) studied the induction of α amylase synthesis in the presence of 5-fluorouracil and came to the conclusion that gibberellin induced synthesis of α amylase in barley aleurone could satisfactorily be explained by a post-transcriptional control point. However, his data have been re-interpreted to show control at the level of transcription! (Chandra, 1974). Furthermore, the synthesis of new ribosomes was thought not to be necessary for α amylase induction by Jacobsen and Gwar (1974).

These points are mentioned to emphasize caution in 'model' building. Whilst a model may be intuitively pleasing, it is difficult to resist a degree of selectivity in examining the relevant evidence, albeit unconscious.

It has been shown then that ribosome synthesis, the aggregation of ribosomes into polyribosomes and the synthesis or at least appearance of membranes are early events influenced by gibberellins. These events have also been shown to be affected by other hormones. We have discussed the evidence for ABA inhibition of many of the events promoted by gibberellin in the aleurone system. The need for cytokinin for cell division in cultured cells of Glycine max has been correlated with a high cellular content of polyribosomes. The increase in the presence of cytokinin was rapid and had no detectable lag period. m-RNA synthesis was not required (Short et al., 1974). Also, the removal of cytokinin from a cytokinin requiring strain of soybean cells leads to a rapid decline in polyribosomes. Associated with this decrease was an increase in phosphorylation of the ribosomal protein (Tepfer and Fosket, 1975). Evidence for the modification of protein phosphorylation in other organelles and ribosomes had previously been reported by Ralph et al. (1973). The role of phosphorylation, or the possible alteration of a kinase responsible for such phosphorylation, is unclear, but in animal cells cyclic AMP is known to be responsible for ribosomal protein phosphorylation (Tepfer and Fosket, 1975). A correlation has been shown between the extent of binding of cytokinins to ribosomes and

the biological effects of various cytokinins (Berridge et al., 1970) and the incorporation of cytokinin into transfer RNA is known (Fox, 1966). How cytokinins might affect the ribosome is unknown but there is considerable evidence that cytokinins can interact with ribosomes. We discussed the role of gibberellins in relation to cell membranes, particularly with respect to the lipid fraction. Membranes will be discussed at a later stage. Suffice to point out that the membranes found in plants also have a polysaccharide component. Zeatin, a naturally occurring cytokinin, has been shown to alter the polysaccharide composition of lettuce pith. The change in pattern was evident in the Golgi fraction at an earlier stage in the induction of the response than in the endoplasmic reticulum (Wright and Bowles, 1974).

The mode of action of auxin has been the subject of a recent review by Davies (1973). The earliest actions of auxin are too rapid to be explained by protein synthesis and most likely involve activation of an ATP'ase proton pump located in a cell membrane. Of all recognised plant hormones, the role of IAA in seed germination is the least well established. It is not clear whether IAA is promotive or inhibitory to germination. IAA has been implicated in the control of poly-ribosome levels in etiolated pea stems and in soybean hypocotyls (Trewavas, 1968; Travis et al., 1973). Whilst Trewavas (1968) believed this control to be dependent on the increase in ribosomal RNA, Travis et al. (1973) have shown that the increase in poly-ribosomes was preceded by, and was dependent upon, the activation of 80s monoribosomes. This activation was related, at least in part, to an increase in the level of peptidyl t-RNA associated with the ribosome population. Travis et al. (1974) reported an identical situation with 80s maize ribosomes from dark grown seedlings where activation was under the control of phytochrome. A similar promotion of polyribosome formation, plus an increase in ribosomes, was observed in IAA treated callus tissue of Cichorium intybus. Furthermore, electron microscope studies showed a parallel increase in endoplasmic reticulum (Gurozdy et al., 1974). Auxin induced growth in expanding apical cells of oat coleoptiles has been shown to be mediated through the activation of the dictyosomes or Golgi apparatus. This effect was rapid (Gawlik and Shen-Miller, 1974). A final point where auxin might exert control is suggested by work by Raa (1973). He indicated that ribosomes of cabbage roots contain

IAA oxidase and peroxidase activity. One of the three peroxidases that could be separated by gel electrophoresis showed allosteric kinetics, suggesting an IAA controlled conformational change of the protein.

When dealing with the possible effect of gibberellin on the synthesis of endoplasmic reticulum, it was suggested by Firn and Kende (1974) that the effect of gibberellin might not be on synthesis. Sargent and Osborne (1975) have shown that ethylene leads to an increase in endoplasmic reticulum, as shown by both electron microscope studies and sucrose gradient centrifugation. They believe that this increase in rough endoplasmic reticulum results from a stabilisation of the membranes brought about by a reduced rate of phospholipid turnover in the presence of ethylene.

It was noted that during the early stages of germination one of the events was an increase in membrane, particularly the endoplasmic reticulum. Speculation was made that this membrane was necessary to provide endoplasmic reticulum for ribosomes to attach. This need not be the only role of the membrane. It has been shown that about 30% of the activity of alkaline lipase in castor bean endosperm is associated with the endoplasmic reticulum. The other 70% is associated with glyoxysomes. The authors suggested that the alkaline lipase depended on the prior action of an acid lipase (Muto and Beevers, 1974). Acid and alkaline lipases have been found in both dormant apple seeds and 'Grand Rapids" lettuce seeds (Rimon, 1957). In apple embryos red light stimulates the action of the alkaline lipase, as does GA_4. Germination shows a correlation with the alkaline lipase activity. The activation of acid lipase is more complex (Smolenska and Lewak, 1974). The work on lettuce seeds is fairly old (Rimon, 1957) and shows that a 'neutral' and an 'acid' lipase develop during germination. It is also relevant that in pea the enzymes for fatty acid synthesis are present in the dry seeds and participate in the synthesis of fatty acids once a critical water content of the seed is achieved (Harwood and Stumpf, 1970). Although purely speculative, it may be suggested that in dormant seeds the enzymes of fatty acid metabolism are present and require to be activated by the trigger previously discussed.

The natural control of germination and the imposition of dormancy in seeds of Grand Rapids lettuce lies in an interaction between temperature and phytochrome. We have discussed what might take place in the seed to impose dormancy over a narrow change in temperature, and Cohen (1958) has suggested that the change may be one of a 'physical' as opposed to chemical nature. The action of phytochrome is rapid (Bewley et al., 1968). Thus, in attempting to explain how phytochrome overcomes thermodormancy we should take into account the rapidity of its action and the requirement for its continued presence. Although Cohen (1958) made the suggestion that temperature changes, leading to the imposition or removal of dormancy in lettuce seeds, could act by an effect on the physical properties of the membrane, the evidence that phytochrome or possible secondary messengers could affect membranes has appeared only in the last decade. It can be proposed that phytochrome changes the properties of a membrane, releasing either some compartmentalised enzyme or hormone, which would then act as the germination inducing agent. The evidence that phytochrome can influence membrane properties has come from a number of different studies. It was first suggested by Hendricks and Borthwick (1967) to explain the rapid action of phytochrome in a number of plant responses. Fondeville et al.,(1966, 1967) clearly demonstrated that the closing of leaflets of Mimosa pudica, the sensitive plant, was under phytochrome control. Leaflet closure could be initiated within five minutes of phototransformation. Phytochrome regulation of nyctinastic leaf closure of Albizzia julibrissin and several other legumes has been reported by Hillman and Koukkari (1967). It was shown that the photoreceptors were situated in the pulvinules, at the base of the closing leaflets, and that the effect was not translocated from an irradiated pulvinule to a non-irradiated one.(Koukkari and Hillman, 1968). Habermann (1973) suggests that phytochrome is involved in the light dependent stomatal opening of non-photosynthetic mutants of Helianthus annuus. This opening is accompanied by a flux of K^+ into the guard cells. The author suggests phytochrome may mediate the change in membrane permeability associated with such an ion flux. Tezuka and Yamamoto (1975) have shown phosphate and potassium ion efflux and influx in segments, excised from hypocotyls of Phaseolus aureus to be under phytochrome control. Yet another rapid response to phytochrome is the ability of red light to change the electrical

charge on cell surfaces, causing them to attach to glass charged with phosphates and become detached in far-red light (Tanada, 1968). Tanada considers that the rapidity of the changes, detachment or attachment of root tips occurring within two minutes, indicates that the initial site of action is probably on the plasmalemma. The permeability of the outer plasma membrane of Mougeotia to water, is also under phytochrome control (Wiesensell and Smeibidl, 1973). An extremely rapid response to phytochrome is the red induced far-red reversible change in conductance in etiolated oat coleoptiles, reported by Newman and Briggs (1973). Changes begin within 15 seconds of red light treatment. The authors suggest that phytochrome acts on membrane permeability or on ion pumps in the membrane. The demonstration of these rapid responses suggests that phytochrome must act by changing the functional properties of membranes. However, the evidence, based on rapid responses, is not conclusive. Other lines of study have further strengthened the concept that phytochrome acts through changes in membrane properties. Using artificial model membranes Roux and Yguerabide (1973) have shown that phytochrome induces photoreversible conductance changes when added to an oxidised cholesterol black lipid membrane. The conductance of the phytochrome modified membrane is increased by red light but decreased with far-red. A strong case for a phytochrome effect on membranes would be obvious, if it were possible to show phytochrome was associated with a membrane. Haupt (1970) has carried out a large number of physiological experiments which lead him to conclude that phytochrome is localised in the plasmalemma of Mougeotia. A strong dichroic orientation is found and, moreover, a change in dichroic orientation is found when phytochrome is converted from P_r to P_{fr} and vice versa. Haupt suggests that this changing dichroic orientation is best understood in terms of a conformational change of the protein moiety of the phytochrome molecule, although failure to show such a change has been reported (Tobin and Briggs, 1973). Welburn and Welburn (1973) have also presented evidence that phytochrome is present either inside the plastid or on the plastid envelope of isolated etioplasts, a report backed up by immunocytochemical studies (Pratt and Coleman, 1971; 1974). Marme and Schäfer (1972), using similar methods to Haupt, believe phytochrome in maize to be associated with the plasmalemma.

Further evidence that phytochrome is bound to a membrane has come from the work on 'pelletable' phytochrome first reported by Rubinstein et al. (1969). A small fraction of the phytochrome extracted from dark grown oat seedlings was pelletable. Indirect evidence that the phytochrome was bound to a membrane comes from the work of Marmé et al. (1971) who showed that there was a correlation between phytochrome binding and the binding of naphthylthalamic acid. Naphthylthalamic acid binding has been proposed as a marker for the plasmalemma (Hertel et al., 1972). Since this first report phytochrome has been shown to bind to particulate cell fractions of a monocotyledon (maize) and a dicotyledon (pumpkin) with P_{fr} binding to a greater extent than P_r (Quail et al., 1973b). The physiological significance of this binding is not known and only under certain conditions, magnesium ion concentration being critical, can red, far-red reversal of binding be shown (Marmé et al., 1973). Electron microscope studies of the phytochrome-containing structure reveals them to be vesicular in nature. These vesicles contain phospholipids, steroids, glycolipids and proteins indicative of their membranous nature (Marmé et al., 1974). Evidence that the structures formed with high magnesium concentrations are vesicles comes from comparing their appearance with that of authentic liposomes of similar size (Miyamoto and Stoeckenius, 1971). Quail (1975a) has shown P_{fr} to be bound to ribosomal material whilst Manabe and Furuya (1975) have shown P_{fr} to be bound to microsomal and mitochondrial fractions. The exact relevance of these results is still not apparent but it is clear that caution must be exercised in interpreting particle bound phytochrome, membrane interactions (Quail, 1975b).

If we assume that phytochrome does interact with a membrane, and exerts its effect through an effect on the membrane, this does not mean that phytochrome does not influence protein synthesis. It has been shown that in intact plant cells ribonuclease and ribosomes are separated by membranes. This allows protein synthesis to continue without indiscriminate attack on the various types of RNA (Dyer and Payne, 1974). The implications of this observation need scarcely be spelled out.

Phytochrome could directly influence germination by releasing a component required for germination such as NADP (Manabe and Furuya, 1974) or by releasing an enzyme from some compartment. Phytochrome has been shown to influence the levels of phenylalanine ammonia

lyase in plastids of Hordeum vulgare (Saunders and McClure, 1975).

We have proposed that phytochrome may be the 'trigger' required to cause germination but that a second messenger brings about the events leading to germination. Evidence was presented that phytochrome leads to the release of gibberellins from plastics (Evans and Smith, 1975; Cooke and Saunders, 1975).

Whilst there is considerable evidence that phytochrome affects the functional properties of membranes, plant hormones have also been shown to affect membranes. Gibberellin stimulates the release of β,1-3 glucanase and stored protein and peptidases in barley aleurone layers (Jones, 1971; Varner and Mense, 1972; Melcher and Varner, 1971). There is no evidence for the participation of secretory vesicles in the release process (Chen and Jones, 1974a,b) and it has been suggested that gibberellin exerts its influence by selectively changing the permeability characteristics of the cell membrane (Jones, 1971). This view is strengthened by the reports of gibberellin dependent ion transport in barley aleurone tissue (Jones, 1973b) and work on model membrane systems (Wood and Paleg, 1972, 1974; Wood et al., 1974). These model membranes or lipid bilayers (liposomes) exhibit a thermal transition point, where essentially the membrane changes from a solid to a liquid state. The temperature at which this transition occurs has been shown to be inversely proportional to gibberellin concentration (Wood and Paleg, 1974).

Kinetin has also been shown to influence membrane permeability (Richmond et al., 1971; Feng and Unger, 1972; Feng, 1973). Ilan (1971) could not determine whether the cytokinin induced ion uptake, exhibited by leaf discs of Helianthus annuus, was the result of a change in the DNA/RNA/protein system or its cause. In the same system IAA and kinetin were found to have different effects of ion movement (Ilan et al., 1971). Ethylene has been shown to enhance the release of α amylase from barley aleurone cells (Jones, 1968) and cellulase from bean petioles (Abeles and Leather, 1971).

Finally, ABA can interact with the previously mentioned phytochrome mediated root tip attachment to glass reported by Tanada (1972). ABA also appears to inhibit membrane permeability in Albizzia julibrissin, where phytochrome has been implicated in the control of leaflet movement (Brown and Sun, 1973).

Clearly, there is good evidence that plant hormones and phytochrome are capable of interacting with membranes in some way, and that the rapidity of some of these responses, indicates that this may be the primary event in the control process. It is not necessary to invoke some change in membrane properties when the critical temperature is reached in seeds of Grand Rapids lettuce, in order to explain the imposition of thermodormancy. We may propose that there is a change in the endogenous hormone content. However, for reasons previously discussed, a pleasing alternative would be that hormones or enzymes required for germination become unavailable above the critical temperature, as a result of some form of compartmentalisation. It was noted that gibberellin was shown to alter the thermal transition point of an artificial membrane (Wood and Paleg, 1972, 1974), and that the 'physical' inclusion of phytochrome in membranes has been suggested (Haupt, 1970). How could these molecules affect the membrane and what is meant by the thermal transition point?

The construction of biological membranes has been reviewed by Green and Brucker (1972). These authors assumed that the same principle of membrane structure would be found in all tissue, an assumption which they thought justified from the literature. However, it is now thought that membrane structure may vary with function (Marmé, 1975). Whatever model of membrane construction is put forward it can be generally accepted that there is a protein and a lipid component. The model described by Green and Brucker (1972) of membrane construction is the 'protein crystal model'. In this theory 'bimodal' protein is packed with 'bimodal' lipid in a continuous membrane. By 'bimodal' we mean a protein or lipid posed at a water lipid interphase with one portion in the aqueous phase and the other in the hydrocarbon sector of the lipid phase. Other lipids are considered to be part of the hydrophobic sector of the bimodal lipid domain. In studying the properties of membranes, artificial membranes may be constructed using phospholipids. In natural membranes the phospholipid is generally considered to exist as a bilayer and it is maintained so that the range of temperature over which gel-to-liquid crystal transition occurs includes the environmental temperature (ref. in Phillips et al. 1975). As a result, clusters of phospholipid molecules in crystalline and liquid crystalline states co-exist in the membrane and the 'lateral compress-

ibility at a specific temperature is enhanced. An increase in compressibility should facilitate the insertion of foreign molecules into the bilayer thereby affecting transport across the membrane. There is evidence that the transport of ions (Papahadjopoulous et al., 1973; Wu and McConnel, 1973), and sugars (Linden et al., 1973; Wood and Paleg, 1974), and penetration of an enzyme (Op Den Kamp et al., 1974) is increased when the chain melting transition of the lipids occurs. Using monolayers Phillips et al. (1975) showed that lateral compressibility was altered by the chain melting transition and that the penetration of a hydrophobic protein was dependent on the compressibility. Besides processes such as membrane transport and biogenesis, the high lateral compressibility arising when a biological membrane is maintained in the range of the gel to liquid transition may be of fundamental importance for membrane processes in general. Wade et al. (1974) showed that changes took place in mitochondria, glyoxysomes and proplastids from germinating castor bean endosperm and in micelles (Liposomes) formed from their membrane phospholipids at the transition temperature.

It could be the case that in lettuce seeds the critical temperature is the same as the 'transition' temperature for membranes. No evidence is available on this point. If this were the case it is easy to envisage how phytochrome could bring about germination. If phytochrome is membrane bound the permeability could be altered by the transformation of P_r to P_{fr}, resulting in either the release of a component which directly causes germination, such as an enzyme or a secondary messenger (gibberellin or cytokinin etc.). Below the critical temperature the membrane would be sufficiently permeable to allow the components required to effect germination, to be moveable. Wood and Paleg (1972) believe that the effect on membranes brought about by gibberellin may be too general to explain the fine control exerted by gibberellins of physiological processes. However, specificity of action could reside in the fact that phytochrome is active by virtue of its localization in the plant. If gibberellins have a general effect, the specificity would lie in where gibberellins were released. Such an explanation, whilst lacking experimental verification, is indeed satisfying. It seems highly unlikely that the seeds of lettuce would require a highly complicated control system involving the activation, or de-repressing,

of a set of genes. The seeds are obviously capable of germinating unless exposed to high temperature and this 'adaptive' change may be likened more to adaptive changes in chilling sensitive plants than to a morphological event which may be considered more fundamental. Germination itself is a fundamental or profound change; release from dormancy merely allows this change to take place.

In summary we have dealt with the life of the seed from its maturation on the mother plant to its eventual germination. The factors involved in determining the qualitative and quantitative elements of dormancy were discussed and it was proposed that dormancy was of adaptive value in enabling the seedling to survive. Although seeds of Grand Rapids lettuce are dormant above a critical temperature, the degree of dormancy varying in a quantitative way between seed batches, it should be understood that the seeds readily germinate given a suitable environment. Thermodormancy may be overcome by a number of factors, light, gibberellins, cytokinins and it is suggested that light might owe its activity to increasing the 'effective' concentration of hormone; the hormone may then be considered a secondary messenger. The evidence for this theory is discussed as is the role of phytochrome in thermodormancy. If we accept that thermodormancy is overcome by phytochrome action we must explain what imposes dormancy above the critical temperature. The role of inhibitors is discussed in relation to this question. Furthermore, the possible involvement of IAA in lettuce seed germination is discussed and it is clear from the literature that little work has been done on this aspect of IAA action.

The way in which phytochrome, directly or via a secondary messenger, overcomes thermodormancy, is discussed. A model of what takes place during the early stages of germination is presented and speculation made about how thermodormancy is imposed and removed. This involved examining the action of phytochrome and hormones on these early events, and finally at their possible role in controlling membrane permeability. A theory is presented which accounts for the imposition of thermodormancy in terms of the effect of temperature on the permeability of membranes. Temperature is proposed to alter permeability to agents essential for germination, this being reversed by the conversion of P_r to P_{fr}. Although the control mechanisms

involved appear complex, it should be realised that:

(a) anything which affects the development of the seedling once cell division has started will be seen to affect 'germination' if the emergence of the radicle is affected;

(b) although the control mechanism may be simple it will be operated against a background of changing metabolism which may make the interpretation of results more complicated.

Obviously, it is beyond the scope of this thesis to deal with more than a few of the problems and questions which exist in the literature dealing with thermodormancy in lettuce seeds. This thesis will consider firstly the role of gibberellins in the removal of thermodormancy, more directly whether red light causes a change in gibberellin activity which may then act as a secondary messenger. Use has been made of both endogenous and exogenous studies. It was clear from the literature that the interactions between gibberellins, cytokinins and ABA in the possible control of seed germination had not been fully investigated in Grand Rapids lettuce seeds. This also has been investigated. Furthermore, if ABA is involved in dormancy then it must be shown to be present or absent in seeds of this variety. This has been studied and the metabolism of exogenous radioactive ABA examined in relation to release from dormancy.

From previous work IAA seems to have been 'ruled out' as being involved in seed germination. This seems unreasonable in face of the evidence for the involvement of IAA in a wide spectrum of plant responses. There is a real need to investigate whether IAA has any role in seed germination. Both exogenous and endogenous studies, plus metabolism studies have been used to investigate this possibility in lettuce seeds. With respect to ABA and IAA, special attention will be paid to the question of whether these compounds affect germination, as defined at the start of this introduction, or merely radicle emergence.

SECTION II

MATERIALS AND METHODS

Seed material

Lettuce seeds, *Lactuca sativa* L. cv Grand Rapids were purchased from either the Page Seed Co., Greene, New York, or William Watt, Cupar, Fife, Scotland. All Grand Rapids seeds used in this work were grown in the United States. Lot numbers and their germination performance are given in Table 1.

Arctic lettuce seeds were purchased from Suttons Seeds Ltd., Reading, England.

Radish seeds, cv long white icicle, were purchased from Hurst, Gunson, Cooper and Taber, Witham, Essex, England.

All seeds were stored in glass screw top bottles at -20°C.

Standard germination procedure

Unless stated otherwise only two lots of Grand Rapids seeds were used in the germination studies, lots 011372 and 011372b. These showed identical germination behaviour. Seeds were counted into 50s ahead of dispensing into 4.5 cm glass petri dishes lined with one Whatman's No. 3, 4.25 cm qualitative filter paper. 1.5 ml of test solution was used to wet the paper. Immediately on dispensing the seeds, the dishes were placed in light tight containers. Seeds were exposed to the stated temperature ± 1°C in thermostatically controlled incubators. Germination, as assessed by radicle emergence was counted after 24 hrs and at 24 hr intervals thereafter. At high concentrations of some of the test solutions while radicle protrusion occurred further elongation did not take place. However, if the radicle had emerged through the restraining layers of the endosperm and testa then germination was considered to have taken place.

In these experiments involving irradiations or the transfer of seeds from one solution to another, the seeds were never exposed for more than a few minutes to a green safe light at 24°C. The green safelight consisted of a green fluorescent tube (Thorn Electrical, 2 ft or 6 ft 1¼" diam.) surrounded by two layers of 'Cinemoid" No. 5, orange, and two layers of 'Cinemoid" No. 24, dark green. This safe light was shown to be photomorphogenetically inactive with respect to thermodormant lettuce seeds.

Table 1 . The average percentage germination of Grand Rapids lettuce seed at 20°C and 30°C.

Seed batch	Temp.	Germination after 24 hrs	Germination after 48 hrs
Page 011372	20°C	53.2 ± 2.0	84.7 ± 3.4
	30°C	13.3 ± 2.6	21.9 ± 2.9
Page 011974	20°C	10.0 ± 1.9	23.7 ± 3.4
	30°C	4.0 ± 2.0	7.7 ± 3.6
Page 014974	20°C	76.7 ± 1.5	92.0 ± 1.3
	30°C	48.7 ± 5.3	51.3 ± 5.1
Page H273	20°C	30.0 ± 3.5	91.3 ± 3.4
	30°C	1.3 ± 0.7	17.3 ± 0.7
William Watt I	20°C	0.7 ± 0.7	31.6 ± 4.4
	30°C	0.8 ± 0.8	0.8 ± 0.8
William Watt II	20°C	5.3 ± 0.7	14.7 ± 1.8
	30°C	0	0

Irradiations with red or far-red regions of the electro-magnetic spectrum (hereafter, red or far-red light) were obtained from a 12 v 100 w tungsten halogen lamp built into a special irradiation unit. This consisted of the condenser unit of a standard 5 cm x 5 cm slide projector and a filter of water, 20 cms in length, to remove any infra-red radiation not absorbed by the 'heat absorbing' glass of the condenser unit. Monochromatic light was obtained using interference filters manufactured by Barr and Stroud (Anniesland, Glasgow). The manufacturers' specifications for these filters are as follows:-

	Maximum transmission	Band width	Percentage at peak wavelength
Red	656 nm	10 nm	70%
Far-red	730 nm	10 nm	70%

However, measurements of transmission characteristics made in the department using a Unicam SP 800 recording spectrophotometer did not confirm the manufacturers' specification, and are given in Figure 7. The red filter as provided by Barr and Stroud had, in addition to a filter with the characteristics specified, a dielectric barrier filter to remove radiation with wavelengths greater than 810 nm.

Petri dishes were exposed to radiation at a distance of 20 cms from the filter. At this distance the incident radiant flux density, as measured by a Kipp and Zonen compensated thermopile, was 12.3×10^3 $\mu J\ m^{-2}\ s^{-1}$ for the red source, and 43.2×10^3 $\mu J\ m^{-2}\ s^{-1}$ for the far-red source. All irradiations were given after 2 hrs imbibition.

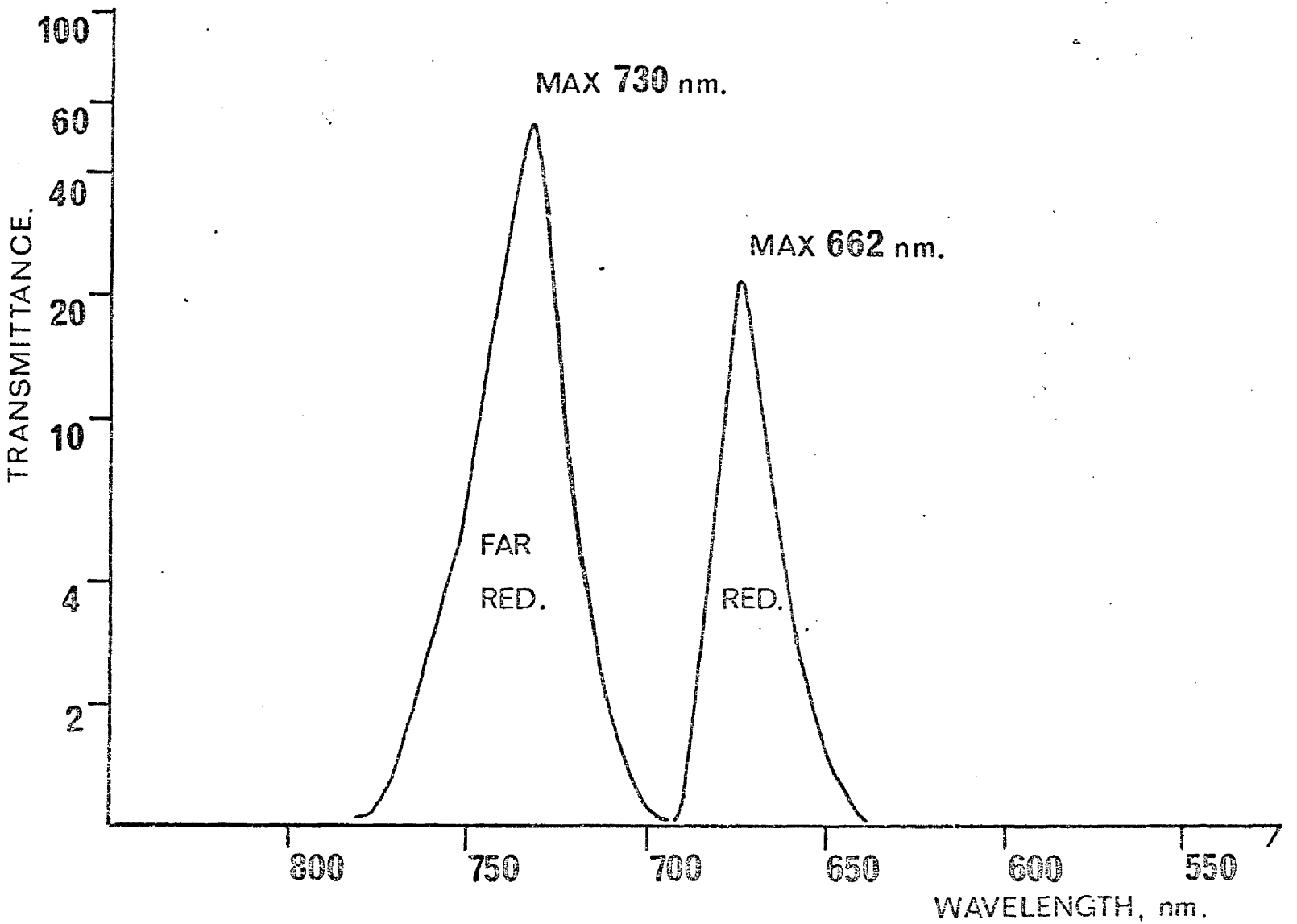
A number of experiments was conducted in which seeds were transferred from one solution to another. In such experiments the seeds were lightly blotted, to remove adhering solution, at the time of transfer. Care was taken not to cause any mechanical damage to the seeds.

All treatments were replicated three times and each experiment was repeated at least twice.

Figure 7

Transmittance spectra for 'far-red' and 'red', Barr and Stroud cut off filters from irradiation unit.

Spectra run on SP800 ultraviolet spectrophotometer.


100
60
40
20
10
4
2
TRANSMITTANCE.
MAX 730 nm.
MAX 662 nm.
FAR
RED.
RED.
800
750
700
650
600
550
WAVELENGTH, nm.

Chemicals

Solutions of all growth regulators used in this study were made up in deionised water. Of the chemicals used, only kinetin at 10^{-4} M could not be dissolved completely in water. Thus, a 10^{-4} M solution is in reality a 10^{-4} M 'saturated' solution. Although kinetin is more soluble in alcohol this was not used as a result of a report by Martin (1972) where he indicates that seeds germinated in dilute ethanol 0.1 molar, did not respond to light treatment in the classical manner, e.g.

RL	90%
RL + FR	3 - 5%
RL + FR + EtOH	26.5 - 50%

The following chemicals were used in this study:-

Chemical	Supplier
Chlormequat (C.C.C.)	Commercial preparation (cyco. cel) containing 40% active ingredient
B995	Mirvale Chemical Co. Ltd.
Phosphon D	-
Amo 1618	Rainbow Color and Chemical Co.
Gibberellin A_3	91.4% pure, I.C.I.
Gibberellin A_{4+7}	46.36% GA_7 + 53.64% GA_4. Calbiochem. Lot No. 80084.
Kinetin	BDH Biochemicals.
Zeatin	Calbiochem. Lot No. 101138.
Benzyladenine	BDH Biochemicals.
SD 8339	Shell Chemicals.
IAA	Calbiochem. Lot No. 001725.
β-indolyl acetic acid-5-^{3}H	Fluorochem Ltd. Batch No. 1074.
(±)ABA mixed isomers	Sigma. Lot No. 620-2230.
(±)ABA cis, trans	Sigma. Lot No. 112C-0680
(±)ABA trans, trans	Sigma. Lot No. 33C-2630
(±)ABA-2-^{14}C	Hoffman La Roche

The structures and chemical names of these compounds are given in Fig. 8.

Figure 8

Chemicals used : their structures.

amo 1618 :- 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenyl piperidine -1-carboxylate.

phosphon D :- tributyl-2,4-dichlorobenzyl-phosphonium chloride.

C.C.C. :- 2-chloroethyltrimethylammonium chloride.

B995 :- N,N-dimethylsuccinamic acid.

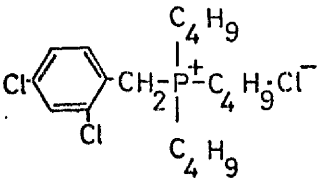
Zeatin :- 6-(4-hydroxy-3-methylbut-trans-2-enyl) amino-purine.

Kinetin :- N^6-furfuryladenine.

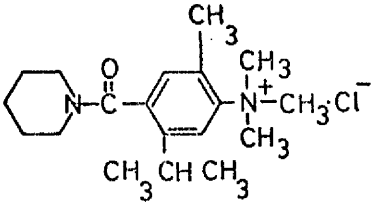
SD8339 :- 6-(Benzylamino)-9-(2-tetrahydropyranyl)-9H-purine.

IAA :- indole acetic acid.

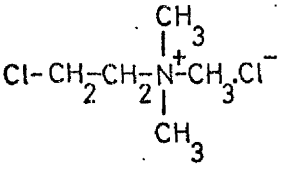
ABA :- abscisic acid.



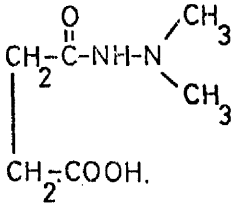
PHOSPHON D.



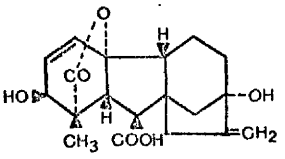
AMO1618.



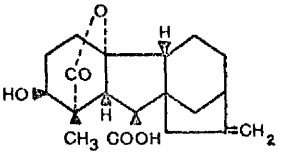
C.C.C.



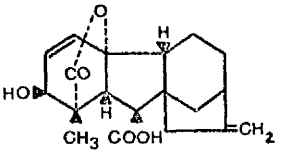
B 995.



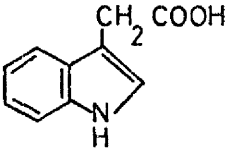
GA$_3$



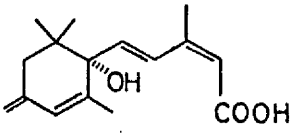
GA$_4$



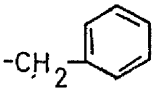
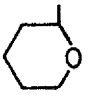
GA$_7$

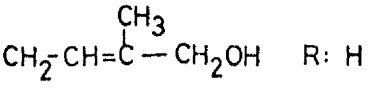


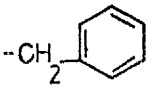
IAA.

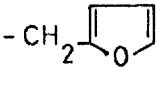


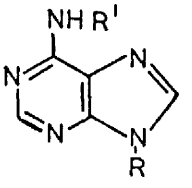
ABA.

SD 8339. R':  R: 

Zeatin. R':  R: H

Benzyladenine. R':  R: H

Kinetin. R':  R: H



Studies on endogenous plant hormones

Extraction and purification

This study was initially aimed at examining the endogenous gibberellin and cytokinin contents of lettuce seed with a view to relating these hormones to the control of thermodormancy. As was discussed on pages 33,34 of the Introduction, the endogenous gibberellins and cytokinins of lettuce seeds remain to be identified. Consequently, in examining these hormone classes one is looking at unidentified compounds.

The classical methods of hormone extraction involve the use of organic solvents to extract the plant material, followed by chemical separation in an attempt to remove interfering compounds and separate hormones which may be present. It is self-evident that only those hormones which are extracted may be identified and/or quantified. Furthermore, it is well documented that during chemical separation losses of hormones can take place (Hemberg and Westlin, 1973; Hemberg, 1974; Letham, 1974; Crozier et al., 1971). These problems will be discussed in more detail in the appropriate section. Suffice to say that, bearing in mind the problem of following unknowns through a chemical separation, it was decided to attempt to exclude, chemical separations when dealing with unidentified compounds, the characteristics of which are unknown.

With ABA and IAA one is working with known compounds where it is possible to follow their movement and losses at each of the separatory steps employed.

(1) Gibberellin and cytokinins like compounds

Only dry lettuce seeds were examined for the presence of gibberellin and cytokinin like compounds. Appropriate size seed samples were extracted in 80% methanol at 4°C for 24 hrs, the methanol removed, and this procedure repeated for a further two 24 hr periods. The extraction solvent was always in a volume to weight ratio of more than 5:1. The combined methanolic extracts were reduced to dryness on a thin film rotary evaporator at a temperature of 35°C. Known weights of extract could then be further purified by chromatography. . Column chromatography using Sephadex LH 20 was employed.

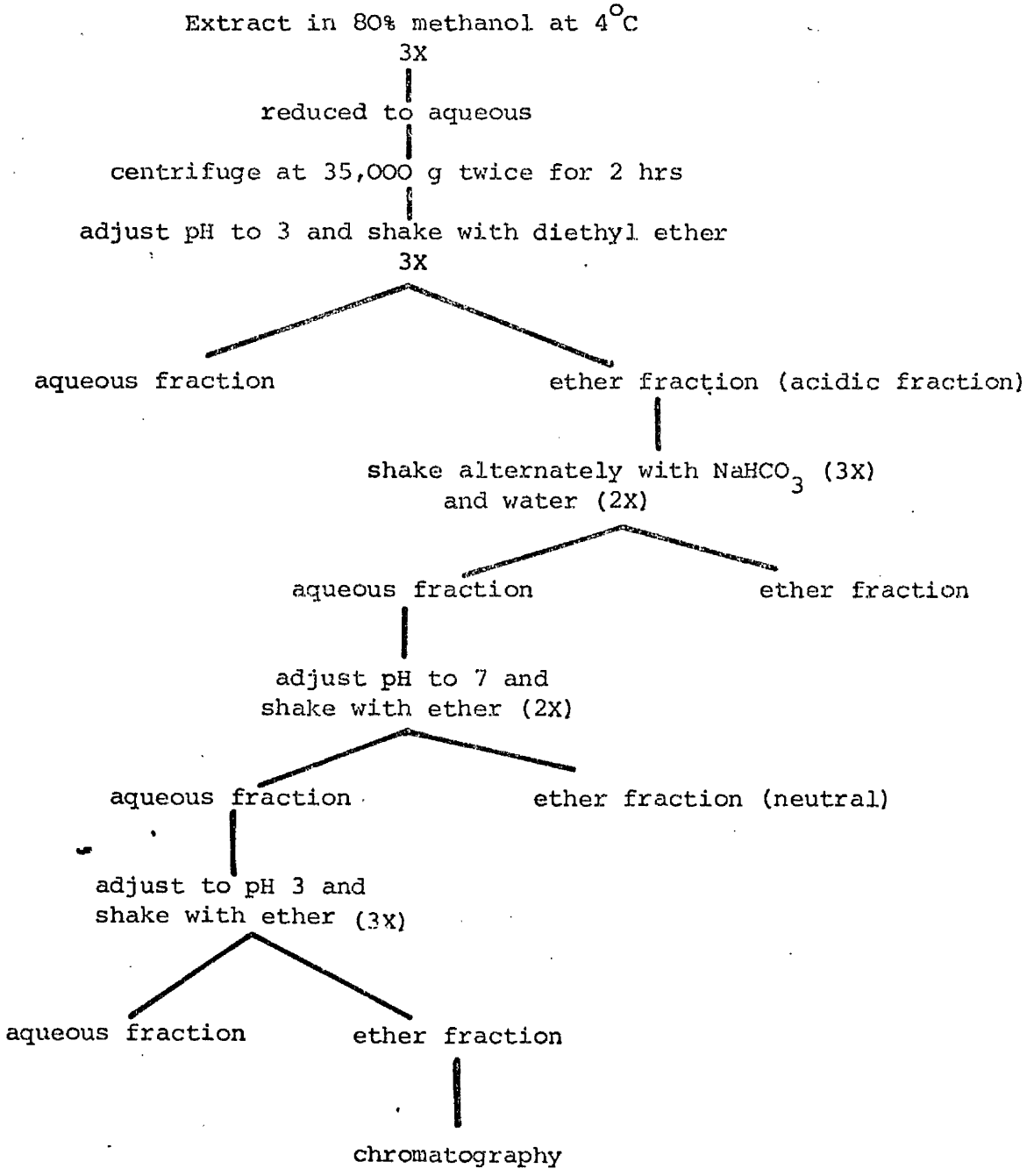
The column was 50 cms in length, with an internal diameter of 5 cms and a bed volume of 900 ml. Sephadex LH20 was slurried in methanol and the column poured and allowed to settle by gravity flow. A disc of thick 'seed test' paper was placed on the top surface to avoid disturbance of the LH20 during sample application. The sample was then applied in a small volume of methanol, allowed to move through the paper disc and eluted with methanol. 720 drop (8 ml) fractions were collected, using a Gilson microcal TDc80 fraction collector, at a rate of 1 drop per second. The fractions were then combined in fives, dried on a thin film rotary evaporator and then subjected to bioassay. The column was calibrated using known weights of standards of ABA, GA_3 and Zeatin. The recovery of standards was absolute. Precautions were taken to ensure extracts and columns were not subjected to long exposures to daylight as it is known this causes photoisomerisation of cis, trans to trans, trans ABA (Milborrow, 1968).

(2) ABA

The extraction procedure used for abscisic acid is the modified procedure of McWha (1973) from Milborrow (1967). Seeds were weighed into 100 g or 500 g lots and extracted as dry seeds or after a specified number of hours imbibition at 20°C or 30°C. Seeds were imbibed in metal trays lined with one layer of filter paper, a layer of muslin and 650 ml of water to each tray. Two trays were used per 500 g seed. Imbibed seeds were gathered in the muslin immersed in liquid nitrogen and then lyophilised prior to solvent extraction. Thus, all the samples for extraction had the same moisture content. The dry or freeze dried seeds were extracted in three changes of 80% methanol, 24 hrs each at 4°C (see Figure 9). The combined methanolic extracts were evaporated on a thin film rotary evaporator at 35°C to give about half the aqueous phase (150 ml). This was centrifuged twice at 35,000 g at 0°C. The pellet obtained could not be shown to contain ABA. This step reduced the problem of emulsion formation during the partitioning procedure. The supernatent aqueous phase was adjusted to pH3 with hydrochloric acid and partitioned twice against one third its own volume of redistilled diethyl ether. At this point the remaining aqueous phase was base hydrolysed by adjusting the pH to 11.5 with sodium hydroxide and heating in a water bath to 60°C

Figure 9

Flow diagram of extraction procedure for abscisic acid.



for 1 hr. According to Milborrow (1970) and Koshimizu et al. (1968) this 'releases' any ABA which is 'bound' The aqueous phase was re-adjusted to pH3 and partitioned against one third its own volume of diethyl ether, three times. The two ether fractions, obtained from the original aqueous phase and the base hydrolysed aqueous phase were then each carried through the remainder of the purification procedure. Each was extracted five times with one-third and one-sixth volumes of 5% sodium bicarbonate and water alternately, and the combined bicarbonate, water phases adjusted to pH 7.5 with sodium hydroxide. Partitioning twice against one quarter volumes of ether removed any neutral compounds. This step was omitted when smaller seed samples were extracted. The bicarbonate, water phase was adjusted to pH 3.0 with 6 N sulphuric acid and this was partitioned against one half volumes of diethyl ether, at least thrice, or until no colour could be seen to move into the ether phase. The combined ethereal solutions were dried by freezing out the water overnight at -20°C and rapid filtering through glass wool. The dried ether was reduced to dryness and samples stored at -20°C in a desiccator, if not further purified immediately.

In early extractions the final ether fractions were further purified by thin layer chromatography, using two solvent systems. In most of the samples a step prior to thin layer chromatography was introduced, column chromatography on polyvinyl pyrrolidone, PVP (Polyclar AT.).

PVP column chromatography

PVP was first used in the purification of ABA from woody plant tissue by Lenton et al. (1971). Model experiments to determine the elution pattern of a number of plant hormones from PVP were reported by Glenn et al. in 1972. It was shown that the elution pattern was dependent on the pH of the buffer. At pH 8.0 ABA was eluted at the same point as GA_1, GA_3 and GA_5. Recovery of standards was more than 99% and plant samples could be reduced in dry weight by up to 50, 60-fold. This is due to the selective retardation of phenols, the degree of retardation being dependent on the number of hydrogen bonding sites on the phenol (Clifford, 1974).

Two sources of PVP were used in our studies, BDH biochemicals and Sigma. To prepare for use in column chromatography, dry PVP powder was slurried in at least five times its volume of water.

Fines were removed by decantation after a settling period of about ½ hr. This was repeated twice and then the appropriate buffer added. The buffer used was a diphosphate buffer (Sorensen, 1909; taken from Geigy scientific tables). The formula is given below:

(A) 1/15 molar monopotassium phosphate KH_2PO_4

(B) 1/15 molar disodium phosphate Na_2HPO_4

pH 8.0 3.7% A + 96.3% B

Decantation to remove fines is required to ensure an adequate flow rate of eluant. The slurry was poured into columns, employing glass wool overlain by glass beads as a support. The column was packed by gravity flow and a disc of filter paper placed over the bed to prevent subsequent disruption. Columns were equilibrated with buffer prior to use by eluting at least one column volume of buffer. Seed extracts were added, in as small a volume of buffer as possible, to the filter disc with a Pasteur pipette.

Three different column types were used with bed volumes of 25 ml, 35 ml and 100 ml. These were standardised for ABA by adding a known amount of standard, collecting fractions and then scanning on a Unicam SP 800 recording spectrophotometer from 200 nm to 400 nm. ABA, under basic conditions, has a maximum absorption at 245 nm (Cornforth et al. 1966 and Figure 10). The amount of ABA recovered was calculated from a graph obtained by running known amounts of ABA against absorbance at 245 nm. The relationship between absorbance at 245 nm and amount of ABA was shown to be linear. The recovery of ABA was over 90%. Figure 11 shows the elution profile of ABA from PVP. Elution volume is expressed as standard elution volume or S.E.V. This is defined as the ratio between measured elution volume and total column volume, multiplied by 100 (Ellingboe et al., 1968). S.E.V. is a dimensionless quantity, numerically equal to 'percentage of total column volume'. The value of SEV is that it serves to correlate results from different sources by reducing them to a standard form. Thus, Table 2 gives data for the elution of ABA from two sources of PVP and the three different columns. In all cases ABA elutes between an SEV of 65-120. When standards were run in the presence of a seed extract, the elution pattern was unaltered. Consequently, the eluate up to an S.E.V. of

Table 2 . Elution of standard (±)-ABA from PVP columns.

PVP	Column	Column bed volume	Start		Peak		End	
			a	b	a	b	a	b
BDH	Pyrex, large	100 ml	65.0	65.0	70.0	70.0	90.0	90.0
	Fisons	35 ml	23.8	68.0	31.5	90.0	34.0	97.1
	Pyrex, small	25 ml	14.3	57.2	16.5	66.0	19.8	79.2
Sigma	Fisons	50 ml	39.0	78.0	45.0	90.0	55.0	110.0

a : elution volume

b : standard elution volume S.E.V.

Figure 10

Ultraviolet (UV) absorbance of abscisic acid at pH 8.0 in phosphate buffer, recorded on a Unicam SP800 spectrophotometer.

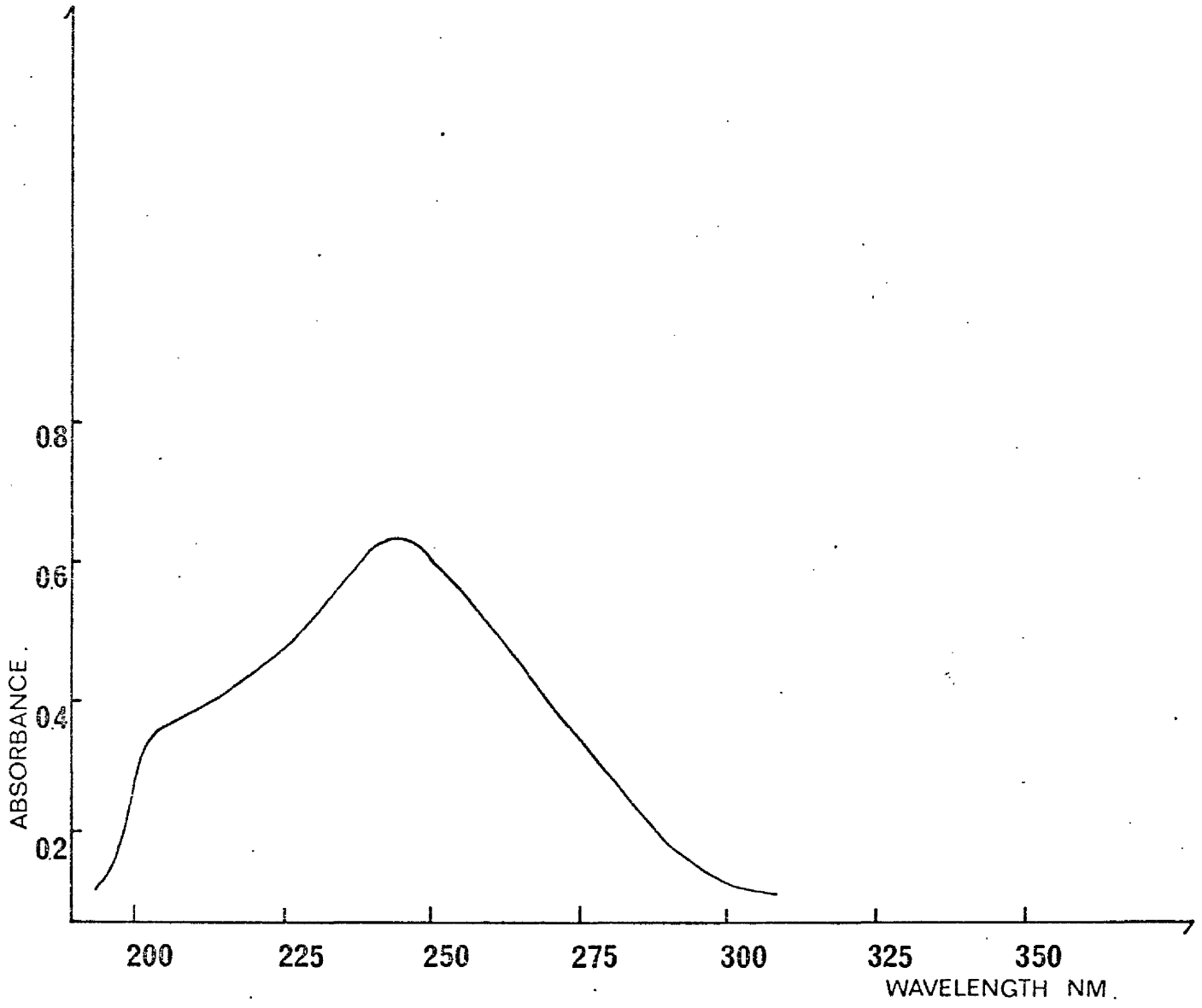
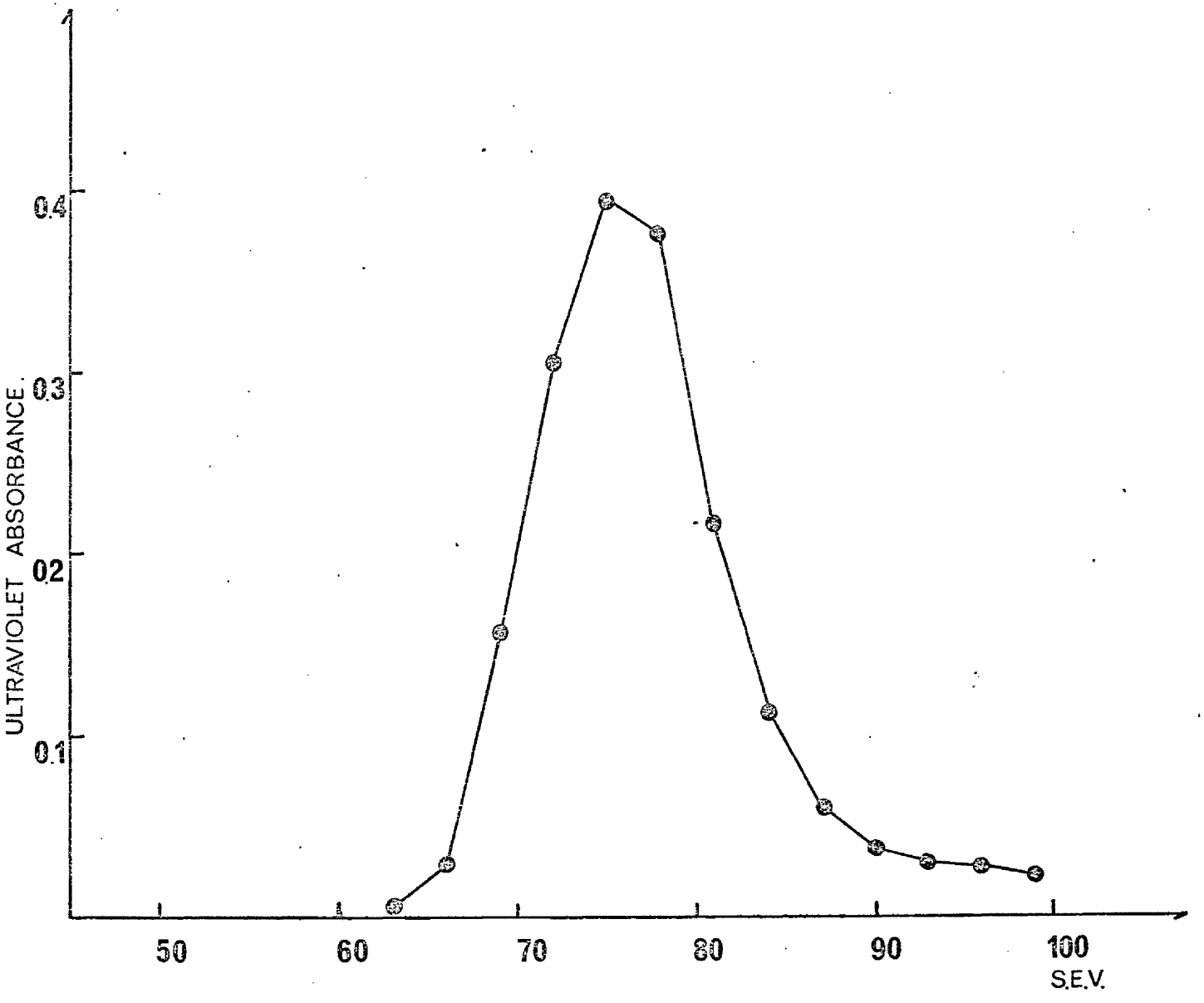

ABSORBANCE.
0.8
0.6
0.4
0.2
200
225
250
275
300
325
350
WAVELENGTH NM.

Figure 11

The elution of abscisic acid from a column of polyvinylpyrrolidone (PVP). Fractions collected and their ultraviolet absorbance measured using a Unicam SD800 spectrophotometer.


ULTRAVIOLET ABSORBANCE.
0.4
0.3
0.2
0.1
50
60
70
80
90
100
S.E.V.

150 was collected. The eluted buffer was acidified to pH 3 with hydrochloric acid and partitioned against diethyl ether, thrice. The combined ether extract was dried by freezing out water and then reduced to dryness, prior to further purification or derivatisation for gas liquid chromatography.

For a seed extract a reduction in dry weight of about 70% can be achieved by the use of PVP column chromatography.

Chromatographic procedures will be discussed following the details for the extraction of endogenous indole acetic acid, IAA.

(3) IAA

The extraction of endogenous IAA follows the method first devised by Elliott and used by Bridges et al. (1973). Figure 12 shows the scheme used. As in the procedure for ABA extraction, seeds were either dry or imbibed. Imbibed seeds were immersed in liquid nitrogen and lyophilised. Seeds were then extracted in three changes of 80% methanol at 4°C. This was followed by reduction on a thin film rotary evaporator, centrifugation, acidification of the supernatant aqueous phase and partitioning, thrice against equal volumes of redistilled diethyl ether. The remaining aqueous phase was discarded. The ether fraction was dried by freezing out water and then taken to dryness on a thin film rotary evaporator at 30°C. This was taken up in a small volume of 50% methanol and applied to a column of diethylaminoethyl cellulose (DEAE cellulose; Whatmans DE1). Preparation of the column is described in detail below. The column used was 40 cms in length, internal diameter 2 cms and 10 grams of cellulose were required to fill the column. The sample was eluted firstly with 300 ml of redistilled water and then with 350 ml of 0.05 M sodium sulphate. The flow rate was in the order of 250 ml per hour. Flow rate is not critical except that it should be neither extremely fast nor slow. The column was run at 4°C in darkness. The sodium sulphate eluate was acidified to pH 3with hydrochloric acid and partitioned, thrice, against equal volumes of diethyl ether. This was dried by freezing out water, followed by rapid filtration through glass wool. The ether fraction was reduced to dryness ready for further purification using thin layer chromatography.

Figure 12

Flow diagram of extraction procedure for indole acetic acid.

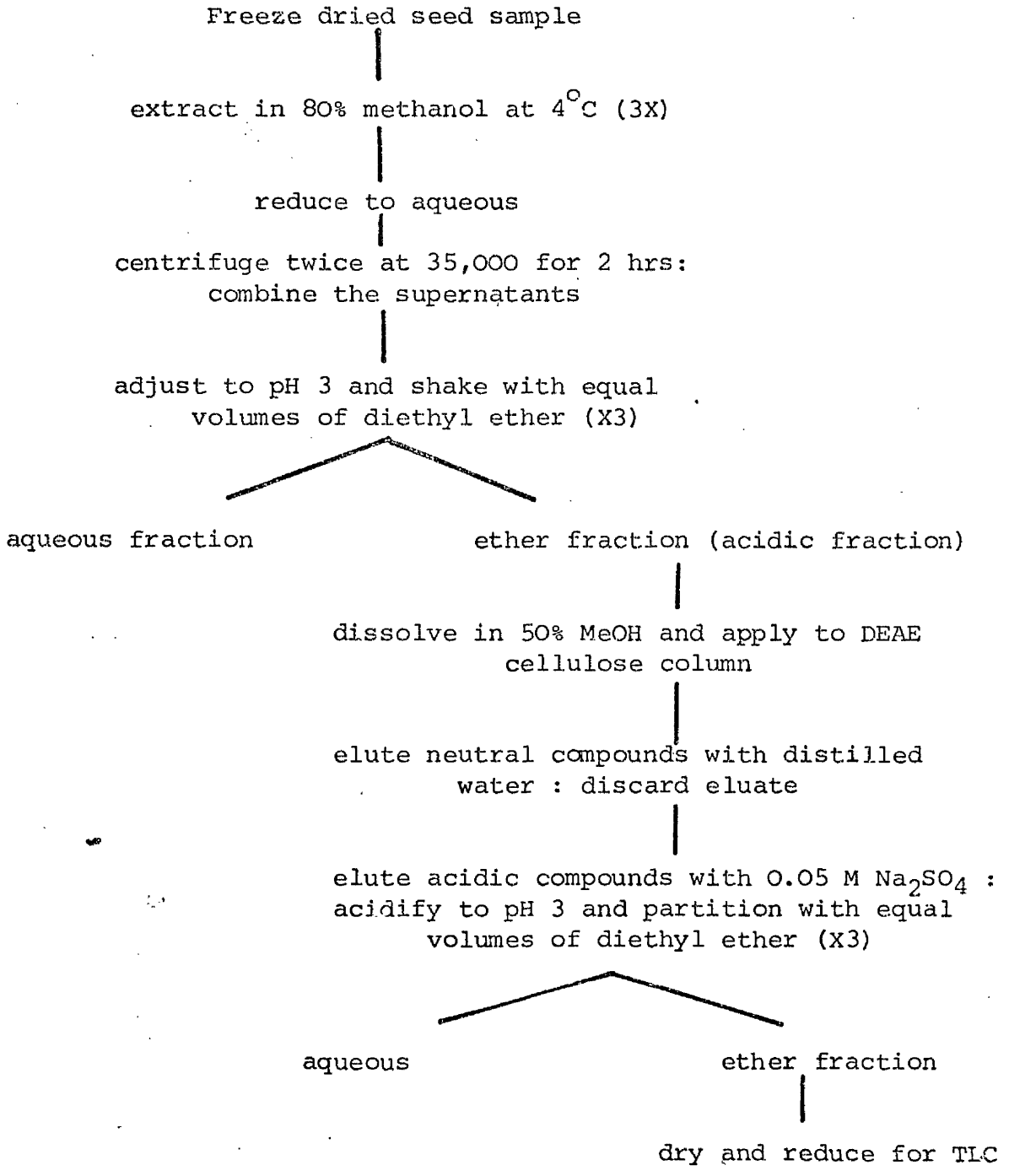


Fig. 13. Precycling of diethylaminoethyl (DEAE) cellulose.

(1) Stir the weighed ion exchanger into 15 volumes (i.e. volume of liquor/dry weight of ion exchanger) of 0.1 M H_2SO_4 and leave for at least 30 minutes, but not more than 2 hrs.

(2) Decant off the supernatant liquor and add more distilled water. Repeat this procedure until a pH of 4 is reached.

(3) Stir ion exchanger into 15 vols of alkali, 0.1 M NaOH and leave for 30 minutes.

(4) Repeat the washing procedure until the pH is near neutral.

Preparation of DEAE cellulose

The type of DEAE cellulose used was Whatman's DE1. This is a dried form and requires to be pre-cycled prior to use. Pre-cycling involves exposing the exchanger to acid and base to ensure that all the ion exchange sites become available. Figure 13 gives details of pre-cycling. Decantation also serves to remove 'fines' which improves the flow qualities of the DEAE cellulose. The column was standardised to check the elution profile and recovery of IAA. Standardisation was carried out by adding a known weight of IAA to a column of DEAE cellulose and collecting fractions. These were then scanned on a Unicam SP 800 recording spectrophotometer from 200 nm to 400 nm. The amount of IAA recovered was calculated from a graph obtained by running known amounts of IAA against absorbance at 281 nm. The relationship between absorbance at 281 nm and the amount of IAA was shown to be linear. Figure 14 shows the elution profile of IAA from DEAE cellulose. All the IAA recoverable was eluted well within the 350 ml sodium sulphate eluate. This was repeated using tritiated IAA (5-[^{3}H]-IAA) alone and in the presence of a seed extract. The presence of seed extract had no effect on the elution pattern. Using [^{3}H]IAA the percentage recovery of IAA was estimated and found to be 90 %.

Thin layer chromatography

Thin layer chromatography plates, 20 x 20 cms (TLC plates) were either spread in the laboratory or purchased pre-coated with a 0.25 mm layer of silica gel which included a fluorescence indicator but did not contain binder. The prepared plates were made by mixing silica gel, HF_{254} (Merck) with water (1:2.1 weight/volume) for two minutes; the slurry was spread over 20 x 20 cm glass plates using a TLC spreader. A 0.2 mm thick layer was found to be suitable. All plates were pre-run in methanol before use. Plates were <u>not</u> activated, as 'pure' adsorption chromatography was not employed. The sample was applied as a 15 cm long narrow band, 2½ cms distant from the edge of the plate, using a new and simple sample applicator described by Malik (1973). Figure 15 shows the improved version of this sample applicator routinely used in this study. Authentic samples were spotted at a 1 cm distance from the sample line, and at the edge of the sample. This was found to be necessary, especially with pre-coated plates where the edges had a thicker layer of silica

Figure 14

The elution of indole acetic acid from a column of diethylaminoethyl (DEAE) cellulose. 8 ml fractions, collected and their ultraviolet absorbance measured using a Unicam SP 800 spectrophotometer.

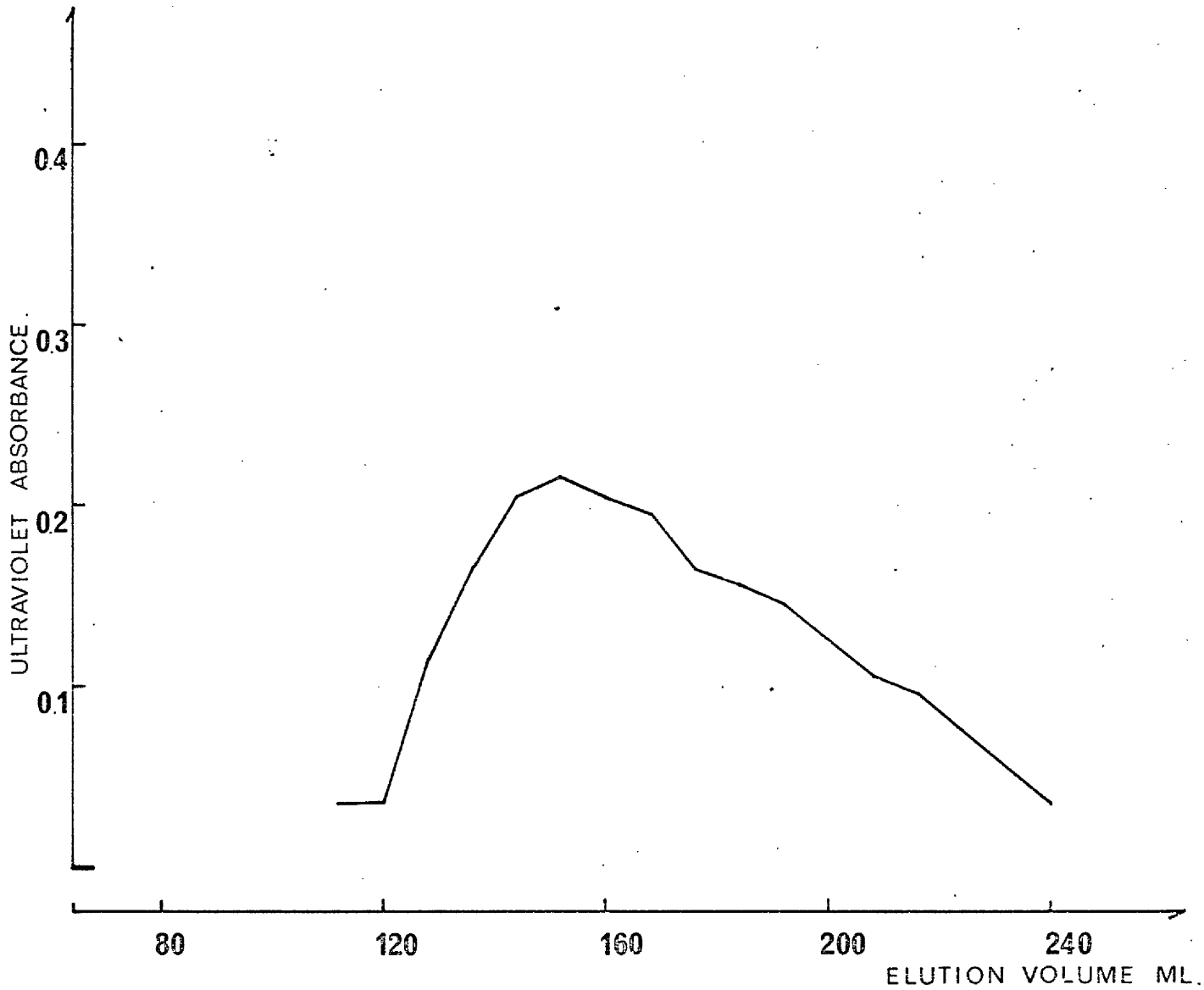
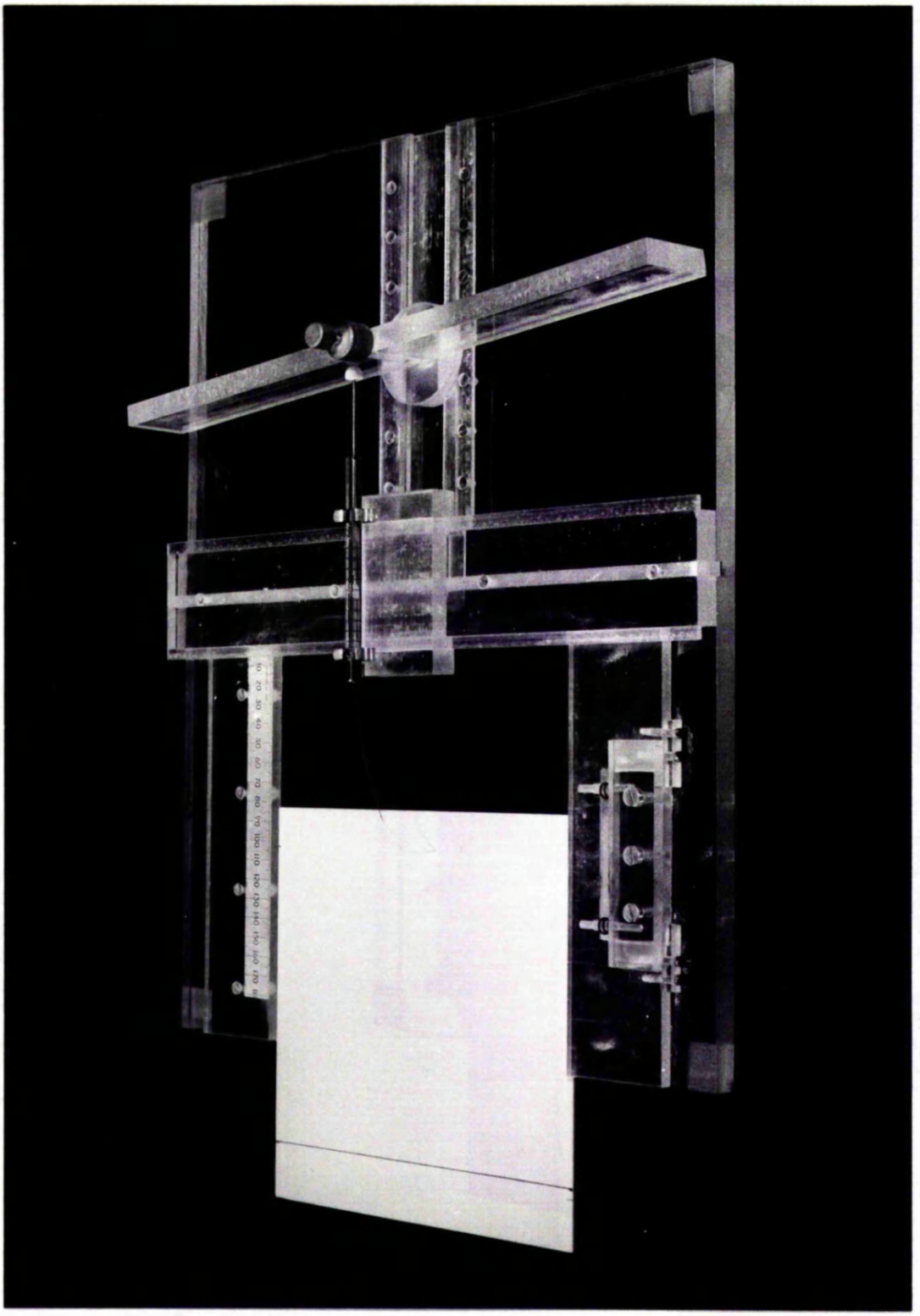

ULTRAVIOLET ABSORBANCE.
0.4
0.3
0.2
0.1
80
120
160
200
240
ELUTION VOLUME ML.

Figure 15

Sample applicator for thin layer chromatography.



gel. This resulted in differential running of standards giving a misleading Rf. A narrow line was drawn with a spatula across the whole plate 10 cms or 15 cms from the origin. The plates were then run in an appropriate solvent system up to the 10 or 15 cm mark. Authentic samples could be detected by their absorbance under a UV lamp at 254 nm. The spots were marked and zones corresponding to the standards scraped off and placed into pyrex eluting tubes (30 cms long), having a sintered glass disc at the bottom. The tubes were eluted with 100 ml of an equal parts mixture of methanol and diethyl ether. The addition of a layer of glass beads was found to improve the flow rate of eluant and prevent clogging of the sintered glass disc by fine particles from the silica gel. The eluates collected were reduced to dryness on a thin film rotary evaporator. It was found that methanol eluted a component from the silica gel as previously reported by Milborrow (1967). If the sample was subjected to a second TLC the eluate was redissolved in methanol, diethyl ether and applied to a TLC plate as before. When the final TLC had been carried out the contamination from the silica gel was removed by adding 20 ml of acidic water (pH 3.0) and partitioning this against equal volumes of diethyl ether. The ether was then dried by freezing out water at -20°C and rapid filtration through glass wool. This ether fraction was then reduced to dryness on a thin film rotary evaporator.

This general procedure was used for both TLC of ABA and IAA. Initially for ABA two sequential TLCs were employed, using the following two solvent systems:

(a) n-hexane:ethyl acetate (1:1 v/v) run 3 times.

(b) chloroform:methanol:water (75:22:3 v/v)

After the introduction of PVP column chromatography only solvent (a) was used. With ABA care was taken to exclude light whenever possible. With IAA two sequential TLCs were routinely used; solvent systems (a) and (b) were found to give good separation of IAA from other components. With IAA elution of zones was carried out at 4°C. Plates were never allowed to remain in a dry condition for more than a few minutes. It has been shown that IAA remains stable on silica gel for up to 2 hrs (Sagi, 1969).

Gas liquid chromatography

Preparation of extracts

Seed extracts to be analysed for the presence of ABA or IAA were

always obtained as final ether fractions. Prior to derivatisation for gas liquid chromatography (GLC) the samples were transferred to small vials and dried under a stream of oxygen free nitrogen. IAA samples were also desiccated prior to derivatisation.

Preparation of derivatives

For GLC the methyl derivative of ABA was prepared by the addition of excess ethereal diazomethane to purified seed extracts. Diazomethane (CH_2N_2) was prepared in the following manner. A quantity of nitrosan was placed in a conical flask fitted with a delivery tube and a layer of ethylene glycol or propylene glycol added. On to this was poured 20 ml of diethyl ether and 5 ml of saturated sodium hydroxide solution. The flask was immediately closed with a foil wrapped cork stopper and immersed in hot water. The delivery tube was placed in a small conical flask containing a little diethyl ether. This flask was held in an ice bath. Diazomethane was distilled over with the ether and could be seen to give a yellow ethereal solution in the small conical flask. Diazomethane in ether was then added to purified seed extracts and left for several hours before being blown down under nitrogen. Methylated samples were taken up in a known volume of solvent prior to GLC analysis. The trimethylsilyl derivative of IAA was prepared for GLC by adding BSA[N,BIS (trimethylsilyl) acetamide] to thoroughly dried samples. The presence of water leads to hydrolysis of the TMSi derivatives.

Columns and running conditions

The analyses of ABA Me esters were carried out using a Perkin Elmer F17 gas chromatograph. The column routinely used was a 2 metre, glass column with an internal diameter of 3 mm, packed with 1½% QF1 (fluorsilicon oil FS-1265) on Chromosorb WAW-DMCS, 80-100 mesh. Oven temperature was either 210°C or 220°C and the injector block was kept at 275°C. Two detectors were fitted to this machine, a flame ionization detector (FID) and an electron capture detector (ECD), the column effluent being split 9:1 between these detectors. Gas flow rate was kept constant at the following pressures:

Carrier gas, nitrogen	140 kilonewtons per metre^2 KN/m^2
Oxygen for FID	165 KN/m^2
Hydrogen for FID	100 KN/m^2
ECD make up gas, nitrogen	150 KN/m^2

Carrier gas flow rate was 45 cubic cms per minute. The detector signals were transmitted to a Servoscribe 2S dual pen recorder set at a full scale deflection of 1 mV. The ECD was operated on a pulse mode at a setting of 6, the last in which linearity was preserved. Normal range and attenuator settings for both the ECD and FID were 100 x 4 or 10 x 32.

Combined gas liquid chromatography, mass spectrometry

Flame ionisation detection was not found to be sensitive enough to quantify endogenous ABA or IAA. Initially single ion monitoring on a combined gas liquid chromatogram, mass spectrometer, GC-MS was employed for the detection and measurement of endogenous ABA or IAA. The mass spectrometer was an AEI MS 30 single beam, double focusing instrument. A Pye 104 GLC was attached to the MS via a permeable membrane. Organic compounds are absorbed or dissolved in a thin elastomer membrane, made of silicone rubber, which is the actual interface between the gas chromatograph and the mass spectrometer. The separator is required as a result of the vast difference in operating pressures (1 atmosphere for GLC and 10^{-9} atmospheres for MS). Furthermore, any organic compound in the GLC effluent is too dilute for MS analysis. The separator enriches the organic component of the gas stream entering the instrument, due to its selective properties, and effects a pressure drop. Two columns were used for analyses, both 9 ft glass columns with internal diameters of 4 mm. One was packed with 2% SE 33 (silicone gum rubber E 303) on chromosorb W, 80-100 mesh, the other with 2% OV 210 (methyltrifluoropropyl silicone) on chromosorb W, 80-100 mesh. Oven temperature was in the range 200-220°C, being isothermal for any one set of analyses. The injector block was not equipped with a pre heater. The carrier gas was helium at a flow rate of 45-60 cubic cms per minute. To obtain the GLC total ion current was monitored and this signal transmitted to a Servoscribe chart recorder. Since the output of the total ion monitor, amplifier system was logarithmic minor components were readily detected and any major

component did not swamp the chromatogram.

For mass spectral analysis of either ABA, ME ester or IAA TMSi the sample was subjected to GLC and the peaks, corresponding to the known retention time of standards, scanned on the MS. This involved either taking a full scan to identify unequivocally the compound present, or a single ion scan. In the latter the MS was focused on a characteristic mass to charge ratio me/e; for ABA Me ester m/e 190 and for IAA TMSi m/e 202. Scanning was carried out from the time of sample injection. In single ion focusing the MS is being used as a selective recorder for the GLC effluent. The area of peaks corresponding to the retention time of standards may be used as a measure of the amount of compound present. The ion source temperature was 200°C and the ionising voltage 70 eV.

On two occasions the direct probe was used for direct application of samples to the MS. Here, the sample was applied to the surface of a small glass holder and this was placed in the probe. The probe was then inserted into the MS where the sample was volatilised, subjected to ionising voltage and a mass spectrum obtained.

The theory of mass spectrometry and problems encountered in quantifying endogenous plant hormones will appear in the relevant experimental sections.

Bioassays

(A) Lettuce hypocotyl bioassay

The lettuce hypocotyl extension bioassay originally described by Frankland and Wareing (1960) was used to measure GA-like activity. Either 'Grand Rapids' or 'Arctic' lettuce were used. Seeds were germinated at 20°C for 24 hrs. Uniform seedlings, showing 2-3 mm radicle elongation, were selected and 9 of these placed in a glass petri dish lined with one Whatmans No. 3 qualitative filter paper containing plant extract or standard or blanks. The dishes were placed in a plastic germinator which maintained a high humidity. The germinators were then placed in a growth cabinet at 22° ± 1°C. under 16 hr daylength conditions (white fluorescent tubes emitting an intensity of 17.02 watts m^{-2} with additional tungsten lamps). After 3 days the length of the hypocotyl was measured to the nearest 0.5 mm.

(B) Inhibition of etiolation of lettuce hypocotyls

This assay, previously described by McWha (1973) was used to measure inhibitor activity. Grand Rapids lettuce seeds were germinated at 20°C for 24 hrs and uniform seedlings, showing 2-3 mm radicle elongation, selected. 9 of these were placed in a glass petri dish as described for the lettuce hypocotyl bioassay. The dishes were then placed in an incubator in darkness at 25°C for 3 days and the hypocotyl measured to the nearest 0.5 mm.

(C) Excised radish cotyledon bioassay

The bioassay technique, used for the measurement of cytokinin like activity, was that of Letham (1968). Radish seeds (Raphanus sativus L. cv. White Icicle) retained by a 10 mesh sieve were germinated at 25°C in darkness on well wetted filter paper in petri dishes for 48 hrs. The smaller cotyledon was excised from each seedling, care being taken to ensure that only cotyledonary material was taken. Cotyledons of uniform size were selected, blotted and weighed to the nearest tenth of a milligram. Five cotyledons were then placed in a 4.5 cm glass petri dish, lined with one layer of

Whatmans No. 3 qualitative filter paper containing the test solution. All test solutions, plant extracts or standards were made up in 2 mM phosphate buffer (pH 5.9) in 10 mM KCl (Radin and Loomis, 1971). The cotyledons were placed with the upper surface facing the solutions. The petri dishes were placed in a growth cabinet as described for the lettuce hypocotyl assay. After three days the cotyledons were removed, blotted dry and weighed.

Radioactive studies

Isotopes

2[^{14}C(±)RS]ABA, specific activity 11.88 mCi/ m mole, was obtained as a gift from Hoffman La Roche. This sample was originally used by McWha (1973) and when checked for purity, using TLC with chloroform:methanol:water (75:22:3 v/v) as the developer solvent, considerable activity remained at the origin. Consequently the sample was purified by TLC and checked for purity at regular intervals (Figure 16).

5[^{3}H]IAA was purchased from Fluorochem Ltd., and had a specific activity of 26.6 Ci/m mole. The purity of the IAA was checked at regular intervals but no degradation took place during this study. [^{3}H]IAA was stored at 4°C in 0.5 ml of ethanol and fresh dilute stock solutions made as required. IAA solutions were used within two days of being made.

[^{14}C]ABA was stored, dry at -20°C in a conical flask. Working stock solutions were prepared twice during the course of this work.

Assay of radioactivity

Radioactive samples were assayed by liquid scintillation spectrometry using either a Packard Tricarb, Model 3380, liquid scintillation spectrometer fitted with an absolute activity analyser, Model 544, or a Tracerlab Corumatic 200 liquid scintillation spectrometer, and the absolute activity calculated.

To determine absolute activity it is necessary to calculate the efficiency of counting. This is determined by the degree of quenching found in the sample. Quenching may take two forms, chemical quenching or colour quenching. In chemical quenching compounds in solution in the liquid scintillant interfere with the transfer of energy from the emitted particle of radiation to the organic phosphor. The energy is degraded by processes which do not produce emission of light. In colour quenching, coloured materials in the liquid scintillant absorb light emitted by the organic phosphor and prevent it being detected by the photomultiplier tube.

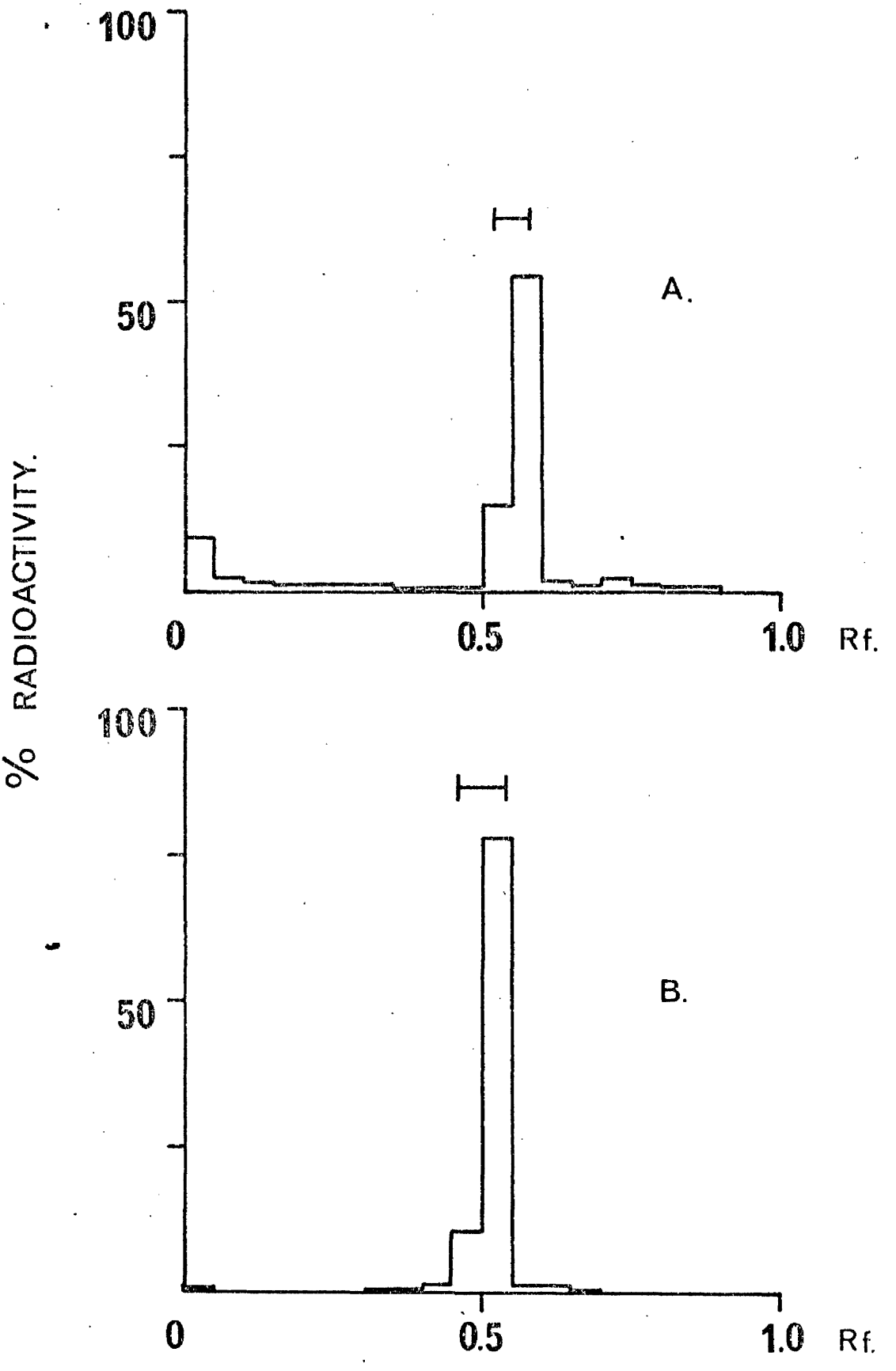
Figure 16

Chromatographic distribution of radioactivity of a stock solution of 2-[^{14}C]-ABA, run in chloroform: methanol:water (75:22:3 v/v).

(A) before 'clean up' procedure.

(B) post 'clean up' procedure.

: Rf of standard ABA.


100
50
0
0.5
1.0
Rf.
A.
% RADIOACTIVITY.
100
50
0
0.5
1.0
Rf.
B.

Three methods may be used to determine the counting efficiency of a sample, internal standard, channels ratio and an absolute external standard (AES).

With both the spectrometers used the external standard method was employed in calculating the efficiency of counting.

In the Tricarb Spectrometer, the degree of quenching is estimated by counting an external standard (americium 241 and radium 226) of known activity through the sample vial and adjusting this electronically to give an AES ratio between 0 and 10. A ratio of 10 indicates that no quenching has occurred, while 0 indicates total quench. The sample is then counted in the absence of the external standard and the resulting cpm (counts per minute) at a given AES ratio converted to dpm (disintegrations per minute) by the Absolute Activity Analyser (AAA). Before this automatic correction is possible, the AAA must be programmed with the counting efficiencies at fixed AES ratios. These were determined by counting samples of n-hexadecane of known activity but quenched to varying degrees by the presence of acetone, methanol or ethanol. The efficiency at the given AES ratio was calculated from the expression:-

$$\text{efficiency} = \frac{\text{net cpm as recorded}}{\text{known dpm as calculated}}$$

By repeating this with samples quenched to different extents, a 'quench curve' was constructed (Figure 17A). The AAA was then programmed by reference to this curve, after which it automatically computed the absolute activities of the samples using the expression:-

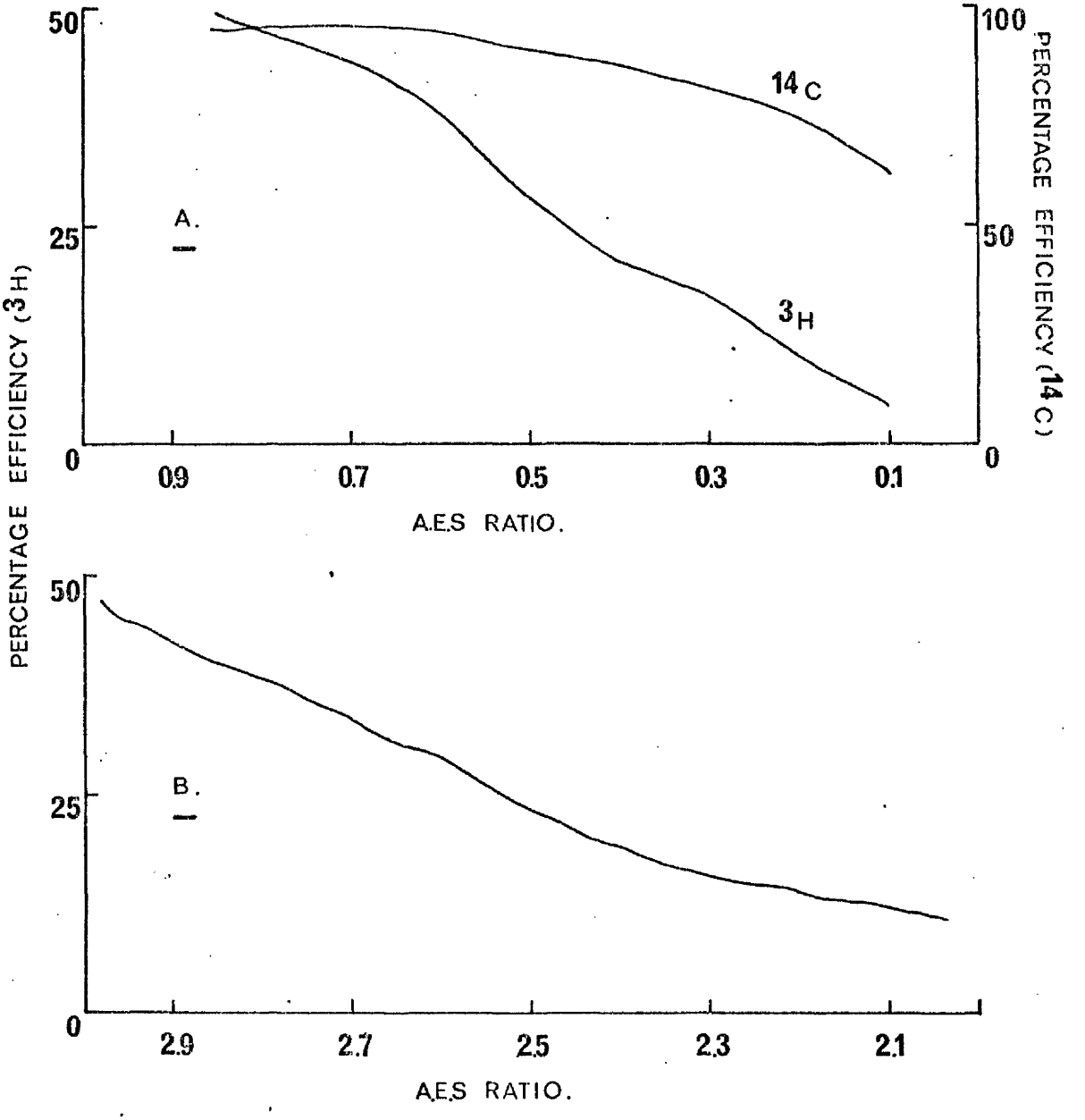
$$\text{dpm} = \frac{\text{cpm}}{\text{efficiency}}$$

The Tracerlab Corumatic 200 utilised a similar system of calculating AES ratios. It was not, however, fitted with a means of automatically converting the activity to an absolute level and each value had to be calculated by reference to a 'quench curve' constructed as described for the Tricarb (Figure 17B). The Tracerlab was used only for [^{3}H] samples, whilst both [^{3}H] and [^{14}C] were counted on the Tricarb.

In one experiment use was made of autoradiography to check the Rfs of [^{3}H]IAA run in different solvent systems on TLC. The TLC

FIGURE 17

Quench correction curves for the liquid scintillation spectrometers. The efficiency of recording disintegrations per minute of ^{14}carbon and tritium 3H at varying AES ratios using (A) the Packard Tricarb liquid scintillation spectrometer; (B) the Tracerlab Corumatic liquid scintillation spectrometer.


50
25
0
100
50
0
A.
14C
3H
0.9
0.7
0.5
0.3
0.1
A.E.S RATIO.
PERCENTAGE EFFICIENCY (3H)
PERCENTAGE EFFICIENCY (14C)
50
25
0
B.
2.9
2.7
2.5
2.3
2.1
A.E.S RATIO.

plates were exposed for four weeks to Ilford double coated red seal x-ray film. All operations were carried out in complete darkness as the x-ray film was sensitive to most photographic safe lights. The film was then developed according to the manufacturer's instructions. Densitometer readings of the film were obtained using a Joyce Loebl Chromoscan.

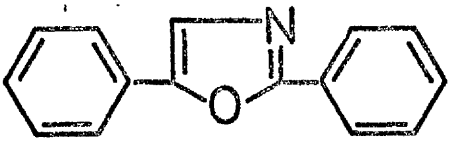
Metabolism of ABA and IAA

Grand Rapids lettuce seeds were counted into batches of 200 prior to dispensing into 4.5 cm glass petri dishes lined with one 4.25 cm Whatmans No. 3 qualitative filter paper and containing 1.5 ml of test solution. On dispensing the seeds in the petri dish, the dishes were immediately placed in light tight containers. After specified times and treatments the seeds were removed, placed in a small muslin bag and external adhering radioactivity washed off of the seed with water. The thermostatically controlled incubators, red light source and details of transfer experiments are as previously described under standard germination conditions. The washed seeds were either extracted whole, or macerated with a pestle in a mortar with a little acidic washed sand, and then extracted. Extraction was in three 25 ml changes of 80% methanol, for 24 hrs each at 4°C. With IAA the seeds were first extracted whole and then macerated and re-extracted.

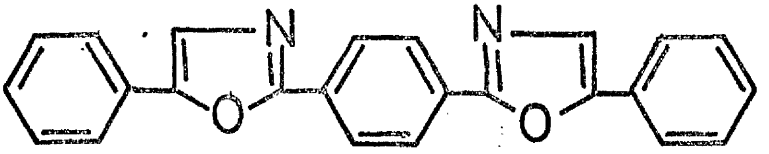
The solvent was reduced on a thin film rotary evaporator and the radioactivity resuspended in a mixture of methanol, diethyl ether. This was applied to pre-coated silica gel TLC plates containing a fluorescent indicator (Merck) which had been prerun in methanol, using the sample applicator shown in Figure 15. Marker spots of standards were applied to the starting line. Plates were developed for 15 cms in solvent systems described in the experimental results. The Rf of the standards was noted by inspection under a UV lamp at 254 nm. They were then marked into 20 equal zones and each zone scraped into a scintillation vial. One ml of methanol was added and the vials left for 1 hr before the addition of scintillant.

The scintillation 'cocktail' consisted of 4 g of 2,5 diphenyl-oxazale (PPO), the primary scintillant, and 0.3 g of 1,4-Bis-

(5-phenyloxazal-2-yl)benzene (POPOP), the secondary scintillant, per ; litre of toluene.



PPO.



POPOP.

10 ml of scintillant was added to each vial. Vials were stored overnight at 4°C in darkness prior to counting on the Tricarb or Tracerlab instruments. Blanks for the calculation of background consisted of vials with one zone of silica gel containing no activity, one ml of methanol and 10 ml of scintillant. Background was always subtracted prior to the calculation of absolute activity.

For [^{3}H]IAA seed extracts were never left in a dry condition even at -20°C. Seed residues after extraction were kept.

Uptake of radioactivity from [^{3}H]IAA or [^{14}C]ABA

Grand Rapids lettuce seeds were counted into batches of 100 prior to dispersing into glass petri dishes as described for the metabolism of ABA or IAA. Externally adhering radioactivity was washed off and the seeds, contained in glass vials, dried in an oven at 120°C. The seeds were then transferred to polycarbonate capsules, and the radioactivity removed by oxidation in an Oxymat JA 101 (Intertechnique) sample oxidiser. The 'Oxymat' is a commercial version of the Peterson apparatus for tube furnace combustion of samples (Peterson, 1969; Peterson et al., 1969). It oxidizes [^{3}H] or [^{14}C] labelled samples automatically, delivering them dissolved in their respective scintillation fluid ready for counting. Recovery for both ^{3}H and ^{14}C is reported to be 97-98% (Wangermann, 1974). According to the manufacturers, cross contamination is less than 1% and residual activity from a sample picked up in the following sample ('memory') is 0.05-1%.

The scintillation 'cocktails' used were:

(1) For tritium samples

1:4 dioxan	700 ml
Toluene	300 ml
PPO	5 g
POPOP	0.3 g

(2) For ^{14}C samples

Toluene	400 ml
2-phenethylamine	330 ml
Methanol	220 ml
Water	50 ml
PPO	7 g

These scintillants were freshly prepared as both have poor storage qualities. Samples were then counted in the Tricarb Liquid Scintillation Spectrometer.

Residual activity in the seed residues, left after extraction during metabolism studies, was also measured using the Oxymat followed by counting on a liquid scintillation spectrometer.

Statistical analysis of results

(A) Standard error

Standard error was calculated by using the following expression:-

$$\text{Standard error (SE)} = \sqrt{\frac{\Sigma x^2 - \Sigma (\frac{x}{n})^2}{n(n-1)}}$$

where Σ is the 'sum of', x = the individual value of each observation, n = number of observations.

Where standard errors are shown on graphs they are represented by a vertical bar through the point to which they refer. The bars normally equal one standard error.

(B) Analysis of variance

A number of experiments reported in this thesis employed a factorial design. The two variables were the two test solutions, each at several concentrations. A factorial design combines these two variables in all different combinations. Where we were interested in examining the possible interaction between these two variables the data were subjected to an analysis of variance. Before this analysis can be carried out on germination data, germination percentages must be transformed by using an 'arc sine transformation'. In scoring germination it is only possible to assess germination in terms of 'either' it does germinate 'or' it doesn't! Thus, the germination of a seed population is binomial, showing a-symmetry at the extremes of the distribution. Analysis of variance can only be applied to data which can be fitted to a normal distribution, that is, one which is symmetrical about a mean. An arc sine transformation converts a binomial distribution to a normal distribution allowing the application of the statistics of the normal distribution.

As an example of how an analysis of variance was carried out the data and calculations for the effect of Amo 1618 and GA_{4+7} at 30°C on germination of Grand Rapids seed, are presented.

Percentage germination of Grand Rapids lettuce seeds treated with Amo 1618 and GA_{4+7} at 30°C after 24 hrs

Concentration Amo 1618 M / GA_{4+7} M	0			10^{-4}			10^{-3}			10^{-2}		
	a	b	c	a	b	c	a	b	c	a	b	c
0	10	4	4	2	4	0	2	2	4	0	0	0
10^{-6}	24	24	26	16	20	24	12	10	10	6	2	2
10^{-5}	56	58	64	50	58	64	46	48	48	6	18	10
10^{-4}	56	64	70	56	74	78	62	58	44	20	8	22

Angular transformation of percentage germination

Concentration Amo 1618 M / GA_{4+7} M	0			10^{-4}			10^{-3}			10^{-2}		
	a	b	c	a	b	c	a	b	c	a	b	c
0	18.44	11.54	11.54	8.13	11.54	0	8.13	8.13	11.54	0	0	0
10^{-6}	29.33	29.33	30.66	23.58	26.57	29.33	20.27	18.43	18.43	14.18	8.13	8.13
10^{-5}	48.45	49.60	53.13	45.00	49.60	53.13	42.71	43.85	43.85	14.18	25.10	18.44
10^{-4}	48.45	53.13	56.79	48.45	59.34	62.03	51.94	49.60	41.55	26.51	16.43	27.97

We require to calculate the following five entities in order to obtain the statistics relating to the above sample.

I. Replicates

Each treatment is replicated three times, a, b and c. Columns a, b and c are summed individually

(a) (18.44 + 29.33 + 48.45 + 48.45 + 8.13 + ... + 14.18 + 26.57) = 447.81

(b) (11.54 + 29.33 + 49.60 + ... + 25.10 + 16.43) = 460.32

(c) (11.54 + 30.66 + 53.13 + ... + 18.44 + 27.97) = 466.52

The individual totals are then squared and summed

$(447.81)^2 + (460.32)^2 + (466.52)^2$

This figure divided by the total number of values contributing to each sub-total gives the value for replicates

$(447.81)^2 + (460.32)^2 + (466.32)^2 \div 16 = \underline{39,379.33}$ ... 1

(II) Treatment category 1 : Amo 1618

The subtotals for treatment category 1 are calculated by summing all values at any concentration of Amo 1618.

Concentration of Amo 1618			
0	10^{-4}	10^{-3}	10^{-2}
(18.44 + 11.54 + 11.54 + 29.33 + ... + 56.79)	(8.13 + 11.54 + 0 + 23.58 + ... +62.03)		
440.39	416.70	358.43	159.13

Now, Σ (subtotal)$^2 \div$ number of treatments per concentration --- II

$\therefore (440.39)^2 + (416.70)^2 + (358.43)^2 + (159.13)^2 \div 12$

$= \underline{43,448.06}$ ... 2

III.Treatment category 2 : GA_{4+7}

As in II the subtotals are calculated by summing all values at any one concentration of, here, GA_{4+7}

$\therefore (88.99)^2 + (256.37)^2 + (487.04)^2 + (542.25)^2 \div 12$

$= \underline{50,407.32}$ ... 3

IV. Interaction

Each sum is the total of the values for each treatment, here 16 treatments, three replicates a, b and c being added to form a subtotal.

(18.44 + 11.54 + 11.54) + (29.33 + 29.33 + 30.66) +

..... + (26.57 + 16.43 + 27.97)

Now, Σ (subtotal)^2 $\div$ number of replicates ---- IV

$\therefore (41.52)^2 + (89.32)^2 + ... + (57.72)^2 + 70.97)^2 \div$

$= \underline{55,272.84}$ ... 4

V. Correction factor

(total of all values)^2 $\div$ total number of values ---- V

$$\frac{(1,374.65)^2}{48} = \underline{39,367.97}.$$ ... 5

The degrees of freedom are calculated as follows:

replicates	df :- no. of replicates -1
treatment category 1	df :- no. of concentrations -1
" " 2	df :- no. of concentrations -1
interaction	df :- no. of df. for treatment cat. 1 x no. of df. for treatment cat. 2
error	df :- (total no. of values -1) - df (replicates + treatment cat. 1 + 2 + interaction).

The sum of squares is calculated as follows:-

replicates	$\div$	(1) - (5)
treatment cat. 1	$\div$	(2) - (5)
" " 2	$\div$	(3) - (5)
interaction	$\div$	[(4) - (5)] - (sum of squares for treatment categories 1 and 2)
error	$\div$	obtained by difference
total	$\div$	total sum of squares - (5)

The mean square is obtained by dividing the sum of squares by the degrees of freedom. The mean squares are 'partial variances'. F is then calculated by dividing the mean square for a particular source by the mean square of the error factor. This can be tabulated as below.

Source	df	Sum of squares	Mean square	F	Significance level
Replicates	2	11.36	5.68	<	N.S.
Amo 1618	3	4,080.09	1,360.3	77.5	****
GA_{4+7}	3	11,039.35	3,679.78	209.7	****
Interaction	9	785.43	87.27	4.97	****
Error	30	526.48	17.55		
Total	47	16,442.71			

The level at which the F values are significant were obtained from statistical tables giving F-distributions where the df of the error factor and the relevant 'source' were used.

The following symbols are used to denote the probability of a difference being significant.

N.S. not significant at the 95% probability level
* greater than 95%
** " " 97.5%
*** " " 99.0%
**** " " 99.9%

SECTION III

EXPERIMENTAL

PART A

Endogenous cytokinin and gibberellin like compounds in Grand Rapids lettuce seeds : some preliminary work.

Introduction

We have noted previously that cytokinin and gibberellin like activities have been shown in seeds of Grand Rapids lettuce (see page 34) and that exogenous cytokinin or gibberellin would promote germination of seeds which were thermodormant. No changes in endogenous gibberellin like compounds could be found in response to the thermodormancy breaking stimulus of red light (Ikuma and Thimann, 1960) whilst the situation with respect to cytokinins is far from being resolved. In the general introduction some of the difficulties, which arise when examining endogenous hormones in seeds, were discussed. These problems are more serious when dealing with unidentified compounds. As a result of these problems it was decided to develop a column system capable of separating the classes of hormones which it was hoped to examine. From previous studies it was thought a likely candidate for this job was the hydroxypropyl derivative of sephadex G-25, sephadex LH20. Sephadex separates compounds by virtue of their size and adsorption properties. Armstrong et al. (1969) showed that four cytokinins were eluted from sephadex LH20 in a tight band in 95% ethanol, but over a broad band in 35% ethanol. Van Staden (1973a) has used sephadex LH20 in 30% ethanol to separate the cytokinins of lettuce seeds. There are also reports of sephadex LH20 being used to separate inhibitors, which could have included abscisic acid (Steen and Eliasson, 1969; Eliasson, 1969). The inhibitors seem to be eluted prior to cytokinins, if the data from these papers are compared.

Since this work was carried out MacMillan and Wels (1973) have reported the use of sephadex LH20 as a partition system used to separate gibberellins. It was hoped to effect a separation of gibberellins from cytokinins on a column of sephadex LH20, in straight methanol, overcoming the inherent problems associated with the use of solvent partitioning.

Results and discussion

The results of this work are shown in Table 3, Figures 18 and 19.

Table 3 shows the length of lettuce seedling hypocotyls in light (gibberellin like activity) or darkness (inhibitor activity), with standard errors. These are shown in histograms A and B respectively in Figure 18. It can be seen that there was considerable gibberellin like activity over a broad band, SEV's 35-120, with two peaks of activity in fractions 12 and 27. In the gibberellin bioassay there is a prominent zone of inhibition which is coincident with the first zone of gibberellin like activity. The zone of inhibition is more clearly shown in Figure 18B at SEV's 38-80. Figure 18B shows that the compound(s) promoting hypocotyl elongation in the gibberellin bioassay (Figure 18A, fractions 26-27) is also capable of causing increased elongation of dark grown hypocotyls. Experiments conducted with standards showed that ABA eluted over the range 35-86. Thus the zone of inhibition is coincident with the elution pattern of ABA. This experiment was repeated twice with similar results. In Figure 19 A and B we can see the cytokinin like activity of non-imbibed lettuce seeds, assayed using the radish cotyledon bioassay (Letham, 1968). Figure 19A corresponds to Figure 18 and a similar zone of inhibition can be seen in fractions 10-17. The vertical bars represent one standard error. Clearly the standard errors are so large that any statistical test would be meaningless. It is possible that there may be zones of cytokinin like activity. This bioassay was repeated on a large number of occasions, using water or buffer to make up test solutions. It was found that there was little difference whether water or buffer was used.

Treatment	Weight increment of radish cotyledon ± SE
Water	10.6 ± 1.0
Buffer	10.1 ± 2.6
Kinetin in water	
10^{-6} M	6.4 ± 1.6
10^{-5} M	7.8 ± 0.8
10^{-4} M	24.1 ± 3.5
Kinetin in buffer	
10^{-6} M	6.2 ± 1.1
10^{-5} M	9.7 ± 1.9
10^{-4} M	25.5 ± 4.8

It was also observed that significant responses to standards could only be obtained when strict selection of cotyledons was made. Cotyledons had to be within a narrow weight range, between 5-6 mgs. Large deviations from this weight, by even 0.2-0.4 mg resulted in large standard errors.

It can be concluded that sephadex LH20 may separate gibberellins plus inhibitors from cytokinins. However, it cannot separate gibberellins from inhibitors. Furthermore, the bioassay selected to follow cytokinin like activity, although rapid, yielding results in 3 days, lacks sensitivity and there are difficulties in obtaining reliable results. In view of these findings it was decided that no further work would be carried out in looking at the endogenous cytokinins and gibberellins of lettuce seeds. As we cannot obtain reliable results with the radish cotyledon bioassay of Letham (1968) and the other 'standard' cytokinin bioassay, the soybean callus bioassay, seems to lack specificity (Van Staden and Drewes, 1975), there seems to be no reliable bioassay for the measurement of cytokinin like activity. Consequently, it is not possible to study this class of hormone. With respect to gibberellins it has already been shown that there are no changes in gibberellin content after red light treatment. As was discussed in the general introduction, this does not rule out a change in the effective gibberellin content of the seeds, via release from a conjugated or bound form, either in a chemical or physical sense, or via qualitative changes in the endogenous gibberellins. Further study in this area would require the prior identification of the endogenous gibberellins of lettuce seeds. At the time this study was started the technology required to carry out this work was not readily available and it was decided to concentrate on studying single compounds such as abscisic acid and indole acetic acid where unequivocal identification of these hormones in lettuce seeds might prove possible. By studying known compounds many of the problems of the classical extraction and purification procedures would also be removed, as the use of standards allows the movement and possible losses of the hormone, during the purification procedure, to be followed.

Table 3 . Methanol extract from 50 g equivalents of unimbibed Grand Rapids lettuce seeds: chromatogramed on Sephadex LH20. Gibberellin and inhibitor like activity.

Fraction number	GA Length of hypocotyl in light (mm) ± S.E.	Inhibitor Inhibition of hypocotyl extension in dark (mm) ± S.E.
1	2.30 ± 0.12	20.80 ± 1.01
2	2.10 ± 0.10	20.00 ± 0.57
3	2.20 ± 0.12	20.40 ± 1.16
4	2.20 ± 0.12	18.80 ± 0.89
5	2.30 ± 0.20	21.10 ± 0.87
6	2.20 ± 0.12	19.90 ± 0.64
7	2.30 ± 0.26	19.80 ± 0.78
8	3.00 ± 0.65	20.90 ± 0.91
9	4.00 ± 0.52	19.20 ± 0.78
10	6.90 ± 1.91	9.70 ± 0.58
11	9.00 ± 0.81	8.60 ± 0.49
12	12.10 ± 0.60	5.90 ± 0.60
13	8.40 ± 0.80	2.50 ± 0.16
14	-	2.40 ± 0.19
15	-	2.40 ± 0.19
16	6.60 ± 0.66	5.30 ± 0.85
17	7.60 ± 0.83	8.30 ± 0.72
18	7.86 ± 0.46	9.80 ± 0.46
19	7.20 ± 0.50	10.20 ± 1.62
20	6.80 ± 1.39	19.60 ± 1.71
21	4.75 ± 0.90	18.80 ± 2.27
22	4.90 ± 0.10	22.60 ± 2.14
23	4.80 ± 0.34	25.70 ± 1.03
24	6.00 ± 1.08	25.10 ± 1.50
25	7.20 ± 0.41	26.40 ± 3.02
26	10.60 ± 0.43	22.80 ± 2.16
27	4.40 ± 0.19	33.00 ± 2.16
28	3.00 ± 0.91	26.60 ± 0.80
29	2.50 ± 0.16	22.20 ± 1.59
30	2.50 ± 0.22	21.40 ± 1.94
31	2.00 ± 0.16	21.10 ± 0.77
32	1.80 ± 0.20	20.30 ± 1.48
Water	2.00 ± 0.19	19.50 ± 0.45
GA_{4+7} M		ABA M
10^{-5}	22.30 ± 0.73	10^{-4} 3.20 ± 0.12
10^{-6}	14.20 ± 0.69	10^{-5} 5.00 ± 0.42
10^{-7}	7.10 ± 0.68	10^{-6} 7.00 ± 0.49

FIGURE 18

Methanol extract from unimbibed Grand Rapids lettuce seeds : chromatographed on sephadex LH20 and bioassayed using Grand Rapids seedlings.

(A) gibberellin like activity.

(B) inhibitor activity.

a 10^{-7} M GA_{4+7}

b 10^{-6}M GA_{4+7}

c 10^{-5} M GA_{4+7}

d water

e 10^{-4} M ABA

f 10^{-5} M ABA

g 10^{-6} M ABA

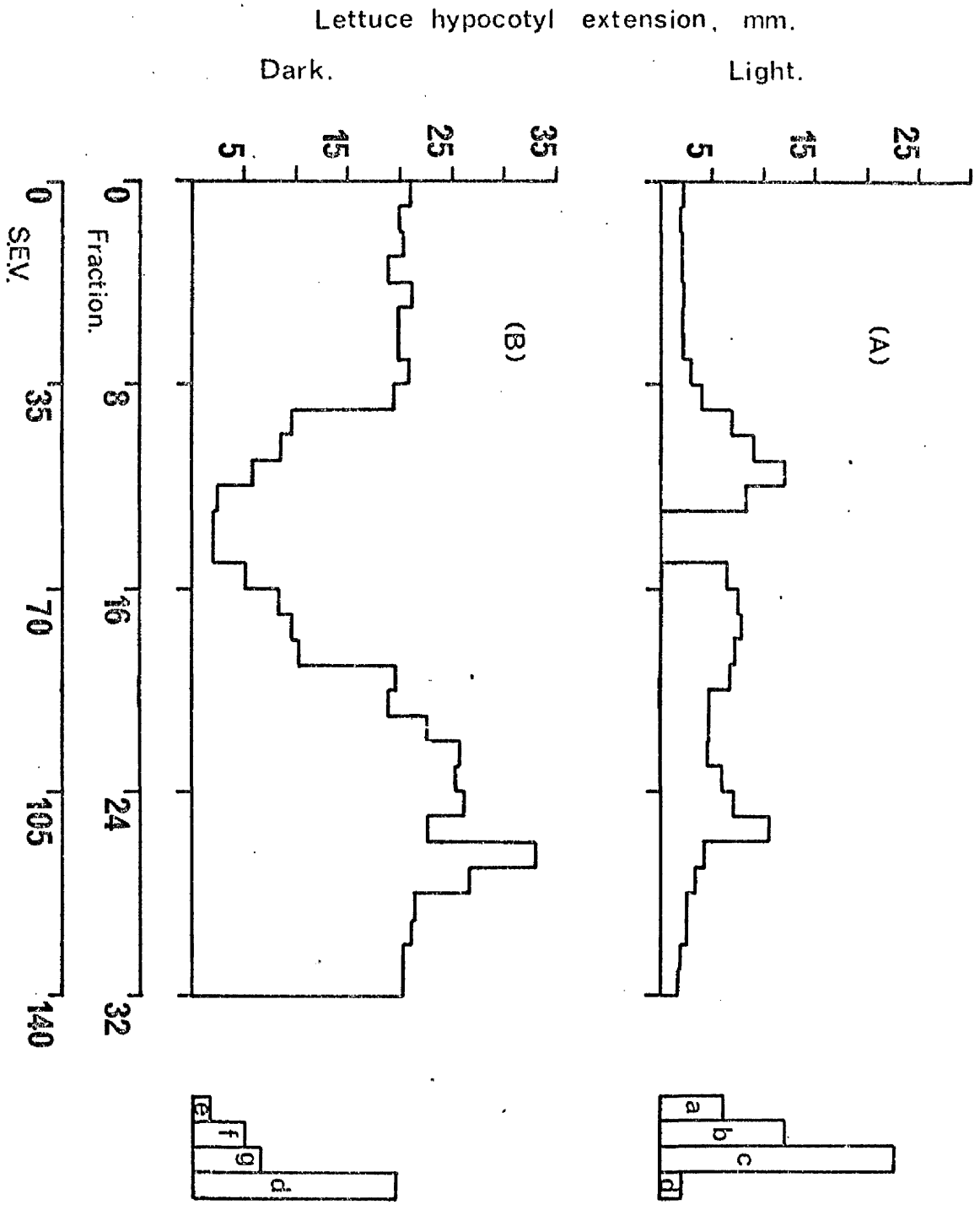

Lettuce hypocotyl extension, mm.
Dark.
Light.
5
15
25
35
(B)
(A)
Fraction.
0
8
16
24
32
S.E.V.
0
35
70
105
140
a
b
c
d
e
f
g
d

FIGURE 19

Methanol extract from unimbibed Grand Rapids lettuce seeds : chromatographed on sephadex LH 20. Radish cotyledon bioassay for cytokinin like activity.

(A) run 1

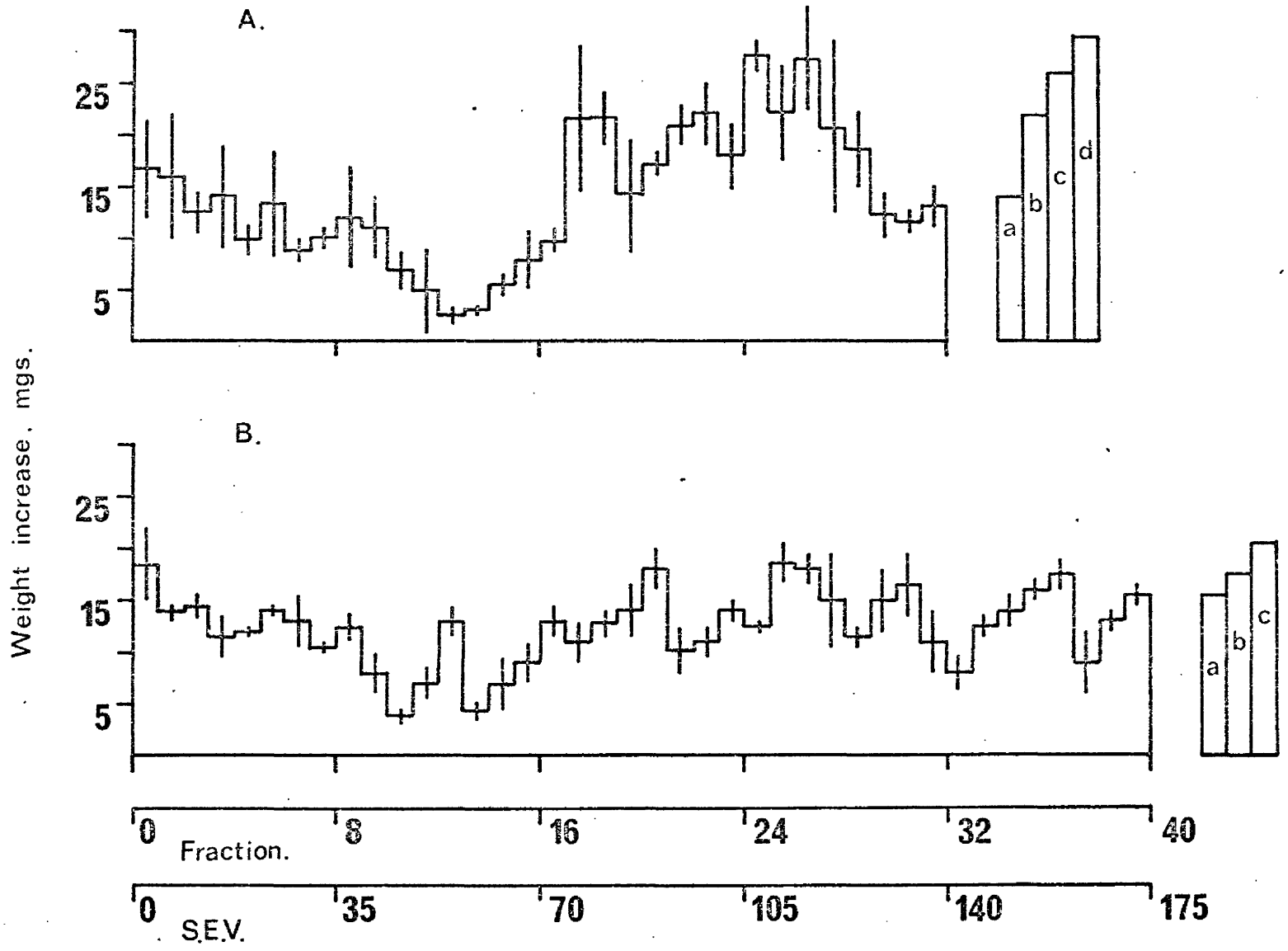
(B) run 2

a buffer control

b 10^{-6} M zeatin

c 10^{-5} M "

d 10^{-4} M "


A.
25
15
5
a
b
c
d
B.
25
15
5
a
b
c
Weight increase, mgs.
0
8
16
24
32
40
Fraction.
0
35
70
105
140
175
S.E.V.

PART B

Does red light cause an increase in endogenous gibberellins in lettuce seeds ?

Introduction

We know that exogenous gibberellins can overcome thermodormancy in seeds of Grand Rapids lettuce (Khan et al., 1957; Brian et al., 1962; Ikuma and Thimann, 1963; Bewley et al., 1968) and it can be argued that the exogenous growth regulator is supplying a requirement that is normally met when synthesis of these compounds is triggered by exposure to red light. Previously the evidence that red light causes an increase in endogenous gibberellins has been discussed and it is clear that there is no evidence to support such a role for red light. However, the numerous problems encountered in examining endogenous gibberellins mean that we cannot completely rule out such an effect. One way of investigating this problem would be to apply compounds which specifically inhibit gibberellin biosynthesis. If these compounds were applied to lettuce seeds and red light were found to be unable to overcome thermodormancy in their presence, this would be good evidence that gibberellin biosynthesis was at least required for red light induction of germination. Such a class of compounds are the 'growth retardants'! Evidence that growth retardants acted by inhibiting gibberellin biosynthesis came from the fact that the decreased rate of stem growth, induced by the retardants, frequently could be overcome by exogenous gibberellin (Lang, 1970). We can propose that in lettuce seeds, if red light causes an increase in gibberellin biosynthesis, exogenous gibberellins should cause germination in the presence of growth retardants, but red light should be ineffective. Wittwer and Tolbert (1960) applied C.C.C. (chlormequat) to Grand Rapids lettuce seeds and found that germination was reduced and that this reduction was prevented either by red light or gibberellins. That red light was effective in overcoming the growth retardant suggests that gibberellin synthesis is not initiated by red light treatment. The aim of this work was to devise experiments to investigate the possibility that the effect of red light in overcoming thermodormancy of seeds of Grand Rapids lettuce, was through the establishment of a mechanism that resulted in enhanced gibberellin production.

We have observed that lettuce seeds should respond to exogenous gibberellin regardless of the presence of growth retardant. In a factorial experiment the results of the interaction between several concentrations of gibberellins and growth retardants may be analysed. From this analysis we can say how significant an effect any growth retardant or promoter has on germination, and whether or not the various compounds interact with each other in a statistically meaningful way. The statistical analyses used in this thesis have been described in materials and methods. It is pertinent to define what is meant by the term interaction, as this term is rather loosely used by many biologists. Drury (1969) points out that 'statisticians define interaction as the failure of a response to one agent to be the same at different amounts of a second agent'. When results are expressed graphically, an interaction will, therefore, express itself as non-parallel lines, i.e. the response to the two agents in combination is not the algebraic sum of the responses obtained when the agents are applied alone. When dealing with germination percentages, the data must be subjected to a transformation prior to the application of the statistics of the normal distribution. Thus, the interactions reported in this work are interactions of the transformed data and not the original germination data. A second critical point, if interactions are to be meaningful, is that the variance of the replicate component is not a major part of the total variance. If such variation were significant any real differences between treatments would not be detected. The definition of interaction subscribed to in this thesis will be that stated by Drury (1969). Consequently, we analyse the data obtained from a factorial experiment, using different concentrations of a growth retardant and a promoter. If gibberellin is the promoter and our hypothesis, that red light increases gibberellin biosynthesis is correct, we would expect no interaction between gibberellin and growth retardant. The seeds should respond to applied gibberellin and be unaffected by the growth retardant.

Results

The germination percentages obtained from seeds treated with one of four growth retardants and one of five promoters of germination, after 24 hrs, are presented in Tables 4-23. A time course study was

carried out using the four growth retardants and gibberellin A_{4+7} (GA_{4+7}), and the data are shown in Tables 24-27 and Figures 20-23. If we examine the data shown in Table 4, which gives the percentage germination of seeds treated with the growth retardant B995 (specific name given in Figure 8) and gibberellin A_3 (GA_3) at either 30°C or 20°C, we can see that dark germination at 30°C is about 10%. Even this is reduced by increasing concentrations of B995 with no germination in the highest B995 concentration, 10^{-2}M. At 20°C B995 also inhibits germination. As would be expected GA_3 promotes dark germination at 30°C and even slightly at 20°C. On the basis of what was stated in the introduction we should not expect an interaction between gibberellin and B995. However, it is clear that B995 can reduce the effect of exogenous GA_3, especially at 30°C. Although the percentage germination figures at 20°C would seem to indicate little interaction, an analysis of variance of the experimental data, used in calculating these averages after transformation, does give significant interactions. The data shown in Table 5 show the effect of combinations B995 and GA_{4+7}. As with GA_3, GA_{4+7} overcomes dark inhibition of germination and even promotes at 20°C. GA_{4+7} is active at an order of magnitude of molarity below that of GA_3. Although a significant interaction is shown at 20°C between GA_{4+7} and B995 no such interaction can be shown at 30°C despite the visible lack of parallelism at different concentrations of GA_{4+7}. This experiment was repeated on more than six occasions without obtaining a significant interaction. Table 6 shows the effect of red light on overcoming B995. If our premise, enunciated in the introduction, is correct, then red light will be unable to overcome the effect of B995. Clearly this is not the case and furthermore, there is a statistical interaction between red light and B995. In view of this result it was decided to examine the effects of cytokinins and B995 in combination. Both kinetin (Table 8) and zeatin (Table 7) were shown to overcome thermodormancy, albeit to a lesser degree than either GA_3 or GA_{4+7}, and both promoted germination in the presence of B995. Zeatin showed no interaction at 30°C or 20°C with B995 whilst kinetin, tested only at 30°C did show an interaction. If we look at Figure 20, Table 24, it can be seen that there is no escape from the inhibiting action of B995 with time, but the effectiveness of GA_{4+7} does increase with time. Thus, with B995 we have shown that the effect can be partially overcome by a number

of treatments, application of GA_3, GA_{4+7}, kinetin or zeatin and by red light. Interactions were shown in all experiments except B995 and GA_{4+7} at 30°C or zeatin at 30°C or 20°C.

Tables 9-13 show a parallel set of experiments using the growth retardant C.C.C., whilst Tables 14-18 show the results using phosphon D and Tables 19-23, Amo 1618. With these three retardants significant interactions were shown with GA_3 and GA_{4+7} in all cases at 30°C and 20°C. This contrasts with the case of B995 where no interaction was shown with GA_{4+7} at 30°C. Figures 21-23 show time course studies for these three retardants with GA_{4+7} at 30°C. As with B995 there is no escape from the inhibiting action of the retardants when seeds were kept in the test solutions, but increased germination was shown in the presence of GA_{4+7} with increasing time. The most effective inhibitor of germination, of these four retardants, was phosphon D. At 10^{-2} M phosphon D inhibition of germination cannot be overcome by any treatment at 30°C. The effect of red light on the depression of germination caused by C.C.C., Amo 1618 or phosphon D is presented in Tables 11, 16 and 21. Germination is promoted, in the presence of growth retardant, by red light, the extent of germination being dose dependent. Interactions can be seen between light and the test compound in all but two cases, these being Amo 1618 at 30°C and phosphon D at 20°C.

Both kinetin and zeatin showed no statistically significant interactions with C.C.C. or Amo 1618 at either 30°C or 20°C (Tables 12, 13, 22, 23). Phosphon D showed a significant interaction with both kinetin and zeatin at 30°C but not with zeatin at 20°C (Tables 17, 18). As was the case with B995, these three retardants, C.C.C., phosphon D and Amo 1618 could have their inhibitory effects on germination reduced by kinetin and zeatin, although to a lesser degree than by red light or gibberellins. Zeatin was more effective than kinetin in reversing the germination inhibition of the growth retardants. Several of these experiments were repeated with a different seed batch and similar results were obtained (data not shown).

Discussion

In the introduction it was assumed that growth retardants owe their actions to an ability to inhibit the biosynthesis of gibberellins.

Before discussing the results presented, it is pertinent to consider the validity of this statement.

The early work on growth retardants was reviewed by Cathey (1964). Evidence that growth retardants inhibited gibberellin biosynthesis came from the observation that the inhibition of stem growth induced by retardants could frequently be overcome by exogenous gibberellin (Cathey and Stuart, 1961; Lane, 1970). With respect to seed germination growth retardants inhibit germination in seeds of a number of species (Wittwer and Tolbert, 1960; Cathey and Stuart, 1961; McDonough, 1965; Kupyl, 1967a,b; Ross and Bradbeer, 1971; Vyas and Garg, 1973). Measurement of extractable gibberellin like activity, after treatment with growth retardants has shown that there is a reduction in the levels of gibberellins in several systems (Zeevaart, 1966; Reid and Carr, 1967; Dale and Filippe, 1968; Cleland and Zeevaart, 1970; Ross and Bradbeer, 1971b; Ryugo and Sansavini, 1972). Furthermore, there is evidence for qualitative changes in the endogenous gibberellins (Reid and Carr, 1967; Snir and Kessler, 1975). It has been proposed that this reduction in the level of gibberellins results from the growth retardant interfering with gibberellin biosynthesis. Evidence in support of this proposal comes from studies on fungi, cell free systems and plant material. Four growth retardants have been used in this study, B995, C.C.C., phosphon D and Amo 1618. There is evidence that all four can inhibit gibberellin biosynthesis. Amo 1618 and C.C.C. have been shown to inhibit the synthesis of gibberellins from mevalonate in Gibberella fujikuroi (Fusarium moniliforme) by preventing the conversion of geranyl-geranyl pyrophosphate to (-)-kaurene (Kende et al, 1963; Barnes et al., 1969; Cross and Myers, 1969). Schechter and West (1969) showed that Amo 1618 and C.C.C. inhibited the action of copalyl pyrophosphate synthetase, an enzyme involved in the conversion of geranyl-geranyl pyrophosphate to copalyl pyrophosphate, this being an intermediate in the cyclization reaction from geranyl-geranyl pyrophosphate to (-)-kaurene. This inhibition was shown in cell free homogenates of Gibberella fujikuroi and Ricinus communis seedlings (Shechter and West, 1969; Robinson and West, 1970). Furthermore phosphon D inhibits the step from copalyl pyrophosphate to (-)-kaurene (Shechter and West, 1969). Amo 1618 has been shown to inhibit kaurene formation in immature pea

seeds (Baldev et al., 1965), in cell free homogenates of pea fruits (Graebe, 1968; Anderson and Moore, 1967), shoot tips of pea (Coolbaugh et al., 1973) and endosperm from Echinocytosis macrocarpa (Barendse and Kok, 1971). C.C.C. has also been shown to inhibit kaurene formation (Anderson and Moore, 1967; Barendse and Kok, 1971) although Dennis et al. (1965) indicated that C.C.C. and B995 were active in inhibiting gibberellin biosynthesis at a step past kaurene formation. B995 has also been reported to disrupt normal gibberellin biosynthesis in pea root tips (Wylie et al., 1970) and the same group showed that the formation of kauren-19-ol was inhibited by B995 in peach ovules (Ryugo and Sachs, 1969). Amo 1618 has no effect on GA_3 induced α-amylase synthesis and subsequent hydrolysis of starch reserves by embryoless barley half seeds (Paleg et al., 1965), implying that Amo 1618 does not interfere with the action of gibberellin. It would appear that growth retardants might well act by inhibiting gibberellin biosynthesis, thus reducing the endogenous gibberellins. It should be pointed out that the biochemical work has in the main been carried out using cell free systems or fungi.

Contrary to the evidence presented, to support the concept that growth retardants interfere with gibberellin biosynthesis, there is increasing evidence to suggest that growth retardants may have other modes of action. Hedley and Stoddart (1971) showed that C.C.C. at a concentration of 10^{-2} M and gibberellic acid (3×10^{-4} M) both decreased leaf alanine aminotransferase activity in intact plants of Lolium temulentum. It would not appear that growth retardants are specific 'anti gibberellins'. Kuypl (1964) showed that C.C.C. could inhibit elongation of etiolated sunflower segments, a response specifically promoted by auxin. The involvement of B995 with auxin has also been proposed by Reed et al. (1965) who demonstrated a correlation between the inhibition of shoot elongation in peas by B995, with the inhibition of the oxidation of trytomine-2-^{14}C to indoleacetaldehyde-2-^{14}C in homogenates of young epicotyls. With respect to lettuce seeds, root growth is inhibited by auxin like compounds, an inhibition which is removed by the addition of C.C.C. (Khan and Tolbert, 1966). Indirect evidence against growth retardants acting solely on gibberellin biosynthesis comes from the effect of growth retardants on a number of systems where the results are difficult to correlate with an effect on gibberellin biosynthesis

(Sachs and Wohlers, 1964; Cleland, 1965; Berry and Smith, 1970; Gaspar et al., 1971; Borchart, 1972; Greenwald, 1972; Crozier et al., 1973; Wellburn et al., 1973).

There is also evidence that growth retardants can increase and not decrease endogenous gibberellins (Bragt, 1969; Halevy and Shilo, 1970; Reid and Crozier, 1970 a, b; Pereira, 1970; Snir and Kessler, 1975). Reid and Crozier (1970 a, b) showed that C.C.C. at 1,000 mg/l applied to pea seedlings caused dwarfing but did not reduce the level of endogenous gibberellins. At 1 mg/l C.C.C. led to a 150-fold increase in gibberellin like compounds, although there was no parallel stimulation in growth. It seems probable that the various growth retardants may have more than one mode of action (Cleland, 1965). If we do not accept an inhibition of gibberellin biosynthesis as a universal mode of action for growth retardants, we should attempt to offer alternatives. Phosphon D at physiological levels, 2×10^{-6} M (1 ppm), has been shown to adversely affect both DNA and protein synthesis. (Borchart, 1972). C.C.C. inhibits chlorophyll synthesis and 3H-leucine incorporation into protein, in barley leaf sections (Berry and Smith, 1970). Thus, there is some evidence that growth retardants can affect the protein synthesising machinery. Whether this is a direct effect, or merely reflects some earlier action is debatable. In work with abscisic acid (Brown and Sun, 1973) we discussed the possibility that effects of abscisic acid on protein synthesis might reflect an earlier action on the membranes. Could it be possible that growth retardants interact in some way with membranes as we have suggested for plant hormones ? Certainly, Wain (1970) believes that as some growth retardants are quaternary ammonium derivatives, their structural properties are ideal for an interaction with plant membranes, perhaps affecting membrane potential. C.C.C. has been shown to enhance the penetration of fungicide into seedlings (Intrieri and Ryngo, 1972), whilst B995 may alter membrane permeability in almond seedlings indirectly by altering respiratory energy necessary for the retention of solutes in the vacuole (Undurraga and Ryugo, 1970). Furthermore, Douglas and Paleg (1974) have reported that growth retardants can inhibit sterol biosynthesis in tobacco seedlings. Sterols have both structural and functional significance in plant membranes !

The evidence for and against growth retardants operating through their influence on gibberellin biosynthesis has been presented, and

it is obvious that no certain conclusion can be reached, in favour or against the hypothesis. What should be clear is that higher plants are much more complex and highly organised than cell free homogenates, from which much of the biochemical evidence in favour of the hypothesis has been gathered. However, there are problems of penetration and breakdown of growth retardants in higher plants which may confuse the interpretation of results. There is no evidence to show the pattern of uptake of growth retardants in lettuce seeds or whether they are metabolised.

If we accept that growth retardants do act by inhibiting gibberellin biosynthesis, the results presented in this study cannot support the view that red light increases the endogenous gibberellins of lettuce seeds. If red light causes the production of gibberellins in seeds we would have expected that this synthesis would have been stopped by the presence of growth retardants. The seeds would then have responded solely to applied gibberellins. However, the interactions shown, indicate that the action of exogenous gibberellin is inhibited by the presence of growth retardants. Furthermore, red light was shown capable of overcoming the growth retardant inhibition of germination.

We have shown that cytokinins are also capable of reversing the effects of growth retardants. This is not unique to lettuce seeds, there being several reports for other seeds (Kupyl, 1967 a, b; Pavevitch et al., 1971; Vyas and Garg, 1973; Chawan and Sen, 1974). Furthermore Skene (1970) has shown that C.C.C. leads to increases in cytokinin like compounds in bleeding sap of grape seedlings. It is suggested that C.C.C. acts directly on the root meristem to increase cytokinin production. Berrie and Robertson (1973) have previously stated that - "it cannot be conceived that the claimed specificity of B995, C.C.C. and phosphon D in the prevention of gibberellin synthesis would extend to include the biosynthetic system for cytokinins". Recent work by Murai et al. (1975) shows that labelled mevalonic acid, a precursor for gibberellins, was also a precursor of transfer-RNA. Approximately 40% of the label was found in the cytokinin component of the transfer-RNA. Whilst it is not suggested that this is specific incorporation of label (the authors suggest it is random incorporation resulting from utilization of degraded ^{14}C-2-mevalonic acid in RNA synthesis) it indicates

that the biosynthetic pathways may be more closely linked than previously thought.

It would appear that any alteration in the utilization of the mevalonate pool results in widespread changes, amongst which might be an alteration of the t-RNA level. The growth retardants by acting on gibberellin biosynthesis will indirectly affect the utilization of the mevalonate pool and their secondary effects could be as important in seed germination as any primary effect.

In conclusion, the evidence does not wholly support the premise on which this work was based, that is, that growth retardants are specific inhibitors of gibberellin biosynthesis. In view of this fact little can be said as regards the role of red light in gibberellin biosynthesis. Even if we accept that the results presented are incompatible with the hypothesis that red light increases endogenous gibberellins via increased synthesis, this work cannot rule out red light causing an increased availability of gibberellin. The results presented do show that growth retardants interact with red light and in most cases with gibberellins, but with cytokinins interaction is the exception rather than the rule.

An interesting observation made during this work was the appearance of a characteristic syndrome after 24 hrs imbibition in phosphon D at 10^{-2} M at 30°C.

The pericarp splits, but the endosperm remains intact, expanding to appear as though gorged with water. Liquid under pressure is contained within this structure, for on piercing with a needle the liquid is forcibly expelled. This phenomenon was previously noted by Ikuma and Thimann (1960).

Table 4. The average percentage germination of lettuce seed treated with gibberellin A_3 and B995 after 24 hrs.

Temp.	Conc^{n}. GA_3 M	Concentration B995 M				Marginal total
		0	10^{-4}	10^{-3}	10^{-2}	
30°C	0	10.7	12.3	7.3	0	30.3
	10^{-5}	24.7	24.0	30.7	26.3	105.7
	10^{-4}	50.3	48.7	65.0	37.7	201.7
	10^{-3}	78.0	74.0	75.3	37.7	265.0
	Marginal total	163.7	159.0	178.3	101.7	602.7
20°C	0	87.3	83.0	79.7	31.0	281.0
	10^{-5}	92.7	95.3	94.0	95.0	377.0
	10^{-4}	96.0	96.3	95.7	95.3	383.3
	10^{-3}	96.3	96.3	95.0	93.3	380.9
	Marginal total	372.3	370.9	364.4	314.6	1,422.2

Analysis of variance; F values, degrees of freedom

		Replicates	GA_3	B995	Interaction	Error
df.		2	3	3	9	30
30°	Exp. a	1	22.0	178.0	5.1	(27.2)
		N.S.	****	****	****	
	Exp. b	2.3	42.0	326.0	12.5	(9.6)
		N.S.	****	****	****	
20°	Exp. a	1	17.3	74.0	10.2	(17.4)
		N.S.	****	****	****	
	Exp. b	1	24.8	102.0	24.0	(9.6)
		N.S.	****	****	****	

Table 5 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7} and B995 after 24 hrs.

Temp.	Conc^{n}. GA_{4+7} M	Concentration B995 M				Marginal total
		0	10^{-4}	10^{-3}	10^{-2}	
30°C	0	6.3	5.7	2.7	0	14.7
	10^{-6}	22.7	24.3	24.0	19.7	90.7
	10^{-5}	62.3	56.0	60.0	31.3	209.6
	10^{-4}	60.7	59.7	48.0	39.0	207.4
	Marginal total	152.0	145.7	134.7	90.0	522.4
20°C	0	80.3	70.0	69.0	24.7	244.0
	10^{-6}	93.3	94.0	96.0	95.3	378.6
	10^{-5}	96.0	95.7	94.7	92.3	378.7
	10^{-4}	96.3	95.0	95.3	93.7	380.3
	Marginal total	365.9	354.7	355.0	306.0	1,381.6

Analysis of variance; F values and degrees of freedom

		Replicates	GA_{4+7}	B995	Interaction	Error
df.		2	3	3	9	30
30°	Exp. a	1.2 N.S.	110.4 ****	11.0 ****	0.7 N.S.	(32.80)
	Exp. b	1.0 N.S.	140.1 ****	12.7 ****	1.9 N.S.	(28.82)
20°	Exp. a	1.0 N.S.	260.0 ****	17.1 ****	14.5 ****	(9.86)
	Exp. b	1.0 N.S.	143.4 ****	32.2 ****	19.8 ****	(9.39)

Table 6 . The average percentage germination of lettuce seed treated with red light and B995 after 24 hours.

Temp.	Seconds red light	Concentration B995 M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C	0.0	10.0	8.0	4.7	0.0	22.7
	30.0	16.0	12.0	9.7	2.3	40.0
	60.0	23.7	25.0	14.7	1.0	64.4
	120.0	47.0	36.7	33.0	4.0	120.7
	Marginal total	96.7	81.7	62.1	7.3	247.8
20°C	0.0	82.3	80.3	79.3	31.0	272.9
	30.0	90.0	90.0	87.3	48.0	315.3
	60.0	90.3	93.3	90.3	68.3	342.2
	120.0	95.3	95.3	94.4	90.3	375.2
	Marginal total	357.9	358.9	351.2	237.6	1,305.6

Analysis of variance; F values and degrees of freedom

		Replicates	Red Light	B995	Interaction	Error
df.		2	3	3	9	30
30°	Exp. a	1.0	83.4	139.2	3.6	(11.2)
		N.S.	****	****	****	
	Exp. b	1.0	92.9	115.4	4.7	(8.7)
		N.S.	****	****	****	
20°	Exp. a	1.0	49.8	78.5	5.8	(15.3)
		N.S.	****	****	****	
	Exp. b	1.0	47.9	71.9	6.9	(17.5)
		N.S.	****	****	****	

Table 7 . The average percentage gerination of lettuce seed treated with Zeatin and B995 after 24 hours.

Temp.	Concentration Zeatin M	Concentration B995 M				Marginal total
		0	10^{-4}	10^{-3}	10^{-2}	
30°C	0	4.2	2.9	3.6	0	10.7
	10^{-6}	32.7	36.9	24.7	2.7	97.0
	10^{-5}	38.9	35.1	39.1	5.8	118.9
	Marginal total	75.8	74.9	67.4	8.5	226.6
20°C	0	72.2	67.3	57.3	20.5	217.3
	10^{-6}	80.7	80.9	74.7	27.1	263.4
	10^{-5}	81.8	81.8	74.9	41.6	280.1
	Marginal total	234.7	230.0	206.9	89.2	760.8

Analysis of variance; F values and degrees of freedom

		df.	Experiment a		Experiment b		Experiment c	
30°	Replicates	2	1.0	N.S.	1.0	N.S.	1.0	N.S.
	B995	3	24.2	****	36.0	****	26.6	****
	Zeatin	2	37.9	****	72.4	****	86.5	****
	Interaction	6	2.9	N.S.	3.3	N.S.	2.8	N.S.
	Error	22	(42.7)		(27.4)		(21.5)	
20°	Replicates	2	1.0	N.S.	1.0	N.S.	1.0	N.S.
	B995	3	142.9	****	98.8	****	45.7	****
	Zeatin	2	17.00	****	20.2	****	13.8	****
	Interaction	6	2.2	N.S.	2.4	N.S.	1.3	N.S.
	Error	22	(19.0)		(17.2)		(26.0)	

Table 8 . The average percentage germination of lettuce seed treated with Kinetin and B995 after 24 hrs.

Temp.	Concentration Kinetin M	Concentration B995 M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C	0	5.0	5.3	2.7	0.7	13.7
	10^{-5}	25.7	31.3	23.7	1.0	81.7
	10^{-4}	39.3	32.3	30.7	5.7	108.0
	Marginal total	70.0	68.9	57.1	7.4	203.4

Analysis of variance; F.values and degrees of freedom

		df.	Experiment a		Experiment b	
	Replicates	2	1.0	N.S.	1.6	N.S.
	B995	3	40.6	****	69.9	****
30°	Kinetin	2	67.2	****	111.5	****
	Interaction	6	6.9	****	4.6	****
	Error	22	(22.7)		(12.2)	

Table 9 . The average percentage germination of lettuce seed treated with gibberellin A_3 and C.C.C. after 24 hrs.

Temp.	Conc^{n}. GA_3 M	Concentration C.C.C. M				Marginal total
		0	$5x10^{-4}$	$5x10^{-3}$	$5x10^{-2}$	
30°C	0	4.3	3.3	3.0	1.0	11.6
	10^{-5}	14.7	11.0	8.3	2.7	36.7
	10^{-4}	42.3	35.7	37.0	3.3	118.3
	10^{-3}	62.0	59.7	46.7	4.3	172.7
	Marginal total	123.3	109.7	95.0	11.3	339.3
20°	0	72.0	69.7	67.7	18.3	227.7
	10^{-5}	88.7	90.0	88.0	34.7	301.4
	10^{-4}	96.0	94.3	93.7	83.3	367.3
	10^{-3}	96.0	95.3	95.7	91.7	378.7
	Marginal total	352.7	349.3	345.1	228.0	1,275.1

Analysis of variance; F values and degrees of freedom

		Replicates	C.C.C.	GA_3	Interaction	Error
df.		2	3	3	9	30
30°	Exp. a	1.0	31.8	58.6	4.2	(37.8)
		N.S.	****	****	****	
	Exp. b	1.0	65.6	93.1	8.5	(25.8)
		N.S.	****	****	****	
20°	Exp. a	1.1	145.2	245.1	13.9	(9.32)
		N.S.	****	****	****	
	Exp. b	1.0	95.9	135.1	17.3	(12.6)
		N.S.	****	****	****	

Table 10 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7} and C.C.C. after 24 hrs.

Temp.	Conc^{n} GA_{4+7}	Concentration C.C.C. M 0	$5x10^{-4}$	$5x10^{-3}$	$5x10^{-2}$	Marginal total
30°C	0	7.7	6.3	3.3	0.7	18.0
	10^{-6}	21.7	19.3	20.0	1.7	62.7
	10^{-5}	55.0	51.0	43.7	2.7	152.4
	10^{-4}	62.3	59.3	50.3	3.7	175.6
	Marginal total	146.7	136.0	117.3	8.8	408.7
20°C	0	67.0	69.7	56.3	20.7	213.7
	10^{-6}	92.7	94.0	87.0	58.0	331.7
	10^{-5}	96.7	93.0	93.7	82.3	365.7
	10^{-4}	95.3	95.0	95.7	90.0	376.0
	Marginal total	351.7	351.7	332.7	251.0	1,287.1

Analysis of variance; F values and degrees of freedom

		Replicates	C.C.C.	GA_{4+7}	Interaction	Error
df.		2	3	3	9	30
30°	Exp. a	1.0	111.3	102.7	6.4	(24.0)
		N.S.	****	****	****	
	Exp. b	1.0	84.8	91.4	7.5	(21.4)
		N.S.	****	****	****	
20°	Exp. a	1.0	92.2	171.8	8.3	(11.2)
		N.S.	****	****	****	
	Exp. b	1.0	68.3	206.1	8.2	(11.5)
		N.S.	****	****	****	

Table 11 . The average percentage germination of lettuce seed treated with red light and C.C.C.

Temp.	Seconds red light	Concentration C.C.C. 0	$5x10^{-4}$	$5x10^{-3}$	$5x10^{-2}$	Marginal total
30°C	0	2.7	3.3	4.3	0.0	10.3
	30	16.3	11.0	7.0	1.3	35.6
	60	32.7	20.7	21.3	0.3	75.0
	120	54.3	55.7	50.3	5.0	165.3
	Marginal total	106.0	90.7	82.9	6.6	286.2
20°C	0	82.3	80.0	74.7	24.0	261.0
	30	87.7	91.0	80.3	44.3	303.3
	60	92.0	90.3	92.7	70.7	345.7
	120	94.3	94.3	92.3	85.7	366.6
	Marginal total	356.3	355.6	340.0	224.7	1,276.6

Analysis of variance; F values and degrees of freedom

		Replicates	C.C.C.	Red light	Interaction	Error
df.		2	3	3	9	30
30°	Exp. a	1.0	45.8	56.4	3.8	(34.1)
		N.S.	****	****	****	
	Exp. b	1.0	74.9	129.2	7.6	(18.4)
		N.S.	****	****	****	
20°	Exp. a	1.4	90.9	70.5	11.8	(12.1)
		N.S.	****	****	****	
	Exp. b	1.0	88.5	46.8	5.3	(18.8)
		N.S.	****	****	****	

Table 12 . The average percentage germination of lettuce seed treated with Zeatin and C.C.C. after 24 hours.

Temp.	Concentration Zeatin M	Concentration C.C.C. M				Marginal total
		0	$5x10^{-4}$	$5x10^{-3}$	$5x10^{-2}$	
30°C	0	5.3	4.4	4.9	0.0	14.6
	10^{-6}	36.0	30.4	26.4	4.0	96.8
	10^{-5}	39.3	37.8	35.1	4.9	117.1
	Marginal total	80.6	72.6	66.4	8.9	228.5

Analysis of variance; F values and degrees of freedom

		df.	Experiment a		Experiment b		Experiment c	
	Replicates	2	1.0	N.S.	1.0	N.S.	1.0	N.S.
	C.C.C.	3	67.9	****	35.6	****	52.8	****
30°	Zeatin	2	118.7	****	62.2	****	126.7	****
	Interaction	6	2.9	N.S.	2.4	N.S.	3.0	N.S.
	Error	22	(14.9)		(25.6)		(15.8)	

Table 13 . The average percentage germination of lettuce seed treated with Kinetin and C.C.C. after 24 hours.

Temp.	Concentration Kinetin M	Concentration C.C.C. M				Marginal total
		0	$5x10^{-4}$	$5x10^{-3}$	$5x10^{-2}$	
30°C	0	4.0	3.7	2.7	0	10.4
	10^{-5}	25.7	26.7	20.3	3.0	75.7
	10^{-4}	41.3	38.0	27.3	4.3	110.9
	Marginal total	71.0	68.4	50.3	7.3	197.0
20°C	0	80.7	73.0	73.0	24.0	250.7
	10^{-5}	81.3	77.3	78.3	28.3	265.2
	10^{-4}	81.3	84.7	80.0	32.0	278.0
	Marginal total	243.3	235.0	231.3	84.3	793.9

Analysis of variance; F values and degrees of freedom

		Replicates	C.C.C.	Kinetin	Interaction	Error
df.		2	3	2	6	22
30°	Exp. a	1.0	38.2	57.2	1.7	(26.8)
		N.S.	****	****	N.S.	
	Exp. b	1.0	21.3	73.2	2.6	(27.1)
		N.S.	****	****	N.S.	
20°	Exp. a	1.1	112.5	4.3	1.1	(18.7)
		N.S.	****	N.S.	N.S.	
	Exp. b	1.0	159.7	3.5	0.6	(14.2)
		N.S.	****	N.S.	N.S.	

Table 14 . The average percentage germination of lettuce seed treated with gibberellin A_3 and Phosphon D after 24 hrs.

Temp.	Concentration GA_3 M	Concentration Phosphon D. M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C	0	21.0	14.3	2.3	0.0	37.6
	10^{-5}	29.7	25.3	4.3	0.0	59.3
	10^{-4}	61.0	40.3	14.7	0.0	116.0
	10^{-3}	80.7	64.7	41.3	0.0	186.7
	Marginal total	192.4	144.6	62.6	0.0	399.6
20°C	0	81.7	85.0	43.7	0.7	211.1
	10^{-5}	92.3	91.3	74.0	3.0	260.6
	10^{-4}	91.0	96.0	81.3	12.3	286.6
	10^{-3}	96.7	96.0	95.0	43.0	330.7
	Marginal total	361.7	368.3	300.0	59.0	1,089.0

Analysis of variance; F. values and degrees of freedom

		Replicates	Phosphon D	GA_3	Interaction	Error
df.		2	3	3	9	30
30°	Exp. a	1.4	691.0	237.8	30.8	(8.7)
		N.S.	****	****	****	
	Exp. b	1.4	290.0	97.7	17.9	(11.3)
		N.S.	****	****	****	
20°	Exp. a	1.0	227.4	29.6	4.5	(36.0)
		N.S.	****	****	****	
	Exp. b	1.0	850.3	156.8	17.5	(10.2)
		N.S.	****	****	****	

Table 15 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7} and Phosphon D after 24 hrs.

Temp.	Concentration GA_{4+7}	Concentration Phosphon D M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C	0	12.0	8.7	1.0	0.0	21.7
	10^{-6}	31.3	28.0	4.0	0.0	63.3
	10^{-5}	62.0	66.7	23.3	0.0	152.0
	10^{-4}	76.7	74.0	24.3	0.0	175.0
	Marginal total	182.0	177.4	52.6	0.0	412.0
20°C	0	81.3	75.0	35.3	3.7	195.3
	10^{-6}	94.3	95.0	75.3	5.3	269.9
	10^{-5}	96.7	95.0	90.0	13.0	294.7
	10^{-4}	97.3	96.3	92.7	27.0	313.3
	Marginal total	369.6	361.3	293.3	49.0	1,074.2

Analysis of variance; F. values, and degrees of freedom

		Replicates	Phosphon D	GA_{4+7}	Interaction	Error
df.		2	3	3	9	30
30°	Exp. a	1.0	364.0	139.7	18.0	(13.8)
		N.S.	****	****	****	
	Exp. b	1:0	304.6	131.1	18.7	(15.3)
		N.S.	****	****	****	
20°	Exp. a	1.0	662.6	113.3	10.7	(9.9)
		N.S.	****	****	****	
	Exp. b	1.13	1,012.6	136.9	12.7	(11.3)
		N.S.	****	****	****	

Table 16 . The average percentage germination of lettuce seed treated with red light and Phosphon D. after 24 hrs.

Temp.	Seconds red light	Concentration Phosphon D M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C	0	9.7	8.0	1.0	0.0	18.7
	30	18.3	11.0	2.7	0.0	32.0
	60	24.7	15.7	3.0	0.0	43.4
	120	46.0	27.7	5.3	0.0	79.0
	Marginal total	98.7	62.4	12.0	0.0	173.1
20°C	0	79.0	73.0	51.3	0.7	204.0
	30	88.7	85.3	63.0	3.3	240.3
	60	90.3	88.0	71.7	8.0	258.0
	120	96.0	93.3	89.3	19.7	298.3
	Marginal total	354.0	339.6	275.3	31.7	1,000.6

Analysis of variance; F. value and degrees of freedom

		Replicates	Phosphon D	Red light	Interaction	Error
df.		2	3	3	9	30
30°	Exp. a	1.0	133.3	23.7	5.2	(16.3)
		N.S.	****	****	****	
	Exp. b	1.0	160.8	22.5	4.0	(11.9)
		N.S.	****	****	****	
20°	Exp. a	1.0	448.4	47.9	0.9	(17.7)
		N.S.	****	****	N.S.	
	Exp. b	1.0	511.6	47.9	2.2	(18.3)
		N.S.	****	****	N.S.	

Table 17 . The average percentage germination of lettuce seed treated with Zeatin and Phosphon D after 24 hours.

Temp.	Concentration Zeatin M	Concentration Phosphon D M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
	0	3.6	5.3	1.1	0.0	10.0
30°C	10^{-6}	34.7	25.8	8.2	0.0	68.7
	10^{-5}	40.4	37.6	12.9	0.0	90.9
	Marginal total	78.7	68.7	22.2	0.0	169.6
	0	69.1	71.6	43.1	0.4	184.2
20°C	10^{-6}	76.7	79.6	54.9	2.2	213.4
	10^{-5}	81.3	85.3	64.0	3.6	234.2
	Marginal total	227.1	236.5	162.0	6.2	631.8

Analysis of variance; F values and degrees of freedom

		df.	Experiment a		Experiment b		Experiment c	
	Replicates	2	1.0	N.S.	1.0	N.S.	1.0	N.S.
	Phosphon D	3	66.4	****	139.5	****	118.8	****
30°C	Zeatin	2	33.8	****	100.1	****	96.1	****
	Interaction	6	6.1	****	15.1	****	13.6	****
	Error	22	(26.4)		(10.2)		(13.5)	
	Replicates	2	1.0	N.S.	1.0	N.S.	1.0	N.S.
	Phosphon D	3	257.7	****	351.0	****	158.7	****
20°C	Zeatin	2	11.6	****	11.5	****	8.4	N.S.
	Interaction	6	0.8	N.S.	1.4	N.S.	0.6	N.S.
	Error	22	(23.4)		(21.3)		(37.6)	

Table 18 . The average percentage germination of lettuce seed treated with Kinetin and Phosphon D.

Temp.	Concentration Kinetin M	Concentration Phosphon D M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C	0	7.3	1.7	0.0	0.0	9.0
	10^{-5}	19.3	16.0	0.7	0.0	36.0
	10^{-4}	38.3	26.3	1.0	0.0	65.6
	Marginal total	64.9	44.0	1.7	o.o	110.6

Analysis of variance; F values and degrees of freedom

		df.	Experiment a		Experiment b	
	Replicates	2	1.0	N.S.	1.0	N.S.
	Phosphon D	3	91.9	****	342.7	****
30°C	Kinetin	2	38.0	****	79.7	****
	Interaction	6	8.8	****	28.5	****
	Error	22	(17.9)		(4.3)	

Table 19 . The average percentage germination of lettuce seed treated with gibberellin A_3 and Amo-1618 after 24 hrs.

Temp.	Concentration GA_3 M	Concentration Amo-1618 M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C.	0	3.0	2.3	0.3	0.0	5.6
	10^{-5}	8.7	9.3	5.7	0.0	23.7
	10^{-4}	37.0	36.3	28.7	3.7	105.7
	10^{-3}	55.7	48.0	56.3	13.3	173.3
	Marginal total	104.4	95.9	91.0	17.0	308.3
20°C	0	80.7	71.3	66.3	38.3	250.6
	10^{-5}	94.0	93.0	92.7	79.3	359.0
	10^{-4}	96.0	95.3	95.3	85.7	372.3
	10^{-3}	96.3	97.0	95.3	89.0	377.6
	Marginal total	367.0	356.6	349.6	292.3	1,365.5

Analysis of variance; F values and degrees of freedom

		Replicates	Amo-1618	GA_3	Interaction	Error
	df.	2	3	3	9	30
30°	Exp. a	1.07	40.39	140.66	4.31	(20.31)
		N.S.	****	****	****	
	Exp. b	1.0	68.42	196.17	4.78	(18.52)
		N.S.	****	****	****	
20°	Exp. a	1.0	42.89	69.34	4.30	(11.95)
		N.S.	****	****	****	
	Exp. b	1.27	78.54	295.57	7.50	(6.9)
		N.S.	****	****	****	

Table 20 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7} and Amo-1618 after 24 hrs.

Temp.	Concentration GA_{4+7} M	Concentration Amo-1618 M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C	0	4.0	2.0	1.3	0.0	7.3
	10^{-6}	23.0	14.7	10.3	2.3	50.3
	10^{-5}	58.0	55.7	47.0	9.3	170.0
	10^{-4}	63.0	62.7	55.7	13.7	195.1
	Marginal total	148.0	135.1	114.3	25.3	422.7
20°C	0	64.0	67.7	39.7	20.0	191.4
	10^{-6}	90.7	88.3	89.0	70.3	338.3
	10^{-5}	93.3	95.3	94.3	88.7	371.6
	10^{-4}	96.0	96.7	94.7	91.7	379.1
	Marginal total	344.0	348.0	317.7	270.7	1,280.4

Analysis of variance; F values and degrees of freedom

		Replicates	Amo-1618	GA_{4+7}	Interaction	Error
	df.	2	3	3	9	30
30°	Exp. a	1.0	77.5	209.7	5.0	(17.5)
		N.S.	****	****	****	
	Exp. b	1.0	65.1	192.9	6.3	(21.2)
		N.S.	****	****	****	
20°	Exp. a	2.0	37.7	273.3	10.0	(12.7)
		N.S.	****	****	****	
	Exp. b	1.0	37.4	169.5	5.9	(13.7)
		N.S.	****	****	****	

Table 21 . The average percentage germination of lettuce seed treated with red light and Amo-1618.

Temp.	Seconds red light	Concentration Amo-1618 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C	0	5.3	4.0	1.7	0.7	11.7
	30	11.0	6.3	5.3	2.0	24.6
	60	26.0	19.3	13.3	3.0	61.6
	120	52.7	50.0	35.0	14.3	152.0
	Marginal total	95.0	79.6	55.3	20.0	249.9
20°C	0	78.7	73.7	58.3	36.0	246.7
	30	89.7	83.3	77.7	53.3	304.0
	60	91.0	90.3	86.3	69.0	336.6
	120	96.0	94.0	94.3	89.0	373.3
	Marginal total	355.4	341.3	316.6	247.3	1,260.6

Analysis of variance; F values and degrees of freedom

		Replicates	Amo-1618	Red light	Interaction	Error
df		2	3	3	9	30
30°	Exp. a	1.0	21.6	64.9	1.3	(33.4)
		N.S.	****	****	N.S.	
	Exp. b	1.0	30.6	84.6	1.5	(20.7)
		N.S.	****	****	N.S.	
20°	Exp. a	1.3	71.0	113.5	4.3	(10.5)
		N.S.	****	****	****	
	Exp. b	1.0	125.9	165.7	7.9	(6.8)
		N.S.	****	****	****	

Table 22 . The average percentage germination of lettuce seed treated with Zeatin and Amo-1618.

Temp.	Concentration Zeatin M	Concentration Amo-1618 M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C	0	5.0	2.7	1.5	0.0	9.2
	10^{-6}	32.0	24.0	12.2	3.3	71.5
	10^{-5}	38.5	27.8	17.5	6.3	90.1
	Marginal total	75.5	54.5	31.2	9.6	170.8

Analysis of variance; F values and degrees of freedom

		Replicates	Amo-1618	Zeatin	Interaction	Error
df		2	3	2	6	22
30°C	Exp. a	1.0	39.81	54.15	1.50	(22.74)
		N.S.	****	****	N.S.	
	Exp. b	1.0	46.15	20.23	1.59	(30.32)
		N.S.	****	****	N.S.	
	Exp. c	1.0	30.26	78.06	2.06	(23.61)
		N.S.	****	****	N.S.	
	Exp. d	1.0	36.20	89.22	2.01	(16.97)
		N.S.	****	****	N.S.	

Table 23 . The average percentage germination of lettuce seed treated with Kinetin and Amo-1618.

Temp.	Concn. Kinetin	Concentration Amo-1618 M 0	10^{-4}	10^{-3}	10^{-2}	Marginal total
30°C	0	5.3	3.0	1.7	0.0	10.0
	10^{-5}	22.0	14.3	13.0	3.0	52.3
	10^{-4}	37.0	28.3	20.0	4.7	90.0
	Marginal total	64.3	45.6	34.7	7.7	152.3
20°C	0	77.0	74.3	51.3	37.3	239.9
	10^{-5}	84.0	76.3	65.3	41.3	266.9
	10^{-4}	85.0	79.3	68.3	45.3	277.9
	Marginal total	246.0	229.9	184.9	123.9	784.7

Analysis of variance; F values and degrees of freedom

		Replicates	Amo-1618	Kinetin	Interaction	Error
df		2	3	2	6	22
30°C	Exp. a	1.0	29.56	64.24	1.3	(20.92)
		N.S.	****	****	N.S.	
	Exp. b	1.0	23.99	45.18	2.14	(26.4)
		N.S.	****	****	N.S.	
20°C	Exp. a	1.0	87.09	9.66	1.41	(12.48)
		N.S.	****	N.S.	N.S.	
	Exp. b	1.0	57.5	6.35	0.4	(21.78)

Table 24 . The average percentage germination of lettuce seed treated with B995 and gibberellin A_{4+7} at 30°C.

Concn. B995 M / GA 4+7	0	10^{-4}	10^{-3}	10^{-2}	
0	10.0 ± 2.1	8.0 ± 1.9	6.0 ± 2.3	0.7 ± 0.4	
10^{-6}	27.0 ± 5.0	30.3 ± 6.4	30.7 ± 6.8	25.7 ± 6.3	
10^{-5}	64.3 ± 7.0	58.3 ± 10.0	61.3 ± 5.8	45.3 ± 9.0	
10^{-4}	72.0 ± 8.6	69.3 ± 4.9	62.3 ± 5.7	40.3 ± 5.3	
					24 hrs
0	20.3 ± 3.8	18.7 ± 4.5	13.3 ± 2.8	0.7 ± 0.4	
10^{-6}	52.3 ± 3.7	53.0 ± 7.2	63.0 ± 5.1	78.3 ± 5.8	
10^{-5}	94.3 ± 1.3	94.0 ± 2.9	97.0 ± 1.4	92.0 ± 1.7	
10^{-4}	97.3 ± 1.4	98.0 ± 1.0	93.3 ± 1.6	89.7 ± 4.4	
					48 hrs
0	21.0 ± 4.8	19.7 ± 4.8	13.7 ± 2.8	1.5 ± 0.5	
10^{-6}	53.0 ± 3.8	54.0 ± 7.1	65.0 ± 4.6	82.3 ± 4.9	
10^{-5}	94.7 ± 0.9	95.0 ± 2.5	96.7 ± 1.1	93.7 ± 0.7	
10^{-4}	98.3 ± 1.0	98.7 ± 0.6	94.7 ± 1.3	92.3 ± 3.0	
					72 hrs

Table. 25 . The average percentage germination of lettuce seed treated with C.C.C. and gibberellin A_{4+7} at 30°C.

Concn. C.C.C. M / GA 4+7	0	10^{-4}	10^{-3}	10^{-2}
0	2.0 ± 0.0	7.3 ± 1.3	2.7 ± 1.3	0
10^{-6}	20.7 ± 2.9	12.7 ± 5.3	10.7 ± 5.7	0
10^{-5}	54.0 ± 4.0	42.0 ± 3.1	26.0 ± 9.2	0
10^{-4}	64.0 ± 6.4	50.0 ± 3.1	24.0 ± 4.2	0
				24 hrs
0	6.7 ± 1.8	15.3 ± 0.7	7.3 ± 1.3	1.3 ± 0.7
10^{-6}	40.0 ± 4.0	47.3 ± 6.4	36.7 ± 7.1	2.0 ± 2.0
10^{-5}	92.0 ± 2.0	97.3 ± 1.5	90.0 ± 2.0	16.0 ± 4.6
10^{-4}	98.7 ± 1.2	95.3 ± 0.9	94.7 ± 0.4	28.0 ± 10.1
				48 hrs
0	6.7 ± 1.8	17.3 ± 1.4	8.7 ± 1.8	2.0 ± 1.2
10^{-6}	41.3 ± 2.5	49.3 ± 8.4	40.7 ± 7.1	4.0 ± 2.0
10^{-5}	92.7 ± 1.2	97.3 ± 1.5	91.3 ± 2.5	24.0 ± 8.3
10^{-4}	98.7 ± 1.2	96.0 ± 0.0	96.7 ± 0.7	56.0 ± 9.2
				72 hrs

Table 26 . The average percentage germination of lettuce seed treated with Phosphon D and gibberellin A_{4+7} at 30°C.

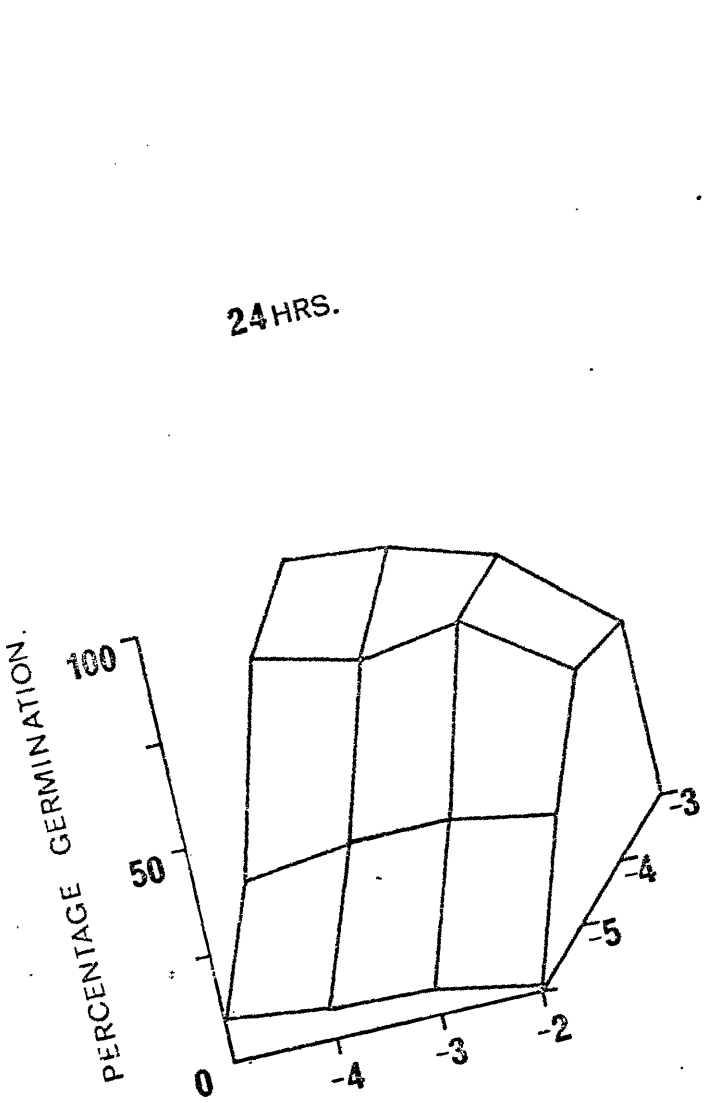
Concn. Phosphon D M / GA_{4+7} M	0	10^{-4}	10^{-3}	10^{-2}
0	4.3 ± 1.7	3.0 ± 1.1	0	0
10^{-6}	16.0 ± 2.2	16.3 ± 2.9	2.0 ± 0.9	0
10^{-5}	42.7 ± 2.3	40.7 ± 5.6	4.7 ± 1.0	0
10^{-4}	65.3 ± 3.1	54.3 ± 4.1	7.0 ± 1.4	0
				24 hrs
0	12.3 ± 2.9	5.3 ± 1.6	0	0
10^{-6}	43.3 ± 3.6	26.7 ± 3.5	2.0 ± 0.9	0
10^{-5}	93.3 ± 1.8	73.0 ± 4.4	6.0 ± 1.4	0
10^{-4}	94.7 ± 1.3	85.7 ± 2.5	7.7 ± 1.8	0
				48 hrs
0	13.3 ± 2.7	6.3 ± 1.2	0.3 ± 0.3	0
10^{-6}	45.3 ± 3.7	27.3 ± 3.6	2.3 ± 0.8	0
10^{-5}	93.7 ± 1.3	75.0 ± 3.8	8.7 ± 1.7	0
10^{-4}	96.0 ± 0.9	88.7 ± 2.0	13.3 ± 2.6	0
				72 hrs

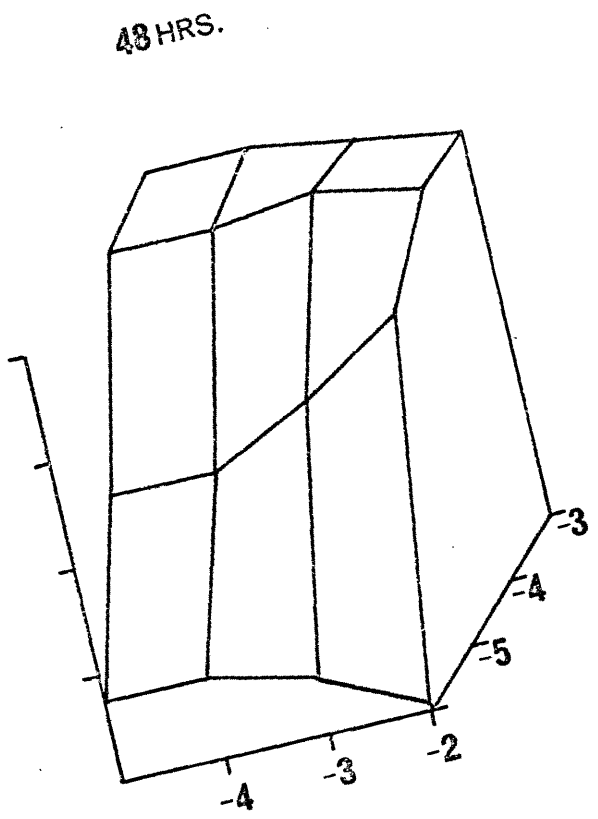
Table 27 . The average percentage germination of lettuce seeds treated with Amo-1618 and gibberellin A_{4+7} at 30°C.

Concn. Amo-1618 M / GA_{4+7} M	0	10^{-4}	10^{-3}	10^{-2}	
0	10.3 ± 1.6	2.7 ± 0.4	3.7 ± 1.6	0.3 ± 0.3	
10^{-6}	29.3 ± 3.4	30.0 ± 2.6	24.3 ± 3.2	3.7 ± 1.2	
10^{-5}	67.0 ± 5.5	69.0 ± 3.3	66.0 ± 2.5	16.7 ± 4.3	
10^{-4}	79.3 ± 3.4	75.0 ± 4.3	74.3 ± 1.9	17.7 ± 2.2	
					24 hrs
0	20.3 ± 4.2	8.7 ± 1.8	6.0 ± 1.6	0.3 ± 0.3	
10^{-6}	61.0 ± 5.2	49.7 ± 3.6	53.7 ± 2.7	6.0 ± 1.3	
10^{-5}	94.3 ± 1.8	96.0 ± 1.4	92.0 ± 1.8	40.7 ± 5.1	
10^{-4}	98.3 ± 1.4	97.0 ± 0.9	95.0 ± 0.7	46.3 ± 6.6	
					48 hrs
0	20.7 ± 4.1	9.0 ± 1.8	6.0 ± 1.6	1.0 ± 0.5	
10^{-6}	61.3 ± 5.0	51.3 ± 4.0	56.7 ± 2.8	6.0 ± 1.3	
10^{-5}	96.0 ± 1.2	96.3 ± 1.1	92.7 ± 1.9	41.3 ± 5.1	
10^{-4}	98.7 ± 1.2	98.0 ± 0.9	95.3 ± 0.7	47.3 ± 6.6	
					72 hrs

FIGURE 20

The percentage germination of seeds of Grand Rapids lettuce in the presence of B995 and GA_{4+7} at 20°C after 24, 48 and 72 hrs.


24 HRS.
PERCENTAGE GERMINATION.
100
50
0
-4
-3
-2
-3
-4
-5


48 HRS.
-4
-3
-2
-3
-4
-5
CONC. B 995. LOG. M.

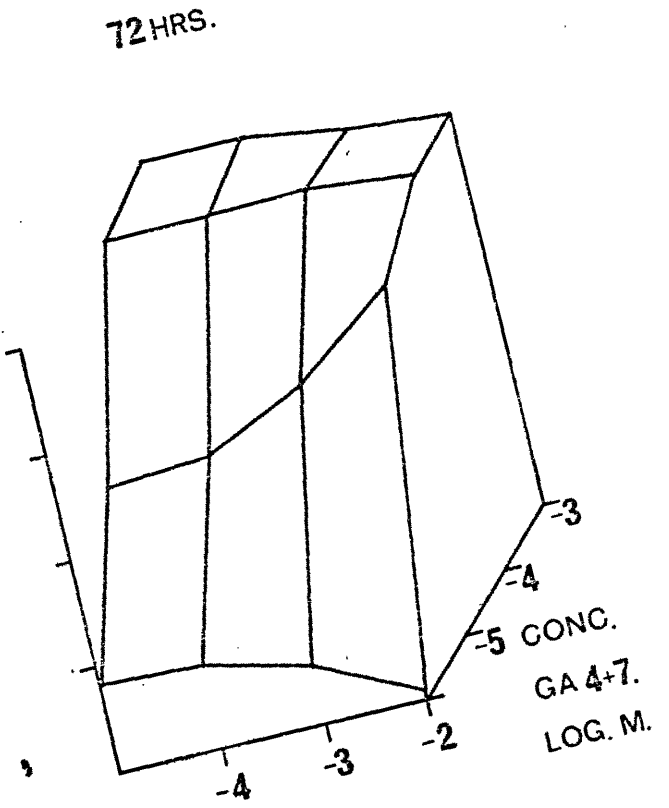

72 HRS.
-4
-3
-2
-3
-4
-5 CONC.
GA 4+7.
LOG. M.

FIGURE 21

The percentage germination of seeds of Grand Rapids lettuce in the presence of C.C.C. and GA_{4+7} at 20°C after 24, 48 and 72 hrs.

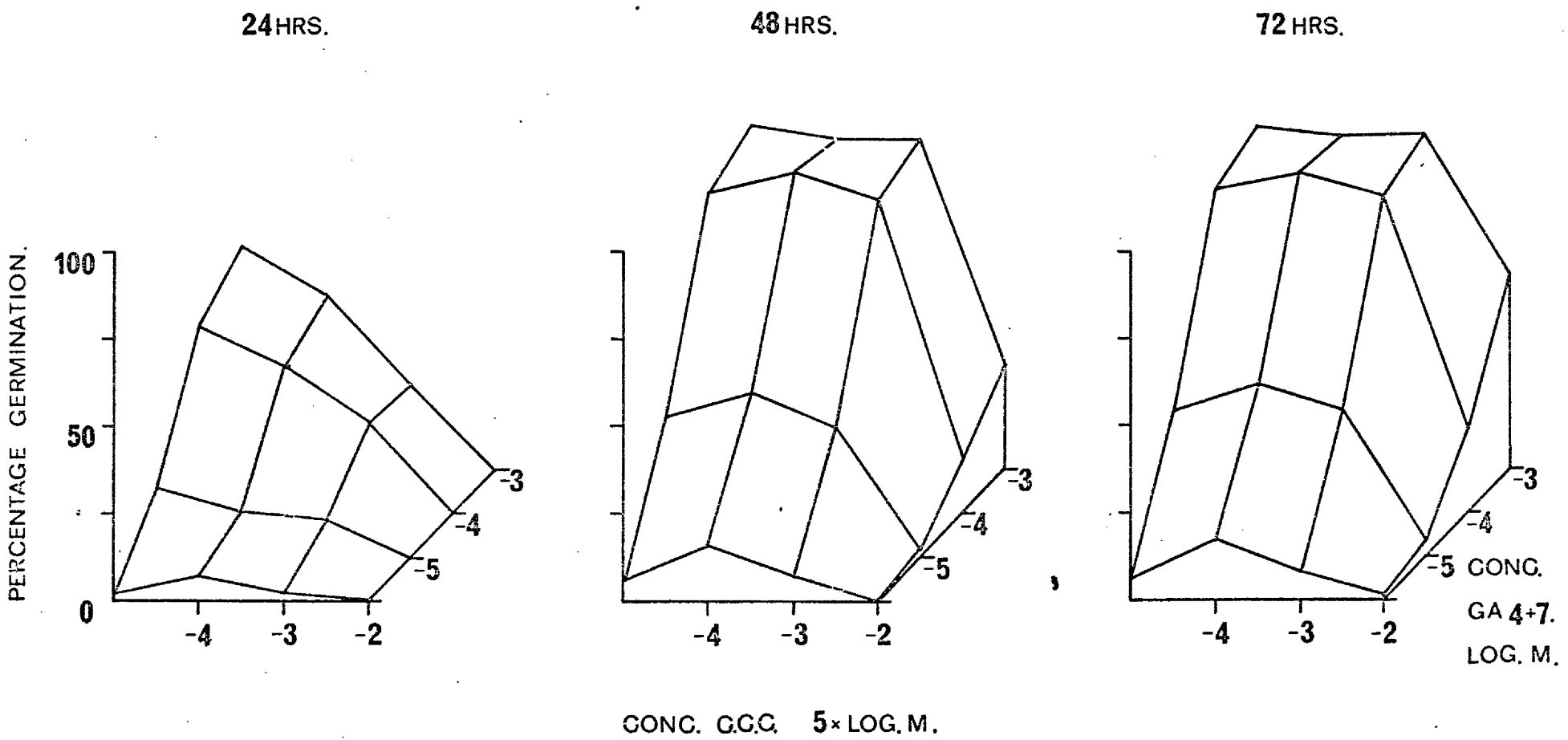

24 HRS.
48 HRS.
72 HRS.
PERCENTAGE GERMINATION.
100
50
0
-4
-3
-2
-3
-4
-5
CONC.
GA 4+7.
LOG. M.
CONC. C.C.C. 5× LOG. M.

FIGURE 22

The percentage germination of seeds of Grand Rapids lettuce in the presence of Phosphon D and GA_{4+7} at 20°C after 24, 48 and 72 hrs.

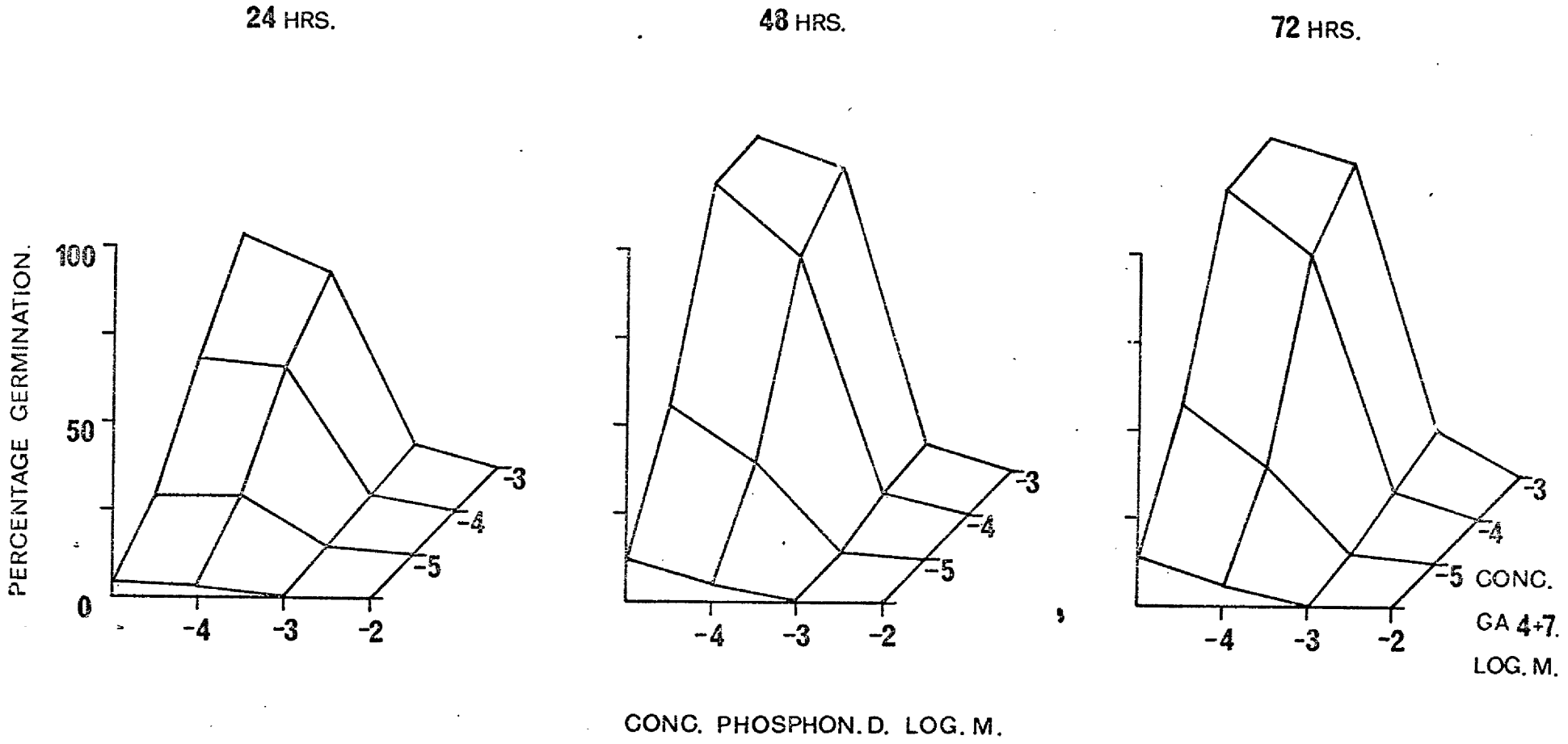
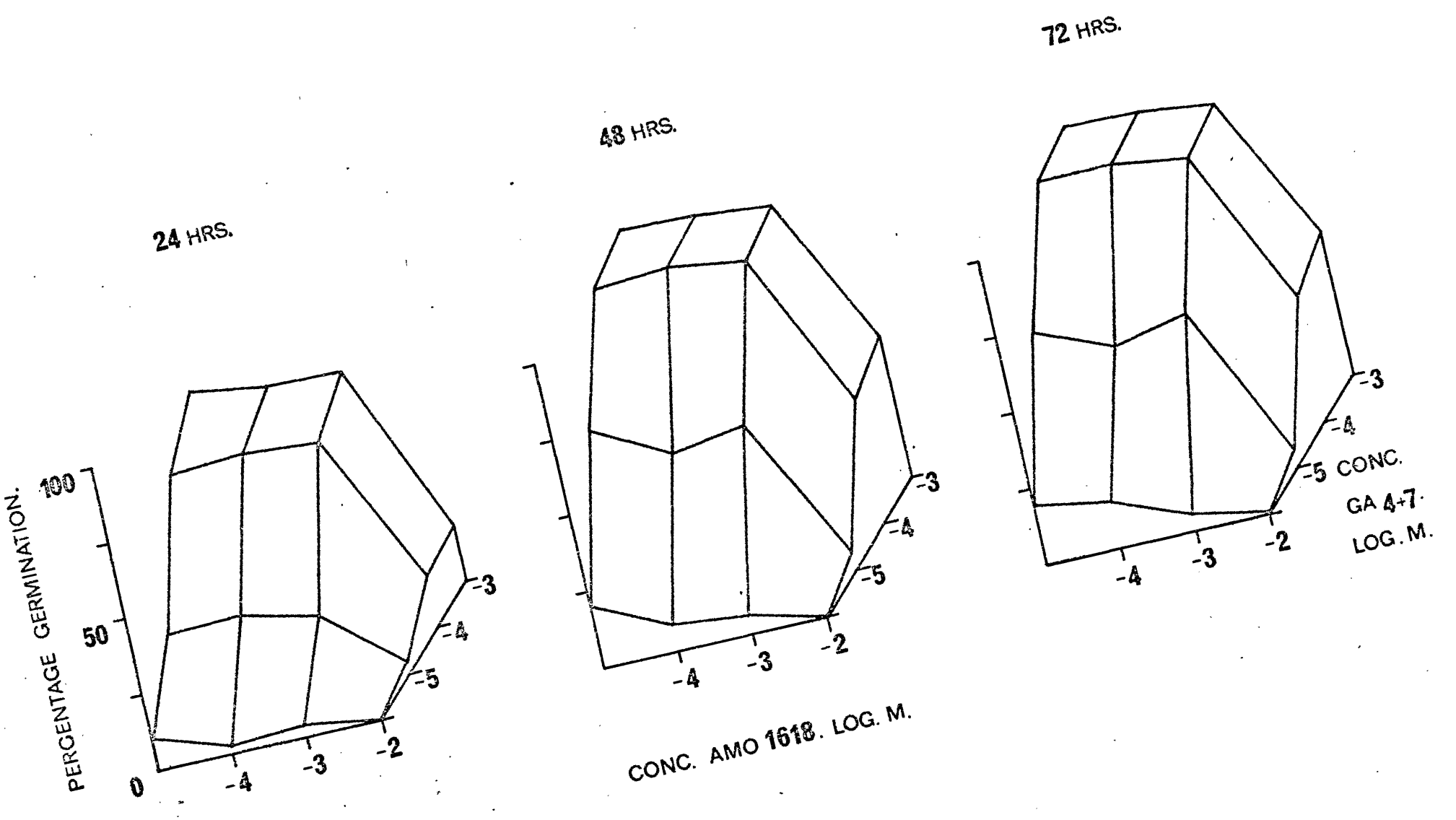

24 HRS.
48 HRS.
72 HRS.
PERCENTAGE GERMINATION.
100
50
0
-4
-3
-2
-3
-4
-5
CONC. PHOSPHON. D. LOG. M.
CONC. GA 4+7. LOG. M.

FIGURE 23

The percentage germination of seeds of Grand Rapids lettuce in the presence of Amo 1618 and GA_{4+7} at 20°C after 24, 48 and 72 hrs.


24 HRS.
48 HRS.
72 HRS.
PERGENTAGE GERMINATION.
100
50
0
-4
-3
-2
-3
-4
-5
CONC. AMO 1618. LOG. M.
-3
-4
-5 CONC.
GA 4+7.
LOG. M.

PART C

The interaction of light, cytokinins and gibberellins in overcoming abscisic acid inhibition of lettuce seed germination.

Introduction

Seeds of Grand Rapids lettuce germinate below the 'critical temperature' for the induction of thermodormancy, given a suitable aqueous environment. It has been proposed that thermodormancy may be maintained as a result of increased inhibitor levels above the critical temperature and that this inhibitor could be abscisic acid (Amen, 1968). Abscisic acid (ABA) has been shown to inhibit the germination of Grand Rapids lettuce seeds below the critical temperature (Khan, 1968). Khan (1971) suggests that the results obtained from applying hormones to seeds may reflect the endogenous hormone balance and has shown that gibberellin cannot alone overcome ABA inhibition of germination. The presence of a cytokinin is required and it was shown that at one concentration of cytokinin, increasing concentrations of gibberellin were then able to cause increasing levels of germination. Khan proposes that the cytokinin plays a 'permissive' role, permitting gibberellin to actually bring about germination. The main reason for using exogenous hormone studies must lie in the difficulties in examining the endogenous hormones of seeds, especially a small seed such as lettuce.

The interaction of cytokinins and gibberellins with ABA in a number of different cultivars of lettuce were fully discussed in the general introduction. In only one case, using Great Lakes lettuce seeds (Aspinall *et al.*, 1967) has the effect of red light been studied. Red light could not overcome ABA induced dormancy. As was pointed out in the general introduction there has been no single, comprehensive study of the interactions of cytokinins, gibberellins, red light and ABA using Grand Rapids lettuce seeds. It was consequently decided to carry out such a study in the hope that several unresolved questions as to the role of cytokinins and gibberellins in overcoming thermodormancy might be answered.

Results

The results of this study are shown in Tables 28-42, Figures 24-34.

In view of the controversy surrounding the effectiveness of cytokinins in overcoming thermodormancy, this was first investigated. Figure 24 shows the effect of a number of cytokinins in overcoming thermodormancy. Seeds were held at 30°C and germination counted after 24 and 48 hrs. It can be seen that the most effective cytokinins at 24 hrs are benzyladenine (BA) and zeatin, a naturally occurring cytokinin. After 24 hrs at 10^{-5} M both these cytokinins have promoted germination from the dark control level of less than fifteen percent to over 35 percent. This effect is even more pronounced after 48 hrs by which time over 75 percent germination is obtained in the presence of cytokinins, against dark controls of less than 20 percent. Care was taken to exclude all light following the reported synergism between light and cytokinins in promoting lettuce seed germination (Miller, 1958). Clearly, cytokinins do promote the germination of lettuce seeds in the absence of light.

Khan (1968) indicated that ABA could inhibit the germination of Grand Rapids lettuce seeds. McWha (1973) using the cultivars Arctic King and Great Lakes, showed that the inhibitory effect of ABA was not permanent, even in the continuous presence of ABA. Figure 25 and Table 40 shows the effect of a racemic mixture of ABA on the germination of Grand Rapids lettuce seeds at 20°C, below the 'critical temperature'. It is clear that increasing the concentration of ABA, increases the inhibition of germination. After 24 hrs no germination can be observed in 10^{-4} M or 2×10^{-4} ABA. As was the case with Arctic King and Great Lakes there is an escape from the inhibiting action of ABA. After 6 days at 2×10^{-4} ABA some 50-60 percent of the seeds have germinated. The ABA used is a mixture of the cis, trans- and trans, trans-isomers. Trans, trans-ABA has been reported to be inactive (Cornforth et al., 1965; Milborrow, 1966; Tamura and Nagao, 1969), or equally active to the cis, trans isomer (Addicott and Lyon, 1969). McWha examined the effect of trans, trans-ABA on the inhibition of germination in seeds of the lettuce cultivars Arctic King and Great Lakes and concluded it had 50% the activity of the cis, trans isomer. Table 41 shows the results of applying trans, trans-, cis, trans-, or mixed isomers

of ABA to seeds of Grand Rapids lettuce. It can be seen that the trans, trans-isomer does inhibit germination but to a lesser degree than the cis, trans-isomer.

We have shown that ABA will inhibit germination in seeds of Grand Rapids lettuce, used in this study. The main part of this work is concerned with examining the interaction of promoters of germination with ABA. Two experiments were first carried out to examine the effect of kinetin or benzyladenine (BA) in combination with GA_{4+7} on the germination of Grand Rapids lettuce seeds. The results are given in Tables 28 and 29. It can be seen that BA is more effective than kinetin in overcoming thermodormancy, and that both do promote germination at 20°C. GA_{4+7} also promotes germination at 20°C and 30°C. In combination an increasing concentration of GA_{4+7} with kinetin or BA leads to greater germination at 30°C. If we now look at Tables 30 and 33 these show the effect of kinetin or BA, respectively, alone and in combination with GA_{4+7} in overcoming ABA inhibition of germination at 20°C. After 24 hrs there is no germination in 6.7×10^{-5} M ABA as opposed to the control level of around 60%. With increasing time kinetin (Table 30) promotes germination in the presence of 6.7×10^{-5} M ABA, germination reaching almost 90% after 7 days. BA (Table 33) is even more active than kinetin. That kinetin can overcome ABA inhibition on its own can be more clearly seen in Figure 30. It is also clear from Tables 30 and 33 that GA_{4+7} can overcome ABA inhibition, on its own: indeed it is more effective than either kinetin or BA. Figure 31 also illustrates this point. If we compare Figures 30 and 31 we can see that the curves are not identical. With kinetin we have a series of almost parallel lines, indicating that the action of kinetin is during the first 48 hrs. Any further increase in germination merely reflects the 'escape' from the inhibitory effects of ABA. However, with GA_{4+7} (Figure 31) we can see that after 72 hrs the increase in germination which occurs during the next 24 hrs far exceeds that shown by the control. Thus, it is possible to distinguish separate roles for cytokinins and gibberellins in overcoming the inhibition of germination by ABA.

As was reported by Khan (1968) increasing concentrations of GA_{4+7} will cause increasing germination at one concentration of cytokinin. Figure 32 shows this effect for BA and GA_{4+7}. If we

accept the observation that non-parallel lines indicate a statistical interaction then cytokinins and GA_{4+7} synergise to overcome the inhibitory action of ABA.

Tables 31 and 34 show the results of identical experiments but carried out at 30°C. In Table 31, which shows the effect of kinetin and GA_{4+7} in overcoming the inhibitory effects of 6.7×10^{-5} M ABA at 30°C, we can see that neither kinetin nor GA_{4+7} can alone overcome ABA. However, in combination the inhibitory effects of ABA to some extent can be removed. This is more striking when BA is used instead of K (Table 34). With BA there is a small amount of germination after 7 days. If we reduce the concentration of ABA to 6.7×10^{-6} M ABA, but still at 30°C, then both kinetin (Table 32) and BA (Table 35) can be seen to overcome the inhibitory effects of ABA as can GA_{4+7}. Together GA_{4+7} and cytokinins are more effective than either hormone alone. It should be noted that in the 30°C treatments in high concentrations of ABA some seeds showed 'cotyledon' rupture of the testa, rather than radicle emergence. Ikuma and Thimann (1963b) have mentioned this phenomenon. These seeds have been included in the percentage germinations shown in the relevant tables.

A further factor which has been shown capable of overcoming thermodormancy in seeds of Grand Rapids lettuce, is red light. There are no reports of investigations of the interaction between red light and ABA in Grand Rapids lettuce seeds. Tables 36 and 37 show the effect of red light on ABA inhibition of germination at 20°C and 30°C respectively. At 20°C it is obvious that ABA inhibits germination, as was previously shown. The dark control germination (no ABA present) of more than 50% is reduced to zero at 24 hrs (Table 36). In the absence of ABA, red light can speed up the germination process and even cause a slightly higher final percentage germination; 99% compared to 91% in the absence of red light after 7 days. ABA inhibition can also be overcome by red light, completely at lower concentrations and partially at higher concentrations of ABA (Table 36 and Figure 33). At 30°C no germination takes place in the highest concentration of ABA used, 10^{-4} M, but at a lower concentration of ABA, 10^{-5} M, red light can overcome the inhibitory action of ABA (Table 37, Figure 34). Ten minutes of red light was as effective as 20 minutes which would indicate that the response was saturated by 10 mins

red light. It has been shown that cis, trans-ABA isomerises with the less active trans, trans-ABA isomer when kept in light (Mousseron-Canet et al., 1966; Lenton et al., 1971) and whilst it would seem unlikely this could explain the effect of red light in overcoming ABA retardation of germination, the possibility is easily tested. Table 39 shows the effect of red light and trans, trans-ABA in combination on the germination of Grand Rapids lettuce seeds at both 20°C and 30°C. Although trans, trans-ABA is less effective in inhibiting germination it is clear that red light can overcome this inhibition. We can also show that the action of red light can be negated if far-red is given immediately following the red light treatment. Table 38 shows that far-red itself has no significant effect, red light promotes germination in the presence of ABA and that far-red can negate the action of red light.

One of the critical questions which must be asked, in attempting to assign a control function for ABA in seed germination and dormancy, is whether ABA really influences germination or merely inhibits radicle formation. If the latter is true then it is possible that we may interpret an effect on seedling growth as one on germination, because the visible manifestation of germination, radicle emergence, has been inhibited. In an attempt to answer this question the following experiment was conducted. Seeds of Grand Rapids lettuce were imbibed in water for 1 to 20 hrs at 20°C and then transferred to either water or solutions of ABA. It was hoped to find out when the seeds had developed sufficient 'potential to germinate' to avoid the inhibitory action of ABA. In these experiments the first visible radicle emergence could be observed after 14 hrs imbibition and germination was in the order of 20-40% by 20 hrs (date not given). Thus, if ABA inhibits germination it must influence events before 14 hrs. The results of this work are presented in Table 42 a, b and c and Figures 26-29. It can be seen from Table 42a that the control treatment, transfer from water to water curve A, has an effect which must be considered when examining the ABA treatments. The seed is sensitive to transfer after 12 hrs. This effect is observable when germination was counted after 24 hrs, but by 48 hrs the control germination had reached a high uniform level at around 95% (Figure 26, curve A and Figure 27, curve A). In Figure 26, curves B and C, which represent the transfer of seeds from water to 10^{-5} and 10^{-4} M ABA

respectively, both show an 'escape' from the inhibiting action of ABA around 12 hrs. However, as the control curve A also shows an upward trend, it is possible that B and C merely reflect this. In order to clarify this question secondary data were derived as follows. If we assume that the control treatment (curve A, Figure 26) should have had no effect on germination, then curve A should have been a straight line indicating uniform germination. Applying this assumption we can take a figure of 65% germination. Any difference between the observed control percentage germination and this expected value, was then added onto the percentage germination for the ABA treatment. Maxima and minima were included to give the range of the expected germination. This manipulation of the data was carried out on curve B, figure 26, transfer from water to 10^{-5} M ABA, and the two curves obtained are given in figure 29. The area between these curves is shaded. It may be considered that this eliminates the control effects and that figure 29 represents a truer reflection of the effect of transfer to ABA. The data in Figure 29 can be interpreted as indicating that ABA affects germination only during the first 8 hrs of imbibition. Thereafter there is a gradual escape from the inhibitory action of ABA. It is possible that ABA may have a dual action, not only inhibiting germination but also subsequent radicle emergence. That ABA seems to inhibit only the first period of imbibition can be more clearly seen from Figure 28 which shows the effect of transfer from water to 10^{-4} ABA, germination being counted after 4 days (see also Table 42 c). Clearly there is an escape from the inhibitory action of ABA after 10-12 hrs, if not earlier. Thus, there would seem to be good evidence that ABA may inhibit the germination of seeds of Grand Rapids lettuce and not only the visible manifestation of germination, radicle emergence.

Discussion

The results presented in this section may be summarized as follows:-

(a) Cytokinins are capable of promoting the dark germination of Grand Rapids lettuce seeds at 20°C and 30°C.

(b) Exogenous ABA, either cis, trans-, trans, trans- or both, inhibit the germination of Grand Rapids lettuce seeds at 20°C, below the critical temperature. There is an 'escape' from the inhibitory action of ABA although at high concentrations

the final germination percentage attained is less than in water controls.

(c) At 20°C in the presence of ABA at a concentration of 6.7×10^{-5} M, both kinetin and BA overcome the inhibition of germination by ABA. GA_{4+7} also overcomes ABA inhibition alone. Together cytokinins and GA_{4+7} are more effective than alone. At one concentration of cytokinin, increasing the concentration of GA_{4+7} elicits an increased germination response. It is possible to distinguish between the effects of cytokinins and gibberellins on germination.

(d) At 30°C in the presence of ABA at a concentration of 6.7×10^{-5} M kinetin and GA_{4+7} are not able to bring about germination. BA has a small but significant effect. Cytokinins and GA_{4+7} together will cause germination. In a lower concentration of ABA, 6.7×10^{-6} M, both cytokinins and GA_{4+7} can overcome the inhibitory action of ABA on germination, on their own.

(e) Red light can promote germination in the presence of exogenous ABA. This effect is far-red reversible and cannot be explained on the basis of photoisomerization of 'active' cis, trans-ABA to the 'less active' trans, trans-ABA.

The results presented are in conflict with several reports in the literature. Firstly, it has been unequivocally shown that cytokinins do promote the germination of seeds of Grand Rapids lettuce in darkness at 30°C, above the critical temperature. Khan (1971) has proposed a theory for the control of dormancy based on cytokinins being a pre-requisite for the action of gibberellins, when an inhibitor is present. He believes that gibberellin controls germination and that cytokinins merely permit the action of gibberellins. He has stated that the results obtained from such exogenous studies may well reflect the endogenous situation. Clearly, the results reported in this section do not support Khan. We have shown that both cytokinins and gibberellins on their own are capable of overcoming the inhibition of germination induced by exogenous ABA, except under a severe inhibitory regime of high temperature and high concentrations of ABA. This does not rule out the possibility that cytokinins are required for the action of gibberellins. It can be argued that the seeds used contained sufficient cytokinin to allow the expression of the promoter. However, what we have shown

is that the role of cytokinins need not be merely that of a 'permitter' for the action of a second promoter. That these effects have been previously missed may result from the concentrations and time courses employed in other studies. We have shown that there is a distinction in the actions of cytokinins and GA_{4+7}; GA_{4+7} acts at a later stage. The physiological significance of this is unclear, but it is possible that hormones, although capable of acting alone, can also act in a sequential fasion. We have confirmed that at one concentration of cytokinin, increasing germination is elicited by increasing concentrations of GA_{4+7}.

For the first time red light has been shown to overcome ABA inhibition of germination; this effect was not as a result of photoisomerization to the less active form of ABA and is far-red reversible. Clearly, we cannot support the concept of a specific interaction between promoters and inhibitors. Such an interaction would certainly imply that a similar situation might occur in the endogenous hormone control of germination. Lack of such an interaction, as we have demonstrated, leaves the question of the value of exogenous studies unanswered. There is no proof that the results obtained from such studies are in any way an accurate reflection of endogenous control. Such a statement could only be made when the endogenous hormones had been identified and meaningful changes shown. It is to this question that much of the remainder of this thesis will be aimed.

Table 28 . Average percentage germination of lettuce seed treated with Gibberellin A_{4+7} and Kinetin.

Temp.	Concentration GA_{4+7} M / Kinetin M	0	6.7×10^{-7}	6.7×10^{-6}	6.7×10^{-5}
20°C	0	60.3 ± 1.9	90.0 ± 3.7	94.7 ± 1.3	96.3 ± 0.7
	6.7×10^{-6}	67.3 ± 4.5	90.3 ± 1.9	96.7 ± 0.9	94.3 ± 1.4
	6.7×10^{-5}	69.3 ± 2.6	89.7 ± 4.0	96.0 ± 0.9	96.7 ± 1.1
					24 hrs
	0	87.7 ± 4.2	96.7 ± 1.2	98.0 ± 0.5	97.7 ± 0.1
	6.7×10^{-6}	91.7 ± 3.0	97.7 ± 1.3	99.0 ± 0.7	97.0 ± 0.5
	6.7×10^{-5}	96.3 ± 1.5	97.3 ± 0.9	98.0 ± 0.7	98.0 ± 0.7
					48 hrs
30°C	0	11.3 ± 2.3	23.3 ± 3.8	58.3 ± 4.5	84.3 ± 1.5
	6.7×10^{-6}	24.0 ± 3.7	34.3 ± 6.7	59.0 ± 9.3	89.7 ± 1.8
	6.7×10^{-5}	35.3 ± 4.4	60.0 ± 4.8	91.0 ± 1.2	95.3 ± 1.1
					24 hrs
	0	20.3 ± 4.0	52.7 ± 7.3	90.7 ± 2.3	96.0 ± 0.5
	6.7×10^{-6}	47.0 ± 8.0	61.3 ± 9.5	93.3 ± 1.5	96.0 ± 0.5
	6.7×10^{-5}	70.7 ± 4.0	92.7 ± 3.0	97.7 ± 0.5	98.0 ± 0.7
					48 hrs

Table 29 . The average percentage germination of lettuce seed treated with Gibberellin A_{4+7} and Benzyladenine.

Temp.	Concentration GA_{4+7} M / BA M	0	6.7×10^{-7}	6.7×10^{-6}	6.7×10^{-5}
20°C	0	46.0 ± 2.1	90.3 ± 3.2	94.3 ± 1.8	95.0 ± 1.4
	6.7×10^{-6}	55.7 ± 3.9	86.7 ± 1.8	94.7 ± 1.5	89.7 ± 2.6
	6.7×10^{-5}	62.0 ± 3.1	88.3 ± 2.6	93.3 ± 1.3	95.3 ± 0.9
					24 hrs
	0	81.7 ± 2.6	98.3 ± 0.9	98.3 ± 0.9	99.0 ± 0.5
	6.7×10^{-6}	91.7 ± 1.4	95.7 ± 1.2	97.0 ± 1.6	96.0 ± 1.2
	6.7×10^{-5}	92.0 ± 1.8	97.3 ± 0.8	96.3 ± 1.1	96.7 ± 1.5
					48 hrs
30°C	0	15.3 ± 2.8	40.7 ± 5.6	78.7 ± 4.0	86.0 ± 2.8
	6.7×10^{-6}	39.3 ± 6.1	70.7 ± 5.0	87.3 ± 2.8	90.7 ± 2.1
	6.7×10^{-5}	45.7 ± 4.8	71.3 ± 3.2	96.0 ± 1.2	90.7 ± 1.7
					24 hrs
	0	23.3 ± 2.8	62.3 ± 3.1	95.7 ± 1.5	94.7 ± 1.3
	6.7×10^{-6}	80.0 ± 4.4	98.0 ± 0.7	96.3 ± 1.1	97.0 ± 0.9
	6.7×10^{-5}	83.7 ± 4.1	95.3 ± 1.1	97.0 ± 1.5	96.0 ± 0.5
					48 hrs

Table 30 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7}, kinetin and abscisic acid at 6.7×10^{-5} M 20°C.

Concentration GA_{4+7} M / Kinetin M	0	6.7×10^{-7}	6.7×10^{-6}	6.7×10^{-5}
0	0	0	0	0
6.7×10^{-6}	0	0	0	0
6.7×10^{-5}	1.7 ± 1.0	0.7 ± 0.4	2.3 ± o.8	2.3 ± 1.0
	64.7 ± 3.6*			24 hrs
0	15.7 ± 2.4	19.0 ± 3.3	24.3 ± .2.9	35.3 ± 6.9
6.7×10^{-6}	14.0 ± 5.4	22.3 ± 1.4	24.7 ± 6.5	20.3 ± 6.2
6.7×10^{-5}	45.7 ± 5.3	51.3 ± 4.3	64.3 ± 5.0	68.7 ± 7.5
	91.0 ± 2.7*			48 hrs
0	34.0 ± 5.2	34.0 ± 3.9	43.3 ± 9.3	60.0 ± 6.6
6.7×10^{-6}	35.3 ± 7.4	45.7 ± 5.7	47.3 ± 8.3	54.7 ± 9.8
6.7×10^{-5}	70.0 ± 5.9	85.3 ± 3.2	92.3 ± 1.9	91.7 ± 3.0
	91.7 ± 2.4*			72 hrs
0	42.0 ± 3.9	43.0 ± 3.6	72.0 ± 5.3	87.2 ± 2.1
6.7×10^{-6}	44.7 ± 5.5	60.0 ± 3.0	77.3 ± 8.1	83.0 ± 7.8
6.7×10^{-5}	81.3 ± 4.6	92.7 ± 2.3	95.3 ± 1.3	95.0 ± 2.1
	93.0 ± 1.8*			96 hrs
0	48.7 ± 4.3	55.3 ± 2.9	90.3 ± 2.7	97.3 ± 0.9
6.7 10^{-6}	54.7 ± 7.1	73.7 ± 4.4	87.3 ± 8.3	97.0 ± 1.2
6.7 10^{-5}	87.7 ± 3.4	94.7 ± 2.1	96.3 ± 0.7	95.3 ± 2.3
				7 days

* water controls

Table 31 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7}, kinetin and abscisic acid at 6.7×10^{-5} M 30°C.

Concentration GA_{4+7} M / Kinetin M	0	6.7×10^{-7}	6.7×10^{-6}	6.7×10^{-5}
0	0	0	0	0
6.7×10^{-6}	0	0	0	0
6.7×10^{-5}	1.0 ± 0.7 7.0 ± 2.4*	0.3 ± 0.3	2.3 ± 0.8	3.7 ± 2.0 48 hrs
0	0	0	0	0
6.7×10^{-6}	0	0	0	0
6.7×10^{-5}	1.3 ± 0.8 7.0 ± 2.4*	2.0 ± 0.7	6.7 ± 1.8	12.7 ± 2.7 72 hrs
0	0	0	0	0
6.7×10^{-6}	0	0	0	0
6.7×10^{-5}	1.3 ± 0.8 7.7 ± 2.5*	2.7 ± 1.0	10.3 ± 2.0	16.0 ± 3.7 96 hrs
0	0	0	0	0
6.7×10^{-6}	0	1.3 ± 1.3	0.7 ± 0.7	1.3 ± 0.7
6.7×10^{-5}	2.7 ± 1.3	2.7 ± 1.3	14.7 ± 0.6	26.0 ± 9.0 7 days
0	0.7 ± 0.7	0	0.3 ± 0.3	1.3 ± 0.7
6.7×10^{-6}	0	1.3 ± 1.3	1.0 ± 0.7	3.3 ± 1.6
6.7×10^{-5}	2.7 ± 1.3	4.0 ± 1.6	15.3 ± 1.2	31.0 ± 6.1 9 days

* water controls

Table 32 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7}, kinetin and abscisic acid 6.7×10^{-6} M 30°C.

Concentration GA_{4+7} M / Kinetin M	0	6.7×10^{-7}	6.7×10^{-6}	6.7×10^{-5}
0	1.1 ± 0.6	4.7 ± 1.3	16.2 ± 3.4	25.1 ± 4.6
6.7×10^{-6}	3.6 ± 1.5	3.8 ± 1.1	20.2 ± 5.6	19.6 ± 5.1
6.7×10^{-5}	20.0 ± 2.6	26.0 ± 3.3	60.9 ± 5.0	54.4 ± 7.8
	8.0 ± 1.2*			24 hrs
0	6.4 ± 1.3	24.4 ± 3.3	67.8 ± 5.9	81.3 ± 3.2
6.7×10^{-6}	22.2 ± 2.9	31.1 ± 4.5	76.9 ± 3.6	76.4 ± 3.8
6.7×10^{-5}	53.6 ± 4.7	87.1 ± 2.8	96.7 ± 0.8	94.7 ± 1.4
	15.3 ± 4.4*			48 hrs
0	7.8 ± 1.5	28.4 ± 4.0	78.9 ± 4.7	86.9 ± 3.3
6.7×10^{-6}	25.8 ± 3.2	38.7 ± 5.3	92.2 ± 1.8	93.3 ± 1.2
6.7×10^{-5}	59.8 ± 4.7	90.7 ± 2.3	98.2 ± 0.7	98.0 ± 0.6
	17.3 ± 4.4*			72 hrs
0	9.1 ± 1.8	30.9 ± 4.9	86.9 ± 3.6	93.3 ± 2.0
6.7×10^{-6}	26.9 ± 3.5	40.2 ± 5.2	96.2 ± 1.4	95.3 ± 1.0
6.7×10^{-5}	60.9 ± 4.7	91.8 ± 2.3	98.7 ± 0.4	98.0 ± 0.6
	17.3 ± 4.4*			96 hrs

* water controls

Table 33 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7}, benzyladenine and abscisic acid at 6.7×10^{-5} M 20°C.

Concentration BA M \ GA_{4+7} M	0	6.7×10^{-7}	6.7×10^{-6}	6.7×10^{-5}
0	0	0.7 ± 0.7	3.0 ± 1.2	0
6.7×10^{-6}	0.7 ± 0.3	0.3 ± 0.3	4.7 ± 1.6	2.7 ± 1.0
6.7×10^{-5}	1.3 ± 1.3	5.7 ± 2.1	9.0 ± 1.5	7.3 ± 2.2
	55.3 ± 2.7*			24 hrs
0	24.7 ± 5.6	35.7 ± 6.8	53.0 ± 8.0	48.7 ± 6.3
6.7×10^{-6}	46.7 ± 6.3	58.3 ± 4.2	80.4 ± 2.4	79.3 ± 4.9
6.7×10^{-5}	39.0 ± 6.1	60.3 ± 9.3	75.7 ± 4.2	76.7 ± 3.9
	94.3 ± 1.7*			48 hrs
0	45.0 ± 7.4	57.7 ± 5.4	83.3 ± 4.0	84.o ± 2.7
6.7×10^{-6}	78.3 ± 3.7	90.7 ± 1.7	96.7 ± 0.9	97.0 ± 0.7
6.7×10^{-5}	72.3 ± 4.6	92.3 ± 2.1	96.7 ± 1.28	97.7 ± 0.7
	94.7 ± 1.6*			72 hrs
0	54.0 ± 7.0	66.3 ± 4.3	92.7 ± 1.7	94.7 ± 1.4
6.7×10^{-6}	89.0 ± 2.7	97.7 ± 0.5	97.7 ± 0.5	98.3 ± 0.9
6.7×10^{-5}	92.0 ± 1.9	97.3 ± 0.8	98.3 ± 0.9	97.7 ± 0.7
				96 hrs

* water controls

Table 34 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7}, benzyladenine and abscisic acid at 6.7×10^{-5} M 30°C.

Concentration GA_{4+7} M / BA M	0	6.7×10^{-7}	6.7×10^{-6}	6.7×10^{-5}
0	0	0	0	0
6.7×10^{-6}	0	0.4 ± 0.3	1.1 ± 0.6	0.4 ± 0.3
6.7×10^{-5}	0.4 ± 0.3	0.7 ± 0.7	1.1 ± 0.9	1.3 ± 0.7
	2.0 ± 1.2*			24 hrs
0	0	0	0	0.2 ± 0.2
6.7×10^{-6}	3.6 ± 1.6	2.7 ± 0.7	12.7 ± 4.4	10.7 ± 2.9
6.7×10^{-5}	2.7 ± 1.4	5.3 ± 1.7	5.8 ± 1.0	18.4 ± 4.5
	8.7 ± 1.8*			48 hrs
0	0	0	0.4 ± 0.3	0.2 ± 0.2
6.7×10^{-6}	5.3 ± 2.2	6.4 ± 1.2	29.3 ± 9.4	28.2 ± 5.7
6.7×10^{-5}	4.7 ± 2.0	10.9 ± 3.3	20.7 ± 2.1	46.0 ± 9.0
	8.7 ± 1.8*			72 hrs
0	0	0	0.4 ± 0.3	0.2 ± 0.2
6.7×10^{-6}	8.0 ± 3.4	8.7 ± 2.8	34.3 ± 15.2	32.3 ± 7.7
6.7×10^{-5}	7.7 ± 3.6	18.7 ± 7.3	39.7 ± 4.3	59.0 ± 14.1
	9.3 ± 1.8*			96 hrs
0	0	0.2 ± 0.2	0.4 ± 0.3	2.7 ± 0.7
6.7×10^{-6}	9.3 ± 2.9	15.6 ± 1.6	56.9 ± 11.5	58.2 ± 10.0
6.7×10^{-5}	12.2 ± 4.3	36.9 ± 7.9	79.1 ± 7.3	90.9 ± 3.8
				7 days

* water controls

Table 35 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7}, benzyladenine and abscisic acid at 6.7×10^{-6} M 30°C.

Concentration GA_{4+7} M / BA M	0	6.7×10^{-7}	6.7×10^{-6}	6.7×10^{-5}
0	0.7 ± 0.7	4.7 ± 2.9	17.3 ± 6.7	25.3 ± 7.5
6.7×10^{-6}	24.3 ± 4.3	40.3 ± 5.0	75.5 ± 4.5	85.3 ± 4.4
6.7×10^{-5}	25.3 ± 5.4	50.3 ± 5.4	81.7 ± 4.8	83.7 ± 2.3
	16.0 ± 4.6*			24 hrs
0	4.3 ± 1.9	12.0 ± 3.2	54.3 ± 11.8	69.3 ± 7.5
6.7×10^{-6}	50.3 ± 5.4	82.7 ± 4.6	94.3 ± 1.5	97.3 ± 0.75
6.7×10^{-5}	55.7 ± 6.1	90.3 ± 2.9	96.7 ± 0.8	95.3 ± 1.3
	24.0 ± 3.1*			48 hrs
0	6.3 ± 1.6	12.3 ± 3.2	60.0 ± 11.1	85.7 ± 2.1
6.7×10^{-6}	58.3 ± 5.8	87.3 ± 3.8	96.0 ± 0.9	98.3 ± 0.7
6.7×10^{-5}	59.3 ± 5.6	91.7 ± 2.1	98.3 ± 0.7	98.0 ± 0.0
	24.0 ± 3.1*			72 hrs
0	6.3 ± 1.6	12.7 ± 2.8	66.0 ± 10.8	90.7 ± 2.0
6.7×10^{-6}	68.7 ± 6.1	90.7 ± 3.1	96.3 ± 1.0	98.3 ± 0.7
6.7×10^{-5}	75.0 ± 4.3	94.3 ± 2.5	98.3 ± 0.7	98.0 ± 0.0
				96 hrs
0	6.7 ± 1.7	16.0 ± 3.0	66.7 ± 11.0	91.7 ± 2.0
6.7×10^{-6}	72.0 ± 6.5	90.7 ± 3.1	96.3 ± 1.0	98.3 ± 0.7
6.7×10^{-5}	94.7 ± 1.3	94.3 ± 2.5	98.3 ± 0.7	98.0 ± 0.0
				7 days

* water controls

Table 36 . The average percentage germination of lettuce seed treated with abscisic acid and red light at 20°C.

Concn. ABA M. \ Red light (mins)	0	2	5
0	52.0 ± 3.7	80.2 ± 3.1	92.4 ± 1.0
10^{-5}	15.8 ± 3.1	48.2 ± 5.9	64.2 ± 5.1
10^{-4}	0	0	0
			24 hrs
0	90.9 ± 0.9	94.7 ± 0.9	97.1 ± 1.0
10^{-5}	74.9 ± 4.1	88.9 ± 1.6	95.3 ± 1.4
10^{-4}	2.9 ± 1.1	7.1 ± 3.2	10.4 ± 4.1
			48 hrs
0	91.1 ± 1.0	95.8 ± 0.7	97.8 ± 0.7
10^{-5}	77.6 ± 3.9	91.1 ± 1.3	96.9 ± 0.9
10^{-4}	13.1 ± 2.8	26.4 ± 5.3	40.3 ± 3.3
			72 hrs
0	91.1 ± 1.0	96.2 ± 0.8	99.1 ± 0.5
10^{-5}	78.9 ± 3.5	92.4 ± 1.2	97.1 ± 0.9
10^{-4}	24.4 ± 4.1	46.7 ± 4.5	66.4 ± 6.8
			96 hrs
0	91.1 ± 1.0	96.5 ± 0.9	99.1 ± 0.5
10^{-5}	78.9 ± 3.5	92.9 ± 0.9	97.1 ± 0.9
10^{-4}	26.7 ± 4.2	55.8 ± 4.8	73.8 ± 5.1
			7 days

Table 37 . The average percentage germination of lettuce seed treated with abscisic acid and red light at 30°C.

Concn. ABA M \ Red light (mins)	0	10	20	
0	6.3 ± 1.2	87.3 ± 2.8	82.7 ± 4.2	
10^{-5}	0	3.3 ± 1.0	3.3 ± 1.3	
10^{-4}	0	0	0	
				24 hrs
0	13.3 ± 2.1	96.7 ± 1.2	92.7 ± 0.8	
10^{-5}	1.3 ± 0.7	23.3 ± 1.6	23.3 ± 3.4	
10^{-4}	0	0	0	
				48 hrs
0	13.3 ± 2.1	97.0 ± 1.1	93.3 ± 1.1	
10^{-5}	1.7 ± 0.8	37.7 ± 3.6	32.7 ± 4.5	
10^{-4}	0	0	0	
				72 hrs
0	13.3 ± 2.1	97.0 ± 1.1	96.3 ± 1.2	
10^{-5}	2.0 ± 0.9	40.3 ± 3.5	34.7 ± 3.8	
10^{-4}	0	0	0	
				96 hrs
0	13.3 ± 2.1	97.0 ± 1.1	96.3 ± 1.2	
10^{-5}	2.0 ± 0.9	41.7 ± 3.8	37.0 ± 3.8	
10^{-4}	0	0	0	
				7 days

Table 38 . The effect of red and far-red light on the average percentage germination of lettuce seed treated with abscisic acid at 20°C.

Light* (mins) / Concn. ABA M	0	5R	10FR	5R + 10FR
0	10.0 ± 1.9	70.0 ± 5.8	3.0 ± 0.9	2.7 ± 0.8
10^{-5}	0	31.3 ± 2.5	0.7 ± 0.4	1.3 ± 0.7
10^{-4}	0	0	0	0
				24 hrs
0	23.7 ± 3.4	89.0 ± 3.5	15.7 ± 3.1	19.7 ± 1.7
10^{-5}	5.0 ± 1.0	79.2 ± 1.5	9.0 ± 2.5	7.3 ± 1.3
10^{-4}	0	4.0 ± 2.3	0	0
				48 hrs
0	25.0 ± 3.5	90.3 ± 3.5	16.7 ± 3.6	21.3 ± 1.2
10^{-5}	5.7 ± 1.0	79.7 ± 1.9	9.3 ± 2.7	8.0 ± 1.7
10^{-4}	0	17.3 ± 4.2	0.3 ± 0.3	0.3 ± 0.3
				72 hrs
0	25.0 ± 3.5	90.7 ± 3.5	17.0 ± 3.6	21.7 ± 1.4
10^{-5}	6.0 ± 0.9	81.0 ± 2.1	10.0 ± 2.7	9.0 ± 1.5
10^{-4}	0.3 ± 0.3	24.0 ± 5.3	1.0 ± 0.7	7.0 ± 0.7
				96 hrs

* R : red
FR : far-red

(Seed batch 011974)

Table 39 . The average percentage germination of lettuce seed treated with trans, trans abscisic acid and red light.

Temp.	Concn. ABA M \ Red light (mins)	0	2	5	
20°C	0	13.3 ± 2.7	73.3 ± 1.8	84.0 ± 2.0	
	10^{-4}	0	1.3 ± 0.7	3.3 ± 0.7	
					24 hrs
	0	18.7 ± 2.4	83.3 ± 4.5	94.7 ± 0.8	
	10^{-4}	3.3 ± 1.8	16.7 ± 2.4	63.3 ± 7.4	
					48 hrs
	0	18.7 ± 2.4	86.0 ± 2.0	96.0 ± 1.2	
	10^{-4}	3.3 ± 1.8	18.0 ± 2.3	68.7 ± 6.9	
					72 hrs
		0	10	20	
30°C	0	4.0 ± 2.0	76.0 ± 1.2	81.3 ± 1.4	
	10^{-5}	0.3 ± 0.3	40.3 ± 8.4	45.0 ± 2.1	
					24 hrs
	0	7.7 ± 3.6	88.7 ± 1.7	92.0 ± 1.2	
	10^{-5}	2.0 ± 0.9	64.3 ± 4.8	71.7 ± 4.3	
					48 hrs
	0	7.7 ± 3.6	88.7 ± 1.7	92.0 ± 1.2	
	10^{-5}	2.0 ± 0.9	67.3 ± 4.8	74.0 ± 5.2	
					72 hrs

(Seed batch 011974)

Table 40 . The average percentage germination of lettuce seed at 20°C treated with racemic abscisic acid, ABA.

Time of count / Concn. ABA M	24 hrs	48 hrs	72 hrs	96 hrs
0	90.0 ± 1.2	100.0 ± 0.0	-	-
10^{-7}	90.0 ± 1.2	98.0 ± 1.2	98.0 ± 1.2	98.0 ± 1.2
2.10^{-7}	84.0 ± 3.1	98.0 ± 1.2	99.3 ± 0.9	99.3 ± 0.9
10^{-6}	77.3 ± 6.6	92.7 ± 3.6	94.0 ± 3.5	94.7 ± 3.6
2.10^{-6}	75.3 ± 9.7	96.0 ± 3.1	96.0 ± 3.1	96.7 ± 3.2
10^{-5}	40.0 ± 12.2	88.7 ± 5.4	91.3 ± 4.8	93.3 ± 3.0
2.10^{-5}	28.0 ± 9.9	90.7 ± 1.9	94.0 ± 3.1	94.7 ± 3.2
10^{-4}	0.0 ± 0.0	10.7 ± 0.7	52.0 ± 8.1	71.3 ± 5.9
2.10^{-4}	0.0 ± 0.0	1.3 ± 0.7	23.3 ± 3.5	56.0 ± 6.9

Table 41 . The average percentage germination of lettuce seed treated with cis, trans, trans, trans or cis, trans and trans, trans abscisic acid at 20°C.

Concentration M		10^{-6}	10^{-5}	10^{-4}	
cis, trans CT		27.6 ± 2.8	0.7 ± 0.3	0	
trans, trans TT		37.8 ± 3.2	23.6 ± 4.2	0	
cis, trans M and trans, trans		30.7 ± 1.9	14.0 ± 2.3	0	
Control H_2O		40.4 ± 3.2			24 hrs
	CT	80.2 ± 4.2	55.3 ± 3.8	0	
	TT	83.3 ± 1.5	75.1 ± 2.0	31.1 ± 7.0	
	M	84.2 ± 2.3	75.8 ± 2.3	0	
Control H_2O		86.4 ± 1.7			48 hrs
	CT	82.2 ± 4.2	60.4 ± 3.5	6.7 ± 1.6	
	TT	87.6 ± 1.6	80.0 ± 1.8	46.9 ± 5.1	
	M	86.4 ± 1.7	78.9 ± 1.9	11.8 ± 3.4	
Control H_2O		88.2 ± 1.2			72 hrs
	CT	82.4 ± 4.3	69.6 ± 3.1	11.1 ± 2.5	
	TT	88.0 ± 1.8	80.9 ± 1.6	53.1 ± 4.2	
	M	87.6 ± 1.7	79.1 ± 1.9	23.3 ± 5.3	
Control H_2O		88.2 ± 1.2			96 hrs

Table 42 . The average percentage germination of Grand Rapids lettuce seed imbibed in water at 20°C and transferred to

A) Water

B) ABA 10^{-5} M

C) ABA 10^{-4} M

after 1, 2, 3, ----- 20 hrs imbibition. Germination counted at 24 hrs intervals from the start of imbibition.

Table 42 A) Water to water.

Germination after	24 hrs.	48 hrs.	72 hrs.
Control H_2O	43.3 ± 3.6	90.7 ± 1.3	92.0 ± 1.6
Transfer after (hrs)			
1	34.3 ± 2.7	88.3 ± 2.5	92.0 ± 1.5
2	35.3 ± 5.7	87.7 ± 1.7	89.3 ± 2.3
3	34.0 ± 3.8	90.0 ± 3.0	92.7 ± 1.9
4	32.3 ± 2.4	91.7 ± 2.6	93.3 ± 2.4
6	32.0 ± 4.4	91.0 ± 2.1	92.3 ± 2.4
8	35.7 ± 2.5	92.7 ± 2.0	94.3 ± 1.4
10	35.3 ± 2.8	94.7 ± 1.1	96.7 ± 1.1
12	34,3 ± 4.1	95.7 ± 0.8	96.7 ± 1.0
16	55.7 ± 6.6	95.7 ± 0.9	96.3 ± 1.0
20	51.0 ± 6.2	97.0 ± 1.1	97.3 ± 1.0

Table 42 B) Water to 10^{-5} M ABA

Germination after	24 hrs	48 hrs	72 hrs	96 hrs
Control H_2O	43.3 ± 3.6	90.7 ± 1.3	92.0 ± 1.4	-
10^{-5} M ABA	12.3 ± 2.3	73.3 ± 2.0	75.0 ± 1.8	78.3 ± 1.9
Transfer after (hrs)				
1	6.0 ± 1.7	64.7 ± 3.8	70.0 ± 3.7	71.3 ± 3.1
2	6.0 ± 1.0	65.0 ± 4.1	70.3 ± 2.7	73.0 ± 1.8
3	8.7 ± 1.1	72.0 ± 4.7	76.3 ± 4.3	77.7 ± 4.4
4	8.7 ± 1.6	68.3 ± 4.3	77.3 ± 2.9	79.0 ± 3.0
6	9.7 ± 2.2	75.0 ± 4.5	84.7 ± 3.8	85.3 ± 4.0
8	11.0 ± 1.8	85.3 ± 2.1	90.0 ± 1.5	93.0 ± 1.3
10	16.3 ± 3.7	90.7 ± 1.6	93.7 ± 1.8	94.7 ± 1.5
12	20.0 ± 3.6	91.3 ± 1.4	94.3 ± 1.3	94.7 ± 1.2
16	44.7 ± 3.1	95.7 ± 0.9	96.3 ± 1.4	96.3 ± 1.4
20	43.0 ± 5.6	95.3 ± 1.6	96.7 ± 1.5	96.7 ± 1.5

Table 42 C) Water to 10^{-4} M ABA

Germination after	24 hrs	48 hrs	72 hrs	96 hrs	7 days
Control H_2O	43.3 ± 3.6	90.7 ± 1.3	92.0 ± 1.4	-	-
10^{-4} M ABA	0	0	7.0 ± 1.7	27.0 ± 3.1	31.3 ± 3.7
Transfer after (hrs)					
1	0	0.7 ± 0.4	7.0 ± 2.7	16.7 ± 1.7	23.0 ± 1.7
2	0	0.7 ± 0.4	6.0 ± 4.1	20.0 ± 8.6	27.0 ± 9.6
3	0	0.7 ± 0.7	8.7 ± 4.6	21.3 ± 6.1	30.3 ± 5.3
4	0	1.0 ± 0.7	4.3 ± 3.6	18.0 ± 4.2	29.7 ± 6.0
6	0	1.0 ± 0.5	6.3 ± 1.9	19.3 ± 2.9	31.7 ± 3.4
8	0	1.0 ± 0.5	13.7 ± 1.7	35.0 ± 3.8	54.3 ± 5.9
10	0	1.7 ± 0.8	11:7 ± 1.2	29.3 ± 3.7	49.0 ± 4.3
12	0	3.9 ± 0.7	12.7 ± 3.4	36.3 ± 4.0	51.3 ± 3.3
16	4.0 ± 1.7	19.0 ± 2.1	50.7 ± 5.9	79.7 ± 2.3	94.0 ± 0.9
20	26.3 ± 10.2	43.7 ± 9.9	69.3 ± 8.4	88.3 ± 1.7	92.7 ± 0.7

FIGURE 24

The percentage germination of seeds of Grand Rapids lettuce at 30°C in the presence of cytokinins.

BA : benzyladenine
K : kinetin
SD8339
Z : zeatin

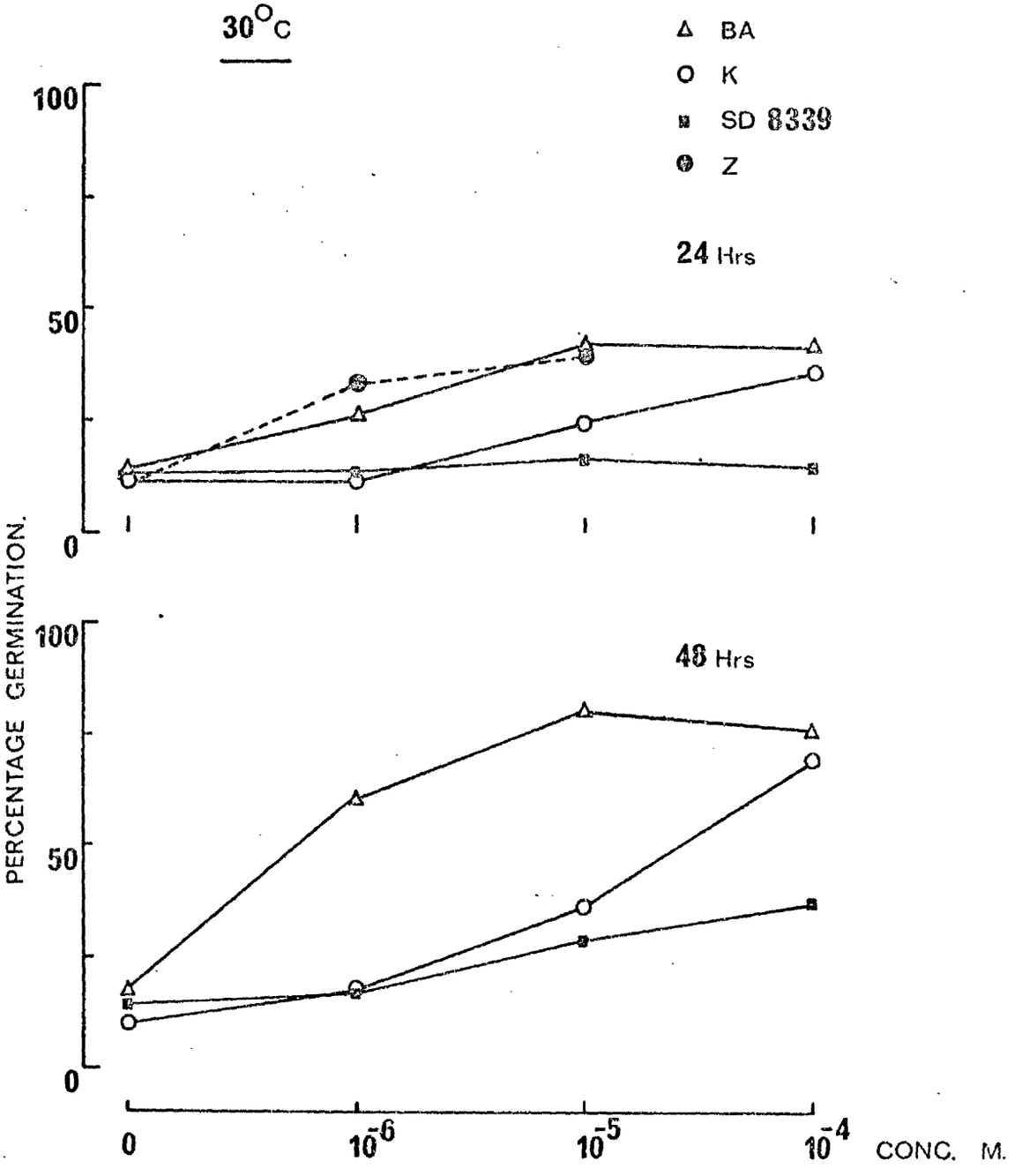

30°C
BA
K
SD 8339
Z
24 Hrs
48 Hrs
100
50
0
PERCENTAGE GERMINATION.
0
10⁻⁶
10⁻⁵
10⁻⁴
CONC. M.

FIGURE 25

The percentage germination of seeds of Grand Rapids lettuce in the presence of (±)-abscisic acid at 20°C.

Horizontal axis : molar concentration of abscisic acid.

Percentage germination in water at 20°C.

24 hrs	48 hrs
90.0 ± 1.2	100.0 ± 0.0

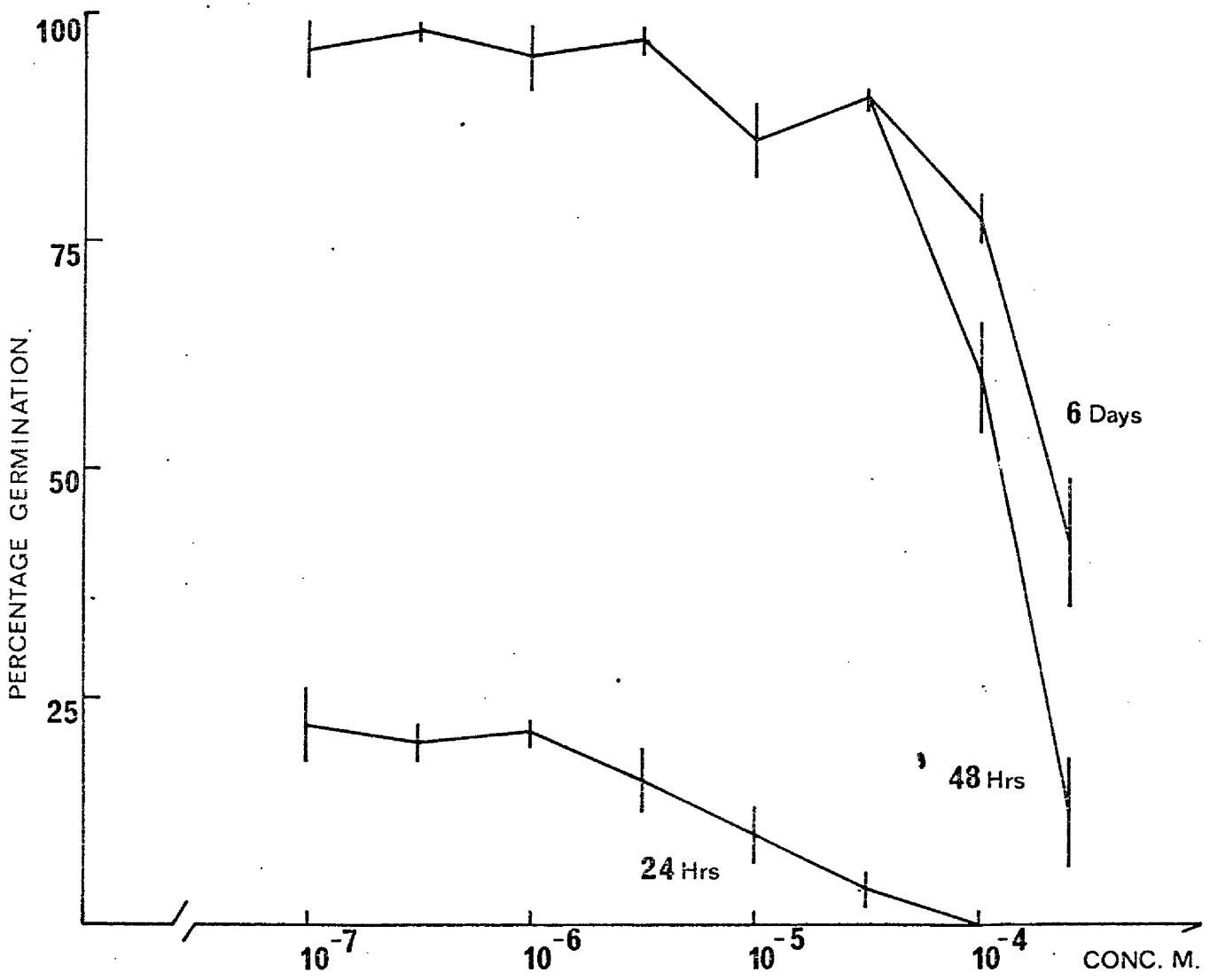

100
75
50
25
PERCENTAGE GERMINATION.
6 Days
48 Hrs
24 Hrs
10^{-7}
10^{-6}
10^{-5}
10^{-4}
CONC. M.

FIGURE 26

The percentage germination after 24 hrs of lettuce seeds imbibed in water at 20°C and transferred to:

(A) water

(B) ABA 10^{-5} M

(C) ABA 10^{-4} M

after 1, 2, 3, ------- 20 hrs imbibition.

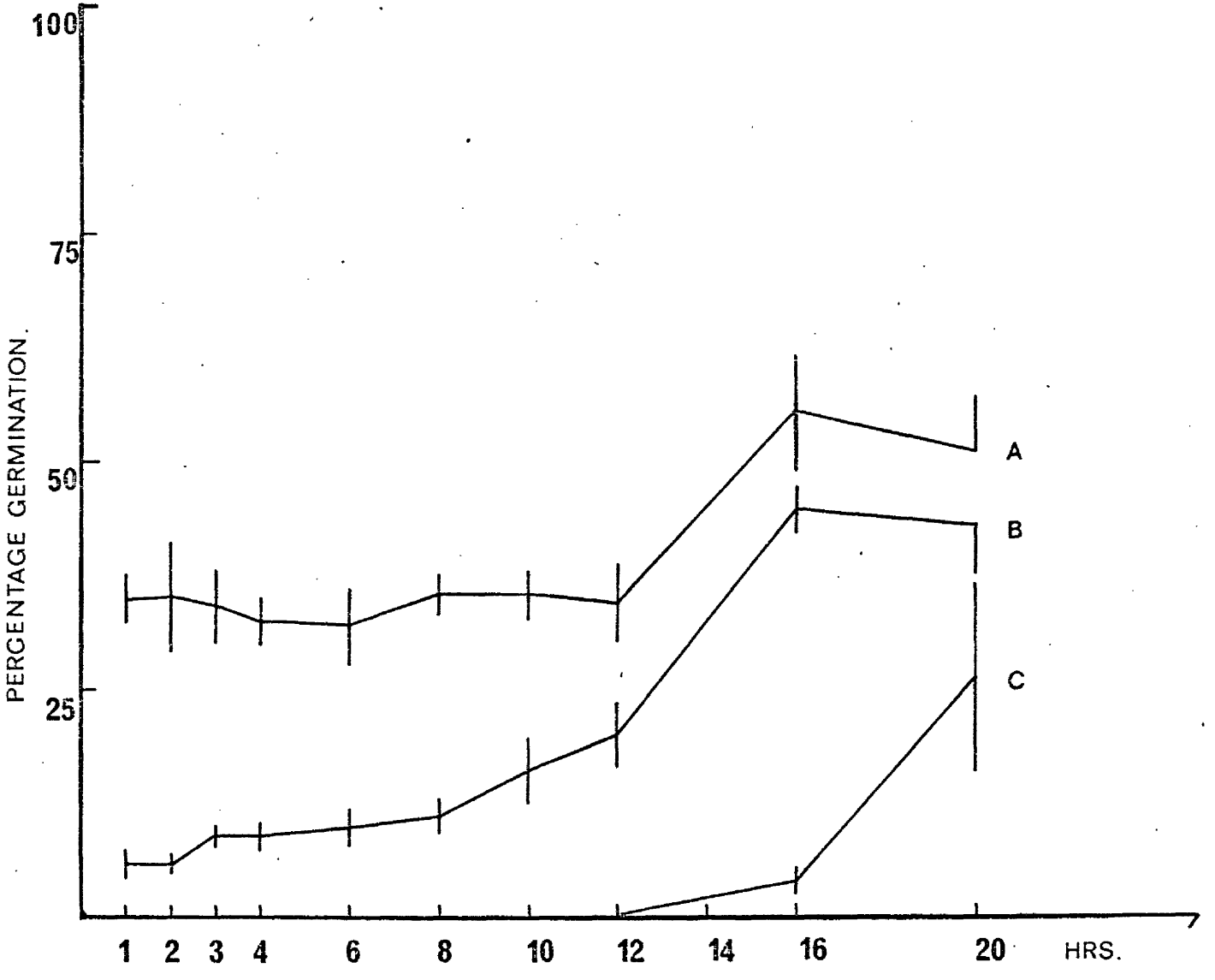

100
75
50
25
PERGENTAGE GERMINATION.
A
B
C
1
2
3
4
6
8
10
12
14
16
20
HRS.

FIGURE 27

The percentage germination after 48 hrs of lettuce seeds imbibed in water at 20°C and transferred to:

(A) water

(B) ABA 10^{-5} M

(C) ABA 10^{-4} M

after 1,2,3, ---------- 20 hrs imbibition.

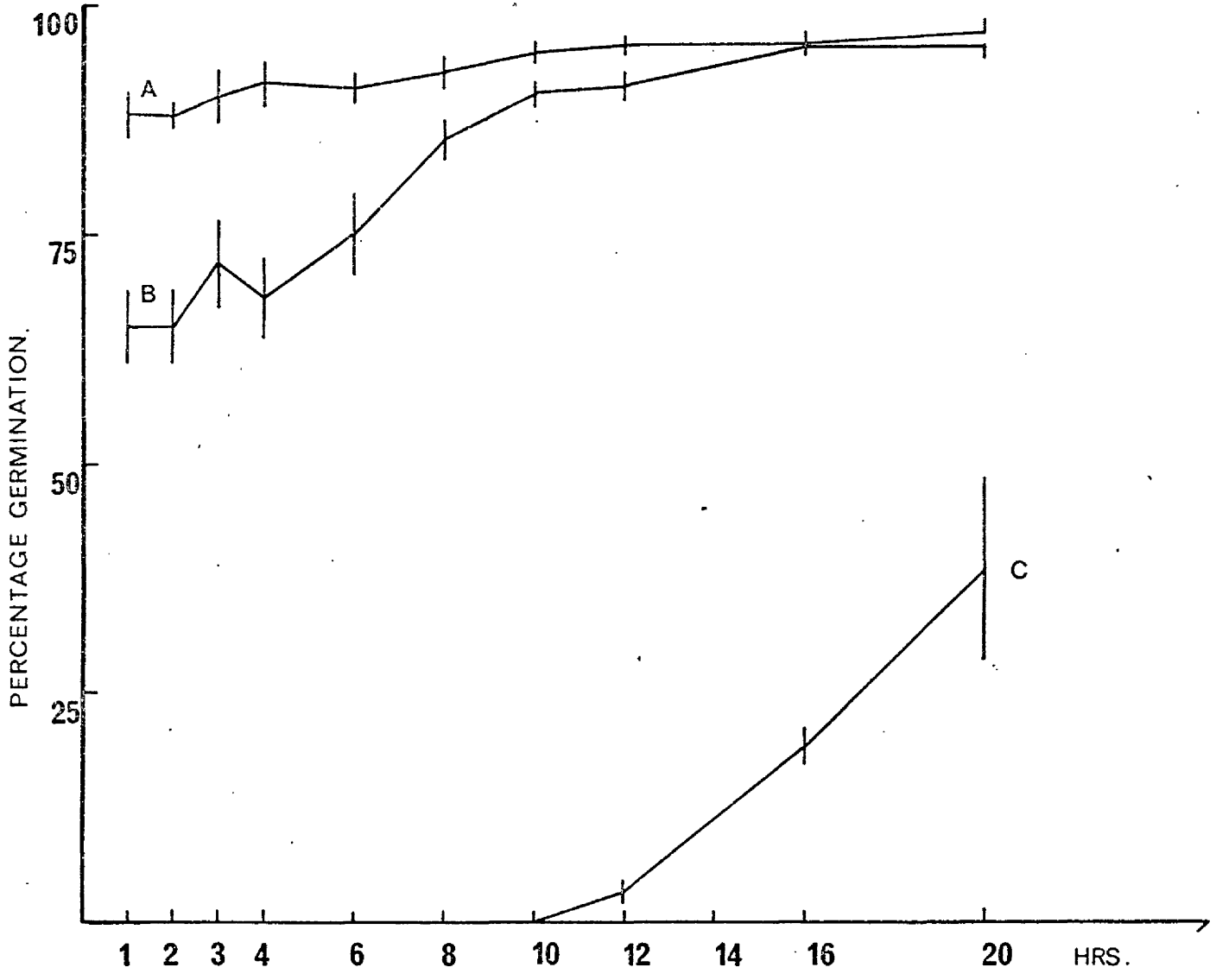

100
75
50
25
PERCENTAGE GERMINATION.
A
B
C
1
2
3
4
6
8
10
12
14
16
20
HRS.

FIGURE 28

The percentage germination after 4 days of lettuce seeds imbibed in water at 20°C and transferred to 10^{-4} M ABA after 1,2,3 ----- hrs imbibition.

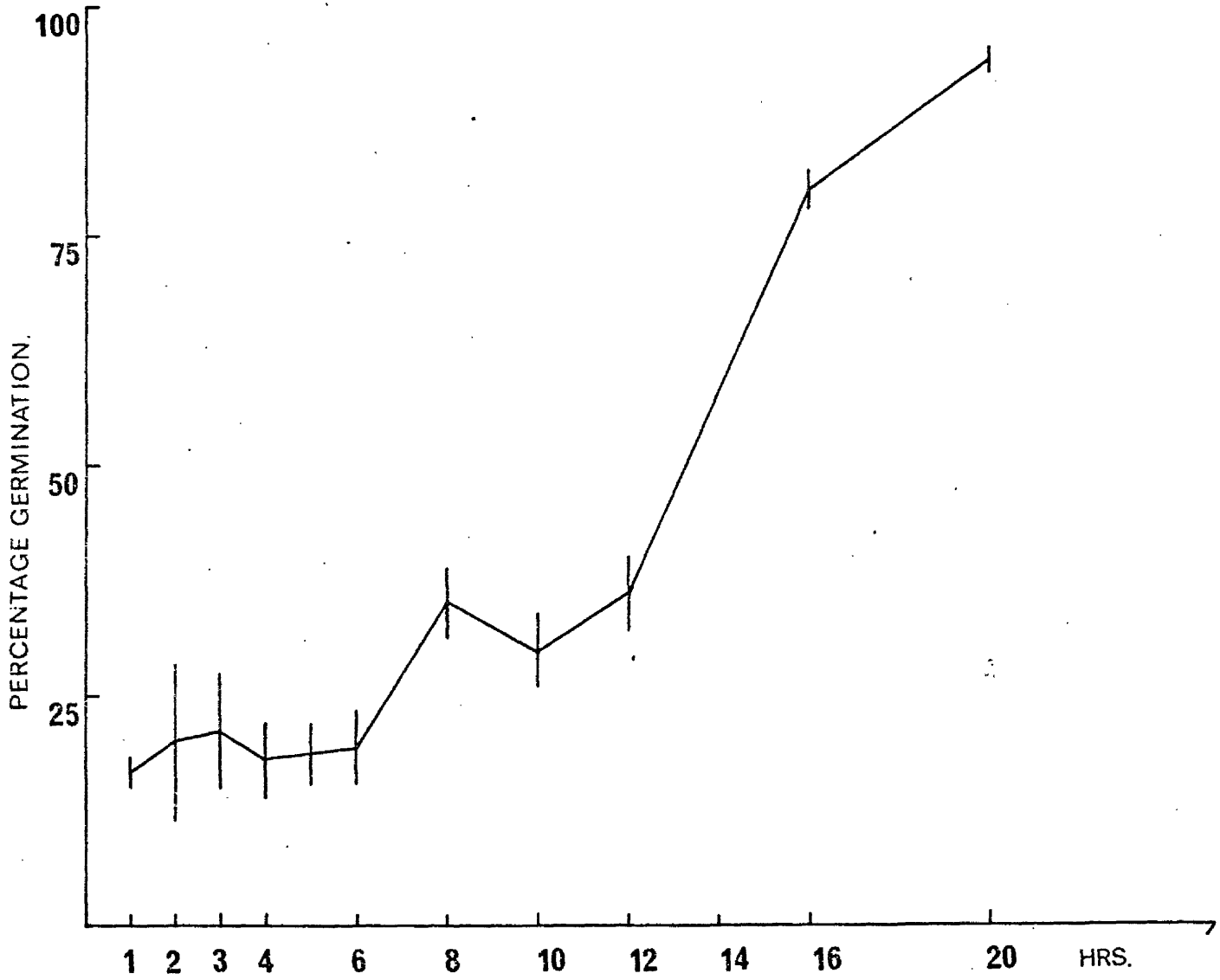

100
75
50
25
PERCENTAGE GERMINATION.
1
2
3
4
6
8
10
12
14
16
20
HRS.

FIGURE 29

The effect on germination of lettuce seeds, of transfer to ABA at 10^{-5} M after 1, 2, 3, ----- 20 hrs imbibition in water. Germination counted after 24 hrs.

The corrected percentage germination was obtained as described in the text.

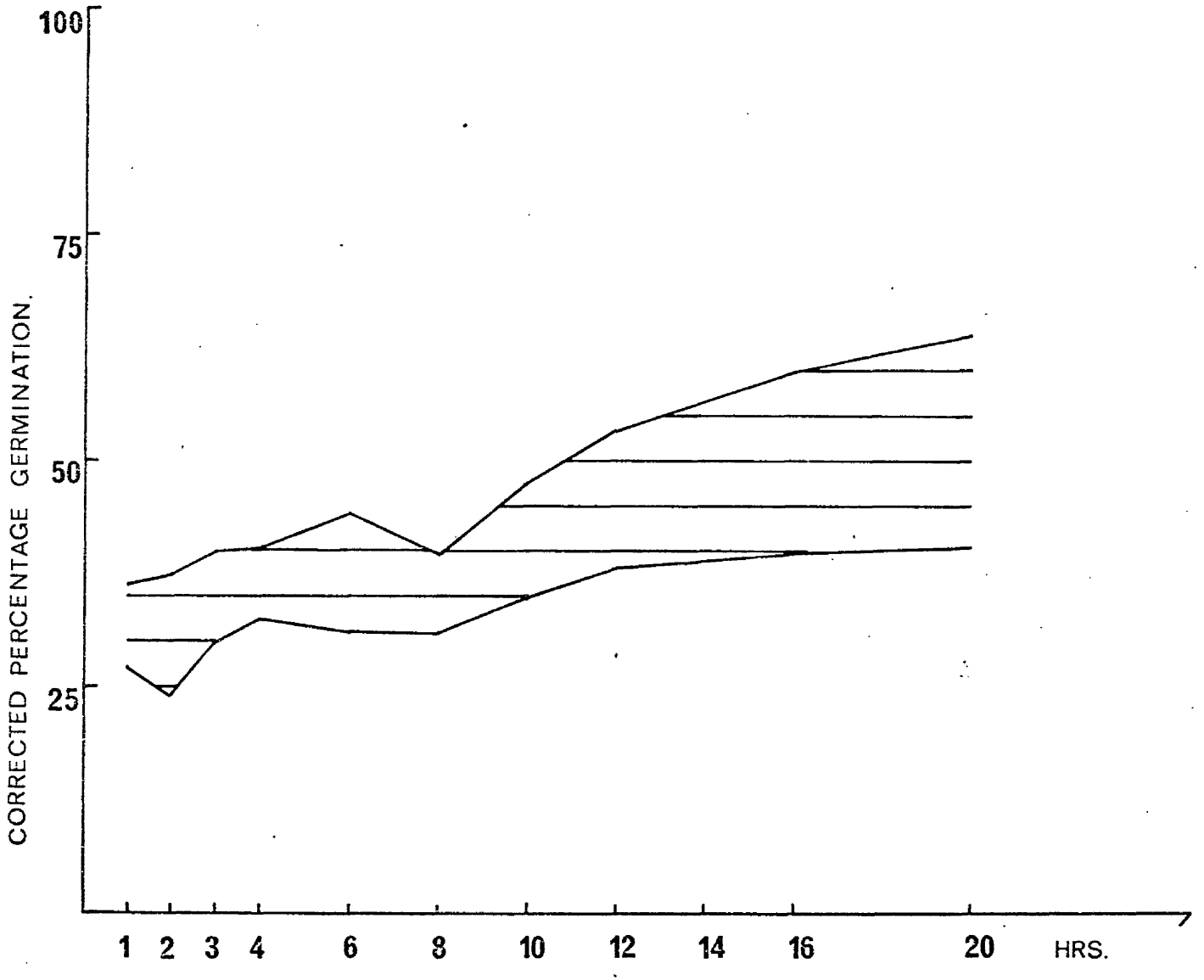

100
75
50
25
CORRECTED PERCENTAGE GERMINATION.
1
2
3
4
6
8
10
12
14
16
20
HRS.

FIGURE 30

The percentage germination of seeds of Grand Rapids lettuce in the presence of kinetin and 6.7×10^{-5} M abscisic acid at 20°C.

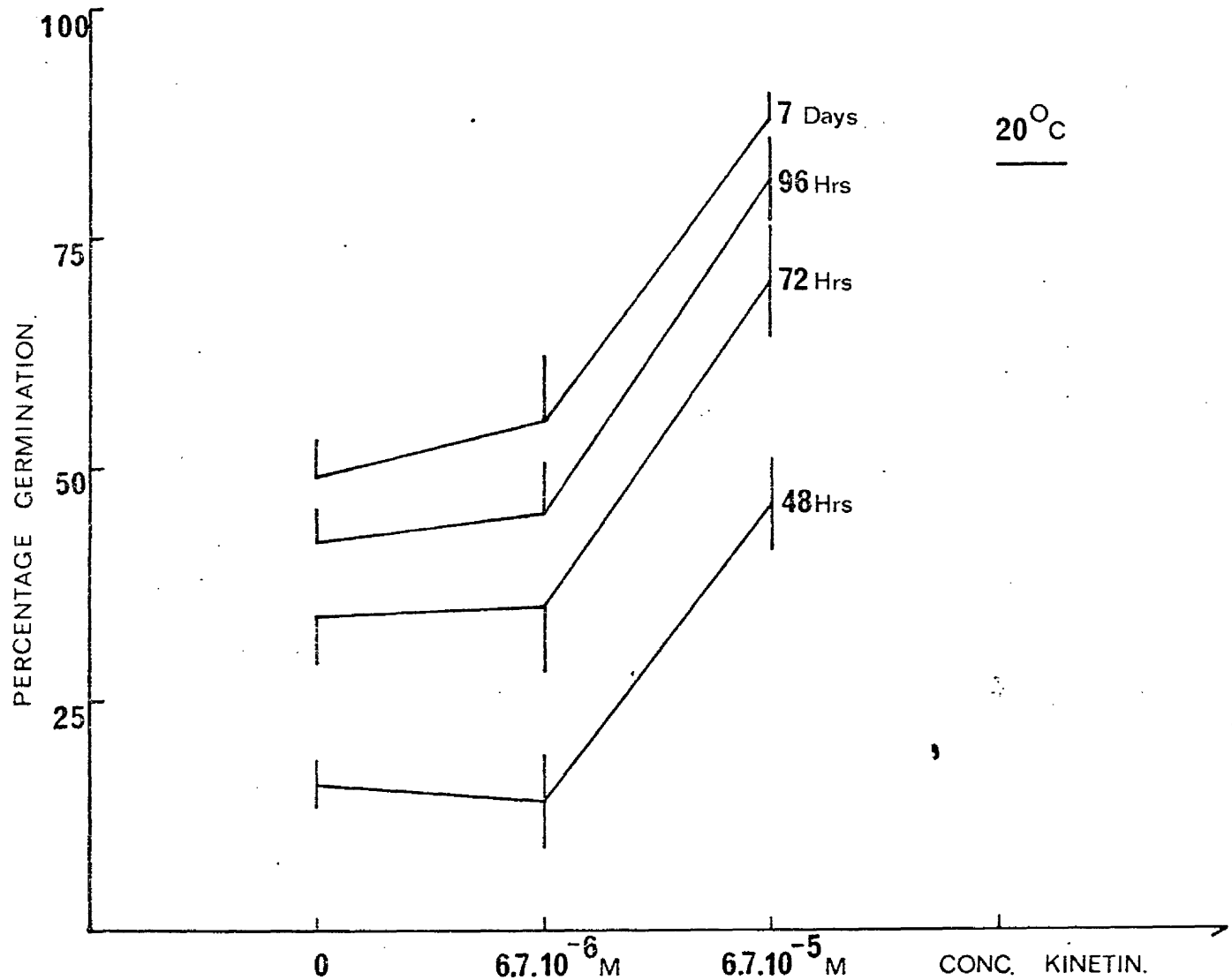

100
75
50
25
PERCENTAGE GERMINATION
7 Days
96 Hrs
72 Hrs
48 Hrs
20°C
0
6.7.10⁻⁶M
6.7.10⁻⁵M
CONC. KINETIN.

FIGURE 31

The percentage germination of seeds of Grand Rapids lettuce in the presence of gibberellin A_{4+7} and 6.7×10^{-5} M abscisic acid at 20°C.

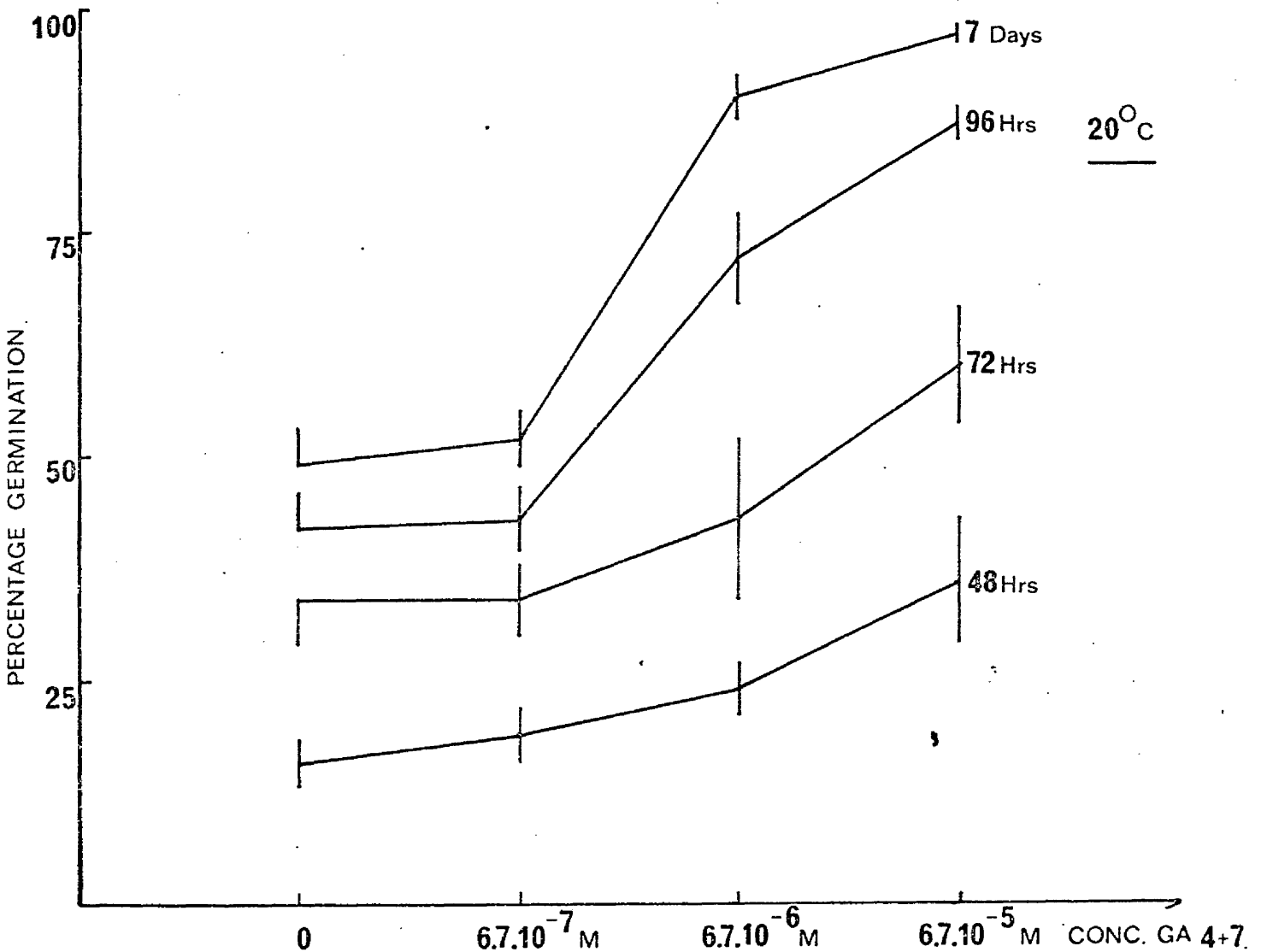

100
75
50
25
PERCENTAGE GERMINATION
7 Days
96 Hrs
72 Hrs
48 Hrs
20°C
0
6.7.10⁻⁷ M
6.7.10⁻⁶ M
6.7.10⁻⁵ M
CONC. GA 4+7

FIGURE 32

The percentage germination of seeds of Grand Rapids lettuce in the presence of gibberellin A_{4+7}, benzyladenine at 6.7×10^{-5} M and abscisic acid at 6.7×10^{-5} M at 20°C.

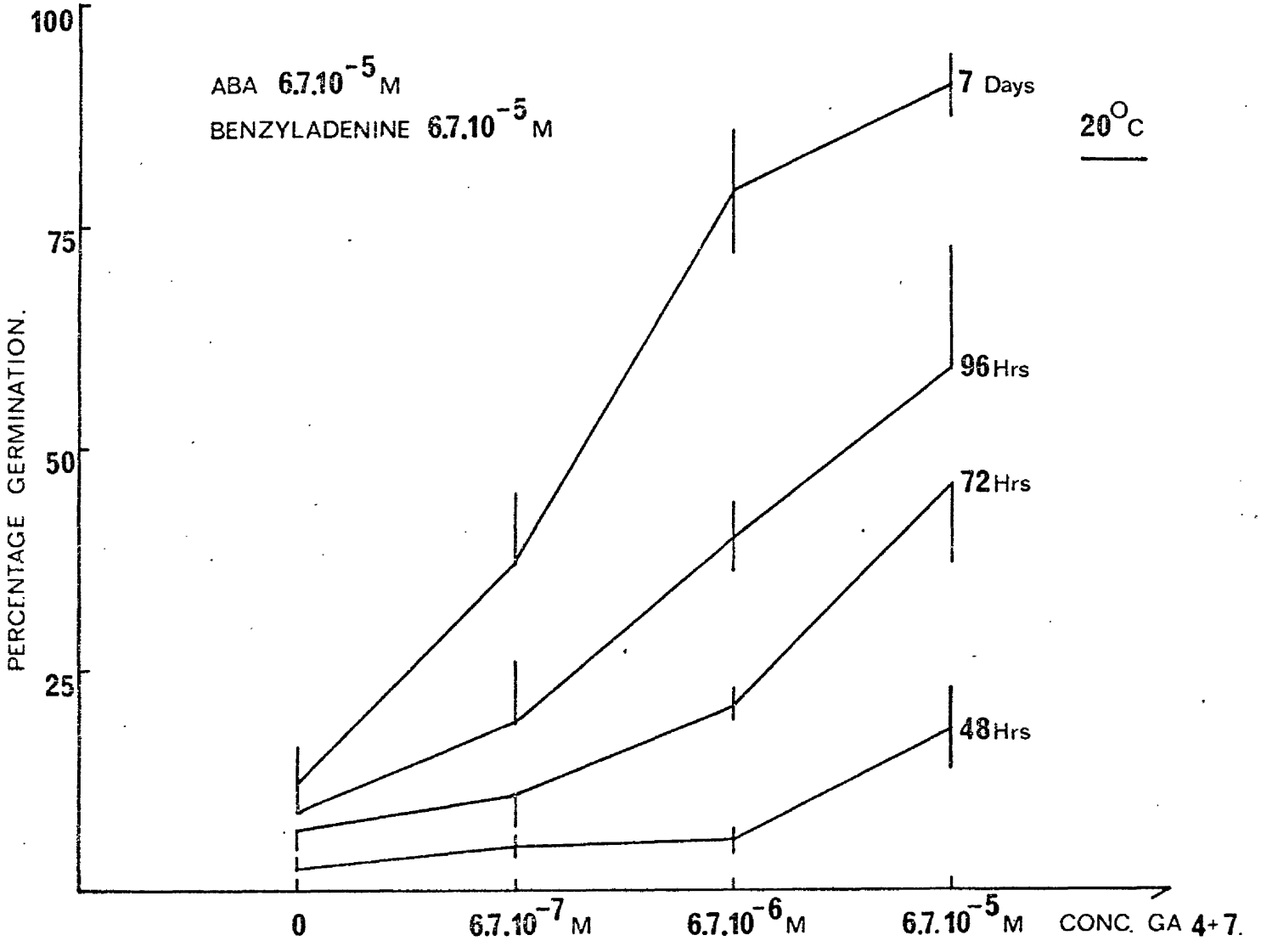

100
75
50
25
PERCENTAGE GERMINATION.
ABA 6.7.10^{-5}M
BENZYLADENINE 6.7.10^{-5}M
7 Days
20^{O}C
96 Hrs
72 Hrs
48 Hrs
0
6.7.10^{-7}M
6.7.10^{-6}M
6.7.10^{-5}M
CONC. GA 4+7.

FIGURE 33

The percentage germination of seeds of Grand Rapids lettuce after 24 hrs at 20°C in the presence of abscisic acid and exposed to red light.

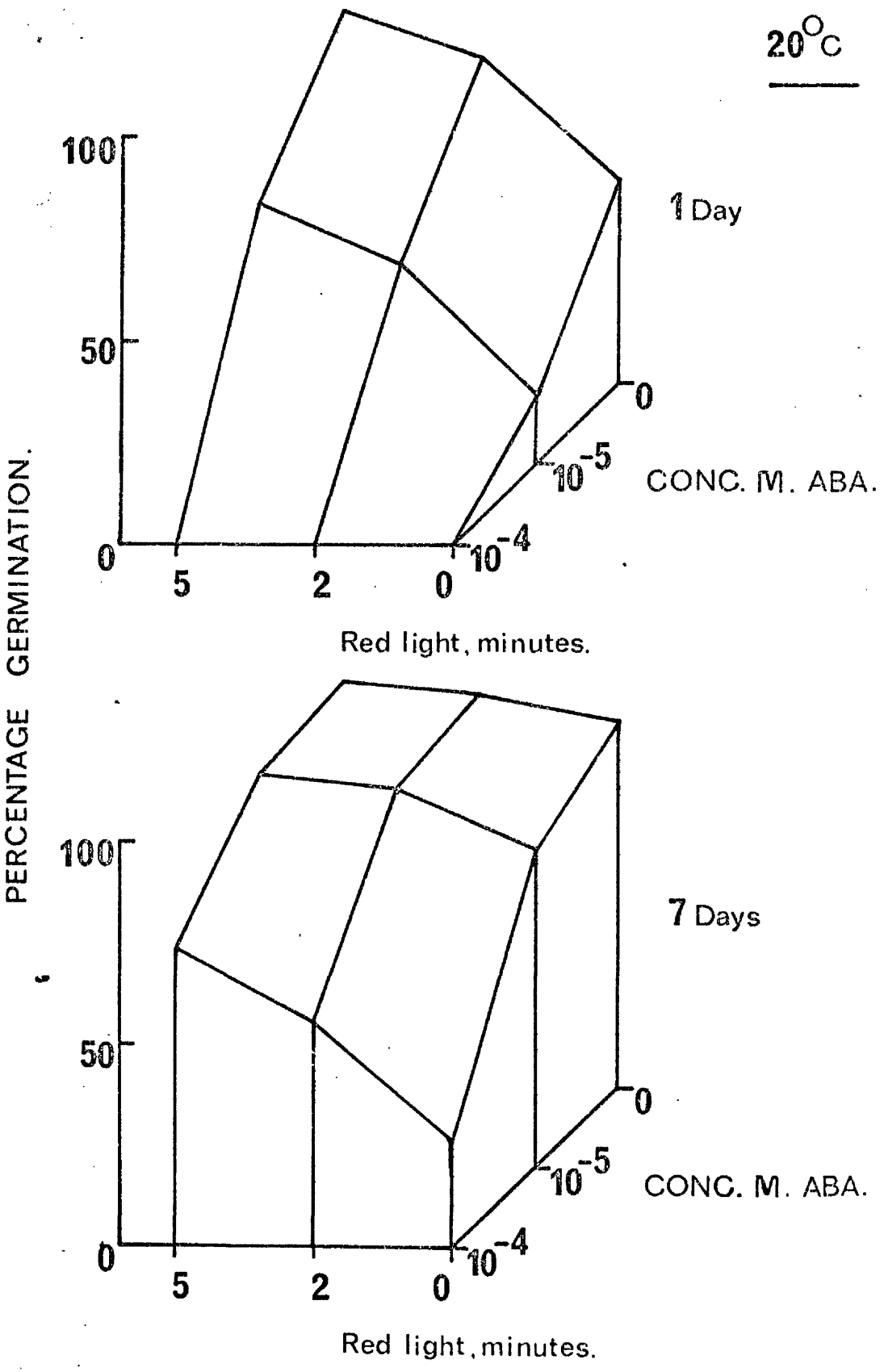
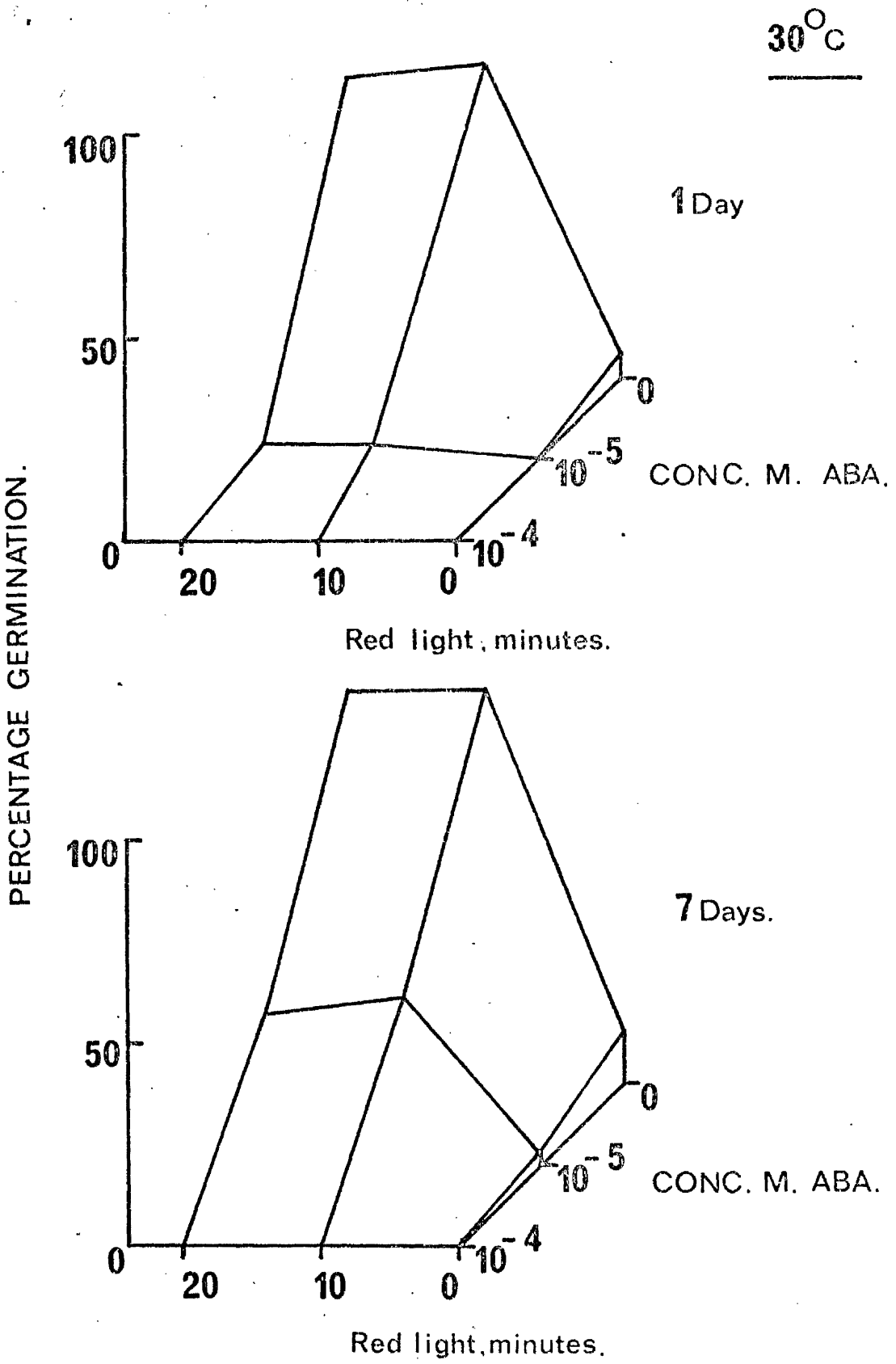

20°C
1 Day
100
50
0
PERCENTAGE GERMINATION.
0
5
2
0
10-4
10-5
0
CONC. M. ABA.
Red light, minutes.
7 Days
100
50
0
0
5
2
0
10-4
10-5
0
CONC. M. ABA.
Red light, minutes.

FIGURE 34

The percentage germination of seeds of Grand Rapids lettuce after 24 hrs at 30°C in the presence of abscisic acid and exposed to red light.


30°C
1 Day
100
50
0
0
10^{-5}
10^{-4}
CONC. M. ABA.
20
10
0
Red light, minutes.
PERCENTAGE GERMINATION.
7 Days.
100
50
0
0
10^{-5}
10^{-4}
CONC. M. ABA.
20
10
0
Red light, minutes.

PART D

Abscisic acid : an endogenous component of seeds of Grand Rapids lettuce ?

Introduction

We have suggested that ABA may be a natural, endogenous inhibitor of lettuce seeds and that it may be involved in the imposition and release from thermodormancy. However, there is no evidence for the presence of ABA in seeds of Grand Rapids lettuce. McWha (1973) has reported that ABA is present in seeds of the lettuce cultivars, Arctic King and Great Lakes, the levels found being 200 and 700 µg/kg in dry seeds and 2.2 µg/kg in 24 hrs imbibed seeds of Great Lakes. Furthermore, there is evidence that the conjugated form of ABA (abscisyl-β-D-glucopyranoside), identified in immature fruits of *Lupinus luteus* by Koshimizu *et al.* (1968), can occur in seeds (Le Page-Degivry, 1973; Bulard *et al.*, 1974), and that a parallel reduction in 'free ABA' and increase in 'bound ABA' can be shown in response to dormancy break (Leshem *et al.*, 1974; Le Page-Degivry, 1973). In the general introduction we proposed that 'bound ABA' might be less active either as an intrinsic property or by virtue of compartmentation (Milborrow, 1974).

This section is concerned with investigating Grand Rapids lettuce seeds for the presence of ABA, 'free' and/or 'bound', and secondly, examining changes in ABA in response to dormancy breaking stimuli in an attempt to assign a controlling function to ABA in thermodormancy.

Results

The extraction and purification procedures employed were described in the materials and methods section. Briefly, seed samples, dry or freeze dried, were extracted in 80% methanol, the methanol removed and the remaining aqueous fraction subjected to acid, base partitioning. The aqueous fraction was also base hydrolysed and then re-partitioned as before. This gave two final ether fractions which were chromatogramed on columns of PVP and then subjected to thin layer chromatography. The losses of ABA during this extraction and purification procedure were calculated by including radioactively labelled ABA

(2-^{14}C-ABA) at the start of the extraction and removing aliquots for assay at each stage. The final purified fractions were methylated using excess ethereal diazomethane. The methyl ester was then analysed by gas liquid chromatography (GLC) or by combined gas liquid chromatography, mass spectrometry (GC-MS). In a small number of occasions, samples were injected in to the mass spectrometer.

Identification of ABA

An extract from 500 g of dry seeds of Grand Rapids lettuce was purified as described and analysed by combined GC-MS. The GLC was a Pye 104 attached to an AEI MS-30, the two being joined via a silicone membrane. The levels of ABA present could not be detected by GLC using the total ion current detection system. Thus, the trace was scanned at the characteristic retention time for standard Me. ABA (cis, trans isomer) on the column in use, routinely 2% SE30. Figure 36 shows the mass spectrum for standard ABA Me ester showing the most abundant ion to be m/e 190, with a parent ion at m/e 278 and characteristic fragmentation ions at m/e 246, 222, 162, 134, 125, 91. Analyses of several samples of extracts of Grand Rapids lettuce seeds gave peaks at m/e 278, 190, 162, 134 and 125. The relative percentage abundance, compared to m/e 190, of one such extract is shown below.

Percentage abundance of major ions in mass spectrum of abscisic acid methyl derivative*

Ion	Standard from Most et al.**	Standard from Sigma	Extract of 500 g dry Grand Rapids lettuce seed
191	11	16	19
190	100	100	100
162	24.5	30	47
134	24	29	46
125	28	38	86
91	19	19	46

* cis, trans isomer

** Planta (Berlin), 92, 41-49. 1970.

Comparison between published spectra and standards with a seed extract confirms the presence of ABA as an endogenous component of Grand Rapids lettuce seeds. Differences in relative abundance of ions may be the result of differences in operating conditions.

Quantification of endogenous ABA

Having identified ABA as an endogenous component of Grand Rapids lettuce seeds, it was essential to measure how the levels of ABA changed in seeds as a result of a number of treatments. It was pointed out that seeds contained too little ABA to be detected as a total ion current, using the GLC attached to the MS. Furthermore a flame ionisation detector (FID) attached to a Pye 104 GLC proved to be of no value, partially as a result of the low levels of ABA present in extracts and also as a result of interfering compounds which gave peaks at identical retention times to ABA. The initial quantification of endogenous ABA was carried out using the technique of 'single ion monitoring'.

When a sample is introduced into the ionisation chamber of the MS it is ionised by electron bombardment by allowing electrons of fixed energy between 5 and 100 eV to collide with the vapourized sample. This produces a molecular ion and fragmentation ions. The electrons are produced by heating a filament. The ions so produced are then separated by their mass to charge (m/e) ratios. The ions are accelerated through a 'source slit'. After this every singly charged ion has approximately the same total energy. In fact, in practice ions have a finite range of kinetic energies. The AEI MS-30 is a double focusing instrument. Thus, the ions are passed through an electrostatic analyser where the field of the analyser acts as an energy monochromator allowing, via a small slit, only ions with a much narrower range of kinetic energy into the magnetic field. In the magnetic field moving, charged particles are deflected. The equation governing the flight of a charged particle is given by:

$$m/e = \frac{H^2R^2}{2E}$$

where E = accelerating voltage

e = charge

R = radius of magnetic field

H = magnetic field force

The 'heavier' the ion the earlier it will come out of the magnetic field and be recorded. This is the basic theory which underlies the operation of the MS. The instrument may be focused on one particular m/e ratio and a record made only of the ion current for this one ratio. We have shown that ABA Me ester produces a number of fragment ions at characteristic m/e ratios. Gray et al. (1974) have presented a pathway for the production of these ions and this is reproduced in Figure 37. The most abundant ion is at m/e 190. By focusing the MS so that it records only ions with an m/e ratio of 190 it is possible to use the MS to monitor the effluent stream of the GLC and obtain a highly sensitive semi-specific detection of endogenous ABA. The area of m/e 190 peaks at the retention time of ABA Me ester can be measured by triangulation to give an estimate of the ABA present. A typical single ion scan is given in Figure 38.

This technique has only been used once previously for ABA by Railton et al. (1974) to show the presence of ABA in chloroplasts of Pisum sativum. However, it is a well-established technique in the study of indole acetic acid (IAA) and derivatives in cerebrospinal fluid (Bertilsson and Palmer, 1972; Bertilsson et al., 1972) and IAA in maize root caps (Rivier and Pilet, 1974) having first been introduced in the late 1960s (Sweeley et al., 1966; Hammer et al., 1968).

There is a number of problems associated with the use of this technique in quantitative analysis which were more apparent with respect to IAA. These will be discussed in more detail when dealing with the measurement of endogenous IAA.

Although single ion monitoring is more sensitive than FID with GLC, it cannot compare with the sensitivity shown by an electron capture detector (ECD) on a gas liquid chromatogram. Some time after this study was started we took delivery of a Perkin-Elmer F17 GLC fitted with an ECD detector. Thereafter this was used to quantify endogenous ABA in seeds. The first reported use of an ECD for Me ABA was by Seely and Powell (1970) who indicated that ECD was several orders of magnitude more sensitive in detecting ABA Me ester than FID. The limit of detection using ECD was reported to be 10^{-6}-10^{-5} µg. The ECD is also semi-selective as it will only respond to certain compounds. As a result smaller seed samples could be used and the samples for GLC did not require as rigorous purification prior

to analysis. It should be pointed out that some purification was still required: if very 'dirty' extracts were subjected to GLC, even using ECD, the response of the ECD could go off scale. This meant that the sensitivity had to be reduced. Thus, if optimal conditions are to be achieved samples must be reasonably pure. The ECD used in this study was operated on the 'pulse mode'. The theory underlying the operation of the ECD and its use in quantitative analyses is fully discussed by Lovelock (1963). Suffice to say that the pulse mode removes many of the problems associated with earlier designs. However, quantification of ABA in a plant extract requires that certain criteria are met with respect to the operation of the ECD. Firstly, the ECD can be 'overloaded' which results in flattened peaks which bear no relation to the real amounts of material present. A more critical factor in the use of ECD is that the response should be shown to be linear with respect to the amount of compound injected into the GLC. Figure 35 shows the results of trials set up to test the linearity of the ECD used in this work. (A) shows the ECD response when different amounts of Me ABA were injected by altering the volume of the solvent containing the sample (e.g. one stock solution). (B) shows the ECD response when the same volume 2 µl, was injected and a series of stock solutions of Me ABA employed. It is clear that the response of the ECD is linear in both cases but that varying the sample size injected could lead to erroneous results. Consequently 2 µl samples were always injected and the ECD was always operated within this linear range. If these conditions were met the ECD was found to be extremely reliable and highly reproducible. Extracts were analysed on several occasions to check reproducibility. It was hoped to include an internal standard to help in the quantification of samples, as it was not possible to analyse all samples at one time and consequently, under exactly identical conditions. However, despite a wide search a suitable compound could not be found. Such a compound would ideally have to have a greater retention time than the trans, trans isomer of ABA. Me ester and also give a good response on the ECD. Lenton et al. (1971) used the trans, trans isomer of ABA as an internal standard. However, this requires that no trans, trans isomer occurs naturally. With respect to Grand Rapids seeds this question was not specifically investigated. However, if care was taken to ensure extracts were not exposed to light, only a very small amount of trans, trans ABA could be detected by GLC. Whether this is naturally occurring or the result of photoisomerisation cannot be answered. The absence

of the trans, trans isomer might be expected as Grand Rapids seeds have a dark, opaque pericarp. Quantification of extracts was by triangulation of the peaks corresponding to the retention time of cis, trans- and trans, trans-ABA, and comparison to the area for known amounts of ABA. Table 50 shows the results obtained for endogenous levels of both 'free' and 'bound' ABA. The levels of ABA were examined in seeds imbibed at 20°C, below the critical temperature for the induction of thermodormancy, 25°C at the critical temperature and 30°C where seeds were thermodormant. Unfortunately, the early work was carried out using very large weights of seed, 500 g which meant the experiments could not be repeated using the same seed batch. Seeds were purchased in 10 kg batches. In later experiments smaller amounts of seed, 100 g lots, were used which enabled repetition.

We can see from the table below and Table 50 that different seed batches contain varying amounts of both 'free' and 'bound' ABA. William Watt I (WWI) contained over five times the level of 'free' ABA of that present in William Watt II (WWII). The ratio of 'free' to'bound' also varied from almost 1:1 (011974 and 014574) to 1:2 (WWII). Thus, there is no similarity between batches of the same cultivar, Grand Rapids.

Levels of abscisic acid in different batches of 'Grand Rapids' lettuce seed

Batch	µg/kg ABA	
	Free	Bound
WWI	381.0	-
011974	184.0	137.0
WWII	70.5	134.0
014574	172.0	160.0

The levels of ABA are shown in Table 50 and have been corrected for losses during extraction and purification. The percentage loss can be calculated from the recovery during extraction up to chromatography 70%, recovery from PVP 90%, and recovery from TLC 66%.

Recovery of radioactivity from 2[^{14}C]ABA during extraction and purification procedure employed for endogenous ABA.

Extraction stage	Phase	% of original radioactivity added to aqueous phase (67,542 dpm)
(1) aqueous pH 3.0 x diethyl ether	aqueous ether	4.5 95.5
(2) ether x H_2O & Na_2HCO_3	ether H_2O & Na_2HCO_3	0.6 85.2
(3) H_2O & Na_2HCO_3 pH 7.5	ether H_2O & Na_2HCO_3	0.2 85.0
(4) H_2O & Na_2HCO_3 x ether pH 3.0	ether H_2O & Na_2HCO_3	66.8 (45,086 dpm) -
(5) PVP chromatography		60.1 (90% recovery)
(6) TLC		40.0 (66% recovery)

(The order of magnitude of variation throughout this procedure was ± 5%.)

If we look at the figures for Page and Co., it is clear that both 'free' and 'bound' ABA levels fall with increasing imbibition at 20°C, where germination occurs. The ratio between 'free' and 'bound' remains constant at about 2.5:1. These data are in agreement with that presented by McWha for Great Lakes except that the reduction in Great Lakes of free ABA was much larger. However, examination of the levels found in WWI at 20°C, whilst showing a reduction in endogenous ABA are not as convincing. Indeed the level of 'bound' ABA seems to increase. At 30°C the pattern with WWI is similar to that shown at 20°C with an increase in 'bound' ABA after 20 hrs imbibition. This reduction in 'bound' ABA followed by a later increase can also be observed in batch No. 04974 from Page & Co. at 20°C.

It is possible to compare the relative levels of ABA of different seed batches, by relating the levels found in any one batch with a figure of 100 for the amount of free ABA found in the dry seeds of that batch. Thus, we can examine the pattern of changes which take place in different seed batches. Such an approach has been used to

produce the data shown in Figures 39 and 40, which show the relative levels of 'free' and 'bound' ABA in different seed batches, respectively. The data for WWII show that the changes which take place can be shown to be repeatable using the same batch. At 20°C the levels of 'free' ABA show a slight increase after 18 hrs which decreases after 24 hrs. There is no consistent pattern in the levels of 'bound' ABA (Table 50, Figure 39). At 30°C and 25°C the level of 'free' ABA shows a large increase, over four-fold, at 12 hrs which then rapidly declines to dry seed levels after 24 hrs. The levels of 'bound' ABA at 30°C show a decrease until after 24 hrs, the ratio of 'free' to 'bound' ABA has altered from 1:2 to 1:1. At 25°C the change in 'bound' ABA is not consistent. Thus, there would not appear to be any uniform pattern between different seed batches, with respect to the change in endogenous ABA levels during imbibition at a temperature where the seeds will germinate or remain dormant.

Furthermore, the changes which do occur cannot be explained in terms of leaching of endogenous ABA into the imbibition medium. The residual water, left after the removal of seeds, was extracted for ABA. Whilst ABA was shown to be present it was in too small amounts to account for the observed changes in ABA levels.

Levels of ABA extracted from water in which lettuce seeds had been imbibed.

Source	Treatment		µg/kg 'free' ABA
	Hrs.	Temp °C	
WWI	12	30	27
WWII	12	20	40
	24	20	30
	12	30	61
	24	30	22

Discussion

From the results presented it is clear that ABA is a naturally occurring component of Grand Rapids lettuce seeds and that it exists both as a 'bound' form as well as 'free'. The 'bound' form was released by base hydrolysis and is presumed identical with the glucosyl ABA previously identified (Koshimizu *et al.*, 1968). An attempt was

made to identify the 'bound' ABA using adsorption on to activated charcoal, elution with aqueous acetone and then PVP column chromatography. However, in the absence of a standard and as a result of the unstable nature of the ester, it proved impossible to unequivocally identify the compound as abscisyl-β-D'-glucopyranoside. The levels of free ABA found in these lettuce seeds compares favourably with those reported for other seeds, e.g.

Walton et al., (1973) dry seed of Phaseolus vulgaris 60 µg/kg.
Williams et al., (1973) non-dormant embryos of Coryllus avellana 24 µg/kg.
McWha (1973) dry seed of lettuce, cv. Great Lakes 700 µg/kg.
cv. Arctic King 200 µg/kg.

When the changes in endogenous ABA during imbibition at 20°C, 25°C or 30°C were studied a clear picture did not emerge. Although it could be argued with WWII that the rapid increase in free ABA at 12 hrs at 30°C could be associated with the induction of thermodormancy, this change could not be found in other seed batches. The results show that in any one seed batch similar changes can be shown in response to a particular stimulus, which gives confidence in interpreting the results. It is difficult to see how ABA might affect germination and dormancy from these data. Certainly, no consistent changes are observed and the theory, that dormancy release may be the result of conversion from 'free' to 'bound' ABA, is not supported by our results. Because Grand Rapids seeds are small and large quantities are required to measure endogenous levels, it was not possible to study the distribution of ABA in the different parts of the seed. Although it is generally thought that only ABA present in the embryo can affect germination (Sondheimer et al., 1968) Rudnicki (1973) believes that ABA may move from the seed coverings into the embryo. In Grand Rapids lettuce seeds we cannot rule out the possibility that the changes in ABA which influence dormancy are too subtle to be discovered by the methods employed. However, in many of the seeds studied for the involvement of ABA in dormancy, the authors have reached the conclusion that ABA is not involved in control, despite correlations being shown which might indicate such a role (Williams et al., 1973; Sondheimer et al., 1974). In both Coryllus avellana and Fraxinus americana ABA levels fell during dormancy release, but similar changes can also be observed under conditions which do not release dormancy (Williams et al., 1973; Sondheimer et al., 1974) !

There is good circumstancial evidence that ABA has no role in the

control of dormancy in Grand Rapids lettuce seeds. The results further indicate that caution should be exercised in reading too much into results obtained not just from one plant species, but from one 'crop' or batch. Before a concept of control can be accepted it must be shown to be of widespread occurrence.

Table 50 . Absolute levels of abscisic acid in seeds of Grand Rapids lettuce.

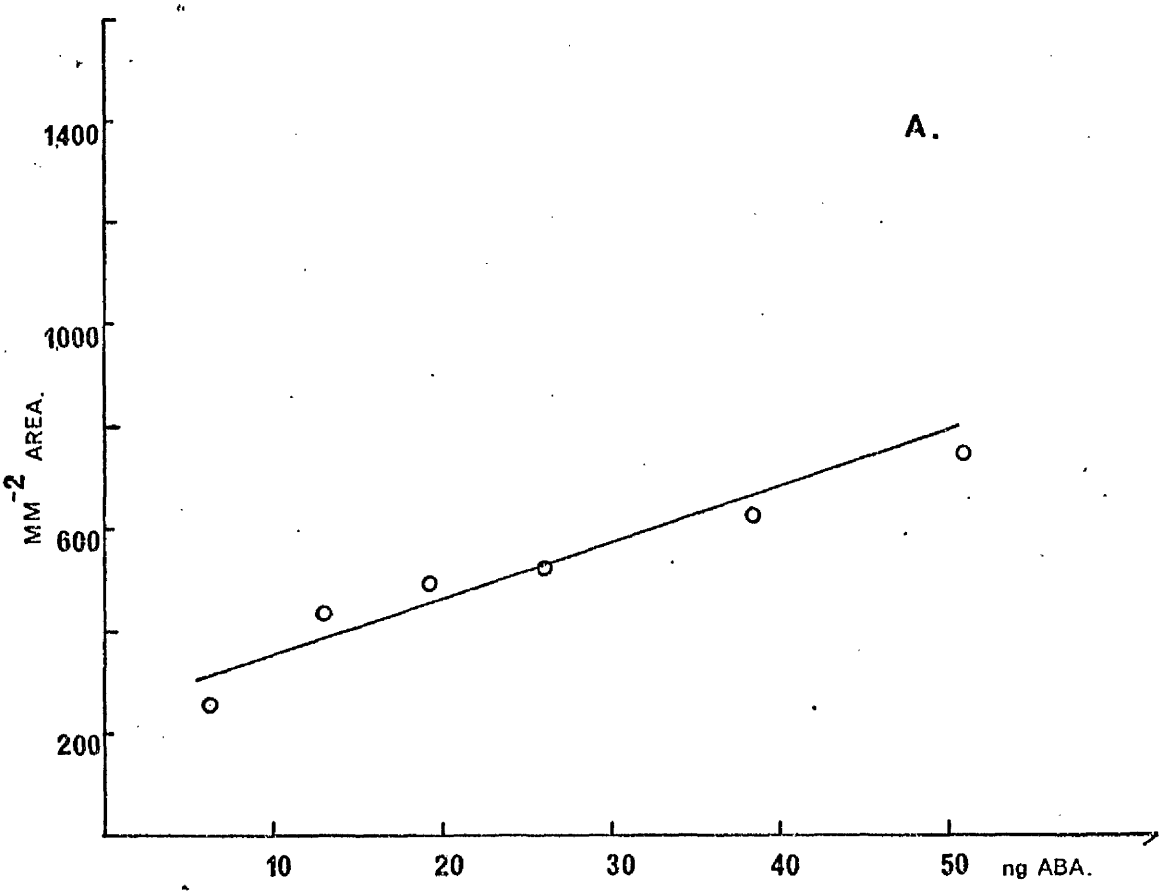
Seed source	Treatment hrs.	temp.	µg/kg 'free'	'bound'	Total
Page & Co.	12	20	700	324	1,024
	24	20	251	101	352
William Watt I	Dry seed		381	-	-
	8	20	398	-	-
	12	20	257	89	346
	48	20	174	101	275
	4	30	294	208	502
	8	30	248	52	300
	12	30	153	73	226
	20	30	168	122	290
Page 011974	Dry seed		184	137	321
	18	20	-	38	-
	30	20	-	76	-
	12	30	-	112	-
	18	30	-	89	-
Page 014574	Dry seed		172	160	332
William Watt II	Dry seed		67	132	199
	Dry seed		74	136	210
A	6	20	52	84	136
	12	20	74	87	161
	18	20	90	89	179
	24	20	73	59	132
B	6	20	79	49	128
	12	20	76	101	177
	18	20	117	-	-
	24	20	83	114	197
A	6	30	86	114	200
	12	30	284	105	389
	18	30	151	67	218
	24	30	74	61	135
B	6	30	-	119	-
	12	30	-	76	-
	18	30	-	70	-
	24	30	-	52	-
	6	25	-	43	-
	12	25	344	129	473
	18	25	136	-	-
	24	25	77	50	127

FIGURE 35

The response of an electron capture detector, attached to a Perkin-Elmer F17 gas liquid chromatogram, to abscisic acid.

A. sample size 2 µl, varying amounts injected by using different stock solutions.

B. varying amounts injected by using one stock solution, and varying sample size.


A.
1400
1000
600
200
MM^{-2} AREA.
10
20
30
40
50
ng ABA.

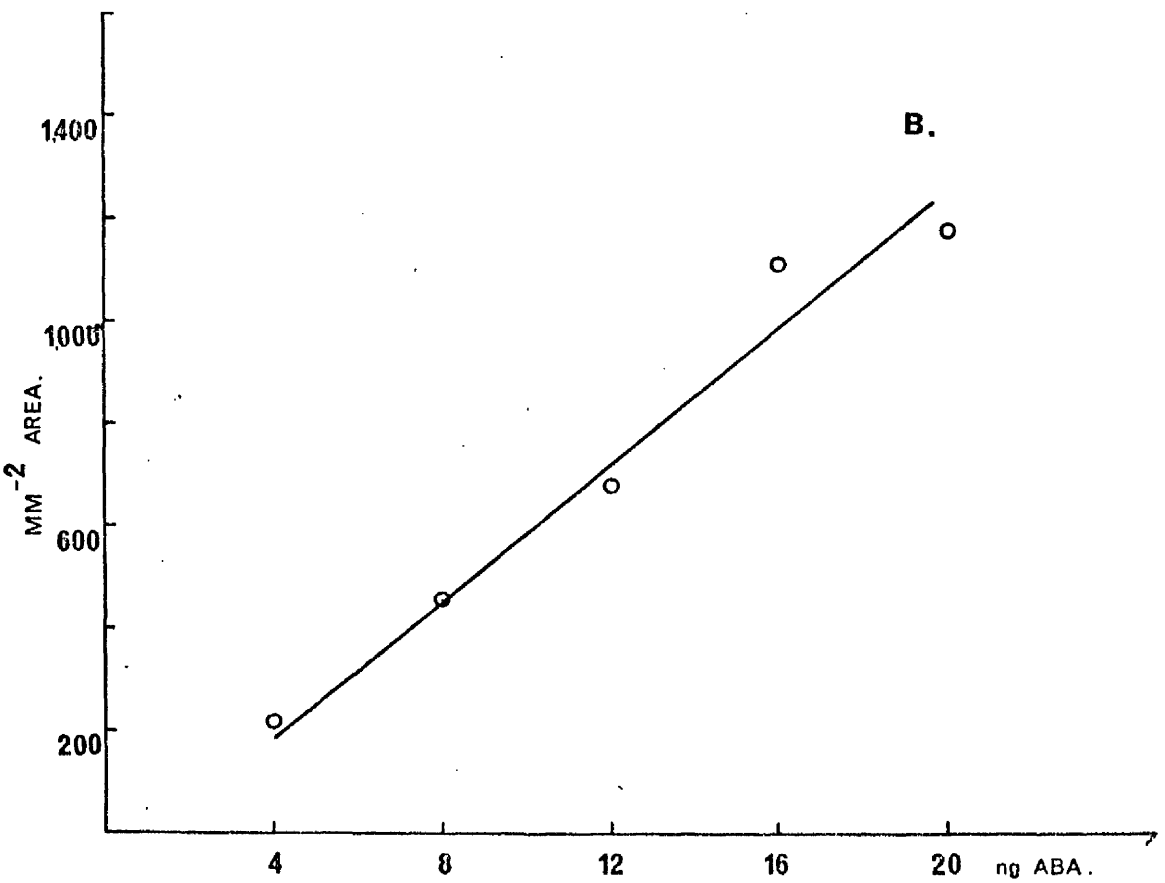

B.
1400
1000
600
200
MM^{-2} AREA.
4
8
12
16
20
ng ABA.

FIGURE 36

Line diagram of a mass spectrum of standard (±) abscisic acid (Sigma) carried out on an AEI-MS 30.

Mass spectrum of ABA. methyl derivative.

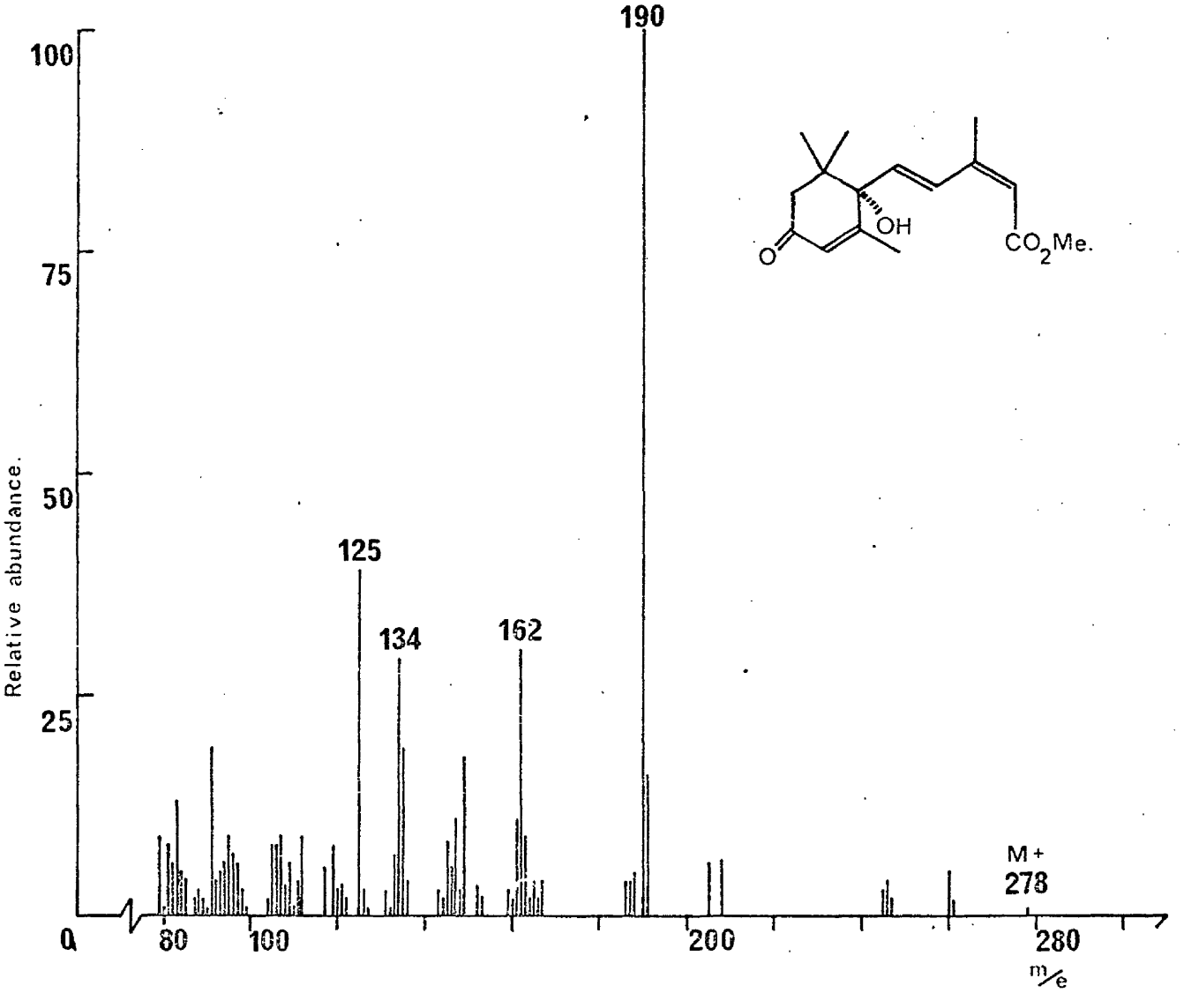
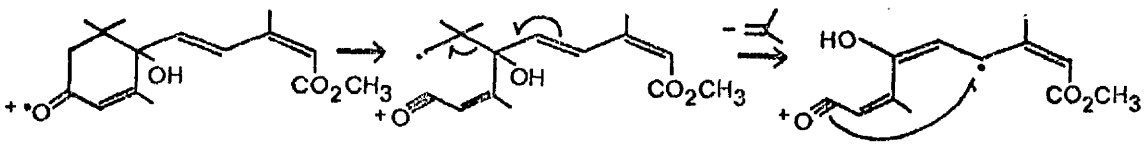


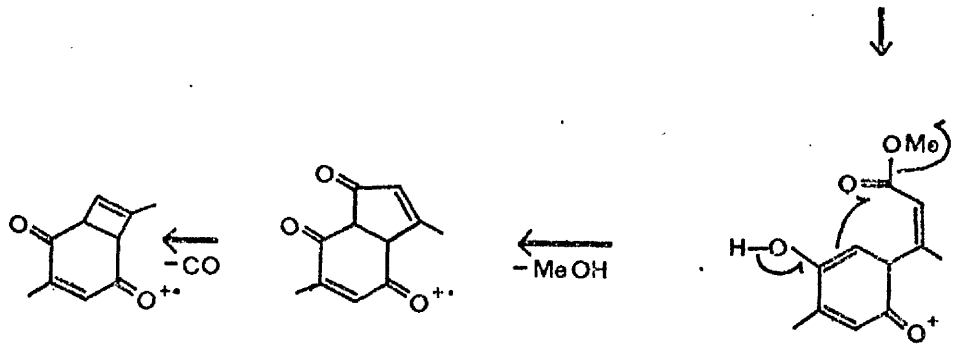
FIGURE 37

The fragmentation pattern of abscisic acid on bombardment with electrons during mass spectral analysis (after Gray *et al.*, 1974).

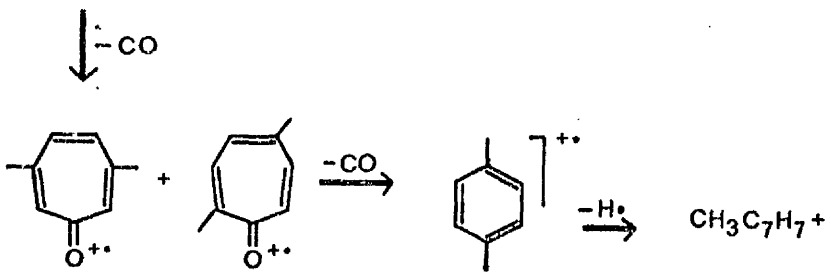
FRAGMENTATION OF METHYL ABSCISATE.



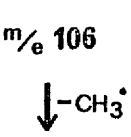
M^+, m/e 278



m/e 162 m/e 190 m/e 222

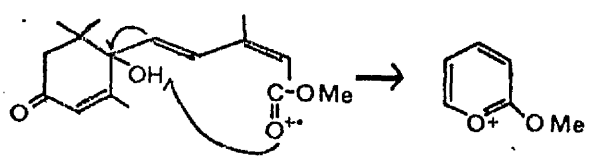


m/e 134 m/e 106 m/e 105



$C_7H_7^+$

m/e 91



m/e 125

FIGURE 38

Single ion scan : continuous monitoring of partial ion current with the MS focused at m/e 190.

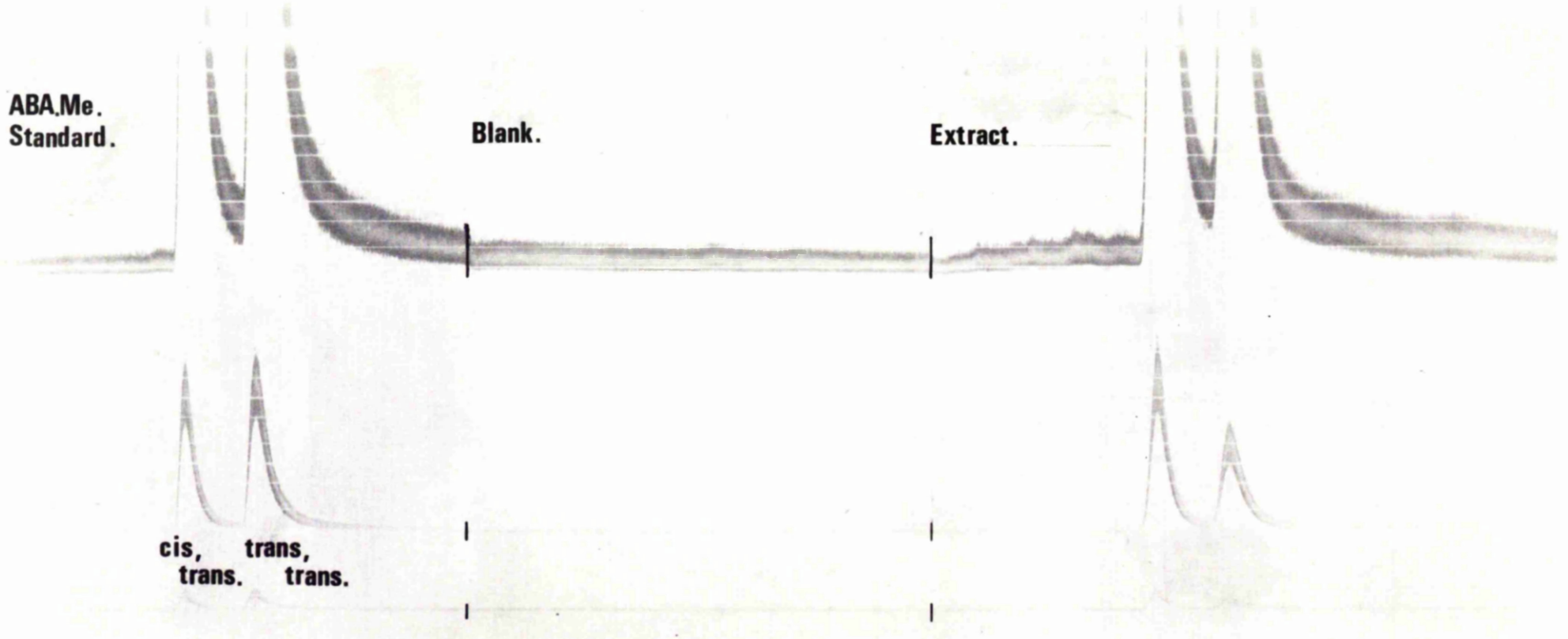

ABA.Me.
Standard.
Blank.
Extract.
cis,
trans.
trans,
trans.

FIGURE 39

The levels of 'free' abscisic acid extracted from seeds of Grand Rapids. Horizontal axis, hours imbibition at 20°C or 30°C.

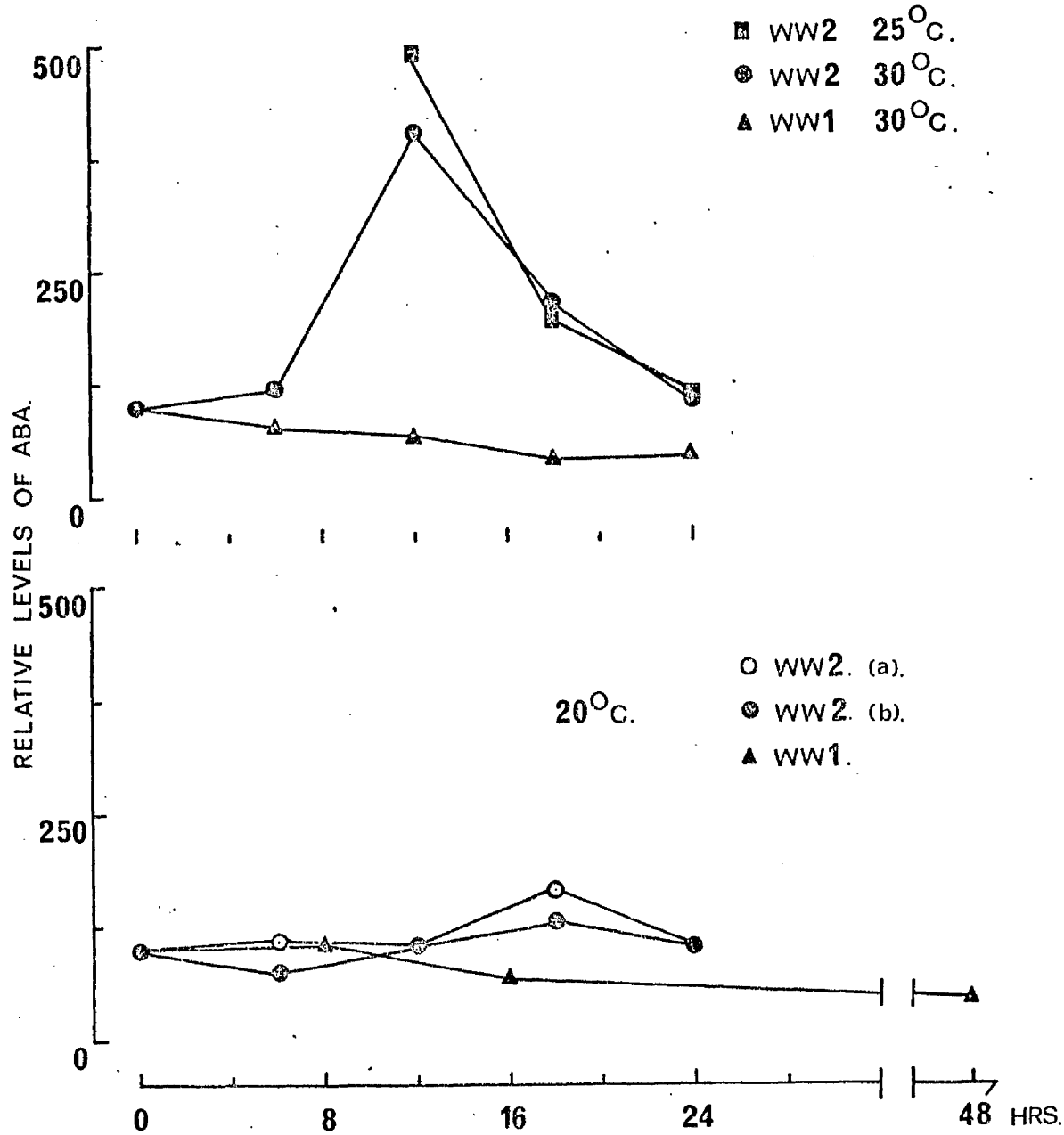
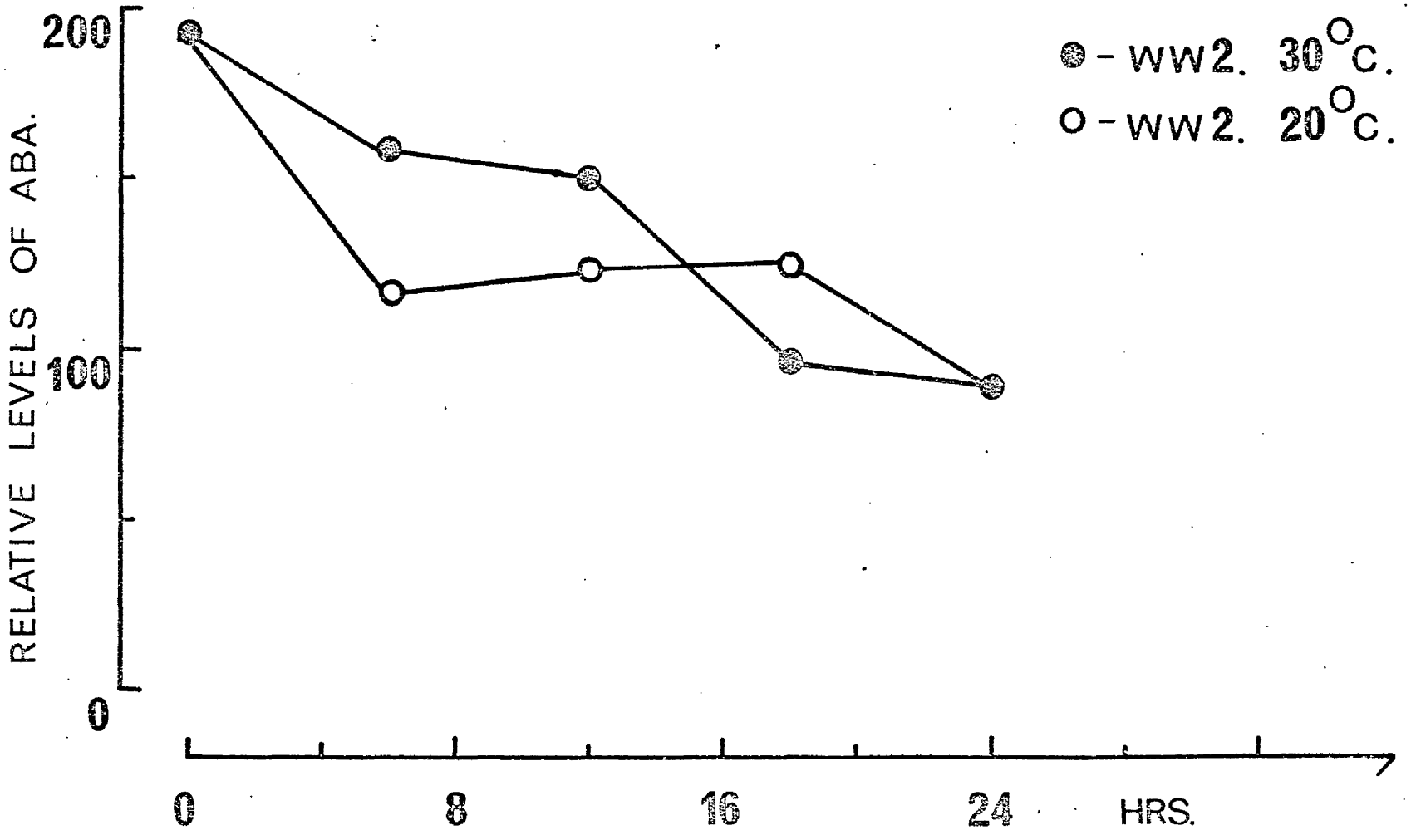

WW2 25°C.
WW2 30°C.
WW1 30°C.
500
250
0
RELATIVE LEVELS OF ABA.
WW2. (a).
WW2. (b).
WW1.
20°C.
500
250
0
0
8
16
24
48
HRS.

FIGURE 40

The levels of 'bound' abscisic acid extracted from seeds of Grand Rapids. Horizontal axis, hours imbibition at 20°C or 30°C.


RELATIVE LEVELS OF ABA.
200
100
0
0
8
16
24
HRS.
● - WW2. 30°C.
○ - WW2. 20°C.

PART E

The involvement of indole acetic acid in the germination and dormancy of seeds of Grand Rapids lettuce.

Introduction

In the general introduction it was pointed out that auxin had been implicated in a number of plant responses, yet a role for the involvement of auxin in seed germination has never been shown. Certainly there is little work on the effects of indole acetic acid (IAA) on seed germination. With respect to seeds of Grand Rapids lettuce, IAA has been shown to cause a small promotion of germination if given in low concentrations to some batches (Poljakoff-Mayber, 1958) but is always inhibitory if given in higher concentrations (Khan and Tolbert, 1966; Sankhla and Sankhla, 1968). In view of the lack of work on this topic it was decided to examine Grand Rapids lettuce seeds for the presence of IAA and also, using exogenous studies, to look for the posssible involvement of IAA in the control and release from thermodormancy.

Results

The results of this work are presented in Tables 43-49 and Figures 41-51. The first part of this work was concerned with establishing a role for IAA in the imposition or release of thermodormancy in Grand Rapids lettuce seeds. Table 43 and Figure 41 show the effect of a series of concentrations of IAA on the germination pattern of Grand Rapids seeds. At 20°C IAA inhibits germination, this inhibition being marked at concentrations of 10^{-5} molar and above. Even in the presence of IAA there is an 'escape' from the action of IAA. After 48 hrs at 20°C, 10^{-5} M IAA shows only slight inhibitory activity (Figure 41). At no concentration tested did IAA promote germination. At 30°C there is a very small promotion of dark germination at a concentration of 10^{-7} M after 48 hrs but this is not statistically significant. There is a significant inhibition of dark germination at 30°C in concentrations above 10^{-5} M. We have shown that IAA inhibits germination but is this inhibition merely of radicle elongation, the ultimate visible sign that germination has occurred ? In an attempt to answer this question we examined when a sufficient 'potential to germinate' had been achieved to avoid inhibition by IAA. The design of this experiment also allowed the possibility of a sensitive

period to IAA, during imbibition, to be investigated. It can be conceived that IAA may be required at some specific point in time, for germination to occur.

The details of the experimental procedure are the same as those described for an identical experiment with ABA (page 167). Lettuce seeds were imbibed in distilled water and transferred after specified times, to either water (control treatment) or IAA at concentrations of 2×10^{-5} or 2×10^{-4} M. The data obtained are presented in Table 44 a, b and c and in Figures 42-45. Figure 42 shows the percentage germination after 24 hrs of seeds transferred from water to (A) water, (B) 2×10^{-5} M or (C) 2×10^{-4} M IAA. It is immediately apparent that the control treatment, transfer from water to water, has an effect. We do not observe uniform germination. In other words, the seeds are sensitive to transfer. Clearly, any analysis of the effect of IAA on germination must be made against a background of this control effect. When percentage germination after 48 hrs is plotted as a function of transfer time (Figure 43) the shape of the control curve is much flatter or more uniform, reflecting the fact that seeds in water at 20°C eventually all reach about 90% germination. If we examine curves B and C in Figure 42, an upward trend can be seen in both. Thus, there is an 'escape' from the inhibitory action of IAA on germination. Curve B, transfer from water to 2.10^{-5} M IAA, shows a sharp upward trend after 6-8 hrs, a plateau between 8-12 hrs and then another upward trend. Curve C, transfer from water to 2.10^{-4} M IAA, shows an upward trend from 12 hrs onwards. In these treatments germination, as assessed by visible radicle emergence, can be observed as early as 14 hrs after the start of imbibition, and is in the order of 20-40% by 20 hrs. When germination is counted after 48 hrs we can observe similar upward trends in germination to those observed after 24 hrs (figure 43). Again in the transfer to 2.10^{-5} M IAA a sharp increase is shown after 6-8 hrs. In the transfer to 2.10^{-4} M IAA the standard errors are too large to assess the significance of any increase at 6-8 hrs but there is a clear increase in germination from 12 hrs onwards, reflecting an 'escape' from the inhibitory effects of IAA. In no case was IAA at these concentrations shown to promote germination. We have shown that the seed develops a potential to germinate which eventually results in IAA being unable to inhibit germination. However, the exact timing of this escape is not clear. The time at which seeds lose their sensitivity to inhibition by IAA

is critical in determining whether IAA affects germination or merely radicle emergence. As was stated the first visible radicle emergence could be seen at about 14 hrs after the start of imbibition. If IAA affects germination it must be shown to affect events prior to this time. It was clear from Figure 42 that the control treatment had an effect which should be taken into account when considering the germination on transfer of seeds to IAA. In an attempt to eliminate the control effect, transfer from water to water, it was considered that this should have had no effect and that uniform germination should have occurred. A figure of 75% germination at 24 hrs and 97.5% germination at 48 hrs was used. The data were then manipulated as previously described for ABA, to give the results shown in Figures 44 and 45. The shaded area in Figure 44 represents the corrected germination after 24 hrs for transfer to 2×10^{-5} M IAA. This shows that rather than there being an increase in germination brought about by IAA after 6-8 hrs, there is a downward trend, from 8-12 hrs which may represent a period when the seed is more sensitive to the presence of exogenous IAA. After 12 hrs there is a steady escape from the inhibitory influence of IAA. When germination is counted after 48 hrs the picture obtained is more complicated (Figure 45). Transfer to 2.10^{-5} M IAA (curve C, Figure 45) shows that there is a sudden increase in germination after 6-8 hrs, the 'flow' then straightening out as more than 90% germination is achieved. In curve D, transfer to 2.10^{-4} M IAA, there is an initial decline followed by an increase or escape from IAA inhibitory action after 12 hrs.

Thus, in lower concentrations of IAA the escape from IAA inhibition of germination seems to be earlier than in higher concentrations of exogenous IAA. The data would suggest that IAA might affect the germination process and not merely radicle growth.

There are certain similarities between the action of IAA and the effect of acid conditions on the elongation of coleoptiles (Cleland, 1973) and it has been suggested that IAA may in part act by virtue of releasing hydrogen ions (Rayle, 1973). If IAA released hydrogen ions into the imbibition medium this could conceivably acidify the solution. The more acid conditions would then inhibit radicle elongation which in turn would be interpreted as IAA inhibiting germination. Some preliminary work was carried out, using IAA made up in buffer

Table 45 we can see that red light promotes germination even in the absence of IAA. Clearly, this promotion is an effect on both the rate of germination and on the final germination attained. In the presence of IAA, red light can be shown to overcome the inhibition imposed by IAA. This is most obvious when germination is counted after 24 hrs. At a concentration of 10^{-5} M IAA germination after 24 hrs is 14.0 ± 5.6 % whilst 5 minutes exposure to red light raises this to over 70%. (Figure 47). At 30°C the effect of red light is even more striking, with 10 minutes red light causing nearly 50% germination in the presence of 10^{-4} M IAA after 24 hrs (Figure 47). The effect of red light can be reversed by far-red light (Table 47) whilst far-red itself has no significant effect.

Thus we can see that the inhibition of germination brought about by exogenous IAA at 20°C or at 30°C can be overcome by cytokinin, gibberellin or red light in a similar manner to that shown for ABA. IAA is not as potent an inhibitor of germination as ABA and in solution IAA quickly loses activity, presumably due to degradation. Consequently, in the work reported here, solutions of IAA were always made up one day prior to use.

It is possible to envisage that IAA could be an inhibitory compound involved in the control of thermodormancy. If IAA has such a role it must be shown to be present as an endogenous component of Grand Rapids lettuce seeds. IAA was extracted and purified as described in the materials and methods section. The final purified fraction was derivatised by the addition of BSA (N,O-bis-(trimethylsilyl)-acetamide) to produce trimethylsilyl (TMSi) derivatives, prior to GLC or combined GC-MS.

Figure 48 shows the mass spectrum of standard IAA. TMSi with a parent molecular ion at m/e 319 and prominent ions at m/e 201 and 202. m/e 202 is the most abundant ion. An identical spectrum was obtained with an extract from 500 g of Grand Rapids lettuce seeds which had been imbibed for 24 hrs at 20°C providing unequivocal evidence for the presence of IAA in lettuce seeds. This is the first evidence for the presence of IAA in lettuce seeds. Having shown that IAA is present in these seeds it was decided to examine the levels of IAA during imbibition at 20°C and 30°C, temperatures below and above that necessary for the imposition of thermodormancy. The endogenous IAA was quantified by use of 'single ion monitoring' on the GC-MS. The theory underlying the operation of the MS in this mode has

solutions, in an attempt to investigate this possibility. However, the buffers used were found to have an effect on germination, being inhibitory. The data presented may be interpreted as showing IAA to affect germination but are not wholly convincing. The possibility must remain that IAA inhibits radicle elongation.

If we accept that IAA affects germination, being perhaps an inhibitor with a functional role in the induction and release from thermodormancy, it is of interest to investigate the interaction of IAA with other factors known to influence the germination of Grand Rapids lettuce seeds. If we evoke IAA as an inhibitor of germination then it is possible that IAA may function as the inhibitor in Amen's (1968) theory of seed dormancy or in the theory proposed by Khan (1971). As there has been so little work carried out, with respect to the involvement of IAA in the germination of seeds, we know nothing of the interaction of GA's or phytochrome with IAA in the germination of Grand Rapids seeds. Kinetin has been reported as not being capable of overcoming IAA inhibition of germination in Grand Rapids seeds (Khan and Tolbert, 1966).

Table 48 shows the effect of benzyladenine (BA) and GA_{4+7}, alone and in combination on IAA induced dormancy at 20°C. IAA was present at a concentration of 6.7×10^{-5} M. It can be seen that both BA and GA_{4+7} are capable of overcoming the inhibition of germination imposed by the presence of IAA, and that BA and GA_{4+7} are almost equally effective after 72 hrs. The action of BA is slower than that of GA_{4+7}. Together BA and GA_{4+7} do elicit a greater response than either alone (see the % germination for 6.7×10^{-7} M GA_{4+7} + 6.7×10^{-6} M BA at 48 hrs, 96.0 ± 1.2% compared with 82.7 ± 1.7 % for GA_{4+7} alone and 52.3 ± 43% for BA alone). If this experiment is repeated at 30°C, where we have the dual inhibitory influence of temperature and exogenous IAA, we can see that once again both BA and GA_{4+7} alone can overcome the inhibitory effects of both temperature and IAA (Table 49). Together they are more effective than alone. The effects of BA and GA_{4+7} at 30°C are more clearly shown in Figure 46. At a concentration of 6.7×10^{-7} M GA_{4+7} can only cause around 11% germination at 30°C after 72 hrs, BA at 6.7×10^{-6} M causes over 80% germination; together over 95% germination is achieved.

The next factor investigated was the effect of phytochrome on IAA induced dormancy. The action of red light on IAA inhibition of germination at 20°C is shown in Table 45, at 30°C in Table 46. From

previously been discussed (page 201). This technique is well established in the study of IAA (Bertilsson and Palmer, 1972; Bertilsson et al., 1972; Rivier and Pilet, 1974). The fragmentation pattern of IAA.TMSi is shown in Figure 49. Only two major ions are produced, the parent ion at m/e 319 and an ion at m/e 202, which was the most abundant ion. The MS was focused on the m/e 202 ion and the area under the peak at the correct retention time for IAA.TMSi taken as a measure of the amount of IAA present. A typical scan obtained with a seed extract is shown in Figure 50. During the course of this work a number of serious problems was found in using single ion monitoring to quantify IAA. It was found that there was great variation in the sensitivity of the MS on different runs. The sensitivity of the MS is dependent on a number of factors and the only sensible way to overcome this problem was by analysing samples on more than one occasion. It should be noted that this problem was so severe that on some runs no IAA could be found whilst in others the same sample gave good responses. A second problem encountered was the lack of linearity of the response of the MS to known amounts of standards. It appears that the detection system employed can be 'swamped' and is not able to differentiate between different amounts of material except over a very narrow range. The point at which linearity of response is lost is not consistent between runs. These problems raise serious doubts over the use of this technique to carry out quantification of compounds. The technique of mass spectrometry was originally used as a tool in identification of compounds. In order to present our results with confidence the above mentioned problems had to be overcome. The most sensible way of doing this appeared to be in analysing each sample on several occasions and comparing them against standards run on different occasions. By doing this it was hoped to eliminate the vagaries of the MS. The results of these studies are presented in Figure 51. This shows the levels of IAA per kg of seed obtained in seeds imbibed at 20°C or 30°C for up to 30 hrs. Several batches of seeds were used. As such large samples of seed were used, 500 g, it was not possible to repeat an experiment with the same seed batch. The levels shown are uncorrected for losses. The percentage efficiency of recovery in this system was only 25%.

Recovery of radioactivity from 5[^{3}H]IAA during extraction and purification procedure employed for endogenous IAA.

Extraction stage	Phase	% of original radioactivity added to aqueous phase (105,900 dpm)
(1) aqueous pH 3.0 x diethyl ether	aqueous	1.3
	ether	98.7
(2) ether phase taken up in methanol; DEAE cellulose chromatography	aqueous Na_2SO_4	-
		84.5 (95% recovery)
(3) Na_2SO_4 eluate x ether pH 3.0	Na_2SO_4	-
	ether	79.8
(4) TLC no (1)		44.6 (56% recovery)
(5) TLC no (2)		25.0 (56% recovery)

(The order of magnitude of variation throughout this procedure was ±5%)

Thus, to obtain absolute levels the values shown should be multiplied by a factor of four. At 20°C there is no consistency in the changes in endogenous IAA shown by the different seed batches, although the levels found in dry seeds were similar, about 0.25 - 0.5 µg IAA/kg seed. It may be that the three seed batches used would have shown the same pattern if analyses of endogenous IAA levels had been continued beyond 30 hrs. With lot WWI and lot 011974 there is a large increase, after 18 hrs for lot 011974 and 24 hrs for lot WWI. In the case of lot 011974 this increase is threefold, in lot WWI ten-fold. If we now look at the data for lot H273 the initial trend is very similar to lot WWI and after 30 hrs there is an indication that the level of IAA is increasing. However, these changes cannot be correlated with germination behaviour. Lot 011974 shows the most rapid, uniform germinationat 20°C, lot WWI the poorest germination. At 30°C the

trends shown by the two lots investigated, lot 011974 and lot WW2 are not dissimilar with an initial decrease, followed by an increase, a further decrease and a second increase. The magnitude of these changes is different with the two seed lots. If we compare the levels shown for lot 011974 at 20°C and 30°C (open circles on Figure 51) we can see that the pattern and indeed the levels of IAA found with respect to time are very similar. Thus, it may be that the changes in IAA are independent of the temperature at which seeds are imbibed.

Discussion

We have presented evidence that exogenous IAA can inhibit the germination of seeds of Grand Rapids lettuce and that there is an 'escape' from this inhibitory action even in the presence of IAA. In an attempt to discover whether IAA owes its action to an effect on germination or merely on radicle elongation, transfer experiments were conducted. The results of these were not unequivocal although it could be considered that IAA acts sufficiently early for its action to be on germination. The interaction of IAA with cytokinin, gibberellins and red light was studied and all of these factors found capable of overcoming the inhibitory effects of IAA. As was the case in the work with ABA, it was possible to distinguish between the effects of cytokinin and gibberellin, cytokinin appearing to act at a later stage. If IAA is the inhibitor component of a promoter/inhibitor complex controlling thermodormancy, we must show its presence as an endogenous component of lettuce seeds. The first unequivocal evidence for the presence of IAA in Grand Rapids lettuce seeds and changes during imbibition of endogenous IAA are presented. It has been shown that material which is not aseptic contains more auxin than sterile material (Libbert et al., 1969) and it could be suggested that the IAA found in lettuce seeds is due to the presence of micro-organisms. However, Elliott and Greenwood (1974) have shown IAA to be present in sterile maize roots. In an attempt to eliminate this possibility in lettuce seeds, samples of seeds were surface-sterilised in 5% chloros solution for 2 minutes, washed and then imbibed for 24 hrs at 20°C prior to extraction for endogenous IAA. The effect of chloros on germination was shown to be negligible provided the seeds were thoroughly washed free of adhering chloros. It was shown that the

'surface sterile' seeds contained IAA. Thus it appears unlikely that the IAA of lettuce seeds can be explained by the production of IAA by micro-organisms. The results presented dealing with the effect of temperature on endogenous IAA show that no correlation exists between germination behaviour, either due to different seed lots or temperature, and the level of endogenous IAA. There has only previously been one report which shows the effect of temperature on endogenous 'auxin' in a seed. The level of IAA (identified by colorimetric assay) in seeds of Hyoscyamus muticus was shown to be increased, prior to germination, by diurnal temperature fluctuation and by the application of kinetin (Elkinaway and Hemberg, 1974). The results we have presented for seeds of Grand Rapids lettuce make it difficult to accept IAA as having a functional role in the release or imposition of thermodormancy. However, this study cannot rule out the possibility, previously discussed, that IAA may act by virtue of its location. Thus, although there is no change in endogenous IAA in response to temperature, there may be the imposition or removal of restrictions to the movement of IAA. A further possibility is that enzymes used in the synthesis or breakdown of IAA may be separated from the endogenous pool of IAA under certain circumstances. There is good evidence that IAA can exist in a vast number of conjugated forms (Schneider and Wightman, 1974). Bandurski and Schulze (1974) have shown that IAA released by mild alkaline hydrolyses far exceeds 'free' IAA in seedlings of Zea mays, although in oat seedlings no conjugated IAA was found. More recently these workers have shown the presence of IAA released by hydrolysis in 7N NaOH for 3 hrs at 100°C. They called this 'peptidic' IAA (Bandurski and Schulze, 1975). This peptidic IAA far exceeded the level of free IAA in oats and pea seedlings. Igoshi et al. (1971) have also shown the presence of a peptidic tryoptophane which could conceivably be 'inactive' precursor pool for the synthesis of IAA.

It can be suggested that 'bound' forms of IAA or precursors of IAA require to be released for the imposition of thermodormancy in seeds of Grand Rapids lettuce, assuming bound IAA to be inactive. The present study cannot distinguish between different alternatives, release from compartments or release from 'bound' forms. Indeed the sample material imposes technical difficulties which may make the investigation of these problems at a direct level impossible. In the

hope that some of these questions might be resolved it was decided to investigate the metabolism of exogenous radioactive IAA under a variety of conditions in lettuce seeds. Because of the possibility that either IAA or ABA could be the inhibitor proposed by Khan (1971) it was further decided to investigate ABA metabolism in an attempt to see whether either IAA or ABA are functional inhibitors involved in the control of thermodormancy.

Table 43 . The average percentage germination of lettuce seed treated with indole acetic acid, IAA.

Temperature	20°C		30°C	
Time of count	24 hrs	48 hrs	24 hrs	48 hrs
Concn. of IAA M.				
0	40.3 ± 3.8	94.7 ± 1.2	1.3 ± 0.7	5.3 ± 1.0
10^{-8}	44.3 ± 5.0	96.0 ± 0.5	2.3 ± 1.1	5.3 ± 0.7
10^{-7}	33.3 ± 2.7	93.0 ± 2.2	3.7 ± 1.1	9.0 ± 1.8
10^{-6}	18.0 ± 3.2	87.3 ± 3.7	1.3 ± 1.0	4.7 ± 1.0
10^{-5}	0.7 ± 0.4	67.7 ± 4.6	0.0 ± 0.0	1.3 ± 0.4
10^{-4}	0.7 ± 0.7	11.7 ± 4.0	0.0 ± 0.0	0.7 ± 0.4
10^{-3}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3

Table 44. The average percentage germination of Grand Rapids lettuce seed imbibed in water at 20°C and transferred to -

A) Water

B) IAA 2.10^{-5} M

C) IAA 2.10^{-4} M

after 1, 2, 3, ----- 20 hrs imbibition. Germination counted at 24 hr intervals from the start of imbibition.

Table 44 A). Water to water.

Germination after	24 hrs	48 hrs	72 hrs	96 hrs
Control H_2O	45.7 ± 4.8	89.0 ± 2.2	89.7 ± 2.3	90.0 ± 2.3
Transfer after (hrs)				
1	38.0 ± 7.1	84.3 ± 4.3	88.7 ± 3.4	89.0 ± 3.2
2	41.3 ± 2.6	86.7 ± 1.5	88.7 ± 2.3	89.0 ± 2.1
3	41.3 ± 3.0	93.3 ± 1.3	95.0 ± 1.3	95.0 ± 1.3
4	42.3 ± 4.6	87.3 ± 2.3	88.3 ± 1.9	88.3 ± 1.9
6	46.7 ± 3.2	92.7 ± 2.1	93.3 ± 1.6	94.0 ± 1.7
8	56.7 ± 2.8	95.0 ± 1.4	96.7 ± 1.0	96.7 ± 1.0
10	61.7 ± 1.0	95.3 ± 0.8	96.7 ± 1.1	96.7 ± 1.1
12	68.3 ± 2.3	96.3 ± 1.0	96.7 ± 0.8	97.3 ± 0.8
14	70.3 ± 4.5	95.7 ± 1.2	96.3 ± 0.7	98.3 ± 0.7
16	69.3 ± 5.7	95.3 ± 0.9	96.3 ± 0.7	96.7 ± 0.7
20	67.3 ± 4.2	98.3 ± 1.0	98.7 ± 0.9	98.7 ± 0.9

Table 44 B). Water to 2.10^{-5} M IAA

Germination after	24 hrs	48 hrs	72 hrs	96 hrs
Controls H_2O	56.7 ± 6.7	90.7 ± 1.7	91.3 ± 1.7	91.6 ± 1.7
2.10^{-5} M IAA	7.1 ± 2.0	71.8 ± 6.9	76.0 ± 5.8	76.4 ± 5.9
Transfer after (hrs)				
1	2.0 ± 1.2	62.4 ± 7.6	66.2 ± 6.8	67.3 ± 7.0
2	5.3 ± 1.4	62.2 ± 5.6	64.9 ± 5.4	65.5 ± 5.4
3	4.7 ± 1.1	57.6 ± 7.6	64.4 ± 6.3	64.7 ± 6.3
4	4.4 ± 1.5	64.9 ± 6.2	69.8 ± 4.6	70.2 ± 4.6
6	4.9 ± 1.2	68.9 ± 4.6	75.6 ± 4.3	76.2 ± 4.3
8	18.7 ± 2.6	86.4 ± 2.3	88.9 ± 2.0	96.4 ± 1.8
10	16.7 ± 2.7	87.3 ± 2.8	88.9 ± 2.7	88.9 ± 2.7
12	21.8 ± 4.4	88.4 ± 3.4	90.2 ± 2.9	90.7 ± 2.8
14	36.2 ± 4.15	93.8 ± 1.2	95.6 ± 0.9	95.7 ± 0.8
16	36.4 ± 6.0	93.8 ± 1.5	94.7 ± 1.9	94.9 ± 1.9
20	53.1 ± 5.5	95.6 ± 1.1	96.4 ± 0.9	96.7 ± 0.9

Table 44 C). Water to 2.10^{-4} M IAA.

Germination after:	24 hrs	48 hrs	72 hrs	96 hrs
Controls H_2O	73.3 ± 5.5	93.6 ± 1.6	94.0 ± 1.5	94.0 ± 1.5
2.10^{-4} M IAA	0.0 ± 0.0	31.6 ± 11.0	43.3 ± 9.2	47.8 ± 8.4
Transfer after (hrs)				
1	0.0 ± 0.0	28.0 ± 8.2	37.1 ± 8.4	46.2 ± 5.4
2	0.0 ± 0.0	20.4 ± 6.6	29.8 ± 7.5	34.2 ± 6.0
3	0.0 ± 0.0	21.6 ± 7.9	41.8 ± 9.2	44.0 ± 8.2
4	0.0 ± 0.0	25.1 ± 8.6	33.8 ± 9.1	43.8 ± 6.1
6	0.0 ± 0.0	26.4 ± 11.3	33.1 ± 11.6	39.6 ± 10.1
8	0.0 ± 0.0	43.6 ± 10.6	52.2 ± 10.0	62.7 ± 6.5
10	0.0 ± 0.0	39.1 ± 8.8	49.8 ± 8.6	57.1 ± 5.8
12	0.0 ± 0.0	43.1 ± 6.1	59.1 ± 5.4	66.7 ± 5.0
14	1.8 ± 0.8	60.0 ± 9.6	69.3 ± 7.9	78.0 ± 4.4
16	3.2 ± 0.9	73.1 ± 6.6	79.8 ± 4.4	84.7 ± 2.6
20	49.8 ± 7.9	90.0 ± 1.8	94.5 ± 0.7	95.3 ± 0.3

Table 45 : The average percentage germination of lettuce seed treated with indole acetic acid and red light at 20°C.

Concn. IAA M \ Red light (mins)	0	2	5	
0	45.3 ± 1.2	75.3 ± 2.9	93.0 ± 1.1	
10^{-5}	14.0 ± 5.6	44.7 ± 8.9	71.3 ± 7.8	
10^{-4}	3.7 ± 1.7	18.5 ± 7.9	33.3 ± 14.4	
				24 hrs
0	90.7 ± 1.2	93.7 ± 1.1	98.4 ± 0.6	
10^{-5}	78.0 ± 5.1	95.0 ± 1.9	96.7 ± 1.0	
10^{-4}	57.0 ± 7.7	87.7 ± 3.8	95.3 ± 0.8	
				48 hrs
0	90.7 ± 1.2	95.0 ± 0.9	98.7 ± 0.4	
10^{-5}	82.7 ± 3.1	96.0 ± 1.0	98.0 ± 0.9	
10^{-4}	65.0 ± 5.9	89.7 ± 3.3	97.3 ± 1.0	
				72 hrs

Table 46 . The average percentage germination of lettuce seed treated with indole acetic acid and red light at 30°C.

Concn. IAA M \ Red light (mins)	0	10	20	
0	6.3 ± 1.2	87.3 ± 2.8	82.7 ± 4.2	
10^{-5}	3.0 ± 0.5	79.0 ± 5.1	77.0 ± 3.3	
10^{-4}	0	46.3 ± 6.8	66.7 ± 4.9	
				24 hrs
0	13.3 ± 2.1	96.7 ± 1.2	92.7 ± 0.8	
10^{-5}	8.0 ± 3.1	92.7 ± 5.5	91.3 ± 1.6	
10^{-4}	1.7 ± 1.0	88.3 ± 2.7	93.7 ± 1.0	
				48 hrs
0	13.3 ± 2.1	96.7 ± 1.2	93.3 ± 1.1	
10^{-5}	8.7 ± 3.1	93.7 ± 1.9	94.7 ± 1.2	
10^{-4}	2.3 ± 1.3	91.3 ± 2.2	94.7 ± 1.2	
				72 hrs

Table 47 . The effect of red and far-red light on the average percentage germination of lettuce seed treated with indole acetic acid at 20°C.

Concn. IAA M \ Light (mins)	0	5R	10FR	5R + 10FR
0	10.0 ± 1.9	70.0 ± 5.8	3.0 ± 0.9	2.7 ± 0.8
10^{-5}	0.3 ± 0.3	37.3 ± 2.8	0	0
10^{-4}	0	0	0	0
				24 hrs
0	23.7 ± 3.4	89.0 ± 3.5	15.7 ± 3.2	19.7 ± 1.7
10^{-5}	9.3 ± 1.6	89.3 ± 1.6	6.3 ± 2.0	6.0 ± 1.0
10^{-4}	0.7 ± 0.7	53.0 ± 3.8	1.7 ± 0.6	0.7 ± 0.4
				48 hrs
0	25.0 ± 3.5	90.3 ± 3.5	16.7 ± 3.6	21.3 ± 1.2
10^{-5}	10.0 ± 2.2	91.3 ± 2.5	9.3 ± 1.6	8.3 ± 1.4
10^{-4}	1.7 ± 1.1	62.3 ± 4.5	2.0 ± 0.9	1.7 ± 0.8
				72 hrs

(Seed batch 011974)

Table 48 .. The average percentage germination of lettuce seed treated with gibberellin A_{4+7}, benzyladenine and indole acetic acid at 6.7×10^{-5} M 20°C.

Concentration GA_{4+7} M / BA M	0	6.7×10^{-7}	6.7×10^{-6}	6.7×10^{-5}	
0	0	0.3 ± 0.3	0.7 ± 0.7	0	
6.7×10^{-6}	0	0	0.3 ± 0.3	0	
6.7×10^{-5}	0.3 ± 0.3	0	0	2.7 ± 1.8	
	30.0 ± 5.0*				24 hrs
0	12.7 ± 2.7	82.7 ± 1.7	97.3 ± 0.9	98.0 ± 1.2	
6.7×10^{-6}	52.3 ± 4.3	96.0 ± 1.2	96.7 ± 1.2	96.7 ± 1.7	
6.7×10^{-5}	57.3 ± 7.5	94.5 ± 1.7	97.3 ± 1.9	96.7 ± 1.2	
	90.0 ± 2.3*				48 hrs
0	32.0 ± 1.2	98.0 ± 1.2	98.7 ± 0.4	99.3 ± 0.9	
6.7×10^{-5}	97.3 ± 1.5	98.0 ± 1.2	98.7 ± 0.4	98.7 ± 1.2	
6.7×10^{-4}	94.7 ± 1.7	96.7 ± 1.2	98.0 ± 1.2	98.0 ± 1.2	
	91.3 ± 1.9*				72 hrs

* water control

Table 49 . The average percentage germination of lettuce seed treated with gibberellin A_{4+7}, benzyladenine and indole acetic acid at 6.7×10^{-5} M 30°C.

Concentration GA_{4+7} M / BA M	0	6.7×10^{-7}	6.7×10^{-6}	6.7×10^{-5}	
0	0	0	0.3 ± 0.3	5.3 ± 1.3	
6.7×10^{-6}	0	2.0 ± 1.2	4.7 ± 1.8	19.3 ± 7.1	
6.7×10^{-5}	3.3 ± 0.7	6.0 ± 2.3	28.0 ± 4.0	39.3 ± 9.7	
	12.7 ± 4.4*				24 hrs
0	0	5.3 ± 0.7	68.0 ± 8.1	96.7 ± 1.2	
6.7×10^{-6}	74.0 ± 4.6	91.3 ± 0.9	94.0 ± 2.0	98.0 ± 1.2	
6.7×10^{-5}	61.3 ± 6.6	98.0 ± 0.0	98.7 ± 0.4	98.7 ± 0.4	
	20.0 ± 4.2*				48 hrs
0	0.3 ± 0.3	10.7 ± 1.8	76.7 ± 7.8	99.3 ± 0.9	
6.7×10^{-6}	81.3 ± 4.4	95.3 ± 1.5	96.0 ± 1.2	98.0 ± 1.2	
6.7×10^{-5}	96.7 ± 0.4	98.7 ± 0.4	99.3 ± 0.9	98.7 ± 0.4	
	20.7 ± 2.5*				72 hrs

* water control

FIGURE 41

The percentage germination of seeds of Grand Rapids lettuce in the presence of indole acetic acid at 20°C.

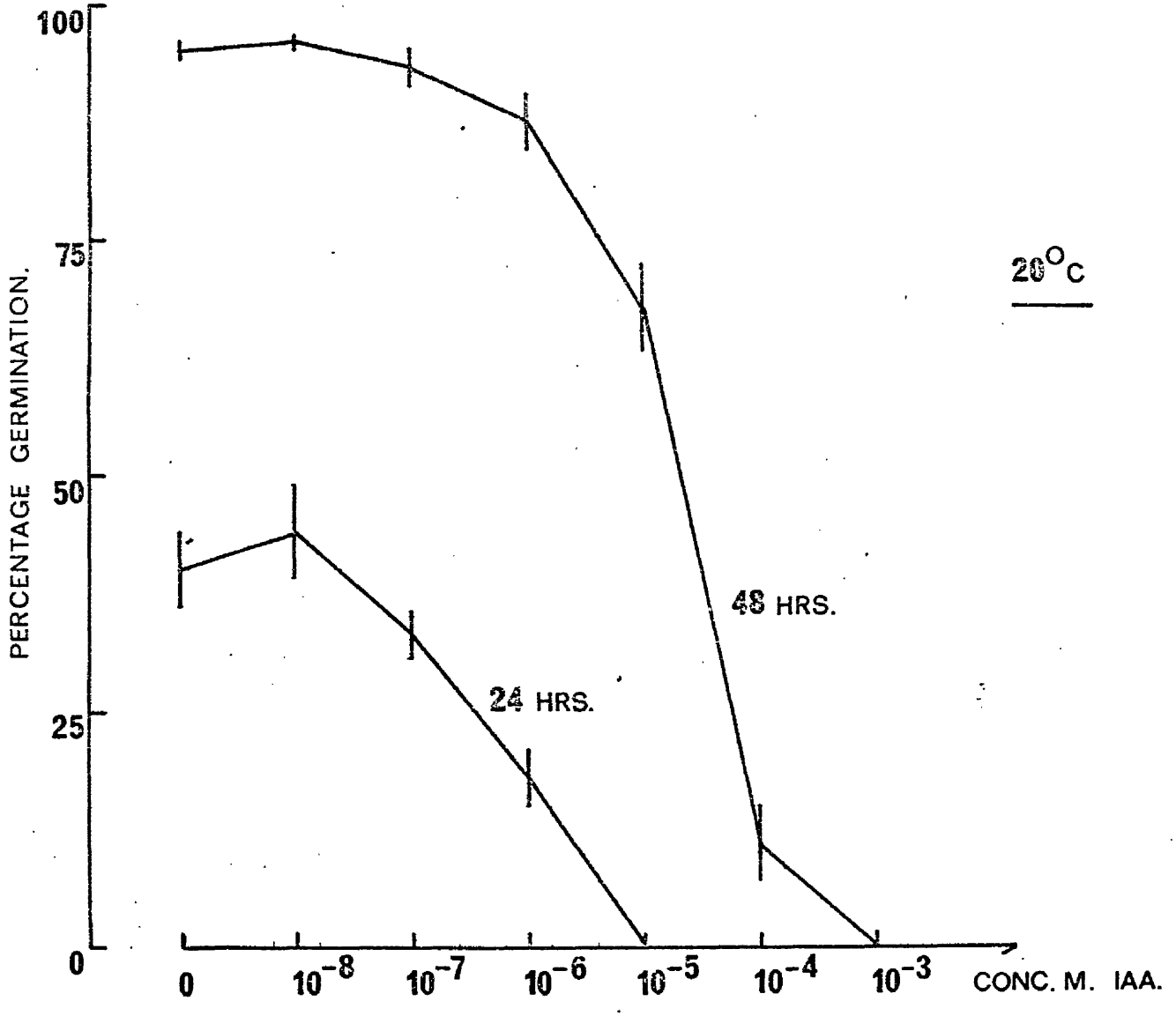

100
75
50
25
0
PERCENTAGE GERMINATION.
20°C
48 HRS.
24 HRS.
0
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
CONC. M. IAA.

FIGURE 42

The percentage germination after 24 hrs of lettuce seeds imbibed in water at 20°C and transferred to:

(A) water

(B) IAA 2.10^{-5} M

(C) IAA 2.10^{-4} M

after 1, 2, 3, ---------- 20 hrs imbibition.

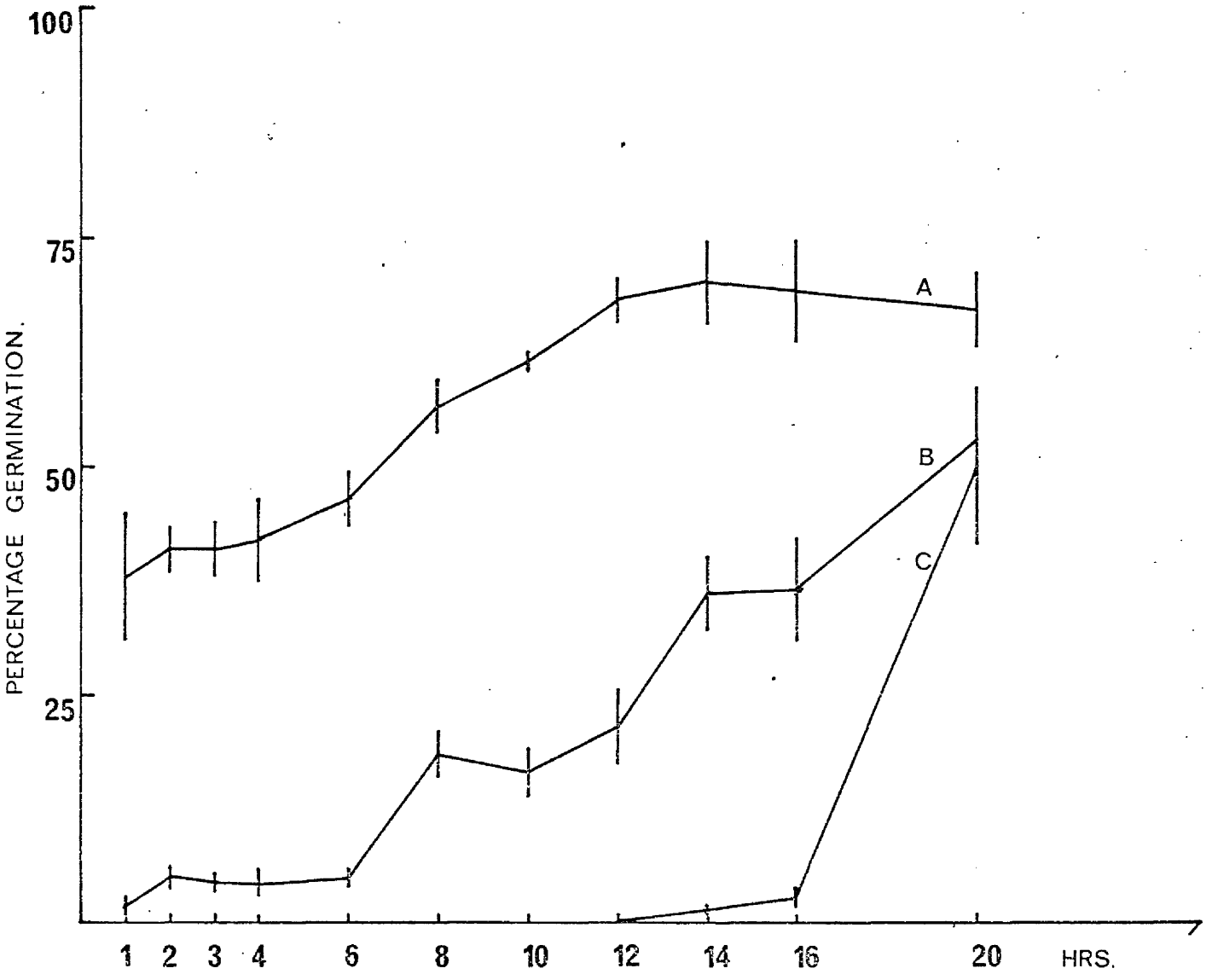

100
75
50
25
PERCENTAGE GERMINATION.
A
B
C
1
2
3
4
6
8
10
12
14
16
20
HRS.

FIGURE 43

The percentage germination after 48 hrs of lettuce seeds imbibed in water at 20°C and transferred to:

(A) water

(B) IAA 2.10^{-5} M

(C) IAA 2.10^{-4} M

after 1, 2, 3, ---------- 20 hrs imbibition.

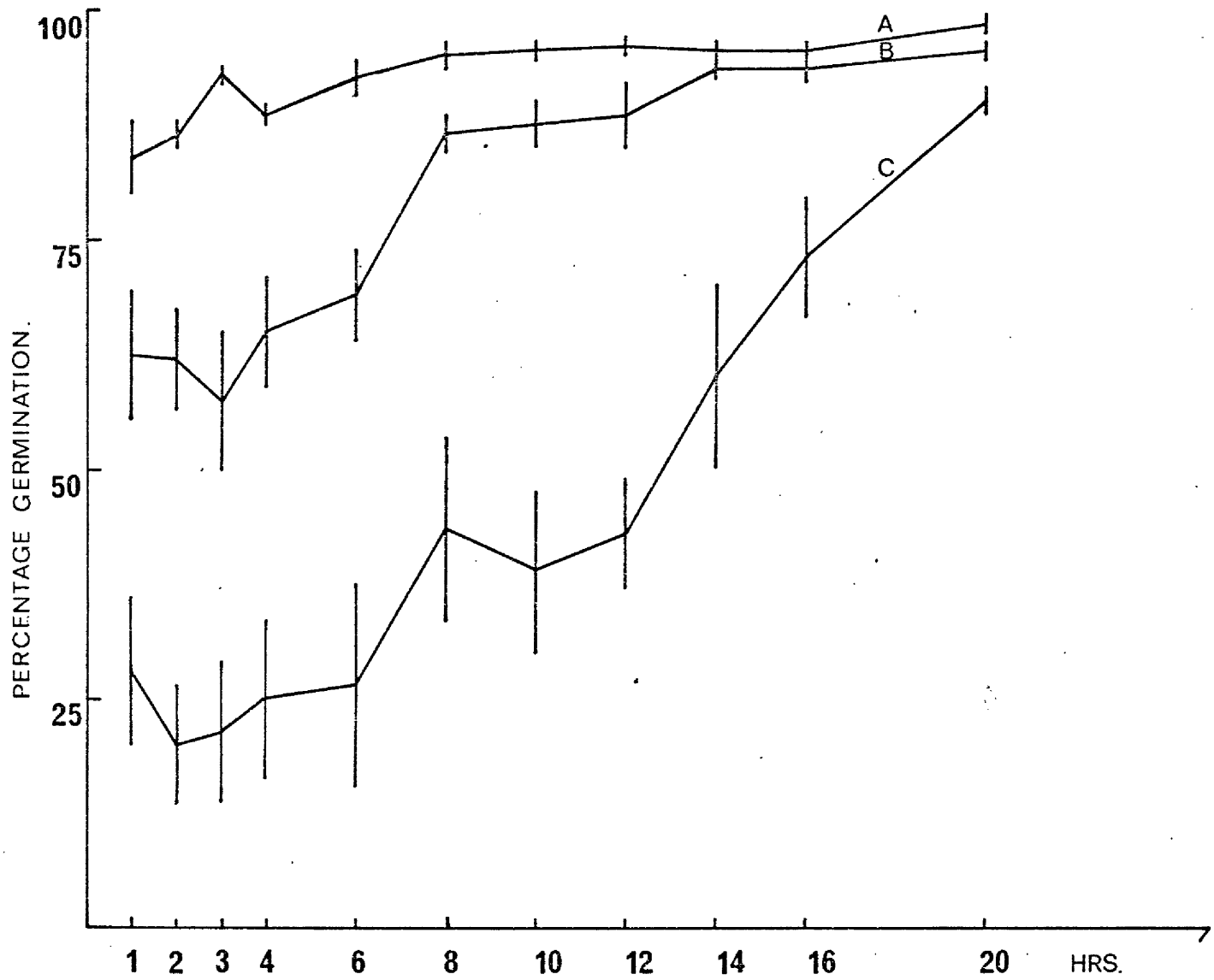

100
75
50
25
PERCENTAGE GERMINATION.
A
B
C
1
2
3
4
6
8
10
12
14
16
20
HRS.

FIGURE 44

The effect on germination of lettuce seeds of transfer to IAA at 2.10^{-5} M after 1, 2, 3, ----- 20 hrs imbibition in water. Germination counted after 24 hrs.

The corrected percentage germination was obtained as described in the text.

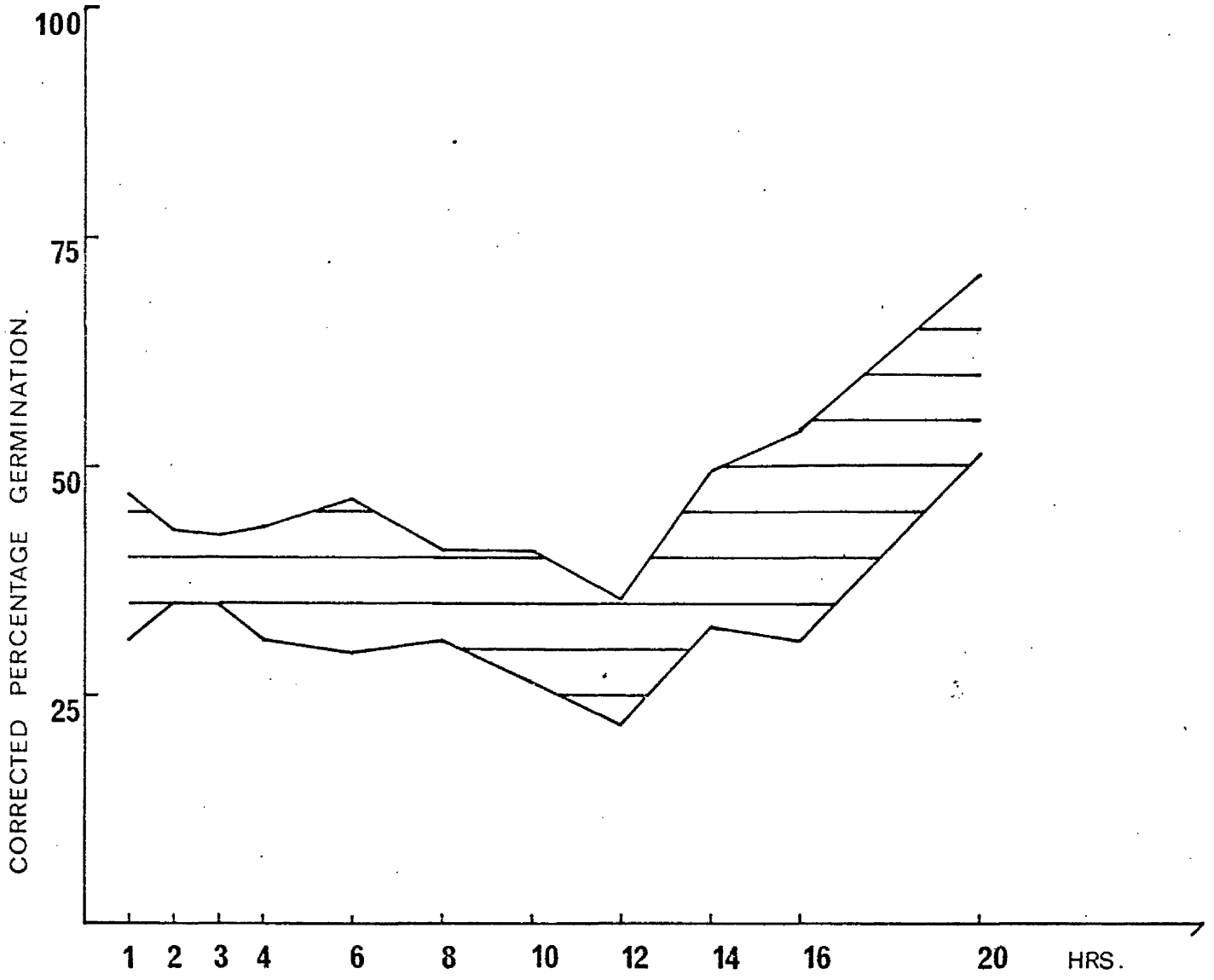

100
75
50
25
CORRECTED PERCENTAGE GERMINATION.
1
2
3
4
6
8
10
12
14
16
20
HRS.

FIGURE 45

As in Fig. 44 except transfer is to:

(C) IAA 2.10^{-5} M

(D) IAA 2.10^{-4} M

Germination counted after 48 hrs.

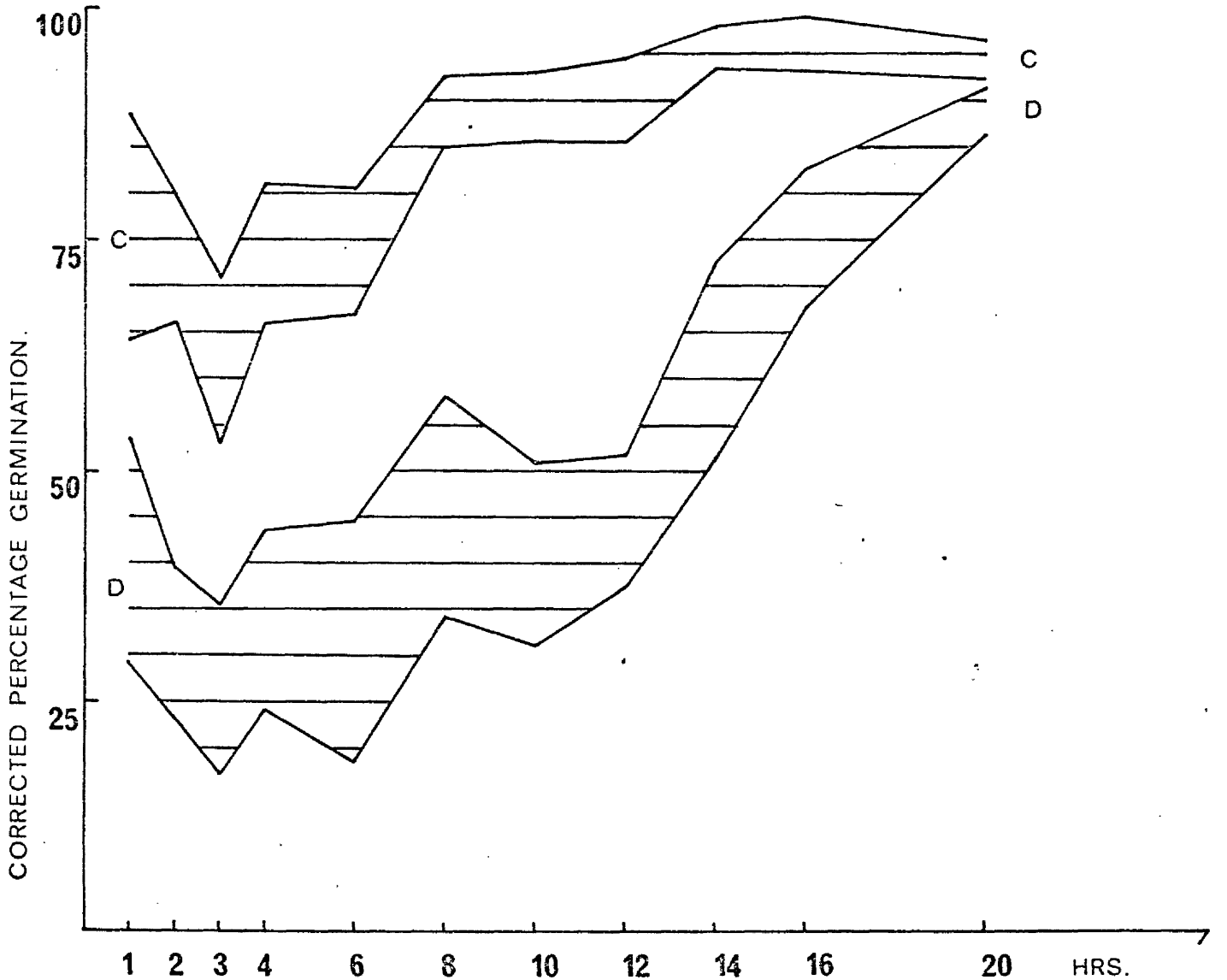

100
75
50
25
C
D
C
D
CORRECTED PERCENTAGE GERMINATION.
1
2
3
4
6
8
10
12
14
16
20
HRS.

FIGURE 46

The percentage germination of Grand Rapids lettuce seeds in the presence of 6.7×10^{-5} M indole acetic acid, benzyladenine and gibberellin A_{4+7} at 30°C.

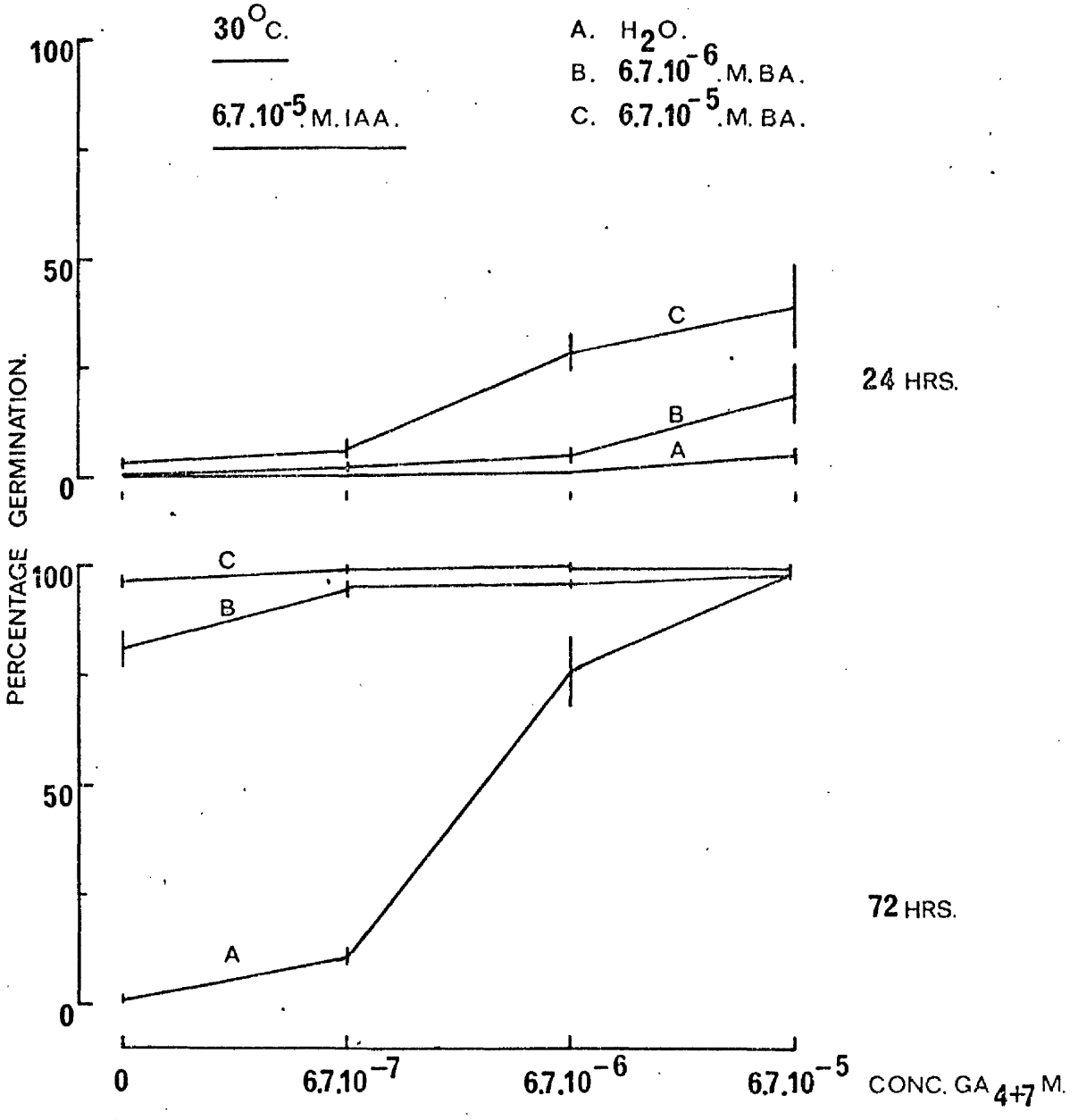

30°C.
6.7.10⁻⁵ M. IAA.
A. H₂O.
B. 6.7.10⁻⁶ M. BA.
C. 6.7.10⁻⁵ M. BA.
PERCENTAGE GERMINATION.
100
50
0
C
B
A
24 HRS.
72 HRS.
0
6.7.10⁻⁷
6.7.10⁻⁶
6.7.10⁻⁵
CONC. GA 4+7 M.

FIGURE 47

The percentage germination of Grand Rapids lettuce seeds in the presence of indole acetic acid and red light at 20°C or 30°C. Germination was counted after 24 hrs.

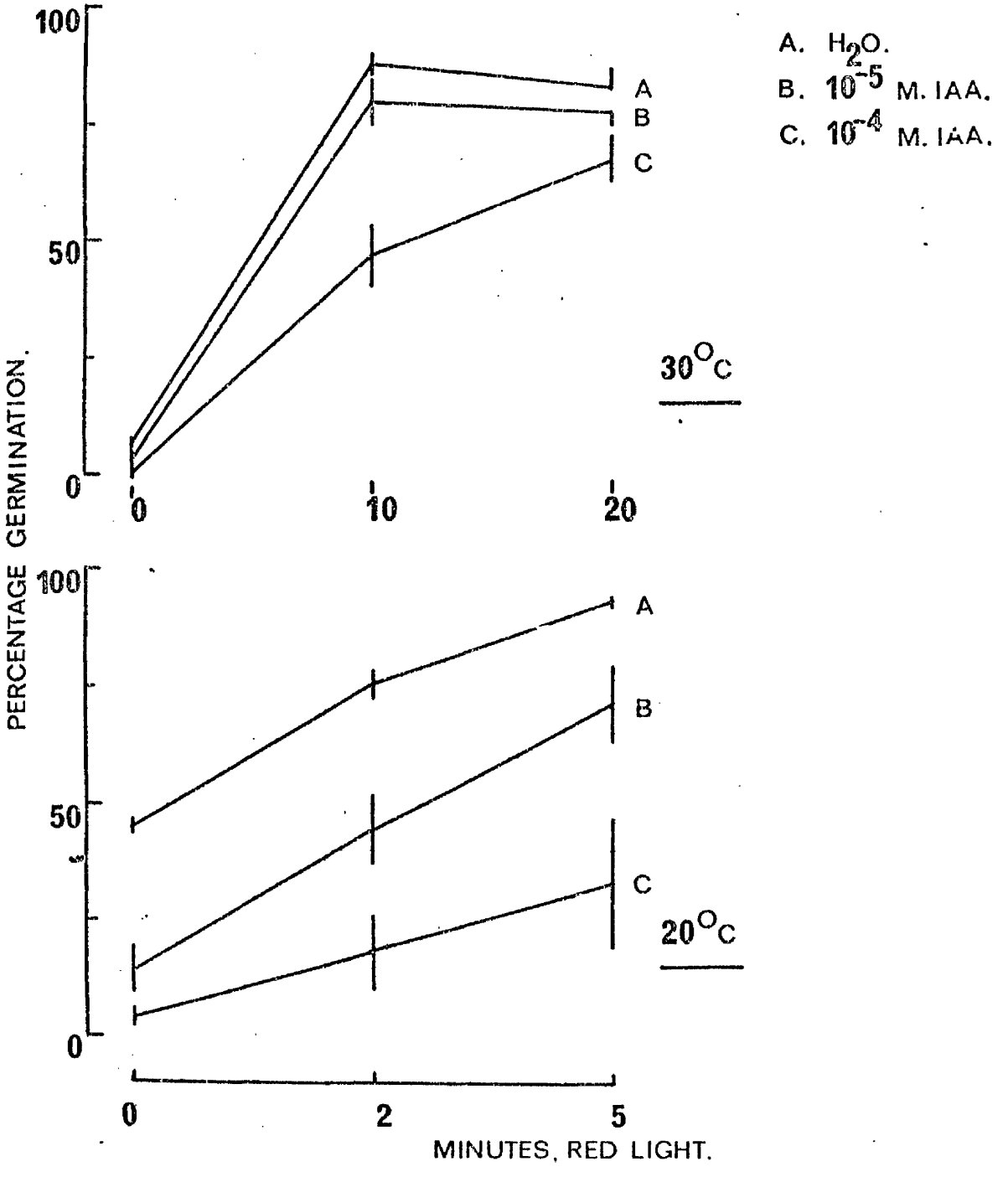

A. H_2O.
B. 10^{-5} M. IAA.
C. 10^{-4} M. IAA.
PERCENTAGE GERMINATION.
100
50
0
A
B
C
30°C
0
10
20
100
50
0
A
B
C
20°C
0
2
5
MINUTES, RED LIGHT.

FIGURE 48

Mass spectrum of standard IAA, TMSi derivative.

Mass spectrum of IAA, TMSi derivative.

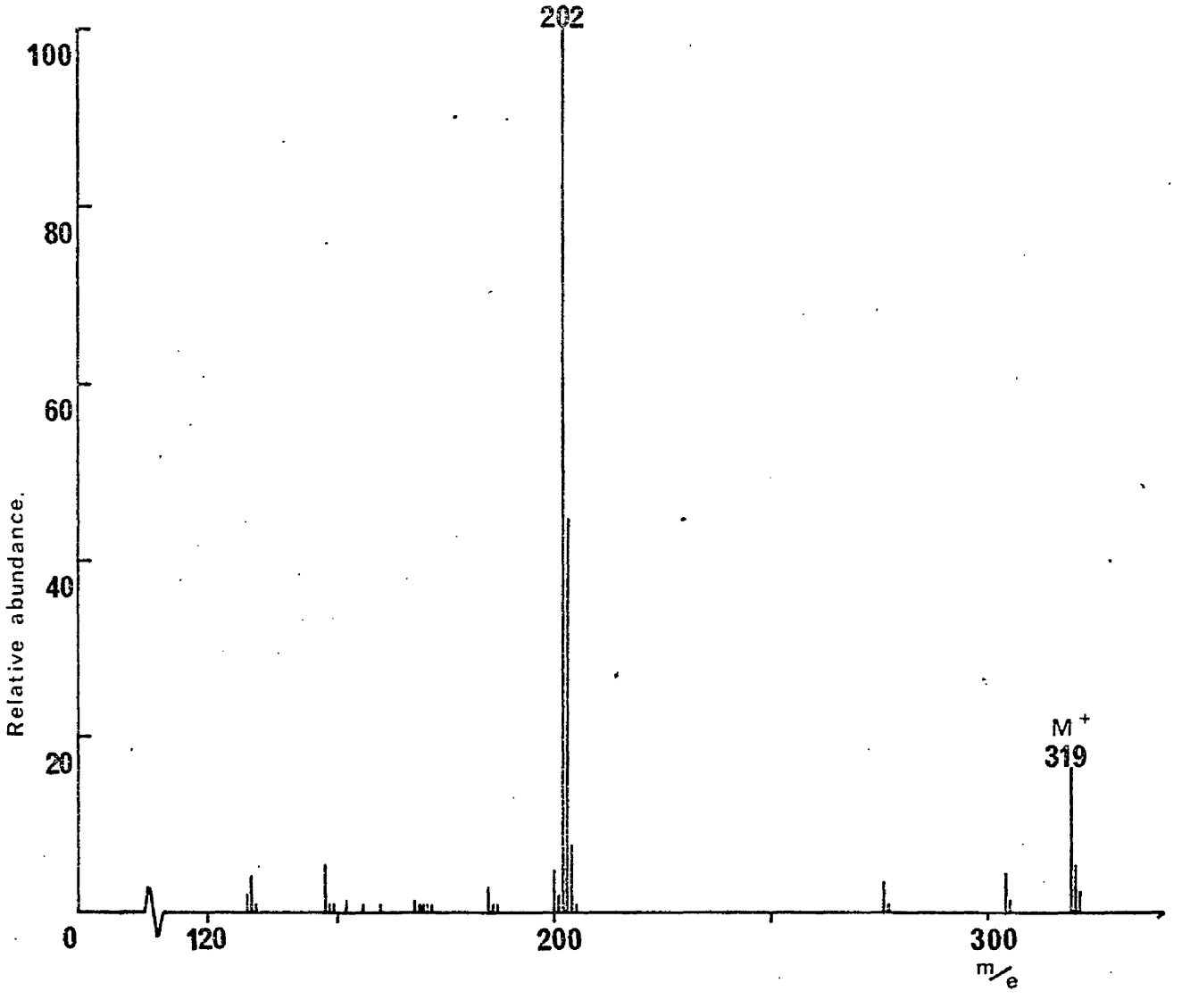


FIGURE 49

The fragmentation pattern of indole acetic acid on bombardment with electrons during mass spectral analysis.

Fragmentation of IAA.

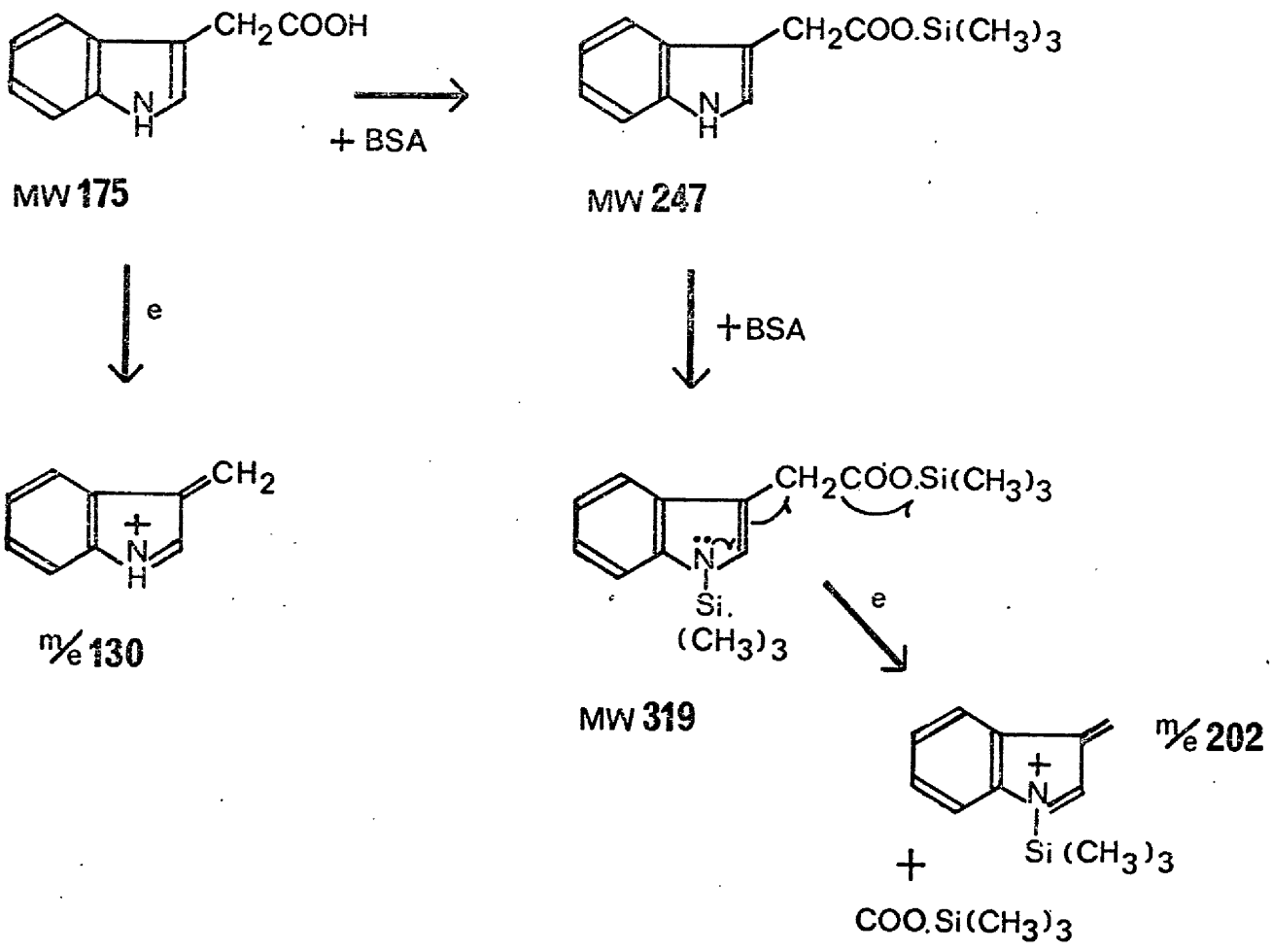


FIGURE 50

Single ion scan : continuous monitoring of partial ion current with the MS focused at m/e 202.

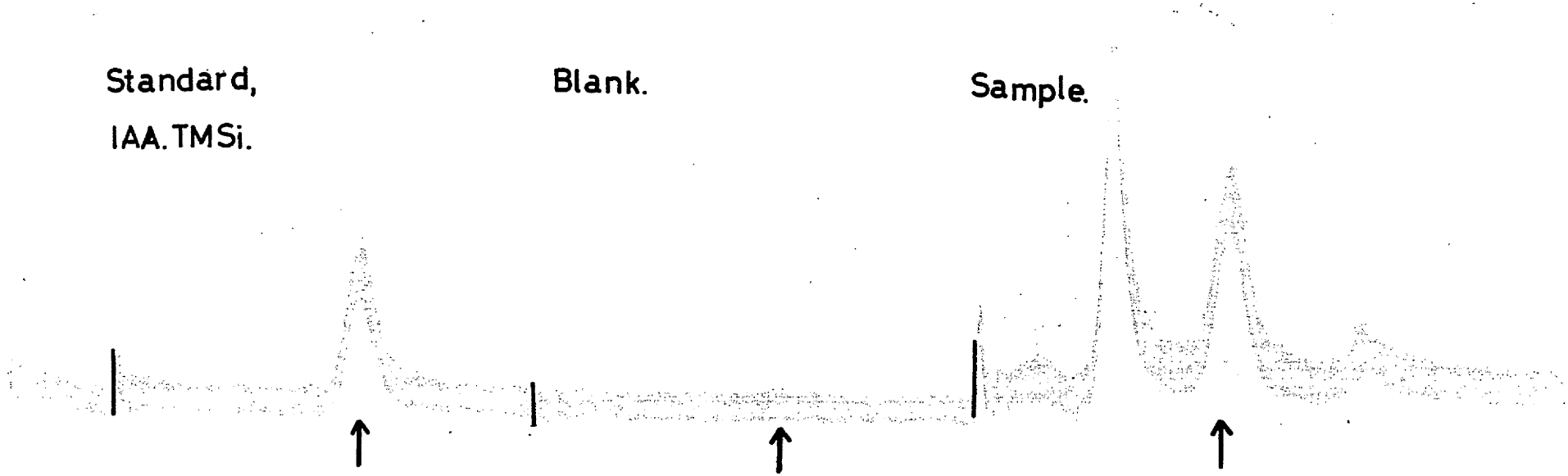
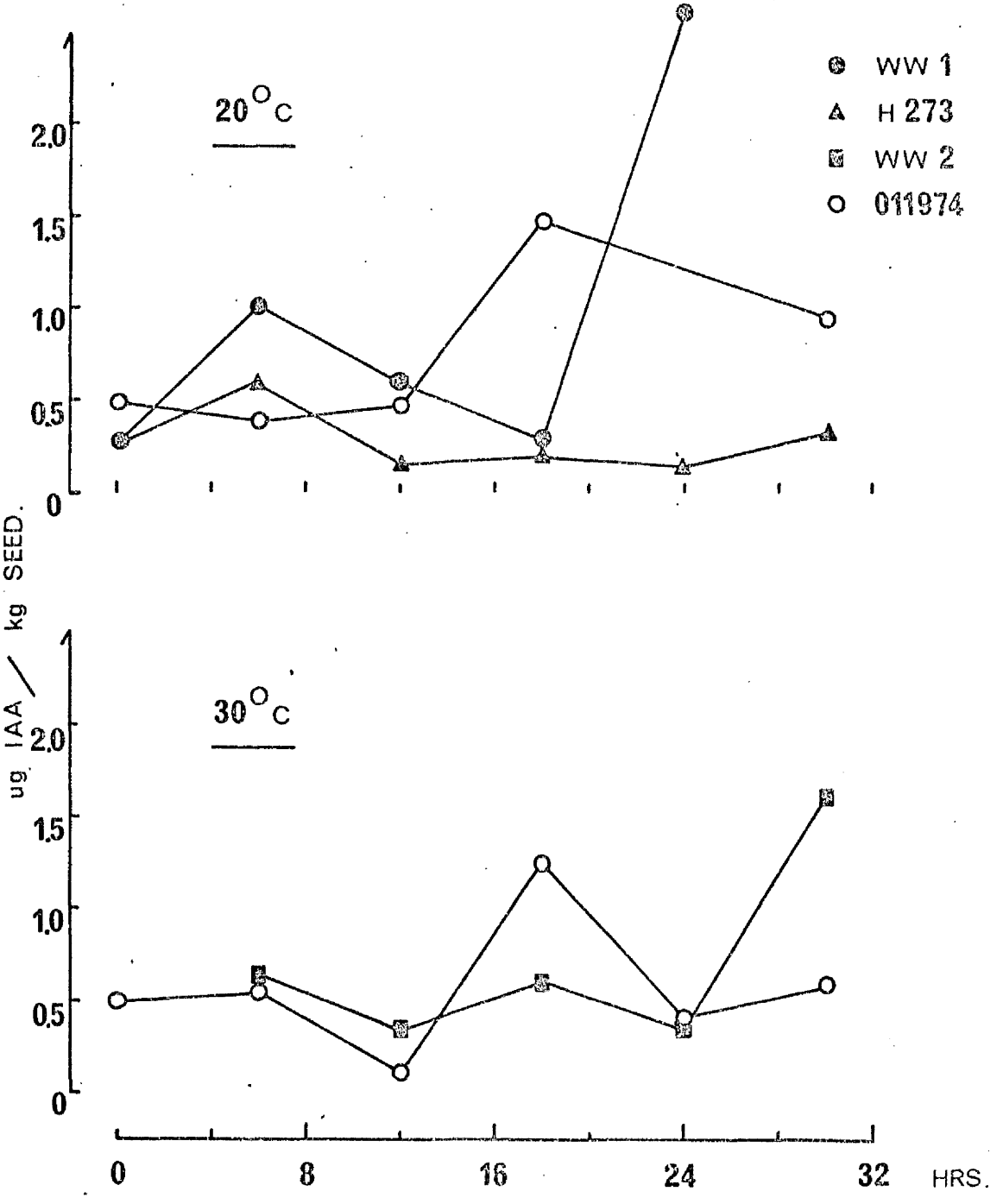

Standard,
IAA.TMSi.
Blank.
Sample.

FIGURE 51

The levels of indole acetic acid in seeds of Grand Rapids lettuce imbibed for 0.30 hrs at 20°C or 30°C. Horizontal axis represents hours imbibition.


20°C
30°C
WW 1
H 273
WW 2
011974
ug IAA / kg SEED.
2.0
1.5
1.0
0.5
0
0
8
16
24
32
HRS.

PART F

The uptake and metabolism of radioactive labelled ABA and IAA by seeds of Grand Rapids lettuce.

Introduction

There are no reports on the metabolism of exogenous radioactive ABA or IAA in the seeds of Grand Rapids lettuce. Previously, McWha (1973) studied the metabolism of 2[^{14}C]-ABA in the lettuce cultivar Great Lakes and found that one metabolite was formed. This metabolite was more polar than ABA and could be first observed after 12 hrs imbibition. The metabolite never exceeded the level of ABA which could be extracted from the seeds, and unlike ABA could not be leached from imbibed seeds. The metabolite could be partitioned into n-butanol at pH 3.0. This metabolite seemed to differ from previously identified ABA metabolites, namely abscisyl-β-D-glucopyranoside, phaseic acid and 4'-dihydrophaseic acid. The metabolism of [^{14}C]-ABA has not been reported in many seed systems. However, the metabolism of labelled IAA in seeds is even less well documented. Minchin and Harvey (1975) did show that barley grains metabolised 1 or 2[^{14}C]-IAA into indoleacetylaspartate and that this metabolism was only carried out by live seeds. However, they failed to show IAA to be present as an endogenous component of barley grains.

The aim of this work was to investigate both the uptake and metabolism of ABA and IAA by seeds of Grand Rapids lettuce, in an attempt to clarify some of the questions raised by the work previously reported in this thesis.

Results

Uptake of 2[^{14}C]-ABA and 5[^{3}H]-IAA by seeds of Grand Rapids lettuce.

The uptake of radioactivity by seeds of Grand Rapids lettuce imbibed in 2[^{14}C]ABA or 5[^{3}H]IAA was studied using the Oxymat as described in the materials and methods. Activity adhering to the outer surfaces of the seeds was removed by washing with water. Figure 54 shows the activity removed by successive 20 ml washes. It is clear that in the case of either ABA or IAA all adhering activity is removed by 3 x 20 ml washes. Routinely five such washes were carried out. The same levels of activity were washed from the surface of seeds imbibed for 1-2 hrs as from seeds imbibed for much

longer times. It has been suggested by McWha (1973) that seeds are more susceptible to leaching at the beginning of imbibition. This factor was one suggested to explain a lag in the uptake of ABA by seeds, shown by McWha, In other words, during the washes to remove surface activity, endogenous activity was removed. The results of the work reported here, would not seem to support this suggestion. These results establish that any activity measured by the oxymat is a real measure of the activity within the seeds. The uptake of radioactive ABA (2[^{14}C]ABA) and IAA (5[^{3}H]IAA) was measured both in live and dead seeds of Grand Rapids lettuce. It must be borne in mind that the uptake of radioactive ABA and 'cold' ABA may not be identical, although it is generally accepted that the biological system does not differentiate between them. Figure 55 shows the uptake of ABA in 10^{-6} M (A) or 10^{-4} M (B) solutions using both live and dead seeds. Standard errors are smaller than the symbols used to indicate activity. Figure 57 shows the uptake of water by Grand Rapids seeds over the same time period. The data in this Figure are from Berrie et al. (1974): the seeds used were obtained from Page & Co., New York and showed similar germination behaviour to those used in this study.

We can see that the uptake of ABA by live seeds closely resembles that of water uptake (Figure 57) with an initial rapid increase during the first 2-3 hrs which levels off after 6-8 hrs. Figure 55 shows that the uptake of ABA in 10^{-6} M or 10^{-4} M ABA solutions is very similar up to 24 hrs; the early uptake of activity seems to reflect the early imbibition of water, an equilibrium being reached after 24 hrs. The activity taken up in these two concentrations of exogenous ABA bathing solution, is identical at about 2,000 dpm per 200 seeds. The activity added to these solutions originally was the same. Thus, there would appear to be no need to invoke active uptake of ABA to explain the uptake pattern. The results can be understood in terms of an activity gradient. It would seem unlikely that active uptake could take place at a time when the seeds show a low level of metabolic activity. This point need not be laboured further as the primary aim of this study was to show that seeds did in fact take up activity.

After 24 hrs the uptake of activity in 10^{-6} M or 10^{-4} ABA shows a distinct difference (Figure 55). This is associated with the occurrence of germination in 10^{-6} M ABA. 10^{-4} M ABA inhibits

germination. Uptake was studied only at 20°C, below the critical temperature for the induction of thermodormancy.

The curves for the uptake of ABA by dead seeds show that there is no difference between uptake in 10^{-6} M or 10^{-4} M ABA solutions. Uptake by dead seeds is greater than in live seeds and continues for 12 hrs. Thereafter there is a fall in activity which would suggest efflux, followed by a further increase and a levelling off into a plateau. This fall in activity after 16 hrs is mirrored by a fall in water content in live seeds at a similar time (Figure 57). The uptake of radioactivity by dead seeds resembles the uptake of water in dead seeds, except that in the latter no fall in water content around 16 hrs was observed (Figure 57).

Figure 56 shows the uptake of activity from a 10^{-4} M solution of IAA by live seeds at 20°C. Clearly, this uptake pattern does not match that shown for water uptake. There is an initial rise during the first 3-4 hours but thereafter the curves for IAA uptake and water uptake diverge. There is a consistent increase in IAA uptake, up to 12 hrs. Thereafter, the increase continues but at a reduced rate up to 32 hrs and then the rate of uptake again increases. This second rapid increase in activity may be associated with germination. The deviation from a purely physical uptake of activity, as was indicated to occur with ABA, could be explained by a requirement for the endogenous concentration of IAA to remain constant, against a background of metabolism. We are measuring activity, not radioactive IAA. This possibility will be investigated when dealing with IAA metabolism.

It is clear that seeds imbibed in either 2[^{14}C]ABA or 5[^{3}H]IAA can take up sufficient activity to allow the investigation of the metabolism of ABA and IAA to be investigated.

The metabolism of exogenous 2[^{14}C]ABA by seeds of Grand Rapids lettuce.

The results of this study are shown in Figures 58-64. Initially seeds were imbibed in 10^{-4} M ABA at 20°C and then extracted, whole, in either methanol or n-butanol. McWha (1973) had indicated that similar results could be obtained from either unmacerated or macerated seeds (McWha, 1973). The reason for including n-butanol was that Sondheimer et al. (1974) have indicated that some of the ABA metabolites shown to occur in many systems, are not very soluble in methanol. The

results of this experiment are presented in Figure 58. After 24 hrs most of the activity co-chromatograms with ABA whether seeds were extracted in n-butanol or methanol (Figure 58). In whole seeds, very little activity was extracted after 48 hrs, and most of the activity imbibed could be detected in the residual seed material. It was obvious that whole seeds of Grand Rapids could not be used to study metabolism. Consequently, seeds were always macerated prior to extraction. Figure 59 shows the activity extracted by methanol from seeds imbibed in either 10^{-6} M or 10^{-4} M ABA at 20°C; the plates were developed in chloroform:methanol:water (75:22:3 v/v). After 1 day most of the radioactivity occurred at the Rf of ABA, in seeds imbibed in 10^{-6} M or 10^{-4} M ABA solutions. In 10^{-6} M ABA there is the gradual appearance of two zones of activity with a reduction in the amount of activity at the Rf for ABA. The zone which can first be seen after 1.5 days occurs at an Rf of 0.05-0.15, near the origin but not at the origin. This zone accounts for more than 30% of the extracted activity after 3 days although it remains constant at this level thereafter. The second zone of activity is found at an Rf 0.30-0.45 and first appears after 2 days. The percentage activity at this zone continues to increase up to 7 days when it accounts for over 30%. After 7 days in 10^{-6} M ABA only 15% of the activity is found at the Rf of ABA.

In 10^{-4} M ABA both these zones of activity can be found although they do not reach the levels found when seeds were imbibed in 10^{-6} M ABA. If this same experiment is repeated at 30°C, where seeds also exhibit thermodormancy, the occurrence of these zones of activity can be observed, although they never reach high levels. There is little difference in the metabolism whether seeds are held in solutions of 10^{-6} M or 10^{-4} M ABA. After 7 days the zone of activity around Rf 0.4 cannot be observed (Figure 60).

Having established that seeds can metabolise exogenous radioactive ABA into two zones of labelled material, it was critical to investigate whether this conversion could be carried out only by live seeds. Figure 61 shows the activity extracted from seeds, which had been killed by heat treatment, when imbibed at 20°C in 10^{-6} ABA for 4 days. If we examine Figure 59 we can see that live seeds produce two zones of activity by this time. No activity could be found in dead seeds except at the zone corresponding to the Rf of ABA. This shows that conversion can only be carried out by live seeds and

and could therefore be thought of as resulting from metabolism.

We have shown that a number of factors can overcome thermodormancy, and ABA-induced dormancy, in Grand Rapids seeds. Amongst these factors is red light. Figure 62 shows the effect of red light on the metabolism of ABA when seeds were held in 10^{-4} M ABA and given red light after 2 hrs. Plates were developed in chloroform: methanol:water (75:22:3 v/v). At 30°C and 20°C both zones of activity are seen after 1 day accounting for about 10% each. At 30°C there is no significant change in the levels of labelled material after 2 or 3 days but after 7 days no activity can be found at the Rf of ABA and almost all the activity can be found near the origin. At 20°C we find a similar situation. Only after 7 days is there any large amount of labelled material at a zone other than the Rf of ABA. We should note that seeds held at 30°C and given red light will germinate only after several days. Certainly, these results indicate red light cannot initiate any rapid alteration in the metabolism of ABA.

In our germination studies we looked at the effect of transfer from water to ABA after 1-20 hours, in an attempt to find if ABA influences germination or seedling growth. This experiment was repeated on a reduced time course with radioactive ABA. Seeds were transferred to 10^{-4} M ABA after 6, 12 or 24 hrs and then extracted after 48 or 72 hrs from the start of imbibition. Figure 63 gives the results obtained, plates being developed in chloroform:methanol: water (75:22:3 v/v). When seeds were extracted after 48 hrs we can see that the transfer of seeds after 6 or 12 hrs had no effect on ABA metabolism. The extracts showed no activity other than at the Rf of ABA. However, when transfer was after 24 hrs both zones of activity previously shown could be found, despite the fact that these seeds had only been in contact with ABA solution for 24 hrs prior to extraction. This effect is more pronounced when the seeds were extracted after 72 hrs; at this time some activity was found near the origin even in seeds transferred after 6 or 12 hours.

No mention has been made of the residual activity in the extracted seeds, the data for which are given in the figure captions. The amounts of residual activity as a proportion of the extracted activity are small, accounting for less than 10%, except in seed which had germinated. When germination had taken place the level of residual activity could be nearly 50% of the extracted activity, i.e. Figure 62. 7 days at 30°C, residual activity 5,191 dpm,

extracted activity 11,658 dpm.

In an attempt to gain more information about the zones of activity found when extracts were applied to TLC plates and developed in chloroform:methanol:water (75:22:3 v/v), extracts of seeds held at 20°C in 10^{-6} M ABA solution for 4 days were chromatogramed using four solvent systems, as shown in Figure 64. From the experiment previously mentioned, the results of which are shown in Figure 59, it would be expected that seeds would produce the two metabolites noted in the earlier work. Figure 64 shows that a lower level of metabolism took place than was expected but that in (A), chloroform: methanol:water (75:22:3 v/v) both zones 1 and 2, plus a zone at the Rf of ABA could be detected. A solvent with similar properties to the former, was (C) n-butanol:acetic acid:water (5:1:2.2 v/v) and in this system zone 1 was at Rf 0.00-0.15 and zone 2 at Rf 0.40-0.50. In solvent (B) isopropanol:ethyl acetate:formic acid (50:40:10 v/v) the activity at Rf 0.50-0.65 probably corresponded to zone 1 whilst zone 2 was probably contained in the peak at the Rf for ABA. Solvent (D) methylene chloride:ethyl acetate: acetic acid (50:5:5 v/v) could not separate the activity, zones 1 and 2 probably were across the Rf 0.55-0.75. Thus, with other solvent developing systems we can see the presence of three labelled zones, and none of the systems used could separate other zones from the two previously shown. Two approaches were used in an attempt to identify the two metabolites. A large scale experiment was set up using 1 g of lettuce seeds in 10^{-6} M [^{14}C]ABA solution for 4 days at 20°C. Half the extract was then run on TLC plates and zones showing activity scraped off and eluted with methanol. The eluates were reduced and the sample material recovered subjected to mass spectrometry using the direct sample probe. The other half was analysed by column chromatography. I should like to thank David Reeve for generously carrying out this work on my behalf. The results of the column chromatography are shown in Figure 75. The methanol extract was dried down and the ethyl acetate soluble fraction chromatogrammed using liquid liquid partition column chromatography. The support material was silica gel (Reeve-Angel, partisil 20) with a stationary phase of 0.5 M formic acid and a mobile phase of a continuous gradient of n-hexane:ethyl acetate, starting with 100% n-hexane and finishing with 100% ethyl acetate. The eluate was collected in 3 ml fractions and subjected to scintillation counting. The results are given in Figure 75A. Four zones of activity can be

discerned; the first two about fraction 100, correspond to the retention time of ABA and in all probability are the cis, trans- and trans, trans- isomers of ABA. The next zones of activity are in fractions 160 and 175 and this activity may be the zone which is found at an Rf of about 0.3-0.4, before ABA on TLC. Considerable activity was found to be insoluble in ethyl acetate and this was taken up in tetrahydrofuran and chromatogramed on a dual column of porous polystyrene. This system of gel permeation chromatography uses two columns, one filled with Biobeads, SX4 (molecular exclusion limit of 1,500; MEI500) and SX8 (ME 1000) and the other with Biobeads SX 12 (ME 400). The radioactivity eluted from this column system was mainly found within a molecular weight range of 450-850 (Figure 75B) with a second smaller peak at MW 300-400. No ABA was found. The liquid liquid chromatography was in the order of 40% efficient whilst the gel permeation columns gave about 65% recovery. This activity probably corresponds to the polar material found near the origin of the TLC system employed. The evidence presented would suggest that this polar material has a molecular weight of over 450. This is close to the molecular weight of ABA glucosyl ester and it can be proposed that this zone contains conjugated forms of ABA. It seems unlikely that the ABA molecule could be specifically split and synthesised into a high molecular weight compound, and it is more likely that ABA combines with other compounds. Certainly, at least two compounds are produced, and we cannot rule out the possibility that there are esters, or ethers with more than one type of compound. It is easily conceived that, for instance, phaseic acid could form an ether linkage with a glucose molecule, although no such linkage has yet been shown. The results from the mass spectrogram also indicate the presence of high molecular weight compounds in the polar metabolite zone, but no further conclusions could be drawn from the spectrum analysis. Thus, whilst the metabolites produced from labelled ABA have not been unequivocally identified it is clear that the polar metabolite zone, contains conjugated compounds, in all probability derived from ABA. Reeve and Crozier from unpublished results suggest that the radioactitity in fractions 160 and 175, collected from the liquid liquid partition chromatography column, is due to di- or tri-hydroxy acids. It is possible, although purely speculative, that these two peaks represent isomers of phaseic or dihydrophaseic acid. Sondheimer et al. (1974) have reported the Rf of dihydrophaseic

acid as 0.32 on TLC using chloroform:methanol:water as the developing solvent. This is a similar Rf to that reported in this work for zone 2.

We have shown that seeds of Grand Rapids lettuce can take up radioactive ABA, apparently by purely physical means and that this can be shown to undergo metabolism. At 20°C, below the critical temperature for the induction of thermodormancy, two zones of activity other than ABA can be shown, these only becoming prominent after 2-3 days when the seeds were in 10^{-6} M ABA solution. In 10^{-4} M ABA solution, the same metabolite zones could be shown but they never reached high levels. At 30°C the same zones were found, again never reaching high levels and zone 2 disappeared with time. Activity could only be extracted from macerated seeds; in this respect Grand Rapids seeds differ from those of Great Lakes lettuce (McWha, 1973). Only live seeds transform ABA. Experiments with red light, and transfer from water to ABA indicate that the metabolism of ABA, observed in this study, is the result of germination and not the cause. Only seeds which had sufficient potential to germinate, possess high levels of metabolism. Some metabolism can take place in the absence of germination. With increasing time in seeds which have germinated there is a build-up of activity which cannot be extracted by methanol. This factor and the metabolism of ABA are not involved in the control of thermodormancy. These metabolites have not been conclusively identified, but circumstantial evidence would suggest that the two zones of activity represent, polar conjugated forms and less polar phaseic and/or dihydrophaseic acids. These are speculative and tentative proposals and further work is necessary to identify the metabolites produced.

The metabolism of exogenous 5[^{3}H]IAA by seeds of Grand Rapids lettuce.

The results of this study are shown in Figures 52, 53 and 65-74. As no previous work had been carried out on the metabolism of IAA by lettuce seeds we had the problem of selecting solvent systems which might prove of value in separating IAA and its possible metabolites by TLC. The solvent systems chosen were:

A chloroform:methanol:water (75:22:3 v/v)

B isopropanol:ethyl acetate:formic acid (50:40:10 v/v)

C n-hexane:ethyl acetate, 3 runs (1:1 v/v)

Solvents A and C were previously used in the study of endogenous IAA whilst with solvent B it was hoped that IAA would be more mobile than in A and C. The results of running standards of IAA can be seen in Figure 52 (autoradiogram) and Figure 53. In solvent B the Rf of IAA was found to be 0.77-0.80, in solvent C 0.24-0.43 and in solvent A 0.37-0.47. Figure 65 shows the results obtained using scintillation counting for solvents A and B. These two solvents were routinely used in the metabolism studies reported in this thesis. A preliminary experiment was conducted where seeds were placed in solutions of IAA at concentrations of 10^{-6} M or 10^{-4} at 20°C. The intact seeds were extracted after 1 or 2 days and the extracts subjected to TLC using solvent system A. The results are presented in Figure 66. Clearly, there is considerable conversion after 1 day in 10^{-6} M IAA and after 2 days most of the activity is near the origin. In 10^{-4} M IAA the production of more polar materials seems to be slower than in 10^{-6} M IAA although after 2 days most of the activity is at an Rf of 0.00-0.15. At 1 day there are two zones of activity, past the Rf of IAA, at Rfs 0.70-0.80 and 0.85-0.90 in 10^{-6} M IAA. From this experiment it can be seen that less activity was extracted in 10^{-6} M IAA after 2 days than after 1 day which would suggest that the activity might be in some way unextractable. A further preliminary experiment was conducted where seeds were held in 10^{-4} M IAA for 1 or 2 days at 20°C and the activity from unmacerated seeds extracted. These seeds were then macerated and re-extracted. As a control the possible breakdown of the IAA bathing solution was studied. The results are shown in Figure 67. IAA was not converted to other zones of activity, in the extract from intact seeds, until the second day when a polar zone of activity appeared. After one day when seeds were macerated far more activity could be extracted than from intact seeds but after 2 days twice the activity could be removed by methanol from intact seeds as opposed to macerated seeds. Furthermore, the residual seed material contained activity, this being 8 times the extractable activity after 2 days, e.g.

		Activity dpm	% total activity
Day 1	Whole seeds	8,200	3.9
	Macerated seeds	171,192	81.1
	Residual	31,576	15.0
Day 2	Whole seeds	35,920	7.6
	Macerated seeds	19,352	4.1
	Residual	418,966	88.3

The activity extracted from macerated seeds was not all IAA, or at least at the Rf of IAA, and as in the whole seed extract, a more polar zone of activity appeared with time. The IAA bathing solution did show some breakdown over the 2 day period but no activity could be found at Rf's less than that of IAA. Thus, the more polar compound observed cannot be as the result of simple IAA degradation. The next experiment conducted was a time course of IAA metabolism at 20°C in 10^{-4} M IAA. The results of two repeats of this experiment are shown in Figures 68A, 68B, 69A and 69B. Repeats of the experiment are included to show that the results obtained are reliable and can be repeated. This was deemed necessary, not only because all experiments are repeated, but because there were disparities between the two preliminary experiments which might be a reflection of differences in the rate of metabolism, rather than the final result. Figure 68A shows the chromatographic distribution of activity extracted from unmacerated seeds treated as described. Two solvents systems A and B were used. The extracts run on these two solvent systems were two separate treatments. From Figure 68A we can see that there is considerable activity at Rf's other than that of IAA using solvent A as early as 6 hrs. There appears to be at least one zone at a greater Rf than IAA, and a very polar zone of activity at Rf's 0.00-0.15. The activity at the origin will be referred to as zone 1 and the activity at Rf's greater than IAA, zone 2. Zone 1 increases up to 32 hrs, appears to fall at 40 hrs but reappears in the extracts at 48 and 72 hrs. IAA does not disappear until 48 hrs. Zone 2 does not appear to increase greatly, except after 48 hrs where some 18% of the activity can be accounted for in this zone. After 72 hrs no activity is associated with zone 2.

Solvent B cannot separate the activity into zones but with

increasing time, especially after 48 hrs, there is a broad spread of activity, much of which is not IAA.

The levels of activity removed do not seem to show any trend. There is an increasing amount of activity with time, which is not extracted by methanol reaching some 150,000 dpm after 72 hrs. This figure far exceeds the level of activity extracted from whole seeds. Figure 68B shows the activity extracted from the macerated seeds of the treatments shown in Figure 68A. Clearly, no activity can be seen beyond the Rf of IAA using solvent A. The activity at the Rf corresponding to IAA increases with time up to 32 hrs, thereafter declining to a low level after 72 hrs. The levels of more polar activity are high at an early stage but decline after 32 hrs before increasing to reach a maximum after 48 hrs. With solvent B there is a broad spectrum of activity which is pronounced throughout the time course of this experiment.

If we now look at Figures 69A and B, which are repeats of the experiments reported in Figures 68A and B, we can see that similar trends are apparent. In figure 69A, solvent A, there is much less polar material at an early stage, but zone 2 shows two areas of activity, reminiscent of the preliminary work. The level of zone 2 does not seem to increase with time but becomes less apparent after 48 hrs and 72 hrs. The level of IAA, as in run 1 of this experiment, decreases after 12 hrs, with only a small increase after 32 hrs and a rapid decrease after 48 hrs. Solvent B shows that considerable activity does not appear at the Rf of IAA after 48 hrs but little else can be said about the results. (The blank at 48 hrs, in solvent B, was due to the TLC plate breaking up.) If we now examine Figure 69B, the activity extracted from macerated seeds, we can see that as before no activity can be found in zone 2 using solvent A. There is an increase with time in the activity near the origin which unlike run 1 does not decline after 32 hrs. The activity corresponding to the Rf of IAA decreases from 6 hrs onwards reaching negligible levels after 24 hrs but there is a reappearance of IAA after 48 hrs. In solvent B, again we can see a gradual broadening of the Rf's of the extracted activity.

Thus, although these two repeats of the same experiment indicate that there is considerable variation between runs, there is a number of general trends which emerge. Firstly, the amount of activity which can be extracted from macerated seeds appears fairly constant

over a long period: certainly, the results do not support the trend shown in the preliminary experiment where after 1 day over 170,000 dpm could be extracted from macerated seeds, but only 19,352 after 2 days. However, both experiments show that with increasing time there is an increasing amount of activity which remains unextracted from macerated seeds. Presumably this activity is 'bound' in some way. As time proceeds with the whole seed extracts we can see the disappearance of activity at the Rf of IAA. Activity at zone 2, using solvent system A, may be one or more areas of activity, but in either case there seems to be little change in the levels of activity associated with this zone until the later stages of this experiment where the activity at this zone disappears. As time increases there is an increase in the activity associated with more polar compounds, although in run 1 this activity may decline around 40 hrs before again becoming prominent. Solvent system 2 did not prove very useful in separating activity into different zones, and only a general trend showing the production of more polar compounds than IAA could be observed.

The extracts from macerated seeds showed that no activity could be observed using solvent system A in what we have called zone 2. The levels of activity at the Rf of IAA fall and then increase with time. In the first experiment this increase is seen after 32 hrs but in the second experiment the increase is not apparent until 48 hrs. Thus, the decrease in experiment 1 after 48 hrs may have been missed in the second experiment. In both experiments there is the accumulation of considerable activity as more polar compounds. After 72 hrs in experiment 2 the zone from Rf's 0.00-0.30 contains 65% of the extracted activity.

At 20°C lettuce seeds do not germinate in 10^{-4} M IAA during the first 24 hrs but after this time there is an 'escape' from the inhibiting actions of IAA, some 40-50% germination being obtained at 72 hrs (Table 44). Could it be that this germination reflects the reduction in IAA with the concominant increase in more polar metabolites shown in the previous two experiments ? One way of investigating this question would be to study the metabolism of exogenous IAA by seeds held at a temperature where seeds exhibit thermodormancy. The results of such an experiment are presented in Figure 70. Seeds were imbibed, at 30°C in either 10^{-5} M or 10^{-4} M IAA solutions and extracted intact and then after being macerated.

The pattern shown in 10^{-5} M or 10^{-4} M IAA solutions is very similar. Extracts from whole seeds show little change with time. There is some activity at the Rf of IAA but this never accounts for more than 25% of the extracted activity. The main zone of activity is less polar than IAA and corresponds to zone 2 previously mentioned in the experiments at 20°C. In the macerated seed extract most of the activity is in the zone near the origin, more polar compounds than IAA, and this activity is greater at 48 hrs than 24 hrs. Unlike the 20°C experiment some activity can be found in zone 2 of the macerated seed extract and this falls with time to a negligible level, especially in 10^{-5} M IAA.

If we examine the residual activity at 30°C, that is the activity which cannot be extracted by methanol, it is clear that the total extractable activity is only a fraction of the total activity taken up by the seed. Furthermore, it can be seen that uptake in 10^{-5} M IAA exceeds that at 10^{-4} M IAA by more than a factor of 3. As equal activity was added to the stock solutions, this implies that the uptake of IAA may not be purely physical.

We can see that at 30°C the same zones of activity can be found as at 20°C and that some of the changes observed at 20°C also take place at 30°C, an increase in polar metabolites, the presence of zone 2 which however, shows little change. Can any of the differences explain how IAA might cause dormancy at 30°C ? Clearly, at 30°C there is less activity at the Rf of IAA than could be seen at 20°C, but when germination had occurred at 20°C, IAA could be seen to disappear. No simple hypothesis emerges from the data presented. Furthermore, we cannot even begin to answer the obvious question of the role of the activity which cannot be extracted by methanol. This activity far exceeds the extractable activity.

So far we have shown only the presence of zones of activity on TLCs, produced in the presence of seeds. A critical experiment in determining whether or not these 'zones' have any real meaning with regard to the control of germination, must be to prove that they are not the product of microbial action. Figure 71 shows the results of an experiment designed to satisfy this requirement. Seeds were washed in 5% chloros solution for 5 minutes and then thoroughly rinsed with water. These seeds were then imbibed in 10^{-4} M IAA and extracted after 48 or 72 hrs. We can see that after 48 hrs and 72 hrs there is activity at the Rf of IAA and at zone 2, beyond the Rf of IAA, but little polar activity. The macerated seed

extracts both show little activity at the Rf of IAA but have large amounts of polar metabolites. Bearing in mind the fact that the chloros treatment 'slows' down the rate of germination, these results are not dissimilar from the experiments conducted at 20°C. Thus, we have some evidence that the production of these zones of activity are from processes which occur within the seed and seem not to involve microbial action.

A second question of critical importance in assigning any significance to the zones of activity, shown to occur in the presence of seeds, must be to prove they are produced by a biological process. The biological integrity of seeds may be destroyed by heating seeds in an oven at 120°C for 24 hrs. Seed s killed in this way were imbibed in 10^{-4} M IAA solutions and extracted as before. The results are presented in Figure 72. It can be clearly seen that there is activity beyond the Rf of IAA in 10^{-5} M or 10^{-4} M IAA. There is not the production of polar activity in the extracts. In the extracts from macerated seeds only 1,222 dpm could be extracted from 48 hrs imbibed seeds, at 10^{-4} M IAA, and 674 dpm at 72 hrs. Some of this activity could be found at Rfs 0.00-0.20. However, the small levels of activity make interpretation of histograms meaningless. Thus, in dead seeds most of the extractable activity can be removed from intact seeds, but the largest part of the activity taken up by the seeds is still not soluble in methanol (Figure 72, residual activity). Whilst the results obtained with dead seeds are not identical with those using live seeds, mainly the absence of very polar metabolites, it is clear that metabolism cannot wholly be explained in biological terms. Whether this means we are only looking at a chemical breakdown of IAA, within seeds, which is of no significance with respect to germination cannot be satisfactorily answered by this work. Although we have shown that the so called metabolism of IAA is not biological, it does require the presence of seeds, it does not seem to be microbial (although this possibility cannot be completely ignored) and the metabolism does appear to show differences at different temperatures.

Two experiments were conducted to investigate the possible involvement of IAA metabolism, in the control of dormancy. Firstly, the effect of red light on IAA metabolism was examined. The results are presented in Figure 73. At 20°C we can see the

presence of the two previously mentioned zones of activity. The levels of activity in these zones do not seem to alter significantly after 48 hrs. The macerated seed extract also shows three zones of activity, although activity is spread over the whole range of Rfs. If anything the polar metabolites are reduced with time, with an increase in activity beyond the Rf of IAA.

At 30°C after 48 hrs there is a very large peak at Rf 0.15-0.20 The significance of this metabolite is not clear, although there is considerable polar activity at all the analysis times, 48 hrs, 72 hrs, and 96 hrs. There is some increase in activity in zone 2 with the percentage activity at the Rf of IAA falling with time. In the macerated seed extract initially there is considerable activity at the Rf of IAA but this falls with time. The more polar activity is always present.

The levels of red light given will cause germination within 48 hrs at 20°C or 30°C.

A second experiment carried out to investigate a possible role for metabolism of IAA in lettuce seeds is a repeat of the transfer experiment, where seeds are moved from water to IAA after specified times. The results of this work are shown in Figure 74. Transfers were made after 6, 12, and 24 hrs imbibition in water and seeds extracted after a total of 48 hrs imbibition. With ABA we have previously noted that metabolism could only be shown in seed transferred after 24 hrs, by which time germination was considered to have taken place. Thus, metabolism of ABA could be conceived to be the result of germination and not the cause. It may be suggested for IAA that the 'important' zone of activity with respect to a controlling role of IAA metabolism, is the polar zone at Rfs 0.00-0.15. If we examine Figure 74, the macerated seed extracts show little differences but the extracts from intact seeds show that after 24 hrs in water the seeds can produce large amounts of activity in zone 2, beyond the Rf of IAA. However, these seeds had only been in contact with IAA solution for 24 hrs. Thus, the results are not directly comparable with the 6 or 12 hr transfers. During a subsequent 12 or 18 hrs the activity at zone 2 could be expected to fall; in the early experiments at 20°C activity in this zone was shown to fall rapidly around 40 hrs (Figure 68A). We have proposed that the activity at zone 1 could be important in the control of dormancy, primarily based on the evidence which suggests that this zone shows changes which could be

involved with germination. However, there are no significant changes in this zone of activity in response to transfer.

The interpretation of zones of activity found by extracting the seeds must be considered against the background of an ever-increasing proportion of the activity, taken up by seeds, being insoluble in methanol. The extractable activity is an ever-decreasing fraction of the total activity within the seeds.

This study has shown that tritiated IAA can be taken up by lettuce seeds, and that uptake differs from a purely physical uptake with water. The possibility exists that there is active uptake of IAA by lettuce seeds. Whether this is the case or not, we can extract some of this activity from intact seeds, and more activity from the macerated residues. However, with time the ratio of extractable to non-extractable activity widens in favour of non-extractable activity. Indeed, after several days only about 1/10th of the activity in the seeds is readily extractable. Studies of metabolism at 20°C, below the critical temperature, showed the presence of two zones of activity, one more polar, one less polar than IAA, and there is evidence that the zone, less polar than IAA (i.e. beyond IAA on TLC) may have at least two components. Changes were shown to take place at 20°C with time, although there was considerable variation between experiments. At 30°C the pattern of IAA metabolism was in some ways similar to that at 20°C, although disparities were also shown. It was further shown that chloros washed seeds were capable of IAA metabolism, indicating that micro-organisms were not solely responsible for the observed IAA metabolism. However, when seeds were heat killed the IAA was still 'broken down' although not in an identical fashion to that at 20°C or 30°C.

Attempts to show some involvement or role for IAA, in the control of thermodormancy, using red light and transfer from water to IAA, merely left the situation unresolved. It is clear that a fuller investigation of these questions would be necessary before unequivocal answers might be found. However, the value of such further studies should be seriously questioned, in view of the demonstration that the conversion of IAA by lettuce seeds, although requiring the presence of seeds, is not biological. We would suggest that the control of thermodormancy must invoke inherent

biological control mechanisms. Perhaps a more interesting line for future investigation could lie in the fact that most of the radioactivity taken up by lettuce seeds is non extractable by methanol. It can be proposed that this IAA is in some way bound. Much of the metabolism shown in the experiments reported in this study cannot be related to germination. For instance, at 20°C we studied metabolism up to 72 hrs. However, as was pointed out germination had reached a significant level by 48 hrs. Thus any later metabolism may reflect seedling metabolism. The changes of IAA may reflect detoxification of the imbibition medium. IAA in solution inhibits germination. IAA is broken down by a wide variety of enzymes (as well as specific IAA oxidase ?). Seeds are rich sources of a variety of enzymes (Mayer, 1974). Clearly, it would be unlikely that an easily destroyed compound such as IAA would not be metabolised, thus reducing the IAA content of the seeds and allowing germination. The removal of IAA into bound forms could also allow germination to proceed. That IAA is found in a wide variety of conjugated forms is known (Schneider and Wightman, 1974), and during germination in seeds there is the release of many compounds with which IAA could form conjugates. Apart from the fact that removal of these compounds by IAA might itself retard the visible signs of germination by removing essential compounds for seedling growth, the removal of IAA as conjugates need not be a normal function of the seeds' metabolism. In conclusion, the involvement of IAA in seed germination and the imposition of thermodormancy is tenuous: the metabolism studies reported throw no light on the previously reported endogenous studies.

FIGURE 52

Autoradiography of TLC's of [^{3}H]-IAA standards using three developing solvent systems.

A chloroform:methanol:water (75:22:3 v/v)

B isopropanol:ethyl acetate:formic acid (50:40:10 v/v)

C n-hexane:ethyl acetate:3 runs (1:1 v/v)

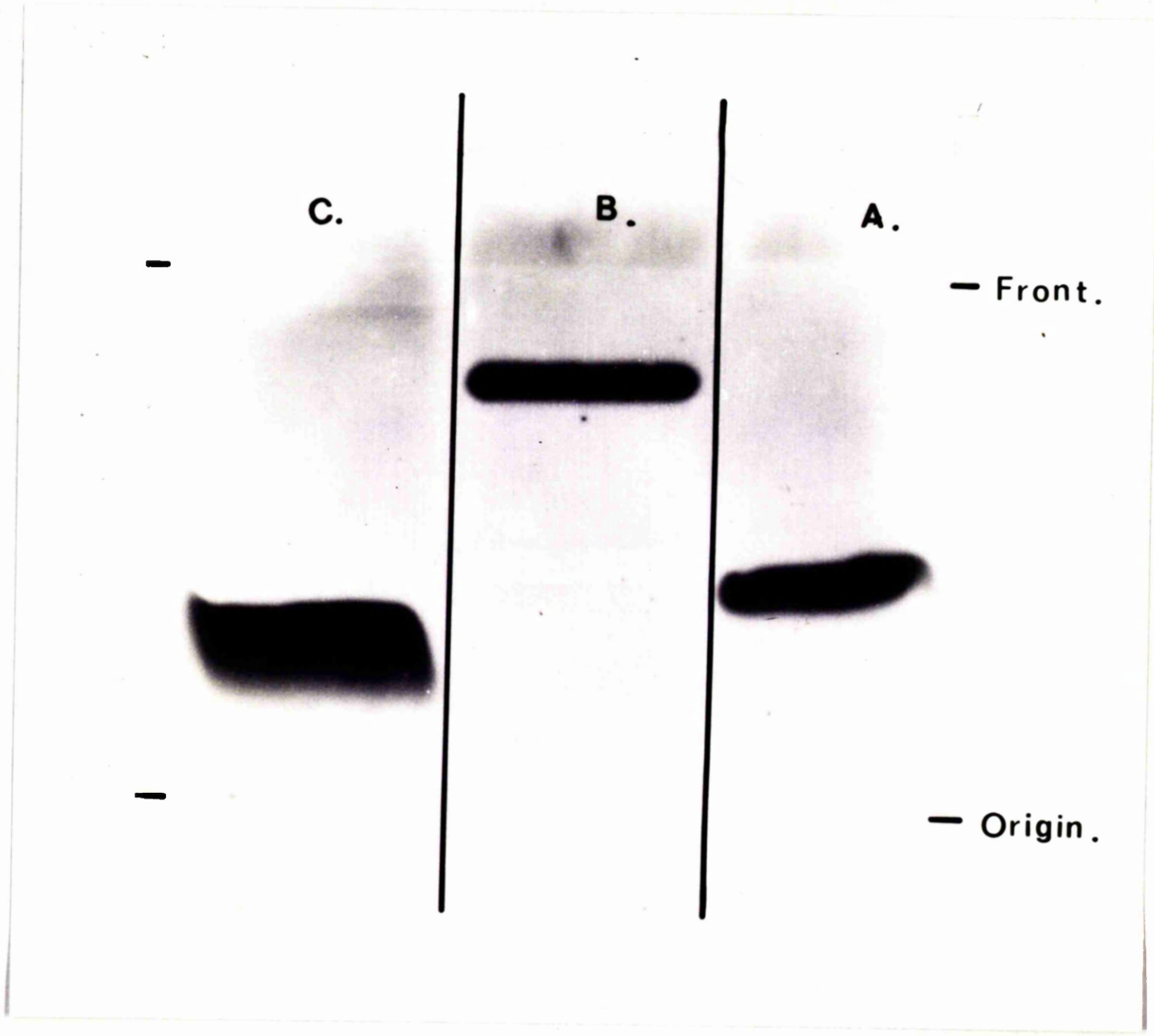
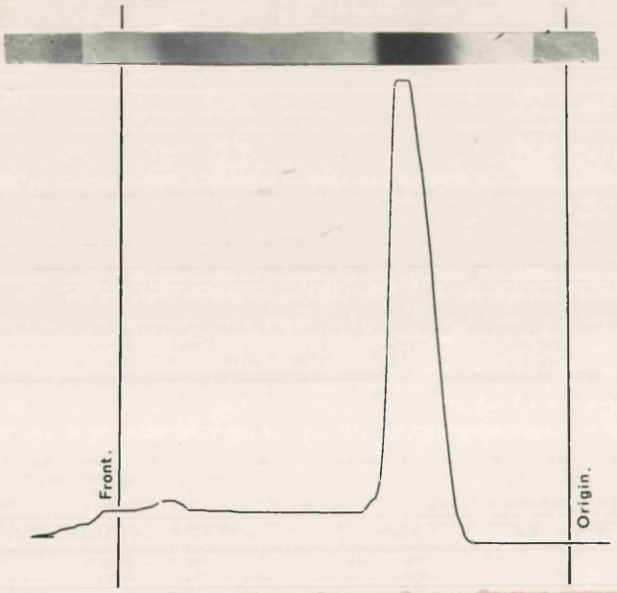
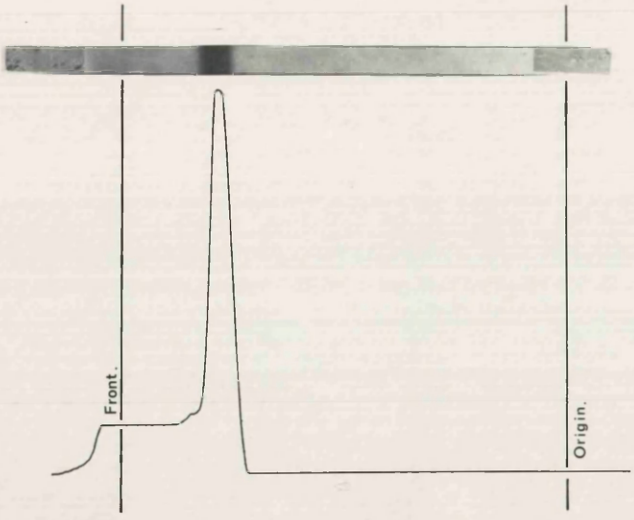

C.
B.
A.
Front.
Origin.

FIGURE 53

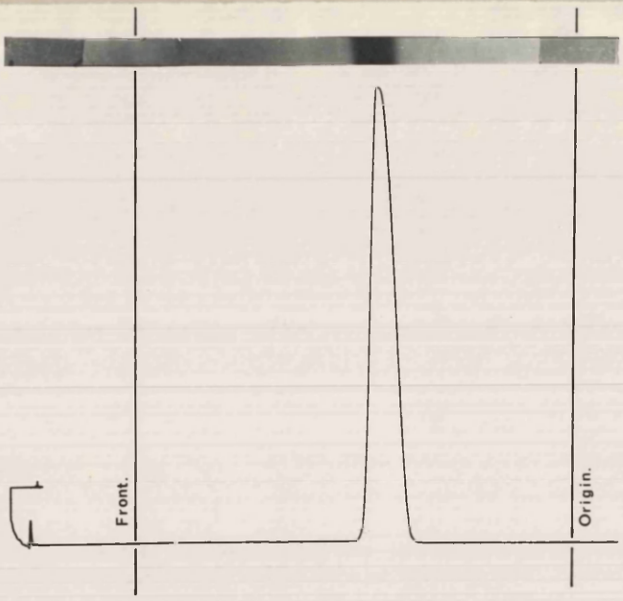
As Figure 52 : autoradiograms scanned on a Joyce-Loebl chromoscan.



C.



B.



A.

FIGURE 54

The radioactivity washed from seeds of Grand Rapids lettuce imbibed in either [^{14}C] ABA or [^{3}H] IAA for 6 hrs at 20°C. The radioactivity is expressed as dpm per 200 seeds.

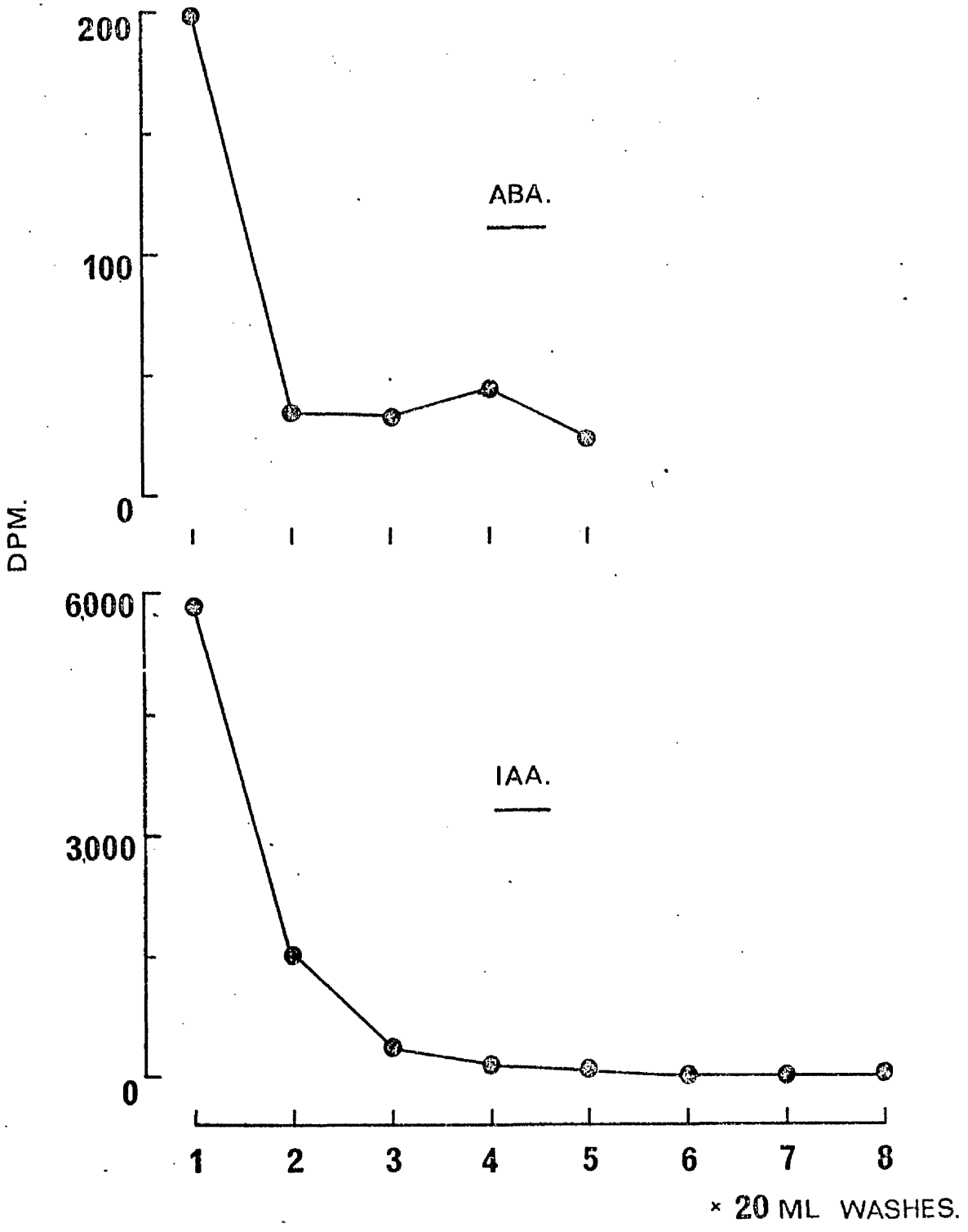

200
100
0
DPM.
ABA.
6000
3000
0
IAA.
1
2
3
4
5
6
7
8
× 20 ML WASHES.

FIGURE 55

The uptake of radioactivity from [^{14}C] ABA solutions, (A) 10^{-6} M (B) 10^{-4} M, by seeds of Grand Rapids lettuce at 20°C. The radioactivity expressed as dpm refers to the levels per 200 seeds.

▲ : dead seeds, 10^{-6} M ABA solution.

■ : live seeds, 10^{-6} M ABA solution.

+ : dead seeds, 10^{-4} M ABA solution.

● : live seeds, 10^{-4} M ABA solution.

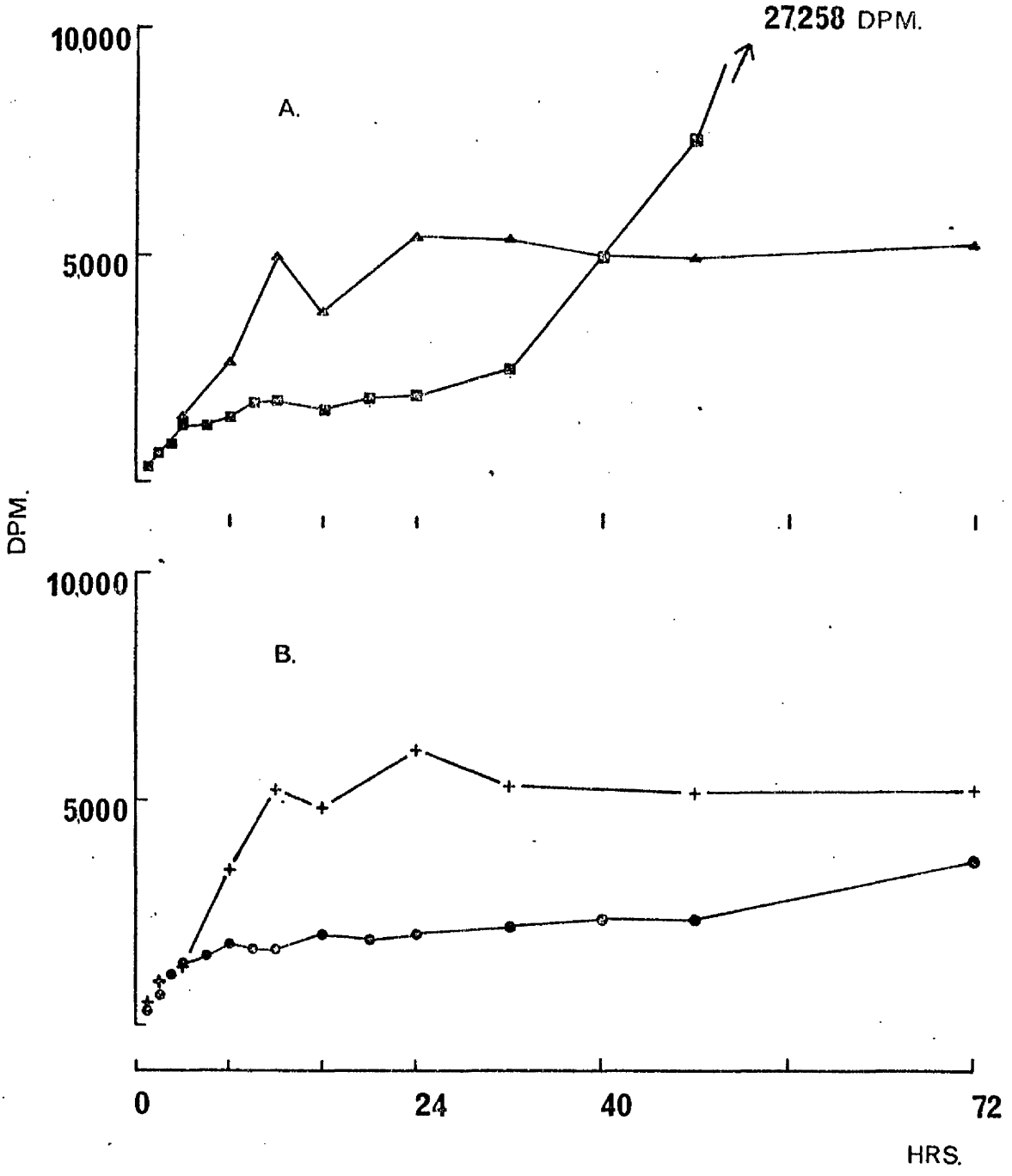

27,258 DPM.
10,000
5,000
A.
DPM.
10,000
5,000
B.
0
24
40
72
HRS.

FIGURE 56

The uptake of radioactivity from [^{3}H] IAA solution at a concentration of 10^{-4} M by seeds of Grand Rapids lettuce at 20°C. The radioactivity expressed as dpm refers to the levels per seed.

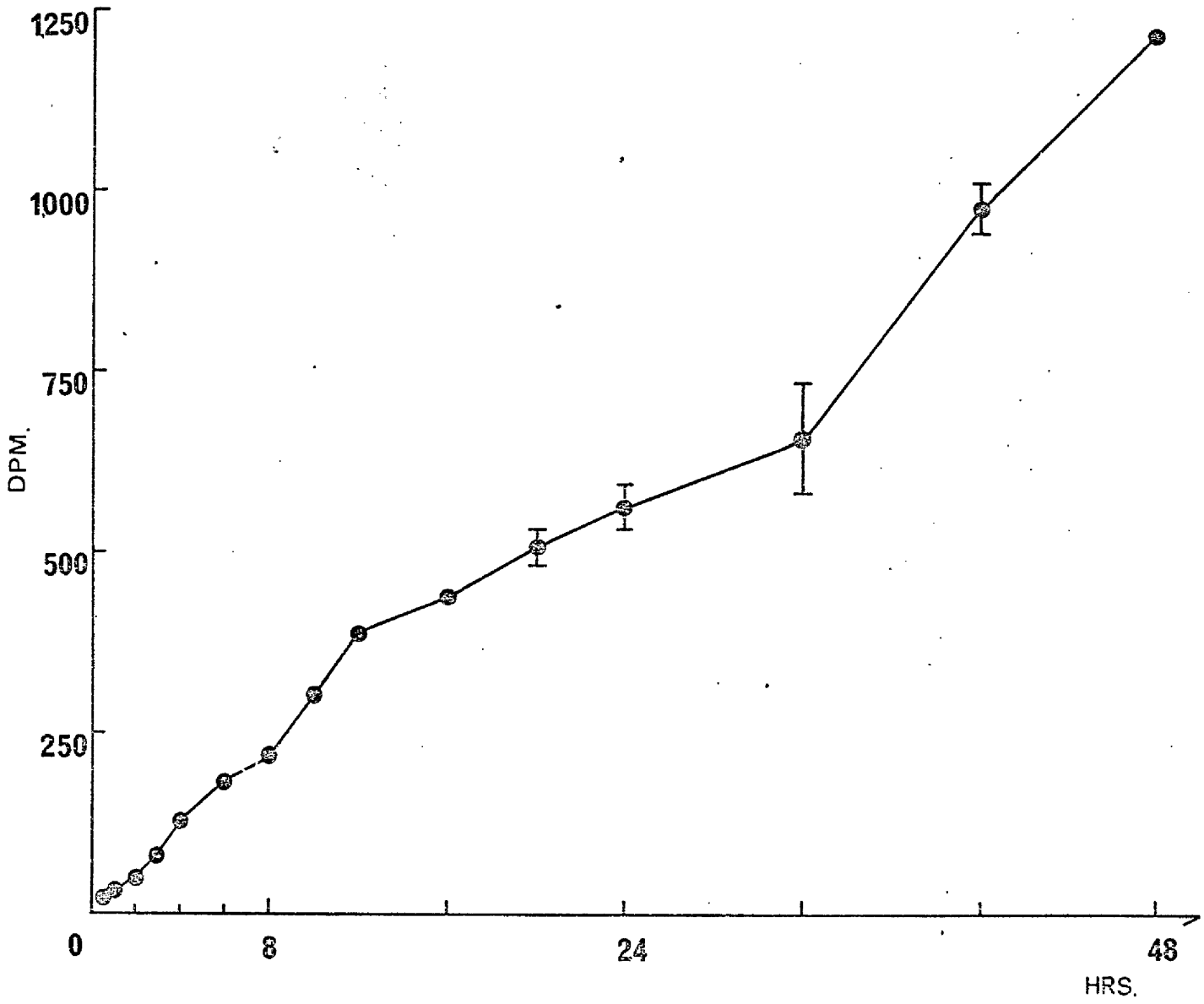

1250
1000
750
500
250
DPM.
0
8
24
48
HRS.

FIGURE 57

The uptake of water and [^{14}C] ABA by seeds of Grand Rapids lettuce at 20°C.

● : live seeds, water uptake.

✚ : dead seeds, water uptake.

■ : uptake of [^{14}C] ABA by live seeds.

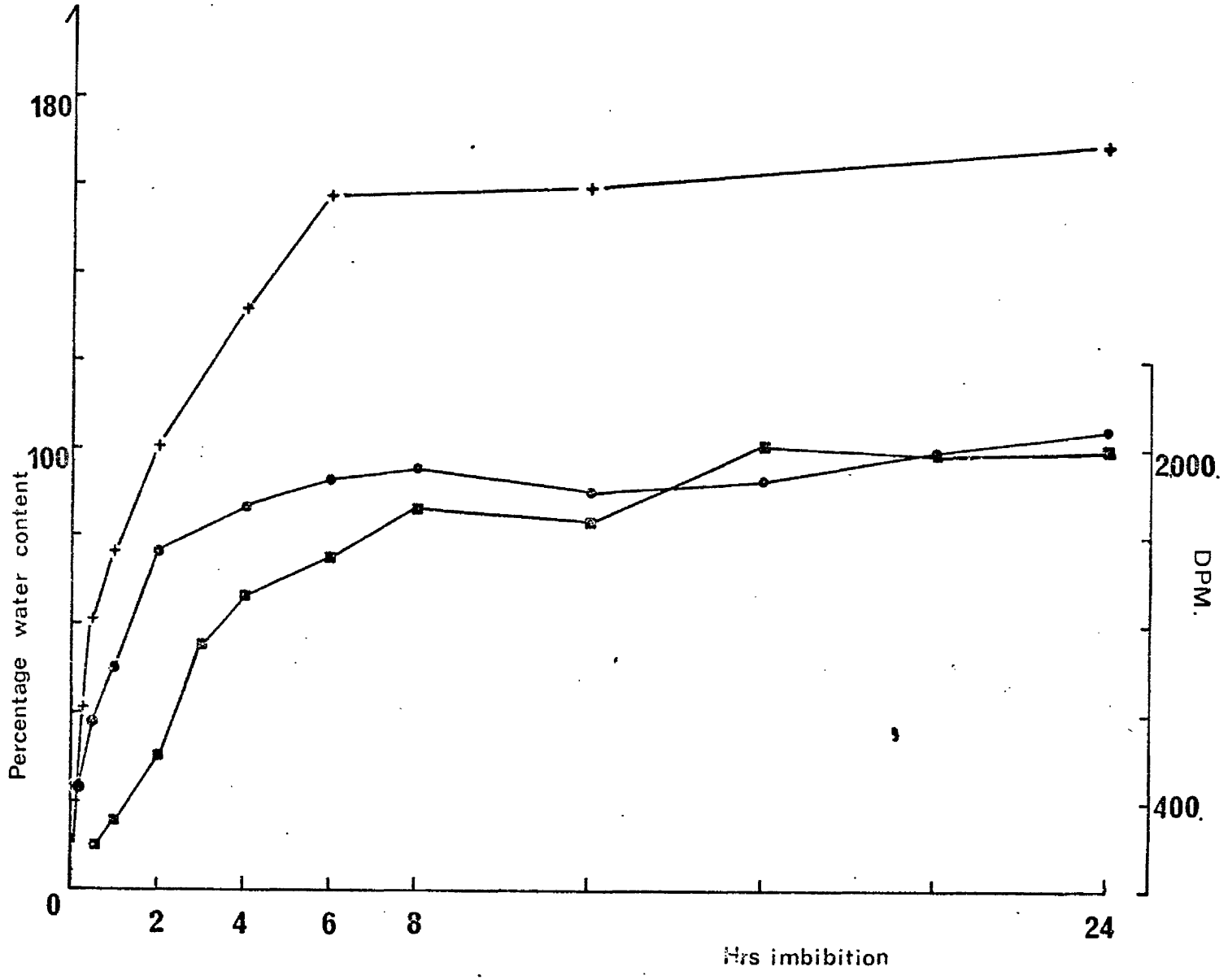

180
100
0
Percentage water content
2000.
400.
DPM.
2
4
6
8
24
Hrs imbibition

FIGURE 58

Chromatographic distribution of the radioactivity extracted from seeds after 24 hrs or 48 hrs imbibition in [^{14}C] ABA (10^{-4}M) at 20°C. Plates developed in chloroform:methanol: water (75:22:3 v/v).

a	extracted in	methanol	:	24	hrs	imbibed
b	"	"	"	:	48	"	"
c	"	"	n-butanol	:	24	"	"
d	"	"	"	:	48	"	"

Residual activity (dpm)

a 1,846.9 b 4,276.2 c 1,973 d 3,867.6

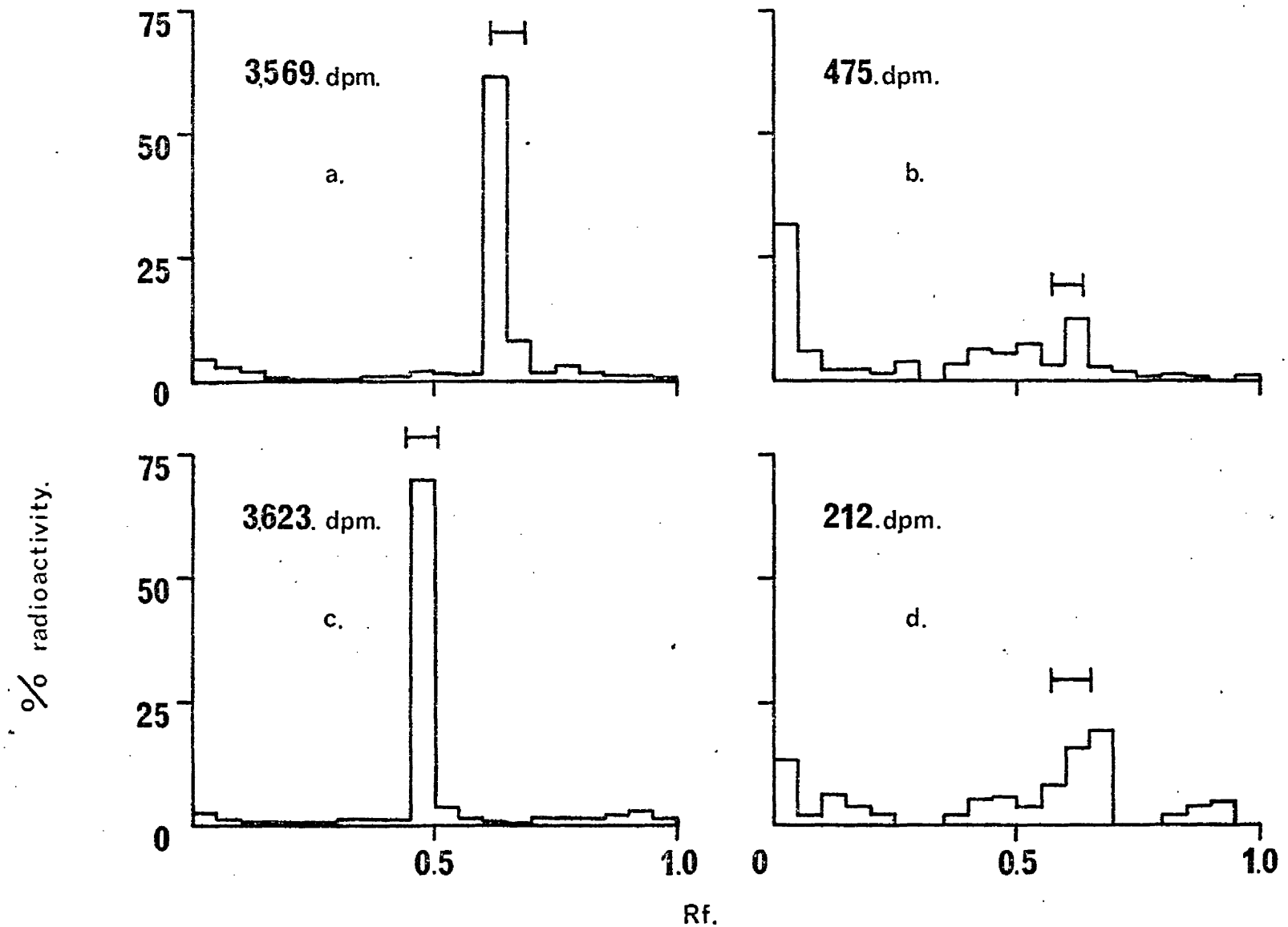
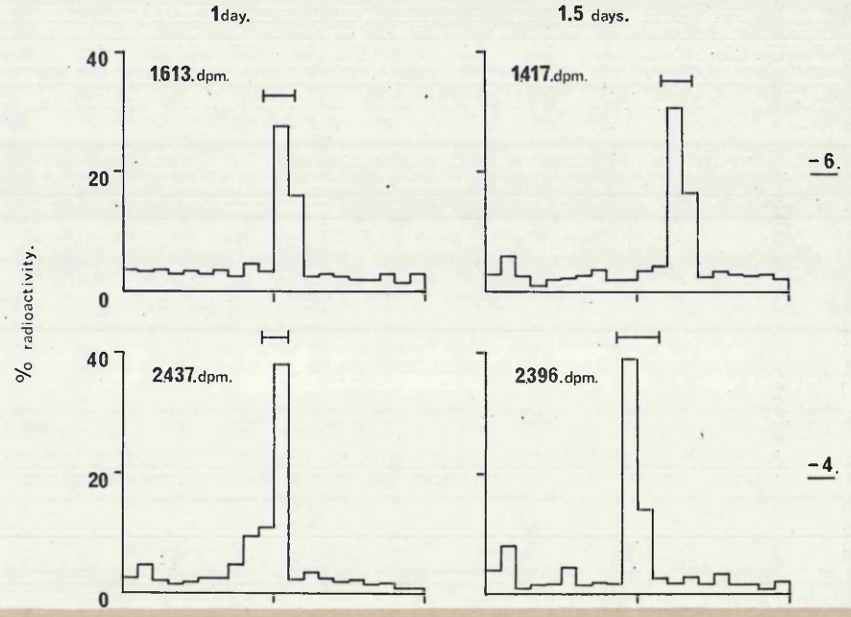

75
50
25
0
3,569. dpm.
a.
475. dpm.
b.
3,623. dpm.
c.
212. dpm.
d.
% radioactivity.
0
0.5
1.0
Rf.

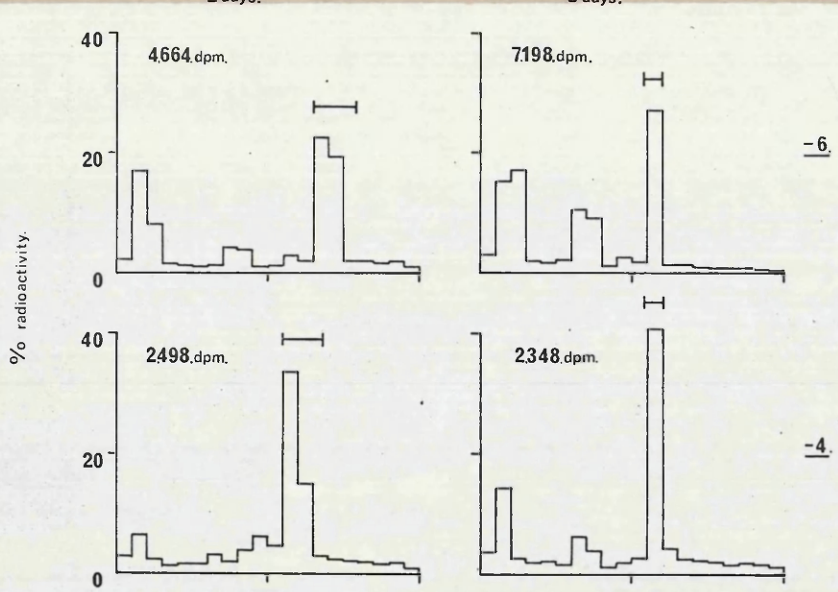
FIGURE 59

Chromatographic distribution of the radioactivity extracted by methanol from seeds after imbibition in [^{14}C] ABA at 20°C. Plates developed in chloroform:methanol:water (75:22:3 v/v). ABA solutions 10^{-6} M or 10^{-4} M.

Residual activity (DPM)

	10^{-4} M	10^{-6} M
1 day	389	349
1.5 days	713	586
2 days	406	426
3 days	248	1,100
4 days	562	1,187
7 days	774	3,233


1 day.
1.5 days.
1613. dpm.
1417. dpm.
2437. dpm.
2396. dpm.
-6.
-4.
% radioactivity.
40
20
0


2 days.
3 days.
4664. dpm.
7198. dpm.
2498. dpm.
2348. dpm.
-6.
-4.
% radioactivity.
40
20
0

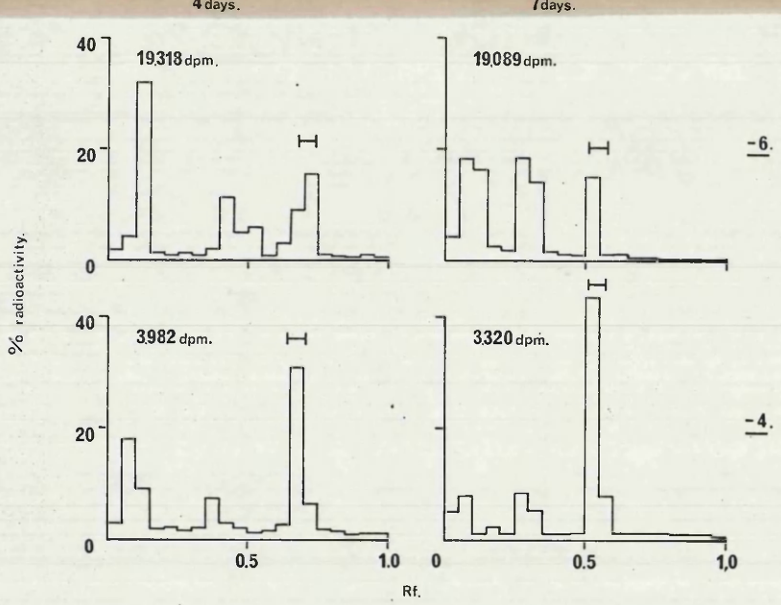

4 days.
7 days.
19318 dpm.
19089 dpm.
3982 dpm.
3320 dpm.
-6.
-4.
% radioactivity.
40
20
0
0.5
1.0
Rf.

FIGURE 60

Chromatographic distribution of the radioactivity extracted by methanol from seeds after imbibition in [^{14}C] ABA at 30°C. Plates developed in chloroform:methanol:water (75:22:3 v/v)/ ABA solutions 10^{-6} M or 10^{-4} M.

Residual activity (DPM)

	10^{-4} M	10^{-6} M
1 day	385	220
2 days	822	346
7 days	5,543	5,302

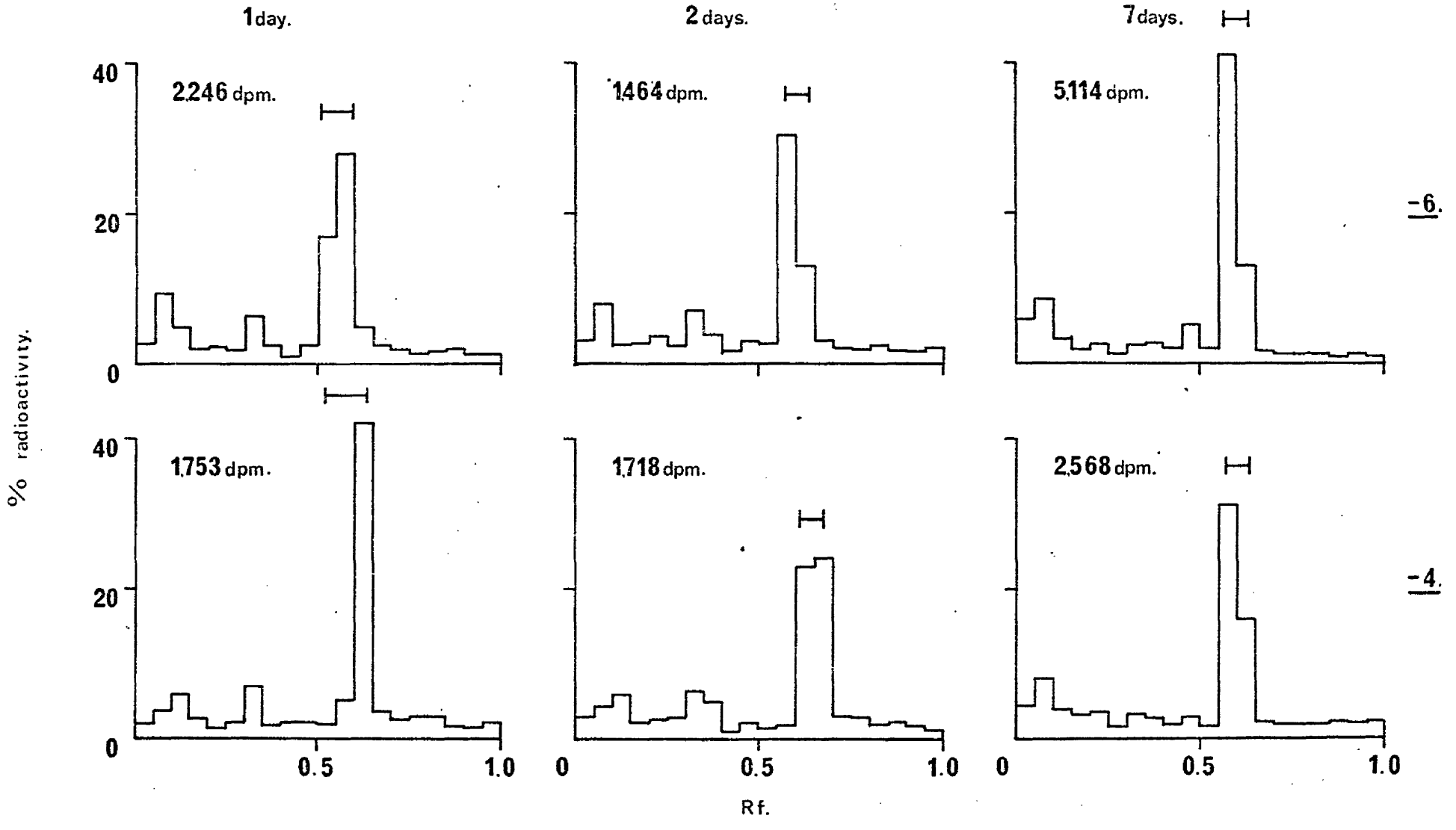

1 day.
2 days.
7 days.
2.246 dpm.
1464 dpm.
5.114 dpm.
-6.
1.753 dpm.
1.718 dpm.
2.568 dpm.
-4.
40
20
0
0.5
1.0
% radioactivity.
Rf.

FIGURE 61

Chromatographic distribution of the radioactivity extracted by methanol from seeds after imbibition in [^{14}C] ABA (10^{-6} M) at 20°C for 4 days. Seeds were killed by heat treatment prior to imbibition. Plates developed in chloroform:methanol:water (75:22:3 v/v).

Residual activity (dpm)

A 833.6

B 616.3

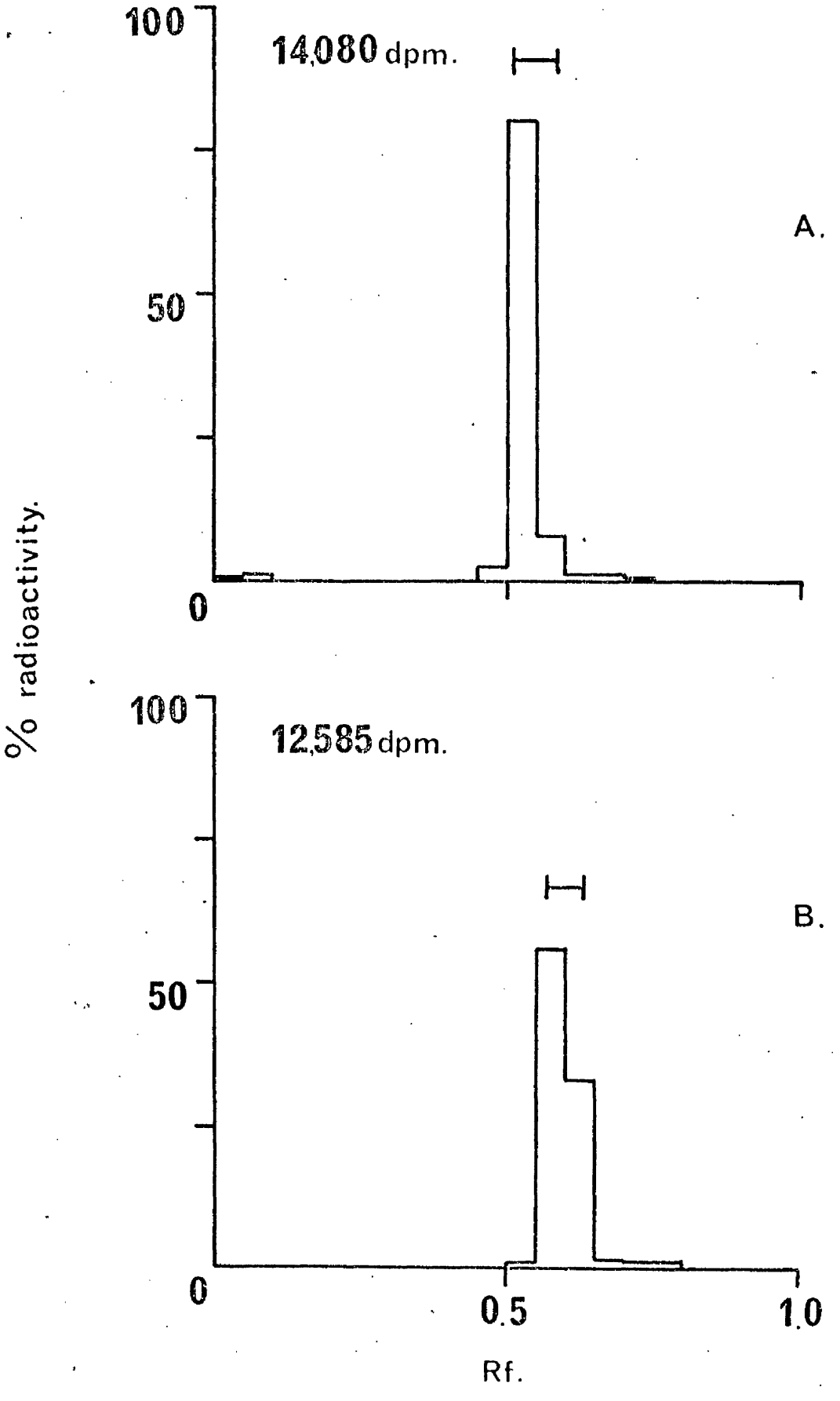

100
14,080 dpm.
A.
50
0
% radioactivity.
100
12,585 dpm.
B.
50
0
0.5
1.0
Rf.

FIGURE 62

Chromatographic distribution of the radioactivity extracted by methanol from seeds after imbibition in [^{14}C] ABA (10^{-4}M): the effect of red light treatment. Plates developed in chloroform:methanol:water (75:22:3 v/v).

20°C : exposure to 10 minutes red light.

30°C : exposure to 20 minutes red light.

Residual activity (DPM)

	20°C	30°C
1 day	203	244
2 days	229	569
3 days	275	976
7 days	933	5,191

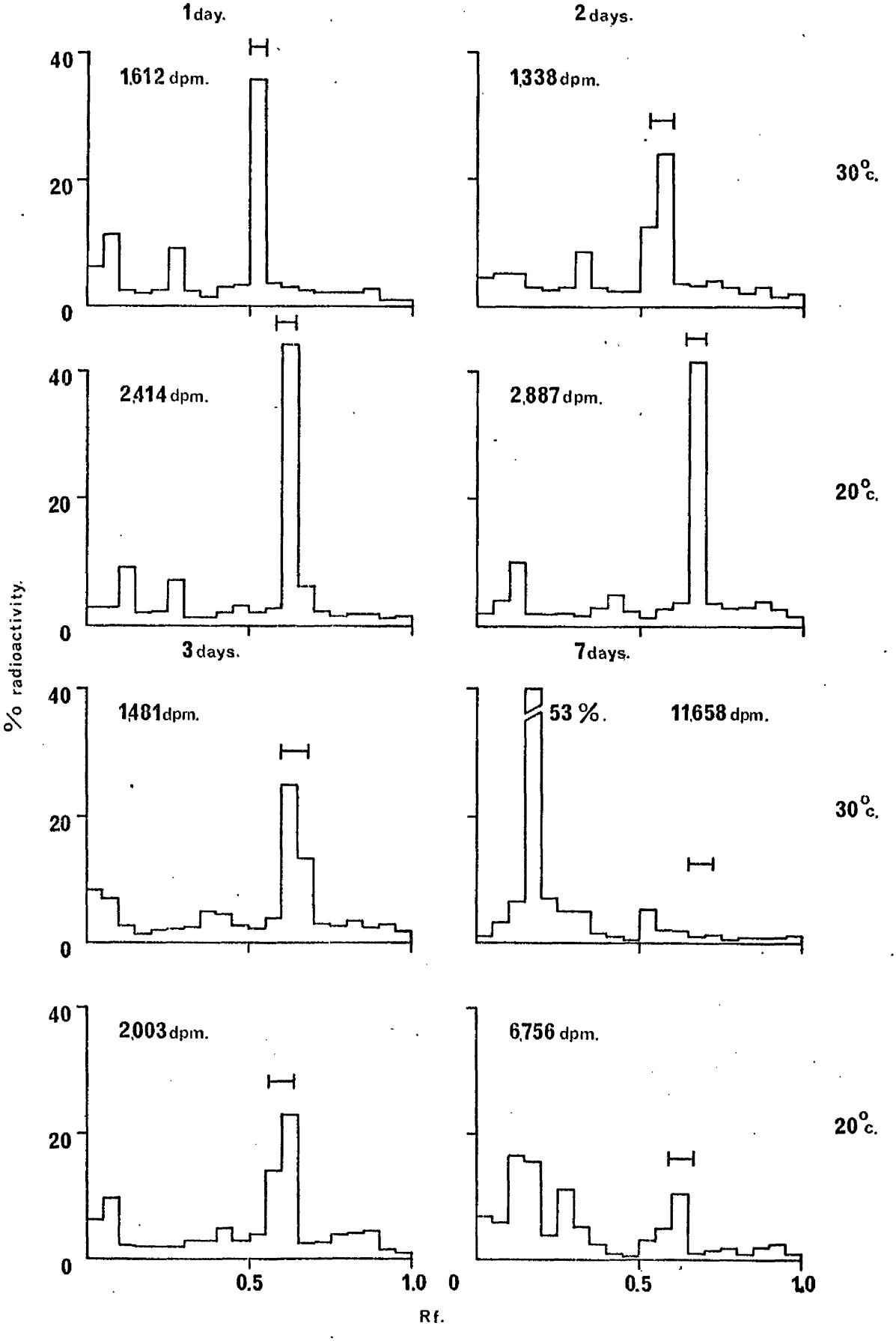

1 day.
2 days.
40
20
0
1,612 dpm.
1,338 dpm.
30°c.
2,414 dpm.
2,887 dpm.
20°c.
% radioactivity.
3 days.
7 days.
1,481 dpm.
53 %.
11,658 dpm.
30°c.
2,003 dpm.
6,756 dpm.
20°c.
0.5
1.0
0
Rf.

FIGURE 63

Chromatographic distribution of the radioactivity extracted by methanol from seeds after the following treatments: transfer from water to [^{14}C] ABA (10^{-4} M) after 6, 12 or 24 hrs imbibition at 20°C. Seeds were extracted in methanol 48 or 72 hrs after the start of imbibition. Plates developed in chloroform:methanol:water (75:22:3 v/v).

(1) extracted after 48 hrs

a transfer after 6 hrs
b " " 12 "
c " " 24 "

(2) extracted after 72 hrs

a transfer after 6 hrs
b " " 12 "
c " " 24 "

Residual activity (dpm)

(1)	a	208	(2)	a	286
	b	125		b	249
	c	218		c	356

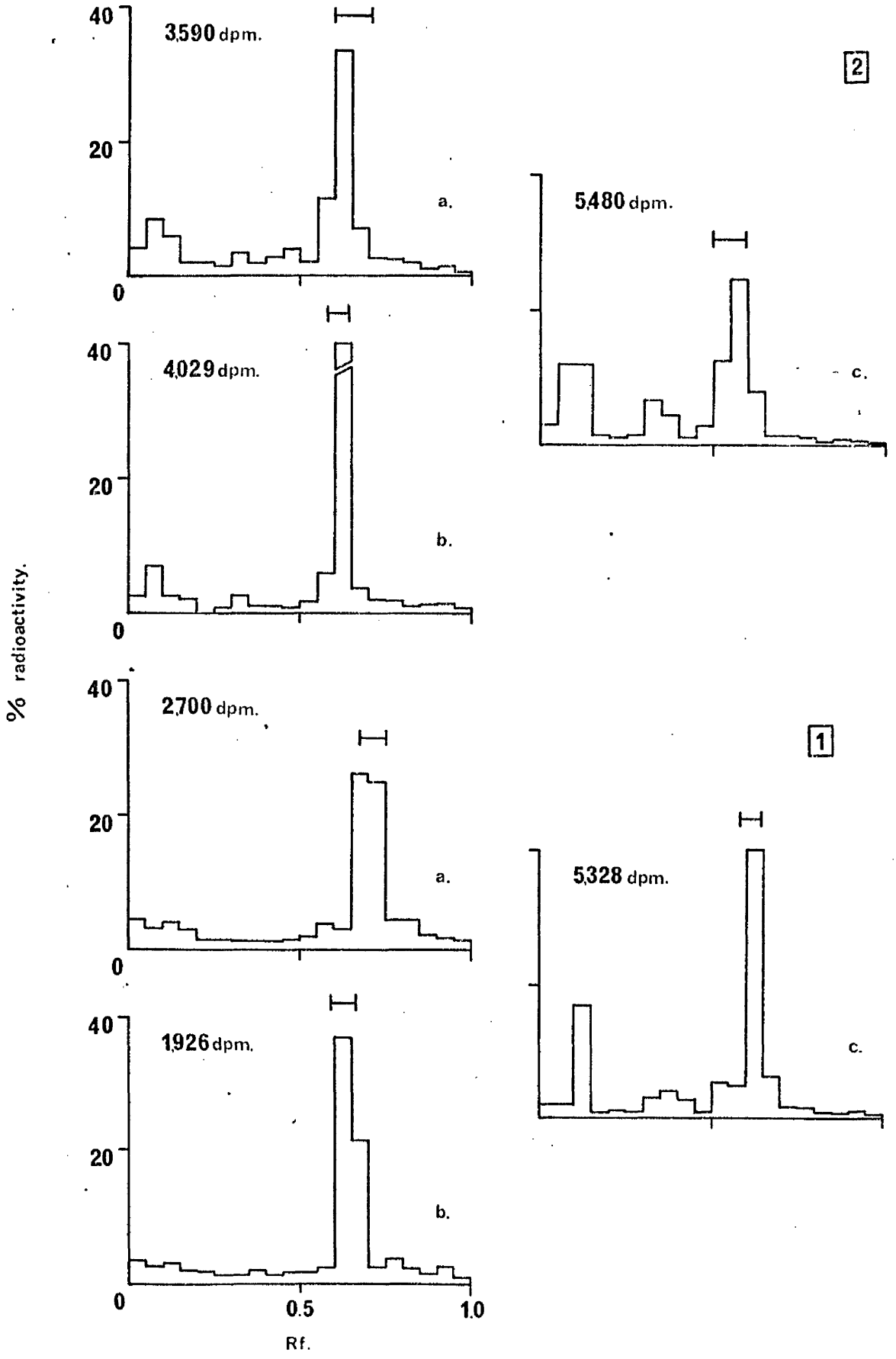

40
20
0
3,590 dpm.
a.
4,029 dpm.
b.
2,700 dpm.
a.
1,926 dpm.
b.
0.5
1.0
Rf.
% radioactivity.
2
5,480 dpm.
c.
1
5,328 dpm.
c.

FIGURE 64

Chromatographic distribution of the radioactivity extracted by methanol from seeds after imbibition in [^{14}C] ABA (10^{-6} M) at 20°C for 4 days. TLC plates developed in:

(A) chloroform:methanol:water (75:22:3 v/v)

(B) isopropanol:ethyl acetate:formic acid (50:40:10 v/v)

(C) n-butanol:acetic acid:water (5:1:2.2 v/v)

(D) methulene chloride:ethyl acetate:acetic acid (50:5:5 v/v)

In solvent system (C) the first bar represents the Rf for trans ABA, the second for cis, trans ABA.

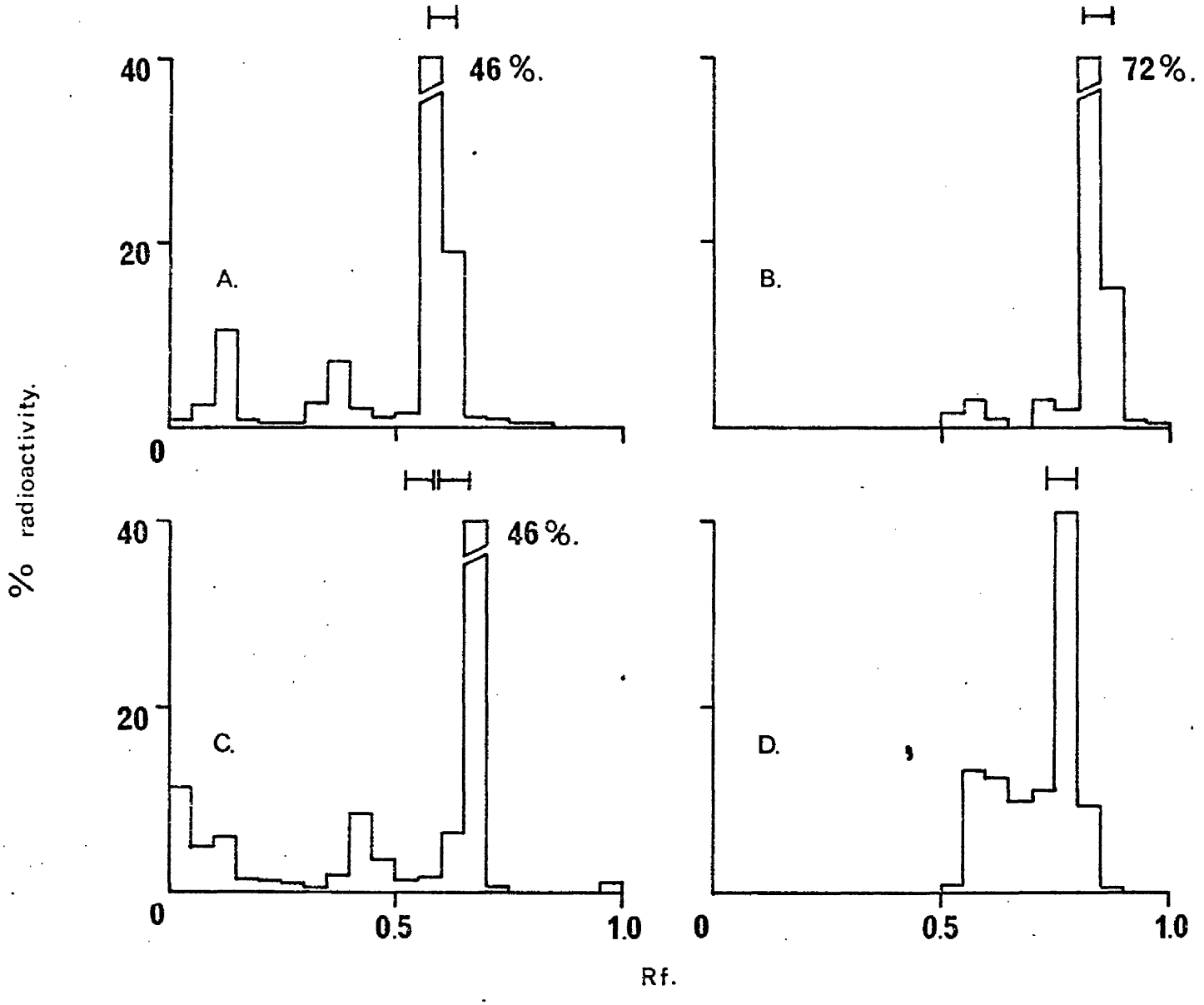

40
20
0
46 %.
A.
B.
72%.
40
20
0
46 %.
C.
D.
0
0.5
1.0
0
0.5
1.0
% radioactivity.
Rf.

FIGURE 65

Chromatographic distribution of [^{3}H] IAA standards. Plates developed in (A) chloroform:methanol:water (75:22:3 v/v) and (B) isopropanol:ethyl acetate:formic acid (50:40:10 v/v).

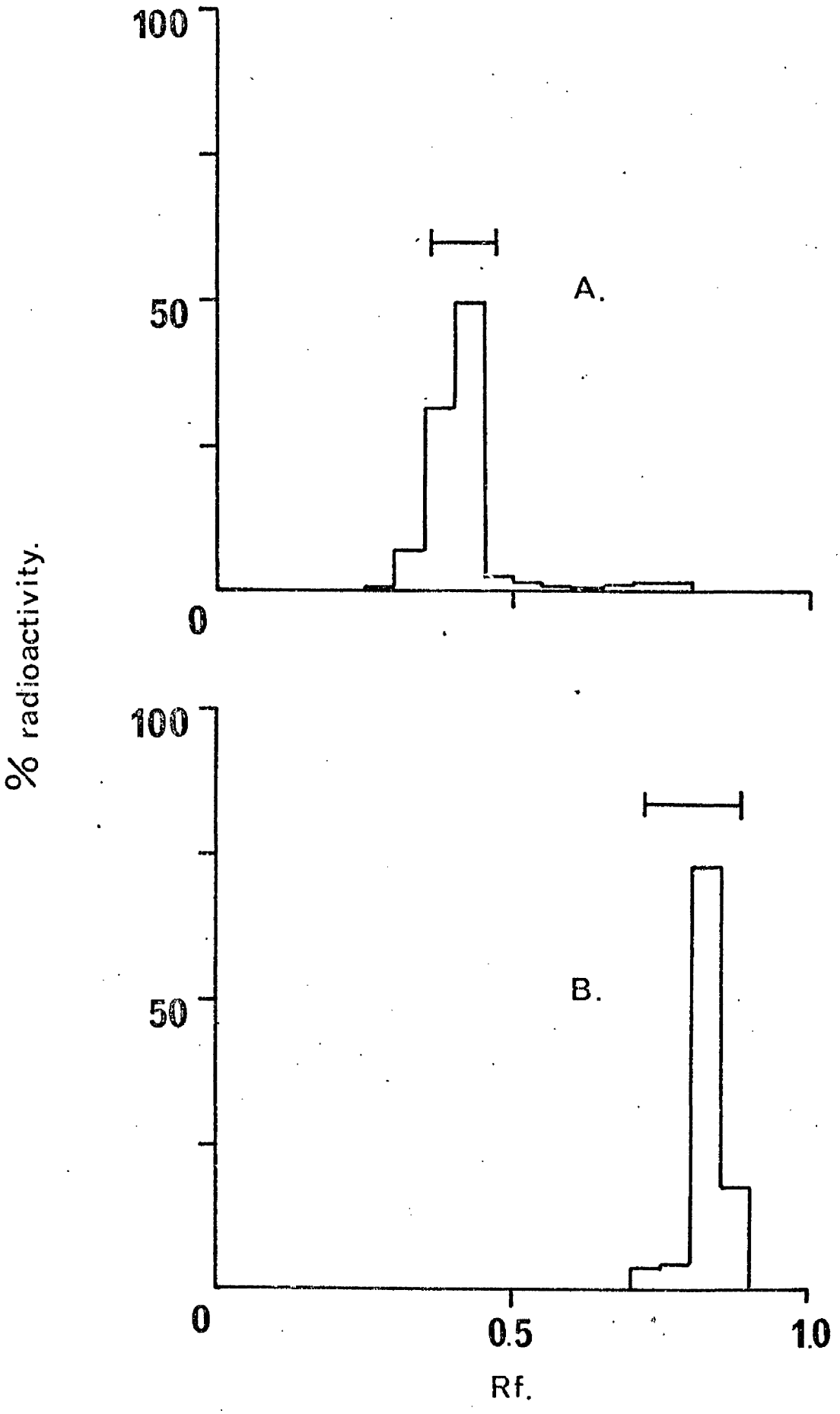

100
50
0
A.
100
50
B.
0
0.5
1.0
Rf.
% radioactivity.

FIGURE 66

Chromatographic distribution of radioactivity extracted by methanol from seeds after imbibition in [^{3}H] IAA at 20°C. Concentration of IAA solutions 10^{-6} M or 10^{-4} M. Plates developed in chloroform:methanol:water (75:22:3 v/v).

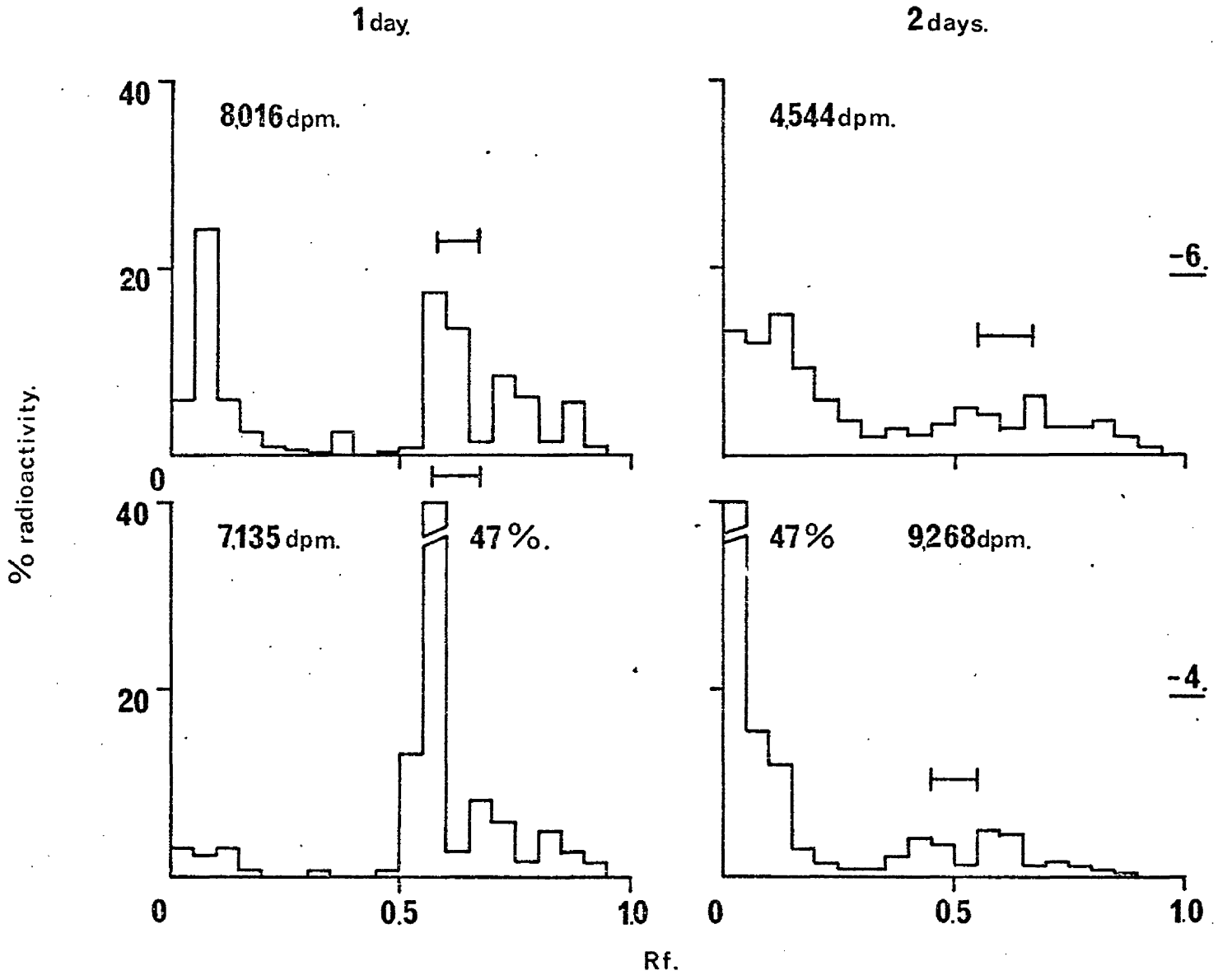

1 day.
2 days.
40
20
0
8,016 dpm.
4,544 dpm.
-6.
7,135 dpm.
47 %.
47 %
9,268 dpm.
-4.
0.5
1.0
% radioactivity.
Rf.

FIGURE 67

Chromatographic distribution of radioactivity, plates developed in chloroform:methanol:water (75:22:3 v/v). The treatments were as follows:

A methanol extract from whole seeds. 1 day at 20°C in [^{3}H] IAA 10^{-4} M.

B methanol extract from whole seeds. 2 days at 20°C in [^{3}H] IAA 10^{-4} M.

C methanol extract from macerated seeds of A.

D " " " " " " B.

E [^{3}H] IAA 10^{-4} M no seeds. 1 day.

F [^{3}H] IAA 10^{-4} M no seeds. 2 days.

Residual activity (DPM):

C 31,575.7

D 418,966

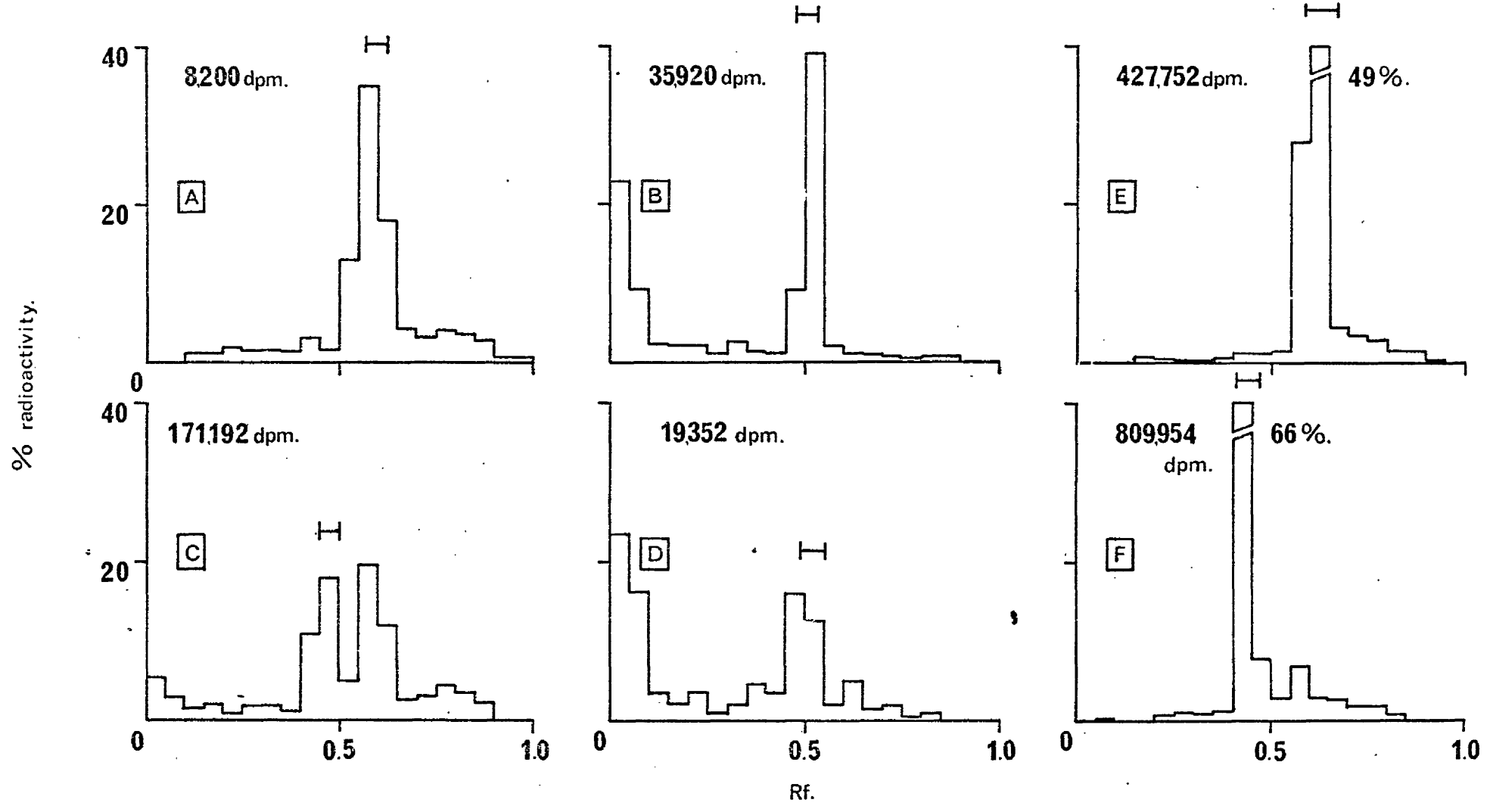

40
20
0
8,200 dpm.
A
35,920 dpm.
B
427,752 dpm.
49 %.
E
171,192 dpm.
C
19,352 dpm.
D
809,954 dpm.
66 %.
F
0.5
1.0
% radioactivity.
Rf.

FIGURE 68A

Chromatographic distribution of the radioactivity extracted by methanol from seeds imbibed in [^{3}H] IAA at 10^{-4} M at 20°C. Seeds were imbibed for 6, 12, 18, 24, 32, 40, 48 or 72 hrs. Plates were developed in two solvent systems, chloroform: methanol:water (75:22:3 v/v) solvent A, or isopropanol:ethyl acetate:formic acid (50:40:10 v/v), solvent B.

Residual activity (DPM):

6A	-
6B	26,096
12A	53,206
12B	55,996
18A	60,592
18B	41,968
24A	105,576
24B	79,133
32A	100,155
32B	129,995
40A	125,898
40B	113,339
48A	123,728
48B	105,979
72A	153,607
72B	157,025

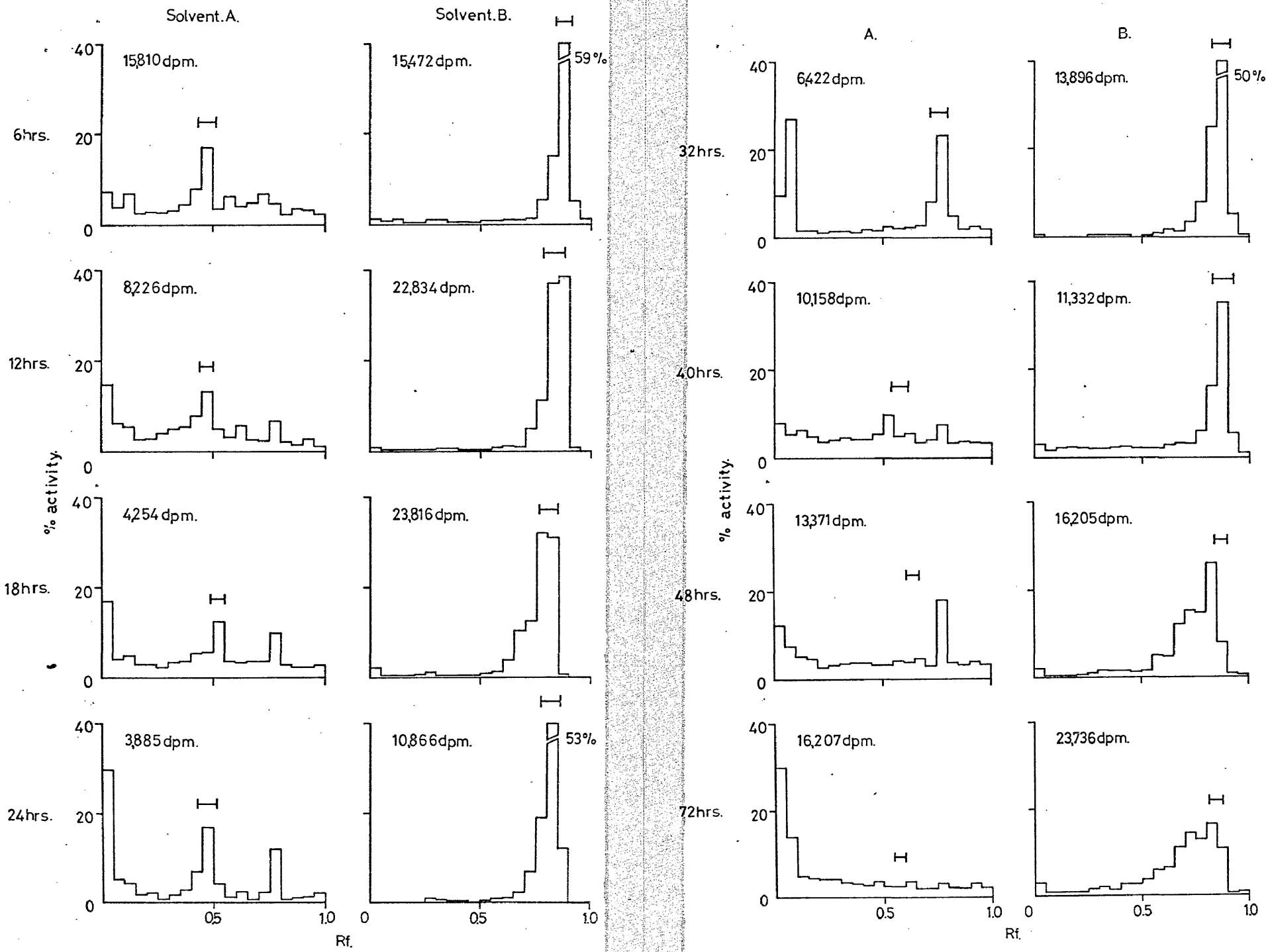

Solvent.A.
Solvent.B.
A.
B.
6hrs.
12hrs.
18hrs.
24hrs.
32hrs.
40hrs.
48hrs.
72hrs.
15,810dpm.
15,472dpm.
59%
6,422dpm.
13,896dpm.
50%
8,226dpm.
22,834dpm.
10,158dpm.
11,332dpm.
4,254dpm.
23,816dpm.
13,371dpm.
16,205dpm.
3,885dpm.
10,866dpm.
53%
16,207dpm.
23,736dpm.
% activity.
Rf.
0
20
40
0.5
1.0

FIGURE 68B

As in Figure 68A except using macerated seeds.

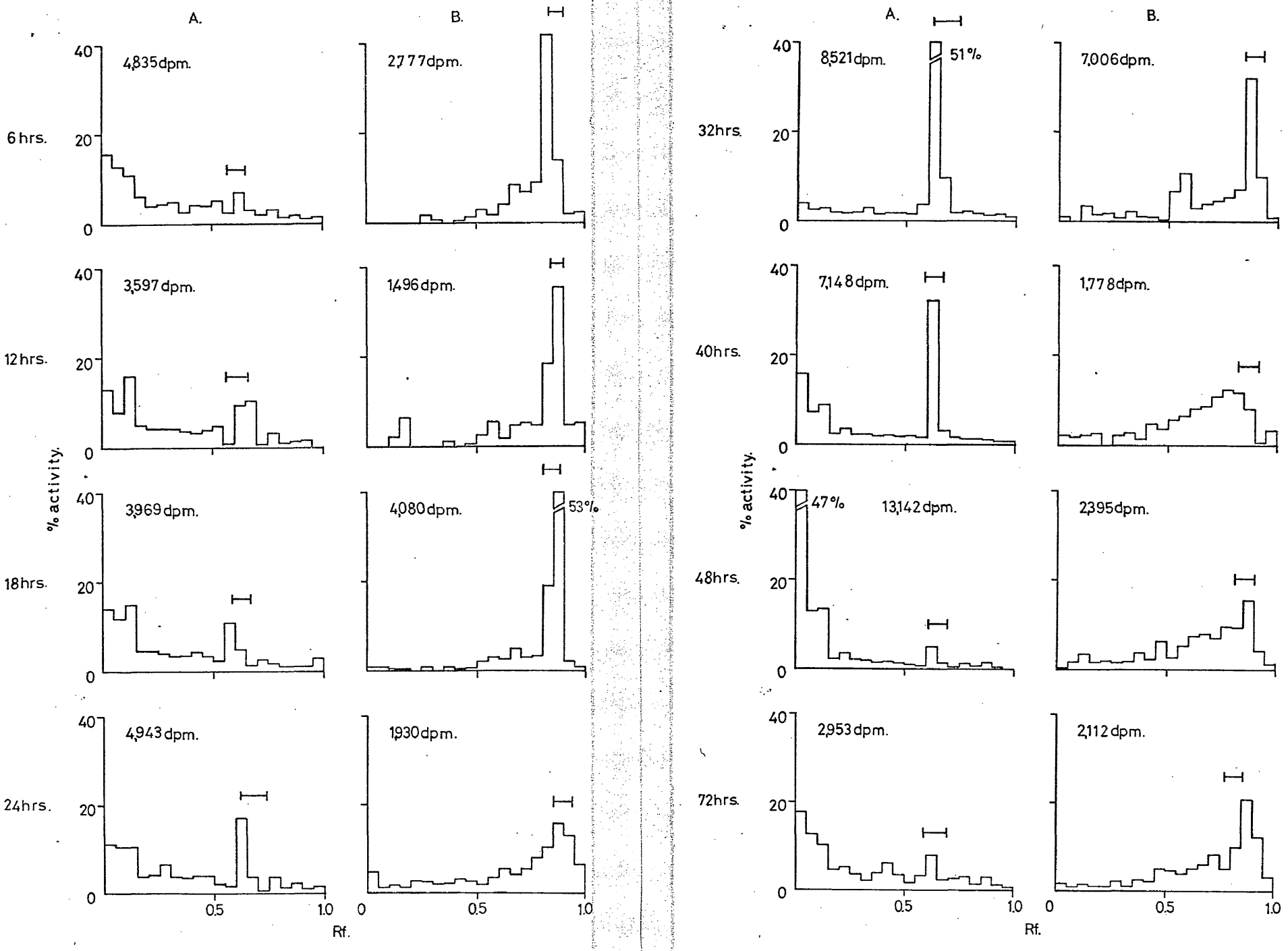

A.
B.
A.
B.
6 hrs.
12 hrs.
18 hrs.
24 hrs.
32 hrs.
40 hrs.
48 hrs.
72 hrs.
4,835 dpm.
2,777 dpm.
8,521 dpm.
51 %
7,006 dpm.
3,597 dpm.
1,496 dpm.
7,148 dpm.
1,778 dpm.
3,969 dpm.
4,080 dpm.
53 %
47 %
13,142 dpm.
2,395 dpm.
4,943 dpm.
1,930 dpm.
2,953 dpm.
2,112 dpm.
% activity.
Rf.
0
20
40
0.5
1.0

FIGURE 69A

As in Figure 68A : repeat of this experiment.

Residual activity (dpm)

6A	30,998
6B	30,680
12A	49,030
12B	53,149
18A	66,001
18B	65,928
24A	90,045
24B	72,744
32A	106,830
32B	100,229
40A	89,316
40B	120,903
48A	113,959
48B	93,452
72A	121,051
72B	117,628

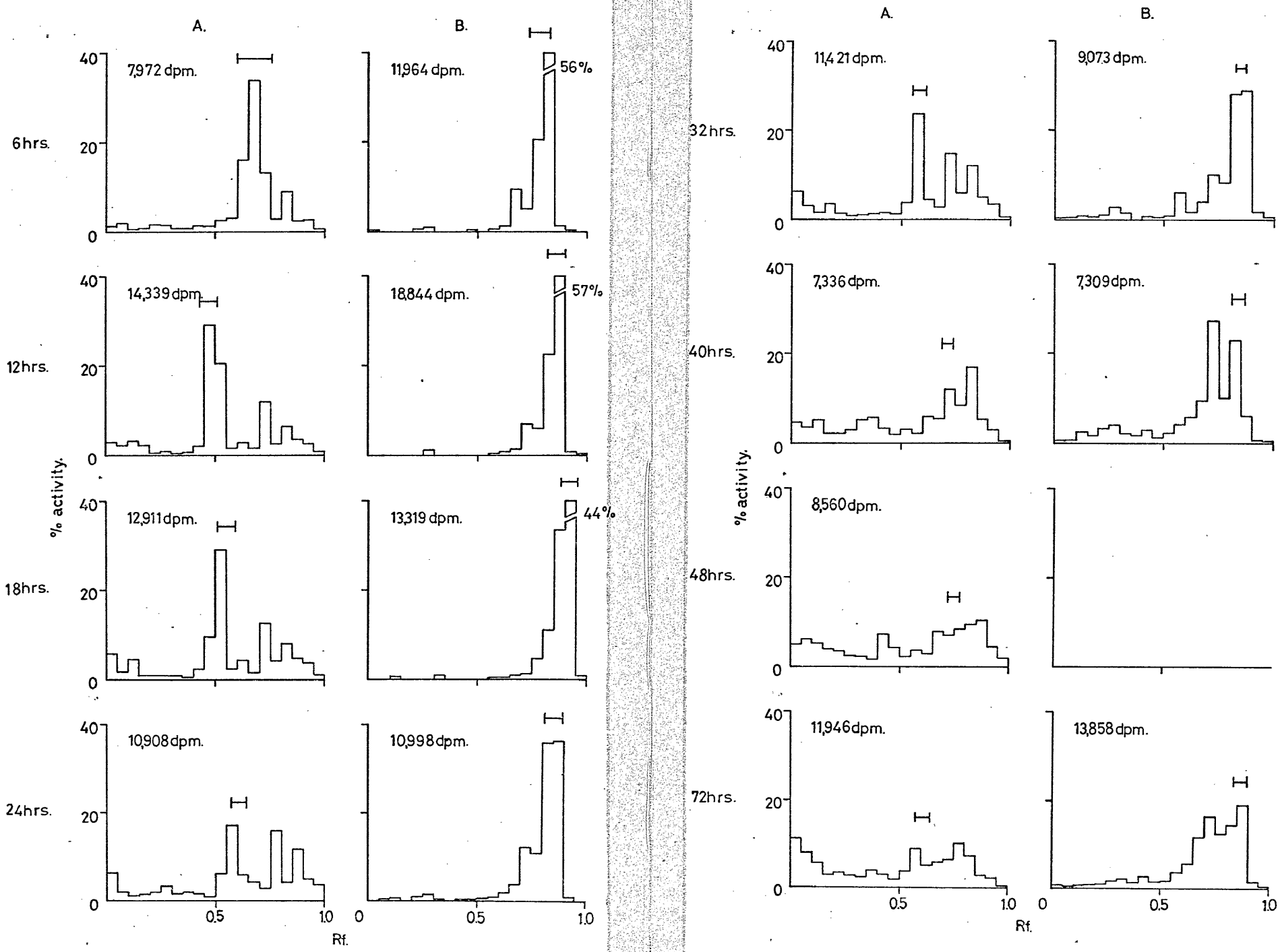

A.
B.
A.
B.
6hrs.
7,972 dpm.
11,964 dpm.
56%
32hrs.
11,421 dpm.
9,073 dpm.
12hrs.
14,339 dpm.
18,844 dpm.
57%
40hrs.
7,336 dpm.
7,309 dpm.
% activity.
18hrs.
12,911 dpm.
13,319 dpm.
44%
48hrs.
8,560 dpm.
24hrs.
10,908 dpm.
10,998 dpm.
72hrs.
11,946 dpm.
13,858 dpm.
40
20
0
0.5
1.0
Rf.

FIGURE 69B

As in Figure 68B.

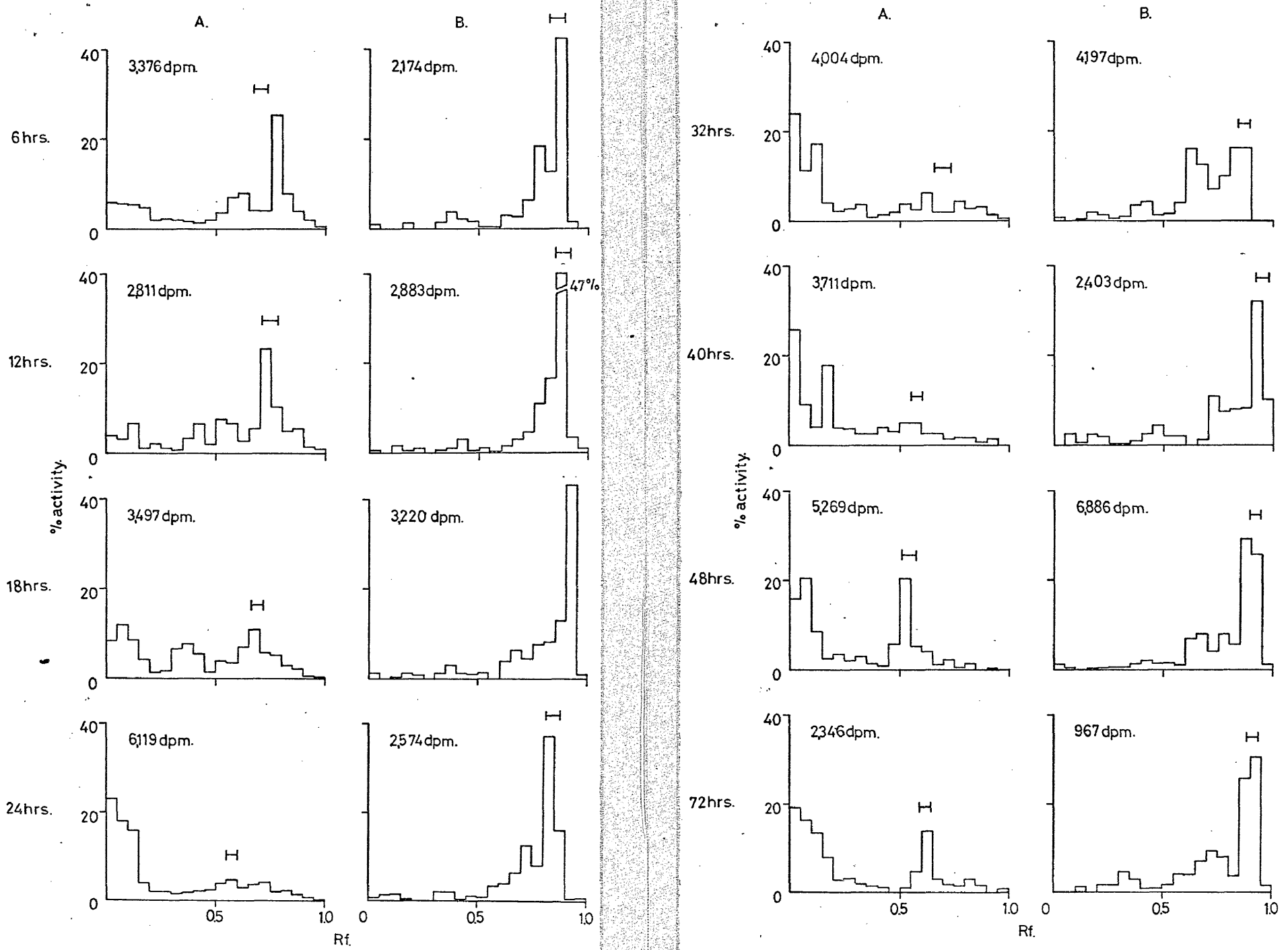

A.
B.
6hrs.
12hrs.
18hrs.
24hrs.
32hrs.
40hrs.
48hrs.
72hrs.
3,376dpm.
2,174dpm.
2,811dpm.
2,883dpm.
47%
3,497dpm.
3,220dpm.
6,119dpm.
2,574dpm.
4,004dpm.
4,197dpm.
3,711dpm.
2,403dpm.
5,269dpm.
6,886dpm.
2,346dpm.
967dpm.
% activity.
Rf.
0
20
40
0.5
1.0

FIGURE 70

Chromatographic distribution of radioactivity extracted by methanol from seeds imbibed in [^{3}H] IAA (10^{-5}M or 10^{-4}M) at 30°C. Plates developed in chloroform:methanol:water (75:22:3 v/v).

Residual activity (dpm)

10^{-4} M IAA	24 hrs.	97,461
	48 "	128,765
	72 "	88,162
10^{-5} M IAA	24 hrs	82,806
	48 "	284,955
	72 "	398,567

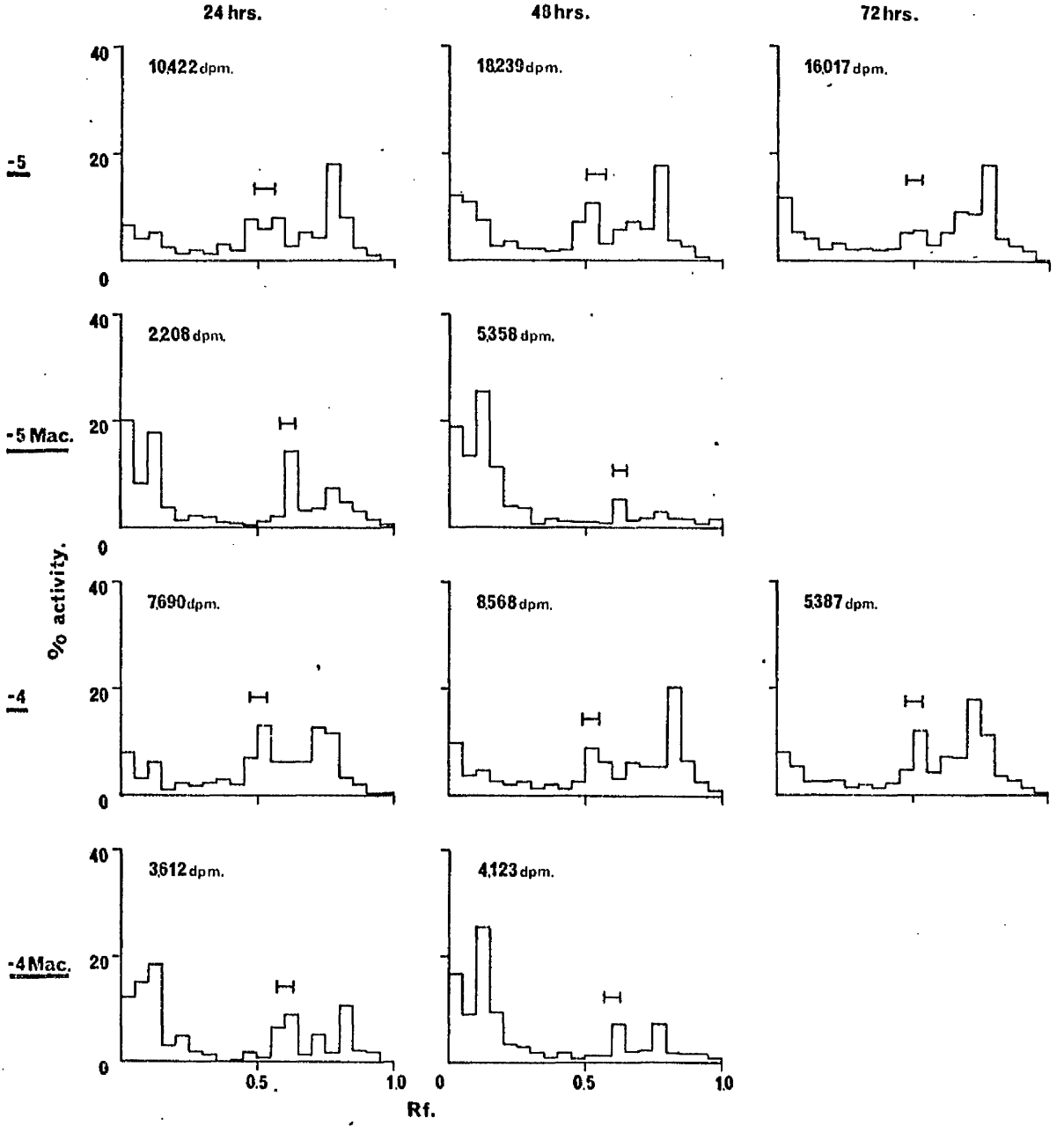

24 hrs.
48 hrs.
72 hrs.
-5
-5 Mac.
-4
-4 Mac.
% activity.
10,422 dpm.
18,239 dpm.
16,017 dpm.
2,208 dpm.
5,358 dpm.
7,690 dpm.
8,568 dpm.
5,387 dpm.
3,612 dpm.
4,123 dpm.
40
20
0
0.5
1.0
Rf.

FIGURE 71

Chromatographic distribution of radioactivity extracted by methanol from seeds, washed with chloros, and imbibed in 10^{-4} M [^{3}H] IAA at 20°C. Plates developed in chloroform:methanol:water (75:22:3 v/v).

Residual activity (dpm)

48 hrs	84,335
72 "	110,809

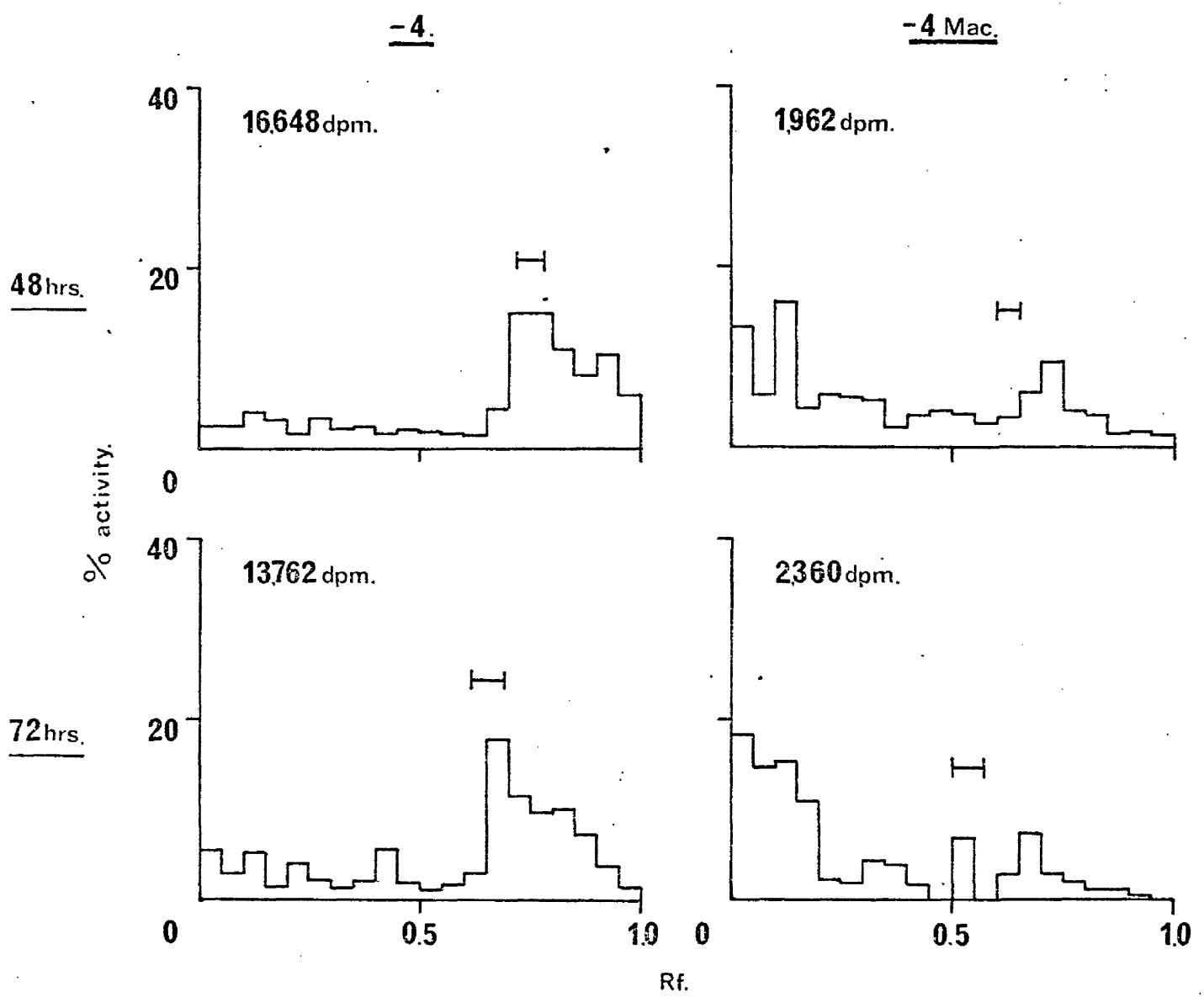

-4.
-4 Mac.
48hrs.
72hrs.
16,648dpm.
1,962dpm.
13,762dpm.
2,360dpm.
40
20
0
% activity.
0.5
1.0
Rf.

FIGURE 72

Chromatographic distribution of radioactivity extracted by methanol from seeds, killed by heat treatment, and imbibed in 10^{-5} M or 10^{-4} M [^{3}H] IAA at 20°C. Plates developed in chloroform:methanol:water (75:22:3 v/v).

Residual activity (dpm)

10^{-5} M	24 hrs	56,904
	48 "	34,131
10^{-4} M	24 "	15,754
	48 "	49,347
	72 "	121,840

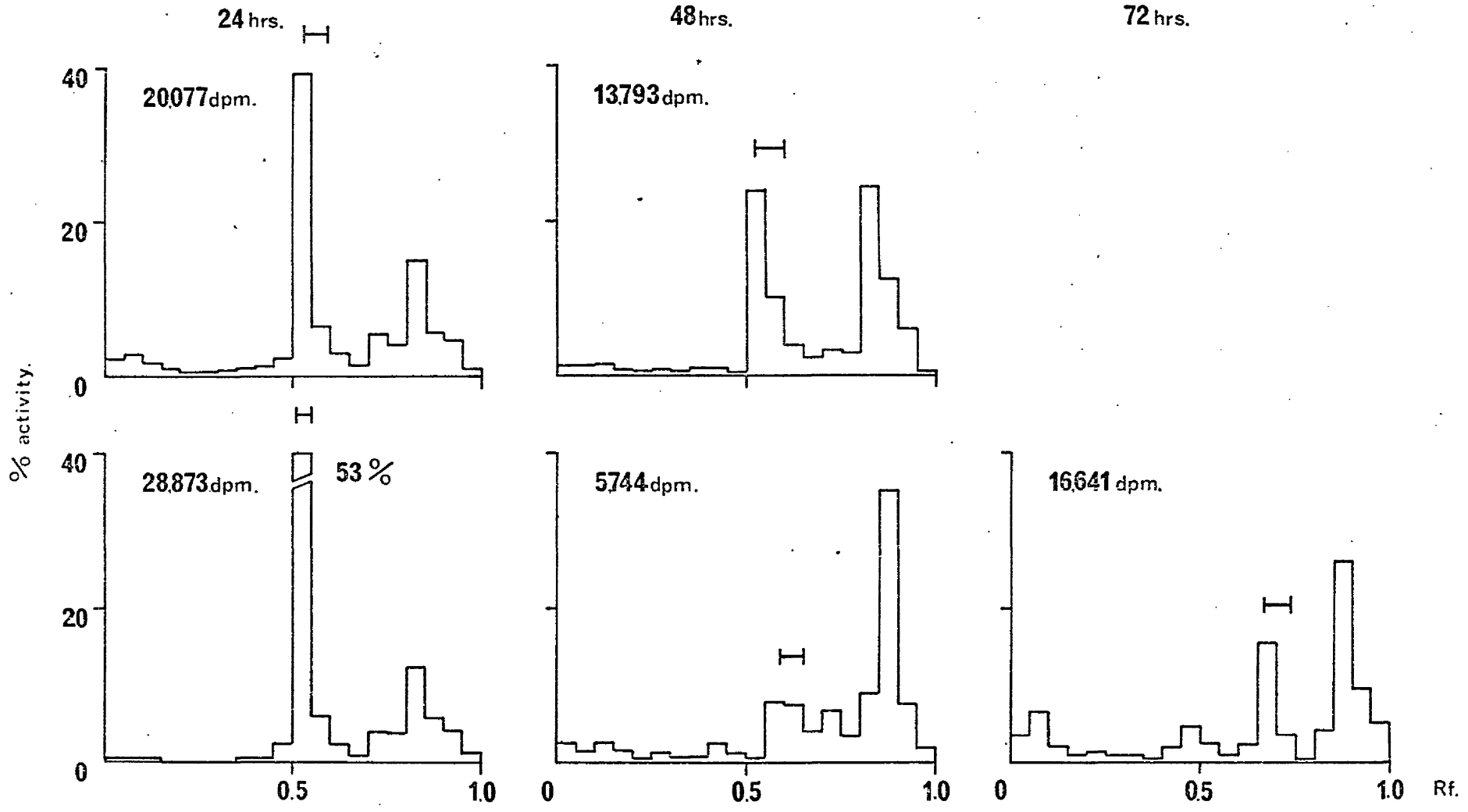
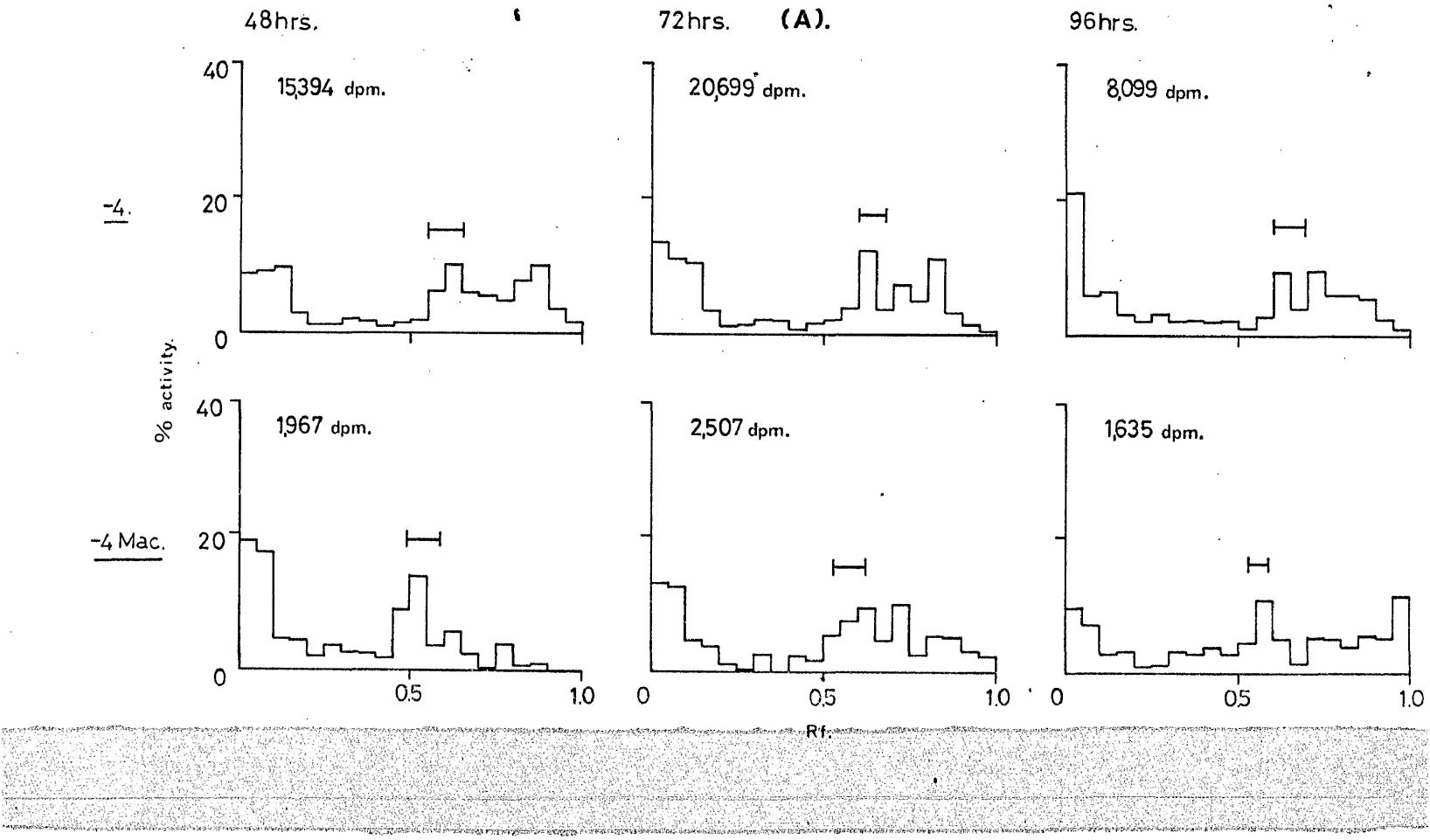

24 hrs.
48 hrs.
72 hrs.
-5
-4
% activity.
40
20
0
20,077 dpm.
13,793 dpm.
28,873 dpm.
53 %
5,744 dpm.
16,641 dpm.
0.5
1.0
Rf.

FIGURE 73

Chromatographic distribution of radioactivity extracted by methanol from seeds imbibed in 10^{-4} M [^{3}H] IAA at 20°C or 30°C and given red light. 20°C : 5 minutes red light. 30°C : 10 minutes red light. Plates developed in chloroform:methanol:water (75:22:3 v/v).

Residual activity (DPM)

A	20°C	48 hrs	162,035	B	30°C	48 hrs	133,858
		72 "	228,659			72 "	284,721
		96 "	503,628			96 "	196,253


48hrs.
72hrs.
(A).
96hrs.
15,394 dpm.
20,699 dpm.
8,099 dpm.
-4.
1,967 dpm.
2,507 dpm.
1,635 dpm.
-4 Mac.
% activity.
40
20
0
0.5
1.0
Rf.

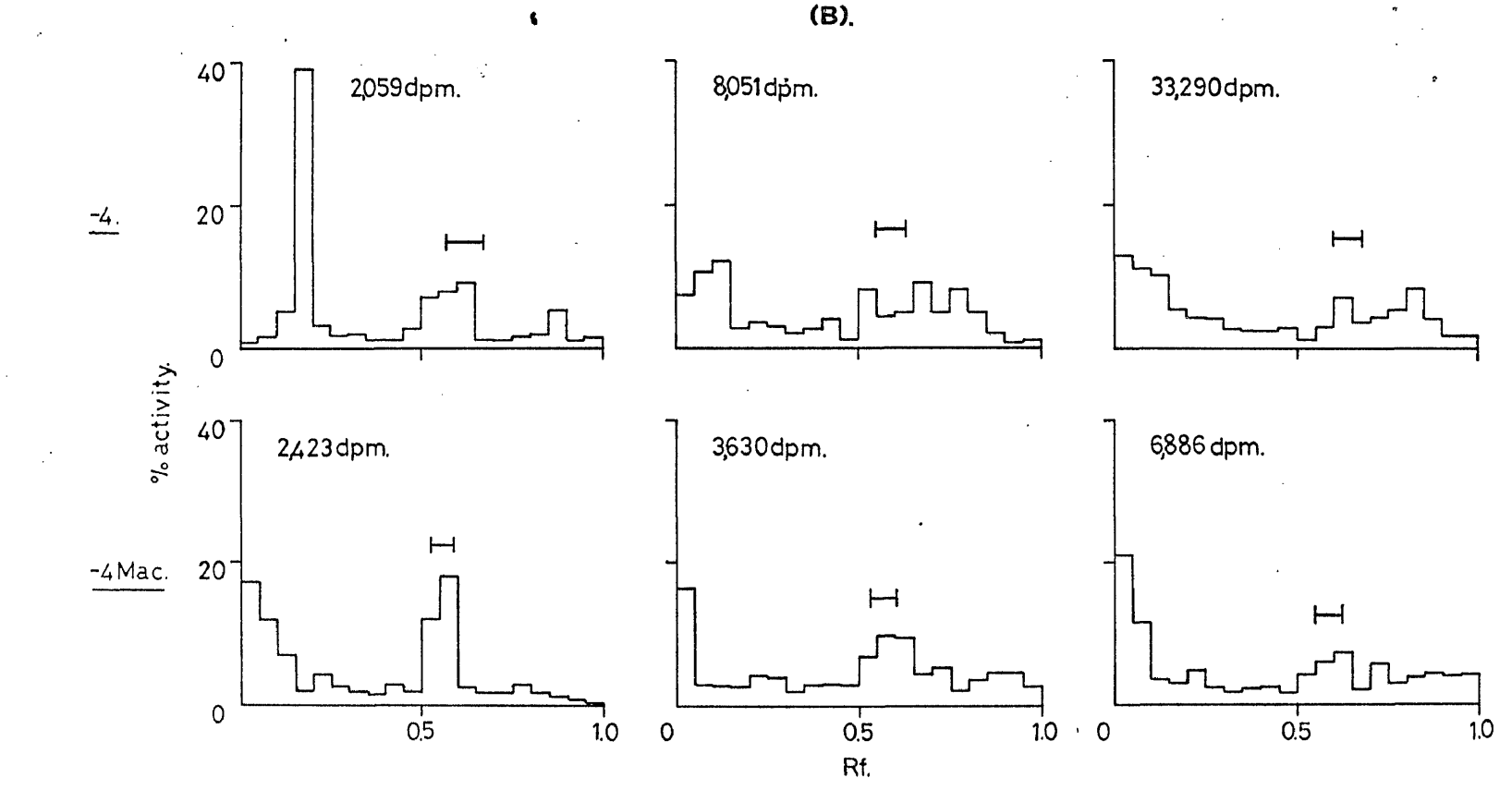

(B).
2,059dpm.
8,051dpm.
33,290dpm.
-4.
2,423dpm.
3,630dpm.
6,886 dpm.
-4Mac.
% activity.
40
20
0
0.5
1.0
Rf.

FIGURE 74

Chromatographic distribution of radioactivity extracted by methanol from seeds imbibed in water and transferred to 10^{-4}M [^{3}H] IAA after 6, 12 or 24 hrs at 20°C. Seeds extracted after 48 hrs. Plates developed in chloroform: methanol:water (75:22:3 v/v).

Residual activity (dpm)

6 hrs	151,316
12 "	115,408
24 "	104,808

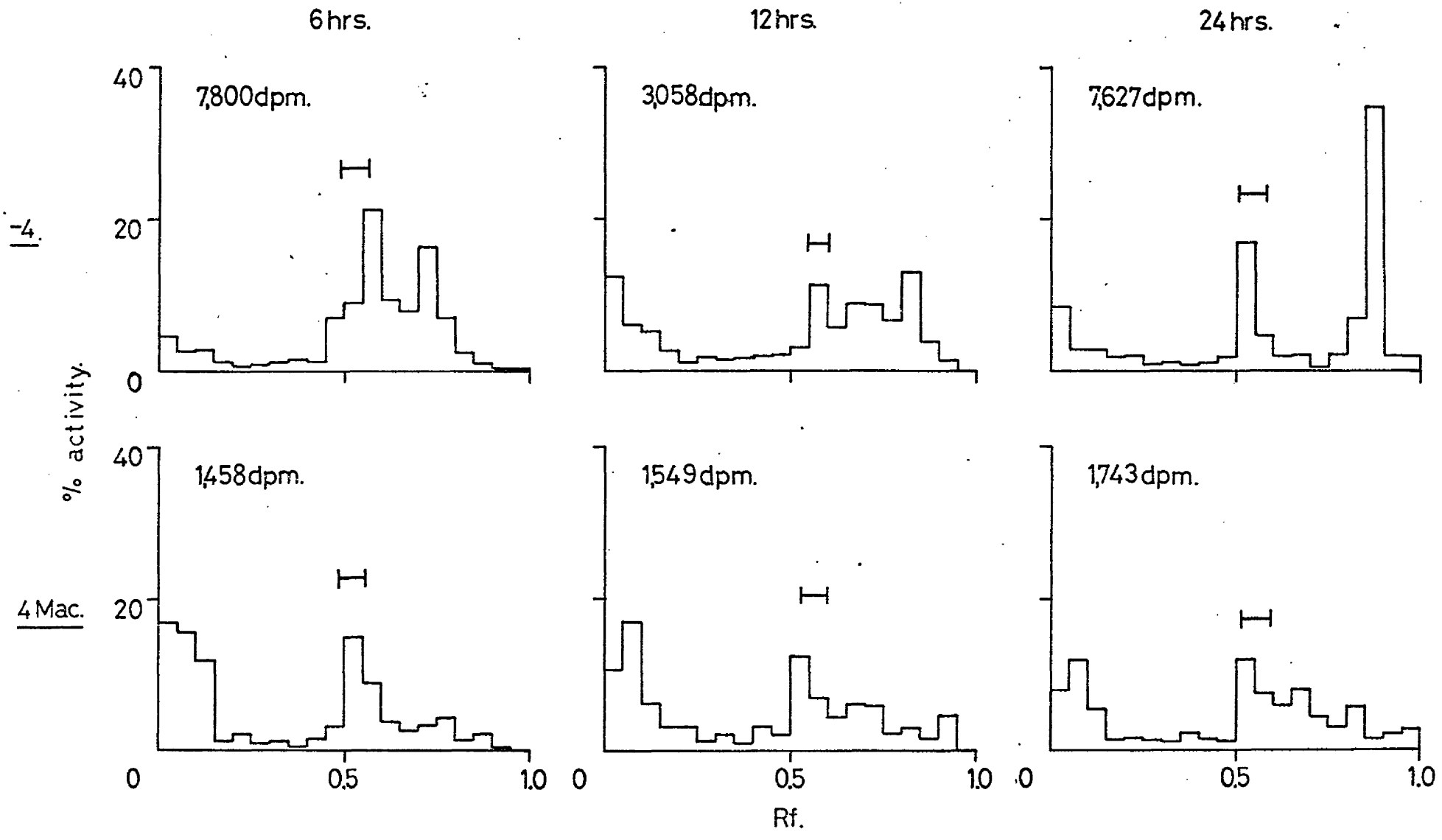

6 hrs.
12 hrs.
24 hrs.
7,800 dpm.
3,058 dpm.
7,627 dpm.
1,458 dpm.
1,549 dpm.
1,743 dpm.
-4.
4 Mac.
% activity.
40
20
0
0.5
1.0
Rf.

FIGURE 75

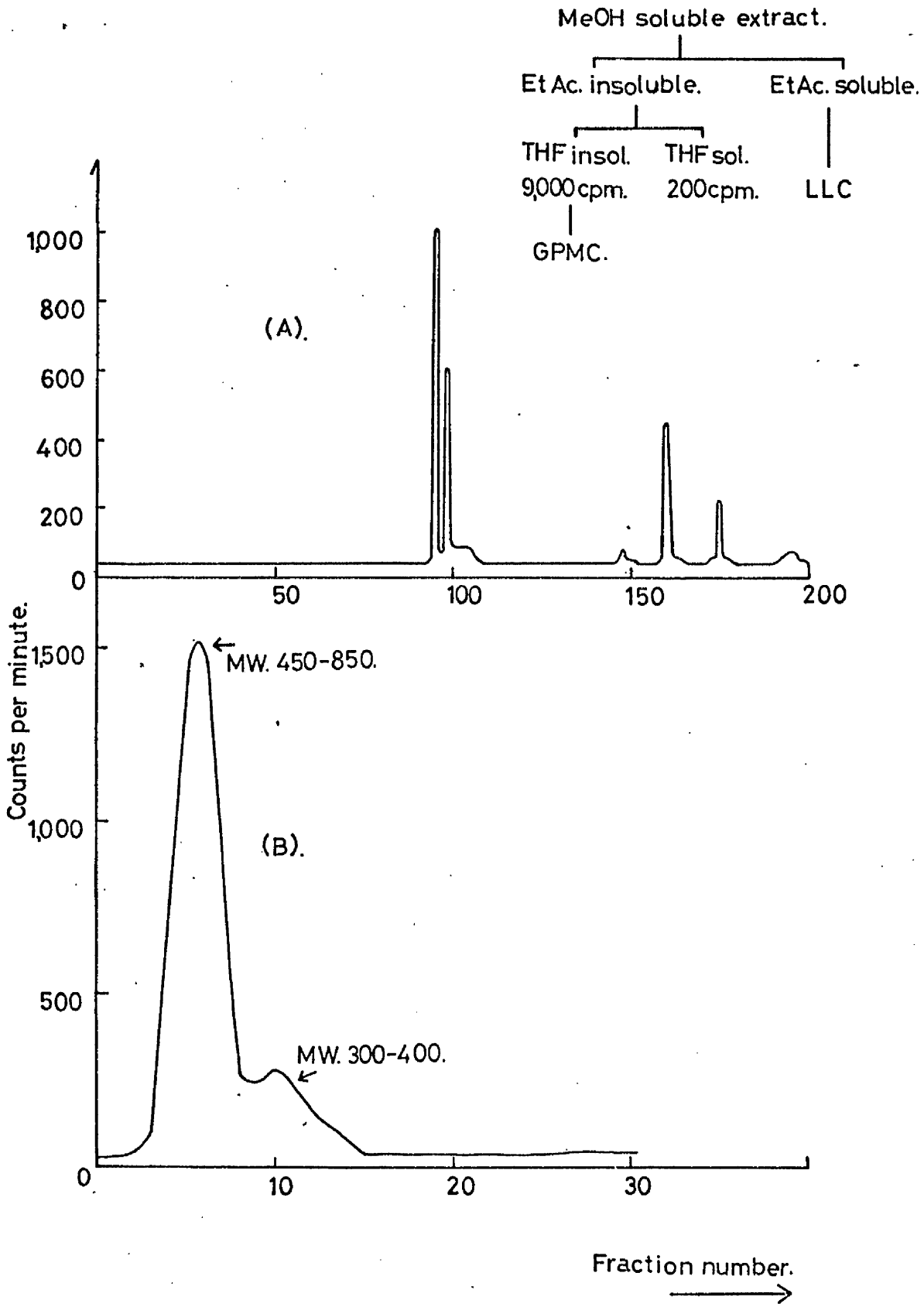
Chromatographic distribution of radioactivity extracted by methanol from seeds of Grand Rapids lettuce imbibed in [^{14}C] ABA, 10^{-6} M, at 20°C for 96 hrs. Methanolic extract chromatogramed using:

(A) liquid liquid partition column chromatography LLC.

(B) gel permeation chromatography GPMC.

Et Ac : ethyl acetate.

THF : tetrahydrofuran.


MeOH soluble extract.
Et Ac. insoluble.
Et Ac. soluble.
THF insol.
9,000 cpm.
THF sol.
200 cpm.
LLC
GPMC.
(A).
1,000
800
600
400
200
0
50
100
150
200
Counts per minute.
1,500
1,000
500
0
MW. 450-850.
(B).
MW. 300-400.
10
20
30
Fraction number.

SECTION IV

GENERAL DISCUSSION AND CONCLUSIONS

An attempt was made in the introduction to present an integrated, overall picture of the present state of knowledge with respect to the germination and thermodormancy found in seeds of Grand Rapids lettuce. Several factors were shown to have probable roles in the imposition and release from thermodormancy and their actions may be intertwined in a web which although apparently confused, is in fact a highly organised structure. The introduction to this thesis opened with a quotation -

> "the obvious truth is that the moment any matter has passed through the human mind it is finally and for ever spoilt for all purposes of science. It has become a thing incurably mysterious and infinite ..."

Clearly Chesterton implies that much of the confusion in understanding scientific events lies not in the events themselves, but in the approach used by the human investigator. The aim of this study has been to examine a few selected points in an effort to untangle a little of the 'web'.

Studies on seed germination may be carried out in several ways. We may examine the endogenous hormones of the seeds and attempt to correlate changes with physiological events, or we may apply hormones or growth regulating compounds to seeds and study their effects, or we may study the metabolism by the seeds of exogenous hormones. All three approaches have been used in this study.

The results of each section have been discussed and it remains to try to draw some conclusions. In Section I the endogenous gibberellin and cytokinin contents of lettuce seeds were examined, but this work could not be continued because of the difficulties in looking at unidentified compounds and the lack of a specific cytokinin bioassay. In an attempt to overcome this problem use was made of growth retardants, thought specifically to inhibit the biosynthesis of gibberellins. It was hoped to examine the possibility that red light promoted germination by virtue of its action on gibberellin biosynthesis. However, the results are interpreted as showing that these growth retardants may not be specific inhibitors of gibberellin biosynthesis, but owe their inhibitory action to some other property. Unlike the case with gibberellins and cytokinins, lettuce seeds cannot 'escape' from the inhibitory action of the growth retardants. In other words lettuce seeds will not germinate if kept in the presence of the retardants.

From the literature it was clear that no single comprehensive study had been carried out to investigate the interaction of gibberellins, cytokinins, red light and ABA on the germination of seeds of Grand Rapids lettuce. Khan (1971) believes that the results obtained by exogenous hormone studies may be an accurate reflection of the endogenous control system. It has also been proposed that abscisic acid might be an inhibitor with a fundamental role in the control of thermodormancy. Our studies did not confirm the previous reports in the literature inasmuch as we found that ABA inhibition of germination could be reversed, under most conditions by cytokinins or gibberellins alone. Furthermore red light could overcome ABA on its own, except where there was the dual inhibitory forces of temperature and high concentrations of ABA. The effect of red light was not to produce a less active form of ABA and it was reversed by far-red light. Thus, ABA does not remove or alter phytochrome action in lettuce seeds. Clearly, our results would not have supported the erection of Khan's hypothesis, that cytokinins play a 'permissive' role, allowing the primary germination - promoting agent, gibberellin, to act. Neither do our results destroy such an hypothesis. What we can say is that 'exogenous' cytokinin can alone overcome ABA-induced dormancy, no more! Do exogenous studies really yield an accurate picture of the endogenous control situation ? Obviously, until the endogenous picture has been more fully investigated this question cannot be answered. However, it does not seem possible that the application of large amounts of growth regulator to seeds can reveal the probable subtlety of control which seems to exist. Furthermore, surely these massive doses of exogenous compounds must override or alter the natural control mechanisms. We can propose that all that is really being studied when we conduct this type of experiment, is the interaction of an inhibitory agent and a promotive agent. If an inhibitory agent is applied, any promotive agent will overcome the inhibition. This does not mean that the growth regulators need necessarily have the same mode of action, or even affect the same part of the seed. There is good evidence that cytokinins and gibberellins can affect different parts of the seed. We have also presented evidence that cytokinins and gibberellins act at different times in overcoming both ABA and IAA inhibition.

It is our opinion that exogenous application of growth regulators will not reveal the subtle endogenous control mechanisms involved and that the value of such studies lies not in understanding the control processes, but in manipulating the germination behaviour of seeds. Grand Rapids lettuce seeds are not unique; there are many species which have small seeds showing photosensitive responses. Thus any knowledge gained using seeds of Grand Rapids lettuce may be applicable to these other species, many of which are important weeds. Manipulation of the germination pattern of such seeds is of immense agricultural importance.

One area in which exogenous application of growth regulators might be of value, would be the application of specific compounds known to inhibit the biosynthesis or the action of particular plant hormones. Use of such a compound would enable the involvement of plant hormones to be studied, although again the possibility should be borne in mind that the control mechanism involved may be flexible. If the production or action of, for instance, gibberellins is stopped, the seeds might compensate by producing more cytokinin. Even so, the only specific set of compounds known to date are the growth retardants, and it is now obvious that in whole plants we must question the mode of action of these substances. Only recently Kuo and Pharis (1975) have provided further evidence that B995 leads to increased levels of gibberellins in seedlings of Cupressus arizonica. Although various interpretations of the data are possible, the very least we can say is that there is some doubt as to how growth retardants act.

It is obvious that the use of exogenous studies is of dubious value in assessing the role of plant hormones in the control of thermodormancy. However, use of these techniques has been made to examine whether ABA owes its inhibitory action to an effect on germination or on the visible manifestation of germination, radicle emergence. Unlike seeds of Chenopodium album (Karssen, 1968) lettuce seeds do not have a two stage germination where it is possible to distinguish between an effect on germination or radicle growth. By transferring seeds from water to ABA it was possible to study at what time the seeds escaped from the inhibitory action of ABA. The evidence suggests that ABA acts only during the first 6-10 hrs which can be interpreted as showing ABA to affect seed germination. At the same time ABA does inhibit the rate of subsequent radicle growth (McWha, 1973). We next investigated the endogenous ABA content of lettuce seeds at temperatures where seeds would germinate or show

thermodormancy. Both the levels of 'free' and 'bound', presumed to be the glucosyl ester of ABA, were studied. However, it was not found possible to correlate changes in endogenous levels with germination behaviour. There did not seem to be a conversion of free to bound ABA which it is proposed to be an inactive form, in response to temperature. Furthermore, a factor which clearly emerged from this work was the variation between different seed batches. This highlights the problems associated with erecting a hypothesis based on results from one set of experiments on one batch. Although both 'free' and 'bound' ABA have been shown to occur in Grand Rapids lettuce seeds the evidence presented makes it difficult to see how endogenous ABA could be involved in the imposition and release from thermodormancy. However, in this work large quantities of whole seeds were extracted. It is possible that ABA may function by virtue of its localization within the individual seeds. In the introduction this point was fully discussed and it is difficult to see how a satisfactory answer to this question can be achieved. The use of compounds which affect membrane permeability could be of interest, but interpretation of results obtained from such studies would be difficult. The effects of such compounds might be too general.

In an attempt to resolve questions regarding the role of ABA, metabolism of exogenous radioactive ABA was studied. It was observed in the earlier work with exogenous ABA that even in the presence of ABA, seeds will eventually germinate. Is this germination the result or cause of ABA metabolism ? The results of the metabolism studies showed the presence of two radioactive zones as well as a zone corresponding to the Rf of ABA. These have been tentatively identified as conjugated forms of ABA, at Rfs 0.00-0.15, and phaseic acid or dihydrophaseic acid. It was shown that the metabolism of ABA took place at a low level even when seeds do not germinate, but when seeds germinate the degree of metabolism is much greater and was not associated with the 'release' from dormancy. Further experiments with red light and transfer experiments indicated that metabolism is due to germination taking place.

From these different lines of attack it seems fair to suggest that the degradation of ABA does not appear to be involved in the release or imposition of thermodormancy. ABA may be the endogenous

inhibitor of germination at temperatures where seeds are thermodormant, but if this is the case it must be as a result of some form of compartmentation. Thus, we may consider that the role of ABA is not fundamental in so far as the events leading to germination are promotive events.

A second hormone which was investigated was IAA. This compound was chosen because of the apparent lack of any previous investigations to examine IAA for a role in the control of thermodormancy in lettuce seeds. As with ABA three experimental approaches were used to investigate this problem. From exogenous studies we showed that IAA was an inhibitor of seed germination, although seeds would eventually germinate even in the presence of it. Transfer experiments failed to determine unequivocally whether IAA inhibits germination or radicle emergence. Furthermore, the possibility that IAA might act by releasing hydrogen ions which would then acidify the imbibition medium resulting in an acid effect on radicle growth, could not be investigated. Weak phosphate buffers, in general use in biological research, were found to inhibit germination. The inhibition caused by IAA at 20°C or 30°C could be overcome by exogenous gibberellin or cytokinin, alone and in combination. Again the action of cytokinins and gibberellins was not at the same time, but here, as opposed to ABA, gibberellins elicited an earlier response than cytokinins. This suggests that cytokinins probably act on the same processes as ABA, or at least on events closer in time to those inhibited by ABA. In the seed there may be sequential action of endogenous hormones. Red light also overcame IAA inhibition and this was reversed by far-red light. Thus, as with ABA, we could evoke IAA as the inhibitor which imposes thermodormancy. However, if we examine endogenous IAA it is clear that no correlations can be made with germination behaviour and endogenous concentrations. The difficulties in quantifying, what are extremely low levels of hormone, preclude any categorical statement about the involvement of IAA, but in one seed batch there seemed to be no difference in the pattern of change at 20°C or 30°C. Furthermore, studies on the metabolism of radioactive IAA showed that although such metabolism required the presence of seeds, it could not be considered biological. Labelled IAA was converted into compounds with chromatographic properties less and more polar than IAA. Considerable metabolism did take place and this could explain why seeds were able to germinate at 20°C even in the presence of IAA solutions. Metabolism could be

observed prior to radicle emergence unlike ABA where the metabolism seemed to be the result of germination. However, IAA was metabolised whether the seeds germinated or not, which would certainly support the proposal that IAA is not involved in germination. As with ABA we cannot rule out the possibility that IAA acts by virtue of its localization. When large amounts of IAA are added to the seeds we can propose that the IAA will be in contact with enzymes which rapidly metabolise it. At 30°C, where the seeds are thermodormant the endogenous IAA and the enzymes involved in its metabolism may be spatially and physically separated. If we propose that red light overcomes dormancy by releasing these enzymes, as was suggested in the introduction, it is conceivable that the IAA would then be removed by rapid metabolism. We have shown that red light overcomes IAA inhibition of germination; however, the effect of red light on the metabolism of radioactive IAA is not striking.

We might have expected that the release of endogenous IAA would lead to a larger pool of IAA in the seeds; the enzymes involved in the metabolism of IAA would then have to deal with this larger pool and the degree of metabolism of radioactive IAA could be less. However, the amount of exogenous IAA could 'swamp' the levels of endogenous IAA present and no effect on rate of metabolism would be seen. Furthermore, seeds continue to take up IAA most of which seems to be converted into some unextractable form. The significant of this with respect to the imposition or release from thermodormancy is not obvious. It may have no relevance as it takes place at 20°C and 30°C.

The experiments carried out with ABA and IAA both arrive at the same point: we can present evidence that ABA and IAA have no obvious role in the imposition and release from thermodormancy but it is not possible to rule out a compartmentation effect on either the compounds themselves of the enzymes involved in their metabolism. The metabolism of ABA indicated in this study is not involved in the control of germination, but this does not rule out the ABA present being of importance in merely maintaining dormancy. The metabolism of IAA, although appearing not to be biological, may be associated with germination, although the evidence that IAA affects germination is less strong than that for ABA. Certainly no answers to these questions will be found until the problem of membrane effects and compartmentalisation are investigated.

In this thesis we have attempted to show that either or both ABA and IAA are inhibitors of germination in lettuce seeds and that there are changes in these hormones which can be correlated with the induction or release from thermodormancy. No such correlations could be shown and if ABA and IAA are inhibitors of germination, being one side of a balance between promoters and inhibitors, it would appear that they may be active only by their localization, or the localization of enzymes used to metabolise them. With ABA or IAA changes occur in endogenous levels at temperatures where the seeds are dormant, or will readily germinate. However, provided there is sufficient endogenous ABA or IAA at a particular site in the seed, this metabolism may be unimportant. The application of exogenous compounds, either 'cold' or radioactive, may not reflect what is happening to the endogenous hormone at that site. Amen (1968) has stated - "a causal or deterministic correlation between the biogenesis and role of hormonal factors and that of inhibitors does not appear warranted at present." He implies that inhibitors, although perhaps maintaining dormancy, dormancy being of adaptive value to seeds, are not involved in control. The fundamental control lies with the endogenous promoters. Whilst my own tendency is to agree with the conclusions reached by Amen, the evidence is neither in favour nor against such an argument. It is quite possible that ABA or IAA interact with membranes, this being a primary effect of plant hormones, to inhibit germination. The effect could be to reduce the effective levels of promotive compounds.

This effect could be reversed by lowering temperature or activation of the phytochrome system. The converse argument is of course also possible. It is also possible that none of the hormones interact with membranes at all, that inhibitors act as a block to germination and that an increase in the effective promoter level is brought about by phytochrome, the promoter then leading to events which culminate in germination.

Clearly this study cannot answer these questions. Although it would appear that IAA is not involved in thermodormancy and that ABA, if involved, is only required to stop the seeds from germinating, the role of inhibitors may only be understood when the role of promoters has been more fully investigated. The problems involved in examining a group of compounds as large as the gibberellins or

cytokinins, where the endogenous components of seeds are unknown, has precluded their study at more than a preliminary level in this thesis. However, future work on the Grand Rapids seed system must lie in the study of the endogenous promoters and if possible, at the level of membrane effects, particularly with respect to the effects of phytochrome. Possibly the most important point to emerge from this work is the need to use more than one sample of even the same species. It is interesting to speculate how many of the accepted 'facts' of plant physiology have been repeated by different groups of workers or how many are from work carried out once !

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