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Kinetic Studies of the Cell Elongation Phenomenon in Etiolated Pisum Stem Segments

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KINETIC STUDIES OF THE CELL ELONGATION PHENOMENON IN ETIOLATED PISUM STEM SEGMENTS

by

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KINETIC STUDIES OF THE CELL ELONGATION PHENOMENON IN ETIOLATED PISUM STEM SEGMENTS

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The short-term growth response of etiolated pea stem segments (Pisum sativum L., var. Alaska) was investigated using a high-resolution growth recording device. The immediate effect of treatment with indoleacetic acid (IAA) is an inhibition of growth. This inhibition lasts about 10 minutes and then the rate of elongation rises abruptly to a new steady rate about 4 times the rate of elongation before auxin treatment. This rapid steady rate of elongation, however, continues for only about 22 minutes before declining suddenly to a lower steady rate of growth about 2 times the rate of elongation before the addition of auxin. Pretreatment of the segments with cycloheximide or actinomycin strongly inhibits both phases of auxin promoted elongation without altering the length of the latent phase in response to the hormone. The triphasic nature of the growth response in relation to the IAA-oxidase system and a possible pathway for elongation is discussed.

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INTRODUCTION

In recent years the stem of the common garden pea (Pisum sativum L.) has become one of the most frequently used object in the study of plant cell elongation. Both the intact stem and small excised stem segments of this plant show rapid and marked growth responses to such environmental factors as light intensity, light quality, photoperiod, and temperature and to such chemical influences as auxins, gibberellins, ethylene, absicissic acid, the cobaltous ion, sugars, alkyl lipids, calcium and adenine. The classical method of measuring the elongation of pea stem segments can be traced back to the work of Thimann and Schneider (1939). They used short segments which were excised serially from the same plant. Later the method was improved by Galston and Hand (1949a), by using longer segments which were excised from individual plants. Since that time a great many workers have used these methods and have made changes in procedure and technique. All methods of investigation, however, have utilized a technique which allows measurement of elongating stem sections after a period of many hours or at best at half-hour or hourly intervals. In this manner, only long term effects of auxins (and other substances) can be measured. This procedure has added valuable information regarding the effects of auxin on growth. However, recent work with sections from monocotyledonous plants

have shown that the first few minutes of auxin-induced growth may be more important in elucidating the mode of action and kinetics of auxin effects. New devices for studying the short term effects of auxin have opened up a new area for research in auxin physiology. Short term measurements have been made with various tissues for many years. The work of Ray and Rusink (1962) seems to have reawakened interest in this manner of growth measurement.

In view of the results obtained with short term measurements of monocotyledonous plants it was then of considerable interest to examine the short term response of dicotyledonous tissue. In this work a short term growth apparatus designed to measure the response of Pisum epicotyl segments has been used to measure the timing and rate of elongation as affected by auxins and various other compounds.

PEA SEGMENT ELONGATION: HISTORICAL APPROACH

Straight-Growth Tests

The physiological basis for straight-growth tests is the simple stimulation of straight growth by auxins. There is no transport limitation and no dependence upon differential growth to produce curvature, as in the split pea stem test originated by Went (1934). The presence of salts, sugars and other substances will alter the results obtained, both in etiolated and light-grown plants.

The straight-growth test using etiolated pea stem segments was first described by Thimann and Schneider (1939). They used the third internode of eight day-old etiolated pea plants. They described the third internode as "being then well developed but still growing." They utilized three sections, each three millimeters long from individual plants, beginning about ten millimeters below the tip.

Since Thimann and Schneider's original work many other researchers have used the etiolated Pisum straight-growth test as a measure of the activity of different auxins. Table 1 presents a chronological compilation of conditions used by various workers both in the growth of etiolated peas and the application of the testing method. During the development of the pea segment straight-growth bioassay a number of modifications have been introduced. It was learned, for example, that added sucrose will cause an enhancement of growth and this sugar is therefore included by many workers along with the standard phosphate buffer. Sucrose can be omitted in work

Table 1. A compilation of conditions used by various workers utilizing etiolated Pisum straight growth. In all cases the third internode was used at day seven or eight and elongation measurement was made. The key to symbols is given below.

					Phosphate Basal Medium		
Worker	Substrate	Temperature	Section Length	Section Location	Molarity	pH	Sucrose
Thimann and Schneider, 1939		24	3	10			
Galston and Hand, 1949b	WS	25	5	SA		6.1	
Christiansen and Thimann, 1950	Sp	25	20	SA		6.5	
Galston and Baker, 1953	Vm	25	5.3	SA	.60	6.1	2%
Miller, 1954	S	25	5.3	SA		6.0	
Purves and Hillman, 1958		26	V	V	.02	6.0	2%
Galston and Warburg, 1959	WVm	27	5	SA	.02	6.1	1-2%
Stowe, 1960			10	SA		5.5	1-2%
Galston and Kaur, 1961	Vm	27	V	2	.01	6.1	1-2%
Winter and Venema, 1965	Vm	25	5.1	1	.02	6.0	V%

Key to symbols: V, variable; S, sand; WS, washed sand; Sp, special device; Vm, vermiculite; SA, subapical; where section location is given the number indicates the distance below the apex in millimeters, section length given in millimeters and temperature in degrees centigrade.

measuring the short term response of excised stem segments, since the endogenous quantities of sugars are sufficient over the length of time that measurements are made (2-3 hours). In other cases workers have used different types of substrate (most common have been sand or vermiculite), growth medium, basal medium[1], germination temperatures, imbibition times and section lengths. All workers, however, have used a portion of the third internode and have harvested the plants at approximately the seventh day.

The procedure for the etiolated Pisum straight-growth test has been outlined by Leopold (1955) and more recently by Mitchell and Livingstone (1968). For the sake of completeness and reference the procedure outlined by Leopold is given below:

> Planting: In a porcelain-ware tray about one-half inch of dry vermiculite or sand. Saturate with water. Scatter seeds of Alaska pea evenly over the vermiculite; cover with dry vermiculite to a depth of about two inches. Place in a darkroom at 25 degrees centigrade. Planting should be eight days before use. The seedlings may be given some red light.
>
> Cutting: Select seedlings in which the stem internodes above the first leaf node is ¼ to ½ inch long. If completely etiolated peas are used, select seedlings in which the internode above the second scale used is important. Cut off the seedling near the base and then cut a section of uniform length (a size between 3 and 5 millimeters is satisfactory) beginning at a uniform place such as ¼ inch below

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[1]Differentiation should be made here between growth medium and basal medium. For the present work basal medium may be defined as the buffer solution in which segments are grown for test purposes (10^{-3} M potassium phosphate buffer, pH 6.3). Growth medium is a basal medium plus any additional substance, e.g. IAA, sucrose or cobaltous ion.

> the leaf node. Place directly in Petri dishes containing solutions. The use of 10 milliliters of solution keeps all sections at the surface. Submerged sections grow crooked.
>
> Reading: Measure after 24 hours when approximately 90% of growth has usually occurred, or at 48 hours when growth is essentially finished. Again, if growth rate is to be studied, readings will have to be made six hours after the test is started, for the rate begins to decline after that time.

As in other tests the results are expressed as the mean per treatment plus or minus the standard error. In general where serial dilutions are used and a smooth curve is obtained repeatedly, the standard error correction may be considered to be unnecessary (Leopold, 1955).

The growth obtained is approximately proportional to the logarithm of the concentration of auxin applied. In the pea straight growth tests the minimum amount of growth regulator detectable is approximately 0.01 mg/liter of indoleacetic acid (IAA). Maximum growth is obtained at approximately 10 mg/liter of IAA.

Since straight-growth tests are not dependent, as is the Avena test, upon polar transport of auxins, they can be used to test the growth regulator activity of compounds without interference from transport characteristics. Buffers and salts can be used as variables in the straight-growth test.

Galston and Baker (1951) described a straight-growth test using green, light-grown Pisum internodes. In their work they used the fifth internode of plants grown for a period of from twelve to fourteen days. The procedure has been adopted by other workers (Brian and Hemming, 1958) and has been further used by

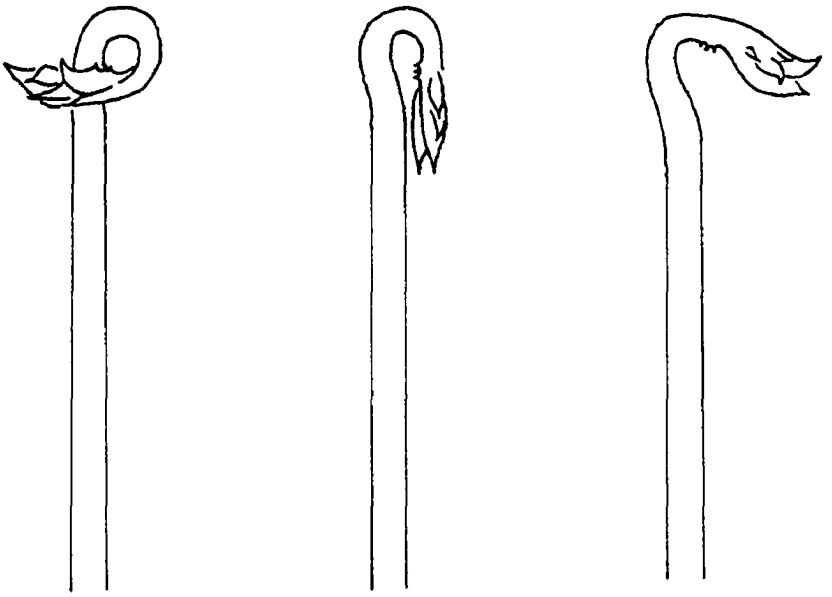
Galston (Galston and Kaur, 1961; Penny and Galston, 1966). Basically the same experimental procedure can be applied to green light grown sections as has been described for etiolated sections.

Morphology of Etiolated Pisum

At an age of seven to eight days after planting, etiolated pea epicotyls are about 20 centimeters tall and have three nodes on the erect portion of the stem. The two lower nodes produce leaves which are pressed scale-like to the internode and comprise 3-5% of the internode surface (Burström, 1964). The scale-leaves are morphologically etiolated stipules in which are found one or more buds which customarily remain dormant (Galston and Hand, 1949b). Galston and Kaur (1961) have described various terminal bud configurations, and have divided these configurations into three patterns of development; (a) the apex of the stem may be recurved, (b) hooked, or (c) open (Figure 1). The open pattern of development may be considered to be the most frequently occurring and the recurved pattern most infrequent. The configuration of the terminal bud is a photosensitive morphological development controlled by a light-regulated opening of the plumular hook (Klein, 1965). Recently it has been shown that repression of the hook opening response in etiolated peas (Goeschl and Pratt, 1965) and in bean (Kang et al., 1967) is related to ethylene production. Inhibition of ethylene production and hence hook opening in the presence of cobaltous ion, cycloheximide and red light have also been demonstrated (Kang et al., 1967). Galston and Kaur (1961) have further stated that no major difference in

Figure 1. Seven day old etiolated Pisum hypocotyl; terminal bud configuration

A, recurved configuration; B, hooked configuration; C, open configuration



A B C

growth occurs between the three conditions of hook configuration, but that the response to auxin in etiolated plants is slightly better with the recurved condition.

The internodes are elliptical or rounded rhombical, in cross section, with a ratio of smallest axis to largest axis equal to 0.89. The stele is central and weakly developed with the axes 400-500 u and occupies only 20% of the cross sectional area. Four leaf traces are faintly indicated. The epidermal cells have a radial diameter of 30 $\pm$ 1.4 u, followed by two parenchymatous layers with small intercellular spaces. The major part is taken up by a seemingly uniform parenchyma with cell diameter 60-100 u and larger intercellular spaces (Burström et al., 1967).

Effect of Calcium During Growth

The procedure for growing Pisum seedlings outlined by Leopold (1955), (see pages 4 and 5 of this work) does not call for the inclusion of nutrients in the watering solution even though certain factors may be necessary for the proper growth and development of the young plant. Calcium, for example, seems to be important in the development of young growing pea plants. Day (1928) in discussing the effect of calcium on the growth of peas states that "in plants deprived of calcium the length of the stem is less than that of plants grown in the presence of calcium." She also noted that the difference in plants grown with and without calcium "is in the elongation rather than in the anatomical structures." Also, the green and dry weight of the plant decreases as the amount of calcium

is decreased. Day also recorded " the calcium in the seed is used for early growth." Burström (1964) working with the etiolated pea has made a more complete study of the effect of calcium. In general his results on etiolated pea agree with those of Day, but can be more specifically applied to the present work. It is observed that the length of internodes grown under Ca-deficient conditions are much less than those supplied with calcium at any time up to nine days, and the quantity of calcium in the internodes increases in response to externally added calcium (Table 2).

Table 2. Calcium content of etiolated pea internodes (umol per 100 mm internode length) at six days (after Burström, 1964).

Internode	Ca-deficient	Ca-supplied
I	0.19	0.83
II	0.12	0.82
III	0.08	0.83

Burström states that the first internode does not react to externally supplied calcium; it is probably sufficiently supplied from the cotyledons. This observation coincides with that made by Day. Burström further remarks that the elongation of a calcium deficient second internode begins to slow down after the seventh day, and the elongation of all three internodes ceases after eight days. It is obvious, from Burströms work, that Ca-deficiency appears about six days after planting. Uhrström (1969) has shown that calcium always increases Young's modulus and gives the cell wall a more rigid structure. Uhrström states that calcium deficiency implies both a decreased

modulus and a decreased growth, and has shown that calcium in certain concentrations is necessary for longitudinal growth.

It may be recommended, considering the above discussion, that when etiolated Pisum is grown for growth studies that a sufficient amount of calcium be added to the watering solution. Other minerals may also become limiting during the first week of Pisum growth. Deficiency symptoms may be such as to go unnoticed. Added minerals may be necessary for maximal and normal elongation of the stem and especially of the third internode. Because of these possible mineral deficiency effects it may be advisable to culture young pea plants in a growth medium containing a complete nutrient solution, such as that described by Hoagland and Arnon (1950). Few reports have appeared in the literature which have utilized an externally applied nutrient solution, prior to growth studies. Most workers utilize half-strength Hoagland's solution or a solution of mineral salts which is essentially similar.

Effect of Internode Length and Plant Age

Sections of etiolated Pisum may obviously be derived from internodes of varying length. Galston and Kaur (1961) have demonstrated that etiolated internodes less than 15 millimeters long are inferior to longer internodes as sources of tissue for growth experiments (Table 3). Despite the fact that the experiment illustrated is atypical, in showing depressed growth with IAA, Galston and Kaur state that this type of result has been generally obtained in other experiments. They selected internodes as near as possible to 30

millimeters in length as the most desirable material for their studies.

Table 3. Effect of length of the third internode on the growth of 5 mm etiolated pea stem segments in the presence of 10^{-6} M IAA, after 20 hours (Galston and Kaur, 1961).

	Growth of Segments (mm)	
Length of Internode	Endogenous	+IAA
15	4.19	4.31
15-30	4.77	4.50
30-50	4.65	4.51

In regard to the age of plants Galston and Kaur state that so long as the morphology of the terminal portion of the plant is kept reasonably constant, age of the plant is not an important determinant of section growth.

Effect of Segment Length and Location

A fair amount of work has been done on the response of pea segments as affected by the length of the segment and distance of the segment from the apex. The most complete study involving length and distance parameters has been reported by Purves and Hillman (1958). In their work they utilized five and ten millimeter sections of etiolated Pisum taken at various distances from the apex. They determined that "the distance from the apex strongly affects the elongation response of dark-grown sections to IAA." They have shown that sections further removed from the apex show less growth at lower concentrations than those close to the apex. In their work with etiolated peas, IAA at a concentration of 10^{-5} M caused maximum fresh weight increase of five and ten millimeter sections one and four

millimeters removed from the apex. Higher concentrations of IAA (10^{-4} M) caused only slight inhibition of elongation. Sections five millimeters long, one millimeter removed from the apex showed maximum elongation at 10^{-7} M IAA. Concentrations greater than 10^{-6} M caused an inhibition of elongation. The absolute response of such sections to optimal IAA was usually extremely small. Other five and ten millimeter long sections, taken at various distances from the apex showed a much greater response to IAA and the optimal concentration was 10^{-6} M. None of these were inhibited in elongation by concentrations up to 10^{-4} M IAA.

Sections five millimeters long, one millimeter removed from the apex, have a number of striking properties; high endogenous growth rate, weak response to IAA, and low IAA optimum. These may be explained in terms of proximity to the site of auxin production and a high endogenous auxin content. Galston and Dalberg (1954) have found that the young actively growing region of etiolated Pisum is very low in IAA-oxidase activity, while progressively older regions have successively higher IAA-oxidase activity. This then explains why segments close to the apical region of etiolated pea have a higher endogenous IAA content and therefore require less exogenous IAA to maximize elongation rates.

Galston and Kaur (1961) have determined the effect of the initial length on the percent increase in length and fresh weight of etiolated Pisum segments in the presence of IAA. Their conclusion was that three to five millimeter long sections of etiolated Pisum

are close to optimal. Longer sections of etiolated Pisum exhibit a much lower efficiency of growth. Galston and Kaur state that "this resembles the situation in the intact plant and probably reflects the fact that cell division is restricted to the apical region in etiolated Pisum." Based on these results they chose five millimeter segments two millimeters removed from the apex for subsequent investigations.

METHODS, MATERIALS AND ELONGATION MEASUREMENT

The first experiments employing magnification devices for the study of plant growth may be traced back to the eminent plant physiologist Kliment Timiryazev, about 1882. He used two devices, one mechanical, another optical, to study the elongation of intact plant parts. His mechanical device provided continuous recording of stem growth utilizing a turning kymograph drum (Timiryazev, 1958). In recent years many devices have been employed in the magnification of plant growth. Ray and Rusink (1962) developed a device for studying the growth kinetics of a single oat coleoptile section. Their method while not providing continuous recording of growth, allowed sufficient precision to measure the growth that occurred in one minute. Recently a new device called the Evans-Ray machine[1] has been described (Evans, 1967; Evans and Ray, 1969). This device provides both automatic and continuous recording of the growth of may coleoptile sections. Perhaps the most recent device which has been developed for measuring the short term responses of plants is an electrical mechanical instrument in use by Warner and Leopold. Their device consists of an electric positioning sensor which permits the measurement of growth rates of whole seedlings of etiolated Pisum in two minute intervals[2].

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[1] Anton Lang in an address on 'Plant Hormones - Advances and Problems,' Notre Dame University, March 11, 1969.

[2] A.C. Leopold, private communication, June 9, 1969.

Plant Material

Alaska peas (Pisum sativum L., var. Alaska) purchased from Asgrow Seed Company, New Haven, Conn., were used for all experiments with Pisum described in this work. Seeds were soaked in distilled water for 4-12 hours and were sown in plastic trays in vermiculite saturated with half-strength Hoagland's solution (solution A, Hoagland and Arnon, 1950). Nutrient solution was considered necessary for the reasons cited in the previous section (pages 10-12). Trays were placed in a dark room in a light-tight cabinet at 23-25 degrees centigrade. Occasional weak red light was employed for watering and other manipulations. A double-bladed cutting tool was used to cut segments five millimeters in length from the third internode of 7-8 day old plants beginning 1-3 millimeters below the apical hook of the third internode. The segments were floated on 10^{-3} M potassium phosphate buffer (basal medium pH 6.3) for at least 30 minutes prior to use.

In a few experiments cucumber hypocotyl segments or oat coleoptile segments were used. Cucumber seeds (Cucumis sativus L., var. Straight-eight) were planted directly on wet filter paper in covered plastic dishes and placed in complete darkness at 23-25 degrees centigrade. When the seedlings were 4-5 days old, segments were cut as described above for peas. Oats (Avena sativa L., var. Victory) were grown, and eight millimeter segments were harvested as described by Evans and Ray (1969).

Segment Retention Device

The high-resolution growth recording device used in these experiments is described in detail in an earlier work (Evans and Ray, 1969). For experiments with pea stem segments modifications were necessary since Pisum is not hollow and does not lend itself to 'stringing' without tissue damage. Therefore, a pea segment retention device has been designed to hold segments. The assembled retention device consists of two basic parts; the segment holder and outer restraining sleeve (Figure 2, A and B). The pea segment holder is a thin walled plastic tube 13.5 centimeters long and 2.5 millimeters in inside diameter. Toward the top of the tube there is an optical slit (S_o) 2 millimeters wide and 22 millimeters long. In addition there are a set of helical slits (S_t) beginning 4 millimeters below the optical slit and running the length of the tube terminating at the sealed end (E). The outer sleeve is a thin walled plastic tube 10.2 centimeters long with an inside diameter of 3 millimeters (Figure 2, A). It is provided with many large openings (O) which are arranged in a helical fashion coincident with those of the holder. In the assembled device the sleeve fits over the lower portion of the holder below the optical slit. The assembled arrangement has proven flexible, to the extent that it will accomodate lateral growth of segments without binding, and segments of different diameter. Flexibility is accomplished by the two helical slits (S_t) and retention is maintained through the use of the plastic sleeve.

The coincident slit arrangement in both the holder and sleeve provide the segment with contact to an external bathing solution.

Stem segments are loaded into the holder (B) one at a time and are pushed gently down the tube until a total of 20 segments (usually) are accomodated. A metal cylinder (M), weighing 150.4 milligrams (6 mm long and 2 mm in diameter) is then inserted into the holder so that it rests upon the uppermost segment of the column and serves to keep the segments in contact. The assembled retention device, as described above, is attached to a glass tube 17 centimeters long, which is fire-polished at one end to provide a snug fit with the plastic segment holder (Figure 2, G). The entire retention device plus the glass extension is then inserted into the specially constructed glass growth measurement chamber (Figure 3) and suspended from the top of the side arm so that the optical slit (S_o) lies in the middle of the cuvette (C) and is held in place with a plastic tube cut to provide a friction fit with the glass extension tube and the wall of the glass measurement chamber side arm.

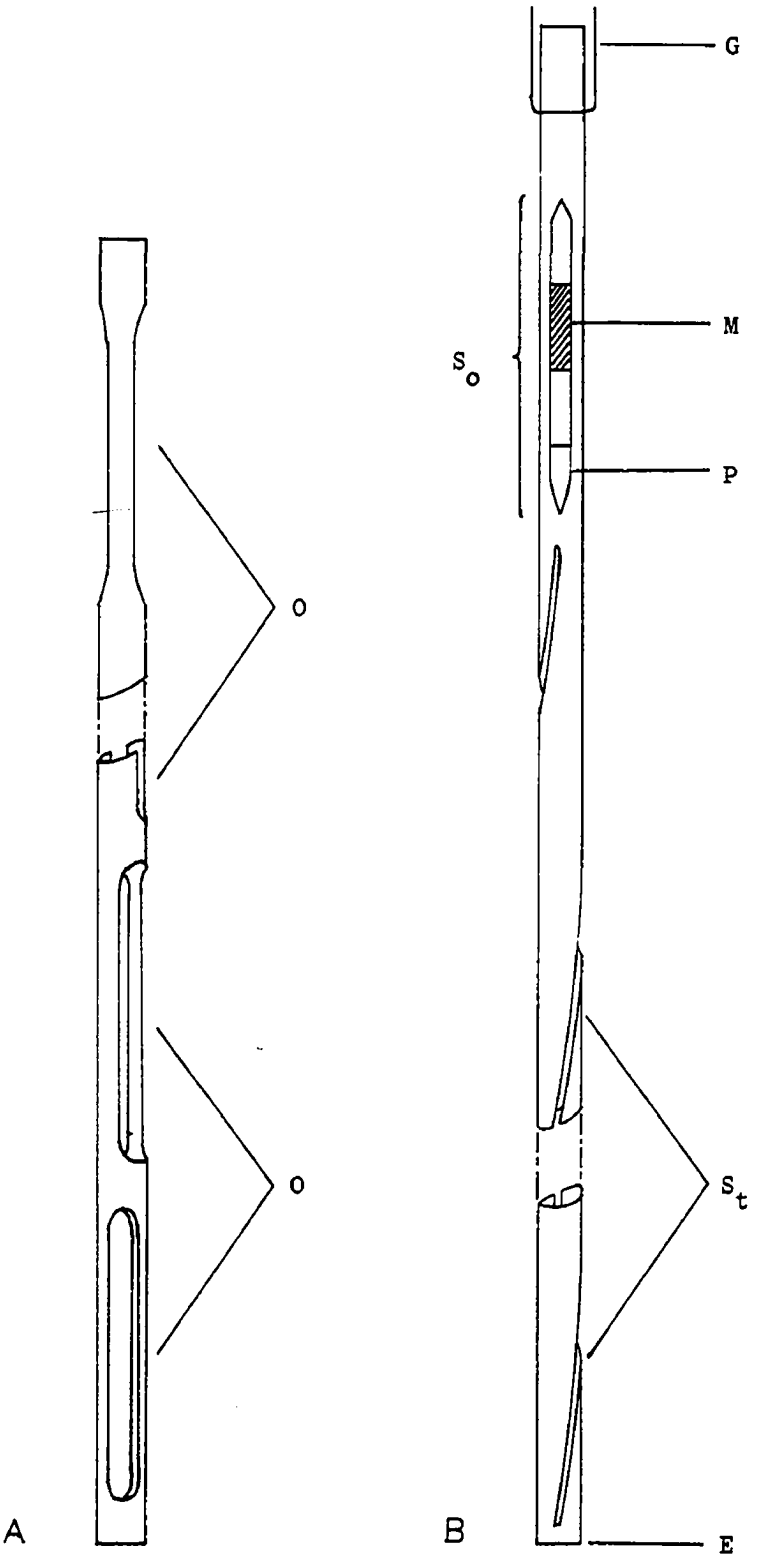
Method of Measurement

The idea for an automatic high-resolution recording device for segments of plant material does not originate here. The equipment and theory has been adopted from the work of Evans (1967) and has been described in detail in his published papers (Evans and Ray, 1969; Evans and Hokanson, 1969) and is here briefly described, including the modifications necessary for work with solid sections of the Pisum type. The original motivation behind the method was to

Figure 2. Pea segment retention device

A: External sleeve; O, pairs of large openings arranged in a helical fashion

B: Segment holder; G, glass extension tube; S_o, optical slit; M, metal weight; P, pea stem segments; E, sealed end; S_t, double helical slit



simplify the technique which had been established so that it could "provide for continuous recording in the absence of the investigator" (Evans, 1967).

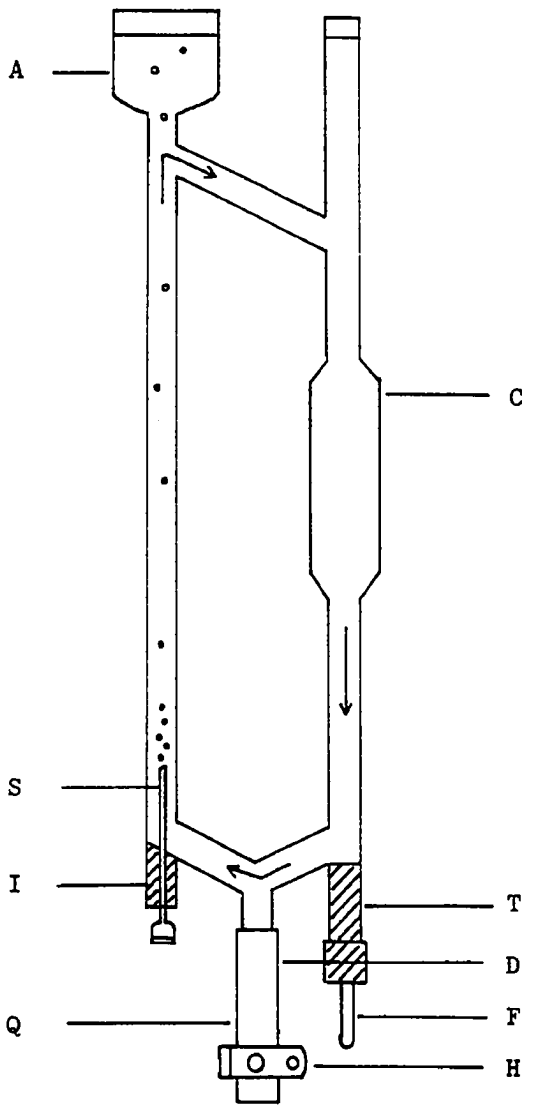
Growth measurement chamber

A glass growth measurement chamber has been designed for Pisum segments and is a modification of that used by Evans (1967) in that its holding volume has been increased from 14 to 25 milliliters. This has been accomplished primarily by an increase in the size of glass tubing used to construct the chamber. The basic design of the chamber has not changed. Figure 3 illustrates the growth measurement chamber used in all experiments described in this work.

The growth measurement chamber is made from pyrex glass tubing and a pyrex glass cuvette. In a typical experiment the open end of the arm containing the cuvette is stoppered with a teflon plug (T). The hole in teflon plug (T) is stoppered with a small glass rod (F). A hose is attached to the bottom of a #15 syringe needle (S) which is set in inert resin glue (I) serving to stopper this arm of the chamber. The syringe needle (S) provides the chamber with prepurified grade oxygen at a rate of approximately 2.5 ml/min. Opening D is fitted with a small piece of tygon tubing (Q) and a pinch clamp (H); this serves as a drain. Solution is poured in the chamber via the reservoir (A) until approximately 20-23 milliliters is accepted. Gassing the chamber causes the solution to circulate as indicated by the arrows in Figure 3. The pea segment retention device is then

Figure 3. Pyrex glass growth measurement chamber

A, reservoir; D, drain; C, cuvette; S, syringe needle; I, inert resin glue; Q, tygon tubing; H, pinch clamp; F, small glass rod; T, teflon plug



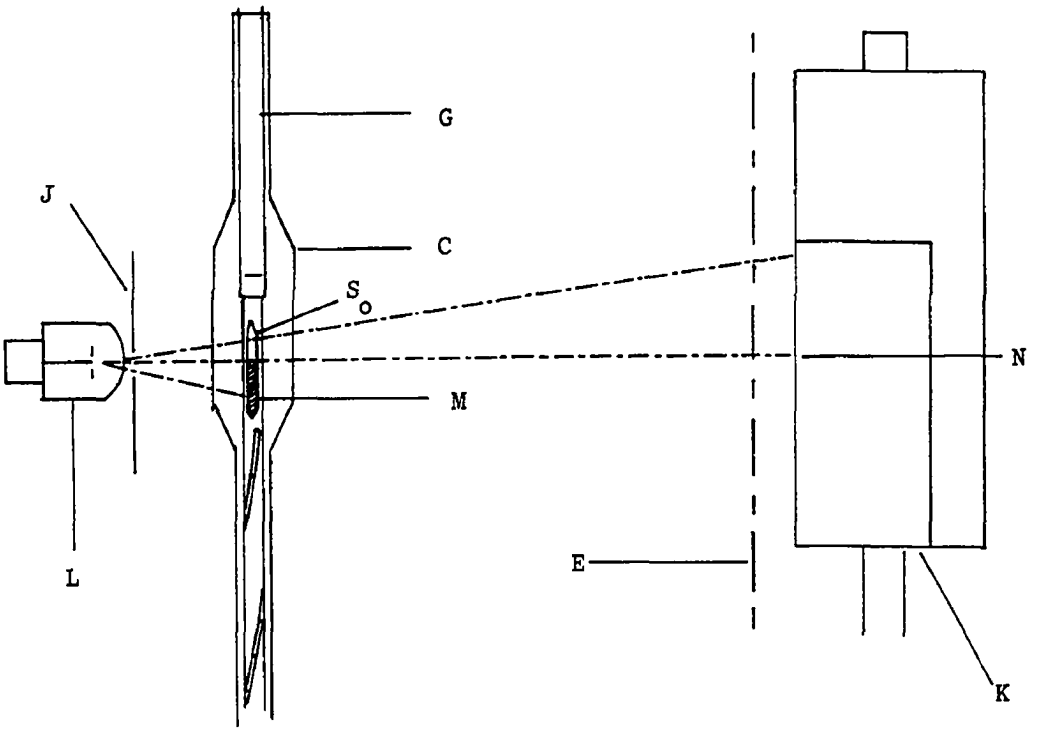
suspended in the chamber as previously described. The chamber may be drained (via opening D) and refilled (via funnel A) with a different growth medium in a minimum time of 30 seconds.

Recording device

The elongation of the entire column of pea stem segments is recorded shadowgraphically as diagrammed in Figure 4. A two watt zirconium arc lamp (L), (Sylvania type GZ2U Model C2) is positioned within the body tube of a horizontal microscope in place of the objective lens. At a distance of about 1.5 meters from the stand supporting the growth chamber, a plywood baffle (E) with vertical slit (U) 2 millimeters wide is placed. Behind the baffle is located a multispeed kymograph drum (K) positioned as indicated in the lower part of Figure 4 and bearing a sheet of Kodak polycontrast photographic paper (203 mm wide). Light from the zirconium arc, which constitutes a nearly point source (0.127 mm in diameter) is limited by the diaphram (J) so that a narrow cone of light passes through the cuvette (C) falling upon weight (M) as it passes through the optical slit (S_o). A sharp shadow (N) of the weight, within the circle of light, is thereby cast upon the slit as shown in Figure 4. The light exposes that portion of the photographic paper that is above the edge of the shadow. As the segments grow the boundary moves upward and traces a record of the elongation which is apparent upon development of the photographic paper. Figure 5 is a diagrammatic half-scale example of such a record. The vertical line (A) is an event marker and was produced by covering the slit during the solution change.

Figure 4. Diagram of the shadowgraphic growth-measuring device

L, zirconium arc lamp; E, plywood baffle; U, vertical slit; K, kymograph drum; J, diaphram; C, cuvette; M, metal weight; So, optical slit



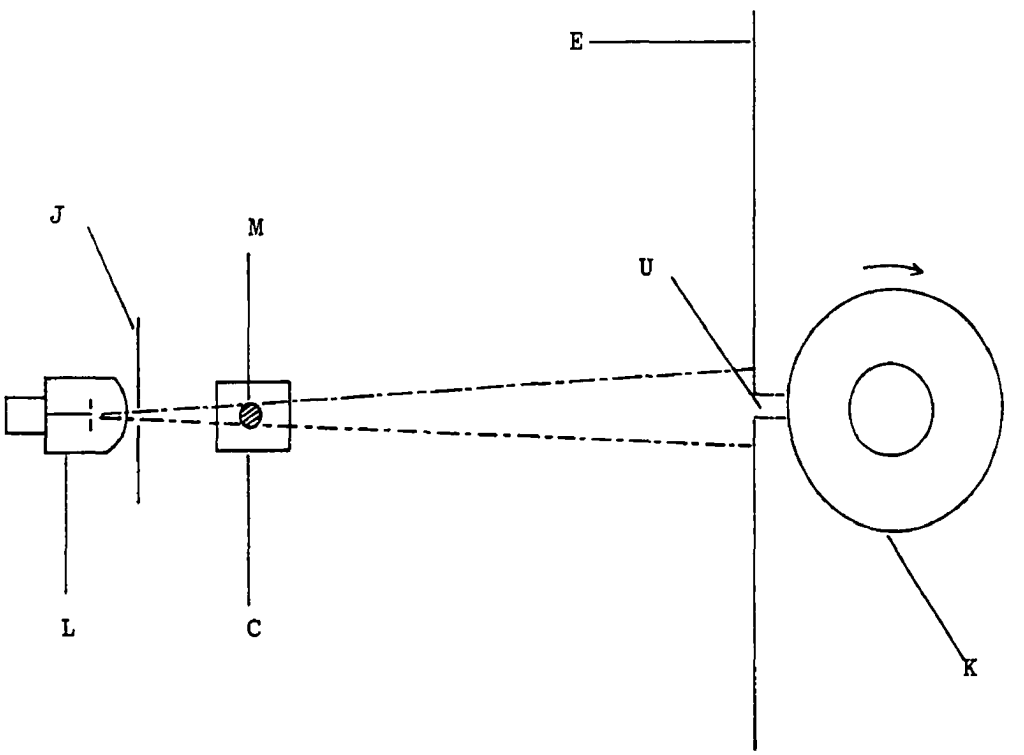
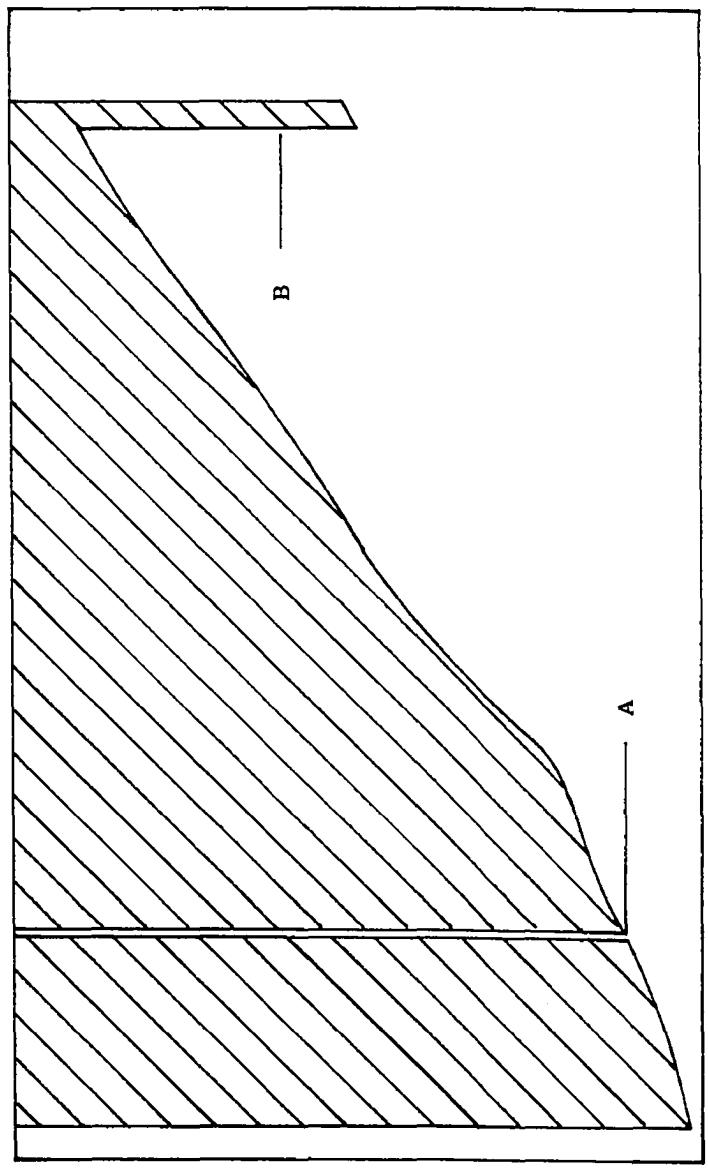


Figure 5. Diagrammatic half-scale example of a typical growth record

A, event marker; B, calibration guide. Hatching indicates the darkened portion of the growth record (see text for explanation).



The darkened extension (B) serves as a calibration guide (see below). All growth curves presented in this paper are exact half-scale reproductions made with a mechanical pantographic device used on original shadowgraphic records.

Calibration of the Growth Measuring Device

Using this recording method the elongation of the column of segments is magnified by the distance of the lamp from the slit, divided by the distance of the lamp from the metal weight. This magnification was calibrated at the end of each experiment by moving the lamp up or down a known distance (usually 2 mm) using a gear mechanism positioned below the mounted light, thus causing a displacement of the shadow boundary which appears on the photographic record (Figure 5, B). Thus, knowing the displacement of the light and magnified displacement of the object (the metal weight) the magnification can be calculated by similar triangles using the equation;

$$\text{Magnification} = \frac{\text{Distance of Shadow Displacement (mm)}}{\text{Distance of Light Displacement (mm)}} + 1 \quad (1)$$

In most experiments the optical magnification is between 27 and 42. This calibration allows the absolute elongation to be calculated from the shadowgraph record. With each curve presented a vertical bar is given which indicates one-half millimeter of elongation (by the entire column of segments) for the particular growth record.

Timing and Rate Calculations

Determination of the timing of various events on the growth record involves a simple measurement of the horizontal displacement of the event from a standard. Conversion is then made to time units, since the kymograph drum revolves at a known rate, utilizing the following equation;

$$\text{Time (min)} = x \,/\, .308 \text{ cm/min} \qquad (2)$$

where x is the horizontal displacement in centimeters and .308 cm/min is the usual rate of revolution of the kymograph drum. This equation is applied to determine the exact time of occurrence of an event. It has been applied to the calculation of the break-time, half-time, steady-state-time and rate-change-time. These events may be defined here for convenience; break-time is that time where the increase in rate is just detectable; steady-state-time is that time where the rate first maintains a rather steady character, and half-time is that time after auxin addition where the rate is one-half the steady-state rate (calculated as that point where the tangent to the curve is defined by an angle, which represents the latent rate plus one-half the difference between the latent-rate and the final steady-state rate).

Rate calculations are made using the calculated magnification (mag) from equation (1), the number of segments used during the experiment (seg) and the tangent of the angle of incline for the particular phase of growth to be measured (Tan θ). The equation

for a rate calculation is;

$$\text{Rate} = \text{Tan}\ \theta \times .308\ \text{cm} \times \frac{1}{\text{mag}} \times \frac{1}{\text{seg}} \times \frac{60\ \text{min}}{\text{hr}} \qquad (3)$$

The rate is expressed as the number of millimeters of growth per segment per hour (mm x seg/hr).

Chemicals

Actinomycin D was purchased from Calbiochem, Los Angeles, California and cycloheximide from Sigma Chemical Company, St. Louis, Missouri. Indoleacetic acid was obtained from Fisher Scientific Company, Chicago, Illinois and indolepropionic and indolebutyric acids were purchased from Nutritional Biochemicals, Cleveland,Ohio. Cobaltous chloride ($CoCl_2.6H_2O$) was obtained from Baker and Adamson Products of the Allied Chemical Corporation, New York.

RESULTS: KINETIC ASPECTS OF THE AUXIN RESPONSE

The response of Pisum stem segments to exogenously supplied auxin is not immediate. As previously reported for monocotyledonous tissue (Ray and Rusink, 1962; Evans, 1967; Evans and Ray, 1969) pea segment tissue responds to IAA only after a latent phase of about 10 minutes. The character of the response in pea segment tissue is much different than that which has been reported for monocotyledonous tissue in that the response which is observed is sigmoidal. This paper constitutes the first report of such a finding in the short term measurement of stem tissue. The results presented deal with the characterization, timing and rate response of pea stem tissue as affected by auxins and various antibiotics.

Timing of the Auxin Response

Immediately after cutting segments and their introduction into the growth measurement chamber a rather rapid rate of elongation is observed. Figure 6, curve A, illustrates the response of five millimeter segments in basal medium immediately following cutting. The initial rapid rate begins to decline until after 30 or 40 minutes the rate of elongation levels off to a rather constant rate. This rate is referred to as the water rate and represents the elongation rate of pea stem segments after the depletion of endogenous levels of IAA. It is after this initial period of 30-40 minutes that the effects of added auxin can be accurately measured. It is for this reason that

Figure 6. Elongation response of five millimeter long pea stem segments

Curve A: endogenous growth rate of segments in basal medium immediately following cutting

Curve B: elongation response of segments immediately following cutting with a change from basal medium to 10^{-5} M IAA at the caret

segments are floated on basal medium for a minimum time of 30 minutes prior to insertion and measurement in the growth measurement chamber.

If the basal medium is replaced by a solution of IAA, there intervenes a latent phase of about 10 minutes followed by a sudden increase in the rate of elongation. Figure 6, curve B, illustrates the response of segments to 10^{-5} M IAA (auxin added at the caret). This experiment has been repeated over 15 times using 10^{-5} M IAA and the results indicate an absolute latent period (period of no significant increase in rate) of about 10 minutes. The latent period is followed by a rapid increase in growth rate within the next two minutes, with the establishment of a new rate within about 11 minutes after auxin addition (Figure 7, curves A and B). This auxin-promoted rate of elongation, however, persists only 11 minutes before the rate begins to drop to a lower value. The time at which the second rate response begins is referred to as the rate-change-time (RCT) and occurs rather rapidly. The rate-change-time occurs consistently, in response to 10^{-5} M IAA, 22 minutes after the addition of auxin. This response is considered the standard response for Pisum tissue presented in this work, since at this concentration the response is most reliable and reproducible.

The time between half-time (HT) and the rate-change-time is referred to as the primary phase of the auxin response and that time after the rate-change-time as the secondary phase of the auxin response. For convenience the latent phase may be defined as that time between the addition of auxin and the half-time.

Figure 7. Elongation response of Pisum segments to 10^{-5} M IAA

Curve A: Basal medium changed to 10^{-5} M IAA at the caret.

Curve B: Basal medium changed to 10^{-5} M IAA at the caret. Extrapolation lines for both curves are explained in the text.

Response to various IAA concentrations

Figure 8 illustrates the timing of the auxin response using various concentrations of IAA. The latent period (measured as half time) remains quite constant through a 100 fold increase (10^{-6} to 10^{-4} M) in IAA concentration, and the sigmoidal response seems to be much stronger as the concentration increases. Table 4 presents the average timing measurements over the concentration range studied. Break-time is observed to increase slightly as the concentration is reduced. This may be explained on the basis of concentration, since it would seem more reasonable that a higher concentration of IAA should penetrate more quickly than a less concentrated one. The half time (latent period) of the response is quite consistent for the range of concentrations observed, and the mean value is 10.37 minutes. Experiments have also been performed at concentrations greater, lesser and intermediate to those presented in Table 4 and show the timing of the latent phase to be the same. Steady-state-time is quite variable and is noted to be highest for the two extremes of concentration. The rate-change-time shows a gradual increase as concentration is decreased. It is of interest to note that the proportionate increase in break-time, with decreasing concentration is very similar to the proportionate increase in rate-change-time. The break-time and the rate change time both may be slightly concentration dependent.

The rate of elongation in response to various concentrations of IAA is presented in Table 5. Variations in the endogenous rate of growth of segments are unavoidable and are probably directly related to growth conditions of seedlings. Very good agreement is obtained

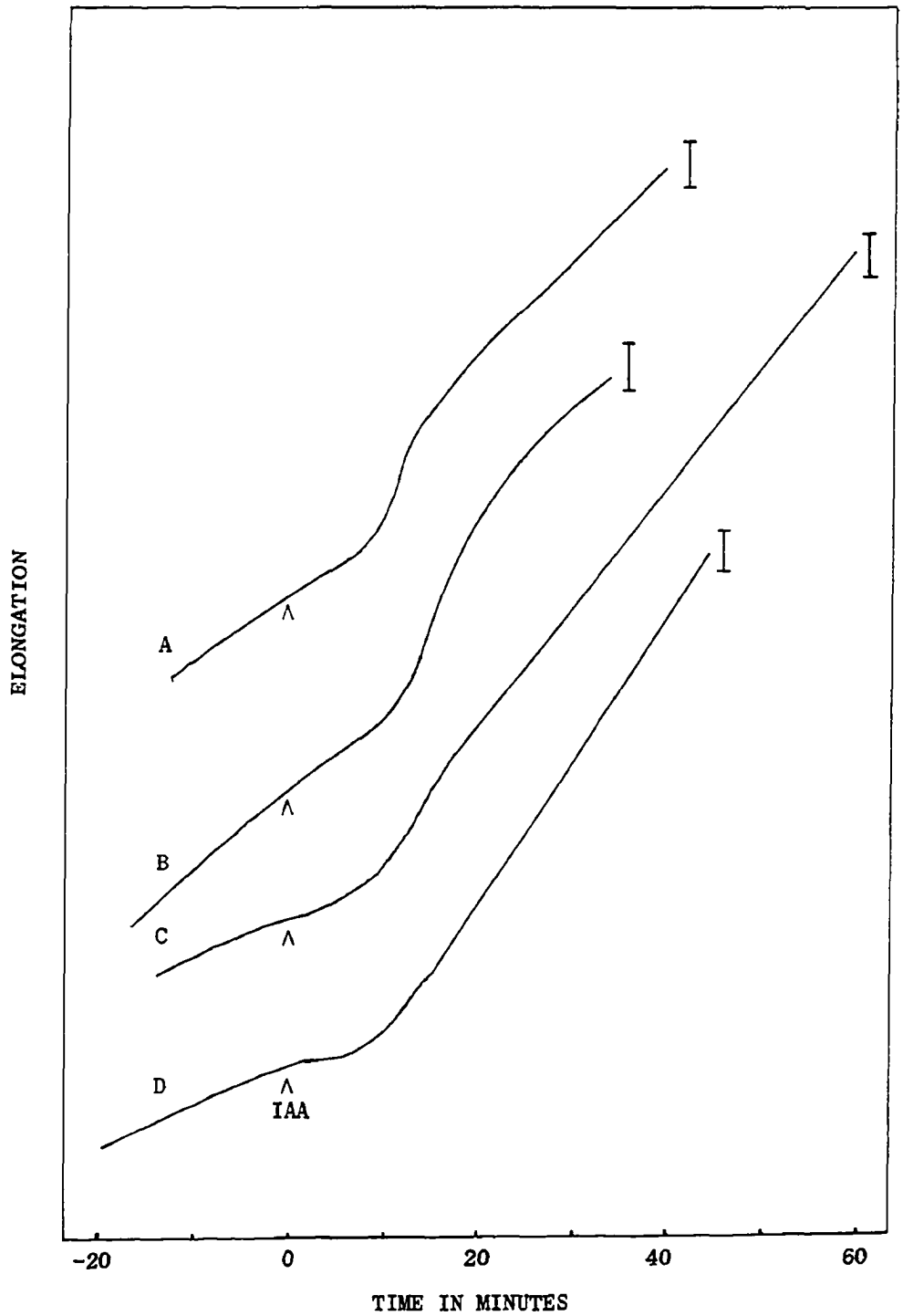
Figure 8. Elongation response of segments to various IAA concentrations

Curve A: Elongation response of Pisum stem segments. Basal medium changed to 10^{-4} M IAA at the caret.

Curve B: Elongation response of cucumber stem segments. Basal medium changed to 10^{-5} M IAA at the caret.

Curve C: Elongation response of Pisum stem segments. Basal medium changed to 10^{-5} M IAA at the caret.

Curve D: Elongation response of Pisum stem segments. Basal medium changed to 10^{-6} M IAA at the caret.



from segments within the same batch, but comparable rates and timing measurements vary somewhat between batches. This variability was also noticed by Audus (1952) and by Thimann (1956).

Table 4. Timing of the auxin response with various IAA concentrations (time in minutes).

	Molar Concentration (IAA)		
	10^{-4}	10^{-5}	10^{-6}
Break-time	8.11	8.42	8.55
Half-time	10.37	10.42	10.55
Steady-state-time	15.72	11.26	14.06
Rate-change-time	20.30	22.11	22.70

As noted from Figure 8 the sigmoidal response seems to increase in strength as the concentration is increased. This is due mainly to the greater reduction in rate in passing from the primary induced phase to the secondary (PR→SR). Table 5 shows that the percent reduction in rate in passing from PR to SR decreases with decreasing concentration. It seems obvious therefore that the lower the concentration of auxin the more closely the PR approaches that of the SR.

Table 5 also shows that the percent increase in the rate of elongation from the latent rate to the primary and secondary rate increases with decreasing concentration of hormone. For this range of concentrations then 10^{-6} M IAA may be considered the optimum, inducing the greatest response. This agrees with other data on the optimum response of segments measured in long-term experiments (Galston and Kaur, 1961; Thimann and Schneider, 1939).

Table 5. Elongation rates during various phases of the response of pea stem segments to various IAA concentrations (rate = mm x seg/hr).

	Molar Concentration (IAA)		
	10^{-4}	10^{-5}	10^{-6}
Water-rate (WR)	.171	.142	.081
Latent-rate (LR)	.139	.105	.071
Primary-rate (PR)	.448	.443	.457
Secondary-rate (SR)	.291	.327	.422
% decrease PR→SR	35.0	26.0	7.5
% increase LR→PR	222	321	543
% increase LR→SR	109	227	459

Initial effect of IAA

The initial effect of IAA over a wide range of concentration is to inhibit elongation. Table 6 shows the average water and latent elongation rates for a range of concentrations. The percent reduction for concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M are 9.7%, 26% and 14% respectfully. Figure 9, curves A, B and C, illustrates the initial response of segments to various concentrations of IAA. The reduced rate of elongation during the latent phase is quite apparent.

The reduction in rate of elongation during the latent phase occurs consistently and appears to be independent of the water rate and of the concentration of applied auxin over the range studied. The inhibition may be as high as 77% in some cases, or as low as 19% (Table 6).

Figure 10 illustrates the effect of increasing the concentration of IAA at intervals of four to five minutes during the latent phase, following the initial treatment with auxin. Each successive higher concentration of auxin results in continued inhibition of

Figure 9. Elongation response of Pisum stem segments to various IAA concentrations, after growth in basal medium and auxins

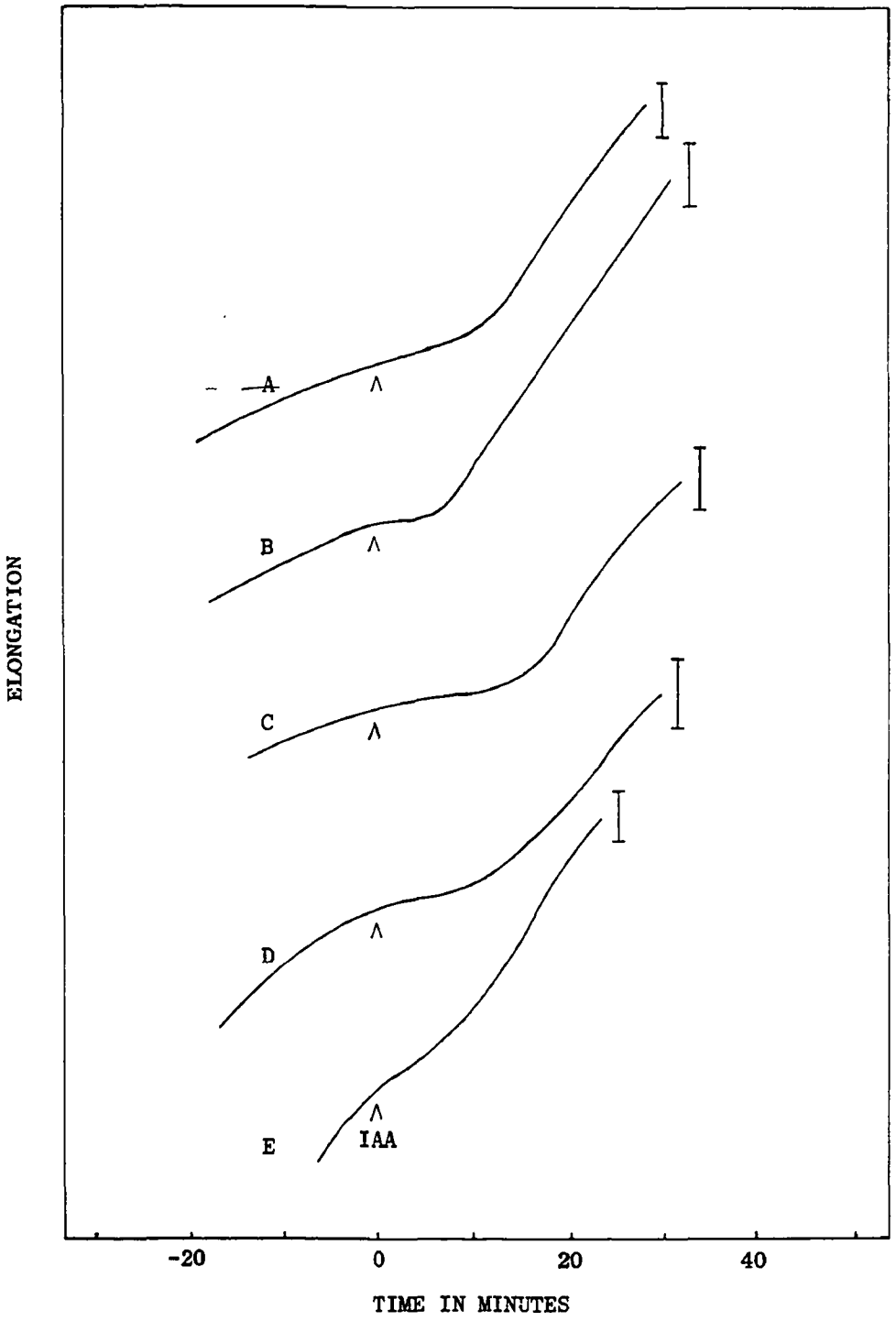
Curve A: Basal medium changed to 10^{-6} M IAA at the caret.

Curve B: Basal medium changed to 10^{-5} M IAA at the caret.

Curve C: Basal medium changed to 10^{-4} M IAA at the caret.

Curve D: Growth medium changed from 5×10^{-7} M IAA to 10^{-3} M IAA at the caret.

Curve E: Growth medium changed from 10^{-4} M IPA to 10^{-4} M IAA at the caret.



elongation and postponement of the positive growth response for a total of 13.6 minutes. The usual 10-minute latent phase follows the final increase in auxin concentration so that the total latent period between initial auxin treatment and the beginning of the positive growth response is 23.6 minutes. This experiment represents a greater than two-fold increase in the timing of the latent phase over the control. It may be possible to extend the latent phase even further by using further stepwise increases in IAA concentration.

That an increase in auxin concentration can cause inhibition of the elongation of segments already promoted by IAA is shown in Figure 9, curve D. Here an established rate of elongation supported by a low concentration of IAA ($5x10^{-7}$ M IAA) was replaced with a high concentration of IAA (10^{-3} M) after the beginning of the secondary phase of the elongation response to the low concentration. This results in an immediate inhibition of elongation that lasts about 10 minutes. This same inhibitory effect was observed when 10^{-4} M IAA was added to segments growing in 10^{-4} M indolepropionic acid (IPA), (Figure 9, curve E).

The initial latency and inhibition exhibited by high concentrations of IAA are not due to supraoptimal inhibitions. Evans (1967) and present experiments show that the initial latency in the response of segments to supraoptimal conditions (10^{-3} M IAA) is equivalent to that seen at optimal auxin concentrations (10^{-6} M IAA). Evans has shown with Avena coleoptile sections that the supraoptimal effects are not apparent in a buffered solution until about 50 minutes after the addition of auxin.

Figure 10. Lag extension experiment using increasing concentrations of IAA

Curve A: Control experiment; basal medium changed to 10^{-3} M IAA at the caret

Curve B: Lag extension experiment; basal medium changed to 10^{-6} M IAA at first caret, replaced by 10^{-5} M IAA at the second caret, replaced by 10^{-4} M IAA at the third caret and replaced by 10^{-3} M IAA at the last caret

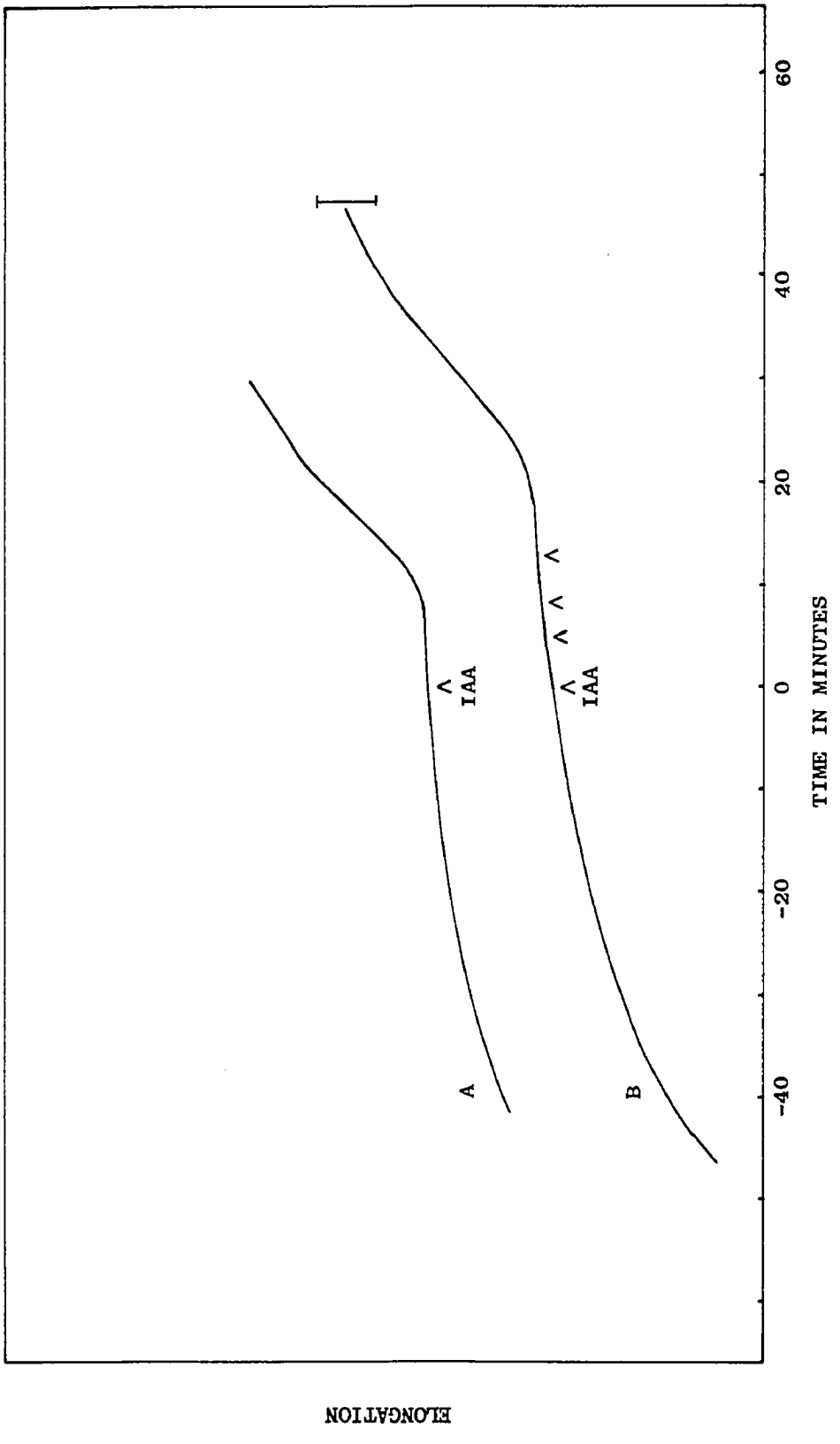


Table 6. Auxin inhibition of the elongation rate during the latent phase (rate = mm x seg/hr).

Experiment Number and Concentration	Water-rate	Latent-rate	Percent Inhibition
IAA (10^{-6} M)			
1	.092	.009	44.5
IAA (10^{-5} M)			
2	.216	.136	37.0
3	.108	.086	20.5
4	.155	.031	67.1
5	.122	.050	77.3
IAA (10^{-4} M)			
6	.128	.104	19.0

Rhythmic oscillations in the auxin response

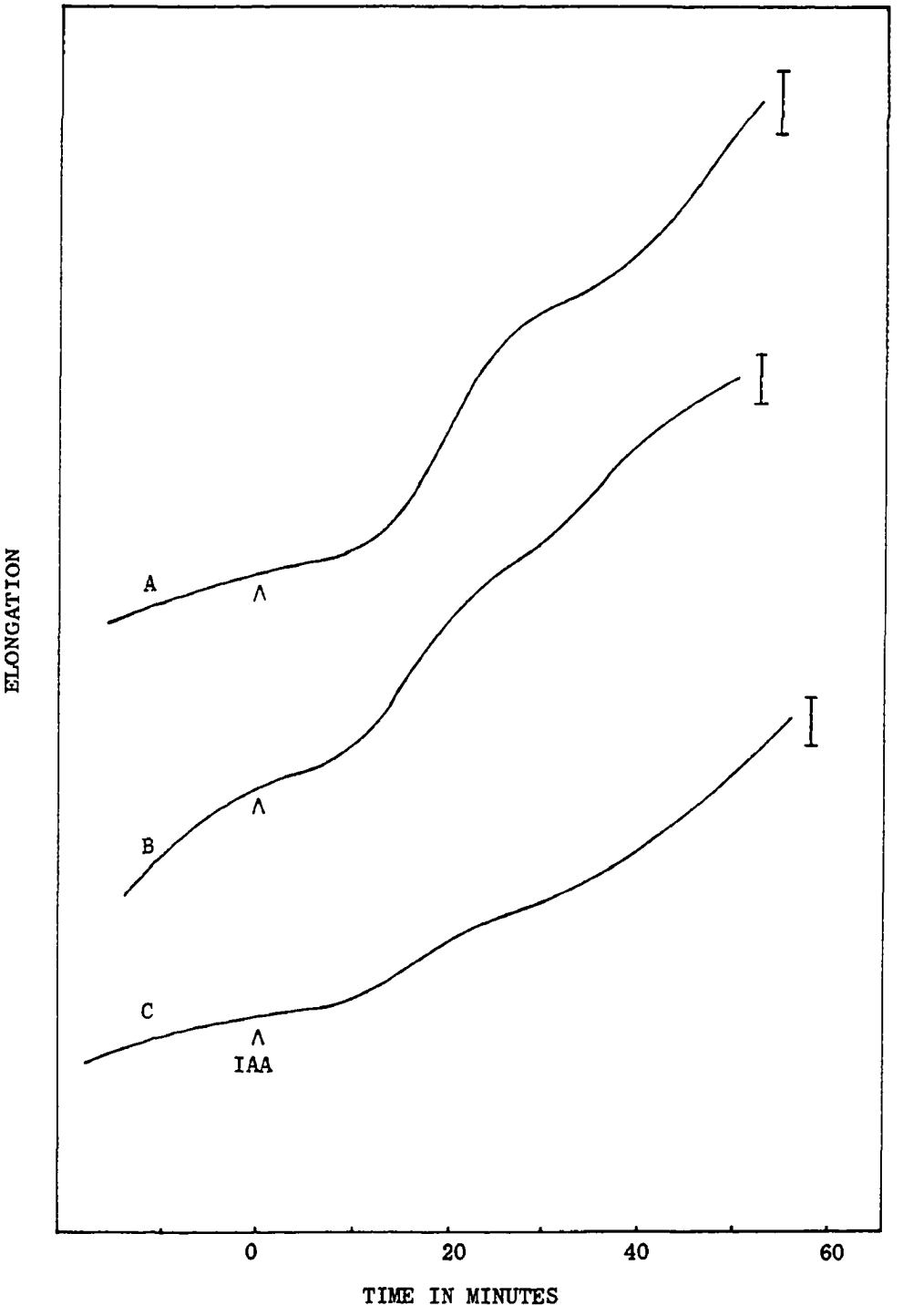
Throughout the present work there have been occasions when the response of pea stem segments to auxin has been considered 'unusual' or atypical. One such response noted may be called the rhythm response since it manifests itself as a rhythmic fluctuation in the rate after the beginning of the secondary phase. The phenomenon does not occur consistently, but may happen one in twenty times, regardless of the manner of treatment. When it does occur it exhibits a rhythmic period of about 20-30 minutes. Figure 11 illustrates some of the rhythm responses which have been observed. It may be that this response is in some way connected with the transport characteristics of the tissue. Hertel and Flory (1968) have shown that auxin transport through coleoptile tissue occurs in rhythmic pulses with a period of 20-30 minutes. Examples of oscillations with a similar

Figure 11. Rhythmic oscillations in the elongation response of Pisum segments

Curve A: Basal medium changed to 10^{-5} M IAA at the caret.

Curve B: Basal medium changed to 10^{-5} M IAA at the caret.

Curve C: Basal medium changed to 10^{-5} M IAA at the caret



period length (c.a. 25 minutes) may be found in numerous auxin connected phenomenon; such as the transient transport and growth inhibition after blue-light stimulus (Thornton and Thimann, 1967) and light-induced potential waves (Newman, 1963).

Effect of Other Auxins

Since the response of Pisum stem segments to IAA is fairly complex it was of interest to examine the response to other indole ring compounds. Figure 12 shows the response of pea stem segments to indolebutyric acid (IBA) and IPA. Neither of these compounds exerts an inhibitory effect during the latent phase or induces the secondary phase which is characteristic of IAA. Table 7 presents comparative data on the timing and magnitude of the growth of pea segment tissue to IAA, IBA and IPA. The length of the latent phase

Table 7. Timing and magnitude of the elongation response of pea stem segments to various auxins (concentration = 10^{-4} M, rate = mm x seg/hr and time in minutes).

	IAA	IPA	IBA
Break-time	6.82	11.00	16.20
Half-time	8.44	13.00	17.50
Steady-state-time	9.75	14.00	18.20
Rate-change-time	15.80		
Water-rate	.249	.147	.239
Latent-rate	.216	.147	.239
Primary-rate	.539	.270	.339
Secondary-rate	.367	.270	.339
% increase LR→PR	150	84	42
% increase LR→SR	70	84	42

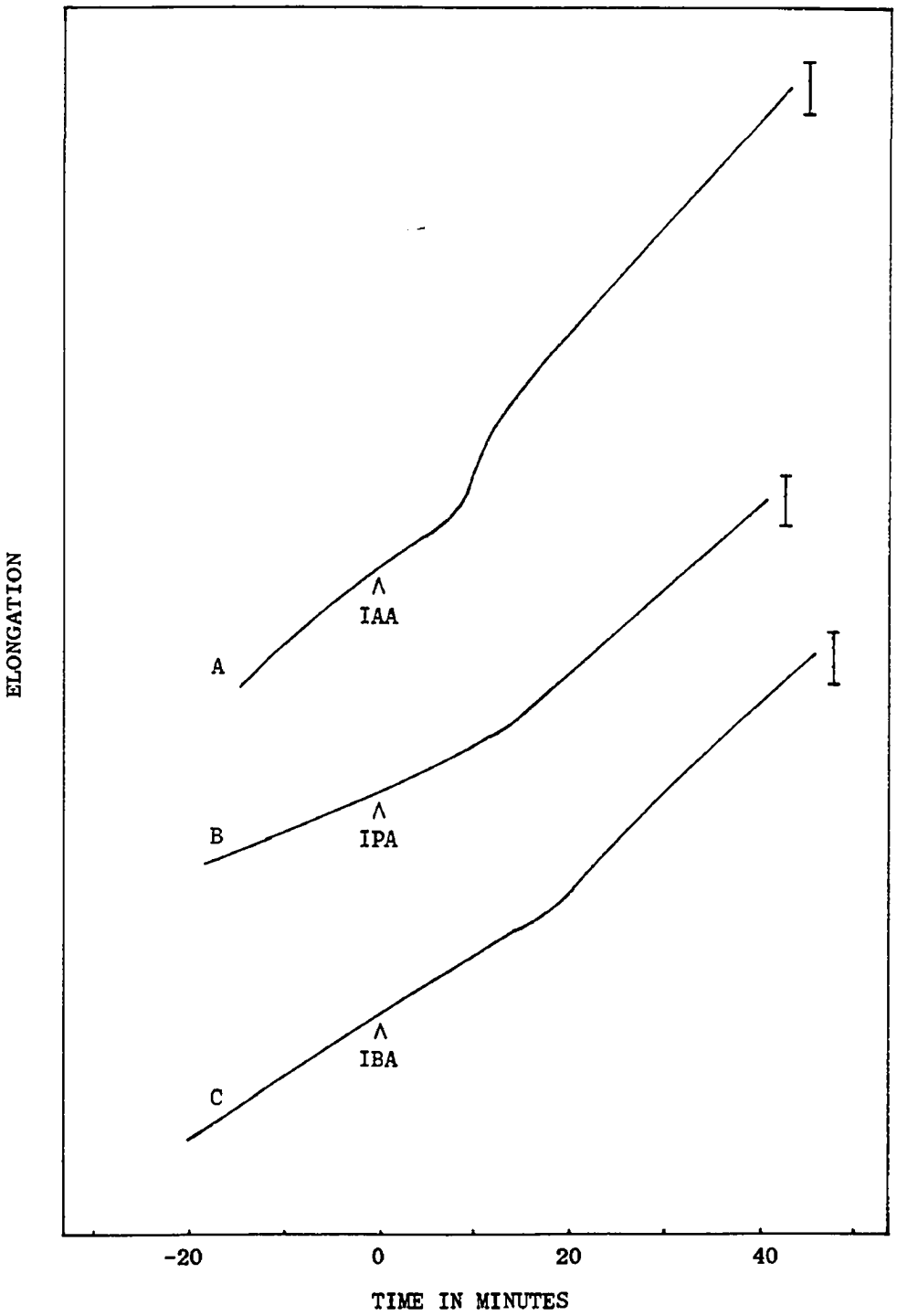
is noted to increase significantly as the length of the side chain on the indole ring is increased. This extension of the latent phase

Figure 12. Elongation response of Pisum stem segments to various auxins

Curve A: Basal medium changed to 10^{-4} M IAA at the caret.

Curve B: Basal medium changed to 10^{-4} M IPA at the caret.

Curve C: Basal medium changed to 10^{-4} M IBA at the caret.



might be due to an increase in the time required for uptake of the compounds bearing longer side chains or to the fact that, once taken up by the tissue IPA and IBA must be biochemically changed into some active form prior to exerting an effect on the growth-sensitive pathway. The percent increase in growth rate shows that growth promoting activity falls off as the side chain of the indole ring is increased.

Effect of Antibiotics

Actinomycin D

Actinomycin D has been shown to inhibit protein synthesis by interfering with DNA dependent RNA synthesis (Reich and Goldberg, 1964). Low concentrations of actinomycin D strongly inhibit growth in a variety of plant tissues (Evans and Ray, 1969; Nooden, 1968; and Penny and Galston, 1966). Figures 13 and 14 show the effect of varying lengths of pretreatment with actinomycin D (10 ug/ml) on the growth response of pea stem segments to auxin. Segments were pretreated in the chamber with 10 ug/ml actinomycin D for a given length of time, then the growth medium was changed to the same concentration of actinomycin D plus 10^{-5} M IAA. A one hour pretreatment with actinomycin D has only a small effect on the growth response of etiolated pea stem segments to auxin during the first 20 minutes, even though the same treatment almost completely eliminates the growth response of oat coleoptile tissue to auxin (Figure 15, curve B). Inhibition of pea stem elongation by actinomycin D begins only about

Figure 13. Effect of one-hour actinomycin D pretreatment on the elongation response of Pisum stem segments

Curve A: Basal medium changed to 10^{-5} M IAA at the caret.

Curve B: Growth medium changed from actinomycin D (10 ug/ml) to actinomycin D (10 ug/ml) plus 10^{-5} M IAA at the caret (total time in actinomycin D prior to the addition of IAA plus actinomycin D = 60 minutes).

Figure 14. Effect of various lengths of actinomycin D pretreatment times on the elongation response of Pisum segments.

Curve A: Growth medium changed from actinomycin D (10 ug/ml) to actinomycin D (10 ug/ml) plus 10^{-5} M IAA at the caret (total time in actinomycin D prior to the addition of IAA plus actinomycin D = 180 minutes)

Curve B: Growth medium changed from actinomycin D (10 ug/ml) to actinomycin D (10 ug/ml) plus 10^{-5} M IAA at the caret (total time in actinomycin D prior to the addition of IAA plus actinomycin D = 360 minutes).

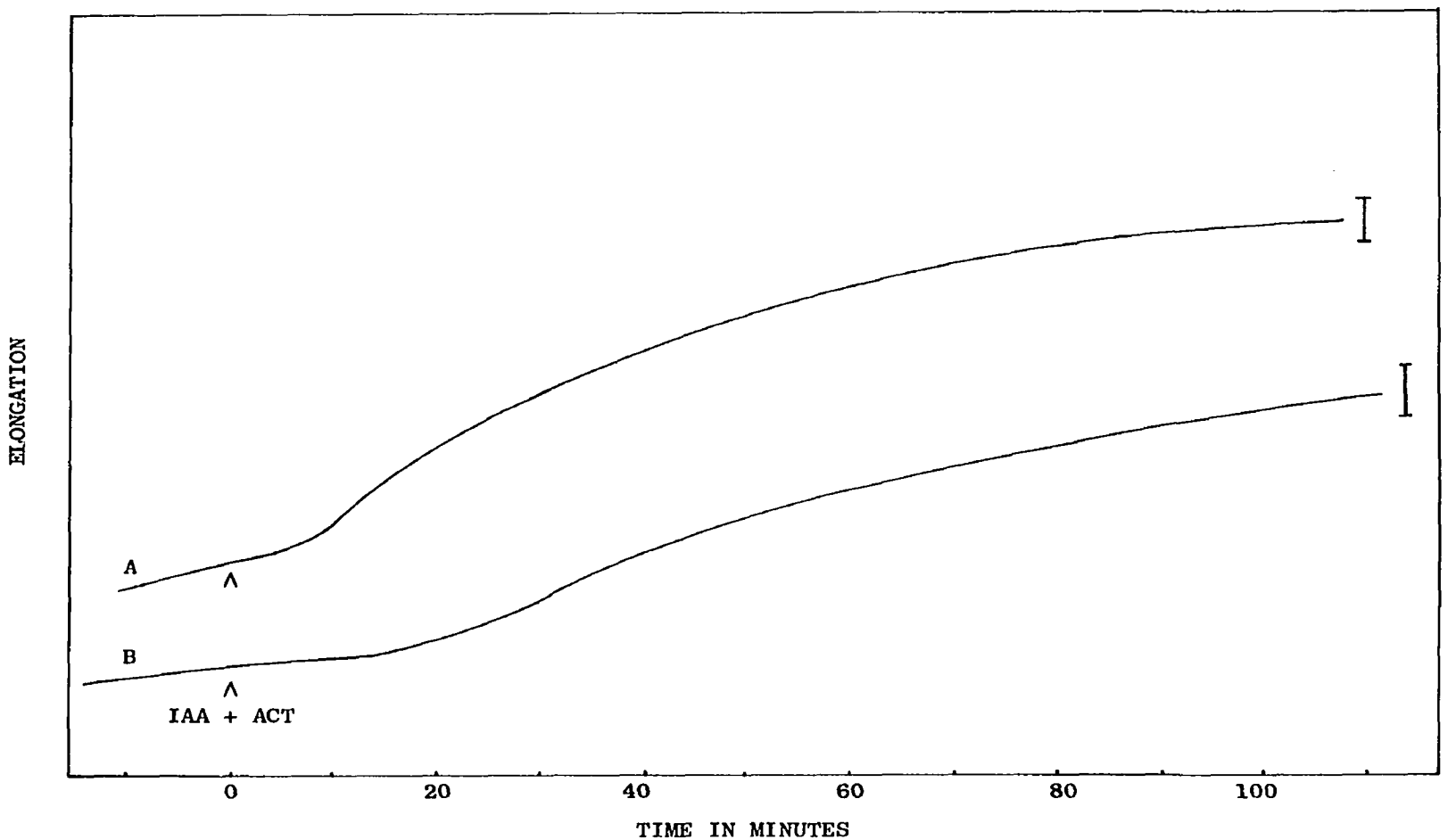
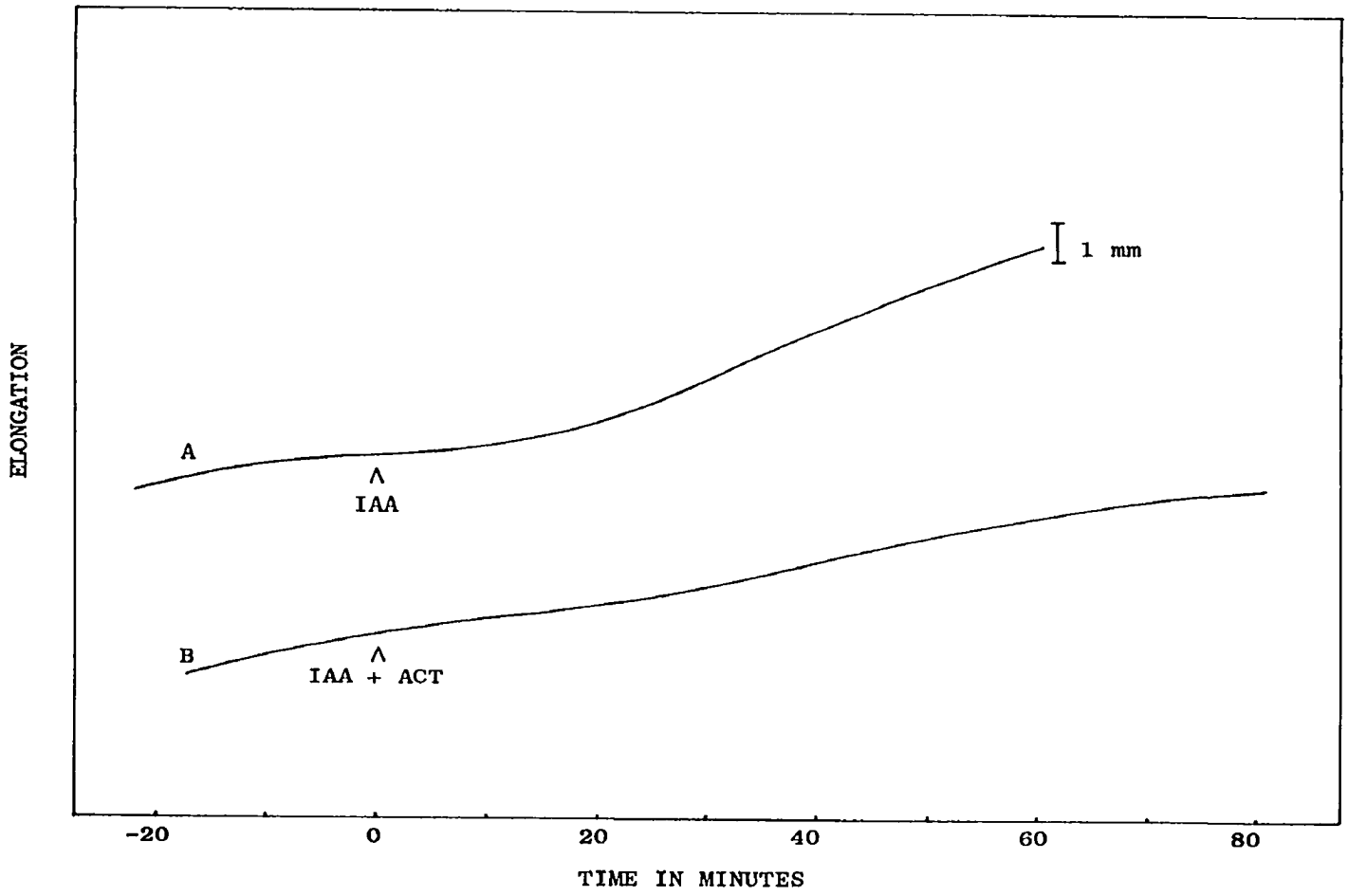


Figure 15. Effect of a one-hour actinomycin D pretreatment on the elongation response of oats (Avena sativa var., Victory)

Curve A: Basal medium changed to 10^{-5} M IAA at the caret.

Curve B: Growth medium changed from actinomycin D (10 ug/ml) to actinomycin D (10 ug/ml) plus 10^{-5} M IAA (total time in actinomycin D prior to addition of IAA plus actinomycin D = 60 minutes)



20 minutes after the addition of IAA (80 minutes total time in actinomycin D). This inhibition is seen as a gradual decline in the rate of elongation over a period of about 25 minutes (Figure 13, curve B).

That the difference in sensitivity of etiolated coleoptile and pea stem tissue to actinomycin D might be due to a difference in permeability to the antibiotic is suggested by the results shown in curves A and B of Figure 14. After a 3 hour pretreatment with 10 ug/ml actinomycin D, the inhibition of the elongation of pea stem segments is visible from the beginning of growth promotion by auxin (Figure 14, curve C). A six hour pretreatment with actinomycin D results in even more severe inhibition of the initial auxin response (Figure 14, curve B).

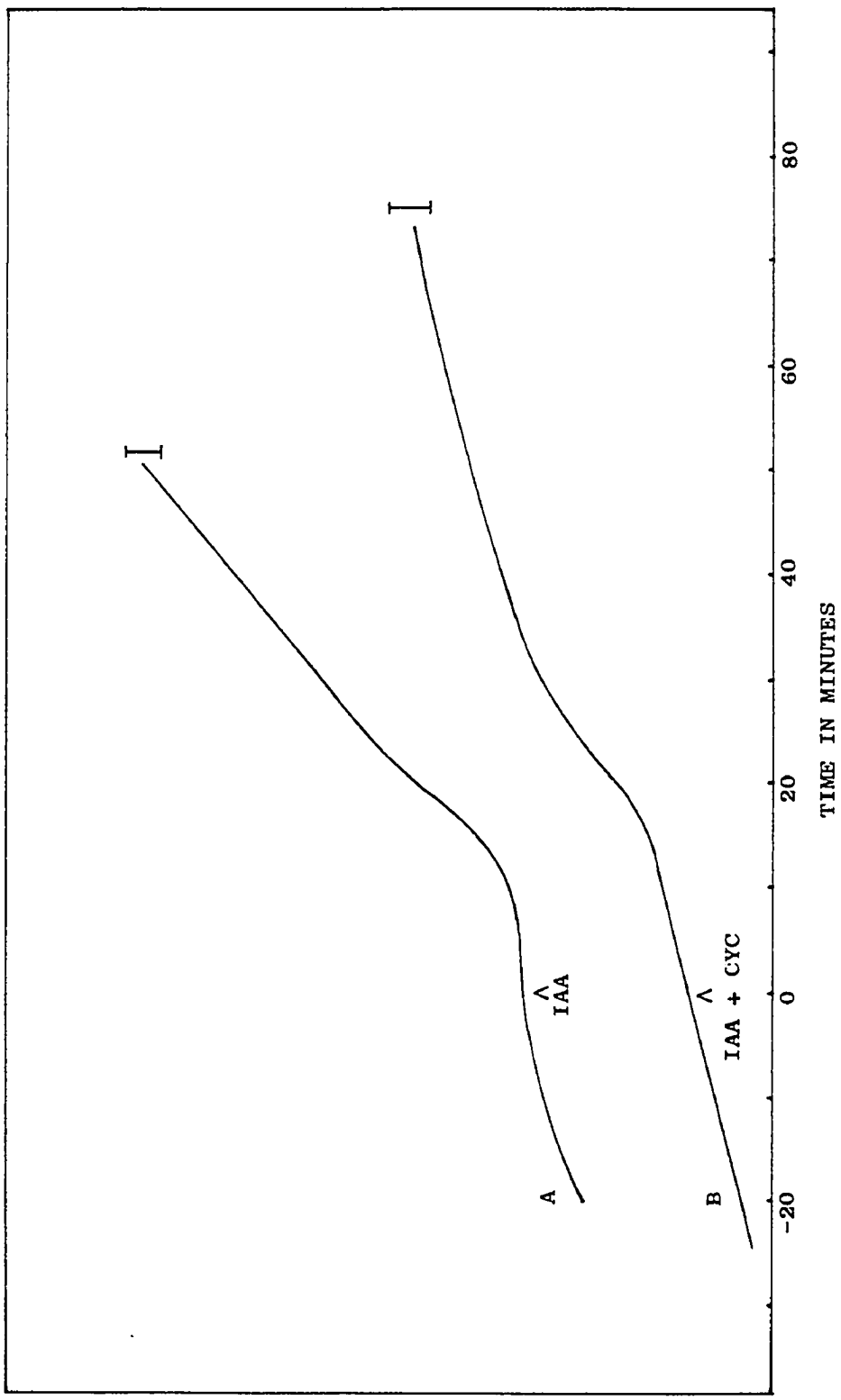
Cycloheximide

Cycloheximide is known to be a potent inhibitor of translation in both plants (Lin and Key, 1967) and animals (Lin et al., 1966). Its ability to inhibit elongation in plants is well documented (Evans and Ray, 1969). Figure 16 shows the effect of cycloheximide pretreatment on the timing of the auxin response in pea stem segments. Segments were pretreated in the chamber with 2 or 3 ug/ml cycloheximide for 35 or 40 minutes, at which time the medium was changed to the same concentration of cycloheximide plus 10^{-5} M IAA. Cycloheximide pretreatment appears to have no significant effect on the length of the latent period in response to auxin even though there is a marked reduction in elongation rate during both the primary and secondary

Figure 16. Effect of cycloheximide pretreatment on the elongation response of Pisum stem segments

Curve A: Basal medium changed to 10^{-5} M IAA at the caret.

Curve B: Growth medium changed from cycloheximide (2 ug/ml) to cycloheximide (2 ug/ml) plus 10^{-5} M IAA at the caret (total time in cycloheximide prior to the addition of cycloheximide plus IAA = 40 minutes)



phases of auxin promoted growth.

Even though both cycloheximide and actinomycin D have no significant effect on the latent phase of the auxin response they both have the effect of reducing the magnitude of the growth response during the primary and secondary phases. It is interesting to note that the auxin response is clearly biphasic after cycloheximide pretreatment, but is much less so after pretreatment with actinomycin D.

Table 8. Timing of the elongation response with 10^{-5} M IAA preceded by antibiotic treatments of varying length (time in minutes).

Antibiotic	Pretreatment Time	BT	HT	SST	RCT
Cycloheximide					
2 ug/ml	35	11.00	15.20	16.90	26.60
3 ug/ml	40	7.15	12.00	18.80	29.50
control	0	11.02	12.40	13.30	22.70
Actinomycin D					
10 ug/ml	360	12.30	18.20	20.80	
10 ug/ml	180	6.17	7.80	9.10	
10 ug/ml	60	7.80	9.10	10.04	
control	0	11.70	12.30	13.30	24.70

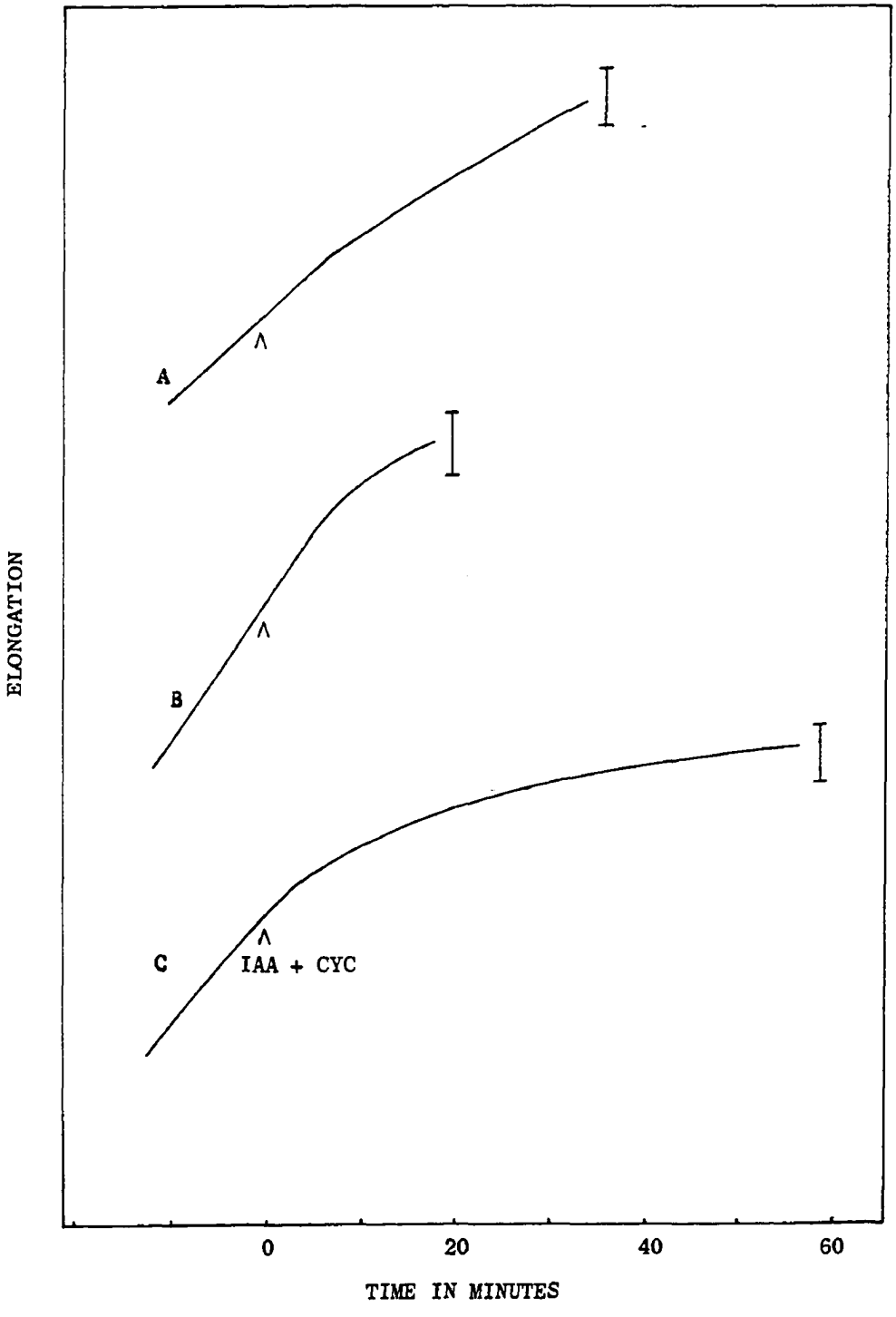
Since short pretreatments with cycloheximide result in a reduction of growth comparable with longer actinomycin D pretreatments, there may be a difference in the uptake or efficiency of inhibition of the two antibiotics. Cycloheximide uptake and inhibition of elongation occurs quite rapidly. Figure 17 illustrates the effect of various concentrations of cycloheximide on the established rate induced by 10^{-5} M IAA. Timing of the inhibition is 8.88 minutes with 2 ug/ml, 8 minutes with 10 ug/ml and 4 minutes with 50 ug/ml.

Figure 17. Effect of cycloheximide on the elongation response to IAA during the secondary phase

Curve A: Change in growth medium from 10^{-5} M IAA to 10^{-5} M IAA plus cycloheximide (2 ug/ml).

Curve B: Change in growth medium from 10^{-5} M IAA to 10^{-5} M IAA plus cycloheximide (10 ug/ml).

Curve C: Change in growth medium from 10^{-5} M IAA to 10^{-5} M IAA plus cycloheximide (50 ug/ml).



Other Effects on the Elongation Response

Citric acid effects

Figure 18, curve A, illustrates the response of pea stem segments to a 40 minute pretreatment with citric acid buffer (pH 4.2). When citric acid buffer is replaced with a solution of 10^{-6} M IAA a rapid rate of growth begins immediately. That auxin is not essential to the immediate increase in elongation is shown in curve B, where a change from citric acid buffer (pH 6.6) to basal medium is accompanied by a rapid and immediate increase in elongation rate. Figure 18, curve C, illustrates another aspect of the citric acid response Here a basal medium has been replaced by a citric acid buffer of high osmolarity (pH 6.3) at the first caret. The citric acid buffer in this case results in an osmotic effect, in that a negative growth rate occurs accompanied by a shrinking of segments. When the citric acid buffer is replaced by IAA (10^{-6} M) at the second caret, a short recovery period occurs (3.2 minutes) followed by a rapid increase in elongation.

Evans (1967) using Avena coleoptile sections has shown that citrate buffer (10^{-3} M) at pH 4.0 brings about a rapid sustained increase in elongation rate. This "acid effect" is apparent even with high unbuffered concentrations of auxin (Evans and Ray, 1969). The effect here may be similar, in that high concentrations of citric acid buffer causes an inhibition of the "acid effect" until it is released by a change to a phosphate buffered growth medium. The

Figure 18. Effect of citric acid buffer pretreatments on the elongation response of Pisum stem segments, with and without auxin addition.

Curve A: Growth medium changed from citric acid buffer (pH 4.2) to 10^{-6} M IAA at the caret (total time in buffer prior to the addition of IAA = 40 minutes).

Curve B: Growth medium changed from citric acid buffer (pH 6.6) to basal medium at the caret

Curve C: Basal medium changed to concentrated citric acid buffer (pH 6.3) at the first caret and then to 10^{-6} M IAA at the second caret.

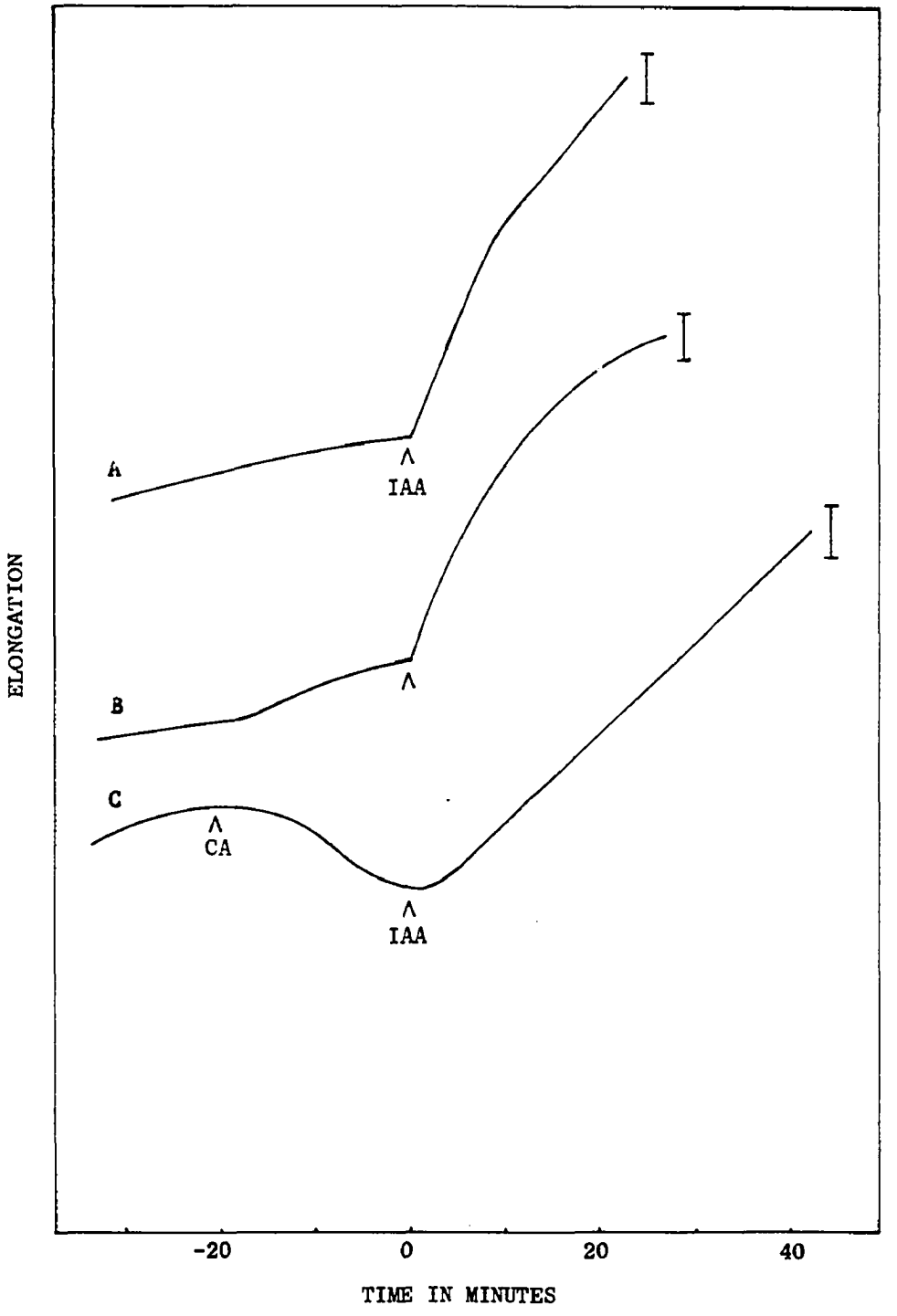
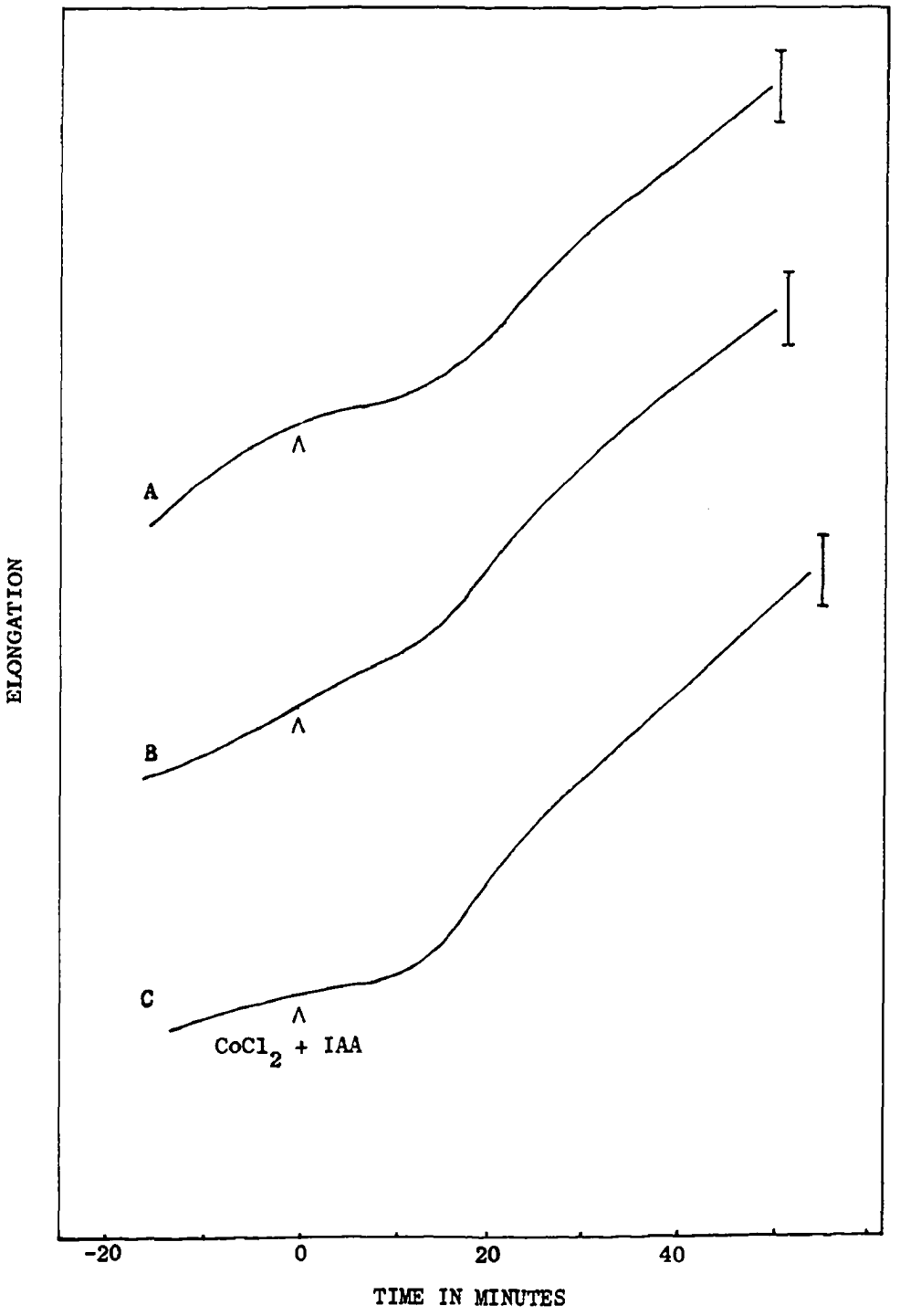


Figure 19. Elongation response of Pisum stem segments to cobaltous ion added with and without IAA in the growth medium

Curve A: Basal medium changed to $CoCl_2$ 10^{-5} M plus 10^{-5} M IAA at the caret.

Curve B: Basal medium changed to 10^{-3} M $CoCl_2$ plus 10^{-5} M IAA at the caret

Curve C: Change in growth medium from 10^{-5} M $CoCl_2$ to 10^{-5} M $CoCl_2$ plus 10^{-5} M IAA at the caret (total time in $CoCl_2$ prior to the addition of $CoCl_2$ plus IAA = 40 minutes)



difference between curves A and B is quite apparent. When auxin is present the rapid promotion of growth is maintained well into the second phase. Without auxin there is a decline in rate of elongation beginning about 10 minutes after the addition of phosphate buffer.

Effect of cobalt

Cobalt has long been recognized for its promotive effect on plant growth. Originally the effect of cobalt in stimulating the expansion of bean leaf disks was reported (Miller, 1951 and 1952). Later cobaltous ion was found to stimulate elongation in Pisum stem segments (Miller, 1954; Thimann, 1956). It was therefore decided to examine the response of pea stem segments to exogenously supplied cobalt, with and without IAA in the growth medium.

Figure 19, curve A, shows that 10^{-5} M $CoCl_2$, when added with IAA (10^{-5} M) has no effect on the elongation response to auxin. Higher concentrations of $CoCl_2$ (10^{-3} M) also have no effect (Figure 19, curve B). Similarly, segments pretreated with 10^{-3} M $CoCl_2$ for 40 minutes, respond to IAA in the usual manner, at least during the first 60 minutes after the addition of auxin (Figure 19, curve C).

DISCUSSION

From the results presented it is quite clear that the nature of the elongation response in Pisum segments may be characterized as tri-phasic. These phases have been designated as the (a) latent phase (b) primary phase and, (c) secondary phase. Each phase has been shown to have definite boundaries and to occur in a precise, predictable and characteristic manner. The regulation of these phases as an integrated system, and their possible relation to the mechanism of auxin-action will be discussed here.

The Latent Phase

The immediate effect of IAA during the latent phase of the auxin response is to inhibit elongation. This is in direct contradiction to previous claims (Penny and Galston, 1967; Purves et al., 1967) that the immediate effect of IAA is to cause a promotion of elongation. The effect of IAA may be immediate in that inhibition of elongation occurs within the first few minutes of its addition, but its promotive effect has a latent period of about 10 minutes. The finding of a 10 minute latent phase in pea segment tissue is in agreement with previous work on monocotyledonous tissue (Ray and Rusink, 1962; Evans, 1967; Evans and Ray, 1969; Evans and Hokanson, 1969). Recent work by Warner and Leopold (unpublished) with whole etiolated pea seedlings (var. Alaska) show that the latent phase is 9.3 ± 0.8 minutes. This is an indication that the method presented here using

the additive response of many segments approaches the in vivo measurement of responses with the same tissue.

The results also offer an explanation for the apparent lack of a latent period in the response of pea stem segments to IAA during long-term experiments. Clearly, elongation measurements made every 30 minutes or every hour after the addition of IAA would fall along a straight line representing the steady rate of elongation during the second phase of auxin promoted growth. The initial biphasic nature of the elongation response would go undetected. Hence, extrapolation of a line drawn through points obtained in long-term experiments would be expected to pass almost exactly through zero on the time axis (see Figure 7, curves A and B) and the 10 minute latent period would not be seen. This example only serves to emphasize the warnings of a number of authors against the practice of extrapolation to zero time of curves obtained using long-term measurements (Audus, 1952; Ball, 1953). That the latent period for auxin action is independent of the exogenously supplied IAA concentration for pea segment tissue (shown here) and for coleoptile tissue (Evans, 1967; Evans and Ray, 1969), and that the rate of IAA absorption is strongly concentration dependent (Christie and Leopold, 1965; Johnson and Bonner, 1956; Reinhold, 1954) argues against the possibility that the latent phase represents primarily time required for uptake of IAA. The latent phase may reflect time required for the completion of a concentration-independent transport of auxin to its site of action or time required to establish a particular concentration ratio between

different cell or tissue compartments. Recent work of Moyed and Tuli (1968) suggests that in pea stem segments conversion of IAA to 3-methyleneoxindole is necessary prior to the growth reaction. If conversion is necessary this would also contribute to the latent phase of the auxin response.

Evans and Ray (1969) have speculated, on the basis of kinetic evidence, that the latent phase may represent time required for the completion of four or more kinetically comparable linear steps that must precede the growth response. They have also stated that another source of latency in the response would be the time required for assembly of complete chains of polymers such as RNA or protein, if these products are involved in the response.

The effect of IAA on the activation of gene transcription and specific protein synthesis in pea stem tissue (Masuda and Tanimoto, 1967; Courtney et al., 1967; Trewavas, 1968a and 1968b) and in other tissues (Key, 1964; Key and Shannon, 1964; Key and Ingle, 1964; Nooden and Thimann, 1966; Nooden, 1968) is well documented. Clearly, if these processes contribute to the established latent period they must be accomplished within a short period of time, since growth promotion by 10^{-4} M IAA is noted to begin in as little time as eight minutes. Since actinomycin D and cycloheximide have been shown to be inhibitors of transcription and translation, respectively, it would be expected that they would extend the latent phase, if these processes were implicated in the elcngation response. These agents, however, did not extend the latent period significantly even though they did

strongly inhibit the final elongation response. This indicates that the gene activation theory of the mechanism of action of auxin in the promotion of elongation may be invalid.

The Primary and Secondary Phases of Auxin Response

The sigmoidal response to IAA and the biphasic nature of the elongation response are not unique to pea segment tissue. This type of response is also clearly illustrated with cucumber (Cucumis sativus var., Straight-eight), (Figure 8, curve B) and with the coleoptile tissues of corn and oats at low concentrations of IAA (Evans, unpublished).

The biphasic nature of the growth response may be explained either as, (a) a burst in the 'normal' rate of growth which occurs in some manner directly related to the latent phase, or (b) as an inhibition phenomenon which may be induced by IAA.

Since the decrease in rate during the secondary phase is clearly concentration dependent (Figure 8; Table 5) both in timing and in magnitude of the decrease, this would implicate the induction, via IAA, of some substance capable of elongation inhibition. Ethylene production would seem to fit the requirements cited. Recently it has been demonstrated by Burg and Burg (1966) that the amount of endogenous ethylene produced by pea segments is directly related to the concentration of applied IAA. They have shown that ethylene production decreases with decrease in the IAA concentration, through the range of 10^{-3} to 10^{-6} M IAA, and that 10^{-6} M IAA was the highest concentration which does not stimulate ethylene production. If

inhibition by induction of ethylene were involved than at any auxin concentration the rate of the secondary phase would approach that of the primary phase in the presence of an inhibitor of ethylene production. Recently it has been shown that cobaltous ion and cycloheximide are strong inhibitors of ethylene production (Kang et al., 1967). From their findings and the results presented here with cobalt and cycloheximide it is quite evident that ethylene cannot be implicated in the rate reduction associated with the secondary phase of IAA induced growth, since the biphasic growth response still occurs after $CoCl_2$ or cycloheximide pretreatment. Also, the work of Burg and Burg (1966) has shown no ethylene production with 10^{-6} M IAA, and the work here clearly shows a secondary phase in response to that concentration of IAA.

Effect of Antibiotics

Elongation of etiolated pea stem segments in response to IAA was strongly reduced by brief pretreatment with cycloheximide. This high degree of sensitivity to cycloheximide has also been demonstrated in coleoptiles (Evans and Ray, 1969). Cycloheximide pretreatment of coleoptile segments has been reported to shorten somewhat the latent phase of response to auxin (Evans and Ray, 1969). This does not seem to be the case with pea segment tissue. Both phases of the positive growth response of pea stem segments to auxin are very sensitive to cycloheximide pretreatment. Each is strongly reduced after 35 minutes in 2 ug/ml of the antibiotic.

That data also show that actinomycin D pretreatment can strongly inhibit the initial growth response of pea stem segments to auxin. However, this requires rather long pretreatments (3 hours or more), suggesting that uptake of actinomycin D by etiolated pea stem segments may be rather sluggish in comparison with the uptake of cycloheximide. These strong inhibitions of the initial auxin response in etiolated pea stem segments by long actinomycin D pretreatment are in contrast to data obtained by Penny and Galston (1966) in similar experiments using green stem tissue of the same species of pea. These workers found that, regardless of the length of pretreatment, actinomycin D did not begin to inhibit the elongation response to auxin until 2 hours after the addition of the hormone.

It is also clear from the results that, as with cycloheximide the inhibition of the auxin response by actinomycin D pretreatment is not accompanied by an extension of the latent period in response to the hormone. Similar data for coleoptile tissue have been presented as evidence that the primary action of IAA in the promotion of elongation probably does not involve a direct stimulation of the synthesis of a growth-specific enzymatic protein (Evans and Ray, 1969).

A Proposed Growth System

It is known that with certain systems that any sudden, continuous or periodic change in the physical or chemical external parameters influencing that system results in a perturbation of the

equilibrium of the steady-state which is followed by re-equilibrium or an approach to a new steady-state. The elongation of plant tissue may be a system influenced in this manner. The rate-change-time may be thought of as the time required for the system to reach a new steady-state or re-equilibrium after initial perturbation by addition of auxin. This time may be considered the relaxation time for the system. Generally this time is specific for a system and is related to the response time and transient of the system. The half time or latent period may be considered the response time. The transient time may be thought of as the time between the response and the relaxation time. Investigation of transient times of various components in cell metabolism indicate that any type of transient can be produced and that the complexity of a transient is based on the nonlinearity of metabolic processes (Hess, 1968). Thus, the transient sigmoidal nature of the growth response to auxin is not unique in biological systems and is only one of several types of systems that may be described.

Generally systems may be epigenetic, metabolic or enzymatic and have monotonic, aperiodic, cyclic or oscillatory transients. All of these types of transient motions are well known to occur in technical systems. It may be that the final solution to the mode of regulation of such biological systems will find their origin in cybernetic theory. Cybernetic theory (cybernetics), developed in recent years in connection with the construction of self-regulating machines has contributed much to the understanding of homeostasis and homeorhesis

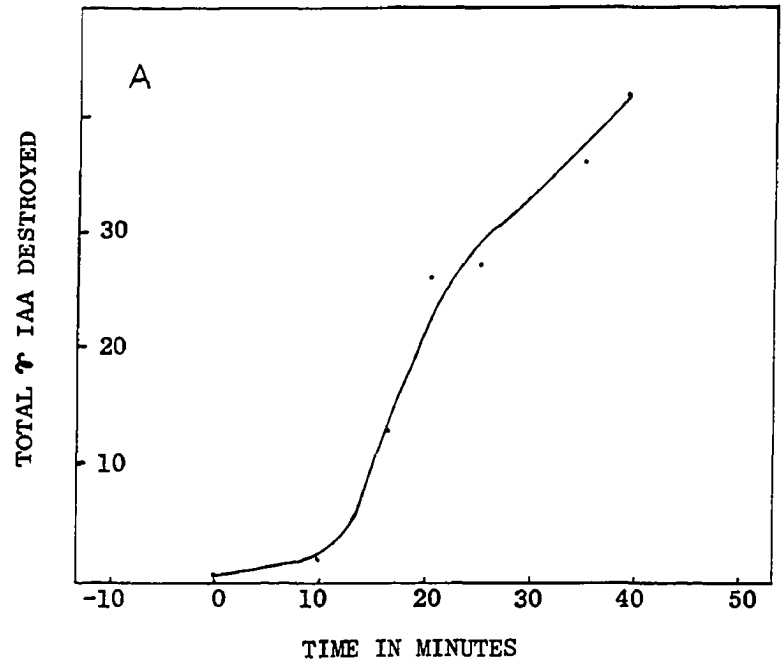
in living organisms. The essence of a self-regulating system is a method of feedback whereby future change in the system is related to the present status of the system. In the regulation of growth and development in higher plants one might confidently expect to find analogous kinds of control, especially where auxins are concerned, depending upon the regulatory systems.

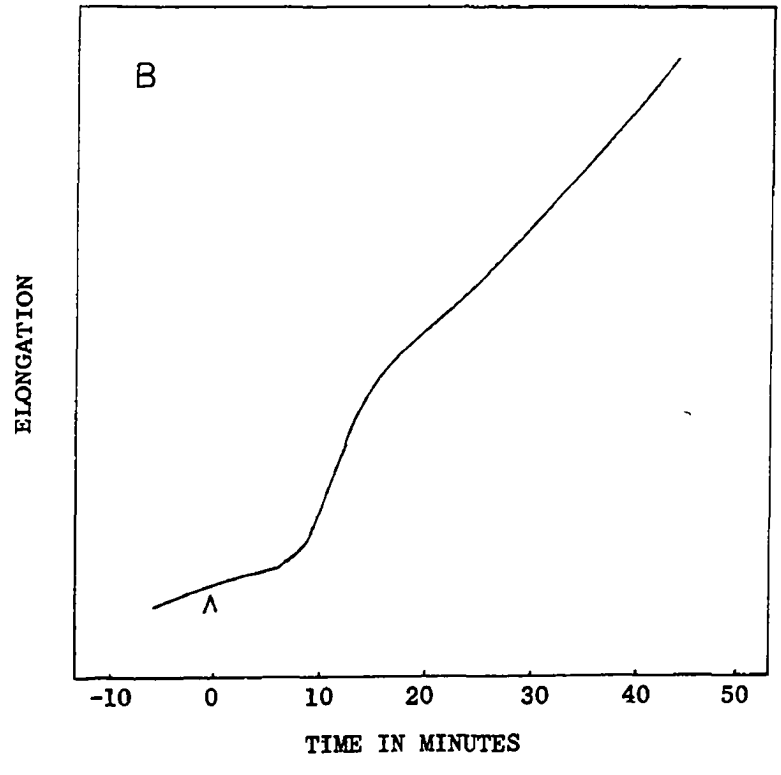
If the time required for the auxin response is in some way related to an enzyme induction phenomenon, this may explain the concentration independent nature of the latent phase, and implicate a role for an adaptive enzyme in the response. Such adaptive enzymes are well known in microbiology (Spiegelman, 1950) and it has been shown that glycolic acid oxidase is adaptive in higher plants (Tolbert and Cohan,1953). Galston and Dalberg (1954) have shown that the nature of the indoleacetic acid oxidase system is adaptive and that the induction time is about 10 minutes. A comparison of the kinetics of IAA destruction in vivo by young rapidly growing etiolated pea epicotyl segments, as shown by Galston and Dalberg (Figure 20, curve A) with the growth kinetics of identical segments given IAA of similar concentration (Figure 20, curve B) shows a striking similarity. Both have sigmoidal curves where the latent phase is about 10 minutes, relaxation times identical (c.a. 20 minutes) and comparable rates of growth and IAA destruction. It is then possible to speculate that a degradation product of IAA may cause the promotion of growth rather than IAA itself. Such speculation is in order with the findings of Moyed and Tuli (1968) that 3-methyleneoxindole induces significant

Figure 20. The comparative kinetics of IAA destruction and IAA growth promotion by Pisum stem segments.

Curve A: The kinetics of IAA destruction in vivo by young rapidly growing etiolated pea epicotyl segments (3rd internode). Residual IAA measurement using Salkowski test on degraded sample of 2×10^{-4} M IAA at five minute intervals (Redrawn from Galston and Dalberg, 1954).

Curve B: The kinetics of growth promotion for segments given 10^{-4} M IAA at the caret.





elongation of etiolated pea stem segments. They have found that the IAA-oxidase pathway leads first to the formation of 3-hydroxymethyl-oxindole, then by dehydration to 3-methyleneoxindole and finally to the inert 3-methyloxindole.

The fact that the latent period of the auxin response is not lengthened by actinomycin D or cycloheximide, argues against the induction of IAA-oxidase as responsible for the formation of a growth specific substance. Also, against this idea is the fact that inducible systems appear to be mainly those which attack exogenous substances, especially in bacterial cells (Stanier, 1951). Since IAA is a natural plant product the necessity for an inducible system is questionable. The endogenous level of IAA-oxidase in the youngest portion of the third internode shows low activity compared with other parts of the etiolated seedling (Galston and Dalberg, 1954), but the presence of the oxidase alone shows that a system is already formed.

Peroxidase and IAA-oxidase activity of tissue may be regulated not only by the amount of enzyme in the tissue, but also by the relative concentrations of phenolic cofactors and inhibitors. This has been shown in many plants including peas. In peas ultimate control over phenolic synthesis resides in the phytochrome system, which determines whether monophenolic (stimulatory) or diphenolic (inhibitory) cofactors shall be produced (Hillman and Galston, 1957; Bottomly et al., 1966). Thus, phytochrome can control overall IAA-oxidase activity without influencing synthesis of the enzyme itself. It is significant to note that Boll (1965) has shown that the work

of Galston and Dalberg (1954) on the adaptive formation of IAA-oxidase is due entirely to these factors.

Fox and Purves (1968) have recently shown, in work with the horse radish perioxidase (HPR) system, that the time course of IAA oxidation reveals two linear phases. The first phase represents the enzyme mediated oxidation of IAA, during which free radicals are initiated. Enzyme inactivation occurs as a result of reactions with the free radicals and, once all of the enzyme is inactivated, IAA is oxidized by a free radical chain mechanism (Phase II). They have further established that cyanide has no effect on phase II of the system and have shown that IAA oxidation is not solely an enzyme catalysed reaction. This implies that an enzyme is necessary only for the beginning of the reaction (Phase I) whereby the formation of free radicals sustains the oxidation system.

Other systems have been shown to be active in the oxidation of IAA. Meudt (1967) has shown that in the absence of added cofactors considerable IAA-oxidase activity was observed and that more than one enzyme system acts to produce similar oxidation products. Also, Leopold and Plummer (1961) and Skoog (1944) have shown that enzymes of the polyphenol oxidase system oxidize IAA.

It is possible to implicate the IAA-oxidase system coupled with production of 3-methyleneoxindole as a possible pathway for growth promotion. In a bacterial model system Tuli and Moyed (1966) have found that 3-methyleneoxindole causes desensitization of several regulatory enzymes to feed-back inhibition by their ultimate end

products. It is suggested that IAA-induced increases in the rate of cellular metabolism and growth may actually be the result of its conversion to methyleneoxindole. This is strengthened by the report of Moyed and Williamson (1967) that the reduction of 3-methyleneoxindole to the inert 3-methyloxindole is catalyzed by four or five reduced triphosphopyridine nucleotide-linked enzymes which are inhibited by various synthetic auxins. The existance of multiple forms of 3-methyleneoxindole reductase and their differential sensitivity to the synthetic auxins may reflect the importance of the reduction, and thereby, inactivation of 3-methyleneoxindole. Most remarkable in this pathway is that even small fractions of IAA degraded in this manner could have far reaching effects since the key intermediate, 3-methyleneoxindole, would be amplified through its reactions with enzymes (Tuli and Moyed, 1967). It has also been found that 3-methyleneoxindole is a powerful sulfydral reagent; it could be expected to have a profound effect on other activities in its immediate surroundings, including perhaps an effect on auxin transport (Moyed and Tuli, 1968).

That the relaxation time of the pea segment growth response to IAA shows greater decrease and earlier occurrence with increased concentration may be explained as some type of feedback inhibition due to the production of the oxidation products of IAA, either 3-hydroxymethyloxindole or 3-methyleneoxindole, or due to the effects of IAA directly. This is strengthened by the fact that growth responses to IPA and IBA are not biphasic. The extended latent phase

in response to these compounds may be due to their breakdown to IAA prior to their introduction into the IAA-oxidase system and subsequent conversion to 3-methyleneoxindole. There is an indication that IAA directly inhibits its own conversion. The IAA produced by conversion might be completely channeled into the oxidase system so excessive amounts would not accumulate and no feed back inhibition of the system would result.

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