AUXIN ACTION AND CELL ELONGATION: A RATIONAL APPROACH.

A thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Physiology at Massey University.

Pauline Elizabeth Penny

1970

To the courteous and well willing readers:

Although my paines hav not beene spent (courteous Reader) in the gracious discoverie of golden Mines, nor in the tracing after silver veines, whereby my native country might be inriched with such merchandise as it hath most in request and admiration; yet hath my Labour (I trust) been otherwise profitably imploied, in descrying of such a harmelesse treasure of herbes, trees, and plants, as the earth frankely without violence offereth unto our most necessary uses. Wherein though myne art be not able to contervaile Nature in her lively portraitures, yet have I counterfeited likeness for life, shapes and shadows for substance. Yet may my blunt attempt serve as a whetstone to set an edge upon sharpey wits, by whom I wish this course Discourse might be both fined and refined. Faults, I confesse have escaped, some through defects in my selfe to perform so great a work, and some by means of the greatness of the Labour, being void of friends to beare some part of the burden. I trust that the best and well minded will not rashly condemne me. Therefore accept this at my hands (loving countrymen) as a token of my goodwill.

John Gerard, 1597

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A B S T R A C T

A method was developed to measure every minute the growth of a single segment excised from the elongating region of a plant. The method was used to determine the short term kinetics of growth in response to auxin addition. The method is not dependant on the use of hollow coleoptile tissue and the results are plotted as a growth rate against time. The technique has a resolution an order of magnitude higher than those in current use.

The results show that there is a latent phase before auxin-induced increase in elongation rate occurs. After this latent phase, there is a rapid rise in rate to a maximum followed by a decrease and then usually a rise to a second maximum. Three hypotheses for explaining the growth rate curve are considered.

It was found that neither RNA nor protein synthesis were required for the initial action of auxin but that protein synthesis became necessary within a few minutes after auxin addition. The apparent half-life of the protein whose synthesis is stimulated by auxin is about 12 min. This short half life suggests that, after the synthesis of the protein, there is a limited time during which it can act with auxin to increase elongation. A model which incorporated these results has been proposed and its relationship to the three hypotheses for explaining the growth rate curve is discussed.

A C K N O W L E D G E M E N T S

I wish to record and acknowledge with thanks the continual encouragement of Prof. R.G. Thomas for research in his department in general and his interest in this project in particular.

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To Ann and Kim I can only say thank-you - you will understand in a few years.

TABLE OF ABBREVIATIONS

DNA	deoxyribonucleic acid
DOC	deoxycholate
IAA	indol-3-yl-acetic acid
NA	numerical aperture
RNA	ribonucleic acid
m-RNA	messenger RNA
r-RNA	ribosomal RNA
s-RNA	soluble RNA
TCA	trichloroacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid

INTRODUCTION

The plant growth hormone, auxin, which was later characterized as indolyl-3-acetic acid was "discovered" by Went in 1928. Aside from cell elongation, auxin is known to play a role, directly or indirectly, in many other processes such as cell division, abscission and apical dominance. However, the precise mechanism of action, or even the locale of action of this hormone is still unknown. The following short historical summary is intended to convey some of the multiplicity of approaches that have been made toward elucidating the mechanism of auxin action in cell elongation. It also is to indicate the background of ideas which led to the present work.

In 1931, several years before IAA was identified as a natural auxin, Heyn proposed that the hormone acted by increasing the plastic or irreversible extensibility of

the cell wall (Heyn, 1940). This was based on the observations that irreversible extensibility closely paralleled growth rate whereas the reversible (elastic) component did not. Also the irreversible extensibility was greatly increased by hormone treatment. Heyn (1940) proposed, in addition, that extension of cell wall area did not directly depend on the production of more cell wall material and that the energy for surface enlargement of the wall was derived from turgor pressure.

By 1933 Bonner had shown that there was some relationship between auxin action and respiration. Bonner (1936) "concluded that processes of a respiratory nature but of relatively small magnitude form one or more integral steps in the chain of reactions by which the plant growth hormone brings about cell elongation." There was considerable controversy over (a) whether auxin-induced respiration occurred and (b) if it did whether it was the cause or the effect of the increased growth. It is now generally accepted that in etiolated tissue there usually is a small increase in respiration due to auxin but that this is an indirect effect of auxin (Bonner and Bandurski, 1952; Audus and Garrard, 1953). The respiration would supply the metabolic energy required for elongation in etiolated tissues.

Sweeney and Thimann (1938) observed that auxin promoted protoplasmic streaming in Avena coleoptile cells.

It is an effect that appeared within 2 minutes of the addition of auxin and continued for 30 minutes but could be maintained for 2 hours on the addition of sugars. This may be explained in part by the observation of Northern (1942) that by within 30 minutes (at the most) after adding auxin there was a marked decrease in the viscosity of the cytoplasm in cortical cells of young navy bean plants. The work of Galston and Kaur (1959, 1961, 1962) and Galston et al (1963) may be another aspect of this phenomenon. They treated etiolated pea stem sections with auxins and auxin analogs (which did not promote growth). They found that after at least 4 hours in auxins, the heat coagulability of extracted proteins decreased. There was no decrease in total protein and it was the protein of the particle-free cytoplasm that was affected in particular. In vitro experiments showed that pectin could decrease the heat coagulability of diverse proteins.

There was a basic evolution of ideas concerning the site and mechanism of the primary action of auxin on elongation. An early idea was that the site was the cell wall (Heyn , 1940, Robbins and Jackson, 1937). However as data accumulated illustrating diverse effects on the cytoplasm which preceded effects on the cell wall, attention was focused on the cytoplasm as the site of action. Auxin is also involved in other processes as well as elongation, such as cell division. differentiation and the expression of sexuality in plants. It seemed a more

logical assumption that there was one site of action, the expression of that action depending on the status of the cell rather than postulating a different site for each of the many participatory roles of auxin.

The plant cell is usually in a hypotonic environment. The presence of the relatively rigid cell wall prevents the lysis of the plant cell. The cell wall exerts pressure in the cytoplasm (turgor pressure, Ψp) which is counterbalanced by the osmotic potential (Ψs) of the cytoplasm. During cell enlargement, deformation of the cell wall (Heyn, 1940) and water absorption occur. In principle, the deformation could be brought about in either of two ways. First, an increased osmotic potential could increase the tendency for water to enter the cell and therefore increase turgor pressure. Second, a change in the physical properties of the cell wall could permit a greater deformation per unit force to occur. Much work has been devoted to trying to distinguish between these alternatives. No consistent effect has been noted on the effect of auxin on the osmotic potential (Galston and Purves, 1960). It now seems probable that auxin affects the cell wall in such a way that it permits a potential increase in cell volume and therefore of the entry of water (Ketellapper, 1953).

By the beginning of the 1960's (Cleland, 1961) it was accepted that with respect to elongation the final

result of auxin action was an effect on the cell wall but that this was mediated by an effect on the protoplasm. Cleland (1968) has concluded that the irreversible wall extensibility due to auxin-induced elongation consists of a series of independent plastic deformations, rather than viscoelastic flow. Each of these plastic deformation events consists of: (a) an auxin induced increase in extensibility by cleavage of wall polymers followed by (b) a conversion of this extensibility into extension by turgor pressure. Lockhart (1967) would tend to agree. Cleland has evidence that the cleavage of wall polymers may be a reversible reaction. The reaction is driven toward cleavage (and therefore wall loosening) by an enzyme in the presence of a sufficient supply of a respiratory product "X~P". In the absence of X~P the polymer reforms. Auxin controls the amount of enzyme in the wall.

The findings of Ray and Ruesink (1962) would tend to support Cleland's position. They studied the effect of temperature on the length of the latent phase - the time between the addition of auxin and the increase in growth rate. They found that the Q_{10}'s indicated that metabolic reactions were more important in the subsequent growth than an alteration of the viscoelastic properties of the cell wall. The relationship of the viscoelastic behaviour has been found to follow the "W.L.F. equation" in which the temperature coefficient increases rather

rapidly as temperature is lowered (Ray and Ruesink, 1962).

Cleland's X~P may be the same as Morré and Eisenger's (1968) unknown secreted factors (SF). It is envisaged by the latter that these factors act with a "transformase complex" of wall loosening enzymes to produce wall loosening or increased extensibility. Turgor, with the possible involvement of an extensase complex would then cause an increase in elongation. The role of IAA would be that of a cofactor, in the transformase complex or it may effect RNA or protein synthesis. The models of Cleland and Morré and Eisenger seem to be very similar, except for differences in terminology.

Cell wall loosening is a major event in auxin-induced elongation. However several biochemical events appear to take place within the protoplasm before the increase in extensibility can occur. It is recognized that even when events occurring in the protoplasm are elucidated, these must be related to changes in the cell wall in order to gain a full understanding of the mechanism of auxin action. The discussion that is to follow will be concerned only with the primary effects of auxin. It is possible to study the mechanism of auxin action by clarifying its effects on the cell wall or by trying to determine the site and primary mechanism of action of

auxin. One approach uses the end result and tries to determine the steps preceding it. The other tries to find the primary mechanism and attempts to clarify the steps following it. This work is concerned with the mechanism of the initial action of auxin and therefore of events which occur in the protoplasm.

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PART I

The Kinetics of Auxin-Induced Cell Elongation

A. Introduction

In much of the early work on the mechanism of auxin action, the actual growth measurements consisted of incubating tissue for a period of time (usually overnight) and then measuring the final length (or weight). When other parameters were studied, for example the effect of auxin on respiration, these were often done at times that bore no relationship to the final growth measurements. Respirometer studies were usually made shortly after excision of the material or within a few hours. The assumption must have been made that the growth rate and the biochemical and physiological status of the tissues were constant. Audus (1952) pointed out "....that an isolated growing segment of a plant organ is not a system of constant properties or potentialities: it is from the moment of excision to the cessation of its growth a constantly changing system, not only from

the point of view of its structure and its metabolism but also in its reactions to the external environment". In his work on arsenate inhibition of growth he studied the effect on growth rate with time as well as on respiration rate with time. His first measurement was at 1.5 hours and the shape of his growth curves (using etiolated peas) was sigmoid. Bennet-Clark and Kefford (1954) obtained similar types of curves, with Avena coleoptile segments, when optimal concentrations of IAA were used. When the concentrations were above the optimum, the initial growth rate was the same, but the segments grew at this rate for increasingly shorter lengths of time as the concentration was increased. Similar experimental results were obtained by Barlow and Hancock (1959) and Bentley and Housley (1953). These results were quite different from those of Bonner and his group, under whose conditions the growth rate was linear from time 0 to 24 hours. They found that the growth rate increased with increasing concentration of IAA up to 5×10^{-6} M (Foster et al., 1952). Beyond this concentration the growth rate decreased with increasing concentration (Bonner and Foster, 1955).

Specific inhibitors of DNA, RNA or protein synthesis have been used by many people (e.g. Noodén and Thimann, 1963; Key, 1966) in order to determine the site of auxin action. The usual method has been to incubate segments

overnight and if the length of the segments was shorter with auxin plus inhibitor than with auxin alone, then the step blocked by the inhibitor was claimed to be required for auxin-induced growth. When kinetic experiments were performed, it was shown that often the claims were unwarranted. For example Penny and Galston (1966) showed that although after 24 hours actinomycin D had severely depressed the IAA-induced growth, the growth for the first two hours was not affected. It was not until the third hour that the growth decreased. Likewise the use of data from overnight incubations with RNase to support the hypothesis of an in vivo-IAA-RNA binding was unwarranted (Galston et al., 1964); the RNase did not exert its effect until after 8 hours (Penny and Galston, 1966). These authors also found that it took 5 hours before puromycin inhibited auxin-induced growth. Evidence with this antibiotic in an overnight treatment was used to support the thesis of Noodén and Thimann, (1963) that the locale of action of auxin was on the nucleic acid system controlling the synthesis of an essential protein.

From the results of their kinetic experiments with green peas, Penny and Galston (1966) concluded that RNA synthesis was not necessary for the primary action of auxin. It was necessary, however, for continued growth. Nelson et al. (1969) using etiolated sunflower epicotyls confirmed these results and their interpretation.

De Hertogh et al. (1965) found that actinomycin D inhibited auxin-induced growth within one hour. They were using at least 5 times the concentration of actinomycin D (50-100 µg/ml) as that used by Penny and Galston, and Nelson (10 µg/ml) and this may have had effects on respiration. Nelson et al. (1969) also used the protein synthesis inhibitor cycloheximide and found that it caused an immediate cessation of auxin-induced growth. They concluded that auxin acts at the translational level.

The foregoing kinetics can be called long term kinetics since the growth was measured over intervals of one hour or more. This work illustrates the information that can be gained by studying the kinetics of growth. However more information may be obtained by the use of short term kinetic experiments. These are experiments in which the growth is measured every minute or every few minutes at the most.

One of the major questions that can be answered by short term kinetic experiments, is "how long after auxin addition does auxin-induced growth occur?" Clearly the answer to the question is important if experiments are to be planned which try to determine the reactions which cause the increase in elongation.

Yamaki (1954) was the first to publish results of short term kinetic experiments. He showed that in Avena coleoptile segments there was a latent phase of about 15 minutes before an auxin induced increase in elongation occurred. Later, Ray and Ruesink (1962) using more precise methods showed that the length of the latent phase was 13 minutes at 23^{0}C and 28 minutes at 13^{0}C. It was therefore temperature dependant, having a Q_{10} of about 2.

In both these cases the work was done with Avena coleoptiles. There is at least one example of a generalization based on work with Avena coleoptiles which was shown not to hold universally. This was the theory of the mechanism of auxin action based on the IAA-induced methylation of pectin. When studied further (Cleland, 1963) it was found that this response occurred only in some monocotyledons and not in dicotyledons.

In the present work, it was therefore important to determine whether the rapid response to the addition of IAA occurs in tissues other than Avena coleoptile segments. Another reason for doing the short term kinetics of auxin action was to establish the timing of the auxin response. This knowledge would then be used to plan experiments to try to elucidate the mechanism of the response.

B. Materials and Methods

1. Materials

Seeds of Alderman pea (Pisum sativum), a tall variety, and Bitter Blue lupin (Lupinus angustifolius) were surface sterilised by immersion in 1% chlorogen for 20 min. They were then planted in pots in pre-washed coarse exploded mica and placed under continuous light in a growth room with a minimum temperature of 22°C. The light intensity was 14.06 W m^{-2} supplied by a combination of 95% fluorescent (Philips TLA 80W/55) and 5% incandescent light. The pots were irrigated with water daily until one week old and then with nutrient solution daily.

Pea plants 10-14 days old were carefully selected so that only those whose apex was barely showing above the folded stipules of the youngest leaf were used. Under our growing conditions the subapical internodes (5th or 6th) were about 20 mm long at this stage.

Lupin hypocotyl segments were excised from plants four days old. At this stage the leaflets of the first leaf were protruding from between the cotyledons by 7 to 10 mm and the hypocotyl length was 50-55 mm.

2. Methods

The plant material was used in several ways and for

each the method of treatment was slightly different. The methods were as follows:

a. For measuring the growth of intact plants

Three day old plants were carefully removed from the vermiculite in which they germinated. Markers were placed on the hypocotyl with an implement which had 11 fine wires 2 mm apart. A fine coating of Gestetner ink in lanolin had been placed on the wires. The plants were then pinned to foam plastic and the roots replaced in the vermiculite. A centimeter ruler was placed next to the plants. At intervals of several hours, the pot was removed from the growth room and photographed. The total growth was determined by measuring the distance between the markers on the negatives using an Olympus stereo microscope. The magnification was adjusted so that the 100 units of the ocular micrometer was equal to 2 cm.

b. For bioassays

5 mm segments were excised from the apical end of the elongating internode of peas or from lupin hypocotyls. Only one segment was excised from a plant. They were pooled in 0.025 M phosphate or tris-maleate buffer pH

6.1. After 1 - 1.5 hours samples of ten segments were then placed in 4 cm diameter petri dishes containing the same buffer and the compounds to be tested. The segments were incubated in the growth room under the same light and temperature conditions under which they were grown. After 6 hours the total length of the segments was measured with an Olympus stereo microscope at 14X magnification and the increase in length computed.

c. For long term kinetics

The method was similar to that for bioassays except that each sample of segments was measured before being placed in the petri dishes. The segments were measured every hour for at least the first 5 hours.

d. For short term kinetic experiments

A segment of pea (20 mm long) or lupin (23 mm long) was cut (as in Ray and Ruesink, 1962) so that a short flap of epidermal and subepidermal cells extended from the apical end. This was to allow focusing on the end wall of a subepidermal cell which was used as the reference point in the growth measurement. The basal end

was clamped in the holder described below. The rate of response to auxins was determined by measuring elongation of sections every minute under a microscope. This apparatus is illustrated in Fig. 1. Sections were set up in a special chamber constructed similarly to that used by Ray and Ruesink (1962). The flow of solution through this chamber avoided localised depletion of auxins or oxygen in the solution around the section. The lucite (plexiglass, perspex) chamber (A on Fig. 2), 5.7 cm long by 2.6 cm wide by 2.0 cm high internally, was mounted on one side of a plate 5.7 x 4.6 cm. The other side (B) was screwed to the moving stage of an Olympus microscope. The solution flowed into the chamber through the inlet tube (C) and out through a trough (D) which had a cylinder (E) at the far end. The cylinder could be screwed to any desired height thus regulating the depth of solution in the chamber. The tissue was held by the section holder (F) shown enlarged in Fig. 3.

The design of the section holder was the major modification of the apparatus used by Ray and Ruesink. The body of the holder was made of lucite and had a grooved, sliding panel which, when pushed down, held the section in place. The panel was held down by a piece of wire (b on Fig. 3), which acted as a spring. To make doubly sure the section (c) did not move, a pin was placed through the holder and section at (d). Because

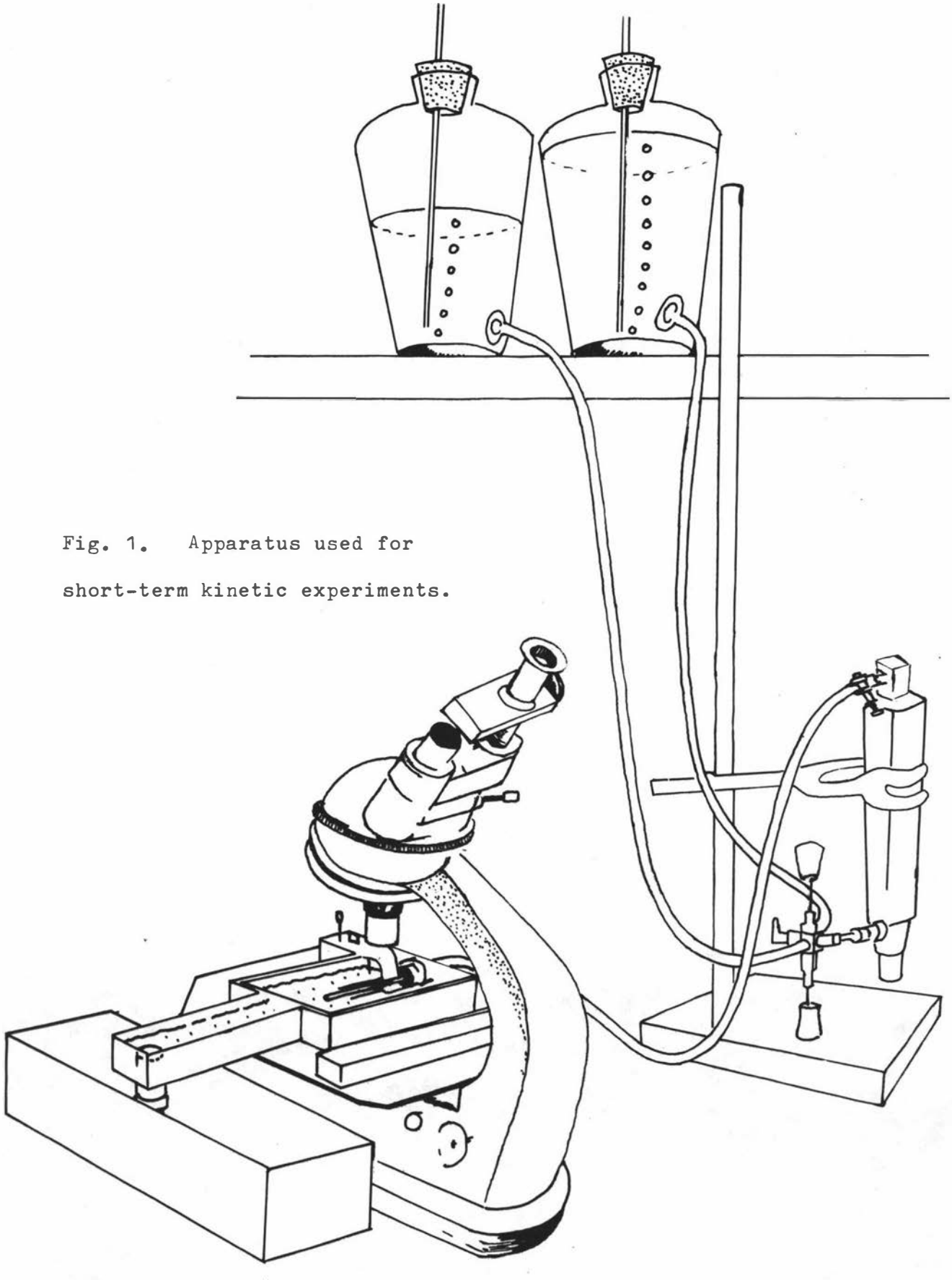


Fig. 1. Apparatus used for short-term kinetic experiments.

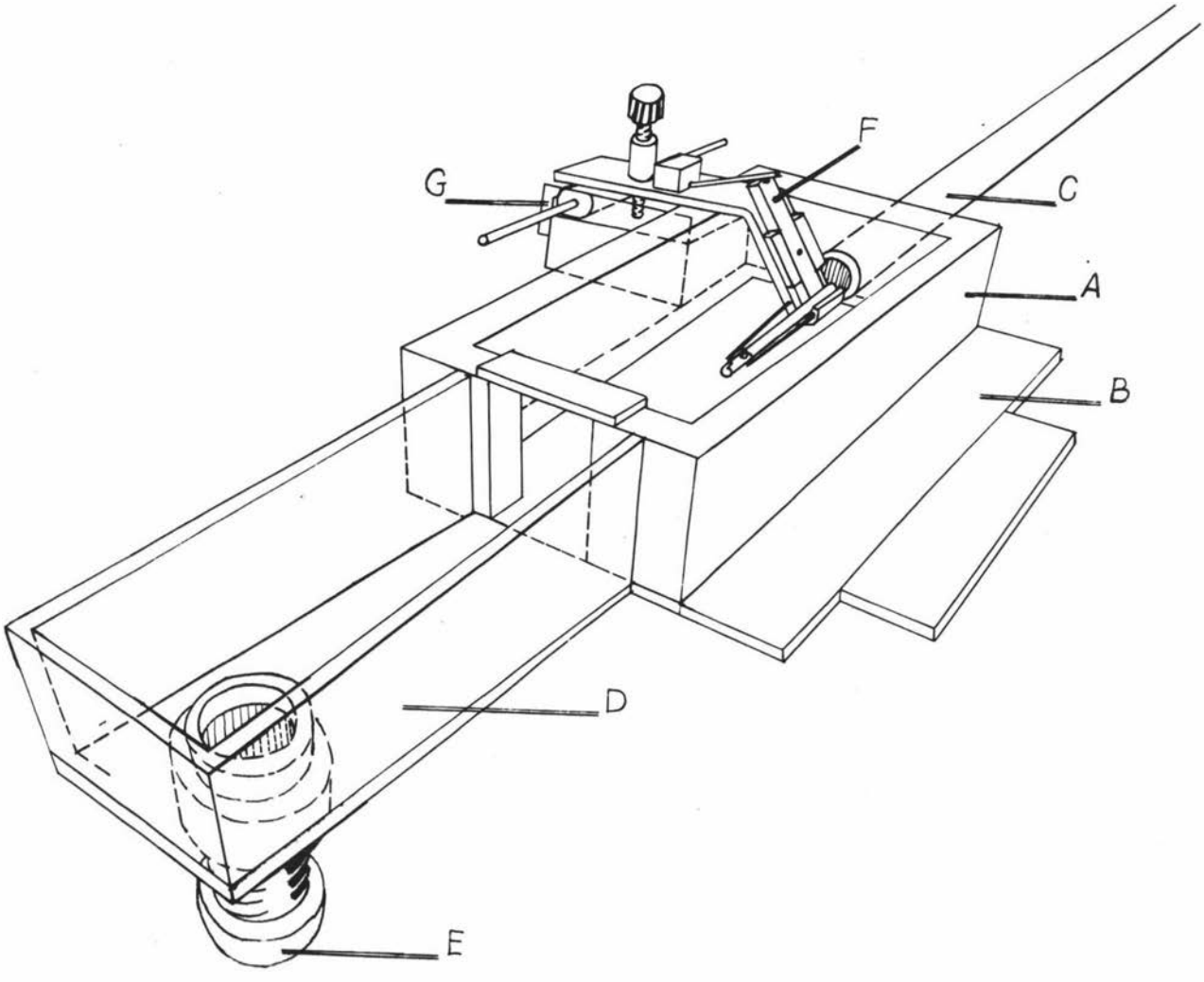


Fig. 2. Chamber used for kinetic experiments.

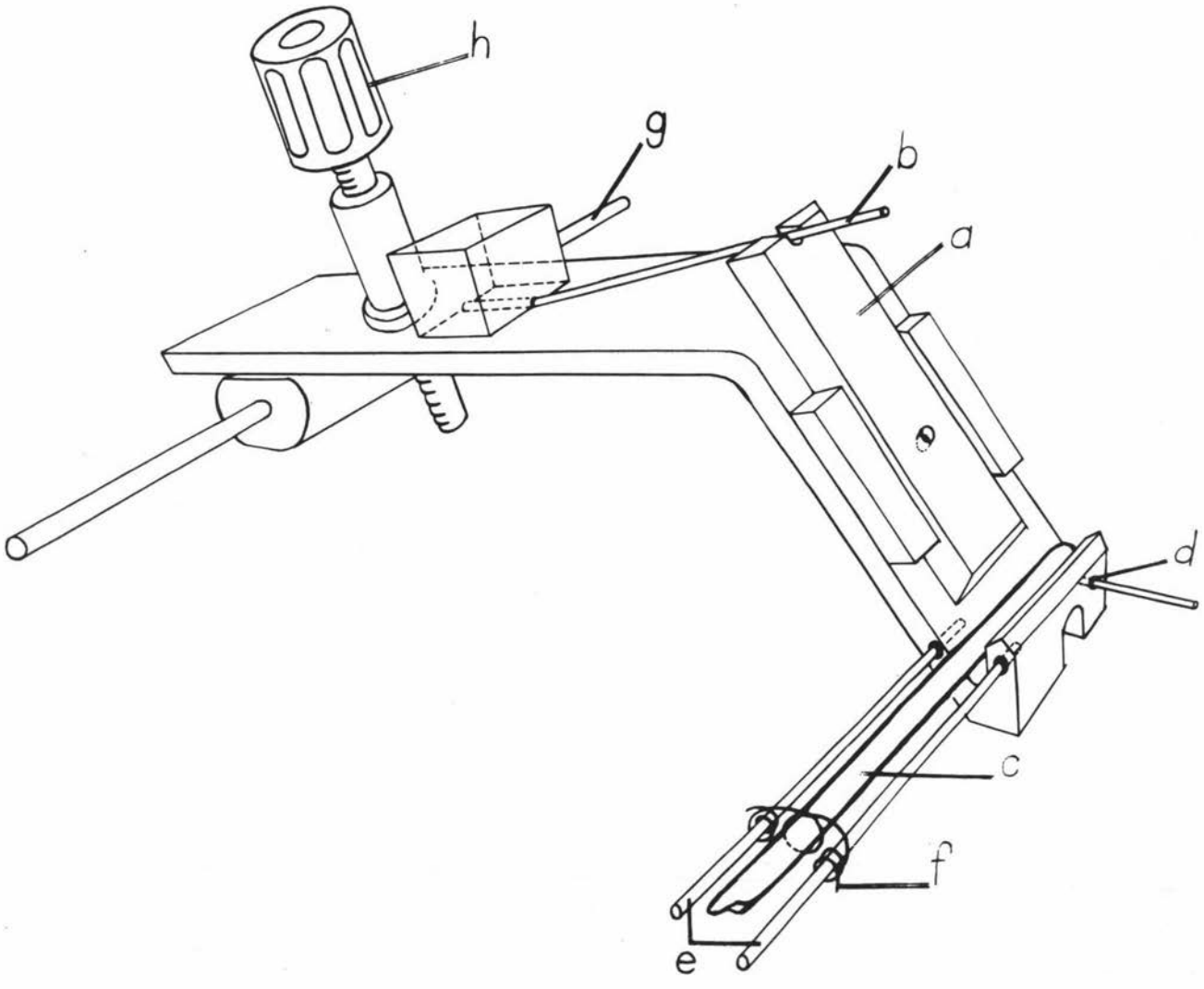


Fig. 3. Detail of segment holder.

it was found that the geotropic response of sections was rather rapid (35-40 min. in lupin under some conditions), two parallel wires were placed with one end in the holder, one either side of the section. Then a fine wire collar (f) was placed around the end of the section. The holder was held in position in the chamber (G on Fig.2) by a metal pin (g). By adjusting the screw (h) the section could be placed at any desired depth in the solution.

An ocular micrometer was found to be sufficiently accurate at 150X magnification to avoid the necessity of submerging the objective. However, a constant fluid level was necessary when the objective was not submerged. This created another problem in the original design in which the flowing buffer outlet was tubing so that siphoning with consequent fluctuation in fluid level occurred. The design of the trough for the outlet solved this problem. Attaching the chamber to the moving stage ensured rapid and precise positioning of the chamber.

In most experiments, two reservoirs were used, one containing 0.025M phosphate buffer, or 0.02M tris-maleate buffer, pH 6.1 and the other buffer plus indolyl-3-acetic acid (IAA) or another auxin. These were connected to a Hamilton four-way valve which allowed rapid and easy changeover from one solution to the other. Between the

valve and the chamber was a Gapmeter flowmeter. After the valve was switched from one solution to the other, the time it took for the new solution to reach the chamber was determined using an aqueous dye solution. It took, for example, 45 sec. at a flow rate of 20 ml/min.

Measurements were made by means of a Watson ocular micrometer (filar or moving micrometer eyepiece) equipped with a slidewire which was manually manipulated by revolving a wheel calibrated into 100 units. With the magnification used (15 X ocular, 10 X objective) each unit was equal to 0.23 μ. The accuracy of measuring a stationary object was ± one unit. The measurements were actually made by choosing a particular point on the tissue as a reference and every minute the slidewire was moved to that reference point. The distance that the wire was moved could then be determined from the reading on the calibrated wheel. Theoretically (Françon, 1961), the setting of the slidewire can be made accurately to a much smaller distance (0.03 μ) than is possible to resolve (2 μ) with the objective used (NA 0.25).

The distance that the wire is moved is determined by the initial point so if there was any error in one reading (slidewire moved too far or not enough), there

would be another error in the next to compensate. Because of this, the growth increment for each minute is presented as the average of the increment for that minute plus that preceding and following it.

All segments were pretreated for about 1.5 to 2 hr in flowing aerated buffer. It was found in preliminary experiments with lupin, examples of which may be seen in Appendix 1, Fig. 1 that the endogenous growth rate had been stabilised by this time. The temperature of the solution varied between 23.5^{O}C and 25.5.^{O}C in different experiments but the variation in each experiment was less than 1^{O}C. In colder weather constant temperature above ambient was obtained by immersing the reservoirs in a constant temperature water bath.

C. Results

1. Long Term Kinetics

a. Growth of intact plants,

Excised hypocotyl segments of 4 day old lupin seedlings were used for most of this work. Some of the characteristics of growth of intact plants at the stage used were determined by photographing plants on which had been placed markers 2 mm apart. Initially the plants were 3 days old. On Fig. 4. is plotted the distance between the markers at various times (usually every 4 hr) for a 24 hr period. The arrow indicates the stage of the plants that were used in all the following work with lupins.

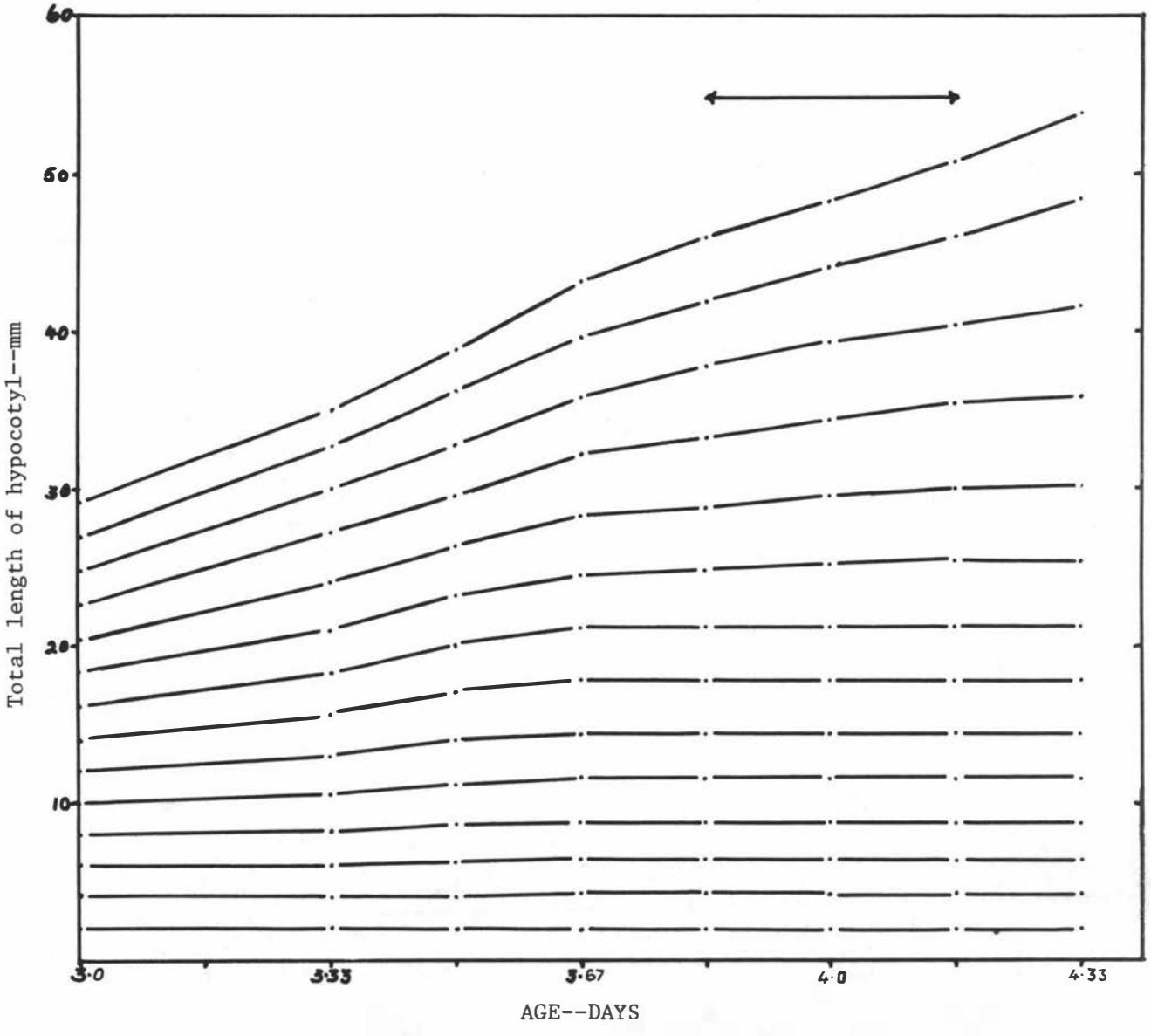


Fig. 4. The growth of the hypocotyl of an intact plant. The lines indicate the position of markers which were initially about 2 mm apart.

The region of most rapid elongation was that portion that initially constituted the upper third of the plant. The bottom portion did not elongate at all, and at this time secondary thickening is taking place (R.G. Thomas, personal communication). The amount of elongation progressively increases from the bottom toward the top of the plant and the length of time during which elongation is taking place also increases toward the top of the plant.

The segments used in the following work were excised from the rapidly elongating region of the plant. At the time at which they were excised the top 11 to 15 mm were elongating at a rate of 2.9% per hour. This is the average of 8 different plants measured during the stages indicated on Fig. 4. Buis (1967) ~~has~~ showed that in etiolated Lupinus albus hypocotyl most of the cell division had occurred by the time the elongation rate had reached a maximum. In the work to be discussed later in which ^{32}P was used to detect RNA synthesis, no plant DNA synthesis was ever detected in the segments. The increase in length of the segments used therefore resulted mainly, if not entirely, from cell expansion.

b. Long term kinetics of excised segments.

When 5 mm segments are cut 2-5 mm below the cotyledons and incubated with or without IAA, the long term kinetics illustrated in Fig. 5 are obtained. Under these conditions the IAA treated segments grow at the rate of about 3% per hour for the first 5 to 10 hrs, a

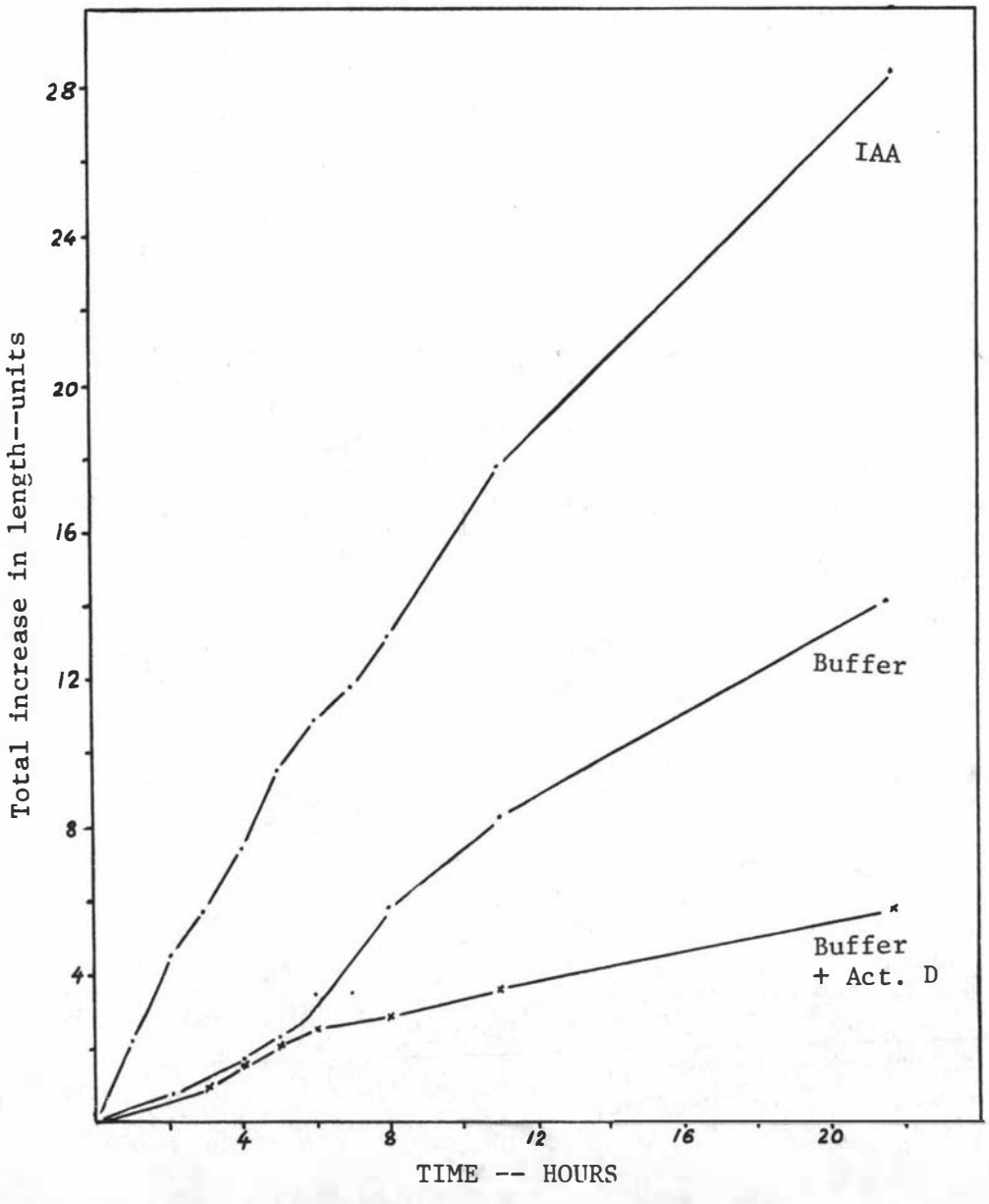


Fig. 5. The long term kinetics of the effect of IAA ($3x10^{-5}$M) or actinomycin D (10 µg/ml) in tris-maleate buffer on the growth of 10 mm lupin hypocotyl segments. 1 unit = 0.23 µm.

rate which is comparable to the growth rate of the same portion of hypocotyl on the intact plant. After 5 to 10 hrs the growth rate falls to an average of about 1% per hour. The effect of actinomycin D is discussed on pages 111 to 114.

2. Short Term Kinetics.

a. Effect of IAA on the growth rate of lupin hypocotyl segments.

i. IAA present continuously

It should be pointed out that all the short-term kinetic results will be presented as a rate of elongation which provides more information than the conventional method of presentation. (i.e. total increase in length see Fig. 35). The effect of IAA on the growth rate of lupin hypocotyl segments is shown in Fig.6. This graph is illustrative of the 11 segments treated with 3×10^{-5}M IAA. The latent phase is defined as the time lapse after the addition of auxin before the growth rate is 3 units above the endogenous rate. The average length of the latent phase for the 11 experiments was 17.5 min. with two segments having a latent phase of 15 min., one of 16 min. and the rest 18 and 19 min. The growth rate rose to a peak at about 32 min. (the range being 28 to 38 min.) and subsequently fell to a lower rate. Using IAA concentrations of 10^{-4}M, 10^{-5}M and 3×10^{-6}M the timing was the same. Further examples of segments treated with different concentrations of IAA may be seen in Appendix Fig. 2.

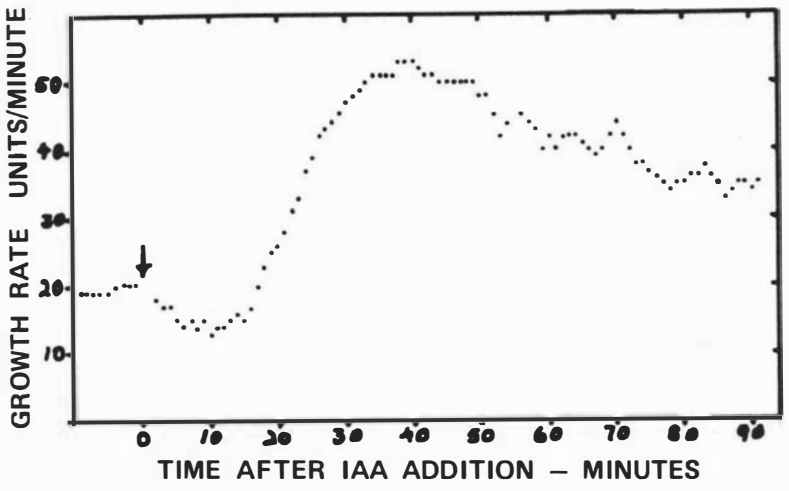


Fig. 6. Effect of IAA ($3x10^{-5}$M) on the growth rate of a lupin hypocotyl segment. Tris-maleate buffer. No supplementary light. 1 unit = 0.23 μm.

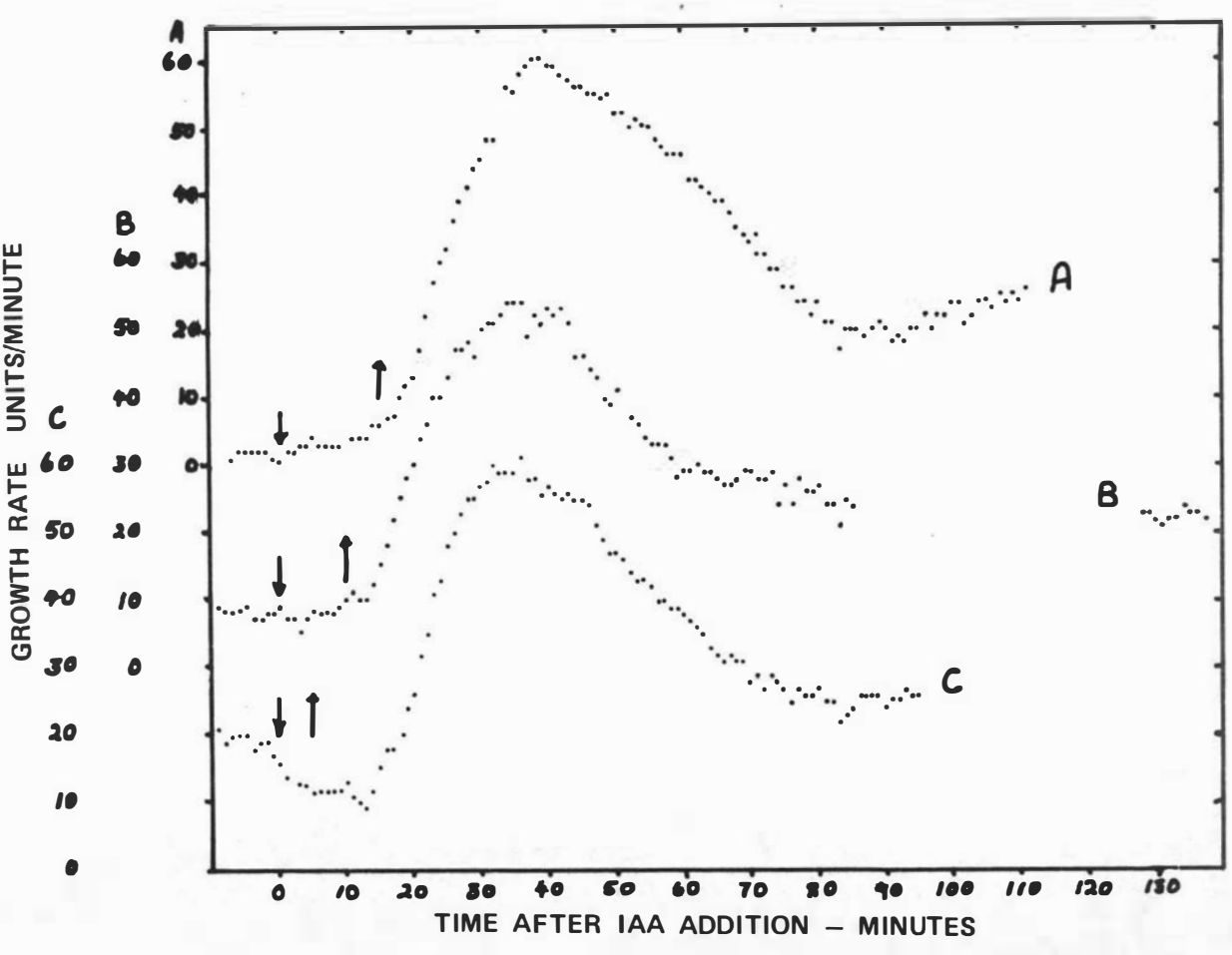


Fig. 7. Effect of short term IAA treatment on the growth rate of lupin hypocotyl segments. $3x10^{-5}$M IAA in tris-maleate buffer. IAA removed after a) 15, b) 10, c) 5 min. First arrow indicates time of addition of IAA. Second, inverted arrow indicates time of removal of IAA. 1 unit = 0.23 μm. No supplementary light.

ii. Short term IAA treatments

Lupin hypocotyl segments were pretreated for 1.5 to 2 hrs in tris-maleate buffer and then exposed to IAA for varying lengths of time, followed by buffer. When the change to IAA was made, the flow rate was increased to about 50 ml/min., 30 sec. before the indicated time, and this flow rate maintained for 45 sec. This was done to obtain an IAA concentration within the chamber approaching that of the reservoir in as short a time as possible. This procedure was repeated when the change to buffer was made.

Fig. 7 and Appendix Fig. 3 illustrate the response in growth rate for 5, 10 or 15 min. exposures to IAA. It can be seen that even though the IAA was removed before an increase in growth rate was expected, the increase nevertheless did occur. There did not seem to be any appreciable effect either on the length of the lag phase, the time at which maximum growth rate occurred or the maximum growth rate. The growth rate curves of the sections given short term exposures to IAA differ mainly from those given IAA continuously in the gradual decline of the growth to about endogenous level. The final growth rate was about the same for all the sections. In the ones shown here it was 20 to 25 units/min., while in two other experiments (of a total of eight) it was 15 to 20 units/min. The endogenous growth rate, immediately after pre-treatment, of segment a. was abnormally low, but the section appeared to have grown as much as usual during pre-treatment. This is probably a manifestation

of the variation in endogenous growth rate. Under the conditions used here the variation never exceeded 10 units in 20 min. and was usually much less. The growth rate of section b. before IAA was added is indicative of the variation, when it occurred.

These results indicate that the time, required for these tissues to reach maximum growth rate, which is longer than for Avena (10-20 min. Ray and Ruesink, 1962; Evans and Ray, 1969), is not due to penetration difficulties. It seems therefore that this is characteristic of lupin hypocotyl and that the timing involved is different for different tissues.

These results show that once IAA has been added to the tissue, even only for five min., it cannot be prevented from eliciting its effect by extreme dilution. Therefore, if the primary action of auxin is a binding to a macromolecule, it is a firm binding. If auxin acts by setting in motion a chain of biochemical events, then this chain is irreversible.

iii. Effect of light on IAA-induced growth rate.

Bioassays with lupin hypocotyl tissue are incubated under the lights in the growth room. In the kinetic experiments the section, apart from the flap, is exposed to a light intensity somewhat below normal laboratory illumination at bench top level. In the following

experiments, a desk lamp with a 40W bulb (tungsten) was placed 6 in. from the section. The lamp increased the light intensity within the chamber from approximately 0.5 w/m^2 to 3 w/m^2.

Using 3×10^{-4}M IAA in tris-maleate buffer, four replicates were carried out under these conditions and one of these is illustrated in Fig. 8 and Appendix Fig.4. The latent period before the IAA-induced increase in growth rate was 17 to 19 min. The growth rate reached a peak in 32 to 36 min. and a minimum in 45 to 54 min. before rising to a second maximum in 69 to 77 min. After up to 20 min. at the second maximum growth rate gradually fell, presumably to that which is detected in bioassays using 5 mm sections (c. 0.2 mm/hr). In these experiments the latent phase has not been altered but there are now two maxima and the overall growth rate is much higher than in those sections not treated with supplementary light (see Fig. 5).

These results confirm our experience with the bioassays. When the dishes were left in the growth room, but not directly under the lights, the IAA-induced growth was small. However, when they were placed under the lights the response was large and the rate maintained for about 20 hr. The cells of hypocotyl segments contain chloroplasts and it is thought that the supplementary light may affect the growth rate by increasing photo-

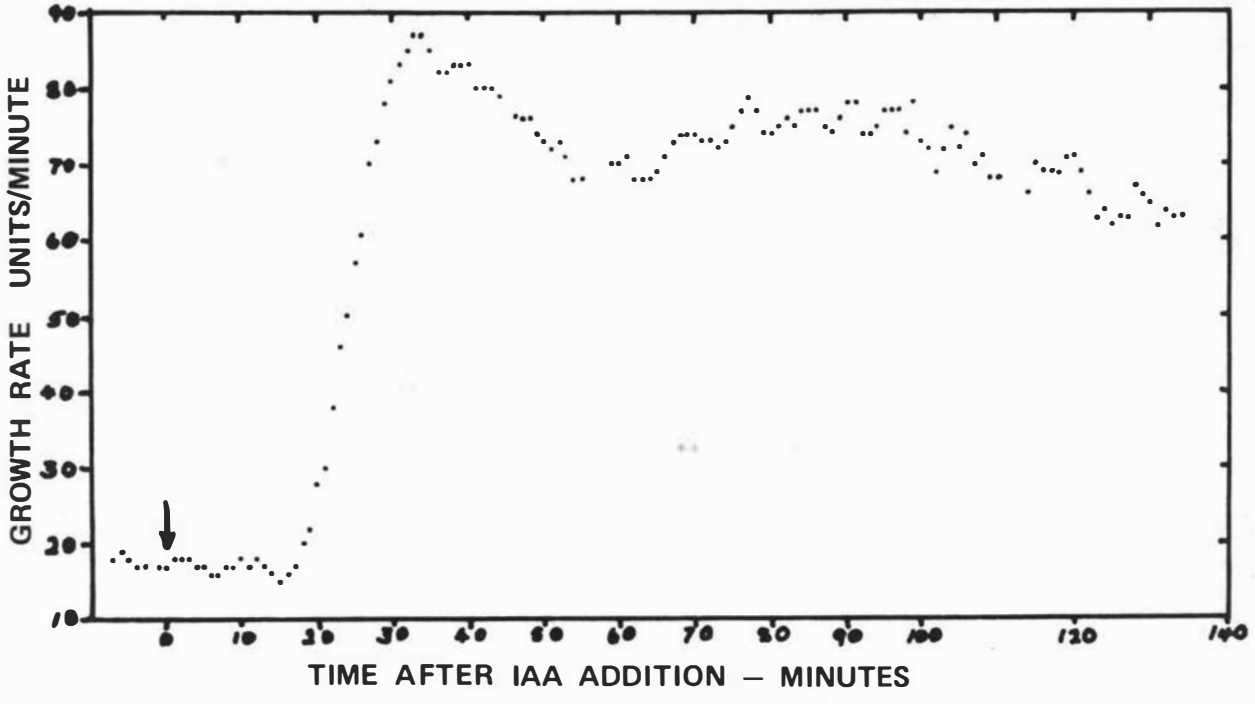


Fig. 8. Effect of IAA ($3x10^{-5}$M in tris-maleate buffer) on the growth rate of lupin hypocotyl segments exposed to supplementary light. 1 unit = 0.23 μm.

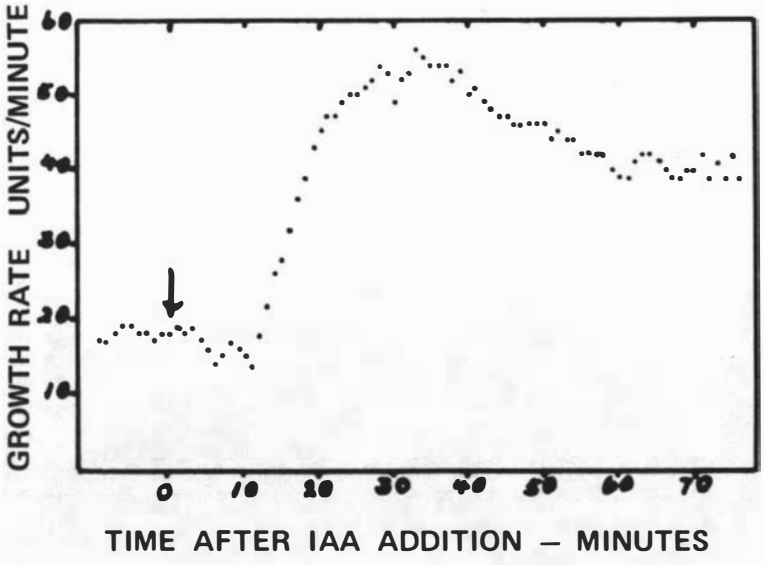


Fig. 9. Effect of $5x10^{-5}$M IAA in phosphate buffer, on the growth rate of a lupin hypocotyl segment. 1 unit = 0.23 μm.

synthesis and thereby available respiratory substrate. In all subsequent short term kinetic experiments (with the exception of those described in the next section on the effect of phosphate buffer), supplementary light was used, unless it is otherwise stated.

iv. Effect of phosphate buffer.

The effect of IAA on the growth rate of lupin hypocotyl segments in phosphate buffer, pH 6.1, 0.02M, is shown in Fig. 9, a plot of a single segment which is typical of the nine replicates using this buffer (other examples are illustrated in Appendix Fig. 5). It can be seen that the main difference of segments treated in tris-maleate buffer from those treated in phosphate buffer is that the latent period is shorter than in tris-maleate buffer. In all cases, the growth rate increased 13-15 min. after the application, rising to a maximum at 28-32 min. and then starting to decrease at 35-40 min. The final growth rate was always somewhat lower than the maximum. These kinetics occurred in all IAA concentrations used (10^{-5}, 3×10^{-5}M, and 10^{-4}M). The decrease in the growth rate immediately after the addition of IAA exhibited by the particular section shown was not usual and generally a growth rate consistent with the basal growth rate occurred.

v. Experiments without a buffer solution.

In the experiments already described a buffer was

always included. It was considered possible that some molecules or ions in the external medium were essential for the segments to respond to auxin, e.g. the segments after addition of auxin could accumulate mineral ions, increase the osmotic potential of the cell and hence increase the pressure exerted against the wall. In several experiments the segments were pre-treated in distilled water and then treated with IAA dissolved in distilled water. The distilled water had a pH of about 6 and so was similar to that of the buffer.

A typical result with a segment in distilled water is shown in Fig. 10. Further results are shown in Appendix Fig. 6. There is no doubt that in this tissue a full response to auxin occurs in the absence of an exogenous supply of nutrients, and it was to decide this point that the experiment was undertaken (there were a total of 5 replicates). However another minor problem arose in that the results with distilled water gave a maximum growth rate 20-30 units a minute higher than when buffer was used. The probable explanation is that the slightly higher osmotic concentration of the buffer results in a slightly lower value of the turgor pressure and consequently a slightly reduced growth rate. The buffer is a 0.02M tris-maleate buffer which gives a freezing point depression of $0.08^{o}C$ which could correspond to an osmotic concentration of .043 osmolar. This could result in a reduction on the growth rate in the order

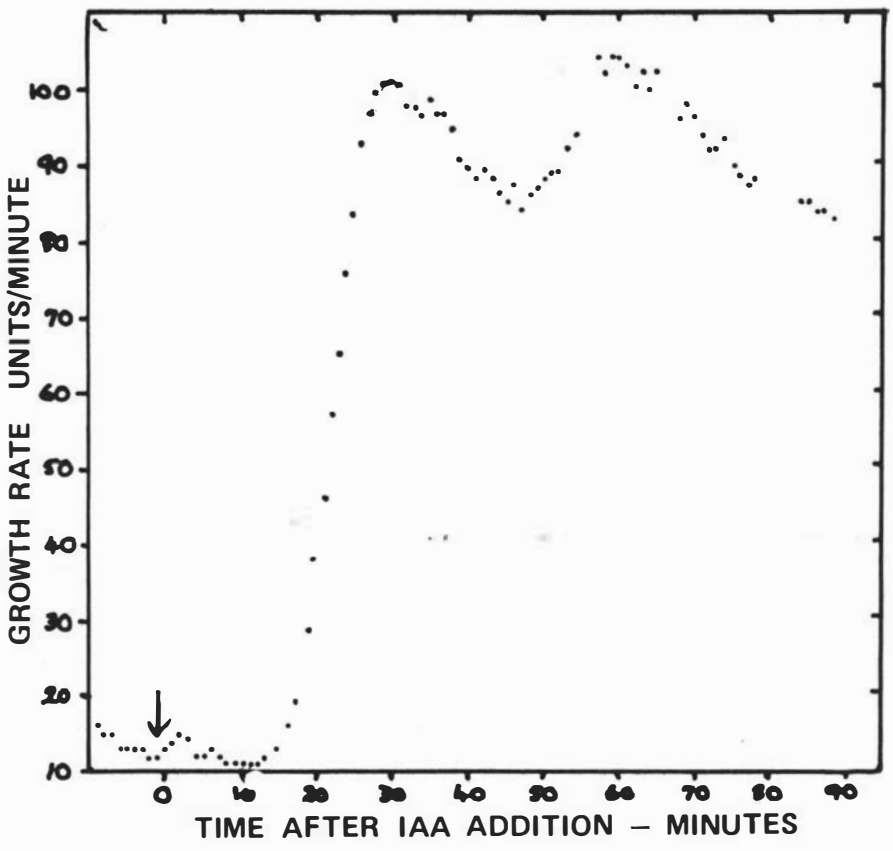


Fig. 10. Effect of $3x10^{-5}$M IAA in distilled water, on the growth rate of a lupin hypocotyl segment. With supplementary light. 1 unit = 0.23 μm.

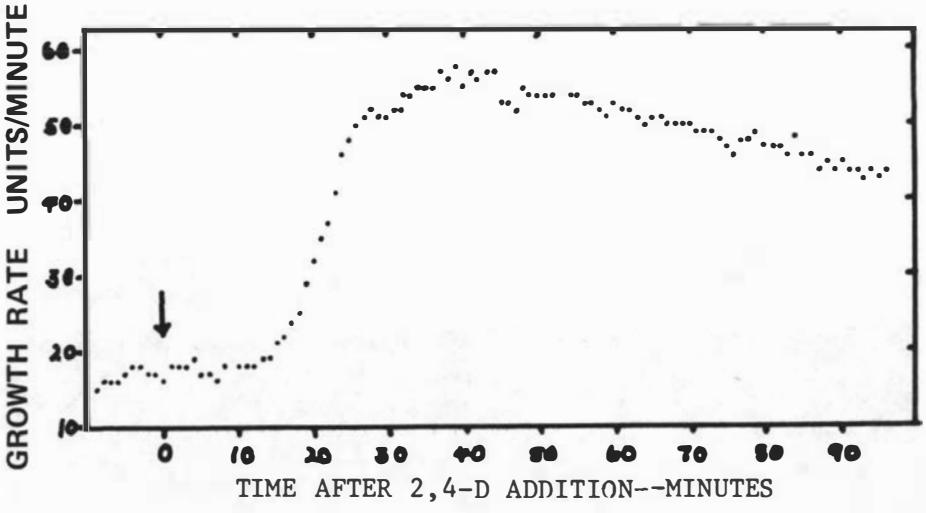


Fig. 11. Effect of 10^{-4}M 2,4-D in phosphate buffer, on the growth rate of a lupin hypocotyl segment. No supplementary light. 1 unit = 0.23 μm.

of 20 - 25% for Avena coleoptiles (Cleland, 1959). Nevertheless, buffer has been used in the majority of experiments because frequently other chemicals, e.g. ^{32}P phosphate or inhibitors are added. However there is no doubt that no exogenous chemicals are needed to get a full response to auxin and this would eliminate theories that required an active uptake from the medium.

b. Effect of other auxins on the growth rate of lupin hypocotyl segments.

It was established that in tris-maleate buffer lupin hypocotyl segments have a latent period of about 18 min. before an IAA-induced increase in growth rate occurs. At this point it was necessary to determine whether this latent period was a general auxin response. Also, compounds postulated to be metabolised to auxin may be expected to have a longer latent period. This could be offered as evidence of the conversion of that compound to a growth active compound.

i. 2,4-dichlorophenoxyacetic acid.

The kinetics of response to 2,4-D are illustrated in Fig. 11 and Appendix Fig. 7. A total of 4 treatments were carried out in phosphate buffer and without supplementary light. This should be compared with the IAA treated segment in Fig. 9. The same general characteristics as with IAA may be seen --- the latent

period is the same length and the final growth rate is lower than the maximum.

ii. Indoleacetamide.

The response to indoleacetamide was rather striking as may be seen in Fig. 12 and Appendix Fig. 8b. A dosage response curve is shown in Appendix Fig. 8a. All 5 replicates were performed with tris-maleate buffer and supplementary light. With indoleacetamide, the timing of the latent period, first maximum, minimum, and second maximum is the same as with IAA. However, the minimum is very marked. This increases the probability that the minimum manifested with IAA treatments, while statistically significant (see Table I) is also biologically significant.

iii. Indolebutyric acid.

In Fig. 13 and Appendix Fig. 9 are plotted the response of lupin hypocotyl segments to 3×10^{-5}M indolebutyric acid. The latent period with this compound is considerably longer than with any of the other compounds tested. It ranged from 22 to 34 min. If allowance is made for the increased length of the latent phase, then the other characteristics of the growth rate curve are similar to those with IAA -- that is the timing of the first maximum, minimum and second maximum. Because the response of the tissue, except for the latent period, is similar to IAA, this compound may be converted to IAA

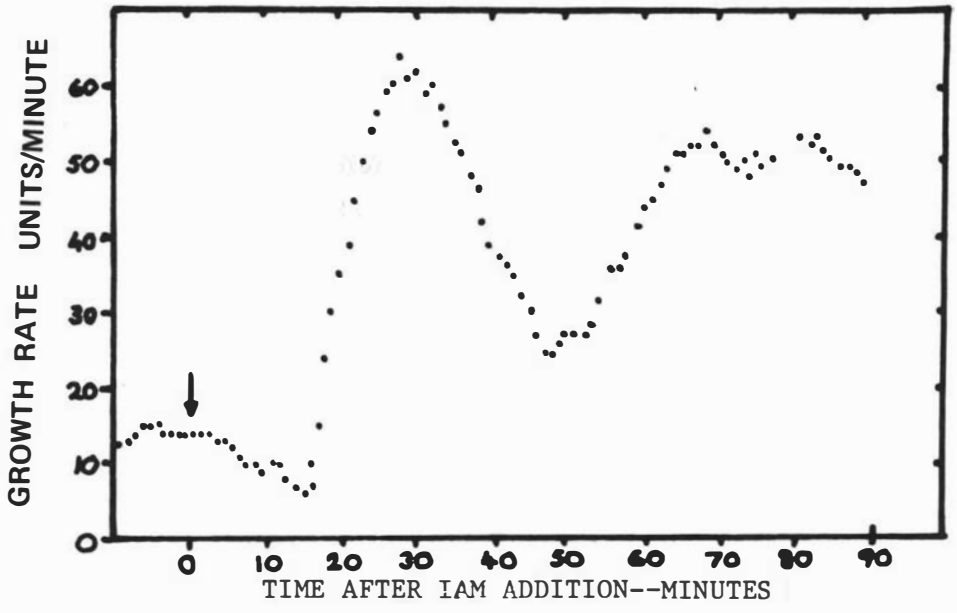


Fig. 12. Effect of 10^{-4}M indoleacetamide in tris-maleate buffer on the growth rate of a lupin hypocotyl segment. With supplementary light. 1 unit = 0.23 μm.

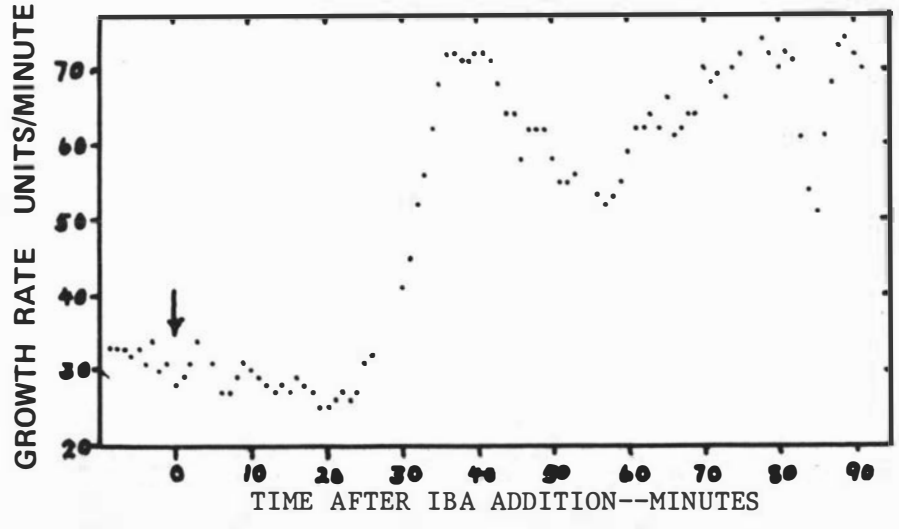


Fig. 13. Effect of $3x10^{-5}$M indolebutyric acid in tris-maleate buffer on the growth rate of a lupin hypocotyl segment. With supplementary light. 1 unit = 0.23 μm.

TABLE 1 Response of Lupin hypocotyl segments to 3 x 10^{-5}M IAA. The values are the duration of (minute) and average rate (units/minute) during the latent phase, the time that the segments first reach either the first maximum, the mihimum or the 2nd maximum rate.

	latent period		1st peak		minimum		2nd maximum	
Expt No.	duration	average rate	time	maximum rate	time	minimum rate	time	maximum rate
125	19	17	35	90	49	73	69	83
129	19	18	36	60	56	53	69	63
130	17	15	38	79	53	65	66	70
133	18	17	33	87	54	68	77	79
≠ 140	16	13	29	101	52	84	64	104
* 143	16	20	39	64	63	55	76	66
191	17	8	34	58	59	46	77	58
≠ 204	15	25	29	105	40	90	57	111
≠ 205	14	23	29	112	42	92	63	117

≠ segments in distilled water plus IAA

* segments in 10 mM phosphate citrate buffer pH 4.8

via a B oxidation mechanism before it can exert an effect.

iv. Indoleacetaldehyde, naphthalacetamide, naphthoxyacetic acid, ethyl-indol-3-yl- acetate.

With the exception of naphthoxyacetic acid, these compounds are thought to be converted to IAA or to the auxin naphthalene acetic acid before they can be active as auxins. It could be expected then that like indolebutyric acid, they may have a longer latent period before an increase in growth rate occurs.

Results of experiments with these compounds are illustrated in Fig. 14. In no case is the latent period longer than that of IAA. While the minimum is less pronounced than with IAA, it is apparent with all the compounds except indoleacetaldehyde. However with indoleacetaldehyde the final growth rate measured is lower than the maximum. There is no evidence that the growth rate curve is very different to that of IAA.

c. Effect of auxins on the growth rate of pea stem segments.

i. IAA.

Plotted in Fig. 15 and Appendix Fig. 10 are the effect of IAA (3×10^{-5}M) on 10 day old pea stem segments. It can be seen that the latent period occurs with this tissue as well as with lupin. However it is about 5 min

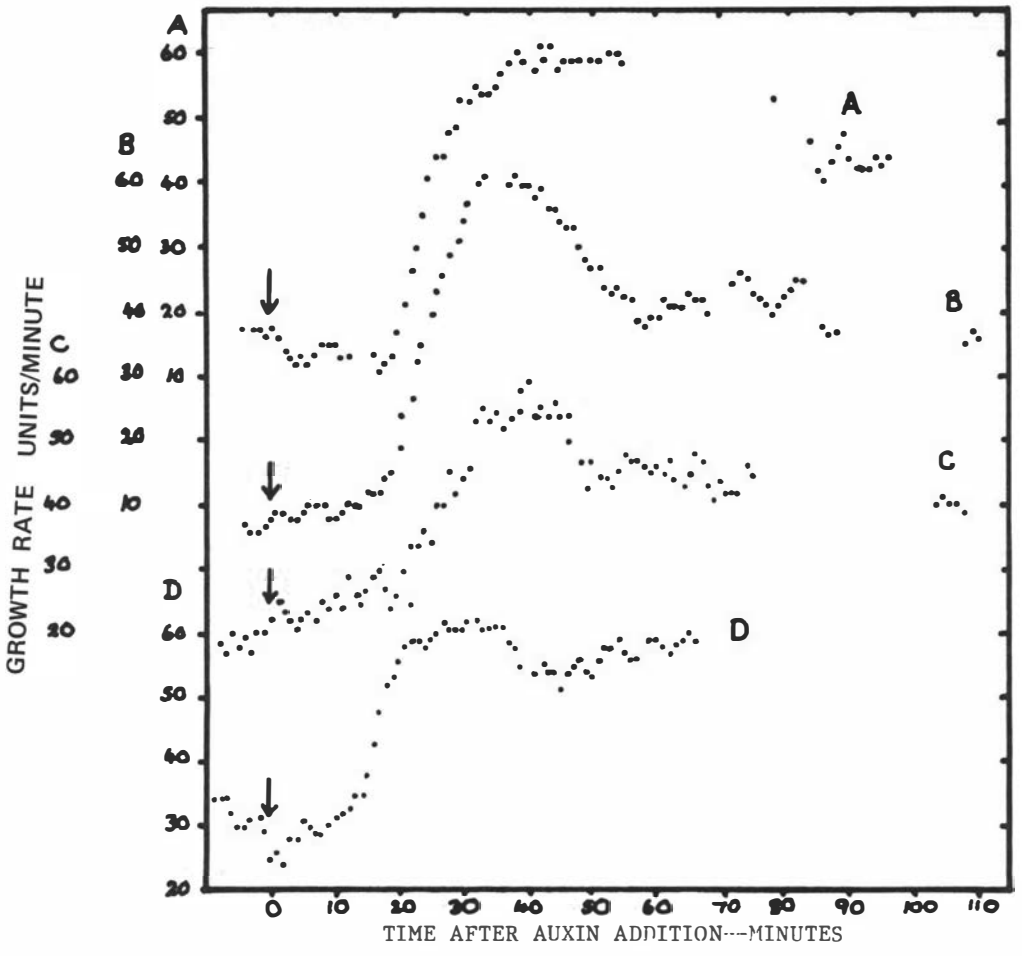


Fig. 14. Effect of different auxins in tris-maleate buffer on the growth rate of lupin hypocotyl segments. With supplementary light. 1 unit = 0.23 μm.

A. $2x10^{-4}$M indoleacetaldehyde
B. $3x10^{-5}$M naphthylacetamide
C. $3x10^{-5}$M naphthoxyacetic acid
D. $3x10^{-5}$M ethyl-indol-3-yl acetate.

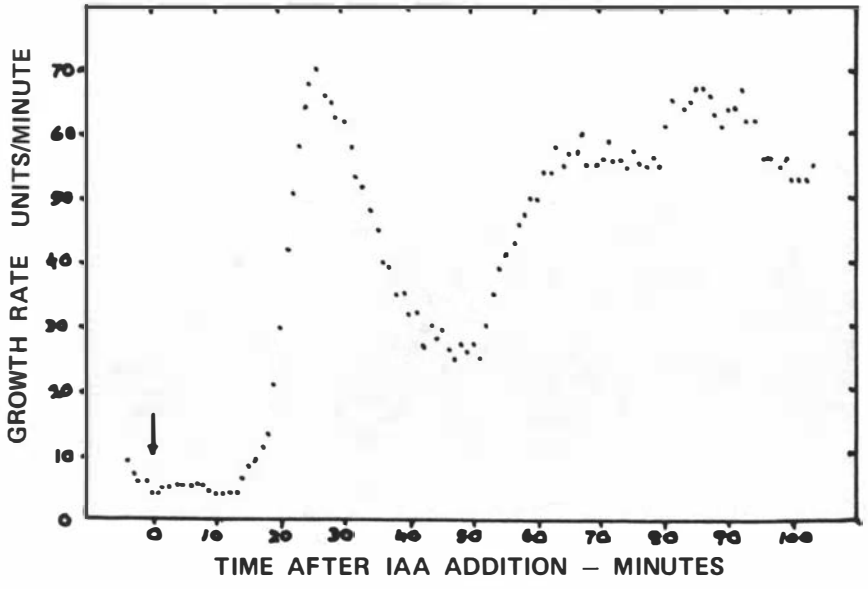


Fig. 15. Effect of $3x10^{-5}$M IAA in tris-maleate buffer on the growth rate of pea stem segments. With supplementary light. 1 unit = 0.23 μm.

shorter, being 12-14 min. Two maxima occur as they do with lupins. The first maximum occurs about 5 min sooner than it does with lupins, perhaps because the latent period is shorter. The main aspect in which the two tissues differ is the more pronounced minimum with peas.

In Appendix Fig. 11 are plotted the results from some early experiments. These were done with phosphate buffer, without supplementary light. The experiments were started 20-90 min. after cutting. The results are an average of 8 individual experiments for 10^{-4}M IAA and 6 experiments for each of 10^{-5}M and no IAA. This procedure was adopted because it was found that the endogenous growth rate was extremely variable. The results do show that the latent period was slightly shorter with phosphate buffer than with tris-maleate buffer. This was also the case with lupins.

ii. 2,4-dichlorophenoxyacetic acid and indoleacetamide.

The results for 2,4-D (10^{-4}M) are plotted in Fig.16 and Appendix Fig. 12 and those for indoleacetamide in Fig. 17 and Appendix Fig. 13. Both these compounds produce effects similar to those of IAA, except that the minimum is not as apparent.

D. Résumé-- The rate of response of segments to auxins.

This work has extended the findings of Yamaki (1954) and Ray and Ruesink (1962) to show that the latent period

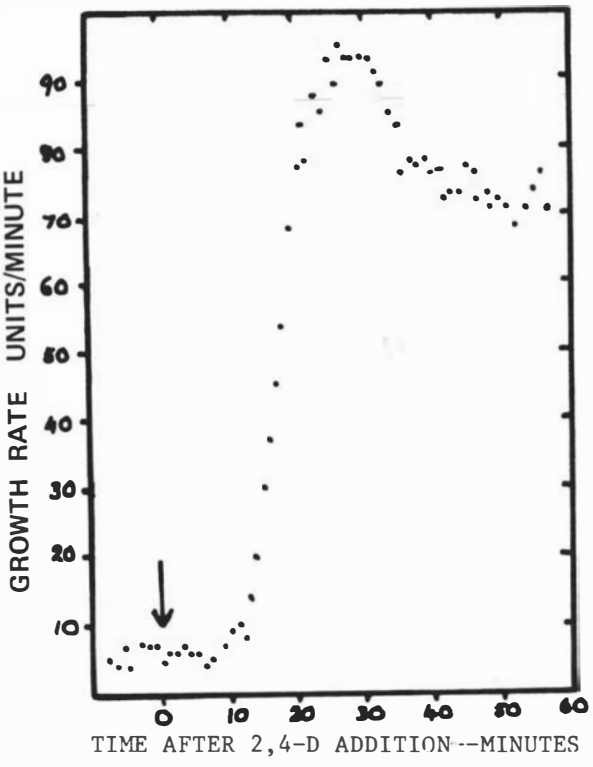


Fig. 16 Effect of 10^{-4}M 2,4-D in phosphate buffer on the growth rate of pea stem segments. Without supplementary light. 1 unit = 0.23 μm.

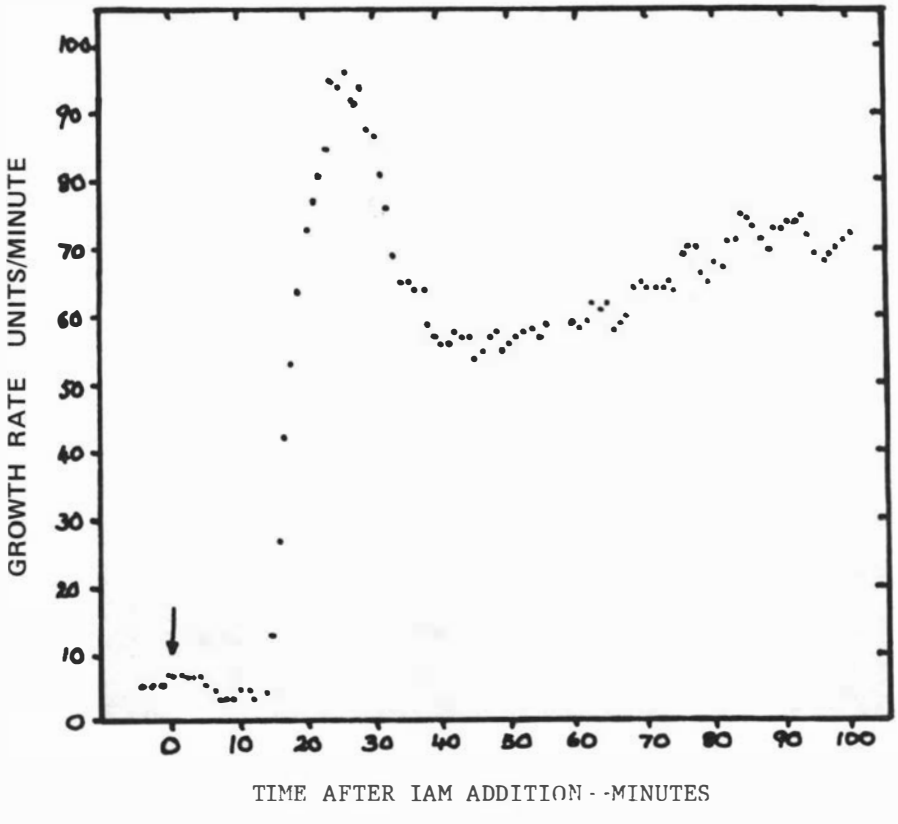


Fig. 17. Effect of $3x10^{-5}$M indoleacetamide in tris-maleate buffer on the growth rate of pea stem segments. With supplementary light. 1 unit = 0.23 μm.

before auxin induced increase in growth rate occurs in hypocotyls and epicotyls. It also occurs in dicotyledonous plants as well as monocotyledonous plants.

Because the technique is more sensitive than that previously used, it was possible to measure the growth rate rather than total increase in length. As a result of the increased sensitivity, it was possible to detect two maxima separated by a minimum which is more apparent with peas treated with IAA than with lupins. However, the minimum in lupins is statistically significant (p 0.001) when a t-test is used with paired samples. Table I gives the results of the 9 most recent experiments with lupin hypocotyls. It can be seen that in each of the experiments, the growth rate falls to a minimum which is 83% of the first maximum.

There are at least three possible explanations of the growth rate curve.

(i) IAA may be affecting two processes which are separated in time. The growth rate curve would then be the resultant of the two processes. Shown in Fig. 18 as a dashed line, the first would be transient and its effect on growth would occur at the end of the latent phase. It would quickly reach a peak and then decline rapidly. This would account for the first peak. The second process would start affecting growth at about

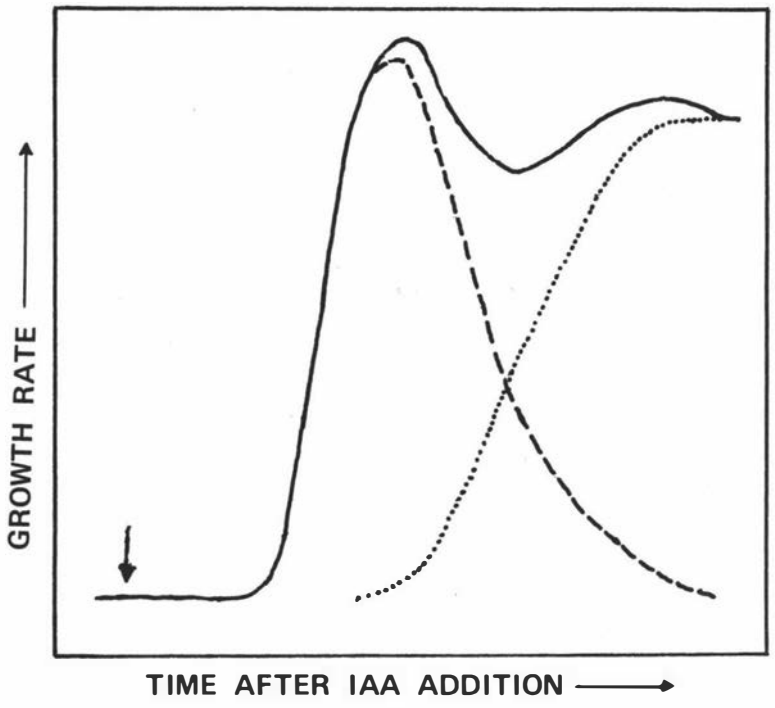


Fig. 18. A hypothetical explanation of the growth rate curve involving two separate processes.

35 to 40 min. after adding auxin and its effect would increase more slowly than that of the first but it would be more long lasting.

(ii) The second hypothesis to account for the growth rate curve is that negative feedback with underdamping is involved. Auxin would act on an existing pool of macromolecules and the first increase in growth rate leading to the first peak would occur. But this would lower the concentration of the macromolecules resulting in an increase in their synthesis. By the time the growth rate has reached the first maximum, the rate of utilization is still greater than the rate of synthesis therefore the growth rate decreases. This would account for the minimum. The growth rate would reach the second maximum as the rate of synthesis of the macromolecule continued to increase to a new steady state level.

(iii) The third hypothesis is most easily visualized by an analogy with the work of Ray (1962). Short term kinetics were used to determine the effect of mannitol on the endogenous growth of Avena coleoptile segments. As seen in Fig. 19a (Ray's data) when mannitol is added the segment shrinks, the growth rate is negative. When after some time the mannitol is replaced by water, the growth quickly resumes. The growth rate (Fig. 19b, Ray's data replotted as a rate)

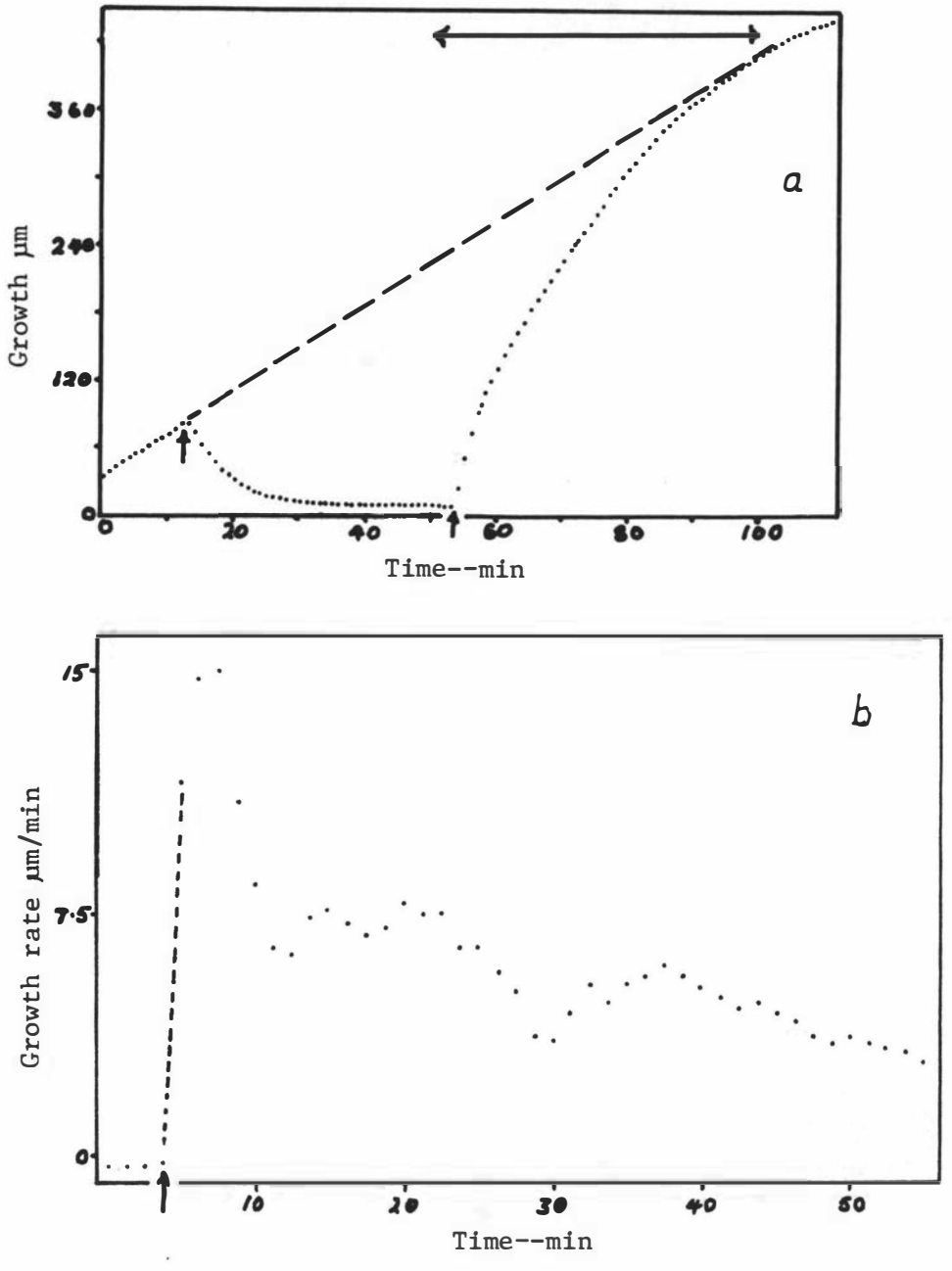


Fig. 19. a) Effect of transfer from water to 0.25M mannitol (first arrow) and back to water (second arrow). Coleoptile 6 mm long. Broken line is an extrapolation of the original growth rate, which may be compared with the actual expansion which occurred after return to water, shown by the experimental points. Data of Ray (1961). b) Points under arrow in a) replotted as growth rate/min.

increases rapidly and reaches a maximum much greater than the original rate. The rate then declines to the previous level. The net result is that the total growth of the segment is the same as it would have been if the segment had not been in mannitol. Ray's explanation was that during the time in mannitol, the protoplasmic processes leading to growth continued. The growth requiring products were "stored" until the turgor pressure was restored. The burst of growth occurring after the mannitol was removed has been called "stored growth". Fig. 19b is the portion of Ray's curve under the arrow. It is plotted with an expanded time scale.

The first peak of the growth rate curve of the response to auxin could be analogous to the increase in growth rate after mannitol removal and/or to the release of stored growth. The rest of the curve would represent an increase in growth rate owing to the metabolic processes affected by auxin.

The first and third hypothesis seem quite similar. However they are separated because the processes leading to the first peak are postulated to be different. In the first hypothesis they are independant of the second. In the third an activation of the processes leading to the second maximum is required for the expression of the first.

These hypotheses have been outlined at this point because they were important in deciding the timing of the biochemical experiments. The latest times (after adding auxin) by which events must have occurred, if it is to be said that they caused the growth are different for the different hypotheses and are as follows:

Hypothesis	Time(s)-min.
1	20 and 40
2	20
3	20 increasing to 60

In theory at least, it should be possible to distinguish between these hypotheses because they all have different timing requirement.

+ + + +

PART II

The effect of Auxin on Macromolecules

A. Introduction

Very low concentrations of auxin induce very large effects on growth. It has been postulated that the mechanism of the primary action of auxin must include an amplification step (e.g. Kefford et al., 1963; Key and Ingle, 1968). If as the result of the action of one molecule of IAA 10 molecules of a substance required for growth are produced then the effect of IAA can be considered amplified. For example one molecule of IAA could stimulate the production of one molecule of m-RNA for a growth active protein. Since each m-RNA molecule maybe "read" many times, then the effect of the IAA would be amplified.

The apparent need for an amplification step is one reason that an action somewhere in the sequence DNA —

RNA — protein synthesis has seemed particularly attractive. It has been suggested that in yeast auxin interacts with RNA near the cell surface to increase permeability to pyronin B (Masuda, 1959). This was originally used as evidence to support the idea that the DNA to protein synthesis sequence is important.

However the fact that this particular sequence has been the centre of attention in biological research for the last 15 years is probably primarily responsible. For instance, the work of Siegel and Galston (1953) showing an interaction between ~~2, 4-D~~ 1AA and a TCA insoluble component of the non particulate portion of the cytoplasm of pea root tips was interpreted at the time as demonstrating an interaction with protein. Later as understanding of the role of RNA increased these results were interpreted as demonstrating an auxin-RNA binding (Galston et al., 1964). For the need of a means of amplification to be satisfied there is no inherent reason for choosing these macromolecules over others, for example polysaccharides, except that little is known about them, the range of techniques is not available and they are not "fashionable".

The current theories of the primary action being within the cytoplasm can be said to postulate onepart or another of the DNA to protein synthesis sequence as being near the target point. The work of Thimann and Beth (1959) showed that in <u>Acetabularia</u> auxin could stimulate

stalk growth and cap differentiation whether or not the plants were enucleated. This demonstrated that the nucleus was not required for auxin action. However, one of the current theories in essence postulates the site of action within the nucleus.

The theory is that ~~proposing that~~ m-RNA synthesis is necessary for an auxin-induced response to occur. (Key et al., 1967). This is based mainly on the effects of various nucleic acid and protein synthesis inhibitors on auxin induced growth. Key and Shannon (1964) showed that after a lag, 2,4-D and IAA increased net RNA synthesis in etiolated soybean hypocotyl. It appeared that the RNA synthesis occurred in the nucleus and then the RNA was transferred to the cytoplasm. Actinomycin D inhibits both growth (after a lag of about 2 hours, Penny and Galston, 1966) and RNA synthesis (with essentially no lag) while 5-fluoruracil inhibits RNA synthesis but not growth (Key and Ingle, 1964; Key, 1964). It was found that 5-fluorouracil inhibits mainly r-RNA and s-RNA synthesis but has little effect on the synthesis of a DNA-like RNA fraction whereas Actinomycin D inhibits this fraction in particular (Key and Ingle, 1968; Key, 1966).

The DNA-like RNA as the name would imply has a nucleotide composition similar to that of DNA. It has an apparent half-like in etiolated soybean hypocotyls of 1.5 to 2 hours (Ingle et al., 1965). It is the synthesis

of this fraction, also called "growth essential RNA", which is postulated to be stimulated by auxin. Specifically, it is thought that the rate of RNA synthesis is limited by RNA polymerase activity and auxin increases this activity (O'Brien et al., 1968) thereby stimulating DNA-like RNA synthesis. This "growth essential RNA" fraction which is assumed to be m-RNA is postulated to code for "growth essential protein" (Key and Ingle, 1968; Masuda and Tanimoto, 1967).

Noodén and Thimann (1963, 1965, 1966) favour the above hypothesis over other possible alternatives. They based their choice on the close correlation between the action of inhibitors on RNA and protein synthesis and the inhibition of growth. Extending the work of Siegel and Galston (1953), Galston et al., (1964) reported IAA is converted by peroxidase to an unknown derivative called IAA'. In the absence of a receptor molecule, the IAA' became IAA". In the presence of RNA however IAA' became bound to the RNA. This was determined by gel filtration on Sephadex G-50 where compounds that absorbed 260 nm radiation and were Salkowski positive eluted together. It was later shown (Kefford et al., 1963) that IAA' by itself eluted at the same volume as RNA. IAA' may have been a polymer of IAA. Although these observations of the interaction of IAA' and RNA proved to be fortuitous, they did serve to direct attention to the possible interaction of IAA

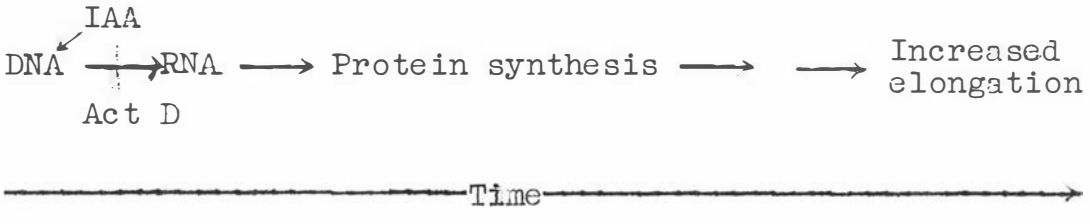
itself with RNA.

Bendaña et al (1965) reported the recovery from green pea stem segments of labelled RNA after the administration of carboxyl labelled ^{14}C-IAA. The label was preferentially incorporated into 4s-RNA, as judged by sucrose density centrifugation. It was possible that the IAA was decarboxylated and the $^{14}CO_2$ subsequently fixed by photosynthesis. Some of the ^{14}C could have been incorporated into RNA by normal anabolic processes. It was found that methylene labelled IAA was slightly less than half as efficient at labelling RNA as IAA-1-^{14}C. Therefore some but not all of the counts may be accounted for by fixation of $^{14}CO_2$ from IAA-1-^{14}C. In a double label experiment using IAA-1-^{14}C and ^{3}H-uridine, it was found that while ^{3}H was incorporated into all the fractions, the ^{14}C was not. Radioactive IAA could be recovered from hydrolysed 4s-RNA. These authors concluded that IAA binds with 4s-RNA. Further work demonstrated the possible significance of this binding. Bendaña and Galston (1965) showed that 4s-RNA extracted from IAA treated green pea stem segments was more resistant to ribonuclease degradation than the same fraction from tissue treated without IAA. Kaur-Sawhney et al., (1967) showed that the binding was covalent and primarily to an RNA fraction. That was resistant to degradation by snake venom phosphodiesterase but not to spleen phosphodiesterase.

Armstrong (1966) has postulated a different function to the binding of IAA to 4s-RNA. According to his model auxin functions as a signal for polypeptide chain initiation. This would require an auxin specific sRNA fraction. Armstrong (1966) assumes the auxin-induced RNA synthesis to be analogous to amino acid dependant RNA synthesis in bacterial systems. Auxin would then play a role similar to that of amino acids in the quantitative regulation of both RNA and protein synthesis.

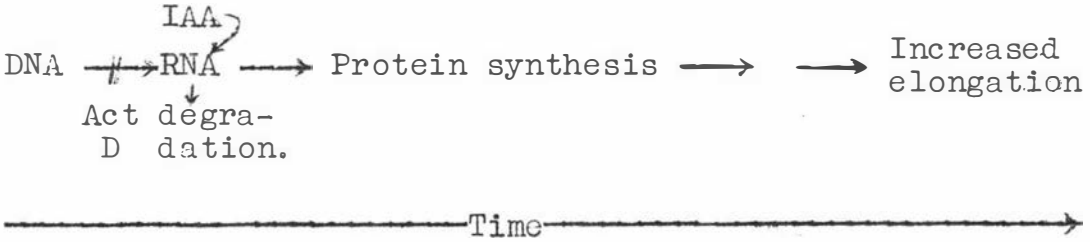
Sarkissian (1966), Sarkissian and Spelsberg (1965) and Mitchell and Sarkissian (1966) have shown that IAA enhances the synthesis of citrate by citrate condensing enzyme purified from castor beans, Phaseolus vulgaris and fresh pig heart. Mitchell and Sarkissian (1966) showed that 1.5 days after irrigation of P.vulgaris plants with 10^{-7}M IAA the citrate content of the shoot had increased by 70% over controls. Sarkissian (1966) proposed that the primary action of IAA was with co-enzyme A and probably with the coenzyme A - substrate - enzyme complex. It was suggested that IAA may elevate the rate constant of the reaction of the enzyme-substrate complex to form products plus enzyme (Michaelis-Menten k_3).

The hypotheses that were current when this work was started could be divided into three main categories. The first was that auxin acted within the nucleus, stimulating RNA synthesis. It can be symbolised as follows:



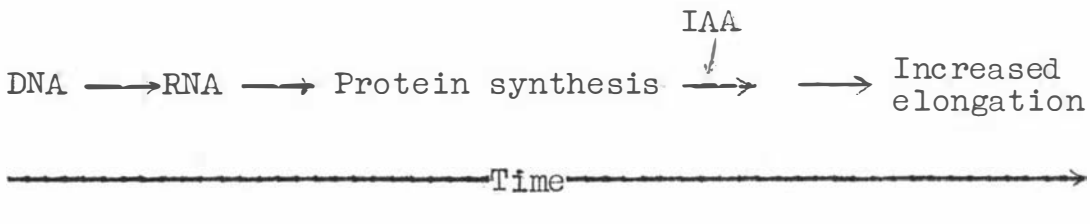
Hypotheses in this category were held by Key and his co-workers, Masuda and Noodén.

The second category included those that auxin exerted its effect on RNA or on subsequent steps. It can be symbolised as follows:



According to these hypotheses, auxin action depends on preexisting RNA (either directly or indirectly) and synthesis of RNA is necessary only after its level falls. e.e. Bendaña et al., 1964; Armstrong, 1966; Nelson et al. 1969). Mechanisms which require protein synthesis (but not RNA synthesis) would be involved after the addition of auxin and before elongation increases.

The third category is that auxin acts on or with existing proteins. It can be symbolised as follows:



Auxin would not directly stimulate protein synthesis. Any stimulation of protein synthesis would be a result of

feedback. Sarkissian's (1966) work would be in this category.

Most of the evidence presented in favour of these hypotheses, particularly those in the first category, was the result of long term experiments of at least an hour's duration or more. In all tissues that have been studied, IAA exerts its effects within 20 min. The evidence therefore is irrelevant and the hypotheses needed to be reexamined in light of known short term kinetics. A decision had to be made as to the best point at which to begin in the DNA, RNA, protein sequence which would yield the most potential in terms of planning future work, and second, having made that decision, the method of approach or the hypothesis to be tested which would give the most clear cut results.

It was decided to start at RNA and to ask the question is RNA synthesis necessary for the initial action of auxin? There were two reasons. Firstly, the answer to the question would point to another part of the sequence which would warrant further attention, so that if the answer was yes one would look to the nucleus and possibly DNA for the site of auxin action but not at the protein synthesis level. Secondly, the theory that auxin induced specific m-RNA synthesis was in vogue at the time.

In order to show that RNA synthesis is required for auxin action, several crucial events have to be demonstrated - and it must be demonstrated that they occur before the end of the latent period. It must be shown that:

1. Auxin-induced synthesis of (plant) "messenger" RNA occurs within the latent period.

(It is in fact difficult to show that the RNA synthesis is "messenger" and bacterial contamination must be eliminated.)

2. This "messenger" RNA is transported from the nucleus to the cytoplasm again within the latent period.

(There are two main difficulties in this step. Apparently much of the RNA synthesised in the nucleus of eukaryotic cells "turn over" i.e. is degraded within the nucleus (Harris, 1963; Loening, 1968), and secondly the time taken to transport RNA from the nucleus to the cytoplasm may be in the order of an hour from the time of synthesis (Barlow, 1970; Tata, 1967; Loening, 1968). Certainly it is inadequate to assume that because RNA is synthesised within the latent period that it will quickly appear in the cytoplasm.

3. During the latent period there is an increase or alteration in protein synthesis and that this

change is dependent on the RNA synthesized in response to auxin and already transported into the cytoplasm.

(It is of course insufficient to show that both protein and RNA synthesis are altered during the latent period. To give any support to the hypothesis it must be shown that the alteration in rate or type of protein synthesised is dependent on the RNA synthesised in response to the addition of auxin and not dependent only on RNA synthesised before auxin addition.)

4. The alteration to the proteins synthesised is directly related to the change in rate of elongation.

(They could be involved in, for example cell division, conjugation of IAA with aspartate, etc.)

The minimum that is necessary to establish that RNA synthesis is not necessary (category 2) would be to show that auxin-induced growth occurred before RNA synthesis was stimulated. Even more clear-cut would be to demonstrate auxin action in the absence of RNA synthesis.

The latter approach was the one taken, but first it was necessary to determine whether the response of lupin hypocotyl segments to actinomycin D was similar to that of other tissue.

B. RNA synthesis.

1. Effect of actinomycin D

a. Long term kinetics

Since the effect of IAA on RNA synthesis was of particular interest, it was necessary to determine whether the response of lupin segments to actinomycin D was similar to that in other tissues. After the usual pre-treatment, 5 mm segments were placed in solutions containing IAA and/or actinomycin D. Additional segments were placed in actinomycin D and after 2, 4 or 6 hr were placed in solutions containing IAA and actinomycin D. Fig. 20 illustrates the results of one experiment in which there were 5 segments per treatment and each treatment was triplicated.

Actinomycin D did not prevent an auxin-induced increase in growth. In fact segments treated with actinomycin D for 6 hr before IAA was added had the same growth rate for the first two hours after IAA addition as those to which IAA and actinomycin D were added concurrently. Compared with these, those segments treated for 2 and 4 hr with actinomycin D before IAA addition had an even higher growth rate for at least the first 2 hr after IAA addition. This may be similar to the case with peas where buffer treatment enhanced the response to auxin (Penny and Galston, 1966). Although actinomycin D does not reduce the initial growth rate, the total amount of growth is reduced with longer

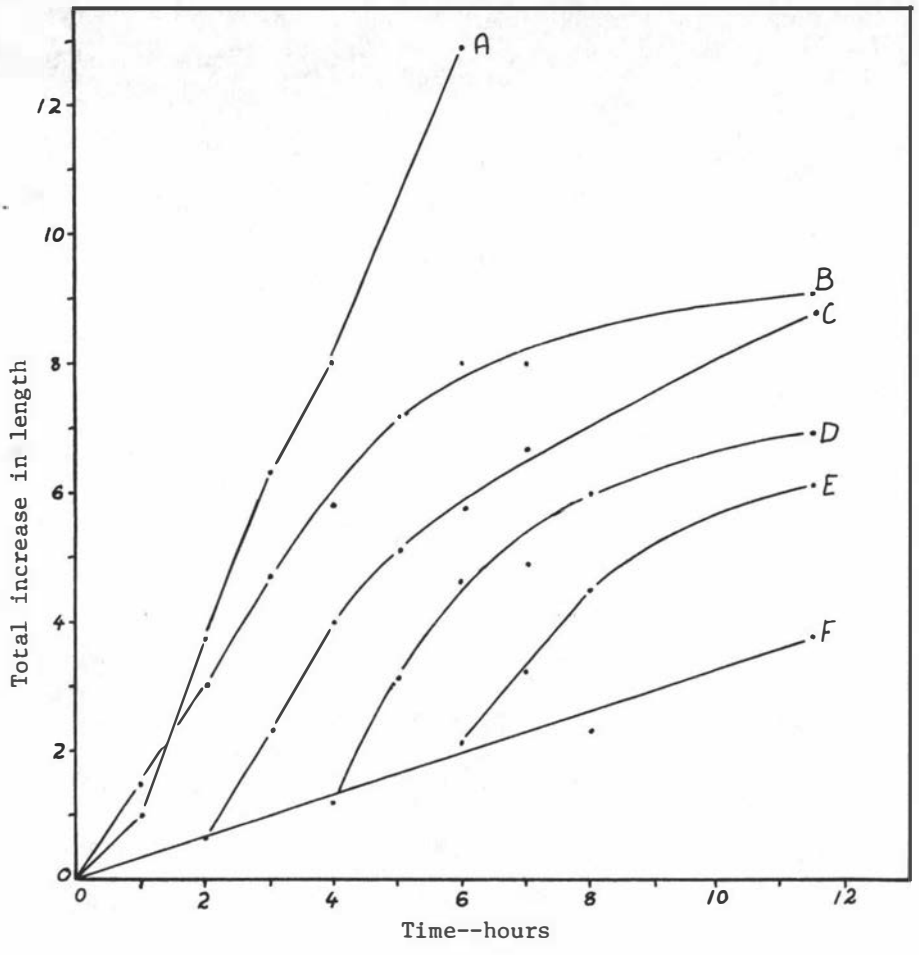


Fig. 20. Long term kinetics of the effect of IAA (3×10^{-5}M) and actinomycin D (10 µg/ml) on the growth of 5 mm lupin hypocotyl sections. a: IAA from time zero. B: IAA and actinomycin D from time 0. C: 2 hr in actinomycin D then IAA and actinomycin D. D: 6 hr in actinomycin D then IAA and actinomycin D. F: actinomycin D alone Treated at 22°C.

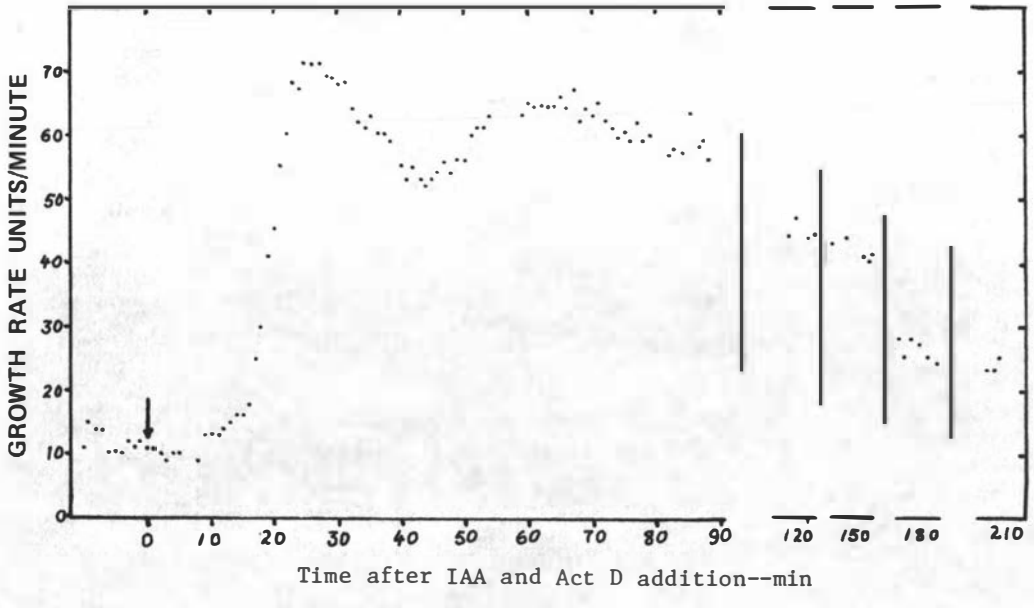


Fig. 21. Short term kinetics of the effect of IAA (3×10^{-5}M) and actinomycin D (10 µg/ml) on the growth rate of a 20 mm lupin hypocotyl segment. IAA and actinomycin D added at arrow. Treated at 24°C. 1 unit = 0.23 µm.

actinomycin D treatments prior to the addition of auxin.

In the experiment shown in Fig. 20, the difference between the lengths of the segments at 2 hr of IAA alone and IAA plus actinomycin D treatments was abnormally high. The difference was 1.1 mm (comparing the 15 segments in each treatment) but the average difference for the 5 experiments that have been performed, including this one, was 0.3 mm. The difference is not statistically significant. ($P > 0.5$). Adding 30 ug/ml actinomycin D concurrently with IAA did not increase actinomycin D inhibition of IAA induced growth.

These results show that the effect of actinomycin D on lupin hypocotyl segments is similar to that shown by Penny and Galston (1966), Cleland (1965) and Nelson et al. (1969) on other tissues.

b. Short term kinetics.

Fig. 21 is a plot of the growth rate per minute of a 23 mm lupin hypocotyl segment treated with IAA and actinomycin D at time 0. For at least the first 90 min the curve is similar to those obtained for IAA alone. The length of the latent period, the time of the minimum and the second maximum are all within the range of those obtained with IAA. The time for the first maximum occurs 4 min sooner (in another experiment, 2 min sooner) than the shortest time of the range for auxin alone (cf Table 1)

A 27% inhibition of the growth rate by actinomycin D occurred by 150 min (in another experiment the inhibition was 15%). It can be seen that the inhibition was much more marked by 180 min.

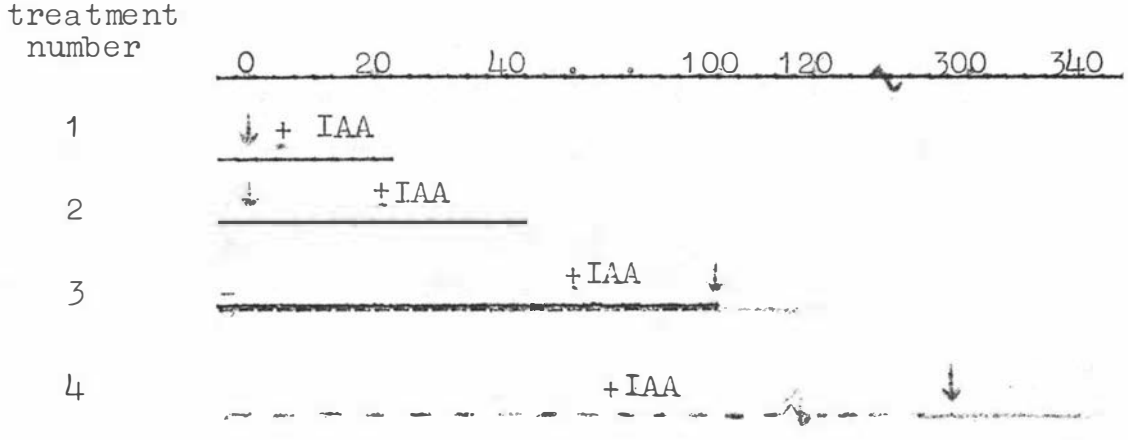
These results strongly support hypotheses in the second and third categories, that auxin acts on existing RNA or on steps after RNA in the DNA to cell elongation sequence. They could possibly be reconciled with those in category 1 in at least 2 ways; actinomycin D could inhibit RNA synthesis only in the presence of auxin or alternatively there could be regions of DNA which do not bind with actinomycin D (Stern and Friedman, 1970),

Since there was a chance that RNA synthesis was necessary for IAA-induced growth, the possibility was investigated in the following experiments.

2. Effect of IAA on RNA synthesis

a. Experimental design

The basic protocol for the experiments was as follows:



The treatments fell into 4 groups. In each group there were 2 samples of 15 segments one of which contained auxin. At the time indicated by the arrow the samples were transferred to solutions containing labelled RNA precurso (either ^{32}P or ^{14}C-uracil) for the length of time indicated by the solid line. The broken line in treatments 3 and 4 indicates the length of treatment time in buffer or auxin plus buffer before the segments were placed in similar solution containing isotope.

The 20 minute period (treatment 1) was chosen because it was the approximate length of the latent period. If RNA synthesis was necessary for auxin action, it would have to occur in this time. (Hypotheses 1 and 2 of page 55). However one of the interpretations of the kinetics of growth of this tissue is that there are two separate effects of auxin, the second becoming apparent at about 40 min (hypothesis 1). It was possible that RNA synthesis was required for the second effect and not the first; hence treatment 2. This time would also test hypothesis 3.

The question that the experiments containing these treatments were designed to answer was "Is RNA synthesis necessary for auxin-induced growth?" The answer could be positive or negative. If it were negative, then the possibility of inadequate technique could not be ruled

out. It was therefore necessary to include treatments to check this possibility. Treatments 3 and 4 were used for this reason. The timing in treatment 3 was chosen because Trewavas (1968) had shown that in etiolated pea IAA-stimulated RNA synthesis could be detected after about 1 hr in IAA. Treatment 4 was chosen because the actinomycin D results indicated that RNA synthesis was necessary for growth at 5 hr after auxin addition. If RNA synthesis could be detected in either of these treatments, then the techniques would be shown to be adequate.

Each experiment consisted of treatments 1 and 2 and either 3 or 4. In some experiments duplicates of some treatments containing 10^{-3}M 6-methyl-purine or 10 ug/ml actinomycin D were included. The methods of RNA extraction, determination and separation are given in Appendix II.

b. The short term effect of IAA on RNA synthesis.

The reason for studying RNA synthesis at short intervals was to determine whether IAA stimulated RNA synthesis either before the end of the latent period or before the second maximum.

Fig. 22 illustrates some of the results of an experiment composed of treatments 1,2 and 4 using ^{32}P as RNA precursor. The top, solid line is the optical

density in UV light of one gel. Superimposed on the graph are the cpm/mm gel of RNA from: curve a, treatment 2 with IAA (40 min IAA and ^{32}P); and curve b, treatment 4 without IAA (^{32}P from 5 hr to 5 hr 40 min). In no experiment was RNA synthesis detected in treatments 1, 2 (e.g. Fig. 22a) or 3, with the exception discussed in the following section. This was so even when 20 mci of ^{32}P were used in each experiment. However it could be readily detected in treatment 4. This shows that a 40 min exposure to labelled precursor is sufficient length of time to detect RNA synthesis in this tissue and therefore that the techniques are adequate.

With respect to treatment 4, it is interesting to note that there is more RNA synthesis in the samples without IAA than those with IAA. In long term kinetic experiments, the growth rate of segments in the buffer controls increases markedly at about 5-6 hrs. RNA synthesis may play an important role in this increase.

The conclusion from this work is that again there is support for the hypotheses in the second category that RNA synthesis is not required for the initial action of auxin on elongation.

Fig. 23 shows RNA separated by MAK column chromatography. It is included to illustrate that the RNA extracted from lupin hypocotyls gives similar types

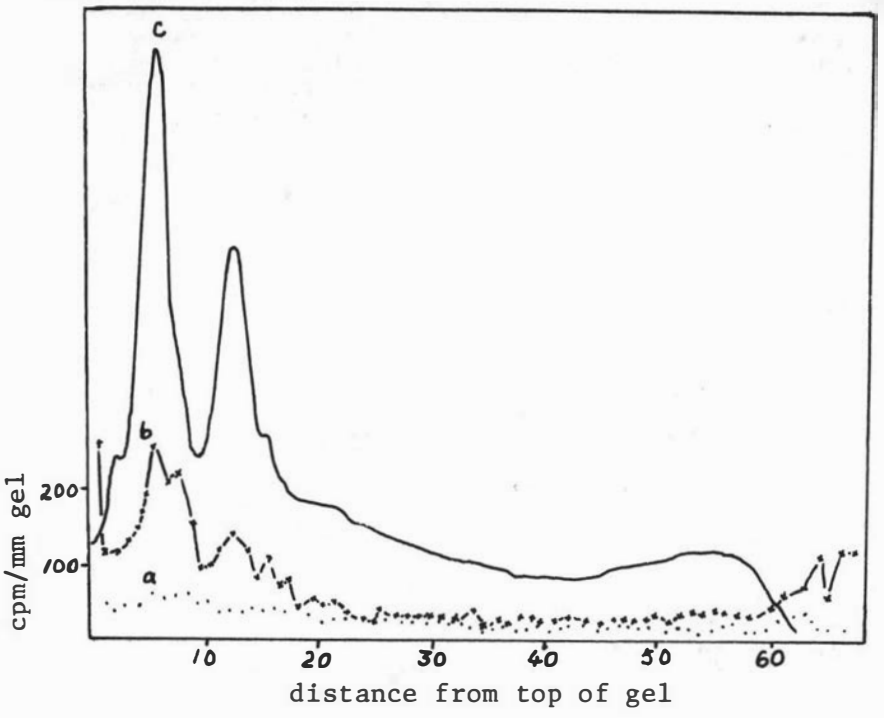


Fig. 22. Acrylamide gel electrophoresis of RNA extracted from tissue treated: a) with IAA (3×10^{-5}M) and ^{32}P for 40 min and b) ^{32}P from 5 hr to 5 hr 40 min after the start of the experiment, buffer present from time zero Curve c) UV optical density of the gels.

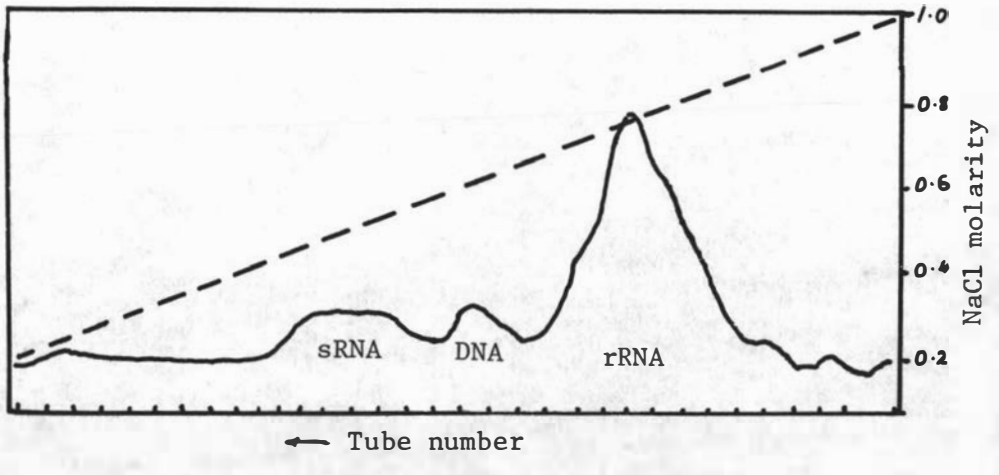


Fig. 23. MAK column chromatography of lupin hypocotyl RNA.
——— UV optical density; ----- NaCl molarity.

of patterns when separated in this manner to those published by other workers.

c. The small radioactivity peaks in the r-RNA region of gels.

In some of the RNA samples there were two small peaks of radioactivity in the r-RNA region of the gels. Fig. 24 is an illustration and other examples may be seen in Appendix III, Fig. 14. In Fig. 24, the continuous line is the reflectance scan of the UV photograph and the dashed line is the cpm/mm gel. The large radioactive peaks on the right hand side (bottom of the gel) ~~is~~ are discussed in Appendix IV.

The small peaks occurred in 11 out of a total of 54 treatments. Their appearance seemed to be random, regardless of the presence or absence of IAA or 6-methyl purine and regardless of the length or time of treatment. In later work when ^{3}H-RNA was run on gels concurrently with experimental samples, it was possible to determine that these peaks corresponded to 23s (1.1×10^{6} daltons) and 16s (0.5×10^{6} daltons) RNA. This is shown in Fig. 25, where the upper line is the ^{3}H-RNA and the lower one the experimental, ^{32}P labelled RNA. It can be seen that the small peaks correspond quite well to the lighter r-RNA. Because of this evidence, it was concluded that the radioactive RNA resulted from that synthesized by bacteria contaminating the segments. The bulk of RNA in

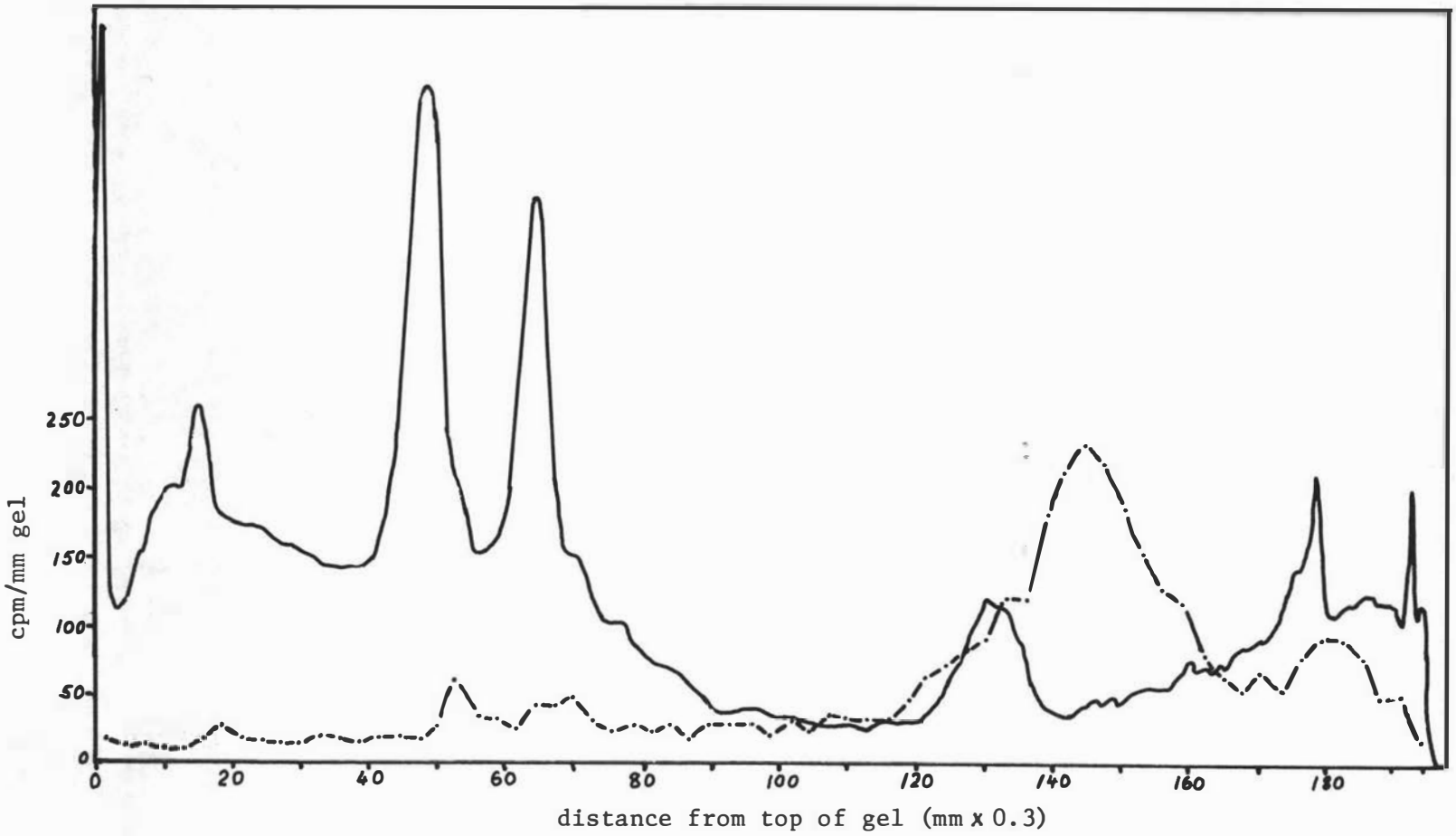


Fig. 24. Acrylamide gel electrophoresis of RNA extracted from tissue which had been pretreated in 6-methyl purine for 1 hr before a 40 min exposure to ^{32}P and 6 methyl-purine. ——— reflectance scan of UV photograph. —·—·— cpm/mm gel.

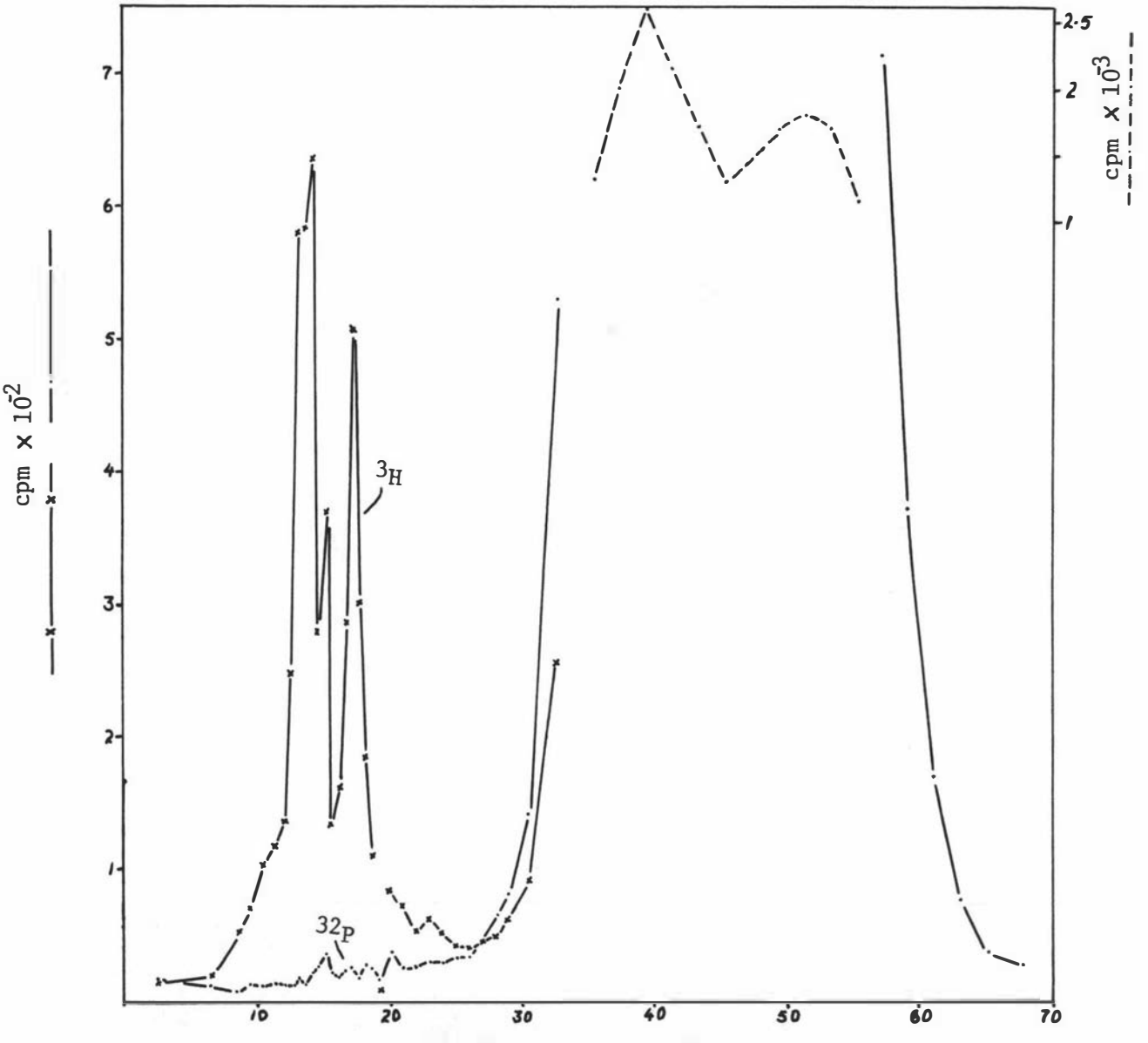


Fig. 25. Electrophoresis of lupin hypocotyl RNA. x——x ^{3}H-RNA extracted from segments treated for 8 hr with ^{3}H uracil. ·——· ^{32}P-RNA extracted from segments treated with ^{32}P for 40 min. Axis for dashed line on right is 10 times greater than axis on the left.

the small UV absorbing peaks was lupin chloroplast and mitochondrial RNA.

In one experiment a sample of segments was accidentally contaminated with water from the water bath about 30 min prior to incubation with ^{32}P. When the RNA fraction from this sample was analysed for radioactivity, the results in Fig. 26 were obtained. Not only were 23s and 16s-RNA labelled and 28s and 18s-RNA not labelled, but the DNA was radioactive too indicating that DNA synthesis had occurred. In this case it is quite clear that bacterial RNA synthesis had occurred.

d. Uptake of ^{32}P.

The inability to detect RNA synthesis at short times could be criticised on the grounds that the ^{32}P did not enter the plant's cells, or if it entered it was not metabolised. This criticism may be countered on several grounds.

In 5 experiments the radioactivity in 5 out of the 15 treatment segments was determined (the other 10 were used for RNA extraction). It should be noted that the segments were rinsed for 30 secs. in 2 x 30 ml of buffer immediately after treatment and before counting. In all experiments there was radioactivity in the segments. An example from one experiment is given in Table II.

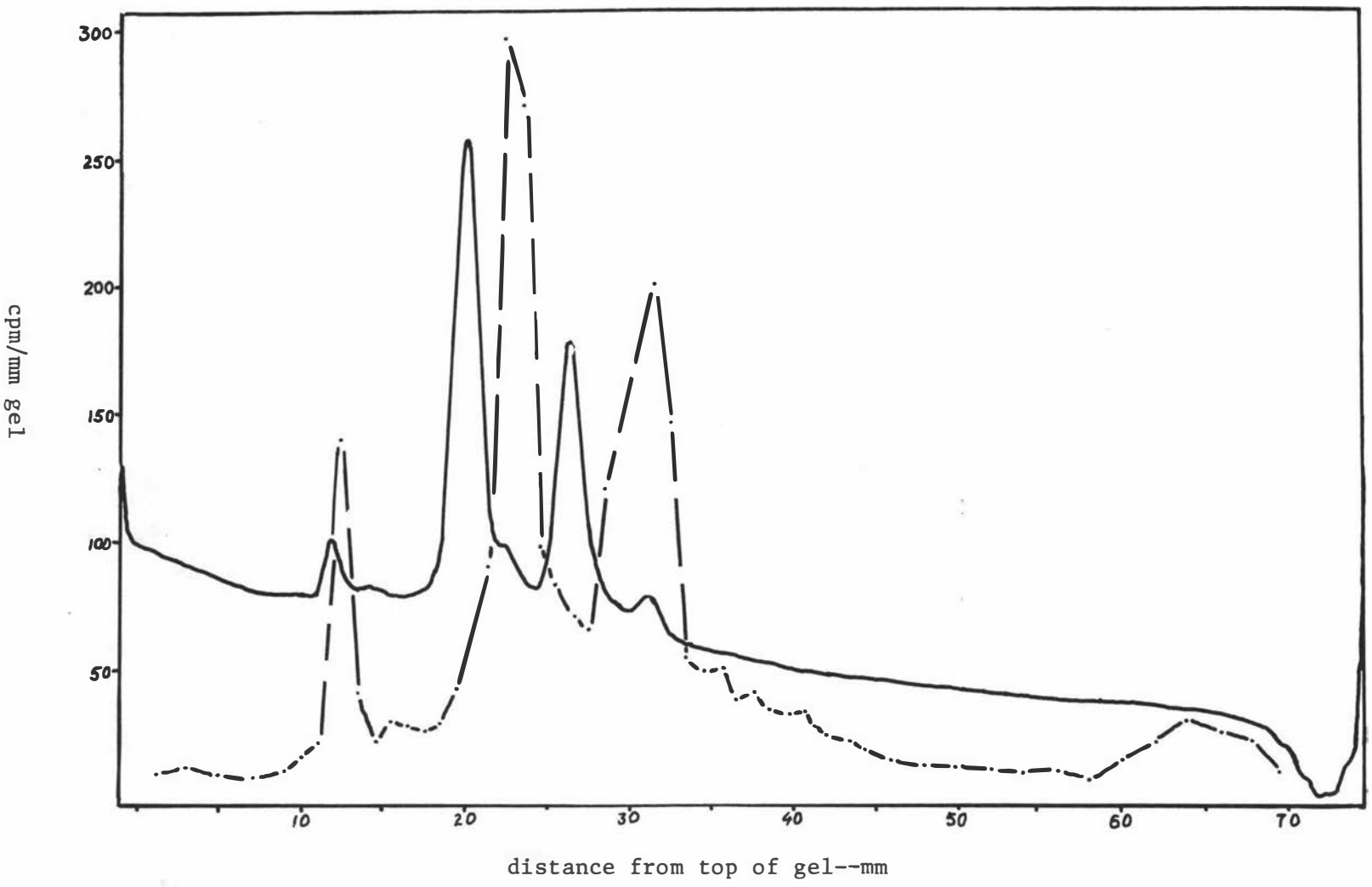


Fig. 26. Electrophoresis of lupin hypocotyl RNA. ———optical density —·—·—·— radioactivity (cpm/mm gel)

Table II Radioactivity per lupin hypocotyl segment after treatment in ^{32}P for various times, with or without IAA.

time of treatment	+ IAA	- IAA
0 to 40 min	287,600 (270,000)	288,200 (18,400)
5 hr to 5 hr 40	460,400 (37,400)	815,400 (118,900)

The figures in brackets are the standard errors of the mean.

It can be seen that ^{32}P was taken up into the tissue. There was more uptake in the treatments at the second time than the first, but IAA did not increase uptake. The segments from which the RNA illustrated in Fig. 22 was extracted were treated for these times. It could be said that the level of RNA synthesis at these times was the same but because there was less tracer taken up in the earlier time, the synthesis was not detected. However in some experiments very high levels of radioactivity were used (3 mci 10 ml medium) and in those experiments the amount of ^{32}P in the segments was greater and still no RNA synthesis was detected at the earlier times.

The nature of the non-UV absorbing ^{32}P polymer is discussed in Appendix IV. Further evidence that ^{32}P was

metabolised is shown in Fig. 27 which is a plot of the total organic phosphates with time. IAA was added at 120 min. It can be seen that the level of organic phosphates increases with time. There is an apparent effect of IAA on the level at 180 min. However, it is more probable that the samples without IAA were low since the curve levels off so abruptly.

C. Protein synthesis

1. The effect of cycloheximide on IAA-induced growth.

It was known from long term kinetics that cycloheximide inhibited auxin-induced elongation in lupin hypocotyl sections. Since cycloheximide is an inhibitor of cytoplasmic protein synthesis, it was used to examine the necessity of protein synthesis for the initial action of auxin. This was done by using short term kinetics. Each of these cycloheximide treatments was repeated at least 4 times, and each line in Fig. 28 and Fig. 29 represents the response of a typical segment. The results are shown in Fig. 28. Curve 28 shows the usual response to IAA alone with the latent period, first peak and second maximum. Curve 28 shows the effect of cycloheximide added together with IAA. The second peak has been eliminated by cycloheximide. It therefore appears that protein synthesis is necessary for the second maximum to occur. The first peak is reduced slightly but is still prominent. With regard to the first peak it can be concluded either (a) that protein

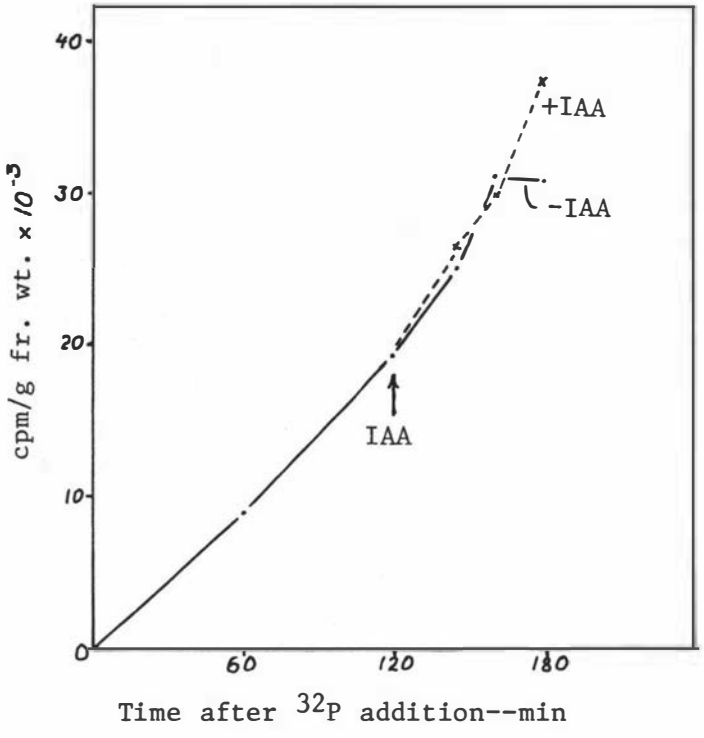


Fig. 27. The incorporation of ^{32}P into organic phosphates of lupin hypocotyl segments. IAA added at arrow. .——. No IAA x——x with 3 10^{-5}M IAA

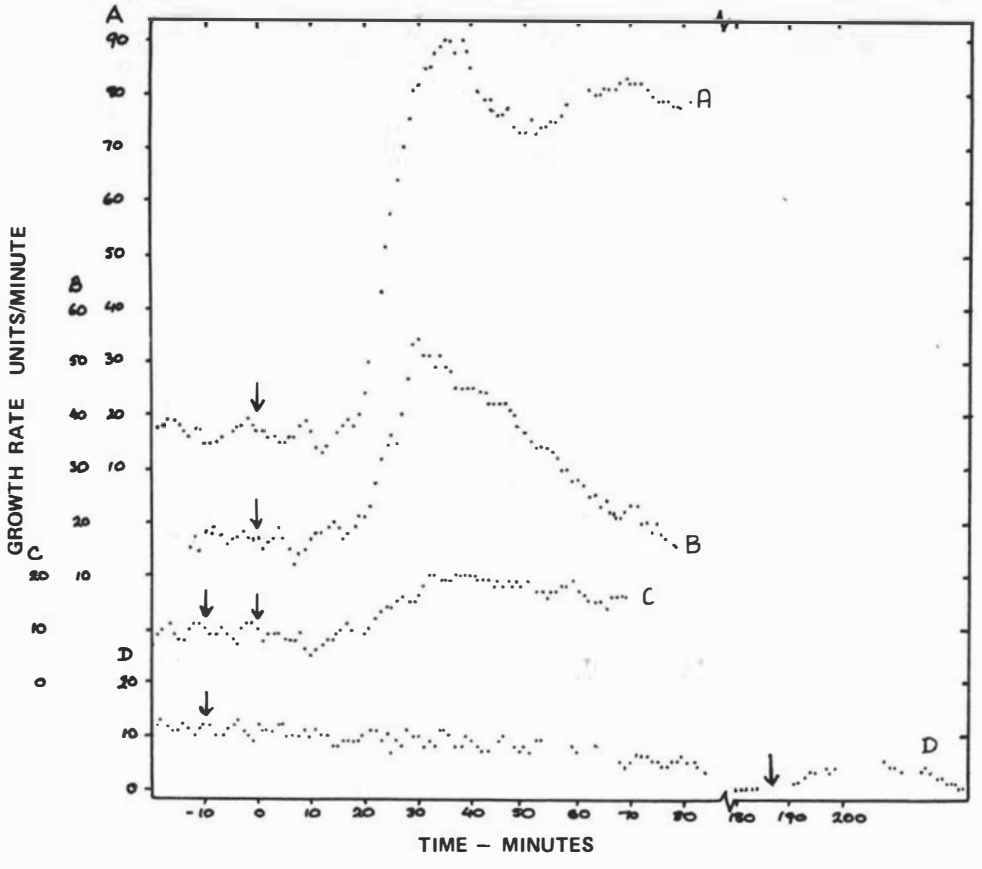


Fig. 28. Effect of cycloheximide (10 µg/ml) and IAA 3×10^{-5}M) on the growth rate of lupin hypocotyl segments. a) IAA added at arrow. b) IAA and cycloheximide added at arrow. c) cycloheximide added at second arrow. d) cycloheximide added at first and IAA at second arrow. 24°C. 1 unit = 0.23 µm.

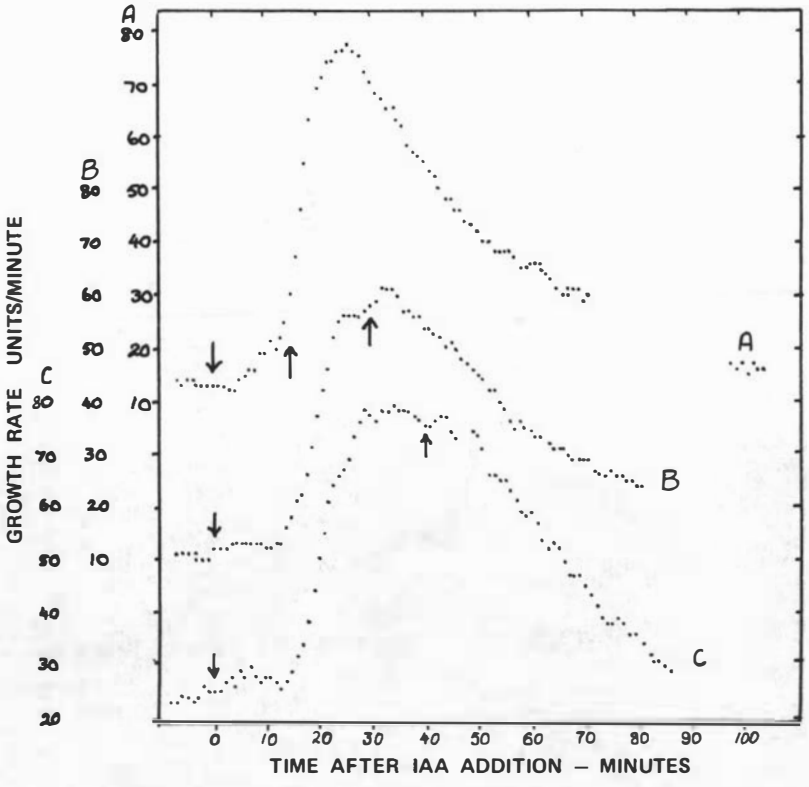


Fig. 29. Effect of cycloheximide (10 ug/ml) on IAA-induced growth rate of lupin hypocotyl segments. IAA addition at first arrow and cycloheximide with IAA addition (second arrow) at: a) 15 min, b) 30 min and c) 40 min after IAA addition. 24°C. 1 unit = 0.23 µm.

synthesis is not necessary, or (b) that for some reason (e.g. slower uptake) cycloheximide takes longer to act than IAA.

In order to help decide between these possibilities, cycloheximide was added 10 min before IAA. Fig. 28 shows that the IAA induced growth is greatly reduced. Fig. 28 is a control with cycloheximide alone and shows a gradual reduction of endogenous growth during the time studied here.

A question now arises which has a bearing on the interpretation of the growth rate curve. It concerns the minimum length of cycloheximide treatment required to prevent the occurrence of the second maximum. Experiments were done in which cycloheximide was added at various times after IAA addition. Fig. 29 illustrates the results. IAA was added at zero time and cycloheximide was added at the second arrow at 15 min in curve A, 30 min in B and 40 min in C. It should be pointed out that the minimum in the growth rate curve can be expected at 45 to 54 min and the second maximum at 69 to 77 min after auxin addition. Cycloheximide added after IAA inhibited completely the second maximum even when the addition was made as close to the minimum as 40 min after IAA addition. It should be emphasized that IAA was present continuously after time zero. In the treatments presented in Fig. 29 the latent period was unusually

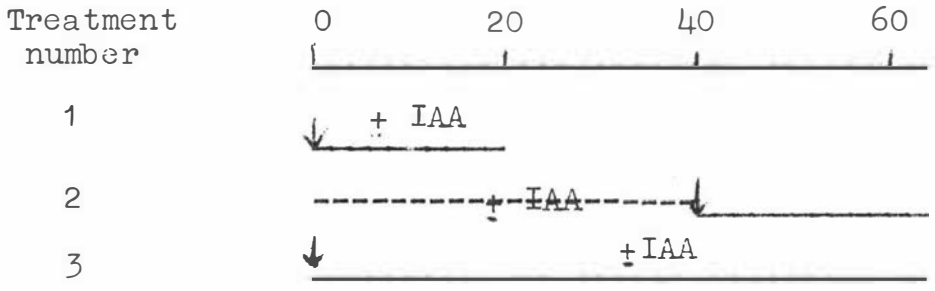
short. This may be owing to the high temperatures (27-30°) in the growth room at the time the plants were grown. The significance (if any) of the slight increase in growth rate after cycloheximide addition in curves B and C is unknown.

The lack of inhibition of the first peak by cycloheximide when added at the same time as IAA may be because protein synthesis is not necessary; i.e. for its initial action IAA acts on an existing pool of protein. The protein would have been synthesized only 10 min before auxin addition. The possibility that cycloheximide takes longer to act than IAA seems less likely since Chrispeels (1970) has recently shown that in carrot tissue inhibition of protein synthesis is 95% complete within 2 min of adding the inhibitor.

2. The effect of IAA on protein synthesis.

a. Experimental design.

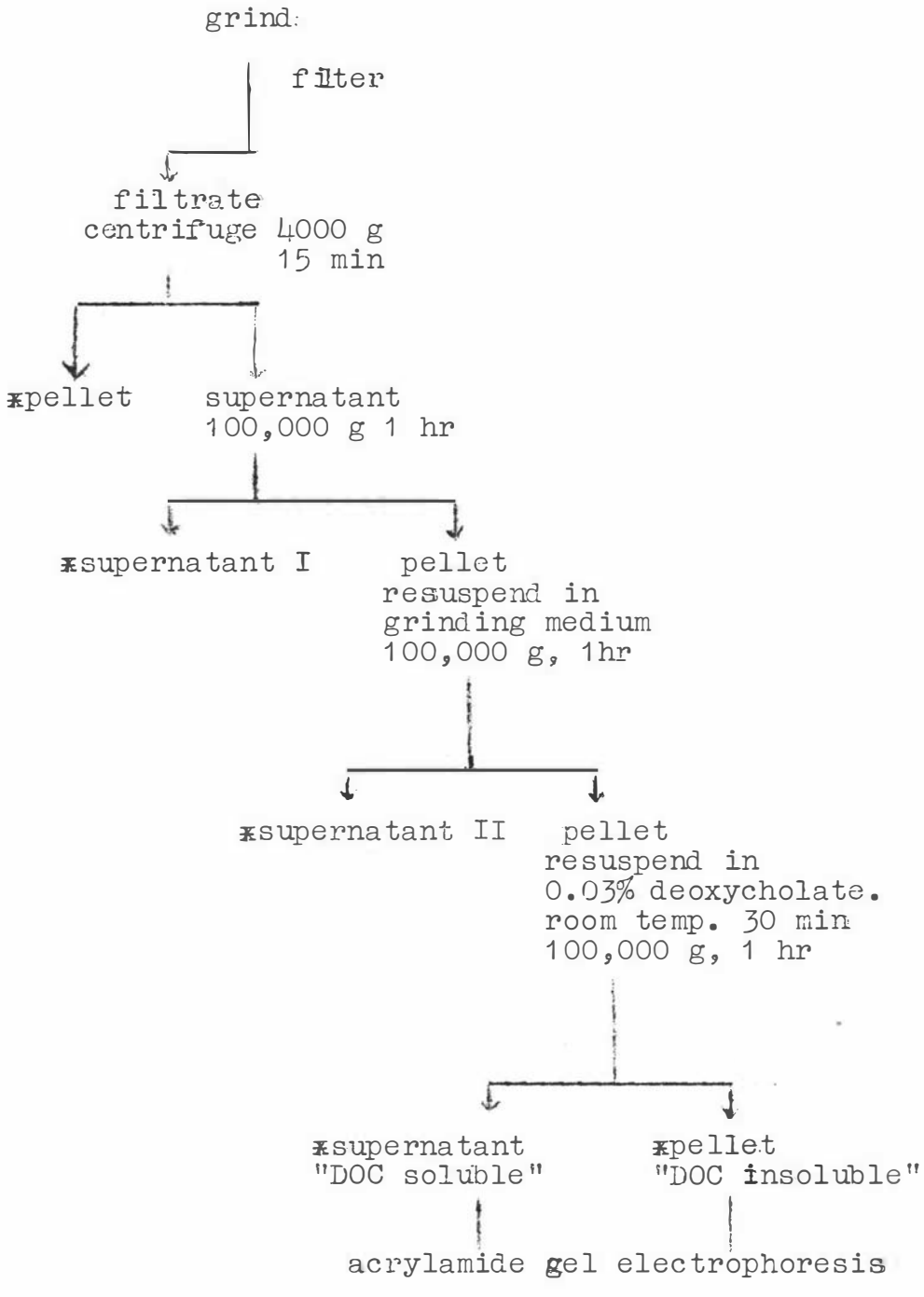
It was recognized that the probability of detecting a single (or a few) protein(s), when none of its properties were known, was slight. However the cycloheximide results were sufficiently encouraging to warrant a few experiments with ^{14}C-leucine in case the task was a simple one. The protocol was as follows.



The segments were pretreated in buffer for about 90 min prior to time zero. The samples were weighed and transferred to solutions with or without auxin at time 0. The arrows indicate the times at which they were placed in fresh medium containing ^{14}C-leucine and the solid lines indicate the length of time before they were removed for extraction. The flow chart on page outlines the procedures used to obtain various fractions. A full description of all the methods occurs in Appendix II.

b. Results

The percentage of radioactivity detected in each fraction are given in Table III. The last lines of the table show the total dpm detected. There was no consistent effect of IAA on any of the fractions. The amount of radioactivity detected localized in the 3rd treatment was proportionally the same as the other treatments. Yet the percentage in both the DOC soluble and insoluble fractions increased. This can be interpreted to mean that a proportionally greater amount of protein was incorporated into the membranes during this time. A greater specific activity of the leucine



* Aliquots removed for radioactivity determination.

Table III. Effect of IAA on the incorporation of ^{14}C-leucine into various centrigual fractions.

	EXPERIMENT #	percentage of total ^{14}C in each fraction					
		period of exposure to ^{14}C-leucine					
		Treatment 1 0-20 min.		Treatment 2 40-60 min.		Treatment 3 0-60 min.	
		-IAA	+IAA	-IAA	+IAA	-IAA	+IAA
4,000g pellet	1	3.7	2.5	2.6	2.1	1.93	3.6
	2	6.3	5.3	5.0	4.8	7.6	6.8
1st 100,000g supernatant	1	90.8	92.0	93.1	93.8	91.6	89.4
	2	86.9	89.4	90.0	89.9	84.0	86.7
2nd 100,000g supernatant	1	2.2	2.6	1.8	1.9	1.9	2.2
	2	2.8	1.7	1.2	1.2	2.2	1.4
2nd 100,000g pellet	1	3.3	2.9	2.54	2.35	4.7	4.8
	2	4.0	3.6	3.9	3.2	6.3	5.1
DOC soluble	1	2.0	2.1	1.8	1.6	3.48	3.4
	2	1.9	1.6	2.0	1.5	2.9	2.1
DOC insoluble	1	1.3	0.8	.74	0.75	1.3	1.4
	2	2.1	2.0	1.9	1.7	3.4	3.0
TOTAL dpm	1	61,450	53,622	65,907	70,871	186,593	188,946
	2	274,786	321,047	444,250	333,897	997,637	991,691

pool of the segments in treatment 3 could account for this, and/or it could be accounted for if the uptake of leucine, synthesis of protein, and protein incorporation into the membrane took a significant proportion of the 20 min in treatments 1 and 2 but not in treatment 3. The results in Table III indicate that the rate of synthesis of the proteins did not increase with time since the proportion of label in the second 100,000g pellet in treatment 2 was not greater than in treatment 1.

However, it was recognized that determination of total protein synthesis in this way may disguise an increase in one protein. Therefore the proteins were separated by acrylamide gel electrophoresis and the radioactivity determined. In experiment 1 only the DOC soluble and insoluble fractions were run. In experiment 2 the first 100,000g supernatant was run as well. Fig.30 illustrates two gels from the DOC soluble fraction of treatment 3. The top (a) gel was the proteins extracted from segments with no IAA whereas those of the bottom gel (b) had IAA (3×10^{-5}M). The arrows point to two peaks of radioactivity present in b but not in a. (They occur at just less than 10% and 40% along the gel length). It appears that there were at least two proteins whose synthesis was stimulated by IAA. These results did not occur in the second experiment, even though conditions were apparently the same. No difference

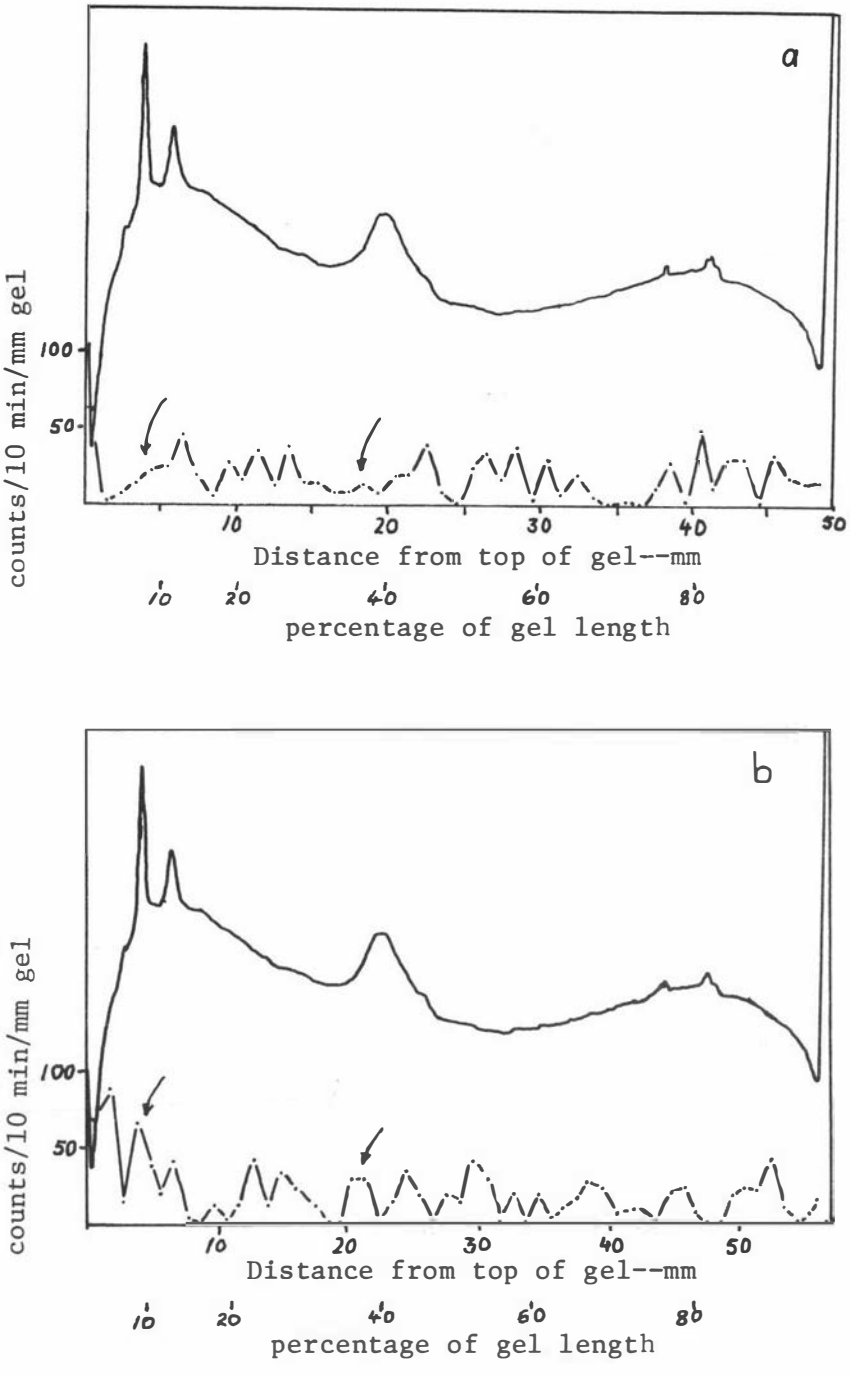


Fig. 30. Acrylamide gel electrophoresis of proteins of the "DOC" soluble fraction of segments treated: a) without IAA and b) with IAA
——— UV optical density of stained gel; —·—·—·— dpm/mm gel.

between IAA and no IAA treatments were detected in gels of any other fraction.

At this point it was decided that another approach to the problem would be more productive in terms of time, energy and laboratory equipment than to continue the quest for the elusive proteins. The approach was to determine the timing of the effect of cycloheximide on growth which could give information about some of the characteristics of the protein. However, before that could be done, the effect of cycloheximide on respiration had to be checked in order to determine whether it was having such non-specific effects on lupin hypocotyl segments.

3. Effect of Cycloheximide on respiration

MacDonald and Ellis (1969) reported that cycloheximide, at concentrations normally used to inhibit protein synthesis (10 ug/ml) also uncoupled respiration in storage tissues such as carrot and beet. Since in this work much weight has been put on results of experiments using cycloheximide, it was imperative to check the effect of cycloheximide in lupin hypocotyl tissue. Using a Gilson differential respirometer the oxygen uptake of segments treatment with IAA (3×10^{-5}M) and various concentrations of cycloheximide (1, 3, 10, 30, 75 ug/ml) was determined. In addition the effect of the presence of 1% sucrose was also checked. There was no effect of sucrose, Fig. 31

illustrates the effect of cycloheximide on oxygen uptake. Each column is the mean of the results with and without sucrose at each concentration. The length of each bar is equivalent to twice the standard error of the mean. There was no apparent effect of cycloheximide on oxygen uptake.

In the experiments the results of which are illustrated in Fig. 31 the tissue was added to solutions already containing cycloheximide. The oxygen uptake was not determined until after the equilibration period during which the segments were in cycloheximide. In order to determine whether these were transient effects of cycloheximide or respiration occurred during the equilibration period, a further experiment was set up. The oxygen uptake of all treatments was measured for one hour before cycloheximide (to give a final concentration of 10 µg/ml) or water was tipped into the vessel from a side arm. The results are presented in Table IV.

Table IV. Effect of cycloheximide (10 µg/ml) on the oxygen uptake of lupin hypocotyl segments treated with 3×10^{-5}M IAA and 1% sucrose.

	µl O_2/gm fr.wt./hr.	
	Solution tipped	
	H_2O	cycloheximide
before tipping	189	193
after tipping	192	193

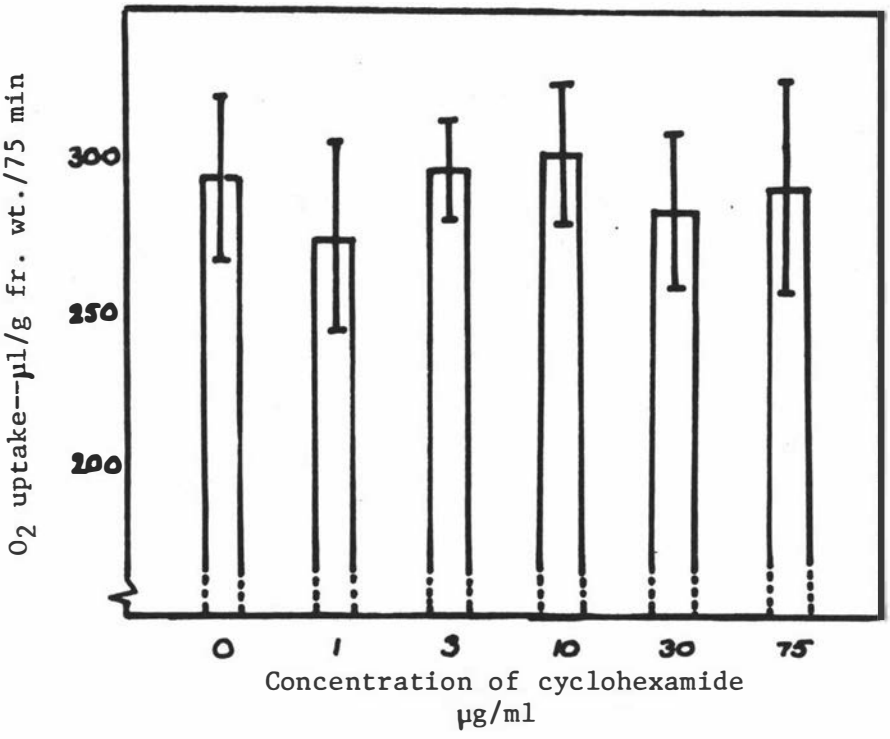


Fig. 31. Effect of various concentration of cycloheximide on the oxygen uptake of lupin hypocotyl segments.

Again it can be concluded that cycloheximide had no effect on oxygen uptake. In fact this table and Fig. 31 illustrates that the variability of the tissue was much greater than the effect of cycloheximide.

4. The timing of the effect of cycloheximide.

It was not possible with centrifugation and electrophoresis to detect directly the protein(s) whose synthesis was stimulated shortly after the initial action of auxin. However, some of the properties of the protein(s) could be determined by indirect means.

The temperature for all the following experiments was maintained at 28°C. The variation in temperature over all the experiments was ± 0.5°C. The relatively even temperature was accomplished by using a peristaltic pump to pump the solutions through the chamber at a rate of about 50 ml/min. When a change of solution was required, the tubing was simply removed from one reservoir to the next. This meant that the tubing was already equilibrated at the desired temperature.

(a) Pretreatment in IAA

Results of experiments in which cycloheximide was added after IAA addition were presented earlier. They indicated that after a certain time in auxin (for example, 40 min.) there was a very rapid effect of cycloheximide on auxin-induced growth. However, the rapidity of the effect

was difficult to judge since the auxin-induced steady-state growth rate had not been achieved.

In order to determine more precisely the timing of the cycloheximide inhibition, the IAA-induced steady state growth rate was obtained by pretreating each segment in IAA (3×10^{-5}M) for 100 min. before cycloheximide (10 µg/ml) was added. Fig. 32a illustrates the results. Plotted is the average of 4 individual segments treated at 28°C. The IAA-induced growth rate is maintained for only 6 min. before it starts abruptly to decrease. The fall in growth rate appears to show two phases. The two phases are more apparent on a semi-log plot as in Fig. 32b. The significance of these phases is unknown, however they could mean that two factors become rate limiting at different time during cycloheximide inhibition of growth.

These results show that the amount of protein available for growth becomes limiting soon after protein synthesis is stopped. It appears that growth may be dependent on two processes each of which require protein synthesis.

(b) Pretreatment in cycloheximide.

One of the properties that can be studied is the half-life during which the protein is available for auxin action and/or growth promotion. This was determined by

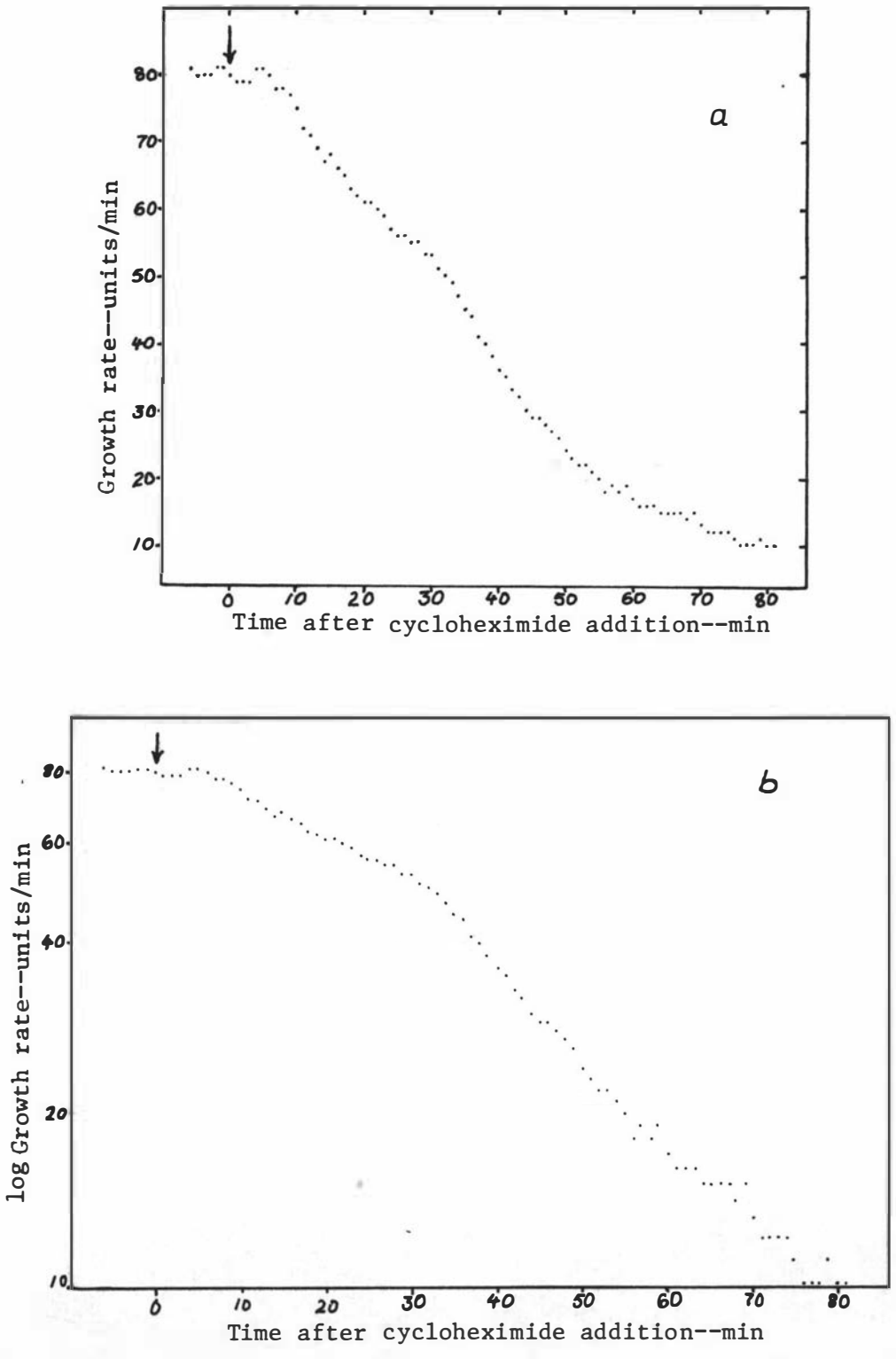


Fig. 32. Effect of cycloheximide on the IAA-induced steady-state growth rate. a) average of four experiments; b) log plot of the data in a). 3×10^{-5}M IAA; 10 µg/ml cycloheximide. 1 unit = 0.23 µm.

stopping protein synthesis for various times before IAA was added. The resulting IAA-induced growth was determined by short term kinetics and was a measure of the amount of protein available for growth promotion.

i) Method

To obtain the total auxin-induced growth, the areas under the peak during the time which the growth was above the initial endogenous growth, are integrated. This was done by subtracting the endogenous rate, as determined from the rates before and during cycloheximide addition, from the rate obtained after auxin stimulation. The decision to use this procedure was made after several experiments had been done and for some of these experiments the entire curve had not been obtained. For these the rates were estimated by extrapolation. The following times of cycloheximide (10 µg/ml) pretreatments were used: 0, 5, 10, 20, 30, 60 min. There were at least 5 replicates for each time and an average IAA-induced growth was obtained for each time.

ii) Results

On Fig. 33 is plotted a curve for each of the pre-treatment times (curves B-H). Each of these was chosen because the IAA induced growth illustrated by them was close to the average obtained for each time. Curve A is IAA (3×10^{-5}M) alone and is given as reference. The latent period is somewhat shorter at 28° than at 24°,

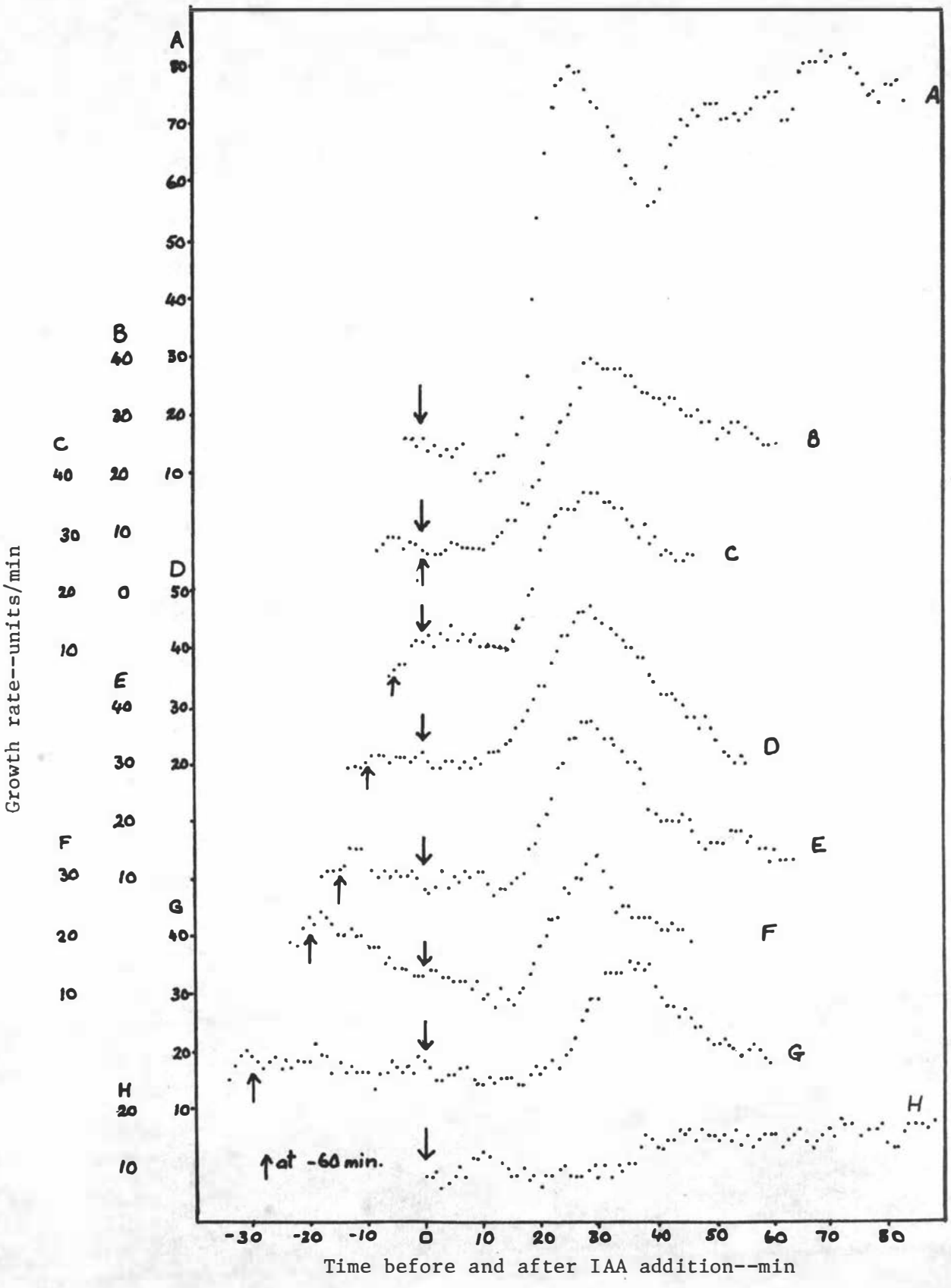


Fig. 33. Effect of various times of pretreatment in cycloheximide on the auxin induced growth rate of lupin hypocotyl segments. Cycloheximide (10 µg/ml) added at upward pointing arrow. IAA (3×10^{-5}M added at downward pointing arrow. Tris-maleate buffer. With supplementary light. 1 unit = 0.23 µm.

14 min. instead of 18 min. With the possible exceptions of the 30 and 60 min. pretreatment (G & H) pretreatment in cycloheximide does not affect the length of the latent period. The IAA-induced growth decreases as the length of pretreatment increases. When a 60 min. pretreatment was used, the growth rate did increase somewhat after auxin addition. However in no case did it decrease once again to the endogenous level. It is not known therefore whether the effect is the same as that being measured for the shorter pretreatment times. The 60 min. pre-treatment was therefore ~~neglected~~ omitted from further analysis.

When the average of auxin-induced growth for each pretreatment period was plotted again pretreatment time, there appeared to be an exponential decay of auxin-induced growth. Such an exponential decay can be described by the equation of the form:

$$A = A_o e^{-kt} \qquad (1)$$

where A is the amount of auxin-induced growth when auxin is added at a given time t after cycloheximide addition,

A_o is the amount of auxin-induced growth when auxin and cycloheximide are added simultaneously,

k rate constant

t time, in minutes, after cycloheximide addition.

To check that the curve was, in fact, exponential a

plot of the auxin-induced growth (average of all the replicates) against pretreatment time in cycloheximide was made. The equation of the line of best fit was calculated by the method of least squares as outline in Remington and Schork (1970). It was:

$$Y = 0.026X + 3.077 \qquad (2)$$

This line and the experimental points are plotted in Fig. 34. Equation (2) permits the evaluation of A_o and k in equation (1):

$$A_o = \text{antilog } 3.077 = 1194$$

$$k = 2.303 \times -0.026 = -.060$$

Equation (1) becomes:

$$A = 1194e^{-.060t} \qquad (3)$$

After one half life the amount of auxin induced growth (A) would be a half of that at zero time. Therefore:

$$A = \frac{A}{2} = \frac{1194}{2} = 597$$

Solving equation (3) t = 11.6.

The average time for interaction of the protein with auxin is called the mean life ($\bar{T}$). The mean life is the reciprocal of the rate constant (Montgomery and Swenson, 1969):

$$\bar{T} = \frac{1}{k} = \frac{1}{.06} = 16.7.$$

Some of the parameters of the interaction of auxin with the protein are now defined. The half life for interaction is about 12 min. and the mean life is about 17 min.

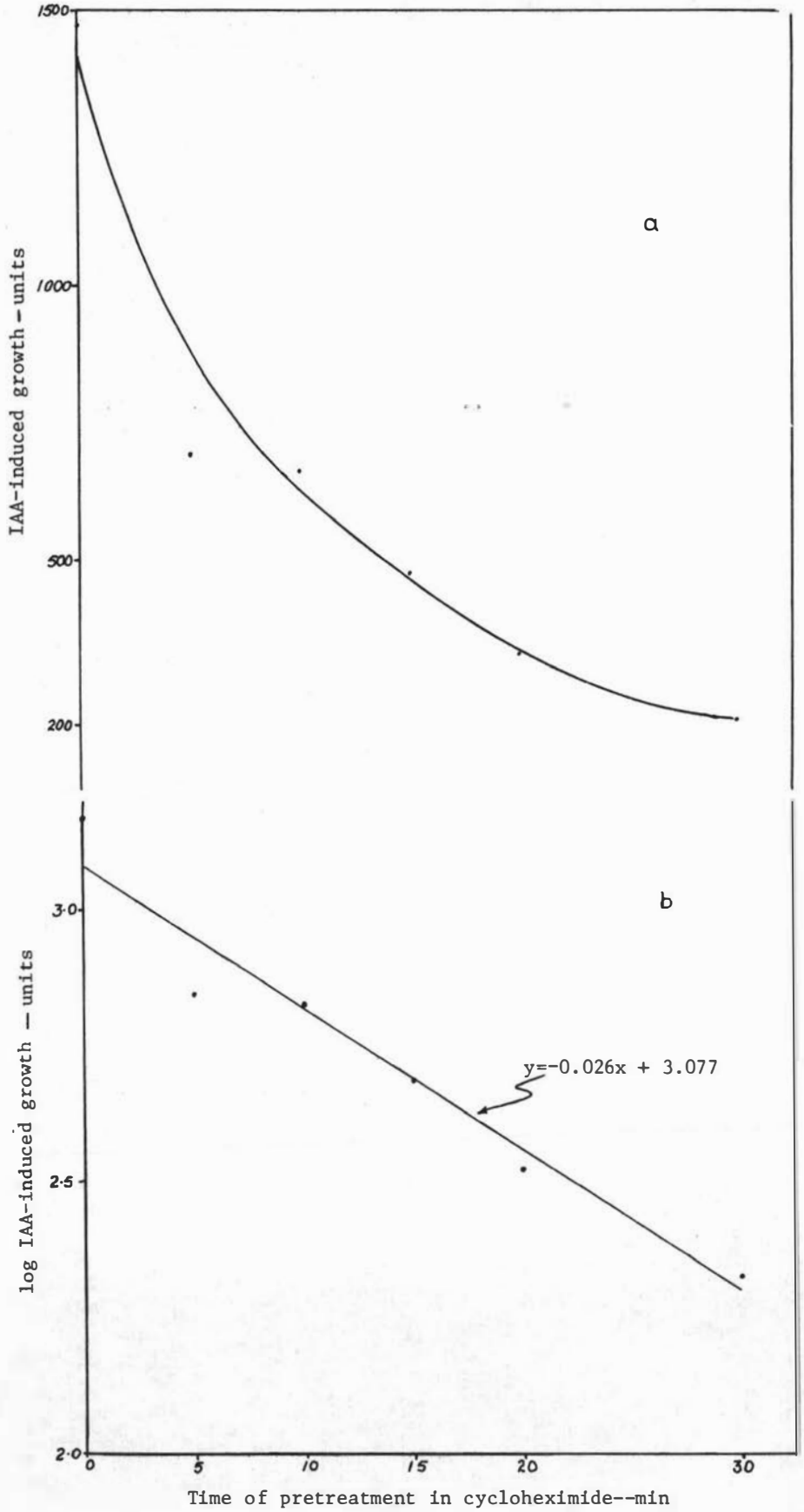


Fig. 34. Effect of various times of pretreatment in cycloheximide on auxin-induced growth. a) linear plot and b) semi-log plot. . . . . experimental points; ——— line fitted by eye in a) and line of best fit in b). 10 μg/ml cycloheximide. $3x10^{-5}$M IAA 1 unit = 0.23 μm.

D I S C U S S I O N

The aim of this work was to elucidate the nature of the primary action of auxin on cell elongation. In order to do so, it was necessary to establish the timing of the response of lupin hypocotyl segments to auxin. The short term kinetic experiments were started with this end in mind. However, once the technique was established it became apparent that it could be used to answer some critical questions regarding the primary effect of auxin. It has been particularly useful in determining the nature of the growth rate curve, and in trying to understand the underlying causes.

A. Short term Kinetics.

1. Comments on methods

There are two basic methods, based on the number of segments used per treatment, that have been used for short term kinetics in the past. The first was the use

of a column of segments. This method was used by Yamaki (1954) and Evans and Ray (1969) and subsequent work by Evans. Yamaki took readings manually every minute using a microscope. Evans' apparatus was automated such that a shadowgraph recording was made in a rotating drum every 24 sec. Yamaki and also Evans and Ray measured total increase in length.

In the second method a single segment was used. This method was employed by Ray and Ruesink (1962), Nissl and Zenk (1969), Köhler (1956) and in this thesis. In all cases readings were taking manually using a microscope. The data of Ray and Ruesink and also of Nissl and Zenk was expressed as the cumulative increase in length, whereas growth rates were immediately derived from the measurements in Köhler's work and in this thesis.

When the data output was a growth rate the determination of the length of the latent period and other characteristics of the growth curve was simple. However these determinations were not so easy when the data ~~was~~ were presented as total increase in length. An extrapolation procedure was used. This is illustrated in Fig. 35 in which the data for a and b were obtained by summation of c and d respectively. It can be seen that the latent period was about 20 min and that it apparently took 5 min in a and 3 min in b before a maximum growth rate was achieved. From c and d the

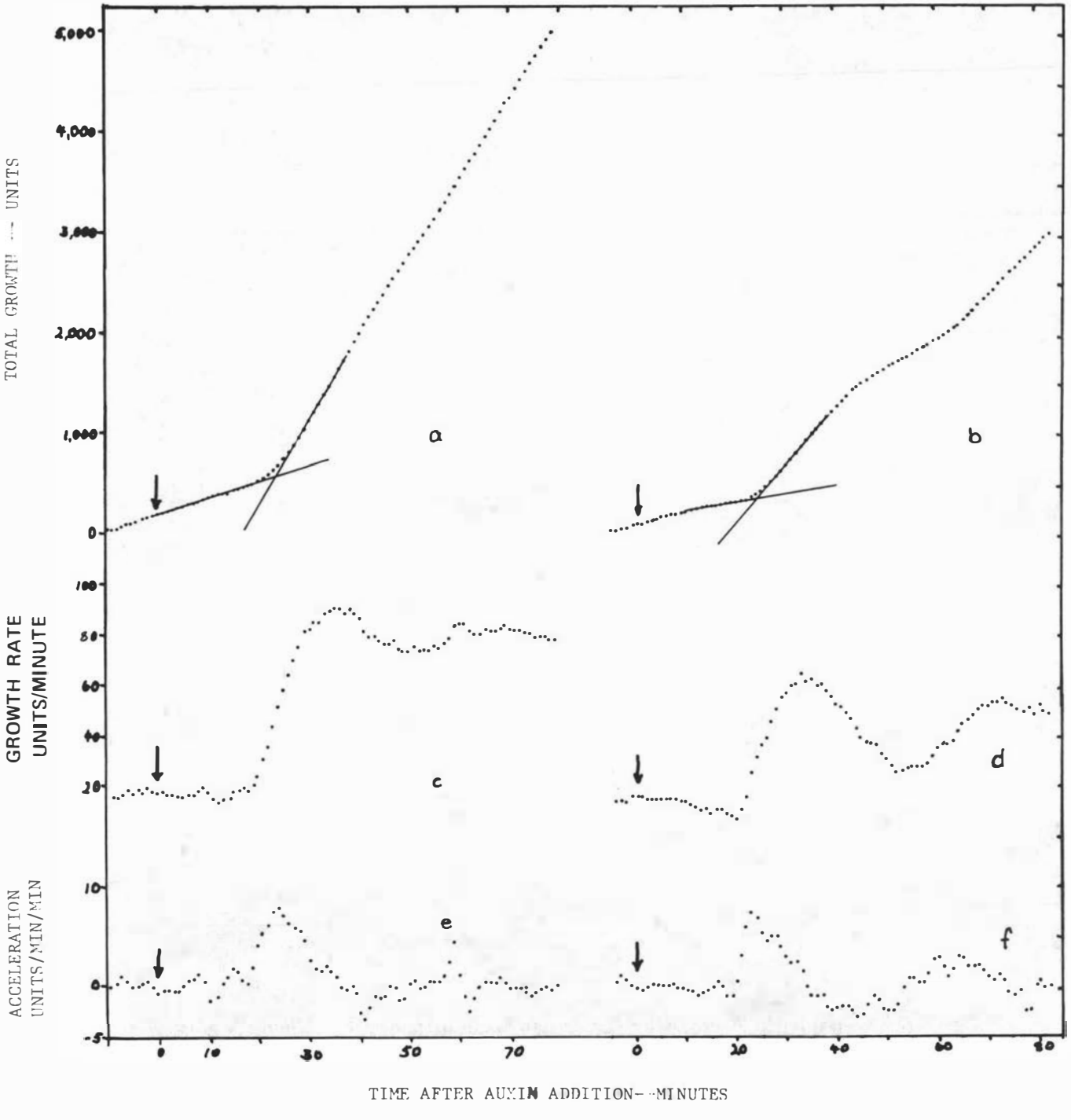


Fig. 35. Effect of IAA (a,c,e) and indoleacetamide (b,d,f) on the growth of lupin hypocotyl segments. a and b, total growth; c and d, growth rate; e and f acceleration. 3×10^{-5}M IAA and indoleacetamide.

growth rate curves it can be seen that these values are under-estimates. This is also illustrated by the acceleration curves shown in e and f. (The acceleration in units/min.$^{-2}$ was determined by averaging the value for each minute with half the value of each of the minutes preceding and following it.) The growth rate is increasing at all times of positive acceleration. From Fig. 35 e and f the actual length of time during which the rate was increasing was 17 and 15 min compared with the apparent values of 5 and 3 min from a and b.

In this thesis, the data ~~was~~ were obtained as a growth rate in digital form and with a higher resolution than previously reported. Such data permitted the characteristics of the growth rate curve to be observed much more easily than the measurements on total increase in length. It may be for this reason that previously the latent period has been studied extensively but other characteristics have passed unnoticed. For example, the data of Barkley and Evans (1970) on etiolated pea stem segments can be replotted to yield a peak in growth rate followed by a lower steady-state growth rate very clearly. This can be seen in Fig. 36A which shows their data converted to rate. The fact that a second maximum is not apparent may be due to the lower resolution of their system or to the errors involved in the procedure used for obtaining the first differential which is difficult and very tedious).

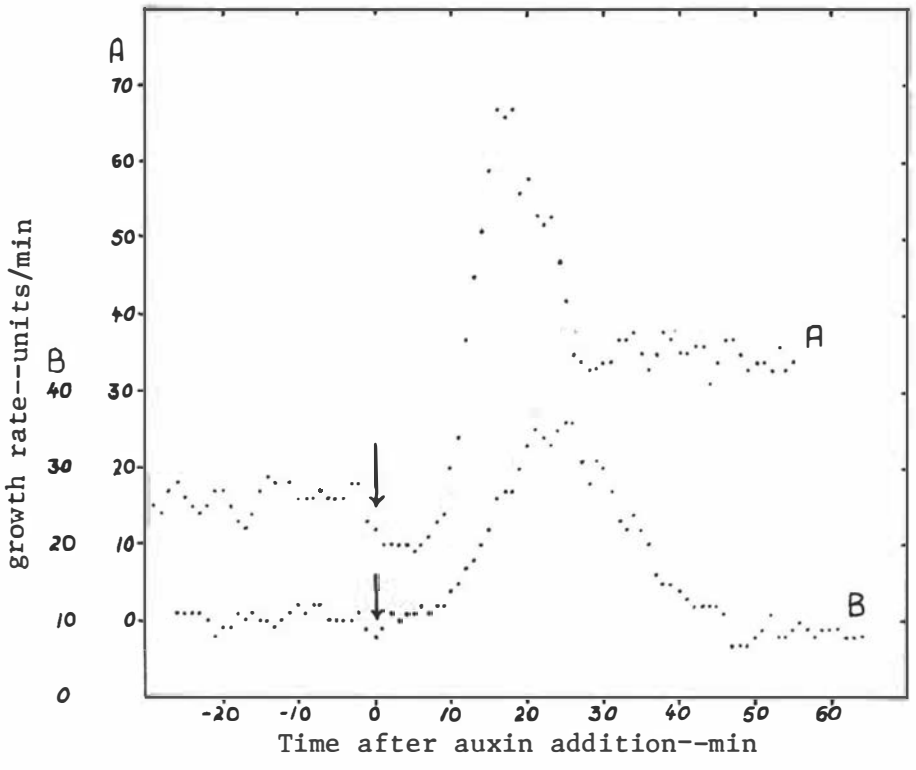


Fig. 36. Effect of IAA (A) and IAA plus cycloheximide (B) on the growth rate of etiolated pea stem segments. 10^{-5}M IAA; 2 µg/ml cycloheximide. Elongation data of Barkley and Evans (1970) differentiated. 1 unit= 2.8 µm.

Rayle et al. (1970) have published growth rate curves for maize which were obtained by calculating rates from the total increase in growth data. These curves illustrate that for maize there is no minimum. That is, the growth rate increases to a maximum rate which is apparently stable. However, the data were obtained for columns of up to 20 segments. Any slight difference between the individual segments in the timing of the peaks would tend to smooth out the curve. This can be seen in Fig. 37a which is a plot of the average growth rate of 7 lupin hypocotyl segments. The growth rate curve of each segment displayed the first and second maxima and the minimum (they were the first 7 segments in Table I). However, the variation of the timing of these characteristics, while small, was almost sufficient to mask the minimum completely. Fig. 37b is the summation of Fig. 37a and illustrates the type of data from which Rayle et al. (1970) derived their growth rates. To obtain from it the graph in Fig. 36a would be difficult, especially if the resolution was reduced by an order of magnitude.

The main disadvantages with the technique used in this thesis are that the measurements were made manually and that the segment is placed horizontally in the chamber. The latter can lead to bending of the segments which would result in loss of accuracy. The bending was minimized by the use of collars (Fig 3), and could be readily

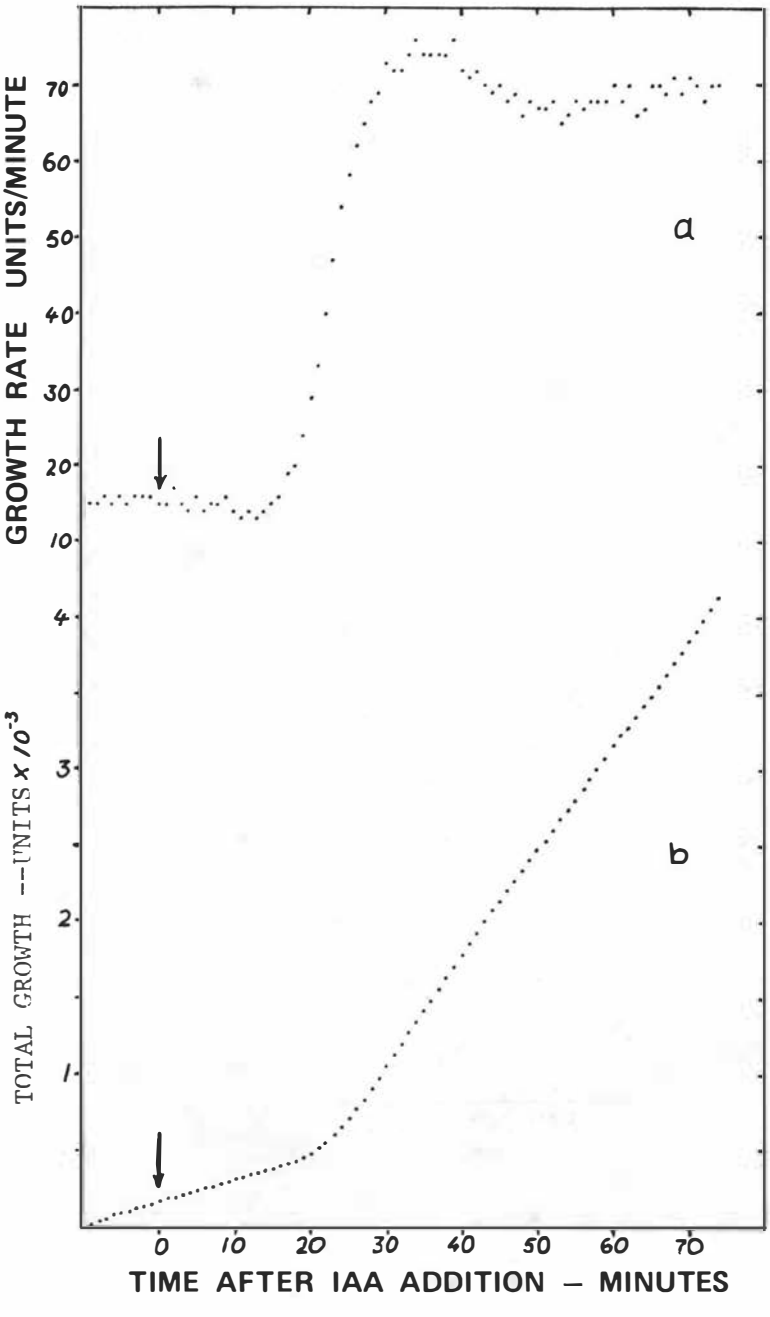


Fig. 37. Effect of averaging the growth rates of 7 segments on the a) growth rate curve and b) on the total growth curve. $3x10^{-5}$M IAA added at arrow. 1 unit = 0.23 μm.

detected because of the amount of refocussing required. The problem of bending should be eliminated by the planned design improvements of the apparatus.

The advantages of using columns of several segments, particularly if the system is automated such as that of Evans is that an average response is easily obtained although the resolution possibly may be lower. The advantages of the system used in this thesis are that the resolution is higher, the output is digital which leads to ease of data handling and individual differences between segments can be obtained. Clearly each system has advantages over the other and it would depend on the type of problem as to which would be most suitable in particular situations.

2. The growth rate curve

The latent period was described by Yamaki (1954), Köhler (1956) Ray and Ruesink (1962) and Evans and Ray (1969) for Avena coleoptiles. To date it has been shown to occur in corn (Evans and Ray, 1969; Rayle et al., 1970) and etiolated pea epicotyl (Barkley and Evans, 1970). This work established that it occurs with lupin hypocotyl and light grown pea epicotyl as well. It appears that the latent period before an increase in growth rate is a characteristic response to auxin under the conditions used here. However, the length of the latent phase is different for different species and it

is also different for the same species treated under different conditions. Under the same conditions, using the same species it is quite reproducable.

Rayle et al. (1970) have reported that the growth rate decreases within a few minutes of adding IAA - that is within the latent period. They have only been able to detect the decrease in about 50% of the samples. It should be noted that their results are obtained in analog form like a graph with the axes increase in length and time. The decrease in growth rate is detected as a decrease in the slope of the line. The results presented here shows that a decrease in growth rate sometimes does occur, but probably not to the extent that would be detectable by their methods. When a drop in growth rate occurred during the present work it was attributed to any or all of the following:-

(a) a poor reference point leading to variability in the position in the slidewire or

(b) a change in temperature or

(c) bending.

In all the work (except for the last experiments with cycloheximide), the reservoirs were kept in a water bath, however the tubing connecting them with the chamber was not. Therefore, when a change to a new solution was made there could be a lowering of temperature (especially in winter) because the first of the new solution was at room temperature and it would take some time for the

tubing to reach the higher temperature.

The length of the latent phase could be used to determine whether a compound which has the properties of an auxin, is actually an auxin or whether it has to be changed to an auxin before it is active. It could also be used to test postulated intermediates in IAA biosynthesis. It would be expected that the closer the compound was to the start of the pathway, the longer would be the latent phase. Several compounds in these categories have been tested and indolebutyric acid was the only one to give a significantly longer latent phase. Possibly this compound is metabolized to IAA before an increase in growth rate occurs. Recently Evans and Rayle (1970) have shown that indoleacetontrile which gives an extended latent phase in corn and what is converted to IAA during the latent period.

There are three known ways of altering the length of the latent phase. One is by altering the temperature. This has been shown by Evans and Ray (1969) and Nissl and Zenk (1969). Ray and Ruesink (1962) showed that the Q_{10} for the length of the latent period was about 1. It has been shown in this thesis that increasing the temperature to 28° decreases the length of the latent period. Hertel et al. (1970) have shown that when segments are treated with very low concentrations of

IAA and short segments (4 mm instead of 8 mm) are used the latent period is shorter than when high concentrations are used. Nissl and Zenk (1970) on the other hand have observed the opposite effect of IAA concentration. That is, the lower the IAA concentration the longer the latent period. The third way of obtaining a decreased latent period is by using the ethyl ester of IAA (ethyl-indol 3-yl-acetate). This has been reported by Polevoy (1967) and Rayle et al. (1970). When this compound was used with lupin (Fig. 14D) no significant alteration of the latent period was noted in the four experiments.

At the end of the latent phase, there is a rapid increase in growth rate for 12-15 min. before the rate declines to a minimum and then increases to a second maximum. This was unexpected because previous reports (Yamaki, 1954; Ray and Ruesink, 1962) had shown that the rate increased for only a short time before a new steady increase in growth (i.e. stable growth rate) was obtained. Later Evans and Ray (1969) also stated that the period of increase in growth rate was 3 min. The conflict with these observations may be for one or both of two reasons. First, their results were all based on hollow coleoptile tissue. The IAA would reach the inner cells sooner in such tissue than in the solid hypocotyls and stems used in this work. This could mean that the individual cells would all respond within a few minutes of each other whereas in lupin hypocotyl tissue they

would take longer. The second reason may be because their output was in terms of increase in length as illustrated in Fig 35 a and b.

The decline in growth rate after the first peak has not yet been reported. Hertle et al. (1970) and Barkley and Evans (1970) have published graphs of total increase in length (tracings of their analog output). The curves for some of their treatments particularly those of etiolated peas are very similar to Fig. 34 a and b. No comment was made about the decrease in growth rate. However when the rate is measured from their graphs and then plotted as in Fig. 36 it can be seen that the resulting curves are similar to those obtained in this work. It is in fact quite difficult (and tedious) to obtain differentials from such data and the errors involved in obtaining the data for Fig. 36 are large. The method is also 10 times less sensitive than that used here. This may account for the fact that a second maximum is not shown in Fig. 36a. Also it may account for the lack of the first peak in those growth rate curves for maize published by Rayle et al. (1970).

It has now been established that the latent period is a typical auxin response. At the end of the latent period there is a rapid increase in elongation rate to a maximum. In some tissues at least there is a decline in growth rate a few minutes after the maximum is

reached so that a lower growth rate is attained. After this minimum the growth rate increases to a second maximum which may be lower than the first maximum.

3. Explanations of the growth rate curve

Evans and Ray (1969) constructed a model to account for the latent phase. Their assumptions, based on their experimental evidence were that there was a latent phase of approximately 10 min after which it took only 2 -3 min before a new steady state rate occurred. (This assumption has now been shown to be too low by a factor of 3-5 for lupins and peas). They also assumed a series of irreversible, first order reaction steps which were at equilibrium. The time courses of concentration change of end product following a step up change at the beginning of the pathway can be predicted. The nature of the time course depends on the number of steps between the initial and final steps -- as they increase a latent phase appears and gets longer, and the length of time before steady-state concentrations are reached increases. They showed that an appreciable absolute latent period is to be expected if four or more kinetically comparable steps are involved in the chain of response.

Another source of delay before an auxin-induced response could be the time required to assemble polymers such as RNA or protein from their monomers (if the synthesis of macromolecules is involved in the response

to auxin). These different sources of latency contributing to the latent period would have additive effects. Evans and Ray (1969) calculated that to reach steady-state concentrations would take about 5 times the half life of the intermediate that has the longest half life.

Having made these conclusions, Evans and Ray (1969) used them to examine critically some of the hypotheses for auxin action. The first two were:
1) the gene activation hypothesis which claims that auxin causes cell enlargement by the induction of certain species of RNA which code for protein(s) that promote cell enlargement, (Category I, p.54) and
2) that auxin acts at the translational level to form a growth specific enzyme (Category II, p.55).
In their view these hypotheses fail because predicted increases in the length of the latent period do not occur with inhibitors of RNA and protein synthesis and because the known half-lives of m-RNA and enzymes are too long to account for the latent period and the rapid attainment of the new steady-state growth rate level.

Evans and Ray (1969) proposed that growth depends on the continual formation of a stable protein which would function to promote growth only at the time of formation. It would therefore be a "structural protein". Further evidence that the protein would be structural rather

than enzymatic came from the experiments of Evans and Hokanson (1969). They found that the growth rate started to decrease about 10 min after removing auxin. If the protein involved were an enzyme, then it would have a half life shorter than that known of higher plant enzymes such as nitrate reductase in cauliflower (Afridi and Hewitt, 1964). Nevertheless Evans and Ray (1969) were still not able to propose a model which would explain both the latent period and the comparatively rapid rise to a new growth rate.

Rayle et al. (1970), as mentioned before, have shown two short term effects of auxin - the reduced latent period with low concentrations, and the transient decrease in growth rate when a large step-up in auxin concentration occurred. Using these facts, (although the latter may be in doubt) Rayle et al. (1970) constructed two alternative hypotheses. The first was that auxin interacted with recognition site X but when a high concentration of auxin was applied it would interact also with a different site Y. The result of the interaction with Y would be an inhibition which diminished with time.

Their second hypothesis again involves the interaction of auxin with a recognition site, but when a large step up in auxin concentration occurred, the binding to the site would cause an overexcitation and

paralysis. They propose that the site may be on a membrane. This is because Hertel et al (1969) have shown that there is a close correlation between the amount of growth induced by auxin analogs and their polar transport. Rayle et al., (1960) have shown that auxin stimulates its own polar transport within 20 min of application. This is another of the early effects of auxin all of which can be explained by auxin acting on the plasma membrane. The binding to the plasma membrane would cause reversible changes to the membrane conformation which could provide a basis for active secretion. (Rayle et al., 1969; Hertel and Flory, 1968). Anker (1953) also proposed that the site of auxin action was on the membrane. He thought that the site was on the lipophilic interfaces making food reserves more accessible to metabolic processes.

Nissl and Zenk (1969) agree with this hypothesis in that the primary site of auxin action causing the initial elongation must be on a preformed system. They postulate that it may involve an allosteric activation of an enzyme such as that proposed by Cleland (1968) which mediates the cleavage and reformation of cell wall polymers.

The results of the kinetic experiments in this thesis will be considered along with the results on RNA and protein synthesis at the end of this discussion where

all the results will be integrated into a model.

4. "Endogenous Growth"

At this point some comment must be made about the relationship of auxin-induced growth to endogenous growth. With respect to excised tissues the term endogenous growth has been used in the literature to describe growth occurring without exogenously supplied auxins. However, what is meant by this term varies from author to author. For example Nissl and Zenk (1969) pretreated their segments for 1 hour before measurement; Evans and his group for up to 0.5hr.; in the present work 1.5 to 2 hr. Segments for bioassays commonly are cut and pooled in a comparatively small volume of medium before the start of the bioassay. It is well known that auxin is transported in a polar manner and that this transport occurs in excised segments. The amount of endogenous auxin in the tissue would decrease with increasing length of pretreatment. The endogenous growth would presumably vary accordingly. It is probable that segments that were being pretreated to deplete the endogenous auxin were being bathed in dilute exogenous auxin. The amount of auxin would depend on the amount of tissue, the volume of medium and the length of time the segments were in the medium.

An implicit assumption is usually made that endogenous growth is due to low endogenous levels of

auxin and that auxin-induced growth is merely a magnification of endogenous growth. In other words, that exogenous auxin acts in the same way and on the same processes as those leading to endogenous growth but increase their rates. The following evidence indicates, however, that the two processes may be different:

1. It takes much longer for cycloheximide to inhibit endogenous growth than auxin-induced growth. Auxin-induced growth requires a protein whose mean life of availability for auxin action is about 17 min. It follows that if the same protein is required for endogenous growth, inhibition after cycloheximide addition should occur within this time. It does not. There is a gradual inhibition becoming apparent at 40-50 min, but growth is still occurring at 200 min.

2. The growth rate of segments treated without auxin is unaffected by actinomycin D for 6-10 hr. However after 6-10 hr segments in buffer without actinomycin D often show an increase in growth rate and this increase is prevented by actinomycin D (see Fig.5). This increase may require additional RNA synthesis and may not therefore be what is normally thoughtof as endogenous growth. Again, this affect occurs after a much longer length of time than the effect on auxin-induced growth.

3. Cleland (1959) has shown that the effect of turgor pressure on the two types of growth is different. For auxin-induced growth there is a critical turgor pressure below which auxin-induced growth does not occur. This is not the case for endogenous growth. Endogenous growth varies directly with turgor pressure as long as it is greater than zero. The effects of turgor pressure on growth are paralleled by effects on cell wall extensibility.

4. Anaerobic conditions reduce the auxin-induced growth rate by about 80% to a lower level of about 10 units/min (2.3 μm/min) (unpublished work from this laboratory). This rate is constant and not due to photosynthetic oxygen evolution because it is not affect by DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). If the endogenous rate is relatively high (30 units/min) then it is reduced to about 10 units/min by anaerobic conditions. Thus the endogenous rate is different from the lower basal rate and may approach it as the endogenous auxin is lost from the segment.

5. Competitive inhibitors of auxin-induced growth do not affect endogenous growth, The results of McRae and Bonner (1953) show

4-chlorophenoxyisobutyric acid, 2,4-dichlorophenoxyisobutyric acid and 2,4-dichloroanisole all inhibit IAA - and 2,4-D- induced growth but have no effect on endogenous growth.

The relationship between auxin-induced growth and endogenous growth remains to be elucidated. In the short-term kinetic experiments in this thesis, each segment (unless otherwise stated) was pretreated for at least 1.5 hr in flowing buffer. Except for the experiments using cycloheximide at 28°C, the buffer was not recirculated. The rate of auxin transport has been reported to be 10-15 mm/hr (Morris et al., 1969), therefore the segments were probably almost completely depleted of diffusible auxin.

B. Biochemical Theories.

1. Category I - RNA synthesis is required.

The current theories of auxin action, based on short-term kinetic evidence, have been outlined. The major theory which is based on biochemical evidence was first propounded by Key and his group (e.g. Key and Ingle,1964,1968) and Noodén and Thimann (1963, 1965, 1966) and later joined and extended by Masuda (e.g. Masuda, 1968; Masuda and Wada, 1966). In essence, IAA is postulated to stimulate the synthesis of DNA-like RNA which is termed "growth essential RNA". (Ingle and Key, 1965a,b). The actual mechanism by which auxin does so, is by stimulating RNA

polymerase activity which is thought to be limiting (O'Brien et al., 1968). The "growth essential RNA" fraction which is assumed to be m-RNA (and has a half-life of 1.5 to 2 hrs (Ingle et al., 1965)) is postulated to code for "growth essential protein"(Key and Ingle, 1968; Masuda and Tanimoto, 1967). Masuda (1968) postulates that this protein is a hemicelulase such as β -1,3 glucanase which loosens the cell wall, increasing the reversible extensibility (Masuda, 1969a). In short term kinetic experiments this enzyme applied externally increased the growth rate of etiolated pea stem segments in 10 min. (Masuda and Wada, 1967) and increases reversible extensibility by 30 min (Masuda, 1969b).

The arguments of Evans and Ray (1969) give theoretical evidence against the stimulation of m-RNA synthesis as one of the first actions of auxin, particularly because of the long half-lives (hours to days) of RNA and proteins of higher organisms. However the work of the groups of Key and Masuda can be criticised on other grounds. The first is based on evidence from the short term kinetics. Most of Key's work, is based on labelling periods of 2 hours - a time in which the auxin-induced elongation has been proceeding for at least 1.5 hours. To determine causal relationships under such circumstances is not possible. The shortest labelling time used by Masuda in which undenatured RNA

was extracted and separated (by MAK column chromatography) is one hour. He has reported work (Masuda and Kamisaka, 1969) in which tissue was treated for 10 minutes and then the total RNA extracted by the perchloric acid method. In this method the RNA is hydrolysed and the radioactivity of the hydrolysate is determined. He found 25% increase due to auxin, but it was not determined whether the label was in the m-RNA fraction. There are, however more serious grounds for criticism.

In this thesis DNA synthesis only occurred when the incubation medium was accidentally contaminated. The results of the experiments of Masuda's group (e.g. Masuda and Tanimoto 1967) shows that DNA synthesis occurred. In fact in those treatments in which mRNA synthesis was enhanced, enhanced DNA synthesis also occurred. Masuda did not use a bacteriostatic compound in the incubation medium. The plant material used by Masuda (Avena coleoptiles and etiolated pea third internode) under their conditions was growing by cell elongation. It would be expected therefore that DNA synthesis would be very low. Therefore it seems probable that the DNA was synthesized by a contaminant. It follows that it would also be synthesising RNA. It is necessary to determine whether the RNA synthesis occurring during the 10 minutes after auxin addition was due to auxin or to a contamination.

The same criticisms can be leveled at the work prior to about 1967 of Key's group, although, this is denied by Sabota et al. (1968). After that time they included streptomycin in the growth medium. Hock (1967) has shown that results similar to those obtained by the groups of Masuda and Key will arise when Pseudomonas is added to the incubation medium of aseptically grown tissue. With these bacteria even high concentrations of penicillin or streptomycin had no effect on bacterial growth.

Trewevas (1968) agrees with Key and Masuda that the action of auxin is on RNA synthesis but disagrees on the fraction that is synthesized. Specifically his hypothesis is that auxin increases the rate of synthesis of r-RNA and therefore increases the level of poly-ribosomes. By DNA hybridization and differential centrifugation techniques Trewevas (1968) showed that the first detectable IAA-induced increase in r-RNA was one hour after the addition of auxin. The first increase in label of RNA was detected in the nuclear fraction and by 2 hours some appeared in the cytoplasm. By 12 hours the levels of polyribosomes had increased (Trewevas, 1968). While in 12 hours there is no increase in total protein (1968b), by 1 hour there is a change in the pattern of proteins as eluted from Sephadex G-100 (Patterson and Trewevas, 1967)

Trewavas has made some significant contributions to the understanding of auxin action, both in the questions he has tried to answer and in the techniques he has used. However his conclusion that an increase in r-RNA causes cell elongation is difficult to understand since he himself has shown that it took 2 hours before it reaches the cytoplasm.

This points to the major criticism of the hypotheses in category 1. Little attempt has been made by the groups of Key or Masuda to determine whether the requirements outlined on p57 are met. It is important that these criteria be met in establishing a theory.

2. Category II - Protein synthesis is required

The present work indicates that the hypotheses in Category 1 are not tenable. The long and short term kinetic experiments with actinomycin D provide substantial indirect evidence against these theories. The failure to detect auxin-stimulated RNA synthesis within 20 min or 40 min seems to rule out the possibility that the stimulation of RNA synthesis can be the cause of either of the maxima of the growth rate curve. Although this is the most direct demonstration that the primary action of auxin does not involve stimulation of RNA synthesis, the weight of indirect evidence has been against it for some time. Penny and Galston (1966), Nelson et al. (1969) and Nissl and Zenk (1969), on the

basis of long term kinetic evidence with actinomycin D, all using different types of tissues came to this conclusion. They all found that protein synthesis inhibitors reduced auxin-induced growth sooner than did actinomycin D. In addition, Cleland (1965) found that actinomycin D had little effect on the extensibility of the cell wall. Taking a more theoretical approach in assessing various theories, Evans and Ray (1969) showed that the stimulation of RNA synthesis was not likely to be the primary action of auxin.

The work of Fan and Maclachlan (1967) can be interpreted as evidence against the hypothesis as well. They replaced the apex of etiolated pea seedlings with lanolin which contained auxin. In some of the treatments, the lanolin also contained actinomycin D or puromycin. The plants were observed over several days. Those plants which had IAA alone formed a large callus just under the lanolin; there was a very large stimulation of RNA and cellulose synthesis, and an increase in fresh weight. On the other hand, those plants which were treated with actinomycin D and puromycin as well showed greatly enhanced elongation, no swelling and little increase in fresh weight. RNA and protein synthesis (in this case cellulase in particular) were required for the increase in fresh weight and for cell division (both of which were stimulated by IAA), but they were not required for elongation. It follows that an "unmasking" of DNA was

required for cell division but not for elongation. Since elongation continued for several days after the decapitation, it follows too that the half-life of the m-RNA for the enzymes required for elongation must be rather long-in the order of days rather than hours. If the DNA - like RNA described by Ingle et al. (1965) does participate in auxin-induced elongation, its short half life may be an illustration of the constantly changing processes occurring after excision that were described by Audus (1952) (see p.8).

A further illustration of Audus' description occurs in the present work. The amount of RNA synthesis changes with time after excision. For example no RNA synthesis could be detected immediately after the pretreatment period (1.5 hr.) but it could be detected readily 5 hr. later (6.5 hr. after cutting). The increase in synthesis was greater in the control segments than those treated with IAA. This may be associated with the increase in growth which often occurs in control segments 6-10 hrs after the start of long term kinetic experiments. The same increase does not seem to occur with IAA treated segments. The same effect on green pea stem segments has been reported before (Penny and Galston, 1966).

The weight of the evidence is against hypotheses in category 1. They are, therefore, improbable even although one of them has reached text-book status

(Thimann, 1969).

The results of experiments investigating the requirement for protein synthesis can be considered in relation to hypotheses in category 2. These postulate IAA action at the RNA level to stimulate synthesis of protein required for growth.

Direct attempts to detect auxin-stimulated protein synthesis led to ambiguous results. According to the postulate of Evans and Ray (1969) auxin stimulated incorporation into membrane fractions (DOC soluble and DOC insoluble) could be expected. In one experiment the synthesis of two proteins in the DOC soluble fraction appeared to be stimulated by auxin. They were not detected in a second experiment. Patterson and Trewavas (1967) have shown that the synthesis of some protein fractions eluted off Sephadex G 150 columns is stimulated by IAA after 1 hr treatment.

The results of the cycloheximide experiments were less ambiguous. When auxin was supplied to segments simultaneously with, or up to 30 min after cycloheximide, auxin-induced growth occurred, but was not sustained for long periods of time. IAA therefore seems to act on pre-existing protein but further protein synthesis is soon required. Similar results with cycloheximide pre-treatment for 35 min have been obtained by Barkley and

Evans (1970) (see Fig. 36b) although they did not conclude that auxin acted on a preexisting protein.

It appears then, that the hypotheses in category II must be rejected. Since protein synthesis is required soon after the initial action of auxin, it can be concluded that

a) the site of primary auxin action is in the DNA ⟶ RNA ⟶ protein --> cell elongation sequence and

b) that any postulate of primary auxin action would be in category III.

By calculating the amount of auxin-induced growth which occurred after protein synthesis had been stopped for various lengths of time in cycloheximide, it was possible to estimate the half-life for the availability of a protein on which auxin acts. This half-life was approximately 12 min. with a mean life of about 17 min. If more than one protein is involved, then the 12 min would represent that of the protein having the shortest half-life, because its concentration would become limiting before the other(s).

These conclusions have been drawn with the assumption that cycloheximide inhibits only protein synthesis. There has been indirect evidence (MacDonald and Ellis, 1969) that cycloheximide may affect other

processes such as uncoupling respiration and thereby decreasing ion uptake in carrot discs, although Kirk (1970) found no such effect in Euglena. However in the present work, no effect on oxygen uptake was detected. In an experiment not reported here, there was very little effect of a 60 min treatment of cycloheximide on the incorporation of ^{32}P into organic phosphates. It appears, then that the assumption was valid for this tissue.

C. The primary site of auxin action.

The hypotheses current at the start of the work ranged from action within the nucleus to direct action on the cell wall. The range of alternatives for experimental investigation was very large. Therefore the basic approach used was to ask a question which divided the range of alternatives into two major groups. The answer to the question would then indicate which of the two groups of alternatives could be discarded as not containing the site of action. The process could then be repeated on the range of alternatives that remained.

The first question that was asked was "Does auxin action depend on an effect on RNA synthesis?" The answer was no. Therefore all processes preceding RNA synthesis, for example effects on DNA transcription, could be ignored. The remaining range of alternatives included the interaction of the various species of RNA

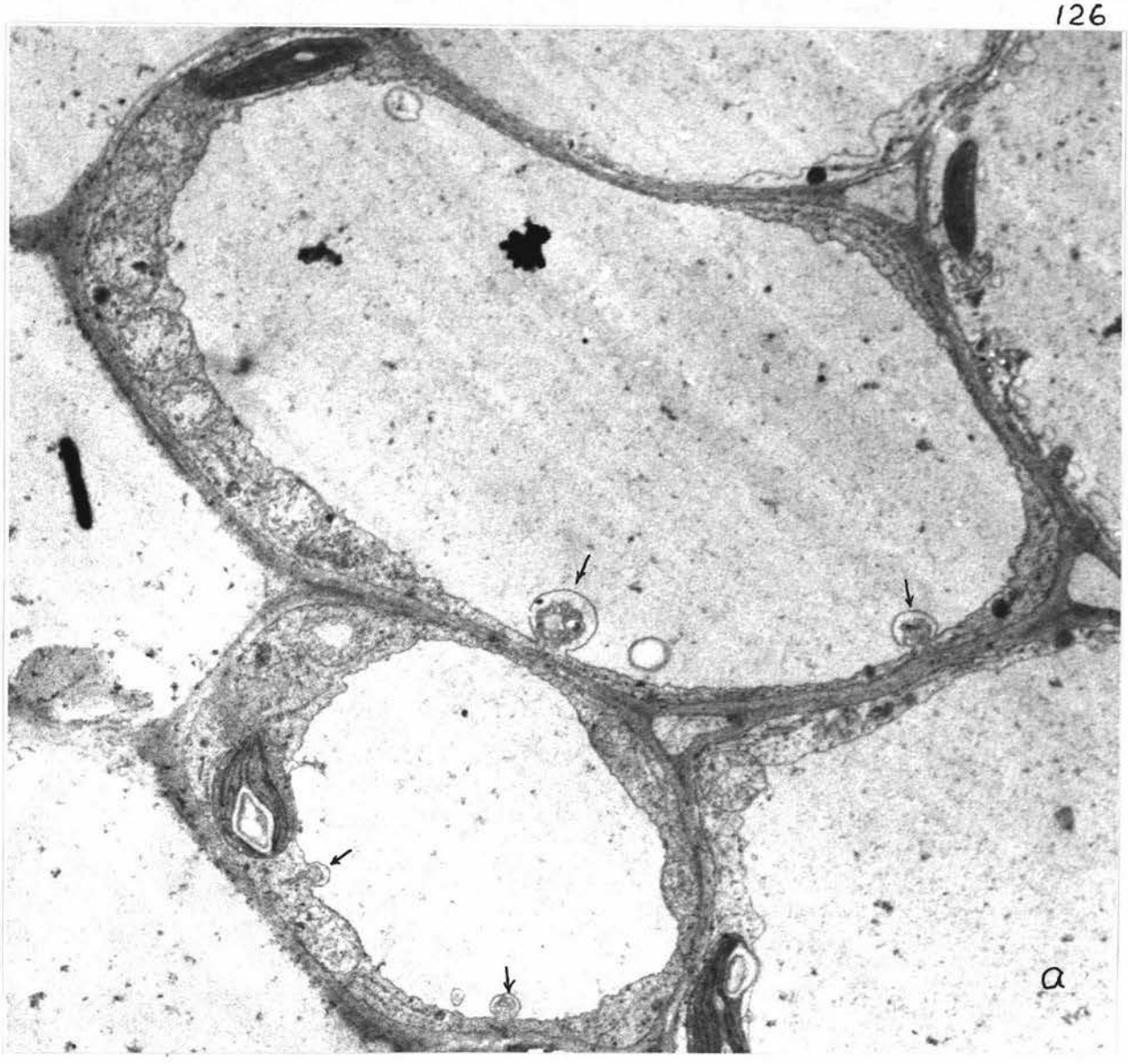
in the synthesis of the protein, synthesis of protein itself or many other possibilities which would not involve these processes such as action in membranes. This range of possibilities was divided into two groups by asking the question "Does auxin action depend on an effect on protein synthesis?" Again the answer was no, but this time it was qualified. Auxin does not require protein synthesis in order that elongation be increased, however protein synthesis is soon required, and some of the characteristics of the protein(s) are known. This precludes auxin acting at the translational level and suggests that it acts on or with a protein.

The half life that was obtained for the protein was unusually short. (It is similar to that estimated by Evans and Hokanson (1969)). This suggests that the half life obtained was a measurement not of rate of degradation of an enzyme (usually much longer for higher organisms Evans and Ray (1969), Dowbin (1969) but of the time that the protein is available to be acted upon or with auxin. In other words after synthesis the protein moves to a compartment where it is not accessible to auxin, and auxin may only act on or with it during the period after synthesis and before entering the compartment. The half life is the average length of that time period for the protein molecules. This interpretation is similar to that of Chrispeels (1970) for the hydroxylation of proline in the

hydroxyproline-rich cell wall protein. Chrispeels has evidence that the proline in the protein can only be hydroxylated for a short time (about 10 mins) before it enters a membrane-bound compartment and becomes inaccessable to hydroxylating enzymes.

Chrispeels was able to isolate membrane-bound organelles which contained the hydroxyproline-rich protein by differential centrifugation. A search was made of electron-micrographs of lupin hypocotyl segments to see if there were organelles which could possibly fulfill a similar role for the protein postulated to act with or to be acted upon by auxin. Fig. 38a illustrates a cell which is typical of the non-vascular cells of lupin hypocotyl. They are highly vacuolated, contain chloroplasts and other structures common to most cells of higher organisms such as nuclei, mitochondria, golgi bodies, ribosomes, endoplasmic reticulum etc. They also contain paramural bodies, structures which are postulated to be involved in cell wall synthesis (Marchant and Robards, 1968). Examples of paramural bodies are indicated in Fig. 38a. Fig. 38b illustrates a paramural body at higher magnification. The structure is enclosed by the plasmalemma and the tonoplast membrane and contains vesicles. The paramural bodies are extremely common. In a low power electron micrographs of sections from two different plants there were approximately 2 per cell. The average cell length

Fig. 38. Electron micrographs of transverse sections of lupin hypocotyl. Magnification (a) 8,500X (b) 53,000X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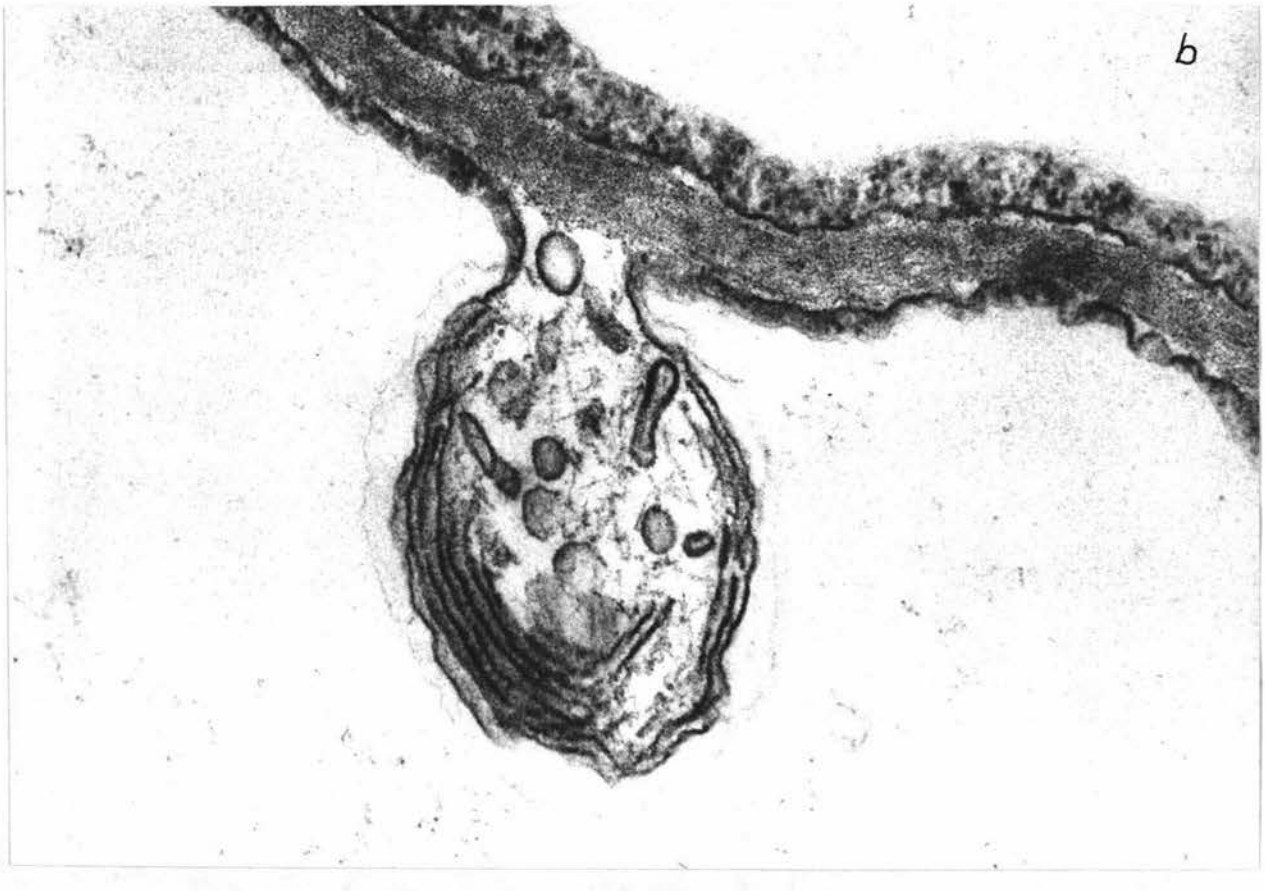
b

was 15 µm. Since the sections were 50 - 70 nm thick there must be about 300 - 500 paramural bodies per cell.

The paramural bodies are abundant in both auxin treated (for 35 min) and non-auxin treated tissue and occur more frequently than golgi bodies. They may play a role in cell elongation in lupin hypocotyl and if so may be the compartment to which the growth-requiring protein is transported.

D. A Model for the Primary Mechanism of Auxin Action.

Fig. 39 is a diagram of a portion of a transverse section of lupin hypocotyl. The organelles which probably do not contain the site of auxin action are indicated with a broken line. The chloroplast is not necessary since auxin-induced elongation occurs in tissues that do not contain chloroplasts. The nucleus ribosomes and endoplasmic reticulum can all be discounted because RNA and protein synthesis are not required for the initial action of auxin. Mitochondria are necessary only to provide the energy for growth and the vacuole supplies the force via turgor pressure.

On p.48 ~~is~~ are outlined three possible hypotheses to account for the growth rate curve. The evidence for each of these will be considered.

The first explanation is that auxin affects two

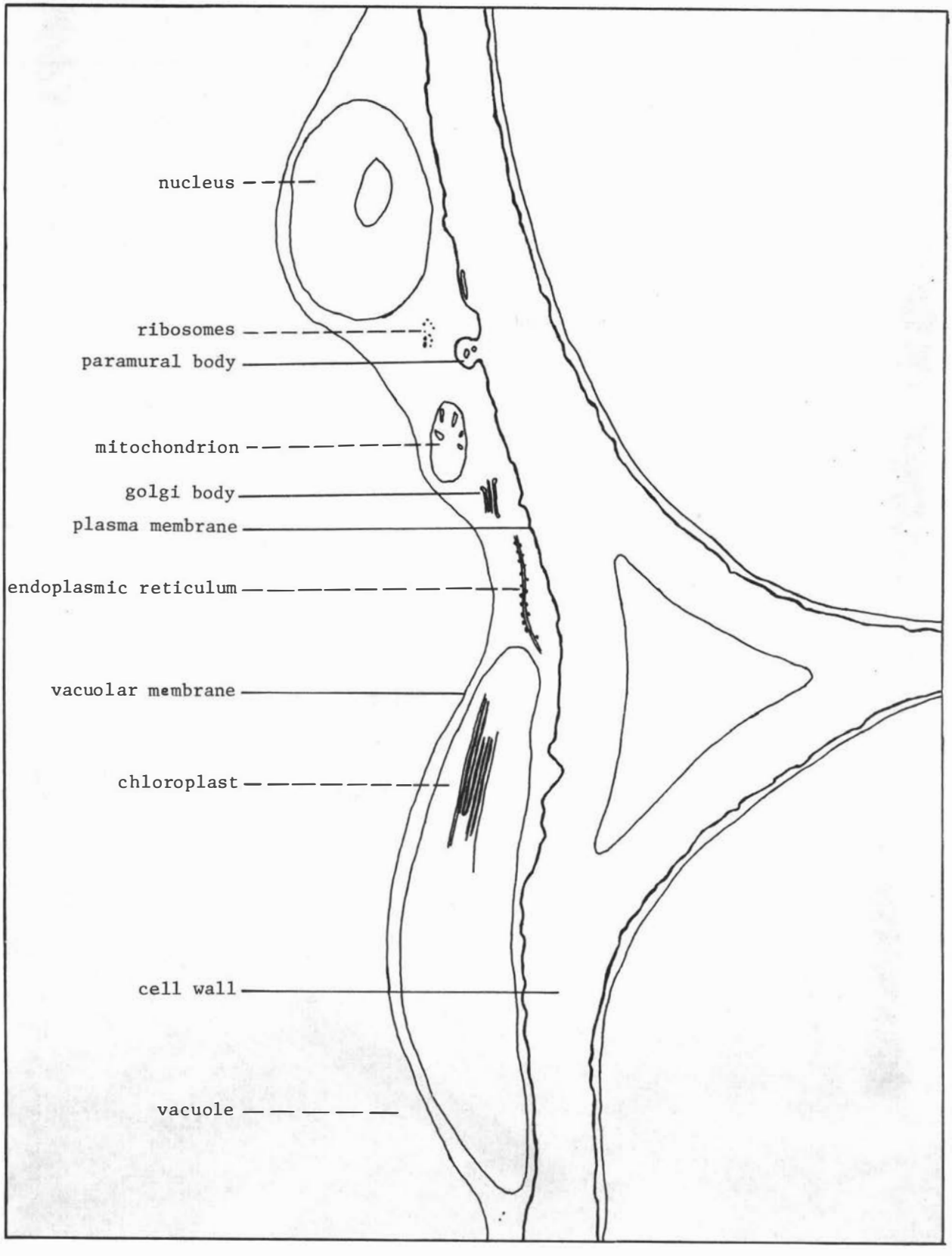

Fig. 39. Diagram of transverse section of lupin hypocotyl. The dashed lines indicate those structures which do not contain the site of primary action of auxin.

processes which are separated in time. The first process would be of short duration and the second would be of much longer duration, and equivalent to that which is measured in long term kinetics. It was Cleland (1967a) who first suggested this possibility to reconcile his findings that maximum plastic extensibility occurred 90-120 min after adding auxin while the growth rate reached a maximum after 10-15 min. He postulated that changes in turgor pressure could have accounted for the difference. He worked with Avena coleoptiles and it has been found that lupins react in a similar way, (Penny et al., 1971). The plastic extensibility starts to increase at about 40 min - a time when the acceleration of growth is at a minimum - reaching a maximum at about 60 min. This could account for the second maximum in growth rate. The work of Yoda (1961) and Yoda and Ashida (1961) demonstrated that in etiolated pea stem segments there were transient changes in osmotic potential after auxin treatment. They found that auxin rapidly increased the osmotic potential until 30 min, after which there was an equally rapid decline to values less than before auxin was added. Although these kinetics do not follow exactly the kinetics of the first growth rate peak, they could be a possible explanation for it. The possibility was checked by determining the freezing point depression of the sap_ various times after adding auxin. This was done by the method of Gross (1954) as modified in Fig.12.6 of Giese

(1968). However no significant differences were detected. Also no significant differences were detected in the pH of the cell sap at various times after auxin addition.

At this stage, it does not seem likely that changes in osmotic potential can account for the first peak. The increase in plastic extensibility occurs at the correct time to account for the second maximum. However, this timing may be coincidental. The segments upon which the measurements were made were killed and dehydrated after auxin treatment and the extensibility of the rehydrated cell walls measured using an Instron stress-strain analyser. There is no evidence that such measurements reflect the dynamic state of the tissue particularly since the element time is not included in the measurement.

The hypothesis has been investigated in a different way. If the two maxima are due to different reactions and if there are different receptors for the two reactions, then it may be possible to find compounds that would elicit one of the reactions more than the other. There have been suggestions to explain results obtained with some synthetic auxins that there may be more than one site that responds to auxins and that some synthetic auxins direct or may activate one more than the other (e.g. Libbert, 1957; Burstrom and Hansen, 1956; Katsumi, 1961). It should be noted that the second hypothesis (the negative feedback hypothesis) would

lead to different predictions in this respect - it should not be possible to find compounds that would elicit one of the two growth maxima to a much greater extent than the other.

No evidence for two discrete sites was found from the small number of auxins tested but there is some evidence that different auxins could result in a different relative heights of the first peak to the second maximum.

The main support for this hypothesis is the increase in plastic extensibility which coincides with the second maximum. As yet there is no explanation for the first maximum since the possibility of an increase in osmotic potential does not seem likely. However, a suggestion of Cleland (1967b) would be worthwhile investigating. He suggests that two types of bond may need to be broken to get deformation of the cell wall. One is strong and the other is weak (hydrogen?) bonds. The Instron technique may only measure the contribution of the strong bonds. The first peak could be the result of breaking the weaker bonds. Uhrström (1969), using a dynamic technique to measure elasticity, found a more rapid increase in the elasticity of the wall and it is possible that this technique could measure a change in these weaker bonds.

The second hypothesis presented on p.48 to explain the growth rate curve was that of negative feedback with underdamping. This model assumes only one effect of auxin, and therefore that the effect on the plastic extensibil ity is not the cause of the actual elongation process but is a consequence of it.

Outlined in Fig,40 is a model incorporating the known facts of the initial action of auxin. The important features of this include an internal pool of IAA in the cytoplasm which changes with the external concentration because the growth rate is proportional to the external concentration of IAA and not to the total IAA taken up by the tissue (i.e. the total internal concentration) (Andreae,1967). It assumes that IAA acts on a pool of recently synthesised protein and causes the first peak. Because of the cycloheximide pretreatment results it is concluded that the protein is in this pool ("active protein pool") for about 10 minutes before it is removed to an "inactive protein pool". Evans and Ray (1969) have pointed out that it is unlikely that in plant cells proteins are synthesised and hydrolysed at such short intervals. For this reason it has been assumed that protein from the "active pool" is transported into an inactive compartment (where IAA cannot act on it) perhaps in a membrane or cell wall or some membrane-bound compartment.

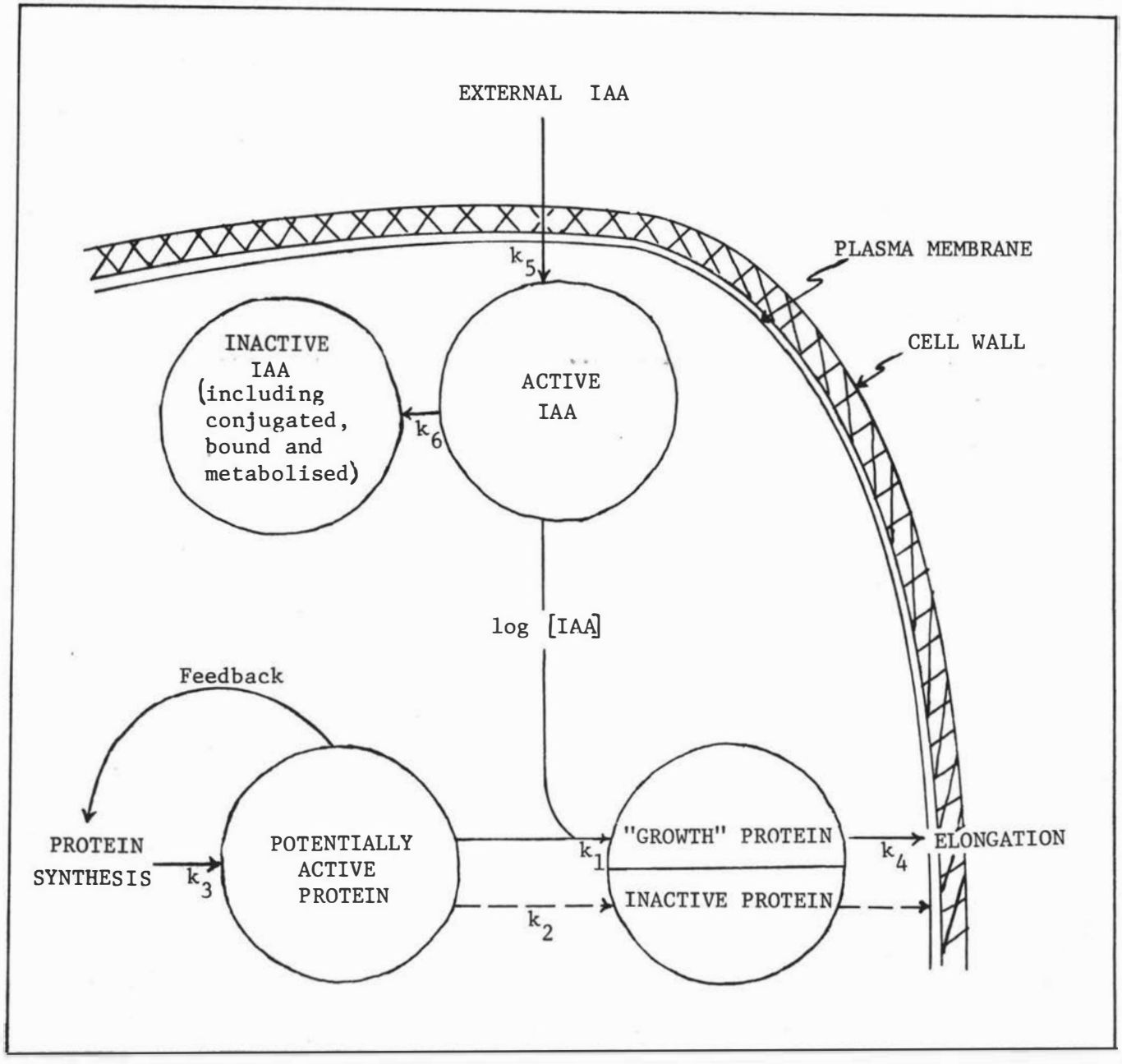

Fig. 40 Schematic representation of a model to account for the interaction of IAA and protein in cell elongation.

The minimum and second maximum in the growth rate (Penny et al., 1971) can be explained if the "active protein pool" inhibits its own synthesis either directly or indirectly. In the absence of IAA a steady state would be attained. The addition of IAA results in a depletion of the "active pool" (hence the minimum) but this reduces the inhibition of synthesis of the protein and a new steady state develops. Once this steady-state has been reached, continuous protein synthesis would be required and the rate of protein synthesis would be limiting. A similar, but less detailed model has been proposed by Galston and Davies (1969).

To be useful a model should meet at least two criteria. It must first be shown that the model is valid in that it can quantitatively and not just qualitatively predict the observed phenomena. And then it should lead to predictions that can be tested by additional experiments. The model proposed here (Fig. 40) has been evaluated quantitatively on a digital computer using a standard digital analog simulator program. By choosing suitable time and rate constants it is possible to duplicate the growth curves for IAA. It should be pointed out that this model could explain the second part of the growth rate curve in hypothesis I.

The third model assumes that the first maximum is the result of "nascent growth" which requires auxin

for expression. The second maximum would be the result of auxin-stimulated biochemical processes, such as those outlined in the model. The main support for the hypothesis comes from the fact that it is possible to obtain the first peak in the absence of the second. This may be done by giving short treatments of IAA - only 5 min is required for the first peak to occur whereas more than 15 min of IAA are required for the second. When cycloheximide is added concurrently with IAA, or at 28° 30 min before IAA only the first peak occurs (albeit greatly reduced). This would imply (according to this model) that the "nascent growth" was a result of protein synthesis which occurred before IAA addition. This in turn would imply that the factor(s) responsible for "nascent growth" were in a steady-state since the height of the first peak does not seem to be associated with length of pretreatment in buffer. If it were not turning over, the height of the peak would be proportional to the length of pretreatment.

The major difficulty with the model is that it is not possible at this time to predict a cause for the first peak - especially one which would require such a long latent period for expression. The analogy that was given was with the return of a segment from mannitol to water and the consequent return of the cells to full turgidity and the expression of stored growth. The former was instantaneous, although the latter may have

taken longer.

A possible cause for the first peak would be an IAA-induced alteration of the reflection coefficient. It is the ratio of:

$$\frac{\text{the flow of water due to permeating solutes}}{\text{the flow of water due to non-permeating solutes}}$$

The reflection coefficient is an important parameter of the membrane and in the equation determining the flow of water across a membrane due to permeating solutes it multiplies the difference in osmotic pressure on either side of the membrane (Dainty 1969). The ratio is negative when the membrane is more permeable to the permeating solute than to water; it is zero when the permeability to the solute and water is the same, and positive when the membrane is less permeable to the solute than to water. An increased reflection coefficient indicates a decreased permeability to permeating solutes. If auxin increased the reflection coefficient then the membrane would be less permeable and the rate of outflow of solutes from the cells decreased. This would increase the osmotic potential, increasing the inflow of water thereby increasing the turgor pressure of the cells.

The second maximum could be caused by any of the processes postulated in models 1 and 2.

So far all attempts to determine the effect of auxin on protein synthesis directly have led to negative results. It was therefore necessary to resort to indirect methods to determine some of the properties of the protein(s) involved. Now that some properties are known, it should be possible to make direct measurements. It should be relatively easy to determine which (if any) of the proposed models is operative because of the strict timing requirements which are imposed on each by the growth rate curve.

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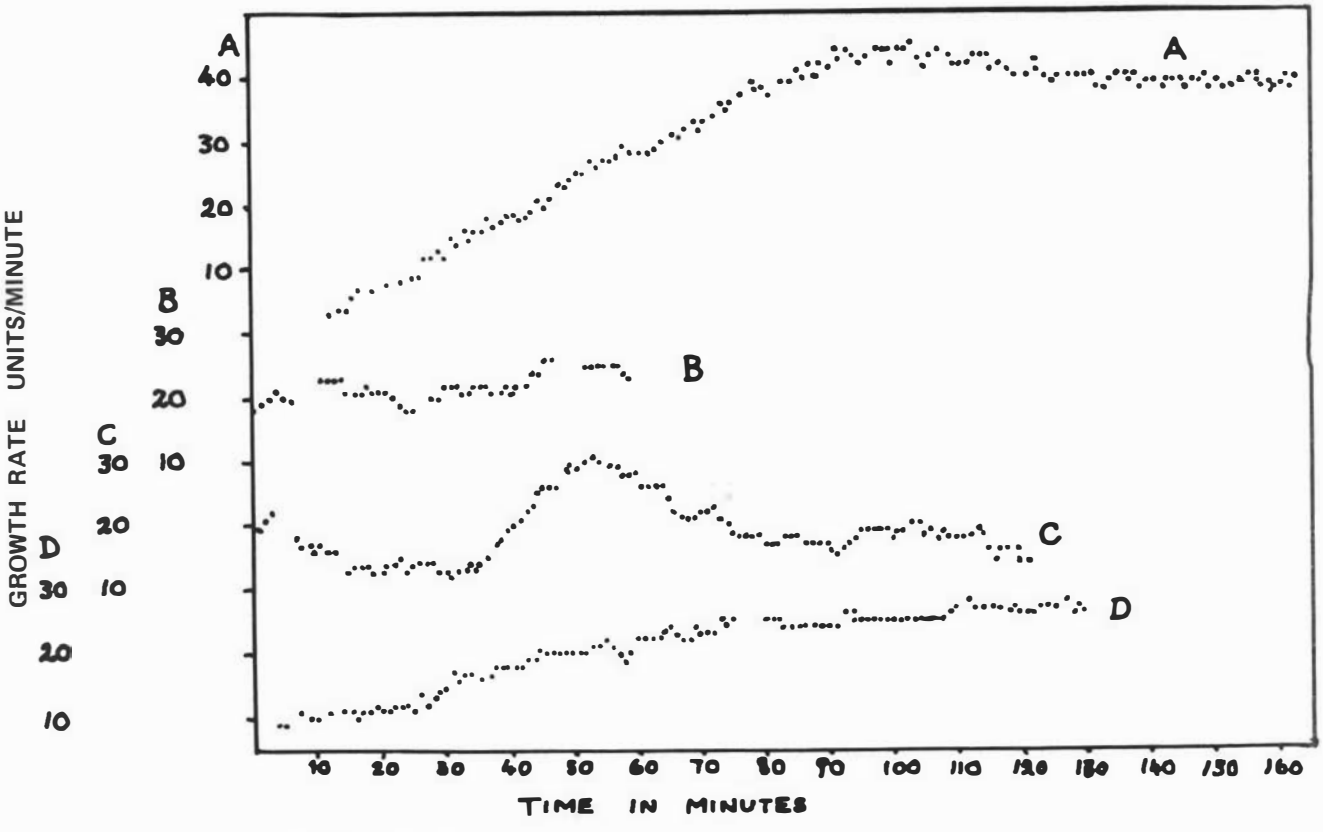
Theories are nets: only he
who casts will catch.

NOVALIS

A P P E N D I X I

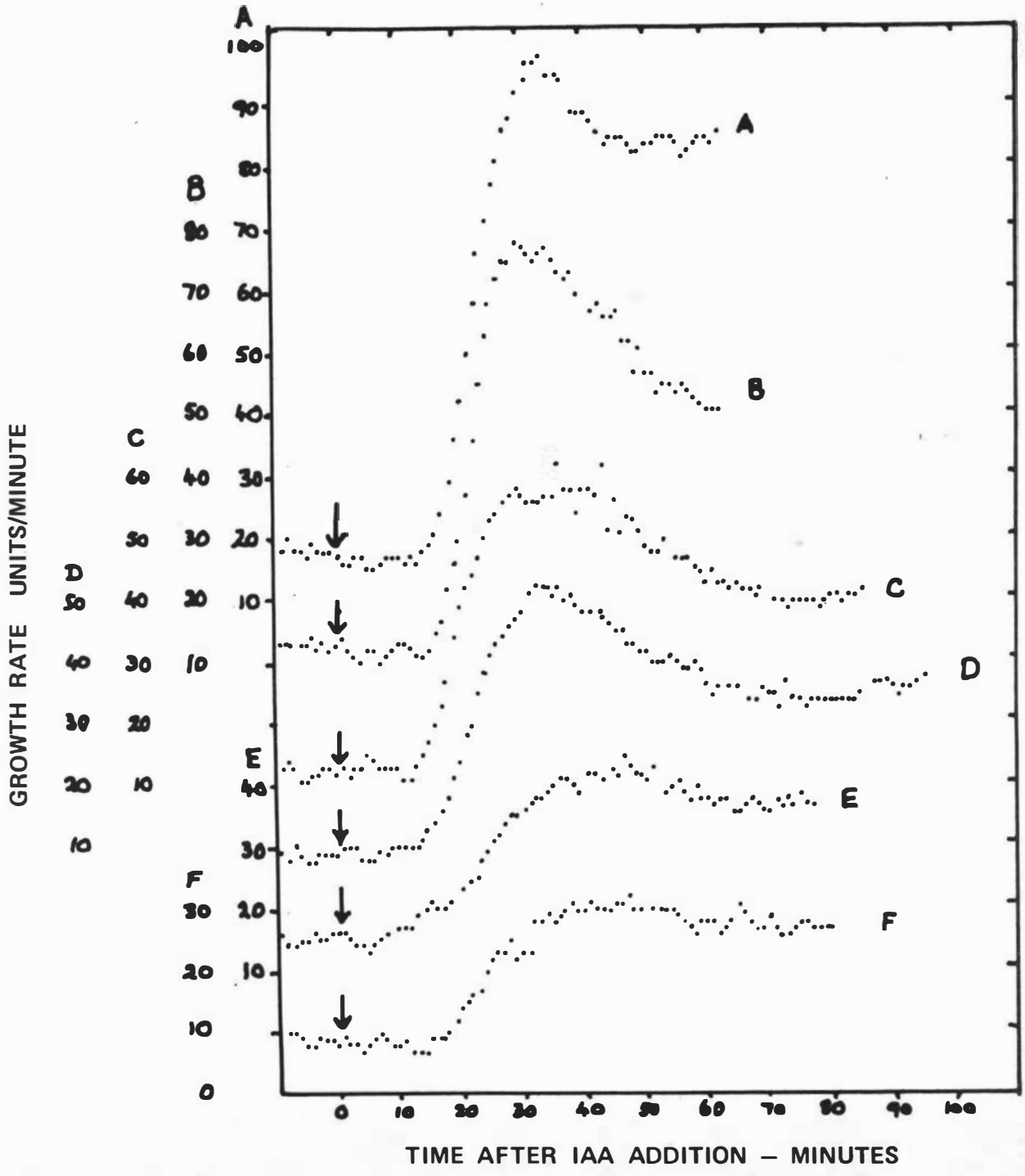
Additional examples of kinetic experiments.

The following figures are included to indicate the range of results obtained in the experiments outlined in Part I.



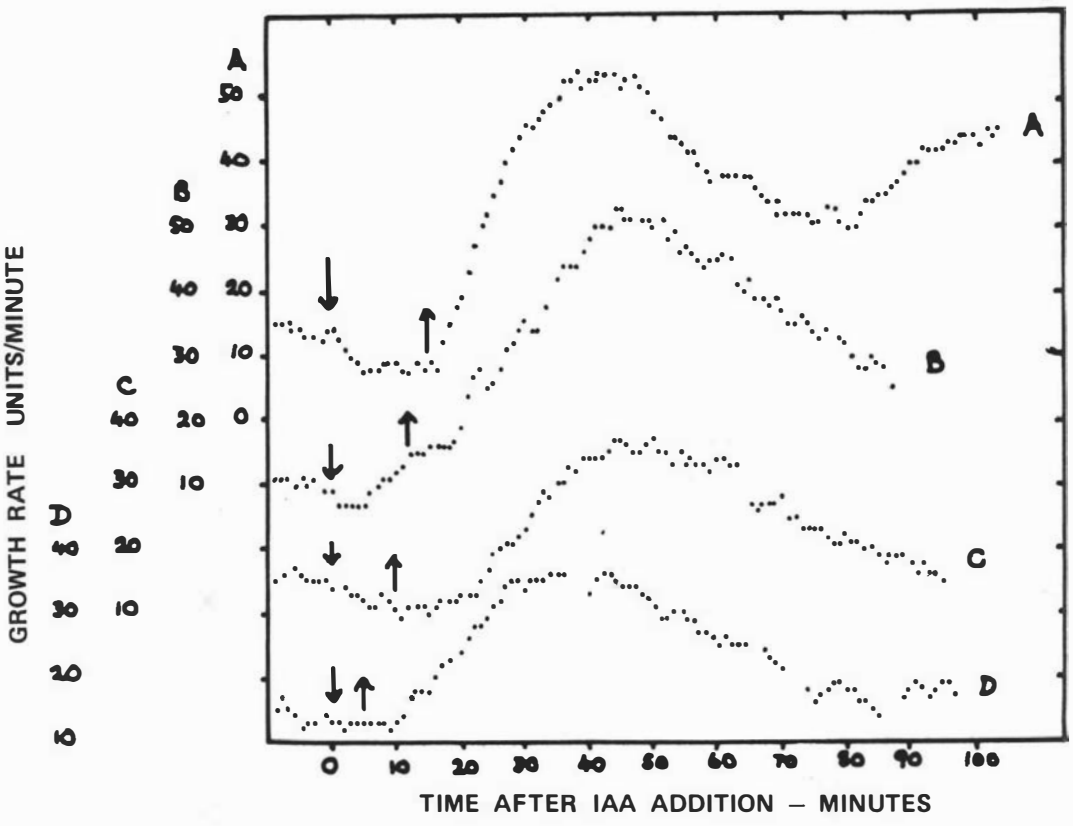
Appendix Fig. 1.
Variations in rate of growth of different lupin hypocotyl segments in tris-maleate buffer. No supplementary light. 1 unit = 0.23 μm.

A. immediately after cutting
B. after 145 min. in flowing, aerated buffer
C. after 130 min. in flowing, aerated buffer
D. after 120 min. in flowing, aerated buffer.

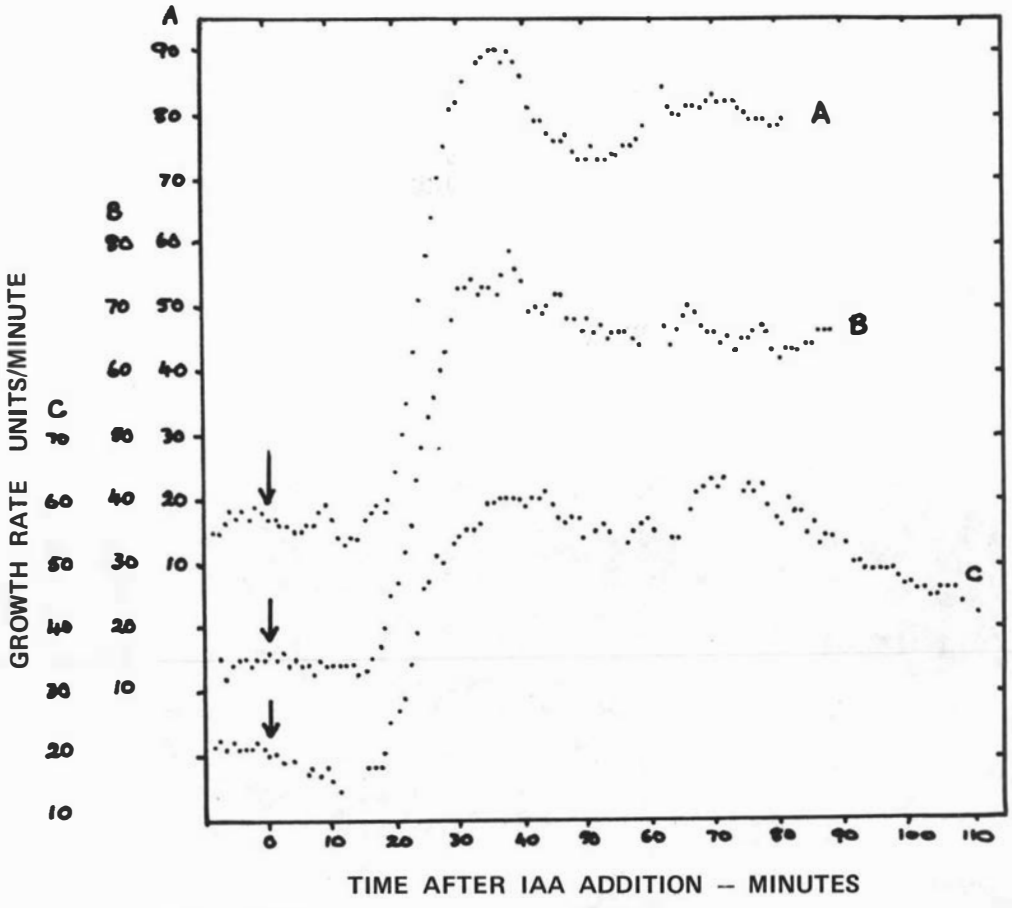


Appendix Fig. 2.
Effect of IAA in tris-maleate buffer on the growth rate of lupin hypocotyl segments. No supplementary light. 1 unit = 0.23 μm.

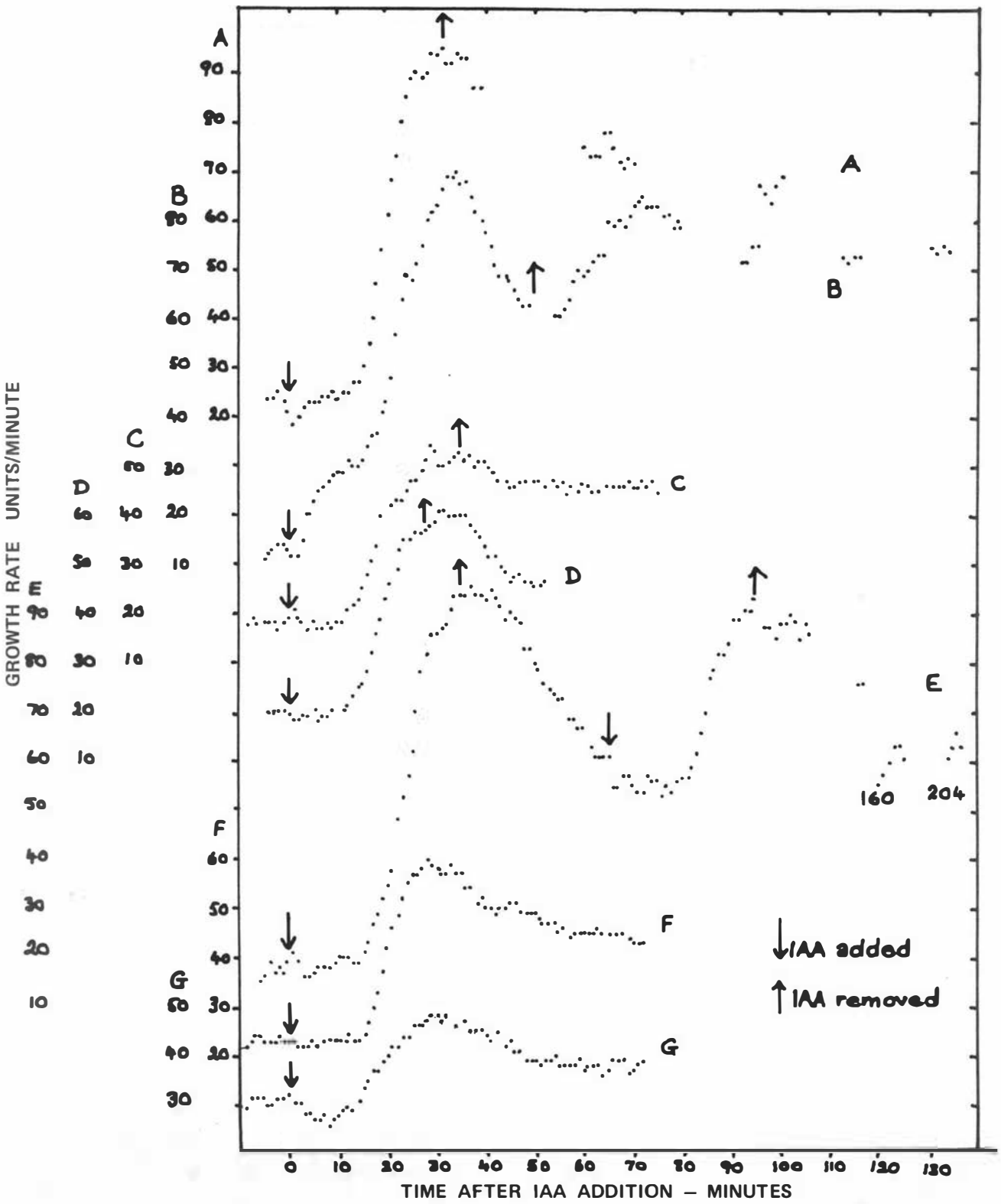
A & B 3×10^{-5}M
C 10^{-5}M 24°C
D & E 3×10^{-6}M 24°C
F 10^{-4}M 23°C



Appendix Fig. 3.
Effect of short term IAA treatment on the growth rate of lupin hypocotyl segments. 3×10^{-5}M IAA in tris-maleate buffer. No supplementary light. IAA removed after: A, 15 min; B, 12 min; C, 10 min; D, 5 min. 1 unit = 0.23 μm.

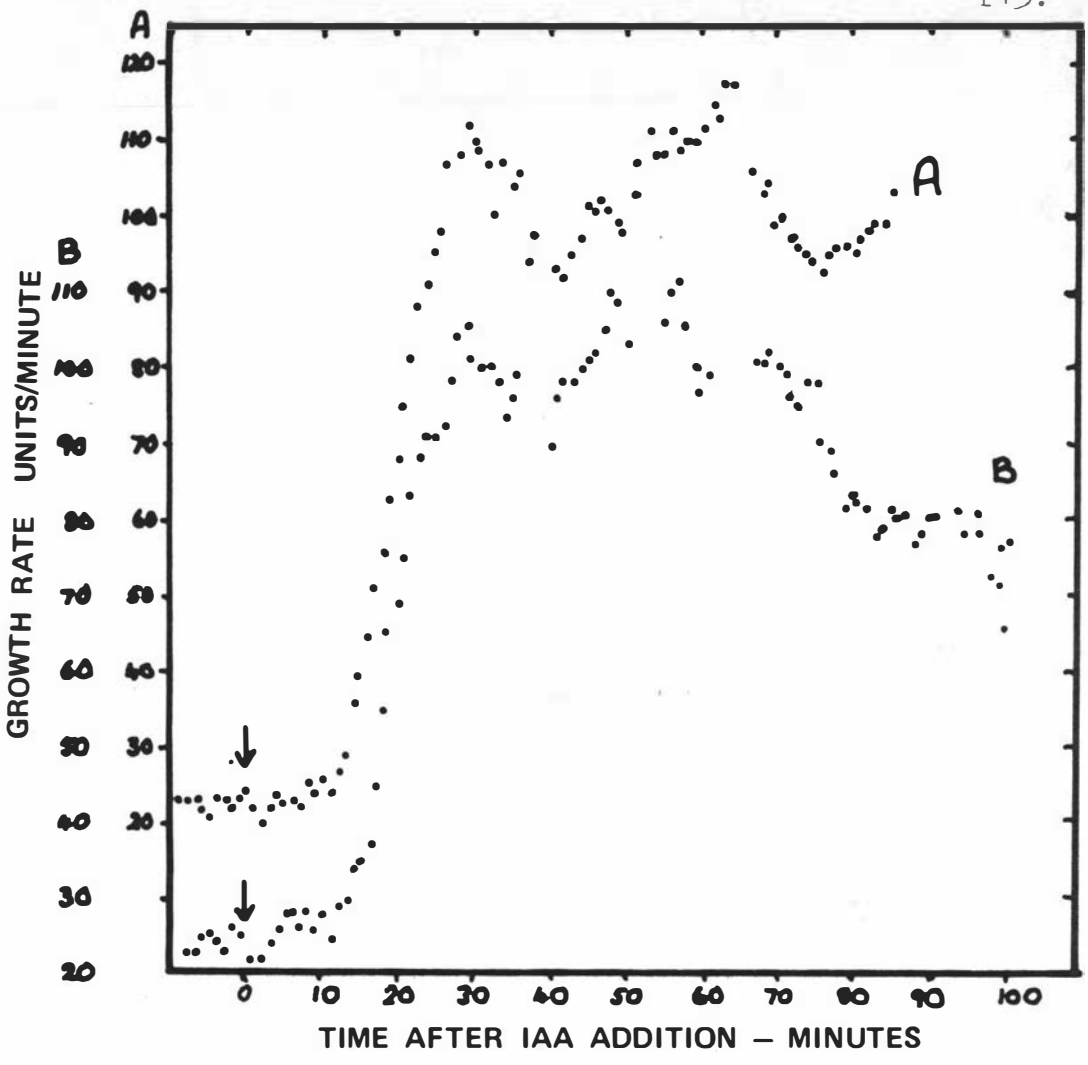


Appendix Fig 4.
Effect of IAA (3×10^{-5}M in tris-maleate buffer) on the growth rate of lupin hypocotyl segments exposed to supplementary light.
1 unit = 0.23 μm.

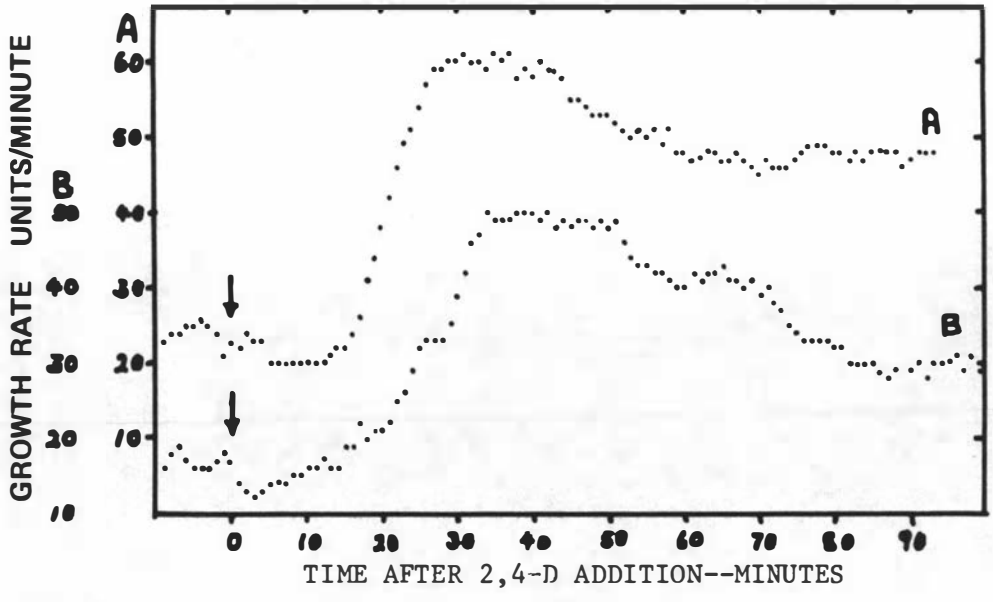


Appendix Fig. 5.
Effect of IAA on the growth rate of lupin hypocotyl segments in phosphate buffer. No supplementary light. 1 unit = 0.23 μm.

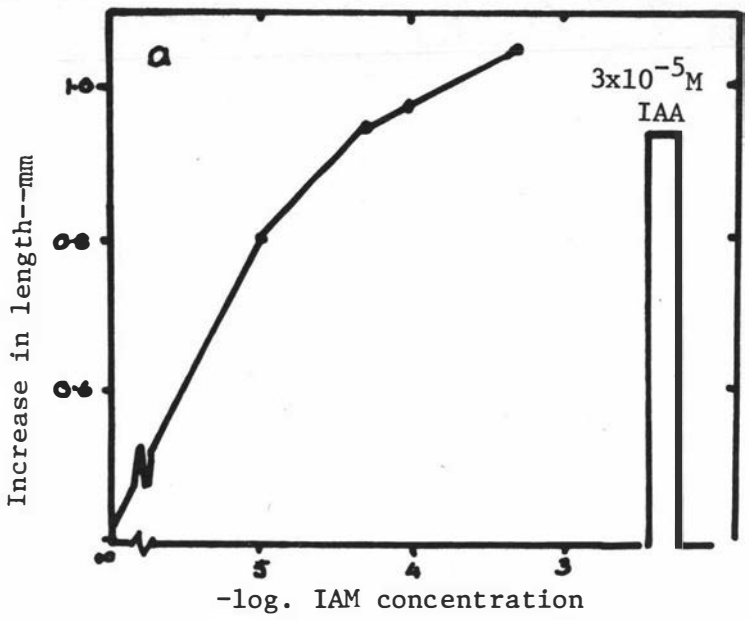
A,B,G 10^{-4}M
C,D 5×10^{-5}M
E,F 10^{-5}M

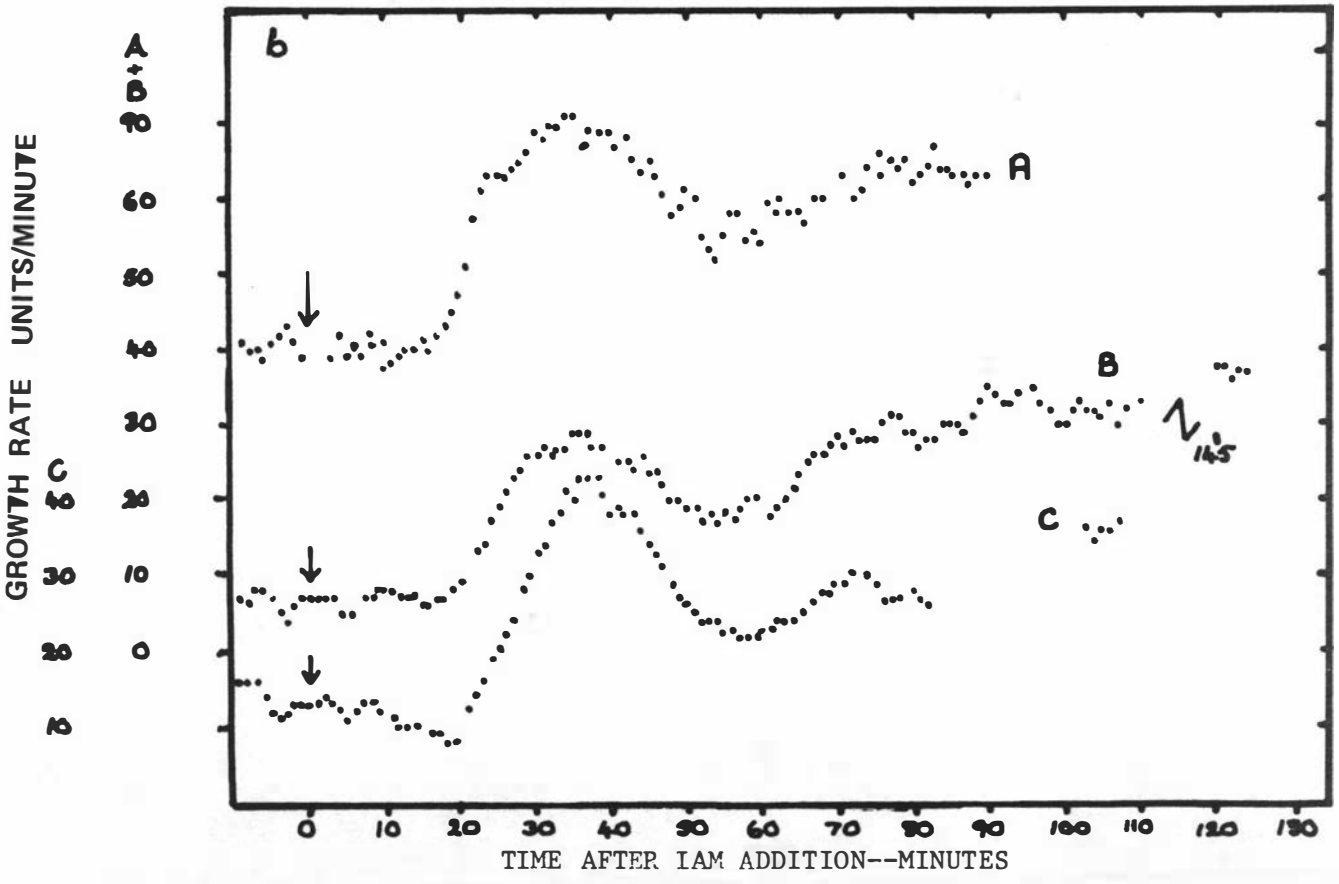


Appendix Fig. 6.
Effect of IAA ($3x10^{-5}$M) in distilled water on the growth rate of lupin hypocotyl segments. With supplementary light. 1 unit = 0.23 μm.



Appendix Fig. 7.
Effect of 2,4-D (10^{-4}M) on the growth rate of lupin hypocotyl segments. Phosphate buffer, without supplementary light. 1 unit = 0.23 μm.
A. 25°C B. 20°C

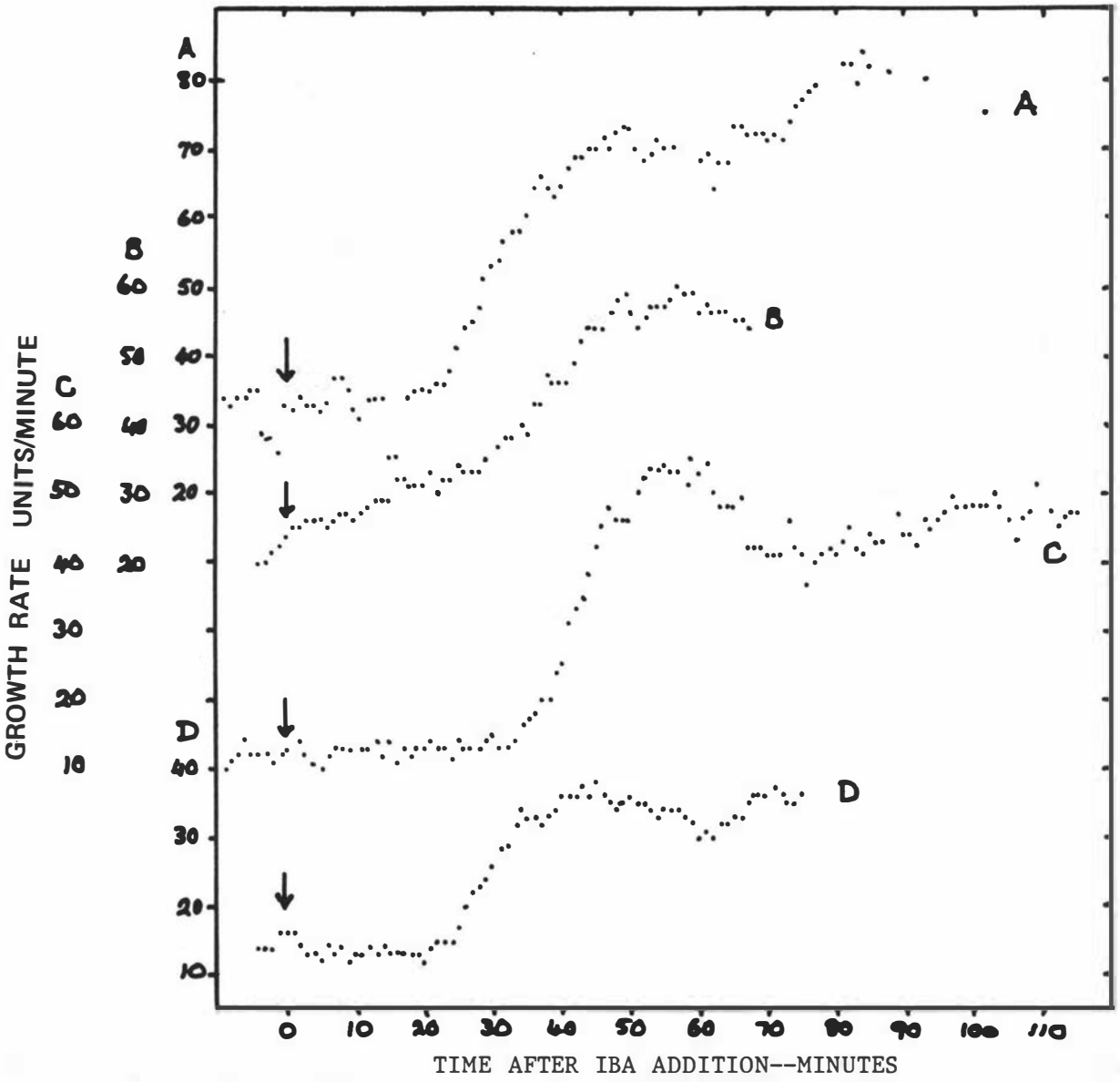




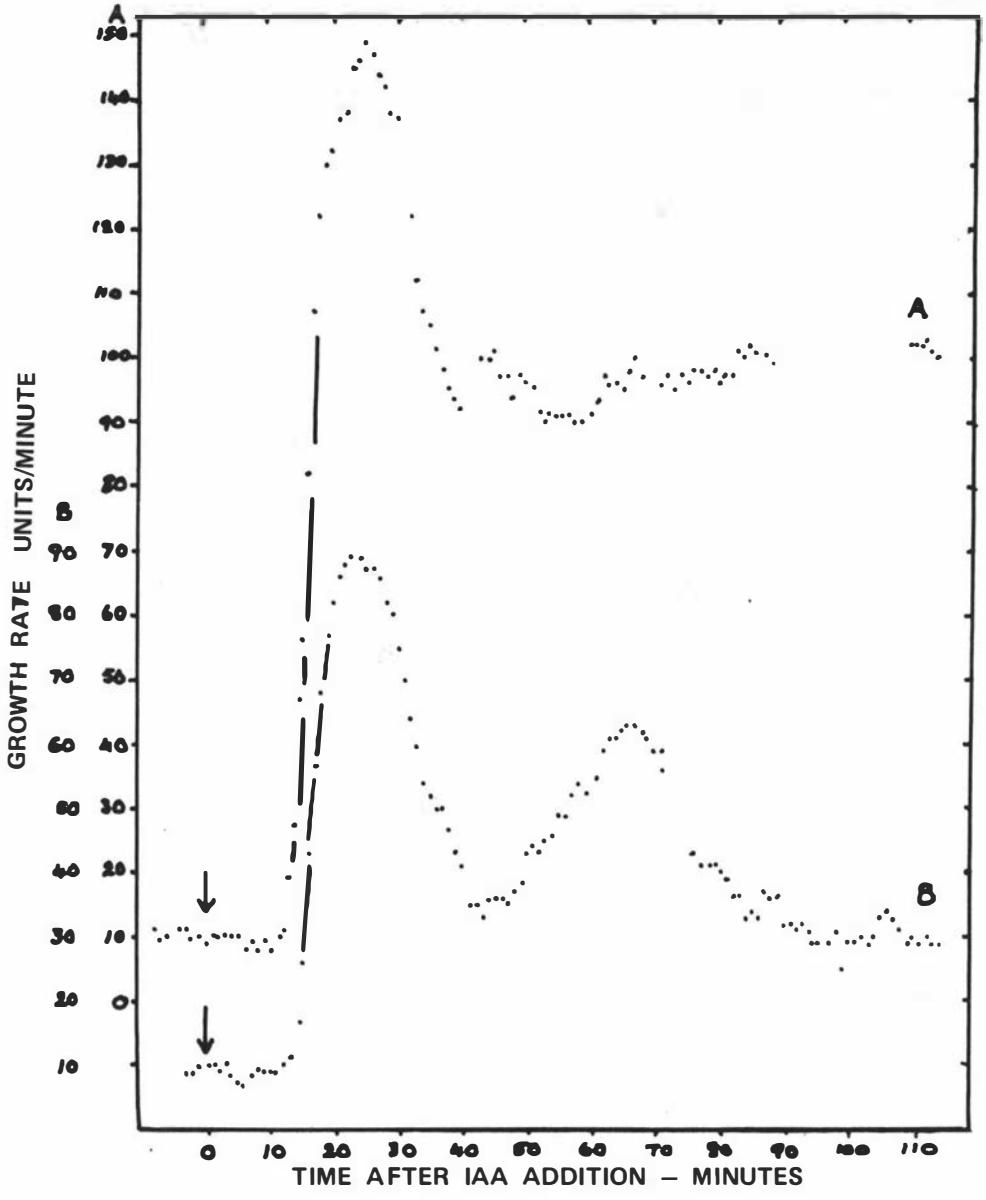
Appendix Fig. 8.
Effect of indoleacetamide on the growth of lupin hypocotyl segments.

a. Total increase in length, in 5 hr, of 5 mm segments treated with various concentrations of indoleacetamide. The bar represents the response of segments treated with $3x10^{-5}M$ IAA in the same experiment.

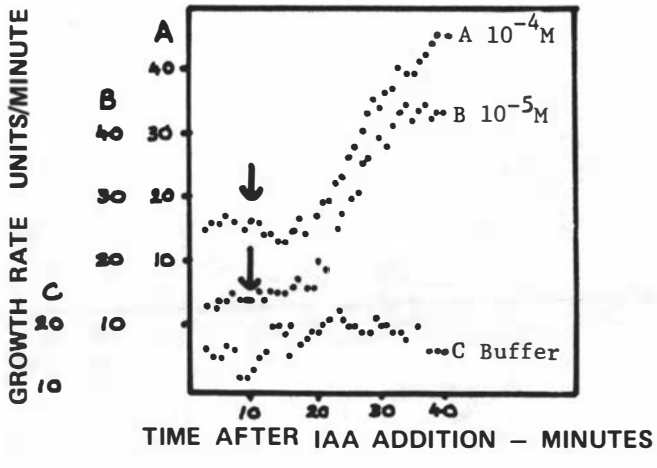
b. Short term kinetics of response to: A. $3x10^{-5}M$
B. $10^{-4}M$
C. $5x10^{-4}M$



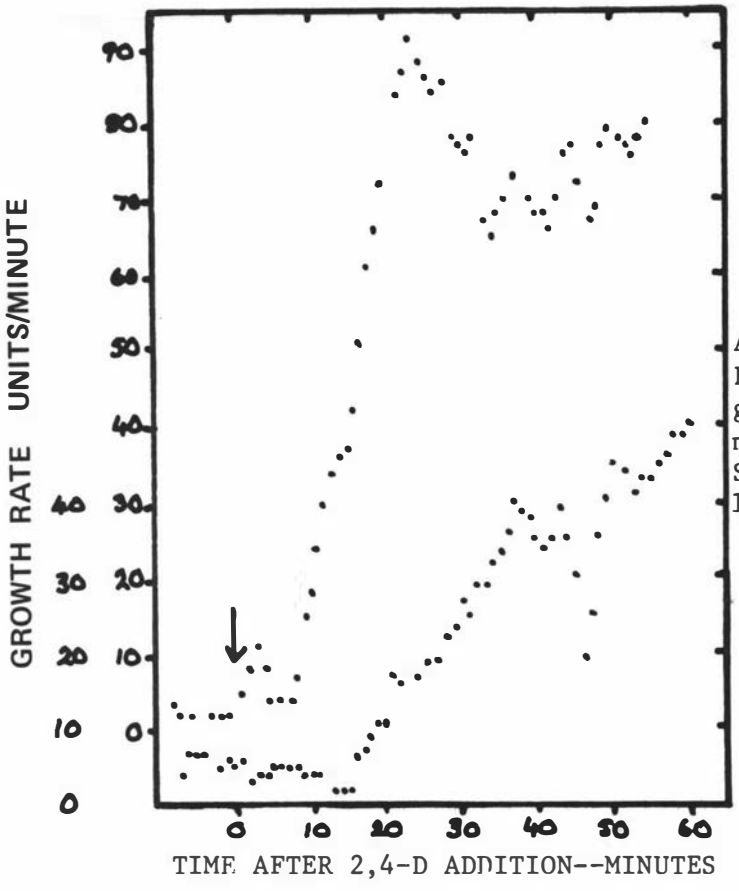
Appendix Fig 9.
Effect of indole butyric acid ($3x10^{-5}$M in tris-maleate buffer) on the growth rate of lupin hypocotyl segments. With supplementary light.
1 unit = 0.23 μm.



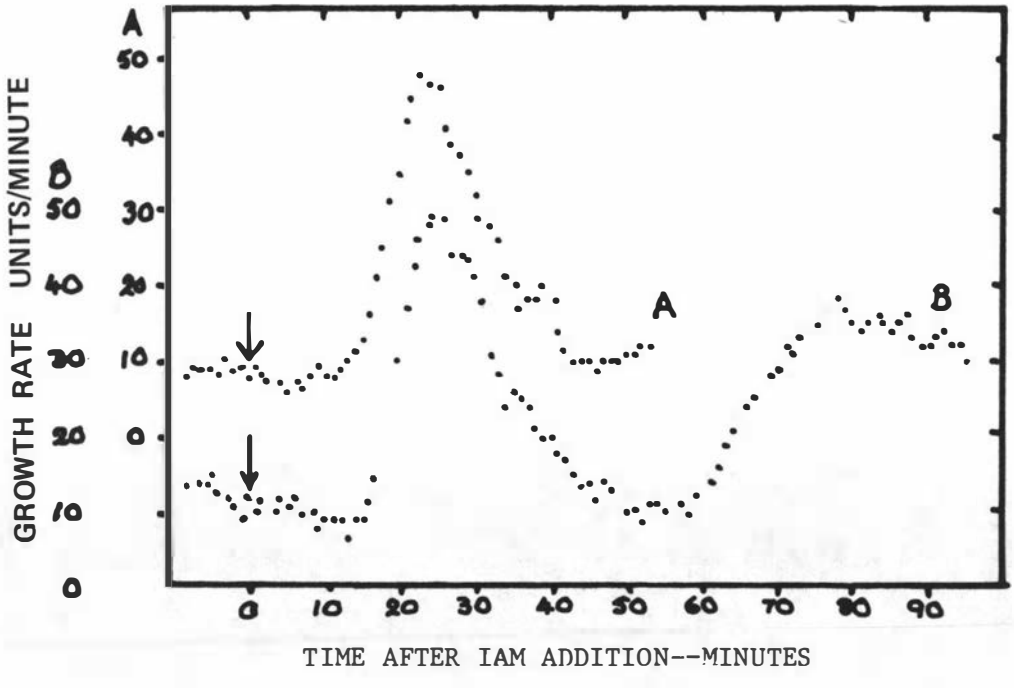
Appendix Fig. 10.
Effect of IAA ($3x10^{-5}$M in tris-maleate buffer) on the growth rate of pea epicotyl segments. With supplementary light. 1 unit = 0.23 μm.



Appendix Fig. 11.
Effect of IAA in phosphate buffer on pea stem segments. Measurements started between 20 and 90 min after cutting. 10^{-4}M IAA, an average of 8 experiments. 10^{-5}M and no IAA an average of 6 experiments. No supplementary light. 1 unit = 0.23 μm.



Appendix Fig. 12.
Effect of 2,4-D (10^{-4}M) on the growth rate of pea stem segments. Phosphate buffer. Supplementary light.
1 unit = 0.23 μm.



Appendix Fig. 13.
Effect of indoleacetamide on the growth rate of pea stem segments. Tris-maleate buffer. Supplementary light. 1 unit = 0.23 μm.
A. 10^{-5}M. B. $2x10^{-5}$M.

A P P E N D I X II

Techniques used in Part II

A. Incubation of plant tissue

After pretreatment in 0.02 M tris-maleate buffer pH 6.1 for 1 - 1.5 hours, the 10 mm segments were placed in 125 ml erlenmyer flasks containing buffer and addenda such as IAA, and radioactive RNA or protein precursor. The incubation took place at 24-25°C in a constant temperature shaking water bath. The segments were 30 cm from a Philips TLA 80W/55 fluorescent tube. After incubation the segments were rinsed twice for 30 seconds in 30 ml tris-maleate buffer pH 6.1.

B. Extraction of macromolecules.

1. Undenatured RNA

The RNA was extracted by a modified form of the method of Pigott and Midgley (1968). The tissue samples (usually consisting of 10 - 10 mm segments) were grown

in a mortar in 2 ml of a phenol mixture and 2 ml of buffer. The compositions of the phenol mixture and buffer solution are listed in Table 1. The mortar and pestle were precooled on ice and the phenol and buffer solutions were kept on ice. After grinding the mixture was transferred to a precooled glass centrifuge tube and stored on ice until it was centrifuged. For all centrifugations a BTL benchtop centrifuge was used and run at, 1000g for 5 minutes. Just prior to centrifugation the tubes were placed in the precooled centrifuge holders and were cushioned with ice and water. By the end of the spin, the tubes were at room temperature.

After the first centrifugation, the phenol layer was removed with a Pasteur pipette. Care was used to prevent the removal of plant material. 1 ml of 2M NaCl and 2 ml of phenol mixture was added, the tube sealed with Parafilm and the contents thoroughly mixed and then recentrifuged. The aqueous layer was transferred to another centrifuge tube, leaving behind the white denatured protein which collects at the interface between the phenolic and aqueous layers. A further 2 ml of phenol mixture was added to the tube and the contents throroughly mixed and centrifuged. The aqueous phase was again removed, mixed with 2 ml of phenol and recentrifuged. This process was repeated (usually three or four times) until the interface between the phenol and aqueous layers was only slightly opaque owing to the denatured protein.

(These steps are known as deproteinizing steps.) The aqueous phase was removed, the volume measured and 2.7 times that volume of absolute ethanol added and stored for at least 1 hr at -10°C. The flocculent precipitate was collected by centrifugation, the ethanol decanted and 1.0 ml of 0.02M sodium acetate added to dissolve the pellet. The solution was then mixed with 3 ml of absolute ethanol and stored at -10°C for at least an hour, but often overnight. The cycle of centrifugation, re-dissolving and precipitation was repeated once more. The final precipitate was again collected and then redissolved in 0.5 ml of electrophoresis running buffer without sodium lauryl sulphate.

2. Total RNA.

The total RNA was extracted by the method of Heyes (1963). The tissue was ground in 2 ml of ice cold water in a chilled mortar and transferred to a centrifuge tube. The mortar and pestle were rinsed with 3 ml cold water and this was added to the tube. The mortar and pestle were then rinsed with 5 ml of cold 2N perchloric acid which was also added to the sample. The sample was then centrifuged at 3,000 rpm at 0°C for 5 minutes in a refrigerated International Centrifuge. The pellet was washed successively with:

0.5N $HClO_4$ at 0°C

80% ethanol at 0°C

Absolute ethanol at 0°C

Absolute ethanol : ether (3:1) at 40°C

Ether at 40°C.

After all the ether had evaporated from the pellet after the last washing, it was resuspended in 0.3N KOH and incubated at 35°C for 15 hours. Perchloric acid was added to a final concentration of 0.2N. The supernatant was removed after centrifugation and UV absorbance spectrum obtained. The heights of the peaks at 260 nm gave estimates of relative RNA concentrations between samples.

This method was preferable to use for estimating total RNA because very little or no loss of RNA during transfer operations would be expected.

3. Organic phosphates.

Aliquots of the 1N perchloric acid supernatant resulting from the total RNA extraction (see above) were used to determine total organic phosphates. The method used was that of Nielsen and Lehninger as outlined in Avron (1960). This method depends on reacting the inorganic phosphate with molybdate reagent and extracting it out of the aqueous phase into an isobutanol: benzine phase. The organic phosphates are left behind in the aqueous layer. Aliquots of this fraction were used for radioactivity determinations.

4. Protein

The protein extraction procedure is shown on the flow chart (p.79). The tissue was ground with 3 ml of "grinding medium" in a homogenizer and then filtered, under suction in a funnel, through $(68\ \mu m)^2$ mesh nylon cloth. The homogenizer was rinsed twice with 1 ml of grinding medium. The composition of the grinding medium was as follows:

0.03 M tris - HCl buffer Ph 7.5

0.001 M Mg Acetate

0.005 M KCl

0.5 M sucrose

10^{-3} M Na diethyldithiocarbamate

The filtrate was collected in a calibrated 15 ml plastic centrifuge tube which was packed in ice and NaCl during filtration. The tube was removed and stored on ice and the cell wall material on the nylon cloth was rinsed with distilled water. The cloth was removed, folded in half with the cell wall material inside. It was blotted dry with paper towel and the weight of the cell wall material determined. Two weights of this material were placed in pre-weighed scintillation vials dried overnight at 80°C and the dry weight determined. The remainder of the cell wall material was placed in 0.3% deoxycholate in grinding medium.

Meanwhile the filtrate was centrifuged at 4,000g

for 15 minutes in a refrigerated MSE centrifuge. After decanting the supernatant, the pellet was resuspended in grinding solution and aliquots removed for radioactivity determinations. The supernatant was centrifuged for 1 hour in a Beckman model L ultracentrifuge using the head 40, at 38K rpm giving a centrifugal force of approximately 100,000 g. (All subsequent centrifugations were under these conditions). After the first 100,000 g spin, the supernatant was decanted, the volume determined, and aliquots removed for radioactivity determinations and electrophoresis. The pellet was resuspended in 5 ml of grinding medium and recentrifuged. Again the supernatant was decanted and aliquots removed for radioactivity determinations. The pellet was resuspended in 0.3% deoxycholate in grinding medium and left at room temperature for 30 minutes after which it was centrifuged for the last time. The supernatant was decanted and the pellet resuspended in 0.03% deoxycholate in grinding medium. Aliquots of both these fractions were removed for radioactivity determinations and electrophoresis.

C. Electrophoresis.

1. Polyacrylamide gel electrophoresis

With two modifications the procedure of Loëning (1967) for the preparation of polyacrylamide gels was followed throughout. The cross linking agent used by Loëning (1967) was bisacrylamide and this was used for some of the work. In the rest of the work ethylene diacrylate,

an alkali-labile cross linker (Weinburg et al., 1967) was used. Electrophoresis was carried out at room temperature.

a RNA

For RNA work, 2.2% gels were used when the cross-linker was bisacrylamide and 2.7% when ethylene diacrylate was used. The composition of the running buffer was:

Tris	36 mM
sodium dihydrogen phosphate	30 mM
disodium EDTA	1 mM
sodium lauryl sulphate	2 mg/l.
glycerol	10% V/V

The gels were generally run for 1.5 to 2 hours.

b Protein

After experimenting with different concentrations of acrylamide for protein separations it was decided to use 7% acrylamide. The method of Davis (1964) was used with the following modifications. The main gel was made first, using ammonium persulphate and TEMED as catalysts. After this gel had set the solution for the spacer gel was placed on top. This was riboflavin photocatalysed. After the spacer gel had set the sample was layered on top.

2. Cellulose acetate.

Cellulose acetate electrophoresis was used for separating the nucleotides of hydrolysed RNA. The RNA

was hydrolysed in 0.3N KOH at 37°C for 18 hours. It was neutralized with "zeocarb 225" in NH_3 form and then dried at 50°C in the air for about 1.5 hour. It was redissolved in water and 5 µl applied to each strip. Ammonium formate buffer, pH 3.05 to 3.1, 0.015M was used. Electrophoresis was stopped when, by means of UV light, it could be seen that the nucleotides had separated.

D. Column chromotography

1. Methylated albumin kieselguhr (MAK)

Kieselguhr coated with methylated albumin was prepared as outlined in Mandell and Hershey (1960). It was placed in 0.5 x 10 cm columns. A Buchler varigrad was used to make the NaCl gradient and the UV absorbance was monitored directly using an ultraviolet analyser made by Isco Instrumentation Specialties Co. Inc.

2. Hydroxyapatite

This type of chromatography was carried out according to the method of Bernardi (1965). It was prepared from brushite by the method of Tiselius et al (1956), and stored at 2.5°C in 0.01M phosphate buffer pH7.0. Care was taken to avoid breaking the large, fragile crystals by pipetting or repeated suspension (Sutton, 1969). The sample, in 0.01M phosphate buffer was applied to the top of the 3 cm x 1 cm diameter column. The column was rinsed with 5 ml 0.01M phosphate buffer and the compounds eluted with a linear gradient starting with 0.01M and

and ending with 0.05M phosphate buffer. Each of the tubes collecting the eluate was assayed for UV absorbance and radioactivity.

E. Determination of RNA.

1 Total RNA in extracts

The final ethanol precipitate containing RNA was usually dissolved in 0.5 ml of electrophoresis running buffer. A 0.1 ml aliquot was removed and diluted (usually 1:30) with water. A UV absorbance scan of this aliquot was obtained using a Hitachi double beam recording spectrophotometer. The ratio of the absorbance at 260 nm to that at 280 nm was used as a check on the purity of the RNA. In this work the ratio was always more than 2. The heights of the peaks at 257 nm were used to give estimates of the relative quantities of RNA in different treatments in an experiment. Thus, the amount of undiluted extracts of each treatment that was placed on the gel could be calculated so that the total number of OD units would be the same for each treatment.

2. RNA in acrylamide gels.

After electrophoresis the gels were scanned by UV light using a Joyce Loebl gel scanner connected to a 5mV recorder (Control Instruments Co.). The gels were then frozen until they were sliced with a McIwain Gel Slicer. The stage of the gel slicer was prepared by placing on it two strips of "cellotape" ("scotchtape"). This was then

covered with a dilute protein solution (saliva) onto which the gel was frozen by means of powdered dry ice. The gel remained in place during cutting as long as it was frozen. The slices were placed individually in scintillation vials, 0.5 ml NH_4OH added (Weinburg et al., 1967) and the ammonia allowed to evaporate before adding scintillation fluid.

The difficulties encountered in trying to localize the radioactivity with respect to a visual recording of optical density are outlined in Appendix III. The following method proved satisfactory. Reference RNA was prepared from lupin hypocotyl segments which were incubated with ^3H-uridine for 8 hr. All the fractions of RNA were labelled. After the RNA obtained from experimental treatments had been placed on top of the acrylamide gel, a 10 µl sample of the reference RNA was added. The reference and the experimental RNA were run together in electrophoresis.

F. Determination of protein in acrylamide gels.

After extrusion, the gels was stained for 20 minutes in 0.1% amido black in 50% acetic acid. After destaining in 7% acetic acid the position of the bands was recorded using the Joyce-Loebl gel scanner. In order to determine the distribution of radioactivity in various portions of the gel, the gel was sliced with a McIlwain gel slicer. The slices were placed individually in scintillation vials

and 0.5 ml of a papain solution added to degrade the protein and release the amino acids into the medium; The solution was saturated with papain and contained 5 mM cystein (which is an activating agent for papain) and 1 mM EDTA (Kimmel and Smith, 1954). They were left overnight before adding scintillation fluid.

G. Determination of Radioactivity.

Most of the determinations of radioactivity were made using a Packard liquid scintillation counter. Two types of scintillation fluid were used. The first was a modified Bray's solution consisting of 60 g naphthalene, 4 g PPO (2,5-diphenyloxazole), 0.2 g POPOP (2,2-p-phenylenebis(5-phenyloxazole)) per litre of dioxane. Water is miscible with this solution. It is, however, comparatively expensive and to reduce cost the system of Patterson and Greene (1965) was used in later work. The mixture is 1 part of the detergent Triton X-100 to 2 parts of toluene. The scintillators (PPO and POPOP) are added to this mixture. Water forms an emulsion with this mixture which is stable at the temperature of the scintillation (3-5°C).

H. Measurement of Respiration.

The oxygen uptake of approximately 0.2g of lupin hypocotyl tissue was determined by standard manometric techniques. A Gilson Differential Respirometer was used and the technique is well described in Dunn and Arditti

(1968). The incubation solutions consisted of 0.02M tris-maleate buffer 3 x 10^{-5}M IAA and 10 µg 1 ml cycloheximide. In some experiments 1% sucrose was also included.

I. Preparation of tissue for electron microscopy.

Pieces of tissue 1-2 mm long were cut from lupin hypocotyl segments which had been pretreated in 0.02M tris-maleate buffer, pH 6.1, for 2 hr and then treated for a further 35 min with or without 3 x 10^{-5}M IAA in tris maleate buffer. The pieces of tissue were fixed overnight in 3% glutaraldehyde in 0.01M phosphate buffer, pH 7.2. They were rinsed three times in phosphate buffer and post-fixed in 1% osmium tetroxide in phosphate buffer. After dehydration through an alcohol series propylene oxide was substituted. They were then infiltrated with araldite (the complete resin mixture, including catalyst). They were sectioned with a diamond knife using a LKB microtome type 2. The sections were stained with uranyl nitrate in water, rinsed and stained with Reynold's lead citrate.

+ + + + +

A P P E N D I X III

Detection of RNA on acrylamide gels.

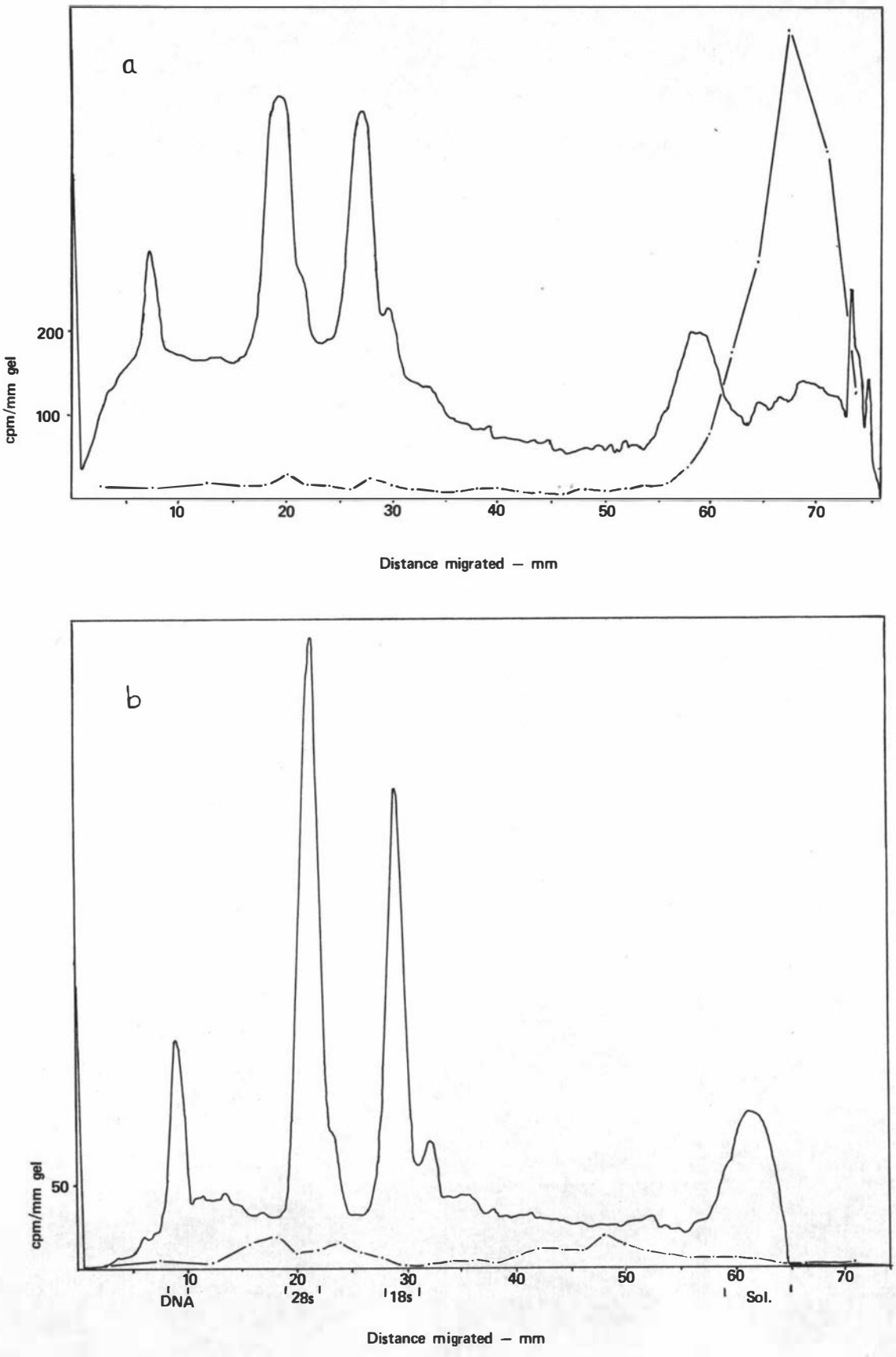
Three methods for the detection of RNA on the gels were used: photography in UV light, staining with toluidine blue, and the addition of ^{3}H RNA marker in the running of the gel and then scanning for UV absorption. A Joyce-Loebl Chromoscan was used to obtain reflectance scans of the UV photographs. The gels were stained in Toluidine blue in 40% methoxyethanol. After one hour in the stain the gels were decolourized over night in 40% methoxyethanol. They were then placed in 30% and then 20% methoxyethanol for one hour and stored in 10% methoxyethanol. Absorbance scans were obtain using a Joyce-Loebl Chromoscan. UV absorbance scans of gels were obtained using a Joyce-Loebl gel scanner.

To determine the radioactivity in various portions of the gels, they were frozen and sliced with a McIlwain

gel slicer made by Mickle Engineering Co., Surrey. The slices were placed in scintillation vials, 0.5 ml NH_4OH added (Weinburg et al., 1967), and the ammonia allowed to evaporate, and then 10 ml of a dioxane based scintillation fluid was added. The vials were counted in a Packard Tri-Carb scintillation counter.

These three methods represent a progression in attempts to determine accurately relative positions of the radioactivity as detected in the gel slices and the RNA as detected on the scans. Appendix Fig. 14a illustrates a reflectance scan and a plot of the radioactivity. It can be seen that there are two small peaks of radioactivity (^{32}P) in the UV absorbing region, but it was impossible to decide whether or not they corresponded exactly to the RNA. This was because the gels were easily distorted when placed on the gel slicer. This was overcome partially by staining the gels with toluidine blue because it was possible to identify those slices which contained the heavily stained r-RNA (1.3 and 0.7 x 10^{-6} daltons) and s-RNA. The counts did not appear to be associated with these fractions. This can be seen in Appendix Fig. 14b (a duplicate gel of Appendix Fig.14a) where the heavily stained regions are indicated on the absicissa.

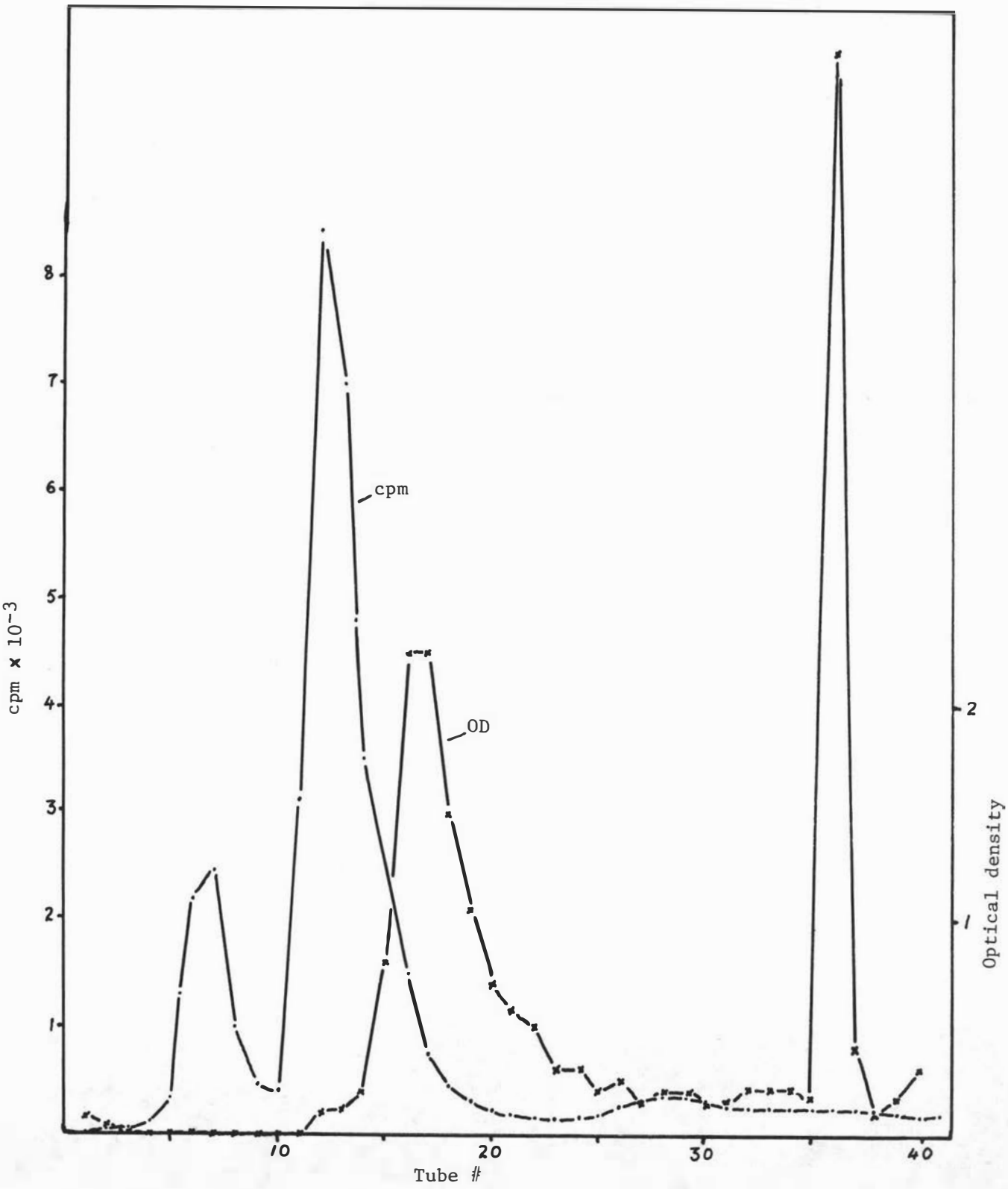
The method using staining was not completely satisfactory and the method described earlier was used unless otherwise stated.



Appendix Fig. 14.
Acrylamide gel electrophoresis of two aliquots of RNA extracted from tissue treated with ^{32}P from 1 hour to 1 hour 20 min. IAA present from 0 time. a) reflectance scan of UV photograph. b) visible light absorbance scan of toluidine blue stained gel. Stained gel slices indicated on the abscissa. ———optical density; .....cpm/mm gel.

A P P E N D I X IV

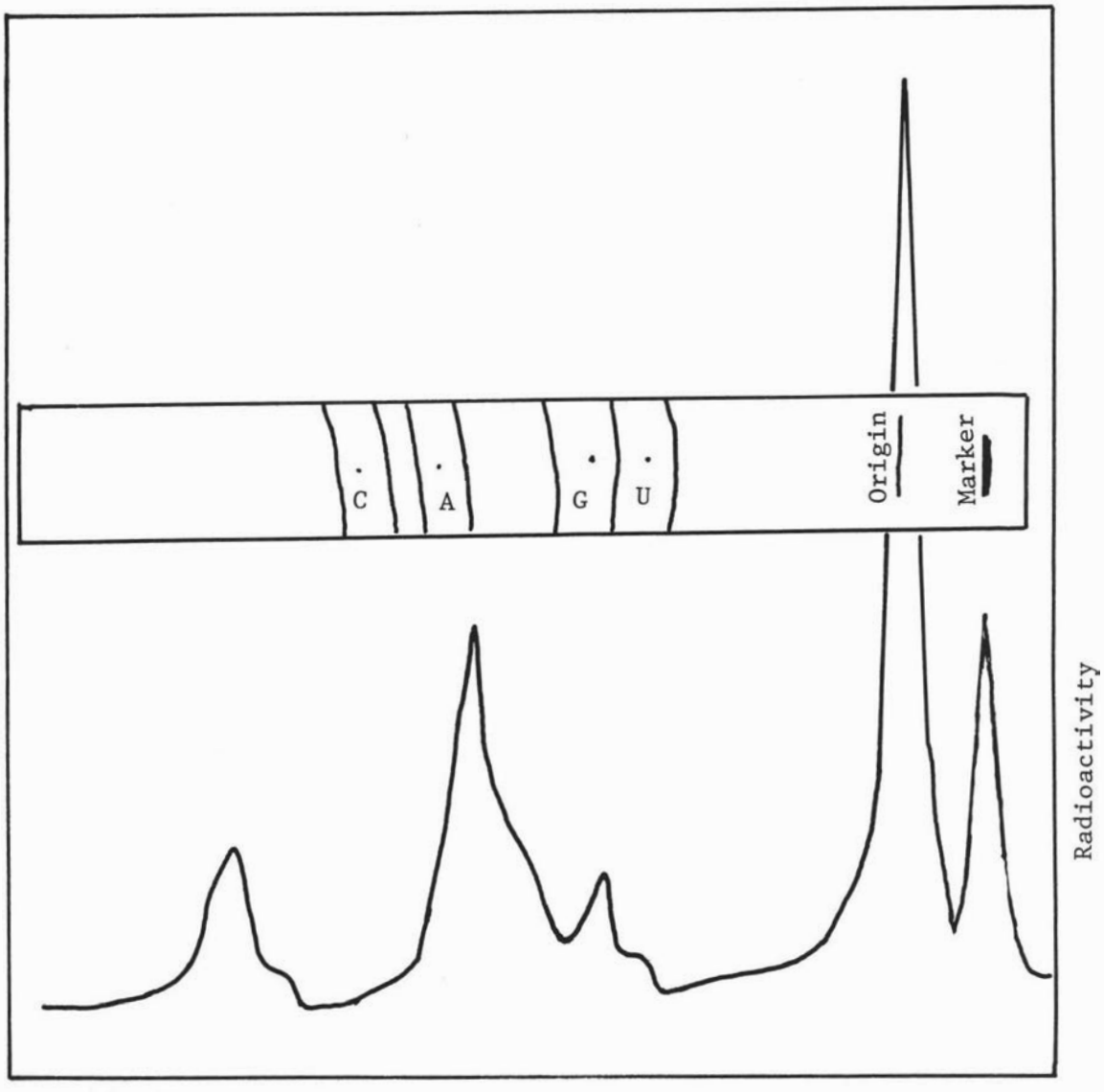
When ^{32}P was used as the RNA precursor, a high proportion of the counts were not associated with RNA. With electrophoresis the majority of the counts move faster than s-RNA as shown in Fig.23 and 25 and Appendix Fig. 14. In gels run for times of 1.5 hr or less, such as Fig. 25, there are two fast running peaks that contain more than 99% of the total radioactivity of the sample. This was confirmed with chromatography in hydroxyaptite columns which were performed as described by Bernardi (1965). As seen in Appendix Fig. 15, the radioactivity eluted as two peaks that did not absorb UV light at lower phosphate molarities than does RNA. The large peak of optical density in tube 36 was identified as phenol (spectral comparison with the authentic phenol). The gel in Appendix Fig. 14b a duplicate of that shown in Fig. 14a, is a stained gel.



Appendix Fig. 15.
Hydroxyapatite chromatography of lupin RNA. Linear 0.01M (left) to 0.5M (right) phosphate gradient. —·—·—·— cpm/ml —x—x— OD at 260 nm.

It can be seen that the radioactivity leached out during the staining procedure. These fast running non- RNA peaks occurred only when ^{32}P was used to label the RNA and not when ^{14}C or ^{3}H-uridine were used.

A sample of 32P labelled RNA was hydrolysed and aliquots applied to cellulose acetate strips for electrophoresis. After the nucleotides had separated the location of the radioactivity was determined using a strip-scanner. This is illustrated in Appendix Fig. 16. The peak on the far right is a radioactive marker applied just before scanning. The peak second from the right is the origin. The position of the nucleotides may be seen on the diagram of the strip. The fast running non-RNA peaks are not located on the gels shown in Fig. 22 because of the length of the run (2hr 40min). They contained approximately 5 times as much radioactivity in the 5 hr 40 min treatment as in the 0 - 40 min treatment. (estimated from duplicate gels run for 1hr 30 min, the heights of the peaks of the former were ca. 20,000 cpm and of the latter were ca. 2,000 cpm). This may indicate a further property of these compounds: namely that their rate of synthesis increases with time. However this may be related to increased ^{32}P uptake (cf. Table II). The portion of greatest UV absorbance is marked with a dot. It can be seen that the three peaks of radioactivity does not correspond to the nucleotides.



Appendix Fig. 16.
Electrophoresis of hydrolysed RNA sample on cellulose acetate (inset). The position of the nucleotides is marked with bands, The position of the radioactivity is indicated by the tracing of the radioactivity scan.

This is further evidence that the ^{32}P is not associated with RNA or nucleotides.

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B I B L I O G R A P H Y

Afridi, M.M.R.K. and E. Hewitt (1964)
The inducible formation and stability of nitrate reductase in higher plants.
J. exp. Bot. 15:251-271.

Andreae, W.A. (1967)
Uptake and metabolism of indoleacetic acid, maphthalene acetic acid, and 2,4-dichlorophenoxy-acetic acid by pea root segments in relation to growth inhibition during and after auxin application.
Can. J. Bot. 45:737-753.

Anker, L. (1953)
The effect of indole acetic acid and other growth promoting substances on the endogenous respiration of the Avena coleoptile.
Acta bot. neerl. 2:22-65.

Armstrong, D.J. (1966)
Hypothesis concerning the mechanism of auxin action.
Proc. natn. Acad. Sci. USA. 56:64-66.

Audus, L.J. (1952)
The time factor in studies of growth inhibition in excised organ sections.
J. exp. Bot. 3:375-392.

Audus, L.J. and A. Garrard (1953)
Studies on the growth and respiration of roots. 1. The effects of stimulatory and inhibitory concentrations of β-indolylacetic acid root sections of Pisum sativum.
J. exp. Bot. 4:330-348.

Avron, M. (1960)
Photophosphorylation by swiss-chard chloroplasts.
Biochem. Biophys. Acta 40:257-272.

Barkley, G.M. and M.L. Evans (1970)
Timing of the auxin response in etiolated pea stem sections.
Pl. Phsyiol., Lancaster 45:143-147.

Barlow, H.W.B. and C.R. Hancock (1959)
Studies on extension growth in coleoptile sections III. The interaction of temperature and β-indolylacetic acid on section growth.
J. exp. Bot. 10:157-168.

Barlow, P.W. (1970)
RNA synthesis in the root apex of Zea mays.
J. exp. Bot. 21:292-299.

Bendaña, F.E. and A.W. Galston (1965)
Hormone-induced stabilization of soluble RNA in pea-stem tissue.
Science, N.Y. 150:69-70.

Bendaña, F.E, A.W. Galston, R.Kaur-Sawhney and P.J. Penny (1965)
Recovery of labeled ribonucleic acid follwing administration of labeled auxin to green pea stem sections.
Plant Physio.. Lancaster, 40:977-983.

Bennet-Clark, T.A. and N.P. Kefford (1954)
The extension growth-time relationship for Avena coleoptile sections.
J.exp.Bot. 5:293-304.

Bentley, J.A. and S. Housley (1953)
Growth of Avena coleoptile sections in solutions of 3-indolylacetic acid and 3-indolylactonitrile.
Physiologia Pl. 6:480-484.

Bernardi, G. (1965)
Chromatography of nucleic acids on hydroxyapatite.
Nature, Lond. 206:779-783.

Bonner, J. (1936)
The growth and respiration of the Avena coleoptile.
J.gen. Physiol. 20:1-11.

Bonner, J. and R.S. Bandurski (1952)
Studies on the physiology, pharmacology and biochemistry of the auxins.
A. Rev. Pl. Physiol. 3:59-86.

Bonner, J. and R.J. Foster (1955)

The growth-time relationships of the auxin-induced growth in Avena coleoptile sections.
J.exp. Bot. 6:293-302.

Buis, R. (1967)
Recherches factorielles sur la régulation de la croissance de l'hypocotyle de Lupin (Lupinus albus L.)
Physiol. Végét. 5:1-36.

Burstrom, H. and B.A.M. Hansen (1956)
The mode of growth action of some naphthoxy compounds in The Chemistry and Mode of Action of Plant Growth Substances. ed. R.L. Wain and F. Wightman.
Butterworths Scientific Publications.

Chrispeels, M.J. (1970)
Synthesis and secretion of hydroxyproline - containing macromolecules in carrots.
Pl. Physiol., Lancaster, 45:223-227.

Cleland, R. (1959)
Effect of osmotic concentration on auxin-action and on irreversible and reversible expansion of the Avena coleoptile.
Physiologia Pl. 12:809-825.

Cleland, R. (1963)
The occurrence of auxin-induced pectin methylation in plant tissues.
Pl. Physiol, Lancaster, 38:738-740.

Cleland, R. (1965)
Auxin-induced cell wall loosening in the presence of actinomycin D.
Pl. Physiol., Lancaster, 40:595-600.

Cleland, R. (1965)
Auxin-induced cell-wall loosening in the presence of actinomycin D.
Pl. Physiol., Lancaster, 40:595-600.

Cleland, R. (1967a)
Extensibility of isolated cell walls: measurement and changes during elongation.
Planta 74:197-209.

Cleland, R. (1967b)
A dual role of turgor pressure in auxin-induced cell elongation in Avena coleoptiles.
Planta 77:182-191.

Cleland, R. (1968)
Wall extensibility and the mechanism of auxin-induced cell elongation.
in Biochemistry and Physiology of Plant Growth Substances.
ed. F. Wightman and G. Setterfield, Runge Press, Ottawa.

Dainty, J. (1969)
The water relations of plants
in Physiology of Plant Growth and Development.
ed. M.B. Wilkins.
McGraw Hill, London.

Davis, B.J. (1964)
Disc electrophoresis II. Method of application to human serum proteins.
Ann. N.Y. Acad. Sci. 121:404-427.

De Hertogh, A.A., D.C. McCune, J.Brown and D. Antoine. (1965)
The effect of antagonists of RNA and protein biosynthesis on IAA and 2,4-D induced growth of green pea stem segments.
Contrib. Boyce Thompson Inst. Pl.Res. 23:23-32.

Dowben, R.M. (1969)
General Physiology, A molecular approach.
Harper and Row. London.

Dunn and J. Arditti. (1968)
Experimental physiology.
Holt, Rinehart and Winston, Inc., London.

Evans, M.L. and Hokanson, R. (1969)
Timing of the response of coleoptiles to the application and withdrawal of various auxins.
Planta 85:85-95.

Evans, M.L. and P.M. Ray. (1969)
Timing of the auxin response in coleoptiles and its implication regarding auxin action.
J. gen. Physiol. 53:1-20.

Fan, D-F. and G.A. MacLachlan (1967)
Massive synthesis of ribonucleic acid and cellulase in the pea epicotyl in response to indoleacetic acid, with and without concurrent cell division.
Pl. Physiol., Lancaster, 42:1114-1122.

Foster,R.J. D.H. McRae, and J. Bonner (1952)
Auxin-induced growth inhibition a natural consequence of two-point attachment.
Proc. natan. Acad. Sci. U.S.A. 38:1014-1022.

Françon, M. (1961)
Progress in Microscopy.
Pergamon Press, London.

Galston, A.W. and Davies P.J. (1969)
Hormonal regulation in higher plants.
Science, N.Y. 163:1288-1297.

Galston, A.W., P. Jackson, R. Kaur-Sawhney, N.P. Kefford and W.J. Meudt. (1964)
Interactions of auxins with macromolecular constituents of pea seedlings.
in Régulateurs Naturels de la Croissance Végétale.
ed. J.P. Nitsch. Edition du Centre National de la Recherche Scientifique, Paris.

Galston, A.W. and R. Kaur. (1959)
An effect of auxins on the heat coagulability of the proteins of growing plant cells.
Proc. natn. Acad. Sci. U.S.A. 45:1587-1590.

Galston, A.W. and R. Kaur (1961)
The intracellular locale of auxin action: An effect of auxin on the physical state of cyloplasmic proteins.
In Plant growth regulation.
Iowa State College Press, Ames, Iowa.

Galston, A.W. and R. Kaur, (1962)
Interactions of pectin and protein in the heat coagulation of proteins.
Science, N.Y. 138:903-904.

Galston, A.W., R. Kaur, N. Maheshwari and S.C. Maheshwari. (1963)
Pectin-protein interaction as a basis for auxin-induced alteration of protein heat coagulability.
Am.J. Bot. 50:487-494.

Galston, A.W., and W.K. Purves, (1960)
The mechanism of action of auxin.
A.Rev.Pl. Physiol. 11:239-276.

Giese, A.C. (1968)
Cell Physiology (3rd edition).
Saunders, Philadelphia.

Goldsmith, M.H.M. (1969)
Transport of plant growth regulators.
in The Phsyiology of Plant Growth and Development.
ed. M.B. Wilkins.
McGraw Hill. London.

Gross, W.J. (1954)
Osmotic responses in the sipunculid Rendrostomum zostericolum.
J.exp.Biol. 31:402-423.

Harris, H. (1963)
Nuclear ribonucleic acid.
in Progress in Nucleic Acid Research. Vol.2. ed. J.N. Davidson and W.E. Cohn. Academic Press. London.

Hertel, R. and R. Flory. (1969)
Auxin movement in corn coleoptiles.
Planta 82:123-144.

Hertel, R., M.L. Evans, A.C. Leopold and H.M. Sell. (1969)
The specificity of the auxin transport system.
Planta 85:238-249.

Heyes, J.K. (1963)
The effects of 8-azaguanine on growth and metabolism in the root.
Proc. R. Soc. B. 158:208-221.

Heyn, A.N.J. (1940)
The physiology of cell elongation.
Bot. Rev. 6:515-574.

Hock, B. (1967)
Nature of a rapidly labeled RNA fraction described in higher plant systems.
Pl.Physiol., Lancaster, 42:1149-1152.

Ingle, J., and J.L. Key. (1965)
A comparative evaluation of the synthesis of DNA-like RNA in excised and intact plant tissues.
Pl.Physiol., Lancaster, 40:1212-1219.

Ingle, J., J.L. Key and R.E. Holm. (1965)
Demonstration and characterization of a DNA-like RNA in excised plant tissue.
J. Molec. Biol. 11:730-746.

Katsumi, M. (1961)
Studies on the mode of action of 1:4 dihydronaphthoic acid.
Bot.Mag., Tokyo. 74:436-441.

Kaur-Sawhney, R., M. Bara and A.W. Galston, (1967)
Analysis of labelling patterns in soluble RNA preparations from green pea stem sections supplied with ^{14}C-carboxyl-labelled indoleacetic acid.
Ann. N.Y. Acad. Sci. 144:63-67.

Kefford, N.P., R. Kaur-Sawhney and A.W. Galston. (1963)
Formation of a complex between a derivative of the plant hormone indoleacetic acid and ribonucleic acid from pea seedlings.
Acta Chem. Scand. 17 (suppl.1) 313-318.

Ketellapper, H.J. (1953)
The mechanism of the action of indole-3-acetic acid on the water absorption by Avena coleoptile sections.
Acta. bot. neerl. 2:387-444.

Key, J.L. (1964)
Ribonucleic acid and protein synthesis as essential processes for cell elongation.
Pl.Physiol., Lancaster, 39:365-370.

Key, J.L. (1966)
The effect of purine and pyrimidine analogues on growth and RNA metabolism in the soybean hypocotyl: the selective action of 5-fluorouracil.
Pl.Physiol., Lancaster, 41:1257-1264.

Key, J.L., N.M. Barnett and C.Y. Lin. (1967)
RNA and protein biosynthesis and the regulation of cell elongation by auxin.
Ann. N.Y. Acad. Sci. 144:49-62.

Key, J.L. and J. Ingle, (1964)
Requirement for the synthesis of DNA-like RNA for the growth of excised plant tissue.
Proc. natn. Acad. Sci. U.S.A. 52:1382-1388.

Key, J.L. and J. Ingle (1965)
Role of RNA metabolism in the regulation of growth of higher plants.
Genes to Genus. Ed. E.A. Greer and T.J. Army.
23 International Minerals and Chemical Corp.
Skokie, Ill.

Key, J.L. and J. Ingle (1968)
RNA metabolism in response to auxin.
Biochemistry and Physiology of Plant Growth Substances.
The Runge Press, Ottawa.

Key, J.L. and J.C. Shannon (1964)
Enhancement by auxin of ribonucleic acid synthesis in excised soybean hypocotyl tissue.
Pl.Physiol., Lancaster, 39:360-364.

Kimmel, J.R. and E.L. Smith (1954)
Crystalline papain. I Preparation, specificity and activation.
J. Biol. Chem. 207:515-531.

Kirk, J.T.O. (1970)
Failure to detect effects of cycloheximide on energy metabolism in Euglena gracilis.
Nature, Lond. 226:182.

Köhler, D. (1956)
Über die Beziehungen zwischen der Länge von Haferkoleoptilen und der Wachstumsgeschwindigkeit ihrer isolierten Ausschnitte.
Planta 47:159-164.

Libbert,E. (1957)
Die Regulation des Wurzelwachstums durch synthetische und Endogene Inhibitoun.
Planta 50:25-40

Lockhart, J.A. (1967)
Physical nature of irreversible deformation of plant cells.
Physiol., Lancaster, 42:1545-1552.

Loening, U.E. (1967)
The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide - gel electrophoresis.
Biochem J. 102:251-257.

Loening, U.E. (1968)
RNA structure and metabolism.
A.Rev. Pl Phsiol. 19:37-70.

MacDonald, I.R. and R.J. Ellis (1969)
Does cycloheximide inhibit protein synthesis specifically in plant tissues?
Nature, Lond. 227:791-792.

Mandell, J.D. and A.D. Hershey (1960)
A fractionating column for analysis of nucleic acids.
Anal. Biochem. 1:66-77.

Marchant, R and A.W. Robards (1968)
Membrane systems associated with the plasmalemma of plant cells.
Ann. Bot. N.S. 32:457-471.

Masuda, Y. (1959)
Role of cellular ribonucleic acid in growth response of Avena coleoptile to auxin.
Physiologia Pl. 12:324-335.

Masuda, Y. (1968)
Requirement of RNA biosynthesis for the auxin-induced elongation of oat coleoptile and pea stem cells.
in Biochemistry and Physiology of Plant Growth Substances.
ed. F. Wightman and G. Setterfield.
The Runge Press. Ottawa. 699-710.

Masuda, Y. (1969a)
Auxin-induced cell expansion in relation to cell wall extensibility.
Pl.Cell Physiol., Tokyo, 10:1-9.

Masuda, Y. (1969b)
Role of cell-wall-degrading enzymes in cell-wall loosening in oat coleoptiles.
Planta 83:171-184.

Masuda, Y and S. Kamisaka (1969)
Rapid stimulation of RNA biosynthesis by auxin.
Pl.Cell Physiol., Tokyo, 10:79-86.

Masuda, Y and E. Tanimoto (1967)
Effect of auxin and antiauxin on the growth and RNA synthesis of etiolated pea internode.
Pl. Cell Phsysiol., Tokyo, 8:459-465.

Masuda, Y and S. Wada (1966)
Requirement of RNA for the auxin induced elongation of oat coleoptile.
Physiologia Plant. 19:1055-1063.

Masuda, Y and S. Wada (1967)
Effect of B-1,3-glucanase on the elongation growth of oat coleoptile.
Bot. Mag., Tokyo 80:100-102.

McRae, D.H. and J. Bonner (1953)
Chemical structure and auxin activity.
Physiologia Pl. 6:485-510.

Mitchell, K.K. and I.V. Sarkissian (1966)
Effects of indol-3-yl acetic acid on the citrate condensing reaction by preparations of root and shoot tissue.
J. exp. Bot. 17:838-843.

Montgomery, R and C.A. Swenson (1969)
Quantitative Problems in the Biochemical Sciences.
W.H. Freeman and Co., San Fransisco.

Morré and Eisenger (1968)
Cell wall extensibility: its control by auxin and relationship to cell elongation.
in Biochemistry and Physiology of Plant Growth Substance.
ed. F. Wightman and G. Setterfield. Runge Press. Ottawa.

Morris, D.A., R.E. Briant and P.G. Thomson (1969)
The transport and metabolism of ^{14}C-labelled indoleacetic acid in intact pea seedlings.
Planta 89: 178-197.

Nelson, H., I.Ilan and L. Reinhold (1969)
The effect of inhibitors of protein and RNA synthesis on auxin-induced growth.
Is.J.Bot. 18:129-134.

Nissl, O. and M.H. Zenk
Evidence against induction of protein synthesis during auxin-induced initial elongation.
Planta 89:323-341.

Noodén, L.D. and K.V. Thimann (1963)
Evidence for a requirement for protein synthesis for auxin induced cell enlargement.
Proc. Natn. Acad. Sci. U.S.A., 50:194-200.

Noodén,L.D. and K.V. Thimann (1965)
Inhibition of protein synthesis and of auxin-induced growth by chloramphenicol.
Pl. Physiol., Lancaster, 40:193-201.

Noodén, L.D. and K.V. Thimann (1966)
Action of inhibitors of RNA and protein synthesis on cell enlargement.
Pl. Physiol., Lancaster 41:157-164.

Northen, H.T. (1942)
Relationship of dissociation of cellular proteins by auxins to growth. Bot. Gaz. 103:668-683.

O'Brien, T.J., B.C. Jarvis, J.H. Cherry and J.B. Hanson (1968)
The effect of 2, 4-D on RNA synthesis in soybean hypocotyl chromatin.
in Biochemistry and Physiology of Plant Growth Substances.
ed. F. Wightman and G. Setterfield.
The Runge Press Ltd., Ottawa.

Patterson, B.D. and A.J. Trewavas (1967)
Changes in the pattern of protein synthesis induced by 3-indolylacetic acid.
Pl. Physiol., Lancaster, 42:1081-1086.

Patterson, M.S. and R.C. Greene. (1965)
Measurement of low energy beta-emetters in aqueous solution by liquid scintillation counting of emulsions.
Analyt. Chem. 37:854-857.

Penny, P. and A.W. Galston (1966)
The kinetics of inhibition of auxin-induced growth in green pea stem segments by actinomycin D and other substances.
Am.J. Bot. 53:1-7.

Penny, P., D. Penny, D. Marshall and J.K. Heyes. 1971. Early responses of excised stem segments to auxins. J. Exp. Bot. In Press.

Pigott, G.H. and J.E.M. Midgley (1968)
Characterization of rapidly labelled ribonucleic acid in Escherichia coli by deoxribonucleic acid hybridization.
Biochem. J. 110: 251-263.

Polevoy, V.V. (1967)
The action of auxin on electropotential, growth, respiration and synthesis of RNA in sections of corn seedlings. Order of reactions. 1
Wiss.Z. Univ. Rostock 16:477-478.

Raper, J.R. and A.S. Flexer (1970)
The road to diploidy with emphasis on a detour.
in Organization and control in prokaryotic and eucaryotic organisms.
Symp.Soc.gen. Microbiol. 20. (1970)

Ray, P.M. (1961)
Hormonal regulation of plant cell growth.
in Control Mechanisms in Cellular Processes.
D.M. Bonner ed. 185-212.
The Ronald Press, New York.

Ray, D.M. and A.W. Ruesink (1962)
Kinetic experiments on the nature of the growth mechanism in oat coleoptile cells.
Devl. Biol. 4:377-397.

Rayle, D.L., M.L. Evans and R. Hertel (1970)
Action of auxin on cell elongation.
Proc. Natn. Acad. Sci.,U.S.A.,65: 184-191.

Rayle, D.L. R. Ouitrakul and R. Hertel (1969)

Effect of auxins on the auxin transport system in coleoptiles.
Planta 87:49-53.

Remington, R.D. and Schork, M.A. (1970)
Statistics with Application to the Biological and Health Sciences.
Prentice-Hall Inc. Englewood Cliffs, N.J.

Robbins, W.J. and J.R. Jackson (1937)
Effect of 3-indole acetic acid on cell walls of stem and root.
Am. J. Bot. 24:83-88.

Sarkissian, I.V. (1966)
Enhancement of enzymatic synthesis of citrate *in vitro* by indole-3-acetic acid.
Physiologia Plant. 19:328-334.

Sarkissian, I.V. and T.C. Spelsberg (1965)
Preliminary studies of the nature of action of indole-3-acetic acid *in vitro*.
Proc. W.Va. Acad. Sci. 37:70-74.

Siegel, S.M. and A.W. Galston (1953)
Experimental coupling of indoleacetic acid to pea root protein in vivo and in vitro.
Proc. Natn. Acad. Sci. U.S.A., 39:1111-1118.

Sobota, A.E., C.J. Leaver and J.L. Key (1968)
A detailed evaluation of the possible contribution of bacteria to radioactive precursor incorporation into nucleic acids of plant tissues.
Pl. Physiol, Lancaster, 43:907-913.

Stern, R and R.M. Friedman (1970)
Double standard DNA synthesised in animal cells in the presence of actinomycin D.
Nature, Lond. 226:612-616.

Sutton, W.D. (1969)
The structure of the bacteriophage alpha DNA molecule Ph.D. thesis. Massey University.

Sweeney, B.M. and K.V. Thimann (1938)
The effect of auxins on protoplasmic streaming. II.
J.gen.Physiol. 21:439-461.

Tata, J.R. (1967)
The function and distribution of ribosomes during hormone-induced growth and development.
Biochem J. 104:1-16.

Thimann, K.V. (1969)
The Auxins
in The Physiology of Plant Growth and Development.
ed. M.B. Wilkins,
McGraw Hill, London.

Thimann, K.V. and K. Beth. (1959)
Action of auxins on *Acetabularia* and the effect of enucleation.
Nature, Lond. 183:946-948.

Tiselius, A., S. Hjerten, and O. Levin. (1956)
Protein chromatography on calcium phosphate columns.
Arch. Biochem. Biophys. 65:132-155.

Trewavas, A.J. (1968)
Effect of IAA on RNA and protein synthesis. Effects of 3-indolylacetic acid in the metabolism of ribonucleic acid and protein in etiolated sub-apical sections of Pisum sativum.
Archs.Biochem.Biophs. 123:324-335.

Uhrström, I. (1969)
The time effect of auxin and calcium on growth and elastic modulus in hypocotyls.
Physiologia Pl. 22:271-287.

Weinburg, R.A. U.Loening, M. Willems and S. Penman (1967)
Acyralamide gel electrophoresis of HeLa cell mucleolar RNA.
Proc. natn. Acad. Sci. U.S.A. 58:1088-1095.

Yamaki, Toshio (1954)
Effect of indoleacetic acid upon oxygen uptake, carbon dioxide fixation and elongation of Avena coleoptile cylinders in the darkness.
Scient.Pap.Cell.gen.Educ. Tokyo. 4:127-154.

Yoda, S. (1961)
Studies on the osmotic change caused by auxin treatment.
Mems.coll.sci.Univ. Kyoto B. 28:389-400.

Yoda, S. and J. Ashida (1961)
Effect of auxin on the osmotic value in pea stem sections.
Nature Lond. 192:577.578.

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