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The Regulation of *Avena* Coleoptile Growth by Abscisic Acid and Indole-acetic Acid

A thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy
in the Faculty of Science

by

J.J. Philipson

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Errata

p.3 line 1	:	van Steveninck (mistake occurs else
p.15 line 18	:	removal of IAA.
p.44 line 25	:	Rank Strand.
p.64 line 25	:	demonstrated.
p.122 line 21	:	85 minutes.
p.156 line 34	:	Endeavour.

SUMMARY

This thesis presents an investigation into the regulation of cell elongation in coleoptile segments of *Avena sativa*. The mode of action of the inhibitor abscisic acid has been examined and a survey of the biological activity of plant growth inhibitors, of potential commercial importance, has been carried out.

Abscisic acid induces a non-toxic inhibition of cell elongation in the *Avena* coleoptile straight growth assay. The use of ABA-analogues has elucidated certain molecular requirements for the inhibitory activity of ABA. The complete carbon skeleton of the 2,4-pentadiene side chain appears to be essential for inhibition and, provided this is present, there are two active moieties in the ABA molecule, *viz.* the ring double bond and an oxygen or nitrogen containing functional group at C-1 in the side chain. The carbonyl and hydroxyl groups attached to the ring appear unnecessary for inhibitory activity. Although 33 ABA-analogues were bioassayed none was more inhibitory than ABA itself.

In view of the importance attached to the promotory effects of indole-acetic acid on cell elongation the effect of ABA, in the presence of IAA, was examined. ABA overcomes the stimulation of growth by IAA and on a mole for mole basis, at 10^{-4}M, ABA is a more powerful inhibitor than IAA is a promoter. Certain presentations of the data from a straight growth assay of IAA and ABA in combination reveal a statistical interaction between these hormones, but transformation of the data reduces the evidence for this interaction. Furthermore, analysis in terms of enzyme reaction kinetics does not demonstrate either a competitive or a non-competitive inhibition of IAA activity by ABA.

A technique was developed which utilizes a linear displacement transducer to continuously monitor the elongation of a column of segments immersed in

flowing solution. The instrumentation is able to detect a latent period before the promotion of a high growth rate with an accuracy of 0.5 minutes and can detect a growth rate of less than 2 μm $min.^{-1}$ for a column of 10 segments.

IAA at 10^{-5} M in phosphate buffer of pH 7.0 promotes a high growth after a latent period of 11 minutes at 25°C, the initial maximum growth rate occurring after 25 minutes. Both the initial maximum growth rate and the duration of the latent period are dependent upon IAA concentration and temperature. The promotion of a high growth rate by IAA is never immediate and a minimum latent period of 7.3 minutes is observed at 25°C with 10^{-3} M IAA in phosphate buffer of pH 7.0. A re-evaluation of a previous report of an immediate response to IAA strongly suggests that this may be attributed to low-pH-induced growth.

Simultaneous treatment with 10^{-5} M ABA and 10^{-5} M IAA does not affect either the latent period or the initial maximum growth rate in response to the IAA treatment alone but subsequently gives rise to an inhibition of growth detectable after 30 minutes. Pretreatment with ABA for 100 minutes, however, increases the duration of the latent period and reduces the initial maximum growth rate. Removal of ABA rapidly relieves the inhibition of IAA-induced growth but a growth rate comparable to that of material treated only with IAA is never attained. Studies using 2-[^{14}C]ABA and 1-[^{14}C]IAA suggest that the latent period before ABA inhibition of growth is detectable is not due to a lag in ABA uptake, and that ABA is not acting by reducing IAA uptake.

CONTENTS

INTRODUCTION

The development of plants involves the growth and differentiation of the component cells. The regulation of development is believed to be under the control of endogenous regulators, as well as environmental parameters. The rôle of these endogenous regulators, first termed hormones by Fitting (1910), has been a major area of research in plant physiology, and particular interest has been attached to the correlative effects of these substances on cell elongation.

Because of the complexity of the processes regulating plant growth it is helpful to choose an apparently simple system in order to study the regulation of growth. The coleoptile of Avena sativa has been employed in investigations of this type because it is a relatively simple organ. Once the Avena coleoptile has grown to approximately 1 cm cell elongation proceeds in the absence of cell differentiation (Avery and Burkholder, 1936). The Avena coleoptile was used by Darwin (1881) and the technique of immersing excised segments in solution and measuring the growth after a known incubation period was pioneered by Bonner (1933). This convenient technique for measuring elongation is termed the straight growth assay. Excising the segments removes both the tip in which growth promoting substances originate (Paál, 1918), and the influence of the remaining seedling.

The growth active substance produced in coleoptile tips has been termed auxin, and the auxin-like properties of urine extracts have been attributed to the presence of indole-acetic acid (IAA) (Kögl et al., 1934). It is widely assumed that IAA is the auxin produced in coleoptile tips; the basis of this evidence rests mainly on the similarity of their growth activity, chromatographic properties and transport properties. Yet there has only recently been an unequivocable affirmation, by the use of direct probe mass spectrometry, that the auxin in a diffusate from Zea coleoptile tips is IAA (Greenwood et al., 1972).

Although initially the theories of hormonal regulation of plant growth concerned only growth promoting compounds, attention quickly turned towards the rôle of inhibitors, and the compound abscisic acid (ABA) has been of notable importance in recent years. Indeed, a large number of theories have implicated a balance between inhibitors and promoters in the regulation of plant development (e.g. Snow, 1937; Wareing et al., 1968b). Consequently, many of the techniques developed during the investigation of IAA action have been used in studies of ABA. The techniques employed include the use of analogues to elucidate the molecular requirements for hormone activity (Porter and Thimann, 1965; McWha et al., 1973), the study of the interactions between hormones (Wareing et al., 1968a), the application of enzyme kinetics in the analysis of growth data (Bonner and Foster, 1956; Rothwell and Wain, 1964) and the continuous monitoring of elongation using sensitive measuring techniques (Ray and Ruesink, 1962; Rehm and Cline, 1973).

The discovery of abscisic acid may be attributed to three research groups, led by Addicott, Wareing and Wain. At the Fifth International Conference on Plant Growth Regulators (1963) they each described the properties of an extracted inhibitor and, following collaboration with organic chemists, the structures of the inhibitors were found to be identical. In the U.S.A., Carns and Addicott were investigating abscission-accelerating substances in cotton fruit, and they isolated abscisin I and abscisin II (Lui and Carns, 1961; Addicott et al., 1964). Subsequently, abscisin II was isolated by Ohkuma et al., (1963) and its structure published (Ohkuma et al., 1965); but abscisin I has not been re-isolated or identified.

At Aberystwyth dormancy in birch and sycamore was being investigated and an inhibitory substance, dormin, was extracted (Eagles and Wareing, 1963; Wareing et al., 1964). Dormin was later shown to be identical to abscisin II (Cornforth et al., 1965a; Robinson and Wareing, 1964) and the structure was confirmed by synthesis (Cornforth et al., 1965b).

In addition, the growth inhibitor in yellow lupin fruit which was first

described by van Steveninck (1959) and also reported by Rothwell and Wain (1964), has been shown to be identical to abscisin II (Cornforth et al., 1966b; Koshimizu et al., 1966). Both dormin and abscisin II are now termed abscisic acid (Addicott et al., 1968); 1969); the abbreviation ABA has been agreed upon and the formula is that given in Table 1.

ABA exhibits both geometrical and optical isomerism. The geometrical isomerism depends upon the configuration of the double bonds in the side chain and the relative positions of specific groups attached to the carbon atoms at either end of the double bond. The groups to be considered are determined by the sequence rule (Cahn, 1964); they are the hydrogen atoms for C-4 and C-5 and for the C-2, C-3 double bond the position of C-1 and the hydrogen atom attached to C-4 are considered. For the proximal double bond the hydrogen atoms are always on opposite sides of the bond which is thus trans (E). The H atom on C-4 is in a fixed position but C-1 may be cis(Z) or trans (E) relative to this. Thus two isomers of ABA exist: trans, trans-(t,t- or E,E-) and cis, trans-(c,t- or Z,E-) (Tables 1 and 2). Both isomers have been extracted and varying biological activity has been reported.

The optical isomerism of ABA is conferred by the asymmetric carbon atom at C-1' (it has four different substituents). Naturally occurring ABA is dextrorotating (+), rotating the plane of polarized light to the right (Cornforth et al., 1966a), but synthetic ABA is a racemic mixture of these enantiomers; it is optically inactive and is referred to as (±) ABA.

Milborrow (1970a), using radioactive ABA, showed that in short term experiments tomato shoots converted the (+) component into 2 major products, but the (±) component gave only one. Thus it might be expected that the (+) and (-) enantiomers have different activity, and first indications were that the synthetic (±) ABA had only about one half the activity of the natural (+) ABA (Wareing and Ryback, 1970). Subsequently, however, there have been varying reports. Pure (±) ABA was prepared (Cornforth et al., 1967) and this was shown to be equally as active as the (+) and (±) forms (Milborrow,

1968) but in a recent report the (+) component was markedly less active than the (-) component in the closure of stomates (Cummins and Sondheimer, 1973). It may well be that the activity depends upon the bioassay selected, as suggested by Sondheimer et al. (1971).

The possible differences in activity of the natural (+) isomer and the synthetic (±)-isomer must be borne in mind when evaluating experiments in which the synthetic ABA was applied to the plant.

The absolute stereochemistry of the natural (+) ABA was originally determined as S (Cornforth et al., 1967) based on Mills' rules (Cahn, 1964), with the side chain in the same plane as the ring. Yet doubt was cast on this configuration by Oritani et al. (1972) and Burden and Taylor (1970); the latter authors showed that violaxanthin will yield t,t-ABA and the stereochemistry of violaxanthin had previously been established as having the side chain at right angles to the ring. Finally, by correlation with malic acid the original configuration was shown to be incorrect (Ryback, 1972). Nevertheless, a reconsideration of the sequence rules (Cahn et al., 1966) has now come to light and the natural (+) ABA, with the side chain at right angles to the ring, is still termed S.

Studies of the physiological rôles of ABA have been facilitated by the availability of synthetic ABA. This inhibitor has been shown to affect many physiological processes (Addicott and Lyon, 1969) and it has been extracted from a wide variety of plant tissues and species (e.g. Milborrow, 1968). Consideration will be given mainly to studies of the mechanism of action of ABA as a plant growth inhibitor.

Analogues of abscisic acid have been used to study the molecular requirements for ABA activity. Because of the restricted availability of these analogues much of the work has been carried out by chemists who have also reported the preparation of these analogues. Consequently, there has frequently been greater emphasis on the preparation of these analogues than on their structure-activity relations (e.g. Oritani and Yamashita, 1970a)

and even where the biological activity of the analogues has been considered the bioassays have frequently been insufficiently described (e.g. Tamura and Nagao, 1969a). An additional problem in appraising the published results is that different bioassays have been used. The Japanese investigators primarily used the rice second leaf sheath bioassay (Oritani and Yamashita, 1970a, 1970b, 1970c, 1970d, 1970e; Tamura and Nagao, 1969a, 1969b, 1969c, 1970; Koshimizu et al., 1966) although Ohkuma (1965, 1966) studied abscission using the cotton explant bioassay, as did Asmundson et al. (1968). Sondheimer and Walton (1970) studied the growth inhibition of excised embryonic axes of Phaseolus vulgaris and Popoff et al. (1972) examined bean stem elongation. In contrast, Taylor and Burdon (1970b, 1972) tested fewer compounds, mainly the extracted compound xanthoxin and the synthetic compounds abscisic aldehyde and alcohol, but they used a range of bioassays. In some publications the authors have discussed the differences in activity of a compound between bioassays as disagreements (e.g. Popoff et al., 1972) yet the most interesting conclusions are probably those that have suggested that the structural requirements for ABA activity depend on the bioassay used (Tamura and Nagao, 1969a; Sondheimer and Walton, 1970). Nevertheless, the data are of interest, both for the agreed structural requirements and for the differences observed.

There have been differing reports over the possible requirement of the ionylidenacetic acid skeleton for inhibitory activity. Both Sondheimer and Walton (1970) and Oritani and Yamashita (1970a) regarded this as necessary but Popoff et al. (1972) listed 9 compounds that have alterations to this skeleton yet possessed activity relatively high compared with that of ABA. The detailed requirements within the ring and side chain should, however, be considered separately.

Consistent reports have indicated that neither the carbonyl group in the ring nor the hydroxyl group are required for inhibitory activity (Tamura and Nagao, 1969a, 1969c, 1970; Sondheimer and Walton, 1970). Indeed, Oritani and Yamashita (1970a) excluded these moieties from the structure which they

concluded possessed the fundamental requirements for activity. Some of these results must be interpreted with care because Sondheimer and Walton (1970) compared the activity of analogues with the activity of a compound that they referred to as active, yet which possessed only 18% of the activity of ABA.

The double bond in the ring between C-2' and C-3' has generally been reported to be an important requirement for inhibition (Tamura and Nagao, 1969c, 1970; Oritania and Yamashita, 1970a; Sondheimer and Walton, 1970) though there are inconsistencies in the literature. For example, Tamura and Nagao (1969a) found that epoxidation of the methyl and ethyl esters of ABA, a process that removed the ring double bond, enhanced the activity of these analogues beyond that of ABA. In addition, there are reports of compounds that are active but which lack the ring double bond (Oritani and Yamashita, 1970a).

Sondheimer and Walton (1970) are the only authors to discuss the structure-activity relations on steric considerations. They suggested that for a fit between ABA and required macromolecules within the cell the C-2' methyl group must be coplanar with four ring carbon atoms, as occurs in compounds that lack the ring double bond.

The cis,trans-2,4-pentadienoic acid side chain of ABA has been considered necessary for inhibition and analogues possessing this were generally found to be more active than the 2-trans isomers (Tamura and Nagao, 1969a, 1970; Sondheimer and Walton, 1970) and Oritani and Yamashita (1970a) included the cis,trans- configuration in their fundamental structure. In contrast, there was no such agreement in publications concerning the geometrical isomerism of ABA itself. There were reports that the 2-trans isomer was inactive (Cornforth et al., 1965b; Milborrow, 1966); that the isomers had similar activity (Nitsch,1967) and that the 2-trans isomer became active only after a lag of about 1 day (Asmundson et al., 1968). The differences possibly reflect the conversion of cis,trans-ABA to a 1:1 equilibrium mixture of

cis,trans-ABA and its 2-trans isomer (Mousseron-Canet et al., 1966; Lenton et al., 1971). Indeed, Milborrow (1970a) suggested that the trans,trans-ABA found in rose leaves was probably formed by photolytic isomerization from cis,trans-ABA.

There have been additional references to specific molecular requirements within the side chain. For example, the unsaturated bonds have been implicated for activity (Oritani and Yamashita, 1970c) although there have been reports of partially active analogues that possess a triple bond in the side chain (Ohkuma, 1965, 1966). Oritani and Yamashita have also presented evidence that the side chain length is critical; in compounds with either shorter side chains (1970b) or longer side chains (1970d) the activity was reduced, although there were other structural changes that may have reduced the activity of these analogues.

The terminal group in the side chain has also been investigated and Tamura and Nagao (1969c, 1970) concluded that this group must be carboxyl; this suggestion is consistent with reports that abscisic aldehyde is active (Oritani and Yamashita, 1970c; Taylor and Burden, 1970b). Furthermore, the methyl ester of ABA possessed similar activity to ABA in the rice leaf sheath assay (Koshimizu et al., 1966) and in the cotton explant bioassay (Asmundson et al., 1968). Jones and Mansfield (1971) recorded that both the methyl and phenyl esters of ABA were slightly more active than ABA in the suppression of stomatal opening and suggested that this activity may be a result of greater penetration into the plant or gradual breakdown to release an active compound.

The activity of one ABA-like compound, xanthoxin, has been related to endogenous occurrence and possible physiological rôles. Xanthoxin has activity similar to that of ABA in the cress seed germination and wheat coleoptile bioassays (Taylor and Burden, 1970b; 1972) and this compound has been extracted from shoots of a wide variety of plants (Taylor and Burden, 1970a; Firn et al., 1972). In addition, photooxidation of violaxanthin,

a common plant xanthophyll, yielded the cis,trans- and trans,trans- isomers of xanthoxin as well as two other inhibitory compounds (Taylor and Smith, 1967; Burden and Taylor, 1970). This light-effect is of particular relevance because Burden et al. (1971) showed that red illumination of dwarf pea seedlings increased the levels of extractable cis,trans- and trans,trans-xanthoxin but had no significant effect on the low level of ABA found in dark grown seedlings. Thus it was suggested that a xanthophyll is a precursor to xanthoxin in some species and that this inhibitor accumulates during illumination.

One technique for studying the mode of action of a growth hormone is to see if it will modify the effect of a different plant hormone. This technique has been used in the study of the action of IAA and was recently employed (Hemberg, 1972; Hemberg and Larsson, 1972). Because IAA stimulates growth the effect of the inhibitor, ABA, on IAA-induced growth, has been studied. Abscisic acid was shown to inhibit growth caused by IAA in Avena mesocotyls (Milborrow, 1966) and Avena coleoptiles (Wareing et al., 1968a). Yet Wareing et al. (1968a) pointed out that the observation that two growth substances have opposite effects in a plant tissue does not necessarily indicate that they act at the same point or on the same pathway; they suggested that an analysis of the responses to the two substances in combination was required, in order to examine the possibility of a statistical interaction. Their results showed that the slope of the ABA response curve was unaffected by the addition of 1 ppm of IAA and they detected no interaction in an analysis of variance. Indeed, both Wareing et al. (1968a,b) and Good (1967) investigated the interaction of ABA with other plant hormones in several bioassays and concluded that there was predominantly no interaction, although in the lettuce germination assay ABA did interact with kinetin.

The problem arises as to the precise meaning of the term interaction and the conclusions that can be drawn from these studies. Furthermore,

consideration is required of the form of the data to be analysed. These problems were discussed by both Drury and Milborrow.

Drury (1969) gave the statisticians definition of an interaction as the failure of a response to one agent to be the same at different amounts of a second agent. Thus graphically the dosage-response curves for one agent at different amounts of the second will not be equidistant, whereas for no interaction the curves are equidistant. Drury claimed that physiologists are inclined to define an interaction as the relationship between two agents which affect the same response in algebraically opposite ways. With consideration to these points Drury (1969) re-evaluated the interaction claimed between GA_3 and ABA (Thomas et al., 1965; Chrispeels and Varner, 1967; Milborrow, 1966; Dey and Sircar, 1968) and decided there was no evidence of a statistical interaction. Drury, however, appeared unaware of the approach of Wareing et al. (1968a) in which both the shape of the response curve and the analysis of variance are considered. Surprisingly, Drury additionally claimed that if there is no interaction the mode of action of the hormones was independent and vice versa.

In reply to these criticisms Milborrow (1970b) regarded Drury's definition to be over simplified, because it did not account for the fact that most plant tissues have a non-linear response to applied substances. He also correctly pointed out that transformation of non-additive data may render them additive and remove the statistical interaction. Consequently, Milborrow suggested that the term interaction should be applied exclusively to measurements and not used for physiological responses. In his defence, Drury (1970) stated that his original definition of interaction did not require revision when transformations are carried out and he explained this in mathematical terms. Yet he admitted that if such transformations are required, e.g. to meet the assumptions of the analyses of variance, then the analysis refers to the transformations and not to the original data. Thus it would seem wise to adopt Milborrow's suggestion and avoid the term inter-

action in considerations of the mode of action of a hormone.

An extension of the statistical analysis of the interaction between IAA and ABA is analysis in terms of enzyme kinetics. Bonner and his co-workers analysed auxin action in these terms and this work is pertinent because it forms the basis for the exploration of the inhibitory activity of ABA.

The enzyme studies to which Bonner drew comparisons are those put forward by Michaelis and Menton (1913). The latter authors adopted the earlier suggestion of Henmi (1902) that an enzyme (E) reversibly forms a complex with its substrate (S) and this complex subsequently breaks down giving the free enzyme and the products of the reaction. In nearly all enzyme reactions a plot of initial reaction velocity (v) against substrate concentration corresponds to a section of a rectangular hyperbola, i.e. at high substrate concentrations no further increase in reaction velocity is obtained if the substrate concentration is increased. The velocity of the reaction obtained at this high substrate concentration is defined as the maximum velocity of the enzyme-catalyzed reaction (V). The substrate concentration required to yield half the maximum velocity ($^{V}/2$) defines the Michaelis constant or Km (Dixon and Webb, 1958).

Certain compounds have the ability to combine with specific enzymes but, because they do not serve as substrates, they block the catalysis by that enzyme. These compounds are called competitive and non-competitive inhibitors. When a compound competes with a substrate for the active site on the enzyme and thereby reduces the catalytic activity of that enzyme, the compound is considered to be a competitive inhibitor. This type of inhibition may be reversed by increasing the concentration of the substrate. Since the active site is directly involved the Km for the enzyme is altered by the competitive inhibitor. Non-competitive inhibition is the type that cannot be reversed by increasing the substrate concentration. The inhibitor combines rather strongly with a site on the enzyme and cannot be displaced by increasing substrate concentration. In this type of inhibition the Km

is not altered by the inhibitor, which simply reduces the maximum reaction velocity (Conn and Stumpf, 1963).

It must be borne in mind, however, that these definitions apply to particular types of inhibition, and intermediate forms of inhibition exist. For example, the inhibition may be partially competitive, partially non-competitive, or mixed (Dixon and Webb, 1958).

Lineweaver and Burk (1934) showed that a double reciprocal plot ($\frac{1}{v}$ VS $\frac{1}{S}$) yields a straight line for an enzyme reaction and the Km and V values for an enzyme reaction may be determined from this plot. Because of the differential effect of competitive and non-competitive inhibitors on the Km and V for an enzymic reaction, the Lineweaver-Burk plot discerns between these two types of inhibitor.

Bonner and his co-workers (Bonner and Foster, 1955, 1956; Foster et al., 1952; McRae and Bonner, 1952; McRae et al., 1953) using Avena coleoptile segments, showed that a graph of segment length VS IAA concentration formed a rectangular hyperbola, and that a double reciprocal plot yielded a straight line. They suggested that the data support the view that the IAA molecule exerts its effect through combination with a suitable receptor site in the cell and that the reversible formation of the auxin-coleoptile complex is analagous to the formation of an enzyme-substrate complex.

By application of enzyme-kinetic theory they demonstrated that the different auxins react within the plant cell as would be expected on the basis that they compete for a common site and that certain diortho-substituted phenoxyacetic acids competitively inhibit the action of IAA. In these studies Bonner and his co-workers estimated the initial reaction velocity from the total growth after 12h or 18h, having shown that in the presence of 3% sucrose and 0.0025M potassium maleate buffer growth was linear for 18h-24h.

Bonner's method was criticised by Bennet-Clark and Kefford (1954) who found that in the absence of buffer the growth rate over 18h is not linear with time. Nevertheless, the application of Michaelis enzyme kinetics to

the Avena coleoptile-IAA system, has been confirmed (Housley et al., 1954) although Marinos (1957) concluded that these studies must be approached with great care because the linear response can be realised only under strictly limited conditions. Housley et al. (1954) pointed out that the function of sucrose in enhancing cell elongation is not understood and that the use of sucrose should be avoided if possible. The effects of sucrose on the IAA dosage-response curve were recently described by Cleland (1972), who suggested that there may be an interaction between sucrose and IAA, either on sucrose uptake or utilization.

Bonner's work forms the basis for the application of enzyme kinetics to the interaction of IAA and growth inhibitors. Using the lupin inhibitor, the active constituent of which has been shown to be ABA (Cornforth et al., 1966b), Rothwell and Wain (1964) demonstrated that a Lineweaver-Burk type of plot ($\frac{1}{S + 0.178}$ VS $\frac{1}{v}$) gave a straight line at all inhibitor levels tested and that because these lines have a common intercept on the $\frac{1}{S + 0.178}$ axis they suggested a non-competitive inhibition was indicated. The growth, however, was not shown to be linear with time and the time period for the experiments was not clearly specified. In addition, the necessity of the constant (0.178) to obtain straight lines has not been commonly used in enzyme kinetics (Capon, pers. comm.; Dixon and Webb, 1958) although the authors suggested that the constant related to an endogenous level of IAA equivalent to an externally supplied solution of 0.178 ppm ($\equiv 10^{-6}$M).

In these enzyme studies the IAA is believed to be acting as a substrate and, apart from the similarity of the graphs drawn to graphs of enzyme reactions, there is no strong evidence to support this. In addition, these considerations assume that there is a single enzyme, as yet unidentified, which binds to the IAA substrate, and this may be an over-simplification in the multi-enzyme plant system (Marinos, 1957).

In one study of the inhibition induced by ABA these assumptions were not required (Saunders and Poulson, 1968) because the enzyme yeast invertase and

its substrate, sucrose, were studied. A Lineweaver-Burk plot of enzyme activity in the presence and absence of ABA indicated that the ABA showed apparently competitive kinetics. Nevertheless, these authors considered it unlikely that the ABA molecule would cause a true competitive inhibition of invertase activity because ABA bears no structural resemblance to the substrate under consideration, sucrose. They suggested that a more detailed kinetic analysis was necessary before definite conclusions could be reached.

Another technique for studying the mechanism of action of a hormone is to continuously monitor its effect on the growth of an organ. During the last decade this technique has been used to study auxin action and recently there have been reports of the timing of the effect of ABA on IAA-induced growth. By the use of sequential and simultaneous hormone treatments, metabolic inhibitors, and comparisons with the time course of biochemical reactions, this technique can be extremely useful.

This approach was probably initiated during early studies of "growth substance" when the growth of a decapitated Avena seedling, treated with acidulated pollen extract, was monitored with an interferometer (Laibach and Kornmann, 1933). These investigations revealed a latent period of approximately 0.25 h before growth was promoted; this latent period is similar to that now accepted for IAA-induced promotion of growth in several organs.

In the mid 1950's it was known that there was a latent period of about 10 minutes before a physiological concentration of IAA would stimulate growth (Bonner and Foster, 1955), although this time was not determined accurately. The first technique which was sufficiently sensitive to time the responses of tissue to applied IAA was developed by Ray and Ruesink (1962). In essence, this technique allowed the growth of a single Avena coleoptile segment, which was maintained in flowing solution, to be monitored with a microscope fitted with a micrometer eyepiece. A similar technique has been used in the study of Avena coleoptile segments (Nissl and Zenk, 1969) and lupin hypocotyl

segments (Penny *et al.*, 1972). A modification of this basic technique has now been more extensively used. This allows the growth of a column of segments to be monitored by casting the shadow of a small weight, placed on the top of the elongating column of segments, on to photographic paper which is moving horizontally. This technique has been exploited in the study of the responses of several tissues to applied hormones; e.g. *Avena* coleoptiles (Evans and Ray, 1969), pea stem segments (Barkley and Evans, 1970) and corn coleoptiles (Evans and Nokanson, 1969). The latter technique has now been developed to allow an electrical transducer to monitor the plant growth (dela Fuente and Leopold, 1970; Rehm and Cline, 1973) and transducer techniques have also been employed to monitor the growth of intact plants, e.g. etiolated pea (Warner and Leopold, 1971) and leaf elongation in intact maize plants (Hsaio *et al.*, 1970). In addition, capacitance auxanometry has been developed whereby the growth of the plant material alters the electrical capacitance between two metal plates (Gordon and Dobra, 1972). This technique is useful for studying light effects on growth as neither light nor contact with the tissue is required to monitor the growth.

The use of these techniques has shown that *Avena* coleoptile segments will respond to 10^{-5}M IAA at 25°C in approximately 10 minutes (Ray and Ruesink, 1962; Evans and Ray, 1969; Nissl and Zenk, 1969). It is now established that the latent period for the IAA response is shortened by increasing the temperature and IAA concentration of the bathing solution (Nissl and Zenk, 1969; Durand and Zenk, 1970) although there were preliminary indications that the latent period was independent of IAA concentration (Ray and Ruesink, 1962; Evans and Ray, 1969). There have been two reports of an immediate growth response of *Avena* coleoptiles to IAA. Firstly at 29°C with 5×10^{-3}M IAA at pH 4.7 (Nissl and Zenk, 1969) and secondly following the removal of an ABA pretreatment, although the latter finding was reported to be obtained in only three out of six trials (Rehm and Cline, 1973).

IAA has been shown to induce a steady growth rate in *Avena* coleoptiles,

probably within 25 minutes of IAA treatment (Nissl and Zenk, 1969) though a shorter lag before the steady state point is reached has been reported (Evans and Ray, 1969). Yet data from a technique which allowed the rate of growth of segments to be calculated directly from the initial readings suggested that the initial maximum in growth rate in lupin hypocotyls was followed by a depression in growth rate and subsequent fluctuations in rate (Penny et al., 1972). Metabolic inhibitors, such as cyanide, reduced the growth rate in response to IAA, as did removal of oxygen (Ray and Ruesink, 1962; Evans and Ray, 1969) and it was suggested that the growth mechanism is controlled by a metabolic process.

The supply of pulses of IAA to Avena coleoptile segments (dela Fuente and Leopold, 1970) showed that the growth rate declined rapidly after the removal of IAA, and it has been suggested that there is an easy association and dissociation of IAA with a growth limiting site in the cell. This theory is consistent with the idea that the coleoptile-IAA complex may behave as an enzyme-substrate complex. Pulse studies with corn yielded similar results and Evans and Hokanson (1969) suggested that the rapid decline in growth rate after the removal if IAA indicated that IAA does not act via the promotion of the synthesis of a protein because the half-lives of proteins in higher organisms are of the order of days or at least several hours. Cleland (1971), however, has presented evidence concerning growth-limiting proteins which are functionally stable for only 20-30 minutes.

The possible involvement of protein synthesis in the initial growth response to IAA has received considerable attention, but the question has not been resolved. The growth response to IAA is certainly within 10 minutes and possibly immediate, yet most reports for IAA action on protein synthesis have indicated a lag of at least 1 hour (Trewavas, 1968; Key, 1969) though a 10-minute latent period for the stimulation of RNA synthesis in Avena coleoptile segments has been reported (Masuda and Kamisaka, 1969). Thus the timing of IAA action on growth and protein synthesis has not elucidated

the problem and neither has the use of inhibitors of protein synthesis. The investigations of Evans and Ray (1969) revealed that pretreatment with actinomycin D, cycloheximide, or puromycin did not affect the latent period for IAA to stimulate growth but reduced the growth rate. Similarly, it has been demonstrated that after a treatment with cycloheximide, which was sufficient to inhibit over 90% of protein synthesis, wheat coleoptile segments exhibit a reduced response to IAA (Pope and Black, 1972) and yet Cleland (1970) reported that such a cycloheximide treatment completely abolished the response of Avena coleoptiles to IAA. Penny et al. (1972) concluded that protein synthesis is not required for the initial action of auxin on elongation though they emphasised that growth does depend on the availability of protein, thus explaining the potent and quick effect of cycloheximide on elongation.

If protein synthesis is not involved in the initial IAA mechanism then an alternative explanation for IAA action is required. Interesting conclusions have been drawn from studies of the effect of low-pH on the promotion of growth. Hydrogen ions at pH 3.0 have been shown to induce an immediate promotion of growth (Rayle and Cleland, 1970) and close similarities between IAA-induced and low-pH-induced growth have been demonstrated (Rayle and Cleland, 1972). The latter authors suggested that IAA induces elongation either by stimulating the release of protons from the protoplast and that these ions hydrolyse acid labile linkages in the cell wall, or by causing the appearance in the cell wall of an enzyme which can hydrolyse the acid-labile linkages.

There have been only a few reports of the accurate timing of ABA action on growth. In etiolated pea seedlings ABA inhibited the endogenous growth in 5 minutes (Warner and Leopold, 1971). ABA also inhibited the growth induced by IAA in Avena coleoptile segments (Zenk, 1970; Rehm and Cline, 1973) when the ABA was added after the IAA had initiated rapid growth and the latent period for the inhibition was shortened as the ABA concentration was increased (Rehm and Cline, 1973). The latter authors also stated that a 30" or 60"

minute pretreatment in 10^{-4}M ABA did not affect the latent period for the response to 10^{-6}M IAA. In addition, they examined the effect of several metabolic inhibitors and concluded that the kinetics of ABA inhibition were not the same as the kinetics of the transcriptional and translational inhibitors tested; they suggested that rigorous biochemical studies are required.

This thesis presents investigations into the mode of action of ABA in inhibiting cell elongation in Avena coleoptile segments. The molecular requirements for inhibitory activity have been examined both in the absence and presence of applied IAA. The action of ABA in the presence of IAA has also been studied. The possible interaction between these hormones was explored by statistical analysis of factorial experiments and by the utilization of the theories of enzyme reaction kinetics in an attempt to establish whether a competitive or non-competitive inhibition was involved. In addition, a transducer technique has been developed to facilitate the continuous monitoring of elongation with sufficient sensitivity to detect minute by minute changes in growth rate. This technique has been used to examine the initial effect of ABA on IAA-induced growth and to study the nature of the initial action of IAA itself. Finally, the uptake of radioactive IAA and ABA into coleoptile segments has been investigated to assist the interpretation of the growth data.

MATERIALS AND METHODS

Unless stated otherwise the following procedures were employed.

1. Plant Material and Cultivation

Experiments were carried out using coleoptile segments of *Avena sativa* L., cv. Svalöf Victory 1, the fruits (seeds) of which were obtained from Svalöf, Sweden.

The fruits were soaked in flowing tap water for 3-6h, sown at a depth of 20 mm in 50-60 mm of damp vermiculite in open polythene boxes, and grown in the dark at 25 ± 1°C. For experiments concerned with the continuous monitoring of the elongation of segments treated with ABA the vermiculite was soaked for 24h in an excess of water and drained for 15-30 minutes before planting the fruits. The latter procedure was used to increase the uniformity of the seedlings which developed. For all other experiments the vermiculite was obtained from a stock which had been mixed with water at least 24h before use.

In certain experiments the growing seedlings were exposed to red light for 24h during the 3rd and 4th days of development. The light source was a 60 watt tungsten filament bulb in a Kodak beehive light filtered with a red filter (Kodak, No. 1). The light was 600 mm above the seedlings.

Coleoptiles were excised from 4-day-old seedlings and placed on damp filter paper in a petri dish. Segments 5 mm in length were excised 2 mm below the tip using a cutting device with parallel blades and were placed on damp filter paper before use. For experiments concerned with the continuous monitoring of the elongation of segments treated with ABA the segments were excised from coleoptiles 15-25 mm in length.

2. Preparation of Solutions

a. Growth substances and analogues. Indole-3-acetic acid (IAA) was supplied by Sigma Chemical Company Ltd., London. RS(±) abscisic acid (ABA) and its analogues (Tables 1 and 2) were donated by Hoffmann-La Roche Ltd., Basle; the cis,trans- (Z,E-) isomer of ABA was used. All compounds were dissolved in a minimal quantity of redistilled organic solvent and dispersed in distilled water at 50°C to provide the stock solutions; working concentrations were prepared from the stocks by serial dilutions with distilled water. The organic solvent concentrations are listed in Table 3.

Table 3. Organic solvent concentrations and pH values for 10^{-5}M solutions.

Compound	ppm Methanol	ppm Acetone	pH of solution
IAA	15		5.4
c,t-ABA	40		5.7
t,t-ABA	40		-
I	100		6.1
II	100		5.0
III	100		5.4
IV	86		6.9
V		26	6.2
VI	42		5.6
VII	100		6.2
VIII	50		6.4
IX	200		6.1
X	100		6.4
XI	400		6.5
XII	24*		6.2
XIII	51		6.2
XIV	159*		6.5
XV	200		5.9
XVI	200		6.2
XVII	200		5.9
XVIII	158		6.4
XIX	200		6.2

Table 3 continued

Compound	ppm Methanol	ppm Acetone	pH of solution
XX	150		6.4
XXI	150		6.4
XXII	150		6.3
XXIII	200		6.4
XXIV	200		6.7
XXV	150		6.6
XXVI	86		6.2
XXVII	146*		6.4
XXVIII		10	6.2
XXIX	50		5.5
XXX	40		5.5
XXXI		200	6.2
XXXII	200		5.9
XXXIII	50		5.5

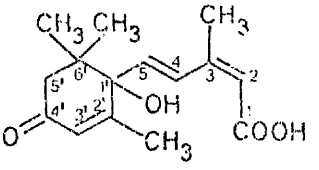
* The stock solution appeared slightly cloudy.

The IAA used in the experiments in which growth was monitored continuously was dissolved directly in distilled water which was stirred rapidly with a Magnetic stirrer and maintained at 50°C. All the stock solutions were stored in the dark at approximately 3°C. Solutions of IAA were kept for a maximum of 2 weeks and 5×10^{-3}M IAA was prepared within 12h of use.

b. Buffer solutions. Three buffer systems were used, all of which were prepared using "Analar" reagents. Phosphate buffer was prepared from aqueous solutions of 0.01M KH_2PO_4 and 0.01M K_2HPO_4 or from these salts at 0.02M. The 0.01M phosphate buffer was used to buffer IAA and ABA solutions to pH 7.0 although 0.01M K_2HPO_4 alone was used to buffer 5×10^{-3}M IAA providing a solution of pH 6.8 - 6.9. Citrate buffer was prepared from citric acid and Na_2HPO_4 with the final concentrations not exceeding 0.01M and 0.02M respectively. Glycine-HCl buffer contained 0.01M glycine (aminoacetic acid) adjusted to the required pH with 2N HCl.

Table 1. The molecular structures of cis, trans-abscisic acid and analogues I to XXII.

The Chemical Abstracts code number for ABA, and the numbering of the carbon atoms in the ABA skeleton, are also shown.



Abscisic Acid (ABA)

Chem Abstr code no -
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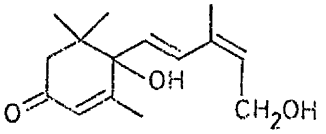
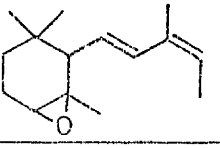
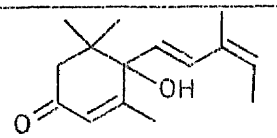
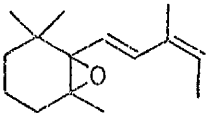
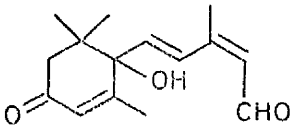
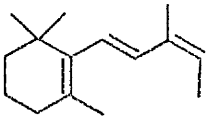
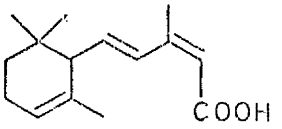
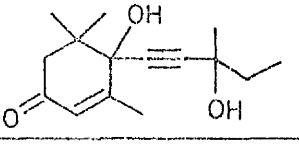
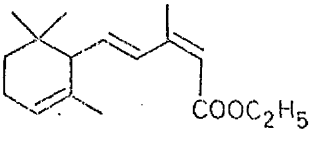
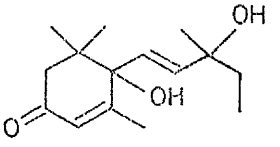
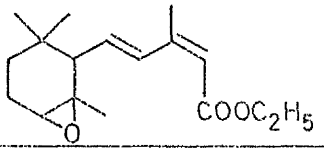
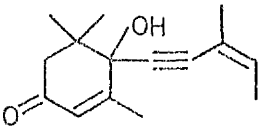
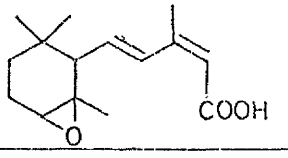
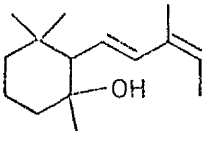
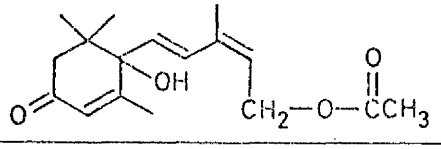
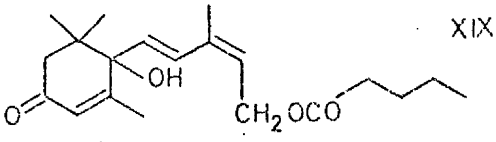
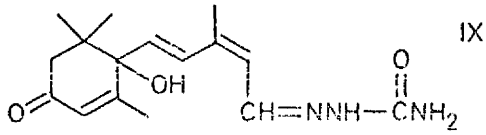
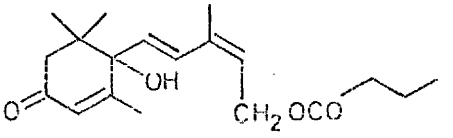
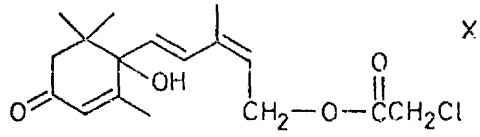
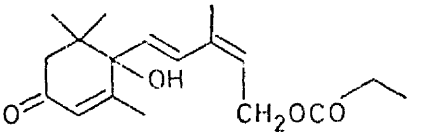
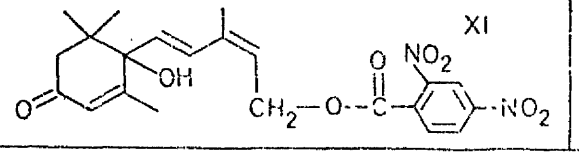
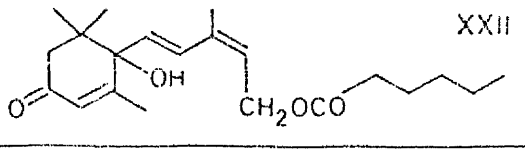
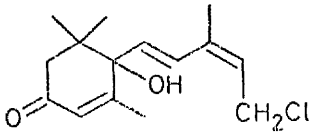
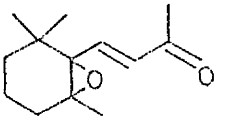
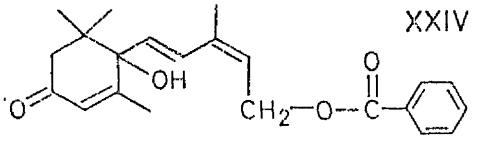
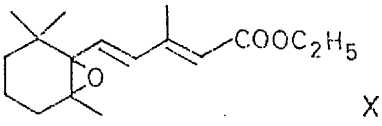
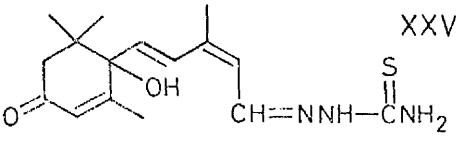
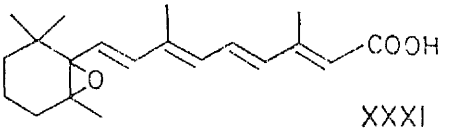
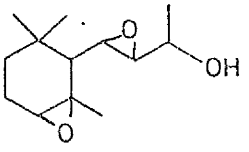
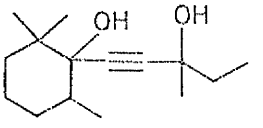
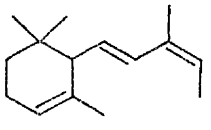
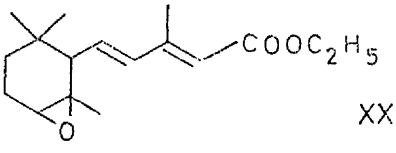
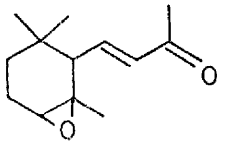
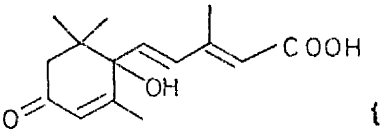
	I		XII
	II		XIII
	III		XIV
	IV		XV
	V		XVI
	VI		XVII
	VII		XVIII
	VIII		XIX
	IX		XX
	X		XXI
	XI		XXII

Table 2. The molecular structures of trans,trans-abscisic acid and analogues XXIII to XXXIII.

 XXIII	 XXIX
 XXIV	 XXX
 XXV	 XXXI
 XXVI	 XXXII
 XXVII	 XXXIII
 XXVIII	 t,t-ABA

3. pH Measurements

The pH of the solutions was measured with a Pye Model 79 or EIL Model 7030 pH meter using Pye pH electrodes. A microelectrode (Tough micro-combination electrode Type CMT, Russell pH Ltd., Fife) connected to the Pye meter was used for pH measurements of solutions contained in petri dishes. In all assessments of pH the meters were standardised using solutions of pH 7.0 and 4.0 prepared from buffer tablets.

4. Radioactive Growth Hormones

1-[^{14}C]IAA at a specific activity of 52 Ci M^{-1} (297 µCi. mg^{-1}) was obtained from the Radiochemical Centre, Amersham, U.K. The solutions used were diluted with distilled water from the laboratory stock which was kept in redistilled absolute methanol; 10^{-5}M 1-[^{14}C]IAA contained 10,000 ppm MeOH. Hoffmann-La Roche Ltd. donated the 2-[^{14}C($\pm$)ABA at a specific activity of 45 µCi mg^{-1}. A stock solution of 4.66×10^{-5}M was prepared containing 5000 ppm MeOH and working solutions were diluted from this using either distilled water or non-radioactive ABA; the latter procedure provided 10^{-5}M 2-[^{14}C]ABA of specific activity 9 µCi. mg^{-1}. All solutions were stored in a refrigerator at approximately 3°C.

5. Straight Growth Assay

Segments were prepared under dim green light; the time between the excision of coleoptiles and the placement of segments in the test solution was typically 10 - 15 minutes. For each replicate ten segments were placed in 10 ml of test solution in a 50 mm plastic petri dish. After 24h dark incubation at 25 $\pm$ 1°C segment lengths were measured to the nearest 0.1 mm using a shadowgraph technique. In experiments to test the activity of analogues in the presence of IAA the analogue solutions were pipetted at

twice the molarity required and diluted with distilled water or IAA. In these experiments, which also indicate the activities of the analogues alone, there were 2 replicates of 10 segments for each treatment. Certain analogues were only tested alone, pipetted at the molarity required with 1 dish of 10 segments for each treatment.

Neither methanol nor acetone have significant effects in this assay over the concentration ranges 0.01 - 500 ppm and 0.1 - 1000 ppm respectively (Fig. 1a and b). Consequently, water controls were used in all assays, arranging at least 1 control for each box of plant material used.

6. Shadowgraph Technique

Segments were filtered from the incubation media, arranged in rows on a glass plate and the assembled plate placed in a photographic enlarger. The images were focused at a fivefold magnification and exposed on photographic paper. Permanent records were prepared by developing the paper in Ilford Contrast Developer (1:4 dilution) and fixing in Kodafix solution (1:4 dilution). After the paper had been washed and glazed on a Kodak glazing machine the images were used for the determination of segment lengths.

7. Continuous Monitoring of Elongation

a. The apparatus. Three techniques were developed to monitor accurately the elongation growth of segments.

Preliminary experiments were carried out using a microscope technique based on that of Ray and Ruesink (1962). The elongation of a single segment, contained in solution in a plastic chamber, was monitored using a high power binocular microscope (Patholux Microscope, Vickers Ltd.) fitted with a micrometer eyepiece. The elongation of individual cells was also examined using this technique.

In the second technique the growth of a column of 10 segments was

Fig. 1. The effect of organic solvent concentration on segment elongation in the Avena coleoptile straight growth assay.

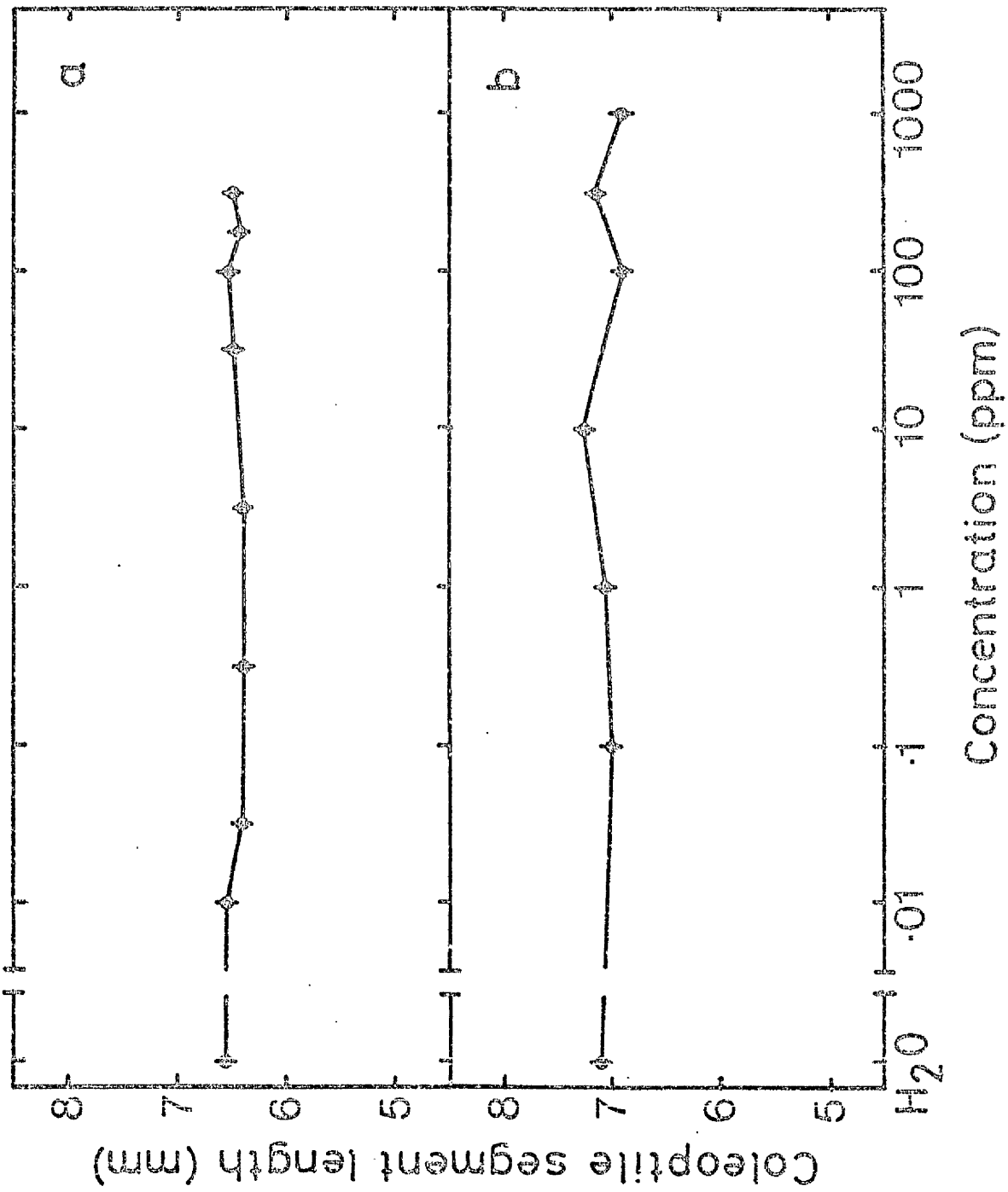
a) Methanol. Mean data from one experiment, 20 segments per treatment.

b) Acetone. Mean data from one experiment, 20 segments per treatment.

Similar data were obtained in two repeats of these treatments.

Statistical Analysis. The t-test was used to test the difference in segment length between the water control in each experiment and the organic solvent concentration indicated.

	Solvent concentration	t value	Significance
a) Methanol	0.01	.090	NS
	0.05	1.467	NS
	0.5	1.376	NS
	5.0	1.559	NS
	50.0	0.438	NS
	100.0	0.102	NS
	250.0	1.142	NS
	500.0	0.043	NS
b) Acetone	0.1	0.698	NS
	1.0	0.400	NS
	10.0	0.996	NS
	100.0	1.268	NS
	500.0	0.278	NS
	1000.0	1.466	NS


a
b
Coleoptile segment length (mm)
8
7
6
5
H_2O
.01
.1
1
10
100
1000
Concentration (ppm)

monitored using a lever system and smoked kymograph drum. The segments were threaded and supported as described for the following transducer technique.

The third technique employed a linear displacement transducer (Type D2/2.00 mm, Sangamo Weston Controls Ltd., Plate 1). This technique was refined during the project and consequently experiments have been carried out with two forms of the apparatus. The basic measuring system and technique was as follows. Ten coleoptile segments were threaded on to a nylon line of 0.4 mm diameter ("Racine Torture" Nylon fishing line, 15 lb or 16 lb) and placed in a 30 ml cylindrical reservoir which was supported in a metal frame. Polythene tubing was threaded above and below the coleoptile segments to provide contact with a perspex lever threaded on the nylon line (Fig. 2A). The lever was counterbalanced with a small weight; the weight acting downwards on the coleoptiles was calculated to be approximately 200 mg. Elongation of the segments resulted in movement of the lever which displaced the central armature of the transducer (Fig. 2B). The transducer operated on a stabilized 12 volt D.C. supply and generated a D.C. output signal proportional to the displacement of the armature; the proportionality was linear for a 2 mm armature displacement (Sangamo Weston Controls, Ltd.). The reservoir containing the threaded segments was held in an adjustable stand allowing the segments to be easily re-positioned relative to the transducer armature. The armature displacement could thus be maintained within the linear range. The output signal was connected to a potentiometric chart recorder and elongation was monitored continuously.

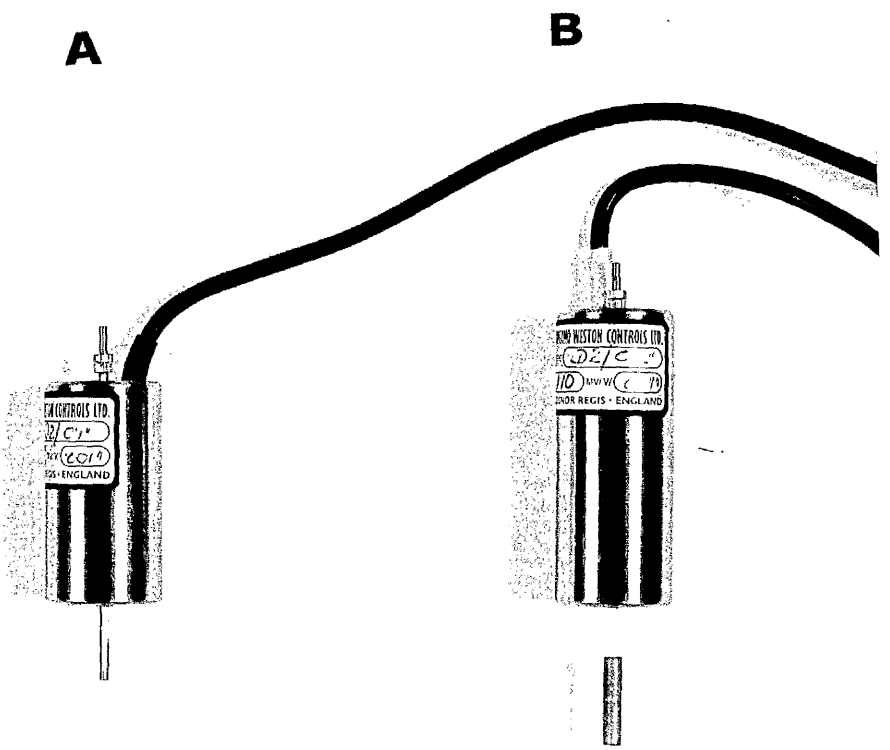
The first measuring system (apparatus I, Plate 2a and b) utilized a reservoir constructed from a plastic measuring cylinder which was supported in a 'Meccano' frame. The counterbalance weight was hung from the lever and solutions were supplied to the reservoir in polythene tubing.

Subsequently, two additional measuring systems were constructed (apparatus II and III, Plate 3a and b). These were of essentially similar

Plate 1. Linear displacement transducers.

A. Transducer with a 2mm linear displacement, as used in the experiments reported in this thesis.

B. Transducer with a 5mm linear displacement.



DISPLACEMENT TRANSDUCERS

Fig. 2. Apparatus for the continuous monitoring of the elongation of coleoptile segments in solution.

A) The reservoir with coleoptile segments in position. The lever acts at right angles to the plane of the diagram.

B) Arrangement of the lever, reservoir and transducer.

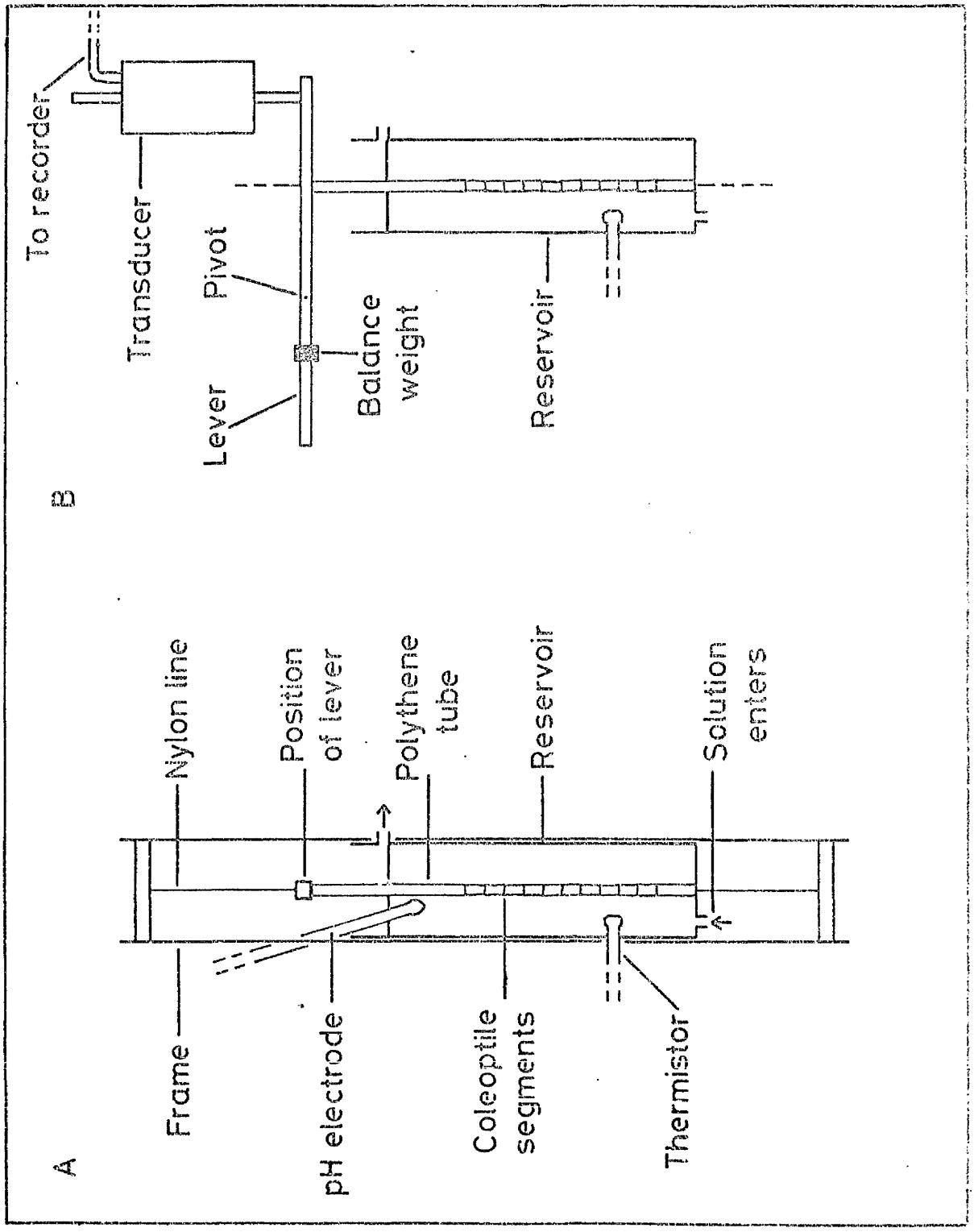
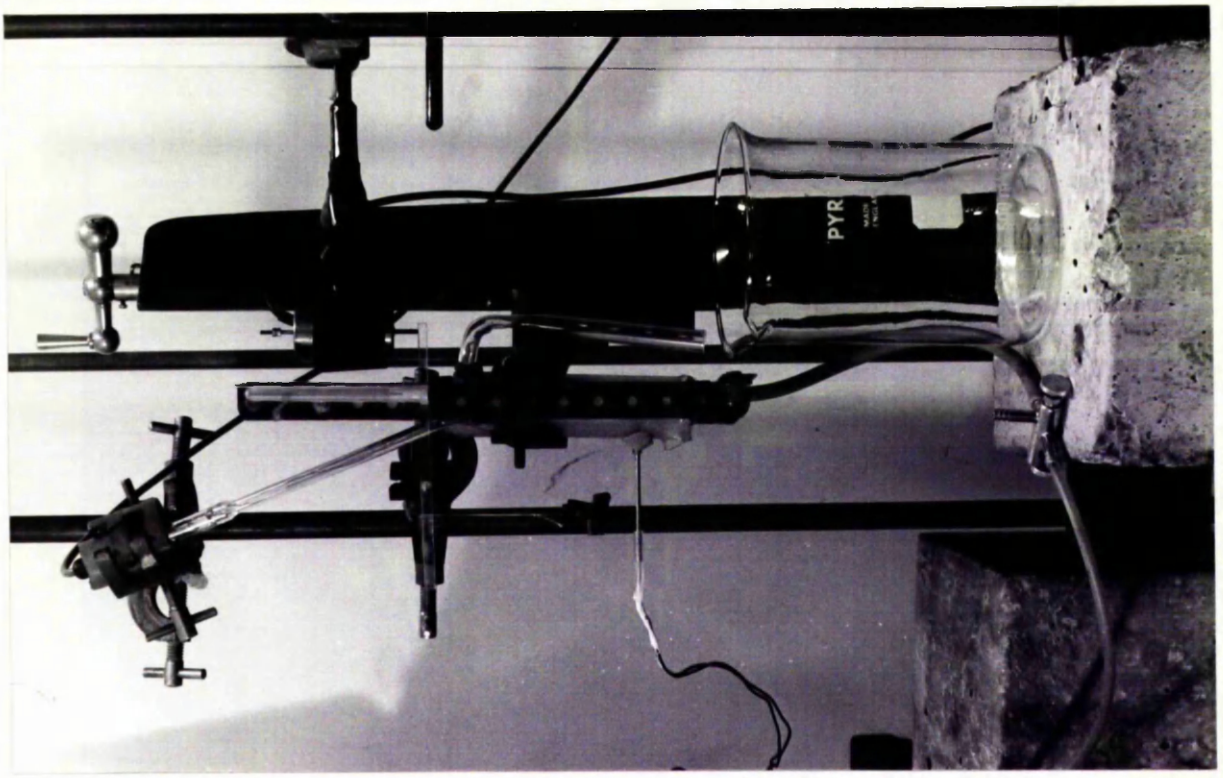

B
To recorder
Transducer
Pivot
Lever
Balance weight
Reservoir
A
Nylon line
Position of lever
Polythene tube
Reservoir
Solution enters
Frame
pH electrode
Coleoptile segments
Thermistor

Plate 2. Apparatus for the continuous monitoring of the elongation of coleoptile segments in solution.

a) Plastic reservoir in frame.

b) Reservoir, lever and transducer arranged for the monitoring of elongation.


PYR

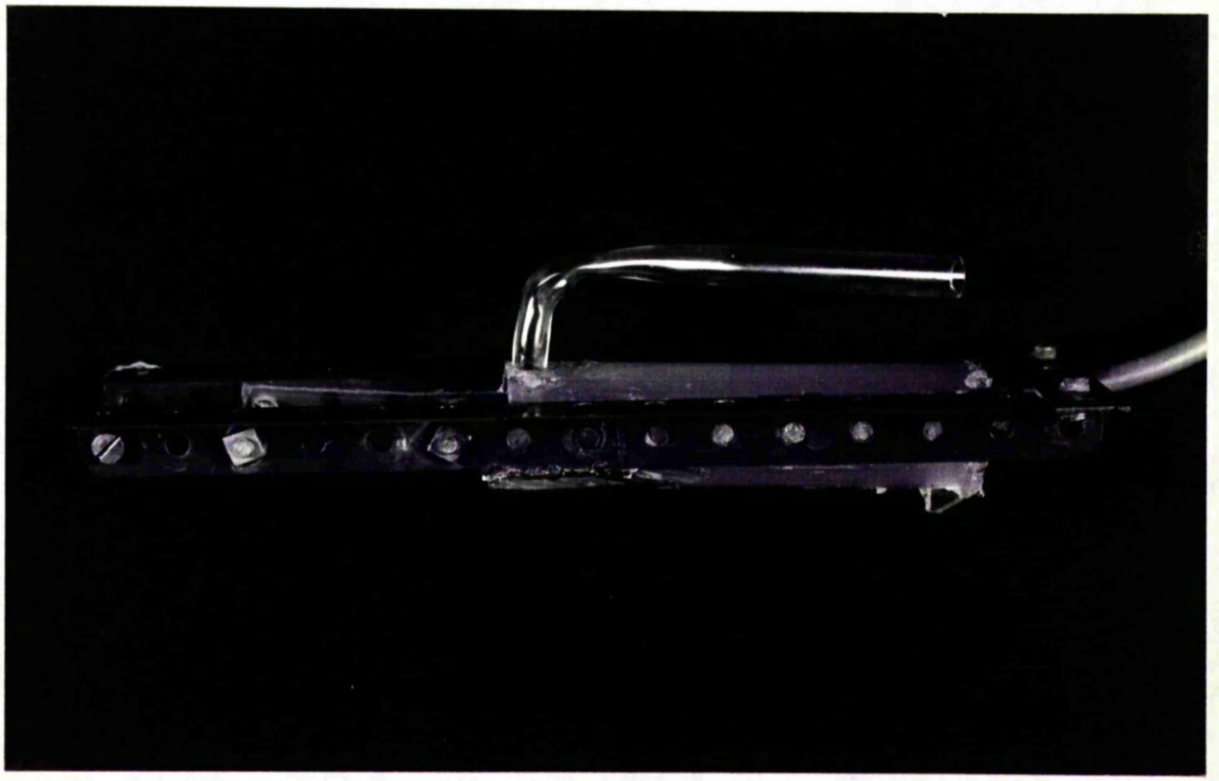
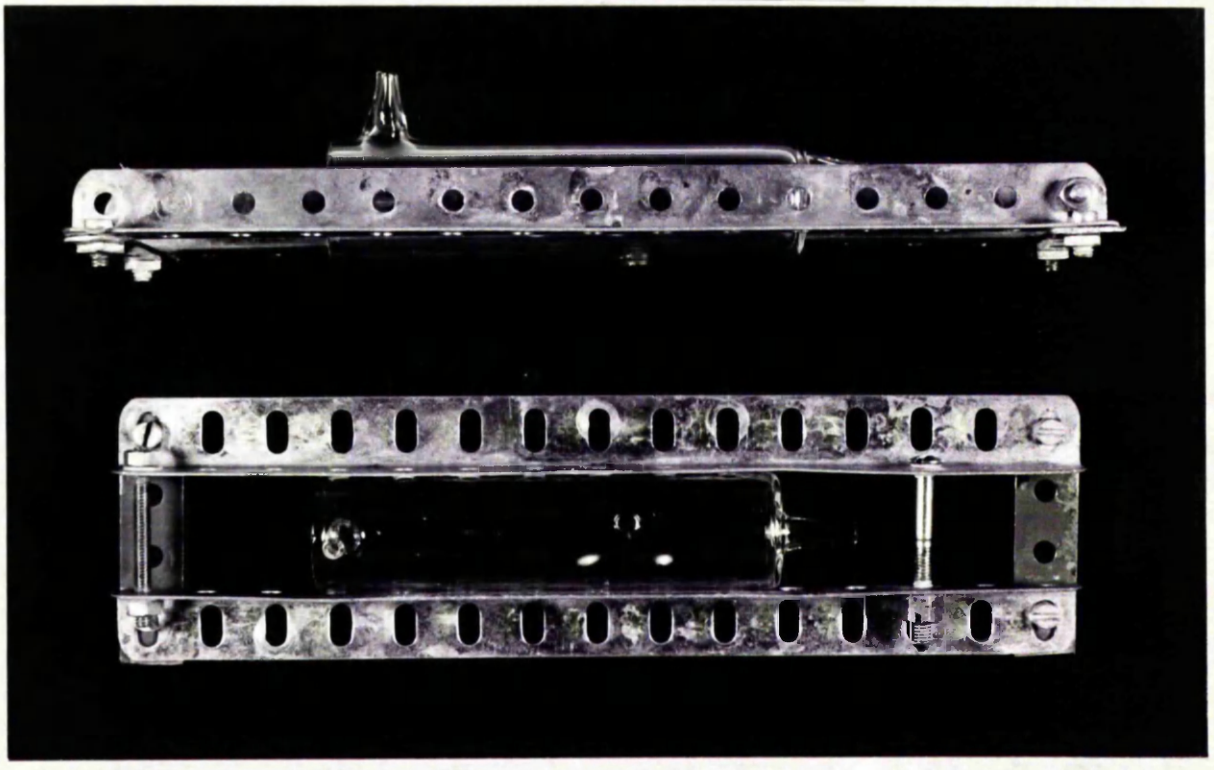


Plate 3. Apparatus for the continuous monitoring of the elongation of coleoptile segments in solution.

a) Glass reservoirs in their frames.

b) Complete equipment set-up for the monitoring of elongation. The plate illustrates the water bath for the incubation of the solutions, rubber tubing which leads the solutions to the reservoirs, the transducers, chart recorders and instruments for the measurement of temperature and pH.



design to apparatus I but the reservoirs were glass, the balance weights were fixed on to the levers and the solutions were supplied to the reservoirs in rubber tubing. The two recording systems enabled treatment and control experiments to be monitored simultaneously; treatment or control were assigned randomly to each apparatus.

The solutions were incubated in conical flasks in a water bath, the temperature of which was regulated. The flow of solutions to the reservoirs was maintained by passing air into the conical flasks, thus providing a pressure feed system. The flow was regulated using three-way taps on the tubes delivering the solutions.

The power supply for the transducers was modified from the mains using the circuit illustrated in Fig. 3a. The transformer and rectifier converted the mains supply to 12 volts D.C. which was stabilized with the Zener diode. The voltage output from this supply was checked at intervals with an "Avometer" and no alterations in voltage were observed.

The temperature of the solution in the reservoir was continuously monitored using a thermistor inserted through a hole in the side of the reservoir; the hole was sealed with plasticine. A naked bead thermistor (Type VA 3102, Mullard Ltd.) of resistance 2.2K at 25°C, was used, with the bead embedded in the end of a thin metal tube using "Araldite". The thermistor was connected to a Wheatstone bridge circuit (Fig. 3b) which was supplied with a constant voltage from a 9 volt battery. As the temperature of the thermistor increased its resistance decreased and this resulted in an increase in current in the circuit. The change in current was monitored on an ammeter. The system was calibrated over the range 10°C - 35°C using water, the temperature of which was measured with a mercury thermometer (Fig. 4).

The pH of the solution in the reservoir was continuously monitored using an EIL pH meter (Model 7030) and Pye pH electrode with a small bulb which was immersed in the solution in the top of the reservoir.

Fig. 3. Diagrams of electrical circuits.

a) The power supply for the transducers.

b) The circuit for the thermistor.

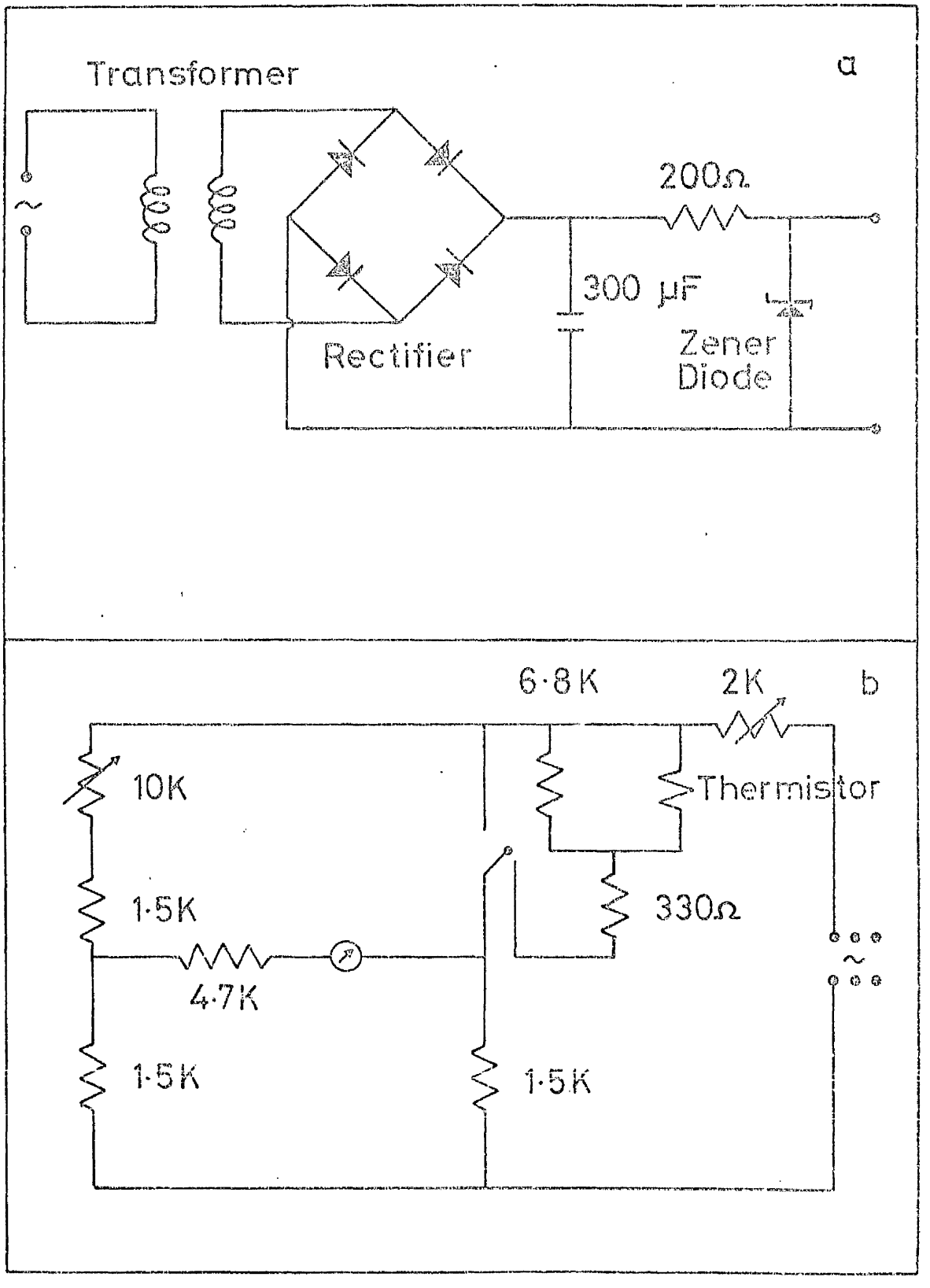
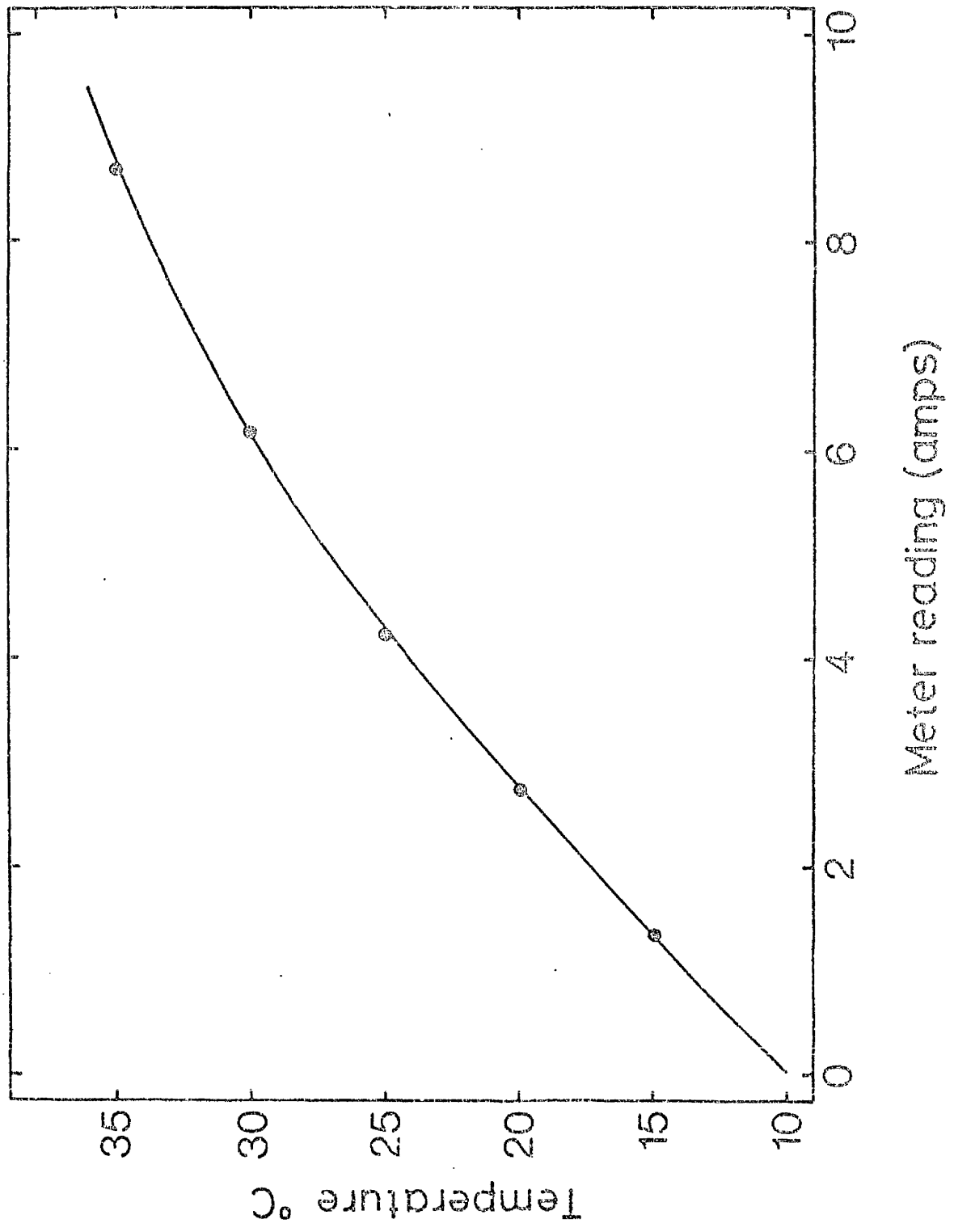

Transformer
a
200Ω
300 µF
Rectifier
Zener
Diode
6·8K
2K
b
10K
Thermistor
1·5K
330Ω
4·7K
1·5K
1·5K

Fig. 4. Calibration curve for thermistor.

The graph shows the effect of increasing the temperature of the water in which the bead of the thermistor was immersed, on the current in the electrical circuit to which the thermistor was connected.


Temperature °C
35
30
25
20
15
10
0
2
4
6
8
10
Meter reading (amps)

Temperature and pH measurements were always made in apparatus I but for paired experiments these measurements were made in apparatus II only.

b. Experimental conditions. Experiments were carried out in dim green light. The ambient temperature was maintained at 25 $\pm$ 1°C for experiments using solutions of this temperature but for experiments at other temperatures the ambient temperature was adjusted to aid regulation of the temperature in the reservoir. In addition, the tubing supplying the solutions to the reservoirs was lagged with cotton wool in experiments using solutions at low and high temperatures. The solution temperature was maintained to within one centigrade degree of the value quoted.

Solutions were passed through the reservoir at 5 ml min.$^{-1}$, the flow was increased to 100 ml min.$^{-1}$ for at least one minute when solutions were changed and this achieved an estimated 98% change in log. concentration of the solution after 1 minute. Flowing solutions were not used for treatments using KCN and their associated controls; the flow of incubation buffer was stopped and the reservoir was drained and refilled twice with the new solution. This solution change was complete within 1 minute.

Solutions of ABA at 10^{-5}M contained 40 ppm MeOH and this concentration of organic solvent was present in all solutions.

The following experiments were carried out with the apparatus indicated:

(i)	The incubation of segments before treatment (Section 6b)	Apparatus I
(ii)	The effect of temperature and IAA concentration on the latent period and rate of IAA-induced elongation (Sections 6d and e)	Apparatus I
(iii)	The response to ABA (Section 6f)	Apparatus II and III
(iv)	The effect of KCN on IAA-induced and low pH-induced elongation (within Section 6d)	Apparatus II and III

c. Calibration and sensitivity of the instrumentation. A separate lever was used in each apparatus which was calibrated independently. The lever was displaced, at the position that the coleoptiles act, specific distances using a micrometer screw and the displacement on the recorder chart measured. The chart displacements produced by 0.5 mm and 0.1 mm micrometer adjustments at recorder sensitivities of 5V and 1V respectively, were measured, over a total lever displacement of 2 mm. The chart displacement was linearly proportional to the lever displacement over this 2 mm range and mean calibration data are presented in Table 4.

Table 4. Column elongation in μm equivalent to 1 division (2 mm) on the recorder chart. The recorder sensitivity refers to the voltage required to produce full scale deflection of the recorder pen.

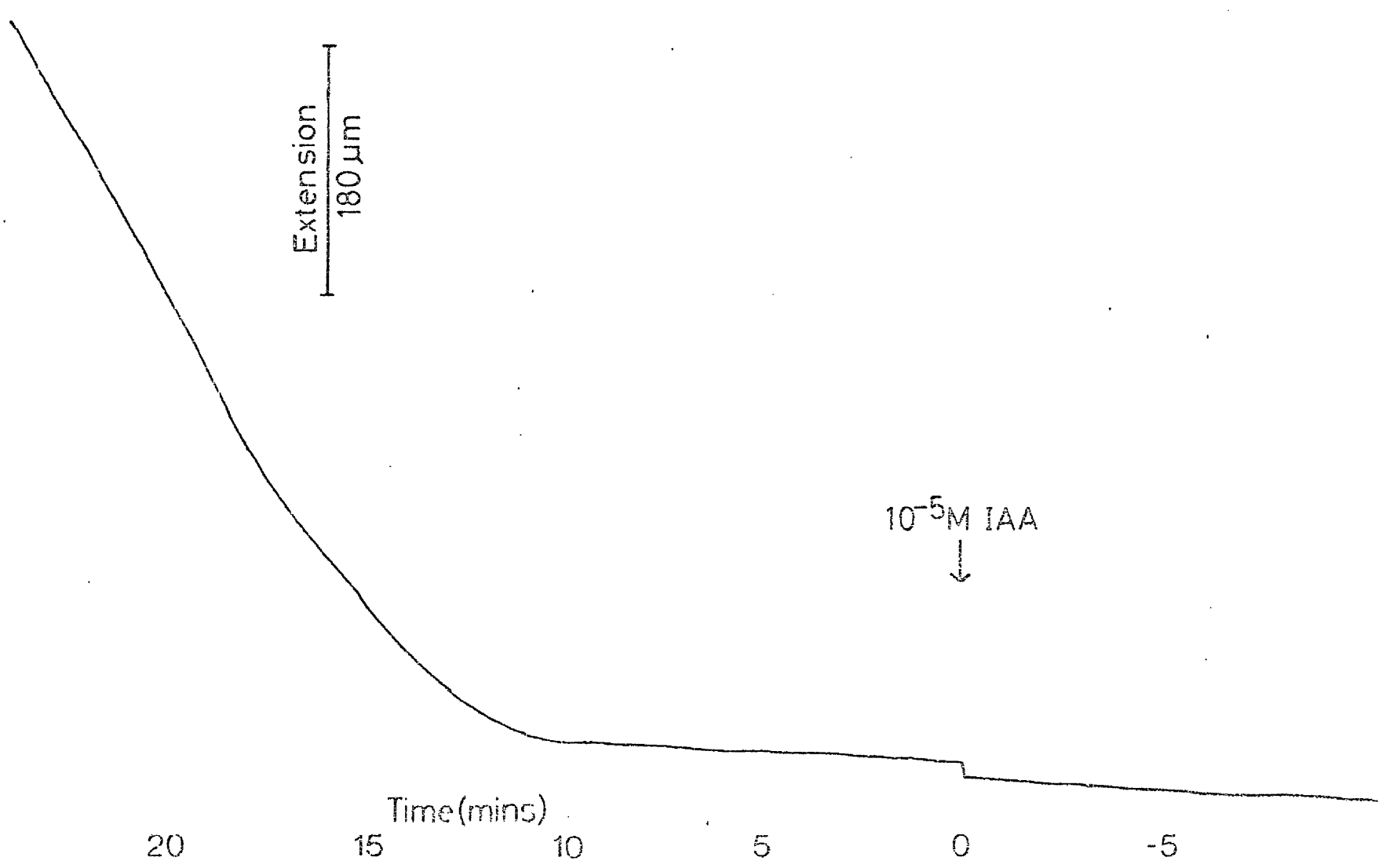
	Column Elongation	
Recorder Sensitivity	5V	1V
Apparatus		
I	33.2	6.6
II	33.2	6.6
III	31.9	6.4

A chart displacement of 1 mm equivalent to approximately 15 μm elongation of the column of segments and a chart speed of 2 mm $min.^{-1}$ were routinely used but both settings were increased fivefold for measurements of latent periods.

Figure 5 illustrates the original trace from one typical experiment. The instrumentation was able to detect a latent period before the IAA response with an accuracy of 0.5 minutes in an individual experiment and could detect a growth rate of less than 2 μm $min.^{-1}$ for the column of 10 segments.

Fig. 5. Illustration of a trace from the recorder during the continuous monitoring of elongation of *Avena* coleoptile segments using the transducer technique.

This is a graph of elongation of the 10 segments in μm as a function of time in minutes. After 100 minutes incubation of segments in phosphate buffer at pH 7.0 and 25°C there is a low steady growth rate, shown from -5 to 0 minutes. At time zero (0 minutes) the buffer was changed for 10^{-5}M IAA at pH 7.0 and 25°C. There is a slight discontinuity in the trace at time zero but the low growth rate continues for a latent period of 10.5 minutes before rapid growth is initiated. A growth rate of 53 μm $min.^{-1}$ for the column of 10 segments is observed after 20 minutes.


Extension
180 µm
10^{-5}M IAA
Time (mins)
20
15
10
5
0
-5

d. Terminology. The term "latent period" refers to the time from the commencement of a treatment to the observation of an effect. For the response to IAA and low pH the latent period was determined from the recorder charts. To determine the point at which significant ABA inhibition occurred a paired analysis on 5 replicates of the treatment and control was frequently carried out.

Time course graphs are presented either as elongation (mm) of the column of 10 segments VS time (minutes) or as rate of elongation (μm min.$^{-1}$) VS time. The rates are presented at 5-minute intervals and the rate at a specific time was calculated over a 10-minute period starting 5 minutes before that point in time.

The term "initial maximum growth rate" refers to the first maximum in growth rate minus the steady growth rate in buffer, the latter having been calculated from -15 minutes to time zero.

e. Estimation of the efficiency of changing the solutions. A solution of 10^{-4}M IAA in buffer, having an absorbence maximum at 280 nm was used to monitor the efficiency of solution changes. A typical U.V. spectrum for this IAA solution is illustrated in Fig. 6. The solution in the reservoir was changed, using flowing solutions, from buffer to IAA and vice versa. Five 25 ml aliquots of solution overflowing from the reservoir were collected immediately the solution change began and their absorbence spectra measured in a Unicam SP 8000 Ultraviolet Recording Spectrophotometer.

The data from the spectra indicate that the absorbence at 280 nm is proportional to the log. IAA concentration (Fig. 7a). The absorbence due to IAA in the aliquots tested is expressed as a percentage of the absorbence due to IAA in the 10^{-4}M stock solution (Fig. 7b and c). The data show there is a rapid change of solutions both when adding and removing IAA. In the 5th 25 ml aliquot, which represents the solution in the reservoir after a 100 ml change of solution, there has been a 98% change in log. IAA concentration in both instances.

Fig. 6. Absorbence spectrum for 10^{-4}M IAA in 0.01M phosphate buffer (pH 7.0). The base line indicates the absorbence due to the 0.01M phosphate buffer alone.

The spectra were obtained with a Unicam SP 8000 Ultraviolet Recording Spectrophotometer, using a distilled water reference sample.

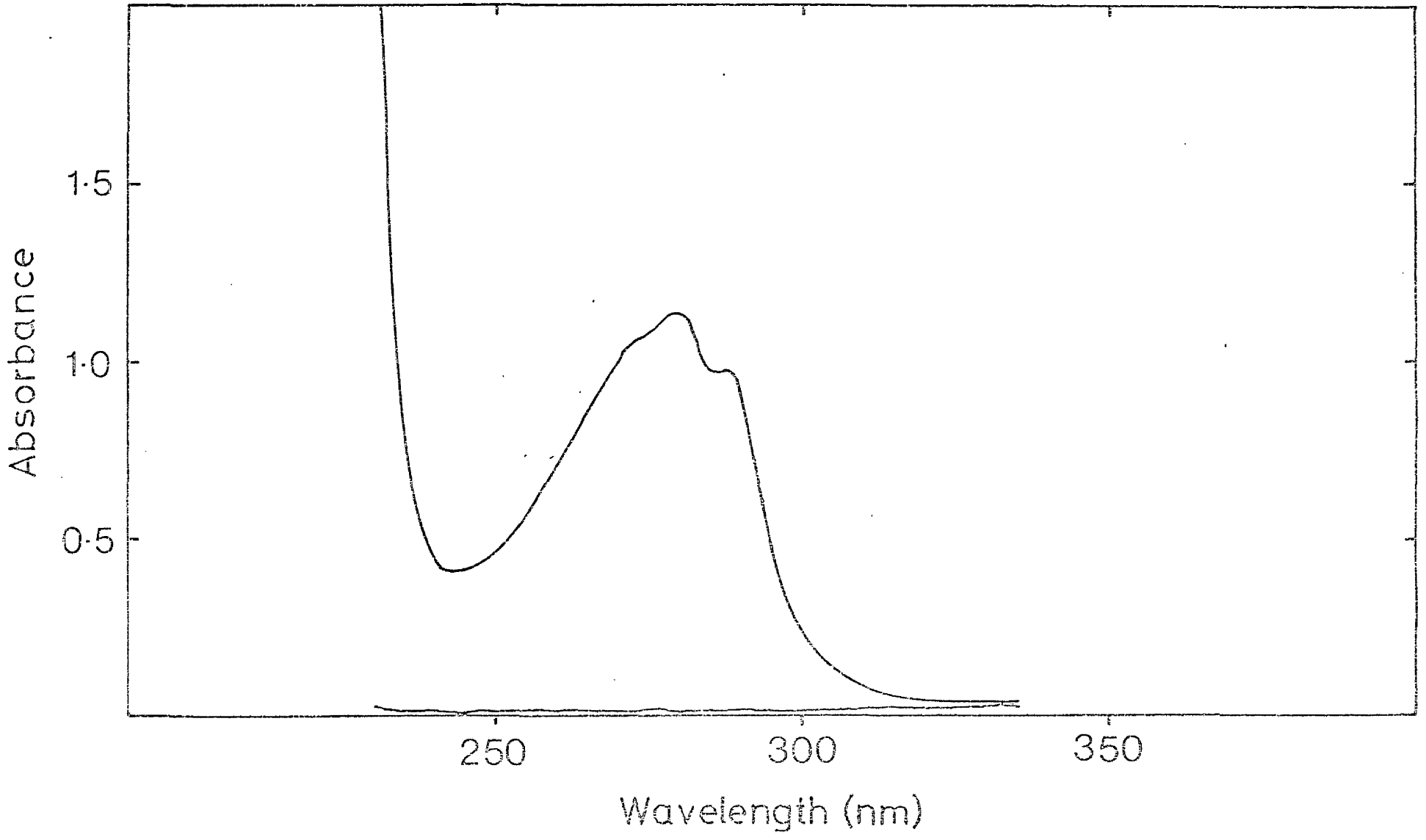

Absorbance
1·5
1·0
0·5
250
300
350
Wavelength (nm)

Fig. 7.

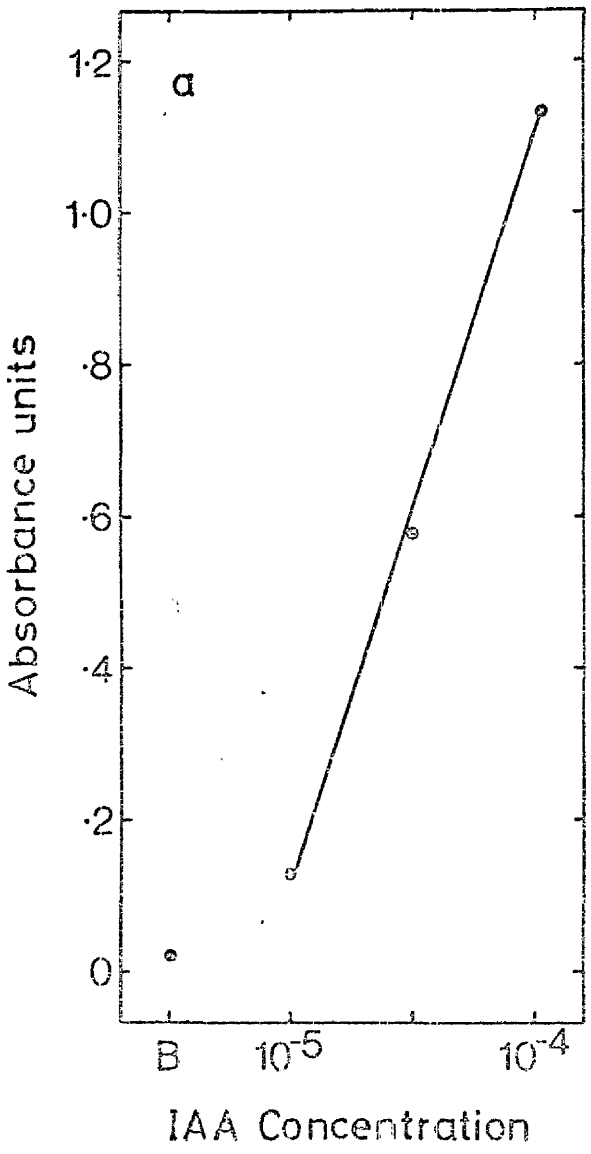
a) U.V. absorbence, at 280 nm, of IAA solutions in 0.01M phosphate buffer (pH 7.0), as a function of IAA concentration. 'B' refers to the buffer solution alone.

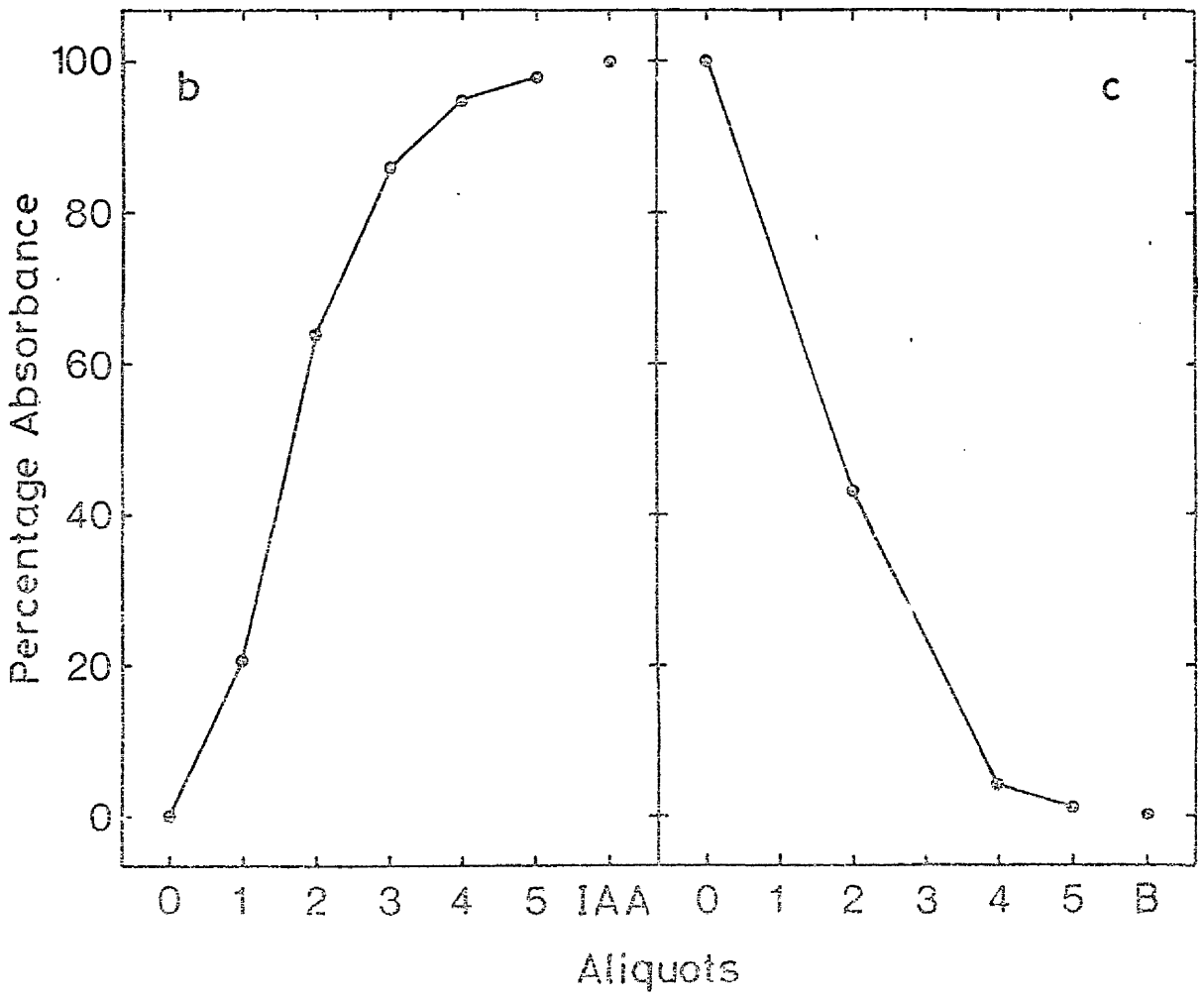
b) and c) Absorbence at 280 nm due to IAA in consecutive 25 ml. aliquots overflowing from the reservoir when changing solution. The absorbence is expressed as a percentage of the absorbence due to 10^{-4}M IAA in 0.01M phosphate buffer.

b) Adding buffered IAA to the reservoir containing buffer.

c) Adding buffer to the reservoir containing buffered IAA.

'0' refers to the solution originally in the reservoir.


a
1·2
1·0
·8
·6
·4
·2
0
Absorbance units
B
10-5
10-4
IAA Concentration


b
c
100
80
60
40
20
0
Percentage Absorbance
0 1 2 3 4 5 IAA 0 1 2 3 4 5 B
Aliquots

9. Uptake of Radioactive Growth Substances

Experiments were carried out at 25 ± 1°C in dim green light using either threaded or floating segments. Radioactive solutions were buffered to pH 7.0 using 0.01M phosphate buffer which was also used as the incubation medium. The methanol concentrations in the radioactive solutions were adjusted to be constant.

For experiments using floating segments the required number of segments were incubated in 25 ml of buffer or ABA for 100 minutes and groups of 10 segments transferred to 1 ml of radioactive solution in a 1 dram vial, using three replicates for each treatment.

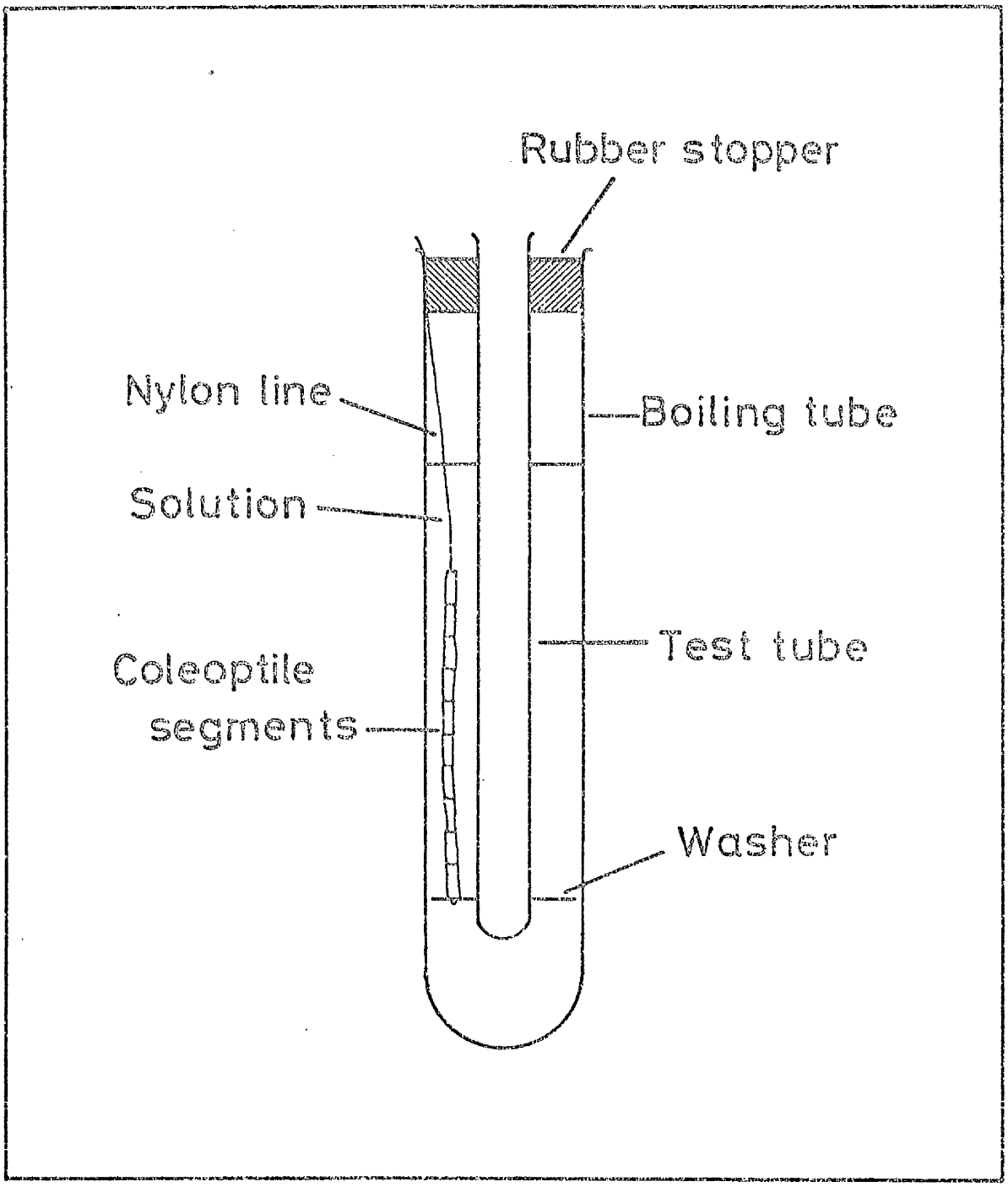
Alternatively, 10 segments were threaded on to a nylon line (0.4 mm diameter) and columns of segments were supported in 25 ml of incubation medium in the space between a boiling tube and a narrow test tube which was supported in the boiling tube (Fig. 8). This was achieved by securing one end of the nylon line to a washer around the lower end of the test tube and supporting the free end of the line against a rubber bung, which positioned the test tube centrally in the boiling tube. This system allowed uptake to be studied under conditions similar to those in the continuous monitoring of growth experiments. In addition, several columns of segments could be incubated in a small volume of solution, the solution could be changed easily by removing the test tube and segments to a boiling tube containing a new solution, and the columns of segments could be removed easily from the solution. Columns of segments were incubated in buffer or ABA for 100 minutes and then transferred to radioactive solution.

Whether floating or threaded segments were used, they were removed from the radioactive solutions after known time periods and the radioactivity in the segments assayed.

To investigate the removal of surface held radioactivity three columns of threaded segments were incubated for 100 minutes in buffer followed by

Fig. 0. Apparatus for examining the uptake of radioactive compounds into threaded coleoptile segments.

One column of 10 segments, immersed in solution, is illustrated.


Rubber stopper
Nylon line
Boiling tube
Solution
Test tube
Coleoptile
segments
Washer

exposure to radioactive ABA. After 1h treatment the segments were removed and each group of 10 segments placed on gauze in a filter funnel and 5 volumes of distilled water consecutively poured over the segments, collecting each volume separately. Radioactivity in the washings was assessed.

9. Assessment of Radioactivity

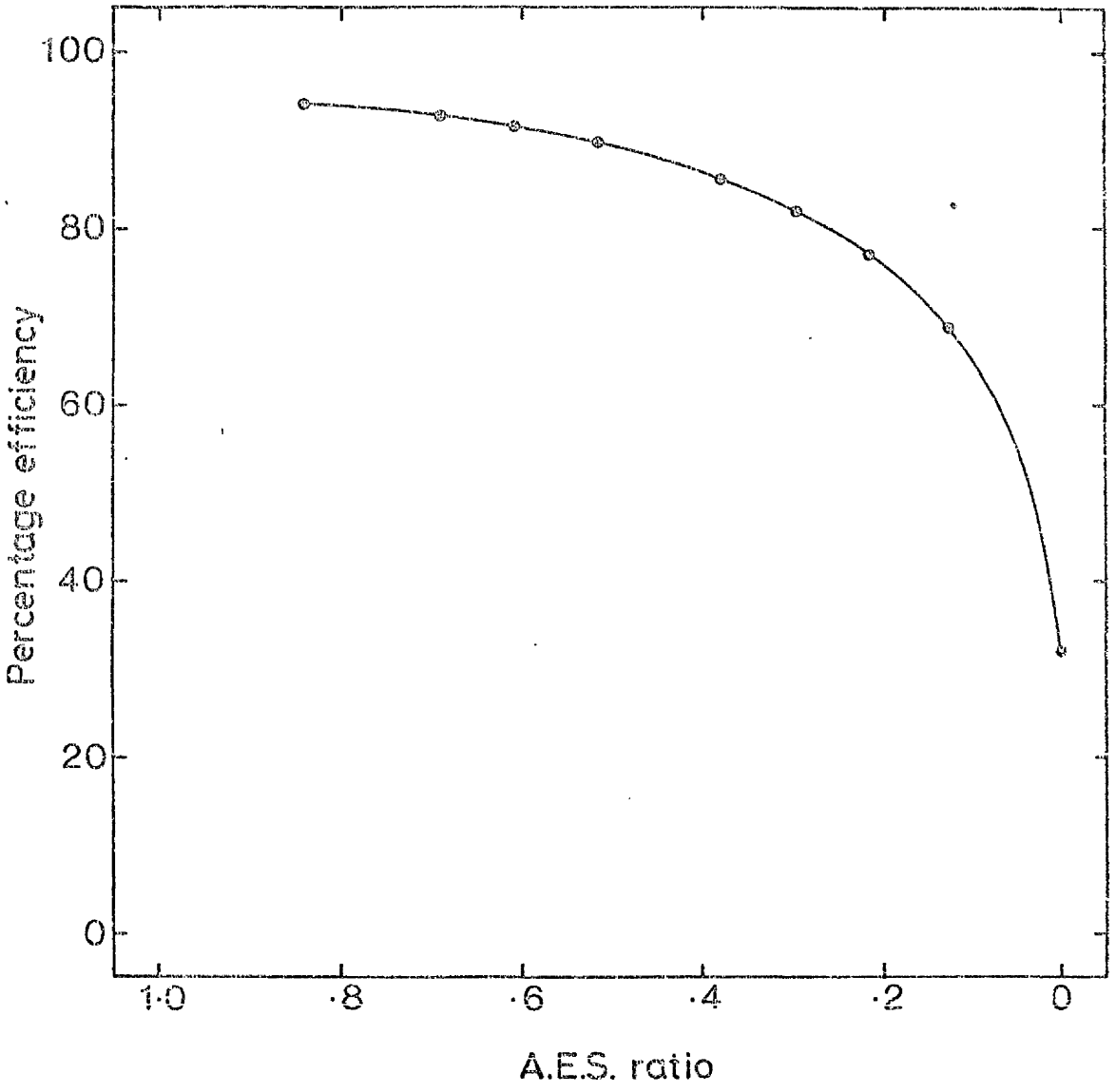
After treatment in radioactive solutions groups of 10 segments were washed in 3 x 10 ml distilled water to remove surface held activity. The segments were placed in 2 ml of 95% ethanol in a glass scintillation vial and kept at 3°C for at least 24h to extract the radioactivity. The ethanol was removed under reduced pressure and 10 ml of scintillation fluid containing 4 g. PPO (2,5 diphenyloxazole) per litre of commercial grade toluene were added to each vial.

The activity in 10 ml distilled water samples which had been used to wash radioactive segments, was assessed with a different procedure. A 3 ml aliquot of each washing was placed in a scintillation vial and 10 ml of Insta-Gel scintillation fluid (Packard Ltd.) were added to form a gel.

Radioactivity was detected using a Packard Tri-carb scintillation spectrometer. The instrument printed radioactivity in counts per minute (cpm) corrected for a preset background and an A.E.S. ratio which indicated the quench level for each vial. An A.E.S. ratio of 0 indicated complete quench and 10 indicated no quench. The efficiency of counting was governed by the quench of the sample. A quench correction curve (Fig. 9) was determined by assessing the cpm from vials which contained N hexadecane-1-^{14}C (specific activity 1.016 µCi g.$^{-1}$; Radiochemical Centre, Amersham, U.K.) of known activity and quenched to different levels using acetone, ethanol and plant material. Radioactivity in disintegrations per minute (dpm) was calculated from this curve.

Fig. 9. Quench correction curve for ^{14}C.

The graph indicates the efficiency of detecting radioactivity as a function of the quench level of the sample (A.E.S. ratio).


100
80
60
40
20
0
Percentage efficiency
1·0
·8
·6
·4
·2
0
A.E.S. ratio

10. Gas Liquid Chromatography of ABA

Gas liquid chromatography was carried out using a Pye Unicam 104 Series Gas Liquid Chromatograph fitted with a 5' OV 210 column. The carrier gas was nitrogen and the temperature was typically 200°C. Samples were methylated with excess diazomethane which was afterwards evaporated. The methyl ester of ABA was resuspended in methanol and injected into the column together with a standard of octokosane (C 28) dissolved in heptane. A 1 µl sample containing 3 µg methyl-ABA together with 1 µg C28 in 1 µl heptane was injected into the column.

a. Ultra-violet irradiation. U.V. irradiation was carried out using a Camag Universal U.V. lamp with a wavelength of 254 nm. A methylated sample of ABA was placed in a silica cell and irradiated for 3h.

b. Preparation of diazomethane. Sodium hydroxide (20g) was dissolved in 50 ml H_2O in a 500 ml narrow necked conical flask and a layer of 100 ml ethylene glycol placed over it. A 25 ml layer of ether was placed on top and a quantity of nitrosane added, rinsing the nitrosane from the neck of the flask with ether. A cork stopper and delivery tube were fitted to the flask and the diazomethane evolved was collected in cold ether until a yellow solution was formed.

11. Green Lights

Two light sources were used, viz: a 3ft. Atlas fluorescent tube (30 watt, Warm White) supported 3ft. above the preparation area, and a Philips 60 watt tungsten filament bulb in a Kodak beehive light which was approximately 1ft. from the material while setting up experiments. Both light sources were filtered with Cinamoid filters (Rank Stand Electric Ltd., London) using 3 green filters (No. 39) and one orange filter (No. 5). Visual examination of

these filtered sources using a hand spectroscope revealed a narrow band of green light but there was also blue and red light visible adjacent to the green. Nevertheless, coleoptile segments responded to IAA at 10^{-7} to 10^{-4}M after setting up a 24-h straight growth assay in the presence of these lights and both treatments and controls received the same exposure to light.

12. Statistical Analysis

a. Replication. Experiments involving straight growth assays and uptake studies were repeated on 3 occasions and when only one parameter, e.g. activity of ABA at different concentrations, was investigated the mean values of the 3 repeats were calculated. Statistical comparisons were made only between treatments and controls where both were carried out on the same 3 occasions. For comparisons between treatments within one experiment, e.g. the activity of ABA at different IAA concentrations, the statistical analysis was carried out on the mean data from 1 experiment only. The analysis was repeated on experiments carried out on different occasions.

There was no replication within experiments on the continuous monitoring of the elongation of segments. The means and standard errors quoted are typically derived from experiments carried out on 5 occasions and comparisons between different treatments are made using these mean values.

b. Standard error. The standard error of the mean value for each series of observations was calculated from the relationship:

$$\text{Standard error} = \frac{\text{Standard deviation}}{\sqrt{\text{number of observations}}}$$

It was calculated using an Olivetti programma 101 desk top computer (British Olivetti Ltd., London).

The standard errors are shown on the graphs as vertical bars, typically drawn symmetrical about the data points and equal to twice the s.e., but where space is limited the bars are drawn on one side of the point and

represent one s.e. value.

c. "Student's" t-test. This t-test was used to ascertain whether the mean values of two samples differed significantly. The Olivetti programma 101 was used to compute the 't' value, and the significance levels obtained from the "Student's" t distribution (Fisher and Yates, 1963) with the degrees of freedom given by (n1 + n2)-2, where n1 and n2 were the numbers of observations in samples 1 and 2 respectively. Differences were referred to as significantly different when the probability value (P) $\leq$ 0.05. For convenience the following notation was used:

P $\not\leq$ 0.05 : N.S. (not significant)

P $\leq$ 0.05 : *

P $\leq$ 0.01 : **

P $\leq$ 0.001 : ***

For experiments in which treatment and control were paired the 't' value was computed by the method of paired comparisons using the Olivetti programma 101; with n paired experiments the degrees of freedom were given by (n-1). In analysis of IAA and ABA treatments from experiments in which growth was monitored continuously physiological considerations lead one to expect IAA to promote growth relative to ABA. Thus, only the hypothesis that the differences were greater than zero needed to be tested (Bailey, 1959). The t-test was consequently based on one tail of the 't' distribution and the significance levels were read from "Student's" t distribution at twice the probability level, i.e. for a true 5% level the table was entered in the 10% column.

d. Analysis of variance. This test was used to examine the effects of replicated treatments of promoters and inhibitors on segment elongation. The data were analysed with an Olivetti programma 101 which computes the sum of squares (SS) and mean square (MS) values for the row items (R), column items (C), interactions between items in rows and columns (I) and error (E).

The variance ratios (F) were computed by dividing the respective mean square values by the error mean square.

$$F \text{ rows} = \frac{RMS}{EMS}$$

$$F \text{ columns} = \frac{CMS}{EMS}$$

$$F \text{ interaction} = \frac{IMS}{EMS}$$

The significance levels for the F ratios were obtained from tables of the variance ratio distribution (Fisher and Yates, 1963).

e. Curve fitting. Time courses of segment elongation in various hormone solutions were carried out (Section 5) and the initial rate of elongation, i.e. the initial slope of the graph, was required to allow analyses in terms of enzyme kinetics to be explored. The Capon-Sutherland initial slope programme (Capon, pers. comm.) was employed to calculate the initial rates. This programmed a KDF 9 computer to fit a polynomial equation, by a least squares method, to the data provided. The programme used was based on the technique described by Wentworth (1965a, 1965b). The computer printed parameters of the equation, including the initial slope of the curve.

The time required by the KDF 9 to calculate an equation which described each time course was dependent partly on the variability of the data. Because of the pressure on computer time a limited period was available for the calculation of each equation. If no equation had been obtained when the time limit was reached the data were rejected and no estimate of the initial slope was available.

EXPERIMENTS AND RESULTS

1. The Elongation Growth of Avena Coleoptile Segments in Response to Abscisic Acid and Indole-acetic Acid

The first experiments were designed to investigate the elongation growth of Avena coleoptile segments in response to exogenously applied ABA and IAA.

Coleoptile segments, 5 mm in length, excised 2 mm below the tip of 4-day-old seedlings, elongate in H_2O, IAA and ABA when using the 24-h straight growth assay (Fig. 10). Promotion of growth is significant ($P < 0.001$) with 10^{-7}M IAA compared with the H_2O control and the promotion increases as the IAA concentration is raised to 10^{-4}M. Little growth occurs in 10^{-3}M IAA, and there is both loss of turgidity of the cells and discolouration of the segments. Significant inhibition is observed with 10^{-7}M ABA ($P < 0.001$) and increases to 78% with 10^{-5}M ABA (Fig. 14a). No further increase in inhibition is achieved using 10^{-4}M ABA.

A time course of growth in 10^{-5}M ABA, 10^{-5}M IAA and distilled water shows that the growth is not linear with time, over the 24-h period, in all treatments (Fig. 11a and b). The results of this experiment indicate that the growth rate of segments exposed to IAA increases to a maximum after 4h, followed by a decline in rate; this maximum, however, is not observed in an 8-h time course although there is an overall decline in rate (Fig. 27b). In H_2O the growth rate is almost constant over the 24-h period and in ABA treated segments there is a slight decline in rate. After only 12h treatment in IAA 83% of the total elongation has occurred, yet in H_2O and ABA treated segments only 47% and 34% respectively, of the elongation is complete. The data suggest that to assess the activity of IAA a 12-h incubation would be adequate. The 24-h period, however, was used because it is convenient and allows the sections to be measured when the growth rate in IAA is low, thereby reducing error. In addition, the 24-h incubation allows greater elongation

Fig. 10. The effect of IAA and ABA on segment elongation in the Avena coleoptile straight growth assay.

The data are the mean from three experiments; at least 10 segments per treatment were used in each experiment.

Statistical Analysis. The t-test was used to test the difference in segment length between the treatments indicated.

Item	t value	Significance
$H_2O/10^{-7}$M IAA	3.549	***
$H_2O/10^{-7}$M ABA	5.031	***

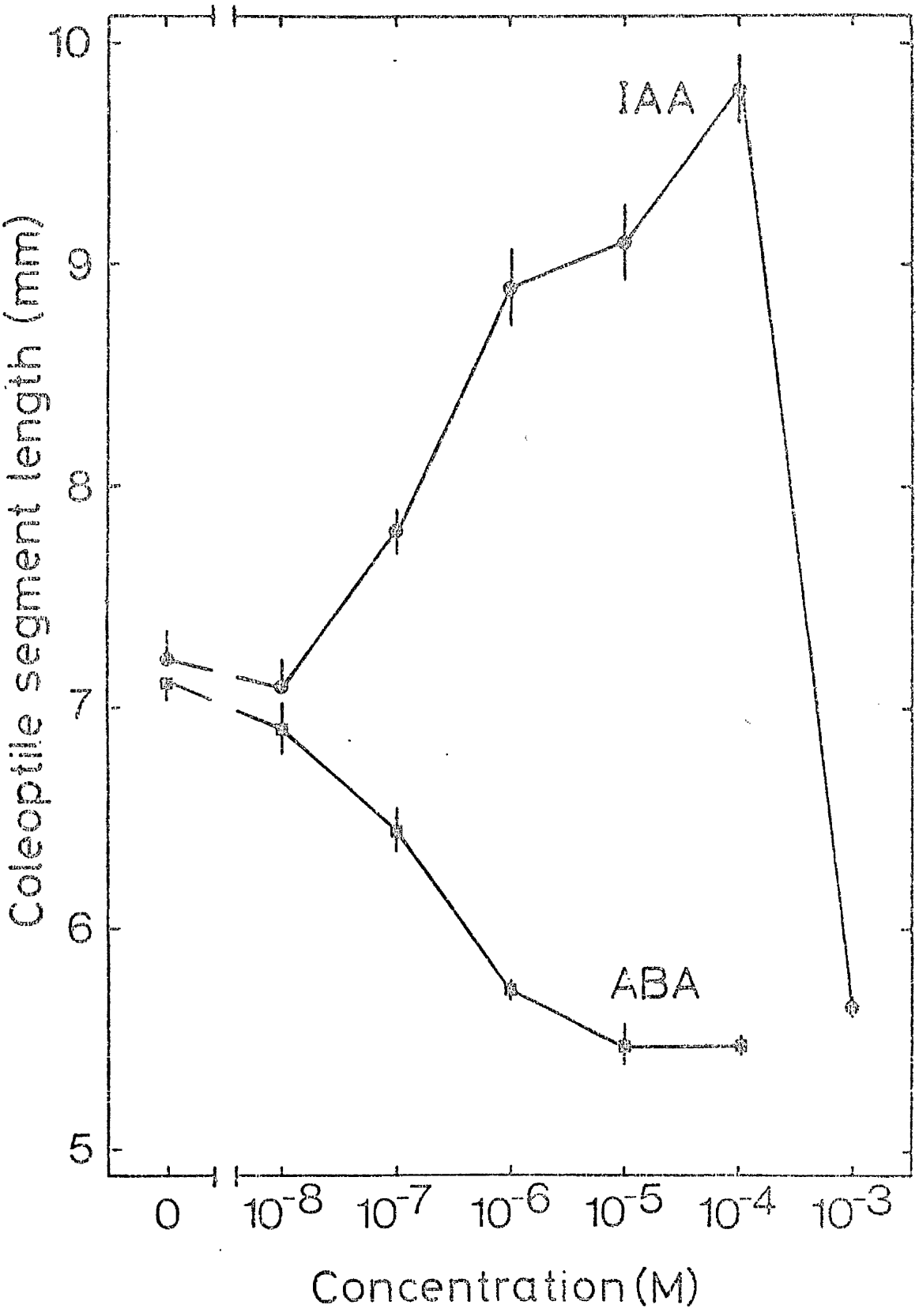

10
9
8
7
6
5
IAA
ABA
Coleoptile segment length (mm)
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Concentration (M)

Fig. 11. 24-h time courses of the elongation of Avena coleoptile segments at 25°C.

Treatments 1 : 10^{-5}M IAA.

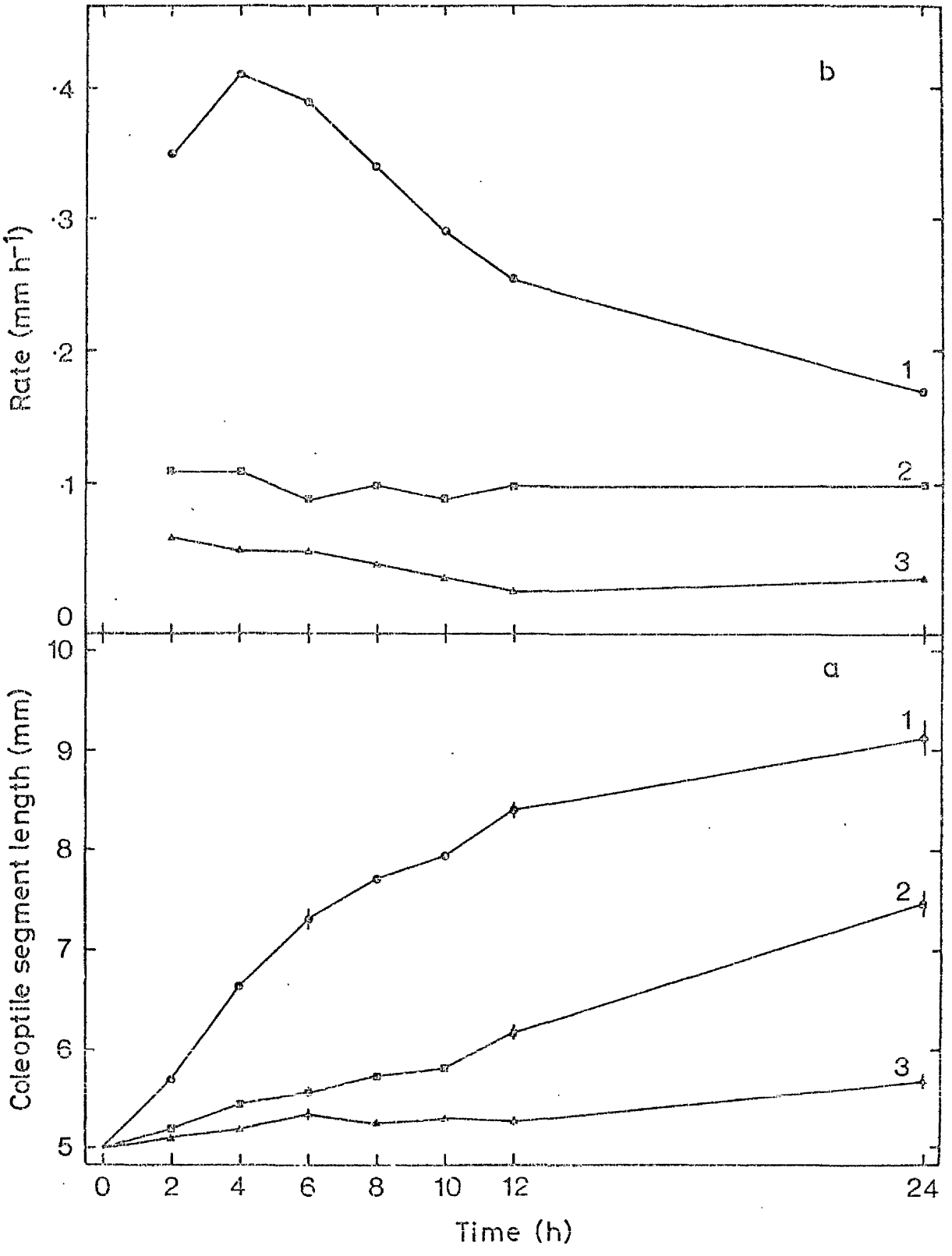
2 : Distilled H_2O.

3 : 10^{-5}M ABA.

a) Coleoptile segment length (mm) as a function of time.

b) Rate of elongation of segments as a function of time. The rates were calculated from time zero to the time at which the segment lengths were measured.

The data are the mean from 1 experiment, employing 20 segments for each time point. Similar results were observed in one repeat of this experiment.


b
Rate (mm h⁻¹)
·4
·3
·2
·1
0
1
2
3
a
Coleoptile segment length (mm)
10
9
8
7
6
5
1
2
3
0
2
4
6
8
10
12
24
Time (h)

in H_2O than a 12-h incubation and is thus more suitable for the assessment of the activity of inhibitors.

The effect of seedling age on the response to 10^{-6}M IAA has been studied (Fig. 12). Segments from 3-day-old seedlings are the most responsive to IAA and they also show the greatest elongation in water. A reduced effect is observed with segments from 4-day seedlings and a further slight reduction is apparent with segments from 5-day seedlings, but no significant difference between 5- and 6-day material is observed. The material grown for 3 days, however, is unsuitable for the straight growth assay because many coleoptiles are shorter than 7 mm and thus a 5 mm segment excised 2 mm below the tip includes the node and part of the mesocotyl. In addition, slight variations in the timing of experiments using 3-day-old material would result in larger variations in section length after treatment than if older material were used.

Thus the 24-h straight growth assay, using segments excised from the coleoptiles of 4-day-old Avena seedlings, is suitable for assaying the biological activity of IAA and ABA.

Abscisic acid does not appear to exert a toxic effect on the coleoptile segments (Fig. 13). To demonstrate this segments were placed in distilled water, 10^{-6} and 10^{-4}M ABA, with 3 replicates of 10 segments for each treatment. After 24-h dark incubation the segments from one replicate of the treatments were shadowgraphed. The remaining 2 replicates were rinsed with 3 x 10 ml distilled water and transferred to either distilled water or 10^{-4}M IAA. Segment lengths were determined after a further 24h.

The growth during 48h incubation in distilled H_2O markedly declines during the second 24h period. Transfer to IAA, after an initial 24h incubation in H_2O, stimulates growth and this observation indicates that endogenous IAA may be limiting growth during the 48-h H_2O treatment. In the presence of IAA growth also continues during the second 24h after exposure to ABA and, in fact, growth is stimulated compared with segments transferred from ABA to H_2O. Thus ABA is not apparently killing the segments during the

Fig. 12. The effect of seedling age on elongation in the Avena coleoptile straight growth assay.

Treatments 1 : 10^{-6}M IAA.

2 : Distilled H_2O.

The data are the mean from 1 experiment; 10 segments per treatment.

Statistical Analysis. The t-test was used to test the difference in segment length between the treatments indicated.

Item		t value	Significance
10^{-6}M IAA	3 day/4 day	7.869	***
	4 day/5 day	3.104	**
	5 day/6 day	1.154	NS
H_2O	3 day/4 day	6.427	***
	4 day/5 day	2.902	**
	5 day/6 day	0.414	NS

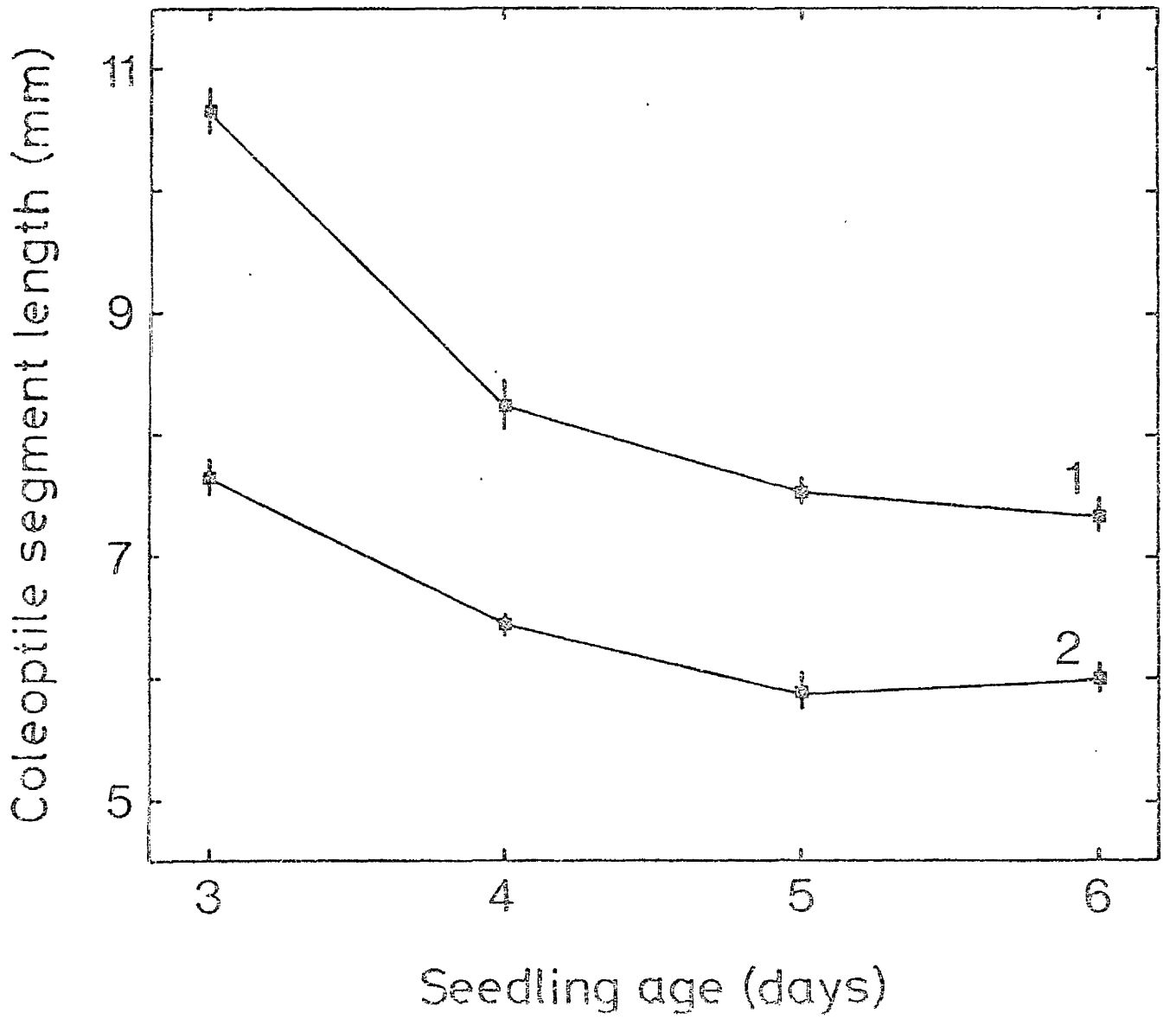
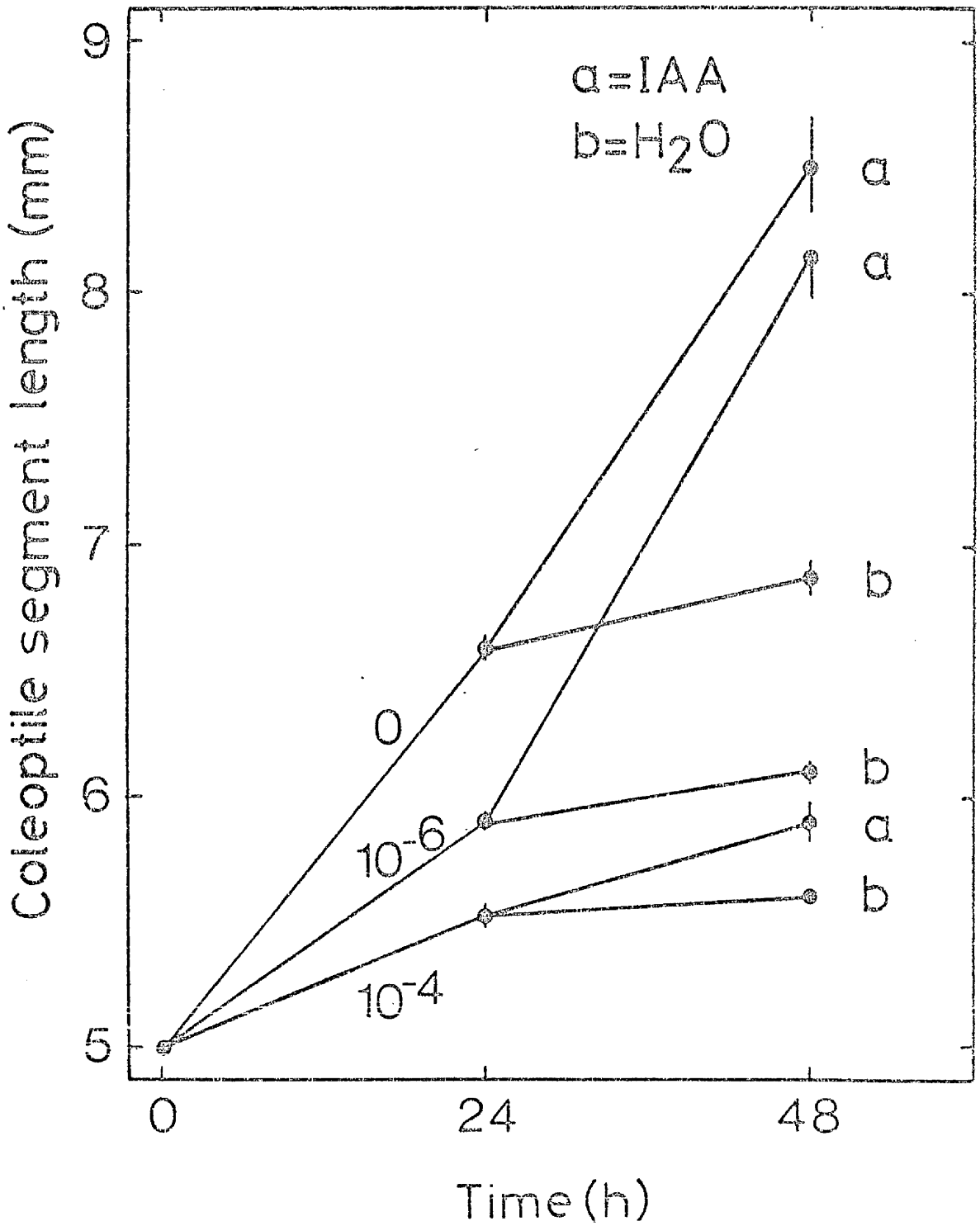

Coleoptile segment length (mm)
11
9
7
5
3
4
5
6
Seedling age (days)
1
2

Fig. 13. Data from 1 experiment to investigate the toxicity of ABA.

Treatment. 24 h incubation of segments in distilled H_2O (0), 10^{-6} M ABA and 10^{-4} M ABA, followed by 24 h incubation in a) 10^{-4} M IAA and b) distilled water. 25°C.

10 segments per sample. Similar results were observed in 2 repeats of this experiment.


a=IAA
b=H2O
Coleoptile segment length (mm)
9
8
7
6
5
0
24
48
Time (h)
0
10-6
10-4
a
a
b
b
b
a
b

first 24h. After segments have been given 10^{-4}M ABA for 24h, however, the growth rate during the subsequent 24h is reduced, even in the presence of 10^{-4}M IAA. Thus an inhibitory effect of this ABA solution is persisting and possibly some of the cells may have been killed. The result may indicate, however, that the ABA has not been satisfactorily removed from the segments either by leaching or metabolism.

2. The Molecular Requirements for Abscisic Acid Activity

The elongation growth of Avena coleoptile segments is inhibited by low concentrations of exogenously applied ABA. The question thus arises as to the mode of action of this inhibitor and one facet of this problem concerns the molecular requirements for the inhibition. One approach to this aspect is to study the activity of analogues of ABA and this has been made possible by the provision of ABA analogues by Hoffmann-La Roche.

The activity of 33 analogues was tested in the 24h Avena coleoptile straight growth assay, using analogue concentrations of 10^{-7}, 10^{-6} and 10^{-5}M with a distilled water control. The maximum concentration used was limited by the availability of the compounds and concentrations of less than 10^{-7}M were not tested because 10^{-8}M ABA does not produce significant inhibition. The significance levels of the inhibition induced are listed in Table 5.

Table 5. The Statistical Significance of the Inhibition of Elongation of Avena Coleoptile Segments. Significance levels were determined using "Student's" t-test, testing the mean length at each concentration against the control used when testing each analogue. The data analysed are from experiments repeated on 3 occasions. 'P' indicates the promotion of elongation.

Table 5.

Compound	Concentration of Compound (M)				
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
c,t-ABA	NS	***	***	***	***
t,t-ABA	NS	NS	***	***	***
I		NS	NS	***	
II		NS	*	***	
III		***	***	***	
IV		***	***	***	
V		***	***	***	
VI		NS	***	***	
VII		NS	NS	***	
VIII		NS	**	***	
IX		*	**	**	
X		NS	NS	***	
XI		NS	NS	***	
XII		NS	NS	NS	
XIII		NS	NS	NS	
XIV		NS	NS	NS	
XV		NS	NS	NS	
SVI		NS	NS	NS	
XVII		NS	NS	*	
XVIII		NS	NS	NS	
XIX		NS	***	***	
XX		NS	***	***	
XXI		NS	***	***	
XXII		NS	***	***	
XXIII		NS	NS	***	
XXIV		*	**	***	
XXV		NS	***	***	
XXVI		NS	NS	NS	
XXVII		NS	NS	*	
XXVIII		NS	**(P)	NS	
XXIX		NS	NS	NS	
XXX		NS	***	***	
XXXI		NS	NS	***(P)	
XXXII		NS	NS	NS	
XXXIII		NS	NS	***	

While expounding these data two considerations were borne in mind. Firstly, that comparisons should ideally be made between compounds that differ in one moiety only and that in cases in which there are additional structural differences these will qualify the conclusions. Secondly, comparisons should be made, directly or indirectly, relative to the inhibitory activity of either ABA or compounds with activity similar to that of ABA. Consequently, only specific compounds are referred to for each inference; however, if the structure-activity relations of another compound qualify the argument reference is always made to that compound.

Comparisons of compounds IV and V (Fig. 14b) with ABA (Fig. 14a) illustrate that the hydroxyl and carbonyl groups of ABA are unimportant for inhibitory activity. Both compounds lack these structures but at 10^{-6}M and 10^{-5}M possess activity similar to ABA at the same concentrations. The activity of V, compared with ABA, also indicates that the acid group of ABA may be replaced by an ethyl ester group without large reductions in activity at 10^{-6}M or 10^{-5}M, although V is much less active at 10^{-7}M than 10^{-7}M ABA. The ester itself may either be active or be hydrolysed to the acid in the plant tissue.

The much reduced activity of VI compared with V, and of VII compared with IV (Fig. 14b) suggests that the ring double bond is important for inhibition because both the compounds which exhibit reduced activity differ in structure from the compound with which their activity is compared only in the loss of the ring double bond. In both VI and VII, however, the ring double bond has been epoxidised and the reduction in activity may be due to addition of the epoxide residue rather than merely to loss of the double bond.

The importance of the ring double bond is also supported by the fact that both XXX and XXXIII (Fig. 15a) have reduced activity compared with V. These compounds differ from V in both the loss of the ring double bond and the fact that the terminal moiety is trans in XXX and XXXIII. The activity of XXXIII, however, is similar to that of VI and the structures of these

Fig. 14 a-d. The inhibitory activity of c,t-ABA and of ABA analogues in the Avena coleoptile straight growth assay.

The mean length of at least 30 segments from 3 experiments was calculated for each treatment.

Percentage inhibition was derived from:-

$$\left(\frac{\text{Mean control increment} - \text{mean treatment increment}}{\text{Mean control increment}}\right) \times 100$$

Statistical analysis is presented in Table 5.

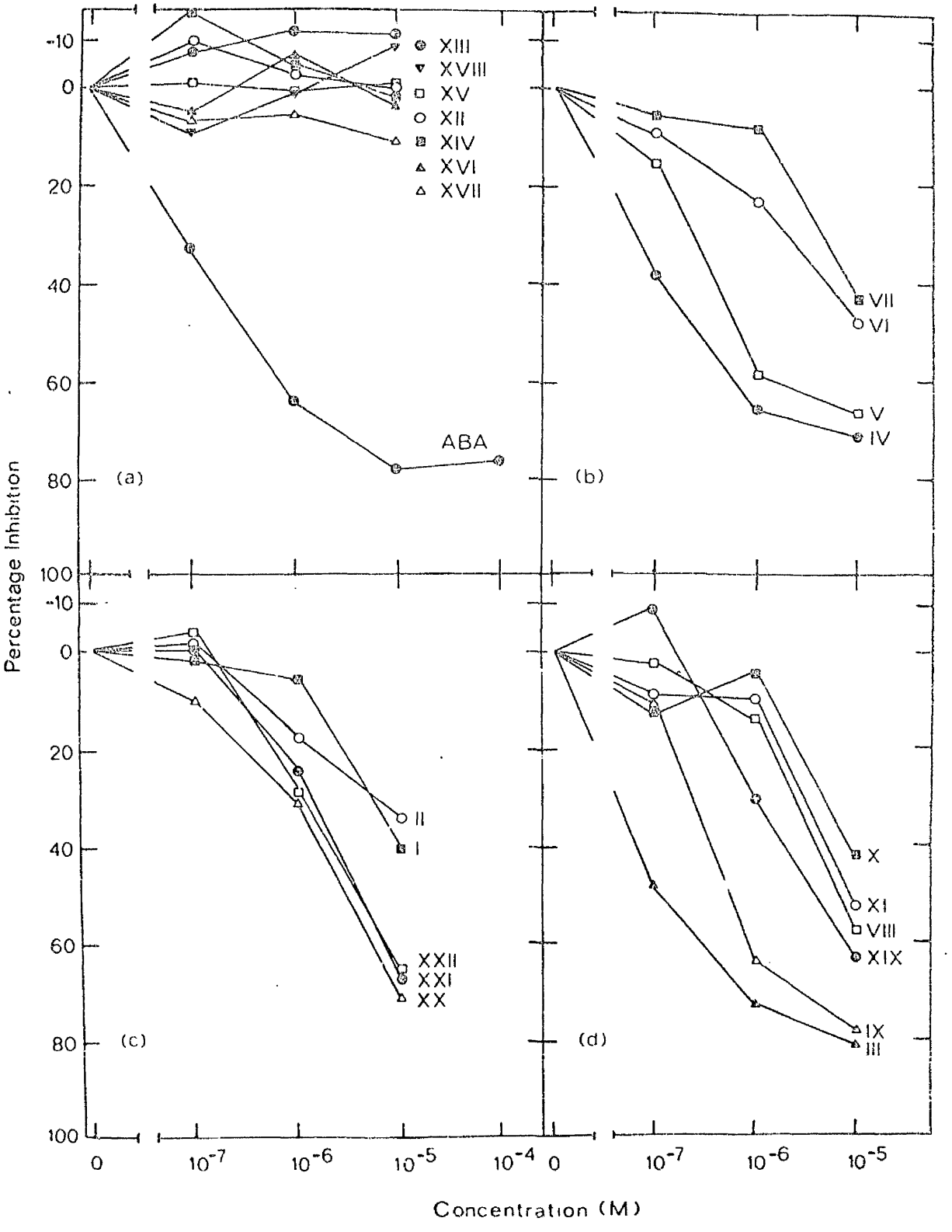

Percentage Inhibition
-10
0
20
40
60
80
100
XIII
XVIII
XV
XII
XIV
XVI
XVII
ABA
(a)
(b)
VII
VI
V
IV
(c)
II
I
XXII
XXI
XX
(d)
X
XI
VIII
XIX
IX
III
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration (M)

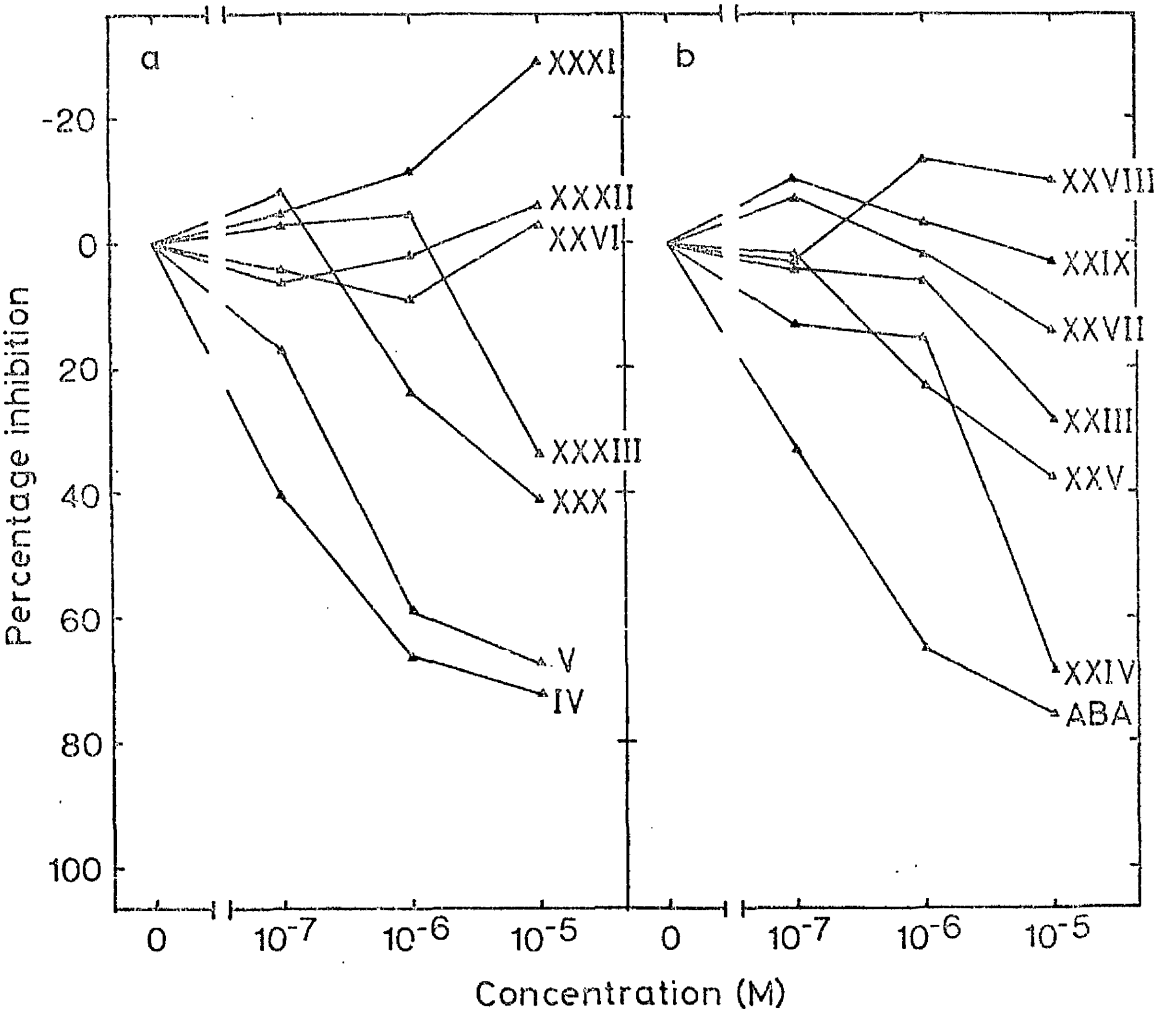
Fig. 15 a,b. The inhibitory activity of c,t-ABA and of ABA analogues in the Avena coleoptile straight growth assay.

The mean length of at least 30 segments from 3 experiments was calculated for each treatment.

Percentage inhibition was derived from:-

$$\left(\frac{\text{mean control increment} - \text{mean treatment increment}}{\text{mean control increment}}\right) \times 100$$

Statistical analysis is presented in Table 5.


a
b
XXXI
XXXII
XXVI
XXXIII
XXX
V
IV
XXVIII
XXIX
XXVII
XXIII
XXV
XXIV
ABA
-20
0
20
40
60
80
100
Percentage inhibition
0
10-7
10-6
10-5
Concentration (M)

compounds differ only in the geometrical isomerism of the side chain; this difference would thus appear to be unimportant for inhibition. Consequently the reduced activity of both XXX and XXXIII, compared with V, may be attributed only to the loss of the ring double bond and not the differences in geometrical isomerism of the side chain. The ring double bond has been epoxidised in XXX and XXXIII but if the epoxide residue itself confers the loss in activity then the fact that the activity of XXX is similar to that of XXXIII suggests that the position of this residue may not be important, although XXX is slightly more active than XXXIII, particularly at 10^{-6}M.

The lack of activity of XIV (Fig. 14a) compared with the partially active II (Fig. 14c) indicates that the presence of a double bond at C-1' in the ring does not confer activity, and thus that the position of the double bond at C-2' in the ring of ABA is important for inhibition. Unfortunately there are no additional compounds to support this inference.

Abscisic acid has an unsaturated functional group at C-1 in the side chain and the structure-activity relations of several analogues indicate that this group is important for inhibitory activity. In the abscisin alcohol (I) and abscisin hydrocarbon (II) (Fig. 14b) C-1 is saturated and the reduced activity of these compounds at 10^{-6} and 10^{-5}M compared with ABA at the same concentrations indicates the importance of the unsaturated functional group at C-1. This inference is supported by the structure-activity relations of VIII, X, XI, XIX, XX, XXI, XXII, XXIII, and XXIV (Figs. 14c and d, 15b). These compounds are also saturated at C-1; at 10^{-6}M they all possess activity which is markedly less than 10^{-6}M ABA and at 10^{-5}M these compounds show a range of activity from 28% to 72% inhibition. This range of activity may reflect differences in penetration or breakdown of these long-chain compounds.

Compound III (Fig. 14d), which has an aldehyde group at C-1, and compound IX (Fig. 14d), which has an unsaturated linkage at C-1 to a nitrogen atom, have similar activity to ABA. Thus an unsaturated linkage of C-1 to a heteroatom may be the important feature of the terminal moiety, rather than

the complete carboxyl group, although the activity of an unsaturated linkage at C-1 to a carbon atom has not been tested. The activity of IX could, however, be the result of hydrolysis to the aldehyde of ABA within the plant tissue.

When both the ring double bond and the unsaturated functional group at C-1 are absent inhibitory activity is lost. This is best illustrated by the non-active compounds XII, XIII and XVIII (Fig. 14a) whose structures lack these moieties. The additional lack of the hydroxyl and carbonyl moieties in these compounds is probably unimportant because the latter moieties are unimportant for inhibitory activity.

Inhibitory activity is also lost when there are changes in the carbon skeleton in the 2,4-pentadiene side chain even though one of the active moieties may be present. This is shown by the lack of activity of compounds XV, XVI andXVII (Fig. 14a) which all contain the ring double bond. The non-inhibitory compound XXXI (Fig. 15a) is particularly interesting because it possesses the unsaturated functional group at C-1 but because XXXI has a side chain with 9 carbon atoms this normally active moiety is at a greater distance from the ring structure than in ABA. This fact indicates that the spatial arrangement of this active moiety may be important for the inhibitory activity of ABA.

It is interesting to note that the presence of an inorganic atom within the molecule of an abscisic acid analogue interferes with the expected activity. Compound XXV, for example, possesses the structural features implicated for inhibitory activity but its activity (Fig. 15b) is reduced compared with that of IX, possibly due to the presence of a sulphur atom in the terminal group of the side chain. Similarly XXIII (Fig. 15b), which possesses a chloride atom, is the least active of those compounds which lack only the unsaturated functional group at C-1.

Compounds XXVI, XXVIII, XXIX and XXXII (Fig. 15a and b) are all non-active, as would be expected since these compounds lack both the active

moieties required for inhibition and have changes to the 2,4-pentadiene side chain of ABA. Compound XXVII (Fig. 15b) may be expected to have had some activity because it has the 2,4-pentadiene side chain and the ring double bond but this compound may not have been dissolved satisfactorily.

Thus, for inhibitory activity of ABA the complete carbon skeleton of the 2,4-pentadiene side chain appears to be essential. Provided this is present there are two active moieties in the ABA molecule; these are the ring double bond and an unsaturated oxygen- or nitrogen-containing functional group at C-1. In addition, there is some evidence that the spatial arrangements of these active moieties within the ABA molecule are important. At 10^{-6}M both moieties are required for inhibitory activity comparable to ABA at the same concentration, e.g. III, IV and V. At 10^{-5}M, however, there is considerable inhibition with only one moiety present, e.g. the ring double bond in I and II, or the unsaturated functional group at C-1 in VI and VII, although this inhibition is reduced in comparison with ABA at 10^{-5}M.

The geometrical isomerism of the 2,4-pentadiene side chain is also of interest. Compounds in which the terminal group is trans possess activity only slightly reduced in comparison with similar compounds with a cis terminal group. For example VI compared with XXXIII (Figs. 14b and 15a) and cis,trans-ABA compared with trans,trans-ABA (Fig.16a and b). The trans,trans-ABA at 10^{-7}M does not produce significant inhibition over the control whereas this concentration of cis,trans-ABA does produce significant inhibition. Examination of the data expressed as % inhibition shows slightly less inhibition at each concentration with trans,trans-ABA than cis,trans-ABA although the inhibition produced by each compound is very similar at 10^{-4}M.

GLC was carried out to ascertain the purity of these geometrical isomers of ABA (Fig. 17). Chromatography of the methylated stock cis,trans-ABA yields one peak whose retention time is 11.70 minutes. The retention time of the octakosane standard is 10.35 minutes and the retention time for Me cis,trans-ABA relative to this standard is 1.132. Chromatography of the

Fig. 16. The inhibitory activity of c,t-ABA and t,t-ABA in the Avena coleoptile straight growth assay.

a) Coleoptile segment length VS hormone concentration.

b) Percentage inhibition VS hormone concentration.

Percentage inhibition was derived from:-

$$\left(\frac{\text{mean control increment} - \text{mean treatment increment}}{\text{mean control increment}}\right) \times 100$$

The mean length of at least 30 segments from 3 experiments was calculated for each treatment.

Statistical analysis is presented in Table 5.

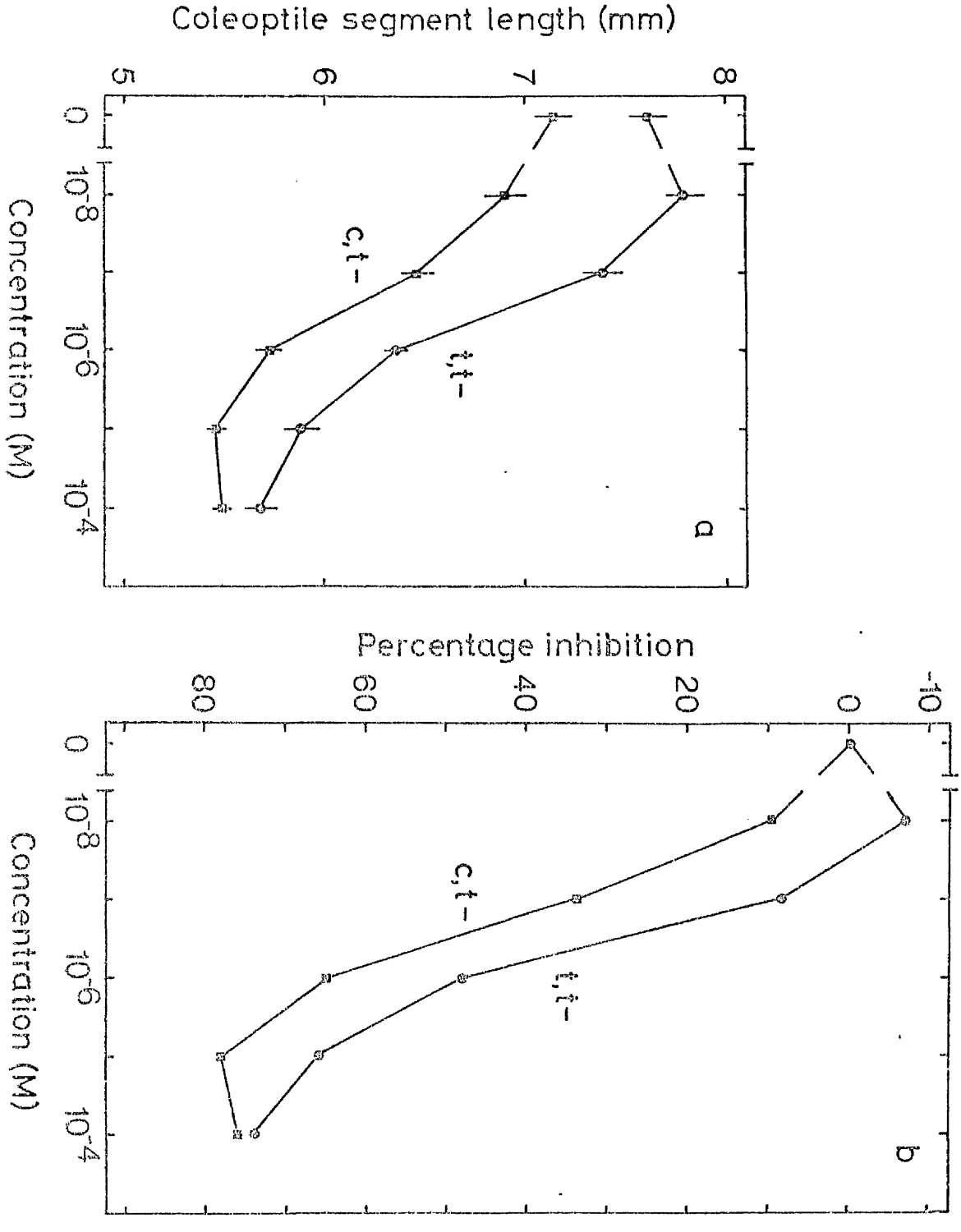

Coleoptile segment length (mm)
5
6
7
8
Concentration (M)
0
10-8
10-6
10-4
c,t-
t,t-
a
Percentage inhibition
80
60
40
20
0
-10
Concentration (M)
0
10-8
10-6
10-4
c,t-
t,t-
b

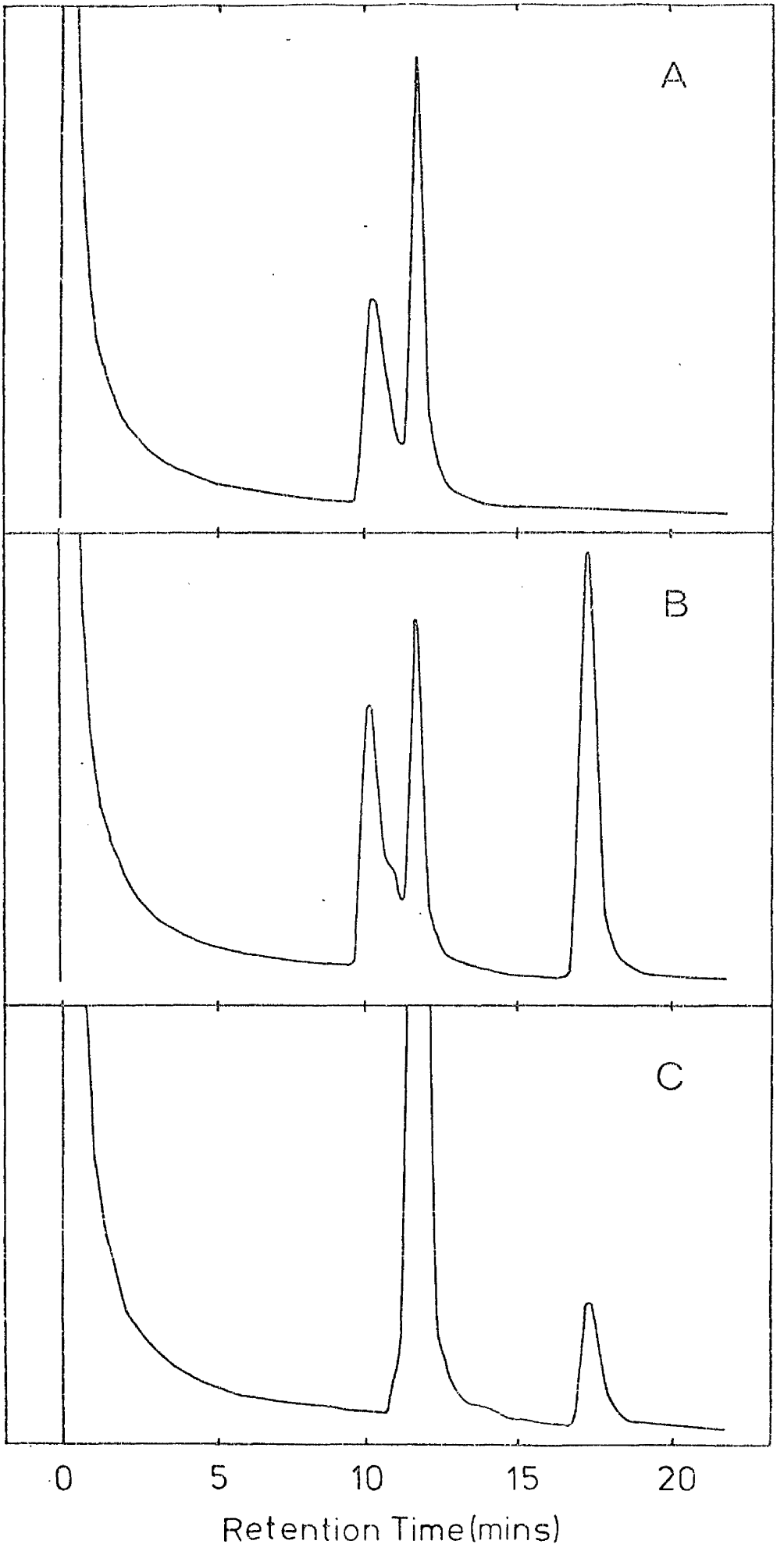
Fig. 17. Gas chromatograms of methylated samples of abscisic acid. Samples were chromatographed at 200°C on a 5ft column of OV 210 using octokosane as a standard.

A. C_{28} and cis,trans-ABA.

B. C_{28} and trans,trans-ABA.

C. Cis,trans-ABA after irradiation by UV light.

Peak attributed to:-		Retention time (mins.)	Retention time sample / Retention time C_{28}
A.	C_{28}	10.35	
	c,t-ABA	11.70	1.132
B.	C_{28}	10.35	
	c,t-ABA	11.70	1.132
	t,t-ABA	17.56	1.697
C.	c,t-ABA	11.70	1.132
	t,t-ABA	17.08	1.650


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

methylated stock trans,trans-ABA gives rise to two peaks whose retention times relative to C20 are 1.132 and 1.697 (Fig. 17B). The first peak thus corresponds to Me cis,trans-ABA and the second is probably due to Me trans, trans-ABA. Confirmation of the latter point is obtained by irradiating Me cis,trans-ABA with U.V. light, a process which results in a second peak with a retention time relative to C20 of 1.650 (Fig. 17C). The conversion of Me cis,trans-ABA to a mixture of this and its 2-trans isomer is well documented (Mousseron-Canet et al., 1966; Lenton et al., 1970). Thus, as the irradiated Me cis,trans-ABA and the methylated stock of trans,trans-ABA both yield peaks with similar retention times relative to C20, these peaks can be attributed to the 2-trans isomer. The peak heights in the trans, trans-ABA are similar to each other, indicating approximately equal quantities of the two isomers.

The trans,trans-ABA stock thus consists of approximately equal quantities of the two geometrical isomers of ABA and has inhibitory activity only slightly reduced compared with pure cis,trans-ABA. This indicates that the trans,trans- component itself possesses considerable inhibitory activity. The effect of U.V. irradiation, however, is a complicating factor and GLC of the bioassay solutions is required before and after the bioassay before firm conclusions may be drawn.

The pH of ABA and its analogues at 10^{-5}M varies between 5.0 and 6.9 (Table 3). It is improbable that these variations in pH contribute to the differences in activity of the analogues because differences in pH within this range have little effect on coleoptile elongation in a 24-h straight growth assay (Fig. 18). The effect of pH on growth is demonstrated with both citrate buffer (0.01M) and phosphate buffer (0.02M). These concentrations of buffers have an inhibitory effect compared with distilled water at the same pH.

The pH values quoted for the analogues are of solutions before use in

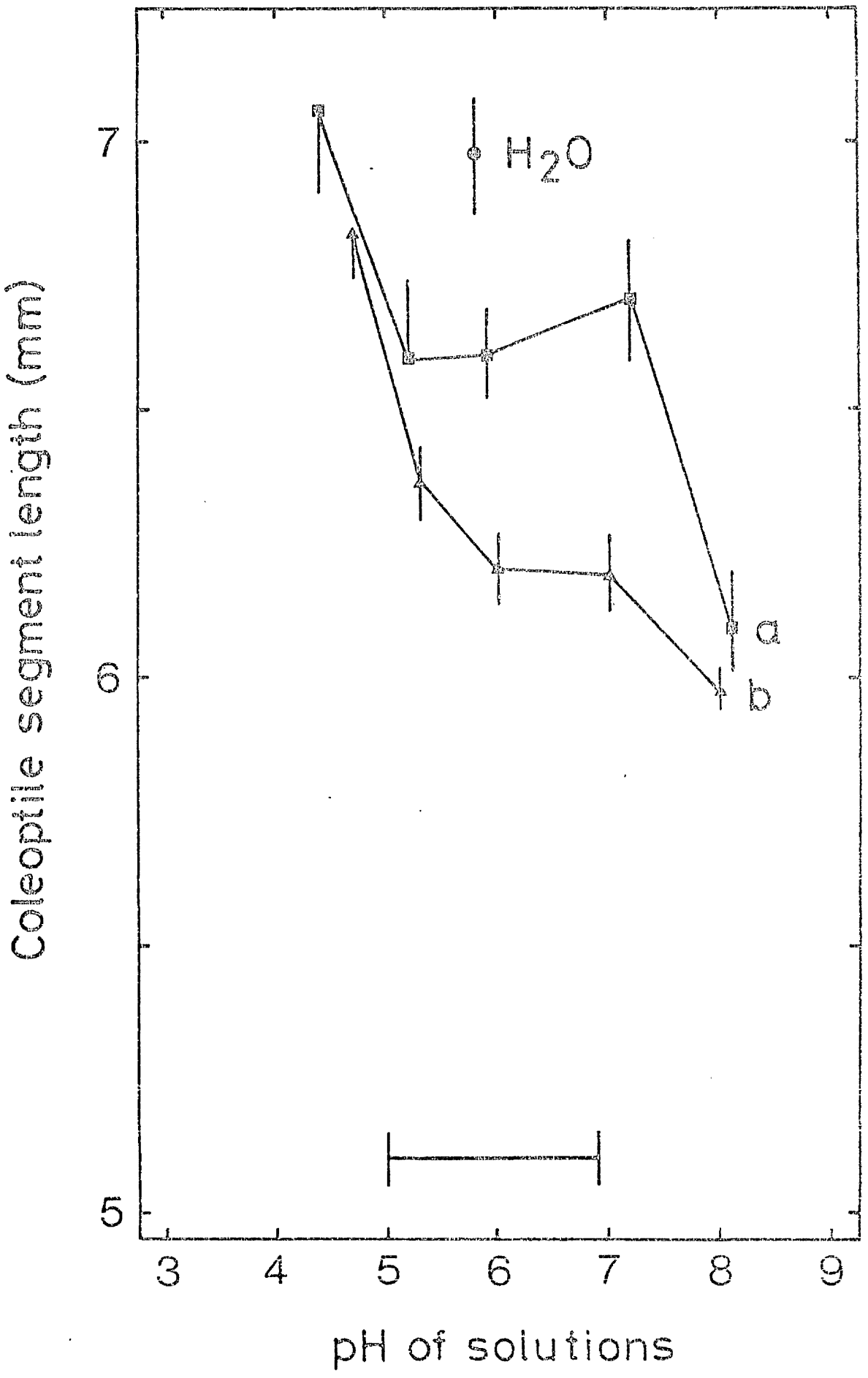
Fig. 18. The effect of the pH of the buffer solution on elongation in the Avena coleoptile straight growth assay.

a) 0.01M Citrate buffer.

b) 0.02M Phosphate buffer.

The data are the mean from 3 experiments, employing 10 segments per treatment. pH values were determined before the straight growth assay.

The horizontal bar indicates the pH range of ABA analogues.


Coleoptile segment length (mm)
7
6
5
H_2O
a
b
3
4
5
6
7
8
9
pH of solutions

the bioassay (Table 3) and it is important to establish whether large changes in pH of the solutions during a bioassay are probable. The pH of IAA and ABA solutions in petri dishes decreases slightly during a 24-h period in the absence of coleoptile segments, though the pH does not decrease lower than 4.5 (Column A, Table 6). This increase in acidity could be due to the

Table 6. The pH of Hormone and Buffer Solutions.

The pH values quoted are of solutions in petri dishes (i) before and (ii) after a 24-h period. 0.02M phosphate buffer was used to buffer the hormone solutions. Similar pH values have been obtained in repeat experiments.

	A		B		C	
Solution	No buffer No segments		No buffer Segments present		Buffer present Segments present	
	(i)	(ii)	(i)	(ii)	(i)	(ii)
IAA 0	5.8	5.4	5.8	5.6	5.3	5.3
10^{-7}	5.9	5.5	5.8	5.6	5.3	5.3
10^{-6}	5.9	5.5	5.8	5.6	5.3	5.3
10^{-5}	5.4	5.3	5.3	5.5	5.3	5.3
10^{-4}	4.5	4.5	4.5	5.0	5.2	5.2
ABA 0	5.8	5.4	5.8	5.6	5.4	5.4
10^{-7}	5.9	5.8	5.9	5.7	5.4	5.4
10^{-6}	5.9	5.7	5.9	5.7	5.4	5.4
10^{-5}	5.9	5.5	5.9	5.7	5.4	5.4
10^{-4}	4.5	4.5	4.5	5.4	5.2	5.2

	Segments present		No segments	
	(i)	(ii)	(i)	(ii)
Phosphate Buffer 0.02M	4.7	4.9	4.7	4.8
	5.3	5.3	5.3	5.3
	6.0	6.0	6.0	6.0
	7.0	6.9	7.0	6.9
	8.0	7.2	8.0	7.3
Citrate Buffer 0.01M	4.4	4.4	4.4	4.3
	5.2	5.2	5.2	5.1
	5.9	5.8	5.9	5.8
	7.2	6.9	7.2	6.9
	8.1	7.3	8.1	7.3

absorption of CO_2 from the air or to a component from the plastic petri dishes. The drop in pH is counteracted by the presence of coleoptiles during a 24-h period and, in fact, solutions originally at pH 4.5, e.g. 10^{-4}M IAA and 10^{-4}M ABA, undergo an increase in pH (Column B, Table 6). Thus, as only small changes in pH of IAA and ABA are observed over a 24-h period and these changes are reduced by the presence of coleoptile segments, it is probably unlikely that there are large changes in the pH of ABA analogue solutions during a bioassay.

The use of 0.02M phosphate buffer to buffer the IAA and ABA solutions is successful because no changes in pH of these buffered hormone solutions are observed after a 24-h straight growth assay (Column C, Table 6). In addition, the pH of the buffer solutions alone is similar before and after the bioassay although the pH of the solutions originally at pH 8.0 decreases slightly.

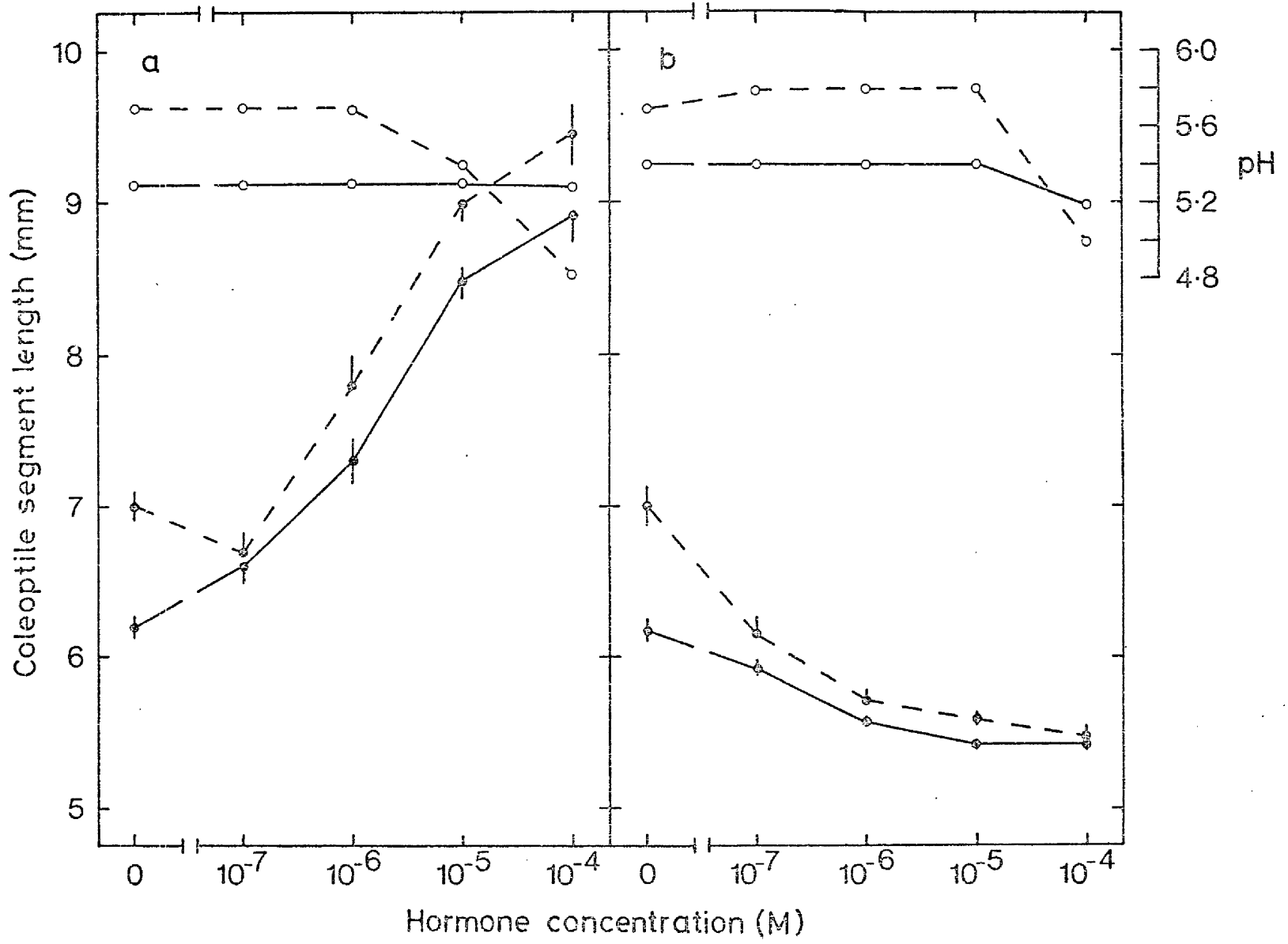
The slight changes in pH of the hormone solutions that are observed in the absence of buffer probably do not modify the activity of IAA or ABA because these hormones produce similar effects on segment elongation over the concentration range 10^{-7} to 10^{-4}M in the presence or absence of a phosphate buffer which maintains a constant pH (Fig. 19a and b). It is also interesting that although the 0.02M phosphate buffer is inhibitory in the absence of applied hormones it has a reduced effect in the presence of either ABA or IAA.

3. The Action of Abscisic Acid in the Presence of Indole-acetic Acid : The Straight Growth Assay

The inhibitory effect of ABA and promotory effect of IAA on Avena coleoptile elongation have already been demonstrated using the 24-h straight growth assay. In attempting to elucidate the rôle of ABA in regulating the growth of Avena coleoptiles the action of ABA in the presence of IAA was studied because endogenous growth may be controlled in part by the action of growth promoters and growth inhibitors. The straight growth assay was

Fig. 19. The effect of a) IAA and b) ABA on elongation in the Avena coleoptile straight growth assay; in the absence of buffer (— — — —) and in the presence of 0.02M phosphate buffer. The solid circles indicate the coleoptile segment lengths and the open circles indicate the pH of the solutions.

The data are the mean of 2 experiments with 10 segments per treatment. The pH values are the mean of the values determined before and after the assay.


a
b
10
9
8
7
6
5
6·0
5·6
5·2
4·8
pH
Coleoptile segment length (mm)
0
10-7
10-6
10-5
10-4
Hormone concentration (M)

initially employed.

A factorial experiment was designed with both ABA and IAA at concentrations of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M, in addition to a water control. This concentration range was chosen to include concentrations at which these hormones alone did not produce significant effects. Hence it was possible to investigate whether a hormone concentration which was non-active alone produced an effect in the presence of a second hormone. An identical concentration range of both hormones enabled comparisons to be made between the activities of these growth substances at equimolar concentrations. The experiment was carried out on 3 occasions and the data from 2 experiments are presented (Exp. A: Fig. 20a; Exp. B: Fig. 20b). Experiment A had 3 replicates of 5 segments for each treatment and B had 2 replicates of 10 segments per treatment.

Comparison of the H_2O controls shows the variation between experiments but in each case the response pattern is similar. Coleoptile elongation is promoted by IAA alone and inhibited by ABA alone. The combination of these hormones shows that ABA is capable of inhibiting the promotion of growth which is induced by IAA alone; for example, with both hormones at 10^{-4}M growth is inhibited relative to the water control. The "Student's" t-test shows this to be significant in both experiments ($P < 0.001$). Thus, on a mole for mole basis ABA at 10^{-4}M is a more powerful inhibitor than IAA is a promoter. This is clearly seen by observing the effects of the hormones combined at equimolar concentrations (Figs. 20a and b; dotted lines).

The effect of ABA on coleoptile segment length is not the same at all IAA concentrations: the elongation inhibited by both 10^{-5} and 10^{-4}M ABA is greater at high IAA concentrations than at low IAA concentrations. Consequently the lines joining data for consecutive increasing IAA concentrations, at the various ABA concentrations, diverge. This is a preliminary indication of a statistical interaction between ABA and IAA. The analysis of variance confirms there is a statistical interaction of these data in experiments A and

Fig. 20. The activity of ABA, in the presence of IAA, in the Avena coleoptile straight growth assay.

a) Experiment A : 3 replicates of 5 segments per treatment.

b) Experiment B : 2 replicates of 10 segments per treatment.

The data are presented as mean coleoptile segment length VS IAA concentration, at five ABA concentrations and in the absence of ABA. The dotted line in each graph joins those treatments in which equimolar concentrations of both hormones are present.

Statistical Analysis. The t-test was used to test the difference in segment length between the treatments indicated.

Item	t value	Significance
Exp. A : $H_2O/10^{-4}$M IAA + 10^{-4}M ABA	7.224	***
Exp. B : $H_2O/10^{-4}$M IAA + 10^{-4}M ABA	3.705	***

For further analysis see Table 7.

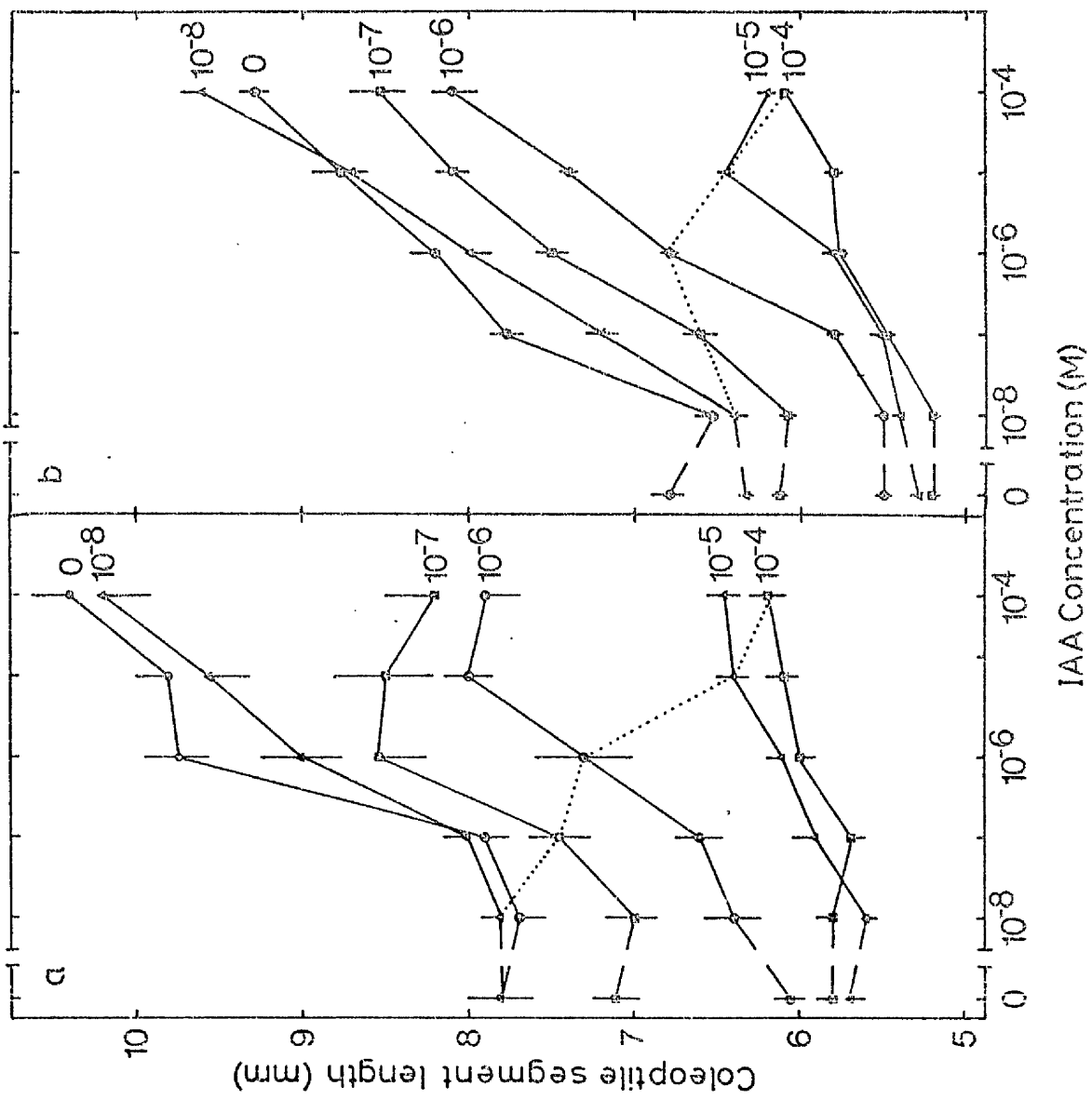

a
b
0
10-8
10-7
10-6
10-5
10-4
Coleoptile segment length (mm)
IAA Concentration (M)

Table 7. Analysis of Variance : ABA Experiments.

(i) Experiment A. 6 concentrations IAA x 6 concentrations ABA.

Item	N	SS	MS	F	P
IAA	5	44.036	8.807	77.254	***
ABA	5	146.854	29.371	257.640	***
Interaction	25	13.466	0.539	4.728	***
Error	72	8.193	0.114	-	-

(ii) Experiment B. 6 concentrations IAA x 6 concentrations ABA.

Item	N	SS	MS	F	P
IAA	5	47.890	9.579	504.158	***
ABA	5	55.318	11.064	582.318	***
Interaction	25	9.294	0.372	19.578	***
Error	36	0.670	0.019	-	-

(iii) Experiment A. 6 concentrations IAA x 4 concentrations ABA.

Item	N	SS	MS	F	P
IAA	5	49.20	9.84	63.076	***
ABA	3	40.37	13.46	86.282	***
Interaction	15	5.15	0.34	2.179	*
Error	48	7.48	0.156	-	-

(iv) Experiment B. 6 concentrations IAA x 4 concentrations ABA.

Item	N	SS	MS	F	P
IAA	5	51.107	10.221	393.115	***
ABA	3	13.211	4.404	169.384	***
Interaction	15	1.107	0.074	2.846	*
Error	24	0.618	0.026	-	-

B (Table 7 (i) and (ii)). The analyses also show that the IAA and ABA effects are significant.

Examination of the data presented in these graphs (Fig. 20a and b) indicates that the effects of 10^{-8}, 10^{-7} and 10^{-6}M ABA are similar at nearly all IAA concentrations. It could be argued that a significant interaction component only develops in the presence of 10^{-5} and 10^{-4}M ABA. On the other hand, an analysis of variance in which the data for 10^{-5} and 10^{-4}M ABA are omitted shows a significant interaction although the level of this significance is reduced ($P < 0.05$; Table 7 (iii) and (iv)). Thus it appears that the significance of the interaction term between ABA and IAA develops with increasing hormone concentration.

The interaction detected may be attributed to a combination of the nature of the assay system and the method of data presentation. Thus it is relevant to consider a different method of presentation of the data. In Fig. 21a and b the growth inhibited by ABA in the presence of IAA is calculated from the growth induced by IAA alone (i.e. growth induced by IAA minus growth induced by IAA and ABA). The lines in these graphs diverge and indicate a statistical interaction. The increased ABA inhibition at increasing IAA concentrations, however, may be a consequence of the greater potential for growth inhibition at higher IAA concentrations, i.e. there is more growth to be inhibited.

The data may be presented on a percentage inhibition basis by calculating the growth inhibited by ABA at each IAA concentration as a percentage of the growth promoted by the appropriate IAA concentration, i.e. % inhibition (Fig. 22a and b). A different response pattern is observed in that the lines are relatively parallel, particularly when comparing 10^{-5} and 10^{-4}M ABA with the zero ABA control. The data from experiments A and B are shown to indicate variation between the experiments. The variation is greater than is observed in the primary data (Fig. 20a and b) because each % datum is dependent on two values, viz. the growth in ABA + IAA and the control growth in IAA alone.

Fig. 21. The activity of ABA, in the presence of IAA, in the Avena coleoptile straight growth assay.

a) Experiment A.

b) Experiment B.

The data are from the same experiments as in Fig. 20 a and b; data presented as the growth inhibited (mm) VS IAA concentration, at five ABA concentrations and in the absence of ABA.

The growth inhibited by ABA at each IAA concentration was derived from:-

mean control increment - mean treatment increment.

The control for each IAA concentration was taken as that treatment with IAA only present.

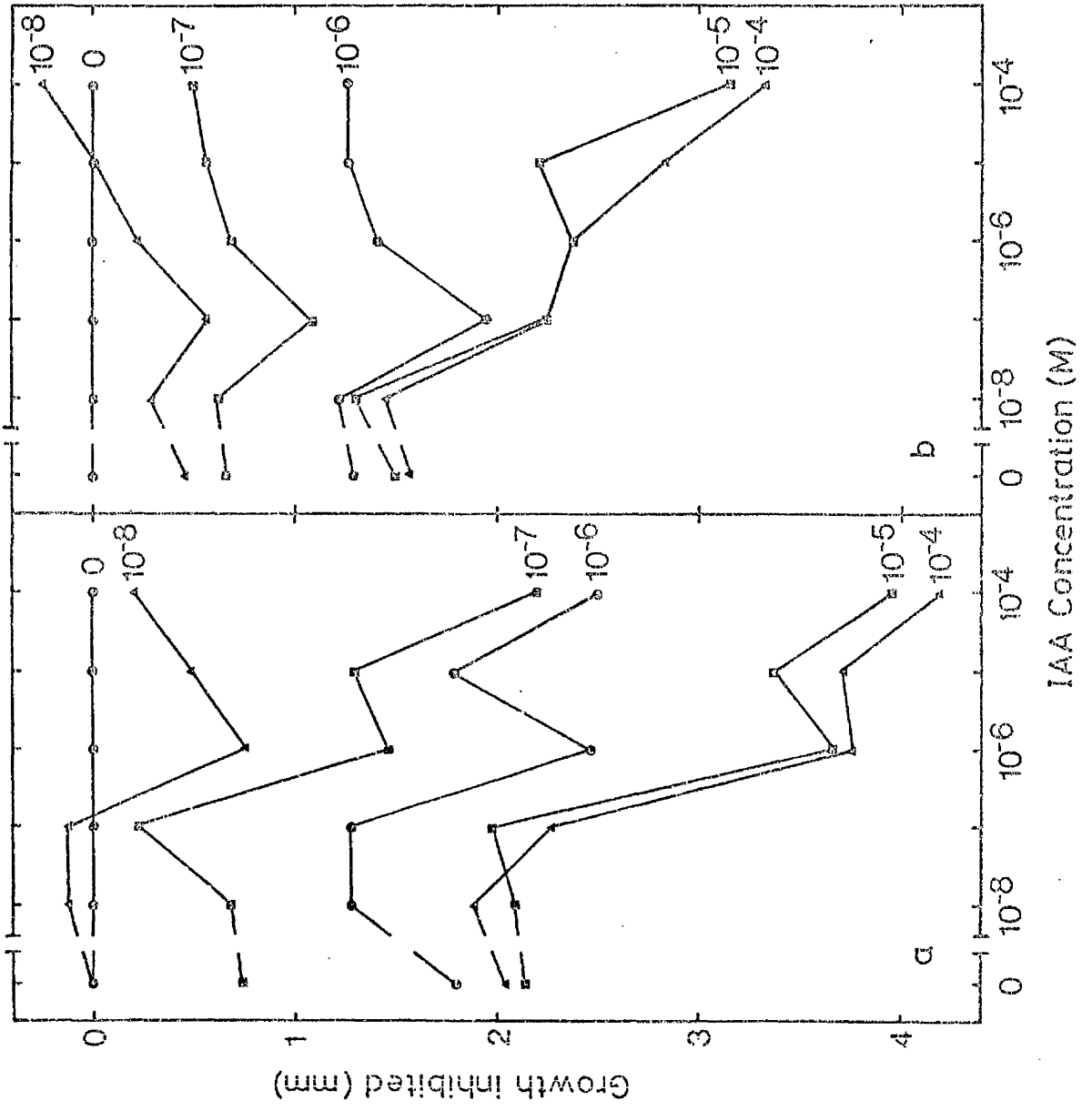

Growth inhibited (mm)
0
1
2
3
4
a
b
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
IAA Concentration (M)

Fig. 22. The activity of ABA, in the presence of IAA, in the Avena coleoptile straight growth assay.

a) Experiment A.

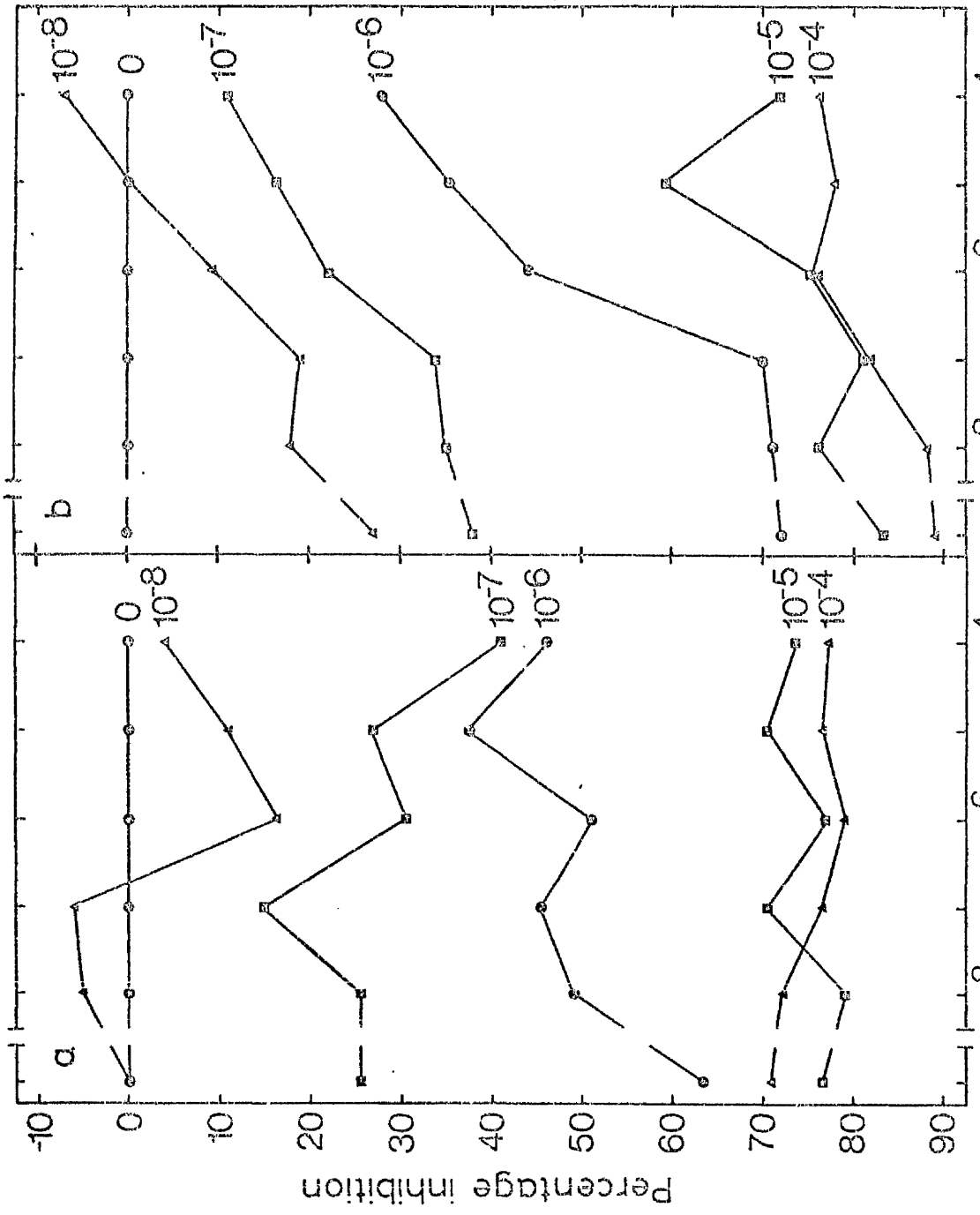
b) Experiment B.

The data are from the same experiments as in Fig. 20 a and b; data presented as percentage inhibition VS IAA concentration at five ABA concentrations and in the absence of ABA.

The percentage inhibition of growth by ABA at each IAA concentration was derived from:-

$$\left(\frac{\text{mean control increment} - \text{mean treatment increment}}{\text{mean control increment}}\right) \times 100$$

The control for each IAA concentration was taken as that treatment with IAA only present.


a
b
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
-10
0
10
20
30
40
50
60
70
80
90
Percentage inhibition
0
10^{-8}
10^{-6}
10^{-4}
IAA Concentration (M)

For example, the % inhibition by each of 6 ABA concentrations in the presence of 10^{-6}M IAA is dependent on the growth in the presence of both hormones and the growth promoted by 10^{-6}M IAA alone. Consequently, the errors within each experiment are not independent and because the independence of errors is one of the assumptions of the analysis of variance this test is not appropriate unless the data are transformed.

These considerations question the relevance of analysing data for a statistical interaction and the meaning of this term is considered more fully in the Discussion. Examination of the primary data, however, is extremely useful to indicate the effects of ABA at different IAA concentrations. For more detailed considerations of these effects analysis in terms of enzyme kinetics and detailed time courses of growth have been carried out (Sections 5 and 6).

4. The Molecular Requirements for Abscisic Acid Activity in the Presence of Indole-acetic Acid: The Straight Growth Assay

Abscisic acid modifies the action of IAA in the Avena coleoptile straight growth assay and ABA has certain molecular requirements for inhibitory activity in the absence of applied IAA. The next problem investigated was whether the molecular requirements for the action of ABA in the presence of IAA are similar to those for ABA alone. The 14 ABA analogues initially available were tested in the presence of IAA in the Avena coleoptile straight growth assay. A factorial experimental design and the analysis of variance were again employed. The analysis of variance was considered to be of value in that it provided an objective test to aid comparisons of the analogues with ABA.

The experiments were designed such that IAA and each analogue were tested at concentrations of 10^{-7}, 10^{-6} and 10^{-5}M with a distilled water control. The analogue concentrations correspond to those employed when assaying the analogues alone. To compare the data obtained with the activity of ABA in

the presence of IAA the ABA/IAA factorial experiments (Section 3) were re-analysed with the analysis of variance selecting those concentrations used in this series of experiments (Table 8).

The data presented in Figs. 23, 24, 25 and Tables 9, 10, 11, represent in each instance the results obtained from one experiment; similar results were obtained when the experiments and analyses were repeated. In all cases the IAA effect is significant ($P < 0.001$), as in previous experiments. The activity of these analogues in modifying the action of IAA reflects the activity of the analogues when assayed alone.

Compounds III, IV, V and IX possess all the structural requirements implied for the inhibitory activity of ABA. The activity of these 4 compounds in modifying IAA action is similar to that of ABA (Fig. 23). The analysis of variance reveals a significant effect of these analogues and a significant interaction with IAA in all cases (Table 9). Similar significance levels were also found in the analysis of the activity of ABA in the presence of IAA, when both hormones were assayed at 0, 10^{-7}, 10^{-6} and 10^{-5}M (Table 8). At 10^{-5}M these analogues overcome the promotory effect of IAA at an equimolar concentration; indeed, compounds III and IV inhibit growth compared with the distilled water control ($P < 0.001$). Thus on a mole for mole basis at 10^{-5}M compounds III and IV are more powerful inhibitors than IAA is a promoter.

Compounds I, II, VIII and XXIV possess inhibitory activity when tested alone, though this is considerably less than that of ABA. This lower activity is accounted for by the lack of the unsaturated functional group at C-1 which is present in ABA. These compounds are all less effective inhibitors of IAA action than is ABA (Fig. 24). Alone, their activity is significant in the analysis of variance but no significant interactions with IAA are detected (Table 10). In addition, a 1:1 molar ratio of each analogue to IAA, with both compounds at 10^{-5}M, clearly results in growth promotion compared with the distilled water controls ($P < 0.001$).

Although XXV possesses the moieties implicated for inhibition when tested

Table 8. Analysis of Variance : ABA Experiments.

IAA and ABA at 0, 10^{-7}, 10^{-6}, 10^{-5}M

(i) Experiment A.

Item	N	SS	MS	F	P
IAA	3	19.54	6.51	68.168	***
ABA	3	51.92	17.31	181.256	***
Interaction	9	3.69	0.41	4.293	**
Error	32	3.058	0.096	-	-

(ii) Experiment B.

Item	N	SS	MS	F	P
IAA	3	20.13	6.71	279.583	***
ABA	3	32.06	10.69	445.417	***
Interaction	9	3.84	0.43	17.917	***
Error	16	0.383	0.024	-	-

Fig. 23. The activity of four ABA analogues, in the presence of IAA, in the Avena coleoptile straight growth assay.

a) Analogue III.

b) Analogue IV.

c) Analogue V.

d) Analogue IX.

Analogue concentrations:-

—●— 0

—○— 10^{-7}M

—■— 10^{-6}M

—□— 10^{-5}M

The data are presented as mean coleoptile segment length VS IAA concentration, at 3 analogue concentrations and in the absence of any analogue. The data for each analogue are the mean from one experiment, employing 2 replicates of 10 segments per treatment. Similar results were obtained in one repeat of these treatments.

Statistical Analysis. The t-test was used to test the difference in segment length between the water control in each experiment and the treatment with 10^{-5}M IAA + 10^{-5}M analogue.

Analogue	t value	Significance
III	7.247	***
IV	6.475	***
V	0.933	NS
IX	1.139	NS

For further analysis see Table 9.

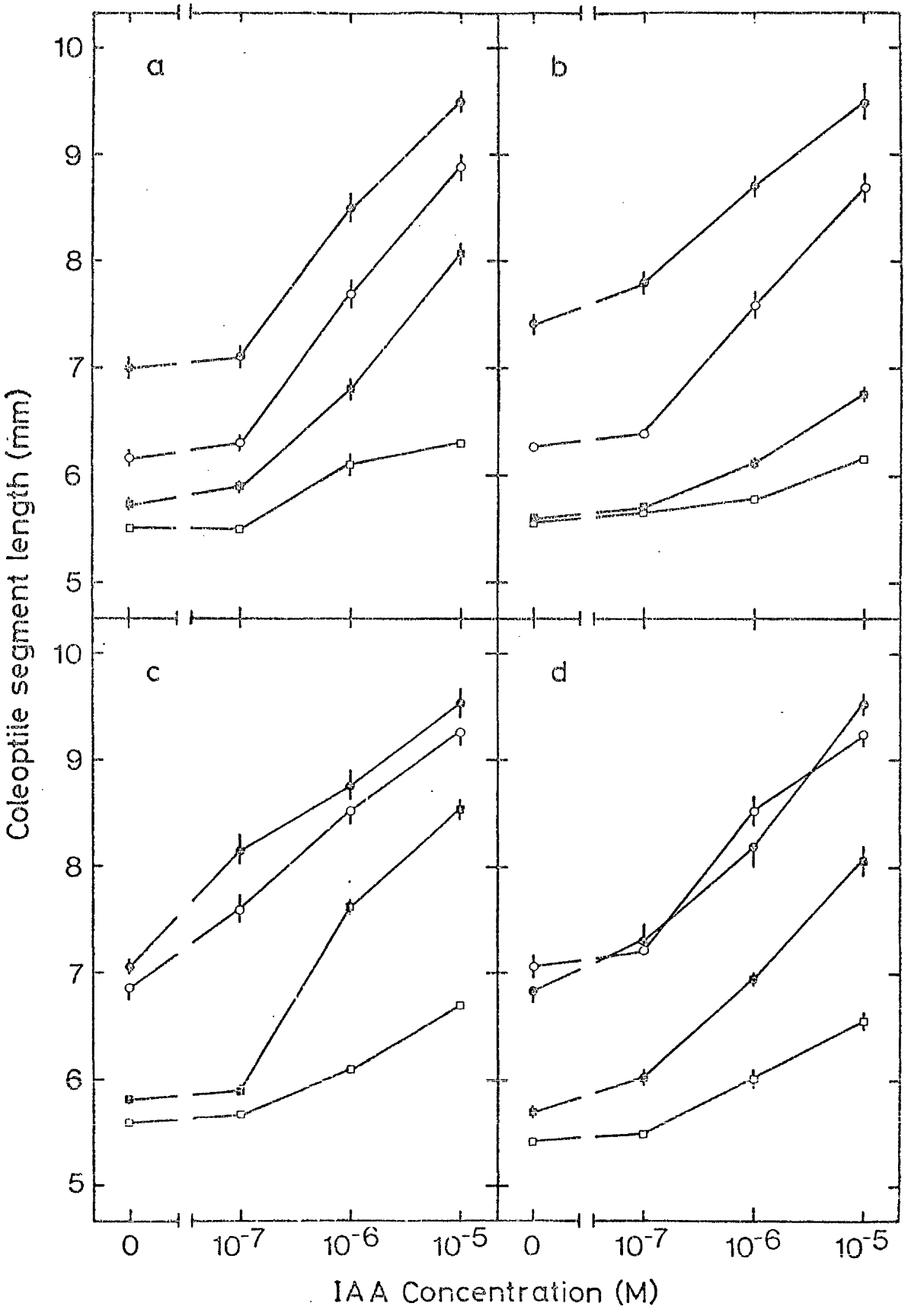

a
b
c
d
10
9
8
7
6
5
0
10^{-7}
10^{-6}
10^{-5}
Coleoptile segment length (mm)
IAA Concentration (M)

Table 9. Analysis of Variance.

(i) Analogue III.

Item	N	SS	MS	F	P
IAA	3	23.870	7.957	568.357	***
III	3	20.958	6.986	499.000	***
Interaction	9	2.937	0.326	23.286	***
Error	16	0.222	0.014	-	-

(ii) Analogue IV.

Item	N	SS	MS	F	P
IAA	3	12.349	4.116	457.333	***
IV	3	33.333	11.111	1234.556	***
Interaction	9	2.978	0.331	36.778	***
Error	16	0.151	0.009	-	-

(iii) Analogue V.

Item	N	SS	MS	F	P
IAA	3	23.130	7.710	350.455	***
V	3	27.362	9.121	414.591	***
Interaction	9	2.729	0.303	13.773	***
Error	16	0.361	0.022	-	-

(iv) Analogue IX.

Item	N	SS	MS	F	P
IAA	3	21.749	7.250	345.238	***
IX	3	26.173	8.724	415.423	***
Interaction	9	1.686	0.187	8.905	***
Error	16	0.338	0.021	-	-

Fig. 24. The activity of four ABA analogues, in the presence of IAA, in the *Avena* coleoptile straight growth assay.

a) Analogue I.

b) Analogue II.

c) Analogue VIII.

d) Analogue XXIV.

Analogue concentrations:-

—●— 0

—○— 10^{-7} M

—■— 10^{-6} M

—□— 10^{-5} M

The data are presented as mean coleoptile segment length VS IAA concentration, at 3 analogue concentrations and in the absence of any analogue. The data for each analogue are the mean from one experiment, employing 2 replicates of 10 segments per treatment. Similar results were obtained in one repeat of these treatments.

Statistical Analysis. The t-test was used to test the difference in segment length between the water control in each experiment and the treatment with 10^{-5} M IAA + 10^{-5} M analogue.

Analogue	t value	Significance
I	10.93	***
II	10.21	***
VIII	8.71	***
XXIV	16.33	***

For further analysis see Table 10.

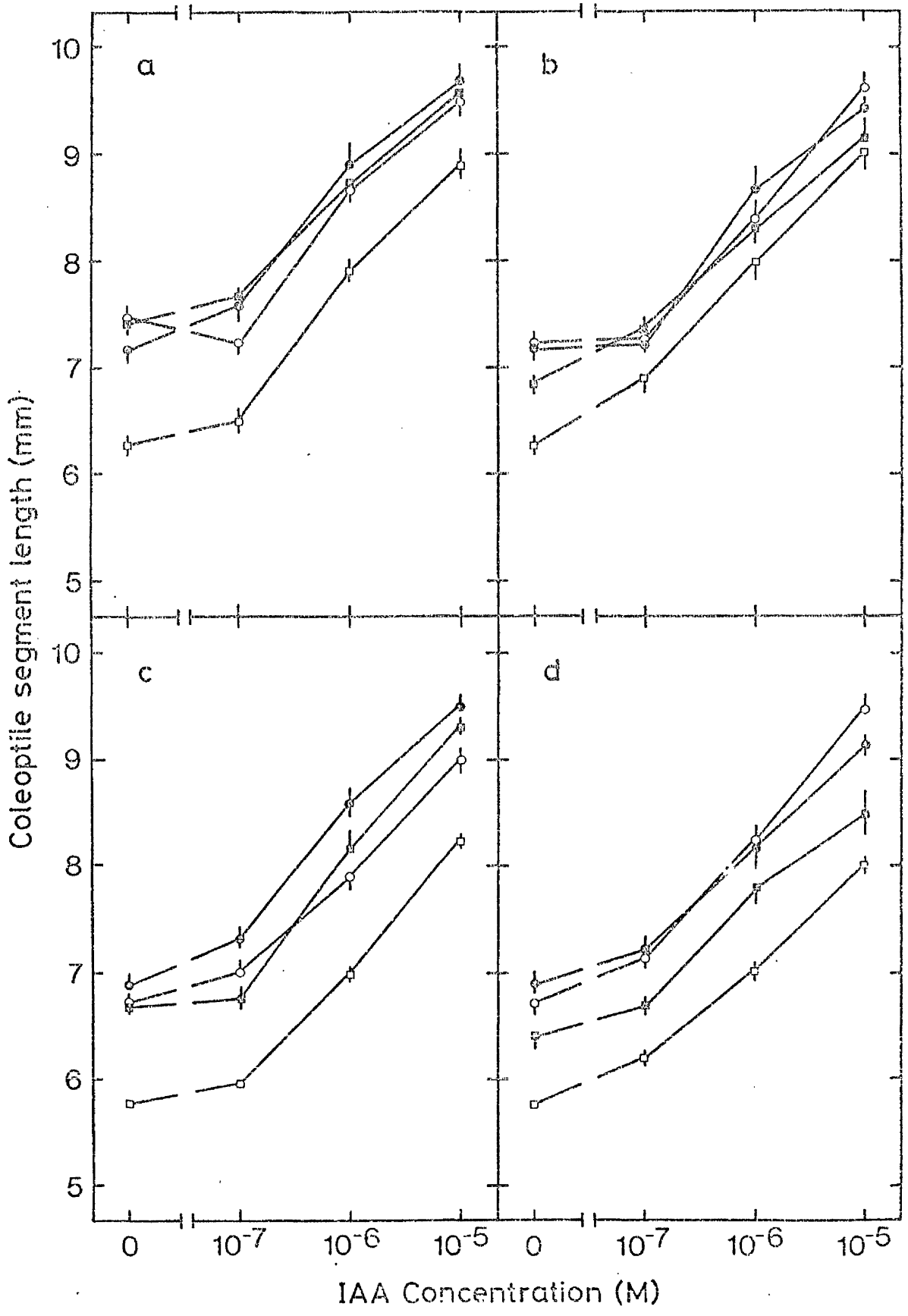

a
b
c
d
10
9
8
7
6
5
0
10^{-7}
10^{-6}
10^{-5}
Coleoptile segment length (mm)
IAA Concentration (M)

Table 10. Analysis of Variance.

(i) Analogue I.

Item	N	SS	MS	F	P
IAA	3	29.585	9.862	328.733	***
I	3	4.896	1.632	54.400	***
Interaction	9	0.433	0.048	1.600	NS
Error	16	0.473	0.030	-	-

(ii) Analogue II.

Item	N	SS	MS	F	P
IAA	3	30.440	10.147	281.861	***
II	3	1.651	0.553	15.361	***
Interaction	9	0.520	0.058	1.611	NS
Error	16	0.581	0.036	-	-

(iii) Analogue VIII.

Item	N	SS	MS	F	P
IAA	3	30.958	10.319	264.590	***
VIII	3	7.823	2.608	66.872	***
Interaction	9	0.364	0.040	1.026	NS
Error	16	0.623	0.039	-	-

(iv) Analogue XXIV.

Item	N	SS	MS	F	P
IAA	3	28.496	9.499	339.250	***
XXIV	3	6.063	2.021	72.179	***
Interaction	9	0.413	0.046	1.643	NS
Error	16	0.449	0.028	-	-

alone it has reduced activity compared with ABA, the reduction in activity is explained by the presence of a sulphur atom within the molecule. This structural modification also reduces the activity of XXV in a factorial experiment with IAA (Fig. 25a). No significant interaction is recorded and in the presence of 10^{-5}M IAA and 10^{-5}M XXV there is promotion of growth compared with the control (Table 11(i)). Compound XXVIII is not active alone and in these experiments it has no effect on IAA-induced growth (Fig. 25b, Table 11(ii)).

Thus the molecular requirements for ABA activity in the presence of IAA appear similar to those for the inhibitory activity of ABA alone. Structural modifications which reduce the inhibitory activity of an analogue when assayed alone also reduce the effectiveness of this analogue in inhibiting the promotory effects of IAA.

In contrast, the activities of analogues VI, XXIX, XXX and XXXIII, in the presence of IAA, are inconsistent with these inferences (Fig. 26). Compounds VI, XXX and XXXIII inhibit growth when tested alone but are less active than ABA; compound XXIX is inactive alone. In the presence of IAA, however, significant interactions are recorded in all cases (Table 12). These interactions are partially due to the unusual, but very variable, dosage-response patterns observed at increasing IAA concentration. Because of the variability obtained in repeat experiments further theoretical consideration is not given to the mode of action of these analogues.

5. The Action of Abscisic Acid in the Presence of Indole-acetic Acid : Enzyme Kinetics

Abscisic acid modifies the promotory effects of IAA in the Avena coleoptile assay and the analysis of variance indicates an interaction between these hormones. It was decided to investigate further the action of ABA in the presence of IAA using experiments which are amenable to analysis in terms of enzyme kinetics. The execution of this type of experiment was stimulated

Fig. 25. The activity of two ABA analogues, in the presence of IAA, in the Avena coleoptile straight growth assay.

a) Analogue XXV.

b) Analogue XXVIII.

Analogue concentrations:-

—●— 0

—○— 10^{-7}M

—■— 10^{-6}M

—□— 10^{-5}M

The data are presented as mean coleoptile segment length VS IAA concentration at 3 analogue concentrations and in the absence of any analogue. The data for both analogues are the mean from one experiment, employing 2 replicates of 10 segments per treatment. Similar results were obtained in one repeat of these treatments.

Statistical Analysis. The t-test was used to test the difference between the segment length for the water control and the treatment with 10^{-5}M IAA + 10^{-5}M analogue.

Analogue	t value	Significance
XXV	8.89	***

For further analysis see Table 11.

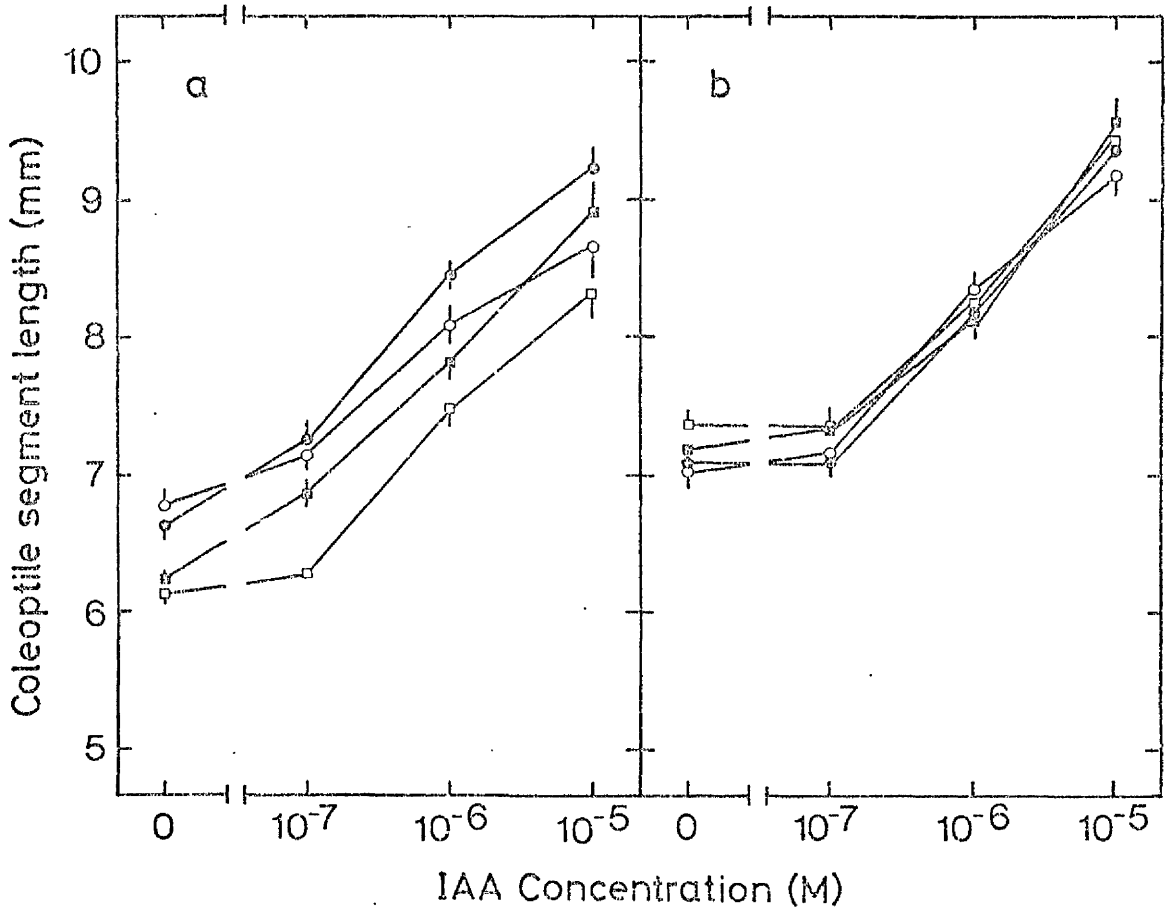

a
b
10
9
8
7
6
5
Coleoptile segment length (mm)
0
10-7
10-6
10-5
0
10-7
10-6
10-5
IAA Concentration (M)

Table 11. Analysis of Variance.

(i) Analogue XXV.

Item	N	SS	MS	F	P
IAA	3	26.985	8.995	121.554	***
XXV	3	3.142	1.047	14.148	***
Interaction	9	0.662	0.074	1.000	NS
Error	16	1.178	0.074	-	-

(ii) Analogue XXVIII.

Item	N	SS	MS	F	P
IAA	3	26.101	8.700	127.941	***
XXVIII	3	0.150	0.050	0.735	NS
Interaction	9	0.294	0.036	0.529	NS
Error	16	1.085	0.068	-	-

Fig. 26. The activity of four ABA analogues, in the presence of IAA, in the Avena coleoptile straight growth assay.

a) Analogue VI.

b) Analogue XXIX.

c) Analogue XXX.

d) Analogue XXXIII.

Analogue concentrations:-

—●— 0

—○— 10^{-7}M

—■— 10^{-6}M

—□— 10^{-5}M

The data are presented as mean coleoptile segment length vs IAA concentration at 3 analogue concentrations and in the absence of any analogue. The data are the mean of 1 experiment, employing 2 replicates of 10 segments per treatment.

Statistical Analysis : see Table 12.

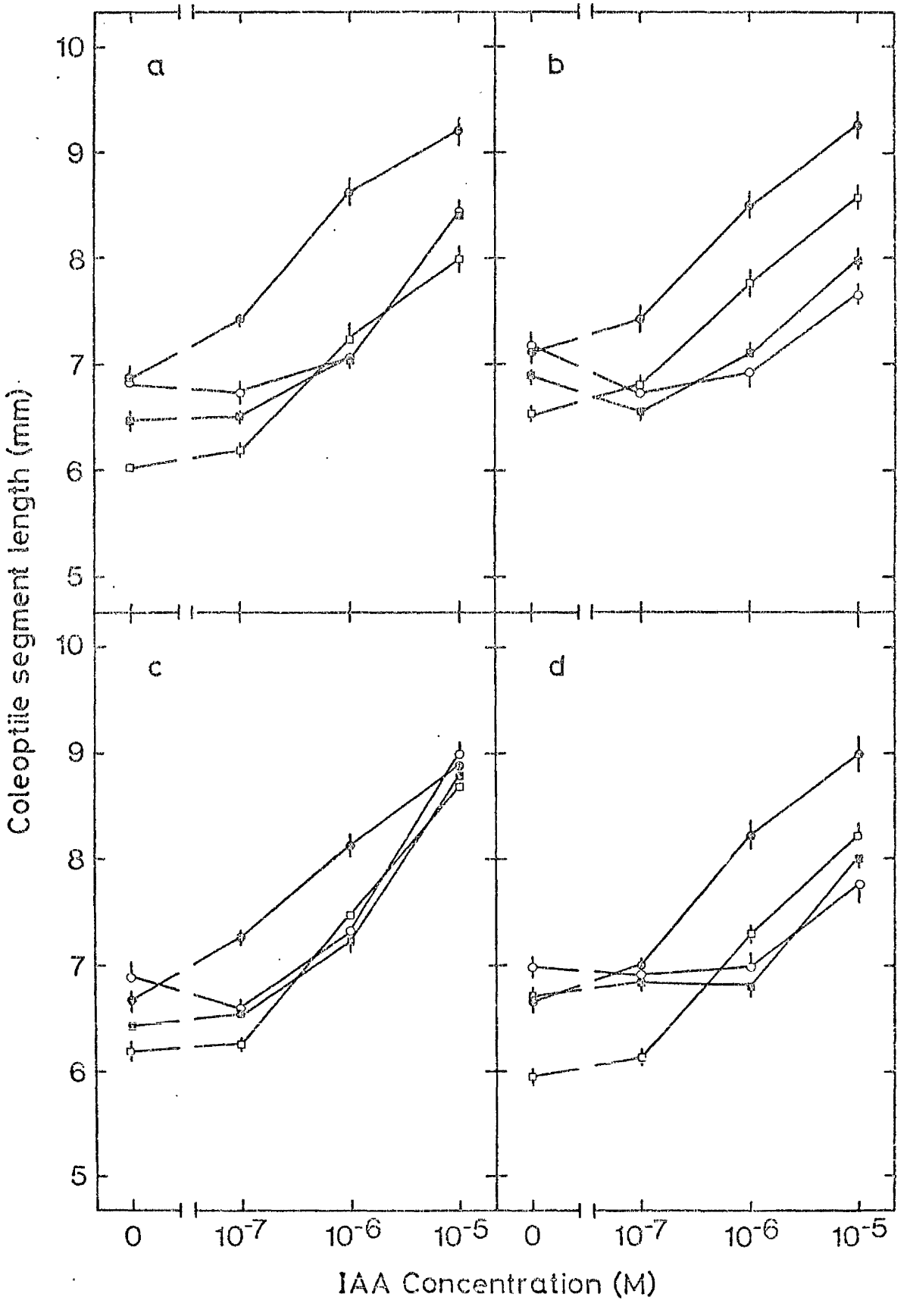

a
b
c
d
10
9
8
7
6
5
0
10^{-7}
10^{-6}
10^{-5}
Coleoptile segment length (mm)
IAA Concentration (M)

Table 12. Analysis of Variance.

(i) Analogue VI.

Item	N	SS	MS	F	P
IAA	3	17.721	5.907	656.333	***
VI	3	5.951	1.983	220.333	***
Interaction	9	1.631	0.181	20.111	***
Error	16	0.152	0.009	-	-

(ii) Analogue XXIX.

Item	N	SS	MS	F	P
IAA	3	11.923	3.974	110.389	***
XXIX	3	4.570	1.523	42.306	***
Interaction	9	2.701	0.300	8.333	***
Error	16	0.582	0.036	-	-

(iii) Analogue XXX.

Item	N	SS	MS	F	P
IAA	3	27.041	9.014	450.700	***
XXX	3	1.670	0.557	27.850	***
Interaction	9	1.115	0.124	6.200	**
Error	16	0.327	0.020	-	-

(iv) Analogue XXXIII.

Item	N	SS	MS	F	P
IAA	3	13.895	4.632	149.419	***
XXXIII	3	2.852	0.951	30.677	***
Interaction	9	3.105	0.345	11.129	***
Error	16	0.502	0.031	-	-

by the findings of Bonner and his colleagues (e.g. Bonner and Foster, 1955) who demonstrated that the kinetics of IAA-induced growth are, under certain conditions, identical to the kinetics of many enzyme reactions. In addition, it could be argued that the molecular requirements for ABA activity relate to the binding of ABA to an enzyme site, thus inhibiting both endogenous growth and the activity of applied IAA. The analysis may indicate whether ABA is acting via competitive or non-competitive inhibition (see Introduction).

In order to maintain the data amenable to enzymic analysis two pre-requisites must be considered when designing these experiments. Firstly, the initial rate of the reaction must be determined. The velocity of an enzyme reaction decreases with time, due for example, to the products of the reaction inhibiting the enzyme. Hence it is necessary to establish the initial rate of the reaction before the factors contributing to the decrease in velocity have time to operate. Secondly, because in nearly all enzymic reactions a graph of initial rates VS substrate concentration yields a section of a rectangular hyperbola then the IAA concentrations selected must be within the correct range to include points on the curve of this hyperbola.

The experiments were carried out in the absence of either buffer or an external energy supply, such as sucrose, to avoid the complicating effects of these additives. Under these conditions and at 25°C the response of coleoptile segments to 10^{-5}M IAA, 10^{-5}M ABA and distilled water was studied over an 8-h period (Fig. 27a and b). In the 3 treatments the initial rate of elongation, i.e. that estimated at 0.5h, is the highest; the rate subsequently declines and there are fluctuations in the growth rate. Indeed, the more pronounced fluctuations are observed in this experiment in which the rate is estimated over a 1-h period than in a previous experiment (Fig. 11b) in which the rate is estimated at 2h intervals. To establish the initial rate of elongation of segments a 4-h time course, in which elongation was measured at 30-minute intervals, was carried out. This provided 8 data for each hormone treatment and enabled the initial rate of response to be calculated.

Fig. 27. 8-h time courses of elongation of Avena coleoptile segments at 25°C.

Treatments 1 : 10^{-5}M IAA.

2 : Distilled H_2O.

3 : 10^{-5}M ABA.

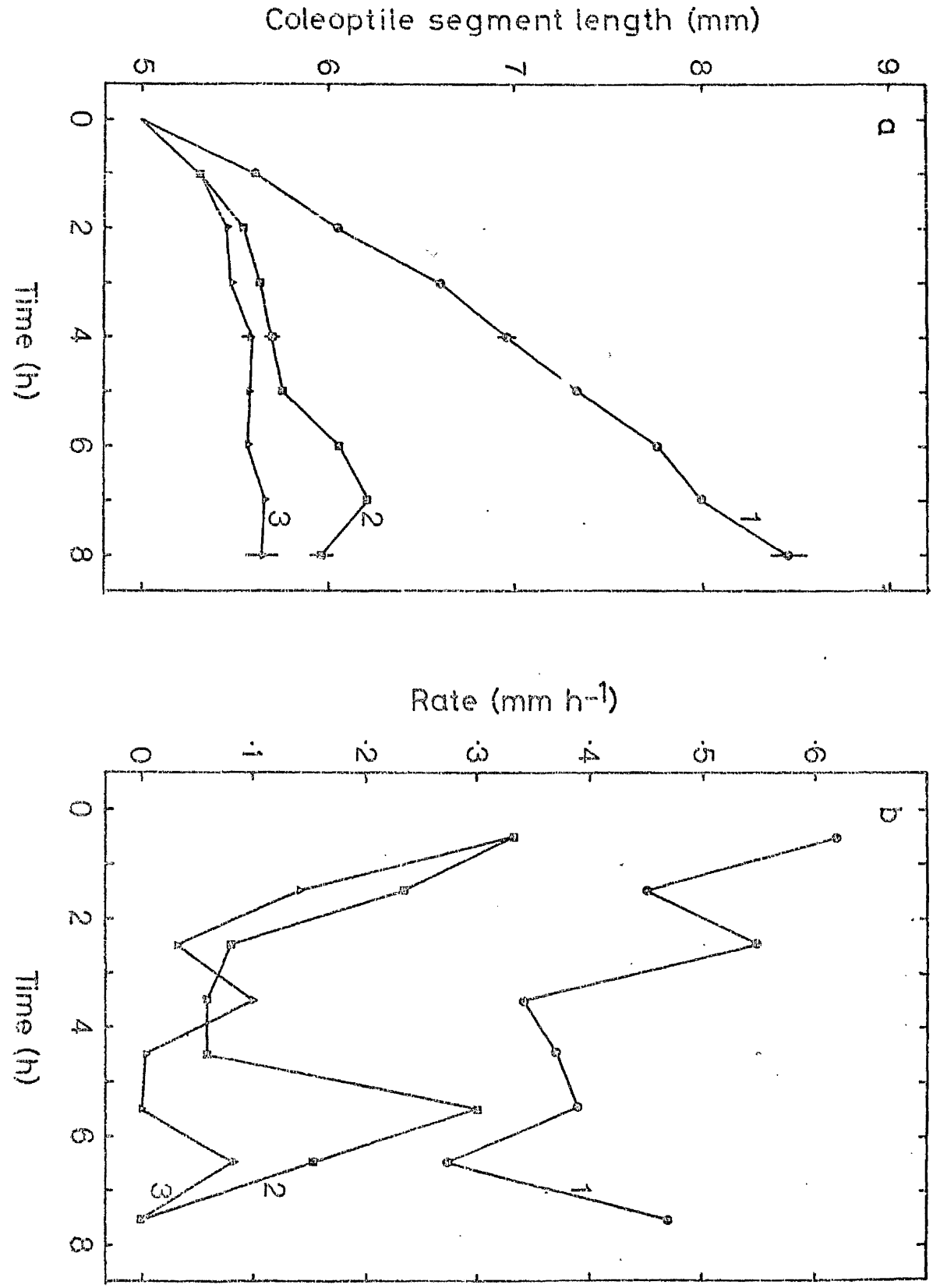
a) Coleoptile segment length (mm) as a function of time.

b) Rate of elongation of segments as a function of time. The rates were calculated at 1-h intervals from the relationship:-

$$\frac{\text{increase in length from time 1 to time 2}}{\text{time interval}}$$

The data are the mean from 1 experiment, employing 20 segments for each time point.

Similar results were obtained in one repeat of this experiment.


Coleoptile segment length (mm)
5
6
7
8
9
a
Time (h)
0
2
4
6
8
1
2
3
Rate (mm h⁻¹)
0
·1
·2
·3
·4
·5
·6
b
Time (h)
0
2
4
6
8
1
2
3

To select the range of substrate concentrations required the data from a 24 h straight growth assay were replotted on a linear scale (Fig. 28). Although these data are not initial rates they indicate that concentrations of approximately 10^{-7} to 10^{-5}M IAA include the curve of the hyperbola. Eight concentrations were selected, 6×10^{-7}, 8×10^{-7}, 1×10^{-6}, 3×10^{-6}, 5×10^{-6}, 7×10^{-6}, 9×10^{-6} and 2×10^{-5}M. Thus, including the water control, there are 9 data contributing to each curve of initial rate at increasing substrate concentrations. Three ABA concentrations were selected to produce a range of inhibition. When the final coleoptile segment lengths, after a 4 h incubation, are presented as a function of IAA concentration, it is apparent that the correct range of IAA concentrations has been selected to embrace the curve of the observed rectangular hyperbola (Fig. 29).

The large number of experimental treatments resulted in time courses for only half the matrix of hormone concentrations being carried out on one day. The matrix was completed over a similar time period on the following day and the complete experiment carried out 3 times. There were 5 segments in a petri dish for each treatment. The mean data are presented in Figs. 30, 31 and 32.

These time courses indicate a burst of growth during the first 30 minute period, possibly the result of endogenous IAA or the stimulating effect of cutting the segments; this is particularly obvious in the absence of IAA treatment (Fig. 30). There is a latent period before the inhibitory effect of ABA is apparent, that is dependent upon the concentration of both hormones. Consequently, the initial rates of elongation at each IAA concentration are similar for the 4 inhibitor concentrations employed and it is irrelevant to estimate these initial rates because the ABA activity is not yet evident. Instead, the initial rates of elongation were estimated over the period 2 to 4 h, on the assumption that the ABA-induced inhibition had begun. Nevertheless, a graph of these initial rates at each ABA concentration, as

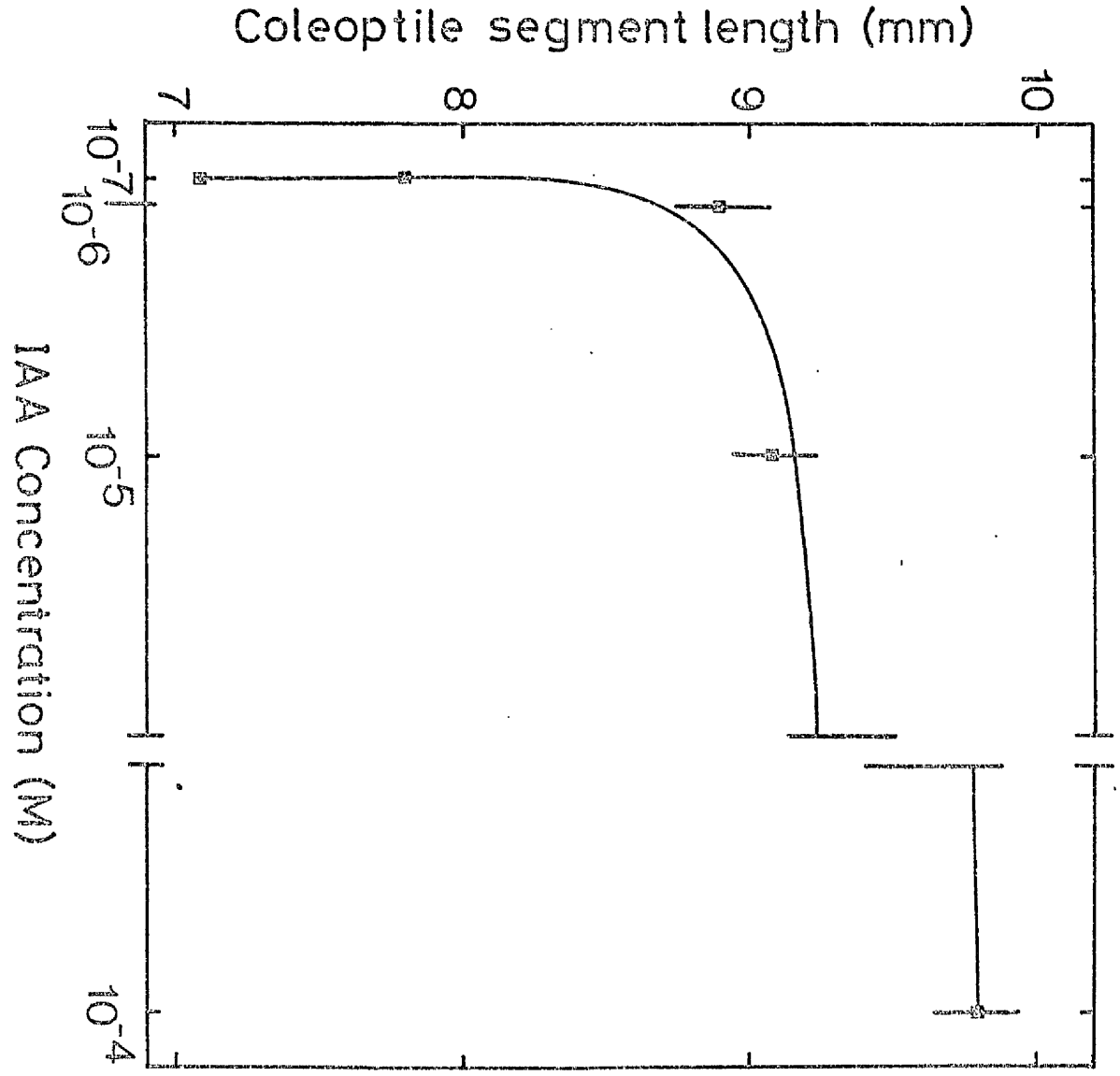

Coleoptile segment length (mm)
7
8
9
10
IAA Concentration (M)
10-7
10-6
10-5
10-4

Fig. 29. The effect of a range of concentrations of IAA, alone and in the presence of 3 concentrations of ABA, on the elongation of *Avena* coleoptile segments, after 4 h incubation at 25°C.

ABA concentrations:

1 : 0

2 : 10^{-7} M

3 : 10^{-6} M

4 : 10^{-5} M

The curves were fitted by eye. The mean length of 15 segments from 3 experiments are presented for each treatment.

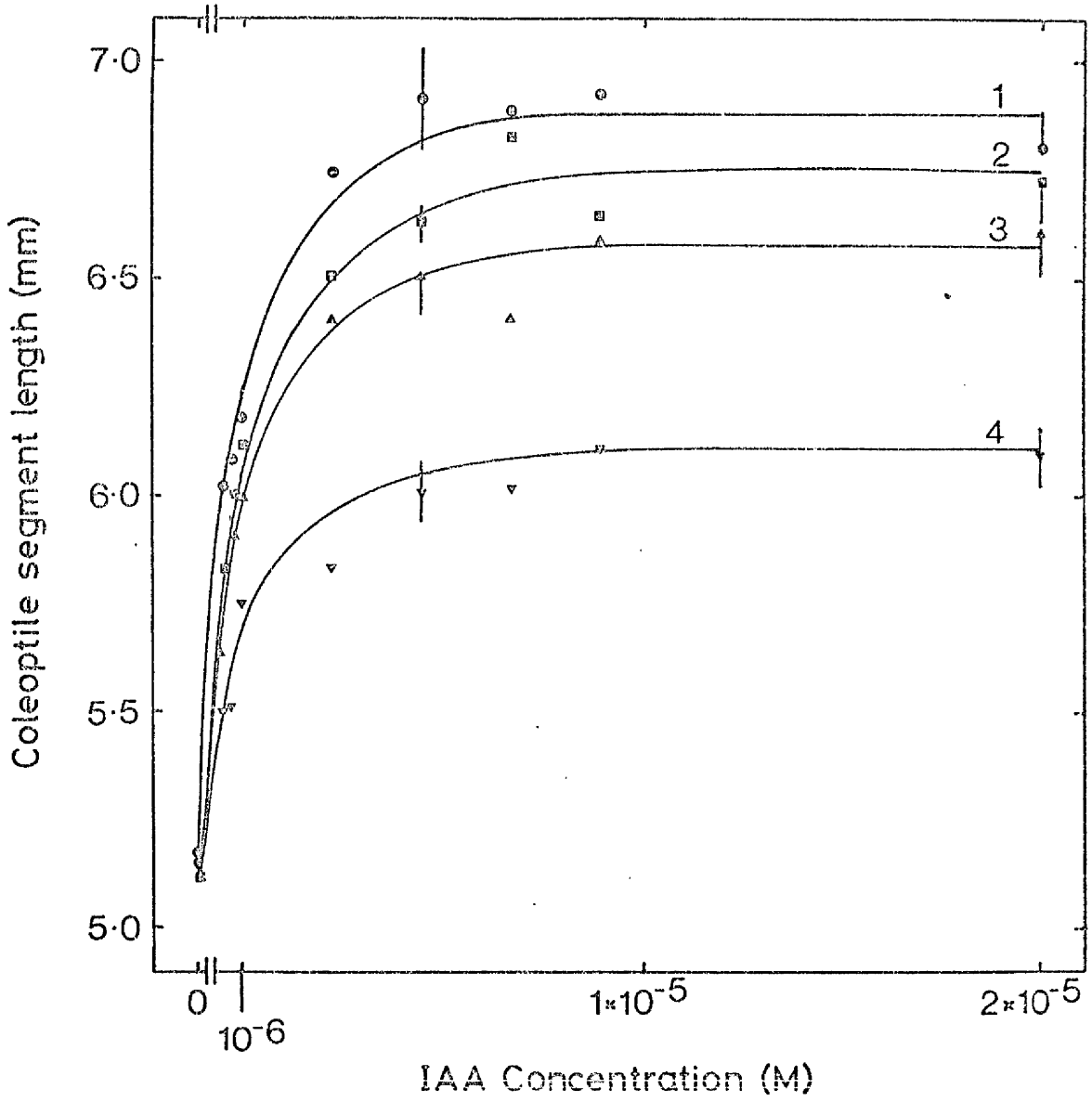

7·0
6·5
6·0
5·5
5·0
Coleoptile segment length (mm)
1
2
3
4
0
10⁻⁶
1×10⁻⁵
2×10⁻⁵
IAA Concentration (M)

Fig. 30. Time courses of the effect of four concentrations of IAA on the elongation of Avena coleoptile segments at 25°C.

a) 0 IAA.

b) 6×10^{-7}M IAA.

c) 8×10^{-7}M IAA.

d) 1×10^{-6}M IAA.

Each IAA concentration was tested alone, and in the presence of 3 concentrations of ABA.

—●— 0 ABA.

—○— 10^{-7}M ABA.

—■— 10^{-6}M ABA.

—□— 10^{-5}M ABA.

The mean length of 15 segments from 3 experiments was calculated for each treatment.

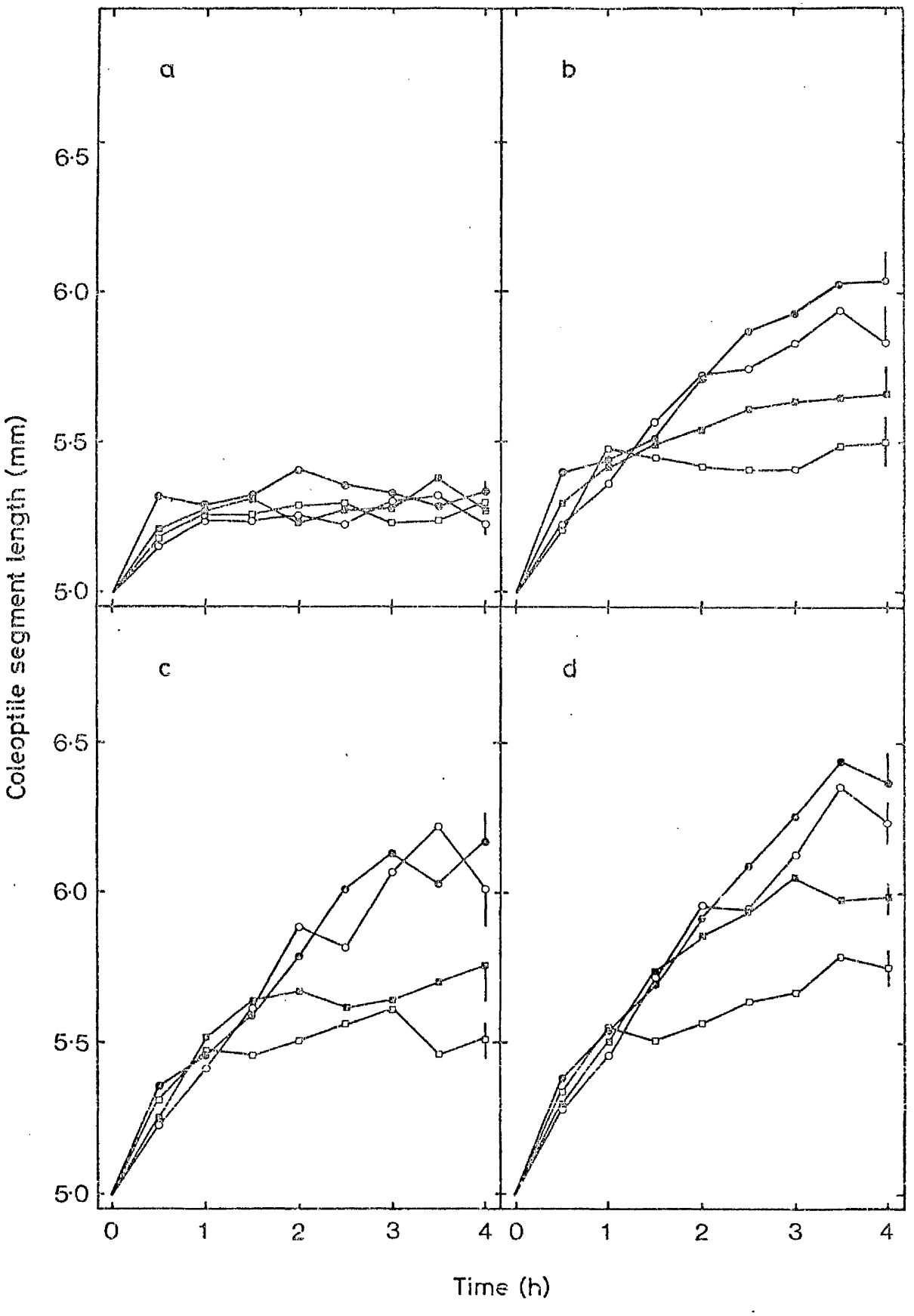

a
b
c
d
6·5
6·0
5·5
5·0
0
1
2
3
4
Coleoptile segment length (mm)
Time (h)

Fig. 31. Time courses of the effect of four concentrations of IAA on the elongation of Avena coleoptile segments at 25°C.

a) 3×10^{-6}M IAA.

b) 5×10^{-6}M IAA.

c) 7×10^{-6}M IAA.

d) 9×10^{-6}M IAA.

Each IAA concentration was tested alone, and in the presence of 3 concentrations of ABA.

—●— 0 ABA.

—○— 10^{-7}M ABA.

—■— 10^{-6}M ABA.

—□— 10^{-5}M ABA.

The mean length of 15 segments from 3 experiments was calculated for each treatment.

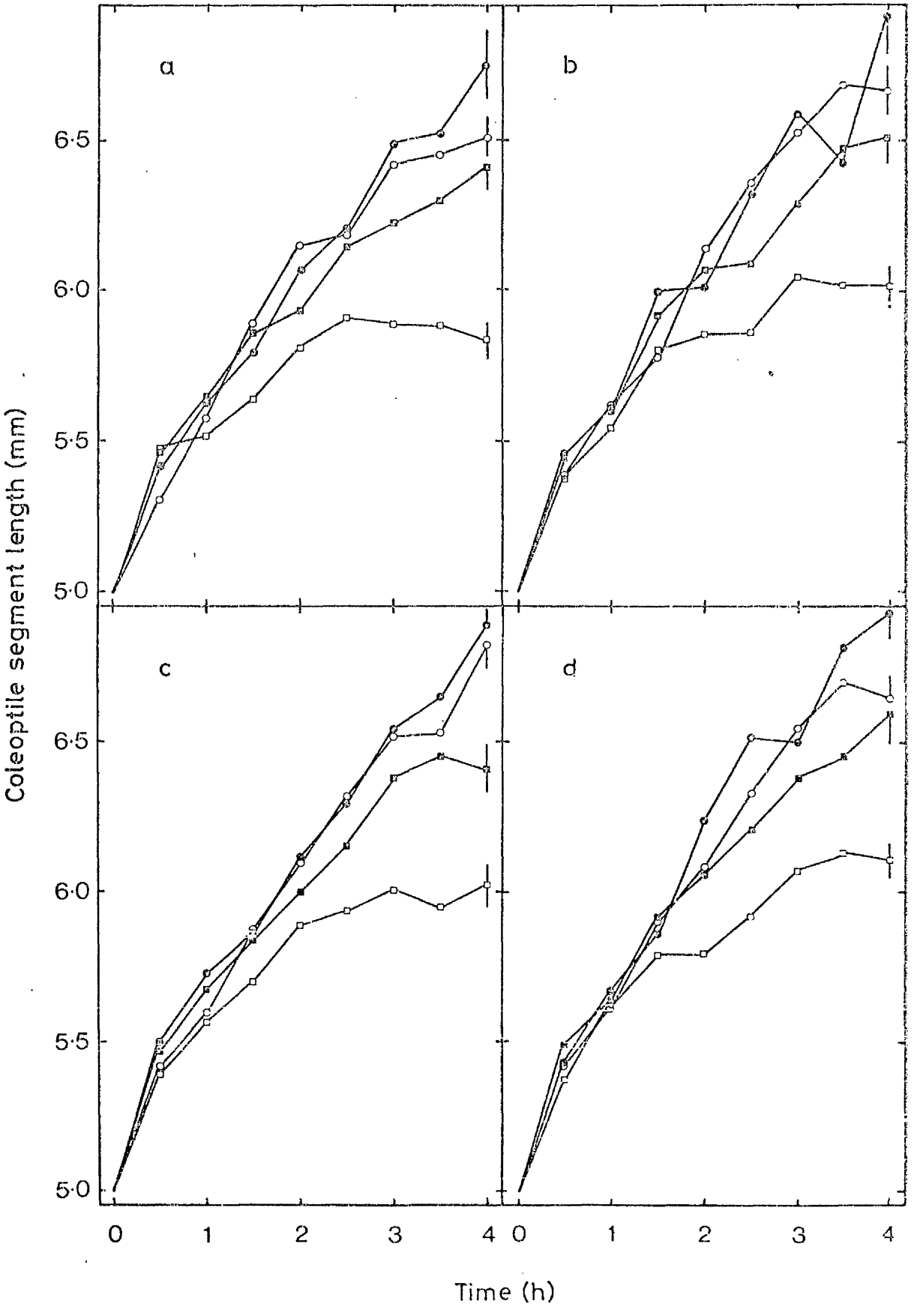
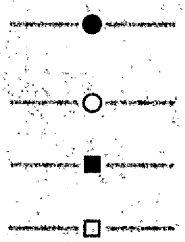

a
b
c
d
6·5
6·0
5·5
5·0
0
1
2
3
4
Coleoptile segment length (mm)
Time (h)

Fig. 32. Time course of the effect of 2 x 10^{-5} M IAA on the elongation of Avena coleoptile segments at 25°C.

The IAA was tested alone, and in the presence of 3 concentrations of ABA.



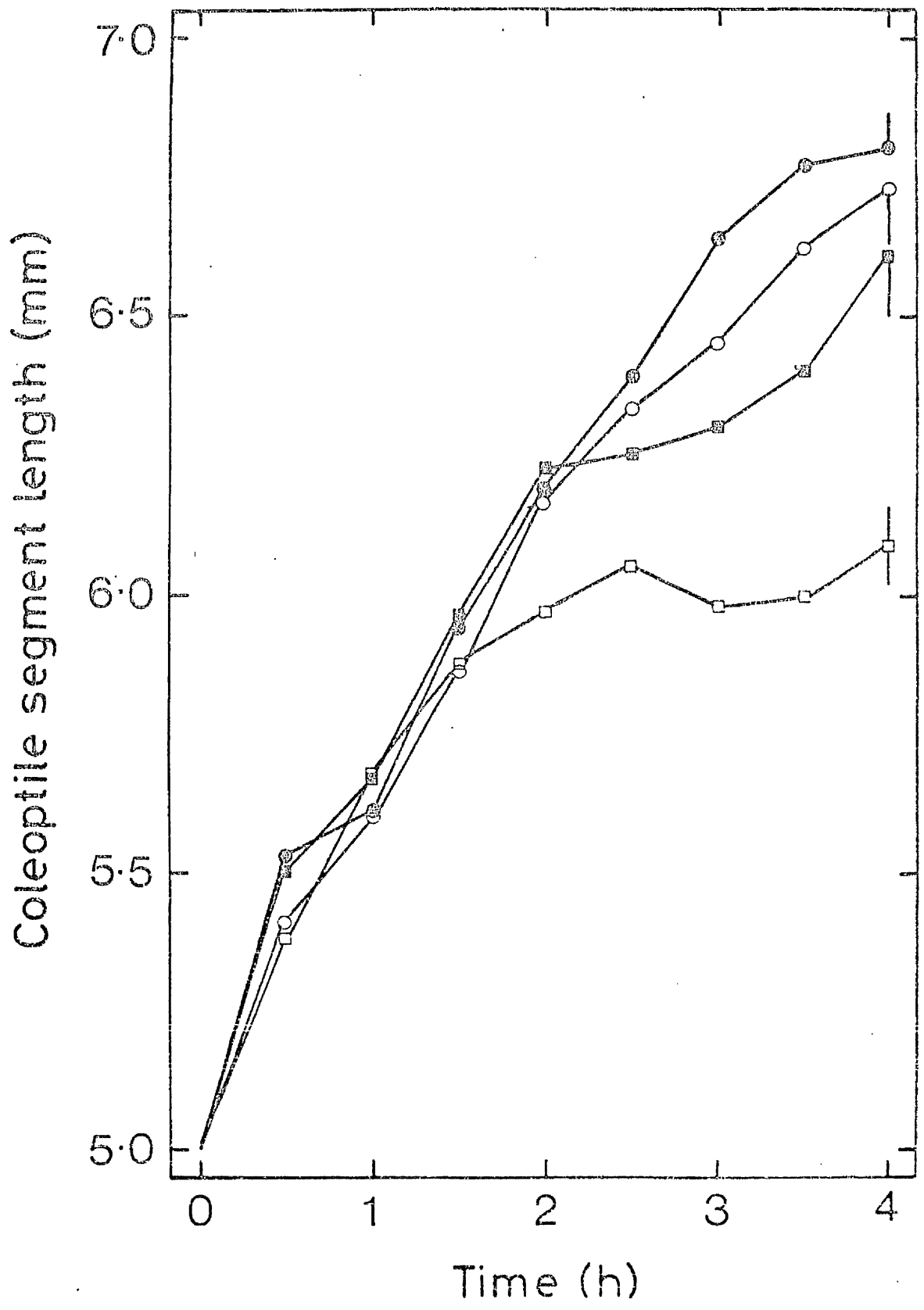
0 ABA.

10^{-7} M ABA.

10^{-6} M ABA.

10^{-5} M ABA.

The mean length of 15 segments from 3 experiments was calculated for each treatment.


7·0
6·5
6·0
5·5
5·0
Coleoptile segment length (mm)
0
1
2
3
4
Time (h)

a function of IAA concentration, does not reveal the anticipated hyperbola which is typical of an enzymic reaction (Fig. 33). The scatter of points is undoubtedly due partially to experimental variation. This suggestion is supported by the fact that the KDF 9 computer, which was used to estimate the initial rates, was unable to fit a polynomial equation to the time course data at some hormone concentrations. Indeed, in certain instances a negative initial rate is observed (fly sheet, Fig. 33). The scatter of points in this graph is probably also attributable to both the fact that ABA inhibition develops at different times with varying hormone concentrations and that growth is stimulated by IAA from 2h before the initial rates are calculated.

Because the graph of initial rates (B) VS IAA concentration (S) yields only a scatter of points the next stage in the analysis, i.e. a double reciprocal plot of $^{1}/_{B}$ VS $^{1}/_{S}$, is not justified. This latter representation of the data may have indicated a competitive or non-competitive inhibition.

If this type of experiment were to be further employed the initial burst of growth after sectioning and the precise latent period for ABA inhibition at all concentrations should be considered. The experimental variation may be reduced if the complete range of treatments are carried out on one occasion. Moreover, it is necessary to measure accurately small differences in elongation and a more sensitive measuring system may be profitable.

6. The Continuous Monitoring of Elongation

The timing of a response is a fundamental aspect required for the understanding of the mode of action of an applied growth substance. Experiments using the shadowgraph technique have given an indication of a latent period for ABA action, but no latent period before IAA promotion of growth was apparent. A latent period of approximately 10 minutes for IAA activity, however, has been reported (e.g. Ray and Ruesink, 1962). In view of the

Fig. 33. Initial rate of elongation of Avena coleoptile segments, as a function of IAA concentration, at 3 ABA concentrations and in the absence of ABA.

ABA concentrations:-

—●— 0 ABA.

—○— 10^{-7}M ABA.

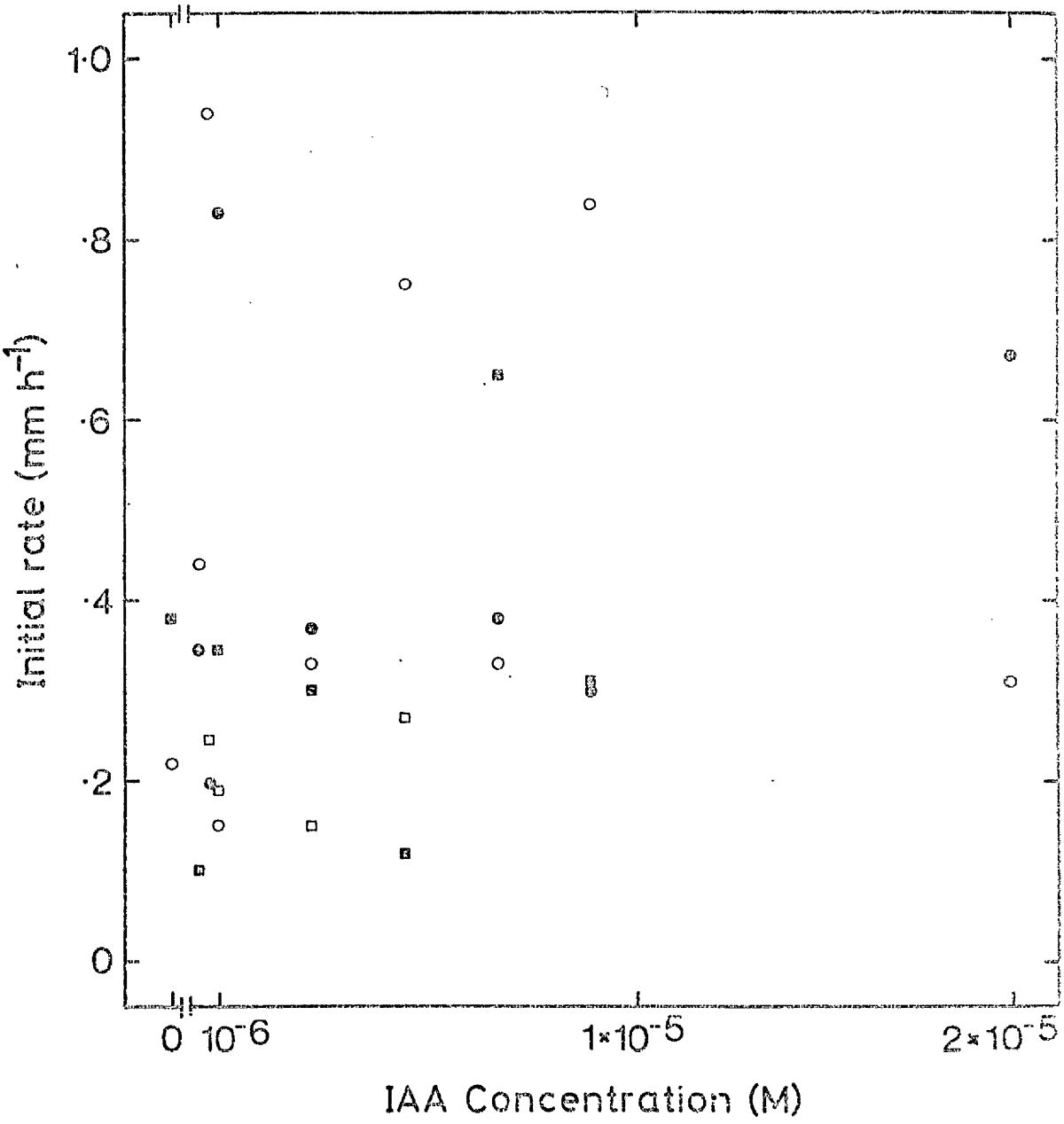
—■— 10^{-6}M ABA.

—□— 10^{-5}M ABA.

The initial rates were estimated from the time course data of Figs. 30, 31, and 32, beginning at time 2 h.

Initial rate of elongation (mm h^{-1})

IAA Conc.(M)	ABA Concentration (M) 0	10^{-7}	10^{-6}	10^{-5}
0	-0.213	0.216	0.387	-0.168
6×10^{-7}	0.343	0.436	0.106	-0.049
8×10^{-7}	0.199	0.939	-0.136	0.245
1×10^{-6}	0.832	0.152	0.345	0.190
3×10^{-6}	0.368	0.324	0.303	0.149
5×10^{-6}	-0.210	0.750	0.122	0.269
7×10^{-6}	0.380	0.333	0.651	-
9×10^{-6}	0.296	0.837	0.309	-
2×10^{-5}	0.668	0.312	-0.019	-


1·0
·8
·6
·4
·2
0
Initial rate (mm h⁻¹)
0 10⁻⁶
1×10⁻⁵
2×10⁻⁵
IAA Concentration (M)

fact that ABA modifies IAA-induced growth it is relevant to develop a technique to study the effect of ABA on both endogenous growth as well as on the initial response to IAA.

a. The Techniques. The first experiments in this series were conducted to evaluate 3 measuring techniques. Initially, a microscope technique was employed that was capable of detecting a growth rate of 1 μm min.$^{-1}$ for a single segment. There were, however, several problems associated with this technique. These problems included difficulty in focusing on the elongating end of the segment, difficulty in securing one end of the segment, in addition to crooked growth of the segment resulting in lateral movement under the micrometer eyepiece. Furthermore, there was large variation in the initial growth of segments in water. This may have been due to variation in the pH of the H_2O in different experiments or due to biological variation between segments.

In an attempt to reduce the variation in growth a technique was tried in which the growth of a column of 10 segments was measured with a lever system and smoked kymograph drum. Mechanical problems were encountered with this technique because a long lever was required to provide sufficient magnification of growth.

Subsequently, the elongation of a column of segments was monitored with a linear transducer. The transducer magnified the elongation and thus a small, easily counterbalanced lever system could be developed. This technique was refined during experimentation and all the data presented in this section were obtained using the transducer technique.

b. The Incubation of Segments before Treatment. Slow steady growth of segments is required before the effects of applied growth substances can be monitored. Thus the growth of segments in buffer was investigated immediately after setting up the experiments.

When segments are placed in the apparatus and 0.01M phosphate buffer at

pH 7.0 and 25°C is introduced there is a high growth rate, typically of 3.0 to 4.0 μm $min.^{-1}$ per segment, though in some instances there was a period of slow growth while the pressure of the coleoptile segments on the lever developed (Fig. 34). The use of this buffer enabled comparisons with published data and it also adequately buffered the hormone solutions.

The growth rate is affected by pH. Using buffer at pH 7.0 the growth rate is typically reduced to 0.2 - 0.3 μm $min.^{-1}$ per segment after 90 minutes whereas with buffers of pH 6 and 5.2 the growth rate is higher after a similar incubation time (Fig. 34). Consequently, a 100-minute incubation in phosphate buffer at pH 7.0 was routinely used at 25°C and growth recorded during this period before IAA treatment was begun at time zero. At 15°C and 20°C a longer incubation period was required to reduce the growth rate in buffer to a low level; a shorter incubation was adequate at higher temperatures.

The growth rate in buffer is at first unaffected by the replacement of the buffer solution at time zero but increases gradually after about 40 minutes (treatment 3, Fig. 46a and b).

c. The Response to Indole-acetic Acid. Prior to examining the effects of ABA on IAA-induced elongation it was necessary to investigate the characteristics of the response to IAA alone. The features of the response to 10^{-5} M IAA at 25°C and pH 7.0 have been demonstrated in experiments investigating the effect of temperature and IAA concentration on the IAA response (section 6d and e) as well as ABA effects (section 6f). These experiments show that 10^{-5} M IAA at 25°C and pH 7.0 stimulates growth with a latent period of approximately 11 minutes (Fig. 35; Fig. 46a and b, treatment 1) and a mean initial maximum rate of elongation of approximately 60 μm $min.^{-1}$ (Fig. 41) occurring 25 minutes after addition of IAA. The experiments in which growth was recorded for a 3-h period following the addition of IAA demonstrate a depression in rate after the initial maximum (Fig. 46b, treatment 1). A

Fig. 34. Continuous time courses of the elongation of Avena coleoptile segments, using the transducer technique.

Treatments 1 : 0.01M phosphate buffer, pH 5.2.

2 : 0.01M phosphate buffer, pH 6.0.

3 : 0.01M phosphate buffer, pH 7.0.

The time courses in this graph begin when the segments were first immersed in the buffer solutions. Each curve shows the elongation of a column of 10 segments and is from one experiment; similar responses were obtained in at least one repeat of each treatment.

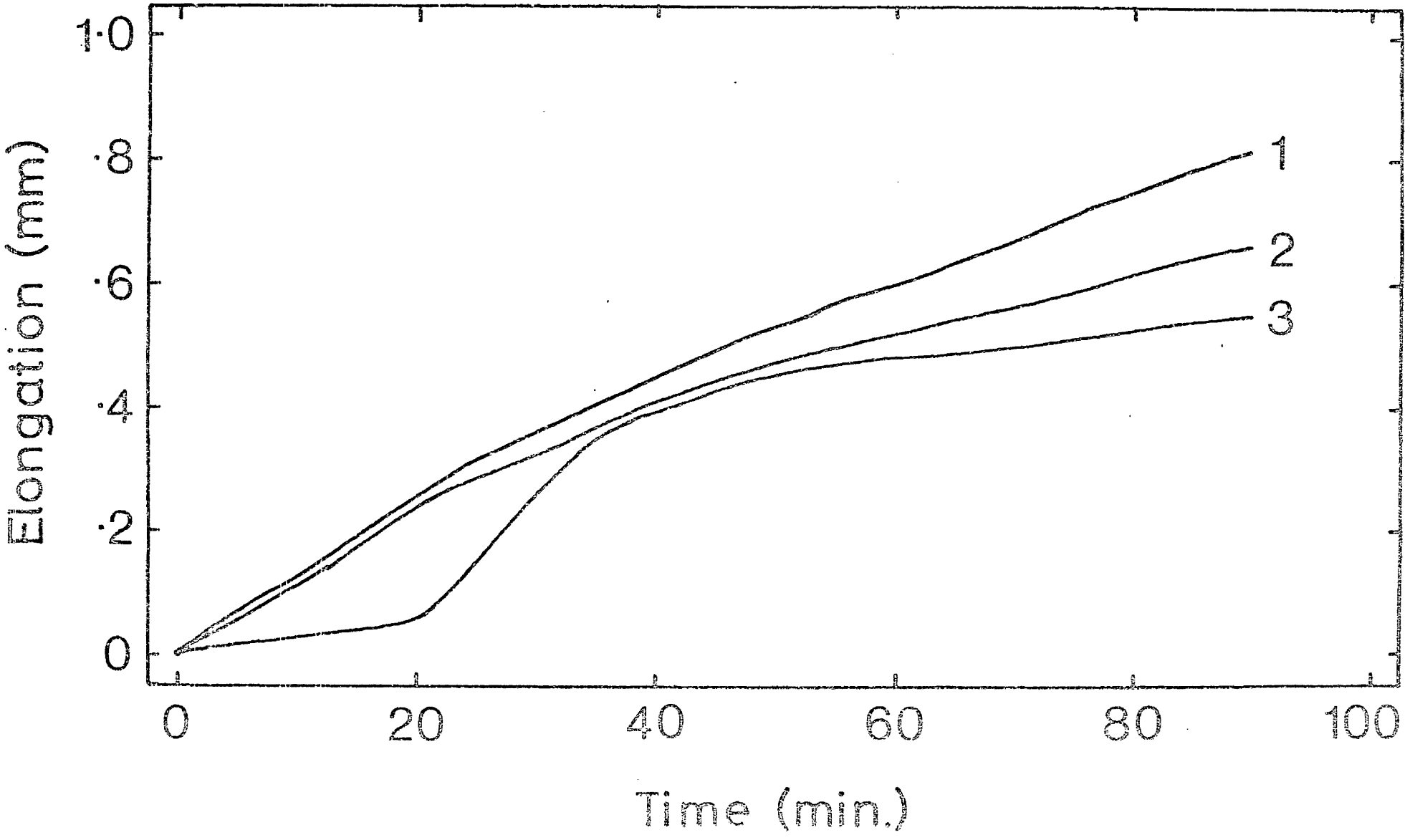
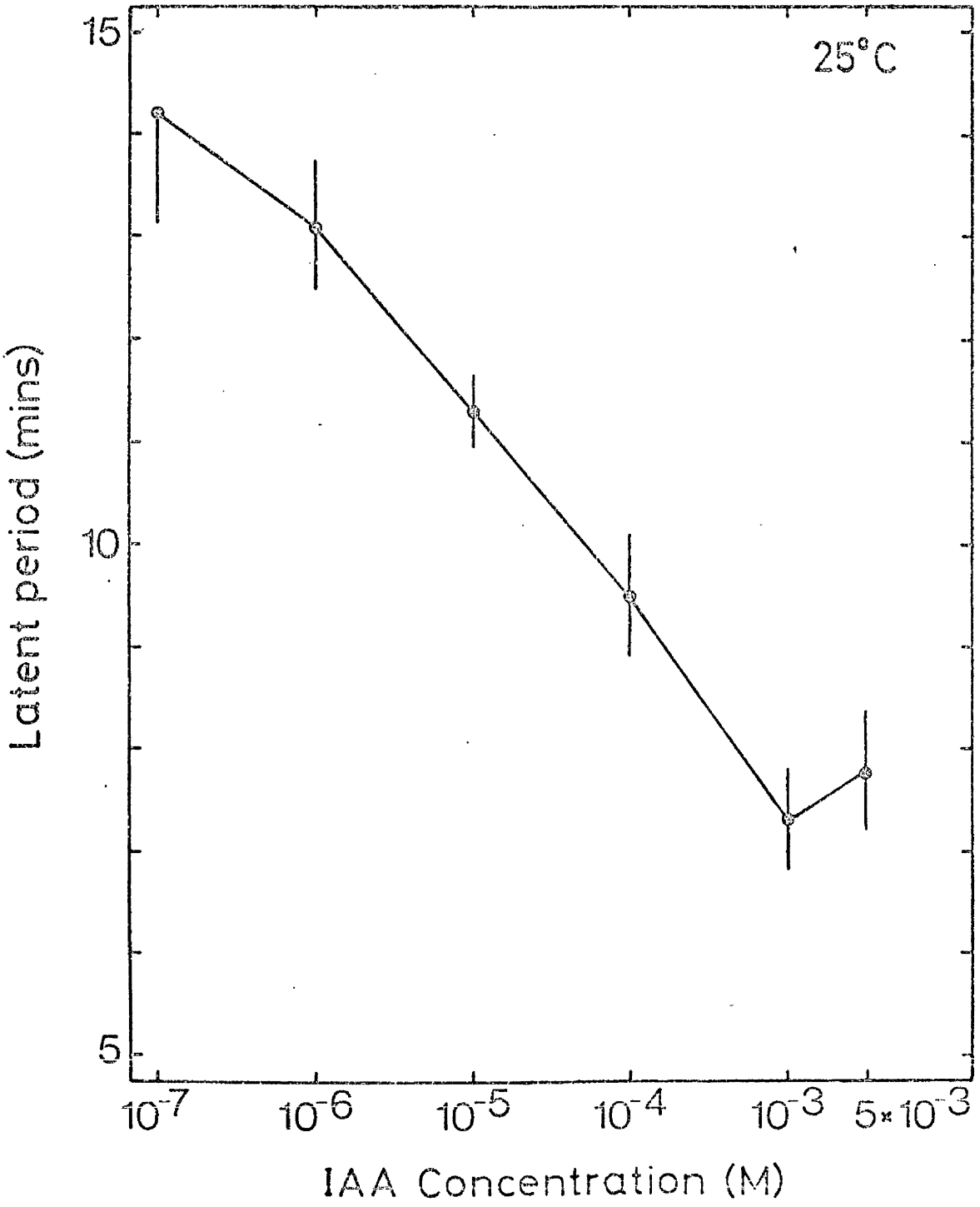

Elongation (mm)
1·0
·8
·6
·4
·2
0
0
20
40
60
80
100
Time (min.)
1
2
3

Fig. 35. The effect of IAA concentration on the latent period for IAA-induced growth of Avena coleoptile segments at pH 7.0, and 25°C.

Each point is the mean of 5 replicates.

Statistical Analysis. The t-test was used to test the difference in latent period between the treatments indicated.

Item	t value	Significance
10^{-4}M/10^{-3}M	2.65	*


25°C
15
10
5
Latent period (mins)
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
5×10^{-3}
IAA Concentration (M)

rate of growth similar to the initial maximum is then maintained until 120 minutes though fluctuations in rate are observed. During this 120 minutes a second maximum greater than the first may occur before the rate begins to decline. The mean data (Fig. 46b, treatment 1) illustrate this greater second maximum but it was not observed in all replicates of this experiment though they all show the fluctuating rate.

The initial maximum rate in separate experiments does not always occur at precisely the same time after addition of IAA. In the 5 replicates contributing to the mean data of treatment 1 in Fig. 46 the initial maximum occurs either 20 or 25 minutes after addition of IAA. Consequently, the mean value of this parameter was calculated from 5 replicates, rather than calculating the mean growth rate at a given time after IAA treatment was initiated.

Thus the IAA response under specific conditions illustrates two diagnostic parameters, viz.: the latent period and initial maximum rate. Both parameters can be readily investigated with the transducer technique and experiments were carried out to investigate the effects of temperature and IAA concentration on these important features of the IAA response.

d. The Latent Period of the IAA response. Increasing the concentration of IAA over the range 10^{-7}M to 10^{-3}M at 25°C using solutions buffered with phosphate buffer at pH 7.0, reduces the latent period to 7.3 $\pm$ 0.48 minutes at 10^{-3}M; raising the IAA concentration to 5 x 10^{-3}M does not further reduce the latent period (Fig. 35). Studies using 10^{-5}M IAA at pH 7.0 show that the latent period is reduced to a minimum of 8.7 $\pm$ 0.43 minutes at 30°C when the temperature is increased in steps from 15°C to 30°C, but a further rise in temperature to 35°C extends the latent period (Fig. 36).

A combination of the conditions that individually produce minimum latent periods in these experiments, i.e. 10^{-3}M IAA and 30°C, does not reduce the latent period, but increases it (Fig. 37). Similarly, extending the temperature range above or below 25°C, using either 10^{-3}M IAA or 5 x 10^{-3}M

Fig. 36. The effect of temperature on the latent period for growth, induced by 10^{-5}M IAA at pH 7.0, of *Avena* coleoptile segments.

Each point is the mean of 5 replicates.

Statistical Analysis. The t-test was used to test the difference in latent period between the treatments indicated.

Item	t value	Significance
25°C/30°C	4.745	**
30°C/35°C	3.472	**

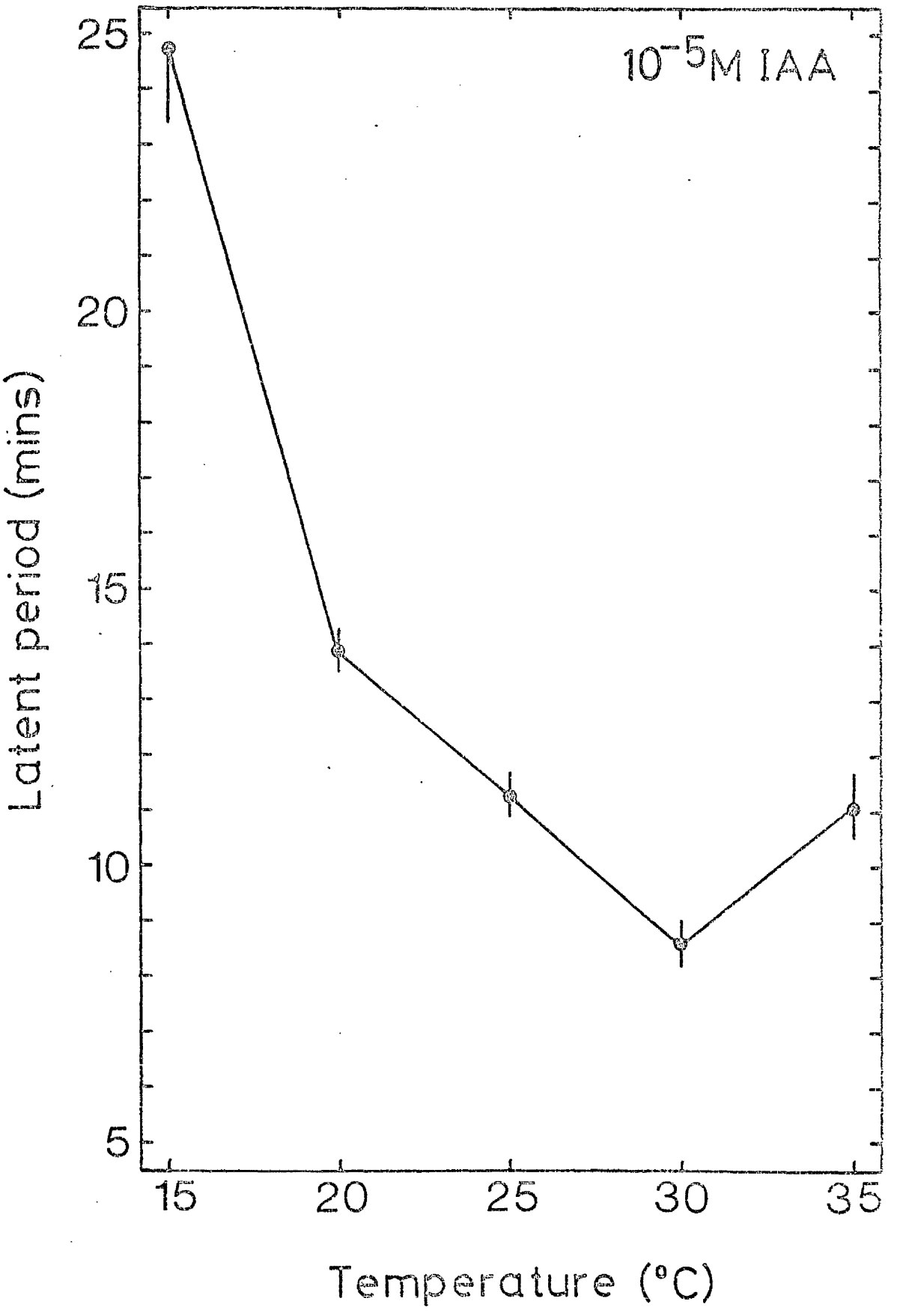
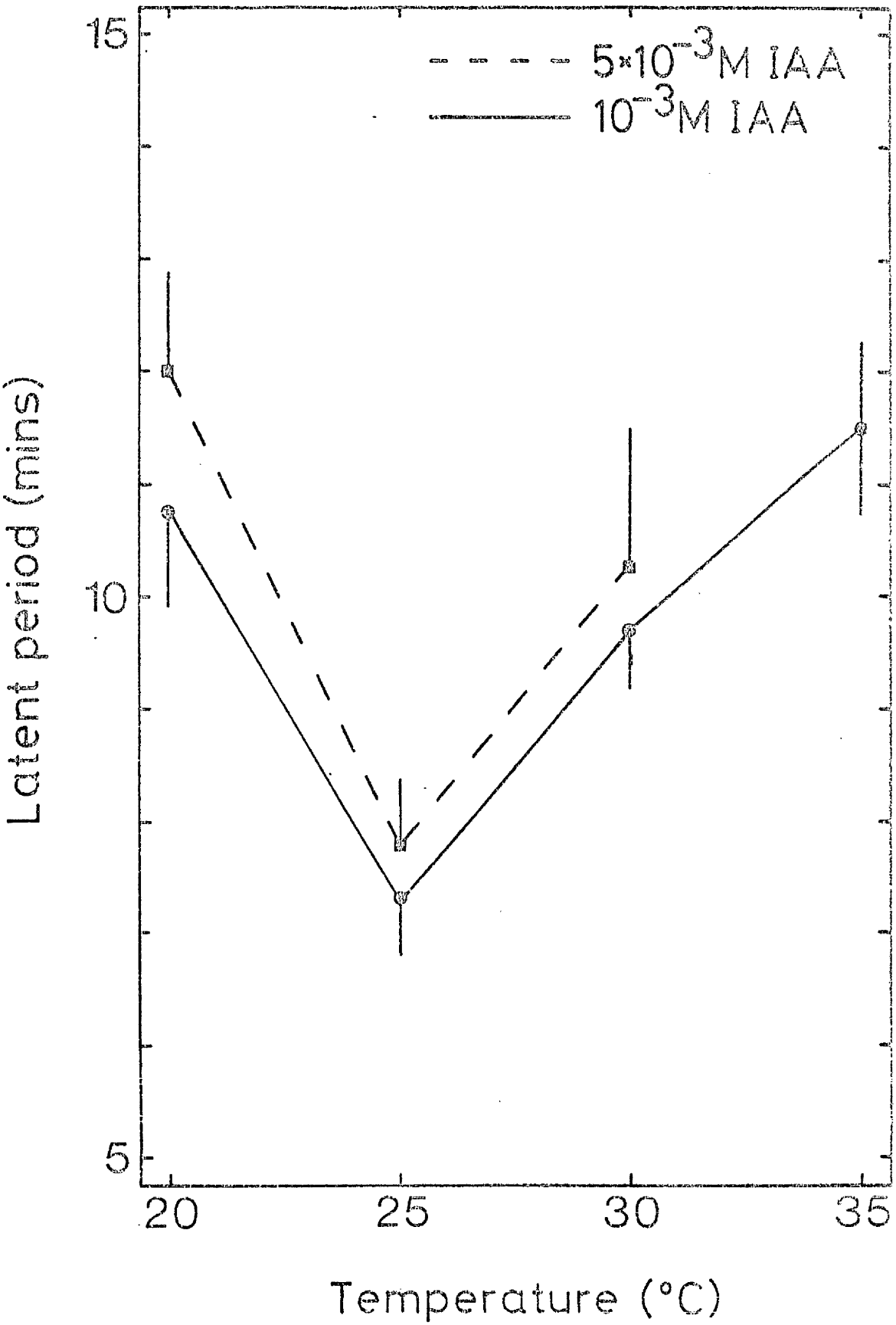

10⁻⁵M IAA
Latent period (mins)
25
20
15
10
5
15
20
25
30
35
Temperature (°C)

Fig. 37. The effect of temperature on the latent period for growth, induced by 10^{-3}M IAA and 5 x 10^{-3}M IAA at pH 7.0 and 6.9 respectively, of Avena coleoptile segments.

Each point is the mean of 5 replicates.

Statistical Analysis. The t-test was used to test the difference in latent periods between the treatments indicated.

Item	t value	Significance
10^{-3}M IAA 25°C/30°C	3.234	*


15
10
5
Latent period (mins)
5×10⁻³M IAA
10⁻³M IAA
20
25
30
35
Temperature (°C)

IAA, does not reduce the latent period beyond the minimum observed at 25°C.

Thus there is no evidence of an immediate response of segments to IAA at pH 7.0 although these experiments embrace those conditions of IAA concentration and temperature which were reported by Nissl and Zenk (1969) to induce rapid elongation with no observable latent period, i.e. 5×10^{-3}M IAA at 29°C. The immediate growth response described by these investigators, however, was obtained with 5×10^{-3}M IAA prepared in 0.01M KH_2PO_4.

In experiments using this solution an almost immediate response is observed, both at 30°C after pretreatment in 0.01M KH_2PO_4 at pH 4.7 (Curve 1, Fig. 38) and at 25°C after pretreatment in phosphate buffer at pH 7.0 (Curve 3, Fig. 38). Although the 0.01M KH_2PO_4 has a pH of 4.7, addition of 5×10^{-3}M IAA reduces this pH to 3.5. Table 13 compares the pH values of a range of IAA concentrations in distilled water and in 0.01M KH_2PO_4. These data show that 0.01M KH_2PO_4 is inadequate to buffer concentrations of IAA in excess of 10^{-4}M. Thus it is possible that the almost immediate response observed may not be due solely to IAA.

Table 13. pH of IAA Solutions. Typical values are quoted. Similar pH readings have been observed in at least 2 separate determinations.

[IAA]M	(a) in H_2O	(b) in 0.01M KH_2PO_4	(c) in 0.01M phosphate buffer
0	6.3	4.7	7.0
10^{-5}	6.0	4.7	7.0
10^{-4}	4.5	4.5	7.0
10^{-3}	3.8	4.0	7.0
5×10^{-3}	3.4	3.5	6.9 (K_2HPO_4 only)

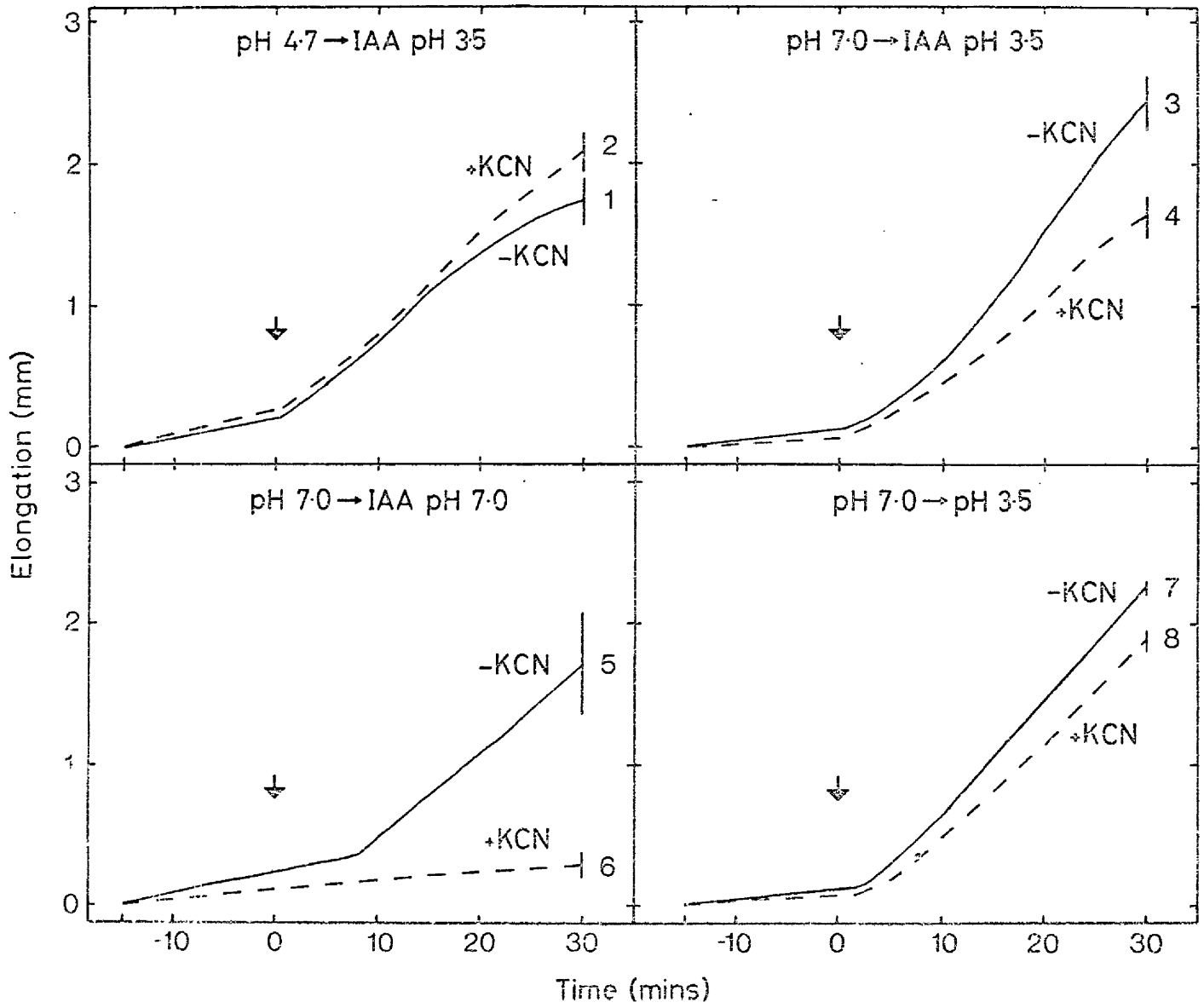
In view of the fact that an incubation of at least 3h was required to produce a slow steady growth rate using 0.01M KH_2PO_4 at pH 4.7, and because an immediate response with 5×10^{-3}M IAA in 0.01M KH_2PO_4 was obtained after

Fig. 38. The response of Avena coleoptile segments, in the presence (– – – –) and absence (————) of 10^{-4}M potassium cyanide (KCN), to treatments involving the lowering of the pH, or the addition of 5×10^{-3}M IAA, at the time shown by the arrow (0 minutes). Cyanide treatment began at -15 minutes and was present throughout subsequent treatments.

The curves show the elongation of a column of 10 segments and each is the mean of 3 separate experiments.

Statistical Analysis. The t-test was used to test the difference in elongation at 30 minutes between the treatments indicated.

Item	t value	Significance
1/2	1.427	NS
3/4	3.037	*
5/6	3.338	*
7/8	3.995	*


pH 4·7 → IAA pH 3·5
+KCN
-KCN
2
1
pH 7·0 → IAA pH 3·5
-KCN
+KCN
3
4
pH 7·0 → IAA pH 7·0
-KCN
+KCN
5
6
pH 7·0 → pH 3·5
-KCN
+KCN
7
8
Elongation (mm)
Time (mins)
0
1
2
3
-10
0
10
20
30

a 100-minute pretreatment in phosphate buffer at pH 7.0, most subsequent experiments were carried out using the more convenient 100-minute incubation in buffer of pH 7.0.

A solution of pH 3.5 can induce an almost immediate promotion of growth in the absence of applied IAA. After a 100-minute incubation at 25°C in citrate buffer at pH 7.0, a citrate buffer of pH 3.5 at 25°C induces a high growth rate with a latent period of only 1 to 2 minutes (curve 7, Fig. 38).

The possibility that the immediate response to 5×10^{-3}M IAA prepared with 0.01M KH_2PO_4 involves an acid response has been further investigated using potassium cyanide. The effect of 10^{-4}M KCN in phosphate buffer at pH 7.0 was studied on segments growing at approximately 2.0 μm $min.^{-1}$ per segment in this buffer; i.e. before a slow steady growth rate in buffer alone is achieved. The growth rate is reduced with a latent period for KCN action of less than 5 minutes (Fig. 39). There is no response of segments to 5×10^{-3}M IAA at pH 7.0 in the presence of KCN after 15 minutes pretreatment in this inhibitor. The cyanide, however, does not irreversibly kill the segments because removal of the KCN after an exposure of 45 minutes results in a high growth rate within 2 to 3 minutes. In subsequent experiments using KCN the inhibitor was added 15 minutes before the low pH or IAA treatment was initiated and the cyanide was maintained in the solution during this treatment.

Fig. 38 shows the action of 5×10^{-3}IAA at pH 7.0 in the presence (Curve 6) and absence (Curve 5) of this cyanide treatment. Although the IAA alone promotes a high growth rate with a latent period of approximately 8 minutes the potassium cyanide completely abolishes this promotion of growth.

In contrast, potassium cyanide has a much less marked effect on the growth induced by low pH. The latent period is not affected by 10^{-4}M KCN added 15 minutes before the introduction of citrate buffer at pH 3.5 and the total elongation after 30 minutes is only slightly reduced, though the reduction is significant ($P < 0.05$; Curve 8, Fig. 38). This cyanide treatment does not abolish the response to 5×10^{-3}M IAA at pH 3.5 either at

Fig. 39. The action of potassium cyanide on the elongation of *Avena* coleoptile segments.

Treatments:-

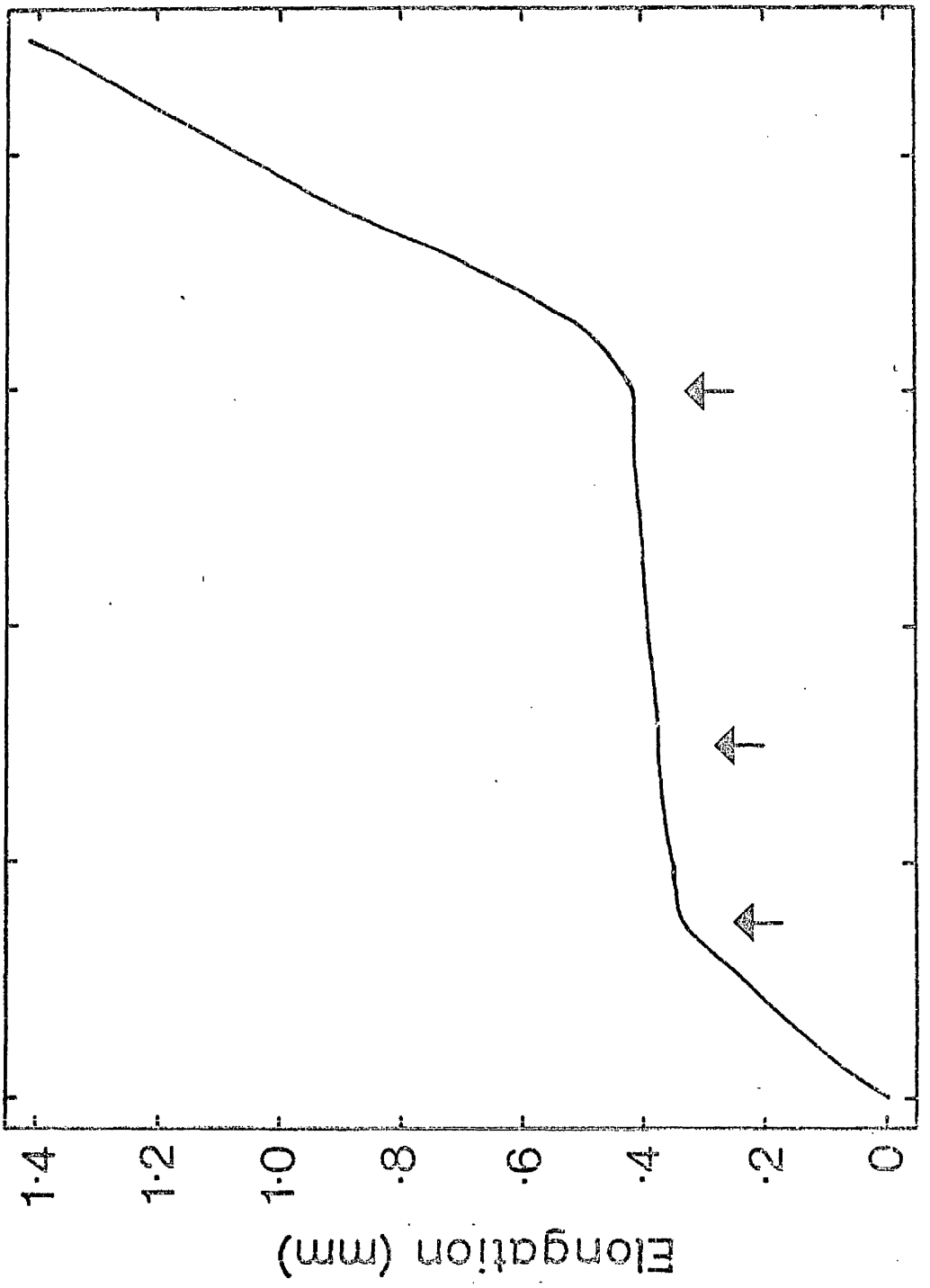
Time 0 : Segments immersed in 0.01M phosphate buffer, pH 7.0.

First arrow (15 minutes) : Solution changed to 10^{-4}M KCN in 0.01M phosphate buffer, pH 7.0.

Second arrow (30 minutes) : Solution changed to 5×10^{-3}M IAA plus 10^{-4}M KCN in 0.01M phosphate buffer, pH 7.0.

Third arrow (60 minutes) : Solution changed to 5×10^{-3}M IAA in 0.01M phosphate buffer, pH 7.0.

The curve shows the elongation of a column of 10 segments and is from 1 experiment.


Elongation (mm)
0
.2
.4
.6
.8
1.0
1.2
1.4
0
20
40
60
80
Time (min.)

30°C after pretreatment in 0.01M KH_2PO_4 at pH 4.7 (Curve 2, Fig. 38) or at 25°C after pretreatment in phosphate buffer at pH 7.0 (Curve 4, Fig. 38). In both instances the response occurs in 2 minutes or less (Table 14); the total elongation after 30 minutes is not significantly affected in Curve 2 compared with Curve 1 ($P > 0.2$) and only slightly, though significantly, reduced in Curve 4 compared with Curve 3 ($P < 0.05$).

Five factors indicate that the almost immediate promotion of growth by 5×10^{-3}M IAA in 0.01M KH_2PO_4 is attributable to low pH:

(1) 5×10^{-3}M IAA in a solution of 0.01M KH_2PO_4 has a pH of 3.5.

(2) 0.01M citrate buffer at pH 3.5 induces an almost immediate promotion of growth.

(3) The quick growth promotion induced by 5×10^{-3}M IAA in 0.01M KH_2PO_4 is not abolished by treatment with KCN.

(4) A KCN treatment abolishes growth induced by 5×10^{-3}M IAA at pH 7.0 but does not annul the low pH response.

(5) The minimum latent period observed with 5×10^{-3}M IAA at pH 7.0 is greater than 7 minutes.

A slight discrepancy exists in the latent periods of these immediate responses: the latent period for the response to IAA at pH 3.5 is slightly less than that for citrate buffer at pH 3.5 which follows incubation of segments in citrate buffer of pH 7.0 (Table 14).

Table 14. Latent Periods (minutes).

The data presented are from individual experiments. Mean values are shown in brackets.

SOLUTION	-KCN	+KCN
A : 5×10^{-3}M IAA in 0.01M KH_2PO_4; pH 3.5.		
(i) after incubation at pH 4.7	0; 2; 0; (0.67)	0; 0; 2; (0.67)
(ii) after incubation at pH 7.0	0; 1; 1; (0.67)	0; 0; 0; (0)
B : Low pH treatment after incubation in citrate buffer pH 7.0.		
(i) Citrate buffer pH 3.5	2; 1.5; 1.5; (1.67)	2; 1.5; 1.5; (1.67)
(ii) Glycine-HCl buffer pH 3.5	0; 0.5; 0; (0.17)	

Nevertheless, glycine-HCl buffer at pH 3.5 induces a high growth rate with a latent period consistently less than 1 minute when this exposure to low pH follows incubation of segments in citrate buffer at pH 7.0. Hence the latent period for promotion of rapid growth by low pH may be affected not only by the pH but also by the constituents of the buffer before and during exposure to low pH.

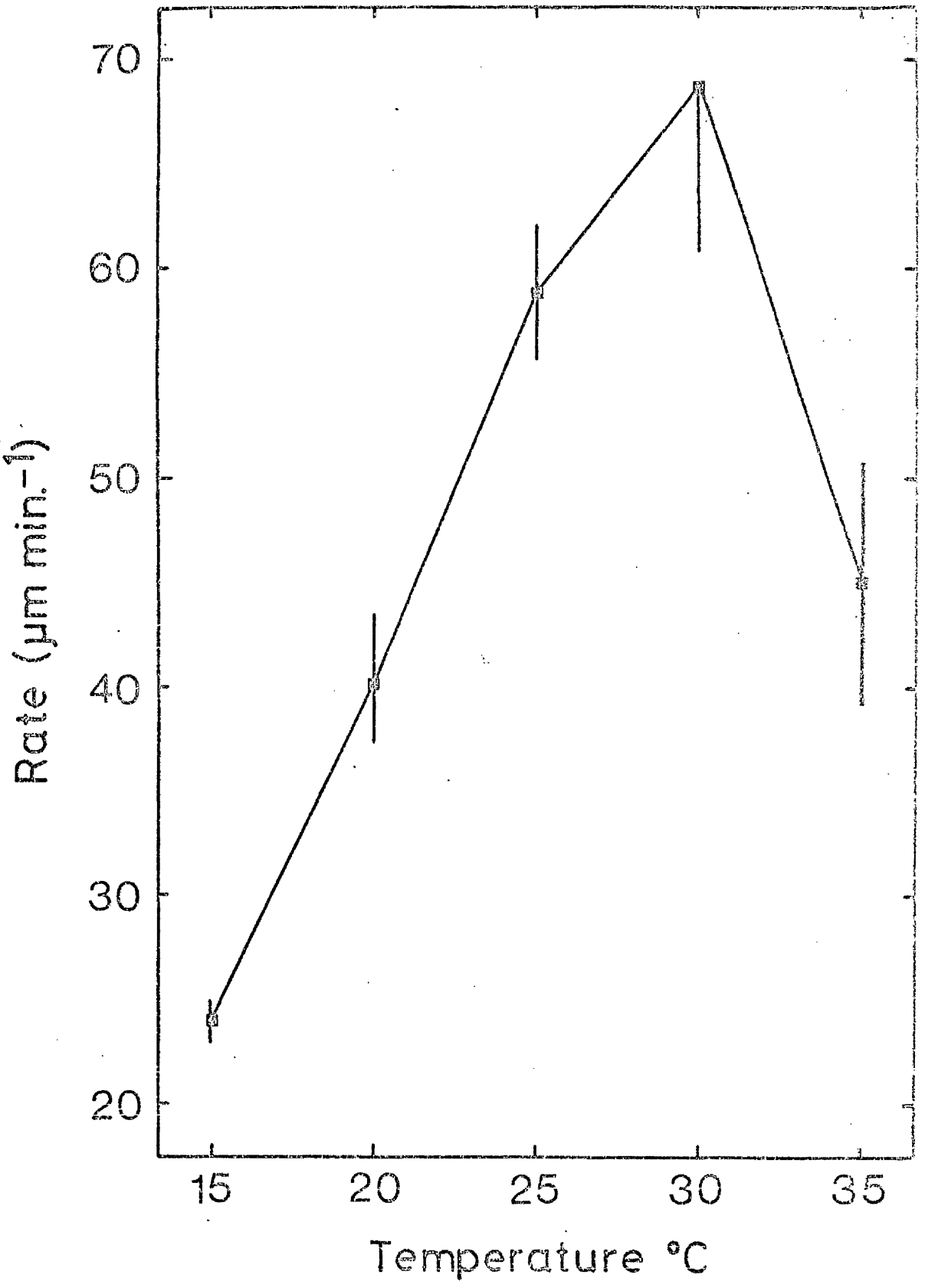
e. The Rate of IAA-induced Elongation. The initial maximum rate of IAA-induced elongation is dependent upon both temperature and IAA concentration when using solutions prepared with 0.01M potassium phosphate buffer at pH 7.0. Using 10^{-5}M IAA the optimum temperature for a high growth rate is between 25 to 30°C (Fig. 40). The rate is greater at 30°C than at 25°C although the difference is not significant. A further increase in temperature beyond 30°C apparently exceeds the physiological optimum and a decrease in initial

Fig. 40. The effect of temperature on the initial maximum growth rate of *Avena* coleoptile segments, induced by 10^{-5}M IAA at pH 7.0.

Each point is the mean of five replicates; the rate of growth refers to a column of 10 segments.

Statistical Analysis. The t-test was used to test the difference in rate between the treatments indicated.

Item	t value	Significance
20°C/25°C	4.254	**
25°C/30°C	1.148	NS
30°C/35°C	2.400	*


70
60
50
40
30
20
Rate (µm min.⁻¹)
15
20
25
30
35
Temperature °C

rate is observed.

The dosage-response curve at 25°C (Fig. 41) does not show the same optimum for a high initial rate as was observed for the minimum latent period (Fig. 35). IAA at 10^{-7}M promotes growth compared with the basal growth rate in buffer alone. The optimum concentration for a high initial growth rate is between 10^{-6} and 10^{-5}M IAA. The maximum rate is observed at 10^{-6}M though this is not significantly greater than that at 10^{-5}M. Increasing the concentration to 10^{-3}M gradually reduces the initial maximum rate. Three experiments using 5×10^{-3}M IAA show extremely variable results. The maximum initial rate, however, is in each case greater than the mean observed with 10^{-3}M and the mean maximum initial rate at 5×10^{-3}M is of similar magnitude to that observed with both 10^{-6} and 10^{-5}M IAA.

The temperature giving rise to the maximum initial growth rate is 30°C at 10^{-5}M IAA and at 10^{-3}M IAA the highest rate is also observed at this temperature (Fig. 42). On the other hand, at 5×10^{-3}M the mean growth rate is higher at 25°C than at 30°C though there is considerable variation within the data.

The dosage-response curve of the initial maximum rate of elongation at 25°C (Fig. 41), between 10^{-7} and 10^{-3}M IAA, rises to a maximum and then declines, as did the dosage-response curve of final segment length after a 24-h assay (Fig. 10). The curve of initial rates, however, is inconsistent with published data estimated from initial rates (Nissl and Zenk, 1969; Cleland, 1972) in that sigmoid response curves were reported.

Both Nissl and Zenk (1969) and Cleland (1972) exposed seedlings to dim red light during development and their experiments were performed at pH 4.7. Consequently, further experiments were carried out to investigate the rôle of light and pH on the dosage-response curve.

In order to allow a variety of treatments to be examined quickly a modified straight growth assay was employed. The required number of segments were sectioned as in previous assays and incubated in buffer for 90 minutes.

Fig. 41. The effect of IAA concentration on the initial maximum growth rate of *Avena* coleoptile segments at pH 7.0, and 25°C.

Each point is the mean of 5 replicates, except that at 5×10^{-3}M IAA which is the mean of 3 replicates. The rate of growth refers to a column of 10 segments.

Statistical Analysis. The t-test was used to test the difference in rates between the treatments indicated.

Item	t value	Significance
$10^{-7}/10^{-6}$	5.482	***
$10^{-6}/10^{-5}$	0.958	NS
$10^{-5}/10^{-4}$	3.096	*
$10^{-4}/10^{-3}$	0.683	NS
$10^{-3}/5 \times 10^{-3}$	1.687	NS

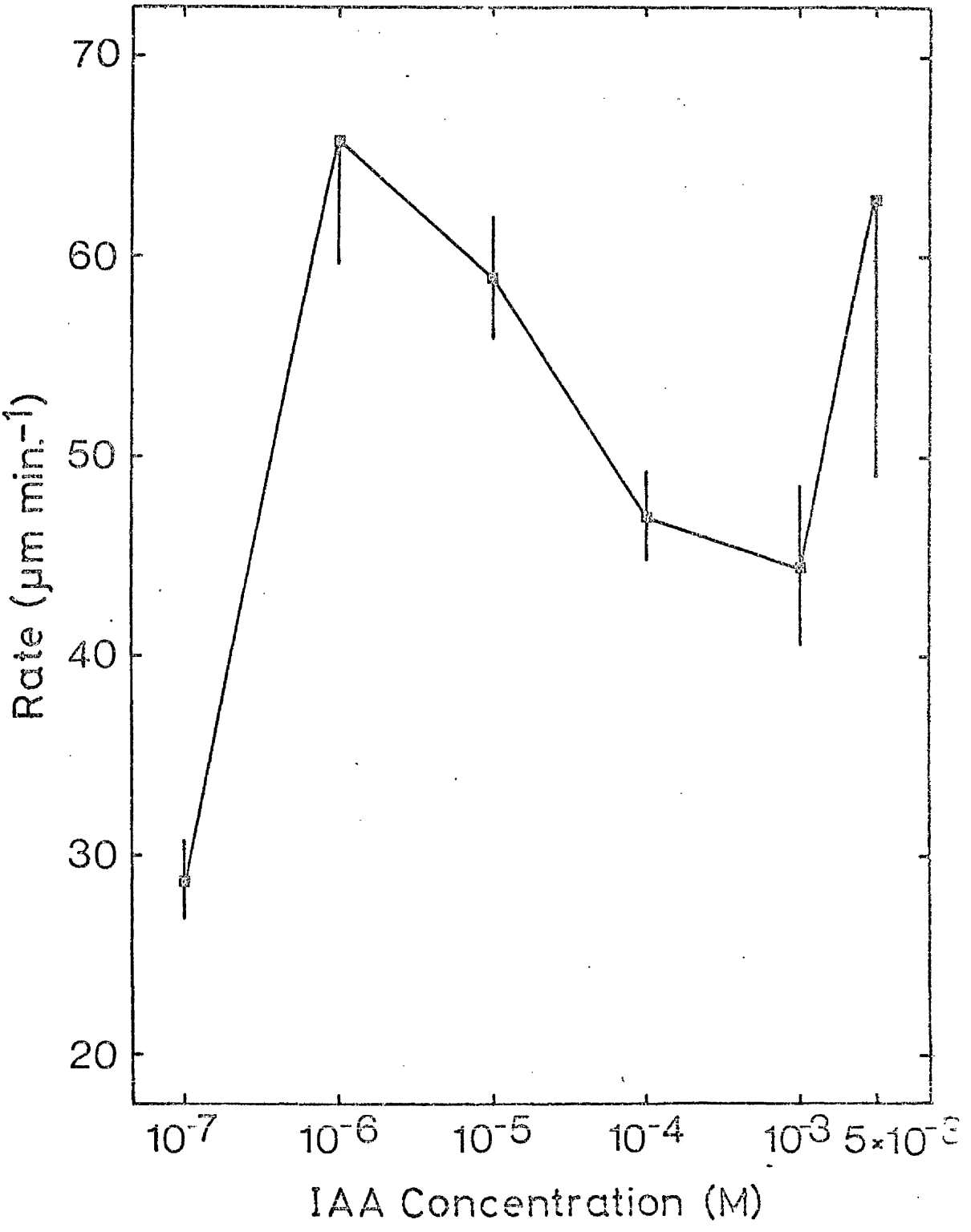

70
60
50
40
30
20
Rate (µm min-1)
10-7
10-6
10-5
10-4
10-3
5×10-3
IAA Concentration (M)

Fig. 42. The effect of temperature on the initial maximum growth rate of *Avena* coleoptile segments, induced by IAA.

Treatments:-

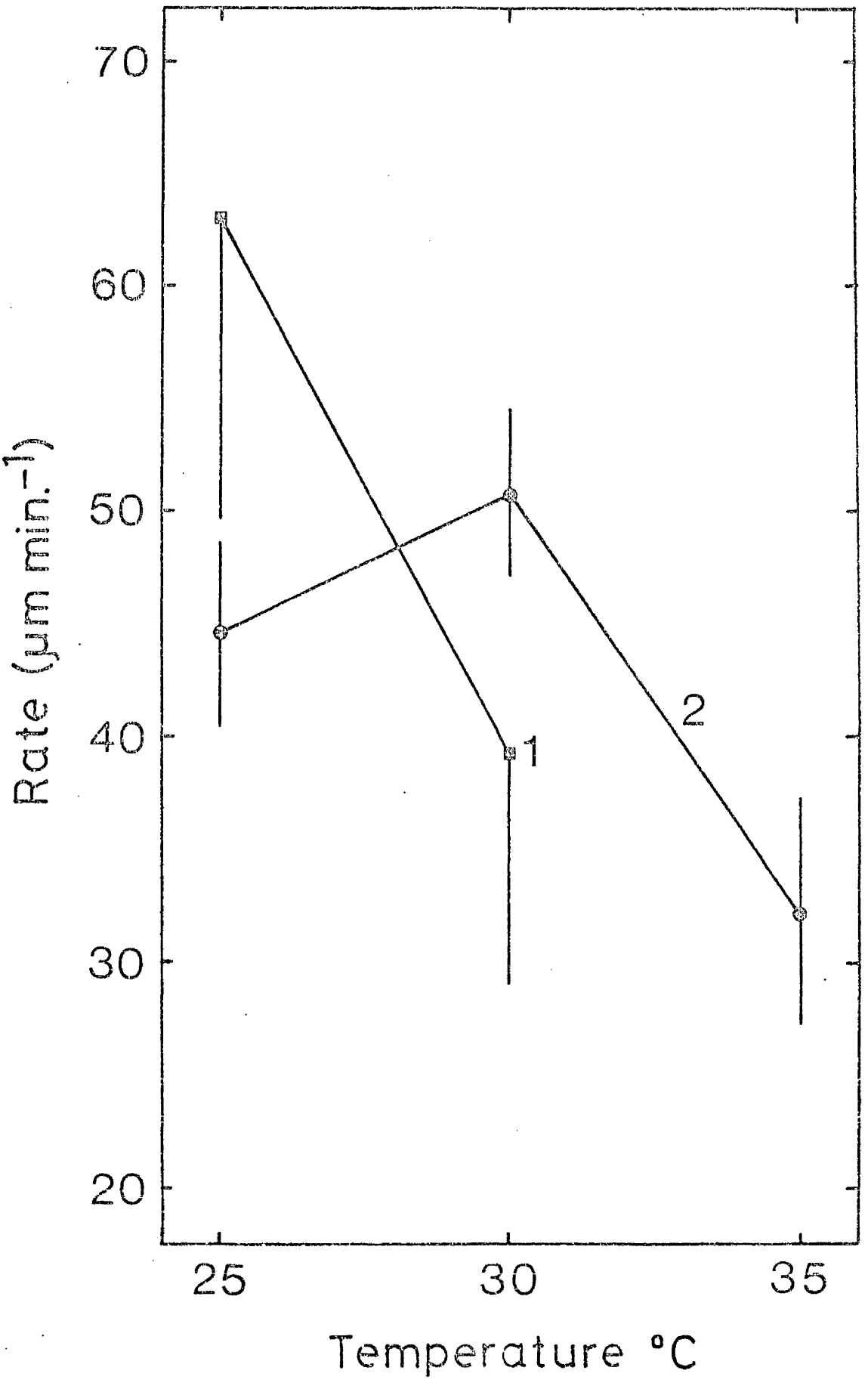
1 : 5×10^{-3}M IAA, pH 6.9. Mean of 3 replicates.

2 : 10^{-3}M IAA, pH 7.0. Mean of 5 replicates.

The rate of growth refers to a column of 10 segments.

Statistical Analysis. The t-test was used to test the difference in rate between the treatments indicated.

Item		t value	Significance
10^{-3}M IAA	25°C/30°C	1.207	NS
—"—	30°C/35°C	2.968	*
5×10^{-3}M IAA	25°C/30°C	1.376	NS


70
60
50
40
30
20
Rate (μm min.-1)
1
2
25
30
35
Temperature °C

The length of 10 segments was determined using the shadowgraph technique and the remaining segments were transferred to IAA treatments for 90 minutes, followed by shadowgraphing. The incubation in buffer allows the endogenous growth, possibly due to a growth stimulating effect of sectioning and to endogenous IAA, to decline to a slow steady rate. The 90-minute treatment was selected because continuous monitoring of segment elongation in 10^{-5}M IAA at 25°C and pH 7.0 shows that a growth rate similar to the initial maximum is maintained for 120 minutes. Hence, the total growth after 90 minutes should be indicative of the maximum initial rate and this treatment yields differences in length sufficiently large to be determined with the shadowgraph technique.

The treatments tested consisted of a 24-h exposure of seedlings to dim red light during the 3rd to 4th days of development and the use of phosphate buffer at pH 7.0 and citrate buffer at pH 4.7.

The red light exposure reduces the response of segments to IAA concentrations between 10^{-6} to 10^{-3}M, both at pH 7.0 and pH 4.7, compared with the elongation of dark grown segments under these conditions (Fig. 43). An exposure to dim red light has frequently been used in experiments investigating the response of Avena coleoptile segments to IAA and a pronounced response has been reported (e.g. Cleland, 1972). It is possible that the intensity or wavelength of the source employed in the experiments reported here differ from those of the light source used by Nissl and Senk and by Cleland. In the absence of details of their lights no further experiments on this aspect were carried out.

The dosage-response curves of dark grown segments at both pH values illustrate an increase in elongation as the IAA concentration is raised. The response curves, however, level off and there is no significant increase in segment length when the IAA concentration is raised from either 10^{-6} to 10^{-3}M at pH 4.7 or 10^{-5} to 10^{-3}M at pH 7.0. Because this response pattern does not confirm that observed in experiments using the transducer technique

Fig. 43. The effect of IAA concentration on elongation of Avena coleoptile segments at 25°C.

Treatments:-

D : Segments from dark grown seedlings.

R : Segments from seedlings exposed to red light during the 3rd to 4th days of development.

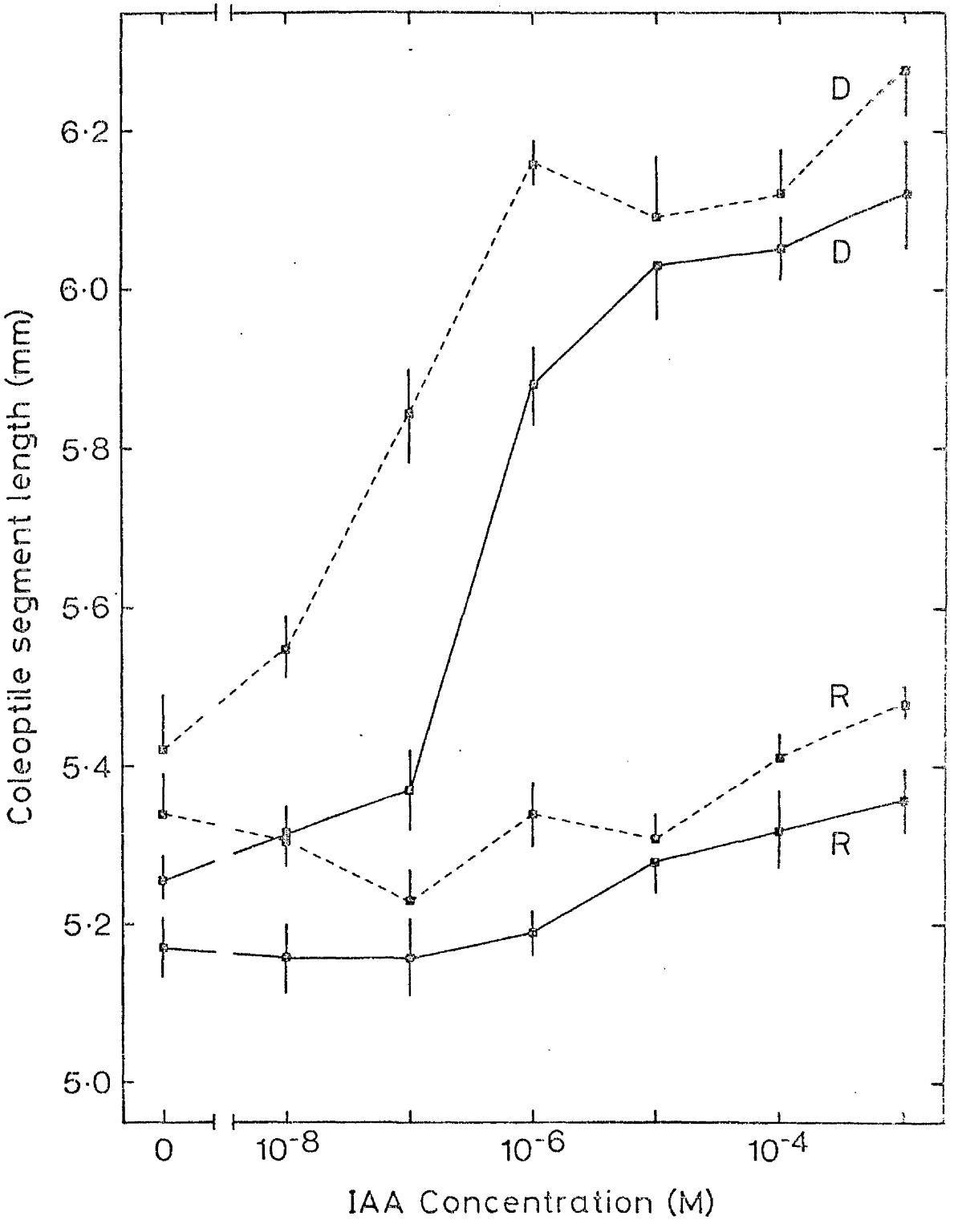
——— : IAA prepared in 0.01M phosphate buffer pH 7.0.

– – – : IAA prepared in 0.01M citrate buffer pH 4.7.

Segments were incubated in buffer for 90 minutes, then transferred to buffered IAA solutions for an additional 90 minutes, and segment lengths measured. The segments were floated on the solutions used. Each point is the mean length of 10 segments from 1 experiment.

Statistical Analysis. The t-test was used to test the difference in segment length between the treatments indicated.

Item		t value	Significance
Dark grown seedlings			
At pH 7.0 :	$10^{-7}/10^{-6}$	7.153	***
	$10^{-5}/10^{-3}$	0.922	NS
At pH 4.7 :	$10^{-7}/10^{-6}$	4.931	***
	$10^{-6}/10^{-3}$	1.683	NS


Coleoptile segment length (mm)
6·2
6·0
5·8
5·6
5·4
5·2
5·0
D
D
R
R
0
10⁻⁸
10⁻⁶
10⁻⁴
IAA Concentration (M)

(Fig. 41) the pH treatments were repeated using batches of 10 segments threaded on nylon line and immersed in 10 ml of solution in a petri dish. This treatment more closely resembles that of the experiments which allow elongation to be monitored continuously. The results obtained are variable and hence the data from the experiments repeated on 3 occasions are presented (Fig. 44 and 45).

At pH 7.0 the dosage-response curve (Fig. 44) is similar to that obtained with the transducer technique. The optimum response occurs between 10^{-6} and 10^{-5}M IAA, and raising the IAA concentration reduces the final segment length. At pH 4.7, however, a different response pattern, and more variable results, are obtained (Fig. 45). In all repeats of the experiment there is initially a peak between 10^{-7} to 10^{-5}M IAA followed by a reduced effect at 10^{-4}M IAA, though this reduction is not significant in experiment 3 in Fig. 45 (see fly sheet). In addition all replicates demonstrate that the elongation at 10^{-3}M exceeds that at 10^{-4}M, though again this is not significant in experiment 3.

In the absence of applied IAA the elongation occurring between the completion of the 90-minute incubation and the final determination of segment length a further 90 minutes later, is greater at pH 4.7 than 7.0. Indeed at pH 4.7 there is a significant difference between the segment lengths at these 2 times in 2 of the 3 experiments (Fig. 45) whereas there are no significant increases at pH 7.0 (Fig. 44).

f. <u>The Response to Abscisic Acid.</u> The shadowgraph technique indicated that there was a latent period for ABA action in the presence of IAA. Although this technique did not reveal a latent period for the IAA response the transducer technique demonstrated a latent period of approximately 10 minutes before IAA promotes elongation. It was decided, therefore, to use the transducer to accurately monitor the response of coleoptile segments to ABA and the effect of ABA on the IAA response.

All experiments were carried out at 25°C using solutions buffered to pH 7.0 with phosphate buffer. A 3-h time course of the growth of segments in

Fig. 44. The effect of IAA concentration on elongation of Avena coleoptile segments at pH 7.0 in 0.01M phosphate buffer at 25°C.

Groups of 10 segments were threaded on nylon line and immersed in buffer for 90 minutes, and then transferred to buffered IAA for an additional 90 minutes. 'B' refers to the segment lengths after the initial 90 minutes in buffer and the other points represent the final segment lengths at each IAA concentration. Each point is the mean length of 10 segments, and each line joining similar symbols represents one experiment.

Statistical Analysis. The t-test was used to test the difference in segment length between the treatments indicated.

Item	t value	Significance
a) To compare the segment lengths at the IAA concentration producing the peak response and the preceding concentration.		
Exp. 1 $10^{-5}/10^{-6}$	2.742	*
Exp. 2 $10^{-5}/10^{-6}$	4.851	***
Exp. 3 $10^{-6}/10^{-7}$	6.319	***
b) To compare the segment lengths at the IAA concentrations producing the peak response and the subsequent minimum response.		
Exp. 1 $10^{-5}/10^{-3}$	3.599	**
Exp. 2 $10^{-5}/10^{-3}$	4.608	***
Exp. 3 $10^{-6}/10^{-4}$	4.139	***

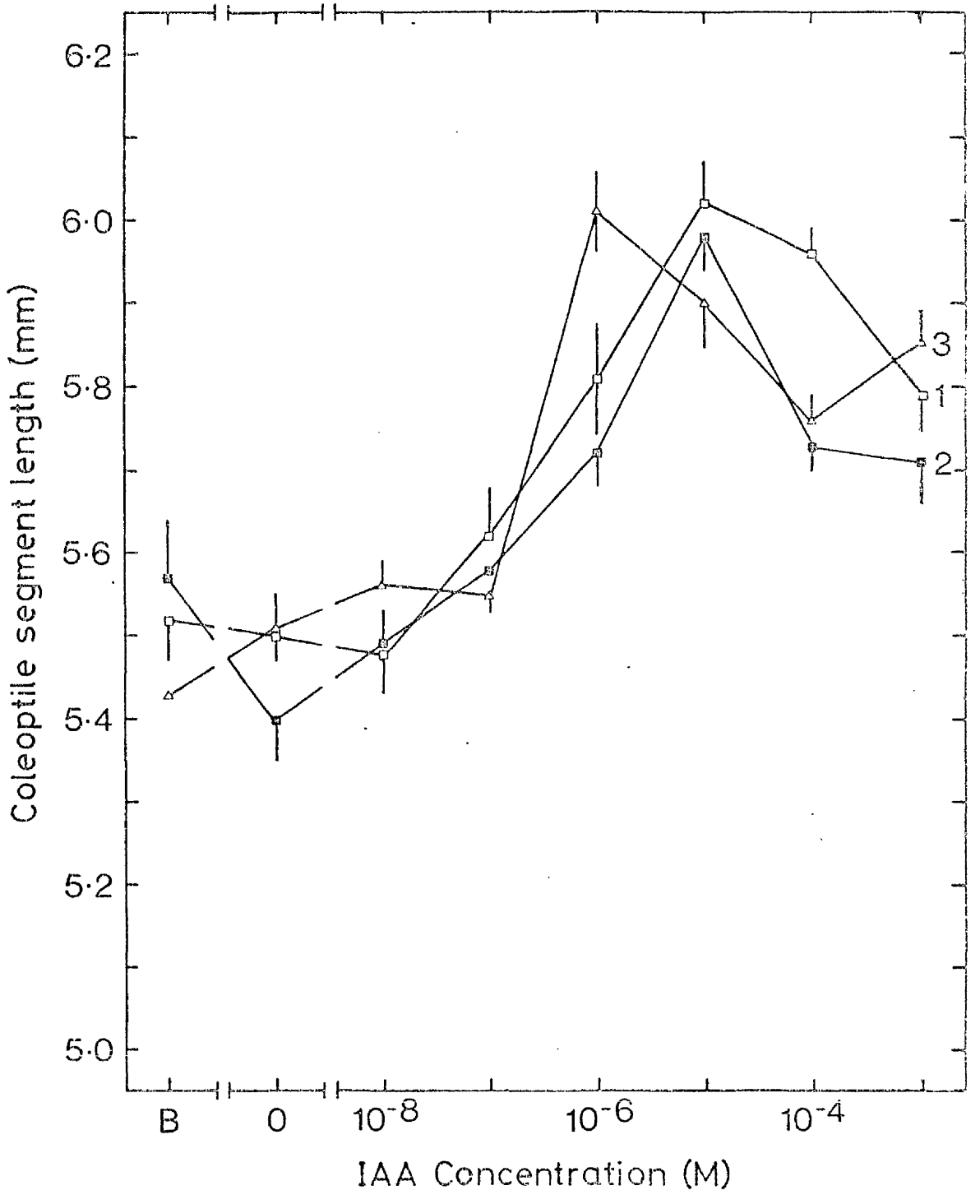
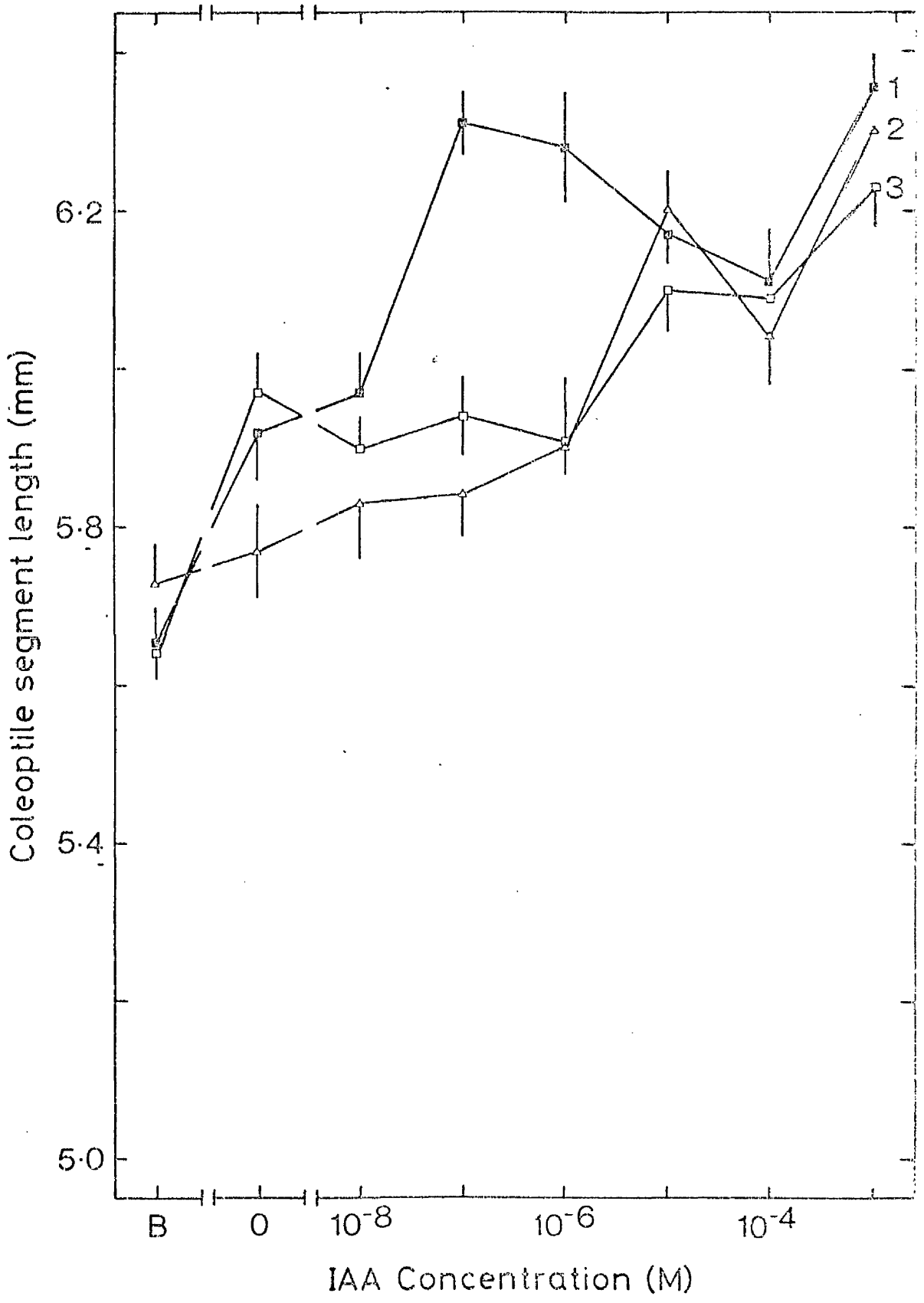

6·2
6·0
5·8
5·6
5·4
5·2
5·0
Coleoptile segment length (mm)
B
0
10⁻⁸
10⁻⁶
10⁻⁴
IAA Concentration (M)
3
1
2

Fig. 45. **The effect of IAA concentration on elongation of *Avena* coleoptile segments at pH 4.7 in 0.01M citrate buffer at 25°C.**

Groups of 10 segments were threaded on nylon line and immersed in buffer for 90 minutes, and then transferred to buffered IAA for an additional 90 minutes. 'B' refers to the segment lengths after the initial 90 minutes in buffer and the other points represent the final segment lengths at each IAA concentration. Each point is the mean length of 10 segments, and each line joining similar symbols represents one experiment.

Statistical Analysis. The t-test was used to test the difference in segment length between the treatments indicated.

Item		t value	Significance
a) To compare segment lengths at the IAA concentration producing the initial peak response and the preceding concentration.			
Exp. 1	$10^{-7}/10^{-8}$	5.052	***
Exp. 2	$10^{-5}/10^{-6}$	4.949	***
Exp. 3	$10^{-5}/10^{-6}$	2.204	*
b) To compare segment lengths at the IAA concentration producing the initial peak response and the minimum at 10^{-4}M IAA.			
Exp. 1	$10^{-7}/10^{-4}$	2.375	*
Exp. 2	$10^{-5}/10^{-4}$	2.150	*
Exp. 3	$10^{-5}/10^{-4}$	0.104	NS
c) To compare the responses at 10^{-4} and 10^{-3}M IAA.			
Exp. 1		2.991	*
Exp. 2		2.995	**
Exp. 3		1.426	NS
d) To compare the segment lengths after 90 minutes in buffer (B) and after an additional 90 minutes in buffer alone (0 IAA).			
(i) pH 7.0	Exp. 1	0.154	NS) data
	Exp. 2	2.335	*) from
	Exp. 3	1.524	NS) Fig.44
(ii) pH 4.7	Exp. 1	3.984	***
	Exp. 2	5.383	***
	Exp. 3	0.528	NS


Coleoptile segment length (mm)
6·2
5·8
5·4
5·0
B
0
10⁻⁸
10⁻⁶
10⁻⁴
IAA Concentration (M)
1
2
3

both phosphate buffer (treatment 3, Fig. 46a and b) and in 10^{-5}M IAA (treatment 1, Fig. 46a and b) has already been described.

Addition of 10^{-5}M ABA to segments in buffer, at time zero, does not initially affect the growth rate but suppresses the increase in rate observed in buffer (treatment 4, Fig. 46a and b), the effect being significant after 90 minutes ($P < 0.05$).

Simultaneous addition of 10^{-5}M ABA with 10^{-5}M IAA does not significantly alter the initial response to IAA (treatment 2, Fig. 46a and b); the latent period is unchanged and the maximum initial growth rate which occurs after 25 minutes is not significantly reduced (Fig. 49a and b). Nevertheless, a significant inhibitory effect of ABA develops after 30 minutes ($P < 0.025$, paired analysis) and the decline in rate continues to 45 minutes. A growth rate of approximately 3.5 μm $min.^{-1}$ per segment is then maintained to 120 minutes after which a further decline takes place. After 150 minutes the growth rate is reduced to a level similar to that of segments maintained in buffer alone and subsequently is less than that of buffer treated segments.

ABA may be inhibiting this IAA-induced growth *via* an inhibition of IAA uptake into the cells. To yield the observed effects, however, it is probable that considerable inhibition of uptake would be required because a similar initial maximum rate is induced by both 10^{-5}M and 10^{-6}M IAA (Fig. 41). Nevertheless, this possible action of ABA was investigated using a 30-minute pretreatment in 10^{-5}M IAA. For 20-30 minutes after this exposure to IAA the growth rate pattern in buffer (treatment 5, Fig. 47) is similar to that obtained with a continuous supply of IAA (treatment 1, Fig. 46), but subsequently the growth rate declines more rapidly. Addition of ABA after the IAA pretreatment (treatment 6, Fig. 47) accelerates the decline in growth rate and a significant inhibition is detected after 40 minutes. ($P < 0.05$, paired analysis.) Thus the time course of ABA-induced inhibition is similar in the presence and absence of an external supply of IAA; hence ABA is probably not acting *via* a reduction in the uptake of IAA. This aspect has

Fig. 46. Continuous time courses of the response of Avena coleoptile segments.

Incubation began at -100 minutes and the solutions were changed at 0 minutes. Elongation (a) refers to the mean elongation of the column of 10 segments calculated from experiments repeated on 5 occasions. Rate (b) refers to the rate of elongation of the column of segments calculated over 10 minute periods from mean elongation data. 'B' refers to 0.01M phosphate buffer, pH 7.0.

Statistical Analysis. The t-test was carried out on the mean elongation data to determine the time at which significant ABA-induced inhibition developed.

Item	t value	Significance
(a) Treatments 3/4 (t-test)		
80 minutes	2.379	NS
90 minutes	3.174	*
100 minutes	3.455	*
(b) Treatments 1/2 (paired t-test)		
25 minutes	1.930	NS
30 minutes	3.565	*
35 minutes	2.930	*

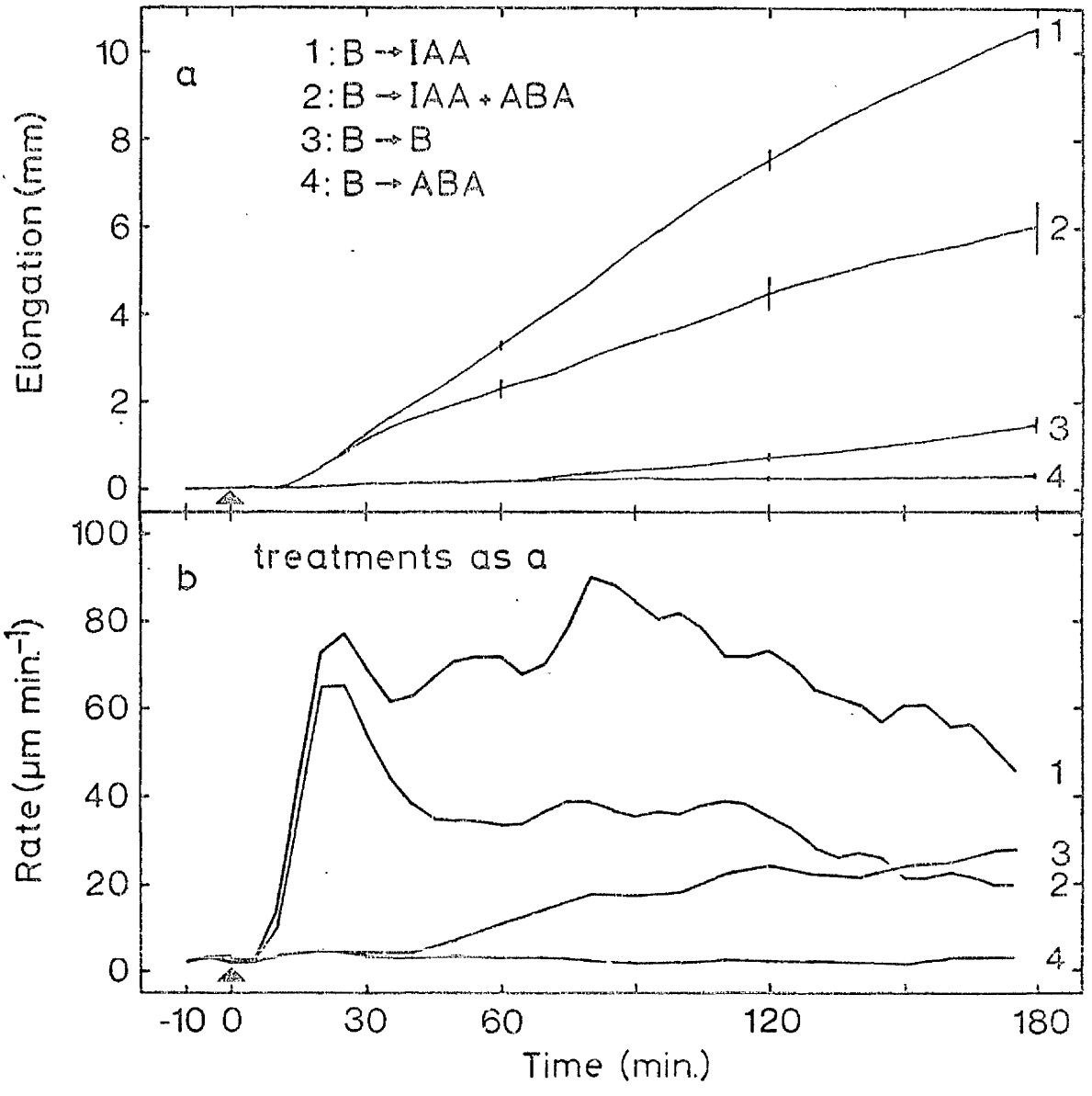
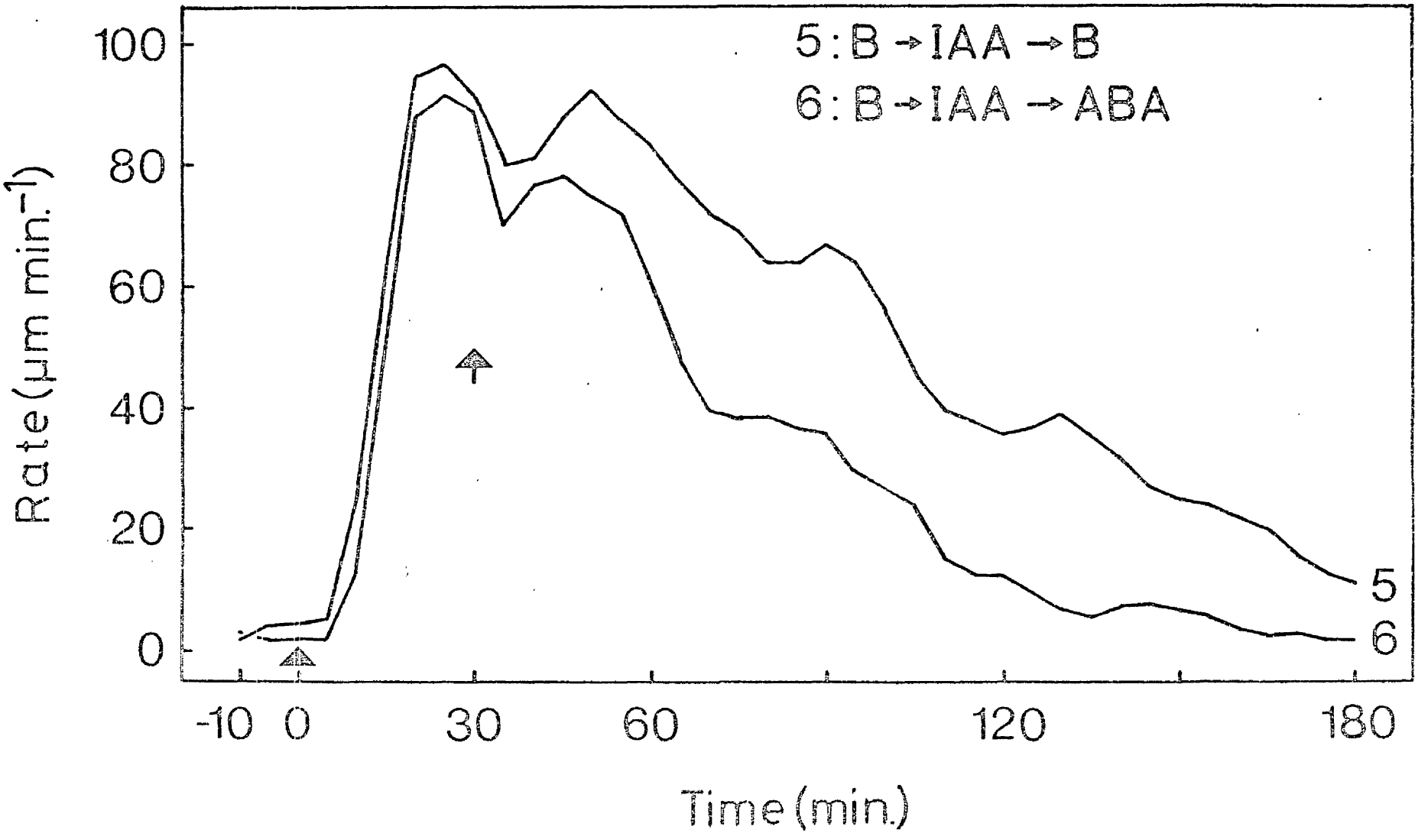

a
1: B → IAA
2: B → IAA + ABA
3: B → B
4: B → ABA
Elongation (mm)
10
8
6
4
2
0
1
2
3
4
b
treatments as a
Rate (μm min⁻¹)
100
80
60
40
20
0
1
3
2
4
-10 0
30
60
120
180
Time (min.)

Fig. 47. Continuous time courses of the response of Avena coleoptile segments.

Incubation began at -100 minutes and solutions were changed at the times indicated by the arrows. The rate refers to the rate of elongation of the column of 10 segments calculated over 10-minute periods from mean elongation data from 5 repeats of the treatments. 'P' refers to 0.01M phosphate buffer, pH 7.0.

Statistical Analysis. The paired t-test was carried out on the mean elongation data to determine the time after the addition of ABA at which significant inhibition developed.

Item	t value	Significance
Treatment 5/6:-		
35 minutes after addition of ABA	1.84	NS
40 "	2.16	*
45 "	2.58	*


5 : B → IAA → B
6 : B → IAA → ABA
Rate (µm min.-1)
100
80
60
40
20
0
-10
0
30
60
120
180
Time (min.)
5
6

also been explored using radioactive hormones (Section 7).

Because of the existence of a latent period before inhibition of IAA-induced growth by ABA it is relevant to investigate whether IAA can promote a high growth rate after a pretreatment in ABA. It was found that IAA at 10^{-5}M will promote the growth of coleoptile segments in the presence of 10^{-5}M ABA even after a 100-minute pretreatment in this concentration of the inhibitor, but the characteristics of the response to IAA are modified (treatment 8, Fig. 48). The latent period is significantly increased to 14.4 $\pm$ 0.8 minutes ($P < 0.02$) and the mean initial maximum growth rate is significantly reduced ($P < 0.001$), although the maximum still occurs after 25 minutes. The latent periods and initial maximum rates of elongation under the conditions studied are also presented in histogram form (Fig. 49a and b).

Having studied the initiation of ABA inhibition the effects of discontinuing the supply of ABA were examined (Fig. 48). Segments were incubated in 10^{-5}M ABA for 100 minutes and then treated with 10^{-5}M IAA in the presence of ABA. After treatment for 20 minutes in both hormones the ABA supply was removed from the solution bathing one batch of segments. For 15 minutes thereafter the growth rate in IAA (treatment 7) is closely similar to that of segments provided with both hormones (treatment 8) but the growth rate in treatment 7 (IAA alone) subsequently increases relative to that in treatment 8 and a higher rate is maintained for 100 minutes. The differences are significant ($P < 0.05$, paired analysis) 80 minutes after the withdrawal of the ABA but the growth rate in treatment 7 never reaches a level comparable to that for IAA-induced growth in the absence of an ABA pretreatment (treatment 1, Fig. 46).

g. Critical Examination of the Transducer Technique. The use of this transducer technique allows continuous monitoring of the elongation of segments in solution. The latent period for a response and the rate of response can be accurately monitored. The use of metabolic inhibitors such

Fig. 48. Continuous time courses of the response of *Avena* coleoptile segments.

Incubation began at -100 minutes and the solutions were changed at the times indicated by the arrows. The rate refers to the rate of elongation of the column of 10 segments calculated over 10-minute periods from mean elongation data from 5 repeats of the treatments.

Statistical Analysis. The paired t-test was carried out on the mean elongation data to determine the time after the removal of IAA from the solution that a significant recovery developed.

Item	t value	Significance
Treatment 7/8:-		
75 minutes after IAA removed	2.04	NS
80 "	2.438	*
100 "	2.75	*

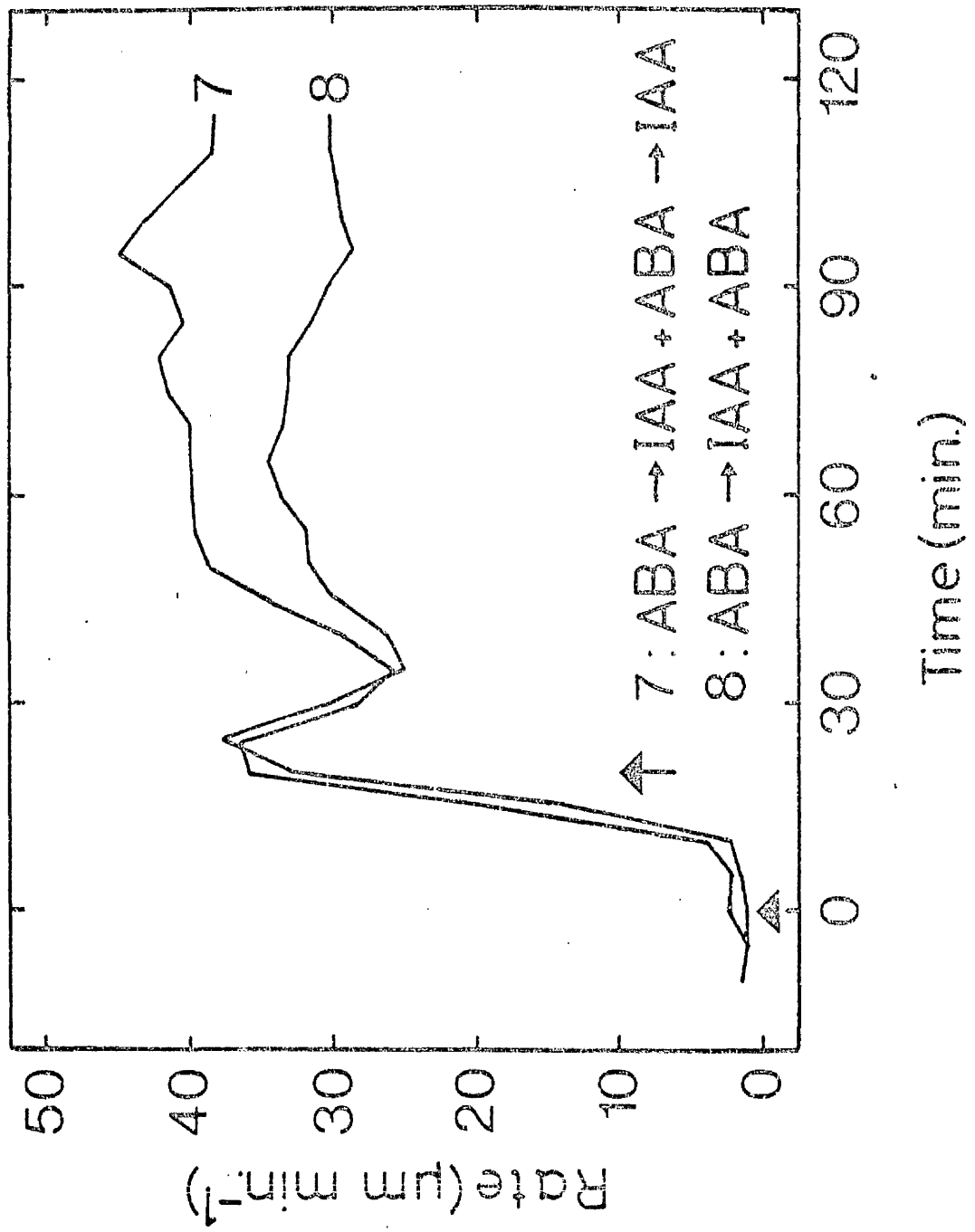

Rate (μm min⁻¹)
50
40
30
20
10
0
0
30
60
90
120
Time (min.)
7
8
7 : ABA → IAA + ABA → IAA
8 : ABA → IAA + ABA

Fig. 49. Characteristics of the response of Avena coleoptile segments (a) initial maximum rate of elongation of the column of 10 segments and (b) the latent period in response to the following treatments at pH 7.0 and 25°C.

1) 10^{-5}M IAA.

2) 10^{-5}M IAA + 10^{-5}M ABA.

3) 10^{-5}M IAA + 10^{-5}M ABA after a 100-minute pretreatment in 10^{-5}M ABA.

Each value represents the mean of 5 repeats of the treatment.

Statistical Analysis. The t-test was used to test the difference between the items indicated.

Item	t value	Significance
a) Rate:		
1/2	1.659	NS
1/3	6.230	***
b) Latent period:		
1/3	2.815	*

initial max. rate (μm min⁻¹)
0
20
40
60
80
a
1
2
3
Treatment

Latent period (min.)
0
4
8
12
16
a
1
2
3
Treatment

as KCN, and sequential and simultaneous hormone treatments allows characterisation of the hormone activity to be elucidated. The variation between replicates for the latent period of a given response is small. Indeed, in the IAA response experiments using the plastic reservoir (apparatus I) and in studies of ABA action using a slightly modified apparatus (II and III) the mean latent periods for the promotion of elongation by 10^{-5}M IAA (25°C, pH 7.0) are 11.3 and 10.9 minutes, respectively. In addition, both forms of the apparatus can detect promotion of a high growth rate by low pH with a latent period of 1 to 2 minutes (Table 15).

Table 15. Latent periods (minutes).

Incubation solution : Phosphate buffer pH 7.0.

Treatment solution : Citrate buffer pH 3.0.

The data are the values obtained in 2 replications of the experiment, carried out at 25°C.

Experiment No.	Apparatus I	Apparatus II
(i)	2.0	2.0
(ii)	1.0	1.0

Considerable variation is observed in the rate of the response. For example, the initial maximum rate of elongation in 10^{-5}M IAA, at 25°C and pH 7.0, studied using apparatus I, ranged from 54.0 to 74.4 μm $min.^{-1}$ per 10 segments, with a mean of 58.9 $\pm$ 3.1 μm $min.^{-1}$ per 10 segments. Because of this variation the transducer technique, in its present state of refinement, is probably unsuitable for the application of enzyme kinetics to hormone studies. The variation observed may be due to the plant material or the experimental apparatus. The preparation of the coleoptile segments is probably not the source of the variation because in the ABA response

experiments (apparatus II and III) greater care was taken in growing the material, e.g. using a precise soaking technique for the vermiculite, and choosing coleoptiles 15-25 mm in length. In the latter experiments the initial rate of elongation recorded in 10^{-5}M IAA (25°C, pH 7.0) is from 70.7 to 94.1 μm $min.^{-1}$ per 10 segments with a mean value of 76.7 $\pm$ 5.0; this variation is similar to that previously described.

The mean initial rates observed under identical conditions of IAA concentration, pH and temperature are different in the 2 forms of the apparatus developed. The source of the differences has not been identified but may be due to one or more of the modifications in the latter apparatus. Because of these disparities it is necessary to make comparisons of the rates observed only between experiments carried out with one form of the apparatus, and if possible paired experiments with a treatment and control should be designed.

7. The Uptake of Radioactivity from 2-[^{14}C]ABA and 1-[^{14}C]IAA

Previous experiments have shown that ABA inhibits IAA-induced growth with a latent period of 30 minutes, when using both hormones at 10^{-5}M. The uptake of radioactivity from 2-[^{14}C]ABA was studied to investigate whether this latent period could be explained by the slow uptake of ABA. In addition, the effect of ABA on the uptake of radioactivity from 1-[^{14}C]IAA has been examined to see whether ABA is acting *via* a reduction in IAA uptake.

Studies of the uptake of radioactive compounds into segments are complicated by the adsorption of radioactivity on to the surface of the material, in addition to the problem of a possible substantial time requirement for the hormone to be transported to active sites or metabolised within the cells.

The efficiency of removal of surface held activity was examined using a 60-minute incubation of threaded segments in 10^{-6}M 2-[^{14}C]ABA. Each of the

five 10 ml distilled water washings given to the segments removed radioactivity and successively smaller quantities of radioactivity were detected in consecutive washings (Fig. 50). The largest amounts of activity were removed by the first 3 aliquots and progressively smaller quantities of radioactivity were present in the 4th and 5th rinses. Consequently, segments were routinely given 3 x 10 ml distilled water rinses before radioactivity was assessed.

a. The Uptake of Radioactivity from 2-[^{14}C]ABA. The uptake of radioactivity from 10^{-6}M 2-[^{14}C]ABA is similar in threaded and floating segments (Fig. 51a and b). In both cases there is substantial uptake of radioactivity during the first ten minutes. Furthermore, the results from both forms of the experiment indicate that the rate of uptake of radioactivity decreases during the incubation period, although this is less apparent in the threaded segments and the observation has not been established statistically. The uptake in threaded segments is generally greater than in those floating on the solution.

Because an essentially similar pattern of uptake is observed with both forms of the apparatus, and because the floating treatment of segments requires less radioactive solution, subsequent experiments were carried out using floating segments.

Confirmation of the rapid uptake of ABA during the first 10 minutes was obtained using 10^{-5}M 2-[^{14}C]ABA. The ABA concentration corresponds to that used in growth studies. For reasons of economy the radioactivity was diluted fivefold with non-radioactive ABA and therefore contained only twice the radioactivity of 10^{-6}M 2-[^{14}C]ABA. The pattern of uptake of radioactivity from the 10^{-5}M 2-[^{14}C]ABA is similar to that described for 10^{-6}M radioactive ABA and again there is no indication of a lag before uptake is detected (Fig. 52). Moreover, the radioactivity detected at each time is approximately twice that observed with the 10^{-6}M 2-[^{14}C]ABA.

Fig. 50. The radioactivity (cpm) in samples from consecutive 10 ml washes of segments which had been treated for 60 minutes in 10^{-6} M 2-[^{14}C]ABA at 25°C.

Each point is the mean cpm of 9 samples from 3 experiments.

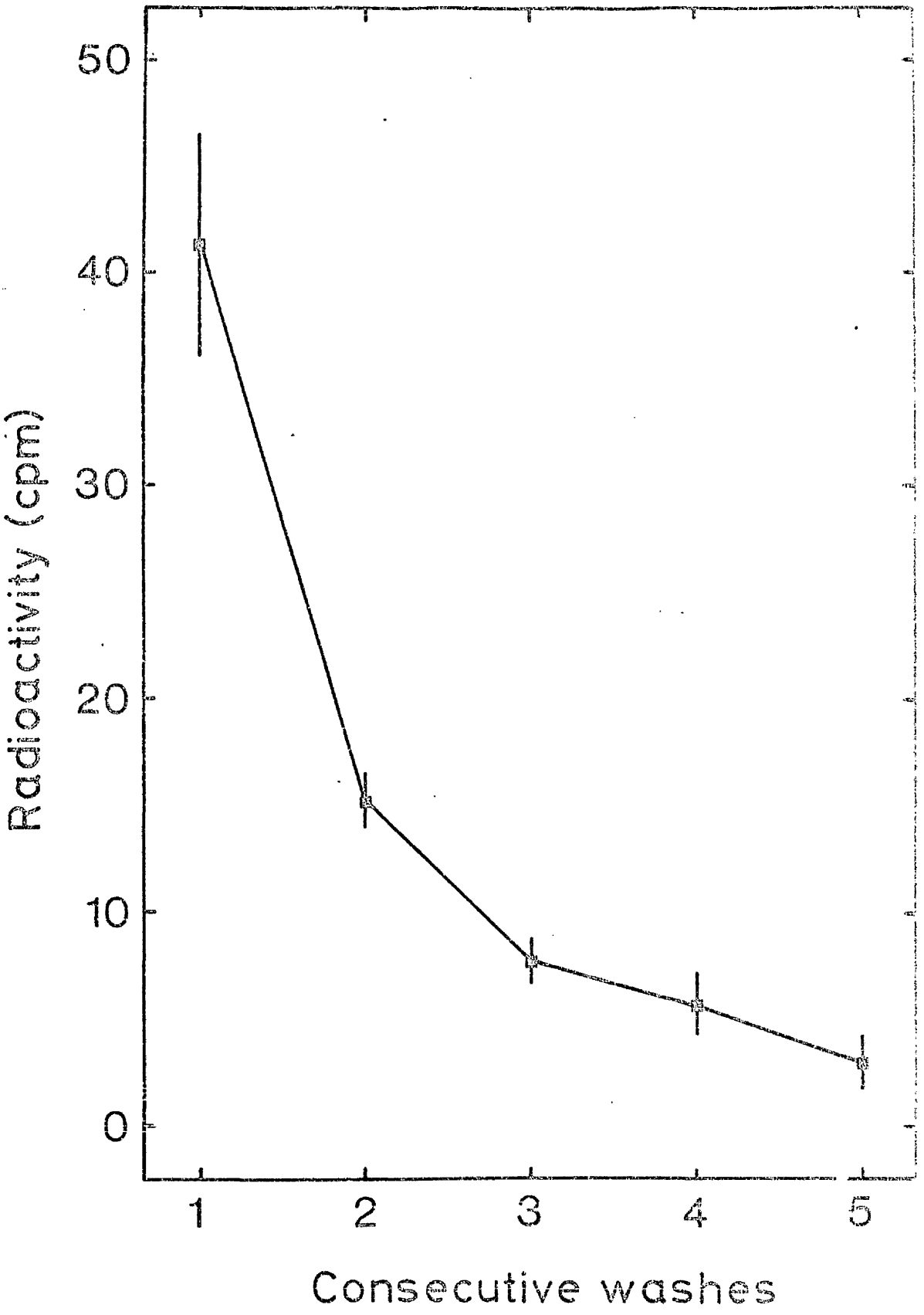

50
40
30
20
10
0
Radioactivity (cpm)
1
2
3
4
5
Consecutive washes

Fig. 51. Time courses of the uptake of radioactivity from 10^{-6} M 2-[^{14}C]ABA into 10 *Avena* coleoptile segments, at 25°C and pH 7.0.

a) Threaded segments. Each point is the mean of 3 samples from 3 experiments.

b) Floating segments. Each point is the mean of 9 samples from 3 experiments.

Efficiency of counting:- 94%.

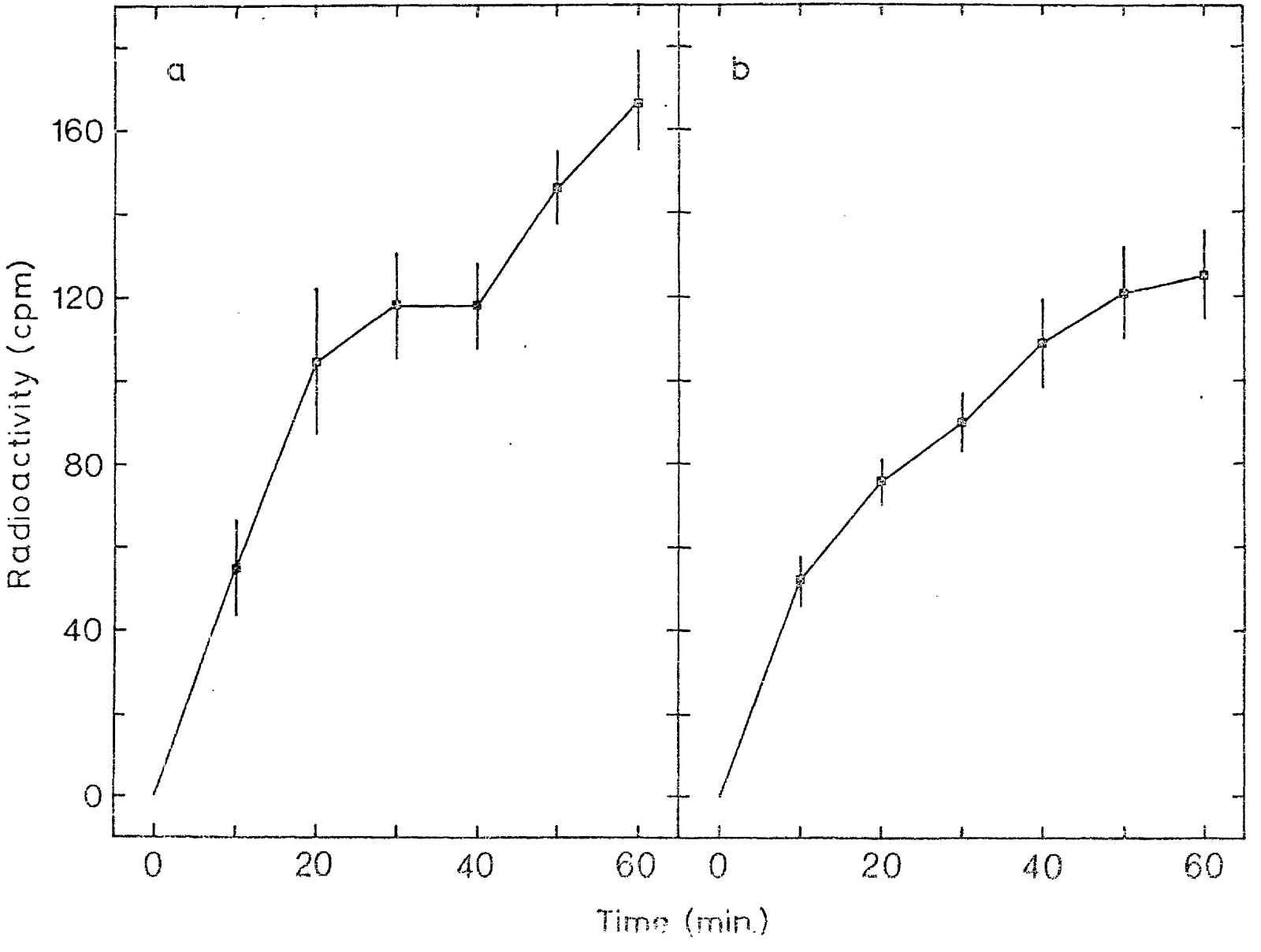
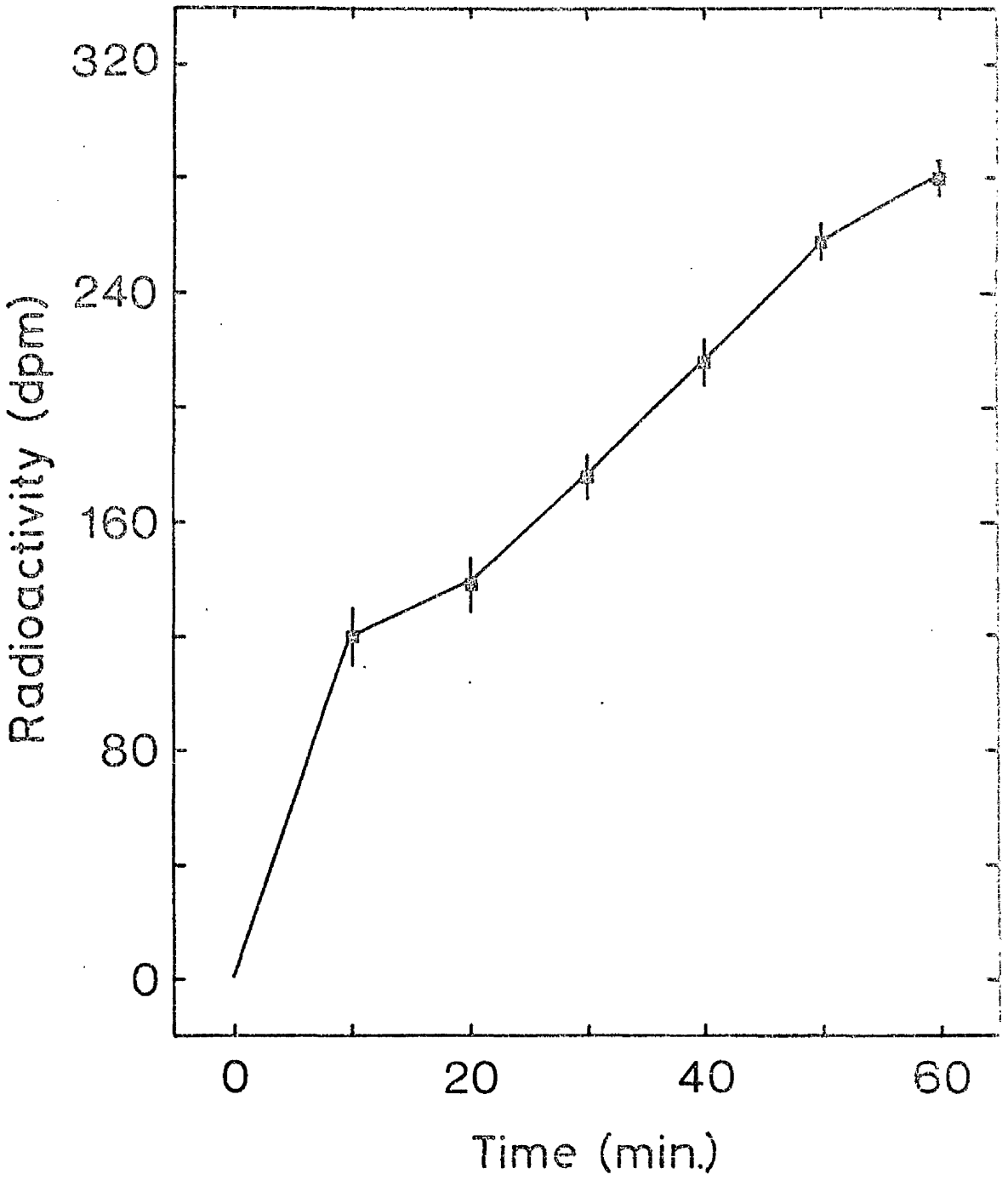

a
b
160
120
80
40
0
Radioactivity (cpm)
0
20
40
60
0
20
40
60
Time (min.)

Fig. 52. Time course of the uptake of radioactivity from 10^{-5} M 2-[^{14}C]IAA into 10 *Avena* coleoptile segments, at 25°C and pH 7.0.

Each point is the mean of 9 samples from 3 experiments.


320
240
160
80
0
Radioactivity (dpm)
0
20
40
60
Time (min.)

b. The Uptake of Radioactivity from 1-[^{14}C]IAA. The uptake of radioactivity from 10^{-5} M 1-[^{14}C]IAA was examined using floating coleoptile segments. A substantial uptake of radioactivity occurs during the first 10 minutes and uptake continues for a further 50 minutes (Fig. 53). The uptake is not significantly reduced in the presence of 10^{-5} M ABA, even after a 100-minute pretreatment in this growth inhibitor.

The experiment thus confirms the growth data and indicates that ABA is not acting directly on IAA uptake. The uptake of IAA may have been slightly greator than indicated by the uptake of radioactivity, due to the loss of ^{14}C as $^{14}CO_2$ following decarboxylation of 1-[^{14}C]IAA. In addition, the observed data could have been obtained were ABA inhibiting uptake of IAA as well as reducing decarboxylation of the radioactive IAA in the cells. The conclusions of this experiment, however, are supported by the growth data.

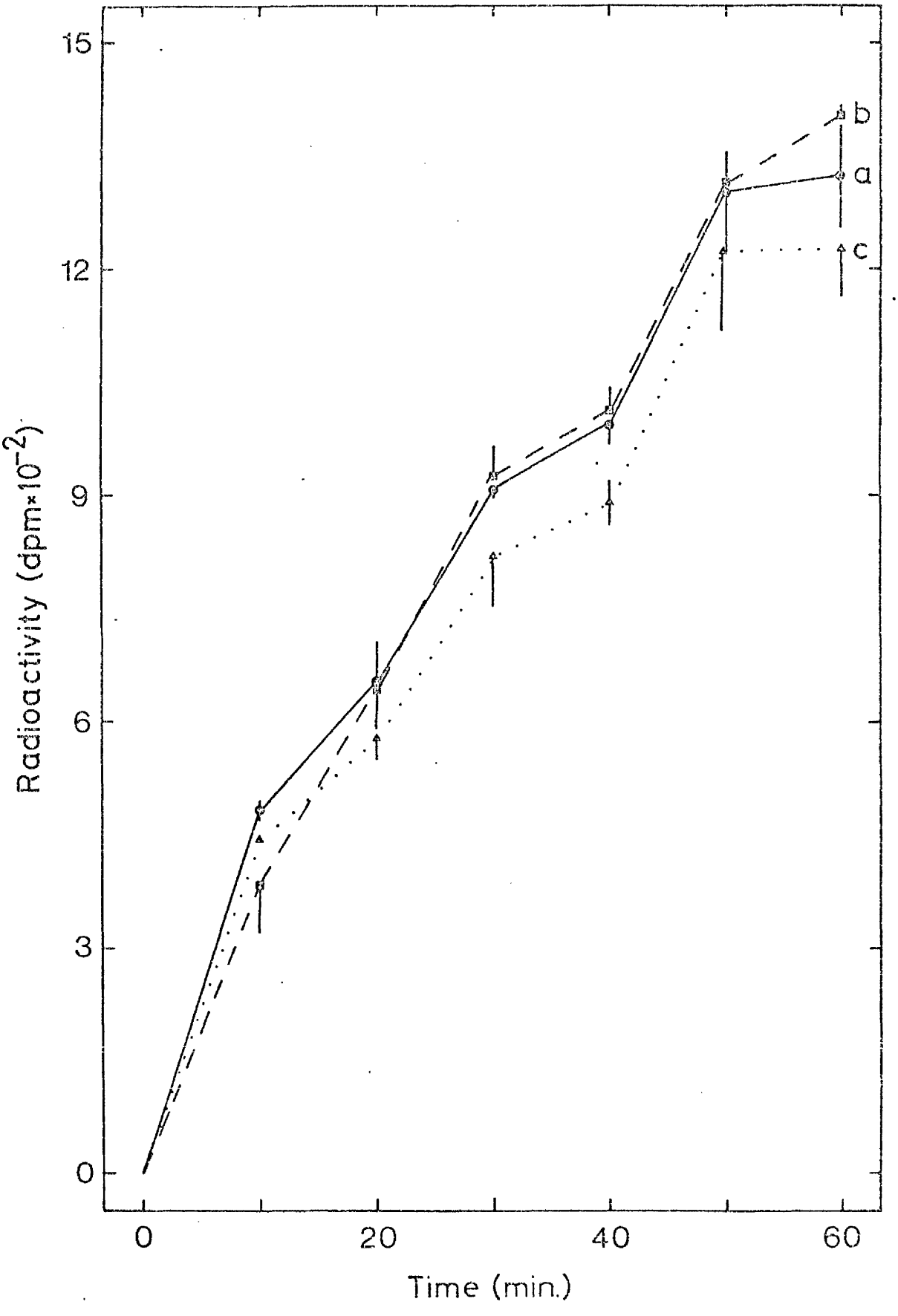
Fig. 53. Time courses of the uptake of radioactivity from 10^{-5} M 1-[^{14}C]IAA into 10 **Avena coleoptile** segments.

(a) in buffer.

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

(c) in 10^{-5} M ABA after a 100-minute pretreatment in 10^{-5} M ABA.

Each point is the mean of 3 samples from 1 experiment. Similar results were obtained in 2 repeats of these treatments.


15
12
9
6
3
0
0
20
40
60
Radioactivity (dpm×10⁻²)
Time (min.)
a
b
c

DISCUSSION

The rôle of abscisic acid in the regulation of cell elongation has been studied using coleoptile segments of Avena sativa. ABA at a concentration of 10^{-7}M inhibits the growth of these segments in distilled water, during a 24-h straight growth assay. This finding supports that of Wareing et al. (1968a), who observed an inhibitory effect of ABA over the concentration range 0.1 to 20 ppm (≡ 3.9×10^{-7} to 7.6×10^{-5}M) when employing this assay. The inhibition of growth by abscisic acid has also been reported in wheat coleoptile segments (Taylor and Burden, 1972), oat mesocotyls (Milborrow, 1966) and other tissues (Addicott and Lyon, 1969). None of the ABA concentrations tested gives rise to a complete inhibition of elongation. Attention was drawn to the latter point by Taylor and Burden (1972), who suggested that this may be due to a lag before ABA inhibition begins; indeed, they noted that ABA did not produce any inhibitory effect for at least 60 minutes in the wheat coleoptile section assay. Experiments using Avena coleoptiles in the straight growth assay demonstrate a similar latent period for ABA-induced inhibition of the growth in distilled water. This latent period, however, will only partially account for the incomplete inhibition because growth does continue during the 24-h period in the presence of ABA, though at a rate which is reduced compared with the water treated segments. The nature of the latent period for ABA activity is further considered when discussing those experiments in which elongation was monitored continuously.

The inhibition induced by ABA over a 24-h period is overcome by transferring segments to IAA, thus indicating that ABA is not killing the segments. With an initial exposure to 10^{-4}M ABA, however, an inhibitory effect did persist even after transference to IAA and hence this concentration of inhibitor may be killing some of the cells. On the other hand, the latter result may indicate that the ABA has not been satisfactorily removed from the segments either by the washing treatment or metabolism. The fact that ABA-

induced inhibition may be reversed by IAA has also been described by Taylor and Burden (1972) and they concluded that this differentiated between inhibitory action and phytotoxicity.

Analogues of abscisic acid have been tested in the Avena coleoptile straight growth assay and these experiments reveal certain molecular requirements for ABA activity. The structure-activity relations of compounds I to XXII have been described by McWha et al. (1973) both in the Avena assay and in the lettuce seed germination assay. The most important point to emerge from that investigation was that an absolute order of activity cannot be ascribed to ABA and its analogues. Analogues active in the inhibition of growth in the Avena assay can be quite inactive in a germination test and vice versa. Hence it is important to look for the main differences between the structural requirements reported for the inhibition of lettuce seed germination and those requirements deduced from the data presented here for the Avena assay, in addition to noting the structural requirements implicated by other authors using different assays and analogues.

The carbonyl and hydroxyl groups in the ring appear unnecessary for inhibitory activity in the Avena coleoptile bioassay and this inference is in general agreement with the structural requirements for the growth inhibition of the second leaf sheath of rice (Tamura and Nagao, 1969a, 1969c, 1970; Oritani and Yamashita, 1970a) and growth inhibition of embryonic axes of bean (Sondheimer and Walton, 1970) although the work of Sondheimer and Walton must be interpreted cautiously (see Introduction). Thus it is particularly interesting that McWha et al. (1973) observed that one or both of the hydroxyl group and carbonyl group are of considerable importance for the inhibition of lettuce germination.

The ring double bond is required for the full inhibitory activity of ABA in the Avena assay and this moiety has been implicated as a requirement in other assays (Tamura and Nagao, 1969c, 1970; Oritani and Yamashita, 1970a; Sondheimer and Walton, 1970; McWha et al. (1973) although there are some

inconsistencies in the literature. Epoxidation of the ethyl and methyl esters, for example, enhanced the activity of these analogues although it removed the ring double bond (Tamura and Nagao, 1969a). In the Avena assay epoxide compounds invariably possess little activity and this observation was also reported in the lettuce assay (McWha et al., 1973). The compound xanthoxin lacked the ring double bond but had activity comparable to ABA in the cress seed germination and wheat coleoptile bioassays (Taylor and Burden, 1970b); the importance of this observation is increased as xanthoxin has been extracted from shoots of many species (see Introduction). Although this observation suggests the ring double bond is not a molecular requirement for the inhibitory activity of ABA in these systems the activity of xanthoxin may be explained in terms of its biological conversion to ABA in these tissues (Taylor and Burden, 1972).

With regard to the side chain, the complete 2,4-pentadiene residue is required for inhibitory activity in the Avena assay and this residue was also implicated as a molecular requirement for inhibition in other systems (Tamura and Nagao, 1970; Oritani and Yamashita, 1970a; Sondheimer and Walton, 1970). In the lettuce germination assay, however, McWha et al. (1973) found that this residue may be altered and activity maintained provided the double bond at C-2 is retained. Furthermore, they reported that for the inhibition of lettuce seed germination the bond at C-4 does not require to be di-unsaturated. Indeed, one compound with a triple bond at C-4 (compound XVII) was more active than ABA; they suggested, therefore, that the spatial configuration of the atoms in this part of the side chain was not important for activity. In contrast, the data for the Avena assay indicate that the spatial arrangements of the ring double bond and the unsaturated functional group in the side chain are important for ABA activity.

The unsaturated functional group at C-1 appears to be a molecular requirement for ABA activity in the Avena system but there have been few reports published concerning the requirement of a specific terminal moiety

in other assays; although Tamura and Nagao (1969c) have suggested that the terminal moiety should be carboxyl or carboalkoxyl and Oritani and Yamashita (1970a) included the acid group in their fundamental structure. McWha et al. (1973), in fact, reported that all the compounds tested that possessed the basic requirements for inhibitory activity in the lettuce assay were active regardless of the terminal moiety, and many of these compounds were more active than ABA in this assay.

The trans,trans- analogues tested here have similar activity to the cis,trans- counterparts, yet other investigators have predominantly reported the t,t-isomers to be the least active (Tamura and Nagao, 1969a, 1970; Sondheimer and Walton, 1970). It is possible that the differences may reflect the interconversion of these isomers by U.V. light (e.g. Lenton et al., 1971). The latter point may also contribute to the conflicting reports concerning the activity of the geometrical isomers of ABA, because although U.V. light will convert t,t-ABA to an equilibrium mixture of the 2 isomers few authors have paid attention to this point. The varying activity of c,t- and t,t-ABA, however, may also be due to the different bioassays used and in this context it is interesting that the inference that these isomers have similar activity was drawn from experiments using the wheat coleoptile bioassay (Nitsch, 1967) and that this inference is consistent with data observed for the Avena assay. Nevertheless, it would be of relevance to pursue these experiments, possibly determining the geometrical structures of the isomers in solution before and after the bioassay and if possible employing pure trans,trans-ABA.

Thus it is important to note that there are particular molecular requirements for the inhibitory activity of ABA in the Avena assay and that these requirements may be modified in different assay systems. Two active moieties, viz. the ring double bond and the unsaturated functional group at C-1, appear essential for complete inhibitory activity in the Avena system. The fact that the spatial arrangement of these moieties is important leads

one to tentatively postulate that there may be two point attachment of ABA to an active site within the cell. Analogues with only one of these moieties, however, possess considerable inhibitory activity at 10^{-5}M, which in some instances is similar to that of 10^{-5}M ABA, and this tends to preclude the two point attachment from being the only mode of action of ABA in the inhibition of elongation of Avena coleoptile segments.

Certain qualifications must be applied to these conclusions which are based on the use of analogues; these qualifications, indeed, apply to the technique of employing analogues in determining the structure-activity relations of any compound. Firstly, no inferences may be drawn about those moieties which have not been altered in any of the analogues. For example, no evidence is presented on the importance of the ring or side chain methyl groups, or on the carbon skeleton of the ring in ABA. Secondly, the differences in activity of the various analogues may be due to differences in uptake or breakdown of the compounds by the tissue, rather than the activity of the molecules per se when they reach the site of action. In spite of these qualifications, most of the inferences made here are based on the structure-activity relations of several compounds and this factor strengthens these inferences.

Abscisic acid overcomes the promotory effects of IAA in the Avena straight growth assay and this basic finding is in agreement with that of Wareing et al. (1968a). The use of molar concentrations of each hormone also indicates that on a mole for mole basis ABA is a more powerful inhibitor than IAA is a promoter. Although Wareing et al. (1968a) quoted hormone concentrations in parts per million, these may be converted to molarities and the data then indicate that with equimolar concentrations of both hormones there is inhibition of growth relative to the water control.

Presentation of the data as segment length at varying hormone concentrations indicates a divergence of lines joining consecutive increasing IAA concentrations at the different ABA concentrations; furthermore, an analysis

of variance indicates a statistical interaction of these data. This is contrary to the conclusions of Wareing et al. (1968a) who found no evidence of an interaction. The differences may be attributable to the fact that data for only one IAA concentration were presented by Wareing et al. (1968a), yet they indicated that various concentrations of both hormones in combination were also assayed and that the analysis of variance revealed no interaction. Alternatively, the differences may reflect the conditions under which the assays were carried out and unfortunately no details were provided.

The transformation of the data in this thesis to percentage inhibition, however, tends to reduce the divergence of the lines. Although the normal analysis of variance is not an appropriate analysis for these data, the percentage inhibition data do not lead one to suspect a statistical interaction. Because the transformation of data can alter the conclusions regarding the possible interaction of data it is advisable to adopt the suggestion of Milborrow (1970b) and not use the term "interaction" when attempting to elucidate physiological mechanisms. Indeed, the existence of a statistical interaction seems only to describe the data that are tested, and analysis of transformed data only characterises these transformed data.

Nevertheless, the technique of examining the effect of one hormone on the response of plant tissue to another hormone is of value and factorial experiments employing a range of concentrations of each hormone are probably the most suitable for growth studies. In addition, these experiments indicate that ABA counteracts the promotive effect of IAA and that the net effect on segment elongation is dependent upon the concentrations of the two hormones.

In an attempt to elucidate the mode of action of ABA inhibition of IAA activity, analyses in terms of enzyme kinetics were carried out. In particular it was hoped these studies would reveal whether ABA was acting via a competitive or a non-competitive inhibition of IAA action. The experiments were carried out in the absence of buffer or sucrose because

complicating effects of these additives have been described (Marinos, 1957; Cleland, 1972). Under the conditions employed it is apparent that growth is not linear with time, and Marinos (1957) has pointed out that a linear response can only be realised under limited conditions. Hence, to apply enzyme-kinetic analysis detailed time courses were carried out and the initial rates of elongation estimated.

In spite of this attention to detail, the technique did not yield a decisive result and several factors appear to have contributed to the lack of success of this technique. For example, the latent period before ABA inhibition begins results in the initial rates of segment elongation, at each IAA concentration, being similar. When the rates are estimated from a given time after the addition of the hormones, however, the variability of the data and the fact that the precise latent period for ABA action depends on the concentrations of both IAA and ABA, obscures the analysis. Further exploitation of this type of analysis may be interesting but these types of consideration may be an oversimplification of the multi-enzyme plant cell (Marinos, 1957). Indeed, the technique of Rothwell and Wain (1964) has been applied in two similar cases and different conclusions reached. In a study of the effect of ABA extracted from lupin pods a purely non-competitive inhibition of IAA action in wheat coleoptiles was observed (Rothwell and Wain, 1964), yet using synthetic ABA a less precise result, described as "largely non-competitive", was reported (Taylor and Burden, 1972). Thus, when applying enzyme-kinetics to the coleoptile-IAA system it seems that the basic premises (see Introduction), and possibly the conclusions, can be insecure.

The investigation into the activity of 14 ABA analogues in combination with IAA indicates that these analogues will counteract the promotory effect of IAA. The analogues whose activity alone is similar to that of ABA alone, show effects in overcoming IAA action which are similar to those of ABA; and the analogues which are less active alone are less effective in

overcoming IAA action. The structure-activity relations of 10 of the analogues indicate that the structural requirements for ABA action in overcoming the promotory effects of applied IAA are similar to the requirements of ABA when assayed alone. Thus it is possible to deduce that the mode of action of ABA is to reduce the effectiveness of endogenous IAA. This suggestion, however, must be regarded as speculative, particularly because the inference is based on experiments with only 10 analogues and because anomalous and variable results were obtained with 4 other analogues.

There are no previous reports that the molecular requirements for ABA activity alone are similar to those for ABA to counteract an IAA effect although some analogues have been shown to be active in the presence of other hormones. Xanthoxin, for example, had similar activity to ABA in the wheat coleoptile bioassay in the presence and absence of applied IAA (Taylor and Burden, 1972). In addition, Koshimizu et al. (1966) have shown that the methyl ester of ABA inhibited the growth promoted by GA_3 in the rice second leaf sheath assay and a similar effect was shown for another ABA analogue which was active alone in this bioassay (Tamura and Nagao, 1969a).

Experiments using apparatus which is sufficiently sensitive to monitor minute by minute changes in the elongation of Avena coleoptile segments have demonstrated that both IAA and ABA exhibit rapid effects on elongation, i.e. latent periods less than 30 minutes (Penny et al., 1972).

At pH 7.0, 10^{-5} M IAA at 25°C promotes a high growth rate in approximately 11 minutes; this supports the finding of Ray and Ruesink (1962) and several other reports (Evans and Ray, 1969; Missl and Zenk, 1969). When the rate of elongation is presented as a function of time it is evident that the maximum initial rate occurs after 25 minutes, as reported by Rehm and Cline (1973), and is not established 3 minutes after the latent period as suggested by Evans and Ray (1969).

This method of data presentation also reveals a depression in growth rate after the initial maximum has been reached and subsequent fluctuations

in rate; in some experiments a second maximum in rate, which is greater than the initial maximum, is observed. Although similar growth patterns have been reported by Penny et al. (1972) for lupin hypocotyl segments and for corn coleoptile segments by dela Fuente and Leopold (1970), data from experiments with Avena coleoptile segments have previously been presented as elongation VS time and have indicated a steady IAA-induced growth rate (Evans and Ray, 1969; Nissl and Zenk, 1969).

The effects of temperature, pH, and IAA concentration on the latent period and rate of the response to IAA are important because they provide information on the mechanism of IAA action. Although the experiments on IAA were intended to form only the basis from which ABA effects could be studied, they have received considerable attention because they indicate that a re-appraisal of some published data is required.

The important point to emerge from the effect of IAA concentration on the rate of elongation, as measured with the transducer technique, is that at pH 7.0 and 25°C a response curve which is roughly bell-shaped is observed between 10^{-7} to 10^{-3} M IAA. This response pattern is confirmed in experiments in which the growth of threaded, floating segments was determined after a 90-minute incubation period. The initial rates show the same trend in response to increasing IAA concentration as did the final segment lengths after a 24-h straight growth assay, i.e. the rate is less than maximal at the highest IAA concentration, although the maximum response is observed at different IAA concentrations. This finding is different from the sigmoid dosage-response curve that has been described at pH 4.7 (Nissl and Zenk, 1969; Cleland, 1972). Hence, it seems probable that the pH of the IAA solution affects the shape of the dosage-response curve, in addition to the factors such as the incubation time and presence of sucrose in the incubation medium as suggested by Cleland (1972). At pH 4.7, using threaded floating segments in experiments in which the elongation is determined after 90 minutes treatment the dosage-response curve more closely resembles the

sigmoid-shape that has been reported at pH 4.7 (Nissl and Zenk, 1969; Cleland, 1972) than the bell-shape reported here at pH 7.0; yet extremely variable results are observed and they must be interpreted cautiously. Furthermore, in the presence and absence of applied IAA there is more growth at pH 4.7 than at pH 7.0. This may indicate that the lower pH is closer to the optimum for both endogenous and IAA-induced growth or that at pH 4.7 an acid-growth mechanism is involved. This is an important point because many published experiments examining IAA effects have been carried out at pH 4.7; this problem certainly merits further investigations.

Two additional observations are apparent from these experiments on the dosage-response curves of initial rates. Firstly, the red light treatment employed almost completely abolishes the response to IAA. This experiment, however, has not been repeated and there are no precise details of the wavelength or intensity of the light source used. Nevertheless, these experiments warrant further attention because an exposure of growing seedlings to red light has frequently been reported in the literature and a pronounced IAA effect observed (e.g. Cleland, 1972). Secondly, the dosage-response pattern at 25°C and pH 7.0 differs in threaded, floating segments and in non-threading segments in which part of the first leaf is present. In threaded segments 10^{-3}M IAA promotes less growth than 10^{-4}M or 10^{-5}M IAA, yet in non-threaded segments a similar effect is observed with these 3 IAA concentrations. This may be due to the fact that in threaded segments in which the first leaf is removed, the inner surface of the coleoptile is moistened and there is a greater area for the uptake of hormones, thus a supra-optimal IAA concentration is more easily attained in these experiments. This suggestion is supported by the fact that there was greater uptake of 2-[^{14}C]ABA in threaded vertically-held segments than in floating segments. Hence, experiments in which the growth rate of threaded segments is measured with the transducer technique should be interpreted with care when high IAA concentrations, e.g. 10^{-3}M, are used.

At pH 7.0 IAA concentration and temperature also affect the duration of the latent period for IAA-induced growth of Avena coleoptile segments. The reduction in latent period with increasing temperature has previously been reported (Ray and Ruesink, 1962; Evans and Ray, 1969; Nissl and Zenk, 1969).

Conflicting evidence, however, has been reported concerning the effect of IAA concentration on the latent period. With Avena coleoptile segments Ray and Ruesink (1962) and Evans and Ray (1969) found that two concentrations of IAA induced growth with similar latent periods, while Nissl and Zenk (1969) observed a dependence of latent period on IAA concentration over the range 10^{-10}M to 10^{-3}M at 21°C and pH 4.7. In addition, Durand and Zenk (1970) have reported a decrease in the latent period in Zea coleoptile segments with increasing IAA concentrations at pH 6.3 and 21°C. The data presented here show that the latent period for IAA-induced growth at pH 7.0 is shortened by increasing IAA concentration but that the latent period at this pH always exceeds 7 minutes.

The observed effects of IAA concentration and temperature on the latent period may be explained in terms of a lag time for IAA uptake and transport to sites of action but with a decreased sensitivity of material to IAA occurring at high temperatures. Thus, at 25°C increasing the concentration of IAA produces a minimum latent period at a concentration of 10^{-3}M. In addition, raising the temperature decreases the latent period with the minimum value occurring at 30°C for 10^{-5}M IAA, and at 25°C for both 10^{-3}M and 5×10^{-3}M IAA. The reduction in sensitivity of material at high temperatures is evident from the fact that the latent period for the response to 10^{-5}M IAA is increased when the temperature is raised from 30°C to 35°C, and using both 10^{-3}M and 5×10^{-3}M IAA the latent periods are lengthened when the temperature is raised from 25°C to 30°C. Furthermore, the initial rate of elongation is decreased when the temperature is raised from 30°C to 35°C with both 10^{-5}M and 10^{-3}M IAA, and with 5×10^{-3}M IAA a slower initial maximum rate is achieved at 30°C than at 25°C.

An almost immediate promotion of elongation is observed with 5×10^{-3}M IAA prepared in 0.01M KH_2PO_4, as used by Nissl and Zenk (1969), who specified that the pH of this solution was 4.7. In contrast, the data presented here show that this solution has a pH of 3.5. Since a buffer of pH 3.5 will promote elongation in coleoptile segments almost immediately in the absence of applied IAA, the low pH may give rise to the immediate increase in growth rate on exposure of the segments to 5×10^{-3}M IAA in KH_2PO_4 at pH 3.5, rather than solely the presence of the IAA.

The differential effects of cyanide on IAA-induced and low-pH-induced growth support this suggestion. Treatment with 10^{-4}M KCN abolishes the response to 5×10^{-3}M IAA in phosphate buffer at pH 7.0, confirming the findings of Ray and Ruesink (1962) who reported that within 5 minutes this concentration of KCN completely inhibits growth induced by 3 µg ml^{-1} IAA. The potassium cyanide treatment, however, does not affect the latent period for low-pH-induced growth and only slightly inhibits the rate of elongation. Thus it is of particular importance that exposure to potassium cyanide does not affect the latent period for the promotion of growth by 5×10^{-3}M IAA in 0.01M KH_2PO_4, the pH of which is 3.5, and only slightly reduces the total elongation after 30 minutes. These data thus led one to the conclusion that the almost immediate promotion of coleoptile segment growth by 5×10^{-3}M IAA in 0.01M KH_2PO_4 is attributable to the change in pH rather than to the presence of IAA.

If protein synthesis were involved in the initial response of coleoptile segments to IAA, then a rapid change in the pattern of synthesis would be required to account for the rapid increase in growth rate. The evidence presented here indicates that this change in synthesis would need to occur within 7.3 minutes under certain conditions. Although there have been some reports of a stimulation of RNA synthesis in Avena coleoptile segments within 10 minutes (Masuda and Kamisaka, 1969) other accounts have indicated a lag of at least one hour (Trewavas, 1968); the differences observed may be due

to difficulties involved in the estimation of changes in protein synthesis over short time periods, and indeed the problems involved in these techniques have been discussed by Trewavas (1968). Thus, on the basis of the present evidence concerning the timing of the effect of IAA on growth and on protein synthesis, one cannot conclude categorically whether the initial elongation response of Avena coleoptile segments involves the promotion of protein synthesis.

Another approach to the problem is the use of inhibitors of protein synthesis, but again conflicting evidence, that was cited in the Introduction, has been reported. This approach also leaves open the possibility that a promotion of protein synthesis is involved in the initial mechanism of IAA action.

The argument of Nissl and Zenk (1969), however, that RNA synthesis is not involved because an immediate IAA effect can be detected, seems untenable. The results presented here strongly suggest that a low-pH-induced response was involved in the immediate response to IAA described by Nissl and Zenk (1969). Nevertheless, these authors specified that their solutions were at pH 4.7 and they also used a different experimental procedure; for example they incubated the coleoptile segments in buffer contained in a shaking flask. Therefore, the differences between their results and those reported here may reflect differences in experimental methods. On the other hand, at pH 7.0 a latent period exceeding 7 minutes was always observed before IAA induces rapid growth in Avena coleoptile segments.

The accurate monitoring of the elongation of coleoptile segments has also shown that ABA produces a rapid inhibition of IAA-induced elongation. Simultaneous application of ABA and IAA does not significantly change the latent period or the initial maximum rate of response to IAA alone but a significant inhibitory effect of ABA develops after 30 minutes. A similar time lag has been reported in experiments using a midcourse addition of 10^{-5}M ABA to material pretreated with IAA and a more rapid inhibition has

been observed with higher ABA concentrations (Zenk, 1970; Rehm and Cline, 1973). In addition, a 5-minute lag for the inhibition of growth of intact etiolated pea seedlings treated with 10^{-5}M ABA has been reported (Warner and Leopold, 1971). With a 100-minute pretreatment in ABA the data presented here show that the IAA response, in the continued presence of ABA, is modified, the initial maximum rate being reduced and the latent period lengthened. Although the increase in the latent period is small it is statistically significant. While Rehm and Cline (1973) reported a reduction in the IAA-induced growth rate after a 30- or 60-minute pretreatment with 10^{-4}M ABA they found no appreciable effect of ABA on the latent period.

There is also a rapid reduction in the inhibition of IAA-induced growth following removal of ABA, but the rate of elongation never recovers to that observed in the absence of ABA pretreatment.

The lag before ABA-induced inhibition is detectable cannot be attributed to slow uptake of ABA because uptake of radioactivity from 2-[^{14}C]ABA takes place during the first 10 minutes treatment. There may, however, be a substantial time requirement for ABA to be transported to active sites or metabolised within the cell. Furthermore, the inhibition of IAA-induced growth by ABA cannot be ascribed to an inhibition of IAA uptake into the segments because ABA does not reduce uptake of radioactivity from 1-[^{14}C]IAA, even after a 100-minute pretreatment in ABA. Further evidence that ABA is not acting directly on IAA uptake is provided by the fact that the latent period for growth inhibition by ABA, following removal of the IAA supply, is similar to that obtained when segments are treated continuously in both hormones.

It is thus possible that if ABA were to control endogenous growth a rapid inhibition of growth could be achieved. ABA levels have been shown to increase at times of water stress (Wright, 1969; Most, 1971) and hence this inhibitor could regulate growth at these times by reducing IAA action. In order to suggest an endogenous regulatory rôle for ABA it is necessary to

demonstrate the existence of this compound in the tissue and to observe changes in the levels of ABA and correlate these changes with alterations in growth rate; yet even this does not establish a cause and effect between ABA levels and a reduction in growth unless the changes in ABA levels are shown to precede the inhibition of growth.

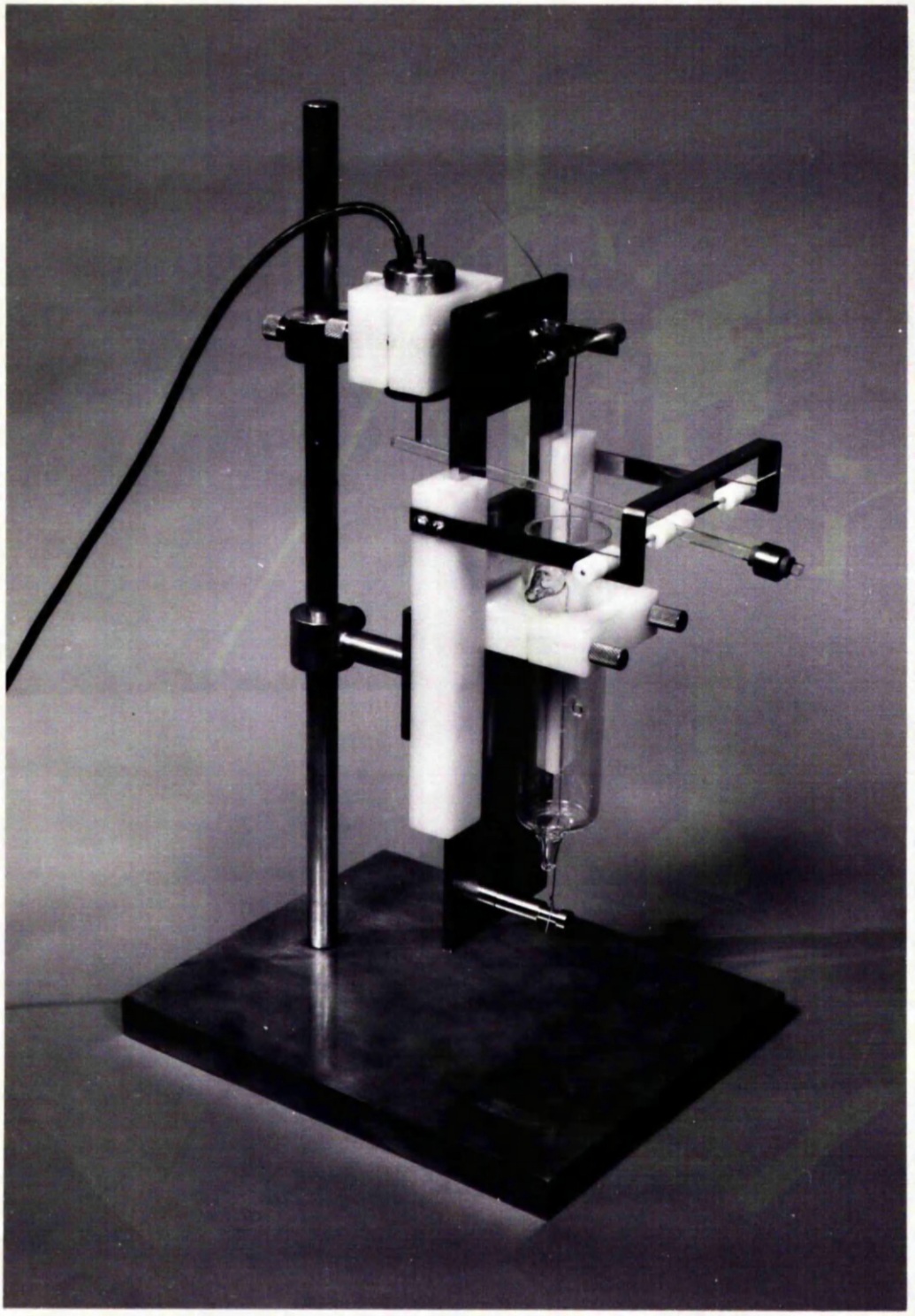
The use of a linear displacement transducer has been successful for the accurate monitoring of elongation growth. The variation in the rates observed may be reduced if the position of the transducer on the lever was more precisely determined. An apparatus in which this is achieved has been designed and built (Plate 4). In this apparatus the lever-pivot and transducer are supported on the same frame and can be accurately moved relative to each other because the boss supporting the transducer is on a screw thread. The fact that the lever, transducer and reservoir are supported on the same frame reduces the size of the apparatus but the segments in the reservoir can still be re-positioned relative to the transducer because the reservoir is held on a brass back-plate that can move in teflon bearings.

Closer attention to the weight acting downwards on the segments may also reduce the variation observed in the rates of growth. Although the counterbalance weight was in a fixed position frictional forces could alter the weight on the coleoptiles. In this context, a position sensing transducer, as used by Rehm and Cline (1973) may prove useful because an accessory lever system is not required.

All the experiments described in this thesis have been carried out with excised coleoptile segments. These are mechanically easier to handle than whole seedlings and were initially selected to allow the analogues of abscisic acid to be assayed. The use of segments also simplifies the interpretation of the data because the source of endogenous IAA in the intact seedling, the tip of the coleoptile, has been removed. Furthermore, the influence of the rest of the seedling has been removed and the complexity of the whole plant system would make interpretation of the data more difficult.

Plate 4. Apparatus for the continuous monitoring of elongation of coleoptile segments in solution.

The brass frame provides a fixed support for the transducer and lever-pivot. The glass reservoir is held on a brass back-plate which can move in teflon bearings and can thus be easily positioned relative to the transducer and lever.



It is difficult, however, to extrapolate the results of experiments with excised tissues to whole plants and it is necessary to correlate changes in endogenous levels of ABA with alterations in growth rate before a natural rôle for ABA in the regulation of cell elongation can be proved.

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Abbreviations

ABA Abscisic acid

A.E.S. (ratio) Automatic external standardization

B Initial reaction rate (enzyme kinetics) or buffer solution

C Column items (analysis of variance)

Ci M^{-1} Curies per mole

cpm Counts per minute

C_{28} Octokosane

c,t-(or Z,E-) Cis,trans-

°C Degrees Centigrade

dpm Disintegrations per minute

E Error (analysis of variance)

F Variance ratio (analysis of variance)

Fig(s) Figure(s)

h Hour(s)

I Interaction (analysis of variance)

IAA Indole-acetic acid

K Ohms x 10^3

Km Michaelis constant (enzyme kinetics)

log. Logarithmic

lb. Pound weight

M Molar

mg Milligram(s)

µg Micro-grams(s)

min. or mins. Minute(s)

ml Millilitre(s)

mm Millimetre(s)

ms Mean square (analysis of variance)

P Probability

pers.comm. Personal communication

ppm Parts per million

R Row items (analysis of variance)

S Substrate (enzyme kinetics)

SS Sum of squares (analysis of variance)

t,t-(or E,E-) trans,trans-

µm Micron

µCi mg^{-1} Micro-curies per milligram

U.V. Ultra-violet

v Initial reaction velocity (enzyme kinetics)

V Maximum reaction velocity (enzyme kinetics)

VS Versus i.e. plotted against

ft. Foot

Me Methyl

N Degrees of freedom

s.e. Standard error