STUDIES ON THE INVOLVEMENT OF ABSCISIC ACID IN THE REGULATION OF DORMANCY IN FRUITS OF *LACTUCA SATIVA* L.

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SUMMARY

In a study of structure-activity relationships, ABA and thirty-two of its analogues have been tested in the lettuce germination, lettuce hypocotyl and pea epicotyl bioassays.

ABA delays germination of lettuce, a delay which cannot be overcome by the application of GA_3 although kinetin is capable of causing at least a partial reversal. In the lettuce hypocotyl and pea epicotyl assays, ABA inhibits GA_3-induced growth but is less effective as an inhibitor in the absence of GA_3. This results in the existence of a statistical interaction between GA_3 and ABA in these assays, although the relevance of this observation to an understanding of physiological mechanisms is debatable.

Twelve of the analogues are more active than ABA as inhibitors of lettuce germination. Assessment of the molecular requirements of ABA for activity revealed that the requirements are similar in all three bioassay systems. The carbonyl and/or hydroxyl groups in the ring are necessary for inhibitory activity as is the unsaturated bond. The double bond at C-2 in the pentadienoic acid side chain is also an important feature of the molecule for ABA-like activity, although other changes in the degree of saturation and to the terminal moiety can be made without destroying the ability of the molecule to act like ABA. The side chain, however, if retaining the pentadiene structure must be the cis, trans-isomer, the trans, trans-isomer being inactive.

There are two phases of 2-[^{14}C]-ABA uptake in lettuce fruits. Although the first phase appears to relate to the imbibition of water, uptake continues for three hours following complete imbibition. The second phase corresponds with the commencement of radicle extension.

Studies of the distribution after uptake of radioactive ABA by soluble compound microautoradiography were affected by the appearance of light reflecting bodies within the cotyledonary cells. The bodies were similar

in size and optical properties to silver grains, rendering the results of automatic grain counting techniques and visual comparisons of the treatments misleading. The appearance in the tissues of these artefacts was caused by fixation in the proprietory fixers, "Kodafix", "Metafix" and "Unifix", but not in either sodium or ammonium thiosulphate. The source of artefacts has been shown, by light and electron microscopy, to be protein storage bodies, many of which contain small inclusions. Treatment of the tissue with photographic fixer causes these inclusions to be released.

The distribution of silver grains, and thus of the radioactivity, could be ascertained by removing the section from the slide and examining the latent image which remained in the emulsion. This revealed that fruits imbibed in ^{14}C-ABA had radioactivity uniformly distributed throughout the cotyledons, although after radicle extension began, a slight accumulation was apparent immediately behind the radicle tip.

Exogenously applied radioactive ABA is metabolised in lettuce fruits to form an unidentified substance which has chromatographic properties different to previously described ABA metabolites. When ABA is present in the bathing solution, the quantity of metabolite never significantly exceeds that of radioactive ABA in the tissues.

Transference to water of fruits imbibed in ABA results in a rapid loss of ABA from the tissues, although no leaching of metabolite occurs. Germination begins shortly after transference and may relate to the lowering of ABA levels within the tissues by either leaching or metabolism.

ABA has been shown to occur endogenously in lettuce fruit tissues vars. Great Lakes and Arctic King by techniques which include circular dichroism, gas liquid chromatography and mass spectrometry. The level of ABA in tissues of Great Lakes has also been shown to fall dramatically up to the time when radicle emergence occurs; the fall cannot be explained in terms only of leaching.

ABBREVIATIONS

ABA	abscisic acid
CD	circular dichroism
DNA	deoxyribonucleic acid
GA_3	gibberellin A_3
GLC	gas liquid chromatography
IAA	indole-acetic acid
MS	mass spectrometry
ORD	optical rotation dispersion
RNA	ribonucleic acid
TLC	thin layer chromatography
UV	ultra violet

Errata

page	line	
1		micron ≡ micrometre.
35	29	ascending → descending.
65	5	three days or less → approximately three days.
70	1	inhibition → inhibitory.
88	7	at 10^{-5}M GA_3, growth was inhibited by 10^{-4}M ABA.
105	1	5×10^{-6}M → 5×10^{-7}M.

INTRODUCTION

The regulation of plant growth and differentiation by naturally occurring substances which exert their effects at low concentrations, has become a major area of research interest in developmental biology. Since the 1940's, interest has been aroused in the presence of compounds which have the general property of inhibiting growth in a non-toxic fashion. Studies on such compounds were stimulated chiefly by consideration of developmental phenomena involving the arrest of growth and the formation of the over-wintering condition.

In 1949 Hemberg suggested that dormancy might be due to the presence of substances inhibitory to growth, and he found that extracts of dormant terminal buds of ash strongly inhibited the elongation of oat coleoptiles. The level of this inhibitory influence decreased as dormancy disappeared.

The isolation of the first inhibitory compound known to alter growth at concentrations similar to those described for the auxins, gibberellins and cytokinins resulted from studies in two independent laboratories. In the U.S.A., Addicott and his co-investigators described the rôle of abscisin-II, a compound which they claimed was responsible for the abscission of cotton bolls. This compound, now known as abscisic acid, was first isolated from young cotton fruits by Ohkuma et al. in 1963, and its structure published in 1965 (Ohkuma et al., 1965). At the same time, investigatons of the induction of bud dormancy in woody plants led to the isolation and identification of "dormin" by Wareing and his associates (Wareing et al., 1964; Eagles and Wareing, 1964). This was later shown to be identical with ABA (Robinson and Wareing, 1964; Cornforth et al., 1965a), and the structure was confirmed by synthesis (Cornforth et al., 1965b). Other investigations on the identity of an abscission accelerating substance in yellow lupins, reported originally by Van Steveninck (1958, 1959), have followed (Rothwell and Wain, 1964), and this too has been shown by Cornforth et al. (1966b) to be ABA.

The widespread occurrence of ABA has subsequently been described in many plant tissues (Milborrow 1967, 1968; Wareing and Ryback 1970), and Milborrow also claims to have shown that ABA is the most active component of the inhibitor β complex.

Abscisic acid is the 'trivial' name for the sesquiterpenoid compound shown in Table 1. The molecule has an asymmetric carbon atom situated at the junction of the side chain and the cyclohexene ring, a feature which confers optical activity upon the compound. The naturally occurring enantiomer has been shown by Oritani and Yamashita (1972) and Ryback (1972) to be (S)-(+)-abscisic acid. The synthetic substance is, however, a racemic mixture and is therefore (RS)-($\pm$)-abscisic acid, an optically inactive compound differing from the naturally occurring enantiomer in having a higher melting point and a lower solubility. Thus, although the components are indistinguishable except by their effect on polarised light, the question arises as to whether or not their biological properties are the same.

Milborrow (1969), using radioactive racemic ABA in short term experiments, showed that tomato shoots converted the (+)-component into two major products, while the (-)-component gave only one. After 24 h, most of the ABA remaining was the (-)-enantiomer. One would thus expect the (+)- and (-)- abscisic acids to have different biological effects and, at first, it appeared that synthetic ABA possessed only half the activity of the natural compound, therefore indicating (-)-ABA was inactive. The resolution of the stereo-isomers (Cornforth et al., 1967) and their subsequent testing by Milborrow (1968) has, however, shown that both R- and S- ABA are equally active.

As well as stereoisomerism, ABA exhibits geometrical isomerism at the unsaturated bond between C-2 and C-3 in the side chain. The ABA most commonly isolated from plant tissues has been the cis,trans- or Z,E- isomer, although the trans,trans- or E,E- isomer has been detected in plant tissues, e.g. cotton and strawberries (Addicott and Lyon, 1969). Reports on the activity of the trans,trans- isomer are conflicting and range from assigning it equal activity to the cis,trans- isomer (Addicott and Lyon, 1969) to regarding it as

totally inactive (Cornforth et al., 1965b; Milborrow 1966; Tamura and Nagao 1969a). The reason for the variable results may be that the trans,trans-ABA can be converted by irradiation with ultraviolet light to a 1:1 equilibrium mixture with its cis,trans- isomer and vice versa (Mousseron-Canet et al. 1966, Lenton et al. 1971). The growth inhibitory activity of trans,trans-abscisic acid in experiments carried out in the light may, therefore, be attributed to the cis,trans- isomer formed by photolysis (Milborrow 1970a).

A number of methods have been used to detect and identify ABA. The original method by which inhibitory activity was detected, relied on chromatography and bioassay techniques (Bennet -Clark and Kefford 1953, and Nitsch and Nitsch 1956). This technique, although satisfactory for the detection of inhibitory activity, is not specific for ABA and no bioassay can, with certainty, separate an ABA response from that of the other components of the inhibitor- β complex which may contain phenolic or aliphatic acids (Lenton et al. 1972).

Methods specific for the detection of ABA usually involve purification by acid/base fractionation and chromatography followed by either spectro-polarimetry (Milborrow 1967,1968,1970a; Zeevaart 1971) or gas-liquid chromatography (Lenton et al. 1968,1971,1972; Seeley and Powell 1970; Most 1971).

ABA contains a centre of asymmetry which confers optical activity on the molecule. Naturally occurring ABA consists only of the dextrorotatory enantiomorph, viz. at the wavelength of the D line of sodium (589 nm), it rotates the plane of polarised light to the right (Cornforth et al. 1966a). The degree of this rotation changes as the wavelength of the incident polarised light varies, and for (+)-ABA the rotation becomes zero at 269 nm, below which it achieves a (-) extremum. This reversal of rotation is the Cotton effect, which for ABA is very marked. ABA has, in fact, one of the highest specific rotations of any known compound. Because of this, the optical rotation dispersion (ORD) curve of (+)-ABA in an extract can be

determined without interference from other optically active compounds unless they are present in very high concentrations. Provided the curve obtained is characteristic of ABA, indicating a lack of interference from other compounds, the quantity of ABA present can be calculated from the rotation, normally at the first (+) extremum. The limit of detection of pure substance in solution is 0.2 µg in 0.7 ml of solvent (Milborrow 1968).

Using this method, the level of ABA in a number of tissues has been determined (Milborrow 1967,1968,1970a; Zeevaart 1971). Corrections for losses occurring during extraction and purification can be achieved by the "racemate dilution method" devised by Milborrow (1967). Synthetic (±)-ABA is added at the beginning of extraction and is determined quantitatively after purification as the difference between the amount of ABA present, as estimated by ultra-violet spectrometry and ORD. Losses of endogenous (+) ABA can therefore be calculated.

Another method of identification and quantification which has better resolution capabilities than ORD, utilises the circular dichroism curve (Milborrow 1967). Circular dichroism (CD), although a related phenomenon to ORD, depends upon the specific absorption properties which optically active compounds possess. Differential absorption of the two circular polarised beams which comprise a plane polarised beam results in a rotated elliptical polarised beam. CD is therefore measured in terms either of the difference between the circular polarised beams or of the ellipticity to which it gives rise, and a curve is constructed by measuring one of these parameters as a function of wavelength. From the peak heights, the quantity of non-racemic ABA present in the solution can be calculated.

Lenton et al. (1968) described the GLC of ABA after formation of its methyl ester with excess diazomethane. The technique has subsequently been used to identify endogenous ABA (Most, 1971; Lenton et al., 1972) and it is capable of separating the cis, trans- and trans, trans- isomers but not the (+) and (-) enantiomers. Originally, the limits of detection by this

technique were between 10^{-2} and 10^{-1} µg of ABA, although Seeley and Powell (1970) claimed limits between 10^{-6} and 10^{-5} µg.

More positive identification of ABA could be made by subjecting an aliquot of the extract to UV light for sufficient time to allow equilibration of the cis, trans- and trans, trans- isomers (Mousseron-Canet et al., 1966). If at this time, the areas under the two curves on the chromatogram are equal, the cis, trans- peak before irradiation must have been the result only of the presence of cis, trans-ABA.

In 1971, Lenton et al. modified their technique such that it could be used to determine ABA quantitatively. These modifications involved the addition of a known quantity of trans, trans-ABA at the beginning of extraction to act as an internal standard. Thus, after GLC, the areas under the respective curves could be measured and the quantity of endogenous, cis, trans-ABA present in the original extract calculated.

Other methods for the detection of ABA include the use of a colour test devised by Mallaby and Ryback (1972) and an immunological assay (Fuchs et al., 1972). The advantages of this latter method are that, after preparation of the modified phage, no specialised equipment is required, and it is repeatable and specific. Since normal purification of extracts was required before immunoassay, no advantage can be gained over spectropolarimetry or GLC, especially where this is associated with mass spectrometry (Jenkins and Shepherd, 1971).

The availability of synthetic, racemic ABA has facilitated the study of its effects on a wide range of plant responses. Much of the work relating to the discovery and characterisation of ABA was a result of its ability to accelerate abscission. Addicott and his associates found that the application of extracts containing ABA to the petioles of cotton seedling "ex-plants" accelerated abscission of the petioles. They also correlated the level of ABA present in young fruits with the time at which they show maximum tendency to abscission, although they were less successful in inducing

abscission in whole plants by the exogenous application of ABA, and Wareing et al. (1967) have confirmed the effects of ABA on abscission in Perilla ocymoides and Coleus blumei. Osborne (1968), on the other hand, was unable to accelerate abscission in bean ex-plants, but it is thought that these tissues may have contained high auxin levels. The ability to accelerate abscission in ex-plants is therefore a well documented property of ABA, but the situation in whole plants is, unfortunately, not so clear. Most reports indicate that to affect abscission, application must be prolonged, and that the tissues must be receptive to the stimulus (Addicott and Lyon, 1969). When the leaves of citrus and olives were sprayed with ABA in summer, abscission occurred, whereas similar applications in winter were ineffective. They concluded that ABA was interacting with other hormones which vary in response to environmental changes, although they do consider that variations in the ability of the tissue to absorb and/or de-activate the ABA may also have been important.

As well as stimulating the formation of an abscission layer, ABA has been shown to cause loss of chlorophyll and turgor in abscising stumps of ex-plants (Addicott and Lyon, 1969). Foliar application has been shown to promote senescence in whole plants, although in young or vigorously growing tissue, a response has not always been observed (Addicott and Lyon, 1969). When the effect of ABA on isolated leaf discs was examined, however, it was found to promote the loss of chlorophyll (El-Antably et al., 1967; Beevers, 1968), and they found that ABA caused even non-foliar tissue such as potato tuber tissue to become soft and senescent. On the other hand, Colquhoun and Hillman (1972) noted that ABA delayed senescence of radish leaf discs. It is thus possible that, under certain conditions, endogenous ABA could exert a controlling influence on leaf senescence.

A recently observed response of plants to ABA is that of reduced transpiration (Little and Eidt, 1968; Mittelheuser and Van Steveninck, 1969, 1971; Jones and Mansfield, 1970,1971; Cooper et al., 1972). Cummins et al. (1971) and Horton (1971) have suggested that the ABA acts directly on the

stomatal apparatus, and Mansfield and Jones (1971) and Squire and Mansfield (1972) have shown that ABA causes major disturbances in the metabolism of guard cells, reducing their osmotic pressures, and hindering potassium accumulation and starch hydrolysis associated with opening. Imber and Tal (1970) showed that ABA caused reversion of a wilty mutant of tomato to the normally transpiring phenotype, and suggested that ABA may play an essential rôle in the stomatal mechanism, by inducing their closure, thereby reducing transpiration. Wright (1969) showed that the inhibitor β content of wheat leaves increased following a period of wilting, while Most (1971) found the ABA content of sugar cane leaves and stem was markedly higher when the plants were wilted. Zeevaart (1971) observed a ten-fold increase in the ABA levels in spinach when the plants were wilted. It is now becoming increasingly apparent that, when plants are subjected to stress, e.g. wilting or salinity, the endogenous ABA levels increase, and there is a marked reduction in transpiration, although a cause and effect relationship between these two has yet to be positively established.

ABA has been implicated in the regulation of apical dominance, i.e. correlative inhibition. Snow (1937) and Libbert (1954) suggested that correlative inhibition may involve an inhibitor. These studies were continued by Dörffling (1963,1966) on Acer pseudoplatanus and Pisum sativum, where he showed changes in endogenous inhibitor level corresponding to the inhibition of the lateral buds, and also showed the presence in Pisum sativum of ABA. Wareing et al. (1967) have shown that exogenous application inhibits lateral bud outgrowth in Ribes nigrum, and concludes that ABA may play a rôle in correlative inhibition.

Wareing et al. (1967) also exposed the long day plant Spinacea oleracea to short days to inhibit flowering, and, under these conditions, the plant tissue was shown to contain increased levels of inhibitor. They also grew Lolium temulentum and Spinacea oleracea, both long day plants, under short days, and then subjected them to a period of long day treatment, and it was

found that, where the plants were sprayed with ABA, during the long day treatment, flower initiation was inhibited. It is thus possible that ABA is the postulated flowering inhibitor produced in long day plants which are subjected to short days. Zeevaart (1971), on the other hand, also using Spinacea oleracea could find no indication of a lowering of ABA levels after transference of plants from short to long days, and concluded that ABA did not function as an inhibitor of flower formation.

ABA has also been shown to inhibit certain aspects of plant growth which are dependent upon both cell division and cell elongation. It has been found to inhibit growth of cereal embryos (Cornforth et al., 1966c), extension of excised oat coleoptiles (Wareing et al., 1968b), cereal mesocotyl extension (Milborrow, 1966), growth of lettuce hypocotyls, pea epicotyls, maize and oat leaf sections (Wareing et al., 1968b), and of small quatic plants such as Lemna and Wolffia (Addicott and Lyon, 1969).

Another physiological response which was responsible for much of the original research on ABA is the induction of resting bud formation in many woody species (Eagles and Wareing, 1964; Wareing et al., 1964). Bud dormancy occurs upon application of both natural and synthetic ABA to the leaves of birch (Betula pubescens), sycamore (Acer pseudoplatanus) and blackcurrant (Ribes nigrum) (El-Antably et al., 1967). Estimation of the endogenous inhibitor levels revealed that transference of many woody species from long to short days results in increases in inhibitor levels and the formation of resting buds, e.g. Phillips and Wareing, 1958. This inhibitor has been shown to be ABA in several species (Cornforth et al., 1966; Milborrow, 1968). On the other hand, conditions such as winter chilling which break bud dormancy, have been shown to lead to a decrease in endogenous inhibitor levels (Wareing et al., 1967). There is thus a good correlation between the level of inhibitor, whether endogenous or exogenously applied, and the state of dormancy of winter resting buds. Nevertheless, Lenton et al. (1972) failed to show an increase in ABA levels under dormancy-inducing

conditions in birch, and thus cast some doubt on the rôle of ABA in inducing bud dormancy of this type.

There are similarities between bud dormancy and certain types of seed dormancy, especially where the seeds have a chilling requirement for germination (Wareing and Saunders, 1971), and in some instances, e.g. birch, the two types can be compared within a single species, although the presence of a seed coat which may result in a "coat-imposed" dormancy, can be a complicating factor. There are, however, other types of dormancy where an analogy cannot so easily be drawn. These include instances where seeds require a period of "after-ripening", or where they are light-sensitive.

The theory that inhibitors may be responsible for preventing the premature germination of the seeds in succulent fruits was first suggested by Molisch (1922), and the presence in a number of such fruits of factors inhibitory to germination has since been demonstrated (Wareing and Saunders, 1971). The inhibitory substances in many of these fruits, and indeed in many non-succulent fruits, have been shown to occur both in the layers covering the embryo, e.g. pericarp, testa, and in the embryo itself. Villiers and Wareing (1960) and Wareing and Saunders (1971) suggested that seed dormancy may be controlled by the balance of endogenous inhibitors such as ABA, and promotors such as the gibberellins. Further theories relating to the nature of dormancy implicate the restriction of oxygen available to the embryo (Wareing and Saunders, 1971) and the inability of the radicles to develop the thrust necessary to penetrate the outer layers (Ikhuma and Thimann, 1963; Esashi and Leopold, 1968; Thimann, 1972).

Application of exogenous ABA to seeds has been found to inhibit the germination of a large range of species (Sankhla and Sankhla, 1968a,1968b; Addicott and Lyon, 1969; Wareing and Saunders, 1971). The effect of ABA on germination, and indeed on many other responses, is however normally transient, and its effect is easily removed by rinsing away the ABA. On the other hand, incubation of Chenopodium album in ABA solution for four weeks

can induce a dormancy which cannot be overcome by transferring the seeds to water (Wareing and Saunders, 1971). The effects of exogenously applied ABA on germination have been especially well reported for lettuce fruits, where it is particularly inhibitory (Aspinall et al., 1967; Khan, 1968; Sankhla and Sankhla, 1968a,1968b; Wareing et al., 1968; Bex, 1972).

Many investigations of the mode of action of ABA have involved studies of either the modification by ABA of the action of other plant hormones or the modification of ABA activity by other hormones. Where an effect has been observed, ABA has frequently been said to interact with the other hormone and to "decrease", "overcome", "reverse", "counteract", "inhibit", etc. its action (Addicott and Lyon, 1969). If the balance of hormones is an important factor in controlling growth, the interactions between these hormones could play a critical rôle.

Wareing et al. (1968b) examined the effects of ABA and IAA alone and in combination on Avena coleoptile extension. They found that the slope of the ABA response curve was not affected by the addition of IAA, and could find no indication of an interaction by analysis of variance. Rothwell and Wain (1964), using the lupin inhibitor which was later shown to be ABA, examined the same response, and by kinetic analysis determined the existence of a non-competitive interaction. Other investigations of the effects of ABA and IAA in wheat coleoptile growth have shown the existence of a "partially competitive" interaction (Addicott and Lyon, 1969). Most experimenters, however, agree that ABA can at least counteract the effect of IAA in most plant systems.

Investigations of the interaction of ABA with other plant hormones in a number of bioassay systems led Wareing et al. (1968b) and Good (1967) to the conclusion that in all but two instances the hormones acted independently. One system in which an interaction occurred was the lettuce germination assay, where the effect of ABA could be overcome by kinetin, but not by GA_3. These results are in agreement with those of Shankla and Shankla (1968b), but

Aspinall *et al.* (1967) were able to reverse the effects of low concentrations of ABA by GA_3, and this feature has also been recorded by other investigators (Addicott and Lyon, 1969).

Kinetic studies on the nature of an interaction are often contradictory, and the finding of a competitive interaction in only a few plant materials emphasises the questionable value of kinetic analysis as a means of investigating these complex multi-enzyme plant materials (Addicott and Lyon, 1969).

The other problem associated with many of these studies is that the criteria used to define an interaction, and in some instances the concepts of interactions, differ. 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 the results are expressed graphically, an interaction, therefore, expresses itself as non-parallel lines, i.e. the response to the two agents in combination is not the sum of the responses obtained when the agents are applied alone. Where no interaction exists, the lines are parallel, *viz.* the responses are additive. No interaction is, therefore, a case of rigid uniformity and any variation will appear as evidence of an interaction, even if the variation is due to error. In many cases, an interaction is defined as the ability of one agent to reverse the response to another agent regardless of whether or not the responses can be summed algebraically.

Taking account of these points, Drury (1969) reconsidered the findings of a number of investigations where GA_3 and ABA were claimed to interact (Thomas *et al.*, 1965; Milborrow, 1966; Chrispeels and Varner, 1967; Dey and Sircar, 1968). After re-assessment, he claims to have shown that no true interaction existed, although many of the supposedly parallel curves show marked inclinations to non-parallelism. He concluded that, if no interaction could be found using this approach, the modes of action of the hormones were independent.

Milborrow (1970b), in reply to the comments of Drury, believed such an approach to be oversimplified and fails to account for the variation in the ability of a tissue to respond to a stimulus at different concentrations of that stimulus, i.e. non-linearity in the response curve. He thus suggested that the term "interaction" should be applied only to the statistical description of measurements because, when physiological responses are non-additive, a transformation of the results can frequently render them additive, thereby removing an apparent interaction.

Drury (1970) said that non-linearity of the response curve was not a problem of interaction studies, although transformation of results might, on occasion, be required to ensure that they complied with the assumptions of analysis of variance. One must, however, remember that any conclusions drawn after such transformation refer to the transformed and not the original results.

Waroing et al. (1968a,1968b) have suggested that ABA may "interact" with GA_3 in the regulation of the growth of maize leaf sections by acting on its biosynthesis. This supports the suggestion of Wright (1968) that ABA and GA_3 may arise from a common precursor such as mevalonic acid, the biosynthetic pathway being later confirmed by Milborrow (1969a) and Milborrow and Noodle (1970).

Other suggested biosynthetic mechanisms include those described by Addicott et al. (1966) where ABA could be formed via an isoprenoid pathway or via a precursor such as violaxanthin. Support for the latter suggestion has been provided by Taylor and Burden (1970a,1970b, 1972) and Burden and Taylor (1971). They showed that violaxanthin can be photo-oxidised to form a neutral inhibitor which they called xanthoxin which, in turn, can be converted to ABA. This work not only led to a re-appraisal of the stereo-chemistry of ABA (Ryback, 1972), but also raises questions as to whether or not ABA is an artefact of extraction.

The use of analogues to elucidate the structure activity relationships

of growth substances is a well established procedure (Porter and Thimann, 1965; Brian et al., 1967; Letham, 1972). Nevertheless, the reports of molecular requirements for abscisic acid (ABA) activity are conflicting and inconclusive. One of the most serious problems in appraising the results is that different bioassay systems have frequently been used, yet none has been examined exhaustively. The Japanese investigators used the rice leaf sheath assay (Oritani and Yamashita, 1970a,1970b,1970c,1970d,1970e; Tamura and Nagao, 1969a,1969b,1970; Nagao and Tamura, 1971), while Sondheimer and Walton (1970) limited themselves mainly to the effect on excised embryonic axes of Phaseolus vulgaris, and Popoff et al. (1972) examined the response of bean stems. Taylor and Burden (1972) examined a range of responses but used only the abscisin aldehyde and the abscisin alcohol.

With the exception of four compounds synthesised by Tamura and Nagao (1969), and the two analogues tested by Taylor and Burden (1972), all the ABA analogues were less active than ABA and, in some cases, much less active (Sondheimer and Walton, 1970). No attempt was made to account for the consistent lack of activity. Many of the investigations are, nevertheless, of considerable interest, and the results are in some respects corroborative.

Sondheimer and Walton (1970) and Oritani and Yamashita (1970a) consider the complete ionylideneacetic acid skeleton of ABA to be of importance for the maintenance of activity, although the cyclohexene ring and the pentadienoic acid side chain may be considered separately. The carbonyl group in the ring is not considered necessary for activity (Sondheimer et al., 1969; Sondheimer and Walton, 1970; Tamura and Nagao, 1969,1970; Oritani and Yamashita, 1970a), nor is the ring hydroxyl group, although Tamura and Nagao do not comment on its functional importance. These results must, however, be interpreted with care since Sondheimer and Walton (1970) list, as active, the analogues lacking these groups yet fail to point out that none exhibited more than 18% of the activity of ABA.

The other important feature of the ring appears to be the unsaturated

bond between C-2' and C-3' (Sondheimer et al., 1969; Sondheimer and Walton, 1970; Oritani and Yamashita, 1970a; Tamura and Nagao, 1970). Some compounds lacking this feature are, nevertheless, active. Koshimizu et al. (1966) reported that the methyl ester has similar activity to ABA in the rice leaf sheath assay, and a similar conclusion was reached by Asmundson et al. (1968) using the cotton ex-plant bioassay. The situation is further obscured by the findings of Oritani and Yamashita (1970a) that analogues with the unsaturated bond between C-1' and C-2' retained some of their activity, and that the activity persisted even when the double bond was epoxidised (Oritani and Yamashita 1970e). Tamura and Nagao (1970) confirmed these findings by substituting the epoxide at the C-1' position and obtaining the only reported analogues which were more active than ABA. Tamura and Nagao (1969) also reported that if the double bond, instead of being in the ring, was in the C-3' side chain, ABA-like activity persisted. These results can be explained on steric grounds, and Sondheimer and Walton (1970) have suggested that, for a fit between the analogues and macromolecules, it may be essential that the C-2' methyl group is co-planar with four of the ring carbon atoms.

The only side chain requirement for ABA-like activity on which there seems to be general agreement is that the cis, trans-2,4-pentadienoic acid is superior to the trans, trans-isomer (Ohkuma, 1966; Tamura and Nagao, 1969, 1970; Sondheimer and Walton, 1970). The results of Oritani and Yamashita also reveal this requirement, but they do not comment on it. These observations on the stereoisomerism of the side chain confirm those described earlier for ABA itself.

The side chain unsaturated bonds appear to be essential for activity (Oritani and Yamashita, 1970c), although Ohkuma (1966,1967), in one of his analogues, replaced the proximal double bond by a triple bond, and retained some activity.

It has also been found by Oritani and Yamashita (1970b,1970c,1970d) that side chain length is critical and that shortening it to remove the

distal unsaturated bond reduces the activity and, although Popoff et al. (1972) do not comment on this, their analogues in which the side chain length was reduced, had appreciably reduced activities. Lengthening the side chain also apparently decreased the activity, although other reasons for the reduced activity could be postulated in most instances. It has also been suggested by Oritani and Yamashita (1970c) and Tamura and Nagao (1970) that, for activity to exist, the terminal group should be a carboxyl or alkocarboxyl or another group which could be converted readily to ABA.

Owing to the limited availability of radioactive ABA, studies of the metabolism of exogenously applied ABA by plant tissues have been limited to a few laboratories. Milborrow (1968,1969b,1970a) has shown that the two major products formed when 2[^{14}C(±)]ABA is supplied to tomato shoots, French bean and sycamore petiole sections are abscisyl-β-D-glucopyranoside and 6'-hydroxymethyl-ABA. He also showed that abscisyl-β-D-glucopyranoside was apparently formed from both enantiomers of ABA, but that 6'-hydroxymethyl-ABA formed only from (+)-ABA. The low levels of trans, trans-ABA detected in tissues could be due to the faster conversion of this isomer to abscisyl-β-D-glucopyranoside. The ABA glucoside has, in fact, been shown to occur naturally in the fruit of Lupinus luteus (Koshimizu, 1968), and in the pseudocarp of Rosa arvensis (Milborrow, 1970a).

In embryonic bean axes, Walton and Sondheimer (1972b) have reported two further metabolites (M1 and M2) which are apparently distinct from those reported earlier. These unidentified metabolites are believed to be part of a de-activation mechanism with M-1 being a precursor of M-2, which is cumulative.

Another method of studying the method of hormone action is to examine the biochemical changes occurring as a result of their application (Phillips, 1972). Although enzyme synthesis and breakdown data would be valuable in this context, routine procedures for the identification and quantification of the enzymes involved in hormone action are not yet possible. Nucleic acid

and total protein levels are, on the other hand, frequently examined.

Van Overbeek (1968) found that ABA at 3.8×10^{-9} M inhibited the growth of Lemna minor, and that this inhibition could be reversed by removing the ABA or by adding benzyladenine. He also observed that ABA inhibited the synthesis of all nucleic acid fractions, with suppression of DNA synthesis appearing most quickly. The addition of benzyladenine caused resumption of nucleic acid synthesis.

Wareing et al. (1968a,1968b) found that ABA pretreatment of radish leaf discs caused a reduction in total RNA synthesis and total phenol extractable RNA. This reduction was associated particularly with the ribosomal RNA. No DNA synthesis could be detected in this system.

Bex (1972b,1972c) observed inhibition of RNA synthesis in maize coleoptiles three hours after treatment in ABA, with the ribosomal RNA again being the most affected. RNA-ase activity also increased in this system, but since this did not occur until 8 h after the beginning of the ABA treatment, it is not likely that it was a primary effect of ABA application.

It has been suggested that the inhibition of RNA synthesis might be the basis of the mode of action of ABA in delaying germination (Villiers, 1968), although many reports suggest that RNA synthesis is not essential for the initial stages of germination (Bex, 1972a). In lettuce, however, RNA synthesis is thought to be an essential prerequisite of germination (Khan, 1966; Smith and Frankland, 1966; Frankland et al., 1971), with ABA inhibiting both RNA synthesis and radicle protrusion. Bex (1972a), in attempting to determine the stage of germination which was being affected by ABA, examined changes occurring during the lag phase of germination and during the early stages of growth. He imbibed lettuce fruits in solutions of radioactive ABA and found uptake of radioactivity to occur within the first six hours, after which no further uptake occurred until radicle emergence. He also assessed the effect of this ABA on respiration, radicle protrusion and RNA synthesis, and detected no effect on total RNA synthesis during the first 8 h between

fruits imbibed in water and those imbibed in ABA solution. After this time, there was significantly less RNA synthesis in the fruits imbibed in ABA solution. The results confirmed those of Walton et al. (1970), who could find no effect of ABA on RNA synthesis in embryonic bean axes before the initiation of cell elongation. Respiration, as assessed by oxygen uptake in lettuce, was unaffected by the presence of ABA, until radicle emergence. On the basis of his findings, Bex (1972a) suggests that ABA affects radicle protrusion merely by reducing its rate of extension.

The investigations of Villiers (1968), in which he examined the effects of ABA on nucleic acid and protein synthesis during germination, utilized a technique by which he could also have examined the site of ABA action. The technique used was microautoradiography, whereby, after "feeding" the tissue with radioactive precursors of nucleic acids and proteins, the location of the radioactive nucleic acids and proteins could be determined from the distribution of silver grains in a photographic emulsion held in close contact with a section of the tissue. The experiments were conducted on radicle tips, and he concluded that ABA acted in inducing dormancy in this species by inhibiting the synthesis of specific types of RNA. That ABA affects nucleic acid synthesis does not, however, demonstrate that the ABA acts primarily and directly on DNA or RNA, since the time between exposure to ABA and observation of a response was sufficient for a great deal of biochemical activity to occur.

The technique of microautoradiography can also be applied, after modification, to examining the distribution, within the tissues, of exogenously applied hormone. Modification of the technique is required because of the water soluble nature of the hormones which could result in massive diffusion across the section and the alteration of the distribution patterns.

A number of methods of maintaining hormone distribution during sectioning and exposure have been adopted (Rodgers, 1969). Lawton and Biddulph (1964) freeze dried tissue and embedded it in wax, believing that this preserved

accurately the *in vivo* distribution of water soluble compounds. Bieleski (1966) and Trip and Gorham (1967), however, considered that partial collapse of the sieve tube contents could occur during freeze drying, resulting in misleading distribution patterns. Fisher (1972) and Fisher and Housley (1972) considered this problem and compared the results obtained with freeze dried tissue with those obtained using freeze substituted tissue. They found that freeze drying did frequently result in artefact formation due to shrinkage of cell contents, but that the technique of freeze substitution gave an accurate account of the distribution of radioactivity.

A technique suggested by Appleton (1964) for maintaining the distribution of water-soluble compounds during microautoradiography involves freezing the tissue before sectioning begins, and maintaining it in this condition until the end of exposure. There is thus no opportunity for a redistribution to occur. Bowen *et al.* (1972) have successfully applied the freezing technique to examining the distribution of IAA in maize root segments.

This investigation relates to studies on the rôle of ABA in the regulation of germination in lettuce.

After establishing the response of lettuce fruits to exogenously applied ABA, an appraisal is made of the molecular requirements for inhibitory activity. This particular aspect was facilitated by the supply of ABA analogues. Further consideration of the mode of action of ABA is made by analysis of interaction experiments using ABA, its analogues and gibberellic acid (GA_3).

The uptake, metabolism and leaching characteristics of 2[^{14}C]ABA are then examined. Using microautoradiography instrumentation, the distribution of the applied radioactivity in the fruits is also considered.

Having studied certain aspects of the physiology of exogenously applied ABA, the concluding experiments are concerned mainly with naturally occurring ABA in lettuce fruits.

MATERIALS AND METHODS

PLANT MATERIALS

Fruits (cypselas or "seeds") of lettuce, Lactuca sativa L., varieties Great Lakes and Arctic King, were supplied in sealed aluminium foil packets by Suttons Seeds Ltd., Reading, U.K. The fruits were stored at -20°C in darkness, until required.

Seeds of Pisum sativum L., var. Meteor, were supplied by Thompson and Morgan, Ipswich, U.K., and stored in a cool, dark and dry area.

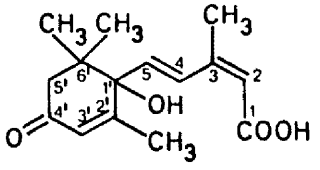
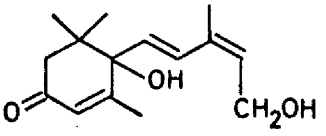
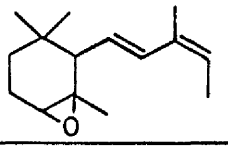
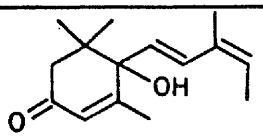
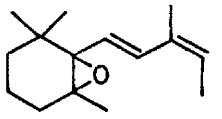
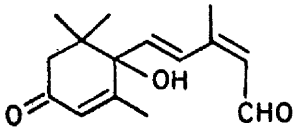
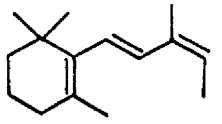
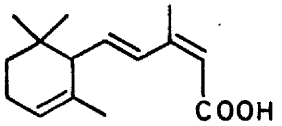
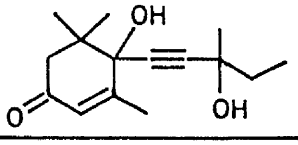
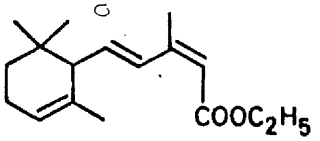
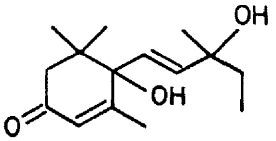
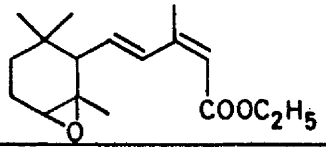
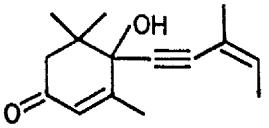
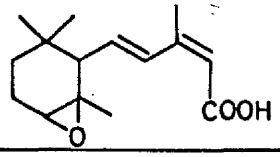
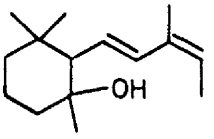
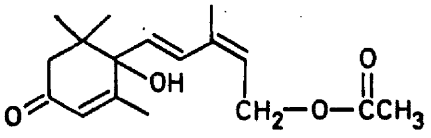
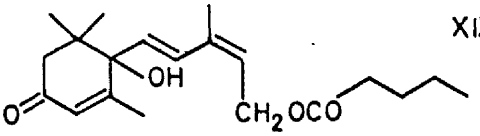
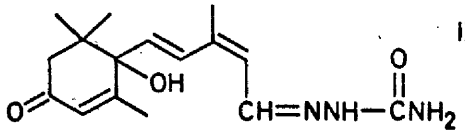
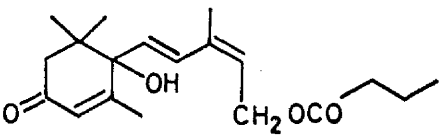
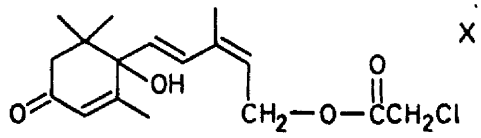
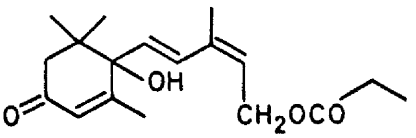
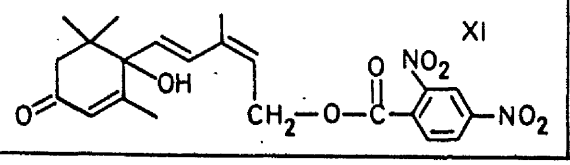
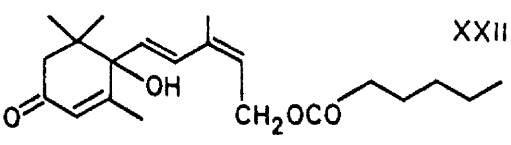
SOLUTION OF COMPOUNDS

Abscisic Acid and its Analogues

RS(±) abscisic acid (molecular weight 264.31) (Table 1) obtained from Hoffman-La Roche, Switzerland, was dissolved in a minimum quantity of redistilled methanol in a small glass container, and dispersed in a large volume of distilled water by vigorous stirring. The volume of methanol used was such that its concentration in the working solutions never exceeded 350 ppm. This method was used to solubilise both the cis, trans- and the trans,trans-isomers of the synthetic racemic ABA as well as the 2[^{14}C(±)]ABA.

A similar technique was used in attempts to obtain aqueous solutions of analogues of ABA which were also supplied by Hoffman-La Roche (Table 1). Three of the analogues were, however, insoluble in methanol and, in these instances, acetone was substituted as the organic solvent. After initial solution in either methanol or acetone it was found that 9 of the analogues would not disperse satisfactorily in water at room temperature, and precipitated even when the water was warmed to 40°C. Where these methods were unsuccessful, the compound was dissolved in the organic solvent and dispersed into a solution of sodium bicarbonate which was subsequently adjusted to the appropriate volume.

Table 1. The molecular structures of abscisic acid and the analogues.

	Abscisic Acid (ABA) Chem Abstr code no - 21293-29-8
I 	XII 
II 	XIII 
III 	XIV 
IV 	XV 
V 	XVI 
VI 	XVII 
VII 	XVIII 
VIII 	XIX 
IX 	XX 
X 	XXI 
XI 	XXII 

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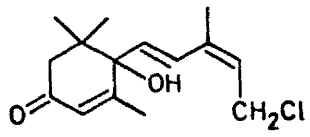
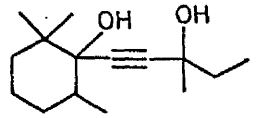
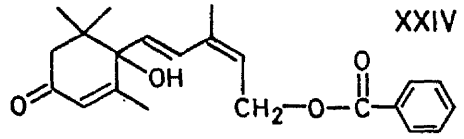
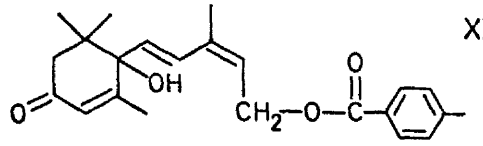
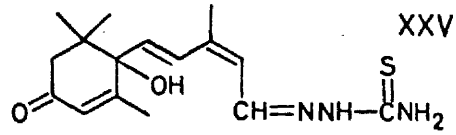
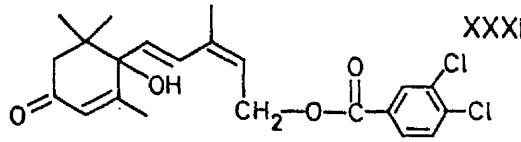
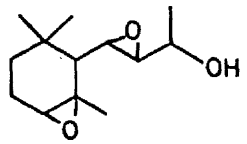
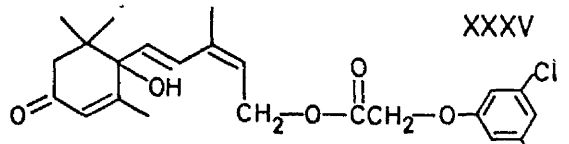
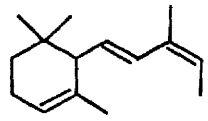
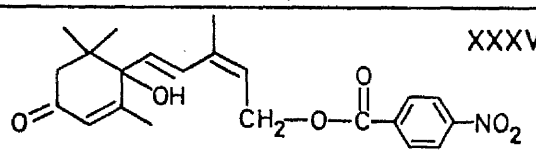
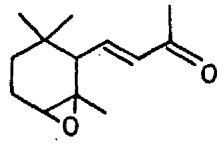
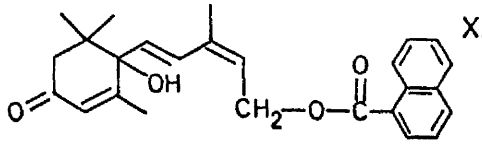
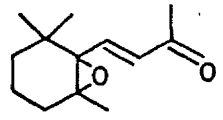
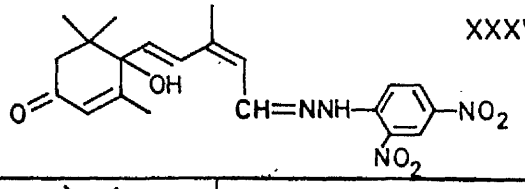
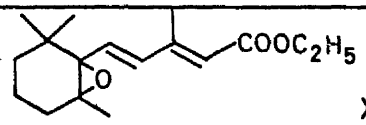
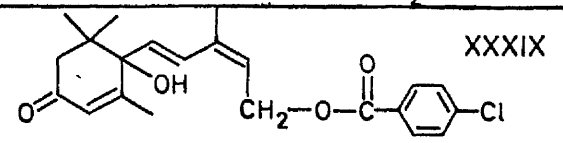
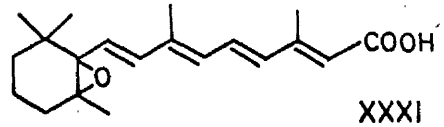
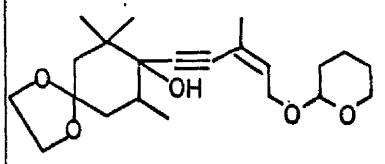
XXIII 	XXXII 
XXIV 	XXXIII 
XXV 	XXXIV 
XXVI 	XXXV 
XXVII 	XXXVI 
XXVIII 	XXXVII 
XXIX 	XXXVIII 
XXX 	XXXIX 
XXXI 	XXXX 

Using these techniques, it was found possible to obtain aqueous solutions of 32 of the analogues (Table 2). Eight could not, however, be dispersed in water using any method which would subsequently permit successful bioassay of their activity. These compounds whose structures are shown in Table 1 have not yet been tested.

Gibberellin A_3

Gibberellin A_3 (MW 346.4) has a limited yet significant solubility in water, and it was found possible to prepare 10^{-4}M solutions by direct dissolution in water. The appropriate weight of crystalline gibberellin A_3 supplied by Sigma Chemical Company, Missouri, U.S.A., was added to water and stirred for a minimum of 12 h in darkness.

Kinetin

Kinetin (6-furfuryl aminopurine MW 215.22) was obtained from BDH Chemicals Ltd., Poole, U.K. An aqueous solution was prepared by dissolving the kinetin in a minimum quantity of N,N'-dimethylformamide at 60-70°C and dispersing this in swirling distilled water at the same temperature.

BIOASSAYS

Lettuce Germination Assay

A 90 mm petri dish was lined with a single circle of Whatman No. 3 filter paper and the paper moistened with 5 ml of test solution. Fifty or 100 fruits of Lactuca sativa L. var. "Great Lakes" were distributed on the surface of the filter paper. The dishes were randomised, placed in plastic boxes (265 mm x 200 mm x 80 mm) and incubated in diffuse white light (0.92 watts m^{-1}, "warm white" fluorescent tubes supplied by Atlas) at 25 ± 1°C. The numbers of fruits germinated were recorded at intervals initially of two hours, increasing to 24 h after the fourth day. Germination was recorded when the radicle had emerged from the fruit coat. All experiments were

Table 2. Dispersion of ABA and its analogues in water.

Compound	Molecular Weight	Concentration in 10^{-5} M solution		Comments
		Methanol (ppm)	Acetone (ppm)	
ABA	264	3.5		
I	250	100.0		
II	234	100.0		
III	248	100.0		
IV	234	100.0		4 mg sodium bicarbonate added
V	262		26.0	
VI	278	42.0		
VII	250	100.0		
VIII	292	50.0		
IX	305	200.0		
X	327	100.0		
XI	444	400.0		
XII	220	23.7		
XIII	220	51.2		
XIV	204	158.7		
XV	250	200.0		
XVI	252	200.0		
XVII	232	200.0		
XVIII	222	157.5		
XIX	334	200.0		
XX	320	150.0		
XXI	306	150.0		
XXII	348	150.0		
XXIII	269	200.0		
XXIV	354	200.0		
XXV	321	150.0		
XXVI	389	86.2		
XXVII	204	145.6		
XXVIII	208		9.8	
XXIX	208	50.0		
XXX	278	40.0		
XXXI	316		400.0	
XXXII	238	200.0		
XXXIII XXXIV XXXV XXXVI XXXVII XXXVIII XXXIX XXXX				Compounds XXXIII to XXXX could not be satisfactorily dispersed in water by any of the applied methods.

terminated after a maximum of 444 h.

Lettuce Hypocotyl Assay

A 90 mm petri dish was lined with one circle of Whatman No. 3 filter paper and the paper moistened with 5 ml of distilled water. Fruits of Lactuca sativa L. var. Arctic King were distributed over the surface of the filter paper such that the fruits were not in physical contact. The petri dish was placed in a controlled temperature room adjusted to 25 ± 1°C and illuminated to an intensity of 2224 watts m^{-1} by a mixture of Atlas "warm white" and "daylight" fluorescent tubes. After 24 h, radicle emergence had taken place in a large proportion of the fruits. Seedlings with radicles measuring 2 mm in length were selected for the bioassay and were transferred to the test solutions. These solutions were also contained in a 90 mm petri dish, prepared as already described. The seedlings were always positioned such that the radicle was directed towards the filter paper.

Ten seedlings were placed in each dish of test solution and distributed such that they were approximately equidistant from each other and from the edge of the filter paper. The dishes were returned to the controlled environment room for 72 h, after which hypocotyl length was recorded to the nearest 0.5 mm. Root length and the cotyledonary leaf dimensions were also recorded in certain experiments. These leaf dimensions were determined by use of a shadowgraph technique where cotyledonary leaves were removed at their junction with the top of the hypocotyl, and arranged in rows between two glass plates which were transferred to a photographic enlarger (Universal Alpha II) adjusted to give a focused image of times four magnification. The image was recorded as a shadow on photographic paper (Ilfobrom 4 1B4 1P) which was then developed in Ilford contrast developer and fixed in a "Kodafix" solution. After drying, the image was measured directly.

Exposure to light suppresses the growth of the hypocotyl. Thus, to detect inhibition of hypocotyl extension, it was necessary either to stimulate

extension by supplying GA_3 to the seedlings or to perform the experiments in darkness. In these latter experiments, the fruits were incubated in darkness for the initial germination period of 24 h and the selection of seedlings suitable for bioassay was carried out under "safe" green lights emitting low intensity illumination. The dishes containing test solution and seedlings were then returned to darkness for the further incubation period of 72 h.

The physiologically "safe" lights were constructed either using a 2 ft Atlas fluorescent tube or beehive lamp fitted with a tungsten filament bulb. Where fluorescent tubes were used, they were of two types. The first was a green tube which was further surrounded by one layer of orange (no. 5) Cinemoid (Rank Strand Electric Ltd., London, U.K.) and 1 layer of primary green (No. 39) Cinemoid. When white tubes were used instead, an extra primary green Cinemoid layer was included. These arrangements provided satisfactory light sources. When a tungsten filament lamp was used, a layer of dark blue Cinemoid (No. 19) was also inserted to guard against high levels of red light.

Pea Epicotyl Assay

Seeds of Pisum sativum L. var. Meteor were grown in darkness for 108 h in moistened vermiculite at 25 ± 1°C. By this time the epicotyls had extended but the first internode remained compressed and the plumular hook reflexed. Epicotyls were selected which were 30-40 mm in length, and the apical 20 mm bearing the plumular hook were excised. Three of these 20 mm portions were placed in a small test tube together with 0.4 ml of the test solution. The tube was plugged with non-absorbent cotton wool to minimise evaporation while not preventing gaseous exchange. The tubes were randomly arranged in racks and placed in a controlled environment room at 25 ± 1°C, illuminated at an intensity of 5530 watts m^{-1} by a mixture of Atlas "warm white" and "daylight" fluorescent tubes. After 48 h the first internode

had begun to extend and its length was recorded to the nearest 0.5 mm. This experiment was terminated after 40 h, rather than 72 h, in order to avoid problems arising from contamination of the culture solutions.

THE EFFECT OF TEMPERATURE ON GERMINATION

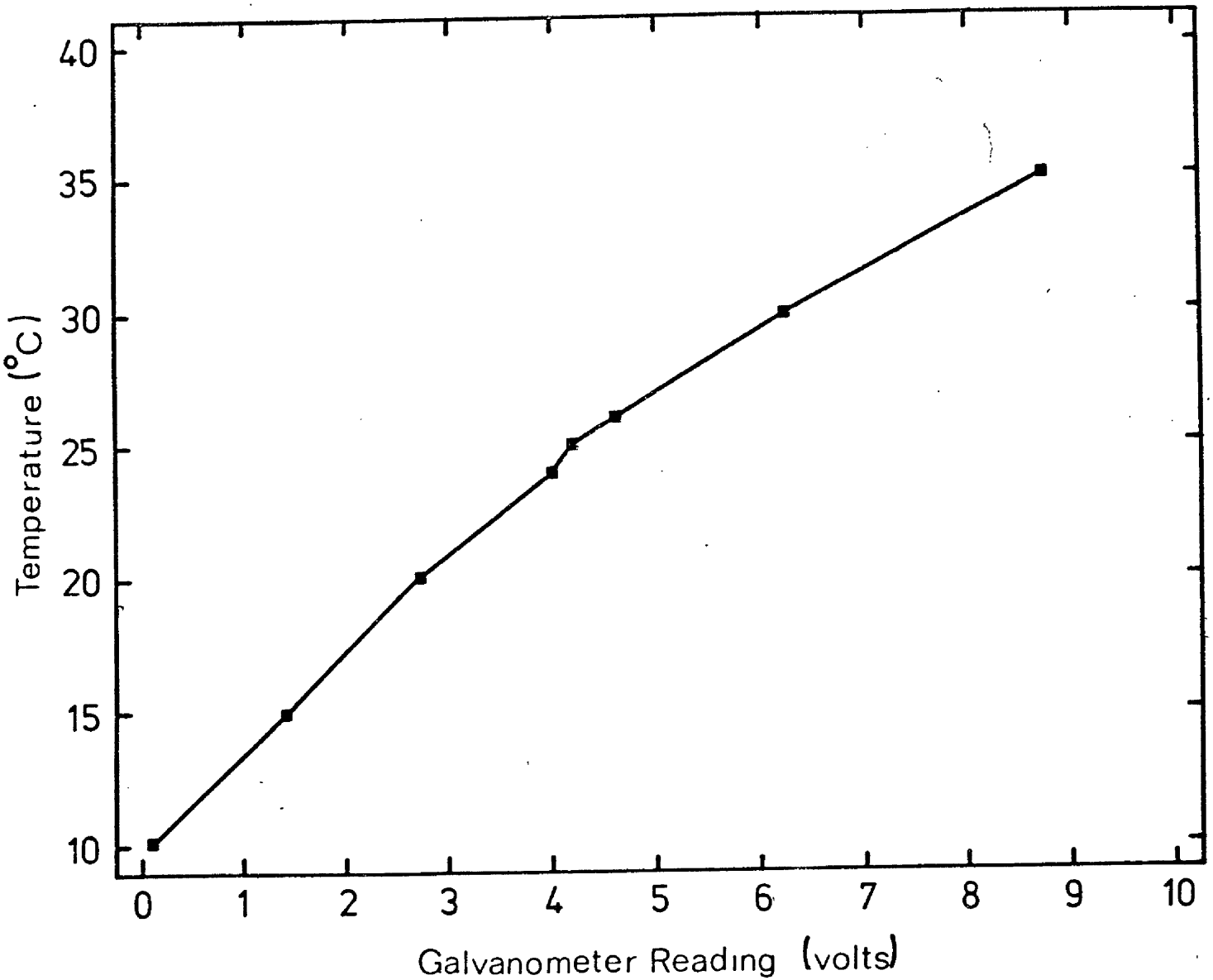
Germination of lettuce fruits (var. Great Lakes) was recorded on a thermogradient bar over a range of temperatures from 5°C - 30°C. The bar consisted of an aluminium plate 290 mm wide and 6 mm thick. A 90° angle was made at either end of the plate such that the horizontal surface was 600 mm x 290 mm, and each of the vertical surfaces 150 mm x 290 mm. One vertical surface was placed in a refrigerated water bath (from Grant Instruments Ltd., Cambridge, U.K.) and the other in a heated water bath. The temperature along the horizontal surface of the bar thus ranged between the two extremes. It was possible, therefore, to control the range by adjusting the temperatures of the water baths. It was in practice found more practical to remove the hot water bath and to control the upper temperature of the bar by regulating the ambient temperature. This was due to temperature drift problems associated with the maintenance of a constant water level in the bath, especially at high temperatures.

Petri dishes, 50 mm in diameter, containing solution were positioned at intervals along the horizontal surface, and the temperature of the solution monitored using a calibrated thermistor. In this way, it was possible to position the dishes at the appropriate temperatures. The thermistor was calibrated using melting ice and boiling water, and the calibration curve is shown in Fig. 1.

LEACHING OF INHIBITORS AND THE EFFECT ON GERMINATION

Lettuce fruits were imbibed in test solutions in a 90 mm petri dish, as described in the germination bioassay. After pre-determined times in the solution, the filter paper bearing the fruits was transferred to a Büchner

Fig. 1. The voltage across the thermister as a function of temperature.


40
35
30
25
20
15
10
Temperature (°C)
0
1
2
3
4
5
6
7
8
9
10
Galvanometer Reading (volts)

funnel attached to an Edward's rotary vacuum pump. The paper was held in the funnel until most of the liquid had been removed and the fruits appeared dry on the surface. They were then scattered in a second petri dish prepared as before, but containing only distilled water.

Germination was recorded at 2-h intervals before and after transference so that the time to 50% germination could again be calculated accurately.

UPTAKE AND LEACHING OF RADIOACTIVITY

The uptake of 2[^{14}C(±)] ABA (specific activity 45 μ Ci mg^{-1}), 8[^{14}C] kinetin (specific activity 80 μ Ci mg^{-1}) and tritiated water into lettuce fruits, and the leaching of the compounds from the fruits by water, were examined using liquid scintillation spectrometry. Radioactive kinetin and water were obtained from the Radiochemical Centre, Amersham, U.K.

Fruits were imbibed in 0.6 ml of the radioactive solutions in a 2 dram vial for known periods of time at 25 ± 1°C in diffuse white light (0.92 watts m^{-1}). After incubation, the fruits were washed in a Büchner funnel under vacuum from an Edward's rotary vacuum pump, and the amount of radioactivity in the tissues assayed.

Where leaching of the radioactivity by water was being examined, the washed fruits were at this point transferred to a second vial containing 2 ml of distilled water. After a pre-determined time, the fruits were removed and the radioactivity in the tissues and the bathing solution assayed. Chromatographic analysis of the radioactivity was also carried out.

ASSAY OF RADIOACTIVITY

(a) Single Label Experiments.

Where the radioactivity was in the form of carbon-14, the tissue was transferred to a scintillation vial and extracted in 2 ml of 80% ethanol for 48 h in the dark at 4°C. The ethanol was removed at room temperature

in a desiccator and 10 ml of toluene containing 4 g l^{-1} of PPO (2,5-diphenyloxazole, BDH Chemicals Ltd., Poole, U.K.) added to each vial. The vials, after equilibration to 4°C, were assayed on a Packard Tricarb Model 3380 liquid scintillation spectrometer fitted with an Absolute Activity Analyser Model 544.

Fruits imbibed in tritiated water were transferred directly to a scintillation vial containing 10 ml of the solution of PPO in toluene, and 4 ml of "Burrough's" 99.9% ethanol added to solubilise the water. No secondary scintillator such as PPOP was added. The vials were then assayed as previously described.

(b) Double Label Experiments.

In a number of experiments, the fruits were imbibed in a solution containing radioactivity in the form of both ^{14}C and ^{3}H. The procedure adopted during preparation of the samples for assay in these experiments was identical with that used when the solution contained only tritiated water. Assay, however, was carried out on a Tracerlab Corumatic 200 liquid scintillation spectrometer, and the absolute activity calculated.

Determination of Absolute Activity

On a liquid scintillation spectrometer, the quench level determines the efficiency of counting. Quenching is caused by either the absorbence properties of coloured materials in solution (colour quenching) or chemical interference with the transfer of energy between the site of an event and a molecule of scintillator (chemical quenching). The result is a reduction in efficiency of counting and a shift towards zero energy.

In the Tricarb Spectrometer, the degree of quenching is estimated by counting an external standard (americum 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 resultant cpm (counts per minute) at a given AES ratio converted to disintegrations per minute (dpm) 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:-

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

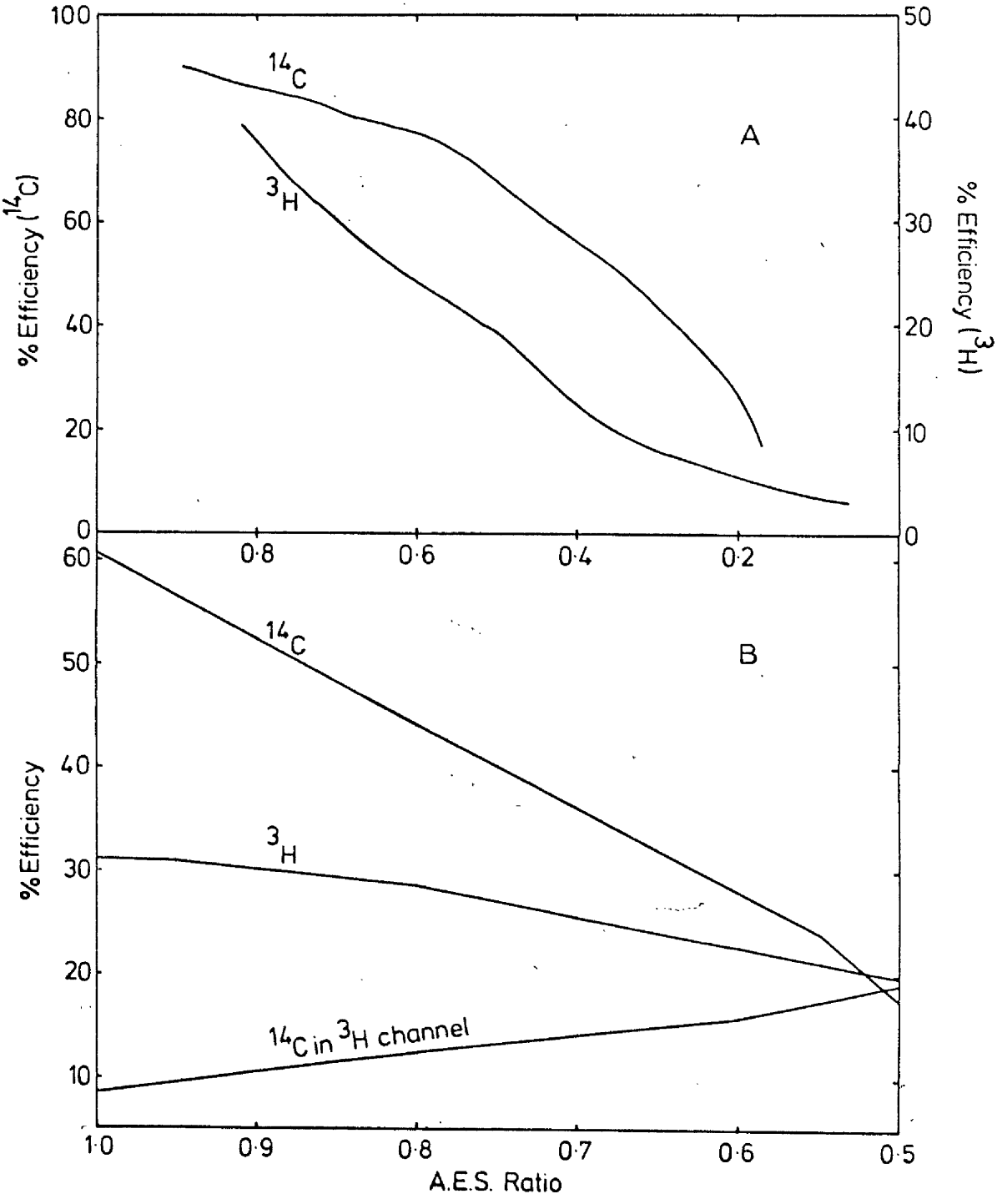
By repeating this a large number of times with samples quenched to different extents, a "quench curve" was constructed (Fig. 2A). The AAA was then programmed by reference to this curve, after which it automatically computed the absolute activities of the samples using the expression:-

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

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 the curve. This machine was used only for the assay of double labelled samples and the appropriate quench curves are contained in Fig. 2B.

When a sample containing both carbon-14 and tritium was counted, a measure of the cpm in the tritium channel was obtained together with the cpm in the carbon-14 channel and the AES ratio. By reference of this AES ratio to the quench curves, the efficiency of counting in both channels could be obtained as could the percentage of ^{14}C counts in the tritium channel. The absolute activity of ^{14}C and the dpm in the ^{3}H channel were thus calculated, and from the latter figure was subtracted the number of dpm of ^{14}C estimated to have registered in the tritium channel. An

Fig. 2. Quench correction curves for the liquid scintillation spectrometers. The efficiency of recording disintegrations per minute of ^{14}carbon and tritium at varying AES ratios using (A) the Packard Tricarb liquid scintillation spectrometer, (B) the Tracerlab Corumatic liquid scintillation spectrometer. The Tracerlab was adjusted for double isotope assay and the proportion of ^{14}carbon which is recorded in the tritium channel has also been plotted.


100
80
60
40
20
0
% Efficiency (^{14}C)
50
40
30
20
10
0
% Efficiency (^{3}H)
^{14}C
^{3}H
A
0·8
0·6
0·4
0·2
60
50
40
30
20
10
% Efficiency
^{14}C
^{3}H
B
^{14}C in ^{3}H channel
1·0
0·9
0·8
0·7
0·6
0·5
A.E.S. Ratio

assessment of the absolute activities of both isotopes in the sample was obtained.

SOLUBLE COMPOUND MICROAUTORADIOGRAPHY

Preparation of Slides

Microscope slides (76 x 25 mm) and glass cover slips (No. 1, 22 x 50 mm) were soaked for 24 h in chromic acid solution prepared by dissolving 100 g of potassium dichromate in 850 ml of water and adding 100 ml of concentrated sulphuric acid. They were then rinsed in flowing tap water and at least six changes of distilled water. A wet slide and cover slip were dipped together into a freshly prepared 5% gelatin solution and allowed to dry. The slide and cover slip thereafter adhered together firmly.

Stripping Film

A solution containing 2% sucrose and 0.05% potassium bromide was held at 25-26°C in a darkroom under a photographic safelight. The plates of stripping film (Kodak AR-10) were maintained at a slightly lower temperature. A 10 mm strip of emulsion was removed from the edge of the plate and the remaining emulsion divided into 10 portions approximately 55 x 15 mm. The strips of film were peeled slowly from the supporting glass plate and floated emulsion side upwards on the sucrose/potassium bromide solution, where they were left for about 2 mins to absorb water. The film was then floated on to the cover slip such that it had an intact covering of emulsion which was dried slowly and stored in a light proof box at -30°C.

Embedding

(a) Liver Technique.

A 1 cm cube of fresh pig's liver was placed excentrically on an aluminium microtome chuck. A slit was cut in the liver and the tissue to be examined, orientated in this slit. The liver was pressed firmly around the

tissue and frozen by immersing the base of the chuck in liquid nitrogen. It was then placed in a cryostat cabinet (Bright's, Huntingdon, U.K.) at -18°C to allow temperature equilibration.

(b) "Tissuetek" Method.

A drop of "Tissuetek" O.C.T. compound (Ames Company, Indiana, U.S.A.) was placed on a chuck and frozen in liquid nitrogen as above. Holes were drilled in the "Tissuetek" and the chuck placed in the cryostat cabinet to permit temperature equilibration. Plant tissue was placed in the holes, covered with fresh "Tissuetek", frozen in liquid nitrogen as before and the chuck replaced in the cryostat for sectioning.

Sectioning

The tissue was sectioned on the freezing microtome at -18 to -20°C with a knife angled at 12° from vertical. Sections approximately 15 microns in thickness were cut, and left on the knife blade. The sections were transferred to the photographic emulsion which had also been brought to -18°C by pressing the emulsion coated cover slip surface against the section. The slides were then replaced in the light proof box at -30°C, where they were stored for a minimum of one month to permit satisfactory exposure to occur.

Development

Development was carried out at 17-18°C using Kodak D19 developer as recommended. "Kodafix" was used initially for fixation, although it was later superseded by other fixers. The procedure used in development and fixation was as follows:-

Kodak D.19 developer	5 mins
Water rinse	15 secs
"Kodafix" fixer	12 mins
Tap water wash I	15 secs
II	15 secs
III	15 secs
Flowing tap water	30 mins

All operations from film stripping to development were carried out under photographically "safe" lighting obtained from a Kodak beehive lamp fitted with a tungsten filament bulb and a Kodak Wratten No. 1 filter giving indirect lighting from a minimum distance of 1.25 metres.

After drying, the cover slip supporting the autoradiograph was removed from the surface of the microscope slide, inverted and mounted on a second slide in a drop of DPX permanent mounting medium supplied by Edward Gurr Ltd., London, U.K. The DPX was hardened on an electrically heated hotplate maintained at a temperature of 50-60°C.

The pattern of silver grains was examined under transmitted light dark field illumination using a Zeiss Photomicroscope II. The silver grains of the autoradiograph showed as small bright spots under this system, while the section was barely visible. Because of this, it was possible to assess the concentration of grains in a given small area by using a Zeiss MPM microscope photometer. When this was combined with the use of a motorised microscope stage (Plate 1) it became possible to obtain a continuous measure of silver grain distribution across a section. The apparatus used is shown in Plate 2 and illustrated diagrammatically in Fig. 3. The light from the tungsten source (2) in the Zeiss Photomicroscope II (4) is powered by a Zeiss GTF 12/60 voltage stabilizer (1). This light is modulated by a light modulator (3) which consists of a rotating sectored disc and is reflected from the silver grains to the photometer head where the detector (6) produces an alternating photoelectric current which is amplified by an AC amplifier

Plate 1. Stage of Zeiss Photomicroscope II fitted with motor.

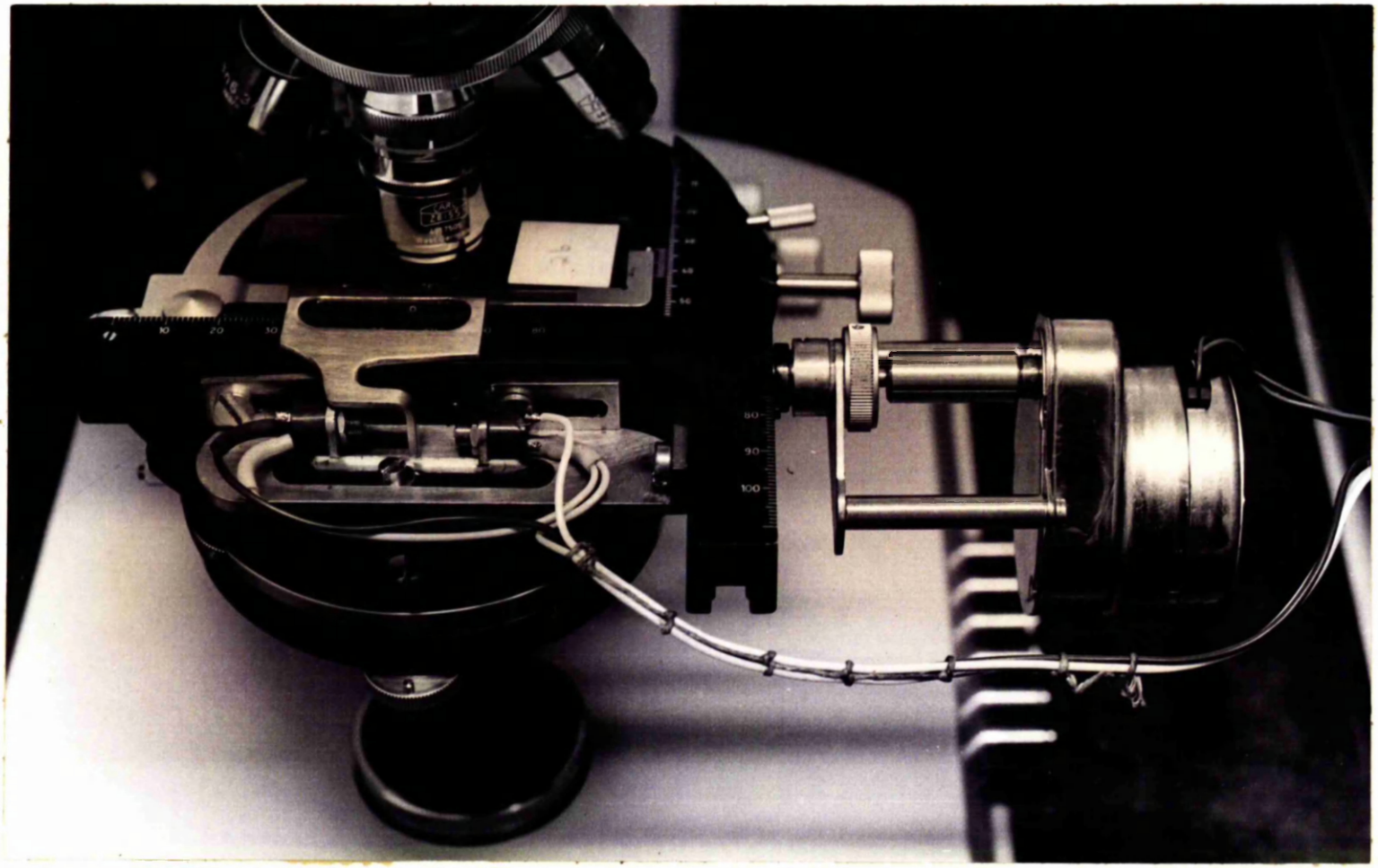


Plate 2. Equipment used in the determination of silver grain density. For explanation see text.

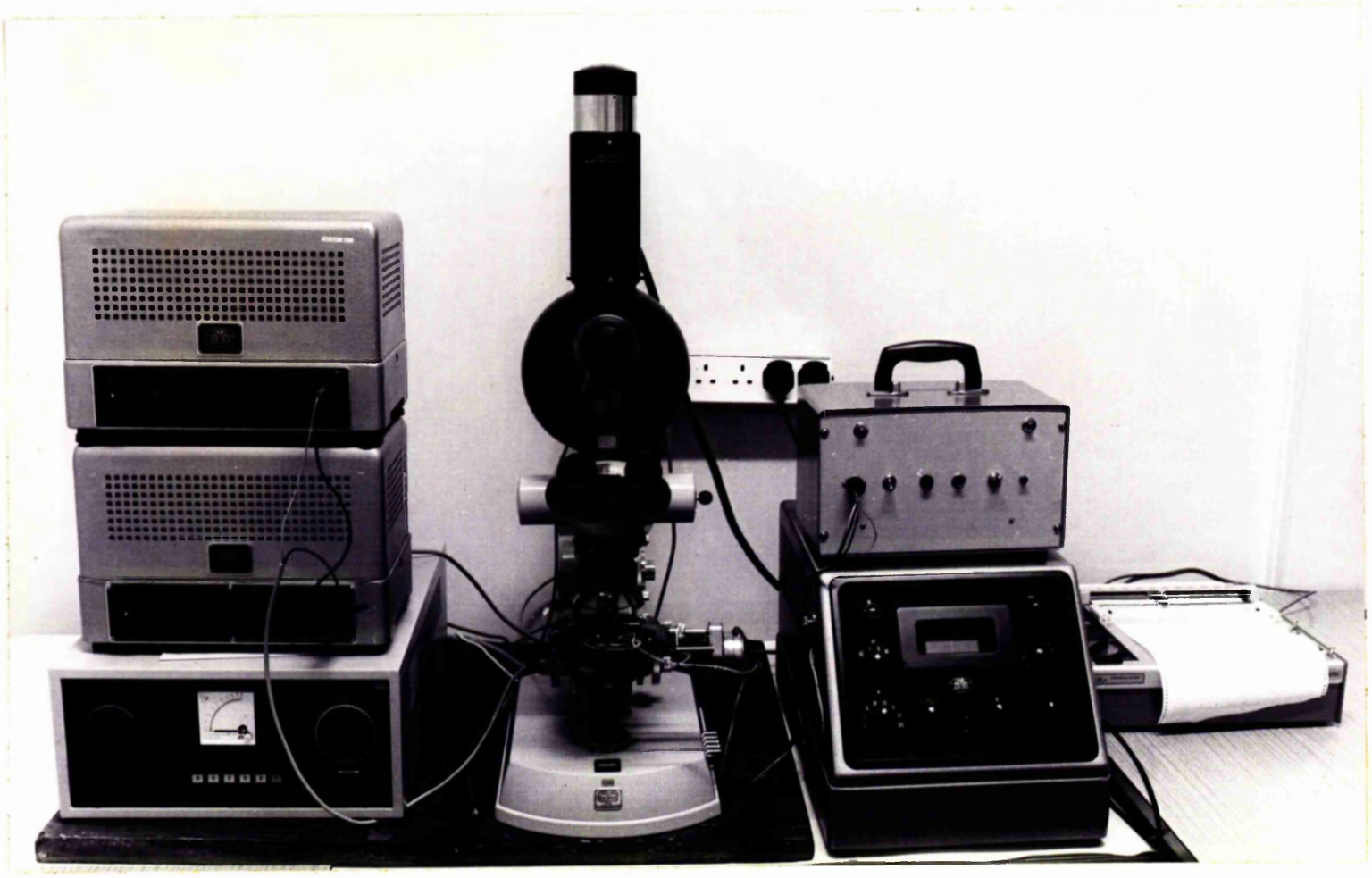


Fig. 3. Block diagram of equipment comprising the Zeiss MPM microscope photometer used in the determination of silver grain density.

(1) GTF 12v/60w voltage stabilizer.

(2) Illuminator containing a 60w tungsten light source.

(3) Light modulator.

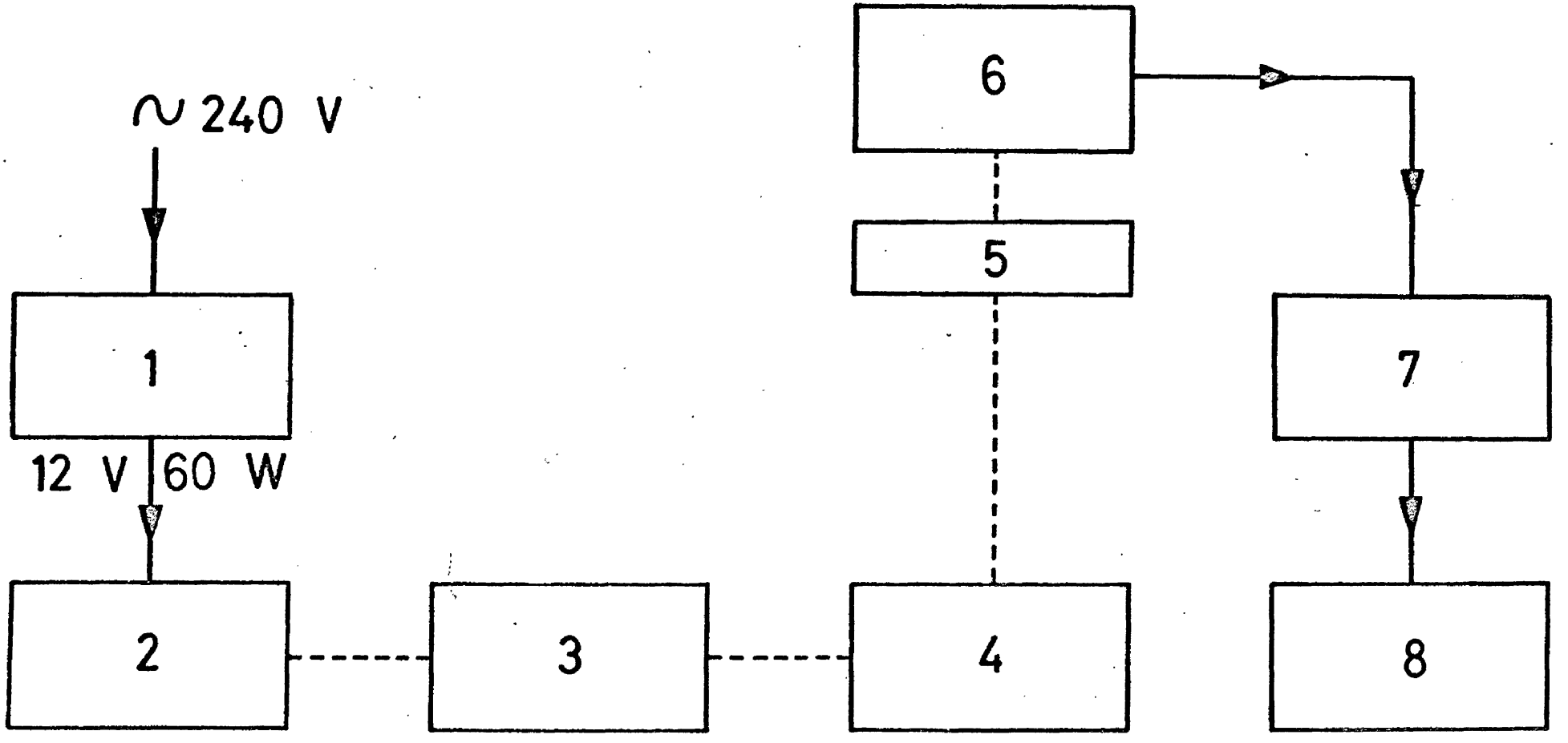
(4) Zeiss Photomicroscope II.

(5) Photometer head.

(6) Detector containing the photomultiplier tube.

(7) Display unit containing A.C. amplifier and galvanometer.

(8) "Telsec" potentiometric chart recorder.


~ 240 V
1
12 V 60 W
2
3
4
5
6
7
8

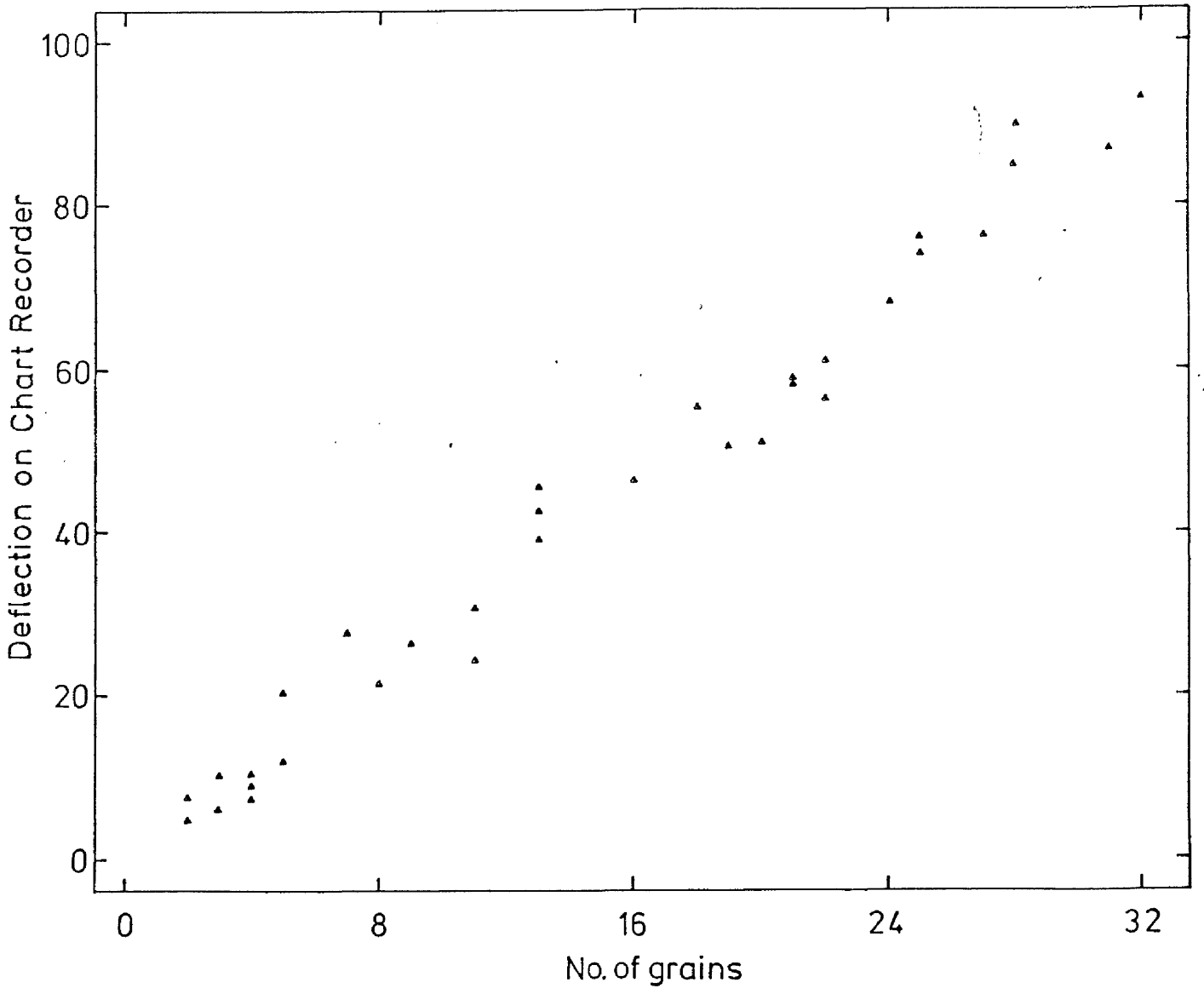
integrated with the display unit (7). AC amplification has a number of advantages which include good linearity, high stability and low zero line drift, in addition to not amplifying the dark DC current from the photomultiplier tube. The display unit in turn drives a Telsec fast response potentiometric chart recorder (8). Thus, when the motorised stage drives the section across the microscope stage at 3.0 mm hr^{-1} a strip of the autoradiograph is scanned. Care had to be exercised during scanning to avoid debris, and for each autoradiograph a large number of scans were made across a given area to ensure representative results.

The relationship between the deflection of the chart recorder and the number of silver grains within the area being observed is shown in Figure 4, and is in keeping with the findings of Bowen et al. (1972) and Shaw (1972).

PREPARATION OF "ARALDITE" EMBEDDED MATERIAL

The plant tissue was fixed in 3% cacodylate glutaraldehyde solution for a minimum of 4 h, and the glutaraldehyde subsequently removed by repeated washings in sodium cacodylate buffer (pH 7.2). The sodium cacodylate buffer was prepared by mixing 100 ml of 0.1M sodium cacodylate (21.4 g/l) with 8.3 ml of 0.1N hydrochloric acid. After careful removal of the glutaraldehyde, the tissue was given a post fixation treatment of 2 h in 1% cacodylate osmic acid. The tissue was again washed for 2-3 h in several changes of cacodylate buffer and dehydrated through an alcohol series (25%[40 mins] → 50%[40 mins] → 95%[40 mins] → 100%[1 h] → 100%[1 h]). This was followed by 1 h in propylene oxide, a process which completes dehydration and assists the infiltration of the "Araldite", and 12-18 h in a 1 : 1 solution of propylene oxide : "Araldite". The dehydrated tissue was then transferred through two changes of pure "Araldite", remaining in each for 2 h, to a final pure "Araldite" solution in which polymerisation took place over 24 h at 60°C under reduced pressure. The pure "Araldite" was prepared by mixing 46 ml of resin, 54 ml of hardener and 1.5 ml of accelerator.

Fig. 4. The relationship between the number of silver grains and the deflection of the chart recorder connected via a galvanometer to a Zeiss MPM photometer. The curve was constructed from an autoradiograph of a lettuce fruit and uses fixed pre- and post-amplification factors.


100
80
60
40
20
0
Deflection on Chart Recorder
0
8
16
24
32
No. of grains

The blocks were trimmed and sectioned on an L.K.B. Ultratome III, using glass knives. The knives were prepared on an L.K.B. Knife Maker Model 7801A and were fitted with a bath containing 15% alcohol solution. The sections, when cut, floated on the alcohol, and their thickness could be assessed by their colour. When relatively thick, they appeared blue, but as progressively thinner sections were cut, they underwent a sequential colour change through green, bronze/gold, silver/gold and silver to silver grey which was the colour of sections ideally suited to electron microscopic examination. These sections were floated on to clean copper grids, dried and stained. If the sections were to be examined on a light microscope, they were cut either 1 or 2 microns in thickness, placed in a drop of 15% alcohol on a microscope slide and dried. Staining and examination were then carried out.

Staining

Electron Microscope.

The staining solutions used were 2% uranyl acetate and lead citrate, both solutions being centrifuged at 2,500 rpm for 30 mins immediately before use to remove particles. The sections were placed in the uranyl acetate solution for 1 h, followed by careful washing in distilled water. Staining in lead citrate was for 30 mins in an atmosphere of low carbon dioxide content. This was achieved by placing the tissue and stain in a closed container with a few pellets of sodium hydroxide. The sections were then washed and dried, after which they were ready for electron microscopic examination.

Light Microscope.

The sections were stained in toluidine blue by one of two techniques. The first method involved immersing the sections in a solution of 0.1% toluidine blue in 1% borax (1 : 1 mix) for 10 mins. The sections were

washed in 1% borax and distilled water and mounted in Canada balsam. The second method was that of Sidman et al., as modified by Feder and O'Brien (1968). It involved staining the sections in a solution of 0.05% toluidine blue in benzoate buffer pH 4.4, for up to 5 mins. The sections were then washed in water and mounted as already described.

METABOLISM OF ABA

Lettuce fruits var. Great Lakes were incubated in a 2 dram vial with 0.6 ml of 10^{-5}M 2[^{14}C(±)] ABA in diffuse white light (0.92 watts m^{-1}) at 25 ± 1°C. After incubation, the fruits were washed in a Büchner funnel as described earlier. They were then extracted for 48 h in the dark, at 4°C with water, absolute methanol, 80% methanol, 80% ethanol or acetone. In most experiments, the fruits were ground using a pestle and mortar, before extraction. The solvent was removed by filtration, and the residue washed and assayed for unextracted activity. The filtrate was reduced to dryness under reduced pressure on a Büchi thin film evaporator. The radioactivity was resuspended in 80% redistilled methanol and separated by either silica gel thin layer or descending paper chromatography. Plastic-backed precoated 0.25 mm silica gel thin layer chromatography (TLC) plates were supplied by Mackeroy-Nagel & Co., Germany and glass-backed plates by E. Merck, Germany. If the TLC plates were not assayed on the radiochromatogram scanner, where it is necessary for the silica gel to be bound to the surface, then they were prepared on a Shandon Unoplan automatic thin layer spreader. The chromatography paper used was either Whatman No. 1, for qualitative work, or Whatman 3MM for preparative work. Aliquots of extract and marker spots of stock solution were applied to the starting line using fine glass pipettes. The thin layer chromatograms were developed for 100 mms in one of three solvent systems, viz. chloroform : methanol : water (75 : 22 : 3), butanol : acetic acid : water (5 : 1 : 2.2) or benzene : acetic acid (50 : 20). The paper chromatograms were developed in an ascending manner for 300-400 mms in

butan-1-ol : propan-1-ol : 0.88 ammonia : water (2 : 6 : 1 : 2).

After development, the chromatograms were scanned on a Panax Radiochromatogram scanner, cut into half Rf zones, and assayed on a Packard Scintillation Spectrometer as already described. The Rf values obtained for peaks of radioactivity were confirmed by elution from the chromatogram after development in one system with subsequent scanning, and redeveloping the radioactivity in a different solvent system. An alternative to elution and respotting was the use of 200 x 200 mm TLC plates which, after initial development, could be dried, turned through 90° and redeveloped.

EXTRACTION OF ENDOGENOUS ABSCISIC ACID

The plant tissue was immersed in each of 3 changes of 80% methanol, for 24 h. The methanol was reduced to approximately half the aqueous volume under reduced pressure on a Büchi thin film rotary evaporator to ensure removal of all traces of methanol. The remaining aqueous portion was centrifuged at 18,000 rpm (35,000 g) for 2 h on an MSE Superspeed 50 Ultracentrifuge, after which the supernatant was poured off, the residue resuspended and centrifuged again. The two supernatant portions were bulked. The pH was adjusted to 3 using 1N H_2SO_4 and extracted three times with one third of its own volume of ether. The ether phase was re-extracted alternately with one third its volume of 5% sodium bicarbonate and one sixth its volume of water (three bicarbonate and 2 water extractions). The water and bicarbonate fractions were then bulked and the pH adjusted to 8. Two further extractions using one quarter of its volume of diethyl ether were carried out, to remove neutral compounds. The pH of the aqueous phase was adjusted to 3 using 6N H_2SO_4 and partitioned three times with half its volume of ether. The ether phase was placed in a deep freeze overnight, to freeze out any water, after which the ether was removed and the extract redissolved in a small volume of methanol.

The extract was then further purified by one of two methods. The first

involved applying the extract to a TLC plate which had been eluted with methanol, and developing the plate in chloroform : methanol : water (75 : 22 : 3). This was followed by further purification in two other solvent systems, viz. benzene : acetic acid (50 : 20) and butanol : propanol : ammonia : water (2 : 6 : 1 : 2), after which the sample was eluted in a small volume of 0.05N methanolic sulphuric acid.

The second method also involved applying the extract to a TLC plate, but this time it was developed repeatedly in hexane : ethyl acetate (1 : 1), after which the zone corresponding with ABA was eluted, methylated with excess diazomethane and rechromatographed in the same system. The chromatographic properties of the methylated ABA were sufficiently different from those of ABA itself to ensure a good purification. The ABA zone was then eluted in methanol.

The efficiency of extraction was ascertained by adding a small quantity of 2[^{14}C(±)] ABA to the extracting tissue at the beginning of extraction. The levels of this were monitored throughout the procedures, thus yielding a good measure of the efficiency. The presence of this synthetic ABA did not complicate the quantitative assessment procedures, since the method used was circular dichroism, which is not sensitive to racemic ABA. A second advantage of the internal standards was that they provided an easy method of locating the ABA zone on the TLC plates. Two other methods of doing this involved the use of a marker spot of 2[^{14}C] ABA or of unlabelled ABA developed on a TLC plate made of Silica Gel GF_{254} where the presence of the fluorescent additive ensured that under ultra violet radiation of wavelength 254 nm, ABA showed as a dark area.

The actual quantification and identification of ABA was achieved by the use of circular dichroism measurements obtained on a Cary Model 6003 Spectropolarimeter with circular dichoism attachment, where the characteristic properties of ABA served as a guide to its presence.

These properties, as described by Milborrow (1967), are a positive peak

at 262 nm, a negative peak at 230 nm and a further small negative at 320 nm. A further characteristic is that the ratio of the ellipticity at 262 nm (ψ_{262}) to the ellipticity at 230 nm (ψ_{230}) is 1.2. Milborrow also quotes the differential molar absorptivity (Δ_E) at 262 nm as +39.5 degrees cm^2 $d\ mol^{-1}$, and from this the concentration of non-racemic ABA in a solution can be calculated using the determined ellipticity values as shown :-

ellipticity ψ = 3,300 ΔA

where ΔA is the differential absorbence of the sample

molar ellipticity $[\psi]$ = 3,300 ΔE

where ΔE is the differential molar absorptivity

$$[\psi] = \frac{\psi}{l \times c}$$

where l = path length in cm

c = concentration in d mol cm^{-3}.

Table 3. Flow Diagram of Extraction Procedure for ABA

Extract in 80% Methanol at 4°C

3X

↓

Reduce to aqueous

↓

Centrifuge at 18,000 rpm for 2 h

↓

Adjust pH to 3 and shake with diethyl ether

3X

↙ ↘

Aqueous fraction — Ether fraction (Acidic fraction)

↓

Shake alternately with $NaHCO_3$ (3X) and water (2X)

↙ ↓

Aqueous fraction — Ether fraction

↓

Adjust pH to 8 and shake with ether

2X

↙ ↓

Aqueous fraction — Ether fraction (neutral)

↓

Adjust to pH3 and shake with ether

3X

↙ ↓

Aqueous fraction — Ether fraction

↓

Chromatography

GAS LIQUID CHROMATOGRAPHY

Gas liquid chromatography was carried out on a Pye Unicam Series 104 Gas Liquid Chromatograph fitted with an OV 210 column. The carrier gas was nitrogen.

Samples of ABA or extracts were methylated by mixing with excess diazomethane which was afterwards evaporated. The methyl ester of abscisic acid was then resuspended in methanol and injected into the column, normally with an appropriate quantity of octakozane as internal standard.

Preparation of Diazomethane

Sodium hydroxide (20 g) was dissolved in 50 ml water in a 500 ml narrow neck conical flask, and a layer of 100 ml of ethylene glycol monoethylether (or ethylene glycol or diethylene glycol) placed over it. A third layer consisting of 25 ml of ether was added. A quantity of nitrosan was also placed in the flask and the liberated gas collected in cold ether in a second conical flask, by means of a delivery tube.

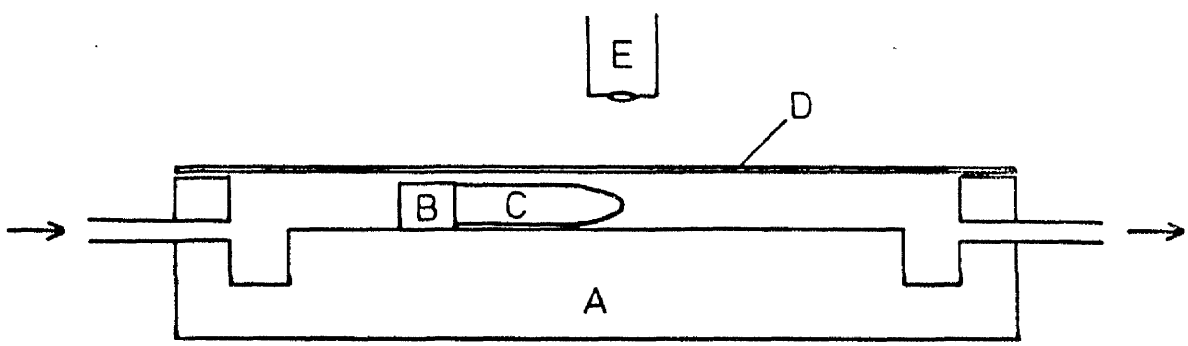
MEASUREMENT OF GROWTH DURING SHORT TIME PERIODS

1. Microscope Technique

A slide tissue culture chamber (Sterilin Ltd.) was modified as shown in Fig. 5. This involved inserting two fine capillary tubes into the cell, one at each side, and fixing a small piece of plastic excentrically on the central platform.

Tissue was positioned such that one end was in a fixed position against the plastic insert while the other could extend freely. The chamber was covered with a cover glass and sealed around its edges by "Vaseline". Solution was passed into the chamber through one of the capillary tubes and allowed to escape from the other by a simple siphon arrangement, hence the tissue was thus continually bathed in solution. The temperature of this

Fig. 5. Diagrammatic representation of the use of the modified slide tissue culture chamber. (A) tissue culture chamber, (B) perspex stop, (C) plant tissue, (D) cover glass, (E) microscope.


E
D
B
C
A

solution was easily controlled and its constitution could be altered by changing the source.

The chamber was positioned on the stage of a Vicker's Photolux Microscope. Elongation of the tissue was measured by means of a calibrated graduated eyepiece.

2. Time Lapse Photography

A Vinten Mark 3 16 mm scientific camera was mounted on an aluminium frame and connected to a Vinten Intervalometer. The aim was to use this equipment to obtain a series of photographs of lettuce fruits during the process of imbibition and germination. After preliminary experiments, the most appropriate experimental design was found to be with the lettuce fruits positioned on moist filter paper inside a chamber constructed from a 90 mm petri dish and with a solution reservoir. For photographic reasons, the light coloured fruits were displayed against a dark background of Whatman No. 29 filter paper. To avoid problems of condensation on the inner surfaces, a square of black filter paper was inserted into a circle of white filter paper.

The dish containing the fruits was orientated at 60° to the horizontal so that the radicle after emergence was not induced to curve, and measurement was made easier. Frames of film were exposed at intervals of five minutes, so that a record of the processes occurring during imbibition and radicle emergence could be constructed.

STATISTICAL ANALYSIS OF RESULTS

An Olivetti Programma 101 desk top computer was used to calculate the standard error of the mean for each series of observations. It utilized the expression :-

$$\text{Standard error (S.E.)} = \frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n(n-1)}$$

x = the individual value of each observation

n = the 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 twice the standard error.

Where the mean values of two randomised groups were to be compared, a student's "t" test was used. This was also calculated using the Olivetti 101.

Significance levels for "t" values were extracted from Fisher and Yates, Statistical Tables p.46, and the following symbols used to denote the probability of a difference being significant :-

N.S.	not significant at the 95% probability level
*	greater than 95%
**	greater than 99%
***	greater than 99.9%

In a number of experiments where tests of significance were carried out on data expressed as percentages, it was essential that an angular transformation was first carried out.

Analysis of Variance

Where the effects of a number of factors were to be compared at the same time, the data were subjected to an Analysis of Variance. In this thesis a number of factorial designs were employed. The analyses were carried out on the Olivetti 101.

EXPERIMENTAL

(1) THE EFFECT OF ABSCISIC ACID ON LETTUCE GERMINATION

Abscisic acid (ABA) inhibits lettuce fruit germination (Aspinall et al., 1967; Khan, 1968; Shankla and Shankla 1968) although the reports do not present a detailed account of precise germination behaviour. In the first instance it was decided to investigate in detail the germination behaviour pattern.

Lettuce fruits var. Great Lakes were incubated in either water or aqueous solutions of abscisic acid (ABA). The germination was recorded at 2-h intervals up to the eighteenth hour, 3-h intervals until the twenty-fourth hour, 12-h intervals until the sixtieth hour, and thereafter at intervals of 24 h. The pattern of germination with time (Fig. 6) revealed that germination of the fruits normally occurred between 10 and 12 h after the start of imbibition. When ABA was present in the incubation medium germination was delayed, and the length of the delay increased with the germination of ABA. After 30 h, 96% had been achieved in 10^{-6}M ABA solution, but in 10^{-5}M and 10^{-4}M this was only 20% and 0% respectively. At all concentrations of ABA, the germination curve was characteristically sigmoidal.

If, however, the results of all germination assays are expressed as shown, any comparison of results would involve the complicated procedure of curve analysis, and in the case of factorial experiments the results would become almost impossible to express graphically because of the presence of four parameters, viz. % germination, time, ABA concentration and GA_3 concentration. A simpler method of expressing the results was therefore sought.

One method is to record germination after a fixed time, normally 24 h, and to assume that, if germination is complete after this time, no inhibition

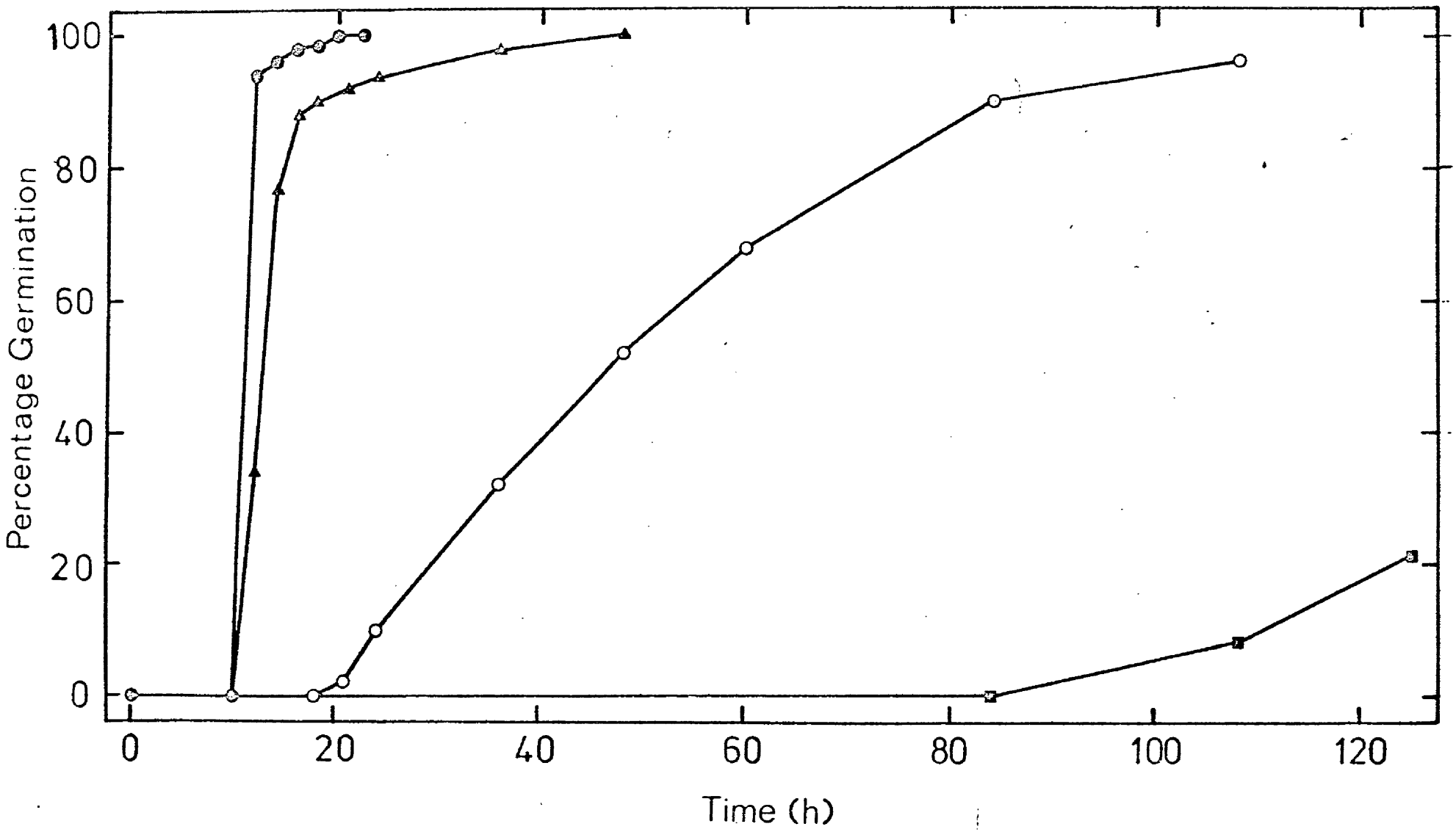
Fig. 6. Germination of lettuce fruits var. Great Lakes as a function of time, and the effect of ABA on that germination.

closed circle :- water.

closed triangle :- 10^{-6}M ABA.

open circle :- 10^{-5}M ABA.

closed square :- 10^{-4}M ABA.


Percentage Germination
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (h)

has occurred. If, on the other hand, inhibition is occurring, the degree of inhibition is assumed to be indicated by the number of fruits still ungerminated. Nevertheless, on adopting this criterion for analysis of the data in Fig. 6, it could be concluded that ABA at 10^{-6}M exhibited no inhibitory effect, and the difference between the effects of 10^{-5}M and 10^{-4}M solutions of ABA would be barely discernible. It was, therefore, obvious that such a method of assessment, although often employed, was too insensitive for the analysis of very small differences in activity.

Another method would be to measure the time taken for all the fruits to germinate, but since 100% germination rarely occurred, this method was clearly impractical. When the procedure was modified, however, such that the time required for 90% of the fruits to germinate was recorded, the results shown in Fig. 7 were obtained. From this figure it can be seen that this is a relatively sensitive method for detecting inhibitory influences, but there was one major disadvantage which was apparent in Fig. 6. At 90% germination, the slope of the curve was very shallow, with the result that small variations on the ordinate (germination percentage axis) would lead to disproportionately large variations on the abscissa (time axis). Bearing this shortcoming in mind, the most logical approach for estimation of inhibitory activity seemed to be to assess the time at the steepest point on the curve, this occurring at about 50% germination. The mean time to 50% germination (MTG) was therefore calculated for each treatment, a procedure carried out with the aid of the Olivetti 101 table top computer, using a purpose designed programme. This programme utilized the expression:-

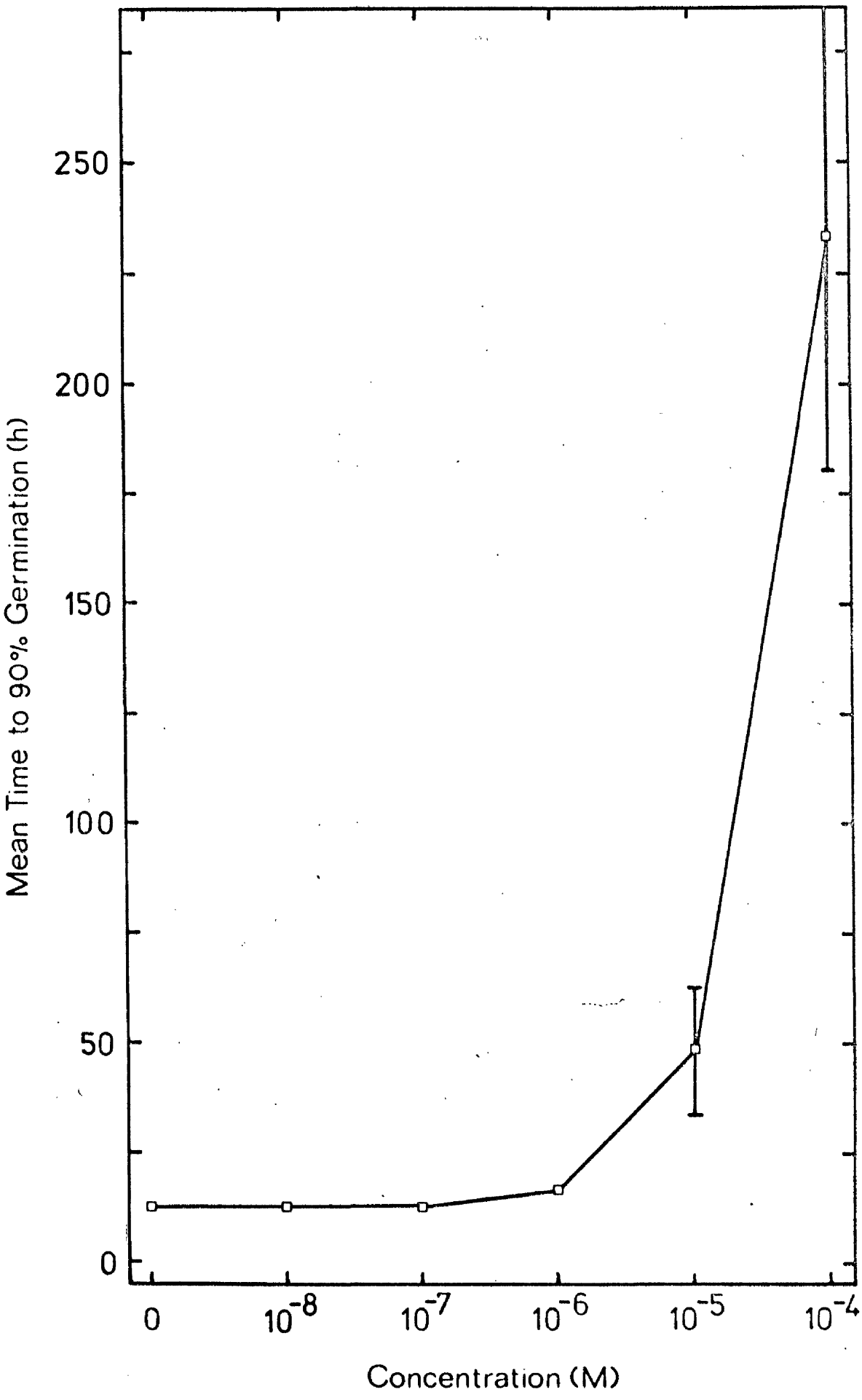
$\frac{-a + b}{c}$ = the slope of the curve at 50% germination.

where a = the number of fruits germinated at the time of assessment immediately before that number exceeded 50% of the total.

b = the number of fruits germinated at the time of assessment immediately after that number exceeded 50% of the total.

c = the interval between the times of assessment of a and b.

Fig. 7. The mean time to 90% germination of lettuce fruits var. Great Lakes as a function of ABA concentration.


250
200
150
100
50
0
Mean Time to 90% Germination (h)
0
10^-8
10^-7
10^-6
10^-5
10^-4
Concentration (M)

$\therefore \frac{x - b}{\text{slope}}$ gave the time required for the number of fruits germinated to increase from "a" to "x"

where x = 50% of the total number of fruits in the test.

It may be argued that a further method of assessing the amount of inhibition is to utilise the slope of the germination curve. In view of the fact that the slope of the curve was constantly changing, this procedure was not adopted.

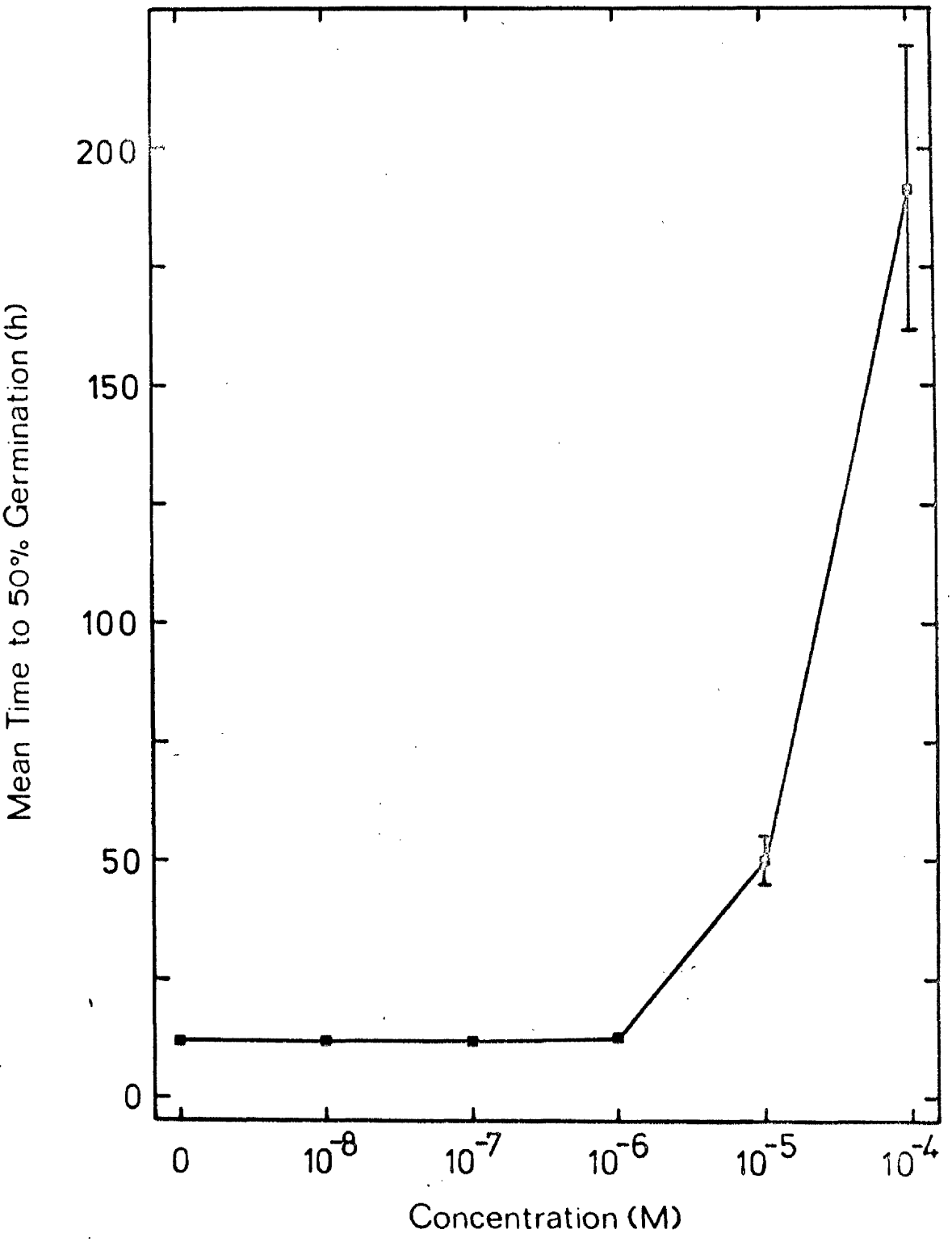
To obtain a more complete picture of the effect of ABA on germination, lettuce fruits were imbibed in 0, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-4}M solutions and germination recorded as described earlier. The mean time to 50% germination was calculated for each treatment, and the results are illustrated in Fig. 8. Each ABA treatment mean was then compared to the mean of the water control by carrying out student's "t" tests (Table 4).

Table 4. The effect of ABA on lettuce germination.

ABA concentration (Molar)	Mean Time to 50% germination (Hours)	Standard Error (±)	Significance of Inhibition (t tests)
0	11.68	0.28	-
10^{-8}	11.47	0.16	NS
10^{-7}	11.63	0.22	NS
10^{-6}	13.00	0.25	**
10^{-5}	49.80	4.44	***
10^{-4}	192.42	30.13	***

Fruits imbibed in 10^{-6}M ABA solution did not germinate until 13.0 ± 0.25 h after the start of imbibition, as compared with 11.68 ± 0.28 h for fruits imbibed in water. This difference was significant at the 1% level of probability. When the concentration of ABA was increased to 10^{-5}M

Fig. 8. The mean time to 50% germination of lettuce fruits var. Great Lakes as a function of ABA concentration.


Mean Time to 50% Germination (h)
200
150
100
50
0
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration (M)

and 10^{-4}M, the mean times to 50% germination also increased markedly to 49.8 ± 4.44 h and 192.42 ± 30.13 h respectively. Concentrations lower than 10^{-6}M did not elicit a significant response. The inhibition was not due to the presence in the incubation medium of organic solvents used for dissolving the ABA because, when solvent controls were assayed, it was found that 350 ppm of methanol in water, the maximum concentration used, had no significant effect on germination (Fig. 9). It can, therefore, be concluded that ABA, when present in sufficient quantity, inhibits the germination of lettuce fruits var. Great Lakes. The inhibition is, however, temporary and is best described as a delay in germination.

(2) THE EFFECT OF ANALOGUES OF ABA ON LETTUCE GERMINATION

Exogenously applied ABA has been shown to delay the germination of lettuce fruits var. Great Lakes. The question thus arises as to the molecular requirements of the ABA molecule for inhibitory activity and to gain an understanding of this, the activities of compounds structurally related to ABA were investigated.

Thirty-two analogues of ABA were, therefore, assayed for their effect on lettuce germination. The structures of the analogues are shown in Table 1 where they are grouped within two major syntagmata, the first of which corresponds with that used by McWha *et al.* (1973). The analogues were tested using the standard assay procedure and at concentrations of 0, 10^{-7}, 10^{-6} and 10^{-5}M. They were dissolved as outlined in Table 2 and solvent, ABA and GA_3 controls included in every experiment. The same batch of lettuce fruits were also used throughout.

The effect of the analogues on germination is shown in Fig. 10. Certain of the analogues, e.g. I, II, XVII, possessed up to 50 times the activity of ABA, when activity is assessed as the quantity of compound required to produce a specific effect, while other, e.g. V, VI, XII, were totally inactive, even at the highest concentrations assayed. The MTG for

Fig. 9. The effects of solvents on lettuce germination. Acetone was assayed at concentrations up to 100 ppm in water and methanol at concentrations up to 700 ppm. Methanol (200 ppm) was also assayed in combination with $NaHCo_3$ (8 ppm). None of the treatments significantly affected the MTG.

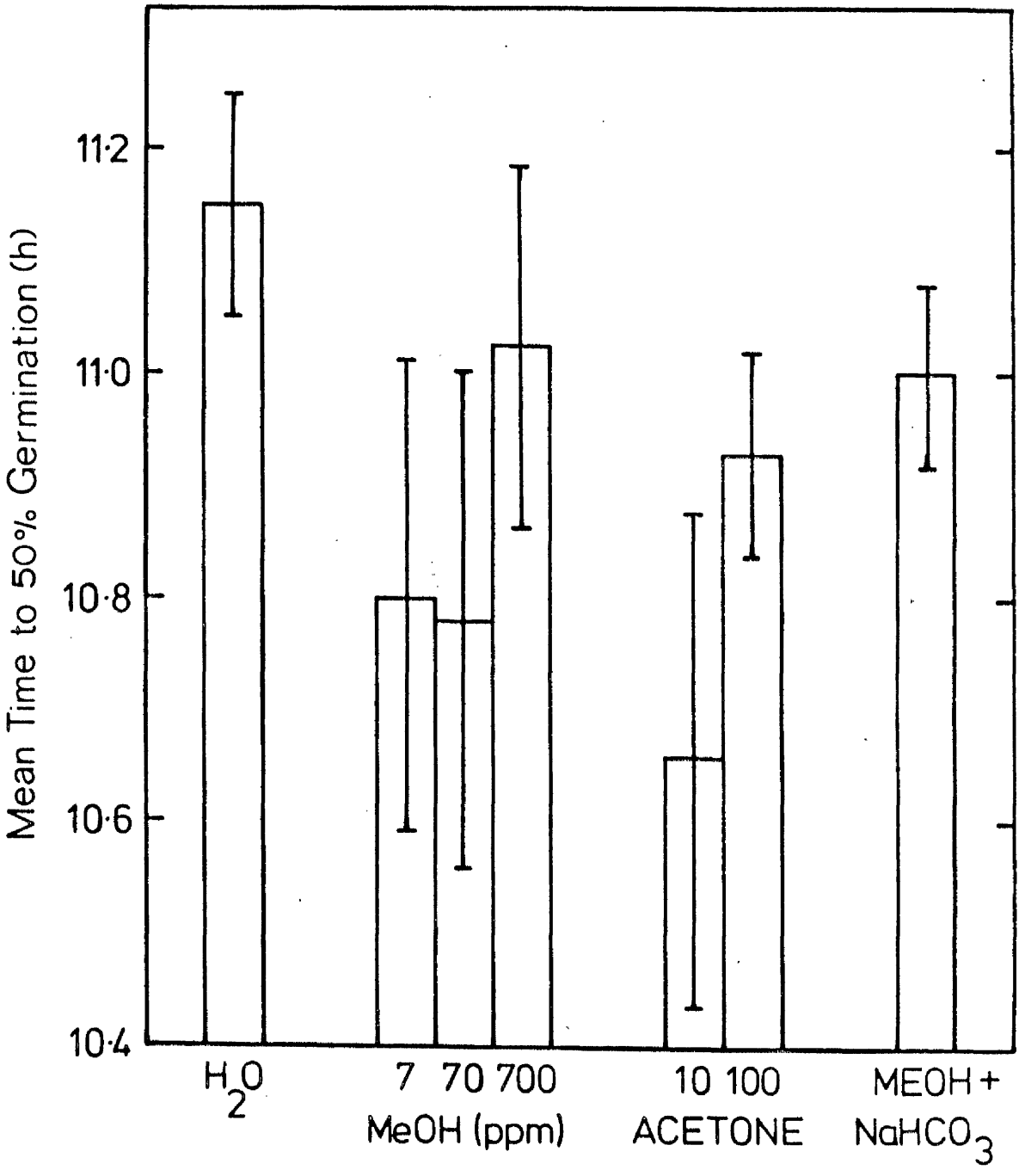
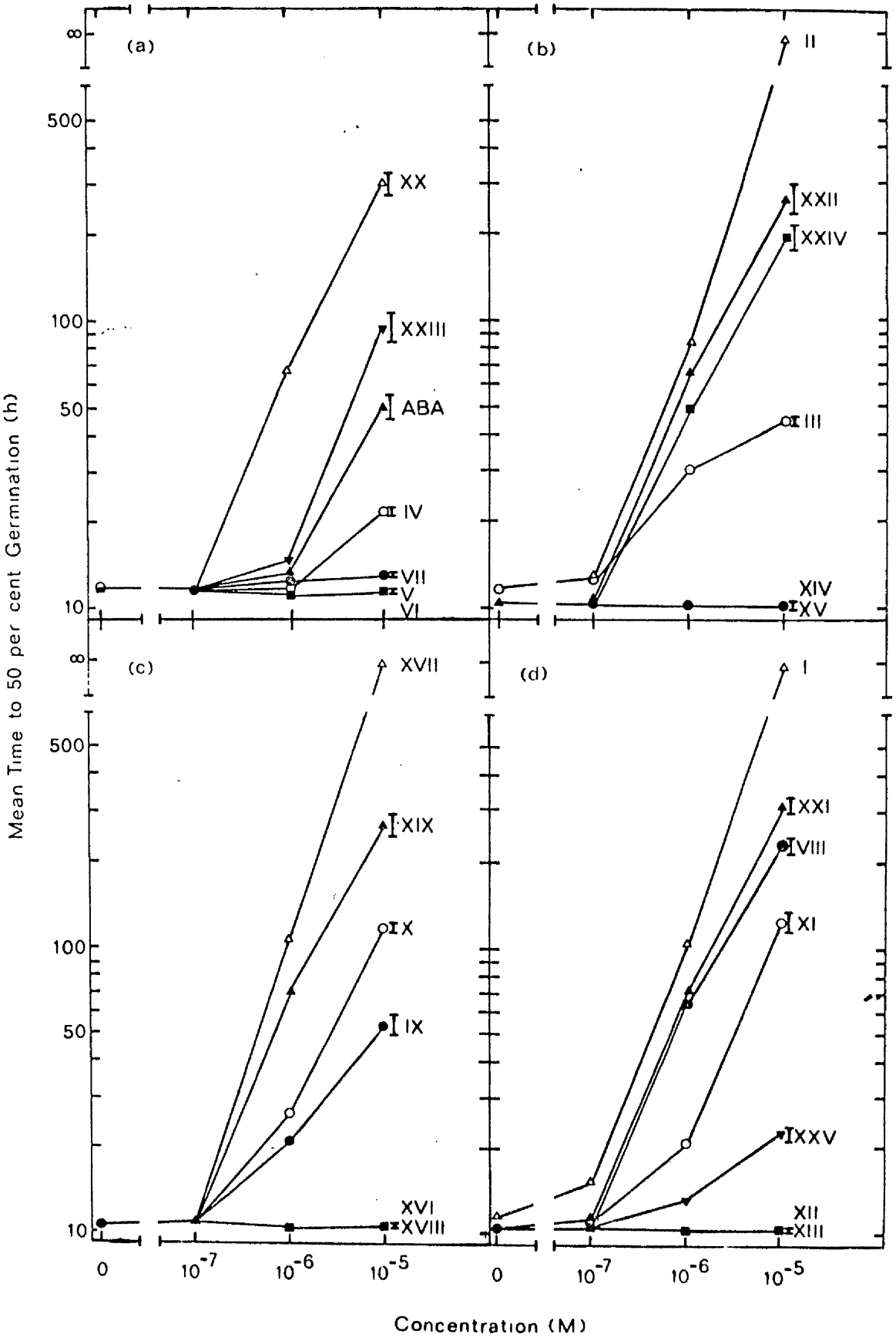

Mean Time to 50% Germination (h)
11·2
11·0
10·8
10·6
10·4
H2O
7 70 700
MeOH (ppm)
10 100
ACETONE
MEOH +
NaHCO3

Fig. 10. The inhibitory activity of ABA and 32 analogues in the lettuce fruit germination assay. Analogues not represented in this figure were among those which did not significantly affect germination.


(a)
(b)
(c)
(d)
∞
500
100
50
10
XX
XXIII
ABA
IV
VII
V
VI
II
XXII
XXIV
III
XIV
XV
XVII
XIX
X
IX
XVI
XVIII
I
XXI
VIII
XI
XXV
XII
XIII
0
10^{-7}
10^{-6}
10^{-5}
Mean Time to 50 per cent Germination (h)
Concentration (M)

the fruits imbibed in each analogue was compared with the MTG for those incubated only in water. This was carried out, using the student's "t" test, for all the analogues at each concentration assayed, and the levels of probability of the compounds being active inhibitors are shown in Table 5.

Table 5. The statistical significance of the inhibition of lettuce germination resulting from the application of ABA and its analogues and the effect of GA_3 on that inhibition.

Compound	Concentration of compound (M)			Substance at 10^{-5}M plus GA at 10^{-5}M vs substance at 10^{-5}M
	10^{-7}	10^{-6}	10^{-5}	
ABA	NS	**	***	NS
I	***	***	——	NS
II	NS	***	——	NS
III	***	***	***	NS
IV	NS	***	***	NS
V	NS	NS	NS	NS
VI	NS	NS	NS	NS
VII	NS	NS	***	NS
VIII	**	***	***	NS
IX	NS	**	***	*
X	*	***	***	NS
XI	NS	***	***	NS
XII	*	NS	NS	NS
XIII	*	NS	NS	NS
XIV	NS	NS	*	NS
XV	NS	NS	NS	NS
XVI	NS	NS	NS	NS
XVII	***	——	——	NS
XVIII	NS	NS	NS	NS
XIX	NS	***	***	NS
XX	NS	***	***	NS
XXI	NS	***	***	NS
XXII	NS	***	***	NS
XXIII	NS	**	***	NS
XXIV	**	***	***	NS
XXV	NS	*	***	NS
XXVI	NS	NS	NS	NS
XXVII	NS	NS	*	NS
XXVIII	NS	NS	*	NS
XXIX	NS	*	NS	NS
XXX	NS	NS	NS	**
XXXI	NS	NS	NS	NS
XXXII	*	NS	NS	NS

From this it can be seen that compounds I, II, VIII, X, XI, XVII, XIX, XX, XXI, XXII, XXIII and XXIV were considerably more active than ABA, at all concentrations tested. ABA at 10^{-5}M delayed germination from 11.68 $\pm$ 0.28 to 49.8 $\pm$ 4.44 hours after the beginning of imbibition and, as reported earlier,

its effect extended over the concentration range to 10^{-6}M. Compound XVII, on the other hand, inhibited germination significantly, even at 10^{-7}M, delaying germination from 9.62 ± 0.08 h to 10.63 ± 0.09. At 10^{-6}M, its effect was so great that the fruits had still not germinated when the experiment was terminated after 444 h. It is of great interest to note that this active compound was not toxic and could be leached from the fruits which then germinated. I, VIII, X and XXIV, although not as active as XVII, were all significantly active at concentrations of 10^{-7}M.

The reason for the greater activity of these compounds is not immediately apparent. It is possible that their structure is an improvement over that of ABA with regard to attachment at the active site(s). On the other hand, it may be that the tissue takes them up faster and/or metabolises them more slowly than ABA. If they are assumed to have the same mode of action as ABA, any moieties absent from these compounds, but present in ABA, can be reasoned to be not essential for ABA-like activity. It must, however, be remembered that an active moiety might have been removed, but its place taken by an equally active or more active replacement, or that an interaction between moieties may be occurring.

Either of these latter interpretations may apply to the carboxyl group where, with the exception of XXV, all compounds in which it was altered in any way were at least as active as ABA. Compounds included in this are the abscisic alcohol, I, where at 10^{-5}M, germination had not occurred even after 444 h, the abscisic hydrocarbon (II) which showed the same germination characteristics at 10^{-5}M as I, and the abscisic aldehyde (III) which delayed germination until 47.22 ± 1.9 h. The inhibitory activity of compound III was statistically significant at 10^{-7}M and was greater than that possessed by ABA at the same concentration. At 10^{-6}M it was again more active than ABA, but at 10^{-5}M the activities of the two were of the same order.

In the other compounds were the carbonyl group was altered, it was replaced by larger moieties, e.g. XI, and activity was maintained. It thus

seems that the carboxyl group is not essential for ABA-like activity and that it may be a factor which limits ability of ABA to inhibit germination, since its replacement frequently resulted in increased activity. The compound which was the exception to the above observations in being less active than ABA was XXV, where only the carboxyl group of ABA had been altered. At 10^{-5}M, it delayed germination until 25.28 $\pm$ 1.80 h after the start of imbibition, compared with 49.80 $\pm$ 4.44 h for ABA. This compound has a structure very similar to IX which delayed germination until 54.03 $\pm$ 5.21 h after the beginning of inhibition. The difference between the two is that the carbonyl group in the side chain of IX is replaced by a thioketone in XXV. This thioketone group may be the reason for the low activity in that steric hindrance or a toxic effect might occur. It should, however, be noted that IX, although as active as ABA, is considerably less active than compounds such as X and XI. The difference between them is in the nature of the moiety replacing the carboxyl group. In IX, and indeed XXV, it contains a number of nitrogen atoms which, possibly, constitute a steric or charge hindrance or, as is more likely, the plant can metabolise it to form ABA. Any difference then occurring would be due to uptake characteristics.

There is, however, other evidence that not all changes are conducive to increased activity. Esterification of the acid group in compounds IV and VII would result in compounds V and VI which lacked significant activity. Compound IV, on the other hand, possessed significant activity at 10^{-6}M and VII at 10^{-5}M. These compounds were also unlike ABA in other respects, differences which in themselves considerably retard activity, especially in the case of VII. Despite this, it appears that esterification of the acid group resulted in a marked loss of ABA-like activity.

The feature by which IV differed from ABA was that it lacked the carbonyl and hydroxyl groups in the cyclohexene ring and, accordingly, its activity was considerably reduced. Fruits imbibed in ABA at 10^{-5}M achieved

50% germination after 44.79 ± 3.36 h while those imbibed in IV at the same concentration required only 21.34 ± 1.20 h. The ring carbonyl and/or hydroxyl groups thus seem essential for ABA-like inhibitory activity. Furthermore, epoxidation of the ring double bond in IV gives rise to a compound (VII) with activity reduced still further. This compound in fact only possessed significant inhibitory activity at 10^{-5}M when the mean time to 50% germination was 12.88 ± 0.1 h. It can therefore be concluded that the ring double bond is also an important molecular constituent for the maintenance of ABA-like activity. Comparisons of XII, XIII, XIV and XVIII with II also illustrate that the ring hydroxyl and/or carbonyl groups, as well as the ring double bond, appear to be moieties of the ABA molecule essential for inhibition in the lettuce germination assay. In these compounds, the ring hydroxyl and carbonyl groups were absent and in all, some alteration was made to the ring double bond. The result was a total loss of inhibitory activity in XII and XIII, while XIV retained significant activity at 10^{-5}M and XVIII significant but low activity at 10^{-7}, 10^{-6} and 10^{-5}M. In XII and XIII, however, an epoxide residue had been introduced and this might have been responsible for the total lack of activity of the compounds as compared with XIV and XVIII where a significant amount of activity persisted. It is possible that the presence of the epoxide residue was in itself detrimental to activity, although this is by no means certain because, if it were true, XIV and XVIII would be expected to be more active than VII because they lack the epoxide and have the carboxyl substituted by a hydrocarbon group. They were, however, found not to be more active than VII and the effect of introducing an epoxide residue must remain ambiguous.

The side chain of the ABA molecule contains two unsaturated bonds. The double bond between C-4 and C-5 does not seem to be essential for activity in view of the fact that, like II, XVII was extremely active but differed in structure in that the double bond was replaced by a triple bond. No compounds in which this bond was saturated were available for testing. Nevertheless,

it is possible that the spatial configuration of the atoms in this part of the side chain is not important for activity, since these two compounds (II) and XVII) differ in arrangement. Only if the mechanisms or the sites of action of the two compounds were different would it be possible for this configuration to be an important contributory factor in activity, and in this case the effect produced would not be truly ABA-like. If, however, the double bond between C-2 and C-3 is saturated, the activity is lost. This can be shown by comparing compounds XV and XVI with XVII and II respectively. XV and XVI were similar to XVII and II respectively, except that, in the former pair, the double bond had been saturated and a hydroxyl group introduced at C-3. Compounds XVII and II were extremely active, e.g. when II was present at 10^{-6}M it delayed the mean time to 50% germination to 87.63 $\pm$ 6.99 h and XVII, at the same concentration, delayed germination for more than 444 h, while XV and XVI were not significantly active even at 10^{-5}M. It is therefore apparent either that the double bond is necessary for ABA-like activity or that the presence of a hydroxyl group at C-3 completely suppresses the activity. Indeed, XV and XVI were in fact the only compounds tested which had a complete ABA ring structure, yet exhibited no activity.

(3) INTERACTION OF ABA AND ITS ANALOGUES WITH GIBBERELLIC ACID IN LETTUCE GERMINATION.

ABA has been shown to delay germination in lettuce and a number of important structural requirements for inhibitory activity have been considered. Gibberellins have been shown to have a basic rôle in the regulation of germination, especially in overcoming natural dormancy. Aspinall et al. (1967) and Addicott and Lyon (1969) have reported that ABA induced dormancy can also be overcome by exogenous gibberellins, but Shaukla and Shaukla (1968) and Wareing et al. (1968) could find no evidence of such an effect. They employed a different type of analysis, however, to that employed in this

study and it was therefore decided to reappraise the situation.

The effects of ABA and GA_3 on lettuce germination were assayed in a factorial experiment of ABA solutions at concentrations of 0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M and GA_3 solutions at concentrations of 0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}M. The assay was set up in diffuse white light at 25°C using the variety Great Lakes. Germination was recorded at intervals up to 444 h and the results displayed in Fig. 11.

An analysis of variance of the results indicated that ABA effects, GA_3 effects, and the interaction between the two were all very highly significant (Table 6A). When, however, the results of only 0, 10^{-7}, 10^{-6} and 10^{-5}M applications of both hormones were considered, it was found that only the ABA effect was significant (at the 0.1% significance level) while the GA_3 effect and the interaction were non-significant (Table 6B).

Table 6. Analysis of variance of the MTG of lettuce fruits exposed to ABA and GA_3 in combination.

(a) 6 x 6 factorial experiment.

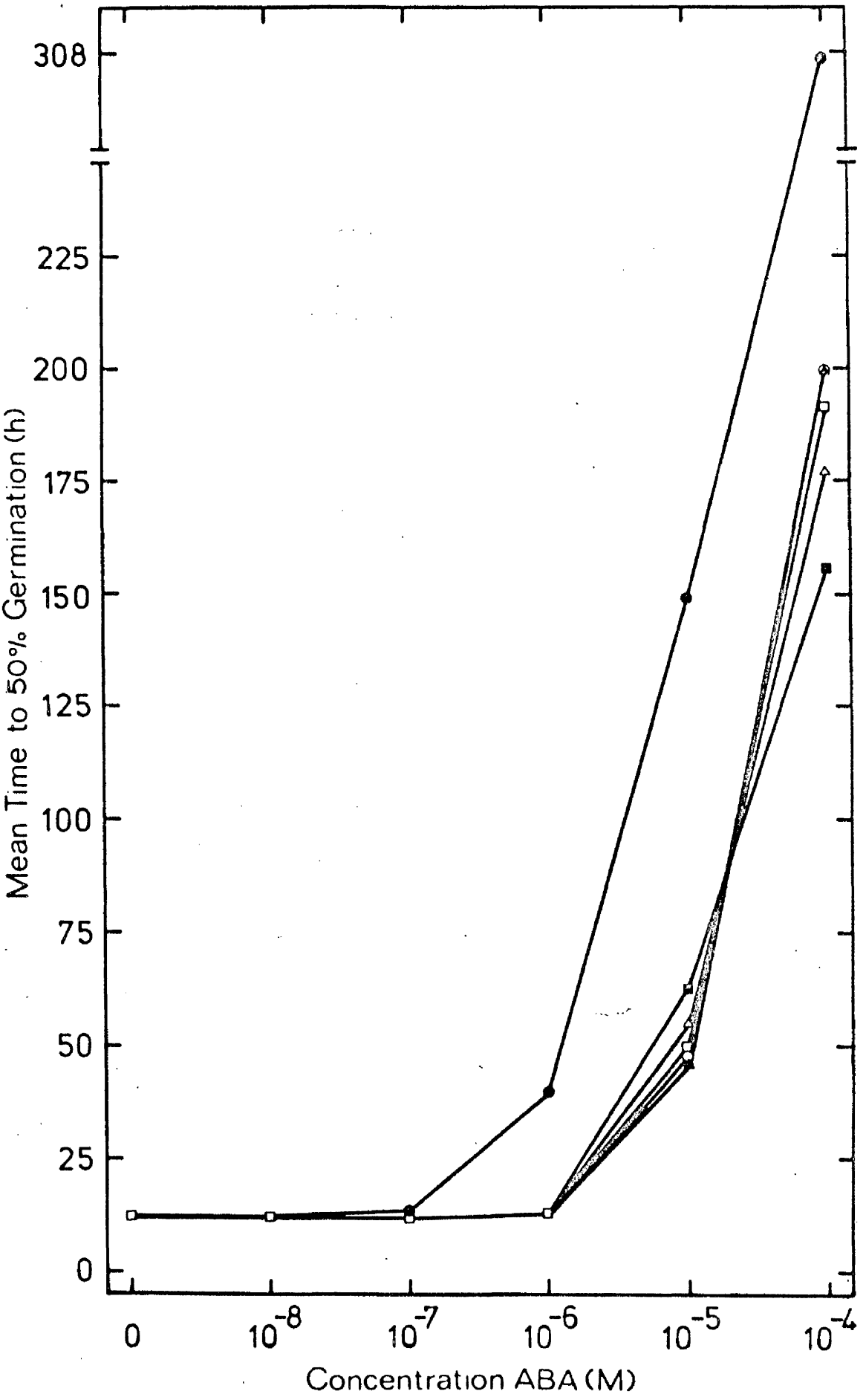
	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
ABA	1072468.3	5	214493.6	274.12	***
GA_3	52410.2	5	10482.0	13.39	***
Interaction	83978.2	25	3359.1	4.29	***
Error	140.846	180	782.4		

(b) 4 x 4 factorial experiment.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
ABA	25634.10	3	8544.70	247.82	***
GA_3	66.30	3	22.10	0.64	N.S.
Interaction	196.91	9	21.88	0.63	N.S.
Error	2758.34	80	34.48		

Fig. 11. The effects and interactions of ABA and GA_3 on lettuce germination.

Open square	:-	No GA_3
Open triangle	:-	10^{-7}M GA_3
Closed triangle	:-	10^{-6}M GA_3
Open circle	:-	10^{-5}M GA_3
Closed square	:-	10^{-4}M GA_3
Closed circle	:-	10^{-3}M GA_3


308
225
200
175
150
125
100
75
50
25
0
Mean Time to 50% Germination (h)
0
10-8
10-7
10-6
10-5
10-4
Concentration ABA (M)

When student's "t" tests were carried out on selected results, it was found that at concentrations of 10^{-6}M and higher, ABA significantly delayed germination (Table 4). Gibberellin A_3, on the other hand, was found to have no significant effect at the concentrations tested except when applied in combination with ABA, both being present in high concentration (Table 7). At 10^{-4}M ABA, the presence of 10^{-3}M GA_3 increased the mean time to 50% germination from 192.42 ± 30.13 h to 308.08 ± 35.00 h (Table 7).

Table 7. The effect of GA_3 on lettuce germination.

(a) No ABA present.

GA_3 concentration (Molar)	Mean time to 50% germination (h)	Standard Error (±)	Significance of Differences ('t' tests)
0	11.68	0.28	-
10^{-7}	11.76	0.23	NS
10^{-6}	11.54	0.16	NS
10^{-5}	11.55	0.21	NS
10^{-4}	11.64	0.30	NS
10^{-3}	11.96	0.32	NS

(b) ABA present at 10^{-5}M.

GA_3 concentration (Molar)	Mean time to 50% germination (h)	Standard Error (±)	Significance of Differences ('t' tests)
0	49.80	4.44	NS
10^{-7}	55.11	4.29	NS
10^{-6}	46.03	3.66	NS
10^{-5}	48.73	6.21	NS
10^{-4}	62.04	5.44	NS
10^{-3}	150.57	20.08	***

As with the ABA effect, this GA_3 induced inhibition could not be attributed to the presence in the incubation medium of organic solvents since

the GA_3 was dissolved directly in water. The effects observed must therefore have been due to the presence, in the incubation medium, of the plant hormones, and it is apparent from the results not only that GA_3 is incapable of reversing the ABA-induced inhibition of germination, but also that above a critical level of between 10^{-4} and 10^{-3}M, GA_3 itself can become inhibitory to germination.

The analogues were also assayed in combination with GA_3 to determine whether the effect on germination produced by many of these compounds was capable of reversal by GA_3. This reversal, if occurring, would indicate either that the ABA effect was made up of a number of individual components, at least one of which was GA_3 reversible, or that the compound in question had an entirely independent mode of action from ABA. The analogues found previously to be inactive were also assayed to determine whether or not they could interact with GA_3 either to promote or to inhibit germination. The compounds and GA_3 were assayed in factorial experiments consisting of the analogues at the concentrations 0, 10^{-7}, 10^{-6} and 10^{-5}M and GA_3 initially at the same concentrations, but in later experiments at only 0 and 10^{-5}M, the number of GA_3 concentrations being reduced once the form of the interactions had been established. The results obtained are illustrated in Figs. 12-19 and are presented, not necessarily in the order in which the compounds were received or tested. Analyses of variance were carried out on all analogues and the results are included with the appropriate figures (Tables 8-15).

In only a few cases were either the GA_3 or the interaction term significant. It can, however, be argued that since GA_3 alone did not produce a significant effect, then the consistent appearance of a GA_3 effect in the presence of an analogue would in itself be indicative of an interaction. In the presence of III, V, IX, XXIV and XXV, GA_3 has a significant effect on germination. Student's "t" tests were also carried out on the mean treatment values and the results abbreviated in Table 5, where the result obtained in the presence of analogue at 10^{-5}M, but in the absence of GA_3, are compared

Fig. 12. The effects and interactions of GA_3 and analogues of ABA on lettuce germination. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound I.

(B) compound II.

(C) compound III.

(D) compound IV.

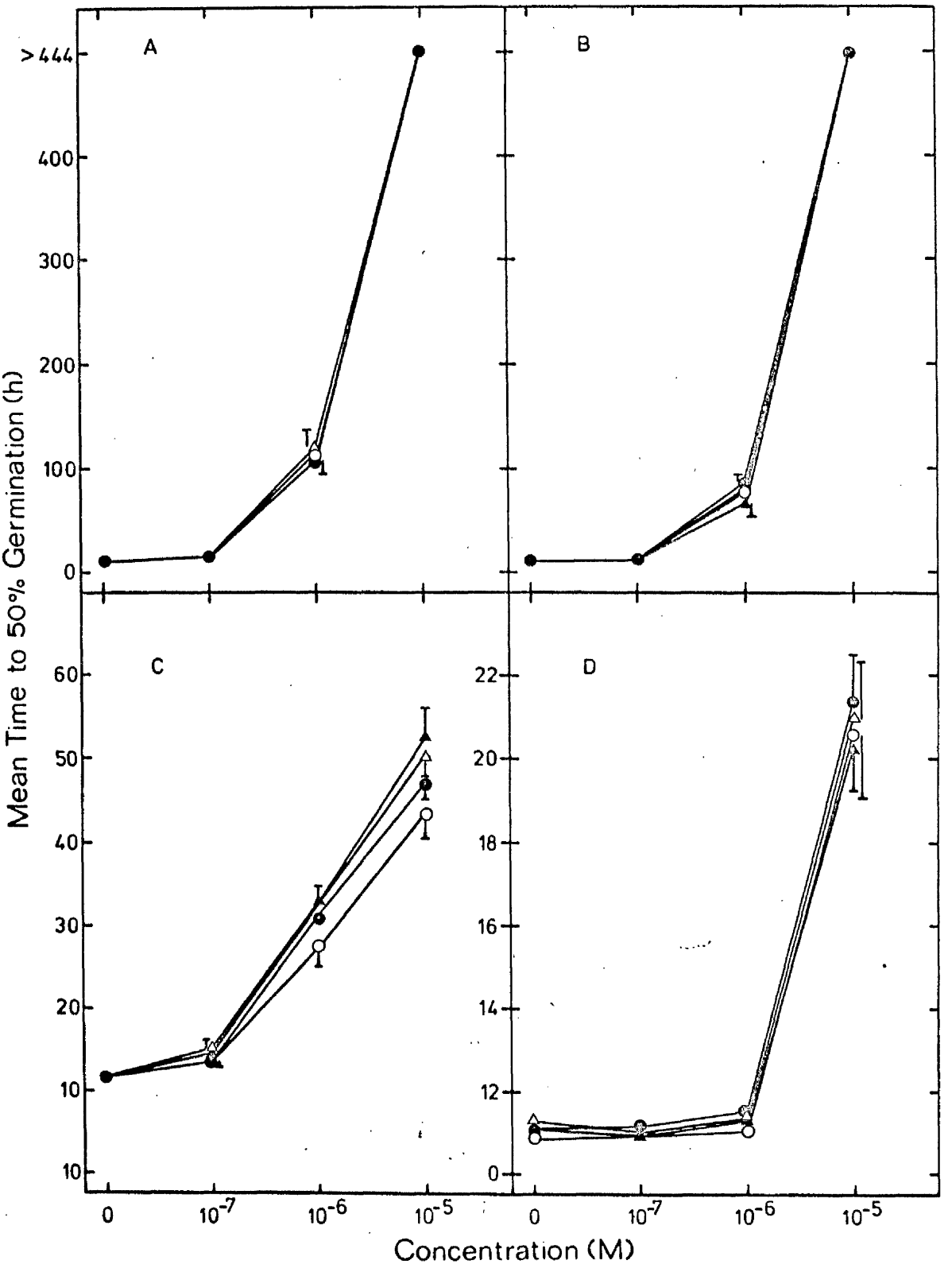

A
B
C
D
> 444
400
300
200
100
0
60
50
40
30
20
10
22
20
18
16
14
12
0
10^{-7}
10^{-6}
10^{-5}
Mean Time to 50% Germination (h)
Concentration (M)

Table 8. Analysis of variance of the MTG of lettuce fruits as a factor of ABA analogue and GA_3 concentrations.

(A) Compound I.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
I	163256.6	2	81628.3	212.67	***
GA_3	158.46	3	52.80	0.14	NS
Interaction	350.4	6	58.40	0.15	NS
Error	23029.70	60	383.83		

(B) Compound II.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
II	69597.39	2	34798.70	206.08	***
GA_3	439.14	3	143.05	0.85	NS
Interaction	874.13	6	145.69	0.86	NS
Error	10131.65	60	168.86		

(C) Compound III.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
III	20687.57	3	6895.86	383.68	***
GA_3	228.86	3	76.29	4.244	**
Interaction	177.32	9	19.70	1.10	NS
Error	1437.90	80	17.97		

(D) Compound IV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
IV	1680.99	3	560.33	271.65	***
GA_3	3.35	3	1.11	0.54	NS
Interaction	2.38	9	0.26	0.13	NS
Error	165.02	80	2.06		

Fig. 13. The effects and interactions of GA_3 and alogues of ABA on lettuce germination. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound V.

(B) compound VI.

(C) compound VII.

(D) compound VIII.

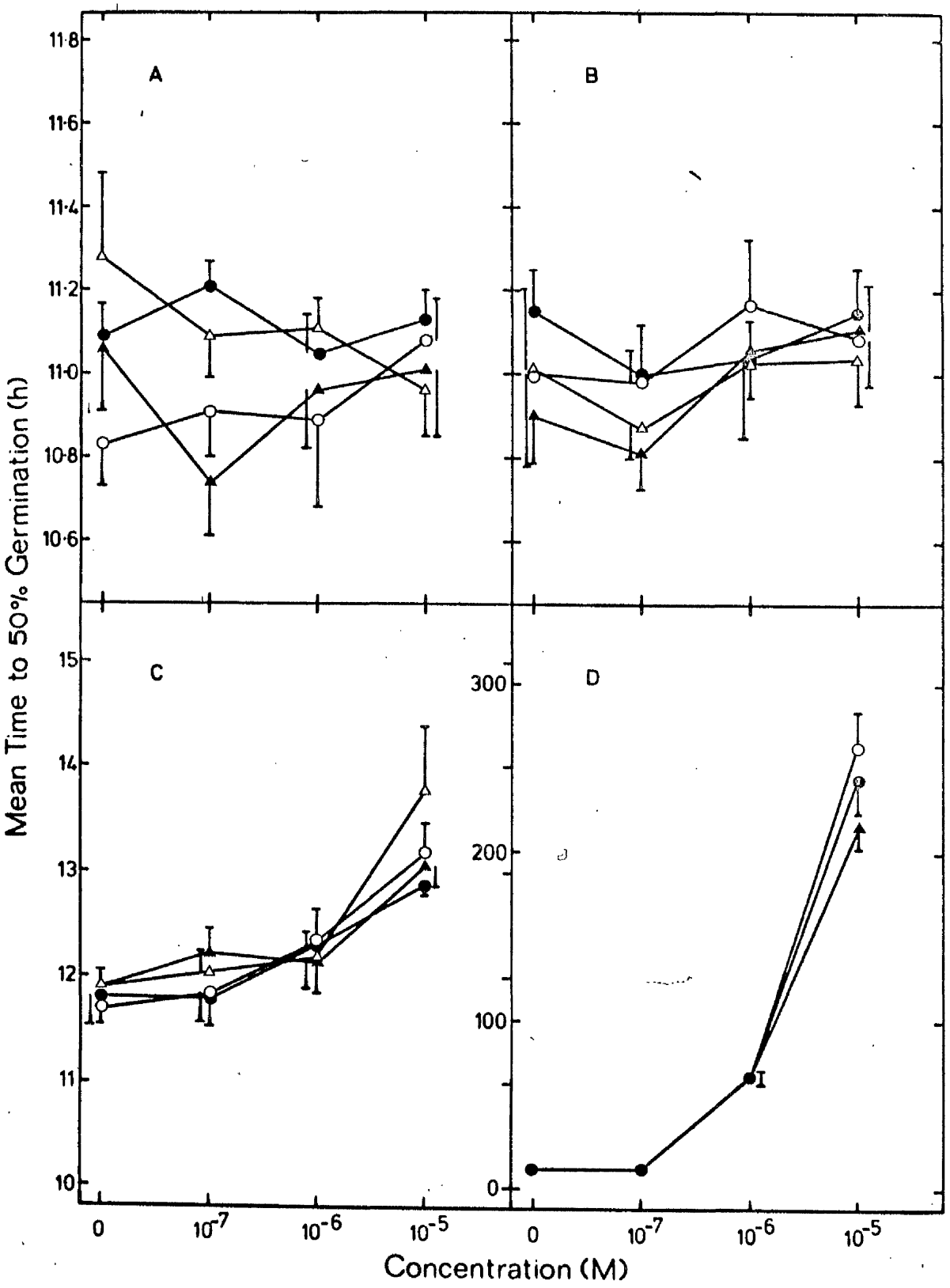

A
B
C
D
11·8
11·6
11·4
11·2
11·0
10·8
10·6
15
14
13
12
11
10
300
200
100
0
0
10^{-7}
10^{-6}
10^{-5}
Mean Time to 50% Germination (h)
Concentration (M)

Table 9. Analysis of variance of the MTG of lettuce fruits as a factor of ABA analogue and GA_3 concentrations.

(A) Compound V.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
V	0.091	3	0.03	0.31	NS
GA_3	0.79	3	0.26	2.72	*
Interaction	0.86	9	0.09	0.98	NS
Error	7.83	80	0.09		

(B) Compound VI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
VI	0.83	3	0.28	2.78	*
GA_3	0.16	3	0.05	0.54	NS
Interaction	0.60	9	0.07	0.67	NS
Error	7.92	80	0.10		

(C) Compound VII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
VII	29.83	3	9.94	13.83	***
GA_3	0.97	3	0.32	0.45	NS
Interaction	3.24	9	0.36	0.50	NS
Error	57.53	80	0.72		

(D) Compound VIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
VIII	898521.16	3	299507.05	977.0	***
GA_3	2232.55	3	744.18	2.43	NS
Interaction	7160.32	9	795.59	2.60	*
Error	24524.71	80	306.56		

Fig. 14. The effects and interactions of GA_3 and analogues of ABA on lettuce germination. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound IX.

(B) compound X.

(C) compound XI.

(D) compound XII.

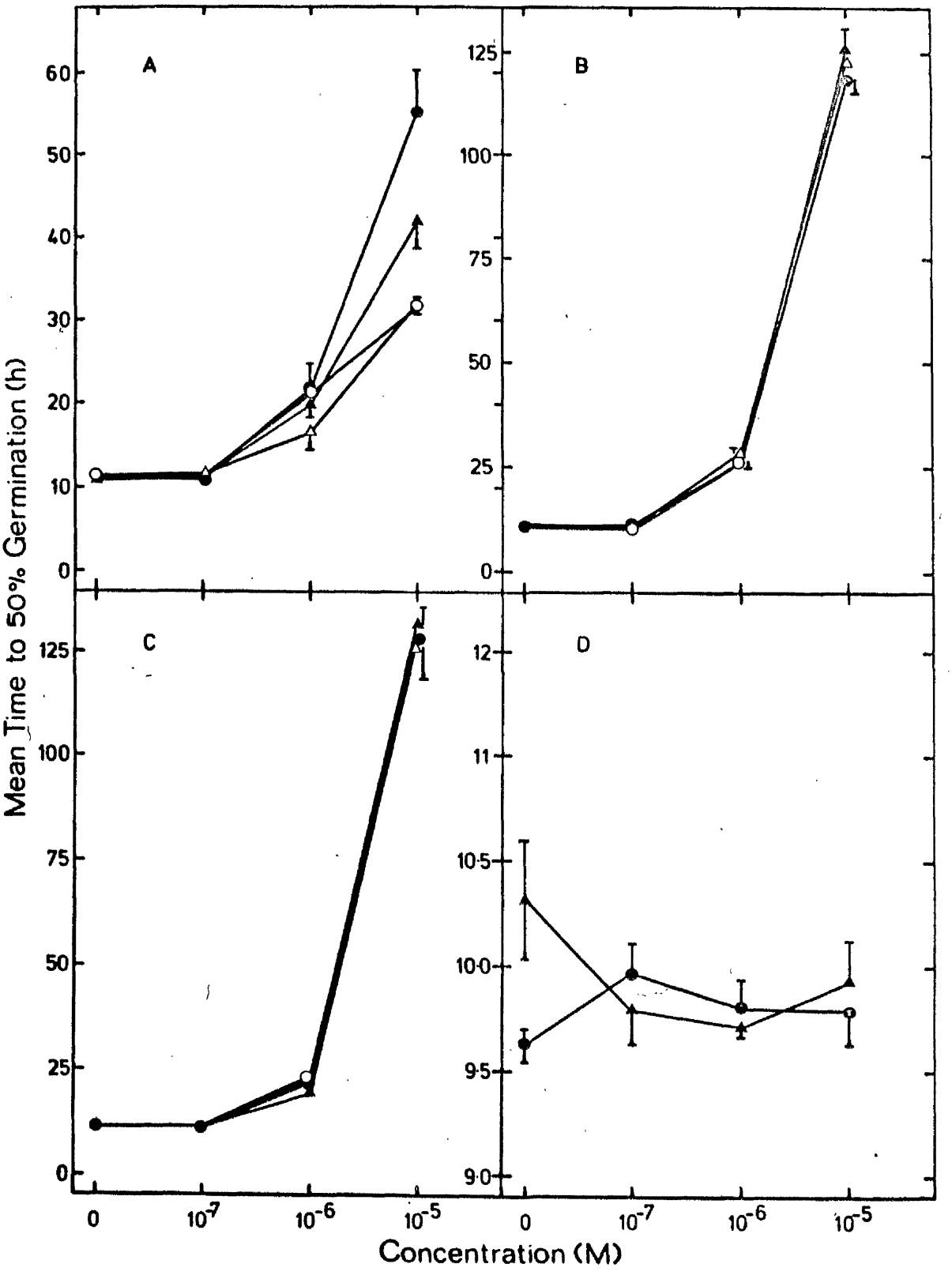

A
B
C
D
Mean Time to 50% Germination (h)
Concentration (M)
0
10-7
10-6
10-5

Table 10. Analysis of variance of the MTG of lettuce fruits as a factor of ABA analogue and GA_3 concentrations.

(A) Compound IX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
IX	13386.17	3	4462.06	323.97	***
GA_3	643.82	3	214.61	15.58	***
Interaction	1669.08	9	185.45	13.46	***
Error	1101.87	80	13.77		

(B) Compound X.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
X	210969.79	3	70323.26	801.67	***
GA_3	56.79	3	18.93	0.21	NS
Interaction	167.96	9	18.66	0.21	NS
Error	7017.59	80	87.71		

(C) Compound XI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XI	242080.99	3	80693.66	730.78	***
GA_3	7.24	3	2.41	0.02	NS
Interaction	63.79	9	7.01	0.06	NS
Error	8833.73	80	110.42		

(D) Compound XII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XII	0.27	3	0.09	0.62	NS
GA_3	0.24	1	0.24	1.67	NS
Interaction	1.45	3	0.48	3.27	*
Error	5.94	40	0.14		

Fig. 15. The effects and interactions of GA_3 and analogues of ABA on lettuce germination. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XIII.

(B) compound XIV.

(C) compound XV.

(D) compound XVI.

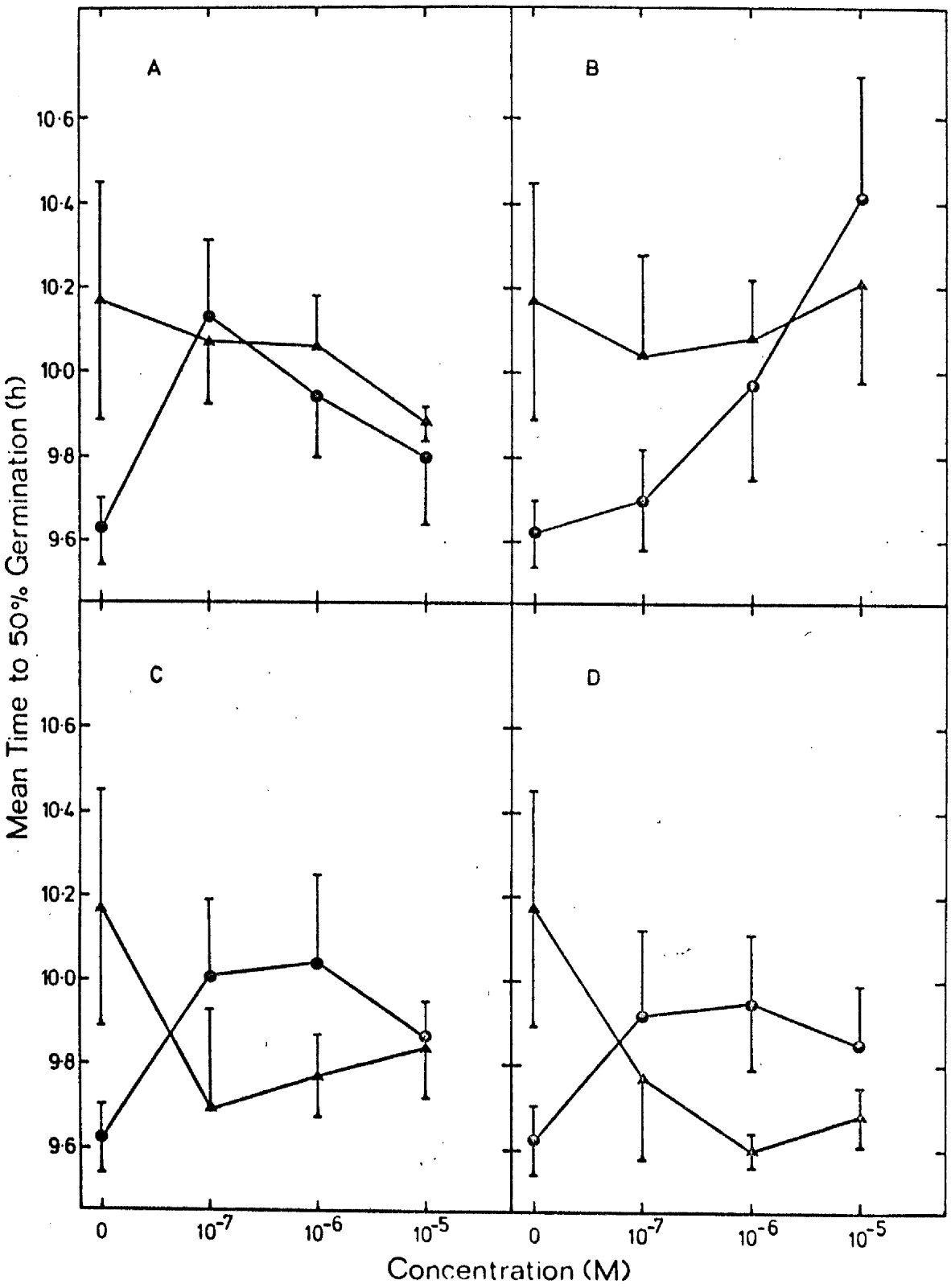

A
B
C
D
10·6
10·4
10·2
10·0
9·8
9·6
Mean Time to 50% Germination (h)
0
10⁻⁷
10⁻⁶
10⁻⁵
Concentration (M)

Table 11. Analysis of variance of the MTG of lettuce fruits as a factor of ABA analogue and GA_3 concentrations.

(A) Compound XIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XIII	0.42	3	0.14	0.91	NS
GA_3	0.56	1	0.56	3.67	NS
Interaction	1.04	3	0.34	2.26	NS
Error	6.13	40	0.15		

(B) Compound XIV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XIV	1.29	3	0.43	1.58	NS
GA_3	0.69	1	0.69	2.55	NS
Interaction	1.33	3	0.44	1.63	NS
Error	10.88	40	0.27		

(C) Compound XV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XV	0.12	3	0.04	0.21	NS
GA_3	0.01	1	0.01	0.09	NS
Interaction	2.07	3	0.69	3.61	*
Error	7.67	40	0.19		

(D) Compound XVI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XVI	0.31	3	0.104	0.64	NS
GA_3	0.02	1	0.02	0.13	NS
Interaction	2.05	3	0.68	4.22	*
Error	6.47	40	0.16		

Fig. 16. **The effects and interactions of GA_3 and analogues of ABA** on lettuce germination. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XVII.

(B) compound XVIII.

(C) compound XIX.

(D) compound XX.

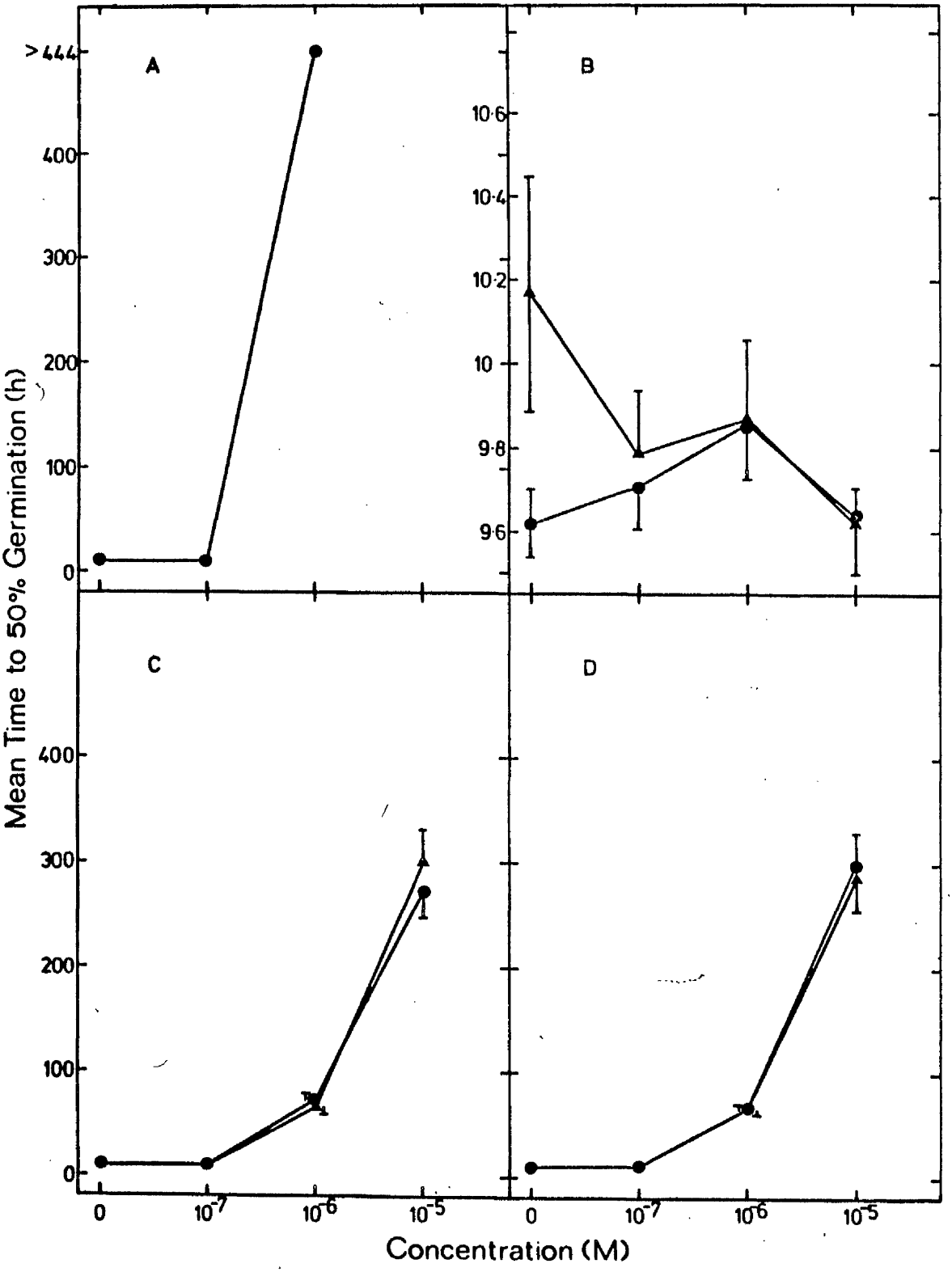

A
B
C
D
>444
400
300
200
100
0
10·6
10·4
10·2
10
9·8
9·6
Mean Time to 50% Germination (h)
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 12. Analysis of variance of the MTG of lettuce fruits as a factor of ABA analogue and GA_3 concentrations.

(A) Compound XVIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XVIII	5.29	3	1.76	14.12	***
GA_3	0.24	1	0.24	1.93	NS
Interaction	0.03	3	0.01	0.08	NS
Error	5.02	40	0.12		

(B) Compound XIX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XIX	621698.44	3	207232.81	160.98	***
GA_3	285.02	1	285.02	0.22	NS
Interaction	1766.39	3	588.79	0.45	NS
Error	51491.41	40	1287.28		

(C) Compound XX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XX	658891.87	3	219630.62	151.75	***
GA_3	192.37	1	192.37	0.13	NS
Interaction	390.07	3	130.02	0.08	NS
Error	57889.24	40	1447.23		

Fig. 17. The effects and interactions of GA_3 and analogues of ABA on lettuce germination. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XXI.

(B) compound XXII.

(C) compound XXIII.

(D) compound XXIV.

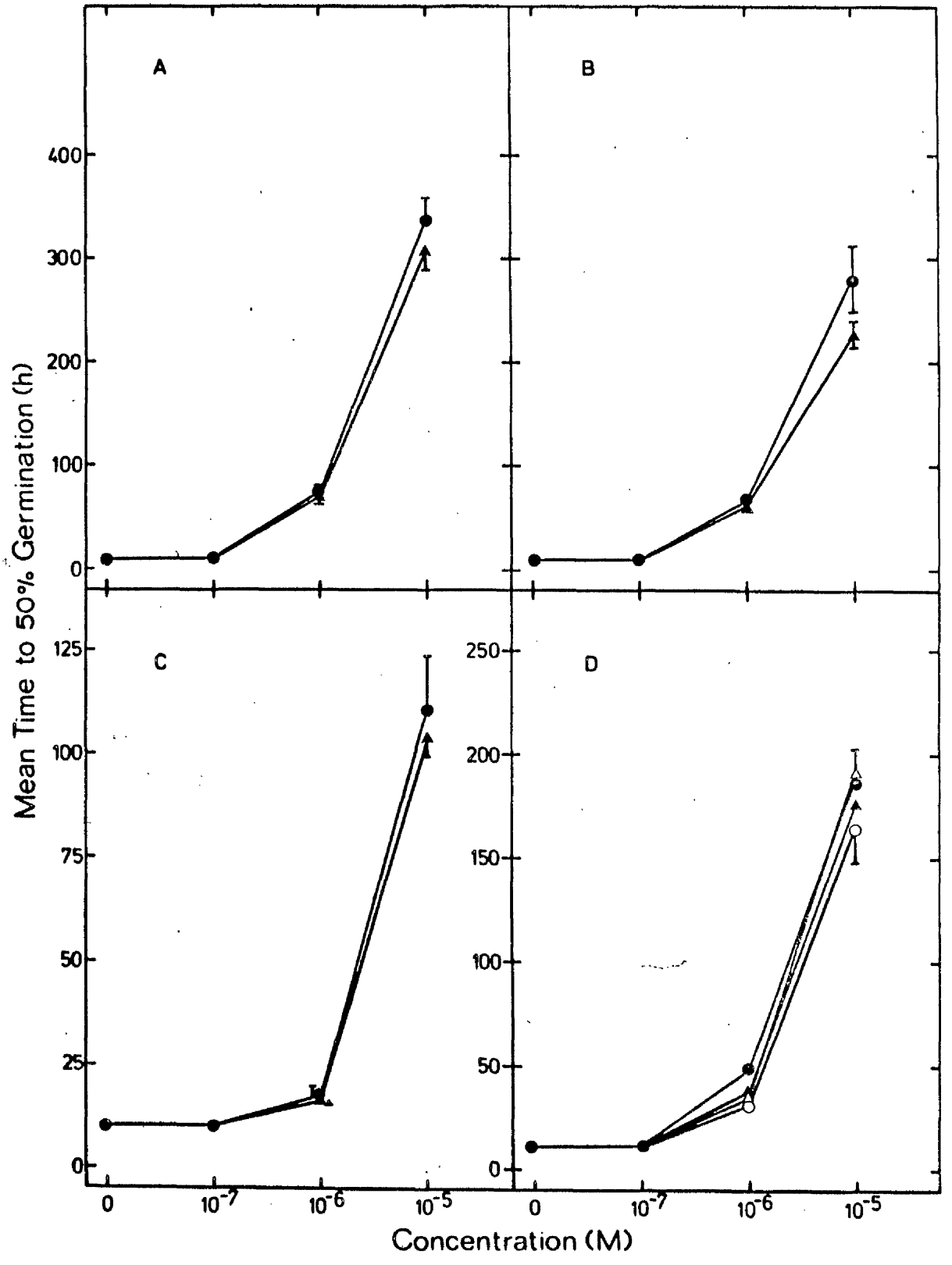

A
B
C
D
Mean Time to 50% Germination (h)
Concentration (M)
0
10⁻⁷
10⁻⁶
10⁻⁵
400
300
200
100
125
75
50
25
250
150

Table 13. Analysis of variance of the MTG of lettuce fruits as a factor of ABA analogue and GA_3 concentrations.

(A) Compound XXI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXI	942424.56	3	314141.52	92.63	***
GA_3	355.21	1	355.21	0.104	NS
Interaction	1659.98	3	553.32	0.16	NS
Error	135653.05	40	3391.32		

(B) Compound XXII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXII	460584.86	3	153528.28	166.08	***
GA_3	1820.24	1	1820.24	1.96	NS
Interaction	4494.27	3	1498.09	1.62	NS
Error	36973.29	40	924.33		

(C) Compound XXIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXIII	81089.62	3	27029.87	185.819	***
GA_3	44.69	1	44.69	0.307	NS
Interaction	85.17	3	27.39	0.195	NS
Error	5818.52	40	145.46		

(D) Compound XXIV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXIV	473558.77	3	157852.92	965.28	***
GA_3	1249.215	3	416.41	2.55	*
Interaction	2128.31	9	236.48	1.45	NS
Error	13082.40	80	163.53		

Fig. 18. The effects and interactions of GA_3 and analogues of ABA on lettuce germination. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XXV.

(B) compound XXVI.

(C) compound XXVII.

(D) compound XXVIII.

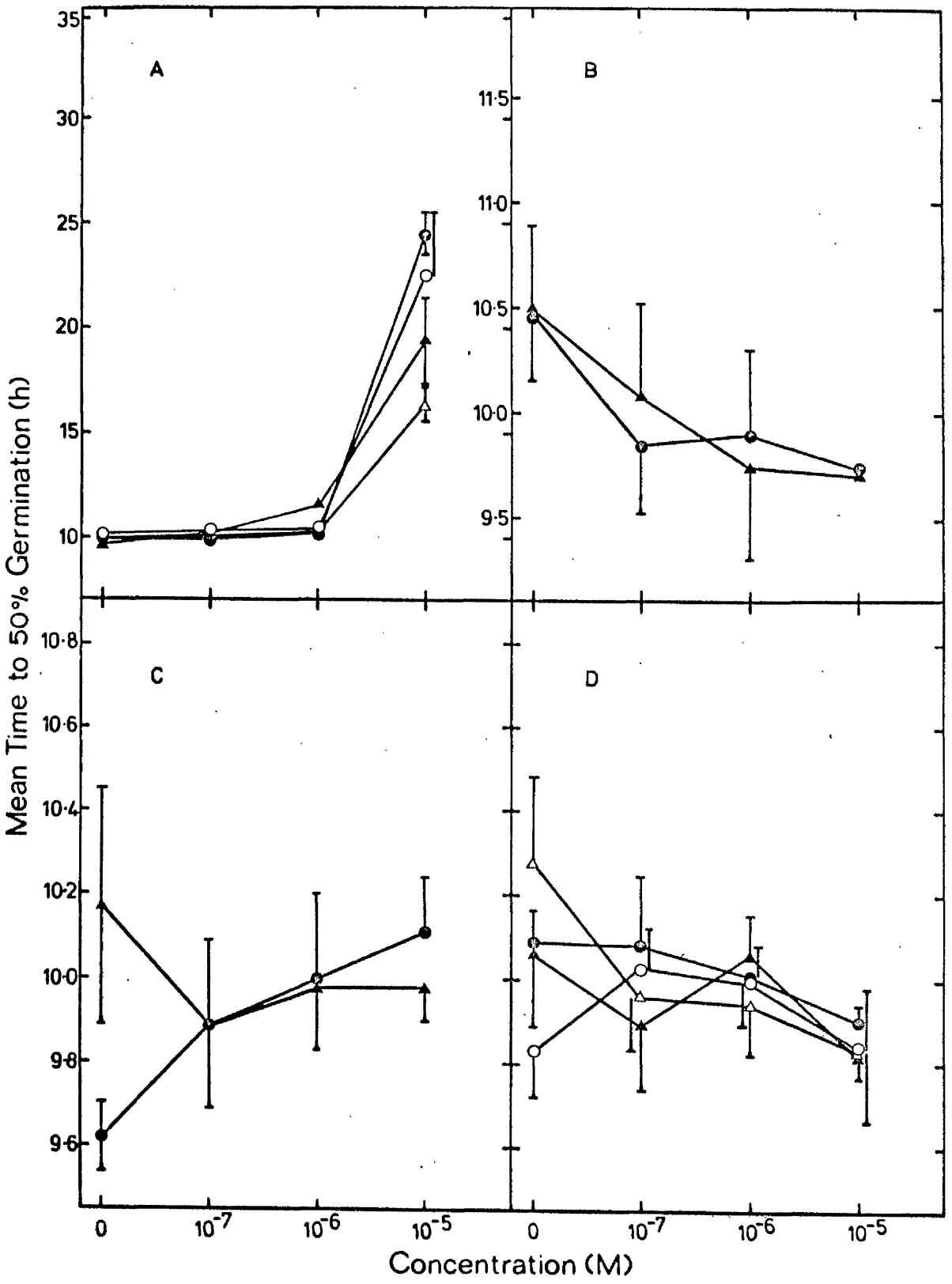

A
B
C
D
35
30
25
20
15
10
11·5
11·0
10·5
10·0
9·5
10·8
10·6
10·4
10·2
10·0
9·8
9·6
0
10^{-7}
10^{-6}
10^{-5}
Mean Time to 50% Germination (h)
Concentration (M)

Table 14. Analysis of variance of the MTG of lettuce fruits as a factor of ABA analogue and GA_3 concentrations.

(A) Compound XXV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXV	2052.60	3	684.20	125.47	***
GA_3	52.81	3	17.60	3.23	*
Interaction	162.25	9	18.03	3.31	**
Error	436.30	80	5.45		

(B) Compound XXVI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXVI	4.33	3	1.44	6.19	**
GA_3	0.24	1	0.24	1.04	NS
Interaction	0.16	3	0.05	0.23	NS
Error	9.33	40	0.23		

(C) Compound XXVII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXVII	0.15	3	0.05	0.27	NS
GA_3	0.27	1	0.27	1.47	NS
Interaction	1.30	3	0.43	2.29	NS
Error	7.55	40	0.18		

(D) Compound XXVIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXVIII	0.598	3	0.199	2.043	NS
GA_3	0.156	3	0.052	0.533	NS
Interaction	0.664	9	0.074	0.756	NS
Error	7.806	80	0.098		

Fig. 19. The effects and interactions of GA_3 and analogues of ABA on lettuce germination. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XXIX.

(B) compound XXX.

(C) compound XXXI.

(D) compound XXXII.

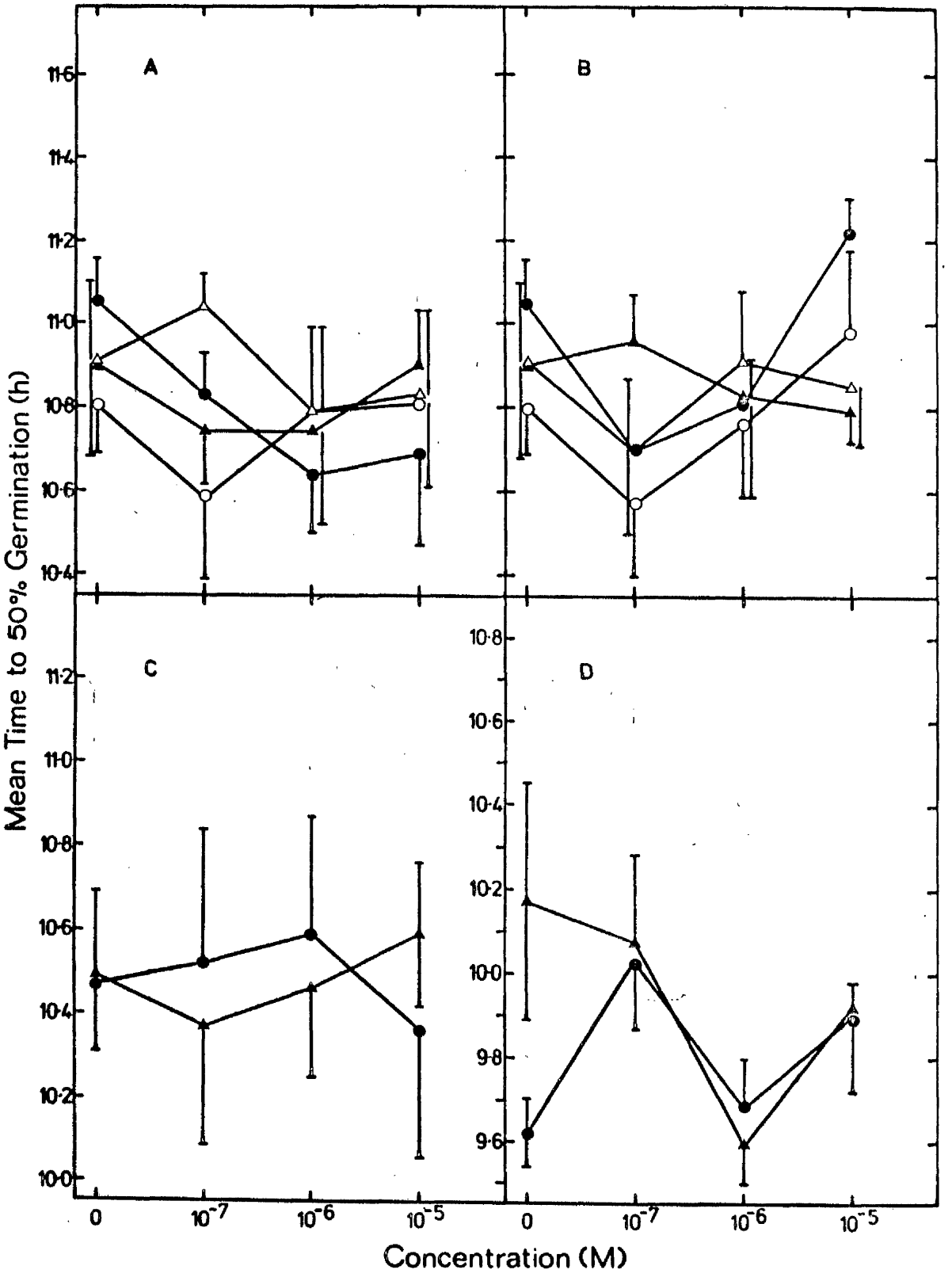

A
B
C
D
Mean Time to 50% Germination (h)
Concentration (M)
0
10⁻⁷
10⁻⁶
10⁻⁵

Table 15. Analysis of variance of the MTG of lettuce fruits as a factor of ABA analogue and GA_3 concentrations.

(A) Compound XXIX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXIX	0.89	3	0.30	1.63	NS
GA_3	0.79	3	0.26	1.43	NS
Interaction	0.70	9	0.08	0.42	NS
Error		80			

(B) Compound XXX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXX	1.25	3	0.42	2.72	*
GA_3	0.86	3	0.29	1.87	NS
Interaction	0.96	9	0.11	0.69	NS
Error	12.25	80	0.15		

(C) Compound XXXI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXXI	0.33	3	0.11	0.29	NS
GA_3	0.025	1	0.02	0.06	NS
Interaction	0.24	3	0.08	0.21	NS
Error	15.08	40	0.37		

(D) Compound XXXII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXXII	1.025	3	0.34	2.09	NS
GA_3	0.10	1	0.10	0.63	NS
Interaction	1.65	3	0.55	3.37	*
Error	6.52	40	0.16		

with the results obtained when GA_3 was present. Where GA_3 was shown by analysis of variance to have an effect, the results were subjected to more comprehensive scrutiny. By this method, the significant GA_3 effect in the presence of V was found to be due only to one treatment, viz. GA_3 present at 10^{-5}M and V at 10^{-7}M. Since the effect was significant only at the 95% level, it is possible that this difference is merely a chance occurrence. The same applies to XXIV, where the response obtained in the presence of XXIV at 10^{-6}M and no GA_3 may, by chance, have been high. In the case of III, although the analysis of variance indicated a significant GA_3 effect, this could not be confirmed by the student's "t" tests. Only in the cases of IX and XXV was the GA_3 effect both highly significant and reasonably consistent, although it was found that the most effective GA_3 concentrations were not the highest concentrations.

The analysis of variance also indicated a significant interaction between certain analogues and GA_3. The analogues involved, and where there was also a significant GA_3 effect, were IX and XXV, although in VIII, XII, XV, XVI and XXXII there was some evidence of an interaction. The significant interaction term in the latter group, however, occurred in the absence of a GA_3 effect and, since they all occurred only at the 95% significance level, it seems unlikely that the effect was real. IX and XXV were thus the only two compounds where the existence of an interaction term could be confirmed. The question next arises as to whether or not this statistical approach does indeed indicate the existence of a true interaction, and this is discussed on page 132. What is, however, evident at this point is that IX and XXV are exhibiting responses different from those of the other analogues. Their unusual behaviour in relation to their activity was also noted in the experiments dealing with the structural requirements for ABA activity. The structures of the two compounds are very similar and it may be that their mode of action differs in some way from that of ABA.

(4) INTERACTION OF ABA WITH KINETIN IN LETTUCE GERMINATION

Gibberellin A_3 is ineffective with regard to both promotive effects on germination and interaction with ABA and its analogues. Kinetin effects, on the other hand, are well documented and this growth substance is believed to interact with ABA (Shankla and Shankla, 1968). It was therefore of interest to analyse possible interactions of kinetin with ABA analogues. Accordingly, preliminary experiments were carried out to confirm the interaction of kinetin with ABA using the germination system employed in this work. Factorial experiments of ABA at 0, 10^{-7}, 10^{-6} and 10^{-5}M and kinetin at 0, 10^{-6}, 10^{-5} and 10^{-4}M were carried out. The MTG was recorded for each treatment, and the results are shown in Fig. 20.

As previously described, ABA significantly delays germination when present at concentrations of 10^{-6} and 10^{-5}M. When kinetin was also present in the incubation medium, no significant effect was exerted at concentrations of 10^{-7} and 10^{-6}M. However, at a concentration of 10^{-5}M kinetin reversed, to a marked extent, the ABA-induced delay of germination. The degree to which this occurred and the statistical significance which can be attached to it are described in Table 16, from which it can be seen that the mean time to 50% germination in 10^{-5}M ABA solution was 32.4 h, but this was reduced to 16.4 h by the simultaneous presence of 10^{-5}M K. These results confirm those of Shankla and Shankla (1968).

Table 16. The effect of kinetin applied in combination with 10^{-5}M ABA on lettuce germination.

Kinetin concentration (Molar)	Mean time to 50% germination (h)	Standard Error (±)	Significance Differences ('t' tests)
0	32.42	0.34	-
10^{-7}	31.9	0.65	NS
10^{-6}	31.14	0.75	NS
10^{-5}	16.40	0.51	***

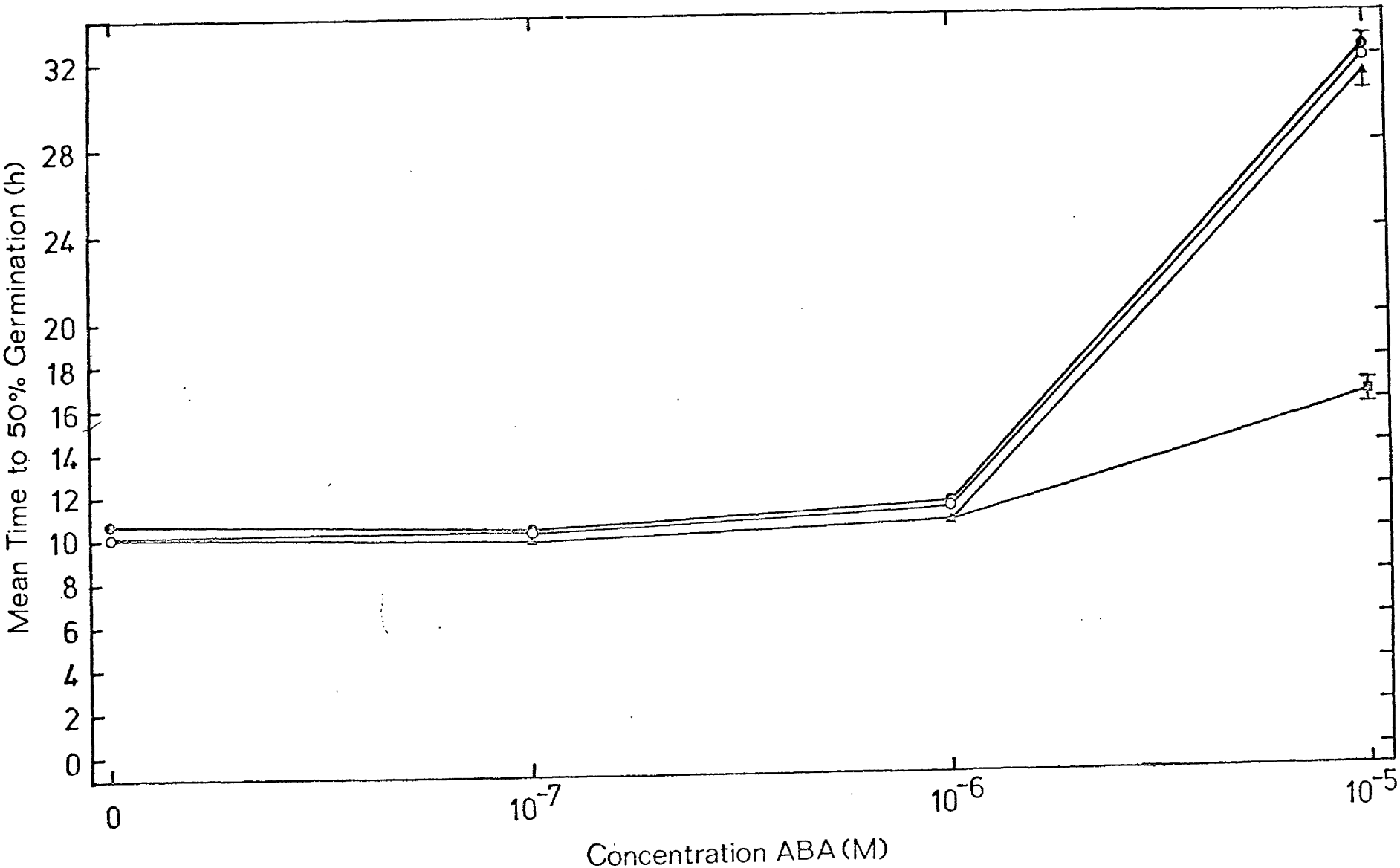
Fig. 20. The effect of ABA and kinetin alone and in combination at various concentrations on lettuce germination.

closed circle :- no kinetin.

open circle :- 10^{-7}M kinetin.

closed triangle :- 10^{-6}M kinetin.

closed square :- 10^{-5}M kinetin.


Mean Time to 50% Germination (h)
32
28
24
20
18
16
14
12
10
8
6
4
2
0
0
10^{-7}
10^{-6}
10^{-5}
Concentration ABA (M)

(5) THE REVERSAL BY KINETIN OF THE INHIBITION IMPOSED BY ABA ANALOGUES

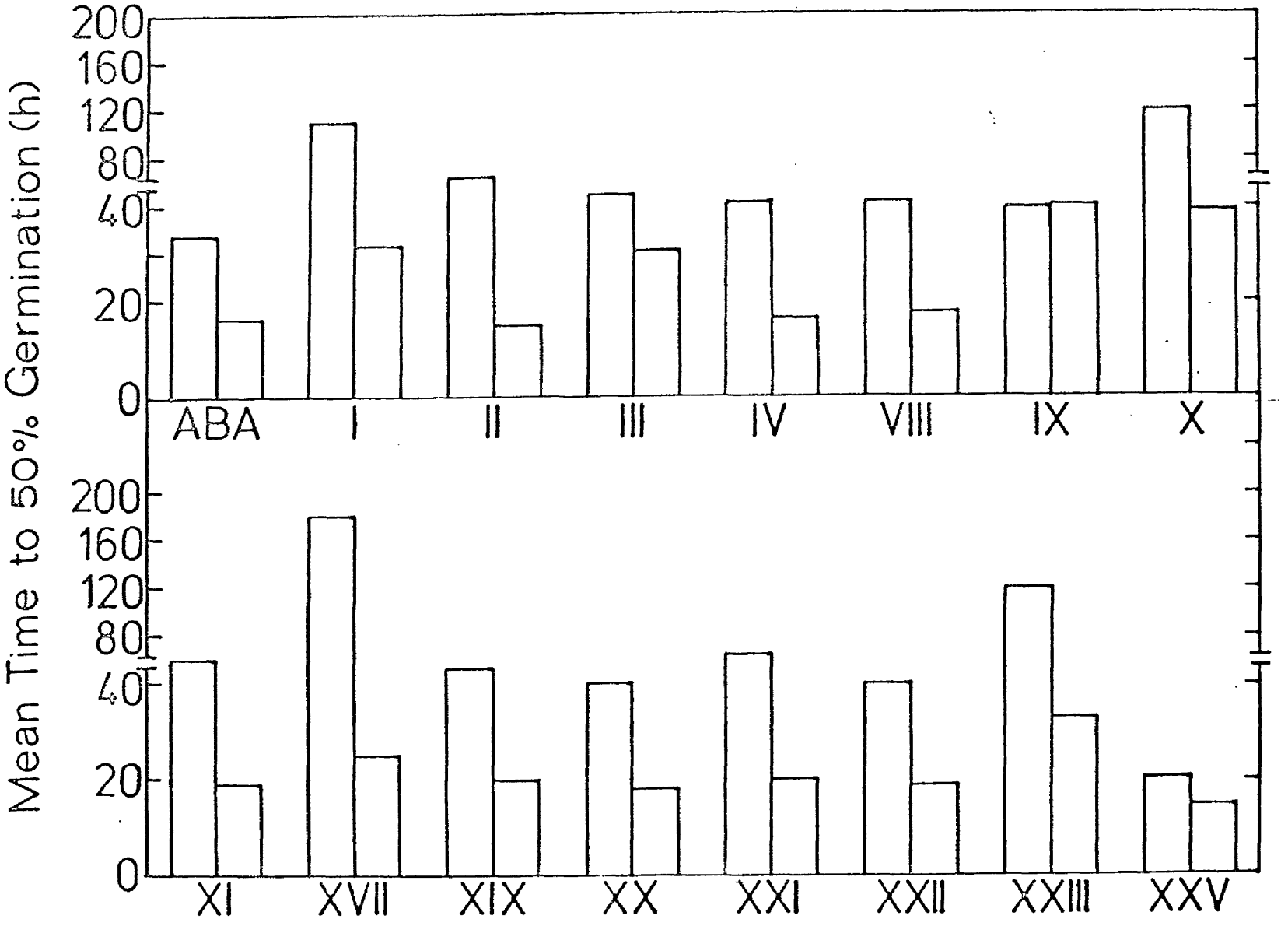
Those ABA analogues which had been shown to be active in inducing a response in terms of a delay in germination were assayed with kinetin at 10^{-5}M. Only one concentration of analogue was examined and this was selected to allow germination within a time of three days or less. The analogues and the concentrations used are described in Table 17. The assay was carried out in the normal way and the mean time to 50% germination recorded for each treatment (Fig. 21 and Table 17).

Table 17. The effect of 10^{-5}M kinetin applied in combination with ABA and certain ABA analogues on lettuce germination.

Compound	Concentration (Molar)	Mean Time to 50% germination (hours)		Significance of differences ('t' tests)
		Compound	Compound + kinetin	
ABA	10^{-5}	33.70 ± 2.44	16.40 ± 0.51	***
I	10^{-6}	115.10 ± 1.64	31.51 ± 0.50	***
II	10^{-6}	64.37 ± 5.83	15.15 ± 0.29	***
III	10^{-5}	50.23 ± 0.62	30.55 ± 0.71	***
IV	10^{-5}	42.73 ± 0.14	16.24 ± 0.22	***
VIII	10^{-6}	43.42 ± 5.22	17.58 ± 0.53	**
IX	10^{-5}	39.47 ± 0.41	40.89 ± 0.63	NS
X	10^{-5}	119.13 ± 0.42	39.27 ± 0.84	***
XI	10^{-6}	49.73 ± 0.09	19.08 ± 0.63	***
XVII	10^{-6}	181.13 ± 35.5	24.58 ± 0.94	***
XIX	10^{-6}	53.57 ± 2.02	19.45 ± 0.42	***
XX	10^{-6}	41.60 ± 2.77	18.17 ± 0.51	***
XXI	10^{-6}	64.96 ± 9.17	20.24 ± 0.45	***
XXII	10^{-6}	40.20 ± 2.32	18.45 ± 0.74	***
XXIII	10^{-5}	100.00 ± 9.24	33.14 ± 1.10	***
XXV	10^{-5}	20.50 ± 0.49	14.59 ± 0.27	***

Kinetin was capable of overcoming the inhibitory effect of all but one of the analogues to a remarkable extent, although there was consider variation in its effectiveness. In compound III, for example, the counteraction from

Fig. 21. The effect of certain active analogues of ABA and kinetin alone and in combination on lettuce germination. The analogue concentrations are described in Table 17; kinetin when present was at a concentration of 10^{-5}M.


Mean Time to 50% Germination (h)
200
160
120
80
40
20
0
ABA
I
II
III
IV
VIII
IX
X
200
160
120
80
40
20
0
XI
XVII
XIX
XX
XXI
XXII
XXIII
XXV

50.23 h to 30.55 h was not as marked as with most other compounds, while in the case of XVII the reversal was much more marked, with the mean time to 50% germination being reduced from 181.13 h to 24.58 h by the addition of kinetin. It is not known to what extent the enhanced effect of kinetin with XVII was due to an increased potential for kinetin action, in that the concentration of XVII used, delayed germination more than did any of the other treatments. If this were, however, the reason, I, X and XXIII would also have been expected to show a greater than average response. Furthermore, this interpretation would not explain why kinetin stimulated germination in XVII to occur after 24-58 h, but not until 30.55 h in III.

The results of the experiments were subjected to the "t" testing and the results of this are shown in Table 17. Kinetin had no significant effect on overcoming the inhibitory effects of IX, whose untypical behaviour has already been remarked upon in the earlier structure-activity and gibberellin interaction studies. In the former studies this compound was less active than anticipated, while in the latter studies it was the only analogue tested which yielded a statistically significant interaction with GA_3. The action of this compound is thus, in some way, distinct from that of ABA and its other analogues. It is possible either that its mode of action is different from that of ABA or that some form of steric hindrance, or a similar phenomenon, is interfering with its action. The reason for the varied activity of kinetin with the other analogues, e.g. the different effects on III and XVII, also warrants further study.

(6) THE EFFECT OF ABA ON THE GROWTH OF LETTUCE SEEDLINGS

The effects of ABA on lettuce germination have been investigated, and it was deemed necessary to consider an alternative assay system in view of the fact that studies of the structure-activity relationships of ABA using different assay systems differ in their findings (McWha *et al.*, 1973). The lettuce hypocotyl extension assay was selected for further studies on ABA and its

analogues.

To ensure that an observed effect was not due to the delayed germination caused by imbibition in ABA, the fruits were imbibed and allowed to germinate in water. After germination, defined as emergence of the radicle through the fruit coat, the seedlings were transferred to dishes containing test solution for a period of 72 h in light. Under these conditions, the hypocotyl of lettuce (var. Arctic King) extends to only 2 mm. Because of this small extension, any inhibitory effects would be difficult to measure accurately. In order to overcome this difficulty it is necessary to stimulate hypocotyl extension by either carrying out the experiments in darkness or by adding a hypocotyl growth stimulant such as GA_3. In order to maintain the conditions as similar as possible to the lettuce germination assay, the latter course of action was chosen. Nevertheless, the analogues were also assayed at 10^{-5}M in darkness.

The effect of ABA in solution at 0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M was assayed in the presence of GA_3 at 0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}M. The parameter most commonly measured in this type of bioassay is the length of the hypocotyl. The mean length of the hypocotyl for recorded for each treatment (Fig. 22) and subjected to an analysis of variance (Table 18) which indicated that GA_3 produced a significant promotory effect. The promotion of growth was shown, by a "t" test, to be significant at GA_3 concentration of 10^{-7}M and higher (Table 19b). At 10^{-5} and 10^{-4}M, however, increases in concentration did not continue to elicit further promotion of elongation, and at 10^{-3}M inhibition of elongation was apparent. This response pattern is comparable with the results obtained in the lettuce germination assay and may indicate that at 10^{-3}M, the optimum dosage for promotion has been exceeded. The optimum dosage occurred between 10^{-5} and 10^{-4}M, and elongation was promoted from 2.18 ± 0.09 mm to 12.03 ± 0.65 mm.

Fig. 22. The effect of various concentrations of ABA and GA_3 alone and in combination on the extension of lettuce hypocotyls. ABA concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols.

open square	:-	no GA_3
open triangle	:-	10^{-7}M GA_3
closed triangle	:-	10^{-6}M GA_3
open circle	:-	10^{-5}M GA_3
closed square	:-	10^{-4}M GA_3
closed circle	:-	10^{-3}M GA_3

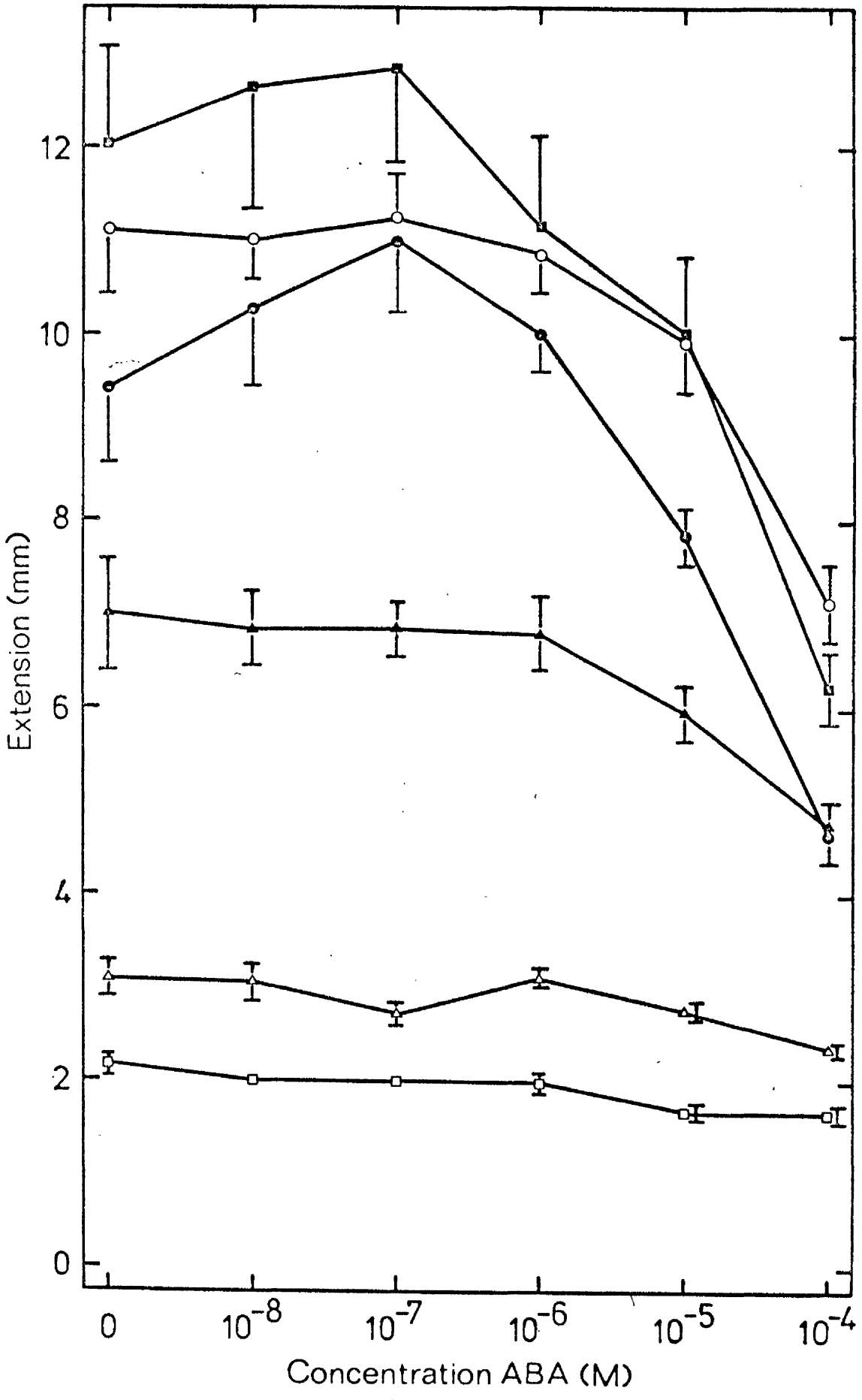

Extension (mm)
0
2
4
6
8
10
12
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration ABA (M)

Table 18. Analysis of variance of the extension of lettuce hypocotyls in response to ABA and GA_3.

(a) 6 x 6 factorial experiment.

	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
ABA	290.54	5	58.11	28.47	***
GA_3	2584.68	5	516.94	253.27	***
Interaction	162.41	25	6.49	3.18	***
Error	367.43	180	2.04		

(b) 4 x 4 factorial experiment.

	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
ABA	7.9	3	2.63	3.10	*
GA_3	1164.09	3	388.03	455.97	***
Interaction	3.87	9	0.43	0.50	NS
Error	68.14	80	0.85		

Table 19. The effects of ABA and GA_3 on lettuce hypocotyl extension.

(a) The effect of ABA on hypocotyl extension induced by 10^{-5}M GA_3.

ABA concentration (Molar)	Hypocotyl length (mm)	Standard Error (±)	Significance of Inhibition ('t' tests)
0	11.13	0.79	-
10^{-8}	11.03	0.83	NS
10^{-7}	11.28	0.76	NS
10^{-6}	10.88	0.41	NS
10^{-5}	9.96	0.29	*
10^{-4}	6.21	0.42	***

(b) The effect of GA_3 on hypocotyl extension.

GA_3 concentration (Molar)	Hypocotyl length (mm)	Standard Error (±)	Significance of Inhibition ('t' tests)
0	2.18	0.09	-
10^{-7}	3.12	0.22	**
10^{-6}	7.02	0.60	***
10^{-5}	11.13	0.79	***
10^{-4}	12.03	0.65	***
10^{-3}	9.43	1.17	***

ABA was found by analysis of variance to exert a significant inhibition effect on growth. Further analysis of the results using the "t" test, however, revealed that this ABA effect could only be detected where GA_3 was also present at a concentration of 10^{-6}M or higher (Table 19a). This inability to detect an ABA effect in the absence of, or at low levels of, GA_3 may have been due to limitations of the mensuration technique. It may, on the other hand, indicate that under the conditions involved, ABA was only capable of inhibiting gibberellin induced growth. The inhibition was significant at 10^{-5}M concentrations of ABA, provided GA_3 was present in concentrations of at least 10^{-6}M.

It is not unexpected, therefore, that the analysis of variance also indicated the presence of an interaction which was significant at the 0.1% level. When, however, the results of only a limited number of concentrations of both hormones were included in the analysis (0, 10^{-7}, 10^{-6} and 10^{-5}M), both the GA_3 and ABA effects were still significant, but there was no longer a significant interaction term.

To ascertain whether or not hypocotyl length was the best parameter, three other features of the seedling were examined, viz. the root length, the cotyledonary leaf length and cotyledonary leaf width (Figs. 23, 24, 25). Root length did not show a significant response to GA_3 until the concentration was increased to 10^{-3}M when the elongation was inhibited. The pattern was the same at all levels of ABA. The response of roots to ABA was similar to that of the hypocotyl, with concentrations of 10^{-5}M and above inhibiting elongation growth (Fig. 23). Leaf length responded to GA_3 concentrations as low as 10^{-7}M, although increasing this concentration beyond 10^{-5}M did not elicit further response (Fig. 24). No response to ABA was recorded until the concentration was increased to 10^{-4}M. Similar results were obtained in the leaf width assay (Fig. 25) where growth was inhibited by ABA at 10^{-4}M. The essential difference in response pattern between the leaf width and leaf length parameters was that leaf width did not respond to GA_3 applications.

Fig. 23. The effect of various concentrations of ABA and GA_3 alone and in combination on the growth of roots of lettuce seedlings.

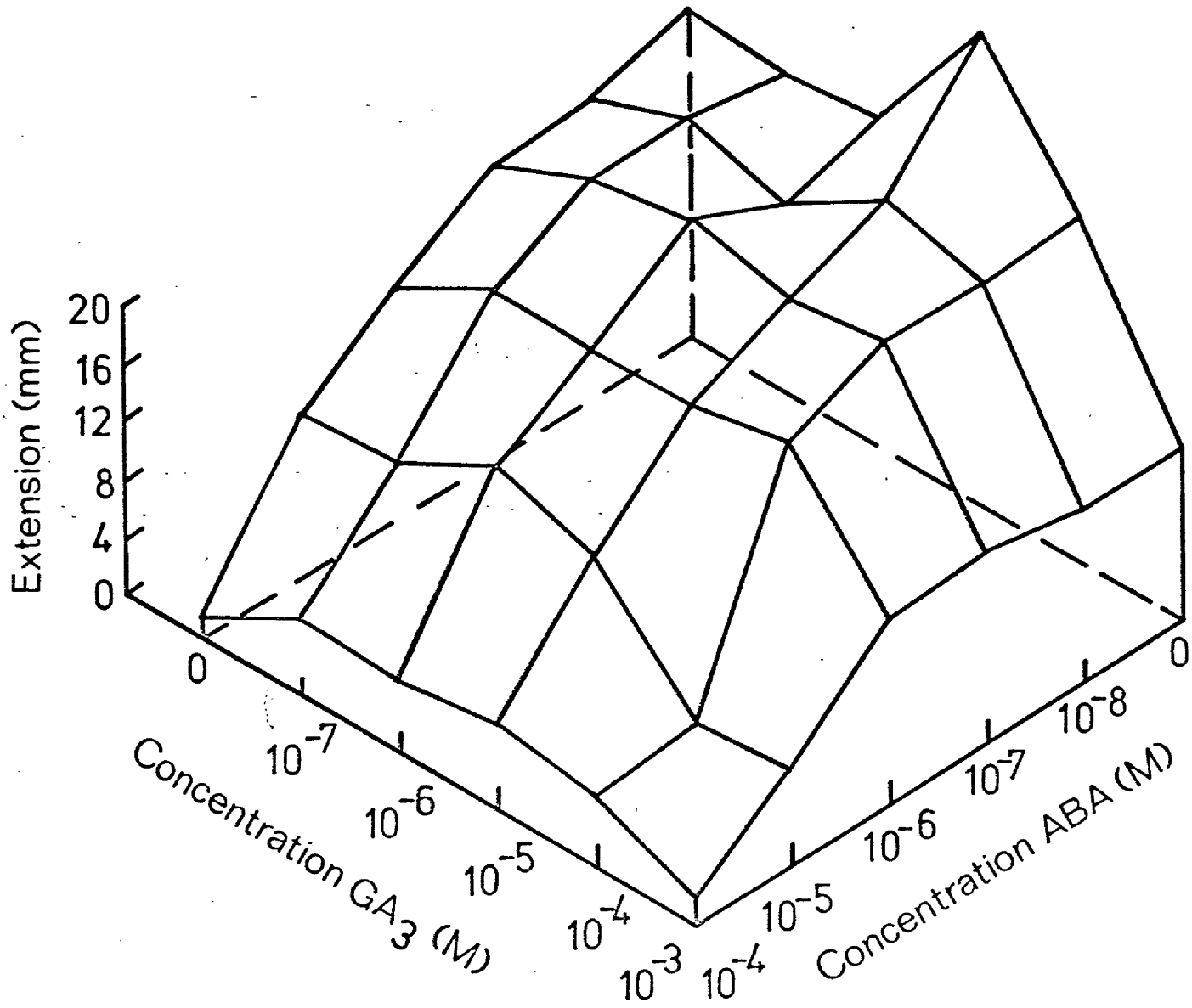

Extension (mm)
20
16
12
8
4
0
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Concentration GA_3 (M)
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
0
Concentration ABA (M)

Fig. 24. The effect of various concentrations of ABA and GA_3 alone and in combination on the length of the cotyledonary leaves of lettuce seedling.

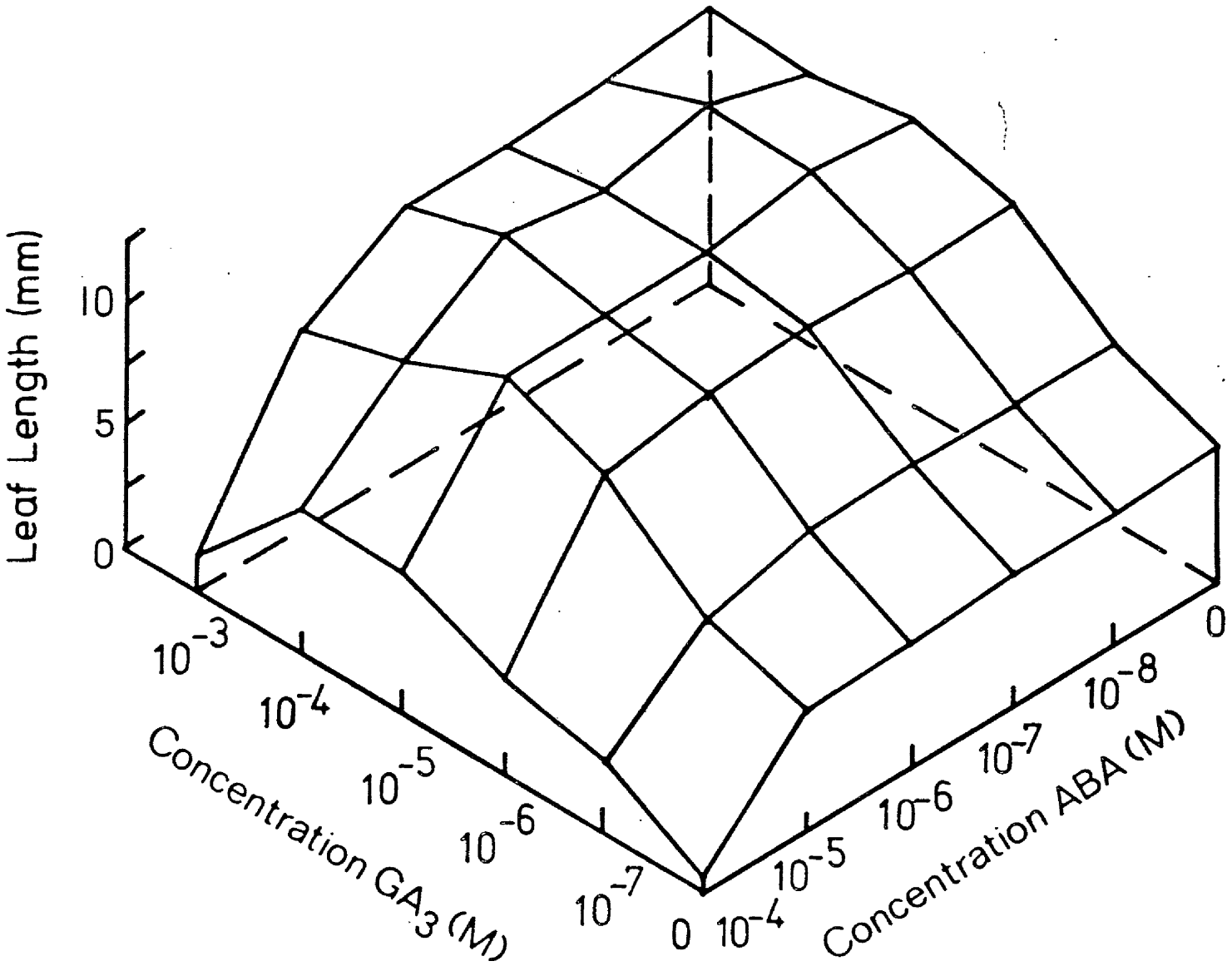
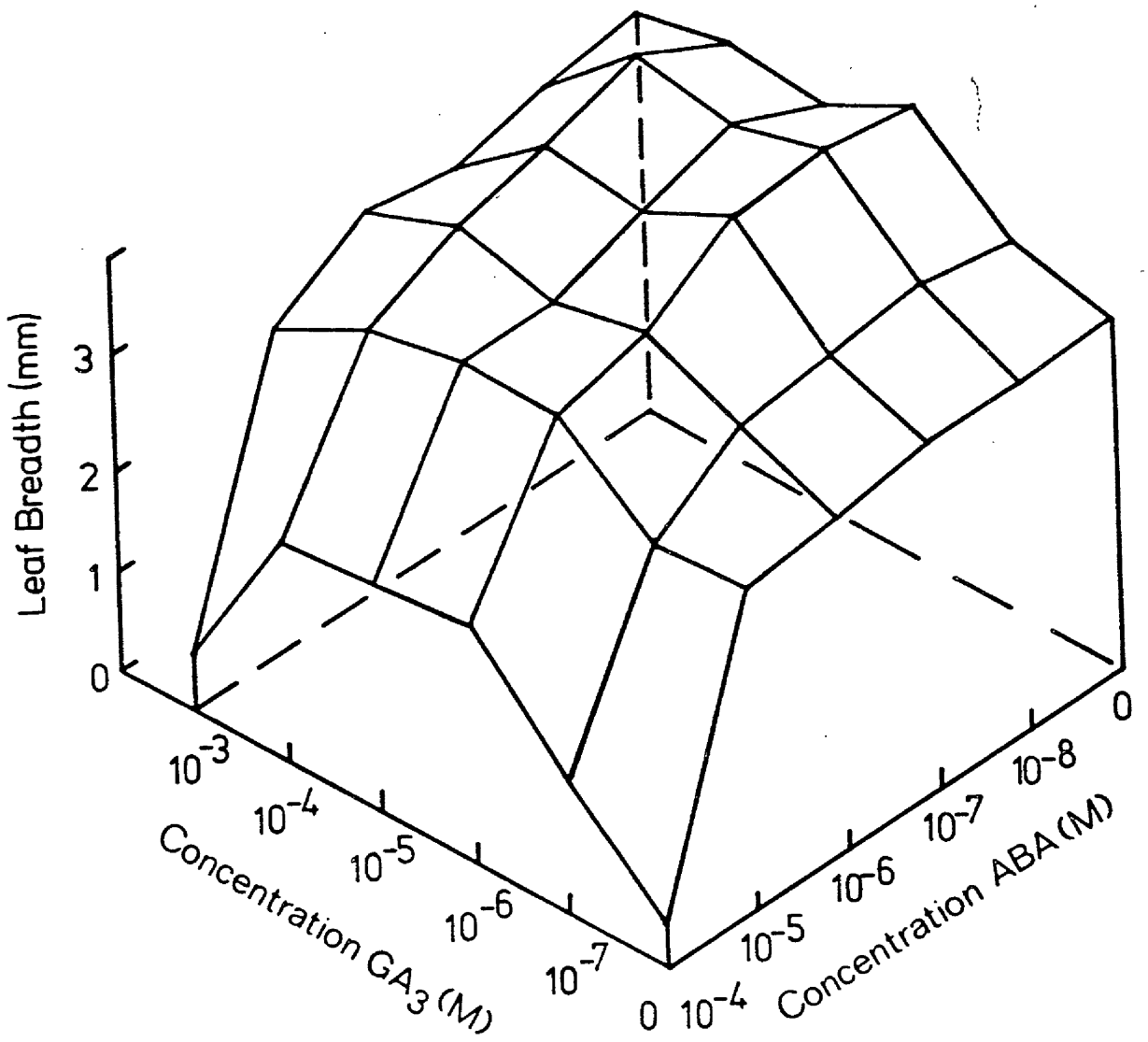

Leaf Length (mm)
10
5
0
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
0
Concentration GA_3 (M)
0
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
0
Concentration ABA (M)

Fig. 25. The effect of various concentrations of ABA and GA_3 alone and in combination on the length of the cotyledonary leaves of lettuce seedlings.


Leaf Breadth (mm)
0
1
2
3
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
Concentration GA_3 (M)
0
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
0
Concentration ABA (M)

It is thus apparent that for significant responses to both GA_3 and ABA, lettuce hypocotyl length was the best and most easily assessed parameter, although the others can provide a guide to activity.

It was observed that the colour of the leaves varied considerably between treatments. Where no gibberellin was present, the leaves were dark green, but as increasing quantities of GA_3 were applied, the leaves became paler and, at high levels, were distinctly yellow. This effect was reversed by the addition of physiologically active concentration of ABA. Were sufficient tissue available, measurement of leaf chlorophyll content could possibly be a suitable assay method for ABA and GA_3. It must, however, be remembered that the change in colour was associated with a simultaneous change in leaf dimension such that the total chlorophyll present would tend to remain constant.

The responses obtained in this assay were not due to the presence of organic solvents in the incubation medium. Acetone, methanol and sodium bicarbonate controls were included in all experiments and a typical set of results is illustrated in Fig. 26 a and b, where 10^{-5}M GA_3 was also included in the incubation medium. The effect of these solvents was not significant even at concentrations far in excess of those actually involved. To ensure that the responses obtained were independent of the arbitrarily selected experimental conditions, three of these conditions were examined. In the first set of experiments, the volume of liquid in the petri dish was varied as was the type of filter paper lining the dish. Four, 5 and 6 mls of solution were added to dishes lined with either Whatman no. 1 or no. 3 filter paper. The solutions contained a range of GA_3 concentrations from 10^{-6}M to 10^{-3}M, with a water control also being included. It was found (Fig. 27) that there was little difference between the treatments at low GA_3 levels, but at 10^{-3}M the seedlings on no. 3 filter paper were more markedly inhibited, especially where 6 mls of solution were added. There was also some evidence that the optimum GA_3 concentration for stimulation of elongation may have been

Fig. 26. The effect of the solvents used in the dispersion of ABA and the analogues on the extension of lettuce hypocotyls in the absence of GA_3 (open triangles) and in the presence of GA_3 at 10^{-5}M (closed squares).

(A) methanol.

(B) acetone.

The histogram illustrates the effect of methanol and sodium bicarbonate on GA_3 induced extension.

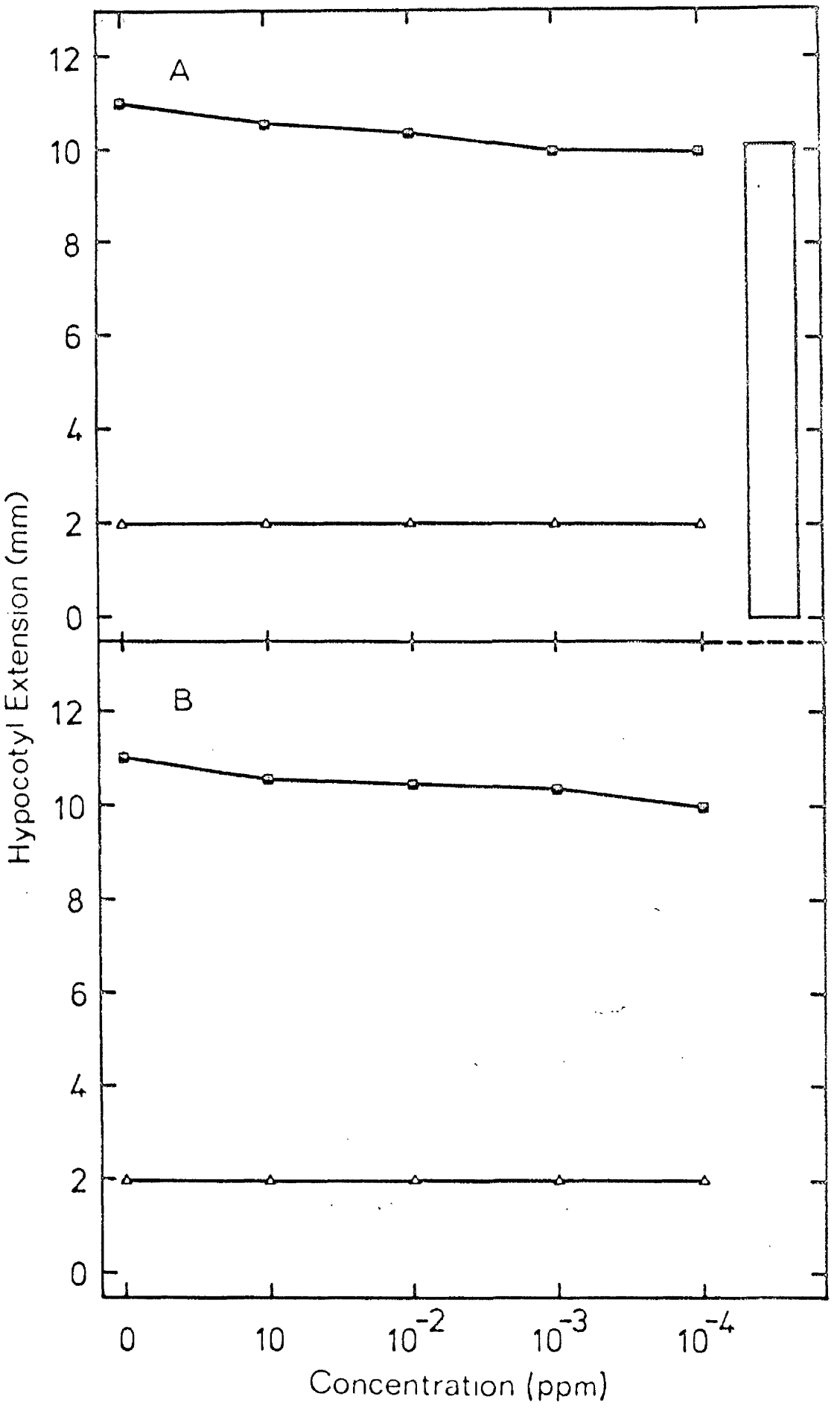

A
B
12
10
8
6
4
2
0
Hypocotyl Extension (mm)
0
10
10^{-2}
10^{-3}
10^{-4}
Concentration (ppm)

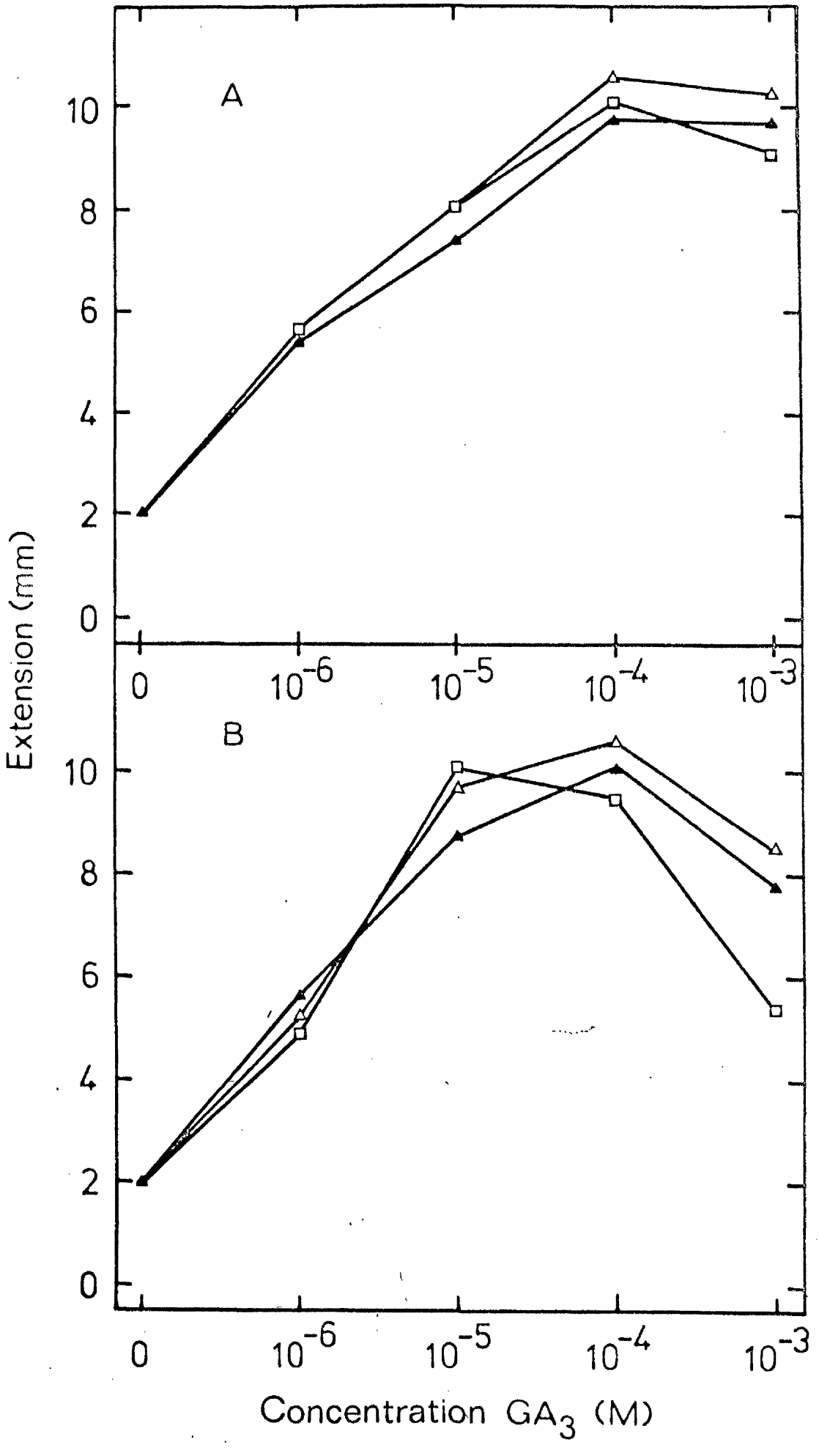
Fig. 27. The effect of the thickness of the paper lining the petri dishes and the volume of test solution on the growth of lettuce hypocotyls.

(A) Whatman no. 1 filter paper, (B) Whatman no. 3 filter paper.

closed triangle - 4 ml of test solution.

open triangle - 5 " " " "

open square - 6 " " " "


A
B
10
8
6
4
2
0
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Extension (mm)
Concentration GA_3 (M)

slightly lower for the no. 3 paper. It was, however, concluded that over the concentration range 10^{-7}M to 10^{-5}M, the volume of solution and the type of paper used were not critical as long as consistency was maintained.

The other point considered, was the length of the radicle at the beginning of the experiment. Using the same gibberellin concentrations as before, experiments were designed in which seedlings with radicles which had emerged by less than 1 mm, approximately 2 mm and more than 3 mm, were assayed. It was found that the effect of radicle length was quite small and only significant at GA_3 concentrations of 10^{-3}M and using seedlings which had been transferred to the GA_3 solution when the radicle had emerged by approximately 2 mm (Fig. 28). The conditions under which the bioassays were conducted were, therefore, shown to be satisfactory.

(7) THE EFFECT OF ANALOGUES OF ABA ON LETTUCE HYPOCOTYL EXTENSION AND THE INTERACTION OF THE ANALOGUES WITH GA_3.

Using concentrations of 0, 10^{-7}, 10^{-6} and 10^{-5}M, the activities of 32 analogues were tested in the lettuce hypocotyl assay (Figs. 29-36). The results of the above factorial experiments were subjected to analyses of variance (Tables 20-27) with the "t" test being applied to selected results. The mean extension at each concentration of analogue was compared to the mean extension when no analogue was present. This procedure was carried out with two of the more active analogues (I and II) at 3 concentrations of GA_3 (Table 28). When GA_3 was present at 10^{-5} or 10^{-6}M, the presence of either of the analogues at 10^{-5}M resulted in a significant reduction in hypocotyl extension, and in the case of I, its presence at 10^{-6}M also caused a significant effect. At the other concentrations of gibberellin, the presence of either analogue, at any concentration examined, did not cause a significant inhibition of hypocotyl extension. For the remaining ABA analogues, the "t" test was applied only to results obtained in the presence of 10^{-5}M GA_3 (Table 29).

Fig. 28. The effect of initial radicle length on GA_3 induced growth of lettuce hypocotyls.

closed square - radicles 2 mm long at start of assay.

open triangle - radicles less than 1 mm long at start of assay.

open square - radicles more than 3 mm long at start of assay.

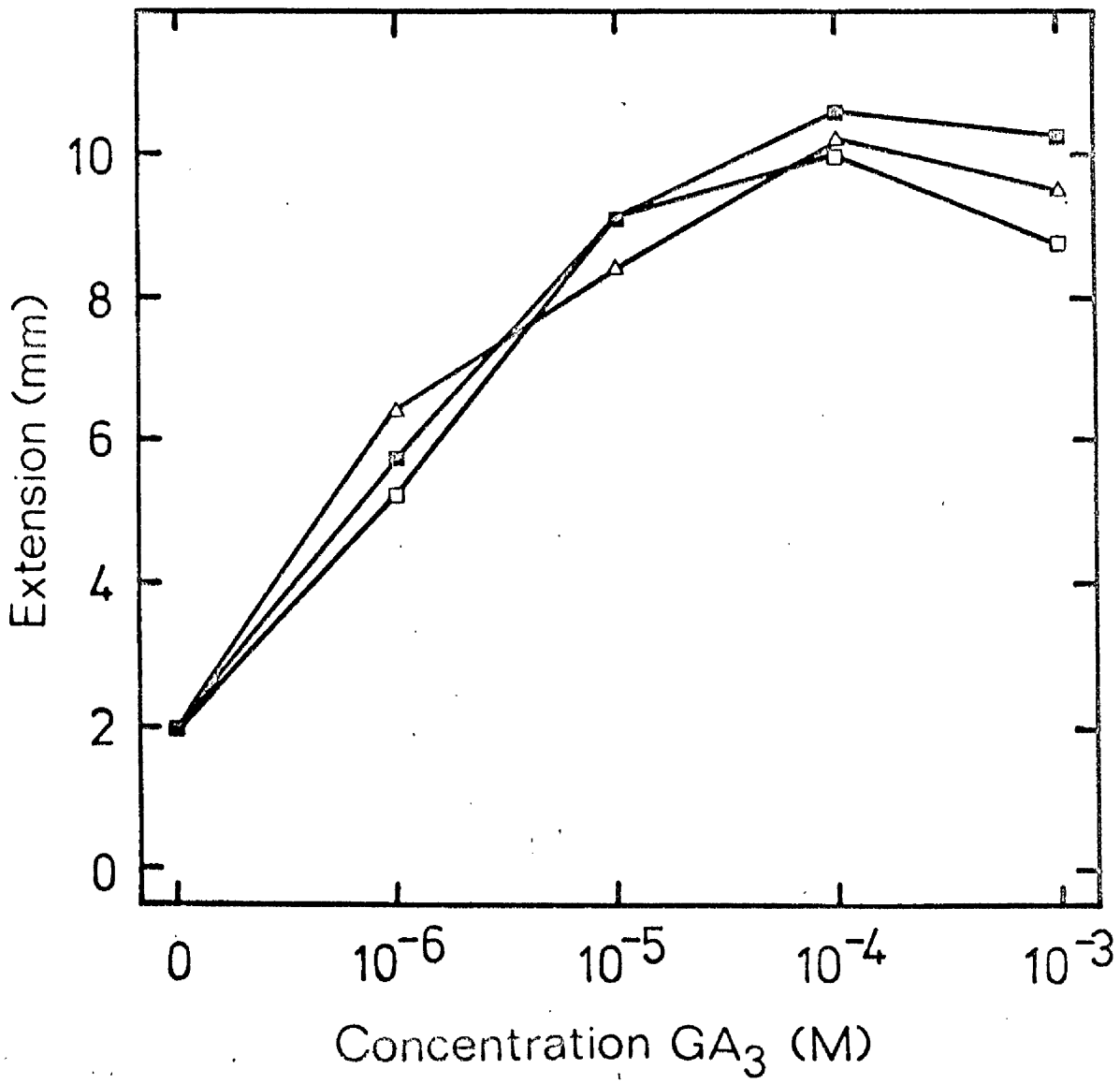

Extension (mm)
0
2
4
6
8
10
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Concentration GA_3 (M)

Fig. 29. The effect of various concentrations of GA_3 and analogues of ABA on the extension of lettuce hypocotyls. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound I.

(B) compound II.

(C) compound III.

(D) compound IV.

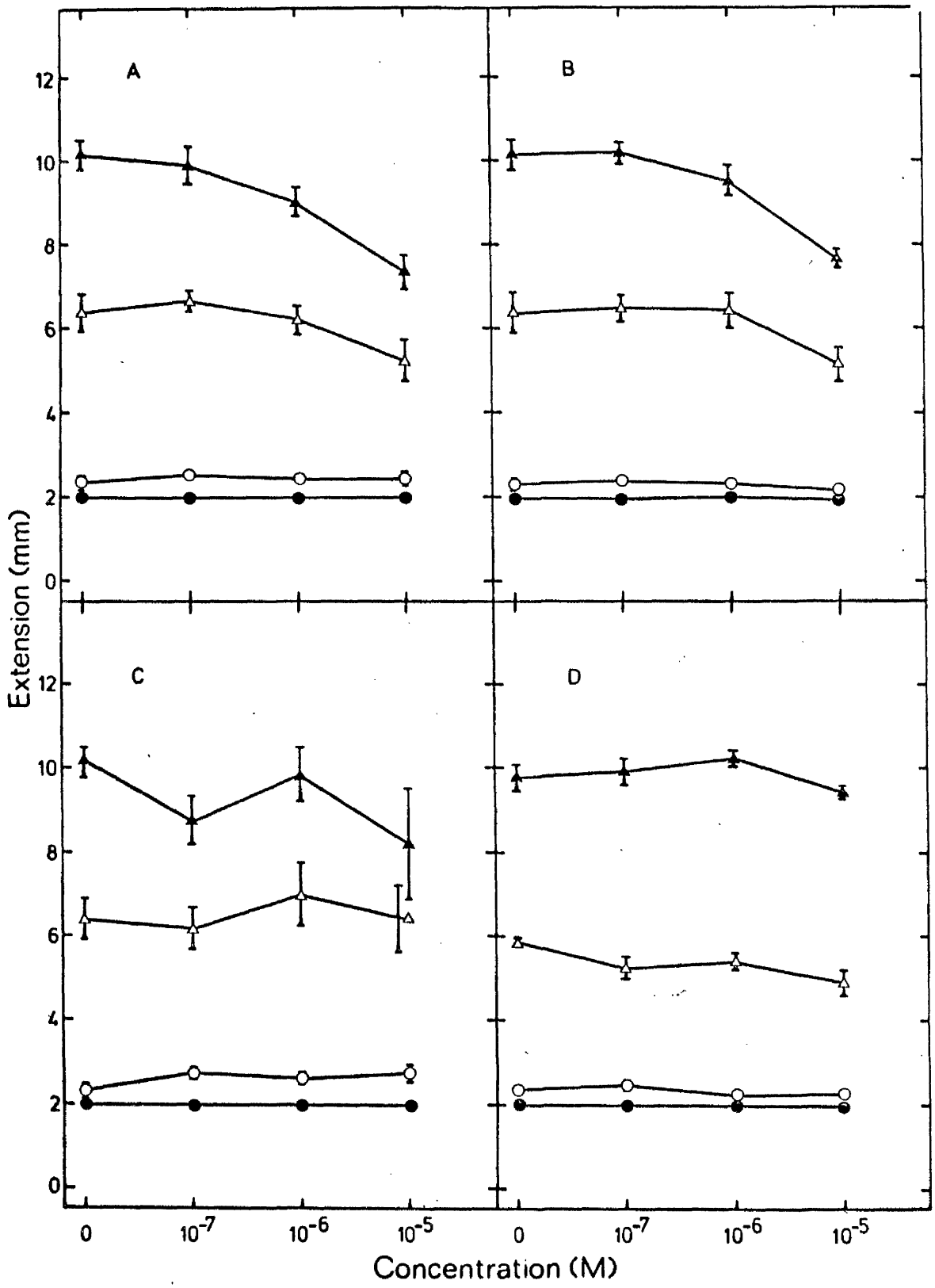

A
B
C
D
Extension (mm)
12
10
8
6
4
2
0
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 20. Analysis of variance of the extension of lettuce hypocotyls in response to GA_3 and analogues of ABA.

(A) Compound I.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
I	16.40	3	5.46	10.96	***
GA_3	814.91	3	271.63	545.02	***
Interaction	20.16	9	2.24	4.49	***
Error	39.87	80	0.49		

(B) Compound II.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
II	17.02	3	5.67	14.20	***
GA_3	890.18	3	296.72	742.75	***
Interaction	16.46	9	1.83	4.58	***
Error	31.96	80	0.40		

(C) Compound III.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
III	4.39	3	1.46	0.87	NS
GA_3	844.98	3	281.66	167.54	***
Interaction	13.90	9	1.54	0.91	NS
Error	134.48	80	1.68		

(D) Compound IV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
IV	1.85	3	0.61	3.49	*
GA_3	957.25	3	319.08	1806.82	***
Interaction	3.36	9	0.37	2.11	*
Error	14.13	80	0.17		

Fig. 30. The effect of various concentrations of GA_3 and analogues of ABA on the extension of lettuce hypocotyls. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound V.

(B) compound VI.

(C) compound VII.

(D) compound VIII.

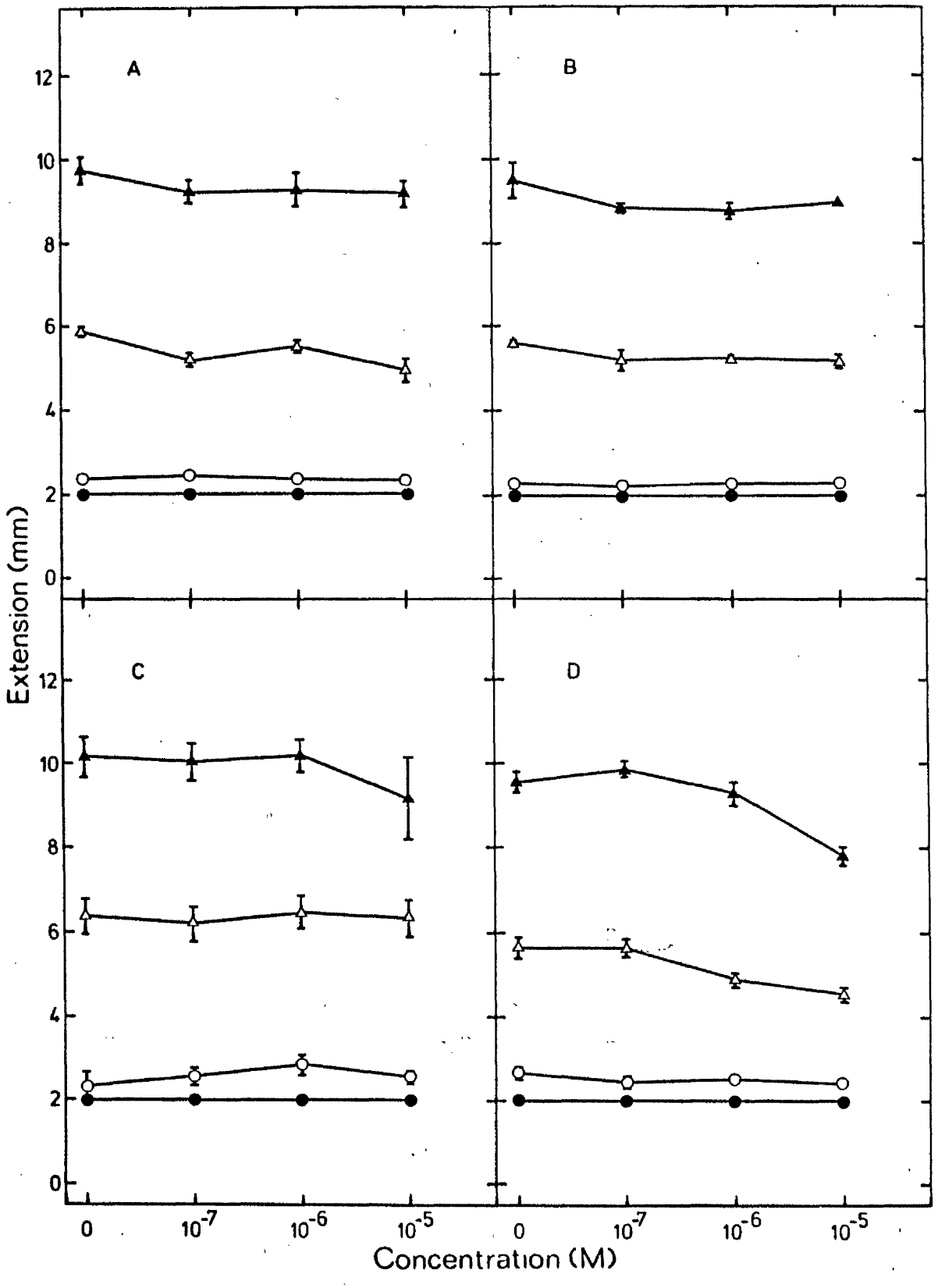

A
B
C
D
Extension (mm)
Concentration (M)
0
2
4
6
8
10
12
0
10^{-7}
10^{-6}
10^{-5}

Table 21. Analysis of variance of the extension of lettuce hypocotyls in response to GA_3 and analogues of ABA.

(A) Compound V.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
V	2.180	3	0.727	3.076	*
GA_3	837.895	3	279.298	1182.465	***
Interaction	2.512	9	0.279	1.182	NS
Error	18.901	80	0.236		

(B) Compound VI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
VI	1.240	3	0.414	3.241	*
GA_3	806.351	3	268.784	2106.455	***
Interaction	1.276	9	0.142	1.111	NS
Error	10.209	80	0.128		

(C) Compound VII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
VII	1.797	3	0.599	0.701	NS
GA_3	976.297	3	325.432	380.845	***
Interaction	3.954	9	0.439	0.514	NS
Error	68.363	80	0.855		

(D) Compound VIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
VIII	9.994	3	3.331	22.555	***
GA_3	771.418	3	257.139	1740.956	***
Interaction	11.081	9	1.231	8.335	***
Error	11.819	80	0.148		

Fig. 31. The effect of various concentrations of GA_3 and analogues of ABA on the extension of lettuce hypocotyls. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound IX.

(B) compound X.

(C) compound XI.

(D) compound XII.

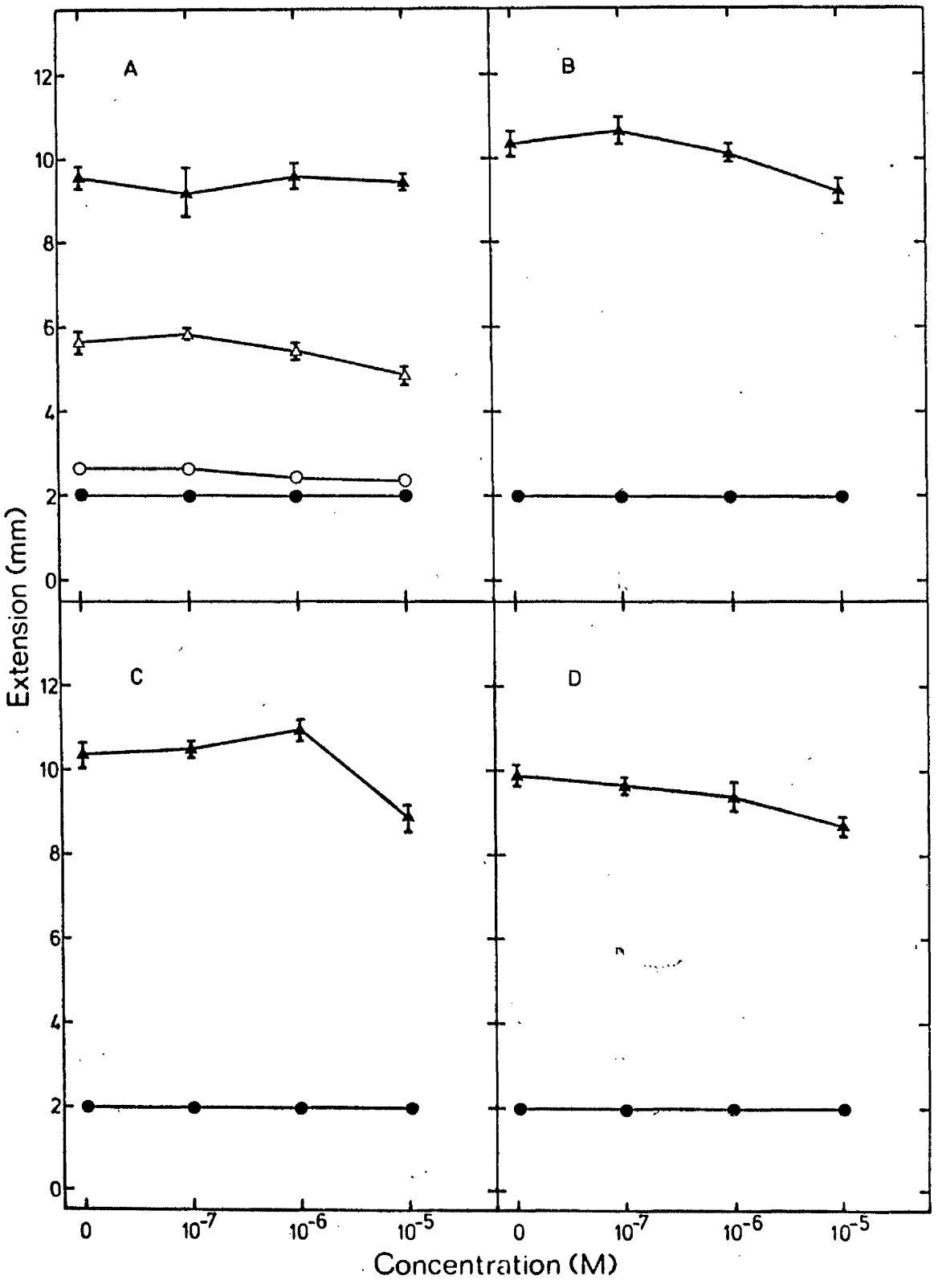

A
B
C
D
Extension (mm)
Concentration (M)
0
2
4
6
8
10
12
0
10^{-7}
10^{-6}
10^{-5}

Table 22. Analysis of variance of the extension of lettuce hypocotyls in response to GA_3 and analogues of ABA.

(A) Compound IX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
IX	1.296	3	0.432	1.529	NS
GA_3	842.448	3	280.816	994.743	***
Interaction	2.931	9	0.326	1.153	NS
Error	22.588	80	0.282		

(B) Compound X.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
X	2.18	3	0.73	1.84	NS
GA_3	797.55	1	797.55	2029.39	***
Interaction	2.55	3	0.85	2.16	NS
Error	15.75	40	0.39		

(C) Compound XI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XI	7.33	3	2.44	11.58	***
GA_3	800.19	1	800.19	3792.39	***
Interaction	7.43	3	2.48	11.73	***
Error	8.47	40	0.21		

(D) Compound XII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XII	0.78	3	0.26	1.24	NS
GA_3	680.49	1	680.49	3255.91	***
Interaction	0.97	3	0.32	1.55	NS
Error	8.38	40	0.21		

Fig. 32. The effect of various concentrations of GA_3 and analogues of ABA on the extension of lettuce hypocotyls. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XIII.

(B) compound XIV.

(C) compound XV.

(D) compound XVI.

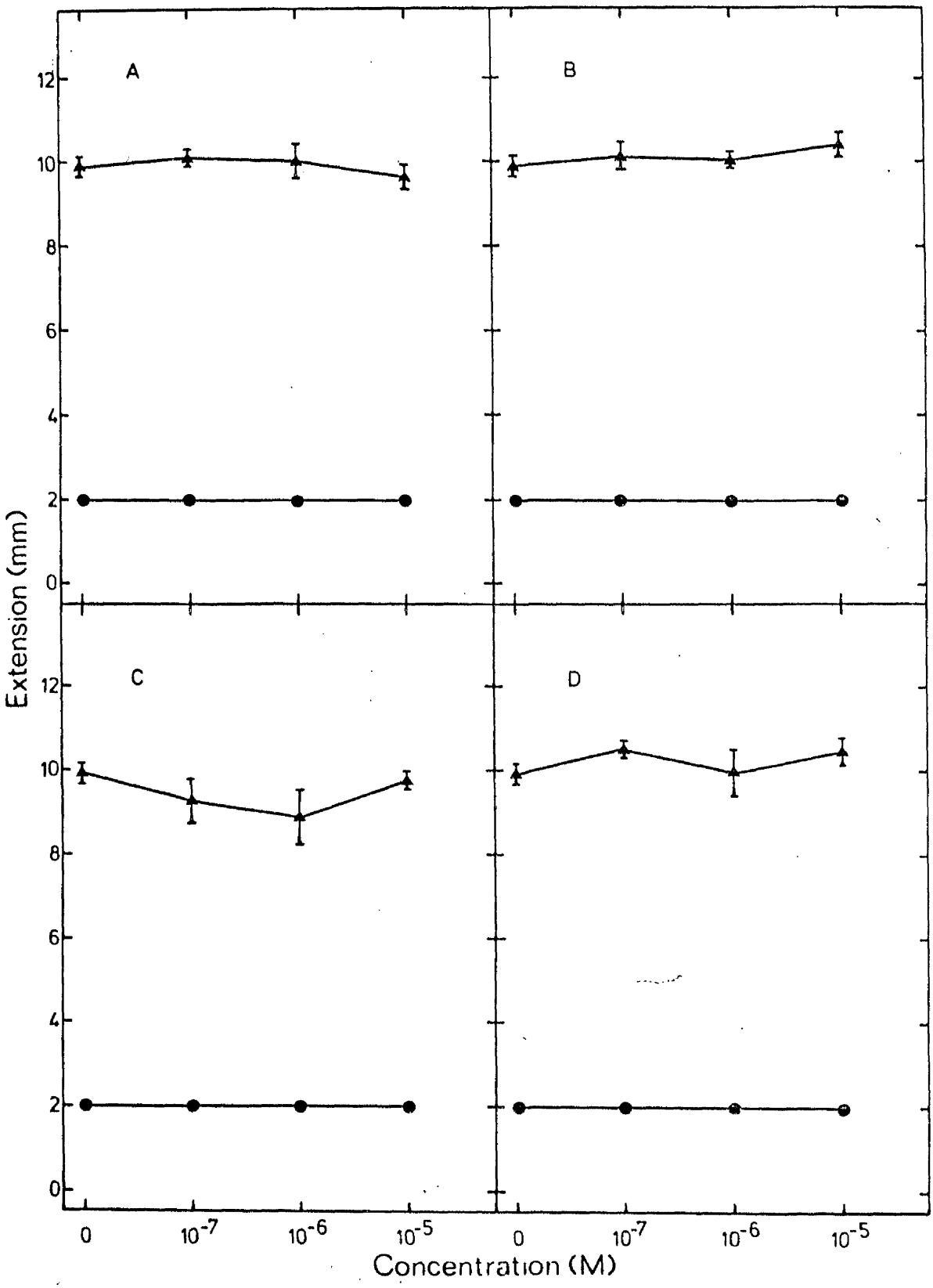

A
B
C
D
Extension (mm)
0
2
4
6
8
10
12
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 23. Analysis of variance of the extension of lettuce hypocotyls in response to GA_3 and analogues of ABA.

(A) Compound XIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XIII	0.38	3	0.13	0.58	NS
GA_3	755.40	1	755.40	3497.20	***
Interaction	0.56	3	0.19	0.86	NS
Error	8.66	40	0.22		

(B) Compound XIV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XIV	0.33	3	0.11	0.54	NS
GA_3	788.16	1	788.16	3882.54	***
Interaction	0.62	3	0.21	1.01	NS
Error	8.14	40	0.20		

(C) Compound XV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XV	1.92	3	0.64	1.61	NS
GA_3	662.13	1	662.13	1667.84	***
Interaction	2.14	3	0.71	1.79	NS
Error	15.89	40	0.40		

(D) Compound XVI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XVI	0.88	3	0.29	0.76	NS
GA_3	795.12	1	795.12	2076.04	***
Interaction	0.73	3	0.24	0.64	NS
Error	15.33	40	0.38		

Fig. 33. The effect of various concentrations of GA_3 and analogues of ABA on the extension of lettuce hypocotyls. The analggue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XVII.

(B) compound XVIII.

(C) compound XIX.

(D) compound XX.

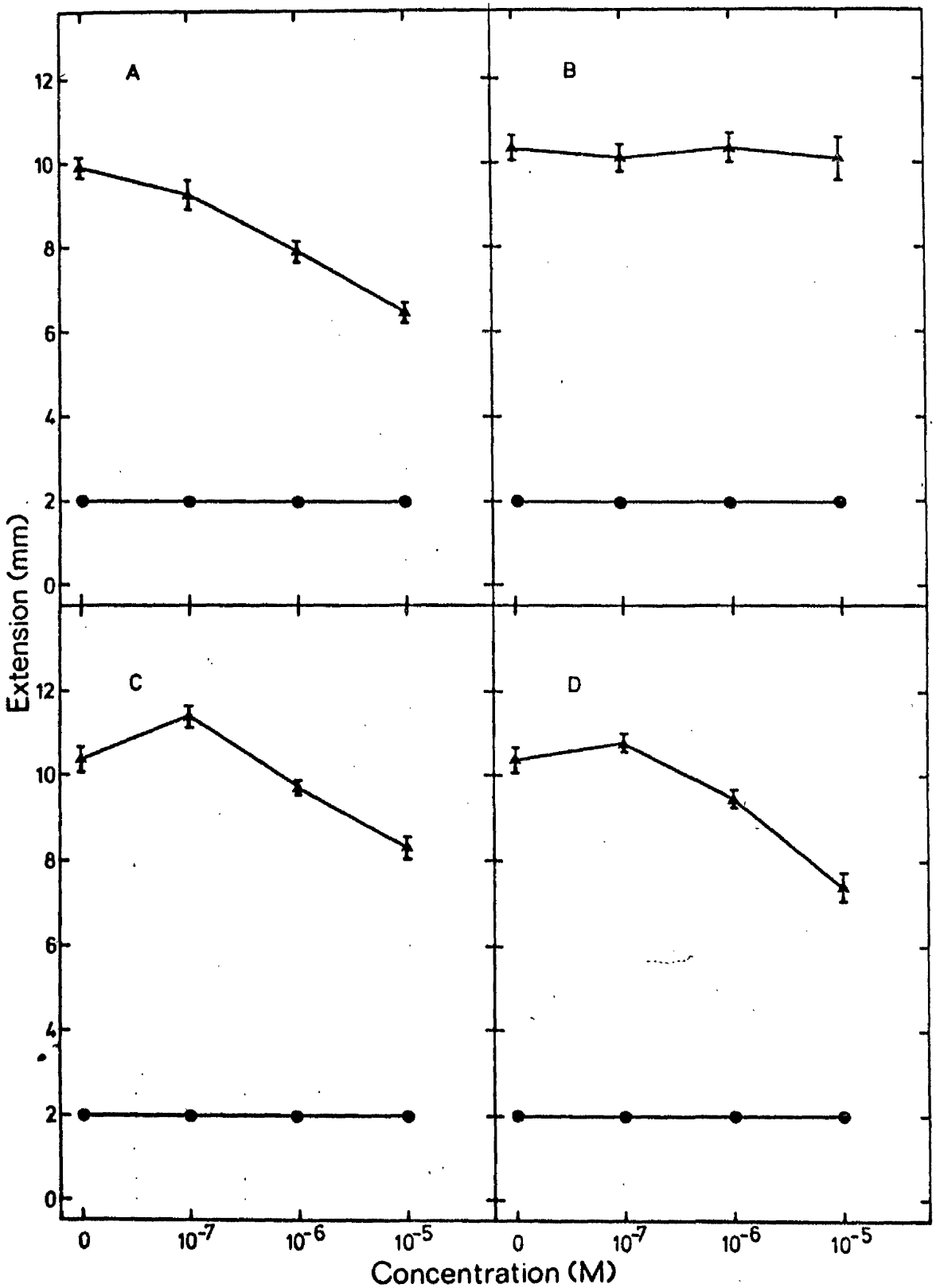

A
B
C
D
Extension (mm)
0
2
4
6
8
10
12
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 24. Analysis of variance of the extension of lettuce hypocotyls in response to GA_3 and analogues of ABA.

(A) Compound XVII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XVII	20.89	3	6.96	28.54	***
GA_3	486.33	1	486.33	1993.16	***
Interaction	21.07	3	7.02	28.79	***
Error	9.78	40	0.24		

(B) Compound XVIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XVIII	0.36	3	0.12	0.26	NS
GA_3	812.44	1	812.44	1817.54	***
Interaction	0.29	3	0.10	0.21	NS
Error	17.90	40	0.45		

(C) Compound XIX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XIX	14.91	3	4.97	24.97	***
GA_3	755.40	1	755.40	3795.96	***
Interaction	15.24	3	5.08	25.53	***
Error	7.99	40	0.20		

(D) Compound XX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XX	20.36	3	6.79	35.53	***
GA_3	673.20	1	673.20	3524.61	***
Interaction	20.65	3	6.88	36.04	***
Error	7.67	40	0.19		

Fig. 34. The effect of various concentrations of GA_3 and analogues of ABA on the extension of lettuce hypocotyls. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XXI.

(B) compound XXII.

(C) compound XXIII.

(D) compound XXIV.

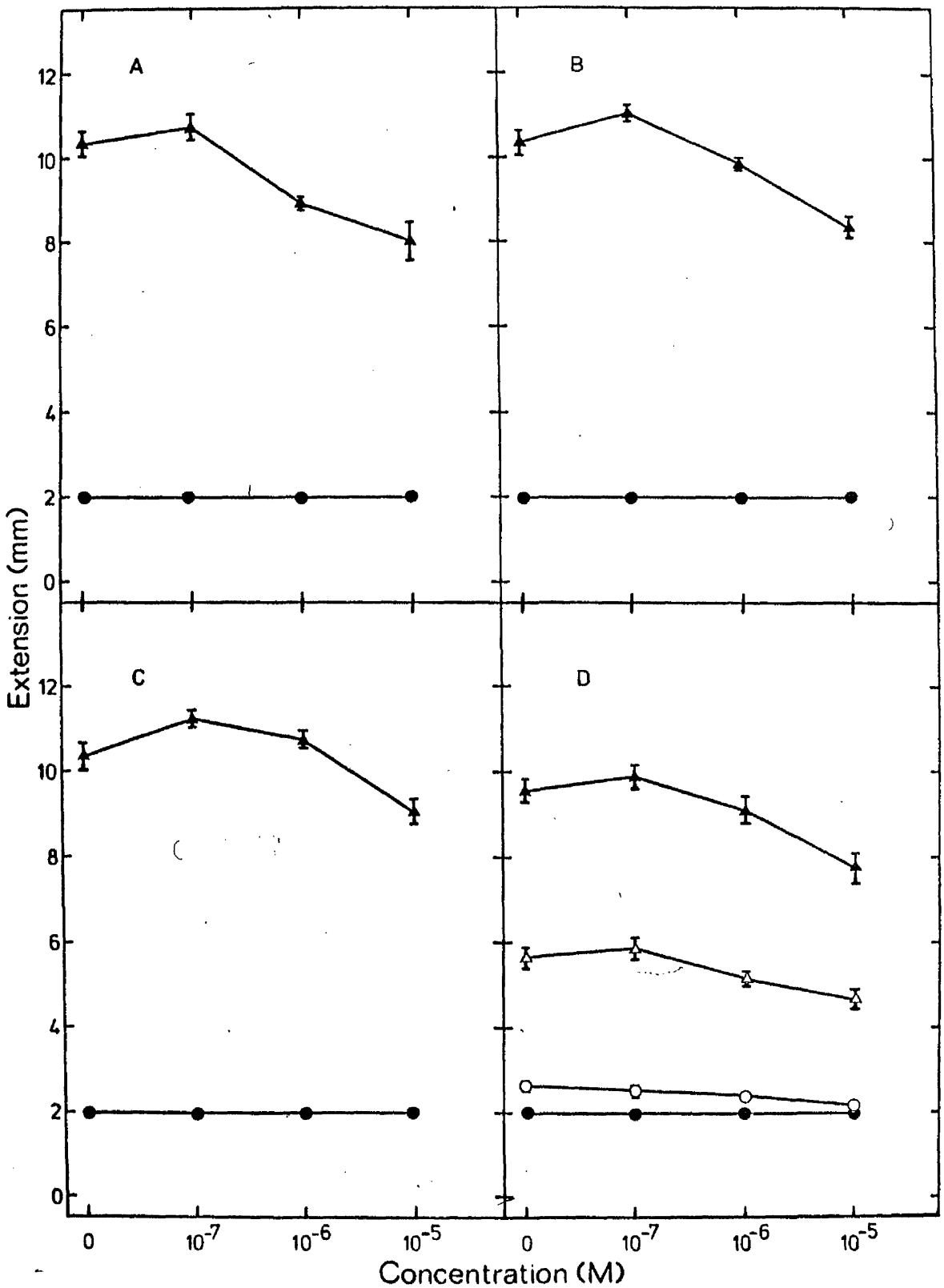

A
B
C
D
Extension (mm)
0
2
4
6
8
10
12
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 25. Analysis of variance of the extension of lettuce hypocotyls in response to GA_3 and analogues of ABA.

(A) Compound XXI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXI	14.34	3	4.78	16.03	***
GA_3	674.64	1	674.64	2263.90	***
Interaction	14.32	3	4.77	16.02	***
Error	11.94	40	0.30		

(B) Compound XXII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXII	11.91	3	3.97	22.43	***
GA_3	748.54	1	748.54	4229.05	***
Interaction	11.90	3	3.97	22.40	***
Error	7.11	40	0.18		

(C) Compound XXIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXIII	7.46	3	2.49	12.19	***
GA_3	827.17	1	827.17	4054.75	***
Interaction	7.69	3	2.56	12.57	***
Error	8.18	40	0.20		

(D) Compound XXIV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXIV	12.201	3	4.067	18.605	***
GA_3	772.857	3	257.619	1178.495	***
Interaction	9.760	9	1.084	4.960	***
Error	17.489	80	0.219		

Fig. 35. The effect of various concentrations of GA_3 and analogues of ABA on the extension of lettuce hypocotyls. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XXV.

(B) compound XXVI.

(C) compound XXVII.

(D) compound XXVIII.

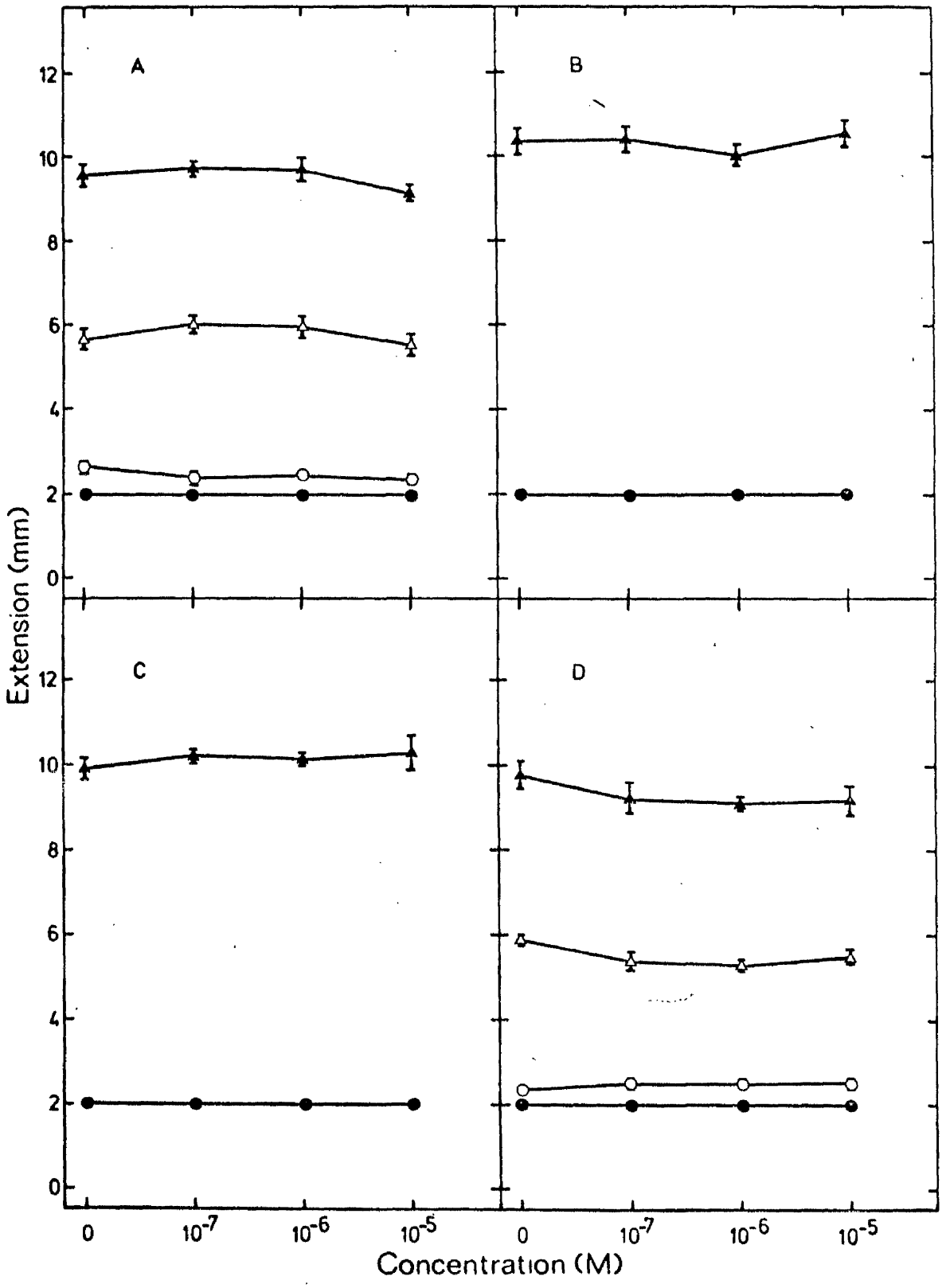

A
B
C
D
Extension (mm)
Concentration (M)
0
2
4
6
8
10
12
0
10^{-7}
10^{-6}
10^{-5}

Table 26. Analysis of variance of the extension of lettuce hypocotyls in response to GA_3 and analogues of ABA.

(A) Compound XXV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXV	1.237	3	0.412	2.412	NS
GA_3	884.024	3	294.675	1724.253	***
Interaction	1.566	9	0.174	1.018	NS
Error	13.674	80	0.171		

(B) Compound XXVI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXVI	0.32	3	0.11	0.44	NS
GA_3	833.12	1	833.12	3359.34	***
Interaction	0.60	3	0.20	0.80	NS
Error	9.94	40	0.25		

(C) Compound XXVII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXVII	0.27	3	0.09	0.46	NS
GA_3	791.10	1	791.10	4016.14	***
Interaction	0.32	3	0.11	0.53	NS
Error	7.89	40	0.20		

(D) Compound XXVIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXVIII	1.119	3	0.373	2.046	NS
GA_3	824.369	3	274.790	1507.348	***
Interaction	1.897	9	0.211	1.156	NS
Error	14.588	80	0.182		

Fig. 36. The effect of various concentrations of GA_3 and analogues of ABA on the extension of lettuce hypocotyls. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XXIX.

(B) compound XXX.

(C) compound XXXI.

(D) compound XXXII.

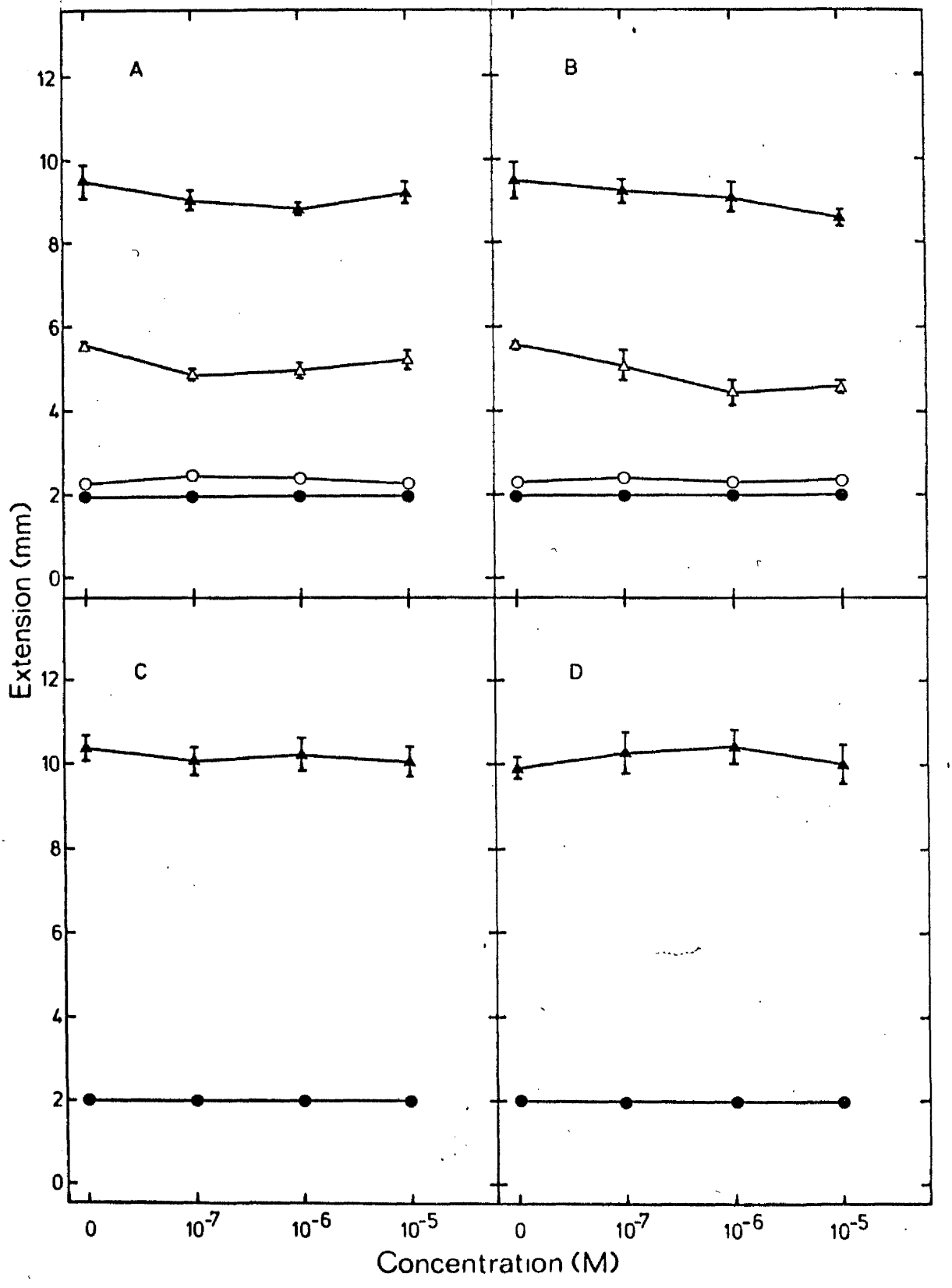

A
B
C
D
Extension (mm)
Concentration (M)
0
2
4
6
8
10
12
0
10^{-7}
10^{-6}
10^{-5}

Table 27. Analysis of variance of the extension of lettuce hypocotyls in response to GA_3 and analogues of ABA.

(A) Compound XXIX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXIX	1.204	3	0.401	2.544	NS
GA_3	792.248	3	264.083	1674.588	***
Interaction	2.436	9	0.271	1.716	NS
Error	12.621	80	0.158		

(B) Compound XXX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXX	2.711	3	0.904	3.711	*
GA_3	779.842	3	259.947	1067.545	***
Interaction	3.094	9	0.344	1.411	NS
Error	19.467	80	0.244		

(C) Compound XXXI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXXI	0.14	3	0.05	0.13	NS
GA_3	791.29	1	791.29	2293.61	***
Interaction	0.49	3	0.16	0.47	NS
Error	13.83	40	0.35		

(D) Compound XXXII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXXII	0.41	3	0.14	0.29	NS
GA_3	795.12	1	795.12	1691.73	***
Interaction	0.70	3	0.23	0.50	NS
Error	18.83	40	0.47		

Table 28. The inhibitory effects of compounds I and II on lettuce hypocotyl extension in the presence of GA_3.

(a) Compound I.

I (M) \ GA_3 (M)	0	10^{-7}	10^{-6}	10^{-5}
0	1.98 ± 0.03	2.32 ± 0.14	6.39 ± 0.47	10.17 ± 0.35
10^{-7}	1.98 ± 0.03^{NS}	2.53 ± 0.16^{NS}	6.68 ± 0.49^{NS}	9.92 ± 0.40^{NS}
10^{-6}	1.97 ± 0.04^{NS}	2.43 ± 0.06^{NS}	6.21 ± 0.36^{NS}	9.03 ± 0.34*
10^{-5}	1.92 ± 0.06^{NS}	2.45 ± 0.08^{NS}	5.21 ± 0.26*	7.36 ± 0.46***

(b) Compound II.

II (M) \ GA_3 (M)	0	10^{-7}	10^{-6}	10^{-5}
0	1.98 ± 0.03	2.32 ± 0.14	6.39 ± 0.47	10.17 ± 0.35
10^{-7}	1.96 ± 0.02^{NS}	2.38 ± 0.12^{NS}	6.50 ± 0.40^{NS}	10.23 ± 0.22^{NS}
10^{-6}	2.01 ± 0.05^{NS}	2.33 ± 0.04^{NS}	6.45 ± 0.43^{NS}	9.55 ± 0.36^{NS}
10^{-5}	1.93 ± 0.04^{NS}	2.17 ± 0.06^{NS}	5.15 ± 0.31*	7.67 ± 0.25***

Table 29. Lettuce hypocotyl extension in solutions containing ABA analogues and GA_3 in combination at 10^{-5}M, and the statistical significance of the inhibition elicited by the analogues in these solutions.

Compound	Hypocotyl Extension (mm)	SE (±)	Significance of inhibition ('t' tests)
I	7.36	0.46	xxx
II	7.67	0.25	xxx
III	8.20	0.55	x
IV	9.44	0.16	NS
V	9.17	0.34	NS
VI	9.01	0.08	NS
VII	9.15	0.99	NS
VIII	7.81	0.19	xxx
IX	9.43	0.21	NS
X	9.48	0.51	NS
XI	8.87	0.30	xx
XII	9.21	0.22	NS
XIII	9.64	0.28	NS
XIV	10.38	0.28	NS
XV	9.74	0.21	NS
XVI	10.22	0.32	NS
XVII	6.45	0.26	xxx
XVIII	10.10	0.52	NS
XIX	8.31	0.27	xxx
XX	7.39	0.33	xxx
XXI	8.02	0.44	xx
XXII	8.34	0.27	xx
XXIII	9.06	0.30	x
XXIV	7.78	0.38	xx
XXV	9.15	0.18	NS
XXVI	10.54	0.28	NS
XXVII	10.28	0.39	NS
XXVIII	9.21	0.35	NS
XXIX	9.24	0.27	NS
XXX	8.58	0.18	NS
XXXI	10.05	0.37	NS
XXXII	9.99	0.43	NS

The compounds which were active in inhibiting GA_3-induced lettuce hypocotyl elongation were, in general, those which also actively inhibited lettuce germination. Eight compounds, (IV, VII, IX, X, XIV, XXV, XXVII and XXVIII), which were active in inhibiting germination were, however, inactive in this assay. Of these, IV, VII, XIV, XXV, XXVII and XXVIII were considerably less active than ABA in inhibiting germination and, since the lettuce hypocotyl bioassay appeared to be much less sensitive than the germination system, the apparent lack of activity can be explained. Allowing for this, the activity of IX and X should, nevertheless, have been detectable if they were relatively as active in this assay as in the germination assay. No activity was detected. In the case of IX, this was in keeping with its atypical behaviour reported earlier, but such is not the case with X. It would appear that the structural requirements for activity are the same in both this assay and the lettuce germination assay.

When interactions with GA_3 were examined it was found that in all cases where the "t" test indicated a significant effect at the analogue concentration of 10^{-5}M in the presence of GA_3 at 10^{-5}M, a significant interaction term was found in the analysis of variance. There were, however, 3 instances (V, VI, XXX) where an analogue effect was significant at the 5% level in the analysis of variance, but no evidence of an interaction existed; these observations may relate merely to chance occurrences in one treatment.

In the absence of GA_3, the growth response to the ABA analogues was constant regardless of the amount of analogue present. Thus, an interaction would exist between the analogues and GA_3, because there was a concentration dependent inhibition of GA_3-induced extension. Thus, in the figures, the "no GA_3" treatment line is parallel to the horizontal axis, but the "10^{-5}M GA_3" treatment line is not parallel to either the "no GA_3" treatment line or the horizontal axis. The exact nature of such interactions and the relevance of a statistical approach is discussed on p.132.

(8) THE EFFECT OF ABA AND ITS ANALOGUES ON LETTUCE HYPOCOTYL EXTENSION IN THE DARK.

ABA and its available analogues were also assayed at 10^{-5}M in the dark for their effect on lettuce hypocotyl extension. It is not clear whether dark extension is brought about by GA_3 or whether other factors are involved.

All manipulations were carried out under physiologically "safe" light and the petri dishes were incubated in complete darkness. The results (Fig. 37) were compared with the extension achieved in water and that achieved in ABA at 10^{-5}M (Table 30). ABA, I, II, III, IV, VIII, IX, X, XI, XVII, XIX, XX, XXI, XXII, XXIII and XXIV significantly inhibited hypocotyl extension, while XXXI produced a small but significant promotion. The degree of inhibition imposed by the analogues ranged from very slight to more than that induced by ABA, which inhibited extension from 21.37 $\pm$ 0.80 mm to 8.47 $\pm$ 1.16 mm. The most active analogue was XVII, which inhibited growth to 1.52 $\pm$ 0.19 mm, and only this analogue and II were significantly more active than ABA, although I, VIII, XIX, XX, XXI and XXII all possessed activity similar to ABA. Compounds III, IV, IX, X, XI and XXIII were also inhibitory, but were significantly less active than ABA.

The only compounds active in the germination assay, but lacking significant activity on dark grown hypocotyls, were VII, XIV, XXV, XXVII and XXVIII, and, with the exception of XXV, these were only slightly active in inhibiting germination. Their activity might not therefore have been detectable in this assay. Compound XXV is similar in structure to IX, which was also active, although not as active as ABA. The unusual behaviour of IX and XXV has already been noted.

The difference between the assays involving the assessment of lettuce hypocotyl extension in the dark and in light plus GA_3, appears to be that in darkness, the assay was moresensitive. The molecular requirements for activity, however, again seem to be the same.

Fig. 37. The effect of ABA and certain of its analogues on the dark extension of lettuce hypocotyls. All compounds were assayed at 10^{-5}M.

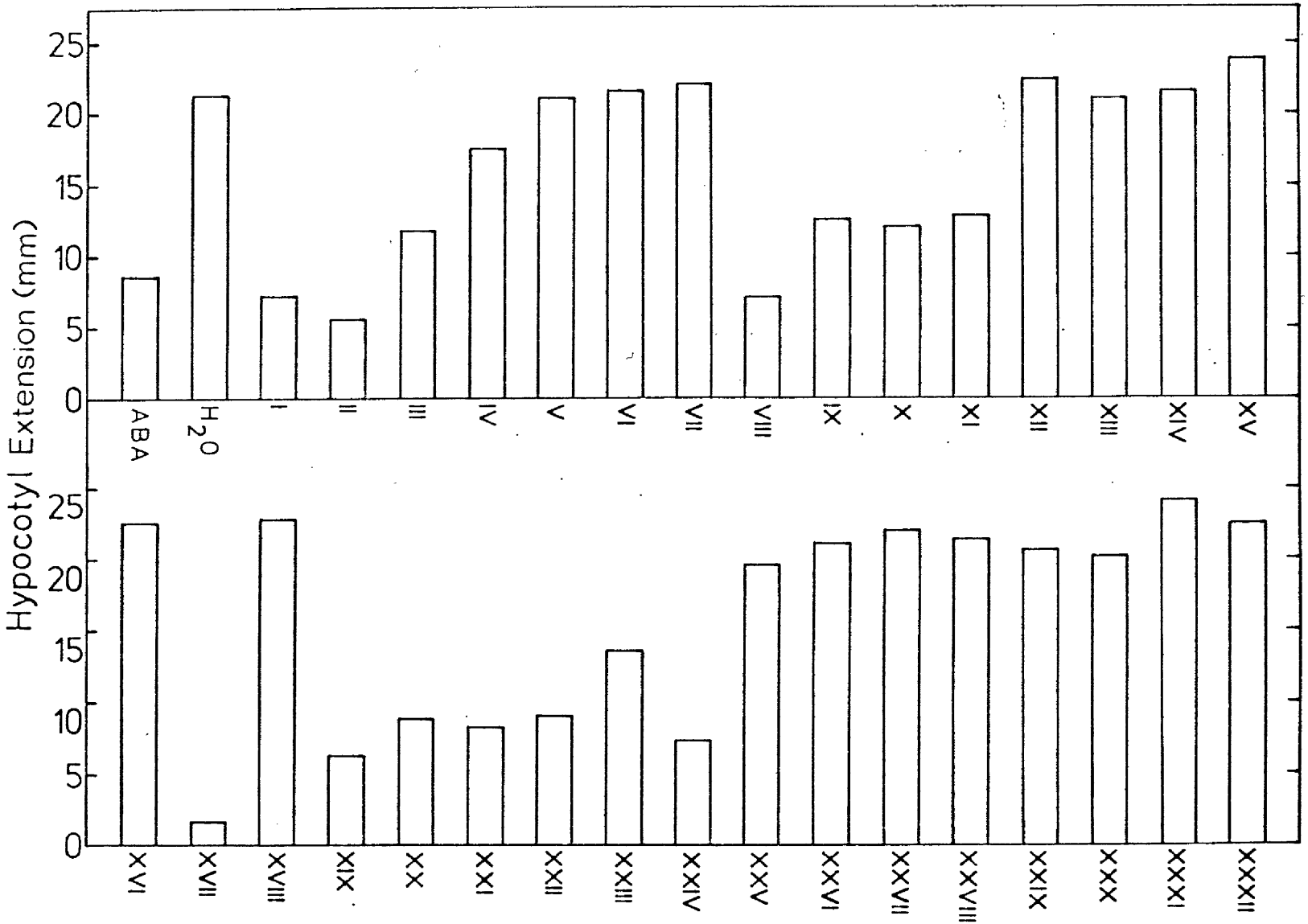

Hypocotyl Extension (mm)
0
5
10
15
20
25
ABA
H_2O
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
XXI
XXII
XXIII
XXIV
XXV
XXVI
XXVII
XXVIII
XXIX
XXX
XXXI
XXXII

Table 30. The inhibition by ABA and its analogues at 10^{-5}M of the dark extension of the lettuce hypocotyl, and the probabilities of these extensions being significantly different from those achieved (A) in water and (B) in 10^{-5}M ABA.

Compound	Hypocotyl length (mm)	Standard Error (±)	A	B
ABA	8.47	1.16	***	-
H_2O	21.37	0.80	-	***
I	7.15	0.38	***	NS
II	5.45	0.76	***	*
III	11.77	0.40	***	*
IV	17.50	1.12	*	***
V	21.05	1.12	NS	***
VI	21.55	0.61	NS	***
VII	21.90	0.96	NS	***
VIII	7.15	0.42	***	NS
IX	12.40	0.42	***	**
X	11.90	0.56	***	**
XI	12.72	0.29	***	**
XII	22.23	0.56	NS	***
XIII	21.00	0.79	NS	***
XIV	21.42	0.61	NS	***
XV	23.78	0.83	NS	***
XVI	22.50	0.46	NS	***
XVII	1.52	0.19	***	***
XVIII	22.80	0.54	NS	***
XIX	6.2	0.92	***	NS
XX	8.78	0.58	***	NS
XXI	8.23	0.60	***	NS
XXII	9.00	0.36	***	NS
XXIII	13.63	0.43	***	**
XXIV	7.20	0.46	***	NS
XXV	19.47	0.56	NS	***
XXVI	20.90	0.71	NS	***
XXVII	21.63	1.40	NS	***
XXVIII	21.33	0.71	NS	***
XXIX	20.48	0.77	NS	***
XXX	20.16	0.86	NS	***
XXXI	23.92	0.72	*	***
XXXII	22.38	0.87	NS	***

Two important points of distinction between this assay, utilizing dark growth of the lettuce hypocotyl, and the previous one, where GA_3 induced light growth was assessed, must be stressed. The hypocotyl in these dark experiments extended in water to 21.37 ± 0.80 mm while in light, at the optimum GA_3 levels of 10^{-4}M it was only 12.03 ± 0.65 mm. It is thus obvious that the dark extension of the lettuce hypocotyl cannot be explained only in terms of endogenous GA_3 levels, and may be due to other hormonal and/or nutritional factors. The second point refers to the degree of inhibition which ABA and its analogues exert. Where the experiments were conducted in darkness, the most active analogue, XVII, inhibited elongation from 21.37 ± 0.80 mm to 1.52 ± 0.19 mm, while in light, the same concentration of analogue was only capable of inhibiting GA_3-induced elongation from 9.88 ± 0.24 mm to 6.45 ± 0.26 mm.

(9) THE EFFECTS OF ABA ON THE PEA EPICOTYL ASSAY AND THE INTERACTION OF THE ABA WITH GA_3.

McWha et al. (1973) have demonstrated that the molecular requirements for ABA-like activity can depend on the assay. Having ascertained the similarity in structural requirements for inhibitory activity in the lettuce germination and lettuce hypocotyl bioassays, a comparable system using a different genus was considered. The system chosen was the pea epicotyl assay. The hypocotyl in lettuce and the epicotyl in pea have similar rôles in sub-soil germination and have root-like anatomy.

Pea seedlings were grown in darkness to stimulate epicotyl growth; exposure to light suppresses epicotyl growth and the assay would be impractical to perform. The apical 20 mm was excised and incubated in the test solution for 2 days, after which the length of the internode was recorded. In order to maintain constant conditions, as far as possible, between this and the previous assays, a dwarf pea variety, "Meteor", which showed little

extension in light was used and the assay was carried out in light. As before, GA_3 was added to the assay to stimulate extension, and any inhibition of this extension brought about by ABA and the analogues was recorded.

ABA was assayed at concentrations of 0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M in the presence of GA_3 at concentrations of 0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3}M (Fig. 38). It was found by 't' testing that GA_3 at 10^{-7}M significantly promoted the extension of the pea internode (Table 31) and that this promotion increased when the concentration of GA_3 was increased to 10^{-6}M. Concentrations higher than this did not, however, elicit further response although high concentrations, even of 10^{-3}M, did not become inhibitory. The response pattern was unlike the lettuce hypocotyl extension assay where GA_3 concentrations of 10^{-4}M and higher produced significantly less extension than at 10^{-5}M. The optimum concentration for extension in both assays was, however, at about 10^{-5}M.

Table 31. The effect of GA_3 on dwarf pea internode extension.

GA_3 concentration (Molar)	Internode Extension (mm)	Standard Error (±)	Significance of Inhibition ('t' tests)
0	6.02	0.38	-
10^{-7}	9.18	0.45	***
10^{-6}	11.74	0.62	***
10^{-5}	12.04	0.45	***
10^{-4}	12.43	0.43	***
10^{-3}	12.49	0.40	***

When ABA was also included in the incubation medium, its effect was dependent both upon the amount added and the presence of GA_3. When no GA_3 was present, significant inhibition only occurred when the shoots were incubated in a solution containing at least 10^{-4}M ABA (Table 32). When ABA

Fig. 38. The effect of various concentrations of ABA and GA_3 alone and in combination on the extension of internodes of dwarf peas var. Meteor. ABA concentrations are shown on the horizontal axis.

open squares :- no GA_3.

open triangles :- 10^{-7}M GA_3.

closed triangle :- 10^{-6}M GA_3.

open circle :- 10^{-5}M GA_3.

closed square :- 10^{-4}M GA_3.

closed circle :- 10^{-3}M GA_3.

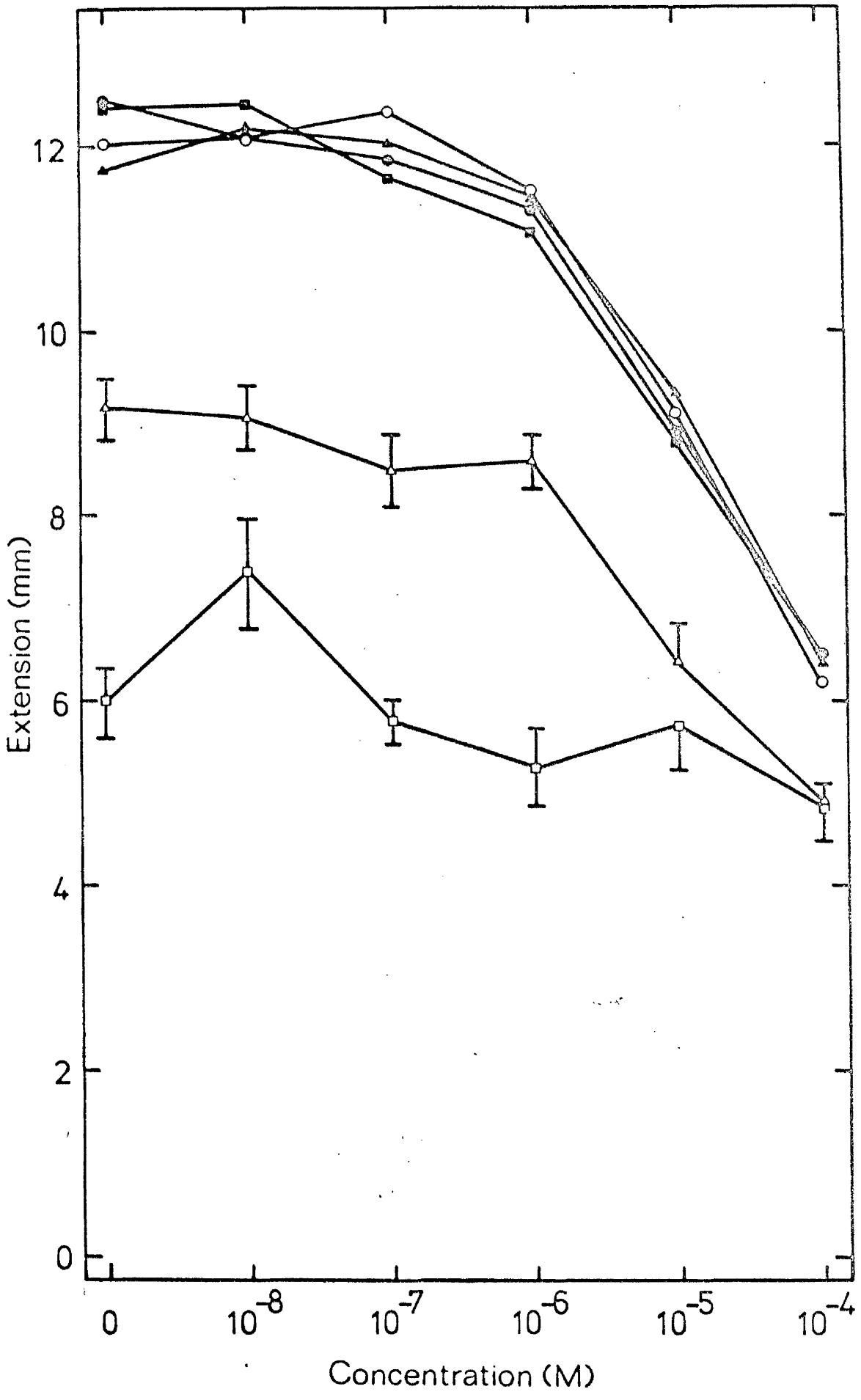

Extension (mm)
12
10
8
6
4
2
0
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration (M)

Table 32. The effect of ABA on dwarf pea internode extension.

ABA concentration (Molar)	Internode Extension (mm)	Standard Error (±)	Significance of Inhibition ('t' tests)
0	6.02	0.38	-
10^{-8}	7.41	0.59	NS
10^{-7}	5.80	0.24	NS
10^{-6}	5.30	0.42	NS
10^{-5}	5.75	0.51	NS
10^{-4}	4.84	0.37	*

was added to incubation medium containing GA_3 at a final concentration of 10^{-7}, 10^{-6} or 10^{-5}M, it was found that ABA at 10^{-5} and 10^{-4}M significantly inhibited extension (Table 33). When no ABA was present, extension in 10^{-7}M GA_3 was 9.18 ± 0.45 mm, but in the presence of 10^{-5}M ABA this was inhibited to 6.42 ± 0.42 mm and in 10^{-4}M ABA to 4.93 ± 0.12 mm. At higher GA_3 levels a similar but more extreme situation existed; at 10^{-6}M GA_3 growth was inhibited by 10^{-5}M ABA from 12.03 ± 0.45 to 6.17 ± 0.35 mm. When these

Table 33. The effect of ABA on the pea internode extension induced by 10^{-5}M GA_3.

ABA concentration (Molar)	Internode Extension (mm)	Standard Error (±)	Significance of Inhibition
0	12.04	0.45	
10^{-8}	12.14	0.48	NS
10^{-7}	12.39	0.30	NS
10^{-6}	11.51	0.38	NS
10^{-5}	9.10	0.42	***
10^{-4}	6.17	0.35	***

results were subjected to an analysis of variance, it was found that, as already shown by the "t" tests, both GA_3 and ABA exerted significant effects, and the interaction term between the two was also significant (Table 34). It thus seems that, as with the lettuce hypocotyl extension, significant inhibition occursmore readily when elongation has been induced by GA_3. The difficulty in detecting an inhibitory effect in the absence of gibberellin may have been attributable to limitation of the mensuration technique. On the other hand, in these experiments 6.02 $\pm$ 0.38 mm of extension occurred in the absence of GA_3; inhibitory effects due to ABA should have been readily detectable at concentrations less than 10^{-4}M ABA.

Table 34. Analysis of variance of pea internode extension in the presence of ABA and GA_3.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
GA_3	1028.64	5	205.73	139.21	**
ABA	1028.64	5	205.73	139.21	**
Interaction	207.51	25	8.30	5.62	**
Error	425.62	288	1.48		

(10) THE EFFECT OF ANALOGUES OF ABA IN THE PEA EPICOTYL ASSAY AND THE INTERACTION OF THE ANALOGUES WITH GA_3.

The analogues of ABA were assayed for their effect in the pea epicotyl assay. They were assayed at concentrations of 0, 10^{-7}, 10^{-6} and 10^{-5}M in the presence of GA_3 initially at the same molar concentrations, but subsequently at 0 and 10^{-5}M. The results of the 4 x 4 factorial assays were subjected to analysis of variance and the analyses are included with the figures (Figs. 39-46 and Tables 35-42). In all cases, selective "t" tests

Fig. 39. The effect of various concentrations of GA_3 and analogues of ABA alone and in combination on the extension of internodes of dwarf peas var. Meteor. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound I.

(B) compound II.

(C) compound III.

(D) compound IV.

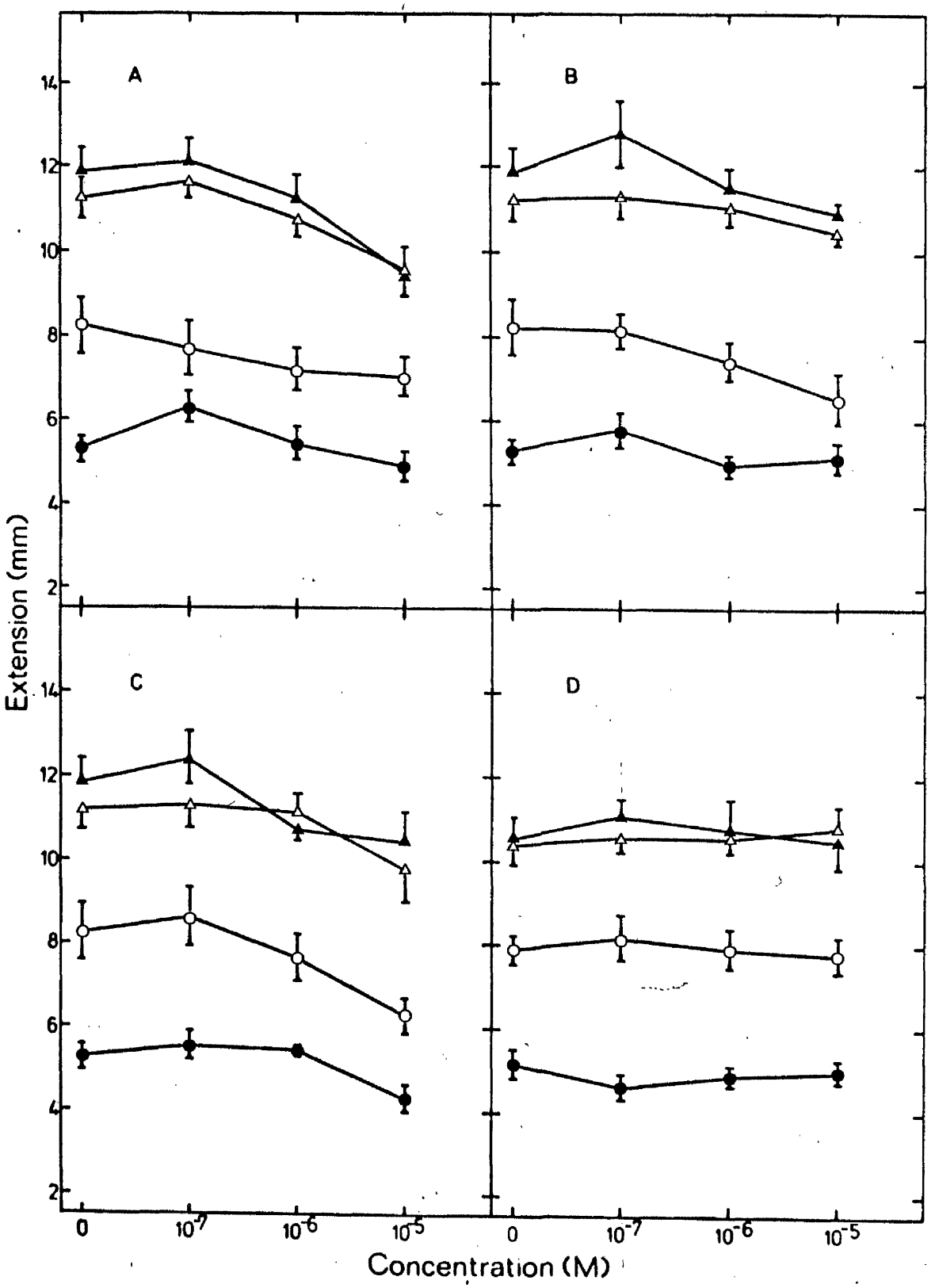

A
B
C
D
Extension (mm)
Concentration (M)
0
10^{-7}
10^{-6}
10^{-5}
2
4
6
8
10
12
14

Table 35. Analysis of variance of pea internode extension in the presence of ABA analogues and GA_3.

(A) Compound I.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
I	53.33	3	17.76	8.2	***
GA_3	833.67	3	277.89	128.12	***
Interaction	32.18	9	3.58	1.65	NS
Error	277.73	128	2.17		

(B) Compound II.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
II	32.93	3	10.98	5.54	***
GA_3	987.66	3	329.22	166.02	***
Interaction	10.15	9	1.13	0.57	NS
Error	253.9	128	1.98		

(C) Compound III.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
III	66.78	3	22.26	9.08	***
GA_3	913.27	3	304.42	124.15	***
Interaction	11.33	9	1.26	0.51	NS
Error	313.87	128	2.45		

(D) Compound IV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
IV	1.21	3	6.4	0.23	NS
GA_3	796.02	3	265.34	153.02	***
Interaction	7.61	9	0.85	0.49	NS
Error	222.04	128	1.73		

Fig. 40. The effect of various concentrations of GA_3 and analogues of ABA alone and in combination on the extension of internodes of dwarf peas var. Meteor. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound V.

(B) compound VI.

(C) compound VII.

(D) compound VIII.

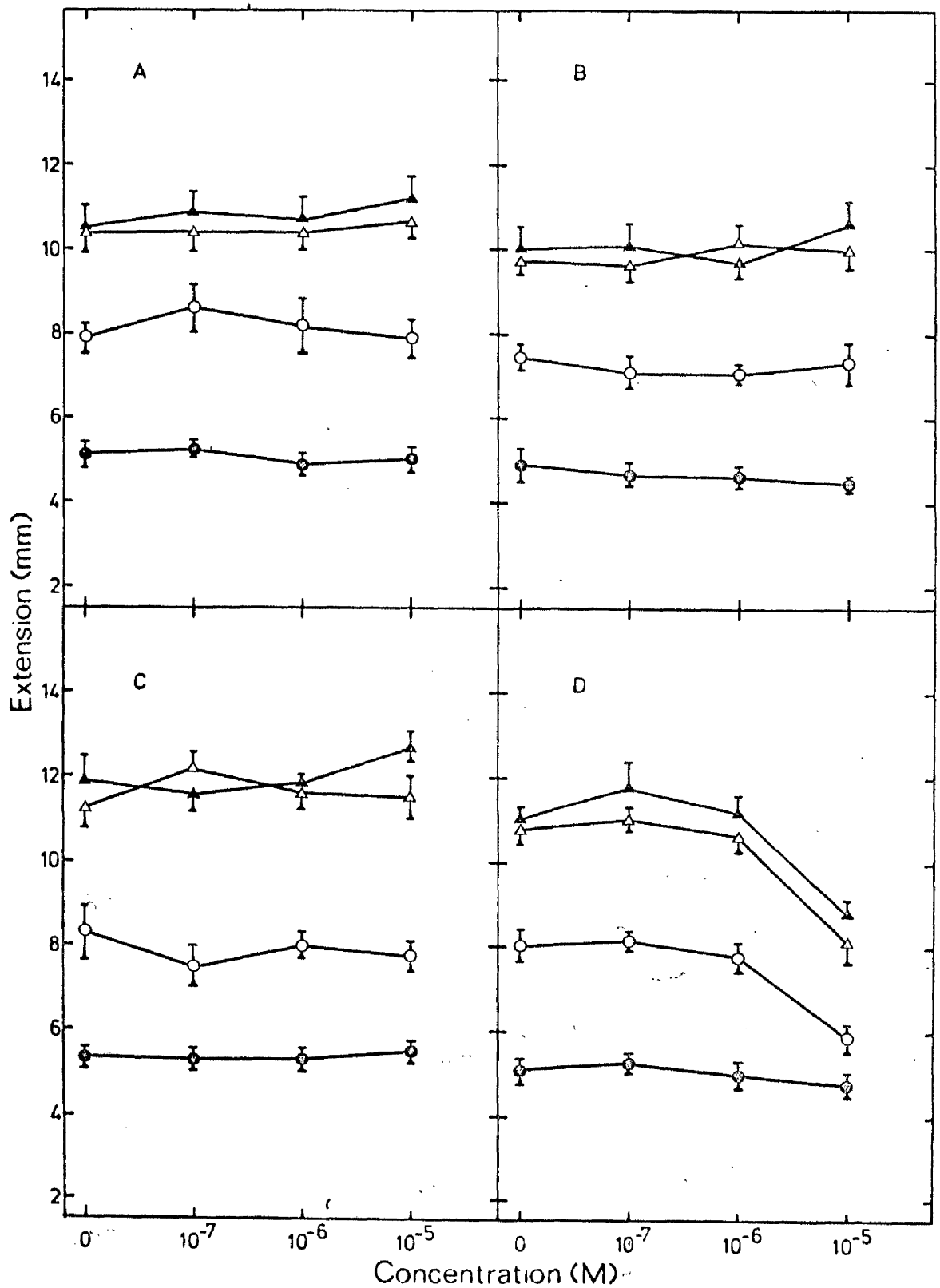

A
B
C
D
Extension (mm)
14
12
10
8
6
4
2
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 36. Analysis of variance of pea internode extension in the presence of ABA analogues and GA_3.

(A) Compound V.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
V	2.02	3	0.67	0.39	NS
GA_3	763.14	3	254.38	147.38	***
Interaction	4.96	9	0.55	0.32	NS
Error	221.02	128	1.73		

(B) Compound VI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
VI	1.73	3	0.57	0.46	NS
GA_3	723.47	3	241.16	192.62	***
Interaction	5.83	9	0.65	0.52	NS
Error	160.29	128	1.25		

(C) Compound VII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
VII	1.04	3	0.35	0.24	NS
GA_3	1091.9	3	363.97	246.76	***
Interaction	13.85	9	1.54	1.04	NS
Error	188.82	128	1.48		

(D) Compound VIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
VIII	102.32	3	34.11	32.86	***
GA_3	743.68	3	247.89	238.82	***
Interaction	25.51	9	2.83	2.73	**
Error	132.94	128	1.04		

Fig. 41. The effect of various concentrations of GA_3 and analogues of ABA alone and in combination on the extension of internodes of dwarf peas var. Meteor. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound IX.

(B) compound X.

(C) compound XI.

(D) compound XII.

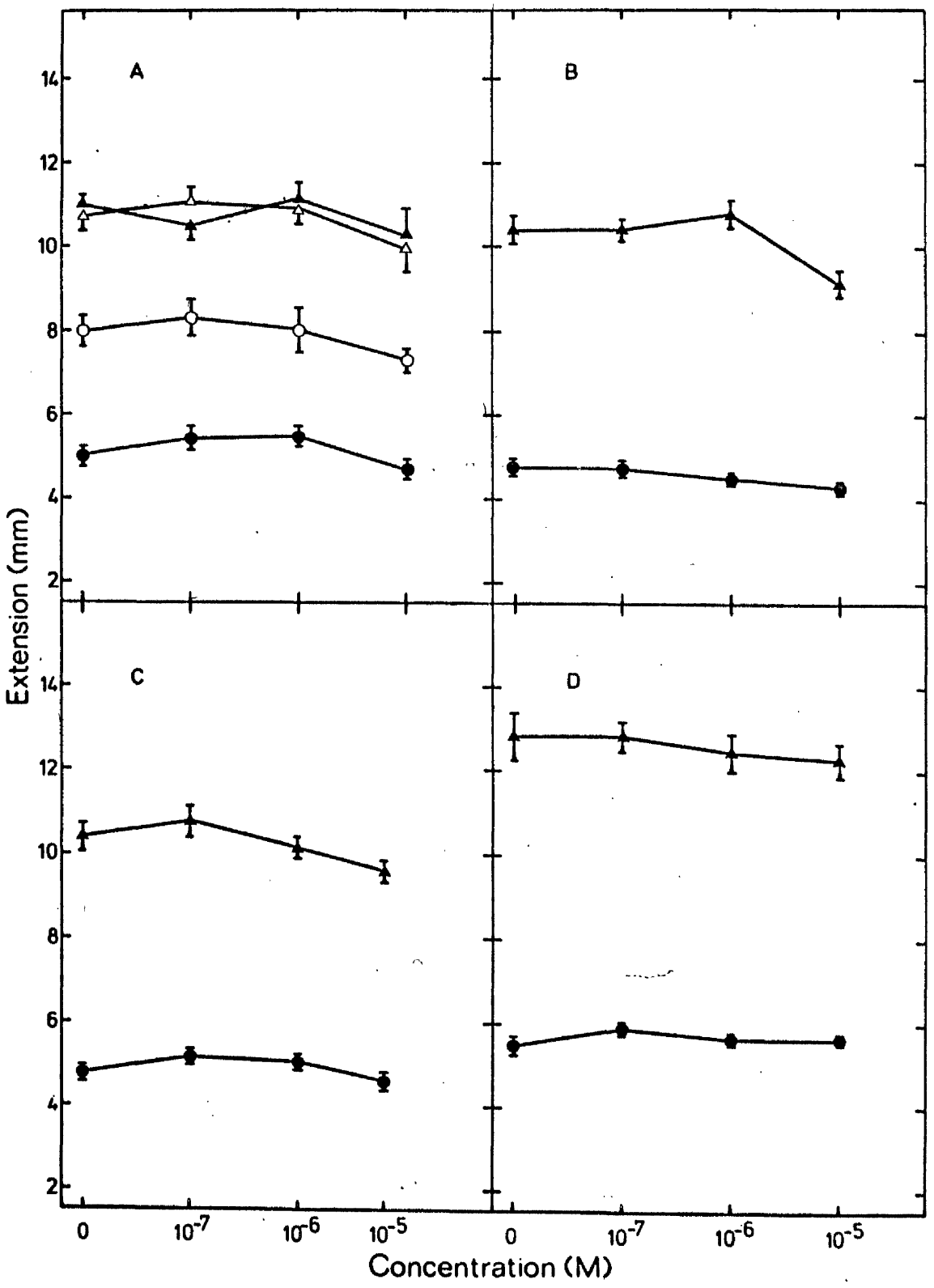

A
B
C
D
Extension (mm)
14
12
10
8
6
4
2
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 37. Analysis of variance of pea internode extension in the presence of ABA analogues and GA_3.

(A) Compound IX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
IX	17.06	3	5.69	4.41	***
GA_3	762.67	3	254.22	197.38	***
Interaction	4.15	9	0.46	0.36	NS
Error	164.98	128	1.29		

(B) Compound X.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
X	11.50	3	3.83	6.35	***
GA_3	554.95	1	554.95	920.31	***
Interaction	6.47	3	2.15	3.57	*
Error	38.61	64	0.60		

(C) Compound XI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XI	6.59	3	2.19	4.22	**
GA_3	510.67	1	510.67	982.06	***
Interaction	1.31	3	0.43	0.83	NS
Error	33.34	64	0.52		

(D) Compound XII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XII	0.81	3	0.27	0.39	NS
GA_3	575.58	1	575.58	840.27	***
Interaction	1.43	3	0.47	0.69	NS
Error	27.4	40	0.68		

Fig. 42. The effect of various concentrations of GA_3 and analogues of ABA alone and in combination on the extension of internodes of dwarf peas var. Meteor. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XIII.

(B) compound XIV.

(C) compound XV.

(D) compound XVI.

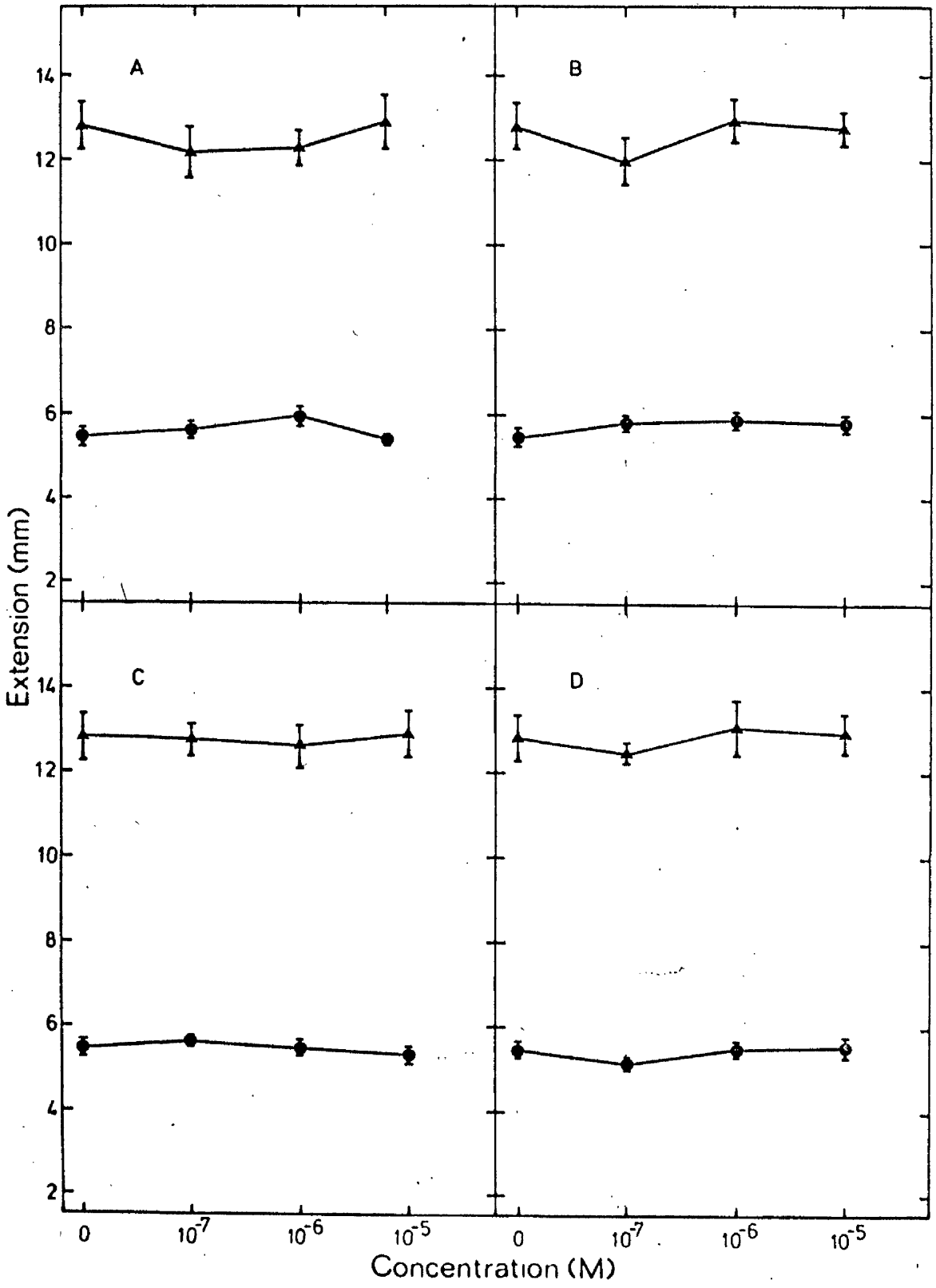

A
B
C
D
Extension (mm)
14
12
10
8
6
4
2
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 38. Analysis of variance of pea internode extension in the presence of ABA analogues and GA_3.

(A) Compound XIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XIII	0.39	3	0.13	0.12	NS
GA_3	577.24	1	577.24	546.11	***
Interaction	3.26	3	1.08	1.03	NS
Error	42.28	40	1.05		

(B) Compound XIV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XIV	2.08	3	0.69	0.75	NS
GA_3	561.69	1	561.69	610.53	***
Interaction	2.50	3	0.83	0.90	NS
Error	36.83	40	0.92		

(C) Compound XV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XV	0.16	3	0.05	0.06	NS
GA_3	633.49	1	633.49	698.44	***
Interaction	0.56	3	0.18	0.21	NS
Error	36.32	40	0.91		

(D) Compound XVI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XVI	1.9	3	0.63	0.71	NS
GA_3	661.78	1	661.78	745.25	***
Interaction	0.116	3	0.038	0.042	NS
Error	35.55	40	0.88		

Fig. 43. The effect of various concentrations of GA_3 and analogues of ABA alone and in combination on the extension of internodes of dwarf peas var. Meteor. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XVII.

(B) compound XVIII.

(C) compound XIX.

(D) compound XX.

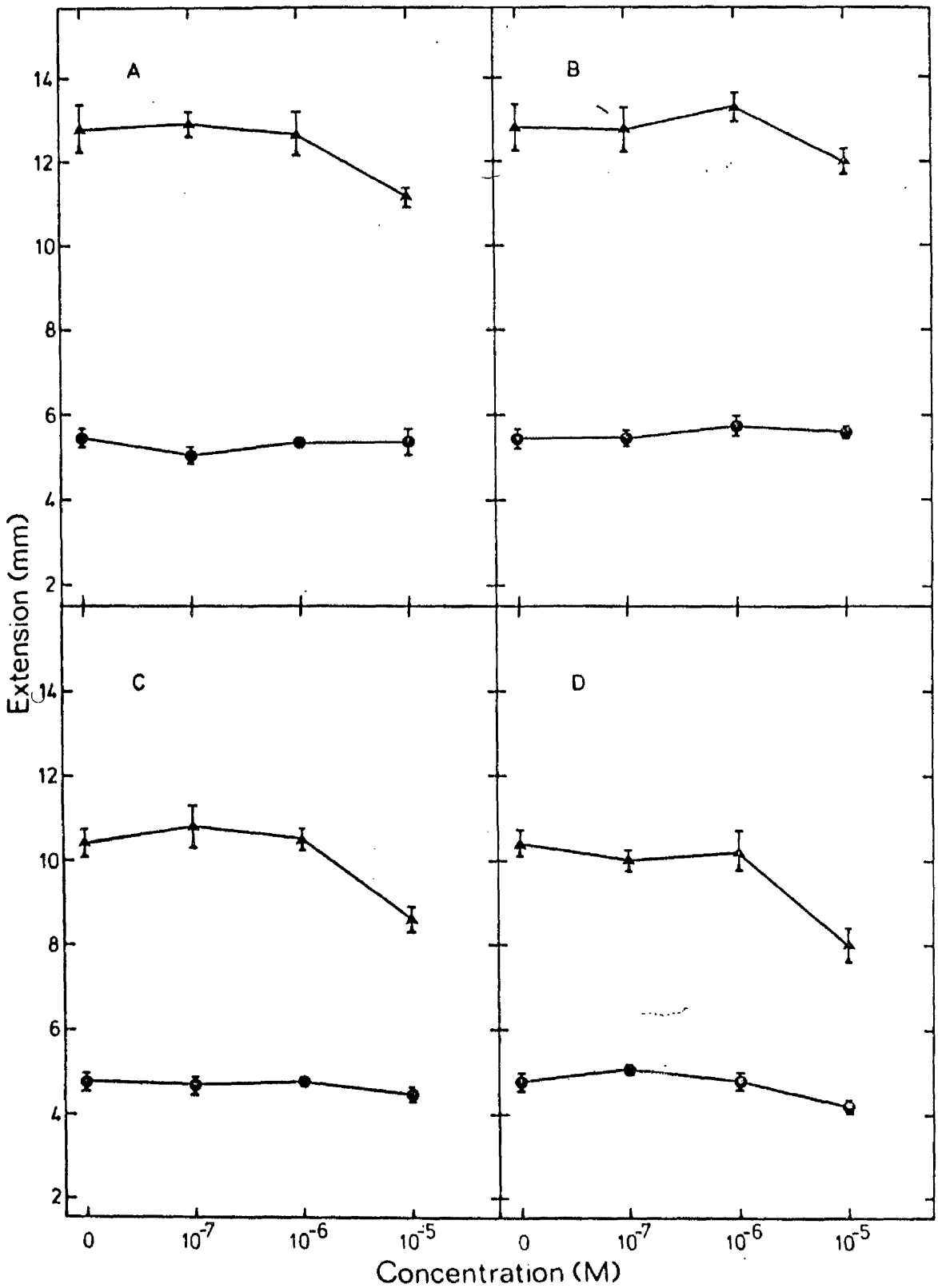

A
B
C
D
Extension (mm)
14
12
10
8
6
4
2
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 39. Analysis of variance of pea internode extension in the presence of ABA analogues and GA_3.

(A) Compound XVII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XVII	5.68	3	1.89	2.76	*
GA_3	601.22	1	601.22	877.7	***
Interaction	7.38	3	2.46	3.59	*
Error	27.4	40	0.68		

(B) Compound XVIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XVIII	3.34	3	1.11	1.58	NS
GA_3	613.84	1	613.84	871.94	***
Interaction	2.33	3	0.77	1.103	NS
Error	28.17	40	0.70		

(C) Compound XIX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XIX	16.80	3	5.6	8.13	***
GA_3	524.14	1	524.14	761.83	***
Interaction	10.34	3	3.44	5.01	**
Error	44.05	64	0.68		

(D) Compound XX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XX	27.59	3	9.19	13.60	***
GA_3	438.49	1	438.49	648.65	***
Interaction	8.92	3	2.97	4.4	**
Error	43.29	64	0.67		

Fig. 44. The effect of various concentrations of GA_3 and analogues of ABA alone and in combination on the extension of internodes of dwarf peas var. Meteor. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XXI.

(B) compound XXII.

(C) compound XXIII.

(D) compound XXIV.

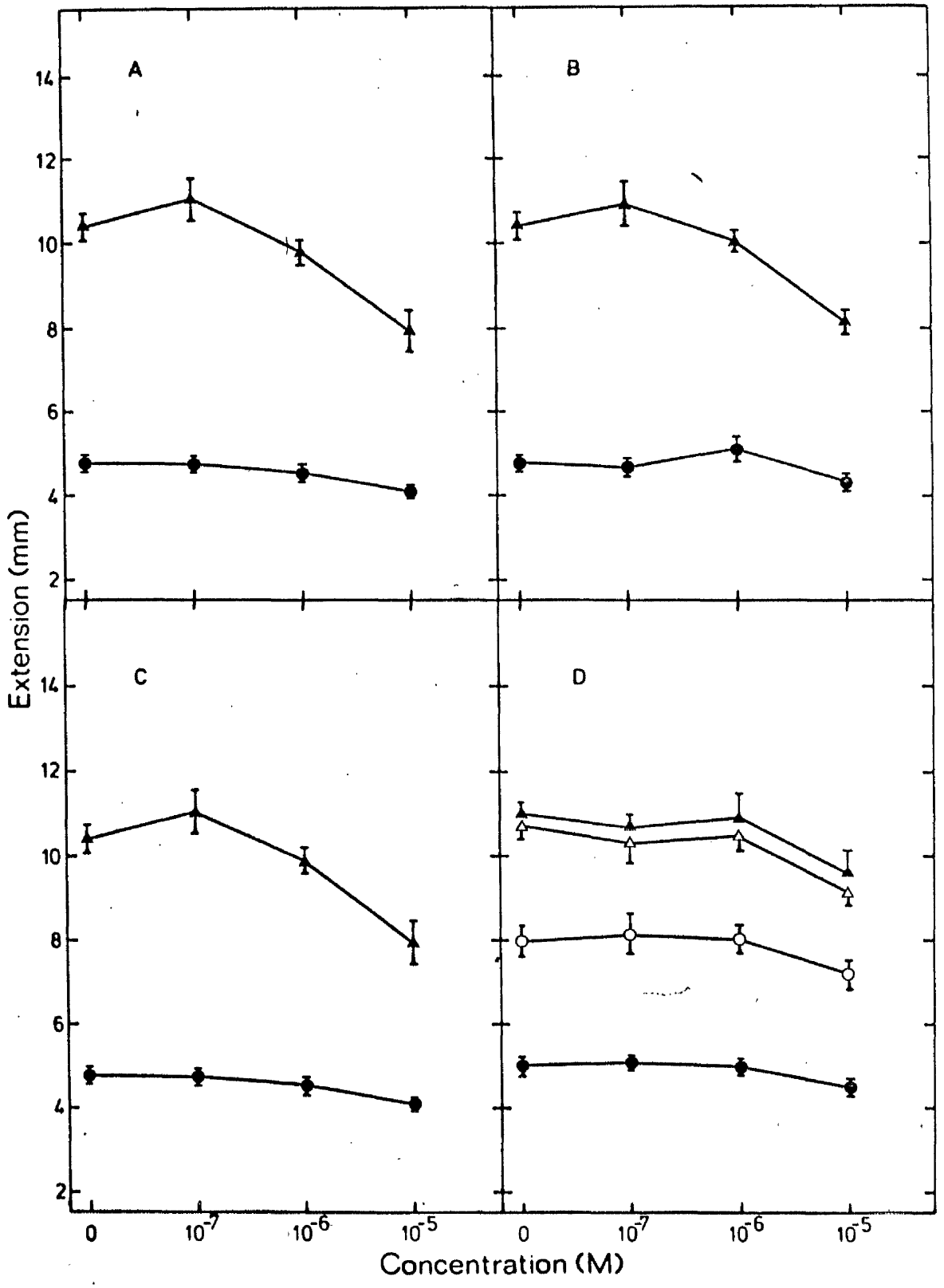

A
B
C
D
Extension (mm)
14
12
10
8
6
4
2
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 40. Analysis of variance of pea internode extension in the presence of ABA analogues and GA_3.

(A) Compound XXI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXI	43.23	3	14.41	8.05	***
GA_3	497.18	1	497.18	277.75	***
Interaction	49.76	3	16.58	9.26	***
Error	114.57	64	1.79		

(B) Compound XXII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXII	28.04	3	9.34	11.10	***
GA_3	479.99	1	479.99	570.06	***
Interaction	14.71	3	4.90	5.82	**
Error	53.94	64	0.84		

(C) Compound XXIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXIII	4.37	3	1.45	2.95	*
GA_3	533.84	1	533.84	1082.84	***
Interaction	1.85	3	0.61	1.25	NS
Error	31.609	64	0.49		

(D) Compound XXIV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXIV	26.8	3	18.93	15.9	***
GA_3	795.48	3	265.16	471.81	***
Interaction	7.4	9	0.82	1.46	NS
Error	72.05	128	0.56		

Fig. 45. The effect of various concentrations of GA_3 and analogues of ABA alone and in combination on the extension of internodes of dwarf peas var. Meteor. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XXV.

(B) compound XXVI.

(C) compound XXVII.

(D) compound XXVIII.

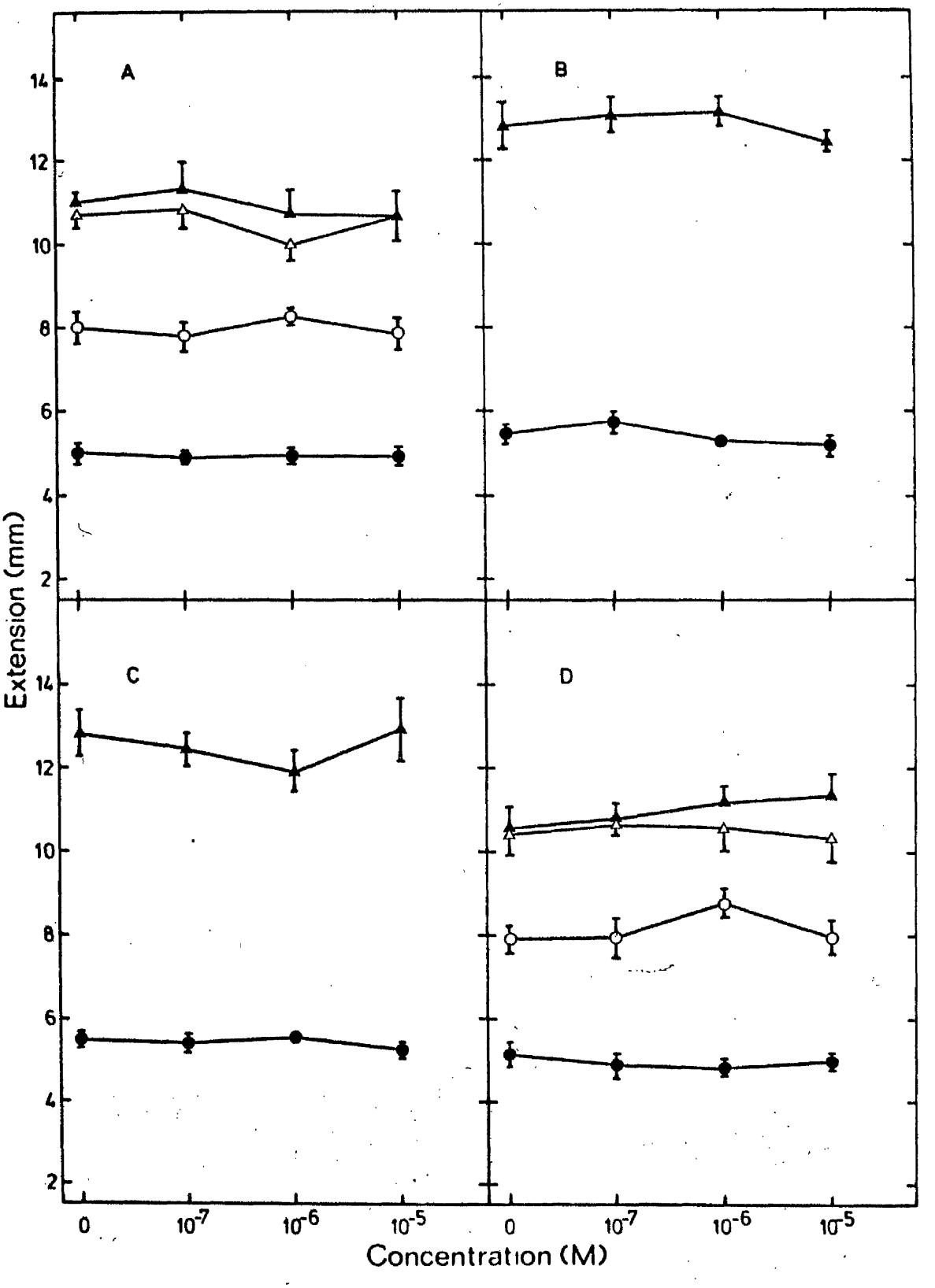

A
B
C
D
Extension (mm)
14
12
10
8
6
4
2
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Table 41. Analysis of variance of pea internode extension in the presence of ABA analogues and GA_3.

(A) Compound XXV.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXV	1.6	3	0.53	0.37	NS
GA_3	842.7	3	280.9	194.26	***
Interaction	6.91	9	0.77	0.53	NS
Error	185.12	128	1.45		

(B) Compound XXVI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXVI	2.059	3	0.68	1.08	NS
GA_3	667.45	1	667.45	1051.107	***
Interaction	0.83	3	0.27	0.43	NS
Error	25.406	40	0.63		

(C) Compound XXVII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXVII	1.24	3	0.41	0.39	NS
GA_3	606.55	1	606.55	584.34	***
Interaction	2.93	3	0.97	0.94	NS
Error	41.55	40	1.03		

(D) Compound XXVIII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXVIII	3.05	3	1.02	0.73	NS
GA_3	823.00	3	274.33	195.95	***
Interaction	7.55	9	0.84	0.6	NS
Error	179.29	128	1.4		

Fig. 46. The effect of various concentrations of GA_3 and analogues of ABA alone and in combination on the extension of internodes of dwarf peas var. Meteor. The analogue concentrations are shown on the horizontal axis; GA_3 concentrations are referred to by the symbols :- closed circle (no GA_3), open circle (10^{-7}M GA_3), open triangle (10^{-6}M GA_3), closed triangle (10^{-5}M GA_3).

(A) compound XXIX.

(B) compound XXX.

(C) compound XXXI.

(D) compound XXXII.

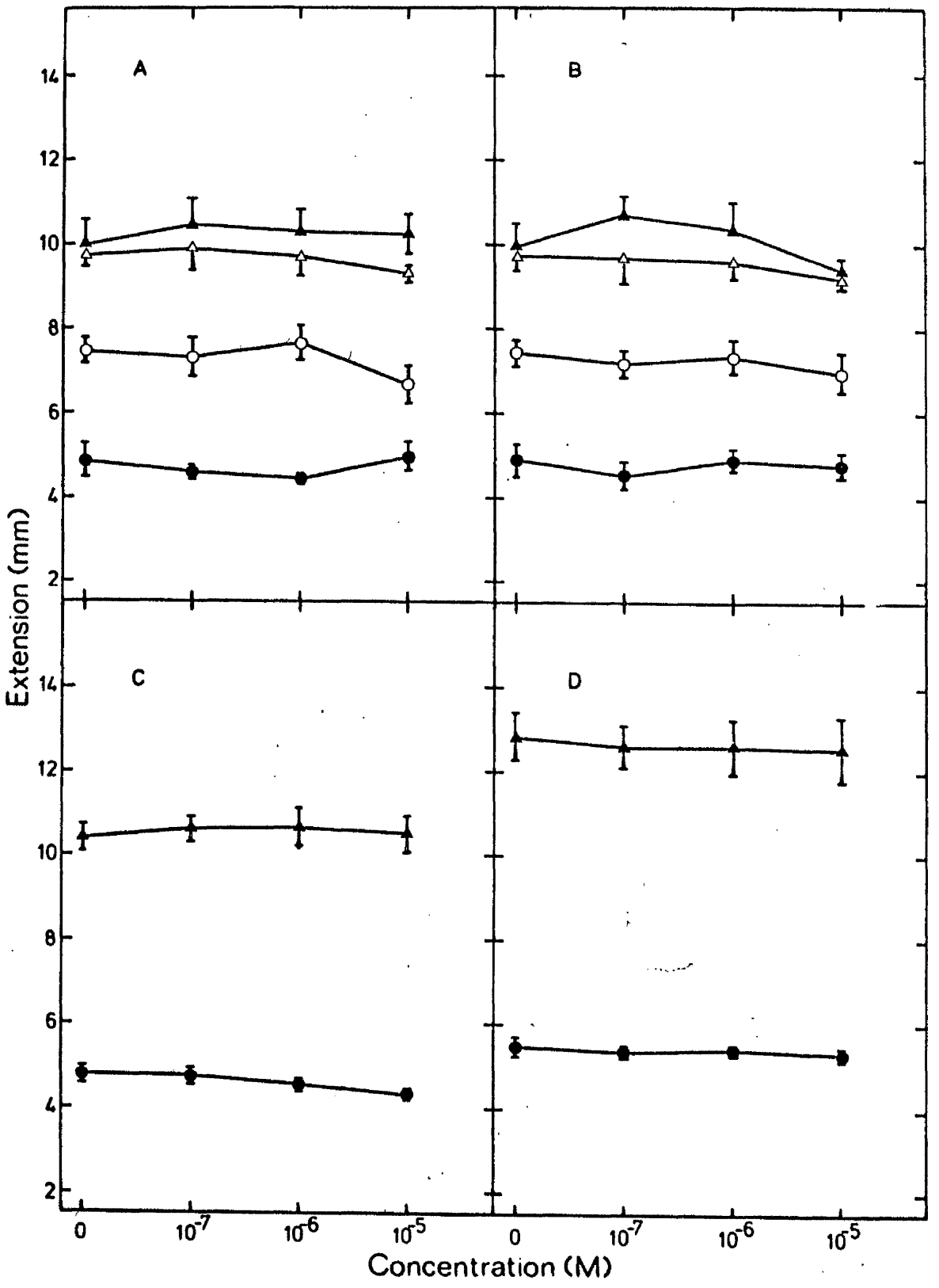

A
B
C
D
Extension (mm)
Concentration (M)
0
10^{-7}
10^{-6}
10^{-5}
2
4
6
8
10
12
14

Table 42. Analysis of variance of pea internode extension in the presence of ABA analoques and GA_3.

(A) Compound XXIX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXIX	1.71	3	0.57	0.38	NS
GA_3	695.08	3	231.69	153.85	***
Interaction	8.07	9	0.9	0.59	NS
Error	192.84	128	1.51		

(B) Compound XXX.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXX	4.77	3	1.59	1.03	NS
GA_3	628.09	3	209.36	135.77	***
Interaction	7.48	9	0.83	0.54	NS
Error	197.42	128	1.54		

(C) Compound XXXI.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXXI	0.49	3	0.16	0.206	NS
GA_3	633.78	1	633.78	803.26	***
Interaction	1.08	3	0.36	0.45	NS
Error	50.53	64	0.789		

(D) Compound XXXII.

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio	Significance
XXXII	0.32	3	0.106	0.08	NS
GA_3	634.53	1	634.53	499.63	***
Interaction	0.55	3	0.184	0.14	NS
Error	50.82	40	1.27		

were also carried out (Table 43). The results of the solvent controls are shown in Figs.47 and 48.

The analyses of variance indicated that I, II, III, VIII, IX, X, XI, XVII, XIX, XX, XXI, XXII, XXIII and XXIV significantly inhibited elongation. Student's "t" tests confirmed that only these compounds were significantly inhibitory although the activity was not always apparent at equimolar concentrations of 10^{-5}M GA_3 and analogue (Table 43). The effects of other analogues concentrations in the presence of 10^{-5}M GA_3, and all analogue concentrations where no exogenously applied GA_3 was present, were also statistically analysed. Only III and XXI were significantly inhibitory to internodal growth in the absence of GA_3. As in the lettuce hypocotyl assay the active analogues were those which showed activity in inhibiting lettuce germination. Thus the molecular requirements for activity are the same in all the biological systems examined. Two notably inactive compounds were IX and XXV and it is possible that some feature of their terminal group limits their ability to act as ABA-like inhibitors.

When the interaction of the analogues with GA_3 was examined in this assay, a significant interaction term was found in the analysis of variance of many of the inhibitory analogues. Inhibition was apparently more marked in the presence of GA_3-induced growth and, because of this, a statistically significant interaction term will inevitably show in the analysis of variance. Whether or not a true interaction exists is discussed on p. 139.

Other methods of expressing the results of the lettuce and pea assays have been considered, e.g. percentage extension occurring in the absence of analogue (Fig. 49) and extension as a percentage of the water control (Fig. 50). Both methods, however, introduce further error while not affecting the interpretations.

Table 43. The inhibitory effect of ABA analogues at 10^{-5}M on dwarf pea internode extension (mm) (A) in the absence of GA_3 and (B) in the presence of GA_3 at 10^{-5}M.

Compound	(A)	S.E. (±)	Sign.	(B)	S.E. (±)	Sign.
I	4.89	0.33	NS	9.50	0.54	**
II	5.15	0.36	NS	10.93	0.24	NS
III	4.30	0.32	*	10.43	0.73	NS
IV	5.00	0.24	NS	10.02	0.64	NS
V	5.02	0.31	NS	11.22	0.51	NS
VI	4.57	0.17	NS	10.61	0.56	NS
VII	5.49	0.27	NS	12.70	0.40	NS
VIII	4.78	0.28	NS	8.80	0.32	***
IX	4.72	0.23	NS	10.28	0.63	NS
X	4.54	0.26	NS	9.13	0.31	*
XI	4.61	0.19	NS	9.61	0.24	*
XII	5.67	0.13	NS	12.28	0.40	NS
XIII	5.42	0.13	NS	12.92	0.63	NS
XIV	5.63	0.18	NS	12.78	0.41	NS
XV	5.33	0.19	NS	12.89	0.57	NS
XVI	5.56	0.24	NS	12.96	0.47	NS
XVII	5.38	0.30	NS	11.16	0.23	*
XVIII	5.61	0.15	NS	12.02	0.28	NS
XIX	4.48	0.18	NS	8.62	0.32	**
XX	4.20	0.15	NS	8.02	0.38	***
XXI	4.11	0.16	*	7.95	0.48	***
XXII	4.32	0.19	NS	8.15	0.32	***
XXIII	4.48	0.09	NS	9.43	0.28	*
XXIV	4.48	0.20	NS	9.61	0.53	*
XXV	4.93	0.23	NS	10.72	0.59	NS
XXVI	5.19	0.26	NS	12.45	0.26	NS
XXVII	5.21	0.19	NS	12.92	0.74	NS
XXVIII	5.00	0.21	NS	11.39	0.49	NS
XXIX	5.00	0.32	NS	10.30	0.46	NS
XXX	4.81	0.31	NS	9.45	0.45	NS
XXXI	4.33	0.13	NS	10.52	0.43	NS
XXXII	5.23	0.16	NS	12.56	0.74	NS

Fig. 47. The effect of low concentrations of methanol on the extension of internodes of dwarf peas var. Meteor in the absence of GA_3 (closed circles) and in its presence at 10^{-5} M (open circles).

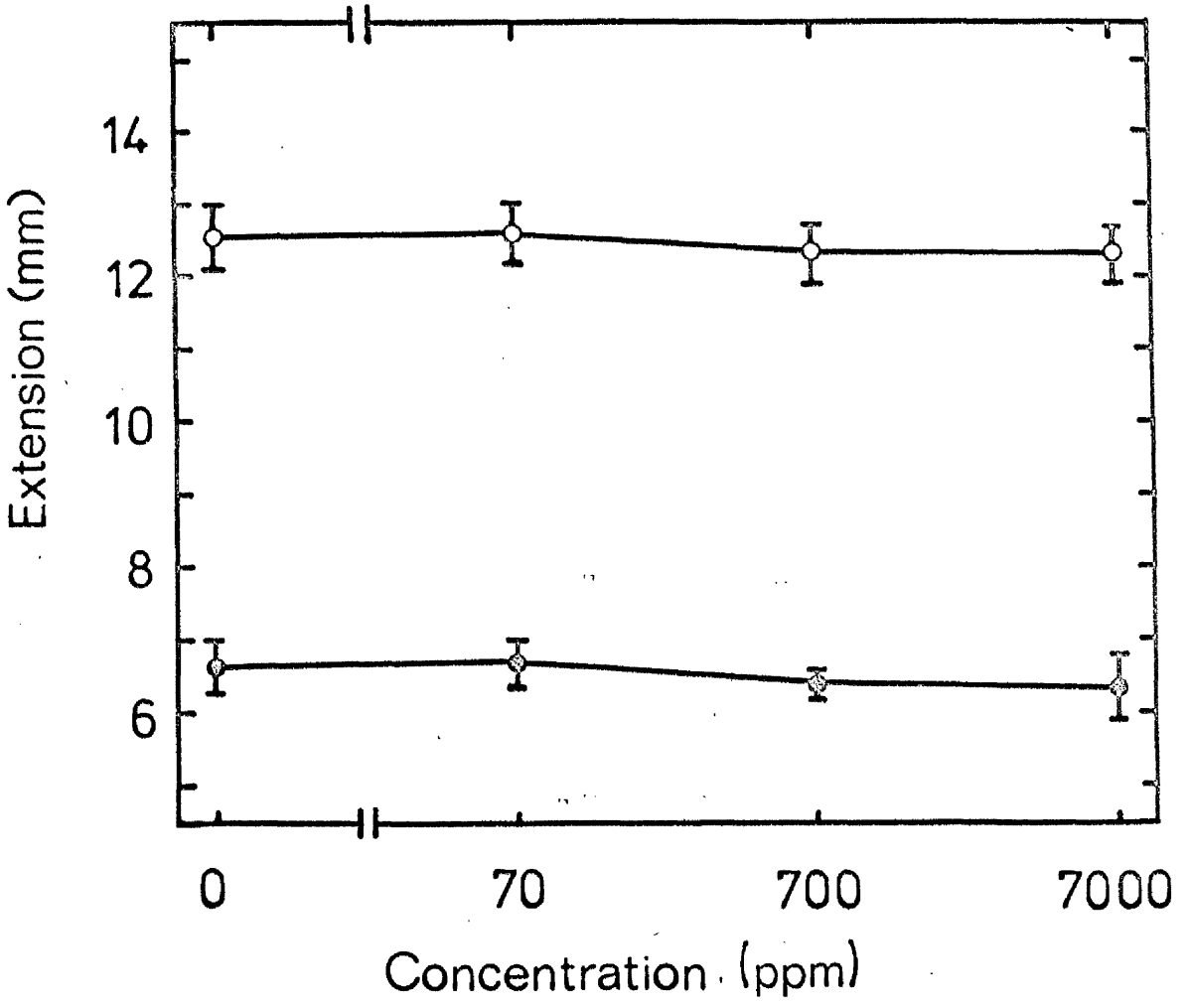

Extension (mm)
14
12
10
8
6
0
70
700
7000
Concentration (ppm)

Fig. 48. The effect of low concentrations of acetone on the extension of internodes of dwarf peas var. Meteor in the absence of GA_3 (closed circles) and in its presence at 10^{-5}M (open circles).

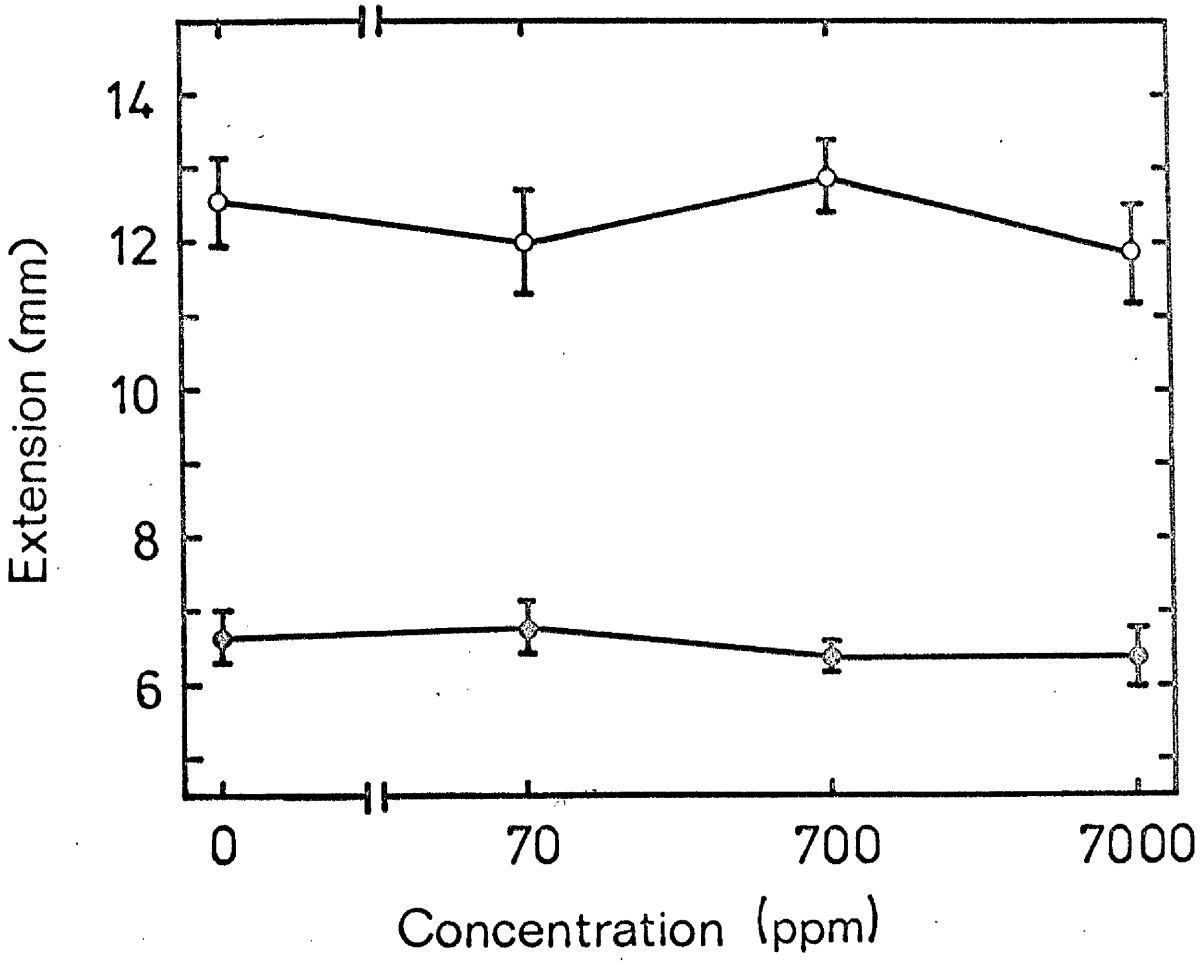

14
12
10
8
6
Extension (mm)
0
70
700
7000
Concentration (ppm)

Fig. 49. The effect of various concentrations of GA_3 and ABA on the extension of internodes of dwarf peas var. Meteor. ABA concentrations are shown on the horizontal axis, and the vertical axis shows the extension as described by:-

$$= \frac{\text{extension in test solution}}{\text{extension in water}} \times 100.$$

closed circle - 0 GA_3

open circle - 10^{-7}M GA_3

closed triangle - 10^{-6}M GA_3

open square - 10^{-5}M GA_3

open triangle - 10^{-4}M GA_3

closed square - 10^{-3}M GA_3

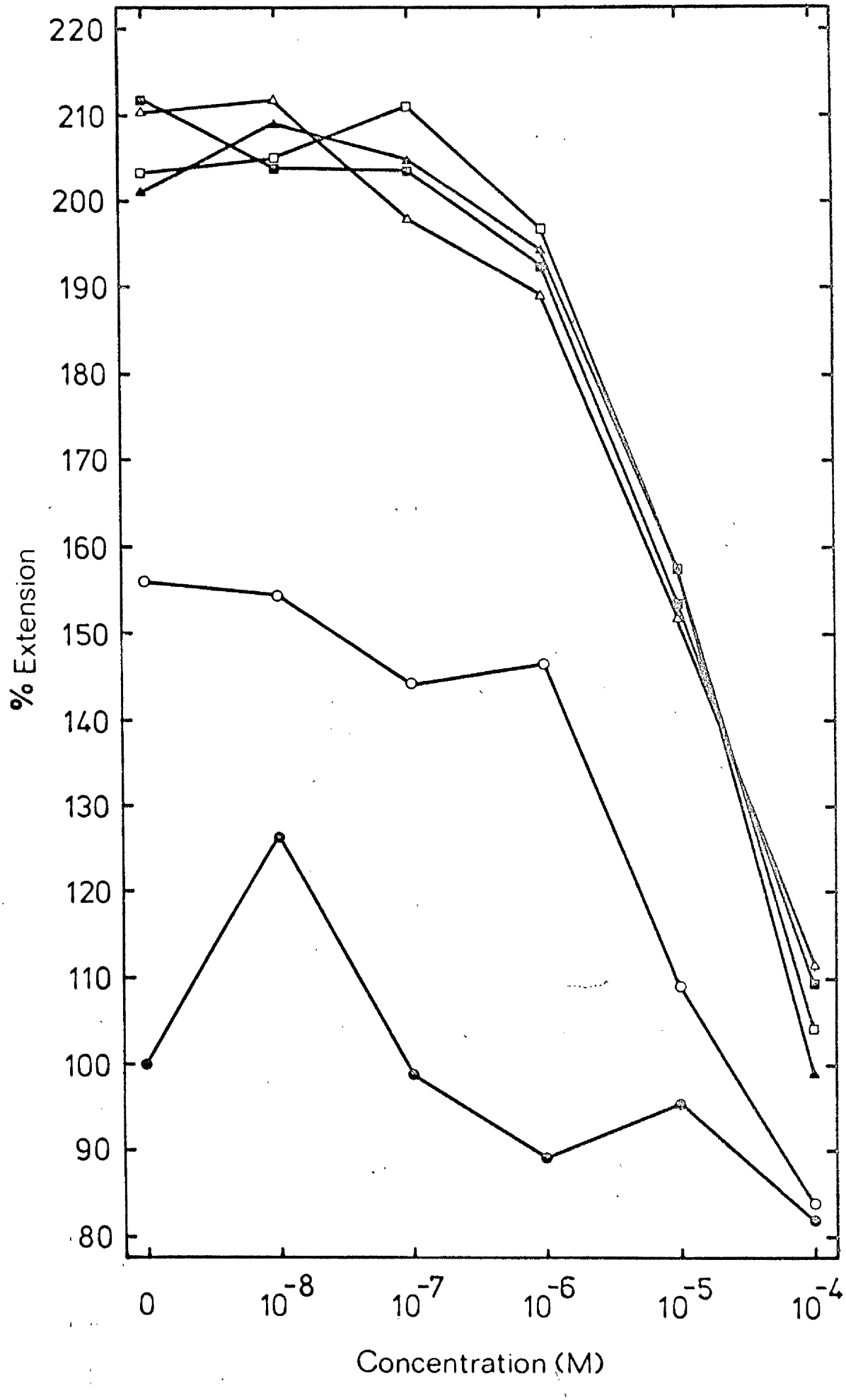

220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
% Extension
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration (M)

Fig. 50. The effect of various concentrations of ABA and GA_3 on the extension of internodes of dwarf peas var. Meteor. ABA concentrations are shown on the horizontal axis, and the vertical axis shows extension obtained from :-

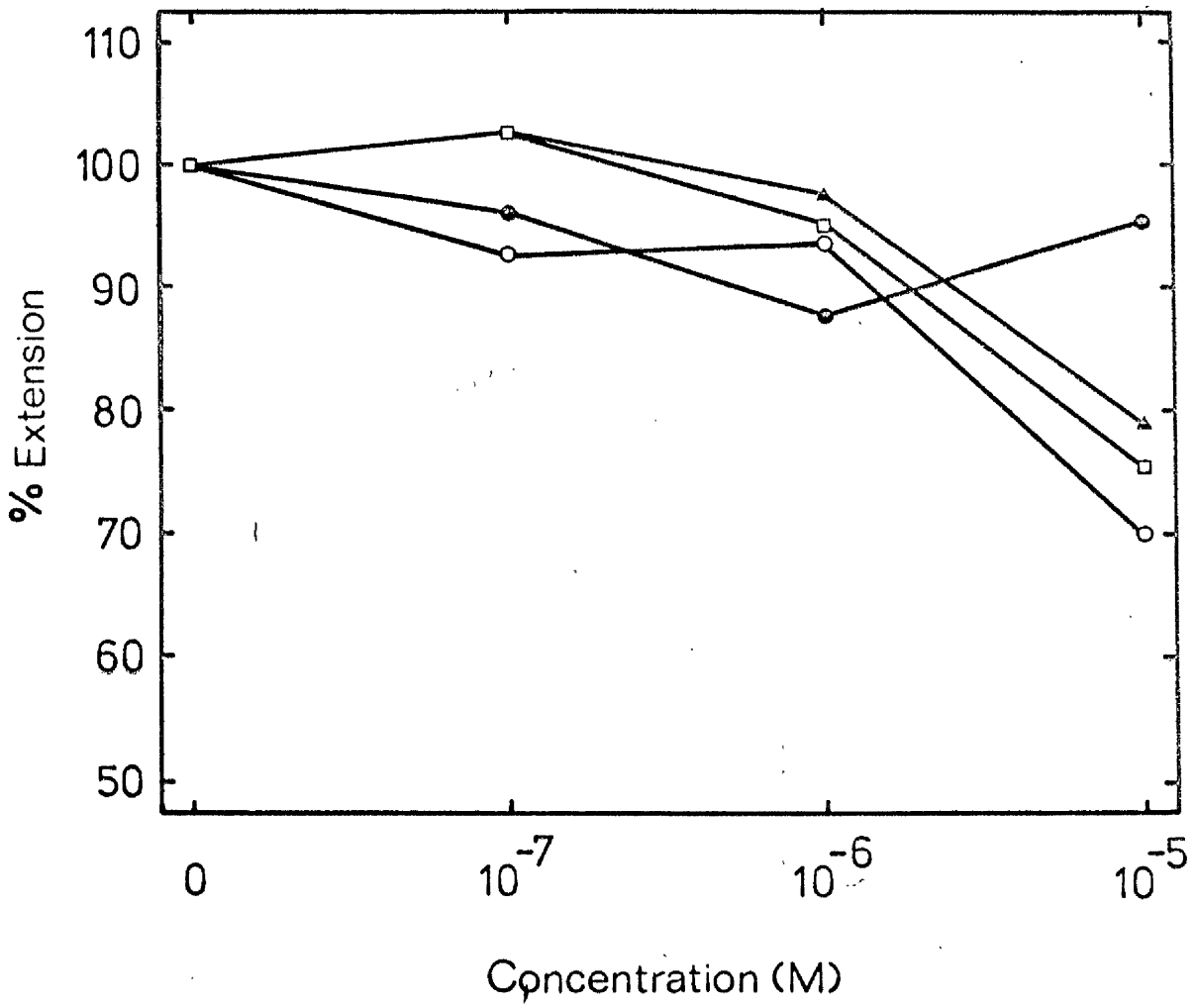
$$\frac{\text{extension in the test solution}}{\text{extension in the absence of ABA}} \times 100.$$

closed circle - no GA_3

open circle - 10^{-7}M GA_3

closed triangle - 10^{-6}M GA_3

open square - 10^{-5}M GA_3


110
100
90
80
70
60
50
% Extension
0
10-7
10-6
10-5
Concentration (M)

(11) BIOLOGICAL ACTIVITY OF TRANS, TRANS (E,E) ABSCISIC ACID.

Trans, trans (E,E) - ABA (tt-ABA) is claimed to be inactive, but there are few data (Milborrow, 1966). With the availability of tt-ABA, supplied by Hoffman-La Roche, it was decided to investigate this important aspect of the structure of ABA in relation to its activity.

The trans, trans (E,E) abscisic acid was assayed for biological activity on lettuce germination, lettuce hypocotyl extension and pea internode extension (Figs. 51-53). Lettuce germination was delayed at 10^{-5}M until 10.53 $\pm$ 0.75 h after the start of imbibition, compared with 46.03 $\pm$ 3.67 h for cis, trans (Z,E) ABA at the same concentration. Solutions of trans, trans ABA and cis, trans ABA at 10^{-4}M inhibited elongation of the lettuce hypocotyl from 10.59 $\pm$ 0.79 to 8.60 $\pm$ 0.35 mm and 7.15 $\pm$ 0.32 mm respectively, while also inhibiting pea internode elongation from 12.56 $\pm$ 0.58 mm to 9.01 $\pm$ 0.31 mm and 6.77 $\pm$ 0.35 mm. The supplied tt-ABA thus exhibited activity in the order of 50% of the activity of the ct-ABA.

To ascertain the purity of the compounds, they were subjected to gas liquid chromatography (GLC) on an OV 210 column. Chromatography of the cis, trans ABA (Fig. 54A) yielded only one peak with a retention time at 200°C of 11.70 mins. An internal standard of octakosane (C-28) was included in the chromatography, and when the retention time of the ABA peak was calculated relative to this standard which had a retention time of 10.35 mins, the ratio was 1.132. When the trans,trans ABA was chromatographed, there were found to be two peaks (Fig. 54B), one of which had a retention time relative to C-28 of 1.132 and the other of 1.697. Chromatographs of both solutions were identical before and after the bioassays were conducted. If, however, the solution of cis,trans ABA was irradiated with ultra-violet light for three hours, a second peak appeared in the chromatograph with a retention time relative to C-28 of 1.650 (Fig. 54C). This could be a result of the conversion of cis trans ABA to trans,trans ABA, a phenomenon already well

Fig. 51. The effect of various concentrations of cis, trans-ABA (open circles) and trans, trans-ABA (closed circles) on the germination of lettuce var. Great Lakes.

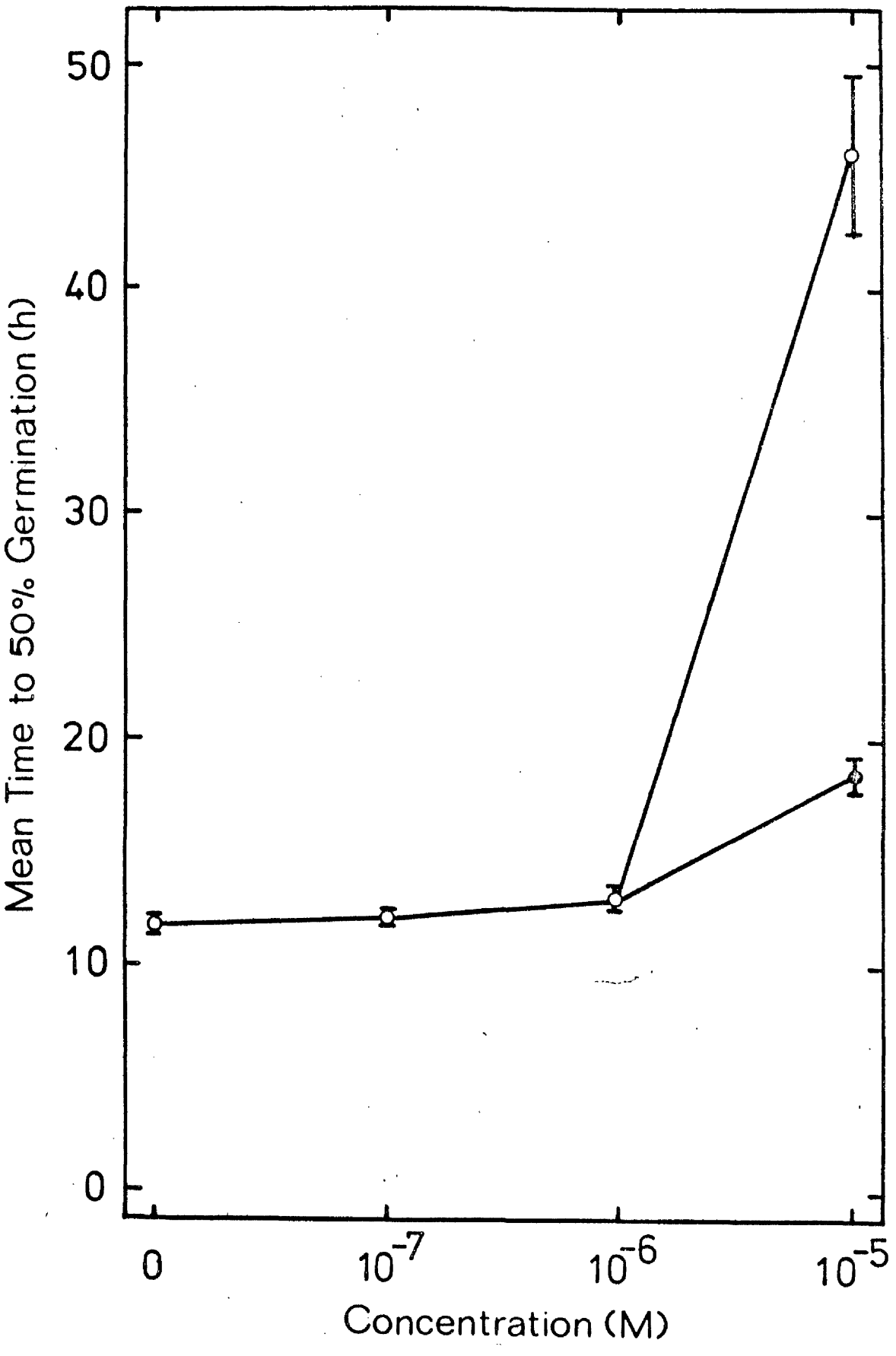

Mean Time to 50% Germination (h)
50
40
30
20
10
0
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Fig. 52. The effect of various concentrations of cis, trans-ABA (closed triangles) and trans, trans-ABA (open triangles) on the extension of hypocotyls of lettuce var. Arctic King.

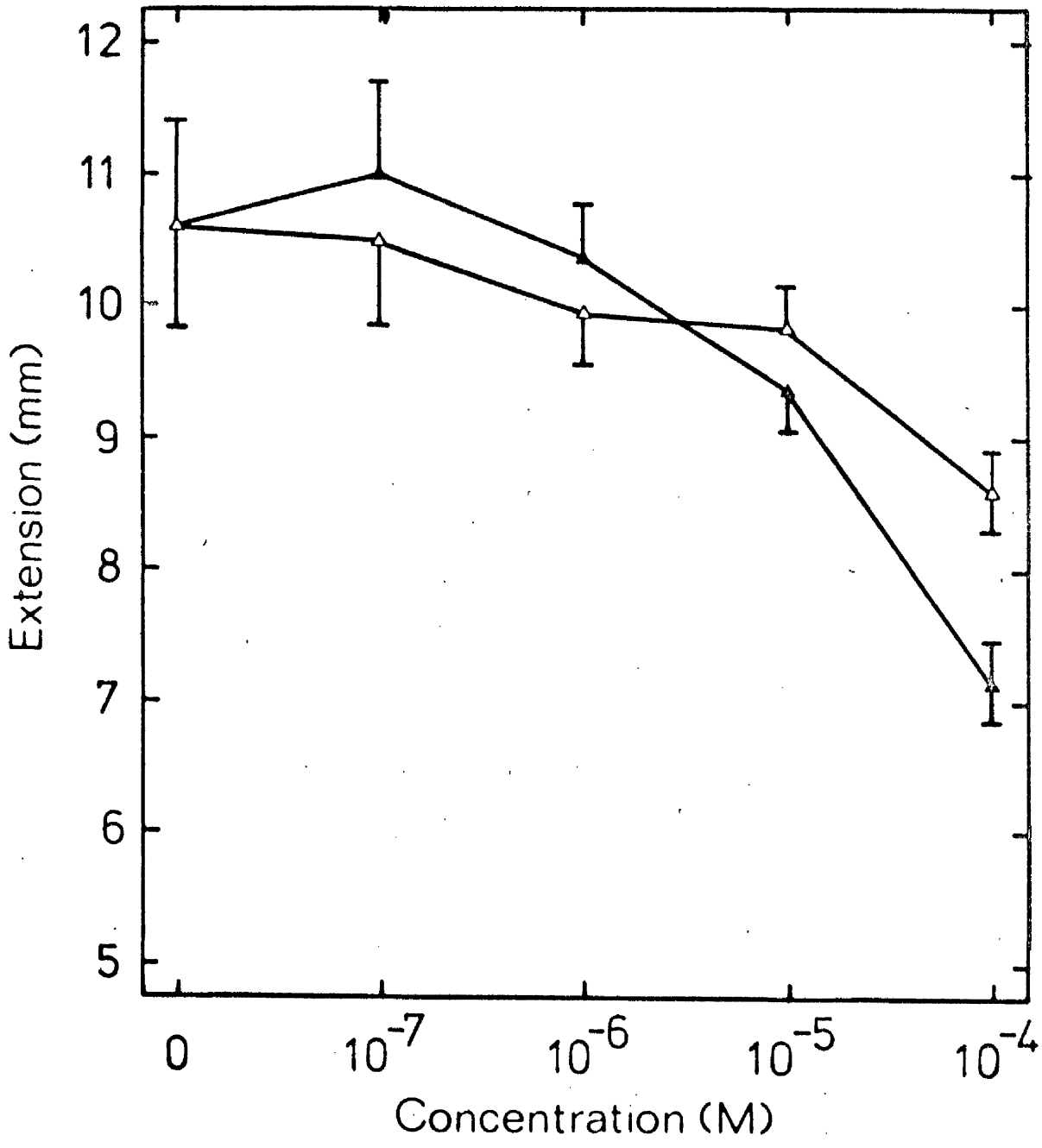

12
11
10
9
8
7
6
5
Extension (mm)
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration (M)

Fig. 53. The effect of various concentrations of cis, trans-ABA (closed circles) and trans, trans-ABA (open circles) on the extension of internodes of dwarf peas var. Meteor.

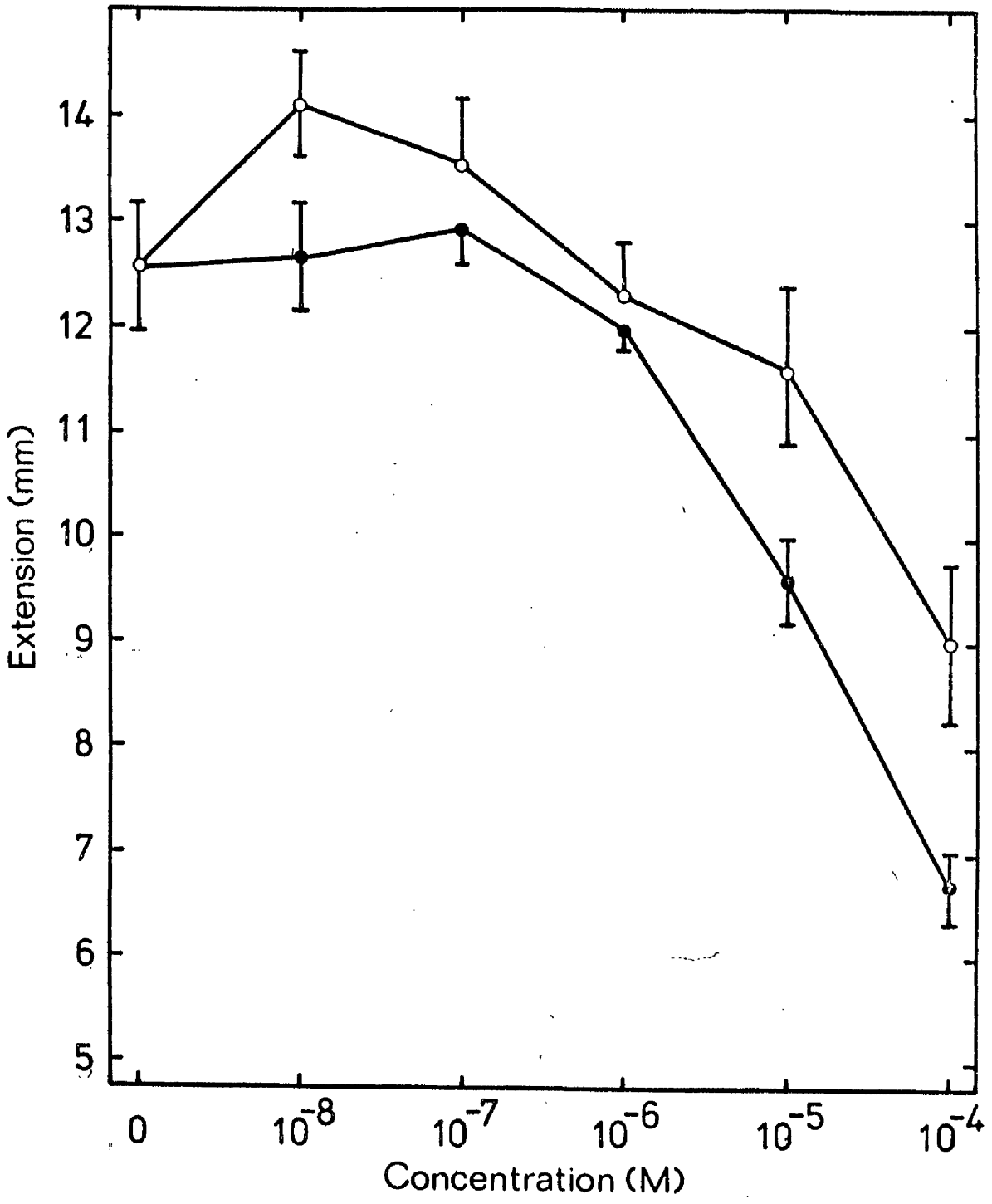
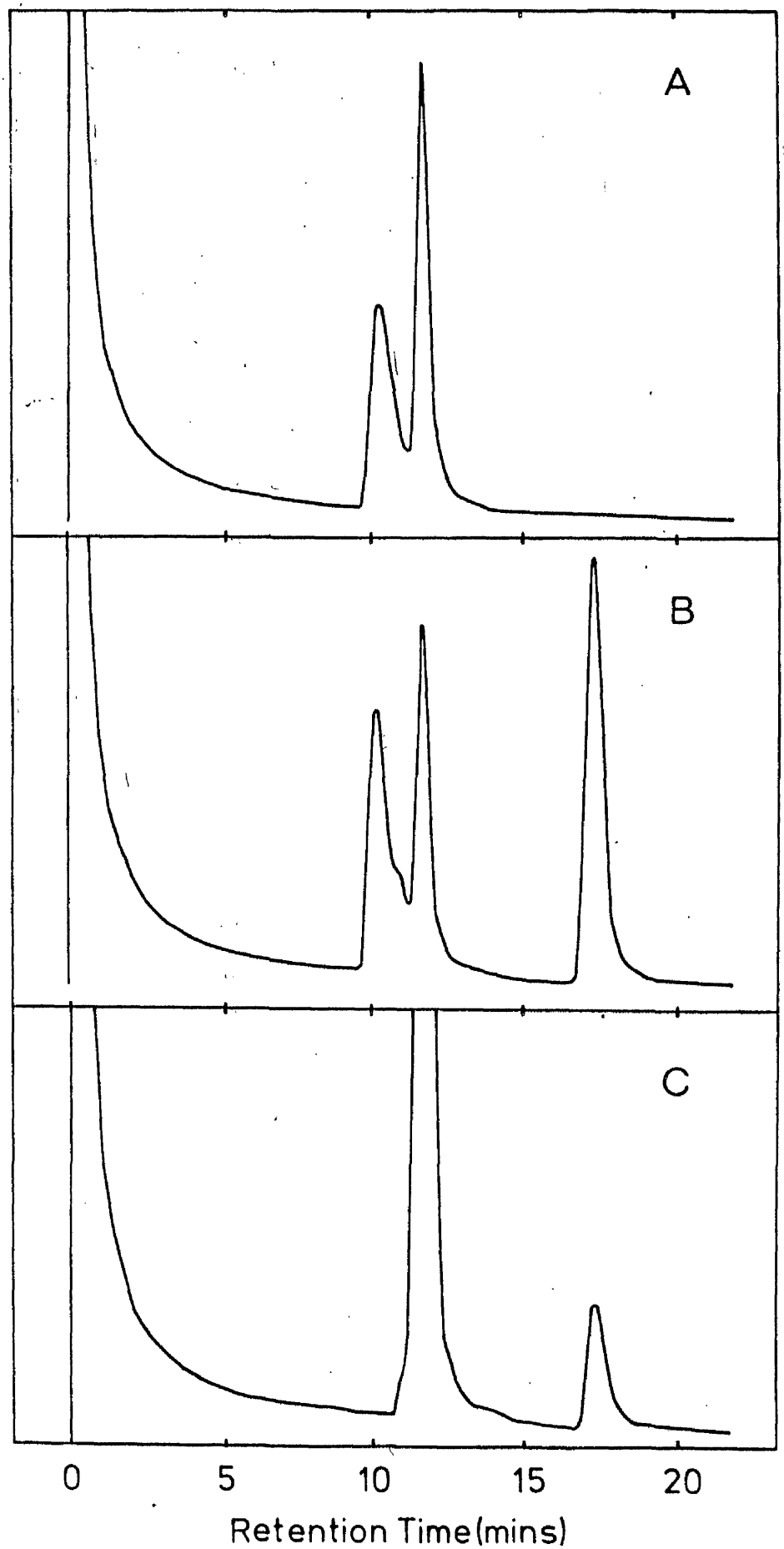

Extension (mm)
14
13
12
11
10
9
8
7
6
5
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration (M)

Fig. 54. Gas chromatogram of methylated samples of stock solutions (A) cis, trans-ABA, (B) trans, trans-ABA, (C) cis, trans-ABA after irradiation by UV light. Samples were chromatographed at 200°C on a 5 ft column of OV 210. Octakozane was included as a reference standard.


A
B
C
0
5
10
15
20
Retention Time (mins)

documented (Mousseron-Canet et al., 1966; Lenton et al., 1971). It would also indicate that the supplied solution of trans,trans ABA was in fact a mixture of both isomers. As a consequence, the results of the bioassays using tt-ABA could be accounted for solely by the presence of the cis,trans isomer, thereby suggesting that the trans,trans isomer possessed no biological activity.

(12) THE EFFECT OF TEMPERATURE ON THE GERMINATION OF LETTUCE VAR. GREAT LAKES.

A series of experiments was undertaken to ensure that the assay using lettuce fruits var. Great Lakes was being carried out at a temperature as near as possible to the optimum for germination, and to examine the effect of the analogues and ABA on that optimum. ABA effects, in lettuce fruits, have already been shown to vary considerably with the temperature at which the experiment is conducted (Reynolds and Thompson, 1971). It was therefore possible that the very marked inhibition of germination produced by some of the analogues might be a result of slightly changing the temperature optimum.

The initial experiments involved the use of a thermogradient bar with a temperature range of 0-30°C. Lettuce fruits and test solutions were placed in petri dishes positioned along the horizontal surface of the bar at points where the temperature was 5, 10, 15, 20, 25 and 30°C. The mean time to 50% germination was recorded for the various treatments of ABA and water (Fig. 55).

A clearly defined optimum temperature for germination existed in this variety at about 25°C. Lowering the temperature from 25°C to 20°C slightly, but significantly, increased the time required for the fruits to germinate. Below 20°C, however, this time increased dramatically with decreasing temperature. This same effect of increased time to 50% germination occurred when the temperature rose above 25°C.

When ABA was also present in the incubation medium, germination was delayed at all temperatures. The response to ABA was greatest at 25°C. It is thus apparent that to reduce the time to 50% germination to a minimum,

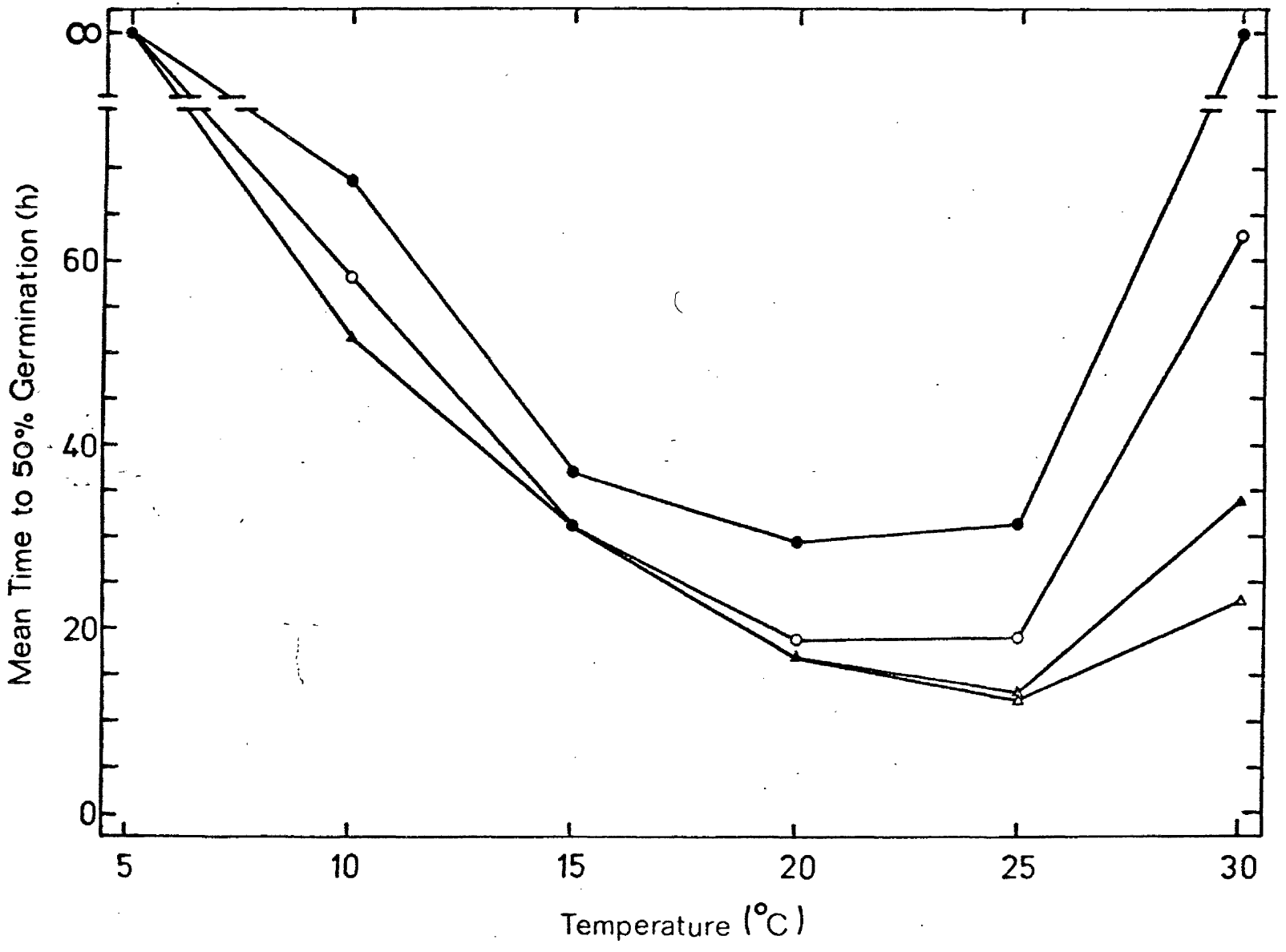
Fig. 55. The mean time taken for lettuce fruits to achieve fifty per cent germination at various temperatures when incubated in the presence of a range of ABA concentrations.

open triangle - water.

closed triangle - 10^{-7}M ABA.

open circle - 10^{-6}M ABA.

closed circle - 10^{-5}M ABA.


Mean Time to 50% Germination (h)
0
20
40
60
80
5
10
15
20
25
30
Temperature (°C)

thereby reducing the possibility of interference from endogenous inhibitors, germination experiments should be carried out at a temperature near the optimum of about 25°C. This is also desirable because it maximises the effects of ABA application.

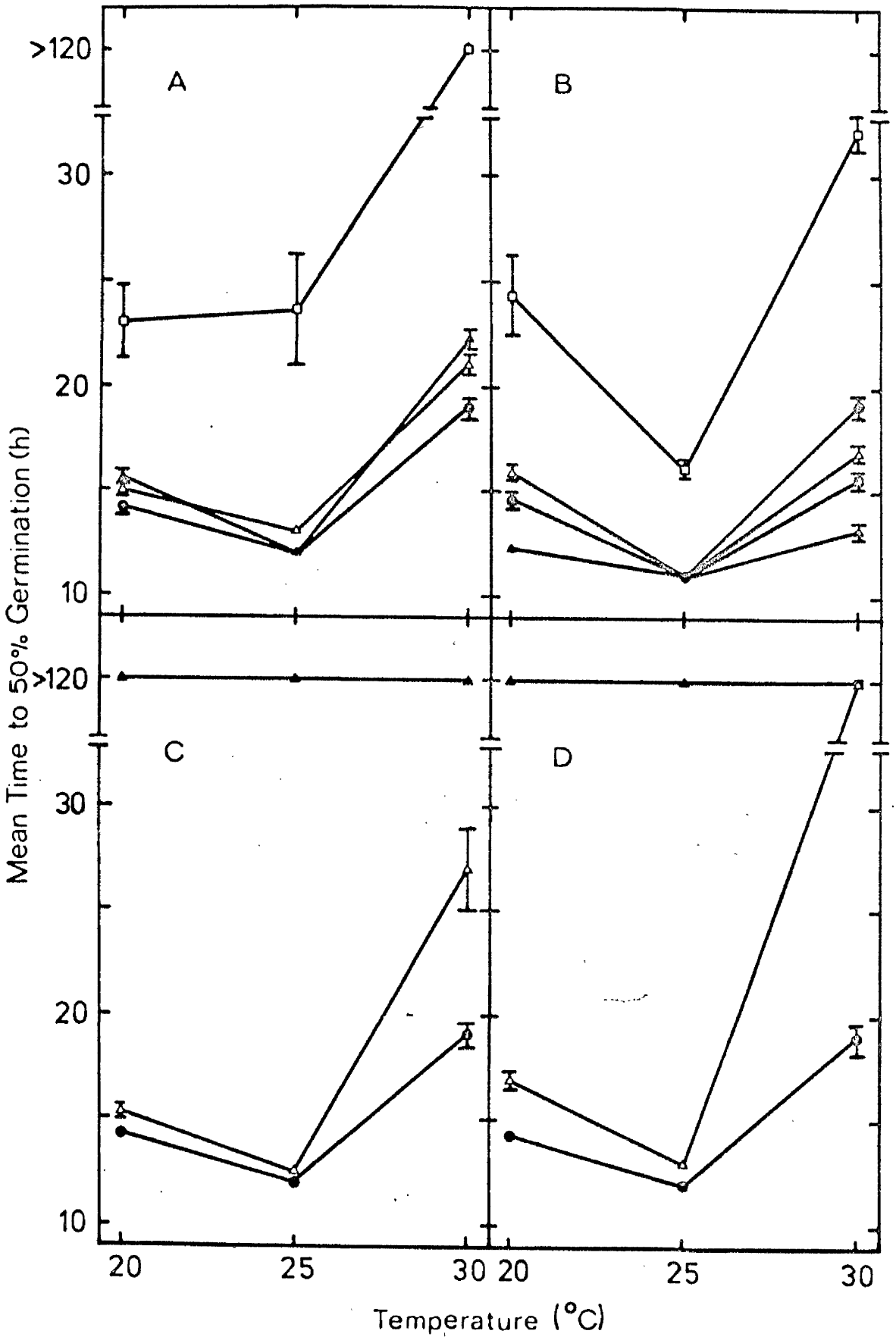
Because of a latent unreliability in the refrigerated water bath, and because of the small number of dishes which could be accommodated in each experiment, a second method of examining temperature effects was designed. The test dishes were placed in a sealed plastic box which was then removed to a controlled temperature incubator. The temperature in the incubator was stable to $\pm$ one centigrade degree and to an even greater extent within the plastic box. To ensure that the results obtained using this arrangement were not a result of the temperature treatments being separated in time, ABA controls were included in each experiment and the results of these compared with the results obtained on the thermogradient bar (Fig. 56A). It was found that the overall effect was the same even though it varied slightly in magnitude. In both assay methods, there was a distinct optimum at 25°C, with the mean time to 50% germination increasing rapidly with increasing temperature.

To determine whether the high activity of some analogues was a result of altering the temperature optimum for germination of the lettuce fruits, compounds II and XVII were examined at 0, 10^{-7}, 10^{-6} and 10^{-5}M at 20, 25 and 30°C (Fig. 56 C and D). Both compounds delayed germination at 10^{-7}M and, at 10^{-6} and 10^{-5}M, this delay exceeded 120 h, the experiment having to be concluded after this time because of contamination in the dishes at 30°C. The form of the temperature response was, however, the same as that observed with ABA. The temperature optimum was still 25°C with marked increases in the MTG at temperatures above and below this optimum. The increased ABA-like activity of these compounds cannot, therefore, be explained in terms of altering the temperature optimum.

The effects of kinetin on the temperature optimum were also considered

Fig. 56. The mean time taken for lettuce fruits to achieve fifty per cent germination at various temperatures when incubated in the presence of a range of concentrations of (A) ABA, (B) kinetin, (C) compound II, (D) compound XVII.

The concentrations of ABA and the two analogues are referred to by :- closed circle (water), open triangle (10^{-7}M), closed triangle (10^{-6}M), open square (10^{-5}M).
Kinetin concentrations were :- closed circle (no kinetin), closed square (10^{-6}M), closed triangle (10^{-5}M), open triangle (10^{-4}M), open square (ABA and kinetin both present at 10^{-5}M).


A
B
C
D
>120
30
20
10
>120
30
20
10
Mean Time to 50% Germination (h)
20
25
30
20
25
30
Temperature (°C)

briefly. When kinetin at 10^{-6}, 10^{-5} and 10^{-4}M was included in the incubation medium there was no effect on the position of the temperature optimum (Fig. 56B), although it may have decreased the sensitivity of the fruits to small changes in temperature near the optimum. This is evidenced by the less rapid rise in MTG when the temperature is moved away from 25°C than occurs in the absence of kinetin.

(13) THE EFFECTS ON LETTUCE GERMINATION OF ABA AND ITS ANALOGUES WHEN PRESENT TOGETHER.

Having described aspects of the interaction of ABA and its analogues with GA_3 in three distinct bioassay systems, and having considered the effects of kinetin in overcoming the inhibitory effects of these compounds in lettuce germination, the effect of applying ABA and its analogues simultaneously was examined. This study could be described as an examination of possible interactions between ABA and its analogues. ABA was applied at 10^{-5}M and the analogue concentrations were again selected such that germination was expected to occur within 3-4 days, i.e. a period over which the mean time to 50% germination could be assessed easily and accurately without the problems of fungal and bacterial contamination. Only those analogues which were active in the lettuce germination bioassay were included in this series of experiments, and the compounds and concentrations are described in Table 45.

The experimental design was similar to that described for the earlier lettuce germination experiments, and the method of assessing inhibitory activity was identical. It was found that ABA, when present alone, delayed the mean time to 50% germination from 12.62 $\pm$ 0.15 h until 33.70 $\pm$ 2.44 h, a period of 21.08 h and, when the concentration was doubled, the delay increased by 25.63 h to 59.33 $\pm$ 5.67 h; these results are comparable to the data presented in Fig. 8. When the analogues were assayed alone, their effects (Fig. 57) were found to be consistent with those shown in Fig. 10,

Fig. 57. The effect of ABA and certain of its active analogues, alone and in combination, on lettuce germination. ABA was present at 10^{-5}M and the analogues at concentrations described in Table 17.

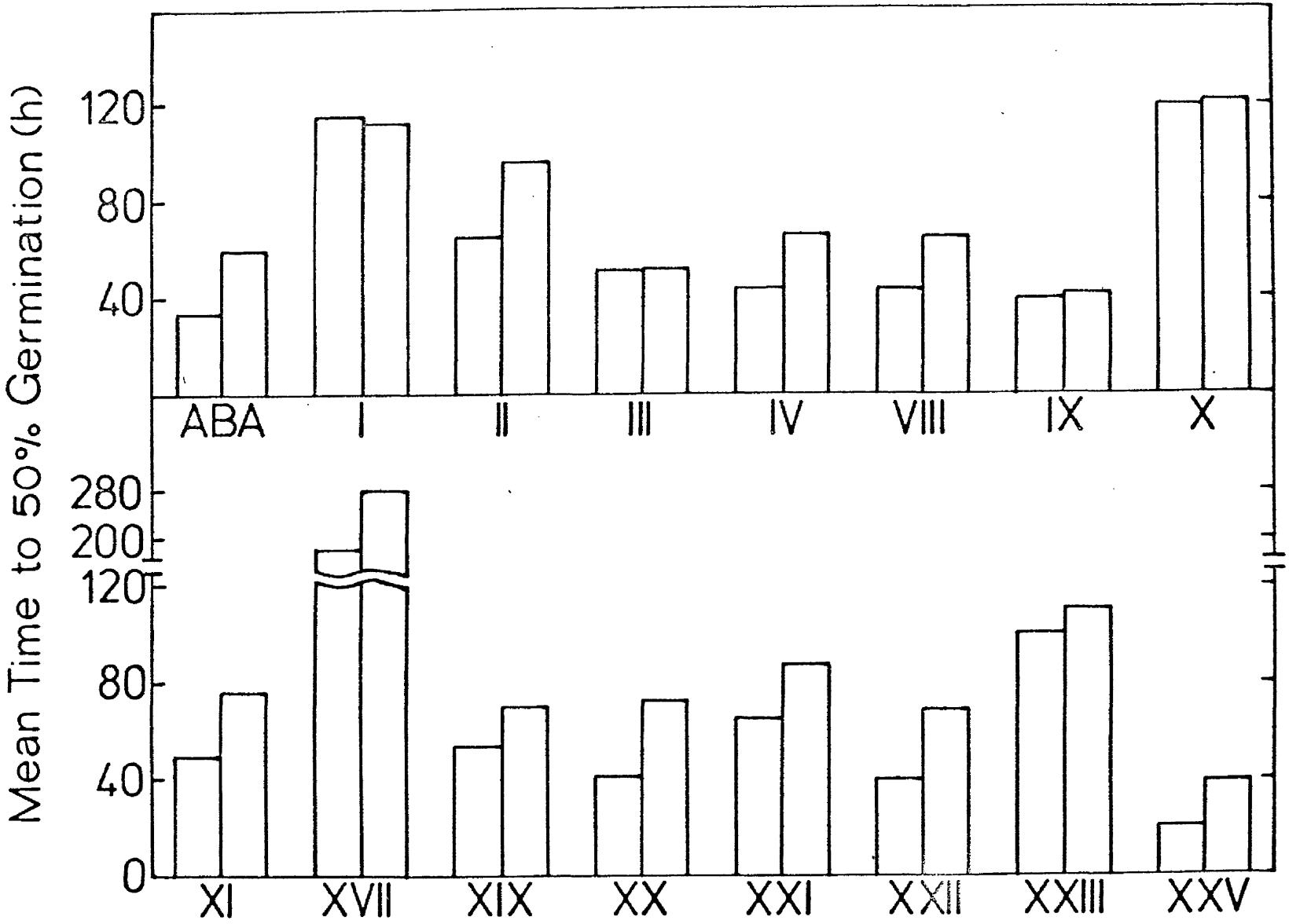

Mean Time to 50% Germination (h)
120
80
40
ABA
I
II
III
IV
VIII
IX
X
280
200
120
80
40
0
XI
XVII
XIX
XX
XXI
XXII
XXIII
XXV

Table 45. The effect on lettuce germination of the active ABA analogues when applied alone, and the statistical significance of the response elicited by the inclusion of 10^{-5}M ABA in the bathing solution.

Compound	Concentration (Molar)	Mean time to 50% germination		Significance of differences ('t' tests)
		Compound	Compound + ABA	
ABA	10^{-5}	33.70 ± 2.44	59.33 ± 5.68	*
I	10^{-6}	115.10 ± 1.64	112.77 ± 4.45	NS
II	10^{-6}	64.37 ± 5.83	96.33 ± 12.73	*
III	10^{-5}	50.23 ± 0.62	50.43 ± 1.60	NS
IV	10^{-5}	42.73 ± 0.14	65.93 ± 1.34	***
VIII	10^{-6}	43.42 ± 5.22	65.55 ± 3.55	**
IX	10^{-5}	39.47 ± 0.41	41.50 ± 2.46	NS
X	10^{-5}	119.13 ± 0.42	121.33 ± 1.09	NS
XI	10^{-6}	49.73 ± 0.09	75.87 ± 2.15	***
XVII	10^{-6}	181.13 ± 35.5	285.00 ± 2.65	*
XIX	10^{-6}	53.57 ± 2.02	69.47 ± 3.36	*
XX	10^{-6}	41.60 ± 2.77	72.73 ± 4.77	***
XXI	10^{-6}	64.96 ± 0.17	86.87 ± 6.03	*
XXII	10^{-6}	40.20 ± 2.32	69.63 ± 2.19	***
XXIII	10^{-5}	100.00 ± 9.24	110.50 ± 0.85	NS
XXV	10^{-5}	20.50 ± 0.49	39.75 ± 1.25	***but

but, when both ABA and the analogues were included in the incubation medium, the analogues could be segregated into two categories or groups (Table 45 and Fig. 57). The presence of 10^{-5}M ABA with II, IV, VIII, XI, XIX, XX, XXI, XXII and XXV delayed the MTG for between 15.90 h and 31.96 h with a mean delay of 24.72 h. The addition of ABA to incubation media containing compounds I, III, IX, X and XXIII did not, on the other hand, affect the MTG. The only compound which did not fall easily into either of these categories was XVII which, when present alone, delayed the MTG until 181.13 ± 35.5 h, and in the presence of ABA until 285 ± 2.65 h, an increase of 103.87 h. However, the variability within treatments was such that even the large apparent difference between these treatments was barely significant. Hence, XVII may belong to the first category of analogues.

To ensure that the analogues in the first group were truly additive with

ABA in their effects, II was re-assayed at 10^{-7}M and XI at 5×10^{-6}M. When present alone at these concentrations, the mean times to 50% germination were 12.62 $\pm$ 0.30 h and 20.53 $\pm$ 1.21 h respectively, and when in combination with ABA at 10^{-5}M, 40.37 $\pm$ 2.35 h and 46.37 $\pm$ 2.68 h respectively. These results indicate that, even where the most active component was ABA, II and XI were still additive with ABA despite being diluted.

Analogues of the second group, when applied together with ABA, delayed germination for a time equal to that induced by the more active component applied alone. Using this criterion, compound XXV would belong to the second category because the MTG was similar to that obtained when ABA alone was applied, and ABA was more inhibitory than XXV at the concentrations tested.

The finding of an additive effect with ABA in only a well defined group of analogues immediately raises doubts as to the similarity of the mode of action of ABA and some of the analogues.

(14) THE RELATIONSHIPS BETWEEN ABA AND WATER UPTAKE IN LETTUCE FRUITS.

In studying the effects and rôle of exogenously applied ABA, it is of essence to evaluate the process of ABA uptake into the tissues. In most seed systems, germination is evoked by the supply of water provided that other environmental conditions are suitable and that the dormancy requirement is satisfied. When water is made available to lettuce fruits, it is taken up by the process of imbibition. Hence, the uptake of ABA, when in aqueous solution, may be simply passive, entering with water during imbibition. In order to establish the patterns of ABA uptake, an investigation of the relationship between water and ABA uptake was undertaken.

The simplest method of determining patterns of water uptake was to weigh a batch of fruits before and after imbibition in test solution. Any increase in weight can be assumed to be a measure of water uptake. By carrying out

this procedure over a time course, it was possible to construct a graphical representation of water uptake (Fig. 58). There were two distinct phases to this uptake, the first phase occurring within two hours and being virtually completed within 4 h. Very little water was then taken up until about 10 h, after which a log linear increase was observed up to 48 h, when the experiment was terminated. The beginning of this second phase coincided with radicle emergence.

When the bathing solution contained ABA at 10^{-5}M, imbibition was unaffected for the first 6-8 h, with neither the rate of uptake nor the total amount of water taken up, being influenced. It was only after 8 h that ABA had a significantly inhibitory effect on water uptake when compared with fruits imbibed in water (Table 46). In the presence of ABA, very little further water was taken up by the fruits before the experiment was terminated after 48 h. This pattern of uptake could be attributed to the delay, by ABA, of growth processes leading to radicle emergence which occurred between 24 and 48 h in the ABA treated fruits, and 8-10 h in the fruits imbibed in water.

Uptake of 2[^{14}C] ABA by Lettuce Fruits.

Lettuce fruits var. Great Lakes were imbibed for known periods of time in a 10^{-5}M solution of 2[^{14}C ($\pm$)] ABA. They were then washed in a Büchner funnel, extracted in 2 ml of 80% ethanol, and the radioactivity assayed by liquid scintillation spectrometry. A number of washing procedures were examined to determine the most efficient method. The results of two such procedures are shown in Fig. 59 A and B. After removal of the excess ABA, 10 ml quantities of water were poured over the fruits, and the number of disintegrations per minute was assayed in each 10 ml washing. The second system was essentially similar, except that 50 ml of water was used in each wash. After four washings of 50 ml, no detectable levels of radioactivity were being removed, whereas even after seven washings of 10 ml, appreciable

Fig. 58. Uptake of water by lettuce fruits var. Great Lakes as assessed by increase in fresh weight.

closed circle - imbibed in distilled water.

closed square - imbibed in 10^{-5} M ABA solution.

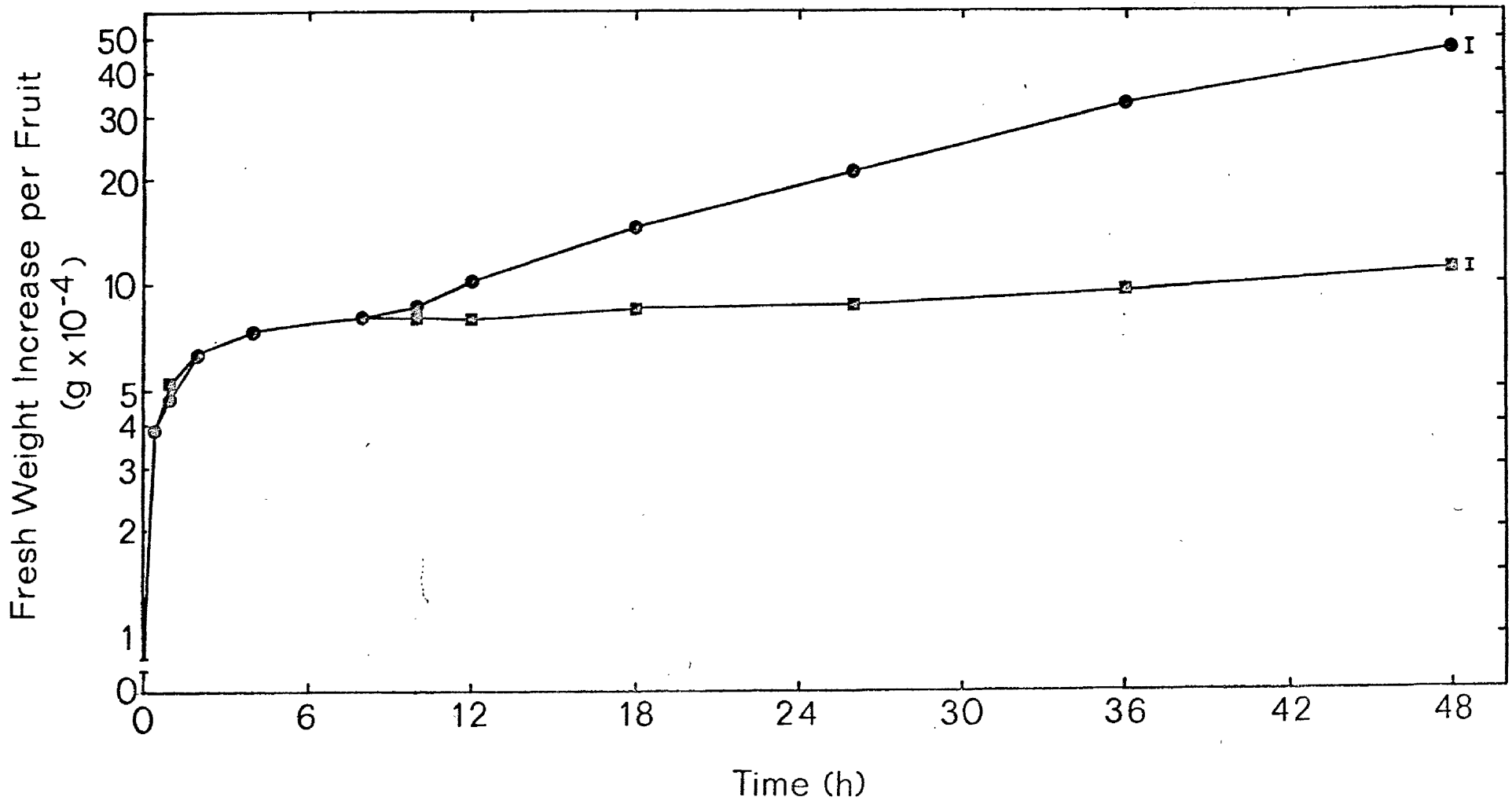

Fresh Weight Increase per Fruit
(g x 10-4)
50
40
30
20
10
5
4
3
2
1
0
0
6
12
18
24
30
36
42
48
Time (h)
I
I

Fig. 59. The efficiency of removal of surface bound radioactive ABA from lettuce fruits by rinsing the fruits with (A) 50 ml, (B) 10 ml amounts of water. The data relate to the amounts of radioactivity removed in consecutive washes.

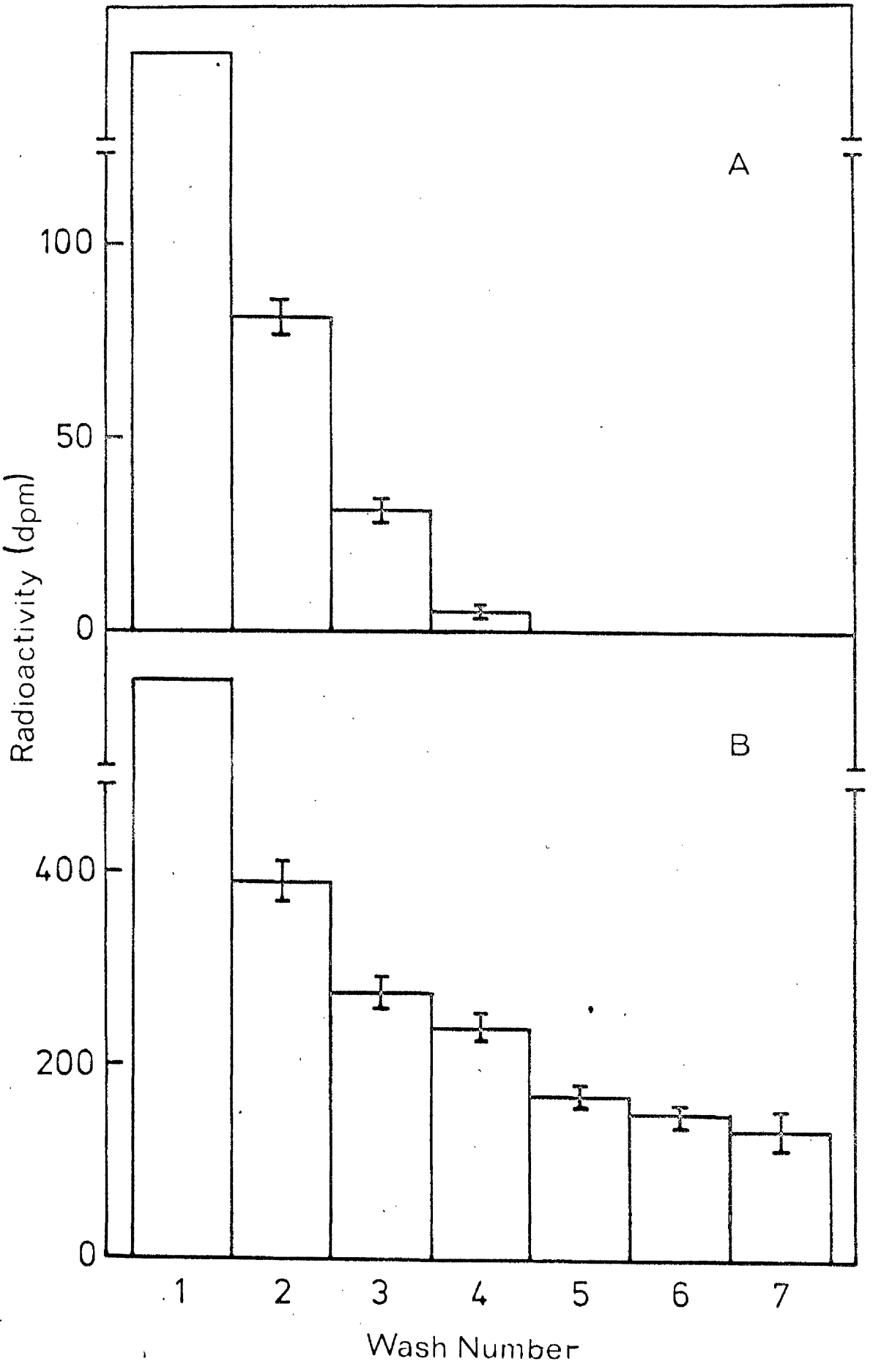

A
B
100
50
0
400
200
0
Radioactivity (dpm)
1
2
3
4
5
6
7
Wash Number

Table 46. The uptake of water by imbibing lettuce fruits and the effect of 10^{-5}M ABA on that uptake.

Imbibition Time	Weight Increase in Water (g) x 10^{-4}	Weight Increase in 10^{-5}M ABA (g) x 10^{-4}	Significance of difference ('t' tests)
0.5	3.86 ± 0.04	3.90 ± 0.18	NS
1.0	4.77 ± 0.07	5.03 ± 0.08	NS
2.0	6.32 ± 0.23	6.39 ± 0.10	NS
4.0	7.32 ± 0.08	7.33 ± 0.12	NS
6.0	7.72 ± 0.08	7.65 ± 0.14	NS
8.0	8.02 ± 0.13	7.90 ± 0.17	NS
10.0	8.65 ± 0.09	8.08 ± 0.10	***
12.0	10.07 ± 0.17	7.85 ± 0.53	***
18.0	14.36 ± 0.33	8.57 ± 0.25	***
24.0	20.87 ± 0.53	8.65 ± 0.04	***
36.0	32.25 ± 0.15	9.65 ± 0.13	***
48.0	45.83 ± 0.25	10.97 ± 0.37	***

quantities of ABA were still being removed. It was therefore obvious that the simplest and most satisfactory procedure was to use four washings of 50 ml.

The results of the uptake experiments indicated that there were again two phases of uptake, and that this uptake of radioactivity from the solution containing 2[^{14}C] ABA closely reflected that of water (Fig. 60). The initial phase, however, continued for 3 h after apparent complete imbibition. Each fruit imbibed approximately 8 x 10^{-4} ml of solution which, at a specific activity of 45 μ Ci mg^{-1} and a concentration of 10^{-5}M, should by calculation have contained 210 dpm, but was found experimentally to contain only 188 dpm. Fig. 61A, however, shows that during imbibition the fruits took up only 45 cpm which, at 94% efficiency of counting, is equivalent to 48 dpm. The

Fig. 60. Uptake of 2-[^{14}C]ABA by lettuce fruits var. Great Lakes as a function of time. Germination began after 24 h.

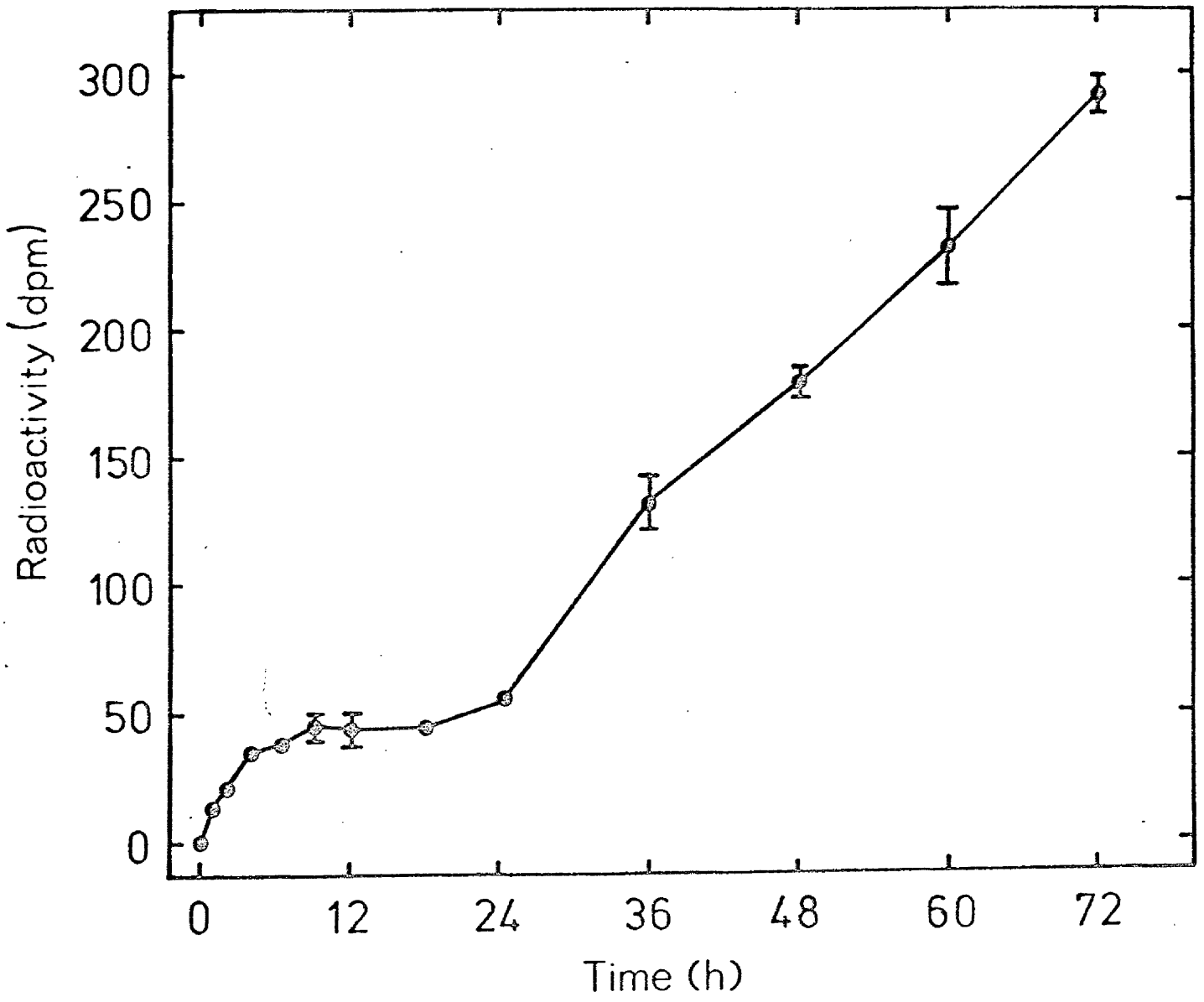

Radioactivity (dpm)
300
250
200
150
100
50
0
0
12
24
36
48
60
72
Time (h)

Fig. 61. Uptake of radioactivity by lettuce fruits var. Great Lakes during imbibition.

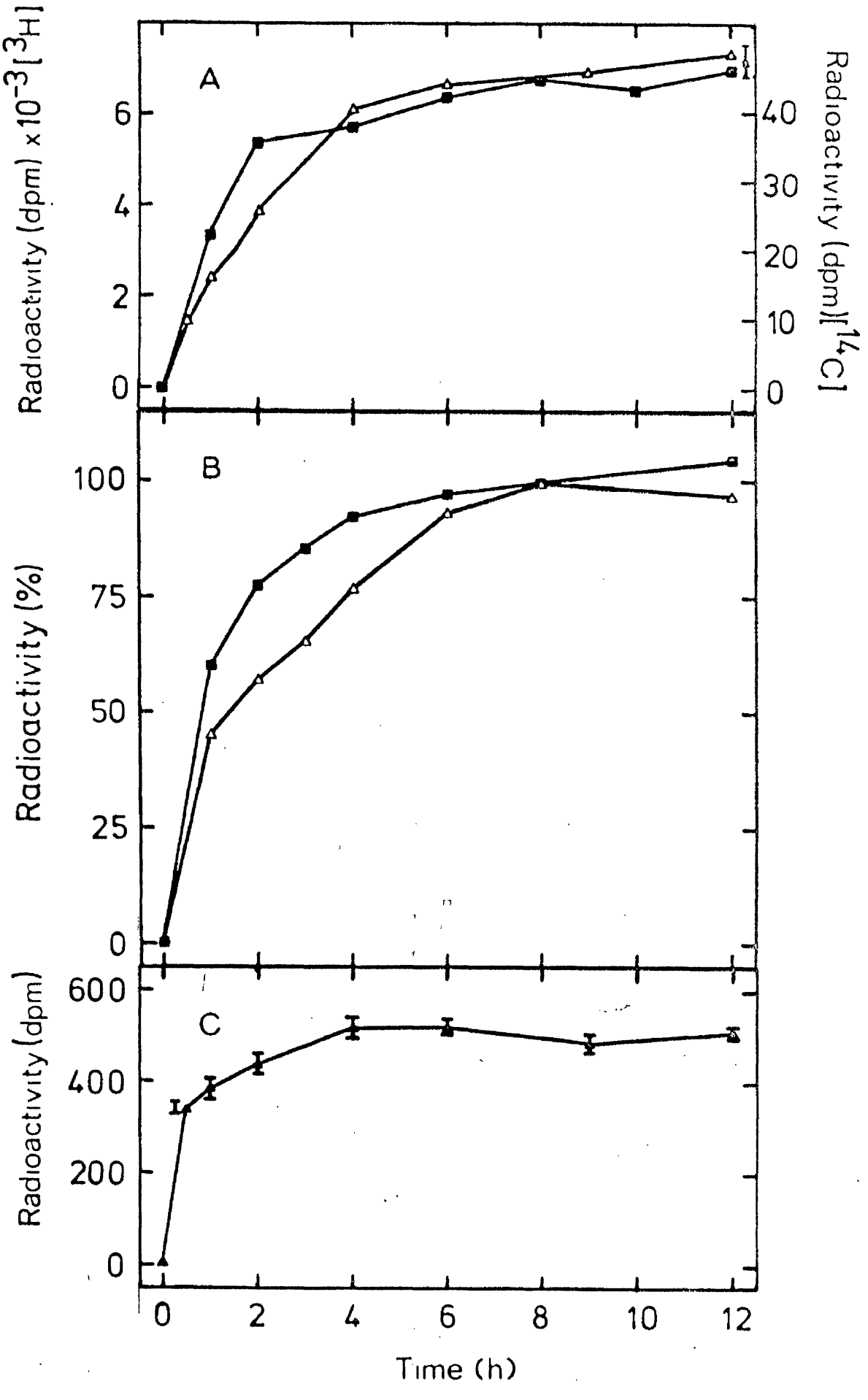
(A) Uptake in d.p.m. per fruit of 2-[^{14}C]ABA (closed squares) and tritiated water (open triangles).

(B) Uptake as a percentage of the amount present after 6 h when imbibition was deemed to be complete. The experiment was carried out using a double isotope technique.

closed squares - 2-[^{14}C] ABA.

open triangles - tritiated water.

(C) Uptake in d.p.m. per fruit of 8-[^{14}C] kinetin.


A
B
C
Radioactivity (dpm) x10⁻³[³H]
Radioactivity (dpm)[¹⁴C]
Radioactivity (%)
Radioactivity (dpm)
Time (h)
0
2
4
6
8
10
12
0
2
4
6
0
10
20
30
40
0
25
50
75
100
0
200
400
600

time lag and uptake inconsistencies could be explained either by there being an active discrimination against the uptake of ABA, or by the washing procedure in some way causing the discrepancies.

Uptake of Tritiated Water.

In an attempt to resolve the cause of the two inconsistencies outlined above, a series of experiments involving the uptake of tritiated water was undertaken. These experiments were carried out in the same way as those investigating the uptake of ABA, the advantage of this over the weight increase method being that the results for water uptake and ABA uptake could be compared directly, since the techniques involved were the same. The fruits were imbibed in tritiated water, washed and assayed. The results showed that the time lag of about 3 h persisted (Fig. 61A). When the quantity of label entering the fruits was examined critically, it was found that 6,000 dpm entered per fruit, while the quantity predicted to enter was 24,000 dpm. The value of 25% of the predicted uptake of $^{3}H_2O$ compares favourably with that found for ABA. The uptake time lag, therefore, appears to be a real phenomenon, while the discrepancy relating to the quantity of label taken up was a feature of the experimental procedure.

Double Label Experiments.

To enable more precise characterisation of the time lag between completion of the first phase of water uptake and of ABA uptake, an experiment was designed where the uptake of both isotopes could be measured simultaneously. Fruits were imbibed in a solution containing 2[^{14}C] ABA dissolved in tritiated water, and then the levels of both isotopes were assayed using a liquid scintillation spectrometer which had been calibrated for "double-label counting". The results were expressed at each time interval as a percentage of the label present after 8 h (Fig. 61B), imbibition of both ABA and water being complete

after this time, while the second uptake phase due to radicle extension had not yet begun. The uptake time lag was still apparent in these results.

Uptake of 8[^{14}C] kinetin.

To ascertain whether or not an uptake lag was a feature confined solely to ABA, the uptake of kinetin was also examined. The technique used was the same as that used for ABA, with 8[^{14}C] kinetin being substituted for the 2[^{14}C] ABA. The results, however, indicate that in this instance, the uptake coincided almost exactly with that of water, no time lag being found (Fig. 61C). Kinetin therefore appears to be taken up passively when supplied in aqueous solution.

(15) MICROAUTORADIOGRAPHIC STUDIES OF THE DISTRIBUTION OF EXOGENOUSLY APPLIED RADIOACTIVITY WITHIN LETTUCE FRUIT TISSUES.

The question arises as to the fate and distribution of exogenously applied ABA after uptake. The availability of radioactive ABA and soluble compound microautoradiographic instrumentation facilitated the initiation of a detailed appraisal of the distribution of exogenously applied 2[^{14}C ($\pm$)]ABA. For comparative purposes, the uptake of another compound which modifies germination, kinetin, was included in this study.

After incubation for 6 h or 30 h in 2[^{14}C ($\pm$)] ABA or 8[^{14}C] kinetin, lettuce fruits were embedded in fresh pig's liver and frozen in liquid nitrogen. Thin sections of the tissue were cut on a freezing microtome and transferred, under photographic "safe" lights, to cover slips which had previously been mounted on microscope slides and coated with photographic emulsion. The slides were then placed in a dark container at -30°C for a minimum time of four weeks to allow exposure of the film to occur. The autoradiographs were developed, fixed and mounted. Observation and scanning of the resultant autoradiographs under dark field illumination yielded an

apparent pattern of silver grain distribution, as shown in Plate 3 and Fig. 62B. Fig. 62A is a scan of an untreated section, as shown in Plate 4. There was an accumulation of bright bodies around the outer and inner edges of the cotyledons with a greater density at the outer than the inner edge. There were no bright areas within the rudimentary vascular tissue (Plate 5). These bodies resembled silver grains both in size and appearance, and may be taken to indicate an accumulation of radioactive materials in the areas.

It is essential that certain controls be included in the experiments to evaluate the degree of interference to the results by positive or negative chemography, or by latent image fading. The controls to detect positive chemography involved exposing the photographic emulsion to sections containing no radioactivity, any silver grains appearing would therefore result from a chemical reaction between the tissue and the film. In the negative chemography controls, film was exposed to daylight and then incubated at -30°C for the duration of the exposure period, in contact with non-radioactive tissue. Any fading of the image was therefore due to negative chemography provided that no latent image fading occurred. To allow for this possibility, autoradiographs were developed at regular intervals to ensure that the image was not fading.

Negative chemography and latent image fading were not a problem, but when the positive chemography controls were developed, a distribution of bright bodies, identical to those described earlier, was observed. It could, therefore, be assumed that the sections were causing silver grains to appear in the photographic emulsion, and that this effect was limited to the edges of the cotyledons. Nevertheless, on detailed examination of the control slides, the apparent silver grains were seen to be artefacts, similar in size and optical properties to silver grains. In Plates 6 and 7 it can be seen that these bodies occur in a slightly different plane of focus to the silver grains, and have a greater tendency to be aggregated. In some autoradiographs, where the section had become detached, it was observed that the

Fig. 62. Autoradiographs of transverse sections of lettuce fruits scanned under dark field illumination using a Zeiss MPM Microscope photometer attached to a Zeiss Photomicroscope II linked through a galvanometer to a flat bed recorder. (a) Control scan of section containing no applied radioactivity, and in which the artifact is not visible; (b) scan of section containing no applied radioactivity but with the artifact apparent owing to the photographic fixation procedures; (c) scan of silver grains in emulsion after removal of section containing applied radioactivity. Pre-amplification factors were 3, 2 and 2 and post-amplification factors 2.5, 4 and 1.6 respectively.

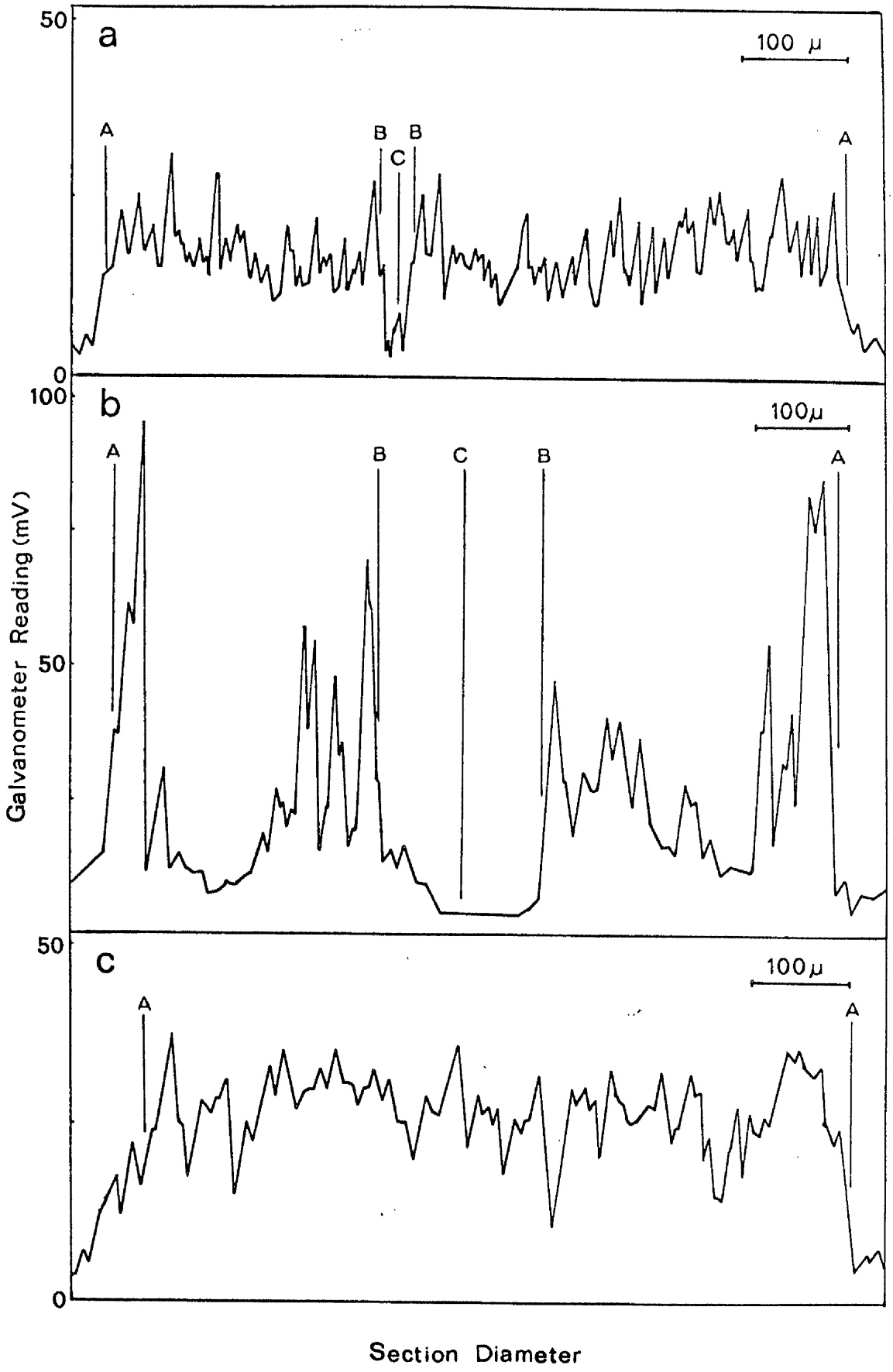
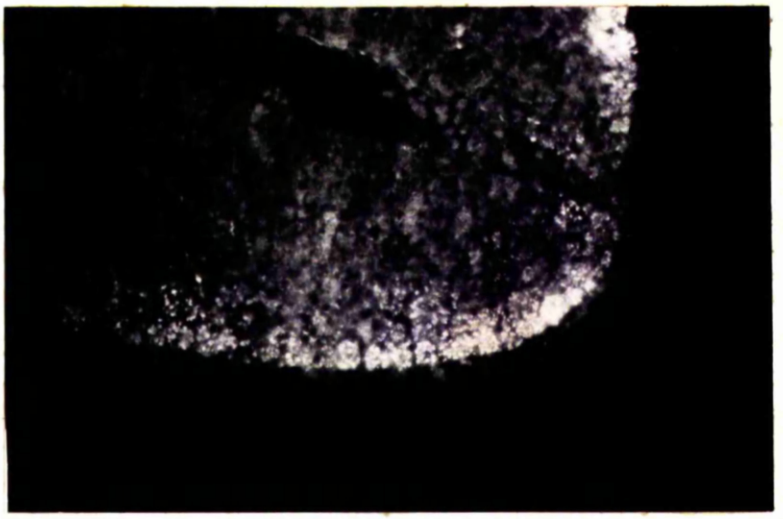
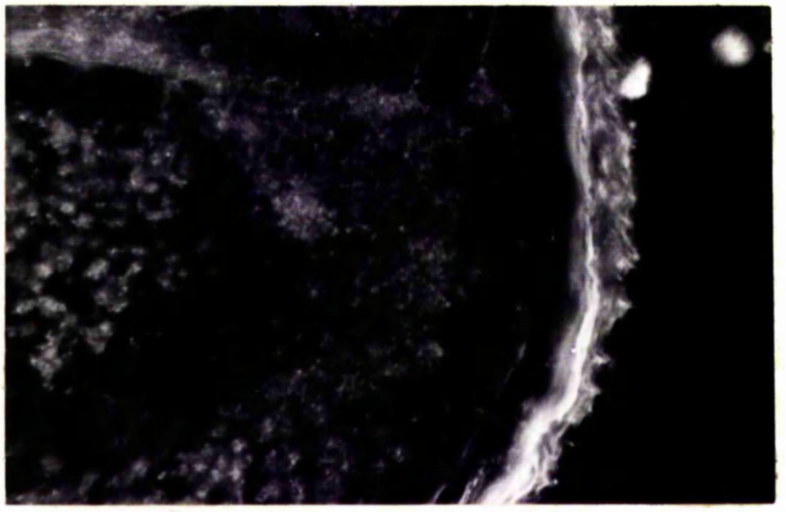

a
100 μ
A
B
B
C
A
50
0
b
100μ
A
B
C
B
A
100
50
c
100μ
A
A
50
0
Galvanometer Reading (mV)
Section Diameter

Plate 3. Control autoradiograph of a 16 µm thick transverse section of lettuce fruit cotyledons containing no applied radioactivity. The section was photographically fixed in "Kodafix" and viewed under dark field illumination.
X50.

Plate 4. Control autoradiograph of a 16 µm thick transverse section of lettuce fruit cotyledons containing no applied radioactivity. The section was not photographically fixed and no artifacts are apparent.
X125.

Plate 5. Longitudinal section of lettuce fruit cotyledon. Note the absence of artifact bodies in the primitive vascular tissue.
X150.





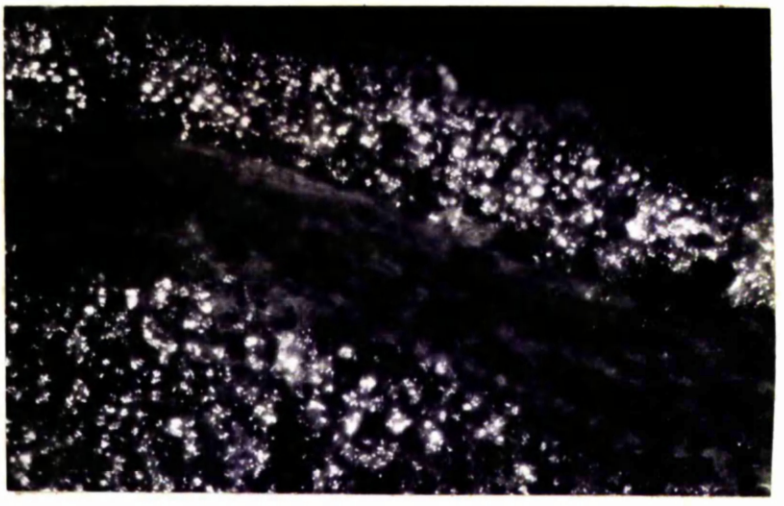


Plate 6. Autoradiograph of transverse section of lettuce fruit cotyledon tissue. (A) Silver grains in focus, (B) A lower focal plane with the artifacts in focus. Note the similarity in appearance between the artifacts and silver grains; the artifact bodies tend, however, to be aggregated. X500.

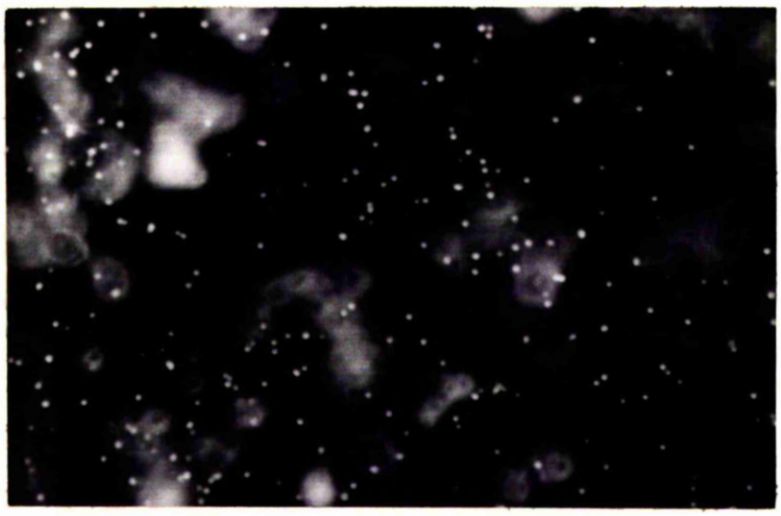
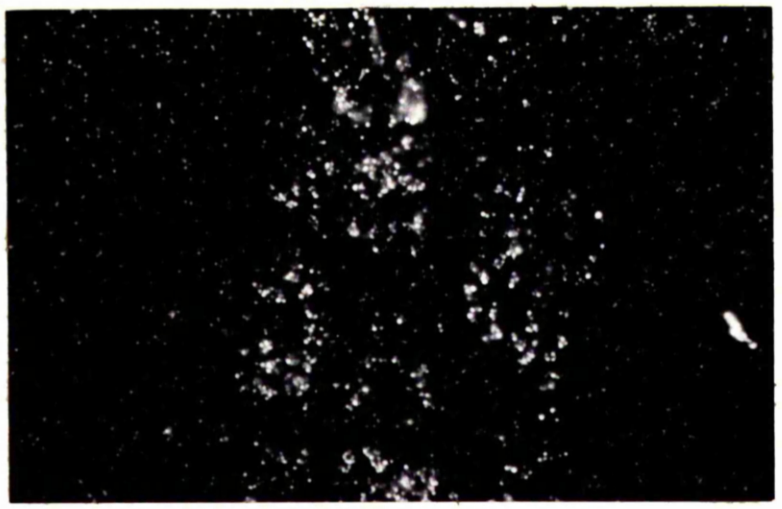
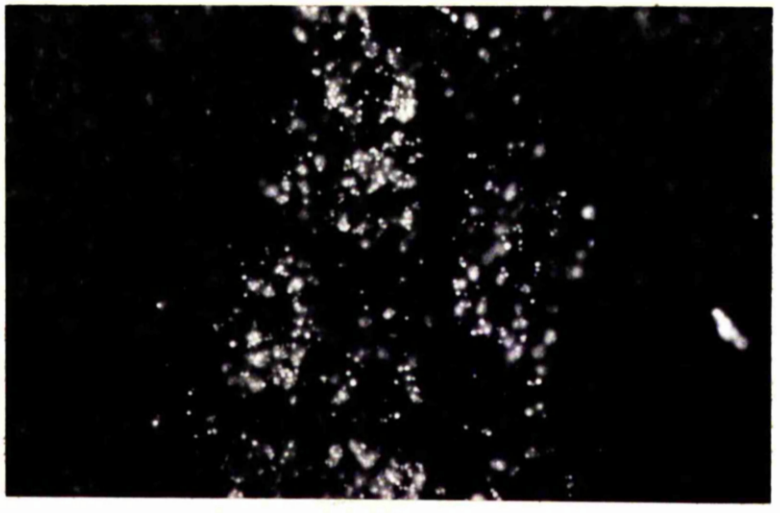


Plate 7. Autoradiograph of transverse section of lettuce radicle. (A) Silver grains in focus. (B) A lower focal plane with the artifacts in focus.

X300.





bodies had also vanished, and it was concluded that they were a type of organelle which changed its properties upon being subjected to the experimental procedures. Because of the similarity of these bodies to silver grains, both automatic scanning and visual inspection of sections were misleading.

In order to overcome this problem, attempts were made to discover the cause of the artefact by separately examining each experimental stage. It was found that the freezing, storage, development and washing stages did not induce artefact formation. Passage of the sections through "Kodafix" fixer, on the other hand, resulted in the appearance of the bodies. It was also noted that the longer the sections remained in "Kodafix", the greater was the area over which the artefact extended and that, after fixation for 20 mins, the whole section was affected, except for those areas of the section corresponding to provascular tissue.

"Kodafix" is a fast fixer based on ammonium thiosulphate, whereas "Metafix" and "Unifix", which are also recommended by Kodak for use with AR.10 stripping film, are based on sodium thiosulphate. Fixation in "Unifix" yielded results similar to those obtained with "Kodafix"; markedly fewer artefacts, however, were noted with "Metafix". When either ammonium or sodium thiosulphate alone was used as a photographic fixer, the lettuce fruit tissue was free of artefacts, although the cell walls in ammonium thiosulphate treated tissue appeared bright under dark field illumination.

An indication of the distribution of silver grains could be obtained by adopting one of three procedures. The first was to count the grains manually, but the presence of the artefacts made this an extremely difficult procedure. The second approach was to fix the autoradiographs in sodium thiosulphate, after which they were scanned automatically. The absence of hardener, however, created difficulties in handling the film, and even when there was no damage, the background brightness was sufficient to mask slight variations in silver grain distribution. The third and most successful method was automatic scanning of the latent image after having dislodged the sections by

vigorous washing during development and fixation. Comparison of the latent image with the section structure of an adjacent serial section enabled correlation to be made between tissue structure and silver grain localisation.

Using this technique, the silver grain distribution in the emulsion covering cotyledonary tissues did not show marked localisation. Plates 8 A and B are autoradiographs of cotyledonary tissue, the section having been removed in 8B. It is apparent, both from visual comparison and from the scan (Fig. 62C), that the radioactivity was uniformly distributed throughout the tissues. Analysis of autoradiographs of radicle sections yielded similar results (Plate 9 A & B). No radioactivity was apparent in the fruit coats. Thus the radioactive ABA and kinetin, when taken up during imbibition, distribute themselves throughout the tissue, although there was some evidence that during the second phase of uptake, the radioactivity accumulated just behind the radicle tip.

Preliminary studies were also carried out on identifying the source of the artefact. Freshly cut sections were stained with osmic acid, iodine and toluidine blue. Iodine gave no staining reaction, but osmic acid produced a dark brown or black coloration of the tissues. The dark coloration was probably due to the presence of large amounts of unsaturated lipids in the tissue, suggesting these lipid bodies might be the source of the artefact. However, staining the freshly cut sections with toluidine blue resulted in blue stained bodies with a distribution different from the lipids. These granular bodies, when viewed under dark field illumination, correlated exactly with the occurrence of the artefact.

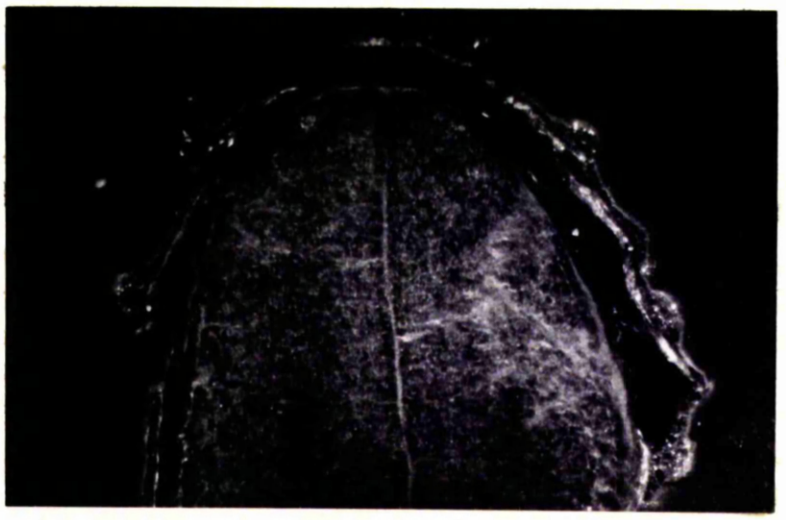
Embryos were dissected out of the endosperms, embedded in "Araldite", sectioned, stained and observed by light and electron microscopy. Two distinct types of body were found to be present in the cells and were apparent under the light microscope as a result of osmic acid and toluidine blue staining (Plates 10 and 11). One type of body had a uniform appearance under both light and electron microscopy, and gave a greyish colour with short

Plate 8. Autoradiographs of serial transverse sections of lettuce fruit cotyledons. The autoradiographs were photographically fixed in "Kodafix" and viewed under dark field illumination.

(A) Section of tissue present on the photographic emulsion.

(B) Section of tissue removed.

X125



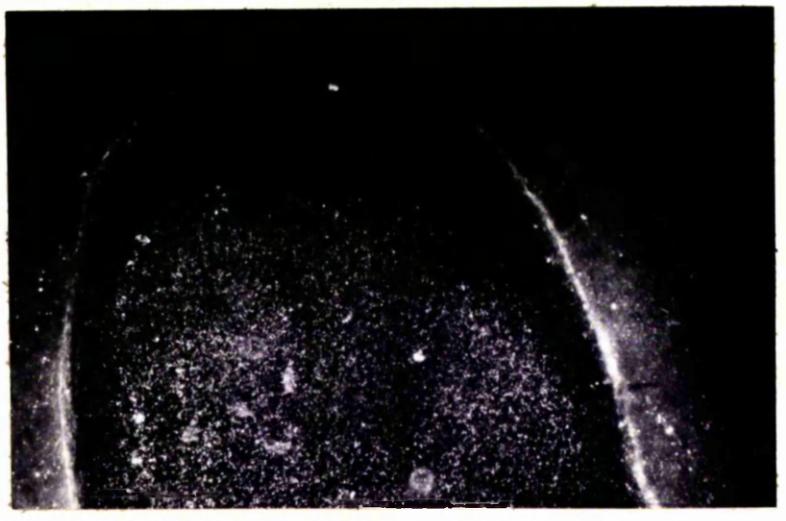


Plate 9. Autoradiographs of serial transverse sections of lettuce radicle. The autoradiographs were photographically fixed in "Kodafix" and viewed under dark field illumination.
(A) Section of tissue present on the photographic emulsion.
(B) Section of tissue removed.
X70.

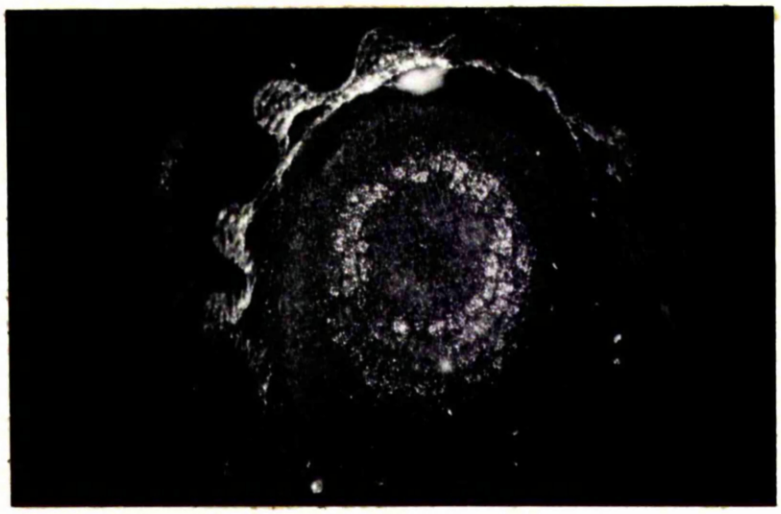


Plate 10. Light micrographs of cotyledonary tissue of lettuce. The tissue was embedded in "Araldite" and stained with toluidine blue.

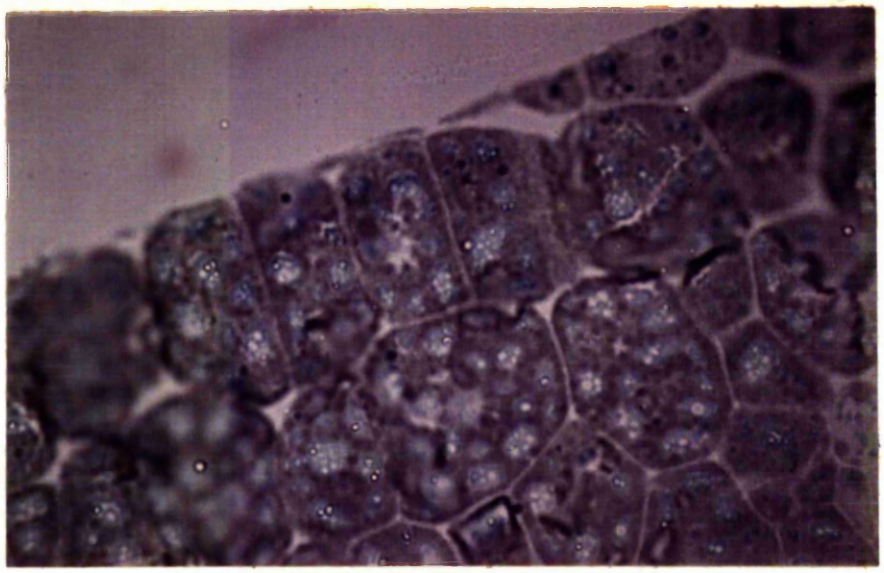
(A) x 1125; (B) x 3150; (C) x 3300.

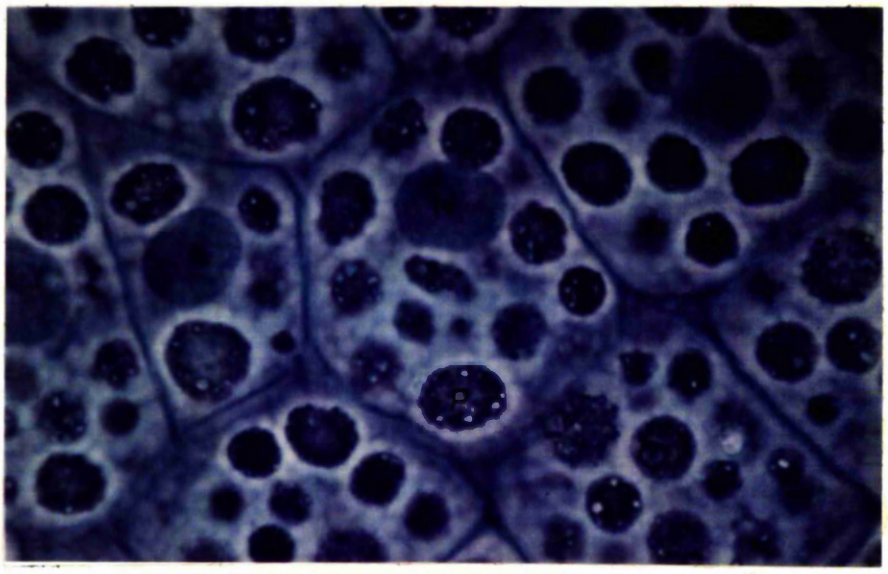
C :- cell wall.

L :- lipid body.

N :- nucleus.

P :- protein body, with inclusions normally visible as unstained areas.




P

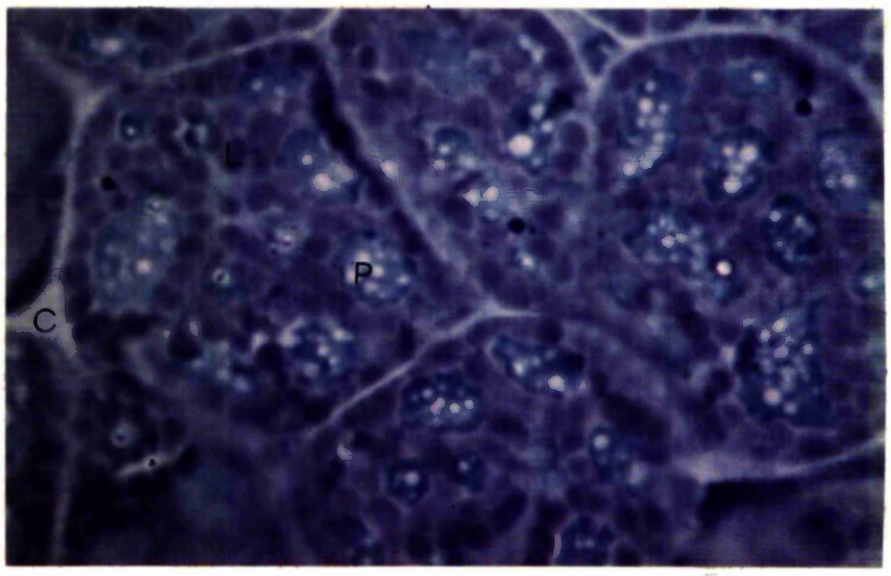

L
P
C

Plate 11. Electron micrographs of cotyledonary cells of lettuce. The tissue was embedded in "Araldite" and stained with uranyl acetate and lead citrate.

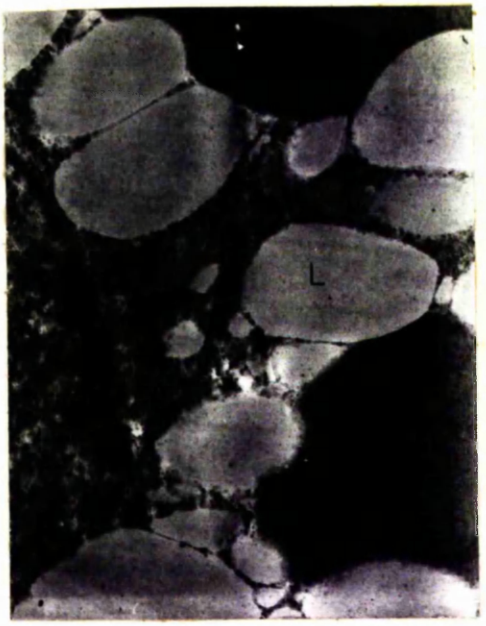
(A) cell of spongy mesophyll. x20,000.

(B) junction of cells of spongy mesophyll. x 15,000.

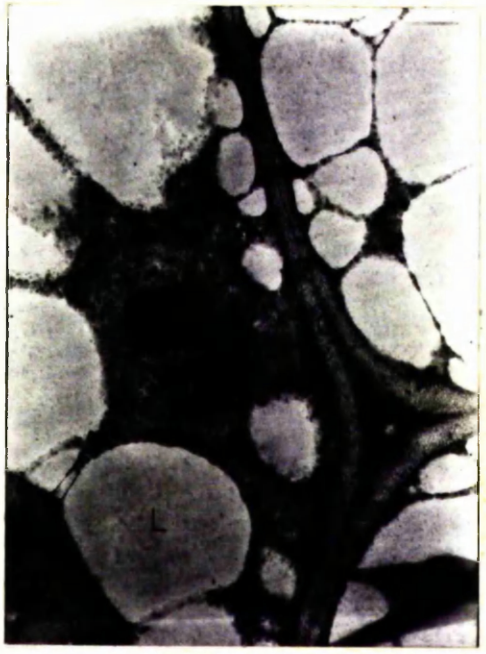
(C) junction of cells of spongy mesophyll. x 20,000.

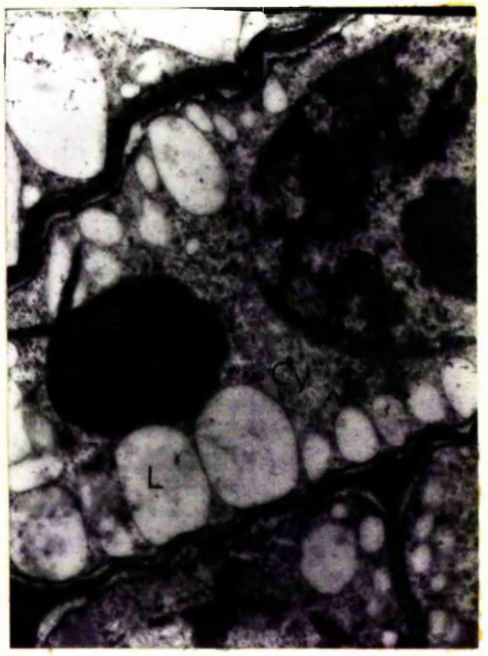
(D) cells of vascular tissue. Note the relatively small number of lipid bodies and the uniform consistency of the protein bodies in which there is no evidence of inclusions. x 30,000.

C :- cell wall; Cy :- cytoplasm; L :- lipid body; N :- nucleus; P :- protein body.


L


L


L


CV
L

exposure to osmic acid, but no staining reaction with toluidine blue. The other type of body had a very dense appearance and gave a blue coloration with both toluidine blue and methylene blue. On the basis of these staining reactions, and by comparison with the findings of previous investigations, especially those of Paulson and Srivastava (1968) and Srivastava and Paulson (1968), these bodies were concluded to be composed essentially of lipid and protein respectively.

The sections examined by light microscopy (Plate 10) also revealed the presence in many of the protein bodies of inclusions which sometimes stained red with toluidine blue. These inclusions rarely occurred in the cells of the vascular tissue, as can be seen in the electron micrographs, and were also frequently absent from the protein bodies of the radicle tip. These protein body inclusions were concluded to be the cause of the artefact which occurred during fixation of the microautoradiographs. The fixation process is thought to disrupt the protein bodies in some way, causing them to release the inclusions. Few membranous bodies could in fact be observed within the cells; dictyosomes, plasmalemma, nucleolemma and mitochondria were all absent. In the spongy mesophyll cells of the cotyledons, the storage bodies occupied so much space that the cytoplasm was visible only as thin strands between the storage bodies.

(16) METABOLISM OF ABA BY LETTUCE FRUITS.

Consideration has been given to the uptake of ABA by lettuce fruits and the subsequent distribution of the ABA within the fruits. It therefore seemed logical to examine the metabolism of ABA by the lettuce fruit tissues.

The 2[^{14}C(±)]-ABA used in these experiments was supplied in two batches. Upon receipt, and at regular intervals, both batches were tested for purity by chromatographing aliquots in at least two separate chromatographic systems. The first batch yielded only one peak of radioactivity in the systems used

(Fig. 63A), while in batch 2 a smear was apparent after development in n-butanol : n-propanol : 0.88 ammonia : water (2 : 6 : 1 : 2), and a second peak after development in chloroform : methanol : water (75 : 22 : 3) (Fig. 63B). Before use, this batch was purified by silica gel T.L.C., in chloroform : methanol : water (75 : 22 : 3).

Lettuce fruits (var. Great Lakes) were imbibed in 10^{-5}M 2[^{14}C(±)]-ABA solution for 24 h to coincide with the time after which germination commenced. The fruits were then washed, ground and extracted for 48 h at 4°C in 50 ml of 80% methanol. The extract was filtered and the filtrate reduced to dryness by thin film evaporation under reduced pressure. The radioactivity was taken up in 80% redistilled methanol and separated on Whatman No. 1 chromatography paper by descending development in butanol : propanol : 0.88 ammonia : water (2 : 6 : 1 : 2). The radioactivity on the chromatogram was located by the use of a radiochromatogram scanner linked to a flat-bed recorder. After scanning (Fig. 64A) the chromatogram was divided into 20 equal portions between the starting line and the solvent front. The portions were placed, one in each of 20 scintillation vials and the radioactivity assayed on a liquid scintillation spectrometer (Fig. 65 A and B). After location by both assay methods, the position of the radioactivity on the chromatogram was described in terms of its Rf value (Table 47). Both assay methods indicated the presence of a radioactive compound or complex not chromatographically the same as ABA. The second compound had an Rf value of 0.359 as ascertained by scanning. The Rf value consistently obtained for ABA was 0.747.

The existence of the second compound was confirmed by developing extracts in other chromatography systems (Figs. 64 and 65) and the chromatographic purity and stability of both the unknown compound and ABA determined by eluting the radioactivity in methanol and developing them individually in 3 other TLC systems, chloroform : methanol : water (75 : 22 : 3); benzene : acetic acid (50 : 20); and butanol : acetic acid : water (5 : 1 : 2.2), with

Fig. 63. Chromatographic distribution of the radioactivity in stock solutions of ^{14}C-ABA. (A) First batch, (B) Second batch before purification, (C) Second batch after purification.

The chromatograms were developed in chloroform : methanol : water (75 : 22 : 3) and assayed on a Panax Radiochromatogram Scanner.

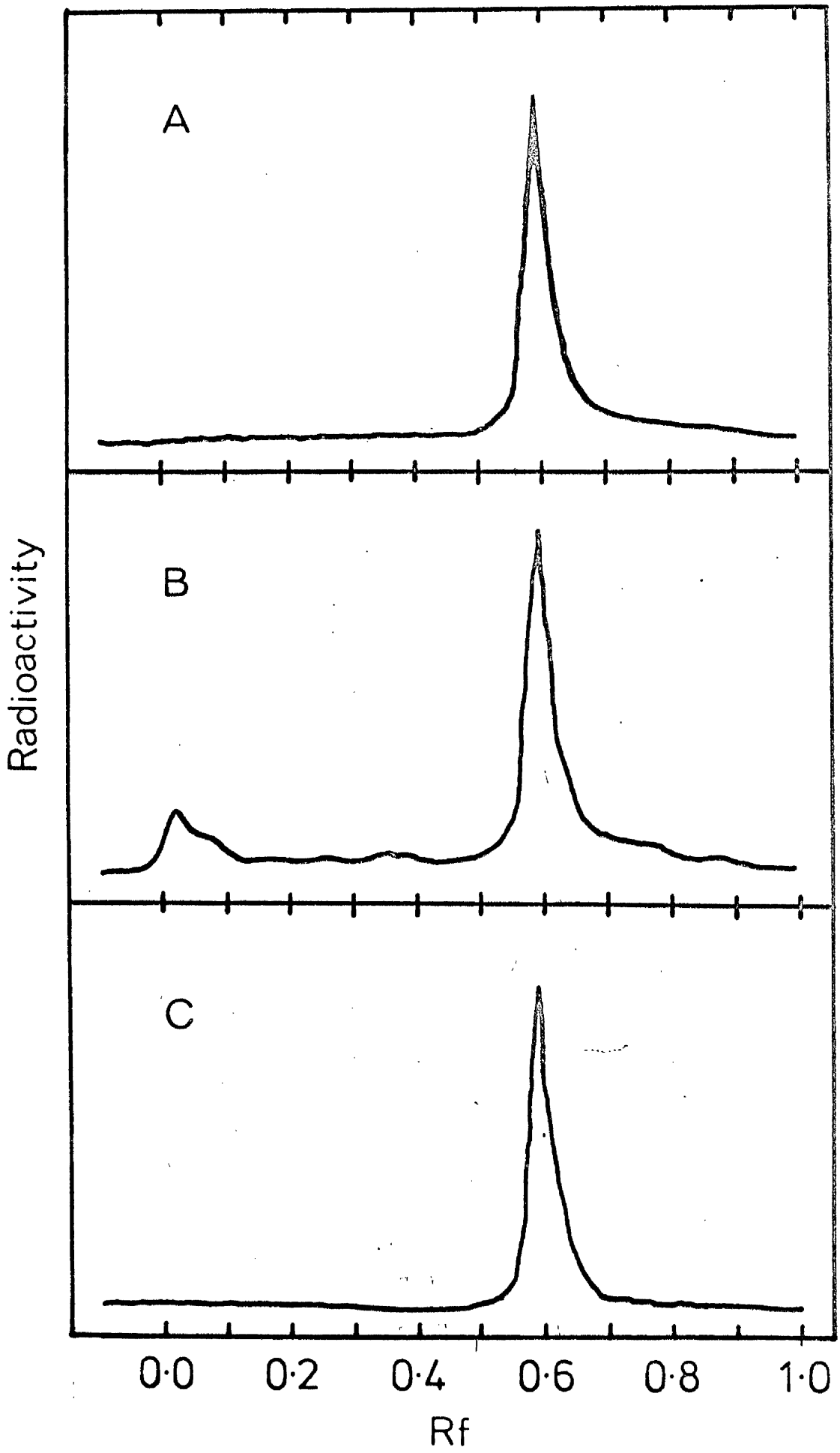

A
B
C
Radioactivity
0·0
0·2
0·4
0·6
0·8
1·0
Rf

Fig. 64. The chromatographic distribution of the radioactivity extracted from lettuce fruits after 24 h imbibition in 2-[^{14}C]-ABA. The chromatograms were assayed on a Panax Radiochromatogram Scanner after development in (A) butanol : propanol : 0.88 ammonia : water, 2 : 6 : 1 : 2; (B) chloroform : methanol : water, 75 : 22 : 3; (C) butanol : acetic acid : water, 5 : 1 : 2.2; (D) benzene : acetic acid, 50 : 20.

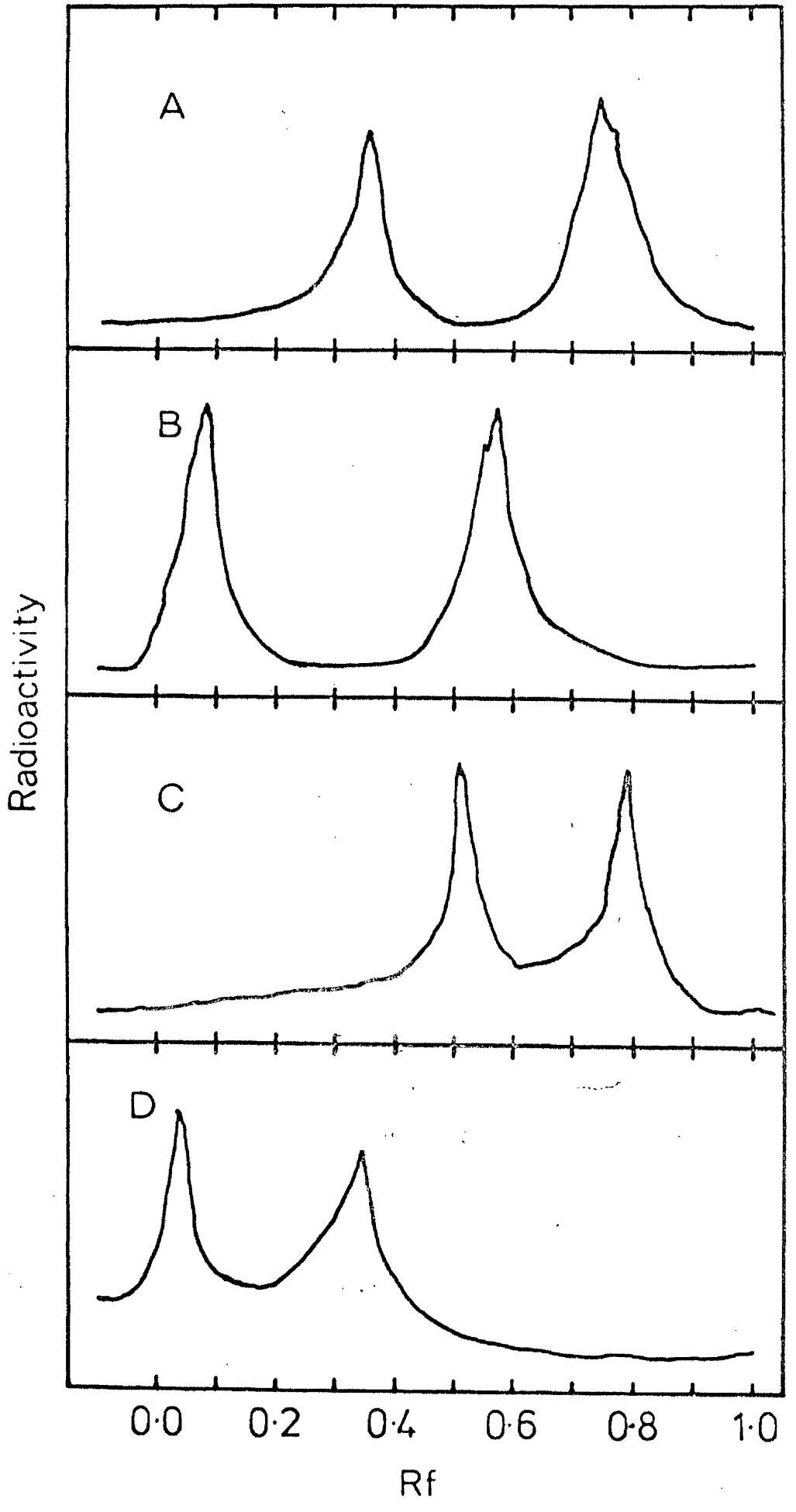

A
B
C
D
Radioactivity
0·0
0·2
0·4
0·6
0·8
1·0
Rf

Fig. 65. The chromatographic distribution of the radioactivity extracted from lettuce fruits after 24 h imbibition in 2-[^{14}C]-ABA, together with the chromatographic distribution of the radioactivity in the stock solution.

A. Stock ABA solution developed on paper in butanol : propanol : ammonia : water, 2 : 6 : 1 : 2.

B. 24 h extract developed as in (A).

C. Stock ABA solution developed on silica gel TLC in chloroform : methanol : water, 75 : 22 : 3.

D. 24 h extract developed as in (C).

E. Stock ABA solution developed on silica gel TLC in butanol : acetic acid : water, 5 : 1 : 2.2.

F. 24 h extract developed as in (E).

G. Stock ABA solution developed on silica gel TLC in benzene : acetic acid, 50 : 20.

H. 24 h extract developed as in (G).

After development, the chromatograms were divided into 20 equal portions and each assayed on a liquid scintillation spectrometer.

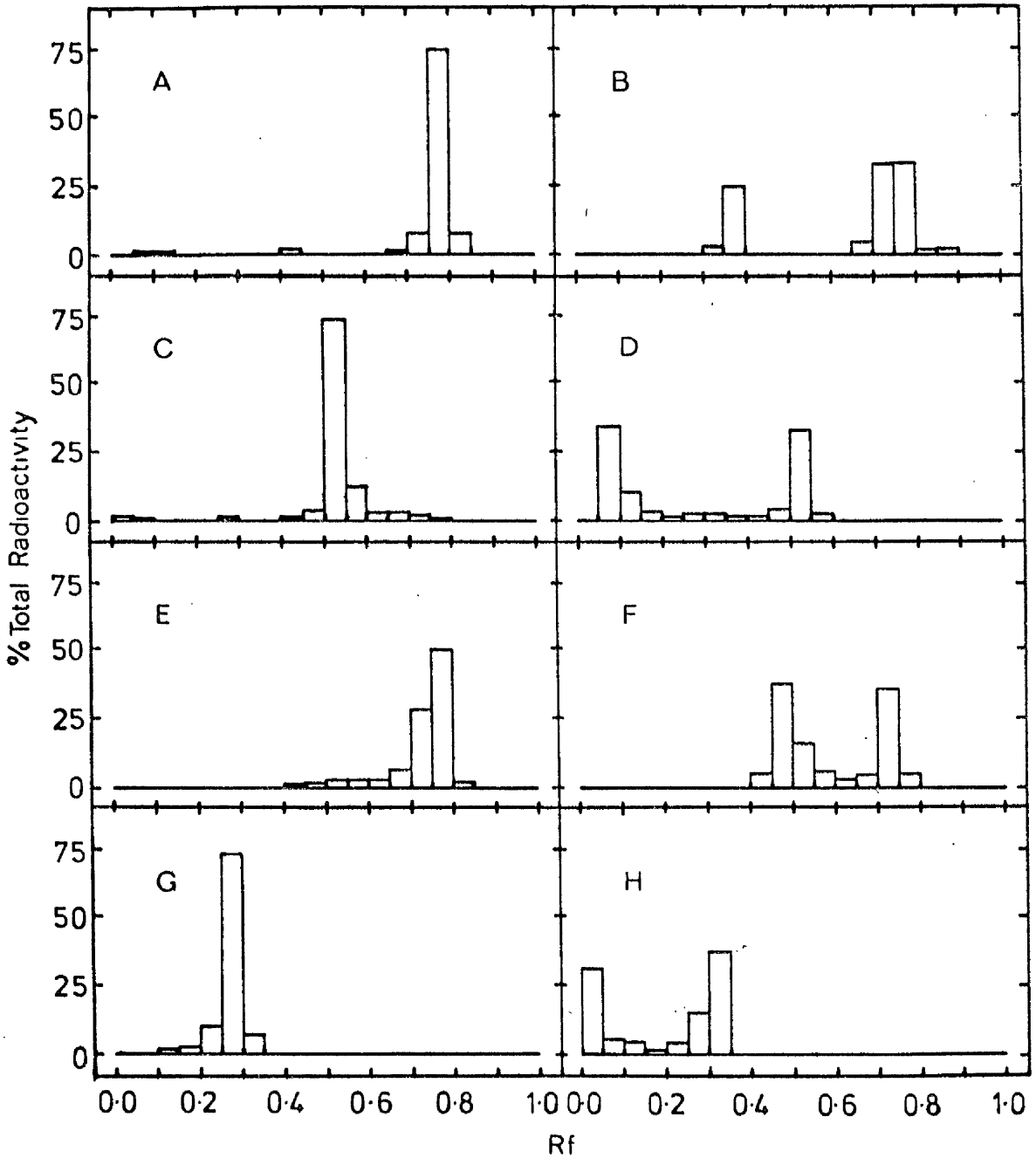

A
B
C
D
E
F
G
H
75
50
25
0
% Total Radioactivity
0·0
0·2
0·4
0·6
0·8
1·0
Rf

Table 47. Chromatographic properties of the unidentified compound and ABA.

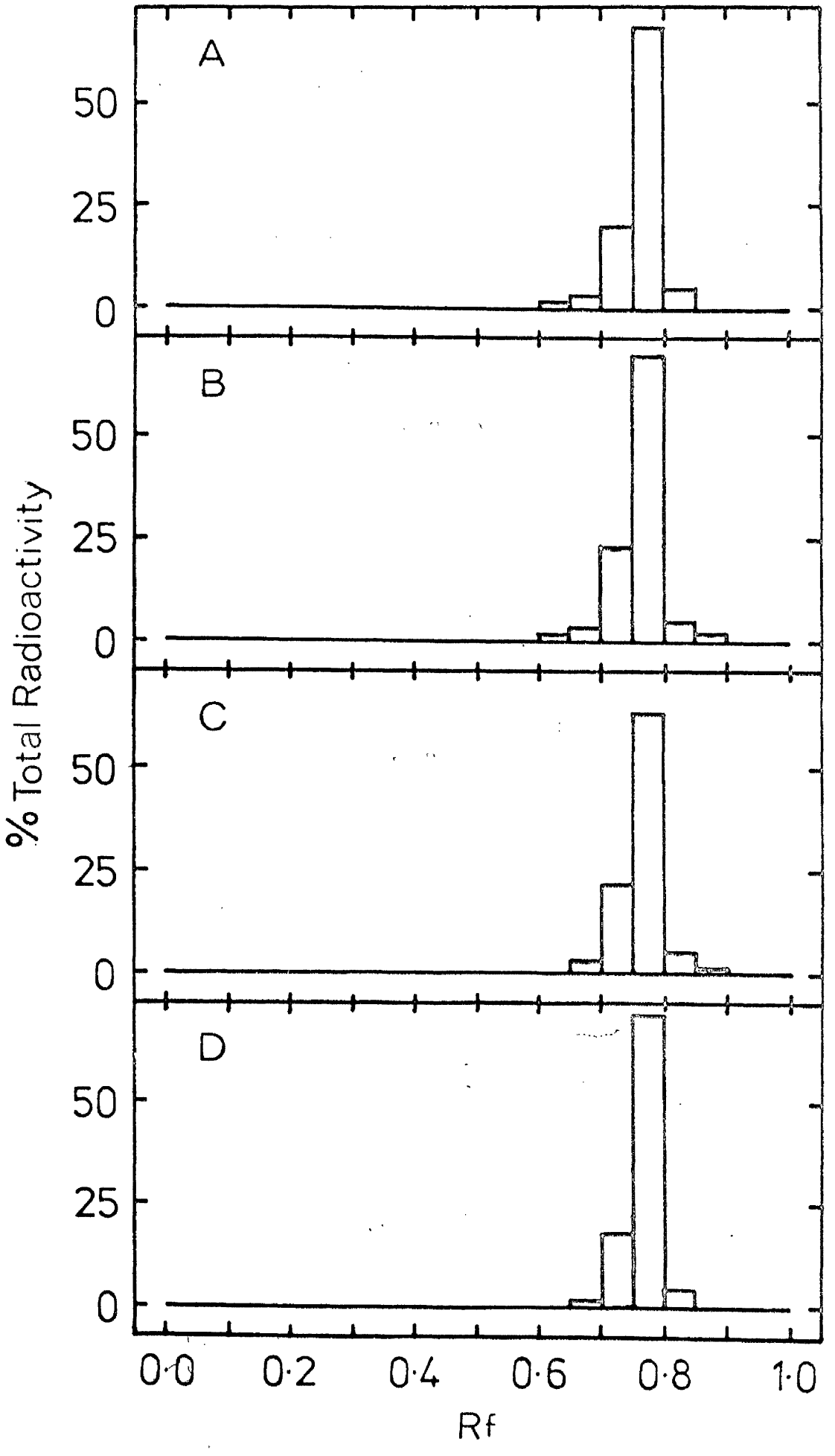
Solvent	butanol:propanol: 0.88 ammonia: water:: 2:6:1:2	chloroform: methanol: water:: 75:22:3	butanol:acetic acid:water:: 5:1:2.2	Benzene:acetic acid:: 50:20
Chromatography procedure	descending paper	T.L.C.	T.L.C.	T.L.C.
Rf of ABA	0.747 ± 0.003	0.591 ± 0.009	0.792 ± 0.005	0.347 ± 0.023
Rf of metabolite	0.359 ± 0.004	0.100 ± 0.020	0.507 ± 0.023	0.027 ± 0.044

silica gel T.L.C. (Figs. 64 and 65). After development in chloroform : methanol : water the ABA was located at Rf 0.591 and the other compound at Rf 0.100. In benzene : acetic acid and butanol : acetic acid : water, ABA moved to Rfs 0.347 and 0.792 respectively, and the unknown compound to 0.027 and 0.507 respectively (Table 47). A proportion of the ABA taken up by lettuce fruits was, therefore, metabolised after 24 h incubation in light at 25°C.

To ensure that the unknown compound did not arise independently of the plant tissue as a result of the breakdown of ABA in the incubation medium, or as a result of a chemical reaction during extraction, a number of control treatments were included. In the first, an aliquot of the medium in which the fruits were incubated was chromatographed. Inevitably there was found to be only one radioactive area on the chromatograms (Fig. 66B). A second type of control involved incubating lettuce fruits for 24 h in non-radioactive ABA, and then washing prior to extraction, during which a small quantity of radioactive ABA was added to the medium. Only one radioactive compound was found and this co-chromatographed with ABA (Fig. 66C). The final type of control involved boiling lettuce fruits until they were considered dead.

Mitochondrial activity, and hence viability, were tested with 2,3,5-

Fig. 66. Chromatographic distribution of the radioactivity after various control treatments of 2-[^{14}C]-ABA. The chromatograms were developed on paper in butanol : propanol : ammonia : water, 2 : 6 : 1 : 2. (A) Stock ABA solution, (B) Aliquot of the ^{14}C-ABA solution in which lettuce fruits were incubated for 24 h, (C) Fruits imbibed in non-radioactive ABA but radioactive ABA added during the extraction, (D) Radioactivity extracted from lettuce fruits which had been boiled prior to incubation.


A
B
C
D
50
25
0
% Total Radioactivity
0·0
0·2
0·4
0·6
0·8
1·0
Rf

triphenyltetrazolium chloride (TTC). Approximately 0.5 g of solid TTC was dissolved in 25 ml of 0.006M phosphate buffer and the boiled fruits infiltrated with this solution. The reduction of TTC to give an insoluble red formazan in active tissues is normally ascribed to activity of a number of dehydrogenases (Smith, 1952), but a lack of red colour with this treatment may be due to limitations of cofactors and substrates rather than inactivation of dehydrogenases (Steponkus and Lanphear, 1967).

The dead tissue was then dried overnight in an oven at 40°C and placed in a solution of ^{14}C-ABA for 48 h. After this time, the fruits were washed, extracted and chromatographed in the usual way, and only one radioactive compound co-chromatographing with ABA could be located (Fig. 66D). The second compound must therefore have been a result of the metabolic processes of the tissue, and hereafter will be referred to as the metabolite.

Other methods of extracting the radioactivity were also examined. When the fruits were not ground before extraction in 80% methanol, less than 4% of the radioactivity remained in the tissue, a value identical to that obtained when the tissue was ground. If, however, the tissue was extracted in absolute methanol or ethanol, a large and variable proportion of the metabolite remained unextracted. Thus, 80% methanol was the best solvent, and grinding the fruits prior to extraction was unnecessary, especially if the extraction procedure was modified such that there were two periods of 24 h in solvent. In all experiments the efficiency of extraction was, nevertheless, determined for certain treatments.

A study of the appearance of the metabolite as a function of time was next undertaken. The fruits were removed from the ^{14}C-ABA solution after pre-determined times varying from 0.5 h to 72 h. They were washed, extracted and chromatographed in butanol : propanol : ammonia : water (2 : 6 : 1 : 2). The resulting chromatograms were scanned, divided into 20 zones and assayed by liquid scintillation spectrometry (Fig. 67). Very little metabolite was present until 9 to 12 h after the beginning of imbibition. After 9 h, 3% of

Fig. 67. Chromatographic distribution of the radioactivity extracted from lettuce fruits at various times after the start of imbibition in 2-[$^{14}C(\pm)$]-ABA. The chromatograms were developed on paper in butanol : propanol : 0.88 ammonia : water, 2 : 6 : 1 : 2.

A :- stock ABA.

B :- 0.5 h after the start of imbibition.

C :- 1.0 h " " " " "

D :- 2.0 h " " " " "

E :- 4.0 h " " " " "

F :- 6.0 h " " " " "

G :- 9.0 h " " " " "

H :- 12.0 h " " " " "

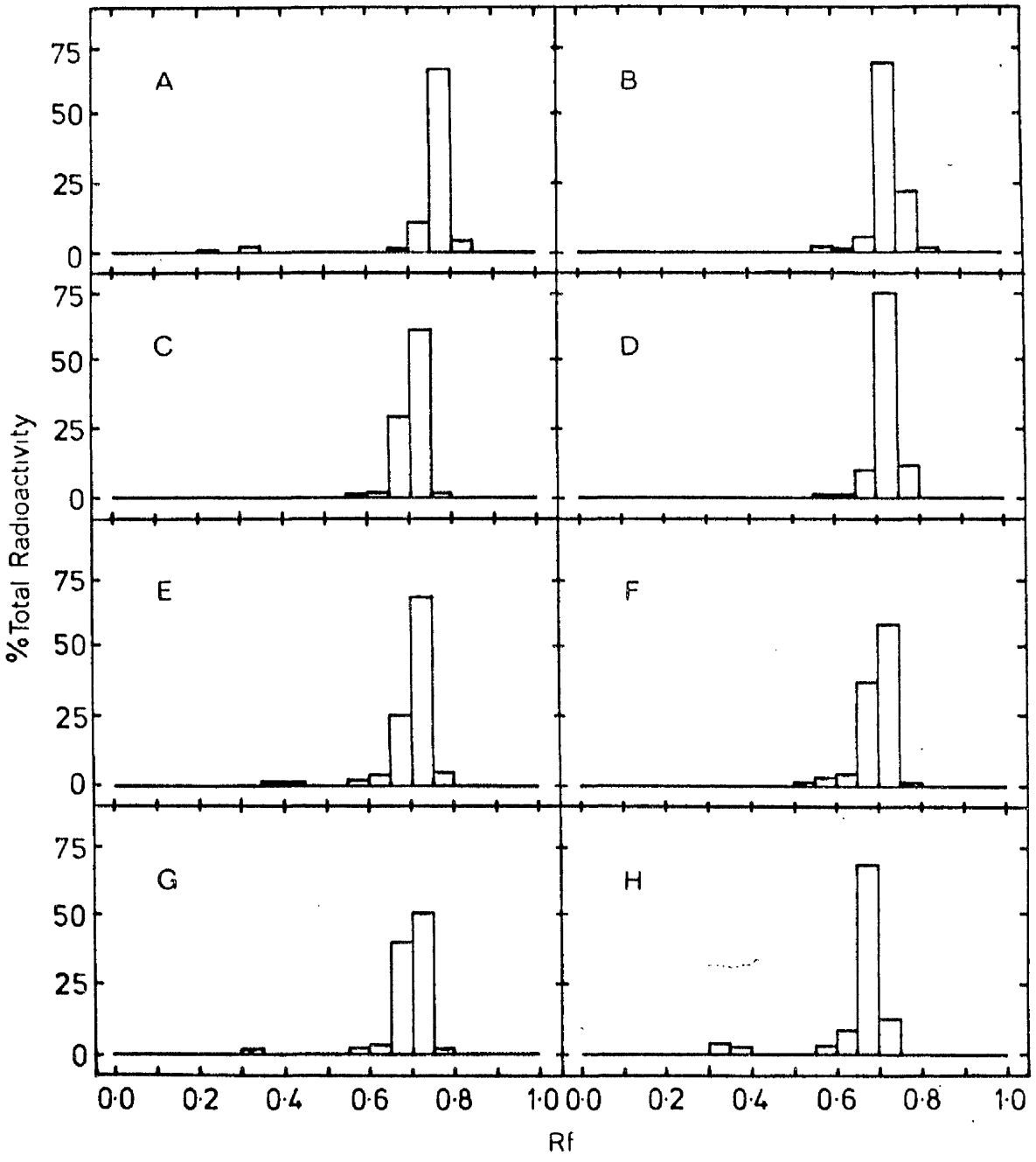

A
B
C
D
E
F
G
H
75
50
25
0
% Total Radioactivity
0·0
0·2
0·4
0·6
0·8
1·0
Rf

Fig. 67 (contd.)

I :- 18.0 h after the start of imbibition.

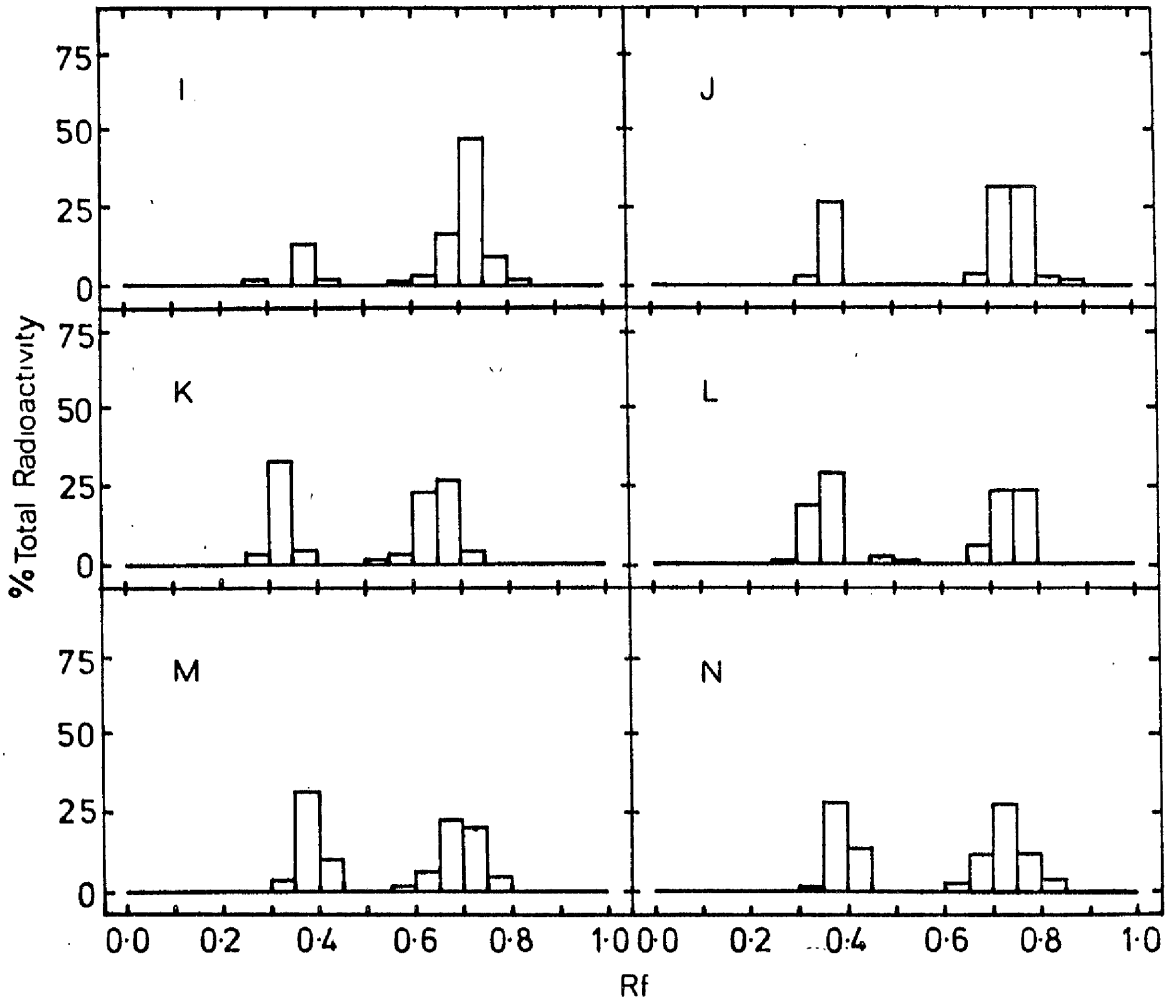
J :- 24.0 h " " " " "

K :- 36.0 h " " " " "

L :- 48.0 h " " " " "

M :- 60.0 h " " " " "

N :- 72.0 h " " " " "


I
J
K
L
M
N
75
50
25
0
% Total Radioactivity
0·0
0·2
0·4
0·6
0·8
1·0
Rf

the total radioactivity was associated with the metabolite, rising to 6% after 12 h. At this time 94% of the total radioactivity co-chromatographed with ABA, while after 18 h the value had fallen to 73%, with 26% occurring in the metabolite fraction. This trend continued until between 36 and 48 h after the start of imbibition when the quantities were approximately equal, 51% co-chromatographing with metabolite after 48 h and 48% with ABA. The balance was maintained thereafter, until the end of the experiment, 72 h after the start of imbibition.

For a more complete appreciation, it was essential that the accumulation of metabolite and the simultaneous decrease in the level of ABA be examined in relation to the total uptake of radioactivity (Fig. 68). The total uptake curve is typical of that described earlier, with imbibition occurring within the first few hours, and no further uptake taking place until the start of germination, which occurred in this experiment ater 24 h, the mean time to 50% germination being c. 30 h. By 24 h, between 25% and 35% of the ABA present had been metabolised. Germination and the second phase of rapid uptake then began, but a position was rapidly established where the ratio of metabolite to ABA remained constant at 1 : 1.

It is, therefore, likely that the length of the ABA-induced delay of germination is linked with the ability of the tissue to metabolise ABA. Attempts to purify samples of metabolite to the stage where it could be bioassayed or subjected to mass spectrometry have, so far, been unsuccessful. The metabolite would not partition into diethyl ether or petroleum ether at acid pH and although, under these conditions, it would pass into butane, so too did the large quantity of oily compounds and the two could not be separated, even by reverse phase column chromatography using a chiral derivative of Sephadex LH-20 (Anderson et al., 1973).

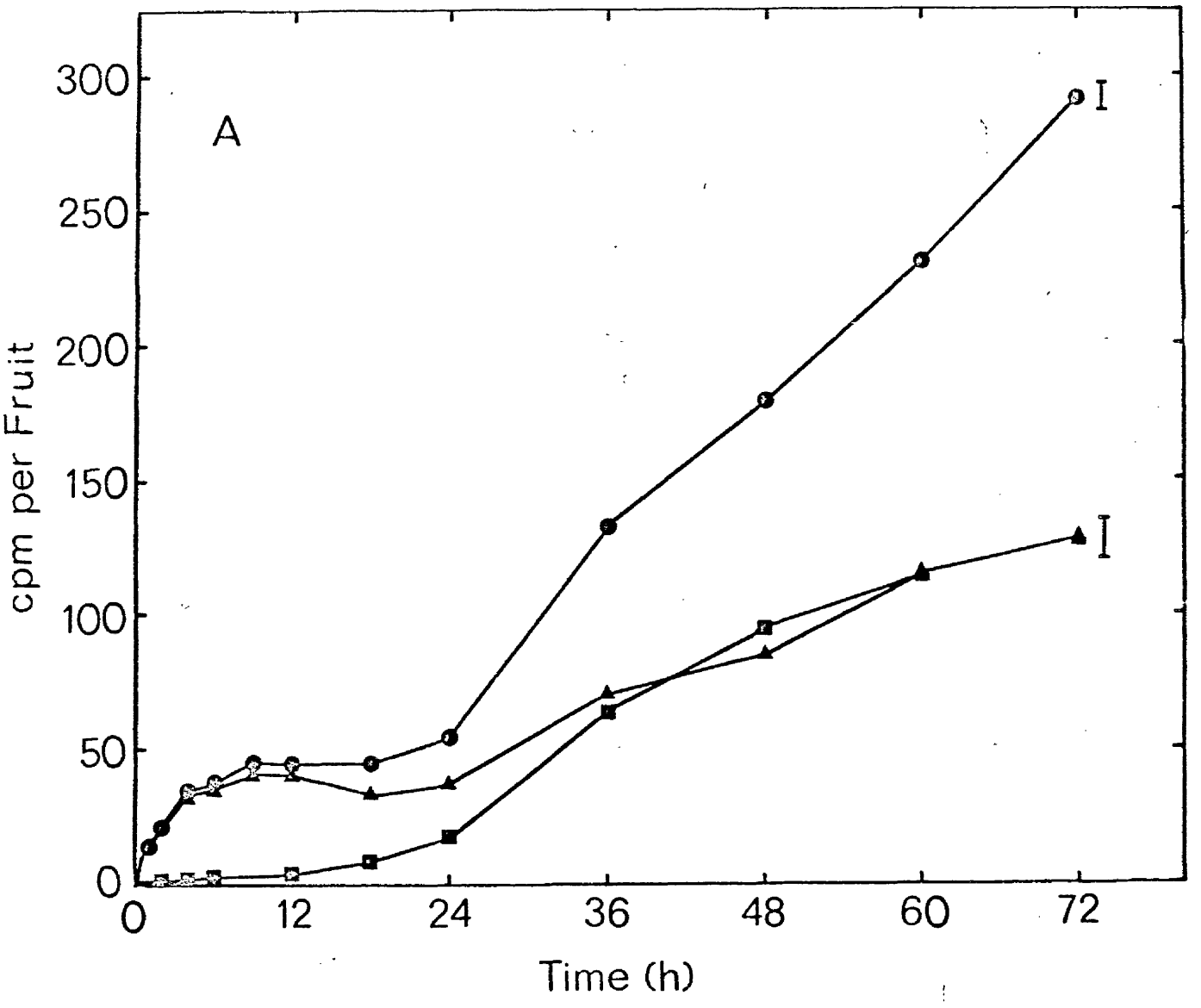
The inhibitory activity of the metabolite can, however, be deduced by an indirect method. At germination, the total amount of radioactivity taken up during imbibition was still present, and was distributed equally between ABA

Fig. 68. Time course of the uptake and metabolism of 2-[^{14}C]-ABA by lettuce fruits.

closed circles - uptake of 2-[^{14}C]-ABA.

closed triangles - extracted radioactivity co-chromatographing with ABA.

closed squares - extracted radioactivity associated with the metabolite of ABA.


A
300
250
200
150
100
50
0
cpm per Fruit
0
12
24
36
48
60
72
Time (h)
I
I

and the metabolite. If the metabolite was inhibitory to germination, the combined effect of it and the remaining ABA should have been sufficient to prevent germination, so the fact that germination did take place must be an indication that the metabolite was inactive with regard to the inhibition of germination.

(17) THE EFFECT ON GERMINATION OF TRANSFERRING FRUITS TO WATER FOLLOWING IMBIBITION IN EITHER ABA OR ITS ANALOGUES.

A pronounced feature of the action of ABA in inhibiting germination is that transference of the fruits from ABA solution to water results in rapid germination. Further information on this aspect was sought by imbibing fruits for 0, 2, 4, 6, 8, 10, 12, 18 and 24 h in 10^{-5}M ABA; they were then blotted dry and transferred to water. Radicle emergence was recorded at two-hour intervals until complete and the MTG calculated for each treatment (Fig. 69). Imbibition for less than 8 h did not affect the MTG, but imbibition for 8 h in ABA delayed the mean time to 50% germination by 1.29 h, as compared with the water controls which germinated 12.15 h after the start of imbibition. Germination therefore occurred 5.44 $\pm$ 0.17 h after transference of the lettuce fruits from ABA to water. Increasing the incubation time in ABA to 12 h reduced still further the delay between transference and germination to 3.9 $\pm$ 0.18 h. After 18 h and 24 h incubation in ABA, the figure decreased to 1.28 $\pm$ 0.14 h and 0.95 $\pm$ 0.05 h respectively. A complete time course of germination after imbibition in ABA for 24 h, with subsequent transference to water, is shown in Fig. 70, together with a control imbibed only in water. Germination in the ABA treated fruits began almost immediately after transference to water.

Incubation of lettuce fruits in ABA solution for 8 or more hours, therefore, delayed germination, even when the fruits were subsequently transferred to water. The longer the incubation in the ABA solution, the more rapidly did germination follow upon transference to water. That

Fig. 69. The effect on the mean time to 50% germination of imbibing lettuce fruits in 10^{-5}M ABA solution for various times and then transferring them to water. The time for which the fruits were maintained in ABA is shown on the horizontal axis.

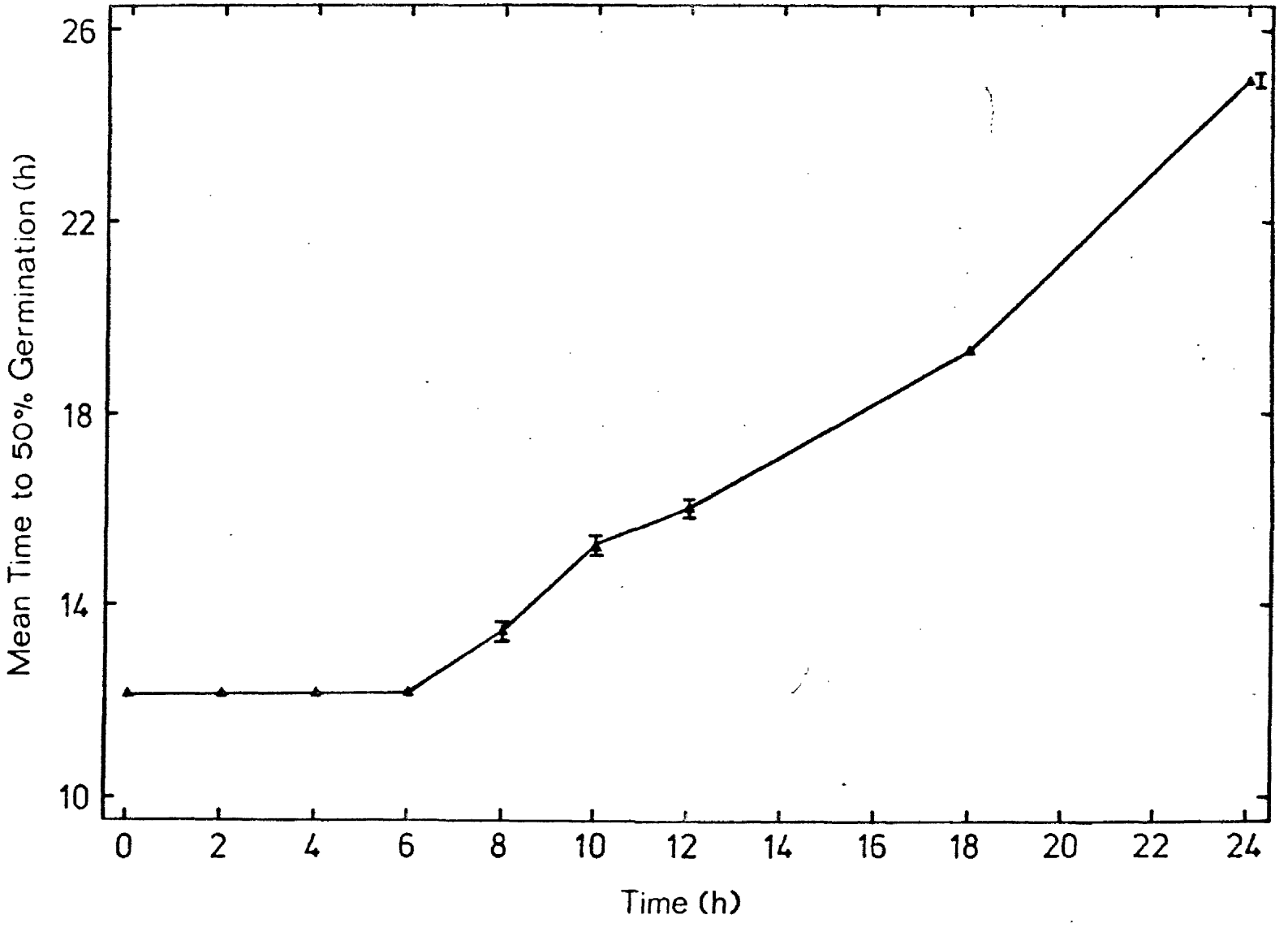
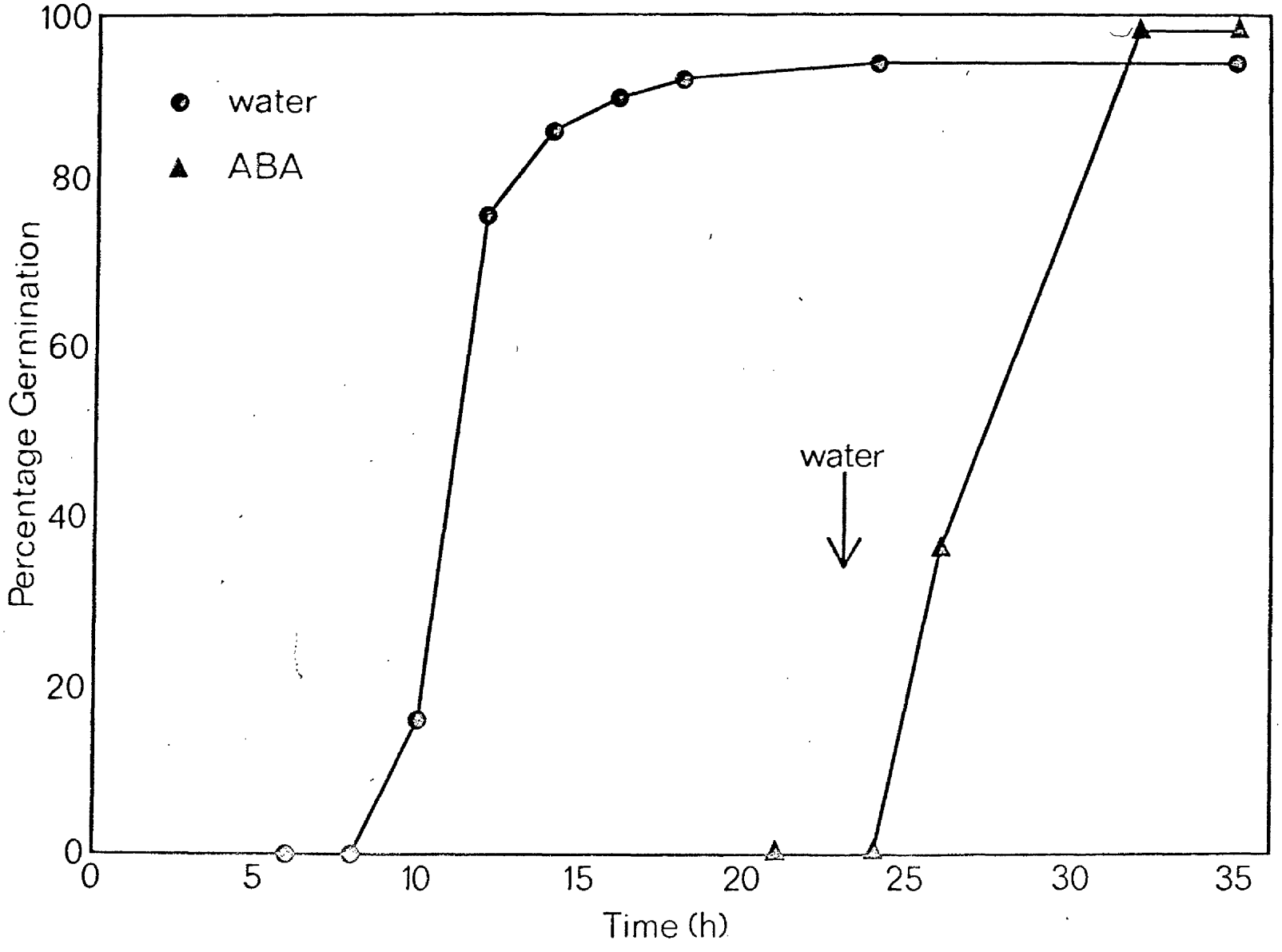

Mean Time to 50% Germination (h)
26
22
18
14
10
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)

Fig. 70. Lettuce germination as a function of time.

closed circles :- fruits incubated in water.

closed triangles:- fruits incubated in 10^{-5} M ABA solution for 24 h and then transferred to water.


100
80
60
40
20
0
Percentage Germination
water
ABA
water
0
5
10
15
20
25
30
35
Time (h)

germination was not caused by the physical process of transferring the fruits from one solution to another, is shown by the results of experiments where fruits were transferred from ABA solution to fresh solutions of ABA. The transfer was carried out at 12-h intervals, beginning after 12 h, and it was found that germination did not occur until 38.25 $\pm$ 2.59 h.

A further series of experiments was carried out to determine whether or not this effect was dependent upon the concentration of ABA in the imbibing solution. Fruits were imbibed in ABA and two of the analogues (II and XVII) most active at inhibiting lettuce germination, using concentrations of 0, 10^{-7}, 10^{-6} and 10^{-5}M. After 10 h in these solutions, the fruits were transferred to water and the MTG recorded. An incubation time of 10 h was selected because it was the shortest time after which an easily detectable effect was present. Fig. 71 and Table 48 show that the effect was concentration dependent, with XVII, the most active compound when present at 10^{-5}M, delaying germination for 95.82 $\pm$ 2.18 h after transference of the fruits to water. Compound II caused a delay of 21.55 $\pm$ 2.35 h and ABA 5.64 $\pm$ 0.34 h when present at 10^{-5}M. At lower concentrations, germination followed more rapidly on transfer to water, and solutions at 10^{-7}M exerted no significant effect. The effect was, therefore, dependent both upon the concentration of the substance in the bathing solution and its biological activity.

When the leaching of the other active analogues was studied, the concentrations tested were selected on the same basis as for the analogue/kinetin and ABA/analogue studies (Table 17). Fruits were imbibed in the solutions for 10 or 18 h before being transferred to water. The maximum imbibition period was chosen to be 18 h because some of the less active compounds, e.g. III, only delayed germination for between 18 and 24 h. The mean time to 50% germination for each treatment is described in Fig. 72. It is interesting to compare the relative inhibitory activities of the analogues at the concentrations used (Fig. 10) with the persistence of the inhibitory influence after transfer of the fruit to water. Whether or not

Fig. 71. Time required for lettuce fruits to achieve 50% germination after transference of the fruits from solutions of ABA, compounds II and XVII in which they imbibed for 10 h. The time to 50% germination is expressed as a function of concentration.

closed square :- ABA.

open circle :- II.

closed triangle :- XVII.

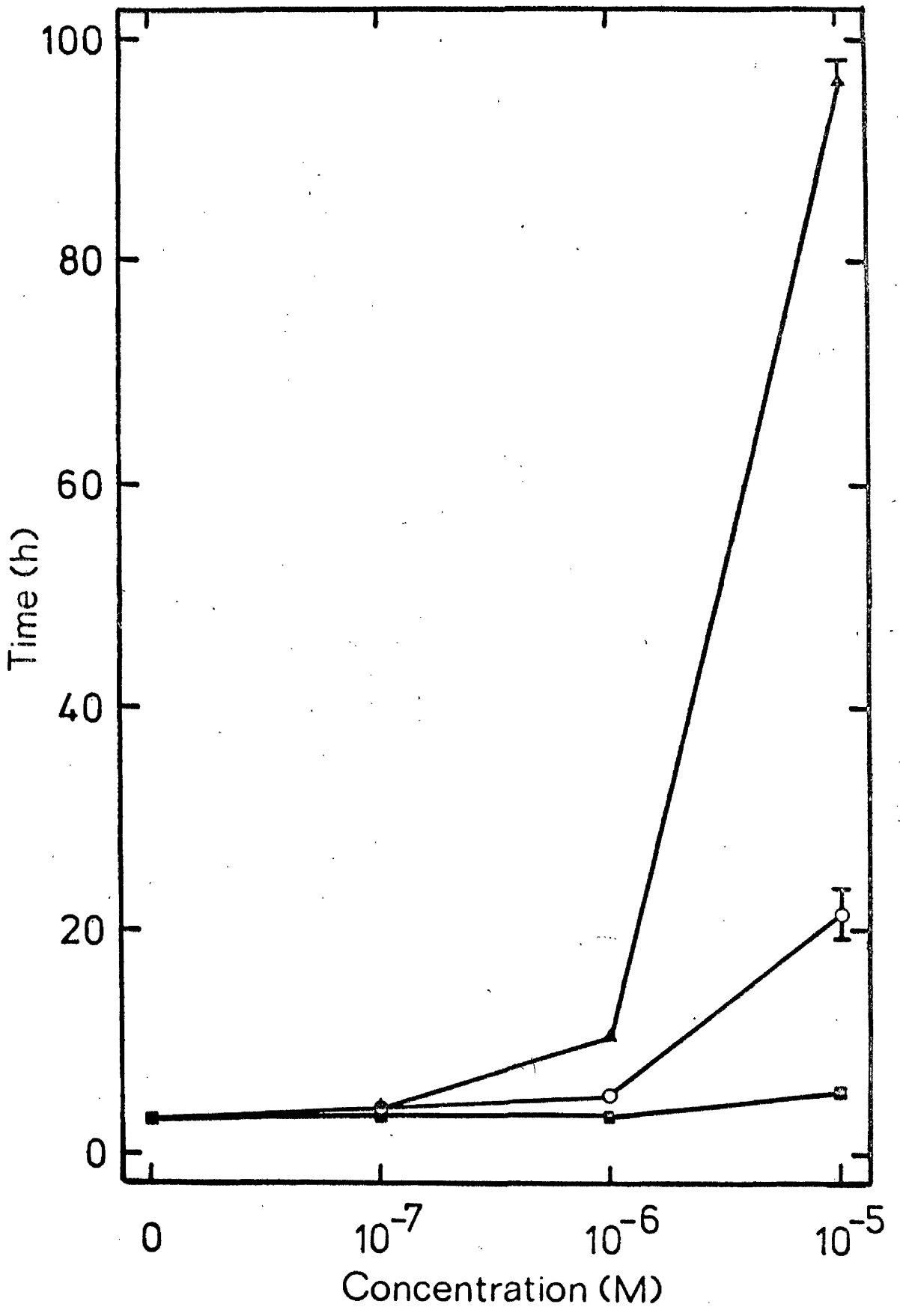

100
80
60
40
20
0
Time (h)
0
10^{-7}
10^{-6}
10^{-5}
Concentration (M)

Fig. 72. The mean time to 50% germination after transference to water of lettuce fruits previously imbibed in ABA or certain of its active analogues for (A) 10 h and (B) 18 h. ABA was assayed at 10^{-5} M and the analogues at concentrations described in Table 17.

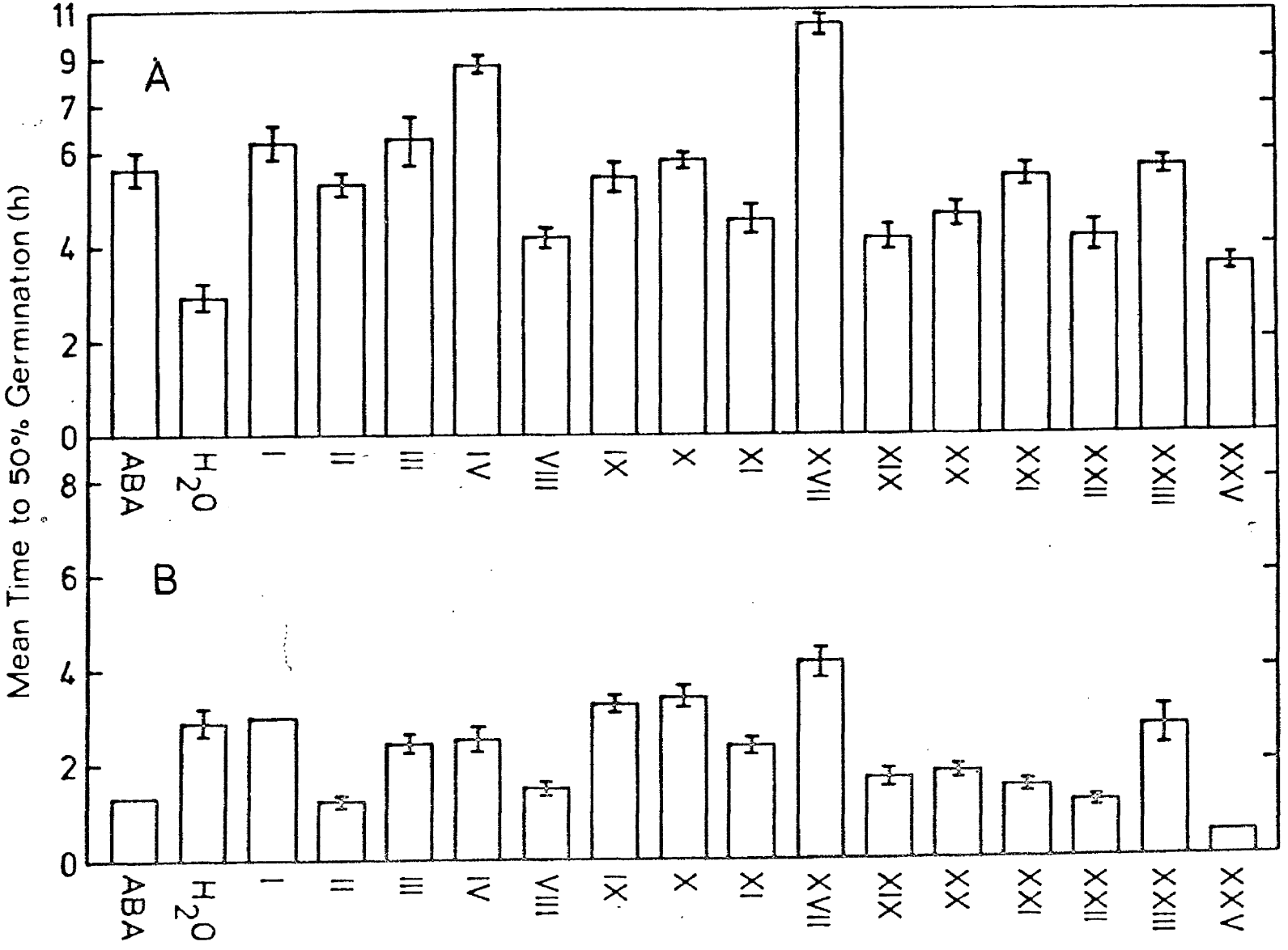

A
B
Mean Time to 50% Germination (h)
11
9
7
6
4
2
0
8
6
4
2
0
ABA
H2O
I
II
III
IV
VIII
XI
X
XI
XVII
XIX
XX
XXI
XXII
XXIII
XXV

Table 48. The MTG of lettuce fruits imbibed for 10 h in ABA, I or XVII and subsequently transferred to water.

Compound	Concentration (Molar)	Mean time to 50% germination (h)	Standard Error (±)	Significance of Inhibition ('t' tests)
Water	-	12.93	0.27	-
ABA	10^{-7}	13.24	0.35	NS
	10^{-6}	13.60	0.11	NS
	10^{-5}	15.64	0.34	**
II	10^{-7}	13.78	0.24	*
	10^{-6}	15.28	0.36	*
	10^{-5}	31.55	2.35	**
XVII	10^{-7}	13.71	0.11	*
	10^{-6}	20.44	0.65	***
	10^{-5}	105.82	2.18	***

the imbibition time in the analogue solutions was 10 or 18 h, XVII exhibited the most persistent inhibitory effect, and III the least. This finding was in keeping with their ability to delay germination, but the compounds of intermediate activity did not show such a rigid hierarchy of persistence effects. Indeed, their relative activities were different in the experiments involving an incubation period of 10 h and those in which the incubation period was 18 h. The variation was such that no simple pattern could be detected, and may indicate differences in, for example, solubility or metabolic rate, which were not determined. It is, however, clear that the inhibitory influence of all the analogues was relatively easily overcome by transferring the fruits to water, although whether this was due to a leaching or a metabolic process is uncertain.

(18) LEACHING OF RADIOACTIVITY FROM LETTUCE FRUITS PREVIOUSLY IMBIBED IN 2[^{14}C(±)] ABA.

In an attempt to ascertain whether the germination of lettuce fruits brought about by transference from ABA to water was the result of a leaching or a metabolic process, fruits were imbibed in 10^{-5}M ^{14}C-ABA solution for 6 h or 24 h. These imbibition times were chosen because, after 6 h, no metabolite of ABA had begun to appear, while after 24 h, a considerable proportion of the radioactivity was in the form of metabolite, indicating that the metabolic pathway was operating freely. The fruits were blotted dry and transferred to water for predetermined times, after which the radioactivity remaining in the tissues was assayed (Fig. 73).

A marked reduction in the level of radioactivity in the tissues occurred in both treatments during the first 2 h, thereafter remaining almost constant. When the fruits were imbibed in ABA for 24 h, the quantity of radioactivity in the tissues was about 50% more than where imbibition was for only 6 h. After leaching, however, the difference between the treatments was much more marked, being more than 100% greater in the fruits which had been imbibed for 24 h in the radioactive ABA solution. The radioactivity was, therefore, being retained within the tissues. To clarify this point, the form of the radioactivity was studied.

When fruits were imbibed in ABA solution for 6 h and extracted before leaching, it was found that all the radioactivity co-chromatographed with ABA (Fig. 74A), while in the fruits imbibed in the radioactive solution for 24 h, c. 30% of the radioactivity was associated with the metabolite (Fig. 74B). After the fruits had been incubated in water for up to 12 h, the water in which they were bathed was reduced to dryness and chromatographed. The radioactivity leached from the 6 h treated fruits co-chromatographed with ABA, as did that leached from the 24 h treated fruits, even at the longest leaching times (Fig. 74C). Extraction and chromatography of the radioactivity

Fig. 73. Leaching of radioactivity from lettuce fruits previously imbibed in 2-[^{14}C]-ABA.

closed triangles - fruits imbibed in 2-[^{14}C]-ABA for 6 h before transference to water.

closed circles - fruits imbibed in radioactive ABA for 24 h before transference to water.

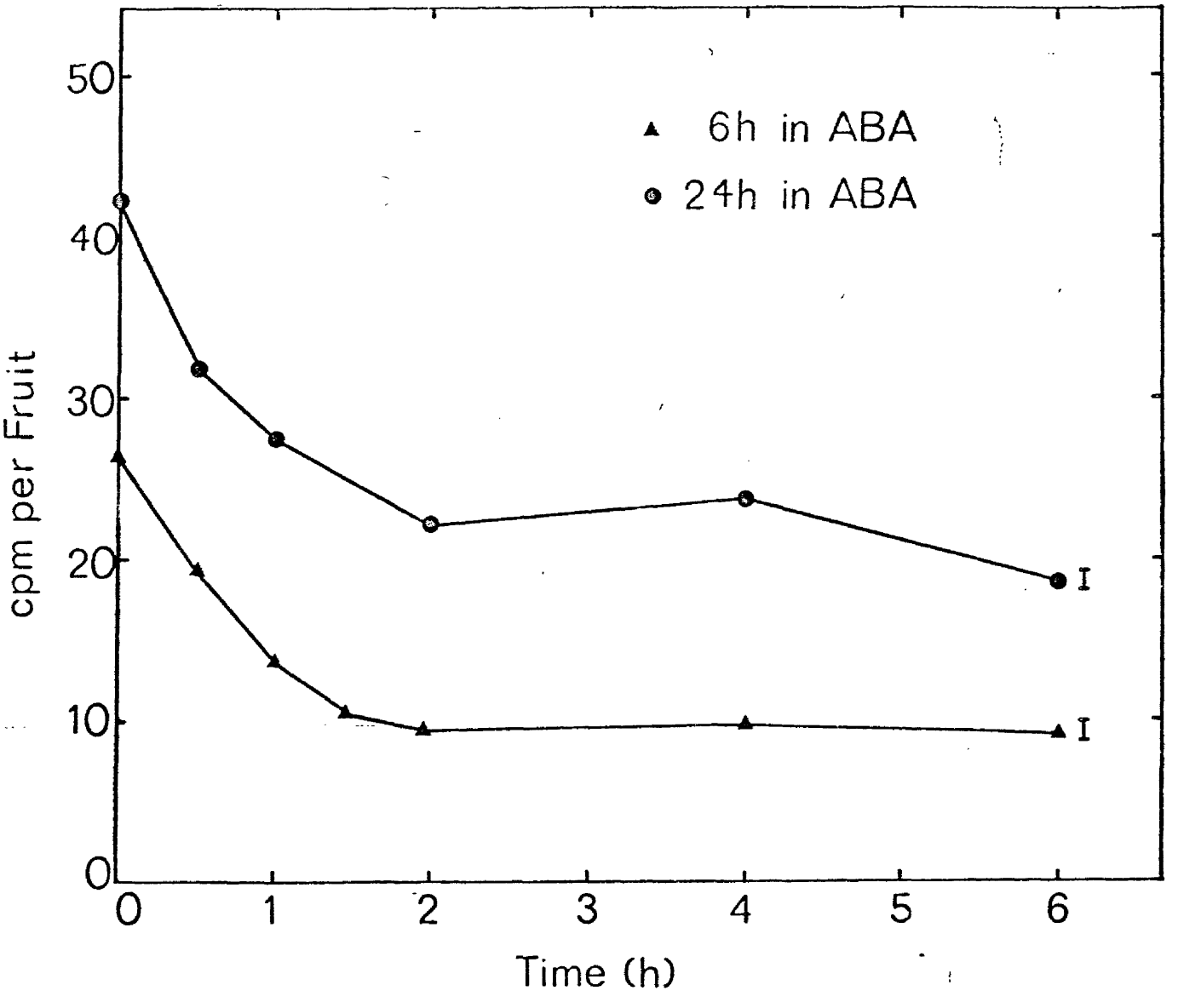
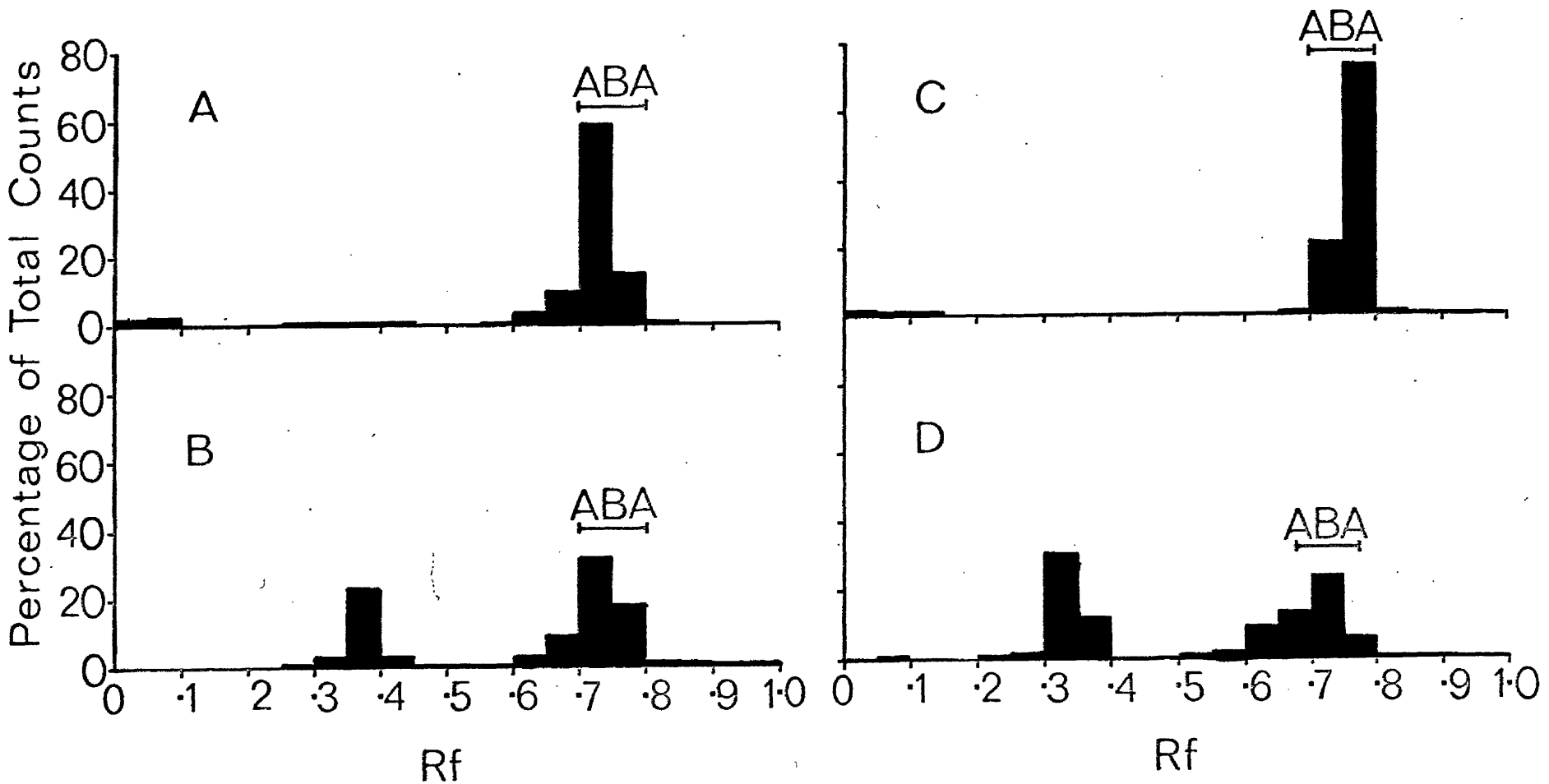

6h in ABA
24h in ABA
cpm per Fruit
Time (h)
0
10
20
30
40
50
0
1
2
3
4
5
6
I
I

Fig. 74. Chromatographic distribution of radioactivity from extracts of lettuce fruits incubated in radioactive ABA. The chromatograms were developed on paper in butanol : propanol : 0.88 ammonia : water, 2 : 6 : 1 : 2. (A) Fruits ground and extracted in 80% methanol after 6 h imbibition in [^{14}C]-ABA. (B) Fruits ground and extracted in 80% methanol after 24 h imbibition in [^{14}C]-ABA. (C) Radioactivity leached during 6 h in water from fruits previously imbibed for 24 h in [^{14}C]-ABA. (D) Radioactivity remaining within the fruits after treatment as outlined in (C).


Percentage of Total Counts
80
60
40
20
0
A
ABA
B
ABA
C
ABA
D
ABA
0
·1
2
·3
·4
·5
·6
·7
·8
·9
1·0
Rf
Rf

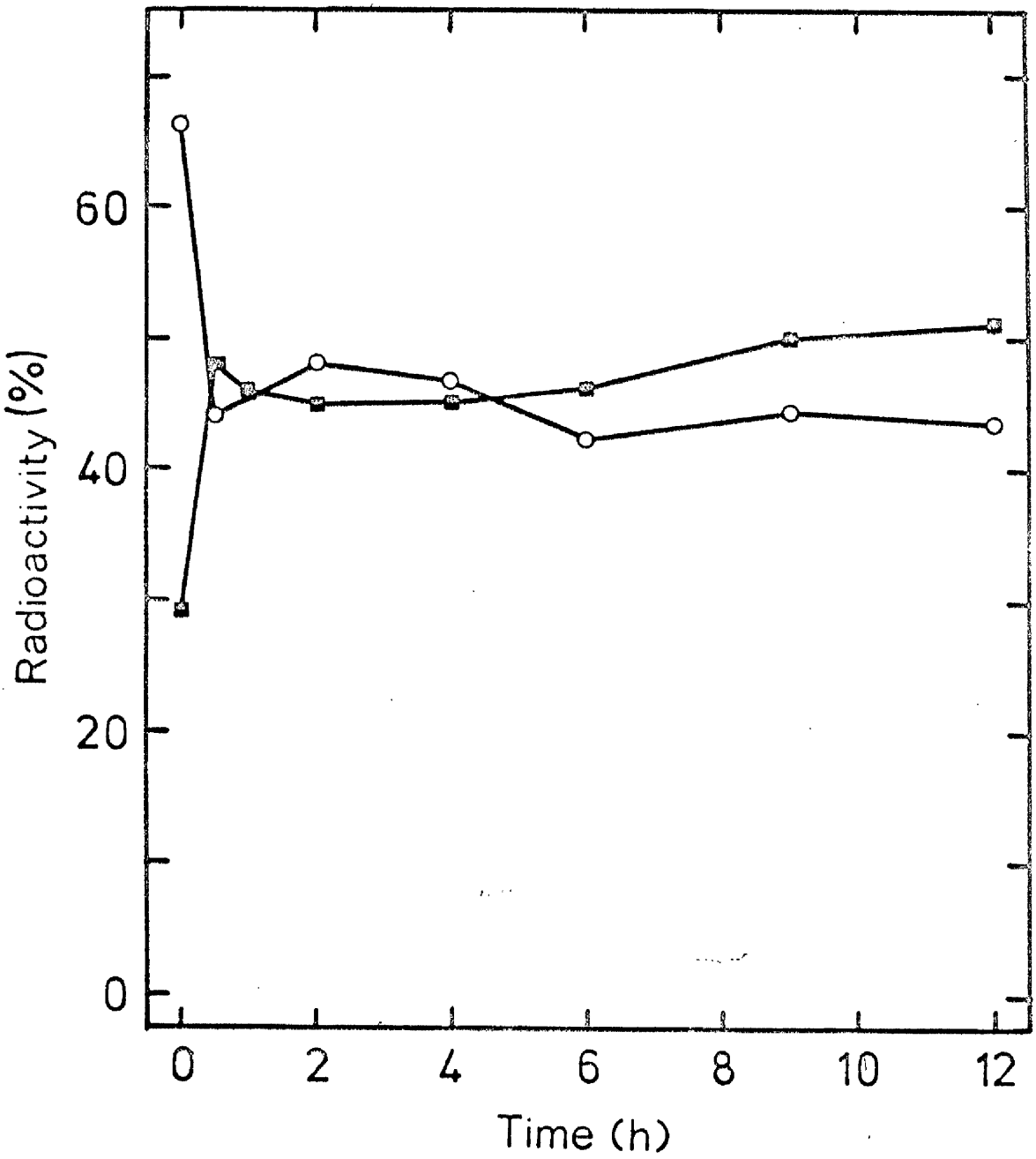
remaining within the 24 h treated fruits (Fig. 74D) indicated that there was an increased proportion of metabolite at the expense of ABA which was being leached from the tissues. Fig. 75 indicates how this change occurred during leaching. At the same time as the total radioactive content was decreasing so was the percentage of radioactivity associated with ABA which remained in the tissues. This latter figure fell from 66% to between 45 and 50%, while the percentage of the total label associated with the metabolite rose from 29% to between 45 and 50%. Moreover, there was no evidence that the absolute quantity of metabolite present increased, i.e. no evidence of continuing metabolism during leaching. The relative proportions of ABA and metabolite again equilibrated within the first few hours to approximately 1 : 1 and, although the leaching treatment was continued for 12 h, did not change significantly. The ABA still within the tissues therefore appears to be unaffected either by metabolic processes or leaching treatments.

(19) EXTRACTION OF ENDOGENOUS ABA FROM FRUITS OF LACTUCA SATIVA L.

Exogenously applied ABA delays germination of lettuce fruits. The structural components of the ABA molecule for inhibitory activity in this system have been examined, as have the patterns of uptake into the tissues, distribution within the tissues and metabolism by the tissues. Although ABA has been found to occur naturally in many propagules, its presence in lettuce fruits has not been reported. Attempts were therefore made to determine its presence in lettuce fruits which, if shown, could indicate a possible regulatory rôle.

Lettuce fruits of the varieties Great Lakes and Arctic King were extracted in 500 g batches and fractions containing ABA-like molecules were purified. Unimbibed fruits of both varieties were extracted, as were fruits of Great Lakes after imbibition for 24 h in water and the water in which they were imbibed. After 24 h imbibition, germination was complete and extension

Fig. 75. Changes in the percentage of total radioactivity associated with ABA (open circles) and the metabolite (closed squares) occurring during leaching. Before transference to water, the fruits were imbibed for 24 h in [^{14}C]-ABA.


Radioactivity (%)
60
40
20
0
0
2
4
6
8
10
12
Time (h)

of the embryonic axis well under way. They were extracted, partitioned and chromatographed, normally by TLC in hexane : ethyl acetate (1 : 1) before and after methylation by excess diazomethane. The circular dichroism curves for the solutions were then determined (Fig. 76).

Those curves, which express ellipticity in degrees as a function of wavelength, were found to show positive peaks at 262 nm, negative peaks at 230 nm and, in the most concentrated solutions, some evidence of a further negative peak at 320 nm. These characteristics agree with those reported by Milborrow (1967) for ABA. The ratio of the ellipticity at 262 nm (ψ 262) to the ellipticity at 230 nm was between 1.35 and 1.5, compared with 1.2 reported by Milborrow. There was, however, considerable variability in the determinations at 230 nm which could easily account for this difference.

The extracts were also subjected to GLC on an OV 210 column and the chromatographs showed the presence, in all cases, of a peak with a retention time relative to C-28 of 1.138 (Fig.-77 A and B). This value is identical with the retention time obtained when the stock solution of cis,trans ABA was chromatographed (Fig. 77C).

There was thus, in all the extracts of lettuce fruits, a compound with the chemical and physical properties of (+) cis,trans ABA as displayed in partitioning, TLC, GLC and circular dichroism (CD).

The ext racts were also subjected to GC-MS on an AEI MS30 single beam instrument. A sample was firstly chromatographed on 1% OV-210 and the peak corresponding to the methyl ester of ABA was diverted to the mass spectrometer via a silicone membrane separator. A trace showing peaks at mass to charge ratios of 190, 162, 134 and 125 were obtained in the correct relative abundance (Jenkins and Shepherd, 1972).

These results show that ABA exists naturally in lettuce fruits vars. Great Lakes and Arctic King.

Fig. 76. Circular dichroism curves of (a) methylated extract of 500 g of lettuce fruits var. Great Lakes; (b) methylated extract of 500 g of lettuce fruits var. Arctic King; (c) solvent base line.

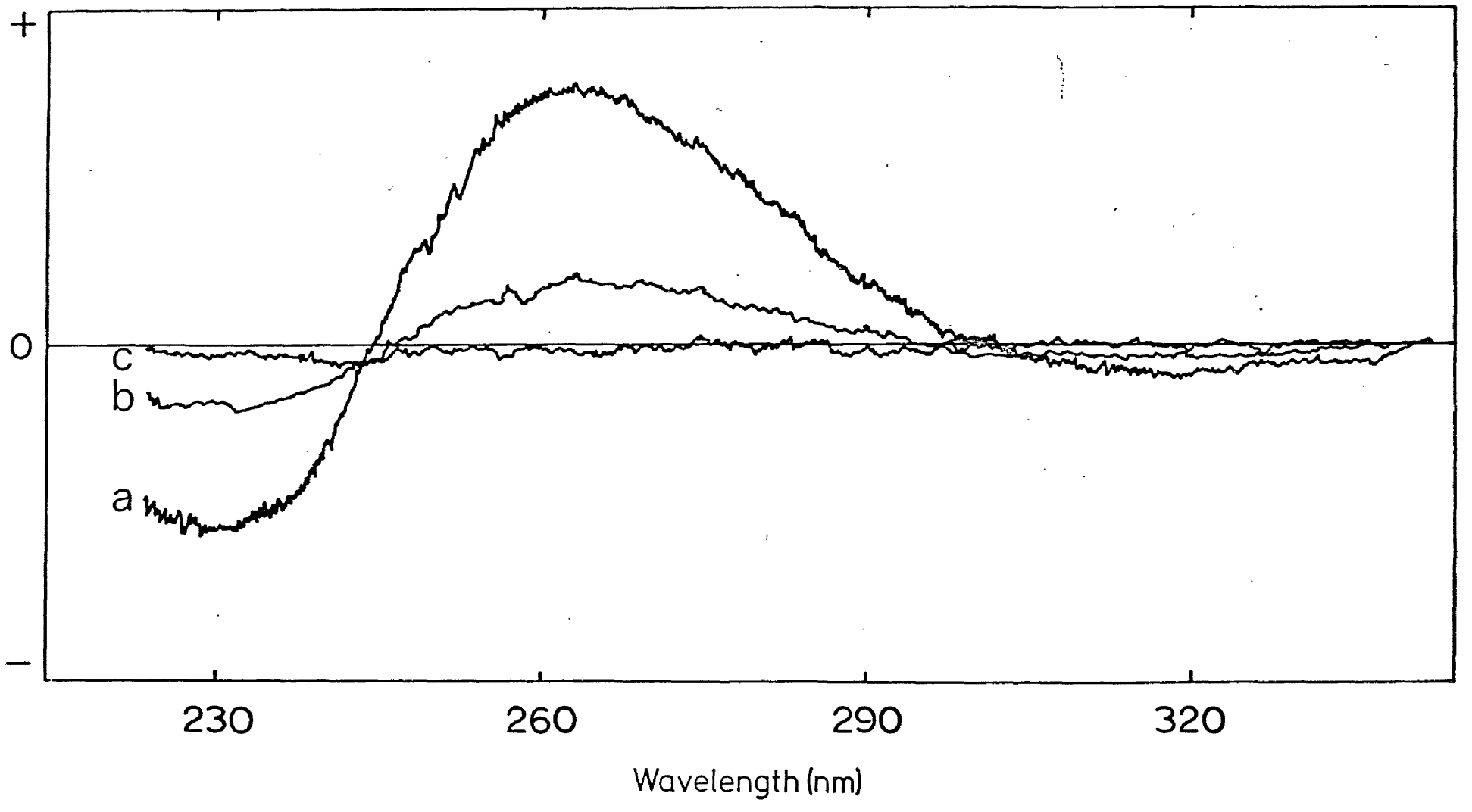
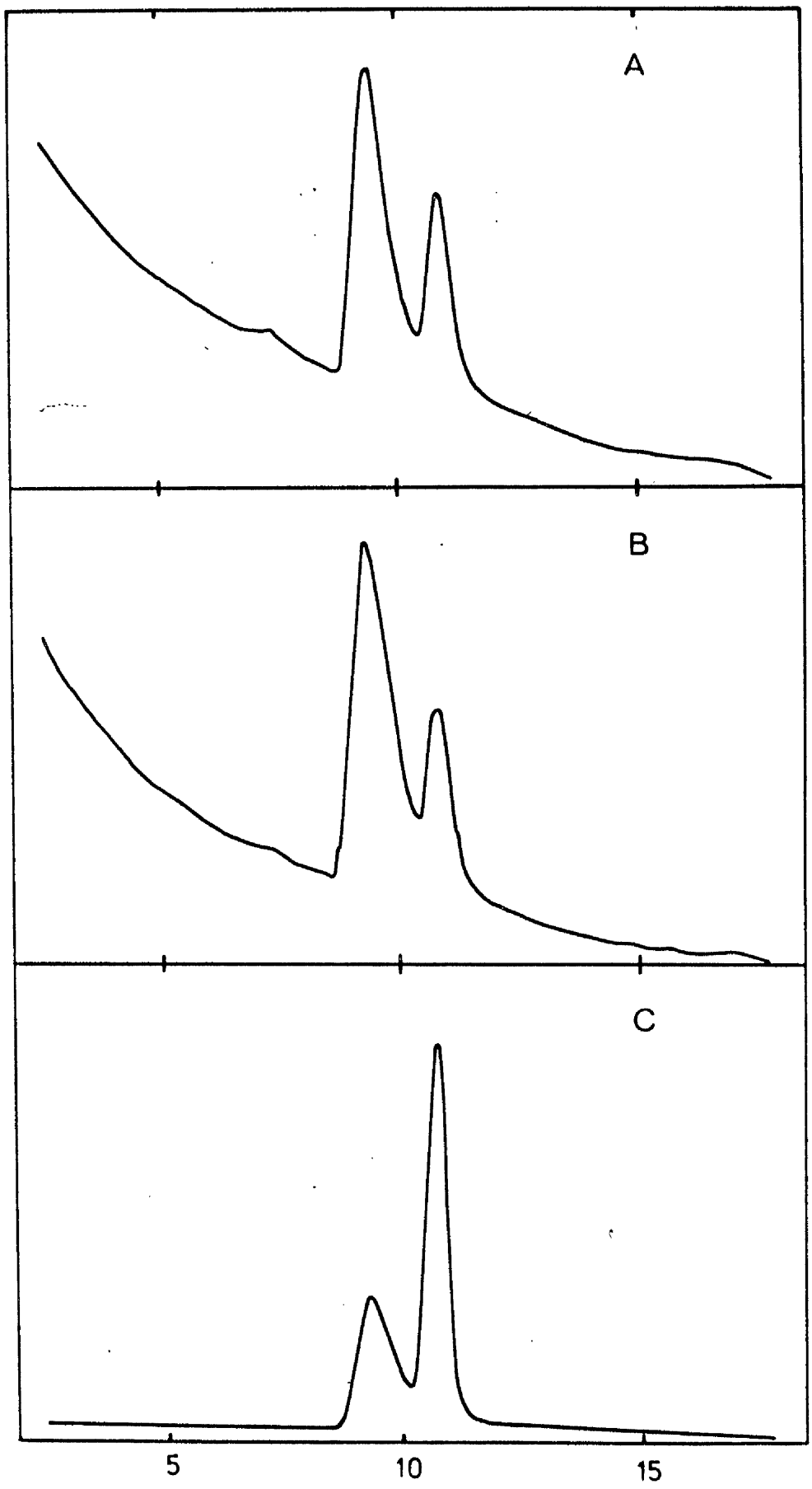

+
0
−
c
b
a
230
260
290
320
Wavelength (nm)

Fig. 77. Gas chromatograms of methylated sample of (A) purified extract from lettuce fruits var. Great Lakes; (B) purified extract of lettuce fruits var. Arctic King; (C) stock solution of cis, trans-ABA.

ABA had a retention time relative to C-28 of 1.138.


A
B
C
5
10
15
Retention Time(mins)

Quantification of Endogenous ABA Levels.

In order to determine the quantity of ABA present in the tissues and the bathing solution, two values had to be determined. It was necessary to determine the efficiency with which the ABA was extracted and purified, as well as the quantity of endogenous ABA present in the extracts after purification.

The efficiency of extraction was determined by including a known quantity of 2[$^{14}C(\pm)$]-ABA in half the extraction procedures. The radioactivity could be monitored throughout and the amount still present after purification determined. Although synthetic ABA would interfere with the GLC of the extracts, it would not affect the CD curves, because it was a racemic mixture. For this reason, the endogenous ABA levels in the purified extracts were calculated from the CD curves.

The concentrations were calculated using the data supplied by Milborrow (1967) and the measurements of ψ 262 (Table 49). Unimbibed fruits of Great Lakes were found to contain approximately four times the quantity of the ABA-like substance than the variety Arctic King. After germination, the levels present in Great Lakes had, however, fallen, but were still appreciable. The medium in which the fruits were germinated also contained ABA, in almost the same concentration as in the extract of the fruits. The loss of ABA from the fruits could not, however, be accounted for by this quantity present in the incubation medium; metabolism must therefore have occurred.

It must be stressed that the assumptions made in this estimation are that the losses of exogenously applied radioactive ABA reflect the losses of endogenous hormone during extraction, and that there is no enzymatic reaction during extraction and purification.

Table 49. Quantity of endogenous ABA in lettuce fruits and the extraction efficiency.

A : 500 g of lettuce fruits var. Great Lakes extracted before imbibition.
B : 500 g of lettuce fruits var. Great Lakes extracted before imbibition.
C : 500 g of lettuce fruits var. Arctic King extracted before imbibition.
D : 500 g of lettuce fruits var. Arctic King extracted before imbibition.
E : 500 g of lettuce fruits var. Great Lakes extracted after imbibition in water for 24 h.
F : water in which "E" incubated.

	Concentration in purified extract ($\times 10^{-5}$M)	Efficiency of Extraction (%)	Quantity of ABA mg/kg fresh weight
A	36.80 $\pm$ 1.22	55.6	0.70
B	30.08 $\pm$ 1.22	50.8	0.63
C	9.20 $\pm$ 1.53	49.1	0.20
D	5.52 $\pm$ 2.46	43.5	0.13
E	1.07 $\pm$ 0.54	51.2	0.022
F	0.92 $\pm$ 0.27	48.7	0.020*

* The weight of tissue was taken as 500 g, since this was the weight which had been incubated in the solution.

(20) ESTIMATION OF NON-RACEMIC ABA IN LETTUCE FRUITS FOLLOWING IMBIBITION IN SYNTHETIC ($\pm$) ABA.

Germination of lettuce fruits imbibed in ABA occurred when the ABA content had been reduced either by metabolism or leaching. Approximately 50% of the imbibed ABA, however, remained intact within the tissues. The reason for this may relate to the synthetic ABA being a racemic mixture of which only the (+) component was biologically active, the tissue being either incapable of metabolising or unresponsive to the unnatural ($\pm$) enantiomorph.

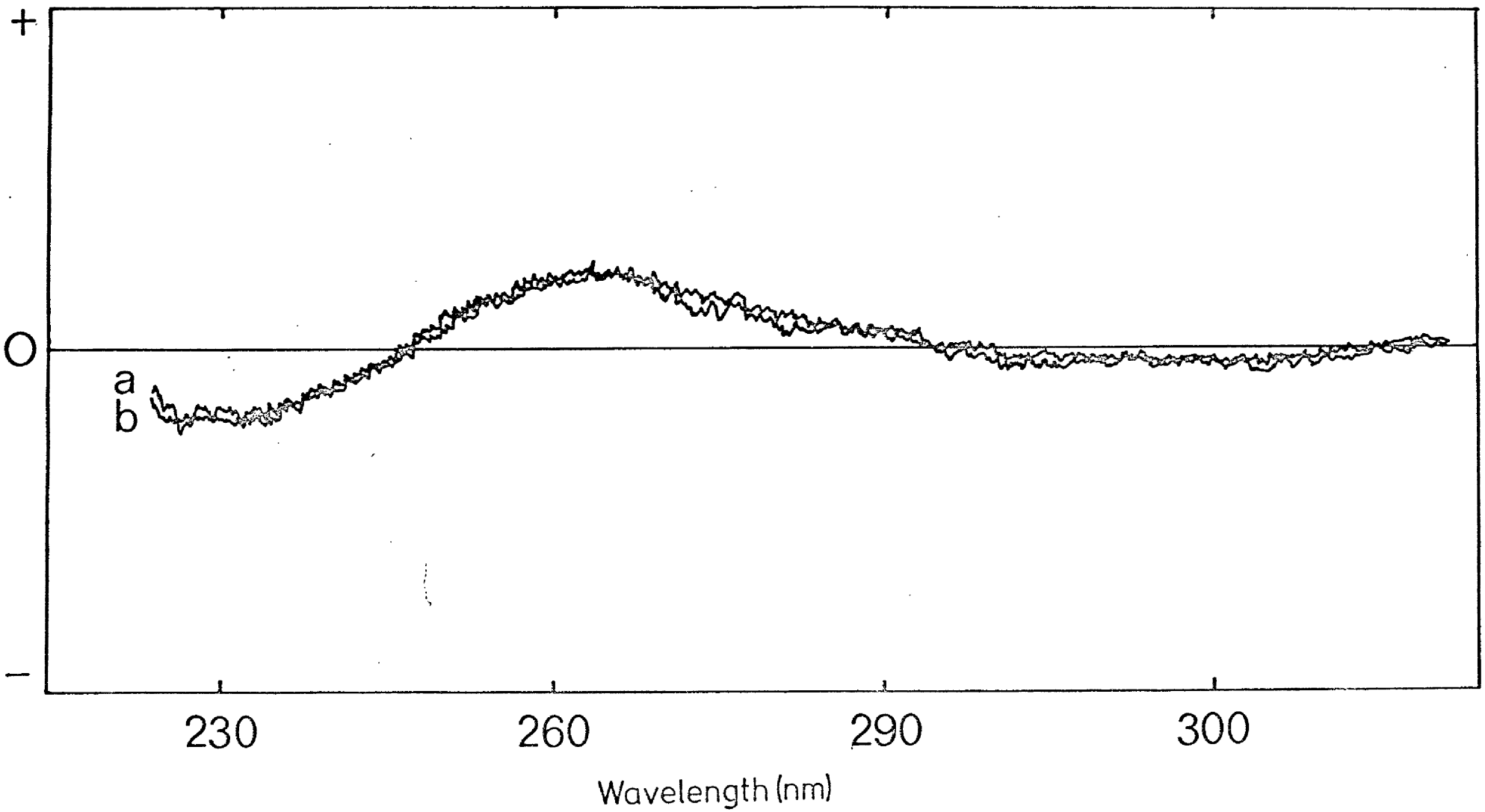
To investigate this possibility, 200 g of lettuce fruits (var. Great Lakes) were imbibed for 24 h in 10^{-5}M (±) ABA, after which they were washed in a Büchner funnel and extracted for three separate 24 h periods in 1.5 l of 80% methanol. The extract was then purified, as described for endogenous ABA samples, viz. TLC in chloroform : methanol : water (75 : 22 2.3), benzene : acetic acid (50 : 20) and butanol : propanol : 0.88 ammonia : water (2 : 6 : 1 : 2), and the circular dichroism curve measured on a spectropolarimeter (Fig. 78). The curve was found to have a positive maximum at 262 nm and a negative maximum at 230 nm. Similar peaks, and with similar amplitudes, were found in the control extract of 200 g of lettuce fruits not previously imbibed in ABA, but were not found in the methanol baseline (Fig. 78). The peaks are characteristic of (+)ABA, and there was no evidence of a preponderance of the (1) enantiomer in the extract of fruits imbibed for 24 h in ABA. The ABA remaining after metabolism does not therefore appear to have been the (±) component of the applied racemic mixture.

(21) THE EFFECT OF ABA ON GROWTH OVER SHORT TIME PERIODS.

To assist in the determination of the precise nature of the effect of ABA on growth, and especially on germination as assessed by radicle emergence, it was essential that a technique be devised whereby growth could be measured over short periods of time. One problem which the development of such a technique could clarify would be whether ABA acts in delaying radicle emergence by slowing the extension of the radicle such that its emergence through the fruit coat is delayed, thereby causing an apparent delay in germination, or whether it acts by some more fundamental process.

The possibilities of a micro-cell technique were firstly investigated. The plant tissues were positioned in a small perspex chamber through which liquid flowed continuously. The chamber was placed on a microscope stage and the extension of the tissue measured at 1 min. intervals using a graduated eyepiece.

Fig. 78. Circular dichroism curves of (a) methylated extract of 200 g of lettuce fruits var. Great Lakes which had been imbibed for 24 h in 10^{-5} M ABA; (b) methylated extract of 200 g of lettuce fruits var. Great Lakes after imbibition for 24 h in water.


+
O
−
a
b
230
260
290
300
Wavelength (nm)

A section of lettuce hypocotyl dissected from a fruit which had just begun to germinate was placed in the chamber. The tissue did not, however, show any extension growth in the chamber, whether the bathing solution was water or GA_3. The same result was obtained with a 2 mm section of radicle tip.

Elongation was observed when the first oat leaf extension was examined. The coleoptile was removed from the first leaf of a 4 day-old seedling, and a 3 mm apical portion of the leaf excised. This was orientated on the raised platform in the microcell so that the cut end rested against the perspex stop. The observed extension was at a rate of between 6 and 12 μm per minute (Fig. 79A) depending on the seedling, and the rate declined slowly until after 2-3 h it was virtually zero. The tissue was allowed 1 h to stabilise, after which 10^{-5}M GA_3 or 10^{-5}M ABA at 25°C was substituted for water (Fig. 79B). No change in the rate of extension was observed up to 1 h after the substitution, this being the maximum time over which it could practically be observed. To ensure that the lack of effect was not due to uptake or penetration problems, the following treatments were also considered:-

(a) Inverted so that the cut end could more easily take up solution (Fig. 79C).

(b) Decapitated and orientated as in (a) (Fig. 79D).

(c) Decapitated and orientated as in (b) (Fig. 79E).

(d) The complete 1st leaf plus approximately 1 mm of mesocotyl excised (Fig. 79F).

In none of these was a GA_3 or ABA response elicited.

It was, therefore, apparent that the successful application of this technique would involve careful buffering and balancing of bathing solutions. The resultant unnatural situation would also have to be used with tissues which would neither resemble embryonic lettuce tissue nor assist in elucidating the mechanism by which ABA acts on germination. The system was therefore abandoned.

Fig. 79. Time course of the extension of the oat first leaf. The tissue was equilibrated in the perspex chamber for 1 h before application of either 10^{-5}M GA_3 or ABA; the time of hormone application is indicated by the arrow.

(A) Oat first leaf incubated in water.

(B) Oat first leaf treated with ABA (upper line) and GA_3 (lower line).

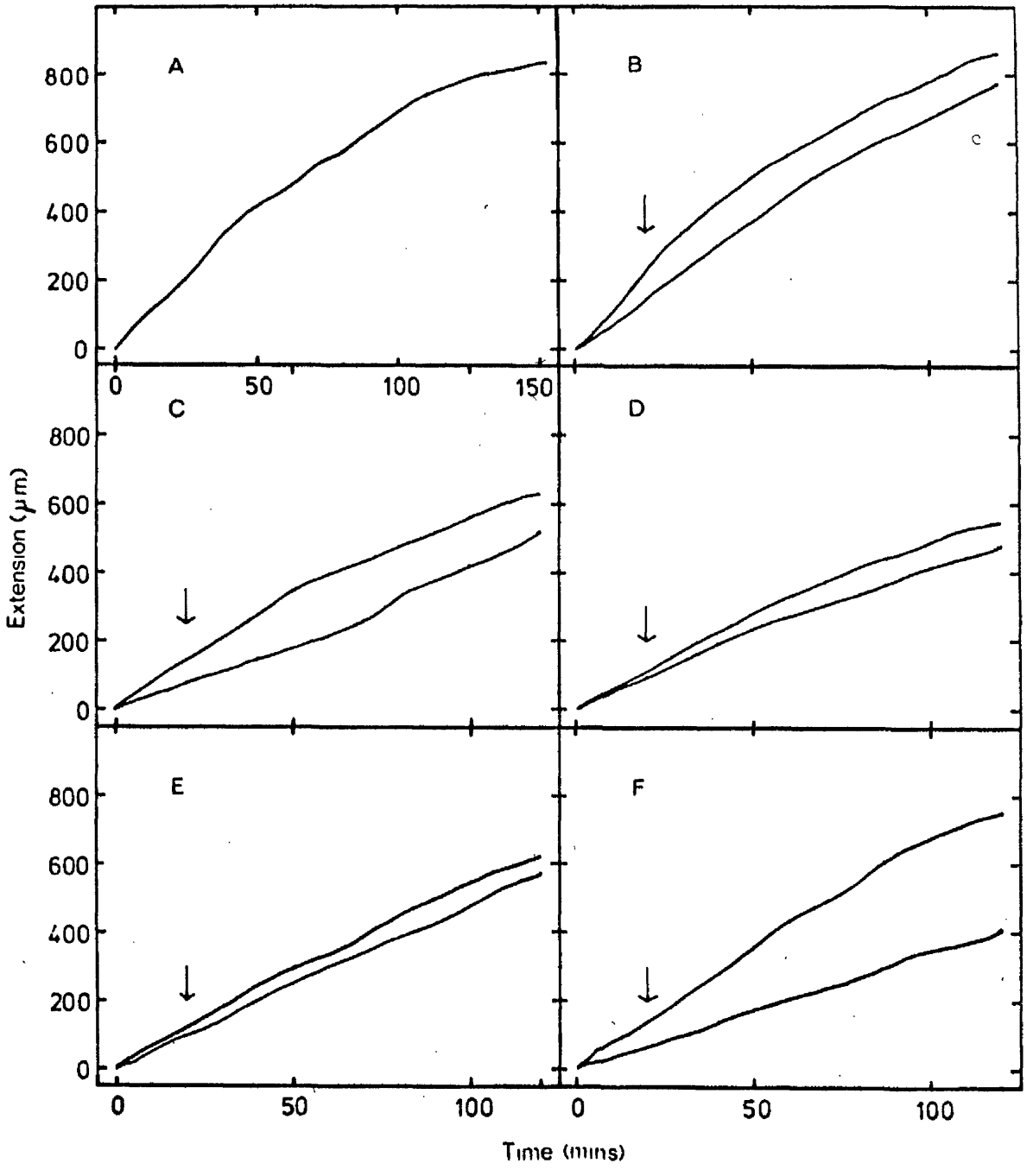
(C) Oat first leaf inverted and treated with ABA (upper line) and GA_3 (lower line).

(D) Oat first leaf decapitated and orientated as in (B). ABA treatment is the lower line and GA_3 treatment the upper.

(E) Oat first leaf decapitated and orientated as in (C). ABA treatment is the upper line and GA_3 treatment the lower.

(F) Oat first leaf + 1 mm of mesocotyl excised and orientated as in (B).

The responses shown are typical results and at no time was a change in the rate of extension detected.


A
B
C
D
E
F
800
600
400
200
0
0
50
100
150
Extension (μm)
Time (mins)

Time lapse photography.

The available time lapse photography equipment enabled small fields of view to be photographed at short time intervals. 4 lettuce fruits were inclined at 60° to the horizontal and images of these fruits and subsequent radicle emergence obtained at intervals of 5 mins. The image was projected on to a screen to give a magnification of x 12.

Radicle emergence was studied in intact fruits imbibed both in water and in 10^{-5}M ABA solution, the whole apparatus being enclosed in a 25°C controlled environment room. Under these experimental conditions, the fruits imbibed in water, germinated after 14-16 h imbibition, and those in ABA, after 26 - 30 h imbibition. The rates of radicle extension immediately after emergence through the fruit coat were recorded. The rate of radicle extension was constant after emergence and the rates in water and 10^{-5}M ABA which were 0.183 mm/h and 0.125 mm/h respectively, were not sufficiently different to account for the differences in the times of radicle emergence (Fig. 80). If, as Bex (1972) suggested, radicle extension began in both treatments after approximately 6 h, the total radicle extension at the time of emergence would, in these treatments, have been 1.65 mm for the water treatment and 2.75 mm for the ABA. Were his hypothesis correct, these two figures would have been equal. It is unlikely that this amount of radicle extension could have occurred within the fruit coat, because the mean imbibed embryo length was 4.08 mm and the mean length of the imbibed fruit, i.e. embryo + fruit coat, was 4.67 mm. The maximum radicle extension which could, therefore, have taken place before radicle emergence through the fruit coat was 0.59 mm.

It was possible, however, that the rate of extension when the radicle was extending within the fruit coat differed from that after emergence. To allow for this possibility, the fruit coats were removed from some of the fruits and the rate of radicle emergence measured as the radicle emerged through the endosperm. These results showed that the rate of extension was the same (Fig. 81).

Fig. 80. The rate of radicle extension in lettuce immediately after emergence of the radicle through the fruit coat.

(A) incubated in water.

(B) incubated in 10^{-5} M ABA solution.

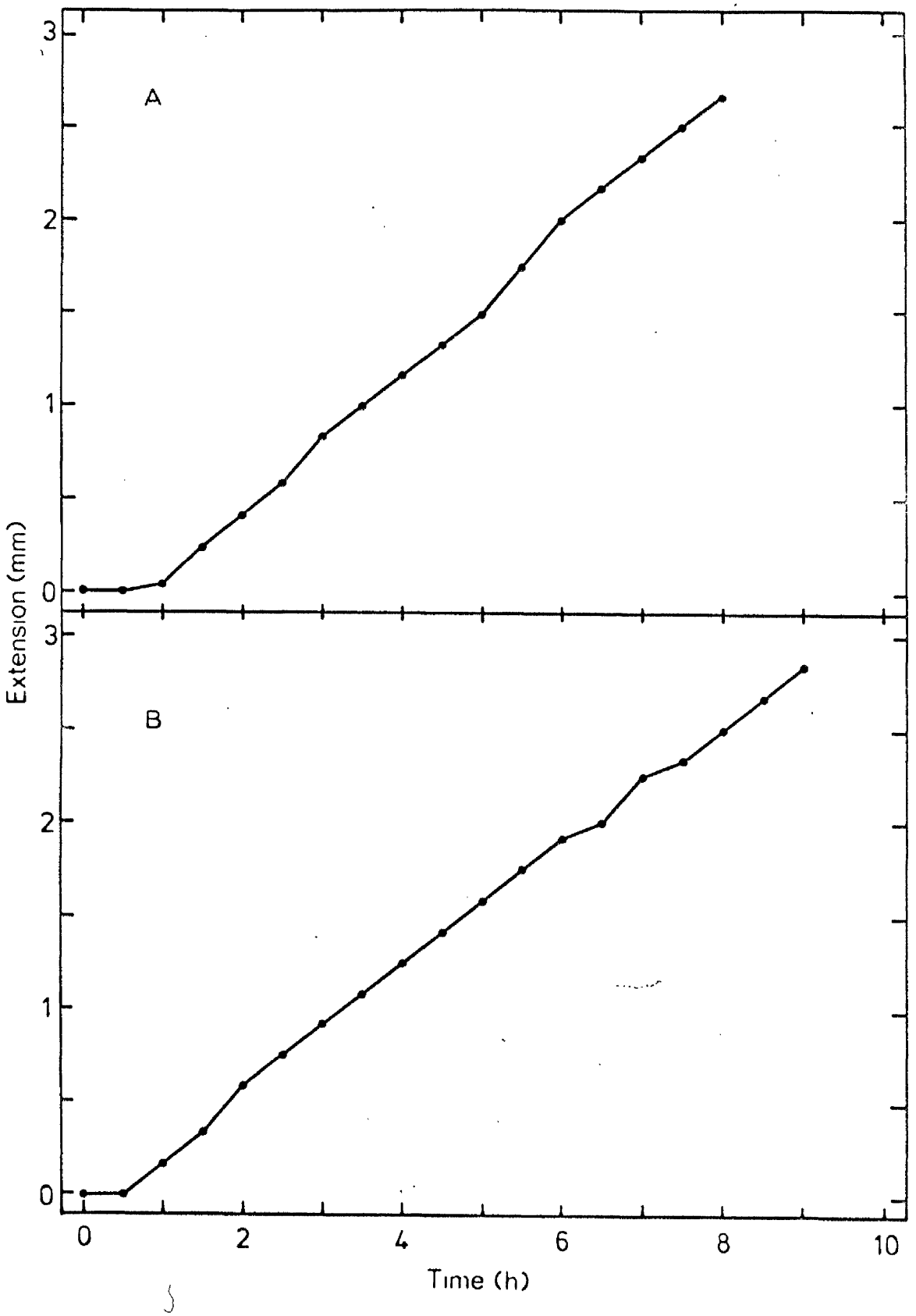
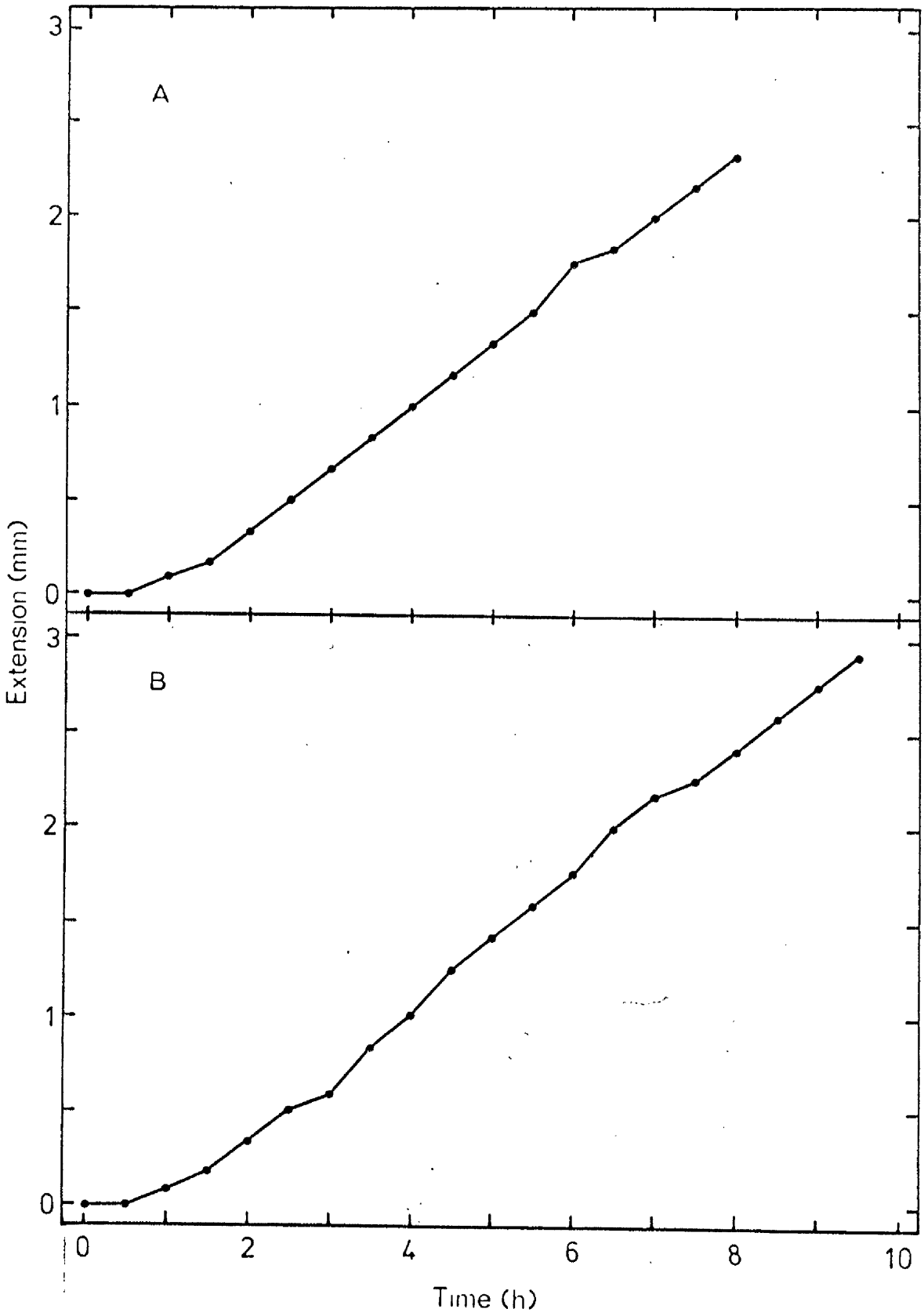

A
B
Extension (mm)
Time (h)
0
1
2
3
0
2
4
6
8
10

Fig. 81. The rate of radicle extension in lettuce immediately after emergence of the radicle through the endosperm, the fruit coat having been removed.

(A) incubated in water.

(B) incubated in 10^{-5}M ABA solution.


A
B
Extension (mm)
Time (h)
0
1
2
3
0
2
4
6
8
10

It seems unlikely, therefore, that ABA acts on germination by slowing radicle extension because, although some slowing of extension did occur when the fruits were imbibed in ABA, it was not sufficient to account for the difference in the time required for radicle extension to occur. The problem does, however, require further investigation, particularly on the effect of removing or damaging the endosperm, which may place a physical restraint on extension. The technique is however satisfactory for studies of this nature, although mounting the camera on a microscope or other magnification device would be a considerable improvement and would permit the assessment of extension over much shorter time periods.

GENERAL DISCUSSION

When lettuce fruits var. Great Lakes are imbibed in a solution containing ABA at a concentration of not less than 10^{-6}M, the emergence of the radicle through the fruit coat is delayed. The length of the delay is dependent on the amount of ABA in the bathing solution, with concentrations lower than 10^{-6}M not significantly affecting germination. These results are in substantial agreement with those of Wareing et al. (1968b), who found that 0.2 ppm of ABA slightly inhibited germination of this variety and that the inhibition increased with increasing ABA concentration. Shankla and Shankla (1968b) and Aspinall et al. (1967) also reported an inhibitory effect of ABA on lettuce germination. The methods adopted by these investigators normally involved assessing the percentage germination after a fixed time and cannot, therefore, be regarded as being as analytical as the mean time to 50% germination (MTG) values used in this study. The effect on germination is not, in fact, a complete inhibition and is best described as a delay.

The inhibitory effects of ABA on lettuce germination are not overcome by the presence of GA_3, whereas Aspinall et al. (1967) reported that the effect of low concentrations of ABA on lettuce germination could be overcome by treatment with GA_3. Shankla and Shankla (1968a,1968b), Khan (1968) and Wareing et al. (1968b), however, reported that GA_3 was incapable of reversing the ABA induced inhibition of lettuce germination. All these investigators, on the other hand, agree that kinetin is capable of overcoming the ABA effect. Aspinall et al. (1967) specified that this occurred only at low concentrations of ABA. The results of Wareing et al. (1968b) also appear to bear this out, with the inhibition or delay of germination resulting from the presence of 20 ppm ABA, not being completely reversed by even the highest concentrations of kinetin applied. In the present study, kinetin partially reversed the ABA induced delay in germination. The results differ from

those of Wareing et al. (1968b) on at least two counts. In these investigations kinetin did not exert a significant effect on germination when the incubation solution contained no ABA, although Wareing et al. found that kinetin promoted the germination of lettuce, even in the absence of ABA. They also found kinetin to be active at 0.2 ppm, a concentration equivalent to 7.6×10^{-7}M, while in this investigation the minimum concentration of kinetin which was found to affect the ABA inhibition of germination was 10^{-5}M. Such differences in results may be attributable to either variations within seed batches or experimental conditions.

When seedlings were maintained under diffuse light at 25°C, and in the absence of GA_3, ABA exerted no significant effect on lettuce hypocotyl extension. When 10^{-5}M GA_3 was applied to the seedlings in the absence of ABA, hypocotyl extension was significantly increased. This GA_3 induced extension could be inhibited by ABA. These results are again in general agreement with those of Wareing et al. (1968b) and Good (1967), but the agreement is incomplete in that they reported that hypocotyl extension was inhibited by ABA both in the presence and absence of GA_3. It is also interesting that the results of Wareing et al. (1968b) indicate that the presence of ABA at 0.25 ppm (9.1×10^{-7}M) exerted an inhibitory effect, and that the inhibition was not greatly affected by a threefold increase in ABA concentration. In these experiments, ABA continued to exert an increasing effect at much higher concentrations. Moreover, Leopold (1971) reported effects of ABA and GA_3 on lettuce hypocotyl extension which agree almost exactly with the results of this study. He observed little extension in the absence of GA_3 but, when 10^{-5}M GA_3 was included in the bathing solution, hypocotyl extension was of the order of 12 mm. This GA_3 induced extension was inhibited by the simultaneous addition of ABA which, at a concentration of 10^{-4}M, gave 50% inhibition.

When the bioassay is carried out in darkness, massive hypocotyl extension occurs in the absence of GA_3 and ABA. The presence of ABA can almost

completely inhibit the extension. Hence the dark extension of the lettuce hypocotyl may be regulated by factors other than endogenous GA_3.

The effects of ABA and GA_3 were also examined using other easily measured parameters of seedling growth. Root length is inhibited by ABA, both in the presence and absence of GA_3, although the latter compound does not significantly affect root growth except at very high concentrations, when it is inhibitory. The effects of ABA and GA_3 on cotyledonary leaf breadth is similar to that recorded for root length. In this case, however, the inhibition by ABA can be partially reversed by the simultaneous addition of GA_3, although the optimum GA_3 concentration is approximately 10^{-6}M, with concentrations higher than this being inhibitory. ABA also inhibits leaf length, both in the presence and absence of GA_3, which promotes leaf length. The simultaneous addition of ABA and GA_3 produces an additive effect, except at high concentrations.

When the effects and interactions of ABA and GA_3 were studied on a related phenomenon, but in a different genus viz. Pisum sativum L., it was found that GA_3 promotes internode extension, and that this effect can be reversed by the simultaneous addition of ABA. In the absence of GA_3, however, ABA does not significantly affect extension at concentrations lower than 10^{-4}M. Wareing et al. (1968b) found that ABA was inhibitory to pea internode extension, both in the presence and absence of GA_3, although Thomas et al. (1965) reported that the sycamore inhibitor "dormin", now referred to as ABA, did not reduce the endogenous growth of either dwarf or tall peas, but that it reduced the GA_3 induced growth in both types. Corcoran and West (1968) made a similar observation on whole peas when testing the inhibitor extracted from carob.

It is debatable whether or not the reversal of the GA_3 effect by ABA in both the pea epicotyl and lettuce hypocotyl assays, and the reversal of the ABA effect on lettuce germination by kinetin, constitute interactions. Wareing et al. (1968b), although conceding that the effect of ABA in many

assays was less marked in the absence of GA_3 than in its presence, ascribed this response pattern to the small amounts of endogenous growth which occur in the absence of GA_3. At the same time, analysis of variance showed no evidence of an interaction between the effects of GA_3 and ABA. The reason for this is not apparent since, if the responses were not additive, an interaction as assessed by this method should have existed. The lack of a significant interaction term can be ascribed only to a large residual variation.

As regards the most satisfactory method of establishing the existence of an interaction, Wareing et al. (1968b) suggested that "the simple observation that two growth substances have opposite effects in a given physiological system does not necessarily indicate that they are acting at the same point, or even on the same pathway". They, therefore, tested for interactions using analysis of variance, a method which Drury (1969,1970) also advocates, but which he erroneously believed not to have been used for the analysis of plant hormone data. He further stated that if no significant interaction term could be found using this approach, the modes of action of the hormones were physiologically independent. This technique was used to analyse the data obtained in this study.

A number of limitations of the technique must be described before the results of these analyses are discussed. The main problem relates to the possible variations in the response of the tissue to a standard stimulus at different points in its response curve, e.g. near the optimum concentration, a unit of stimulus will not elicit the same response as when the concentration is only half the optimum value. This limitation was argued in full by Drury (1969,1970) and Milborrow (1970b), and although Milborrow suggested that attempts should always be made to ensure linearity of response by, if necessary, subjecting the data to a suitable transformation, there is no practical method of selecting this transformation such that the final result is not merely a feature of the transformation rather than of the physiological

phenomenon under consideration. The most satisfactory technique is probably to analyse the original results, as suggested earlier, but always to bear in mind this non-linearity of response.

Analysis of variance describes in statistical terms the tendency of the treatments to vary from additivity, or of the lines in graphical representations to vary from parallelism (Kato, 1968). Thus, when the results of this investigation were subjected to analysis of variance, a term indicating a significant interaction between ABA and GA_3 was obtained in the lettuce hypocotyl and pea epicotyl assays. No interaction between ABA and GA_3 was indicated in the germination assay, in contrast to the apparent relationship between ABA and kinetin. The evidence of interactions in the lettuce hypocotyl and pea epicotyl assays is in disagreement with the findings of Wareing et al. (1968b), who found no evidence of an interaction in these systems. It is, however, apparent that an interaction, as determined by analysis of variance, must exist in all cases where ABA exerted no significant effect in the absence of GA_3, but where it did inhibit GA_3 induced elongation (Corcoran and West, 1968; Thomas et al., 1965). It can be argued that this is not a true interaction because, due to the extremely small amount of growth occurring in the absence of GA_3, there was not an equal capacity for the tissue to respond to inhibitory influences. Similarly, in the lettuce germination assay, germination in the absence of ABA may have occurred in the shortest possible time, and there may not have been an equal opportunity for kinetin to promote germination. At the same time, these are features of the natural system under investigation, and can be taken to indicate that the modes of action of the substances may not be physiologically independent in the system.

The activity of trans, trans-ABA in all three bioassay systems was found initially to be approximately half that of synthetic cis, trans-ABA. These results agree with the original reports of the activity of t, t-ABA (Addicott and Lyon, 1969). However, when samples of the t, t-ABA were subjected to

GLC, only half co-chromatographed with t, t-ABA, while the rest co-chromatographed with c, t-ABA. Taking account of this, all the inhibitory activity observed in the bioassays can be explained in terms of the amount of c, t-ABA present. The trans, trans-isomer must therefore totally lack activity in these bioassay systems, a result which agrees with those of Cornforth et al. (1965b), Milborrow (1966) and Tamura and Nagao (1969a). The presence of c, t-ABA in the test solution may have been the result of the photolytic transformation of the trans, trans- to the cis, trans-isomer, or it may have been a result of the method of synthesis. It seems unlikely that photolysis occurred between solubilisation and chromatography, since these procedures were carried out in darkness or in dim light, as quickly as was practical. Also, the stock cis, trans-ABA would have undergone photolytic conversion to a 1:1 mixture of the two isomers. There are no appreciable quantities of t, t-ABA in this solution, even after a three-day treatment under fluorescent lighting, although a few hours exposure to UV radiation is sufficient for the build-up of t, t-ABA to begin, as described by Mousseron Canet et al. (1966) and Lenton et al. (1971).

When other features of the ABA molecule are considered with regard to their importance in determining ABA-like inhibitory activity, the relative activities of the analogues tested are the same in all the assays considered in this study, although they have been shown to differ considerably in the Avena coleoptile assay (McWha et al., 1973). The molecular requirements for ABA activity are thus the same for inhibition of lettuce germination, hypocotyl extension and pea epicotyl extension, but different for inhibition of Avena coleoptile extension. The carbonyl and hydroxyl groups were unnecessary for inhibitory activity in the Avena coleoptile bioassay, but one or both of the groups is of considerable importance in the assay systems used here. These groups have not been considered necessary by other investigators (Oritani and Yamashita, 1970a; Tamura and Nagao, 1969b,1970; Sondheimer and Walton, 1970; Popoff et al., 1972), although Tamura and Nagao (1969b,1970)

did not comment on the functional importance of the hydroxyl group. Some of the findings of Sondheimer and Walton (1970) are, however, difficult to interpret, since their most active compound differed from ABA only by the absence of the carbonyl and hydroxyl groups and the position of the ring double bond, but possessed only 18% of the activity of ABA.

The ring double bond is necessary for ABA-like inhibition in the bioassays studied. This is in general agreement with the findings of Tamura and Nagao (1969b,1970) and Sondheimer and Walton (1970). Oritani and Yamashita (1970a), indeed, included it in their fundamental structure for ABA-like inhibitory compounds. On the other hand, they found that analogues unsaturated at C-1' or at C-1' and C-3' retained some activity which persisted even when the double bonds were epoxidised. These findings cannot be confirmed in this study where the epoxide compounds inevitably possessed little activity, as they also did in the Avena coleoptile assay (McWha et al., 1973). It is, nevertheless, possible that these tissues lack the ability to convert the epoxides to active forms. It is interesting to note that xanthoxin, which was extracted from Phaseolus vulgaris (Taylor and Burden, 1970a) had activity comparable to ABA in the wheat coleoptile and cress seed germination assays (Taylor and Burden, 1970b), yet the unsaturated functional group in the ring was absent. Taylor and Burden (1972) suggested that the activity of xanthoxin may reflect its biological conversion to ABA.

With regard to the side chain, Tamura and Nagao (1970) considered the complete cis, trans,2,4-pentadiene residue necessary for inhibitory activity, and the results of Oritani and Yamashita (1970a) and Sondheimer and Walton (1970) were in general agreement. McWha et al. (1973) found this residue to be a general requirement in the Avena coleoptile assay, but in the lettuce germination assays the residue may be altered and activity maintained, provided the double bond at C-2 is retained. Popoff et al. (1972), on the other hand, considered that the pentadienoic acid side chain could be shortened to a butenoic chain without losing much of the inhibitory activity,

and suggested that an enzymatic degradation of this type might be part of the mechanism of ABA action. Such a postulation is not supported by these investigations where the double band at C-2 has been found necessary for ABA-like activity, although the double bond at C-4 does not require to be di-unsaturated.

The terminal carboxyl group, or the moiety replacing it, are also of the utmost importance in determining activity. Many of the compounds where this group was altered were more active than ABA in inhibiting lettuce germination, although none was as active as ABA ~~in the other assays in this study, or~~ in the Avena coleoptile assay (McWha et al., 1973). In the lettuce germination assay, esterification of the acid group appears to be the only change which reduces the ABA-like activity. Taylor and Burden (1972) also noted that the abscisic alcohol and abscisic aldehyde were both more active than ABA in a germination assay, with the alcohol being easily the most active, while neither was as active as ABA in inhibiting coleoptile extension.

An assumption which is made throughout the studies of the molecular requirements of the ABA molecule for biological activity is that the inhibition observed in the presence of an analogue of ABA is a feature of the inherent activity of that compound, and results from its molecular structure. Nevertheless, the activity could result from the conversion by the tissues of the analogues to ABA, especially in those analogues where the carboxyl group has been replaced. The observed activity would, therefore, be dependent upon either the rate of conversion, or the competition between this conversion and an ABA de-activation process in the tissues. The activity of an analogue could also depend on the rate of its metabolism to an active compound other than ABA, or upon the rate of catabolism of the compound by the tissues, resulting in inactive products. Finally, it is possible that the activity of certain analogues may be a feature of uptake or of penetration of the compound to an active site in the tissues. The theory of the possible conversion of analogues to ABA cannot explain the activity of

those analogues which are more inhibitory than ABA in the germination assay, although the increased activity could relate either to greater uptake or to slower breakdown than ABA.

Walton and Sondheimer (1972a) found that the methyl and ethyl esters, and the cis- and trans-1', 4'-diols of 2[^{14}C]-abscisic acid were converted to ABA by embryonic bean axes, and that the apparent growth inhibitory activity of the compounds could be explained in terms of the rates of conversion. They also reported that the rate of catabolism of the compounds was much less than for ABA, and suggested that the structural requirements for catabolism were the same as for inhibitory activity. The methyl and ethyl esters possessed inhibitory activity significantly less than that of ABA, a finding which has been confirmed in these studies. Other investigators, however, have claimed that the esters possessed activity equal to or greater than ABA (Koshimizu et al., 1966; Tamura and Nagao, 1969a), but Walton and Sondheimer (1972a) ascribe this to an increased esterase activity in their test tissues and to faster uptake. In these studies, esterification of the carboxyl group was found to reduce activity very markedly in all three bioassay systems, and this may indicate a lack of esterase activity in these systems.

As already described, the carbonyl group in the C-4' position is also necessary for ABA-like activity in some bioassay systems (Mousseron-Canet et al., 1970; McWha et al., 1972). Milborrow and Noodle (1970) found that an active epoxide compound which lacked this carbonyl group was converted to ABA by tomato fruits and wheat leaves; this may explain the inhibitory activity of the compound. It is therefore important to appreciate all aspects of uptake and metabolism of analogues before drawing positive conclusions regarding the structural requirements for activity.

The variable activity of the ABA analogues, especially where the activity was greater than that of ABA in the lettuce germination assay, might be the result of a change in the temperature response of the fruits. Such a response was described by Thompson and Reynolds (1971) for the variety Arctic

King, and by Khan (1968) for Grand Rapids. In the variety Great Lakes, a marked varietal difference exists with the optimum temperature occurring at 25°C, which contrasts with 18°C for Arctic King. Thompson and Reynolds (1971) also reported that the presence of ABA in the incubation medium caused an alteration in the slope of the temperature response curve. In Great Lakes, ABA delays germination at all temperatures, and the increased effect of certain analogues cannot be explained in terms of alterations in the temperature response of the fruits.

When the interactions of the ABA analogues with GA_3 were examined, it was found that only two of the compounds (IX and XXV) gave a significant interaction term in the lettuce germination assay. This observation, in conjunction with the results of a number of other experiments in which these two compounds also behaved atypically, led to the conclusion that they may have a different mode of action from ABA. Compound IX was the only analogue examined whose inhibitory effect could not be at least partially reversed by the application of kinetin. Since kinetin does not affect germination when applied alone, its ability to reverse the effects of all but one of the analogues must indicate that it interacts with the analogues.

In the lettuce hypocotyl and pea epicotyl assays, those analogues which exert a significant effect on GA_3 induced growth also interact with GA_3. This would be expected, especially in the lettuce hypocotyl assay where ABA and its analogues were incapable of inhibiting germination in the absence of GA_3. Thus, where an inhibition occurs in the presence of GA_3, the response curves are non-parallel, and a statistical interaction exists. As with the interactions observed with ABA, this statistical interaction may or may not represent a true interaction. It is significant that all but two of the analogues behave in the same way as ABA in the bioassay systems examined, possibly indicating similar modes of action.

When the effect of applying ABA and its active analogues in combination was considered, it was found that the analogues can be divided into two

distinct groups. One group containing most of the active analogues is characterised by compounds showing additive effects with ABA; the second group contains analogues which, when applied together with ABA, delay germination for a time equal to that induced by the more active component applied alone. These observations immediately raise doubts as to the similarity of the mode of action of ABA and some of the analogues. Two possibilities can be readily envisaged, the first of which is that the compounds of the two groups act by quite distinct modes of action, the first group being similar to ABA. The second possibility is that the metabolic pathways determine whether or not the effects are additive. Where ABA and the analogue share a rate limiting pathway, the time to germination is the sum of the times required for the concentrations to be reduced to their respective critical levels. The effects will therefore be additive. Where there is no common rate limiting point in the pathway, the metabolism of the compounds will proceed independently and the effects will not be additive. The embryonic tissues will require only the time necessary to reduce the level of the more slowly metabolised compound to a critical concentration before germination will occur, and the presence of other substances with a faster rate of metabolism will not significantly affect the MTG.

For ABA and its analogues to delay radicle emergence, they must either slow radicle extension, as suggested by Bex (1972a), or induce a state of dormancy involving the cessation of growth processes. If the latter process operates, the tissue must be capable of metabolising or in some way negating the effects of ABA, thus allowing germination to occur, and ABA uptake will either be a discrete process or the metabolic capabilities of the tissue will not allow it to accumulate in active form.

Time lapse photographic investigations showed that the delay in radicle emergence caused by application of ABA cannot be explained in terms of slowing of radicle extension. The investigations indicated that embryo growth is totally inhibited for a time, after which extension begins and,

although ABA reduces the rate of extension, no evidence could be found that the differences in the rate of extension of the radicle in ABA and in water could account for the total delay in radicle emergence. It may be that the endosperm acts as a physical restraint to expansion, and that ABA prevents its rupture. Ikhuma and Thimann (1963) suggested that "seed coat" digestion, possibly by hydrolytic enzymes, was brought about by the photoactivated form of phytochrome, Pfr, in light sensitive lettuce fruits. A similar process may be inhibited by ABA, although the inability of the GA_3 to reverse ABA inhibition must indicate some differences. A further detailed examination of this process is required, but it seems unlikely that ABA acts in the way suggested by Bex (1972a).

Uptake of both water and ABA prior to germination occur during the first few hours of imbibition. Maximal water uptake by lettuce fruits occurs within the first 2 h of imbibition and falls to almost zero by 4-6 h. No further uptake is observed until radicle emergence. This occurs between 8 and 10 hours for those fruits imbibed in water, but not until after 24 h for those imbibed in ABA solution. The two phases of water uptake, therefore, can be ascribed to imbibition by the dry fruits, followed by the uptake of water required for growth. This pattern of water uptake is similar to that observed by Hallam *et al.* (1972) in rye, where they classify three phases *viz.* wetting, hydration and active imbibition. They do not, however, specify when germination occurs, and it is possible that if this was considered, an even closer correlation could be made. Ikhuma and Thimann (1964), studying water uptake by lettuce fruits, determined that initial uptake during imbibition was not influenced by anaerobic conditions, and Esashi and Leopold (1968) observed two phases of water uptake by *Xanthium* seeds, the first phase passive and the second occurring with growth.

The uptake of water is reflected to a considerable extent by the uptake of ^{14}C-ABA. Indeed, Bex (1972a) demonstrated two phases of ABA uptake in lettuce fruits. The findings herein indicate that, whereas the pattern of

uptake of ^{14}C-ABA is similar to that of water, there are, nevertheless, two inconsistencies. Firstly, the imbibition phase for ABA lags behind that for water by about 3 h, indicating that there is either an active factor involved in ABA uptake, or the fruits are more susceptible to leaching at the beginning of imbibition. Leaching could occur during washing, with radioactivity removed either from the intercellular spaces or from within the cells which lack a highly organised membrane system at this stage. The observations of Simon (personal communication), who recorded the leaching of cellular sugars from pea seeds, supports this view, as does the level of membrane organisation found by Paulson and Srivastava (1968) and Srivastava and Paulson (1968) in unimbibed and imbibing lettuce fruits. Those findings have been confirmed in this study.

Investigations of the uptake of tritiated water, and double label experiments have eliminated the possibility that the lag phase can be accounted for in terms of leaching, by revealing that the pattern of uptake of radioactivity is identical with that obtained when water uptake is assessed by increase in weight. The lag period of 3 h in the imbibition of radioactive ABA is still apparent. The lag is thus a real physiological feature of the uptake of ABA and does not occur with radioactive kinetin, the uptake of which almost exactly coincides with that of water.

A second inconsistency observed in the initial uptake experiments was that, when the amount of ^{14}C-ABA taken up was compared with the water uptake as indicated by weight increase, the fruits took up less radioactivity than calculated for passive uptake in aqueous solution. The lettuce fruits may actively discriminate against the uptake of ABA or, on the other hand, a proportion of the ABA could be metabolised such that the radioactive carbon atom is released as $^{14}CO_2$ and lost from the experimental system. The latter explanation is the less likely because of the position of the "label" in the ABA molecule. In fact, the experiments with tritiated water indicated that the discrepancy in the amount of radioactivity taken up was a result of the

washing procedures. A similar discrepancy exists between the weight of water taken up and the amount of radioactivity associated with that volume of water.

Microautoradiographic investigation of the distribution of exogenously applied ABA within the tissues revealed the presence of a misleading artefact within the lettuce fruits. This artefact, which becomes apparent on treating the tissues with photographic fixer, so closely resembles silver grains that both automatic silver grain counting techniques and visual analysis of the silver grain distribution become extremely difficult.

The artefact is thought to be due to the presence of protein bodies within the cells of the lettuce embryo, the bodies in many of the cells containing small spherical inclusions. Treatment of the tissue with photographic fixer apparently results in the disruption of the protein bodies, thereby liberating the spherical inclusions which appear as small bright bodies under dark field illumination. Paulson and Srivastava (1968) and Srivastava and Paulson (1966), investigating the ultra-structure of lettuce embryos, noted the presence of lipid and protein bodies within the cells, the bodies giving similar staining reactions to those observed in this study, and the marked lack of membranous structures. They also recorded that the protein bodies, especially of the spongy mesophyll,contained two types of inclusion which they distinguished by their staining reaction with toluidine blue; the crystalloids apparently gave a reddish reaction, while the globoids gave no reaction. The protein bodies of the radicle tip frequently contained few or no inclusions. Similar observations were made during this investigation, with the protein bodies of the spongy mesophyll of the cotyledons containing inclusions, some of which gave a red reaction with toluidine blue. That these and the inclusions which gave no reaction were distinct could not, however, be confirmed, since the absence of a staining reaction is no indication of the presence of a body, and may merely indicate incomplete retention of the organelle during fixation and sectioning. A

study of the distribution of the inclusions indicated that they were absent from the protein bodies of many of the cells of the radicle tip and of the vascular tissue. The distribution of the "crystalloids" and "globoids" is therefore identical with that observed for the microautoradiographic artefact, and they can also be directly correlated by light microscopic observation.

The investigations of Swift and O'Brien (1971a,1971b) on the wheat scutellum, Rost (1971,1972) on embryos of Setaria lutescens and Rost and Vaughan (1971) on seeds of Sinapis alba L. are among many others which yielded light and electron micrographs in which the oil and protein bodies appeared almost identical with those observed in this study. In all cases, they also recorded a marked lack of membranous structures in the unimbibed embryonic tissues. It thus seems likely that the problem encountered during microautoradiographic studies of lettuce fruit tissues could occur in relation to most embryonic tissues, and the adoption of one of the methods developed during these investigations will be essential for avoidance of the artefact and accurate quantification of silver grain distribution.

Scans of actual silver grain distribution could best be obtained by first removing the sections from the autoradiographs. Patterns of distribution obtained using this technique indicated that the radioactivity, and thus by implication, the ABA, was uniformly distributed throughout the cotyledons, with a slight accumulation occurring just behind the radicle tip, after the radicle began to elongate.

The radioactive ABA can be extracted from the lettuce fruits apparently unchanged up to 12 h later. Beyond this time, however, the label becomes associated with a compound or complex other than ABA. This compound is believed to be a metabolite of ABA and has been characterised chromatographically in four solvent systems. It appears to be distinct from the metabolites reported by Milborrow (1968) in tomato shoots, French bean and sycamore, and Walton and Sondheimer (1972b) in embryonic bean axes. On the other hand, the Rf values obtained for ABA by Walton and Sondheimer could

not be reproduced in this study. It may therefore be argued that the unknown metabolite described in this article could be similar to their metabolite M-2. The fact that M-2 partitions into ether at pH 2.5 taken in conjunction with the very different chromatographic properties, however, must indicate that M-2 is distinct from the lettuce fruit metabolite.

The amount of metabolite increases up to the time when germination, or more precisely, the emergence of the radicle through the fruit coat, takes place at between 36 and 48 h. Associated with the increase is a reciprocal decrease in the ABA content. In these experiments, germination appears to occur when the original ABA content has been reduced by half. This may reflect that either a threshold level of ABA in the tissues is achieved, or that the ABA remaining is incapable of inhibiting germination. It was thought that this might be due to the inability of the tissue to metabolise the (-)-ABA which constitutes 50% of the applied synthetic ABA, and that this (-)-ABA might not inhibit lettuce germination. The activity of (-)-ABA has not been established in this plant system, although it has been established as active in other plant systems (Milborrow, 1968). Milborrow (1968,1969), however, showed that tomato shoots were capable of metabolising (+)-ABA to yield two major products, while yielding only one with (-)-ABA. Nevertheless, thethe lettuce fruit system no evidence of an accumulation of (-)-ABA could be found after 50% metabolism of exogenously applied ^{14}C-ABA by the plant tissues. It may be that there are other reasons why the remaining ABA does not inhibit germination. The ABA may be compartmented within the tissues in such a way that it has no access to the site of action, or it may be that it is the biologically inactive trans, trans-isomer of ABA which has been formed from the cis, trans by photolysis as described by Mousseron-Canet et al. (1966) and Lenton et al. (1971).

After radicle emergence, further uptake of water occurs, with an accompanying uptake of ^{14}C-ABA. The total amount of radioactivity in the fruits thus increases, with both ABA and the metabolite accumulating in

approximately equal proportions. At the time of radicle emergence, lettuce tissues possess a very marked ability to metabolise ABA.

It was also found that if after imbibition of fruits in ^{14}C-ABA, they are transferred to water, a considerable proportion of the radioactivity is leached into the water during the first two hours and germination proceeds. The radioactivity leached into the water is associated exclusively with ABA, the metabolite remaining within the tissues even after prolonged soaking in water. The rapid germination can be attributed, at least in part, to the leaching of ABA from the tissues. This postulation, however, is dependent upon establishing rather than inferring that the metabolite is not inhibitory to germination.

On examination of the effects of imbibition in the analogues followed by transference to water, it was found that imbibition caused a distinct delay in germination, but that transference to water induced germination to occur after only a short delay. The length of this delay was related both to the inhibitory activity of the analogues as assayed in the germination tests, and to the concentration of the imbibed solution. None of the analogues was however toxic, and accordingly they could have modes of action comparable to, if not the same as, ABA.

ABA has thus been shown to be an active inhibitor of lettuce germination, with identical molecular requirements for activity in three distinct bioassay systems. It has also been shown to be taken up by lettuce fruits during imbibition and during active growth. After imbibition it is distributed uniformly throughout the plant tissues, which are capable of metabolising it until the level of ABA in the tissues is sufficiently low to permit germination to occur. This exogenously applied ABA can be leached from the fruits by transferring them to water.

These findings, however, neither imply a regulatory rôle for ABA in lettuce germination nor implicate ABA metabolism or leaching in the breaking of natural dormancy. In order to evaluate the rôle of ABA, it is first

necessary to show that it occurs naturally within the lettuce fruit tissues, and then to establish a correlation between the amount of ABA in the tissues and the state of germination. It has been shown in this study that ABA occurs in relatively high concentrations in lettuce fruits of the varieties Great Lakes and Arctic King. The recorded levels for unimbibed fruits were 0.67 and 0.17 mg of ABA per kg fresh weight, respectively, after extraction at 49.8% efficiency. No reason for the different levels in the two varieties is apparent. Further examination of the germination characteristics and ABA metabolic capabilities is required. The levels of ABA compare favourably with those obtained by Milborrow (1967,1968), who also applied a spectropolarimetric technique to a range of tissues. Using the racemate dilution method to estimate efficiency of extraction, he calculated that achenes of Rosa canina and Rosa arvensis contained 0.53 and 0.16 mg of ABA per kg fresh weight of tissue. In a subsequent paper he described the efficiency of extraction, estimated using ^{14}C-ABA, as 37.4%. There are, however, discrepancies between these findings and those of Lenton et al. (1971,1972) who, using GLC, estimated that leaves of Betula pubescens contain ABA at a level of the order of 1.24 mg per kg fresh weight of tissue, extracted with 47% efficiency. Milborrow (1967,1968), however, found similar plant material to contain 0.042 mg per kg fresh weight. A particularly high value was obtained by Fuchs and Mayak (1972) who, using an immunological assay, found that tissues of Pisum sativum, var. Alaska, contained 25 mg of ABA per kg fresh weight of tissue. Thus, there appears to be a lack of consistency between assay techniques, but good agreement of results within assays.

It was also found during these studies that the level of ABA in the tissues dropped to 0.022 mg per kg fresh weight of tissue after radicle emergence, a loss of 0.648 mg of ABA per kg fresh weight. Only 0.020 mg per kg of this was recovered from the water in which the fruits were imbibed; the loss cannot, therefore, be explained in terms of leaching. There is,

thus, evidence of the presence of abscisic acid in lettuce fruits, and of a correlation between the decrease in the level of this ABA and germination, the decrease resulting both from metabolism and leaching. The data do not, however, indicate a cause and effect relationship.

Williams et al. (1973) found that the ABA content of the pericarp and testa of Corylus avellana, although decreasing by 61% during imbibition in water, was not the sole factor controlling dormancy. They recognised two types of dormancy. The first they described as seed dormancy, and concluded that it was a result of the presence of ABA in the pericarp and testa. This type of dormancy may be similar to that described by Webb and Wareing (1972) in Acer pseudoplatanus, where germination was prevented by the ability of the testa to prevent leaching of inhibitors from the embryo. Williams et al. also recognised an "embryo dormancy" which they believed to be controlled by factors other than ABA, and which develops slowly during seed storage. They concluded that ABA maintains seed dormancy prior to the development of embryo dormancy. Lettuce fruits examined in this study, and which apparently lack dormancy, may possess the dormancy described by Williams et al. as seed dormancy and lack only the embryo dormancy. Soaking of the fruits in water, which results in a reduction in endogenous ABA levels, would therefore be expected to cause rapid germination.

In view of the fact that ABA was present in aqueous as well as methanolic extracts, it can be argued that the occurrence of the compound was not attributable simply to modification of other compounds by organic solvents. Nevertheless, to fully resolve the question of whether or not ABA is an artefact of extraction, it is essential to evaluate critically all the extraction procedures.

In conclusion, abscisic acid exerts profound and intriguing effects on the germination of lettuce fruits, but a complete understanding of its basic rôle in regulating embryo growth requires a considerable amount of sophisticated investigation.

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