

" STUDIES IN THE AUXIN RELATIONSHIP OF
GEOTROPICALLY STIMULATED ROOTS "

by

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ABSTRACT

The effect of geotropic stimulation on auxin production in Vicia faba root tips was studied. Paper chromatography techniques were used to separate the growth substances. Three assay methods (oat first internode, oat coleoptile and pea root section tests) were used for the detection and estimation of growth substances on chromatograms.

An assessment was made of a number of sources of error in the preparation of material and the extraction, purification and chromatography of extracts.

Geotropic stimulation brought about an increased synthesis of an ether soluble acid auxin in root tips. The maximum amount was formed at 20 minutes stimulation and this fell to the minimum value, recorded after 40 minutes stimulation.

The auxin content from longitudinally split root tips was less than the auxin content from whole root tips. This decrease was thought to be an artefact caused by longitudinal cutting of the roots. No difference was found in the auxin content between the upper and lower half of the tip.

The water soluble auxins of whole and split roots were investigated. Geotropic stimulation produced a change in content

of all the auxins.

Colour tests and fluorimetric analyses were made on the water soluble fraction. 3, 4 -dihydroxyphenylalanine and tryptophan were identified from the water soluble fraction. Substances giving a phenolic reaction coincided with areas of high growth activity. Alkaline hydrolysis revealed the presence of water soluble indole complexes.

IAA	Indolyl - 3 - acetic acid
IAA'	Indolyl - 3 - acetylamide
IPyA	Indolyl - 3 - pyruvic acid
IAA''	Methyl ester of IAA
IAA'''	3 - indolylacetyl aspartic acid

ABBREVIATIONS USED :

IAA	Indolyl - 3 - acetic acid
IAN	Indolyl - 3 - acetonitrile
IPyA	Indolyl - 3 - pyruvic acid
IAEt	Ethyl ester of IAA
IAcAsp.	3 - indolylacetyl aspartic acid.

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CHAPTER IINTRODUCTION

Modern physical and chemical methods of analysis, together with bioassay techniques, have revealed the presence of many growth regulating substances in plants. Many of these are known only by virtue of their R_f values in specific chromatographic solvents, colour reactions and bioassay response. Relatively few have been identified chemically because of the extremely low concentration of these growth substances in plants. The specific manner in which they regulate growth is still unresolved. From this wide range of compounds with growth regulating activity one group has stood pre-eminent for many years. These are the indole compounds, two of which are now recognised as auxins, i.e. a plant growth substance characterised by the property of stimulating extension growth in cells in bioassay tests, Audus (1959). For many years 3-indolylacetic acid (IAA) has been thought of as an auxin, indeed the only auxin in plants. The relationship of the growth regulating activity of IAA to the extension growth of shoot and root cells has been extensively studied. 3-indolylacetonitrile (IAN) is now recognised as an auxin since, in a few tissues only, it does fulfil the functions of an auxin.

Despite much work little is known about many aspects of root growth and tropisms. It was Darwin (1880) who showed it was the tip alone that was sensitive to geotropic stimulus and that when

it was stimulated it caused the regions behind it to bend. In general removal of 1.0-1.5 mm. of the tip was sufficient to prevent bending of the radicle. He also found that cutting off a tip of a radicle placed horizontal for 1-2 hours did not prevent the radicles from bending. From these experiments Darwin concluded that the root tip alone was sensitive to gravity. He thought that the tip transmitted some influence or stimulus to a part of the radicle which grows quickest and under the influence of gravity bends most. Following this work were the decapitation experiments of Weisner (1884), Cholodny (1924 and 1926) and Bunning (1928) which showed that removal of the root tip gave a small growth stimulation. Cholodny (1926) replaced the tip on maize roots and found growth was retarded again. Previously he had found, Cholodny (1924), he could retard the growth of decapitated roots by reheading them with coleoptile tips. In addition he confirmed that decapitated roots were insensitive to gravity and that the roots would regain most of their sensitivity to gravity if reheaded with root or coleoptile tips. Keeble, Nelson and Snow (1931a) confirmed Cholodny's work. From these experiments and work on coleoptile decapitation experiments by Soding (1925) the following conclusions were drawn. Both the root tip and the coleoptile tip "secrete" a substance which retarded root growth. By a comparison of the activity of the substance in root tips with the activity of auxin from coleoptiles in the Avena curvature test, Went (1928) the growth active substance in roots was thought to be an auxin. Lastly this auxin in the root tip enabled the root to respond to gravity.

Cholodny (1926) came to a number of conclusions and these were: "Growth hormones play an essential role in the mechanism of the geotropic reaction." "In vertically placed stems and roots the growth regulating substances are equally distributed on both sides." "As soon as these organs are placed in a horizontal position the normal diffusion of the hormones is disturbed; the upper and lower cortical cells now obtain different amounts of these substances." This unequal distribution is ascribed to a physiological polarity induced by gravity. Went (1926) had likewise come to the same conclusions. "Geotropic perception is caused by a polar alteration in the coleoptile cells instead of moving rectilinearly the growth regulators are more strongly conveyed towards that side which under geotropic stimulation was turned downwards." From the work of Cholodny and Went a general theory of plant tropisms was developed and was called the Cholodny-Went theory. This is as follows. "Growth-curvatures whether induced by internal or external factors, are due to an unequal distribution of auxin between the two sides of the curving organ. In the tropisms induced by light and gravity the unequal auxin distribution is brought about by a polarisation of the cells, which results in lateral transport of the auxin."

Experimental evidence which seemed to confirm the Cholodny-Went theory was found by Hawker (1932). She placed Zea mays roots horizontally. After 3 hours she cut off the tips, separated them into upper and lower halves and placed each half onto half a

decapitated root and measured the curvature induced. The half tip which had been lowermost during stimulation gave three times as big a curvature as was given by the half tip which had been uppermost during the stimulation period. Finally Boysen Jenson (1933b) placed seedlings of Vicia faba horizontally for 4 hours, cut off the root tips and placed these horizontally against 2 agar blocks for 2-5 hours. When these blocks were placed uni-laterally on decapitated Avena coleoptiles a greater curvature was obtained from the blocks in contact with the under half of horizontal root tips.

The isolation of IAA from various sources by Kogl et al (1934), Thimann (1935a) and Avery Burger and Shaluka (1941) were important advances (in growth work). This latter isolation of IAA was of interest for two reasons. It was the first isolation from a higher plant and the IAA had to be released from a bound inactive form by alkaline hydrolysis. A major contribution to the study of endogenous growth substances was provided by Bennet-Clark, Tambiah and Kefford (1951). They applied the principles of paper chromatography to the problem of the separation and isolation of growth active substances in plants.

In the ether soluble acid fraction of roots Bennet-Clark and Kefford (1953) and Kefford (1955) found two other growth regulating compounds in addition to IAA. Using isopropanol, ammonia, water as chromatography solvent they found "accelerator α " near the starting line and "inhibitor β " between IAA and the solvent front.

These two new substances have also been found by Lexander (1953), Thurman and Street (1960). With a different solvent, isobutanol, methanol, water, Lahiri and Audus (1960) have found AP(i) at the "accelerator α " position, "inhibitor β " and a new growth promoter AP(iii) at the solvent front. "Accelerator α " and AP(i) both showed the same characteristic behaviour of promoting both coleoptile and root segment growth. AP(iii) was more characteristic of known auxins in that it promoted coleoptile growth but inhibited root segment growth at the same concentration.

Recently work has been concentrated on the water soluble ether insoluble fraction of root extracts which have been found to have substantial auxin activity, Britton, Housley and Bentley (1956), Audus and Gunning (1958), Lahiri and Audus (1960), Thurman and Street (1960). The auxins of the water soluble fraction showed a spontaneous interconvertibility on elution and rechromatography, Britton et al (1956), Audus and Gunning (1958). Lahiri and Audus (1960) were able to repeat this interconvertibility using a neutral chromatography solvent. Thurman and Street were unable to find this phenomenon and suggested it was due to "excessively loaded chromatograms." Britton et al did use an ammoniacal solvent which rendered suspect their results on interconvertibility, because it has been shown that ammoniacal solvents lead to a destruction of water soluble growth substances, Housley and Bentley (1956). The Nitsch chromatography artefact should also be taken into account.

What this artefact is will be discussed both in Chapter II, in the section on chromatography and in Chapter III under the heading of "The Nitsch chromatography artefact."

The values recorded for the concentration of auxin in roots, with special reference to the auxin at the IAA position, show a large variation. Audus and Thresh (1956) found $1.0 \mu\text{g}/\text{Kg}$ in Pisum roots. Linser and Maschek (1953) gave a value of $2.0 \times 10^{-4}\text{M}$ or approximately $35 \mu\text{g}/\text{Kg}$ in Brassica roots. Lahiri and Audus (1961) reported a figure of $50 \mu\text{g}/\text{Kg}$ from Vicia faba roots. Pilet (1951 and 1952) using Lens roots and Moewus et al (1952) working on Lepidium both reported values of 10^{-3}M or approximately $170 \mu\text{g}/\text{Kg}$. At the moment the physiological significance of this wide range of values for the concentration of IAA or IAA-like substance is not clear. It is necessary that the chemical identity of this auxin in root tissues be settled beyond doubt. Then standardised extraction, purification and bioassay techniques could be used and useful comparative studies could be made. It also seems apparent that, for comparison purposes, root tissue of similar physiological age should be used from which the growth substances could be extracted.

Ever since the isolation of IAA there have been many papers on the effect of IAA on root growth. These experiments have included work on intact root systems, decapitated whole roots and excised root sections. The general response of root systems and root sections to exogenous IAA was an inhibition of growth. However in many cases

roots have been reported as responding to an extremely low concentration (circa 10^{-11} M) by a slight growth stimulation, Audus and Garrard (1953), Audus and Thresh (1953), Moewus (1949), Swanson (1946) and Libbert (1957a).

The implications of this small stimulation by IAA on root growth were important. Some people, Aberg (1957), doubted the reality of the IAA induced stimulation and suggested it was due to adaptation and after effect. Larsen (1961) regarded the stimulation as real. Because of the high concentration of endogenous auxin in roots reported by various workers, Aberg (1957) found it difficult to understand how further addition of IAA could stimulate root growth since presumably the concentration of auxin was supra optimal in root cells. Recent work however has suggested that the assumption of there being a supra optimal concentration of auxin in roots may not be correct. Audus and Das (1954) have indicated that the level of auxin in roots may not be supra optimal. The careful decapitation experiments of Younis (1954) have shown that removal of the root meristem has not led to an increase in root extension growth. Humphries (1958) revealed that removal of up to half of the root of rye and barley did not change the growth rate from that of the intact plant. All this dissension seems to suggest that our knowledge of the growth regulating processes in roots is insufficient.

The relationship of geotropism to endogenous growth regulating substances needs much clarification. If the Cholodny-Went

theory is accepted then the geotropic response was due to a lateral redistribution of the total free auxin content within the root. This concept was supported by Hawker (1932), Boysen Jensen (1933c) and Larsen (1956). Audus and Lahiri (1961) however have cast doubt on the work of Hawker and Boysen Jensen for not having proper controls, i.e. results from vertical roots. Other workers meanwhile had found evidence which suggested that geotropic stimulation did induce increased auxin production. Schmitz (1933) found more auxin in the lower half of geotropically stimulated grass nodes. Brain (1942) found more auxin in the horizontal hypocotyls of Lupinus and van Overbeek et al (1945) found an auxin increase in the nodes of horizontal sugar cane especially in the growth rings.

Larsen (1956) criticised the conclusions drawn from the results of Schmitz and Brain on the grounds that increased auxin production could only be demonstrated in experiments of comparatively long duration, i.e. three to four hours, whereas some geotropic curvatures become visible after a few minutes. This criticism certainly could not be applied to the results of Audus and Lahiri (1961) who found an increased auxin production within 45 minutes in Vicia faba. The reaction time for Vicia roots was found to be 36 minutes. Rufelt (1957) suggested that geotropic curvatures in diageotropic roots could not be due to auxin redistribution because there was a total decrease in growth rate during the geotropic reaction. This decrease in growth rate was also found by

Bennet-Clark et al (1959). The results of Audus and Brownbridge (1957) led to their suggesting a different mechanism to account for the overall reduction in growth rate and growth curvature that they observed in their experiments. They suggested a "de novo" production of a root growth inhibitor in the lower half of the root tip. Bennet-Clark et al (1959) supported the suggestion of Audus and Brownbridge but were not able to demonstrate a "de novo" production of growth substance. Audus and Lahiri (1961) were able to show a large and rapid production of an IAA like auxin in the root tip as a whole.

The purpose of this investigation was to check the rapid production of auxin found by Audus and Lahiri (1961). In addition the relationship of AP(iii) to AP(ii) was to be investigated. Audus and Lahiri had thought that AP(ii) might have been released from AP(iii) by an enzyme generated during geotropic stimulation. It was hoped to extend the investigation to longitudinally split roots. From these experiments it might be possible to locate the large and rapid production of the IAA-like auxin. The amounts of this auxin in upper and lower halves of roots during geotropic stimulation could be recorded. From these results some indication as to the validity of the Cholodny-Went theory as applied to roots could be gained.

CHAPTER II

MATERIALS AND METHODS

(i) Plant material used for extraction of growth substances

All extractions were done on broad beans. Vicia faba var. Leviathan were bought from Messrs. Carters Tested Seeds Ltd.

The seeds were soaked in water prior to planting out. In summer the seeds were soaked for 24 hours in aerated running water in a plastic bowl. In winter they were soaked for 10 hours in still water at 23-25°C. The water was renewed every 2 hours. They were then placed in running tap water for approximately 36 hours. These procedures ensured turgid seeds with the radicle showing signs of growth. The seeds were planted radicle downward in washed sterilised moist sand in seed pans, 25 cm. x 25 cm. x 10 cm. A layer of damp sand was spread over the seeds and then covered with a glass plate to reduce evaporation. The seedlings were grown in the dark at 25°C for 72 hours.

(ii) Material used for the bioassay techniques

Three bioassay methods were used in assaying for growth active substances. These were (a) Avena first internode test (b) Avena coleoptile test (c) Pea root assay.

(a) The Avena first internode assay

Avena sativa var. (Blenda) seeds were soaked in tap water for two hours in darkness. The seeds were sown on moist washed

sterilised sand in glass half bricks. A thin layer of sand was sprinkled over the seeds and another half brick placed on top to reduce evaporation. The seedlings were grown in complete darkness at 25°C for 72 hours.

(b) The *Avena* coleoptile test

The material was prepared and grown as for the first internode assay except that sixteen hours before harvesting the seedlings were exposed to red light for ten minutes from a safelight covered with a Kodak series 1 red filter. This procedure reduced first internode elongation and promoted coleoptile growth.

(c) Pea root assay

Pisum sativum var. (*Meteor*) seeds were soaked in running tap water in a plastic bowl for 36 hours. The seeds were planted radicle downward in sand to give straight root growth. The growth period was 72 hours in darkness at 25°C.

(iii) Extraction of growth active substances

Original methods

Three day old *Vicia faba* seedlings with uniform straight roots were selected and a 7.0 mm. tip cut off from each root with a special cutter. (Fig. 1). Each sample consisted of 100 roots. The tips were collected in 50 ml. industrial methylated spirits. The flask and alcohol were kept at 0°C in an ice bath. The root material was macerated in a Magimix at 0°C, the final volume made up to 150 ml. and the macerate stored in the deep freeze at -18°C in darkness for 18 hours.

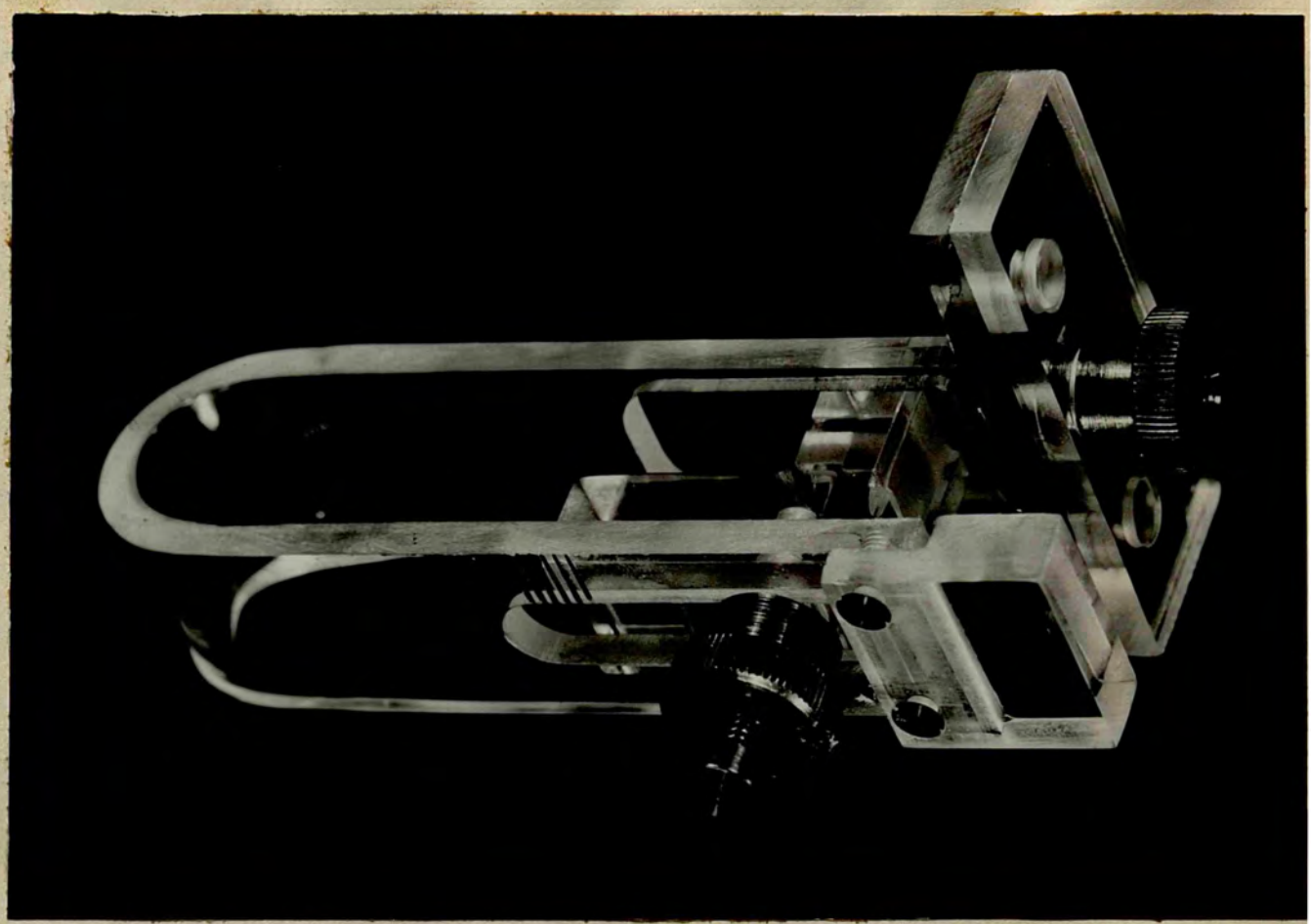
After approximately one year it was decided this method did

appears in writing all these roots like a brown
 uniform length. There is an irregularity to
 permit the exclusion of what is not in

Fig. 1.

Apparatus for cutting off whole root tips of a known uniform length. There is an adjustable stop to permit the excision of variable lengths of root tip.

FIG 1



not freeze the roots sufficiently quickly and it was modified as follows. A dry 500 ml. wide mouth beaker was suspended in a vacuum flask containing a freezing mixture of solid CO_2 and ethanol at -70°C . The root tips were cut into this beaker and froze within 15 seconds by heat transfer through the wall of the beaker. The frozen material was quickly transferred to a cold pestle and mortar. Two ml. cold alcohol was added to the roots and they were rapidly and finely ground. The brei was washed into a conical flask and the final volume made up to 70 ml. The flask was corked and stored in darkness in the deep freeze at -18°C for 18 hours.

Present method

The method was finally modified for use with a freeze-dry apparatus. The roots were frozen as described and crushed rapidly in a dry pestle and mortar. The crushed frozen root material was transferred to cold freeze dry flasks and stored at -18°C until ready for freeze drying. At no time was the frozen material allowed to thaw. With four flasks each containing 1.0 g. fresh weight root material the freeze drying period was six hours. The dried material was put into a dry weighing bottle, corked and stored in a dessicator at $2-3^\circ\text{C}$ in the dark. Each sample of freeze dried material equivalent to 100 roots was extracted with 70 ml. methanol for 18 hours at -18°C . When the freeze dried material was taken out of the refrigerator the methanol was added within 1-2 minutes. This was to prevent the enzymes in the freeze dried material from becoming active with the rise in temperature from $2-3^\circ\text{C}$ to approximately 20°C .

At the beginning of the experiments, industrial methylated spirits was used as the extractant. Following a discussion with Dr. J.P. Nitsch pure methanol was used to extract growth substances. The reason for the change was the presence of an impurity in the methylated spirits. This will be mentioned again in Chapter III in the section on industrial methylated spirits.

(iv) Organic solvents and methods of purification

During the course of the investigation the following solvents were used. Industrial methylated spirits, rectified spirits, methanol (G.P.R. standard), isobutanol (Analar) and diethyl ether (Analar, peroxide free). Some of these had to be purified. The methanol was purified by distillation, the ether by distillation over anhydrous ferrous sulphate.

(v) Purification of growth active fractions

(a) Ether soluble acid fraction

The methanol extract was filtered under vacuum into a Buchner flask and the plant residue washed with 2.0 x 10 ml. aliquots of methanol. The methanol was flash evaporated off at 26°C under vacuum and 5.0 ml. distilled water added to the residue in the flask. This aqueous fraction was acidified to pH 3.0 with $N/10$ H_3PO_4 and then shaken with four successive 15.0 ml. aliquots of ether. The ether fractions were pooled and partitioned four times against 5% $NaHCO_3$. The acids were removed from the bicarbonate fraction by acidifying to pH 3.0 with H_3PO_4 (S.G. 1.75) and extracting four times with 15.0 ml. aliquots of ether. The acid ether fractions were combined and dried overnight over anhydrous sodium sulphate. The ethereal fractions

were reduced to dryness before being taken up in a small volume of ether and spotted onto a chromatogram. This fraction will be referred to as "The ether soluble acid fraction".

(b) The water soluble (ether insoluble) fraction

The aqueous residue at pH 3.0 after extraction with ether was neutralised to pH 5.8-6.0 with $\text{Ba}(\text{OH})_2$. The neutralised residue was centrifuged at 2000 g. for five minutes and the supernatant liquid poured off. The precipitate was washed with 5.0 ml. distilled water and spun down again. The washings were added to their respective aqueous fractions. Each aqueous fraction was flash evaporated in a rotary flash evaporator under vacuum at 26°C to a final volume of approximately 0.5 ml. This gummy extract was taken up in 5.0 ml. alcohol. The reason for dissolving the gum in alcohol were two fold. Firstly the gum was in a ring around the inside of the flask and could not be got off unless taken up in ethyl alcohol. Secondly when the gum was dissolved in alcohol it could be spotted more efficiently onto a chromatogram because the alcohol dried more quickly than water. After the residual gum had been dissolved in ethanol it was shaken for one hour and then reduced to approximately 0.75 ml. under vacuum at 26°C in a modified Thunberg tube. This tube was shaken vigorously for half an hour on a mechanical shaker and then stored overnight at $2-3^\circ\text{C}$. This extract was known as: "The water soluble fraction".

(vi) Hydrolysis methods

- (a) Alkaline hydrolysis with 7.0 N NaOH. (From Bennet-Clark and Wheeler (1959) - slightly modified).

The water soluble fraction after extraction with ether at

pH 3.0 was brought to pH 6.0 with $\text{Ba}(\text{OH})_2$ and the precipitate centrifuged down. The supernatant liquid was decanted off and reduced under vacuum to a volume of approximately 5.0 ml. One and a half ml. 7.0 N NaOH was added to the extract in the boiling tube. The tube was lightly plugged with non-absorbent cotton wool and immersed, above the level of liquid in the tube, in a boiling water bath for one hour.

The extract was brought to pH 3.0 with H_3PO_4 (S.G. 1.75) and extracted with ether to give the ether soluble acid and neutral fractions by a previously described method. The residual aqueous fraction was neutralised with $\text{Ba}(\text{OH})_2$ and then treated as described for the water soluble fraction.

(b) Alkaline hydrolysis of Tryptophan with 7.0 N NaOH

One point five ml. 7.0 N NaOH was added to 60 μg tryptophan in 1.5 ml. H_2O contained in a boiling tube. The tube was immersed in a boiling water bath for one hour. The aqueous extract was brought to pH 3.0 and extracted for ether soluble acids. The residual aqueous fraction was brought to pH 6.0 and then prepared for chromatography as described for the water soluble fraction.

(c) Acid hydrolysis with 2.0 N HCl. (From Bennet-Clark and Wheeler (1959 - slightly modified).

The same procedure as used in the alkaline hydrolysis was adopted except that 1.5 ml. 2.0 N HCl was added to the water soluble in the boiling tube in place of NaOH. The pH after hydrolysis was adjusted to 3.0 with $\text{Ba}(\text{OH})_2$. The extract was partitioned against ether to give the acid and neutral fractions by previously described

methods. The residual aqueous fraction was treated as described for the water soluble fraction.

(vii) Chromatography

Paper partition chromatography was used extensively for the separation of growth active substances. Two grades of paper were used; Whatman No. 2 and 3 M.M. The latter paper was used most frequently especially for gummy extracts and because it was unlikely the chromatograms could be "overloaded". The overloading of chromatograms will be discussed in Chapter III. A neutral chromatographic solvent: isobutanol, methanol, water (80-5-15) was used, Nitsch (1956). Initially the existing descending chromatography apparatus was used. After a series of accidents the method was abandoned and the ascending method used instead. For this a stainless steel tank top was designed and made. (Fig. 2). This top enabled up to four chromatograms to be run simultaneously in each tank.

The growth active extracts were dissolved in 0.75 ml. ether (ether soluble acids) or 0.75 ml. absolute alcohol (water soluble fraction) and spotted as a strip 4.0 cm. from the end of a 2.0 x 36.0 cm. chromatogram. The chromatograms were equilibrated for six hours above the solvent in a glass tank within a larger glass tank and both covered by a light proof wooden box. The chromatograms were then lowered into the solvent and run for 15 hours. At the end of this time the solvent front had moved 22-24 cms. Separate marker chromatograms were run concurrently. The solvent in the

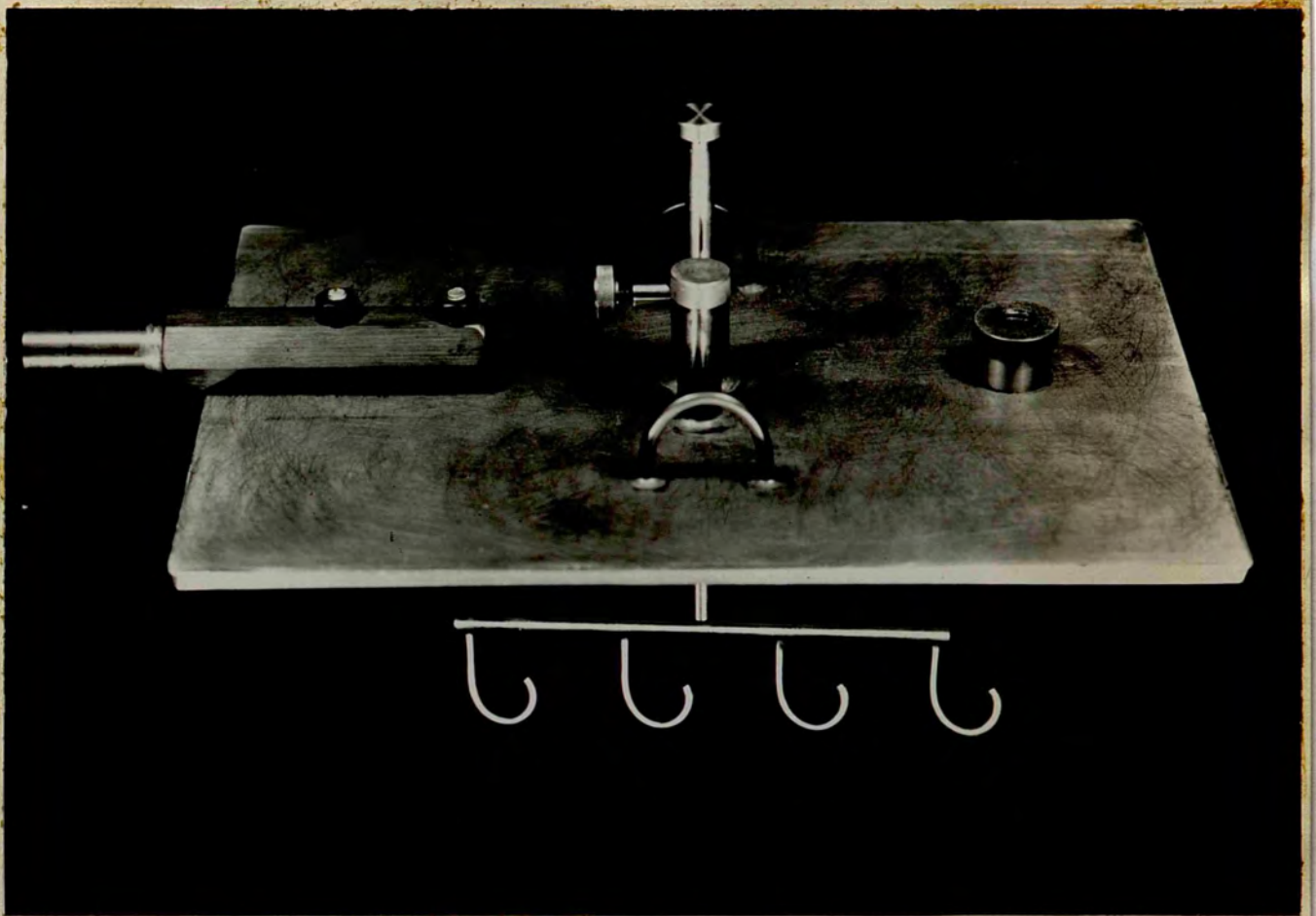
Stainless steel chromatography tank for use in

secondary chromatography.

Fig. 2.

Stainless steel chromatography tank top for use in ascending chromatography.

FIG 2



bottom of the tank was mostly changed after each experiment. A maximum of two sets of chromatograms was put through each lot of chromatography solvent and then it was discarded. Nitsch and Nitsch (1960) demonstrated a chromatographic artefact caused by the transfer of indoles from the marker chromatogram to nearby chromatograms. This will be discussed in Chapter III.

It was found that both grades of paper had to be washed. The reason for this will be given in Chapter III. The papers were washed for 2 days with isobutanol, methanol, water by descending chromatography. The papers were dried overnight and stored in a polythene bag.

(viii) Root stimulation apparatus and method

Originally the roots were horizontally stimulated in the following manner. Pins were pushed through the cotyledons to hold the seedlings in position on cork mats covered with moist filter paper. The roots were orientated in a vertical position and not touching the filter paper. Each cork mat stood in a container with water in the bottom. The roots were brought into a horizontal position by turning the mat through 90° . This method was discarded for two reasons. Firstly the proximity of the roots to the cork mats prevented the two newly designed root cutters from being used. Secondly the wear and tear on the thumb pushing pins through the cotyledons became excessive. The following method was substituted. A length of perspex 84.0 cm. long 1.2 cm. wide and 2.0 cm. deep was mounted on a length of wood slightly longer, broader and deeper.

Slots 1.4 cm. wide and 1.3 cm. deep were cut in the perspex with a gap of 2.0 cm. between each slot. Sticking up through each slot to a height of 1.2 cm. were three pins equidistantly spaced. Part of the apparatus is shown in (Fig. 3). The apparatus was mounted horizontally and so that it could rotate about its own axis through 90° . Seedlings with uniform straight roots were washed and the cotyledons pushed onto the pins with the roots pointing vertically downward. Drops of water on the root tips were gently blotted off. The apparatus was rotated through 90° bringing the roots into a horizontal position. A three sided rectangular perspex box 90.0 cm. x 7.5 cm. x 5.0 cm. was lined on the inner vertical and lower surfaces with wet cottonwool. This cover was placed in position with the roots in the cavity. Polythene sheeting covered the open face helping to reduce water loss from evaporation. Once every 10 minutes the cavity was sprayed with water from an atomiser to keep the atmosphere near 100% relative humidity.

(ix)(a) Apparatus for cutting whole root tips

A cutter was designed for cutting off whole root tips of a known length. This is shown in (Fig. 1). The stop could be adjusted permitting root tips from 5.0 to 10.0 mms. in length to be cut off. The blade was a Wilkinson stainless steel Swordedge. The apparatus proved easy to use and rapid in operation.

(b) Apparatus and method for cutting roots in half longitudinally

The cutter consisted of two hinged pieces of perspex, each 1.8 cm. square and 6.0 mm. thick. From the edge of the middle of

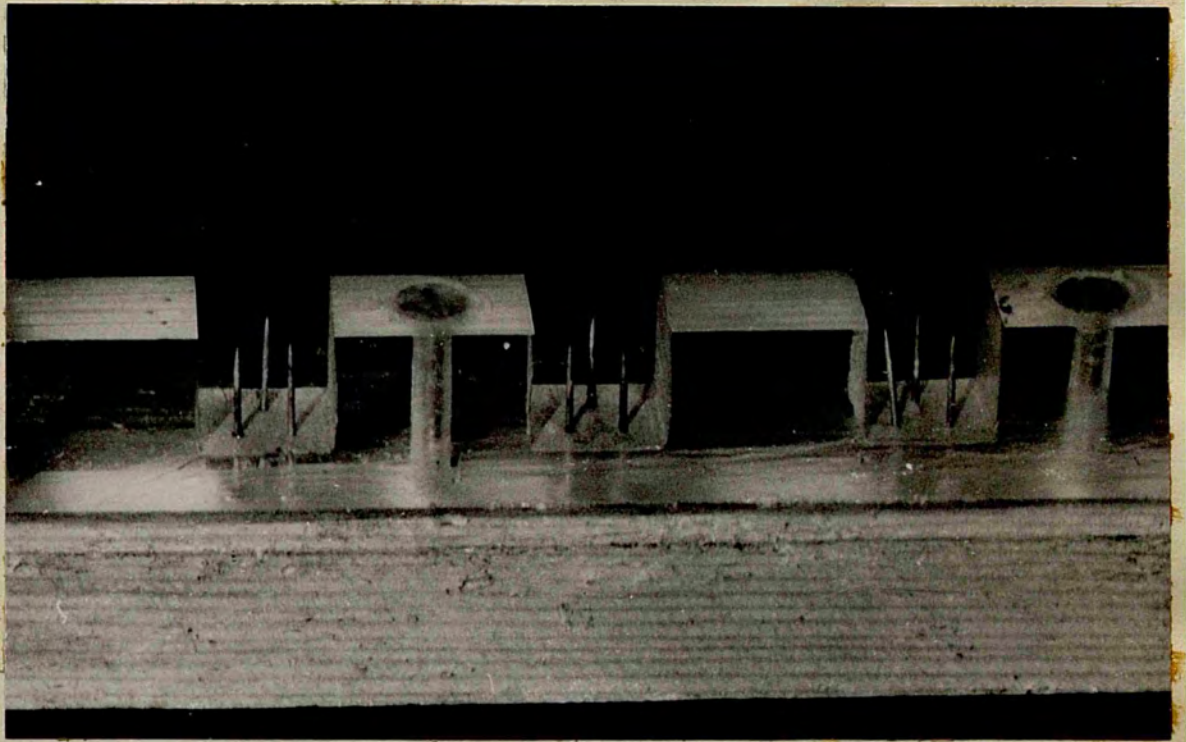
First of the root stimulation apparatus showing the
aloha in the center and the position of the bars
in the aloha. The apparatus was mounted horizontally
and as that it could be rotated through any
angle.

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Fig. 3.

Part of the root stimulation apparatus showing the slots in the perspex and the position of the pins in the slots. The apparatus was mounted horizontally and so that it could be rotated through any angle.

FIG 3



each inner face a tapering groove was cut. This is shown in (Fig. 4), when the faces were closed the grooves joined to form a tapering hole. An "average diameter" root, i.e. 1.5-2.0 mm. in diameter, could be pushed into the hole a distance of 7.0-8.0 mm. The remainder of the root was cut off leaving a 7.00 mm. tip in the hole. By sliding a Wilkinson Swordedge blade between the junction of the two faces the root tip was held in the hole and cut into two longitudinal halves. By marking a few roots it was proved that the orientation of the roots was not lost during cutting. The upper and lower halves were put into separate beakers each immersed in a freezing mixture at -70°C . The time taken from the beginning of cutting to the time the sections dropped into the beakers was approximately 30 seconds.

(x) Bioassay methods

The Avena first internode extension test, the Avena coleoptile test and the pea root extension assay were used to estimate the growth activity of plant extracts. The Avena first internode assay was used to assay the ether soluble acid fraction. The coleoptile test was used to assay for activity in the water soluble fractions. Pea root assays were used to determine whether growth active substances would effect extension in roots in addition to extension in coleoptiles.

(a) Avena first internode extension test

The technique used was based on the method described by Nitsch and Nitsch (1956). The cutter comprised two Durham Duplex blades with a perspex spacer separating the blades. The blades and

Fig. 1.

Apparatus used to split roots in half along the longitudinal axis. The apparatus has been oriented to show the groove in each face. When the faces were closed the grooves formed the hole into which the root tip was pushed.

Fig. 4.

Apparatus used to split roots in half along the longitudinal axis. The apparatus has been orientated to show the groove in each face. When the faces were closed the grooves formed the hole into which the root tip was pushed.

FIG 4



spacer were bolted to an aluminium holder. The two parallel blades cut sections 3.0 mm. in length. The cutter was used in conjunction with a perspex guide grooved on two faces. (Fig 5a).

An 18.5 cm. Whatman No. 1 filter paper was folded around a used clean dry photographic quarter plate. Two parallel pencil lines were drawn 2.0 mm. apart and parallel to the main axis of the plate. The first internodes were arranged with the nodes on line A as shown in (Fig. 5b). The guide was arranged along line B and straddling the internodes. The cutter was placed against the face of the guide along line B and the sections cut with a downward slicing motion. The technique gave sections 3.0 mm. in length and cut 2.0 mm. from the node. Thirty to thirty-five sections were cut at each stroke and a cutting rate of approximately 300 per hour was attained. The first internode sections were soaked for one hour on muslin stretched over glass distilled water in a beaker. After soaking the sections were randomly distributed into vials. All the manipulations were done under a Kodak Safelight with a Wratten O.A. yellow green filter covering a 60 watt bulb.

(b) Avena coleoptile test (after Nitsch and Nitsch 1956)

In this test the two pencil lines A and B were drawn 3.0 mm. apart. The coleoptiles were placed at right angles to the parallel lines and with the tips just touching line A. The perspex guide was placed along line B and the sections cut. This resulted in 3.0 mm. coleoptile sections cut 3.0 mm. from the tip of the coleoptile. The sections were soaked for one hour on

Fig. 1

(a) Diagram of the cutting of
colleptic and first internode sections.

(b) Method of cutting out first internode
and colleptic sections.

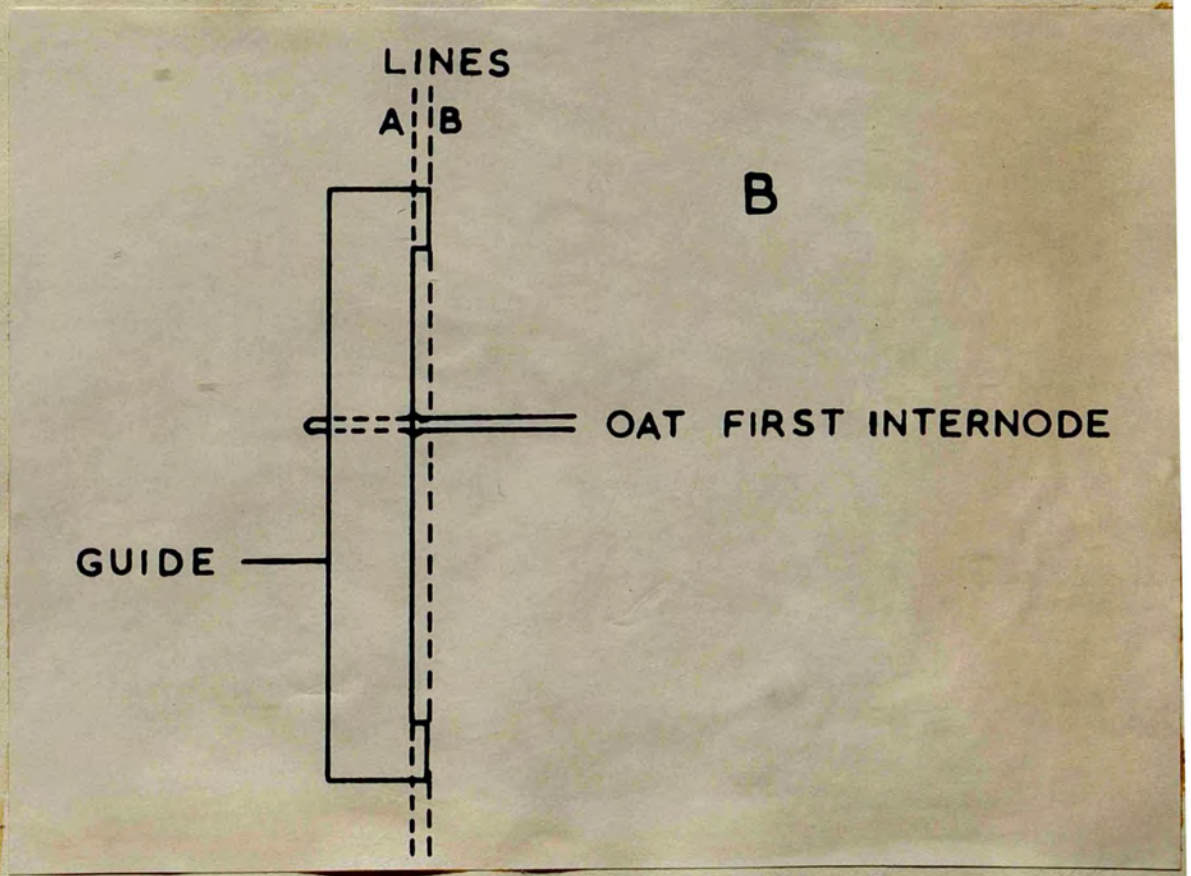
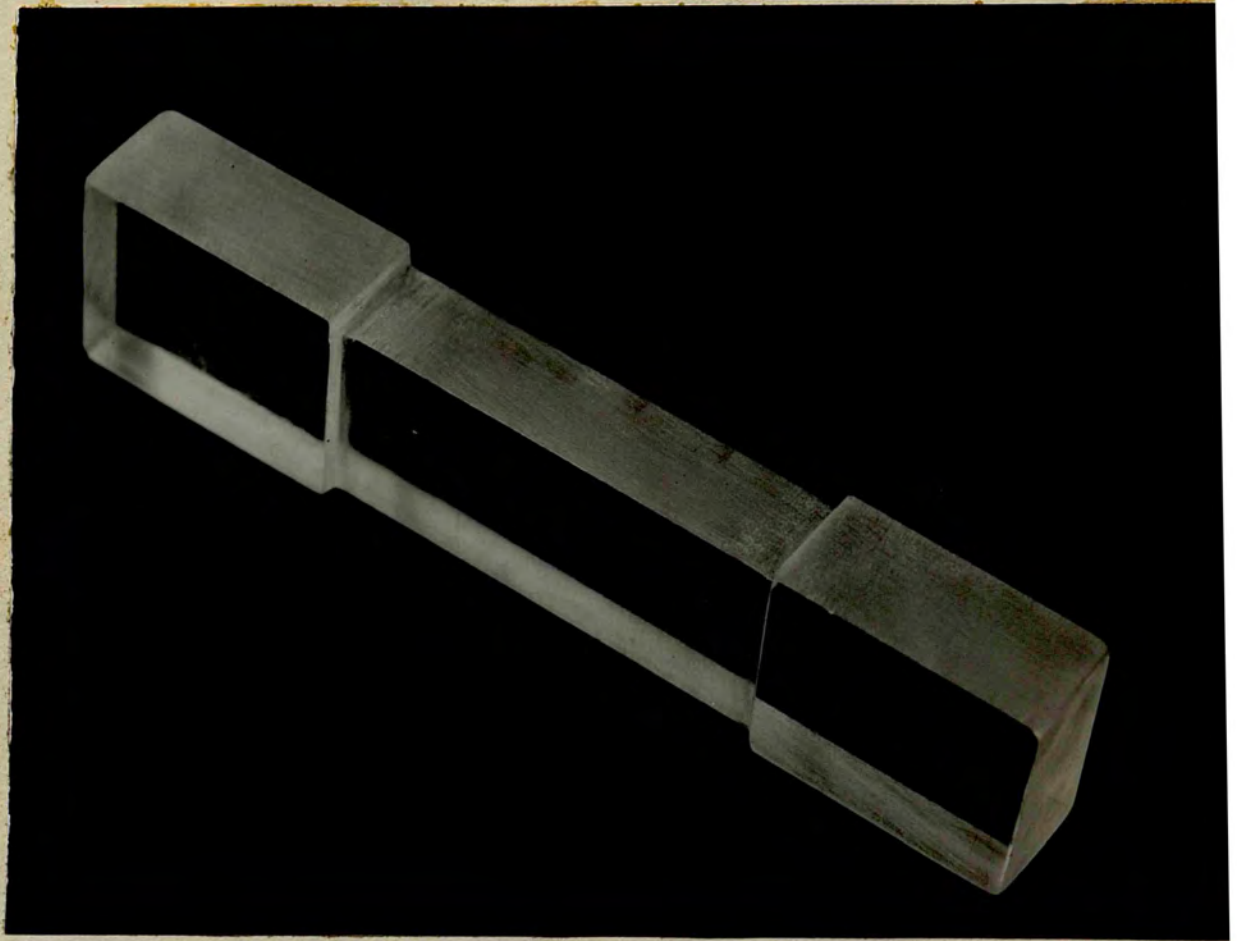
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Fig. 5.

- (a) Perspex guide used in the cutting of
coleoptile and first internode sections.

- (b) Method of cutting out first internode
and coleoptile sections

A



muslin over glass distilled water before random distribution into vials.

(c) Pea root assay

The roots were washed, selected for uniform straight growth and the apical 15-20 mm. cut off. These apices were placed in a special guillotine, (Audus and Shipton (1952)) and the apical 3.0 mms. excised, collected and floated on a thin film of water in a Petri dish. Ten 3.0 mm sections were randomly distributed into each 5.0 cm. Petri dish containing 4.0 mm. 0.5% sucrose.

(xi) Conditions of growth, measurement and presentation of results

The first internode and coleoptile bioassays were done in cylindrical glass vials 2.5 cm. in diameter and 2.5 cm. in depth. The buffer into which the chromatograms were eluted had a pH of 5.0 and was composed as follows:

dipotassium hydrogen phosphate	$1.0 \times 10^{-2} M.$
citric acid	$0.5 \times 10^{-2} M.$
sucrose	2.0%

The dry chromatogram was divided into 20 equal parts. Each twentieth part was shredded into a vial and 1.0 ml. sucrose phosphate citrate buffer added. The vials were stoppered with corks, each cork having a hole in the centre plugged with cotton wool.

For the pea root test the chromatograms were eluted into 0.5% sucrose in place of the sucrose phosphate citrate buffer. The pea root test suffered from the limitation of showing a growth

acceleration over a small concentration range. To overcome this difficulty each twentieth part of the chromatogram was eluted into 0.5% sucrose. Then one in ten and one in a hundred dilutions were made on each primary elution. In this way the effect of the growth substance on dilution could be observed on the growth of the sections.

For each internode and coleoptile experiment ten vials each containing ten sections were used as controls. In later experiments the number of sections in the experimental vials was reduced to five. The control vials each contained 1.0 ml. buffer together with solvent run chromatography paper taken from below the starting line. The control Petri dishes contained 4.0 ml. 0.5% sucrose together with solvent washed chromatography paper. The vials and Petri dishes were rocked on a shaker, 2-3 shakes per second, in darkness at 25°C for 20 hours. Growth was recorded photographically. The sections from each vial were arranged in rows on a clean "quarter plate". The quarter plates were placed in the negative carrier of a photographic enlarger. Shadowgraphs enlarged four times on photographic paper were made of the sections. The total length of the sections from each vial was measured to the nearest 0.5 mm.

(xii) Location reagents and Fluorimetric methods

(a) Location reagents

The following location reagents have all been taken from Chromatographic Techniques, Vol. I. (I. Smith, 1960).

1. Ultra violet light. (Woods light)
2. Ultra violet light after fuming with ammonia.
3. Ninhydrin (Smith 1953)
0.2% in acetone.
4. Ninhydrin acetic acid (Jepson and Stevens 1953)
Ninhydrin 0.2% in acetone 9 vol.
Glacial acetic acid 1 vol.
5. Ehrlich (Jepson 1960)
p - dimethylamino-benzaldehyde 10% W/V
in conc. HCl 1 vol.
Acetone 4 vol.
6. Nitrite - Nitric acid reagent (Jepson 1960)
HNO₃ concentrated 5 vol.
Acetone 45 vol.
NaNO₂ 5% in water (added last) 1 vol.
7. Acidic oxidation reagent (Jepson 1960)
Potassium persulphate (fresh
saturated solution) 1 drop
HCl concentrated 10 ml.
Acetone 40 ml.
8. Alkaline silver nitrate reagent (Jepson 1960)
One drop saturated solution AgNO₃ in water mixed
with 10.0 ml. acetone and the chromatogram dipped.
It is then dipped in 0.5% NaOH in 80% ethanol.
9. Diazotised p - nitraniline (Bray, Thorpe and White 1950)
p - nitraniline 1.5 g. in 45 ml. conc. HCl
and 950 ml. water 10 vol.

- NaNO_2 in water 5% 0.2 vol.
 Na_2CO_3 anhydrous, 10% in water 10 vol.
10. Diazotised sulphanic acid (Jepson 1960)
- (a) Sulphanilic acid 9.0 g.
 Conc. HCl 90 ml. and water 900 ml. 1 vol.
 NaNO_2 5% in water
- (b) Na_2CO_3 anhydrous, 10 in water 1 vol.
11. Ferric chloride - Ferricyanide Reagent (Barton, Evans and Gardner 1952)
- FeCl_3 3% in water
 $\text{K}_3\text{Fe}(\text{CN})_6$, 3% in water
12. Ekman reagent (Jepson 1960)
- (a) HCl 2.0 N 5 vol.
 Acetone 45 vol.
 NaNO_2 5% in water (added last) 1 vol.
- (b) Ethyl naphthylamine 5% in ethanol
 (Ekman reagent)
13. p - anisidine reagent (Jepson and Smith 1953)
- (a) p - anisidine 1% in ethanol containing 1%
 V/V concentrated HCl 1 vol.
- (b) Amyl nitrite 2% in ethanol 1 vol.
- (c) 0.5 N NaOH
14. Aniline diphenylamine reagent (Harris and MacWilliam 1954)
- Aniline 1% (1.0 ml.) plus diphenylamine 1%
 in acetone 10 vol.
 Phosphoric acid 85% 1 vol.

(b) Function of the location reagents

Ultra violet light alone or after fuming with ammonia was

used to locate fluorescent zones. The colour, intensity and change in colour and intensity after fuming with ammonia were noted. This was particularly useful for phenolic compounds which show characterised reactions under these conditions.

Ninhydrin was used to detect amino acids and heating was necessary. Ninhydrin acetic acid was used to detect certain tryptamines. This form of the Ehrlich reagent was used as a dip to detect indole compounds the majority of which will react with it. The nitrite nitric acid reagent was used to locate specific indoles like IAN and Jepson's acidic oxidising reagent was used in place of Salkowski to confirm certain Ehrlich positive spots.

Alkaline silver nitrate was used as a general location reagent to detect reducing groups. Despite its lack of specificity it was useful as a general indicator.

Diazotised p - nitraniline and sulphanilic acid were used to locate phenols especially mono /and dihydroxyphenolic compounds. The different colour reactions of the two location reagents for various phenolic compounds was an additional aid to identification. They were more useful than the ferric chloride-ferricyanide reagent which gave a similar reaction for all phenolic areas. The two diazo reagents were most useful when used as sprays and not as dips as suggested by Smith, p.296-297. p - Anisidine was used to locate polyphenolic compounds.

The Ekman reagent was used to locate aromatic amines and aniline diphenylamine was used to detect sugars but was not found to be very effective.

(c) Spectrophotofluorimetric analyses

Instrumentation for the fluorimetric analyses is as follows. The Aminco Bowman Spectrophotofluorimeter was equipped with a high intensity 150 watt Hanovia Xenon arc lamp. The 2.0 ml. sample is placed in a clear sided quartz cell and can be irradiated at any desired wavelength between 220-650 millemicron. The monochromator which analyses the emitted fluorescent light can be used at any chosen wavelength in the same range. The defining slits were $\frac{3}{16}$ " giving maximum sensitivity and an IP.21 R.C.A. multiplier phototube was used for all fluorescent measurements. Quinine sulphate in 0.1 N H_2SO_4 was used to calibrate the instrument. The activation maximum of Quinine sulphate is at 350 $m\mu$ with a minor peak at 250 $m\mu$ and a maximum emission (fluorescence at 450 $m\mu$.) The activation wavelength peak and the fluorescent wavelength maximum peak were recorded for each unknown sample. It has been found that certain groups of compounds show a characteristic change in fluorescent intensity with a change from acidic to alkaline pH. This characteristic has been used as an aid to identification of unknown compounds eluted from chromatograms. The change in fluorescent intensity with pH was studied using the following buffer systems, Clark (1928); sulphuric acid, pH 0.1 - 1.0; citrate phosphate, pH 2.2 - 8.0; Glycine NaCl/NaOH, pH 8.93 - 12.97 and NaOH, pH 13.2 - 14.0.

(xiii) Preparation of a stock aqueous solution of IAA for calibration curves

Standard growth response curves to known concentrations of IAA were made at intervals throughout the project. An aqueous solution of IAA; 100 mg./litre was prepared by dissolving 0.005 g. IAA in 0.5 ml. pure methanol and making up to 50 ml. with water. The stock solution was also prepared by dissolving the IAA in 1.0 ml. 0.1 N NaOH, adding 40 ml. water, neutralising with 1.0 ml. 0.1 N HCl and making up to volume with water. No difference in growth promoting properties was found between the two methods. From this stock solution the required number of ten times dilutions in sucrose phosphate citrate buffer were made.

(xiv) Statistical Treatments

The growth of sections was expressed in two ways. Firstly as the total extension and secondly as a percentage of the growth of the controls. The second method may be expressed as follows

$$\frac{\text{final length of treated sections} - \text{initial length}}{\text{final length of controls} - \text{initial length}} \times 100$$

The fiducial limits at the 5% level were calculated. Any growth response greater than the fiducial limits was considered to be statistically significant. The fiducial limits at the 5% level = t.S.D.

where t with 9 degrees of freedom

$$= 2.26$$

The standard deviation can be calculated from the formula:

$$\text{S.D.} = \sqrt{\frac{(x - \bar{x})^2}{n - 1}}$$

where

x = mean growth of all ten sections per vial

\bar{x} = grand mean of all ten vials

n = number of control vials

It would have been more accurate to have incorporated other controls each containing different concentrations of IAA. This would have established the sensitivity of the assay material for each experiment in terms of IAA equivalents. Since this was not done the results of all the calibration experiments were pooled and a mean result obtained. This was done for both the first internode assay and the coleoptile assay. To check on this procedure an analysis of variance was made on the first internode results.

Regression of x (as log concentration of IAA)

on y (as % growth). $n = 39$.

$$\sum x = -37$$

$$\sum x^2 = 111$$

$$\sum y = 8667$$

$$\sum y^2 = 2193995$$

$$\sum xy = -3751$$

$$\bar{x} = -0.949$$

$$\bar{y} = 222.23$$

$$\sum (x - \bar{x})^2 = 75.95$$

$$\sum (y - \bar{y})^2 = 267921$$

$$\sum (x - \bar{x})(y - \bar{y}) = 4471.9$$

$$\frac{4471.9}{267921} = 0.01669$$

The regression of x on y = -a + 0.01669y

$$(\text{ where } a = -0.949 - 0.01669 \times 222.23$$

$$= -0.949 - 3.7090$$

$$= -4.658)$$

$$= -4.658 + 0.01669y$$

Therefore when y = 100, x = -2.989

and when y = 335, x = 0.833

These two values of x and y have been plotted on the IAA internode calibration curve.

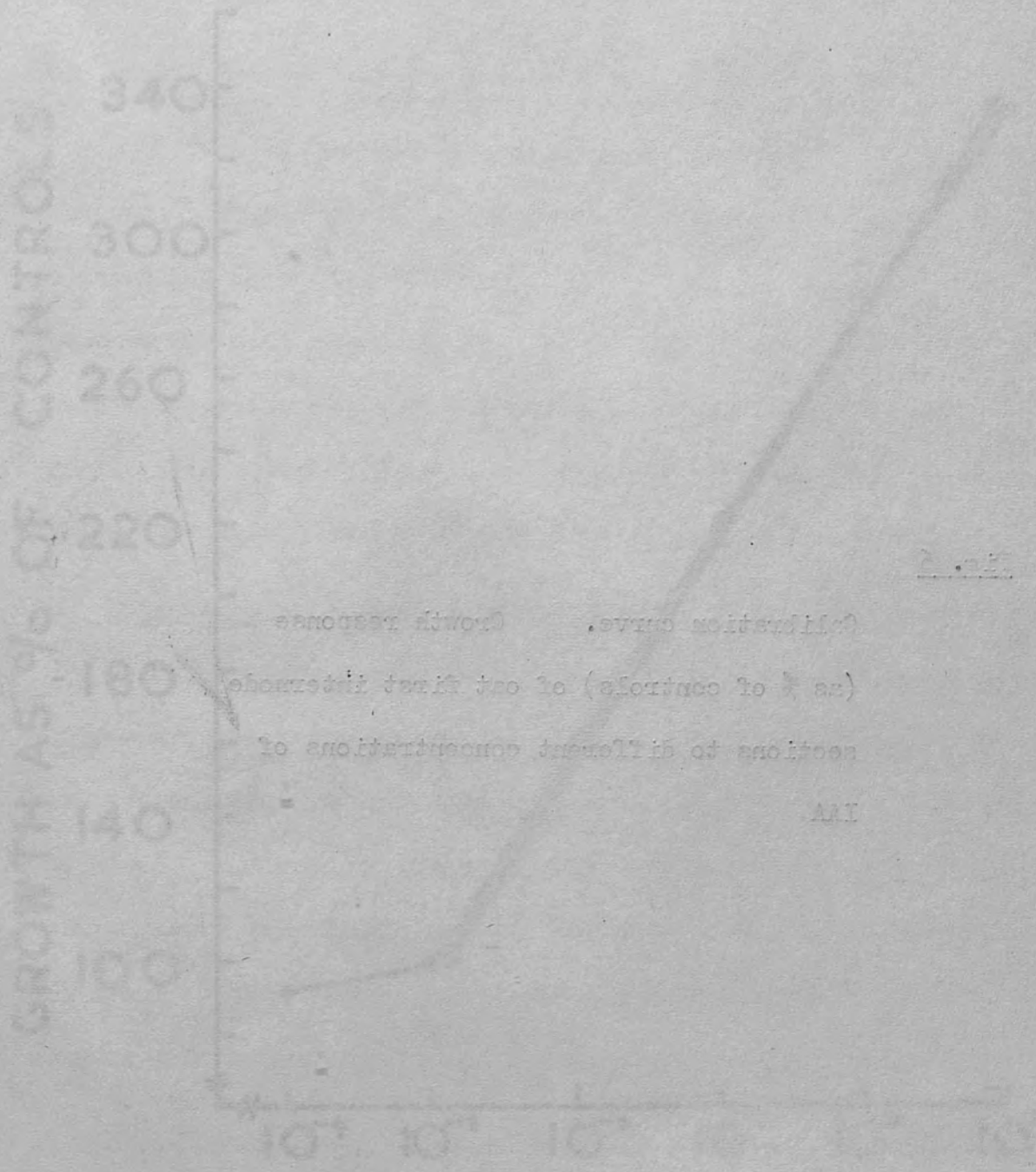
(xv) Growth response of internode and coleoptile sections to IAA

(a) Growth response of *Avena* first internode sections to IAA

Ten first internode sections, presoaked on muslin stretched over glass distilled water, were put into each vial. Each vial contained 1.0 ml. IAA sucrose phosphate citrate buffer plus 1.0 cm. finely-cut solvent-washed chromatography paper. Growth was measured after 20 hours.

The growth responses to added IAA have been tabulated in Table 1, and the mean curve is shown in (Fig. 6). The threshold of sensitivity of the sections to IAA was found to be approximately

FIG. 5

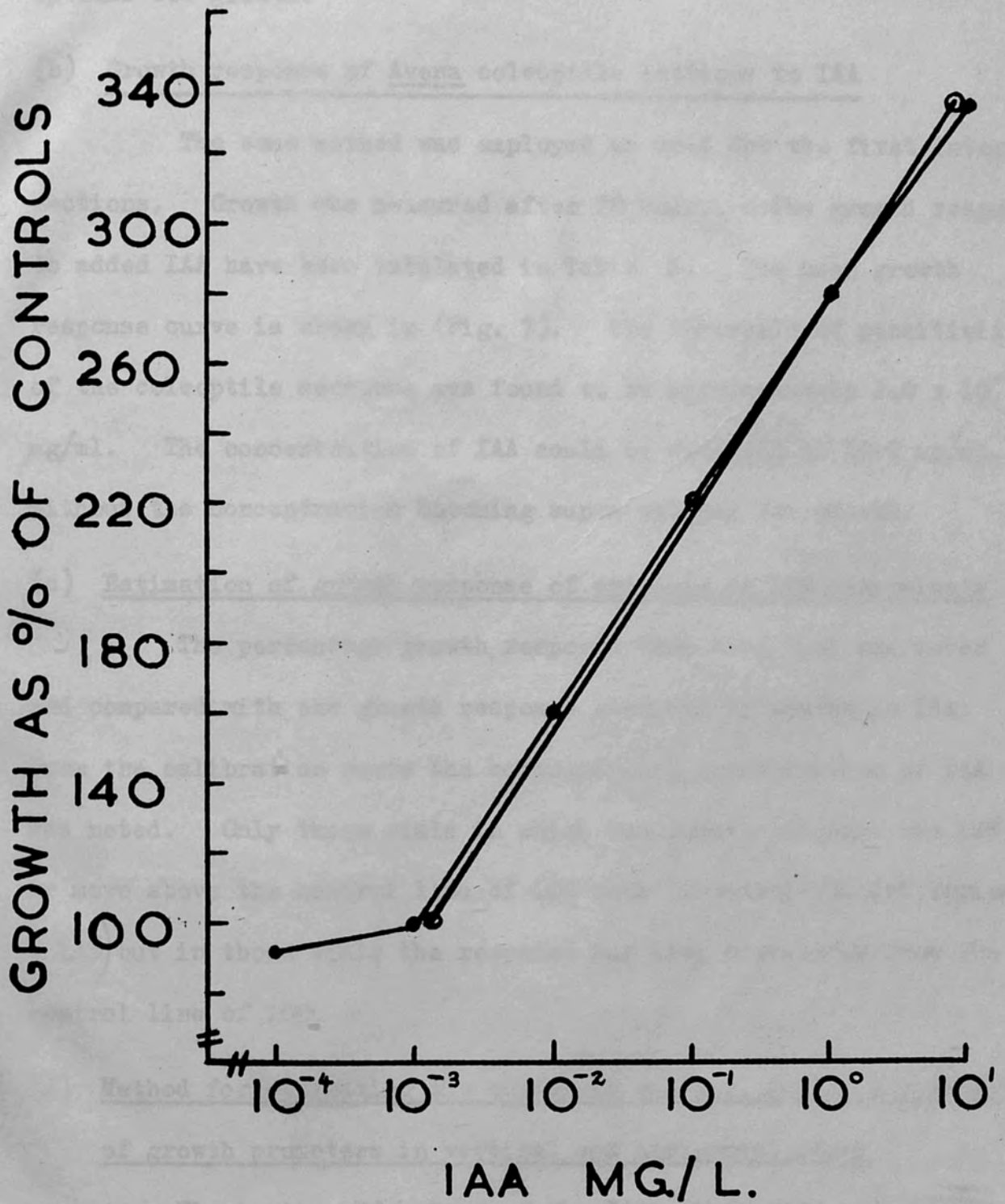


Growth response
inhibition curve
(as % of controls) of out plant
to different concentrations of
IAA

Fig. 6

Calibration curve. Growth response
(as % of controls) of oat first internode
sections to different concentrations of
IAA.

FIG 6



$0.15 \times 10^{-3} \mu\text{g/ml}$. It was possible to extend the concentration in the upper range to $10.0 \mu\text{g}$ without the concentration becoming supra-optimal for growth.

(b) Growth response of *Avena* coleoptile sections to IAA

The same method was employed as used for the first internode sections. Growth was measured after 20 hours. The growth response to added IAA have been tabulated in Table 2. The mean growth response curve is shown in (Fig. 7). The threshold of sensitivity of the coleoptile sections was found to be approximately $1.0 \times 10^{-2} \mu\text{g/ml}$. The concentration of IAA could be extended to $10.0 \mu\text{g/ml}$ without the concentration becoming supra-optimal for growth.

(c) Estimation of growth response of extracts as IAA equivalents

The percentage growth response from each vial was noted and compared with the growth response produced by synthetic IAA. From the calibration curve the corresponding concentration of IAA was noted. Only those vials in which the growth response was 14% or more above the control line of 100 were included (fiducial limits - 14%) but in those vials the response has been calculated from the control line of 100.

(d) Method for estimating the change in content, in IAA equivalents, of growth promoters in vertical and horizontal roots.

Fluctuating Rf values of the IAA marker spot made this estimation rather difficult. The following method was employed. The vials in which the response was obtained were noted and the IAA equivalents recorded and added. This gave the total content

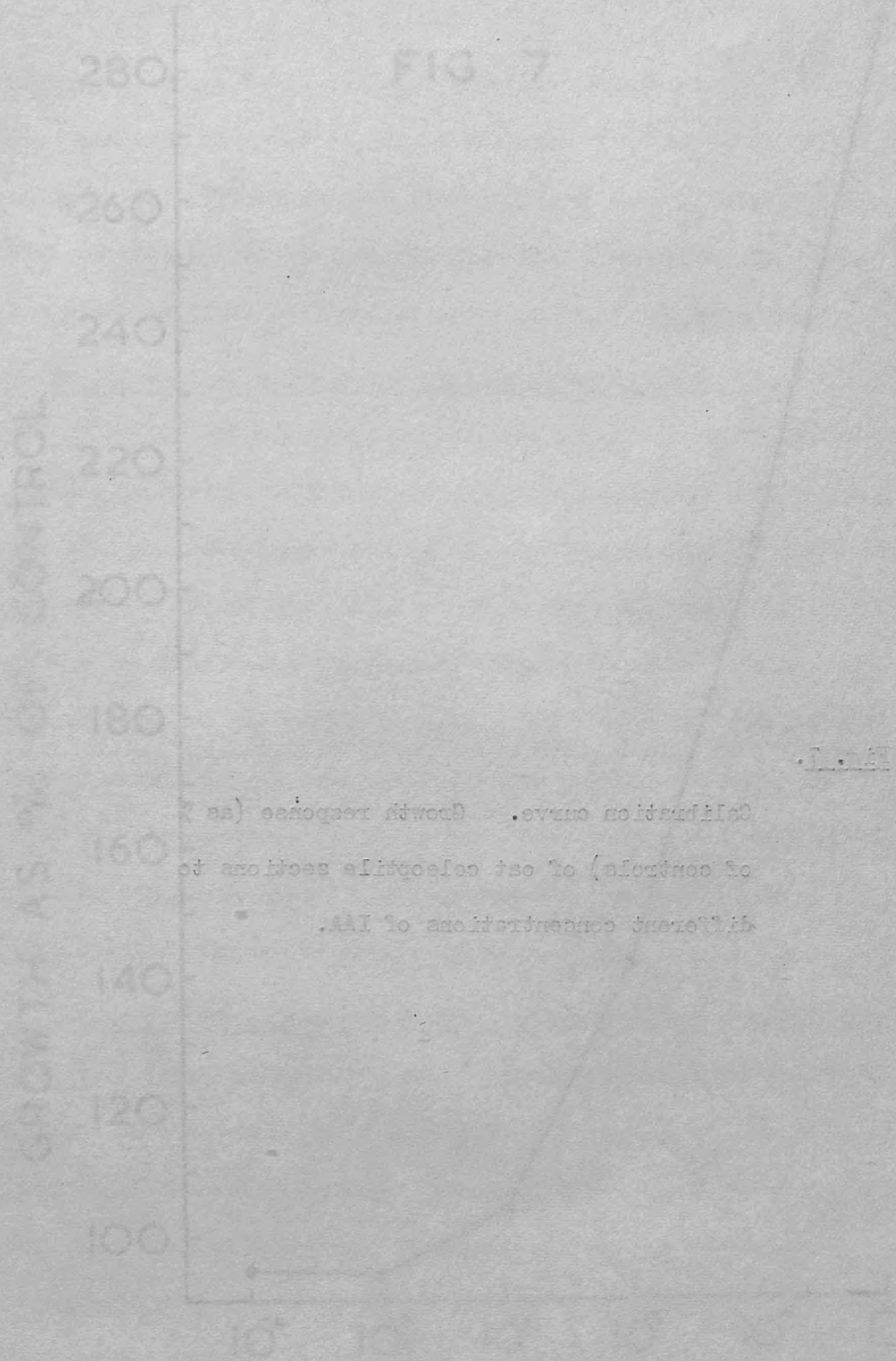
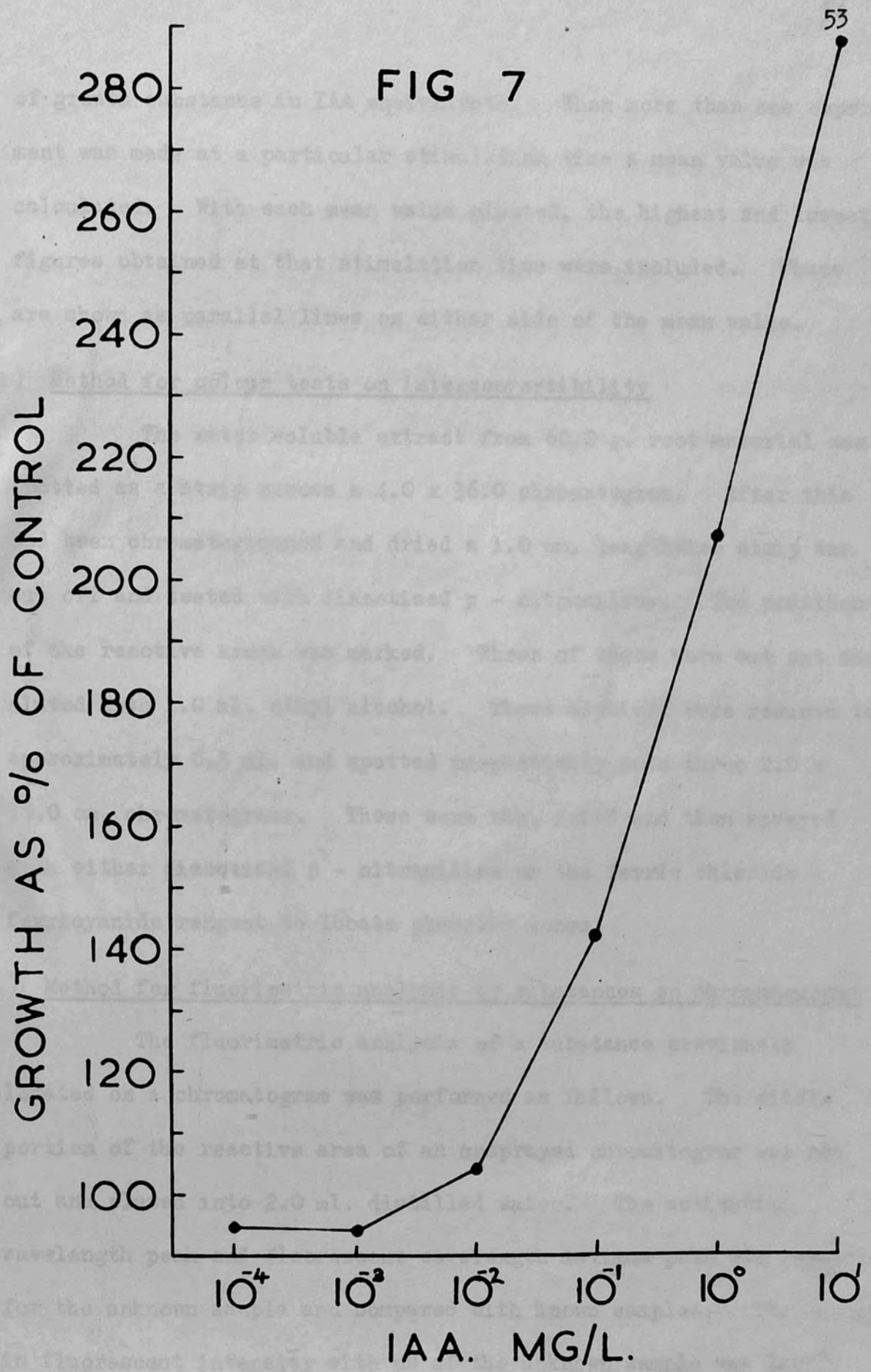


TABLE I
 Calibration curve. Growth response (as % of control) of oat coleoptile sections to different concentrations of IAA.

Fig. 7.

Calibration curve. Growth response (as % of controls) of oat coleoptile sections to different concentrations of IAA.



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of growth substance in IAA equivalents. When more than one experiment was made at a particular stimulation time a mean value was calculated. With each mean value plotted, the highest and lowest figures obtained at that stimulation time were included. These are shown as parallel lines on either side of the mean value.

(xvi) Method for colour tests on interconvertibility

The water soluble extract from 60.0 g. root material was spotted as a strip across a 4.0 x 36.0 chromatogram. After this had been chromatogrammed and dried a 1.0 cm. lengthwise strip was cut off and tested with diazotised p - nitraniline. The position of the reactive areas was marked. Three of these were cut out and eluted into 1.0 ml. ethyl alcohol. These elutions were reduced to approximately 0.5 ml. and spotted respectively onto three 2.0 x 36.0 cm. chromatograms. These were run, dried and then sprayed with either diazotised p - nitraniline or the ferric chloride - ferricyanide reagent to locate phenolic zones.

(xvii) Method for fluorimetric analysis of substances on chromatograms

The fluorimetric analysis of a substance previously located on a chromatogram was performed as follows. The middle portion of the reactive area of an unsprayed chromatogram was cut out and eluted into 2.0 ml. distilled water. The activation wavelength peak and fluorescent wavelength maximum peak was recorded for the unknown sample and compared with known samples. The change in fluorescent intensity with pH of the unknown sample was recorded.

From the figures for the activation and fluorescent wavelength maxima and from the shape of the fluorescent intensity / pH curve a guess could be made as to the nature of the unknown compound. Similar sets of readings were then made on a number of known pure compounds to which the unknown might belong. A comparison was made between the data from the known samples and the unknown sample to help establish the identity of the unknown compound. The methods used have been described in paragraph (xii - c)

(i) The breakdown or release of growth active substances

during the preparation of the material and its activation

and purification and chromatography and separation of

growth substances.

(ii) The effect of chromatography artifact.

(iii) Inhibitors in the extraction solvent.

(iv) Inhibitors in unactivated chromatography paper.

(v) Inhibitors in the chromatography solvent.

(vi) Biological variability.

These points will now be discussed in detail.

(i) The breakdown or release of growth active substances

It was appreciated that growth active substances could

be inactivated or released at various stages in the handling of

biological material and extracts.

CHAPTER III

ASSESSMENT OF ERRORS IN THE PREPARATION, CHROMATOGRAPHY AND ASSAY OF GROWTH SUBSTANCES

In the analysis of the activity of a growth hormones profile from a chromatogram a number of possible sources of error have to be taken into account. Some of these were:

- (i) The breakdown or release of growth active substances during the preparation of the material and in extraction, purification and chromatographic separation of growth substances.
- (ii) The Nitsch chromatography artefact.
- (iii) Inhibitors in the extraction solvent.
- (iv) Inhibitors in unwashed chromatography paper.
- (v) Inhibitors in the chromatography room air.
- (vi) Biological variability.

These points will now be discussed more fully.

(i) The breakdown or release of growth substances

It was appreciated that growth active substances could be inactivated or released at various stages in the handling of biological material and extracts.

Many methods have been suggested for the preparation of plant tissue preparatory to extraction. Freeze-drying of tissue was suggested by Link et al (1941), van Overbeek (1945) and Wildman and Muir (1947). Heat drying was suggested by Gustafson (1941), Thimann et al (1942) and Cooke (1954). Thimann and Skoog (1940), Vlitos (1956) and Bennet-Clark et al (1959) recommended boiling of tissues so as to destroy further enzyme action. Of these suggested methods, rapid freezing of the tissue within 15 seconds followed by freeze-drying and storage in a closed container at 2-3°C was adopted. Every care was taken after the tissue was frozen to prevent it thawing before it was freeze-dried. Link et al (1941) showed that auxin in frozen tissues was rapidly destroyed if the tissue thawed. This rapid freezing method was less likely to produce artefacts than the slow freezing at 0°C (ice water freezing bath) used by Lahiri (1959).

The choice of extraction solvent, extraction time and temperature was beset with difficulties. Ether and ethyl acetate did not appear suitable since they did not inhibit the activity of the polyphenolase enzyme system. Nitsch (1956). Various authors have shown that when ether was used as an extractant, auxin could be released from plant tissue over a long period of time. Thimann et al (1940, 1942), Link et al (1941), van Overbeek (1947). Wildman and Muir (1949) showed that the release of auxin increased with temperature and could be stopped by boiling and therefore it was

presumably enzymatic. The extractant should also be free from water. Vlitos and Meudt (1954) showed that tryptophan could be converted into IAA in tissue extracts even at low temperatures when water was added to the extractant, ethanol. To overcome these various difficulties methanol was used as the extractant for the following reasons:

- (a) It completely inhibited the polyphenolase enzyme system.
- (b) It was apparently more efficient than ethanol or ethyl acetate in extracting growth substances. Nitsch (1956).

Therefore methanol was added to the freeze-dried material and the extraction time was approximately 18 hours at -18°C in darkness.

Since high temperatures could accelerate the breakdown of labile compounds, the temperature during flash evaporation and all other manipulations was kept to 26°C or below. In addition all experiments were performed in diffuse light. Jerchel and Muller (1951) showed that pigments in extracts could lead to photolytic decomposition of IAA.

A neutral solvent, isobutanol, methanol, water, was used for chromatography in place of the more commonly used solvent, isopropanol, ammonia, water. The ammoniacal solvent has been found to decompose a few indoles, Nitsch (1956), Bentley (1956), and also water soluble growth substances, Housley and Bentley (1956). In addition, Gunning (1961) has found that malic acid, when chromatographed in an ammoniacal solvent, formed the half ammonium salt.

This salt was slightly growth active and had an Rf near the starting line. This discovery prompts the query: How many ether soluble dicarboxylic acids could form the half ammonium salt when chromatographed in an ammoniacal solvent. Would any of these salts show any growth promoting activity?

The phenomenon of spontaneous interconvertibility (among the auxins of the water soluble fraction) has been demonstrated by several workers, Britton et al (1956), Audus and Gunning (1958), Lahiri and Audus (1960), Bentley (1961a). Criticism has been levelled at these results. The work of Britton et al has been criticised because they used an ammoniacal solvent. It has been shown that ammoniacal solvents can bring about the decomposition of water soluble growth substances, Housley and Bentley (1956). This criticism could also be levelled at the work of Bentley (1961a). Nitsch and Nitsch (1960) who showed that certain indoles can be transferred from the marker chromatogram to other chromatograms nearby have offered this phenomenon as an explanation of the interconvertibility found by Britton et al. Britton ran IAN marker chromatograms in conjunction with the experimental chromatograms.

Thurman and Street (1960) could not find any interconvertibility and made the general criticism that it might follow erroneously from the elution and rechromatography of zones containing massive amounts of several constituents arising from heavily loaded chromato-

grams. The elution would effect some purification and the re-chromatography would permit more effective separation. It is difficult to see how this criticism could apply to some work. Audus and Gunning (1958) used 0.1 ml. aliquots equivalent to 5.4 g. original tissue. Lahiri and Audus (1960) used an extract from 10.0 g. tissue for the original chromatogram. In both cases the original peaks were separate and clear cut. On elution and re-chromatography the same three or four clear cut peaks at the same positions as the originals were found though the activity was lower, often only just significant. Both Audus and Gunning and Lahiri and Audus used a neutral solvent, isobutanol, methanol, water. Thus the conclusion can be arrived at, that some work on interconvertibility of the water soluble growth substances is open to criticism. Other work, especially that of Audus and Gunning, Lahiri and Audus, shows that interconvertibility is a real and, as yet, unexplained phenomenon.

(ii) The Nitsch Chromatography artefact

Yet another artefact arising out of chromatographic techniques has been pointed out, this time by Nitsch and Nitsch (1960). They demonstrated that indole compounds spotted on a marker chromatogram could transfer to an adjacent chromatogram and give rise to biological activity on the adjacent chromatogram. This transfer of indole auxins occurred during the equilibration period. It was most pronounced with IAN and the ethyl ester of IAA; (IAEt) and

with solvents containing petroleum ether or hexane. The transfer could even take place over isopropanol, ammonia, water. However, Nitsch and Nitsch found that in general IAA did not appear to be transferred.

An experiment was performed to test the possibility that IAA could be "transferred" from the marker chromatogram to other chromatograms 4.0 cm. apart during the equilibration period. The marker chromatogram spotted with 5.0 μ g. IAA was flanked by 2 blank chromatograms, one of unwashed paper and the other of washed paper. The chromatograms were equilibrated for 6 hours over isobutanol, methanol, water, and then chromatogrammed. After drying the chromatograms were assayed with the first internode test. The results are shown in (Fig. 8). This result confirmed the report of Nitsch and Nitsch (1960) that IAA generally did not appear to be transferred. IAA only was used in this test because it was the primary marker substance. IAN was used rarely and then in a separate tank.

The unwashed and washed papers in this experiment were also used to demonstrate the presence of unhibitors in unwashed paper and their absence in washed paper.

(iii) Inhibitors in the extraction solvent

A feature not appreciated at the beginning of this work was that growth inhibitors were present in the industrial methylated spirits used as the extractant. A constant and strong inhibition

FIG 8



Fig. 8

Experiments to show the transfer of IAA from the
 master chromatogram to adjacent chromatograms,
 during the equilibration period was not occurring.
 On first internode assay.

(a) Solvent washed paper.

(b) Unwashed chromatography paper.

These results also demonstrate the absence of
 growth inhibitors in washed paper (a) and the
 presence of inhibitors in unwashed paper (b).

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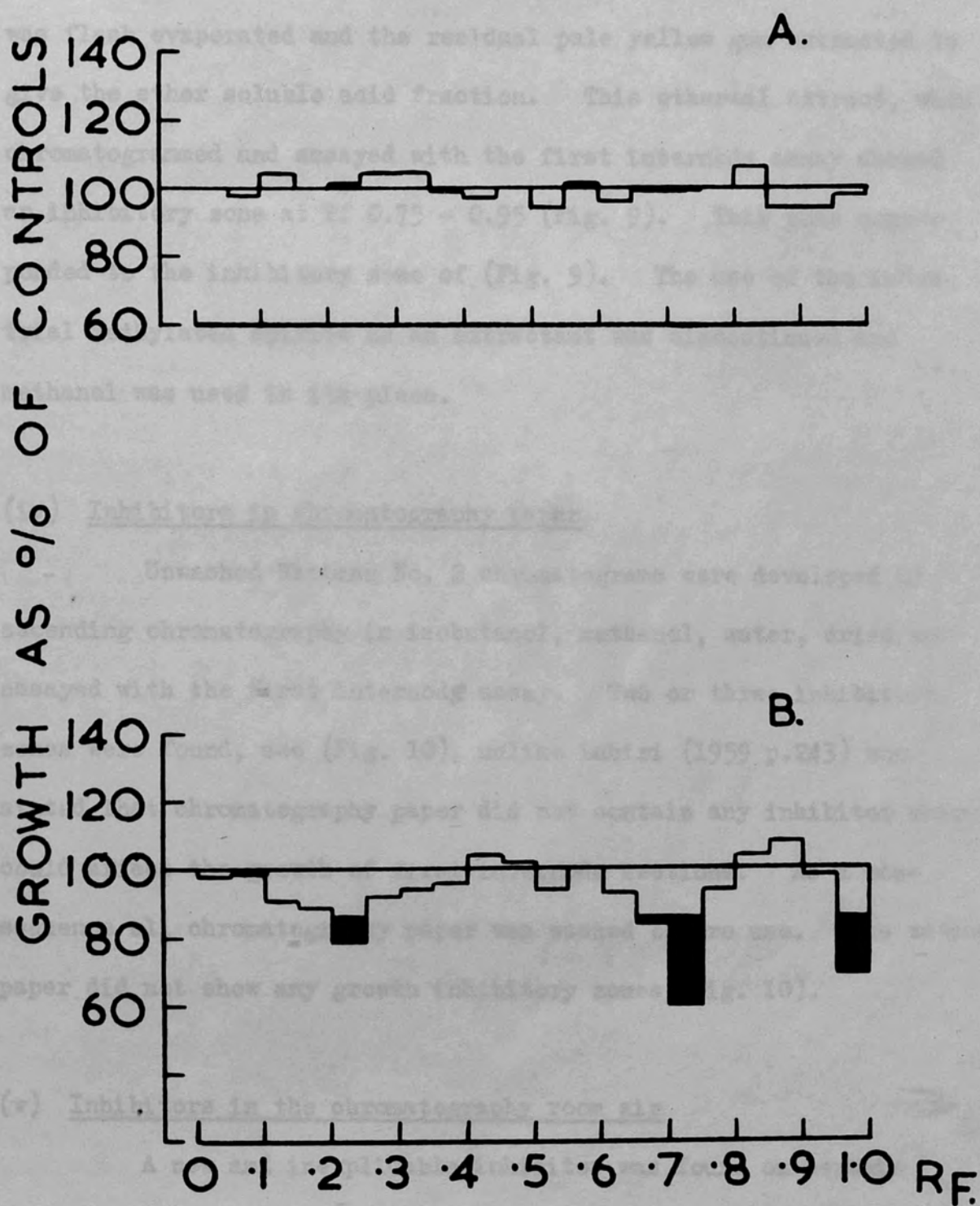
Fig. 8.

Experiments to show the transfer of IAA, from the marker chromatogram to adjacent chromatograms, during the equilibration period was not occurring. Oat first internode assay.

- (a) Solvent washed paper.
- (b) Unwashed chromatography paper.

These results also demonstrate the absence of growth inhibitors in washed paper (a) and the presence of inhibitors in unwashed paper (b).

FIG 8



was found at Rf 0.65 - 0.95 in all experiments. An example is shown in (Fig. 9). Washed chromatography paper was being used and consequently the suspicion arose that the inhibitor might be in the methylated spirits. One hundred and fifty ml. methylated spirits was flash evaporated and the residual pale yellow gum extracted to give the ether soluble acid fraction. This ethereal extract, when chromatogrammed and assayed with the first internode assay showed an inhibitory zone at Rf 0.75 - 0.95 (Fig. 9). This zone corresponded to the inhibitory zone of (Fig. 9). The use of the industrial methylated spirits as an extractant was discontinued and methanol was used in its place.

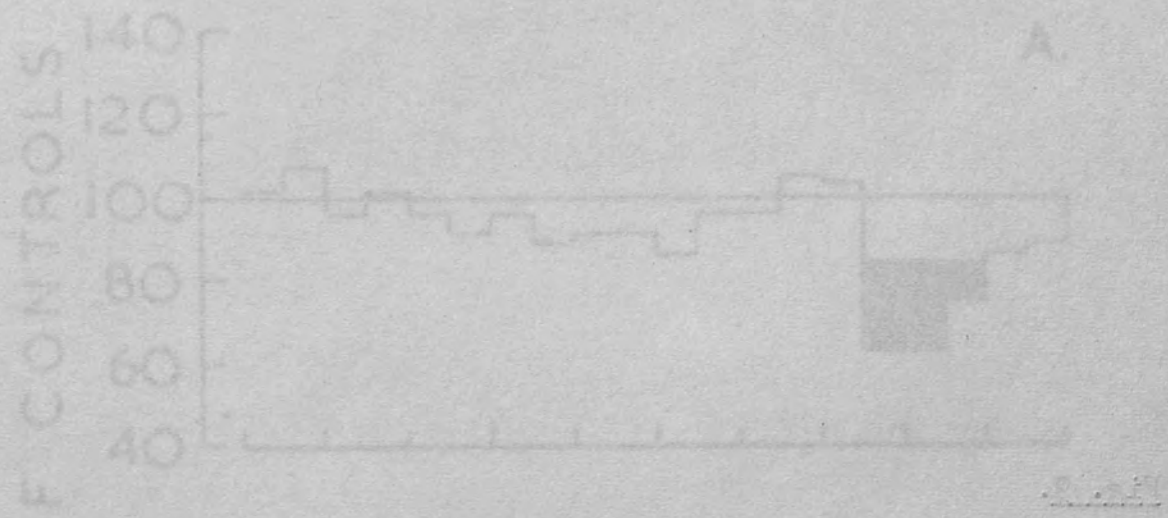
(iv) Inhibitors in chromatography paper

Unwashed Whatman No. 2 chromatograms were developed by ascending chromatography in isobutanol, methanol, water, dried and assayed with the first internode assay. Two or three inhibitory zones were found, see (Fig. 10), unlike Lahiri (1959 p.243) who stated that chromatography paper did not contain any inhibitor which could affect the growth of first internode sections. As a consequence all chromatography paper was washed before use. The washed paper did not show any growth inhibitory zones (Fig. 10).

(v) Inhibitors in the chromatography room air

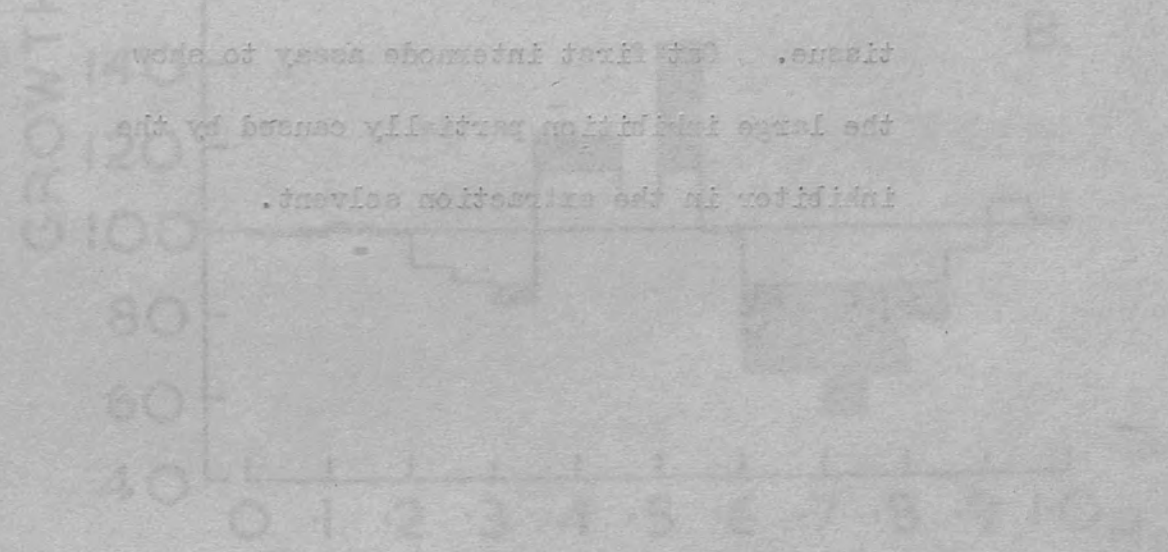
A new and inexplicable inhibitor was found on certain chromatograms at an Rf of 0.85 - 1.0. At the same time it was

FIG 9



(a) Get first intermediate assay of an ether soluble acid extract to demonstrate the inhibitors in 100 ml industrial methylated spirits.

(b) Ether soluble acid fraction from an industrial methylated spirits extract of 2.0 g. root tissue.



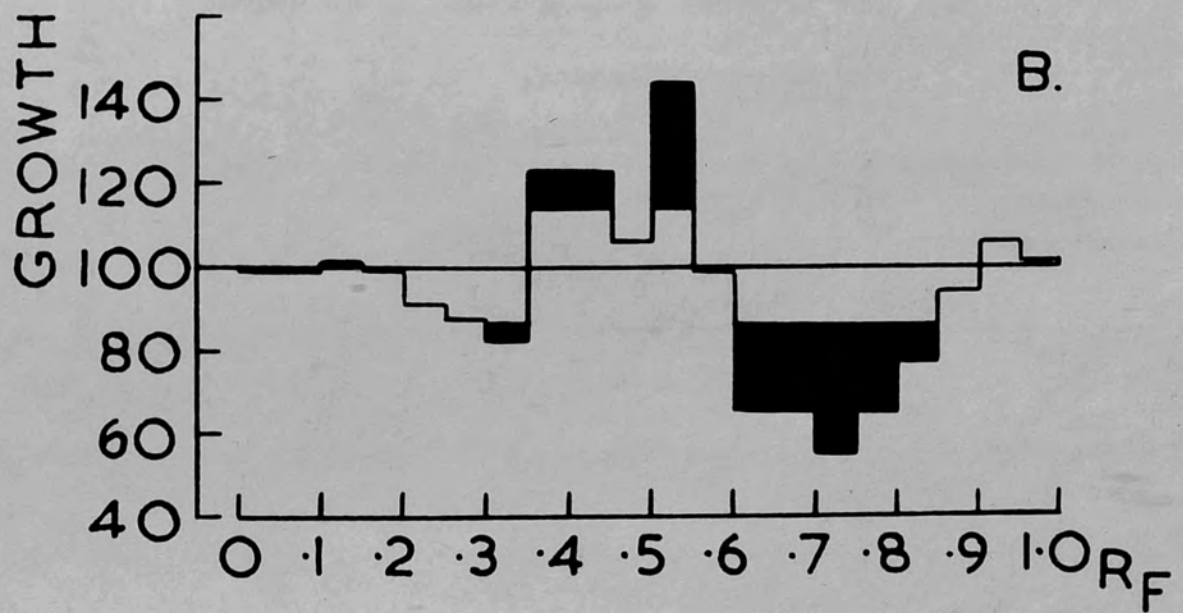
(a) Get first intermediate assay to show the large inhibition partially caused by the inhibitor in the extraction solvent.

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Fig. 9.

- (a) Oat first internode assay of an ether soluble acid extract to demonstrate the inhibitors in 150 ml industrial methylated spirits.
- (b) Ether soluble acid fraction from an industrial methylated spirits extract of 2.0 g. root tissue. Oat first internode assay to show the large inhibition partially caused by the inhibitor in the extraction solvent.

FIG 9



GROWTH AS % OF CONTROLS

FIG 10

140
120
100
80



Fig. 10

Get first intermediate easy to show.

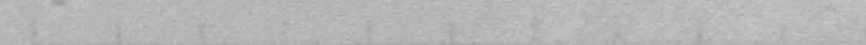
(a) The absence of growth inhibitors in solvent

washed chromatography paper.

(b) The presence of inhibitors in unwashed

chromatography paper.

140
120
100
80



0 1 2 3 4 5 6 7 8 9 10

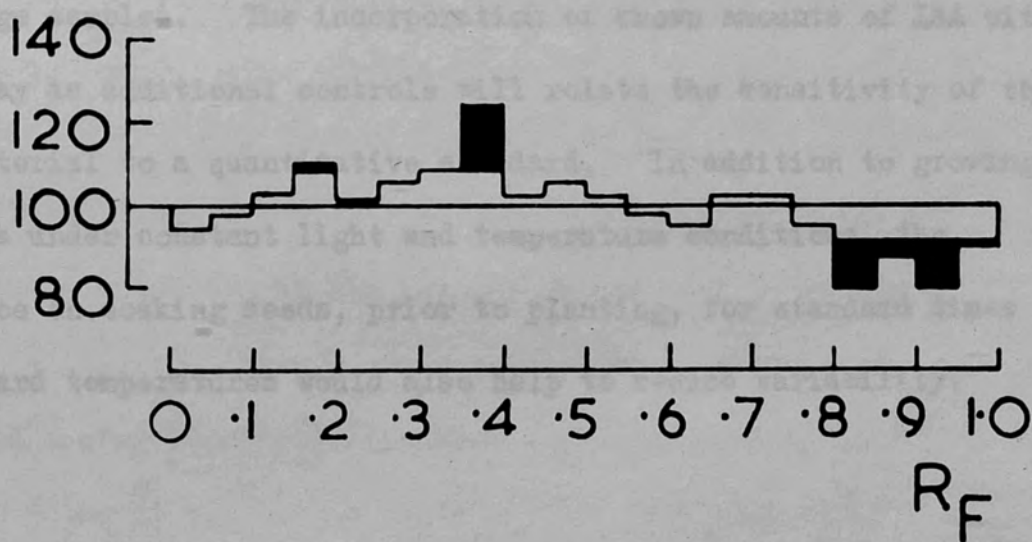
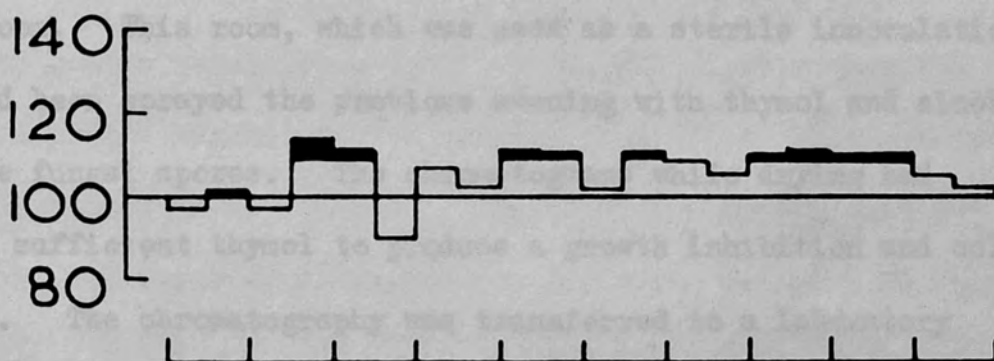
Fig. 10

Oat first internode assay to show.

- (a) The absence of growth inhibitors in solvent washed chromatography paper.
- (b) The presence of inhibitors in unwashed chromatography paper.

FIG 10

GROWTH AS % OF CONTROLS



noticed that marker chromatograms dipped in the nitrite-nitric acid reagent had developed a green spot. This spot had an Rf of 0.85. The green reaction was found to be due to thymol, and which was responsible for the inhibition also. It was found when the washed chromatography paper was hung up to dry overnight in the chromatography room. This room, which was used as a sterile inoculation room, had been sprayed the previous evening with thymol and alcohol to remove fungal spores. The chromatograms while drying had absorbed sufficient thymol to produce a growth inhibition and colour reaction. The chromatography was transferred to a laboratory separate from the main building and free from interfering chemicals.

(vi) Biological variability

Both the experimental material and the assay material will show a variability. This can be partially overcome by experiments with large samples. The incorporation of known amounts of IAA with each assay as additional controls will relate the sensitivity of the assay material to a quantitative standard. In addition to growing seedlings under constant light and temperature conditions, the insistence on soaking seeds, prior to planting, for standard times at standard temperatures would also help to reduce variability.

CHAPTER IV

RESULTS

(A) COLORIMETRIC AND FLUORIMETRIC METHODS FOR THE LOCATION AND IDENTIFICATION OF COMPOUNDS ON CHROMATOGRAMS.

Two points should be noted. These are firstly that the Rf of pure compounds may not be the same as that of the same substance when chromatogrammed in an extract. The Rf of the compound in the extract may be depressed or elevated from the value obtained for the pure compound. The Rf of pure DL tryptophan in isobutanol, methanol, water, was 0.20 - 0.28. The Rf of tryptophan in water soluble extracts in the same solvent was 0.10 - 0.20.

The second point concerned whether tryptophan would yield IAA on alkaline hydrolysis. Gordon and Wildman (1943) obtained IAA from tryptophan on hydrolysis with 0.5 NaOH. However Bentley (1958) was not able to obtain ether soluble auxins from tryptophan after strong alkaline hydrolysis, N NaOH (15 lbs pressure 15 mins.) Thurman and Street (1960) disagreed with Bentley's results. They found Ehrlich positive reactions at the IAA region and also strong growth stimulating activity. However, Thurman and Street hydrolysed their tryptophan at a neutral or slightly acidic pH. From a hydrolysis performed on 60 μ g tryptophan no ether soluble Ehrlich positive acids were detected. In the water soluble residue the only Ehrlich positive spot recorded was that of the tryptophan itself. Therefore in alkaline hydrolysis experiments it has been assumed that any new Ehrlich positive areas could not have come from tryptophan.

(i) Key to the identification of colour reactions shown in Fig's 11, 13, 14, 15, 16.

colours BL = blue, BLK = black, BRO = brown, GR = green, GY = grey, LI = lilac, MAU = mauve, OR = orange, PI = pink, PU = purple, R = red, VI = violet, Y = yellow.

intensity etc. b = bright, d = deep, dk = dark, f = faint,

fd = fading, im = immediate, int = intense, l = light,

p = pale, rp = rapid, sl = slow, v = very, ht = heat to 110°C.,

U.V. = ultra violet light, U.V.A. = UV after fuming with ammonia.

(ii) Colour reactions of the ether soluble acid fraction

Four ether soluble growth promoters, E1, R_f 0.0 - 0.25, E2, 0.3 - 0.55, E3, 0.55 - 0.75 and E4, 0.85 - 1.00 were detected in the ether soluble acid fraction. IAA had an R_f of 0.6 - 0.7 approximately. The growth activity of these promoters will be considered in Chapter IVB.

An extract from 120 g. whole roots approximately 6.0 cm. long failed to give a positive colour reaction to Ehrlich and to the acid oxidising agent. Diazotised p-nitraniline gave a reaction at two zones. The first at R_f 0.3 - 0.45, was a pale yellow, the second at R_f 0.7 - 0.8 was a pinkish brown. The second reaction, characteristic of a phenolic compound, occurred at the inhibitor^β position R_f 0.7 - 0.8 and extended back partially into

the IAA marker region. Diazotised sulphanilic acid gave no reaction at this region. There is a variety of evidence that the inhibitor complex contains a phenolic component. Koves (1957) found the inhibitor β complex from fleshy fruits to contain derivatives of cinnamic, salicylic and o- and p-coumaric acids. Housley and Taylor (1958) isolated azelaic acid, scopoletin and acid A from potato peel. Torrey (1959) recorded the presence from pea roots of substances giving phenolic reactions at the β position.

These results indicate the absence of any indole compounds, though it is possible an insufficient weight of tissue was used for the colour test. These results also suggest that the inhibitor β complex, Rf 0.7 - 0.8, from Vicia roots may contain a phenolic component. The significance of the presence of a phenolic substance at the "inhibitor β " position on the growth response of E3, Rf, 0.40 - 0.75 will be considered more fully in Chapter IVB and Chapter V.

(iii) Colour reactions and fluorimetric analysis of the unhydrolysed water soluble fraction

High growth activity in the water soluble fraction was found at the following Rf's: 0.0 - 0.15, promoter W1, Rf 0.20 - 0.30, promoter W2, and Rf 0.40 - 0.80, promoter W3 & 4. The growth activity of promoters W1, W2, W3 & 4 will be considered more fully in Chapter IVB. The promoters W1, W2, W3 & 4 and their Rf's have been given so that reference can be made to them when the colour reactions are discussed.

Chromatograms of the water soluble fraction when dipped into ninhydrin acetone gave strong purple colours on heating to 110°C (Fig.11a). A red purple was located at Rf 0.0 - 0.15, and a blue purple at 0.15 - 0.22. These colours were permanent. Purple reactors were also found at Rf 0.28 - 0.40 but these colours faded after some hours.

Dipping the chromatogram into Ehrlich acetone to locate indoles revealed 3 positive areas only one of which was purple (Fig.11b). An immediate bright golden yellow was formed at Rf 0.0 - 0.1. A purple reactor was located at Rf 0.12 - 0.20. This reacted within 5 minutes in the cold and faded after 2 hours. It was thought to be tryptophan. Underlying the purple reactor and appearing as it faded was a substance reacting with Ehrlich to give a pale lemon yellow, half an hour to three hours after dipping. The Rf of this region was 0.1 - 0.38. Two amino acids, tyrosine and β phenylalanine were tested for Rf and colour reaction to Ehrlich. Tyrosine had an Rf of 0.0 and gave a pale yellow reaction after 3-4 hours. β Phenylalanine had an Rf of 0.26 and gave a pale yellow colour after 4 hours. However no ninhydrin positive reaction was found at Rf 0.26, the nearest being 0.28 - 0.40. Despite the slight difference in Rf it was thought likely that β phenylalanine could be part of the extensive pale yellow Ehrlich positive zone. This yellow zone was associated with a zone of high growth activity Rf 0.2 - 0.3. Britton et al (1956) reported a pale yellow reaction to Ehrlich also associated with activity in the coleoptile test. They did not state how rapidly the yellow reaction took place.

Fig. 11. a - Standard; b - Fraction; c - and d -
 (cont.)
 acid; e - alkaline; f - AgNO₃ treatment; g -

	a	b	c	d	e	f	g
Color reactions of the hydrolyzed water-soluble fraction from milk whey extract							
of 48 day old roots. The color reactions are tabulated.							
(a) Hydrolyzed extract							
(b) Fraction							
(c) Jackson's nitric oxidant reagent							
(d) U.V. light and UV (after treating with carotene)							
(e) Dissocted - a - nitramine							
(f) Dissocted sulphuric acid							
(g) Alkaline silver nitrate							
(h) Silver nitrate and phosphoric blue							

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Fig. 11.

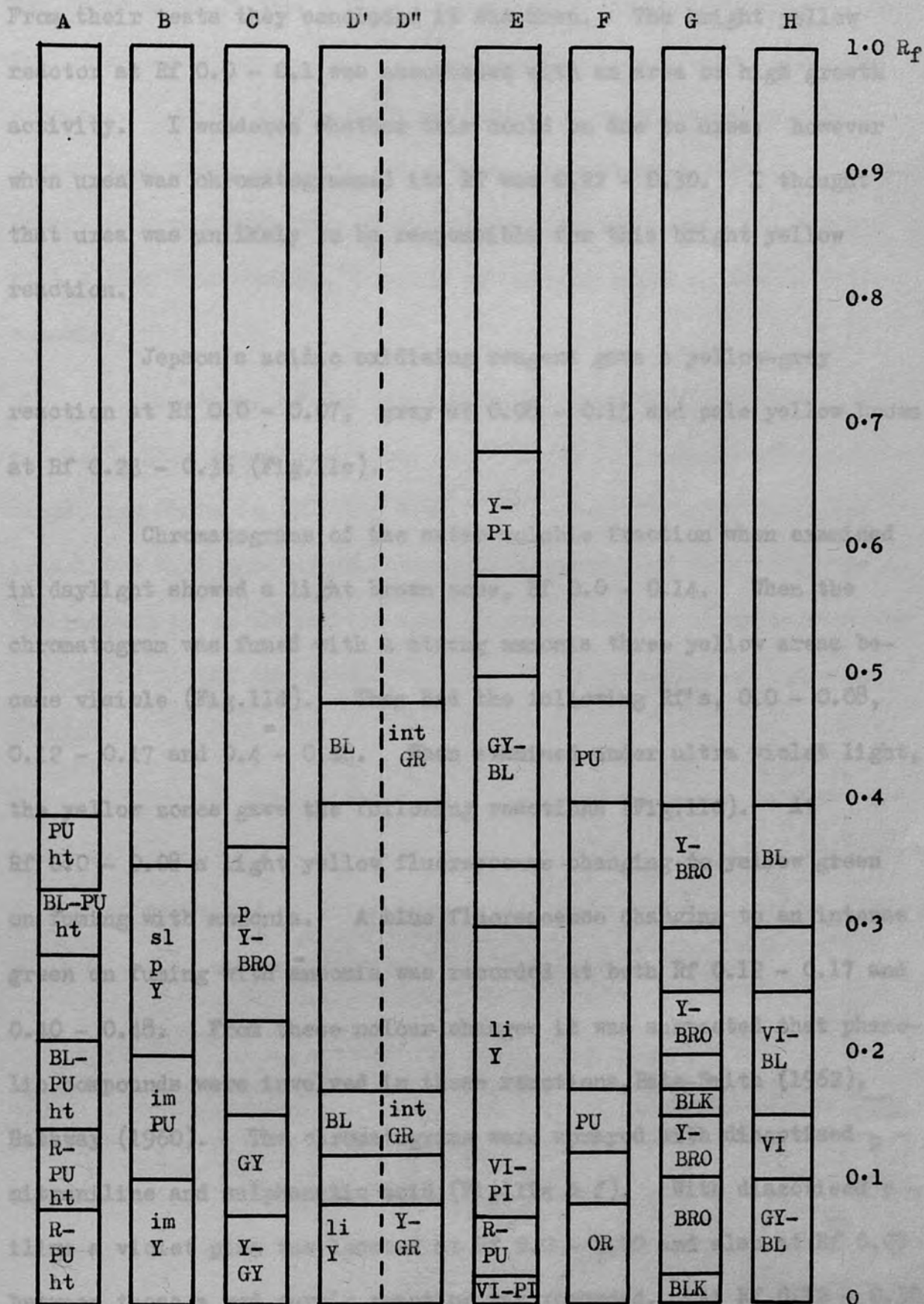
Colour reactions of the unhydrolysed water soluble fraction from bulk methanol extracts of 48 day old roots. The colour reagents are tabulated.

- (a) Ninhydrin acetone.
- (b) Ehrlich.
- (c) Jepson's acidic oxidising reagent.
- (d) U.V. light and UVA (after fuming with ammonia.)
- (e) diazotised p - nitraniline.
- (f) diazotised sulphanilic acid.
- (g) alkaline silver nitrate.
- (h) Silver nitrate and bromphenol blue.

Fig 11: A = Ninhydrin; B = Ehrlich; C = Acid oxidising

agent; D' = U.V.; D'' = U.V.A.; E = p-Nitraline; F = Sulphanilic

acid; G = Alkaline AgNO₃; H = AgNO₃ Bromphenol blue.



Thurman and Street (1960) reported an immediate yellow Ehrlich reactor running just in front of a growth stimulatory zone. From their tests they concluded it was urea. The bright yellow reactor at Rf 0.0 - 0.1 was associated with an area of high growth activity. I wondered whether this could be due to urea; however when urea was chromatogrammed its Rf was 0.22 - 0.30. I thought that urea was unlikely to be responsible for this bright yellow reaction.

Jepson's acidic oxidising reagent gave a yellow-grey reaction at Rf 0.0 - 0.07, grey at 0.08 - 0.15 and pale yellow brown at Rf 0.23 - 0.36 (Fig.11c).

Chromatograms of the water soluble fraction when examined in daylight showed a light brown zone, Rf 0.0 - 0.14. When the chromatogram was fumed with a strong ammonia three yellow areas became visible (Fig.11d). They had the following Rf's, 0.0 - 0.08, 0.12 - 0.17 and 0.4 - 0.48. When examined under ultra violet light, the yellow zones gave the following reactions (Fig.11d). At Rf 0.0 - 0.08 a light yellow fluorescence changing to yellow green on fuming with ammonia. A blue fluorescence changing to an intense green on fuming with ammonia was recorded at both Rf 0.12 - 0.17 and 0.40 - 0.48. From these colour changes it was suspected that phenolic compounds were involved in these reactions, Bate-Smith (1962), Hathway (1960). The chromatograms were sprayed with diazotised p - nitraniline and sulphanilic acid (Fig.11e & f). With diazotised p - nitraniline a violet pink was located at Rf 0.0 - 0.02 and also at Rf 0.07 - 0.12, between these a red purple reaction was recorded. At Rf 0.12 - 0.30 a

light yellow reaction was detected and at Rf 0.35 - 0.50 a grey blue reaction was recorded. In one extract only a yellow pink reaction was found at Rf 0.58 - 0.68. Diazotised sulphanilic acid gave the following reactions. At Rf 0.0 - 0.08 a stable orange colour was produced. At Rf 0.12 - 0.17 and 0.40 - 0.48 an intense purple reaction took place and then faded within two minutes leaving no sign. These two zones with this characteristic reaction were the regions with a blue fluorescence changing to intense green on fuming with ammonia.

Reactions of the water soluble fraction to alkaline AgNO_3 and AgNO_3 plus bromphenol blue, the reagent for purines and pyrimidines, are shown in (Fig. 11g & h). The alkaline AgNO_3 reactions are associated with some of the phenolic reactions and with other tests. The reagent is so unspecific that on these complex chromatograms the test is of little use except to indicate the presence of reducing groups.

Between Rf 0.0 and 0.30 a number of compounds were associated on the chromatogram of which two have been identified with some degree of certainty. These were 3, 4 dihydroxyphenylalanine, (DOPA), Rf 0.07 - 0.13 and tryptophan, Rf 0.12 - 0.20.

A region was found at Rf 0.07 - 0.13 which gave a yellow brown to brown reaction to alkaline AgNO_3 and a red purple with diazotised p - nitraniline. It appeared to be associated with a rapidly fading purple reaction to diazotised sulphanilic acid. This substance was thought to be DOPA. Pure DOPA had the same Rf and gave

a purple reaction with sulphanilic acid, the colour however was stable. The activation and fluorescent wavelength maxima of the unknown sample were compared with those from a known pure sample. These figures are given in (Table 5).

Table 5

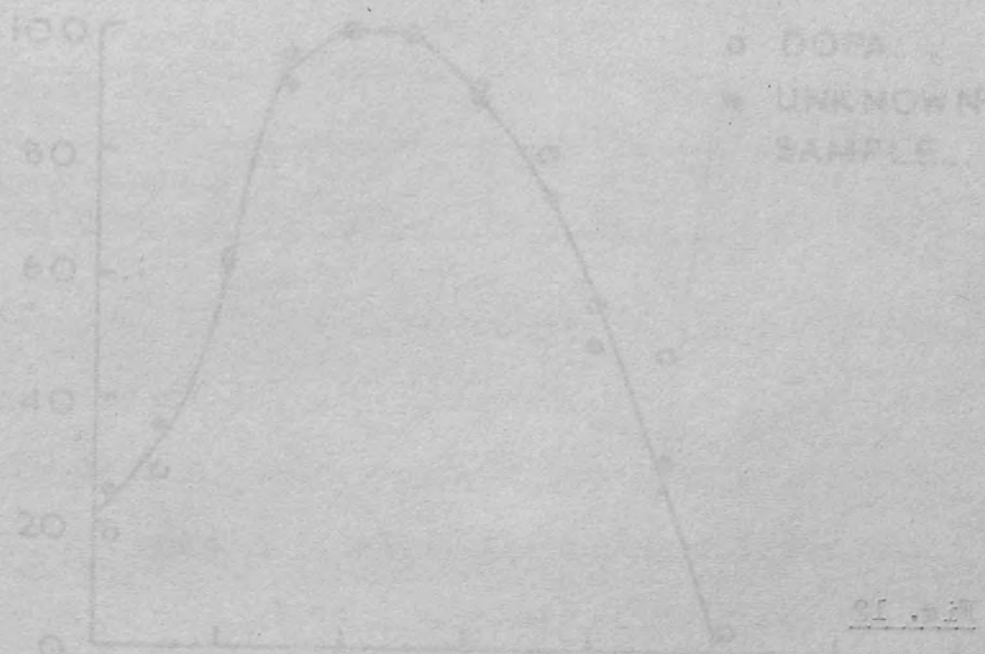
	Activation wavelength	Fluorescent wavelength.
Known sample of DOPA	285	330
Unknown sample	285	330

The change in fluorescent intensity with pH was plotted. The curve comparing the change in fluorescent intensity with pH of the unknown sample with a known sample of DOPA is shown in (Fig.12a). The fluorescent readings were not taken above pH 10.0 because of the formation of a new and highly fluorescent substance in solution with the DOPA. The fluorescent readings were kindly made by D. Burnett, Research Assistant to Prof. Audus in the Botany Department. From the fluorescence data it would appear that the substance at Rf 0.07 - 0.13 was DOPA or a substance very similar to DOPA.

The second compound with an Rf of 0.12 - 0.20 gave an immediate purple colour with Ehrlich. The activation and fluorescent wavelength maxima were compared with those of a pure sample of D.L. tryptophan and are given in (Table 6).

FIG. 12

ARBITRARY UNITS OF FLUORESCENT INTENSITY

(a) The effect of pH on the fluorescent intensity of a known sample of DOPA compared with an unknown sample. The unknown sample was eluted from the unhydrolyzed water soluble fraction.

Graph (b) shows the effect of pH on the fluorescent intensity of tryptophan and an unknown sample. The y-axis is 'ARBITRARY UNITS OF FLUORESCENT INTENSITY' (0 to 100) and the x-axis is 'pH' (0 to 14). Both TRYPTOPHAN (open circles) and UNKNOWN SAMPLE (filled circles) show a peak at pH 10.0.

pH	TRYPTOPHAN Intensity	UNKNOWN SAMPLE Intensity
6.0	20	20
7.0	30	30
8.0	40	40
9.0	50	50
10.0	100	100
11.0	90	90
12.0	60	60
13.0	20	20

(b) The effect of pH on the fluorescent intensity of a known sample of tryptophan compared with an unknown sample. The unknown sample was eluted from the unhydrolyzed water soluble fraction.

Graph (c) shows the effect of pH on the fluorescent intensity of the unhydrolyzed water soluble fraction. The y-axis is 'ARBITRARY UNITS OF FLUORESCENT INTENSITY' (0 to 100) and the x-axis is 'pH' (0 to 14). The curve shows a peak at pH 10.0.

pH	Intensity
6.0	20
7.0	30
8.0	40
9.0	50
10.0	100
11.0	90
12.0	60
13.0	20

(c) The effect of pH on the fluorescent intensity of the unhydrolyzed water soluble fraction.

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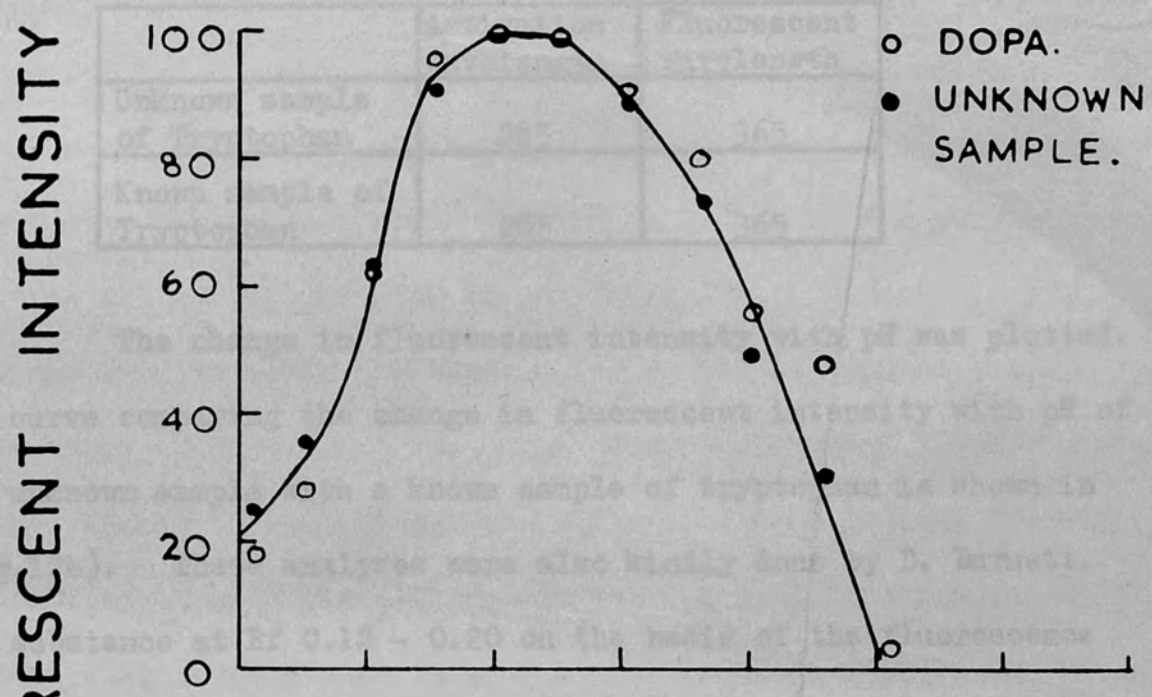
Fig. 12

(a) The effect of pH on the fluorescent intensity of a known sample of DOPA compared with an unknown sample. The unknown sample was eluted from Rf 0.07 - 0.13 of a chromatogram of the unhydrolysed water soluble fraction.

12 (b) The effect on the fluorescent intensity of a known sample of tryptophan compared with an unknown sample. The unknown sample was eluted from Rf 0.12 - 0.20 of a chromatogram of the unhydrolysed water soluble fraction.

FIG. 12.

A



B

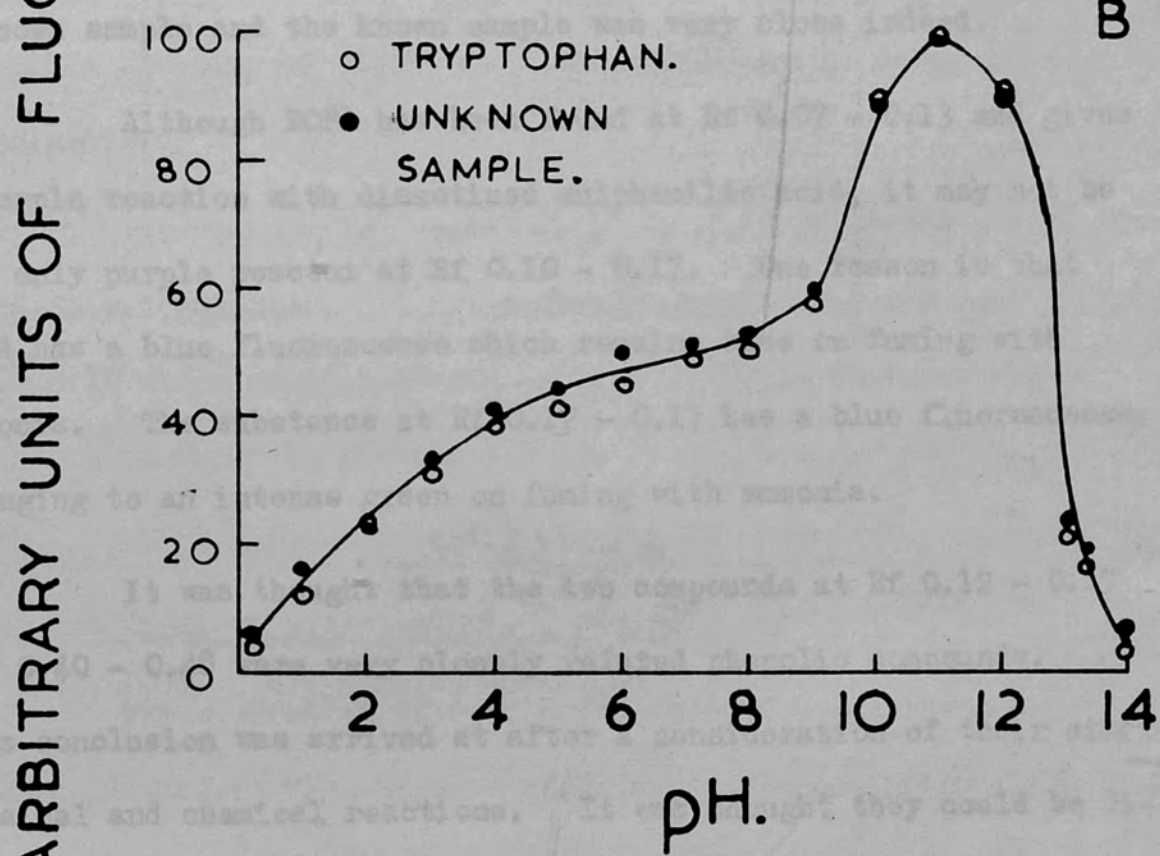


Table 6

	Activation wavelength	Fluorescent wavelength
Unknown sample of Tryptophan	285	365
Known sample of Tryptophan	285	365

The change in fluorescent intensity with pH was plotted. The curve comparing the change in fluorescent intensity with pH of the unknown sample with a known sample of tryptophan is shown in (Fig.12b). These analyses were also kindly done by D. Burnett. The substance at Rf 0.12 - 0.20 on the basis of the fluorescence data was regarded as tryptophan, since the agreement between the unknown sample and the known sample was very close indeed.

Although DOPA has been found at Rf 0.07 - 0.13 and gives a purple reaction with diazotised sulphanilic acid, it may not be the only purple reactor at Rf 0.10 - 0.17. One reason is that DOPA has a blue fluorescence which remains blue on fuming with ammonia. The substance at Rf 0.12 - 0.17 has a blue fluorescence changing to an intense green on fuming with ammonia.

It was thought that the two compounds at Rf 0.12 - 0.17 and 0.40 - 0.48 were very closely related phenolic compounds. This conclusion was arrived at after a consideration of their similar physical and chemical reactions. It was thought they could be dihydroxy phenolic compounds and especially ortho dihydroxy substances.

The phenolic substance at Rf 0.0 with a yellow fluorescence changing to yellow green on fuming with ammonia was thought to be a flavone. Two flavones, quercetin and kaempferol are mentioned as occurring in Vicia faba, Bate-Smith (1962). Quercetin and kaempferol were tested with diazotised p - nitraniline but gave a yellow brown reaction and not violet pink as was found for this region. Coleoptile sections used to assay the water soluble fraction showed a brown stain at the ends at Rf 0.0 - 0.15. The substance causing the brown stain did not appear to inhibit growth. The substance causing the stain was thought to be a phenolic substance. This impression was confirmed by fact that caffeic acid induced a similar brown stain at a concentration between 1.0 and 50.0 $\mu\text{g/ml}$. Barlow and Hancock (1961) found a similar staining of the ends of coleoptile sections which was caused by phloridzin.

The widespread occurrence of substances giving a phenolic reaction was observed. Some of these regions were apparently associated with areas of high growth activity. This feature will be considered more fully in the discussion.

(iv) Colour reactions following elution and rechromatography of the unhydrolysed water soluble fraction

Three zones at Rf 0.0, 0.40 and 0.60 were eluted and rechromatographed. When sprayed with diazotised p - nitraniline only one reaction was found at Rf 0.0 for the fraction eluted from this Rf originally. The same was found at Rf 0.60, i.e. the zone eluted from Rf 0.60 gave a reaction at 0.60 on rechromatography. However, the

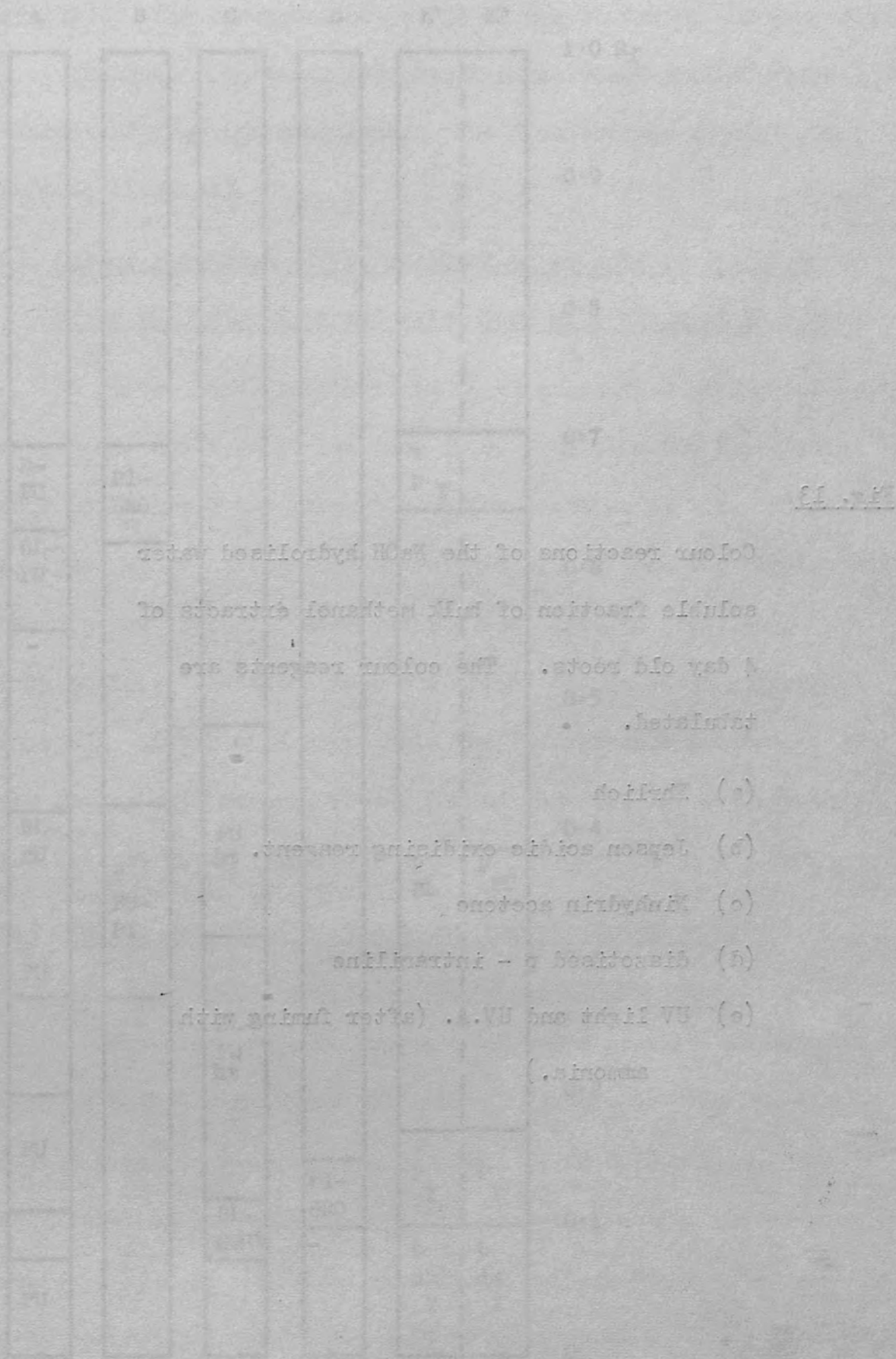
spot eluted from Rf 0.40 when rechromatogrammed gave two spots, one at Rf 0.0 and the original spot at Rf 0.40. This reaction was confirmed with the ferric chloride-ferricyanide reagent. This result gave an indication of a possible interconvertibility amongst phenolic reactive substances.

(v) Colour reactions of the residual water soluble fraction after NaOH hydrolysis and extraction with ether at pH 3.0

After alkaline hydrolysis an array of strong purple Ehrlich positive reactions was recorded (Fig.13a). Reactions were found at Rf 0.0 - 0.07, 0.12 - 0.20, presumably tryptophan, 0.27 - 0.42 and 0.56 - 0.70. These must have been released by alkaline hydrolysis and could not have come from tryptophan. Jepson's acidic oxidising reagent gave a faint pink purple at Rf 0.28 - 0.43 corresponding to a purple Ehrlich zone. At Rf 0.63 - 0.70 a pink brown reaction was noted and this corresponded to a red purple Ehrlich (Fig. 13b). The presence of purple Ehrlich positive reactors (appearing in less than 5 minutes in the cold) following alkaline hydrolysis of the water soluble fraction would seem to indicate the presence of water soluble indole complexes in Vicia faba roots. These indole complexes give no reaction to Ehrlich until hydrolysed. The complex is unlikely to be an acetyl peptide because these give a purple reaction to Ehrlich.

Reactions to ninhydrin, (Fig.13c), showed that one substance at the starting line had disappeared but otherwise the picture was substantially the same.

Fig. 10. *S. aureus* (ATCC 4961) on agar plates containing 0.1% of the following substances: (a) 10% NaOH; (b) 10% Na₂S₂O₄; (c) 10% Na₂S₂O₃; (d) 10% Na₂SO₃; (e) 10% Na₂CO₃; (f) 10% Na₂HPO₄.



Colon reactions of the NaOH hydrolyzed water soluble fraction of milk methanol extracts of 4 day old roots. The column reactions are tabulated.

(a) Positive
 (b) Lysogen sulfate oxidizing reagent.
 (c) Methylene acetone
 (d) Disassociated α -ketoglutarate
 (e) UV light and UV-A (after fuming with ammonia).

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Fig. 13.

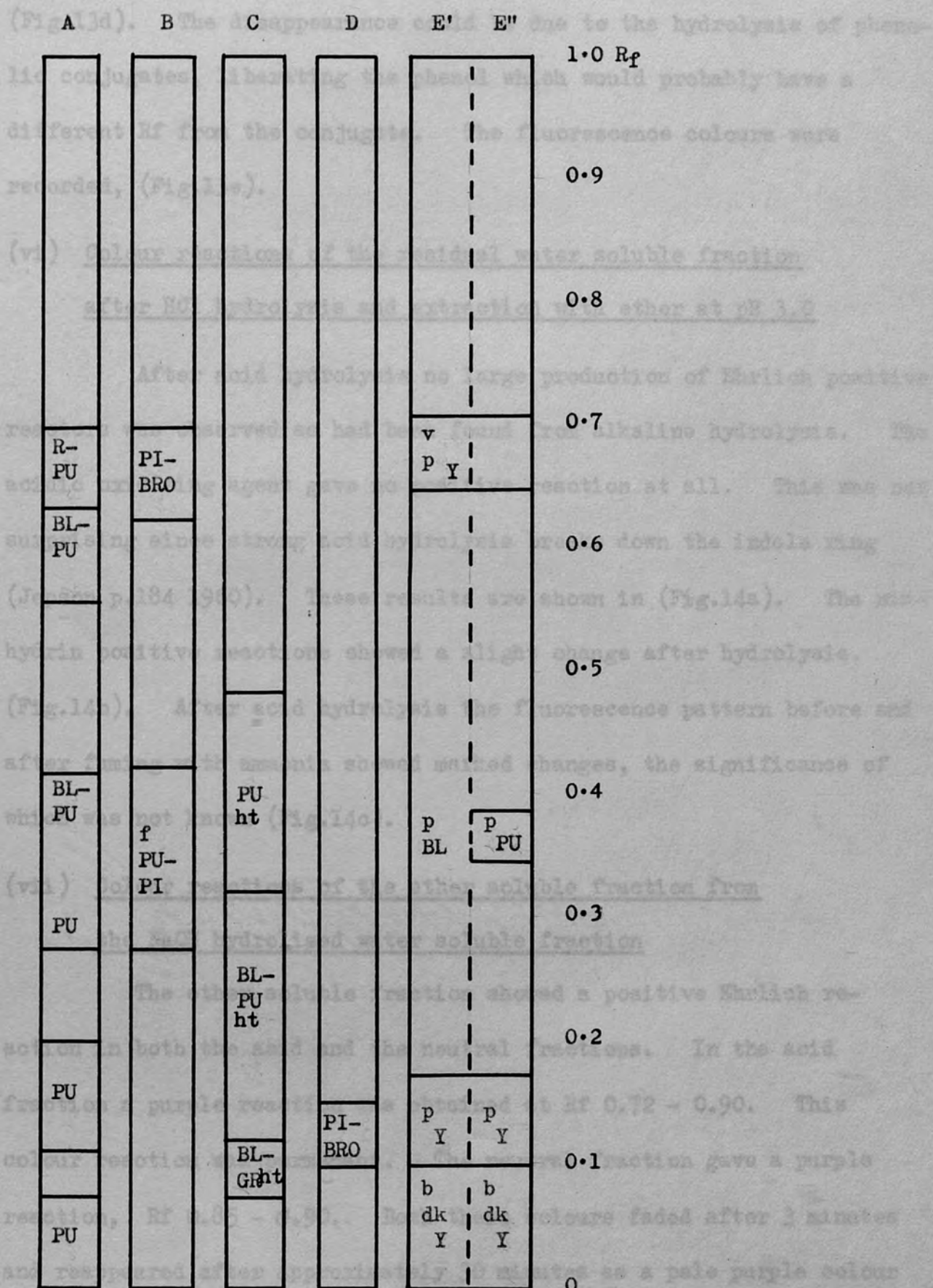
Colour reactions of the NaOH hydrolised water soluble fraction of bulk methanol extracts of 4 day old roots. The colour reagents are tabulated.

- (a) Ehrlich
- (b) Jepson acidic oxidising reagent.
- (c) Ninhydrin acetone
- (d) diazotised p - intraniline
- (e) UV light and UV.A. (after fuming with ammonia.)

Fig 13: A = Ehrlich; B = Acid oxidising agent;

C = Ninhydrin; D = p-Nitraniline; E' = U.V.;

E'' = U.V.A.



The phenolic substances reacting to diazotised p-nitraniline of the unhydrolysed fraction have disappeared after alkaline hydrolysis (Fig.13d). The disappearance could be due to the hydrolysis of phenolic conjugates, liberating the phenol which would probably have a different Rf from the conjugate. The fluorescence colours were recorded, (Fig.13e).

(vi) Colour reactions of the residual water soluble fraction after HCl hydrolysis and extraction with ether at pH 3.0

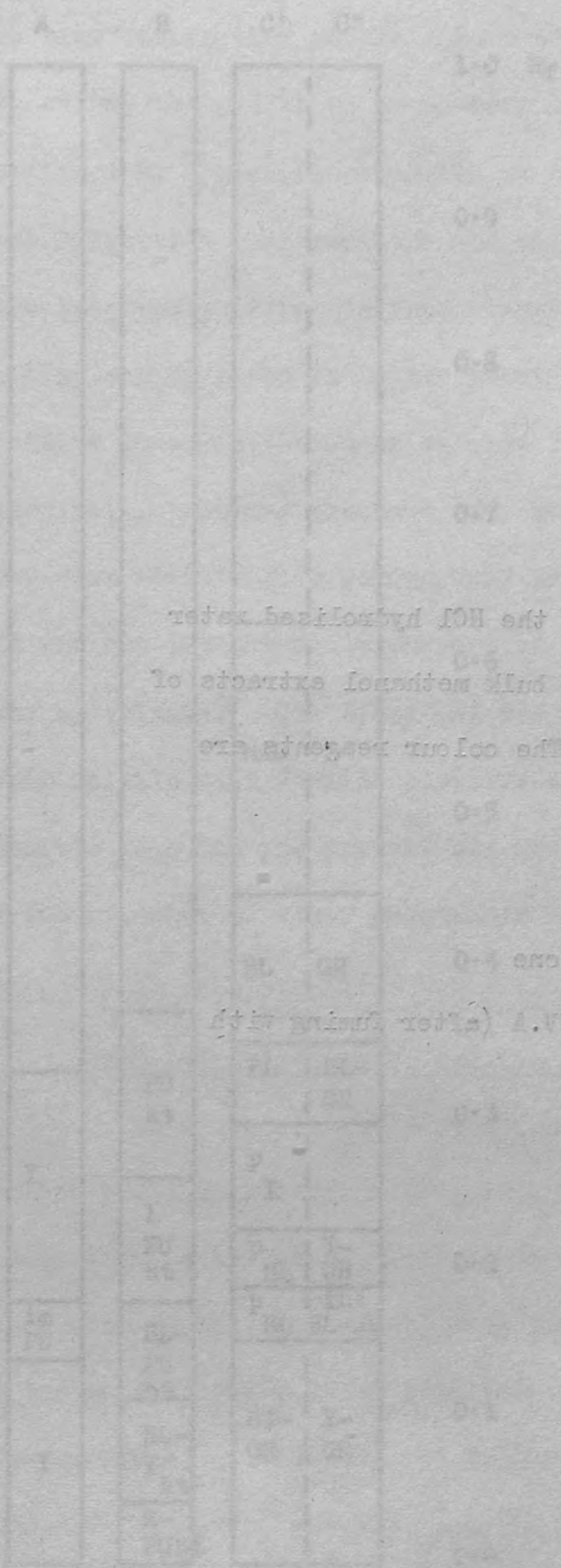
After acid hydrolysis no large production of Ehrlich positive reactors was observed as had been found from alkaline hydrolysis. The acidic oxidising agent gave no positive reaction at all. This was not surprising since strong acid hydrolysis breaks down the indole ring (Jepson p.184 1960). These results are shown in (Fig.14a). The ninhydrin positive reactions showed a slight change after hydrolysis. (Fig.14b). After acid hydrolysis the fluorescence pattern before and after fuming with ammonia showed marked changes, the significance of which was not known (Fig.14c).

(vii) Colour reactions of the ether soluble fraction from the NaOH hydrolysed water soluble fraction

The ether soluble fraction showed a positive Ehrlich reaction in both the acid and the neutral fractions. In the acid fraction a purple reaction was obtained at Rf 0.72 - 0.90. This colour reaction was permanent. The neutral fraction gave a purple reaction, Rf 0.85 - 0.90. Both these colours faded after 3 minutes and reappeared after approximately 30 minutes as a pale purple colour

Fig. 14. A = Solis; B = Malyrin; C = U.V.; D = U.V.

C₁ = U.V.; C₂ = U.V.



Colour reactions of the HCl hydrolysed water soluble fraction of bulk material extracts of 4 day old roots. The colour reactions are tabulated.

- (a) Iodine
- (b) Malyrin acetone 1-0
- (c) UV light and UV.A (after heating with ammonia.)

(ammonia.)

Fig. 14.

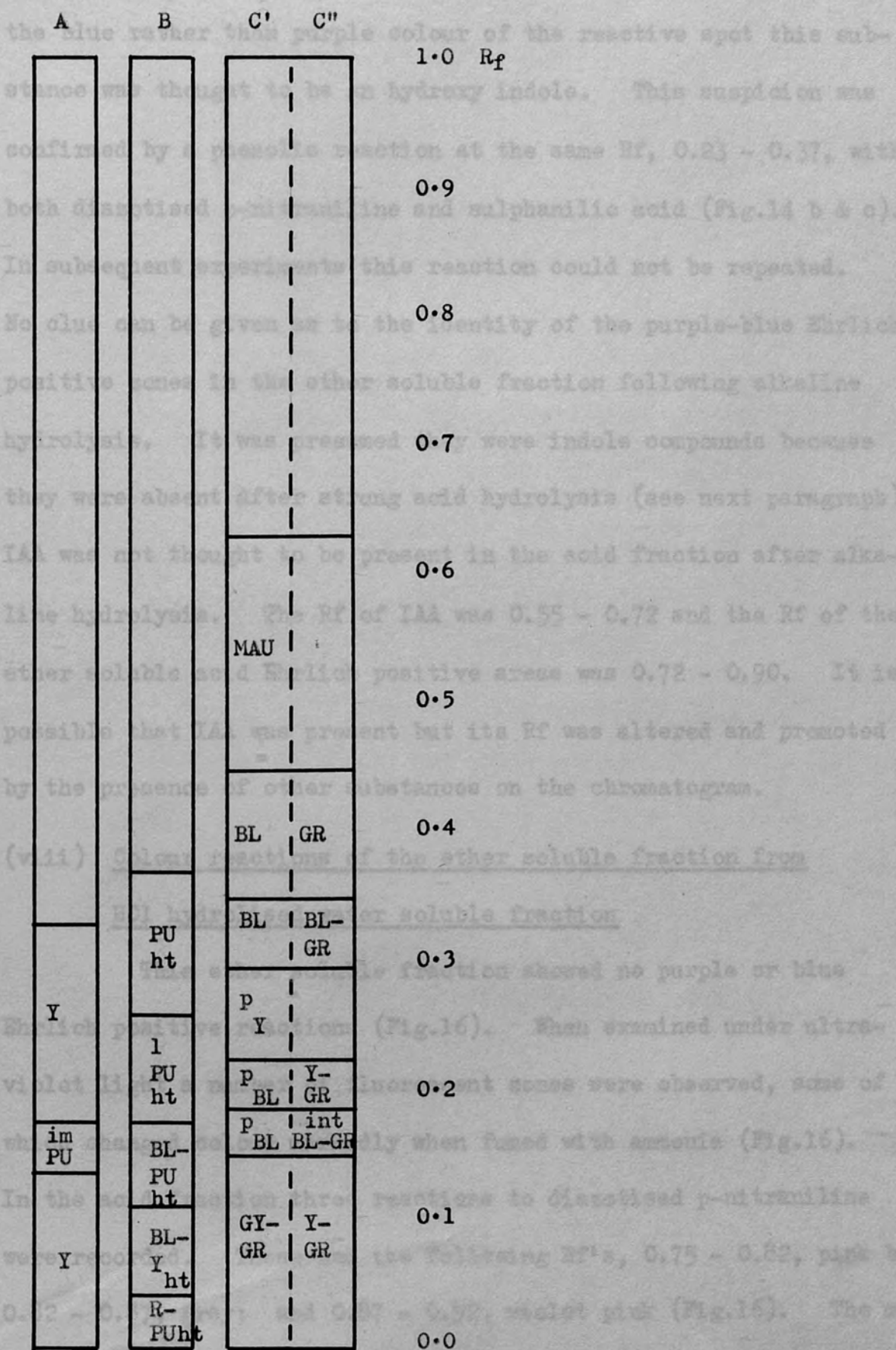
Colour reactions of the HCl hydrolysed water soluble fraction of bulk methanol extracts of 4 day old roots. The colour reagents are tabulated.

- (a) Ehrlich
- (b) Ninhydrin acetone
- (c) UV light and UV.A (after fuming with ammonia.)

C' = U.V.; C'' = U.V.A.

(Fig. 15). In one extract Ehrlich produced a light blue reaction changing to blue grey after 10 minutes, at Rf 0.23 - 0.37. From the blue rather than purple colour of the reactive spot this substance was thought to be a hydroxy indole. This suspicion was confirmed by a positive reaction at the same Rf, 0.23 - 0.37, with both diazotised p-nitroaniline and sulpharilic acid (Fig. 14 b & c). In subsequent experiments this reaction could not be repeated. No clue can be given as to the identity of the purple-blue Ehrlich positive zones in the other soluble fraction following alkaline hydrolysis. It was presumed that these were indole compounds because they were absent after strong acid hydrolysis (see next paragraph). IAA was not thought to be present in the acid fraction after alkaline hydrolysis. The Rf of IAA was 0.55 - 0.72 and the Rf of the other soluble Ehrlich positive zones was 0.72 - 0.90. It is possible that IAA was present but its Rf was altered and promoted by the presence of other substances on the chromatogram.

(vii) Solvent reactions of the other soluble fraction from BL hydrolysis. This fraction showed no purple or blue Ehrlich positive reaction (Fig. 16). When examined under ultraviolet light, fluorescent zones were observed, some of which were not fluorescent when fixed with ammonia (Fig. 15). In the acid fraction, reactions to diazotised p-nitroaniline were observed at the following Rf's, 0.75 - 0.82, pink brown 0.72 - 0.78, violet pink 0.67 - 0.72, violet pink (Fig. 16). The middle



(Fig.15). In one extract Ehrlich produced a light blue reaction changing to blue grey after 10 minutes, at Rf 0.23 - 0.37. From the blue rather than purple colour of the reactive spot this substance was thought to be an hydroxy indole. This suspicion was confirmed by a phenolic reaction at the same Rf, 0.23 - 0.37, with both diazotised p-nitraniline and sulphanic acid (Fig.14 b & c). In subsequent experiments this reaction could not be repeated. No clue can be given as to the identity of the purple-blue Ehrlich positive zones in the ether soluble fraction following alkaline hydrolysis. It was presumed they were indole compounds because they were absent after strong acid hydrolysis (see next paragraph). IAA was not thought to be present in the acid fraction after alkaline hydrolysis. The Rf of IAA was 0.55 - 0.72 and the Rf of the ether soluble acid Ehrlich positive areas was 0.72 - 0.90. It is possible that IAA was present but its Rf was altered and promoted by the presence of other substances on the chromatogram.

(viii) Colour reactions of the ether soluble fraction from HCl hydrolysed water soluble fraction

This ether soluble fraction showed no purple or blue Ehrlich positive reactions (Fig.16). When examined under ultra-violet light a number of fluorescent zones were observed, some of which changed colour markedly when fumed with ammonia (Fig.16). In the acid fraction three reactions to diazotised p-nitraniline were recorded. These had the following Rf's, 0.75 - 0.82, pink brown; 0.82 - 0.87, grey; and 0.87 - 0.92, violet pink (Fig.16). The middle

02

Fig. 15.

Colour reactions of the ether soluble acid and neutral fractions extracted from the NaOH hydrolysed water soluble fraction of bulk methanol extracts of 4 day old roots. The colour reagents are tabulated.

- (a) Ehrlich
- (b) diazotised p - nitraniline
- (c) diazotised sulphanilic acid
- (d) UV light and UV.A (after fuming with ammonia.)
 - (i) Neutral fraction
 - (ii) Acid fraction
 - (iii) Acid + Neutral fraction.

Fig 15: Aⁱ = Ehrlich; Aⁱⁱ = Ehrlich; Aⁱⁱⁱ = Ehrlich;

B = p-Nitraniline; C = Sulphanilic acid; D' = U.V.;

D'' = U.V.A.

A ⁱ	A ⁱⁱ	A ⁱⁱⁱ	B ⁱⁱⁱ	C ⁱⁱⁱ	D' ⁱⁱⁱ D'' ⁱⁱⁱ	R _F
						1.0
			PU-PI	OR-BRO		
PU fd		PU	R-PI		Y-GR	0.9
	PU	R-PU		BRO sl	GY-BL	
		PU			dk GY	0.8
					b ⁱⁱ Y	0.7
					P Y	
			Y-BRO		l GY v1 GY	0.6
		Y-BRO	Y		GY GR-GY	0.5
		l BL	PI-BRO			
		BL-GR		PU-R	b BL b BL	0.3
						0.2
						0.1
			BLK			0

$\text{A}^{111} = \text{U.V.A.}$ $\text{A}^{112} = \text{U.V.A.}$ $\text{A}^{113} = \text{U.V.A.}$
 $\text{A}^{114} = \text{U.V.A.}$ $\text{H} = \text{p-nitrophenol}$ $\text{O} = \text{Salicylic acid}$ $\text{D} = \text{Methyl}$

Time	UV	UV	UV	UV	UV
0-1					
0-2					
0-3					
0-4					
0-5					
0-6					
0-7					
0-8					
0-9					
1-0					

Fig. 10

Colour reactions of the ether soluble acid and neutral fractions extracted from the HCl hydrolysed water soluble fraction of milk methanol extract of 4 day old roots. The colour reactions are tabulated.

(a) UV light and U.V.A. after treatment with ammonia.

(b) Dissolved p - nitrophenol.

(c) Dissolved salicylic acid.

(d) Methyl.

(i) Neutral fraction.

(ii) Acid fraction.

(iii) Acid + neutral fraction.

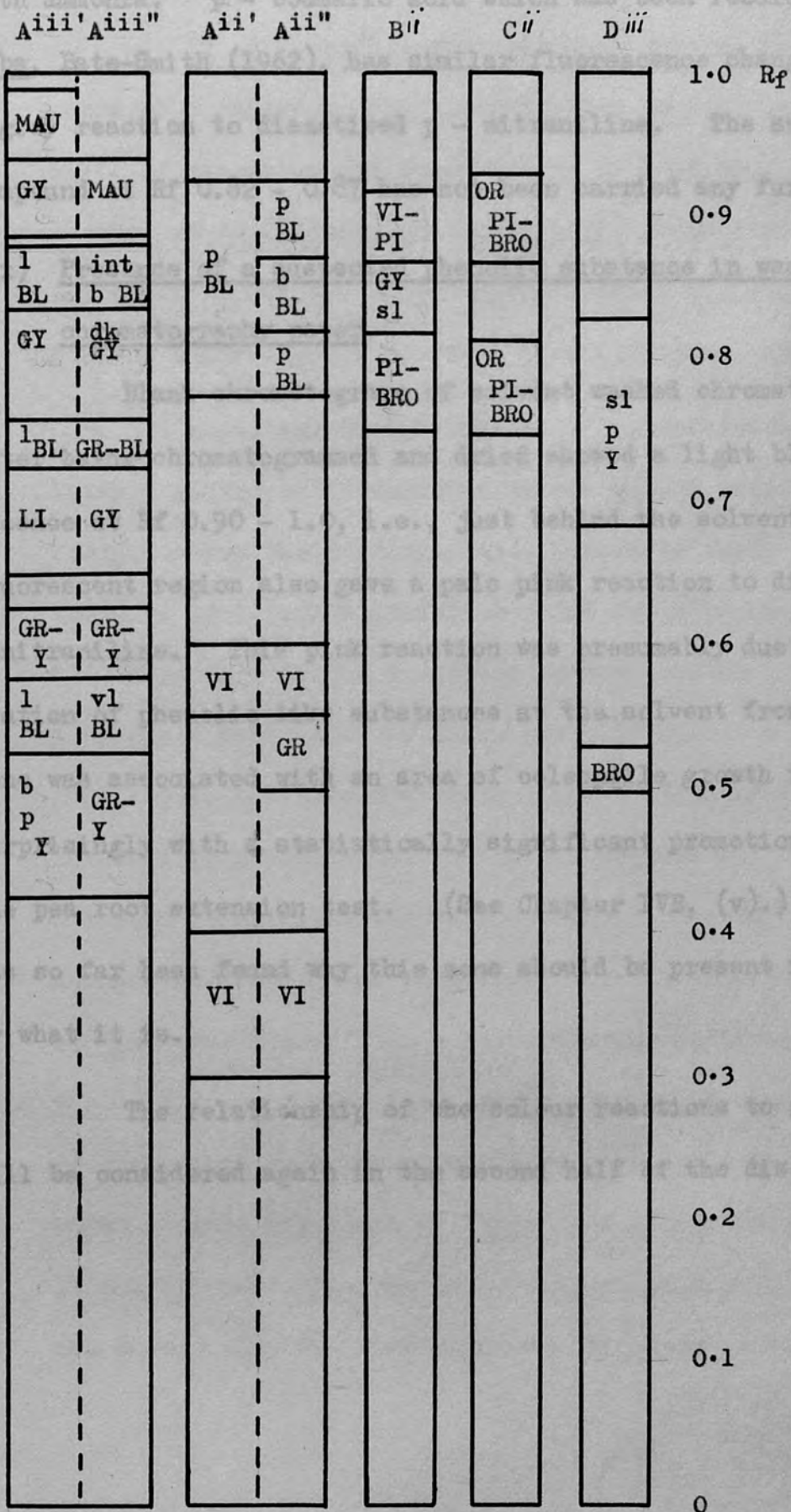
Fig. 16

Colour reactions of the ether soluble acid and neutral fractions extracted from the HCl hydrolysed water soluble fraction of bulk methanol extracts of 4 day old roots. The colour reagents are tabulated.

- (a) UV light and UV.A. (after fuming with ammonia.)
- (b) diazotised p - nitraniline
- (c) diazotised sulphanic acid.
- (d) Ehrlich.
 - (i) Neutral fraction.
 - (ii) Acid fraction
 - (iii) Acid + neutral fraction.

Fig 16: A^{iii'} = U.V.; A^{iii''} = U.V.A.; A^{ii'} = U.V.;

A^{ii''} = U.V.A.; B = p-Nitraniline; C = Sulphanilic acid; D = Ehrlich.



zone had a pale blue fluorescence changing to bright blue when fumed with ammonia. p - Coumaric acid which has been recorded for Vicia faba, Bate-Smith (1962), has similar fluorescence changes and gives a grey reaction to diazotised p - nitraniline. The analysis of this compound at Rf 0.82 - 0.87 has not been carried any further at present.

(ix) Presence of a suspected phenolic substance in washed chromatography paper.

Blank chromatograms of solvent washed chromatography paper after being chromatogrammed and dried showed a light blue white fluorescence at Rf 0.90 - 1.0, i.e., just behind the solvent front. This fluorescent region also gave a pale pink reaction to diazotised p-nitraniline. This pink reaction was presumably due to the accumulation of phenolic like substances at the solvent front. The pink zone was associated with an area of coleoptile growth inhibition and surprisingly with a statistically significant promotion of growth in the pea root extension test. (See Chapter IVB, (v).) No indication has so far been found why this zone should be present in washed paper or what it is.

The relationship of the colour reactions to growth activity will be considered again in the second half of the discussion.

(B) EFFECT OF GEOTROPIC STIMULATION ON THE AUXIN CONTENT
OF ROOT TIPS

It is generally accepted that lateral geotropic stimulation produces a change in the concentration or distribution of auxin in cells of the upper and lower halves of root tips. The classical theory due to Cholodny and Went (1927) postulated a transverse distribution of auxin from the upper side of the root tip to the lower surface under the influence of a gravitational stimulus.

Audus and Brownbridge (1957) investigated the effects of lateral geotropic stimulation on the growth response of Pisum sativum roots. From their results they concluded there was a rapid production of a root growth inhibitor which could be an auxin but not IAA in the lower half of the extension zone of roots. They suggested that this rapid production of auxin could be due to a sudden liberation of free active auxin from a bound inactive form or to a burst of synthesis from a precursor. This latter idea was also suggested by van Overbeek et al (1945). This hypothesis had to be proved by chromatographic separation and bioassay. It was found that the narrow root of Pisum was too difficult to handle for split root experiments and Vicia faba roots were used in its place. A study therefore was made of the growth substances in the ether soluble and ether insoluble, water soluble fractions of Vicia root tips, Lahiri and Audus (1960). It was observed that the basic pattern of growth active substances was essentially the same as found for Pisum, Audus and Gunning (1958).

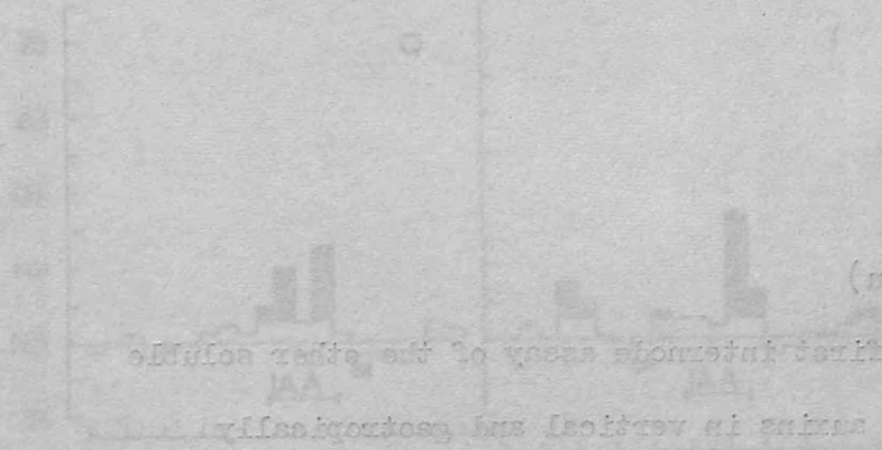
The root tips of Vicia when geotropically stimulated showed an extremely large and rapid production of an ether soluble acid growth promoter AP(ii), Audus and Lahiri (1961). This growth substance which was a root growth inhibitor occurred at the IAA position on the chromatograms. The production of AP(ii) built up to a maximum at 40 minutes and then diminished rapidly. When experiments with split root tips were performed to position the formation of AP(ii) it was found that more was apparently produced in the upper half than in the lower half of the root tip, Lahiri (1959). This was contrary to theoretical expectation.

(i) Ether soluble acid auxins of vertical and geotropically stimulated whole roots

In all experiments 100 root tips, approximately 1.0 g. root material, was used. The results are shown in (Fig.17a-u).

It was evident that geotropic stimulation produced distinctive changes in the auxin content of Vicia roots. Altogether four promoters and one inhibitor were found. The promoters have been named E1, E2, E3 and E4. The E indicated ether solubility and the number indicated the position of the promoter relative to the starting line. The Rf values for the promoters were E1, 0.0 - 0.25; E2, 0.35 - 0.55; E3, 0.55 - 0.75; E4, 0.85 - 1.0. The inhibitor was located at approximately 0.75 - 0.85.

GROWTH AS % OF CONTROL



Get first intermediate assay of the ether soluble
 acid amines in vertical and geotropically
 stimulated whole root tips. 0.7 mm root tips
 and 1.0 mm root tissue was used in all
 experiments.

(growth response as % of controls plotted
 against \log_{10} values)

(n - s) Table II

0 1 2 3 4 5 6 7 8 9 10

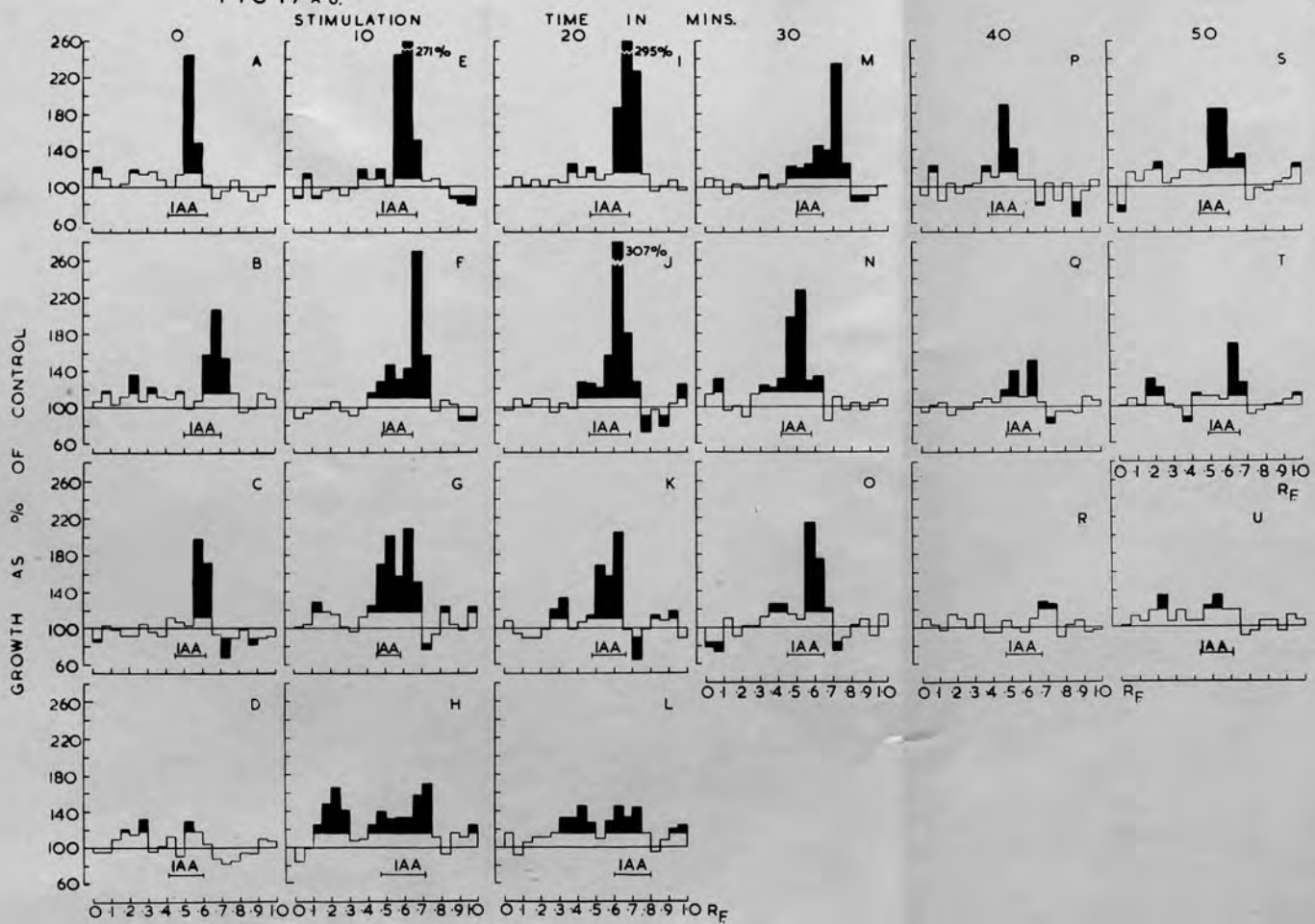
FR

Fig. 17 (a - u)

Oat first internode assay of the ether soluble acid auxins in vertical and geotropically stimulated whole root tips. 0.7 mm root tips and 1.0 g. root tissue was used in all experiments.

(Growth response as % of controls plotted against Rf values)

FIG 17 A-U.



Where more than one experiment has been performed at any specific stimulation time the upper and lower limits of auxin content from the experiments has been recorded. This has been shown by putting in the maximum and minimum values as parallel lines above and below the mean value. Where the figure 0 has been placed below the minimum value this means that a significant growth response was not recorded for at least one experiment at the specific stimulation time.

Promoter E1 was not found regularly in either vertical or horizontally stimulated roots. The content of E1 in vertical roots in IAA equivalents was 5.85×10^{-3} /ug/g. root tissue. The change in content of E1 with stimulation is shown in (Fig.18). No large change in content resulting from horizontal stimulation was observed.

E1 has an Rf approximately the same as AP(i), Lahiri and Audus (1960), who have in turn compared AP(i) to "accelerator" α Bennet-Clark and Kefford (1953), Kefford (1955a). The comparison was based partially upon similar Rf values although AP(i) was located when using a neutral solvent and "accelerator" α was found with an ammoniacal solvent. The most important similarity between AP(i) and "accelerator" α was their ability to promote both coleoptile solvent and pea root section extension growth. This is not a characteristic of IAA which will promote root section growth only at very dilute concentrations. Stowe and Thimann (1953) suggested that "accelerator" α was indolyl ~~acetyl~~ pyruvic acid (IPyA). However, this was unlikely because IPyA inhibited root growth at all concentrations at which it promoted coleoptile growth, Bentley (1956). Audus and Gunning (1958) suggested that indolyl acetylaspartic acid (IAc Asp) could be causing

the growth promotion at the "accelerator" position. It was growth active and its Rf in isopropanol, ammonia, water, was near the starting line. Bennet-Clark and Wheeler (1959) hydrolysed the "accelerator" zone with 7.0 N NaOH and found no release of IAA. Under the same experimental conditions IAc Asp did yield IAA. From these results Bennet-Clark and Wheeler decided that "accelerator" was not IAA.

Promoter E4 was located near the solvent front, Rf 0.85 - 1.0 and by virtue of its Rf in the same solvent it could be similar to AP(iii) found by Lahiri and Audus (1960). It was not found regularly and the growth stimulation at this region was small, not exceeding 35% above the controls. No large change in content resulting from increasing horizontal stimulation was observed (Fig.18). This observation is in contrast to the observations of Audus and Lahiri (1961) who found a significant change in the concentration of AP(iii) with increasing stimulation time. They suggested a correlation between the fall in concentration of AP(iii) and the rise in concentration of AP(ii). No such correlation could be made between E4 and the major growth promoter E3.

The growth promoter in the IAA region showed the most interesting changes. This growth promoting region frequently displayed a marked depression approximately in the middle of the growth active zone e.g. (Fig.17 F,G,J,P,Q). The question was raised: was an impurity or growth inhibitor of similar Rf interfering with the growth response or were two promoters of nearly similar Rf but different growth activities interacting with one another? For the purposes of

FIG 18

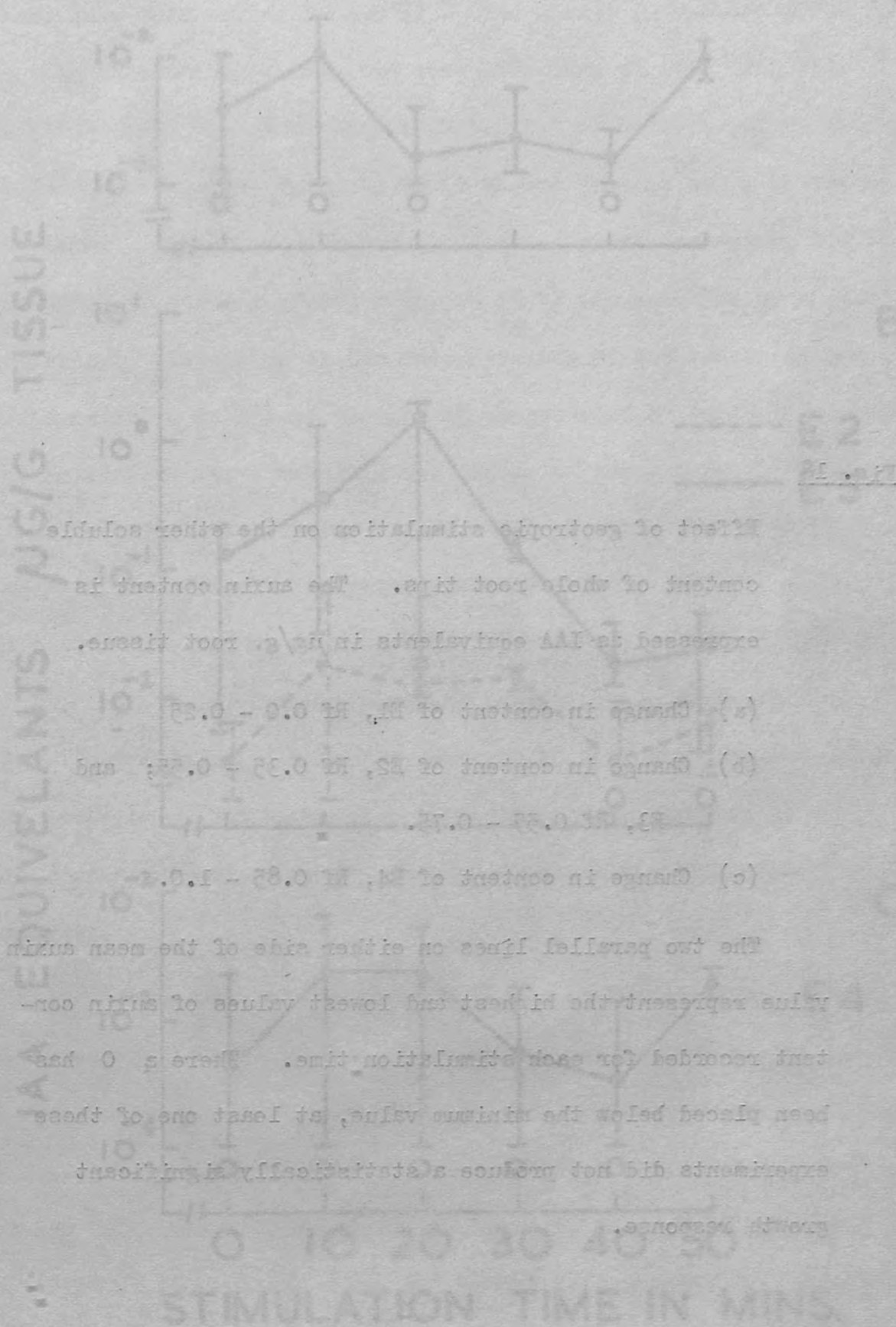


Fig. 18

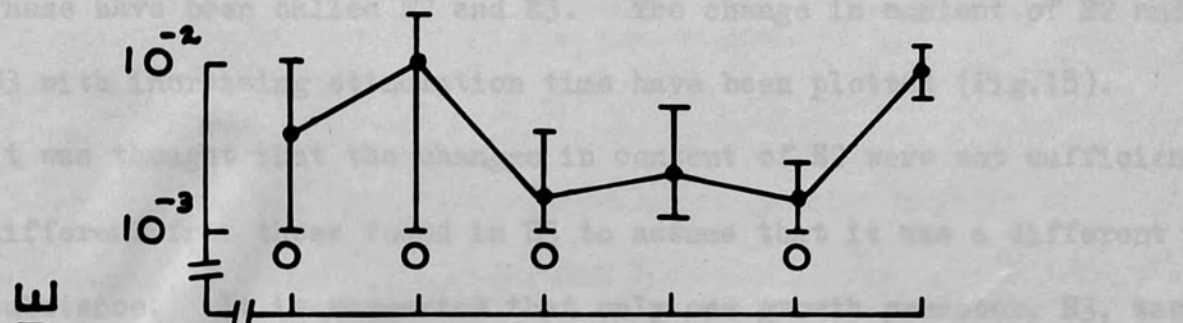
Effect of geotropic stimulation on the ether soluble content of whole root tips. The auxin content is expressed as IAA equivalents in $\mu\text{g/g}$. root tissue.

- (a) Change in content of E1, Rf 0.0 - 0.25
- (b) Change in content of E2, Rf 0.35 - 0.55; and
E3, Rf 0.55 - 0.75.
- (c) Change in content of E4, Rf 0.85 - 1.0.

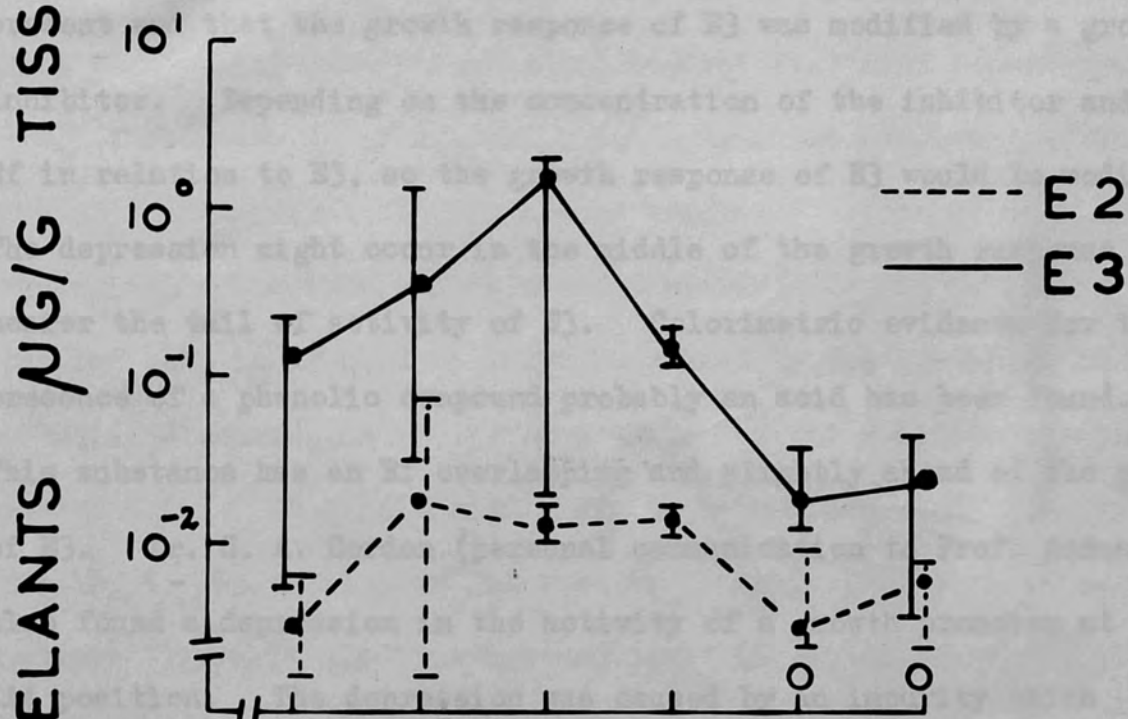
The two parallel lines on either side of the mean auxin value represent the highest and lowest values of auxin content recorded for each stimulation time. Where a 0 has been placed below the minimum value, at least one of these experiments did not produce a statistically significant growth response.

FIG 18

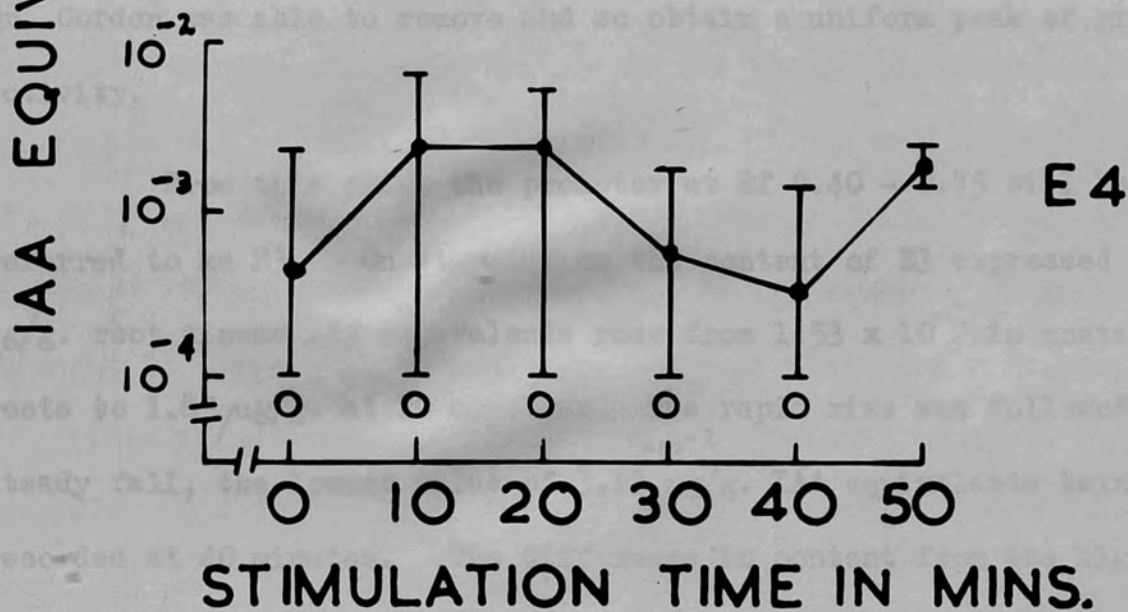
A



B



C



discussion it has been assumed that two promoters might be present. These have been called E2 and E3. The change in content of E2 and E3 with increasing stimulation time have been plotted (Fig.18). It was thought that the changes in content of E2 were not sufficiently different from those found in E3 to assume that it was a different substance. It is suggested that only one growth promoter, E3, was present and that the growth response of E3 was modified by a growth inhibitor. Depending on the concentration of the inhibitor and its Rf in relation to E3, so the growth response of E3 would be modified. The depression might occur in the middle of the growth response or nearer the tail of activity of E3. Colorimetric evidence for the presence of a phenolic compound probably an acid has been found. This substance has an Rf overlapping and slightly ahead of the position of E3. Dr. S. A. Gordon (personal communication to Prof. Audus) has also found a depression in the activity of a growth promoter at the IAA position. The depression was caused by an impurity which Dr. Gordon was able to remove and so obtain a uniform peak of growth activity.

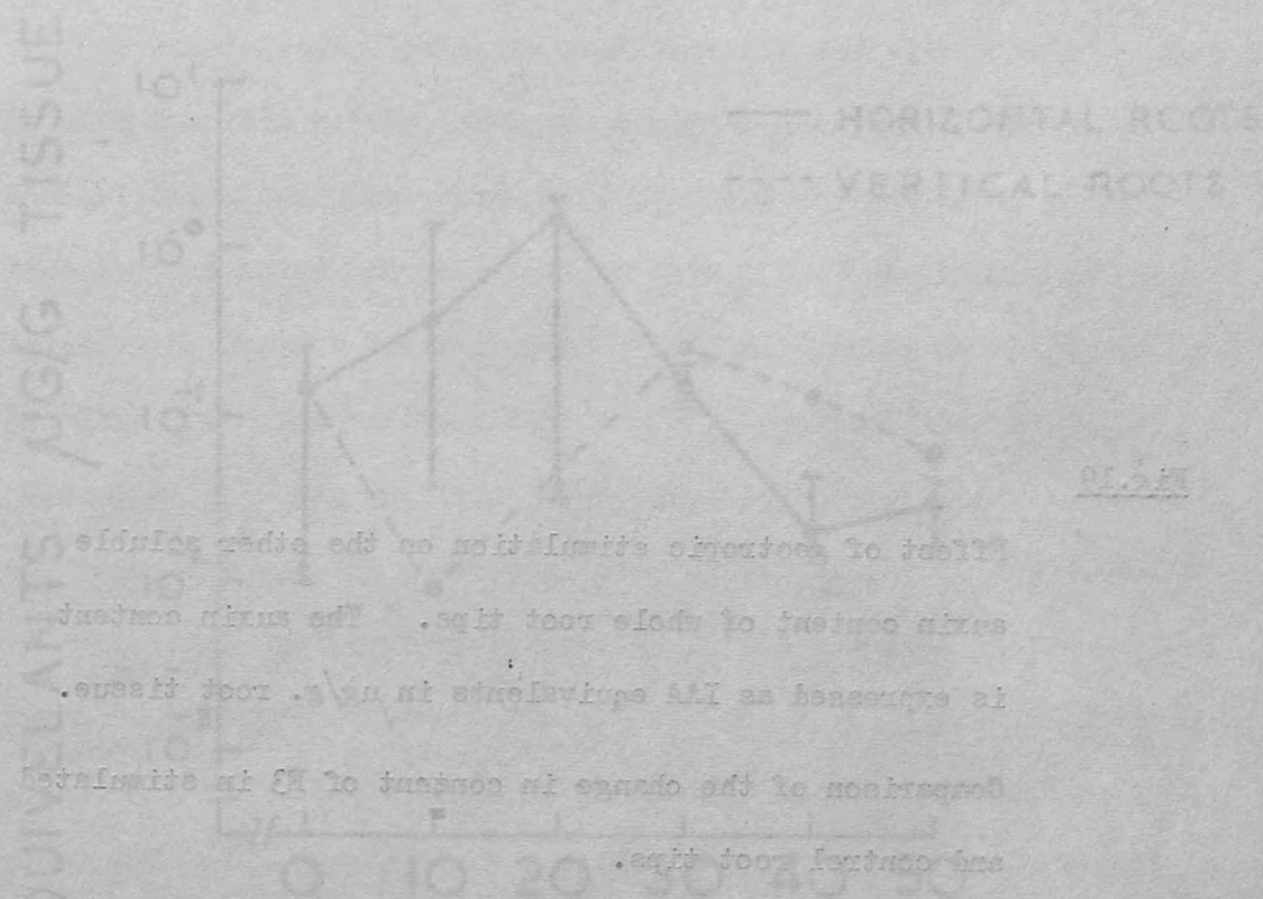
From this point the promoter at Rf 0.40 - 0.75 will be referred to as E3. On stimulation the content of E3 expressed in $\mu\text{g/g}$. root tissue IAA equivalents rose from 1.53×10^{-1} in unstimulated roots to $1.85 \mu\text{g/g}$. at 20 minutes. The rapid rise was followed by a steady fall, the lowest value of $3.13 \times 10^{-2} \mu\text{g/g}$. IAA equivalents being recorded at 40 minutes. The difference in content from the highest value recorded and the lowest represented approximately a 60-fold

decrease. These changes are shown in (Fig.19). The values of E₃ in control roots is shown in the same figure.

The histograms A, E, I, J, N, P, S should be noted. In these experiments the level of E₃ appears greater than in other experiments at similar stimulation times. It was recorded that these experiments were different from the others in one respect. They were stored with the acid and neutral fractions together at -18°C for 6 days at pH 3.0 while other work was completed. Normally the acid and neutral fractions were separated before storage and the storage time was rarely more than 16 hours. It was suspected that the increase in E₃ content might be caused by the breakdown of an inactive precursor in the ether soluble neutral fraction to give an active acid. If this should prove to be correct then it would be similar to the release of IAA from an inactive labile ether soluble neutral or weakly acidic substance found in oat coleoptile tips, Raadts and Soding (1957).

A characteristic feature of the ether soluble chromatograms was an inhibitor at R_f 0.75 - 0.85. This inhibitor β was found by Lahiri and Audus (1960) and by Bennet-Clark and Kefford (1953). By its position relative to the IAA marker spot it is thought to correspond to "inhibitor β ". When sprayed with diazotised p - nitraniline a pinkish spot denoting a phenolic reaction was found at this R_f. A phenolic reaction for the inhibitor β complex has also been found by Torrey (1959).

FIG 19



IAA EQUIVALENTS / µG/G TISSUE

— HORIZONTAL ROOTS
 - - - VERTICAL ROOTS

STIMULATION TIME IN MINS.

The two parallel lines on either side of the mean IAA values represent the highest and lowest values recorded for each stimulation time.

Departure of the change in content of IAA in stimulated and control root tips.

is expressed as IAA equivalents in µg/g. root tissue.

The mean content of whole root tips. The mean content of root tips on the other side of the

201

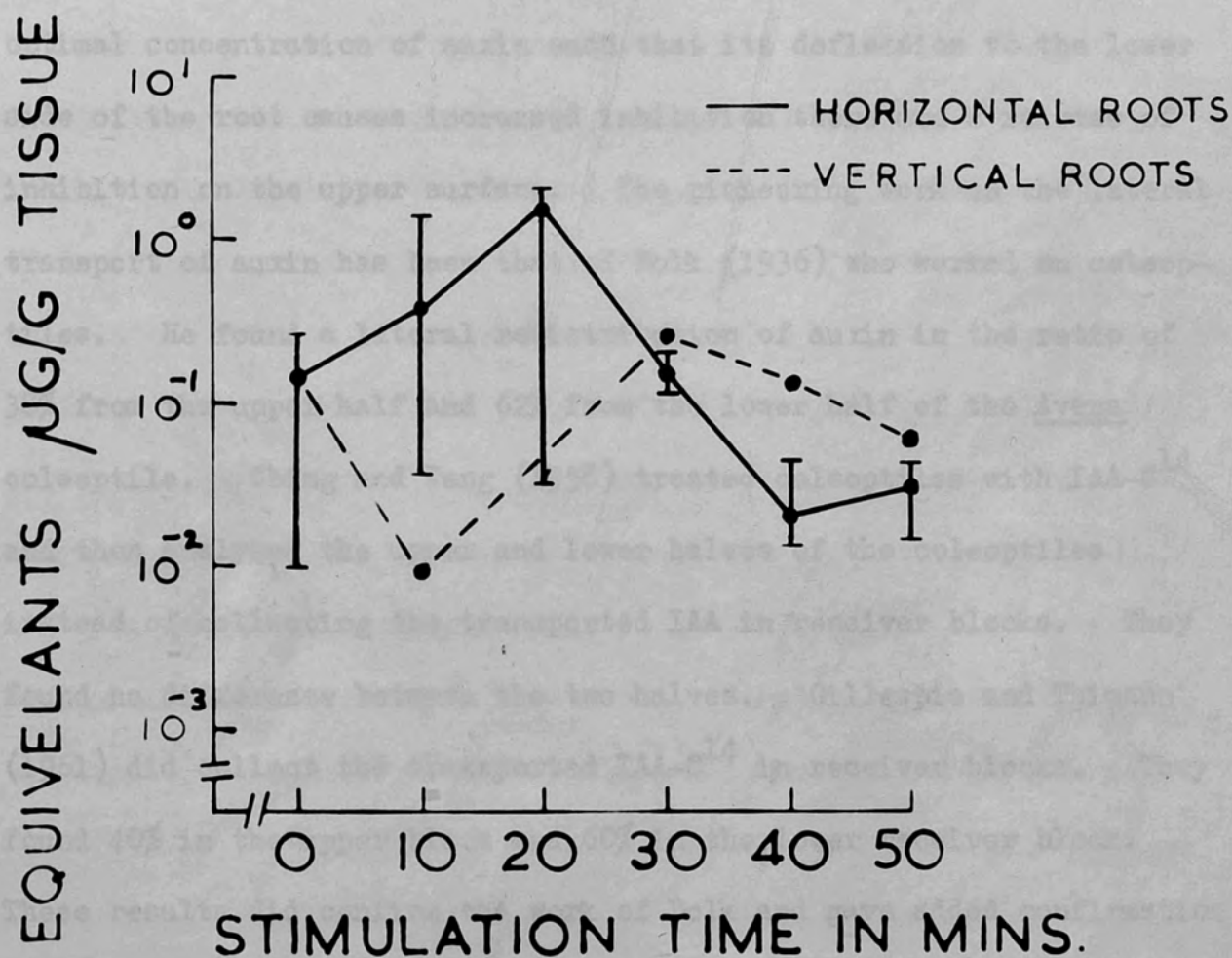
Fig.19

Effect of geotropic stimulation on the ether soluble auxin content of whole root tips. The auxin content is expressed as IAA equivalents in $\mu\text{g/g}$. root tissue.

Comparison of the change in content of E3 in stimulated and control root tips.

The two parallel lines on either side of the mean auxin values represent the highest and lowest values recorded for each stimulation time.

FIG 19



The effect of geotropic stimulation on the lateral redistribution of auxin in root tips.

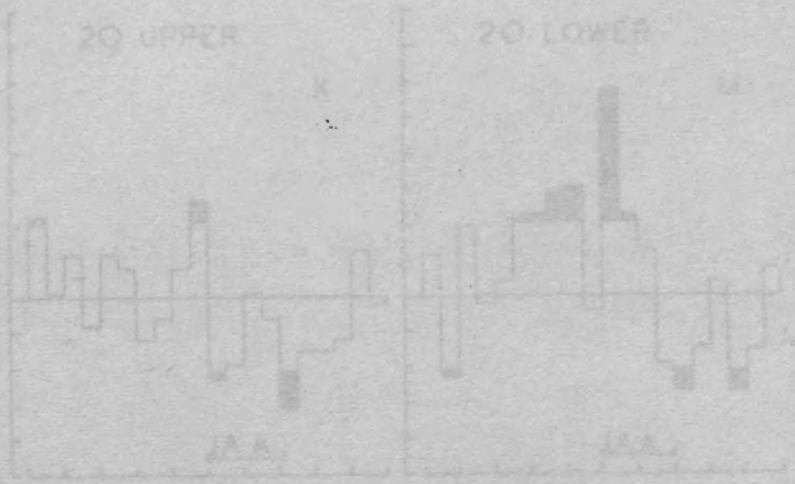
The Cholodny-Went theory of geotropism has been accepted as a basic principle by many plant physiologists. This theory depends on there being in positively reacting roots an optimal or supra optimal concentration of auxin such that its deflection to the lower side of the root causes increased inhibition there and a release of inhibition on the upper surface. The pioneering work on the lateral transport of auxin has been that of Dolk (1936) who worked on coleoptiles. He found a lateral redistribution of auxin in the ratio of 38% from the upper half and 62% from the lower half of the Avena coleoptile. Ching and Fang (1958) treated coleoptiles with IAA-C¹⁴ and then analysed the upper and lower halves of the coleoptiles instead of collecting the transported IAA in receiver blocks. They found no difference between the two halves. Gillespie and Thimann (1961) did collect the transported IAA-C¹⁴ in receiver blocks. They found 40% in the upper block and 60% in the lower receiver block. These results did confirm the work of Dolk and gave added confirmation to the lateral transport theory. In the following experiments it was hoped to locate the position of the increased auxin production and if possible to throw some light on the lateral transport theory.

(ii) Ether soluble acid auxins of vertical and geotropically stimulated longitudinally split roots

Each experimental sample consisted of 100 half root tips. Because of the importance of the change in content of E3, only the

changes in this region will be considered. The results of the experiments are shown in (Fig.20a-b).

The assumption has been made that the auxin content from each longitudinal half of the root when added should approximately equal the content of the whole root tips. The change in content of E₃, resulting from geotropic stimulation, of the combined upper and lower halves has been compared with the values recorded for whole roots. These results are shown in (Fig.21a). The auxin content of the upper half relative to the lower half of vertical and geotropically stimulated roots is shown in (Fig.21b). The E₃ content from split roots remained at a uniform level from 0-30 minutes and then fell slightly. The amount of E₃ from split roots was one fifth the amount recorded for whole roots. No change in content with increasing stimulation time was seen. The auxin content in split roots was more or less evenly distributed between the two halves both in vertical and geotropically stimulated roots. No large accumulation of E₃ in either the upper or lower half was recorded. These results suggested that an extremely rapid inactivation or destruction of auxin was taking place in longitudinally split roots. It was likely the inactivation or destruction was occurring at the cut surface. There have been reports of the destruction of IAA in tissues, Bonner and Thimann (1935), Tang and Bonner (1948), and especially at cut surfaces of tissues, van Overbeek (1938). Galston and Dahlberg (1954) show that the destruction of added IAA in etiolated pea epicotyl tissue occurs immediately in older tissue but there is a lag in younger tissue. No reports have been found of the rate of destruction or inactivation of IAA occurring



(a - b) Fig. 20

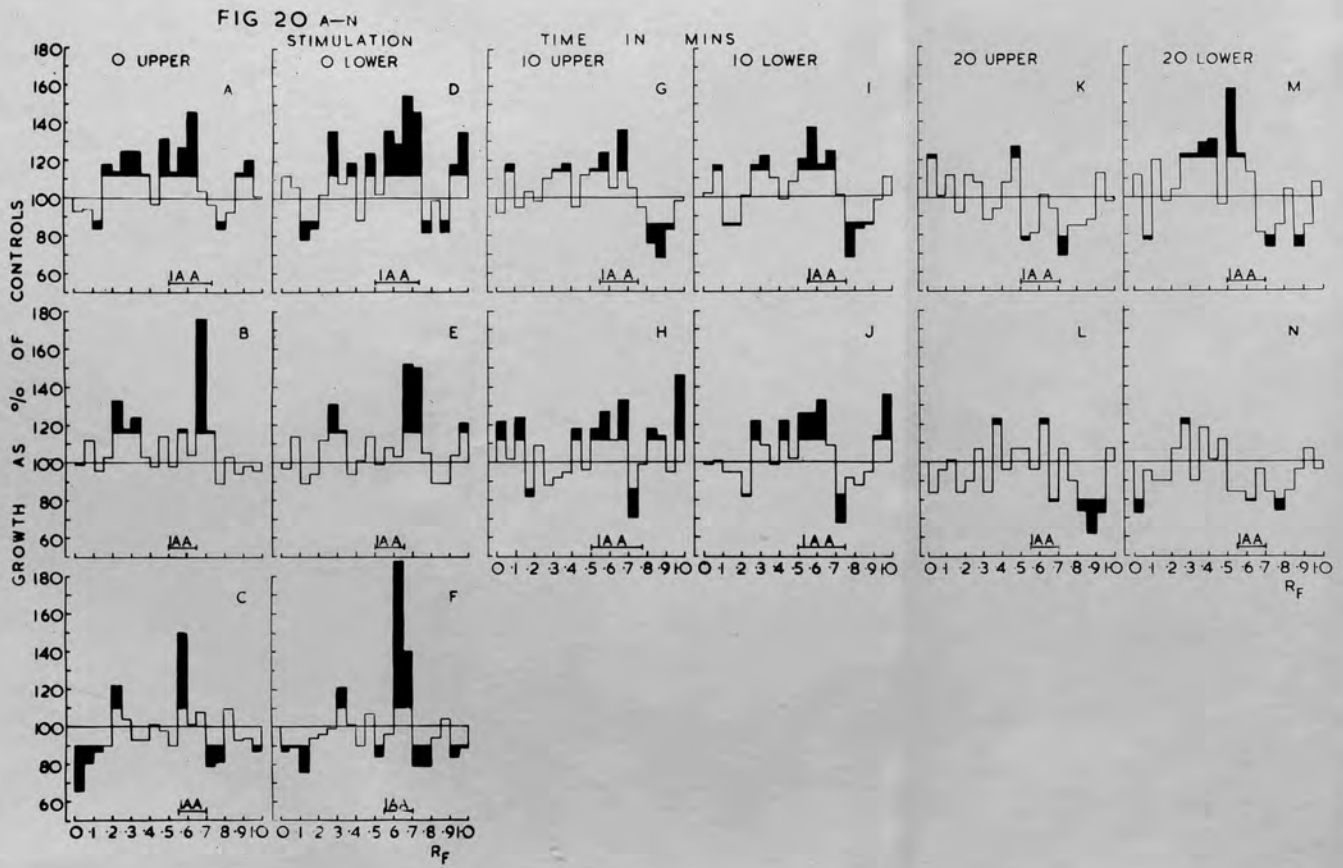
On first interbody assay of the ether soluble acid
 auxins in vertical and geotropically stimulated
 longitudinally split root tips (0.7 m. tips)
 Growth response rate of controls plotted against
 12 values.

The words upper and lower next to each stimulation
 time indicate from which half of the root tip the
 assay was made.

Fig. 20. (a - $\frac{7}{4}$)

Oat first internode assay of the ether soluble acid auxins in vertical and geotropically stimulated longitudinally split root tips. (0.7 mm. tips) Growth response as % of controls plotted against Rf values.

The words upper and lower next to each stimulation time indicate from which half of the root tip the assay was made.



GROWTH OF CONTROL

(cont.) (t - o) Fig. 20

Get first interode assay of the ether soluble
acid units in vertical and geotropically stim-
lated longitudinally split root tips. Growth
response as % of controls plotted against H₂
values.

Fig. 20 (o - t) (cont.)

Oat first internode assay of the ether soluble acid auxins in vertical and geotropically stimulated longitudinally split root tips. Growth response as % of controls plotted against Rf values.

FIG 20 O-T

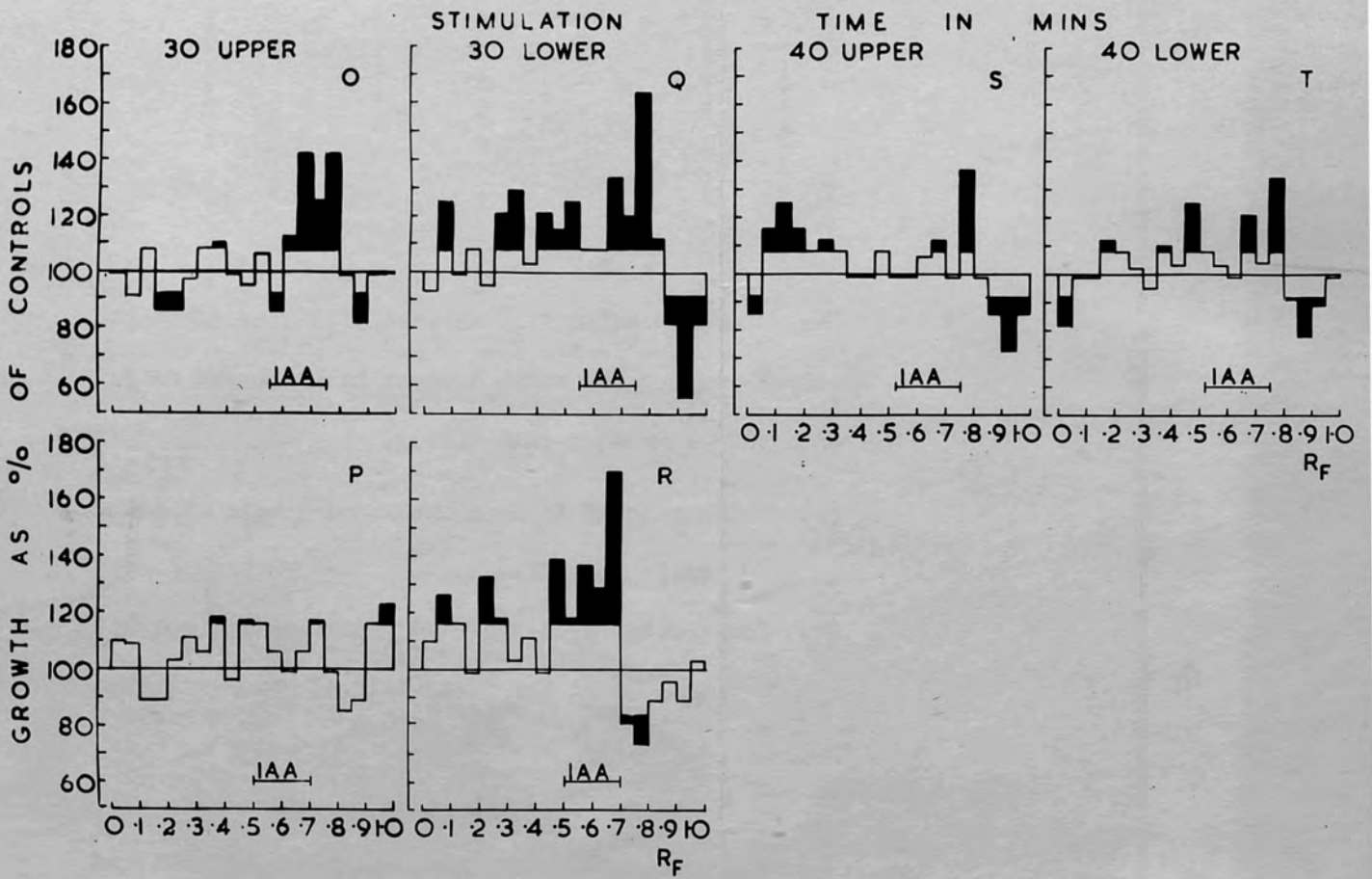
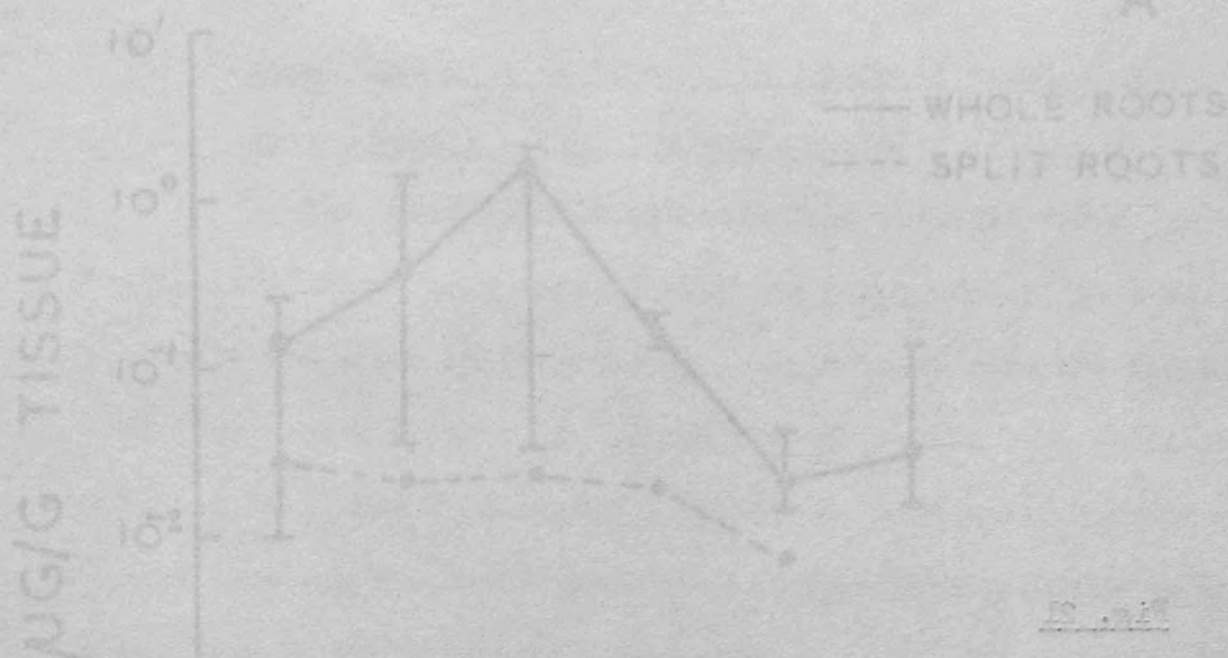


FIG 21



The change in content of IAA resulting from root tip stimulation. The axis content is expressed as IAA equivalents in ng/g tissue.

(a) Comparison of IAA from whole and longitudinally split root tips.

(b) Comparison of IAA from the upper half in relation to IAA from the lower half of root tip.

The two parallel lines on either side of the mean axis represent the highest and lowest values of axis content recorded for each stimulation time.

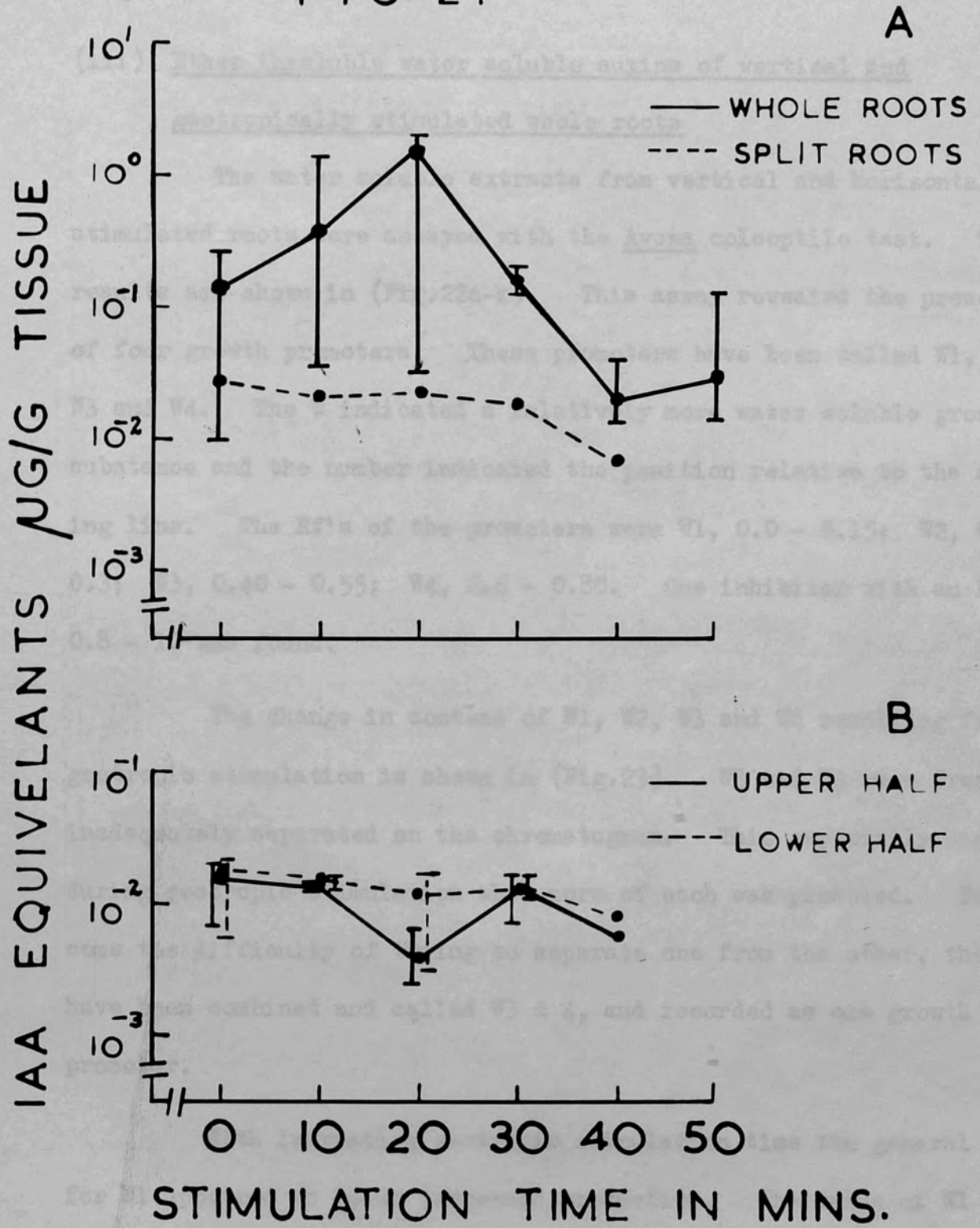
Fig. 21

The change in content of E3 resulting from geotropic stimulation. The auxin content is expressed as IAA equivalents in $\mu\text{g/g}$ root tissue.

- (a) Comparison of E3 from whole and longitudinally split root tips.
- (b) Comparison of E3 from the upper half in relation to E3 from the lower half of geotropically stimulated root tips.

The two parallel lines on either side of the mean auxin values represent the highest and lowest values of auxin content recorded for each stimulation time.

FIG 21



in less than one minute. These aspects will be considered at greater length in the discussion.

(iii) Ether insoluble water soluble auxins of vertical and geotropically stimulated whole roots

The water soluble extracts from vertical and horizontally stimulated roots were assayed with the Avena coleoptile test. The results are shown in (Fig.22a-k). This assay revealed the presence of four growth promoters. These promoters have been called W1, W2, W3 and W4. The W indicated a relatively more water soluble growth substance and the number indicated the position relative to the starting line. The Rf's of the promoters were W1, 0.0 - 0.15; W2, 0.2 - 0.3; W3, 0.40 - 0.55; W4, 0.6 - 0.80. One inhibitor with an Rf of 0.8 - 1.0 was found.

The change in content of W1, W2, W3 and W4 resulting from geotropic stimulation is shown in (Fig.23). W3 and W4 were frequently inadequately separated on the chromatogram. This especially happened during geotropic stimulation when more of each was produced. To overcome the difficulty of trying to separate one from the other, they have been combined and called W3 & 4, and recorded as one growth promoter.

With increasing geotropic stimulation time the general trend for W1 appeared to be an increased production. The value of W1 in vertical roots was $0.55 \mu\text{g/g}$. root tissue, IAA equivalents rising to a maximum, after 30 minutes horizontal stimulation, of $1.17 \mu\text{g/g}$. IAA



Fig. 22 (a - d)

Get complete assay of the other insoluble water soluble extracts in vertical and horizontally stimulated whole root tips. Growth response as of controls plotted against values.

012345678910

311

Fig. 22 (a - h)

Oat coleoptile assay of the ether insoluble water soluble auxins in vertical and geotropically stimulated whole root tips. Growth response as % of controls plotted against Rf values.

FIG 22 A-K

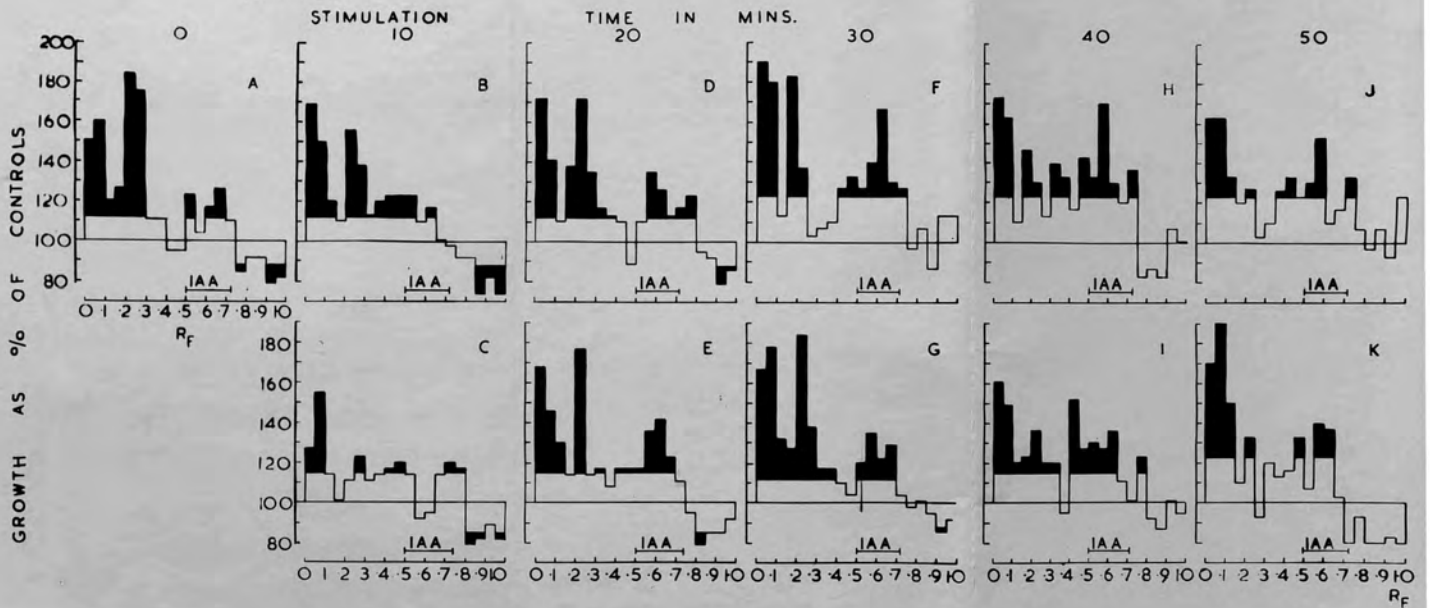
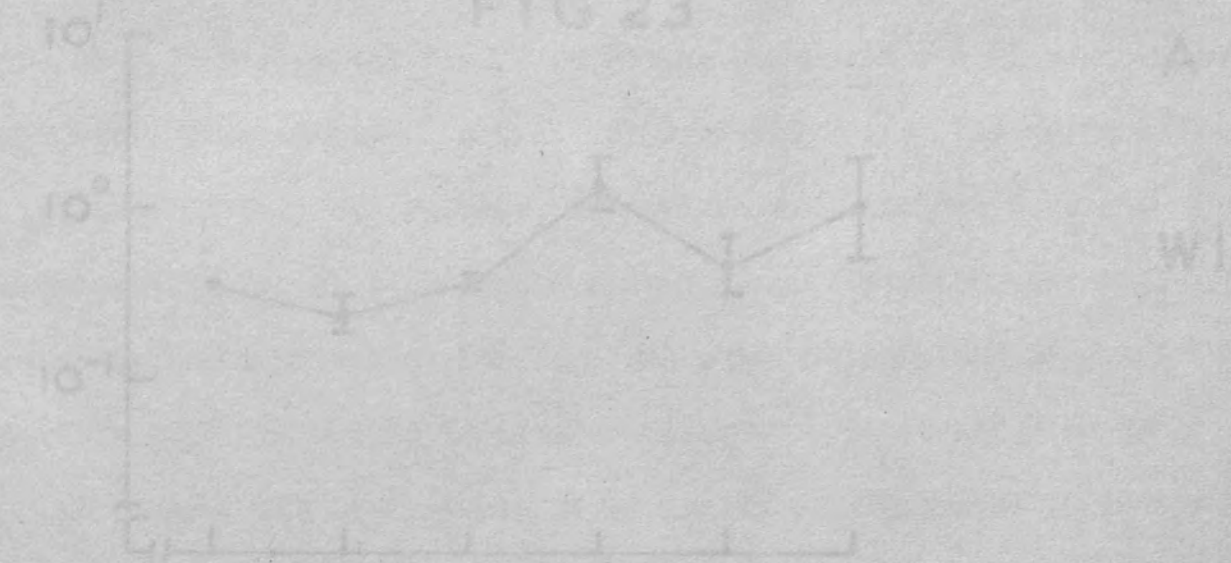
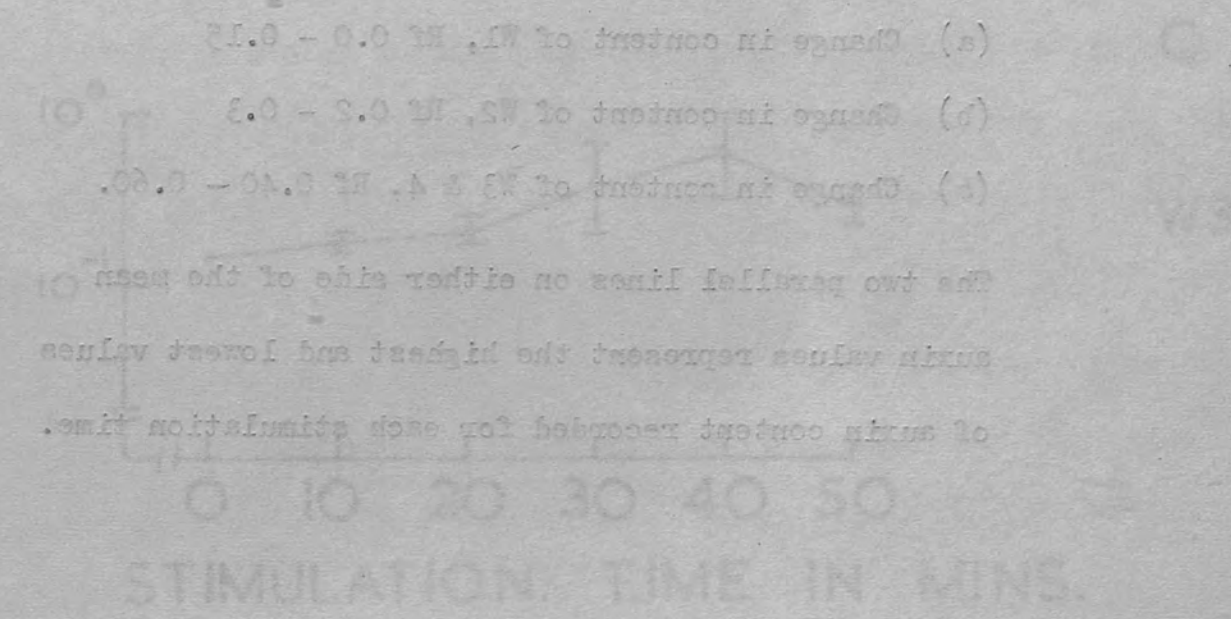


FIG 23

IAA EQUIVALENTS / µG/G TISSUE



Effect of tropic stimulation on the ether insoluble water soluble auxin content of whole root tips. The auxin content is expressed as IAA equivalents in µg/g root tissue.



(a) Change in content of W1, W1 0.0 - 0.15
 (b) Change in content of W2, W2 0.2 - 0.3
 (c) Change in content of W3 & 4, W3 & 4 0.40 - 0.60.

The two parallel lines on either side of the mean auxin values represent the highest and lowest values of auxin content recorded for each stimulation time.

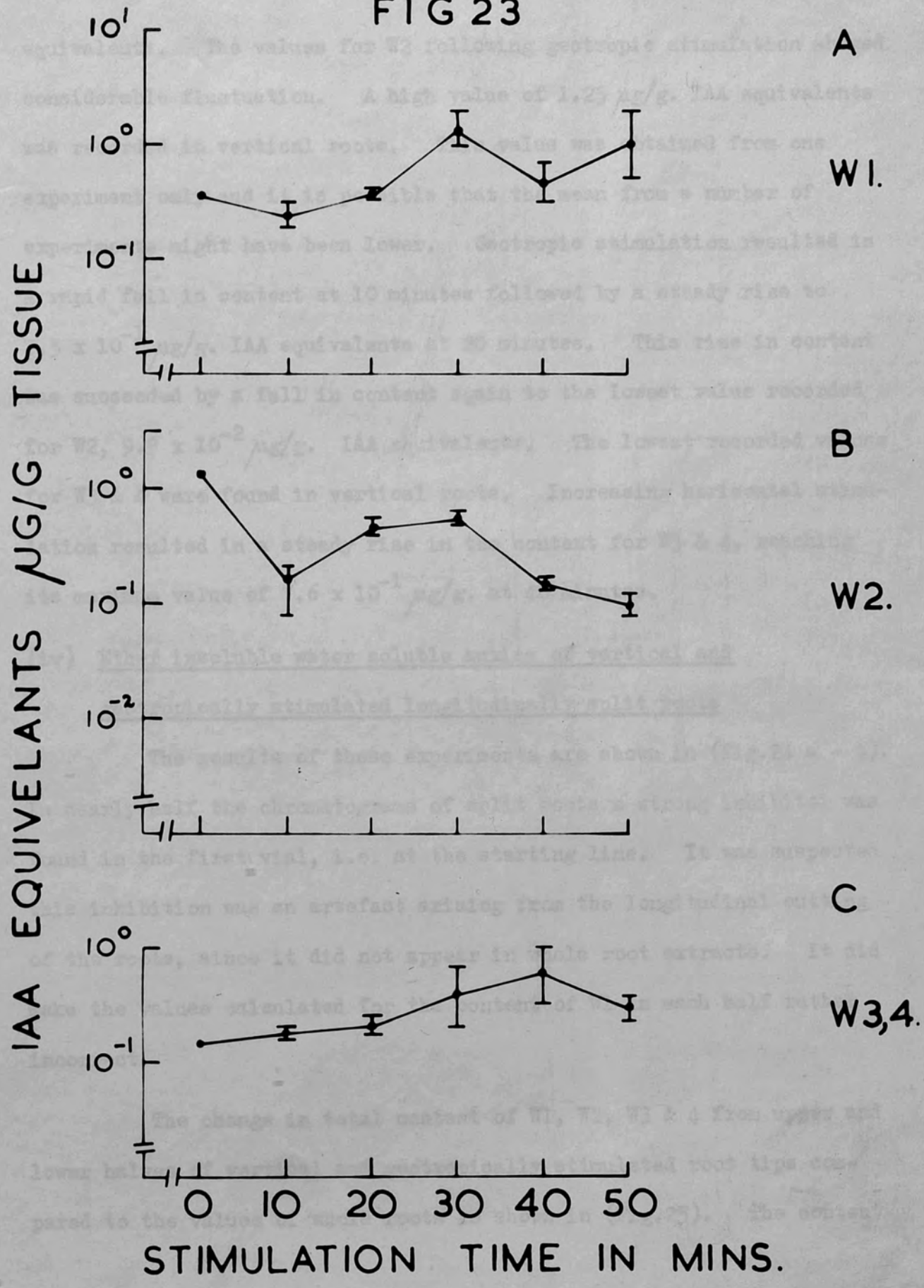
Fig. 23

Effect of geotropic stimulation on the ether insoluble water soluble auxin content of whole root tips. The auxin content is expressed as IAA equivalents in $\mu\text{g/g}$. root tissue.

- (a) Change in content of W1, Rf 0.0 - 0.15
- (b) Change in content of W2, Rf 0.2 - 0.3
- (c) Change in content of W3 & 4, Rf 0.40 - 0.60.

The two parallel lines on either side of the mean auxin values represent the highest and lowest values of auxin content recorded for each stimulation time.

FIG 23



equivalents. The values for W2 following geotropic stimulation showed considerable fluctuation. A high value of $1.25 \mu\text{g/g}$. IAA equivalents was recorded in vertical roots. This value was obtained from one experiment only and it is possible that the mean from a number of experiments might have been lower. Geotropic stimulation resulted in a rapid fall in content at 10 minutes followed by a steady rise to $7.5 \times 10^{-1} \mu\text{g/g}$. IAA equivalents at 20 minutes. This rise in content was succeeded by a fall in content again to the lowest value recorded for W2, $9.9 \times 10^{-2} \mu\text{g/g}$. IAA equivalents. The lowest recorded values for W3 & 4 were found in vertical roots. Increasing horizontal stimulation resulted in a steady rise in the content for W3 & 4, reaching its maximum value of $7.6 \times 10^{-1} \mu\text{g/g}$. at 40 minutes.

(iv) Ether insoluble water soluble auxins of vertical and geotropically stimulated longitudinally split roots

The results of these experiments are shown in (Fig.24 a - t). In nearly half the chromatograms of split roots a strong inhibitor was found in the first vial, i.e. at the starting line. It was suspected this inhibition was an artefact arising from the longitudinal cutting of the roots, since it did not appear in whole root extracts. It did make the values calculated for the content of W1 in each half rather incorrect.

The change in total content of W1, W2, W3 & 4 from upper and lower halves of vertical and geotropically stimulated root tips compared to the values of whole roots is shown in (Fig.25). The content

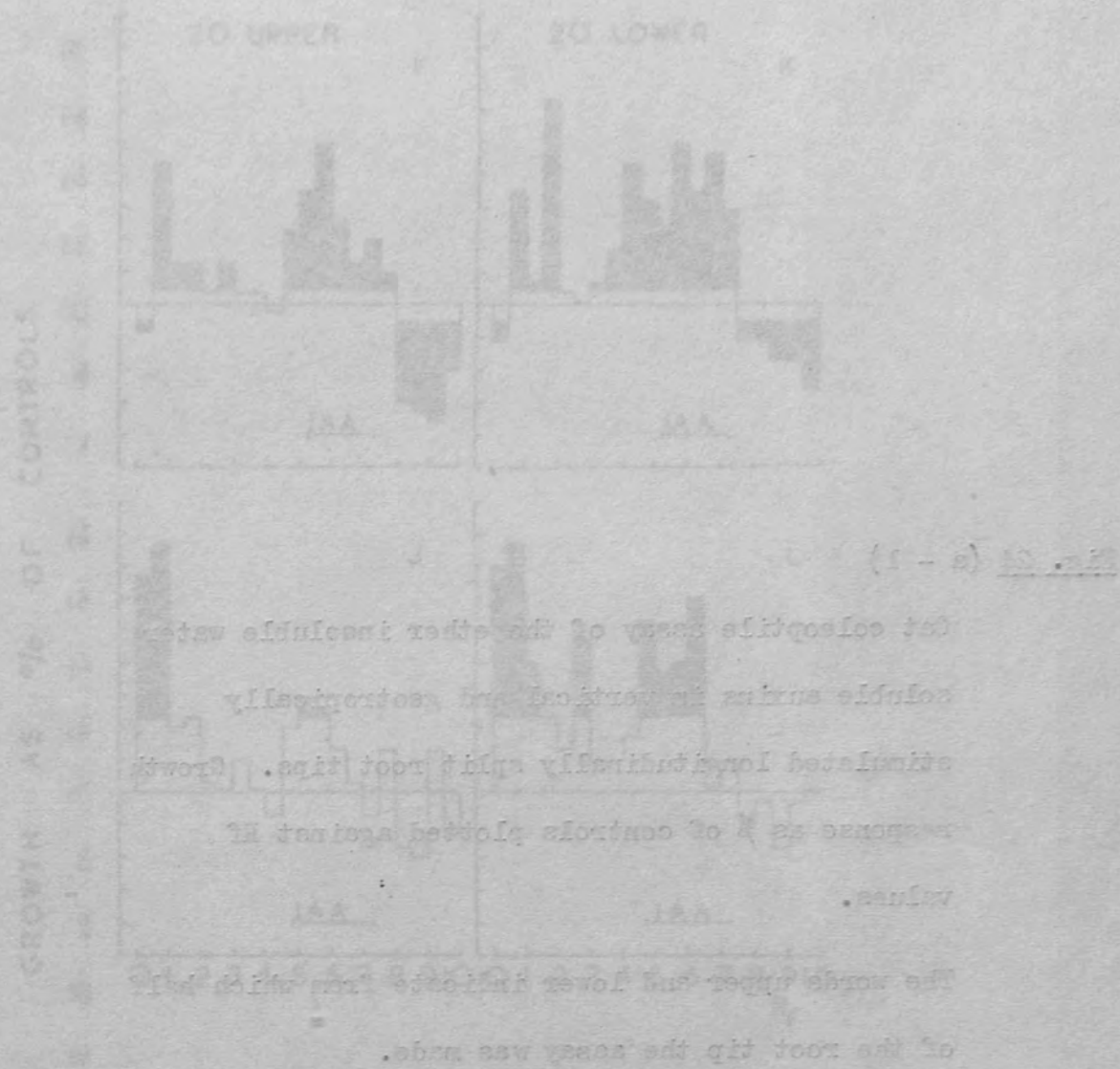
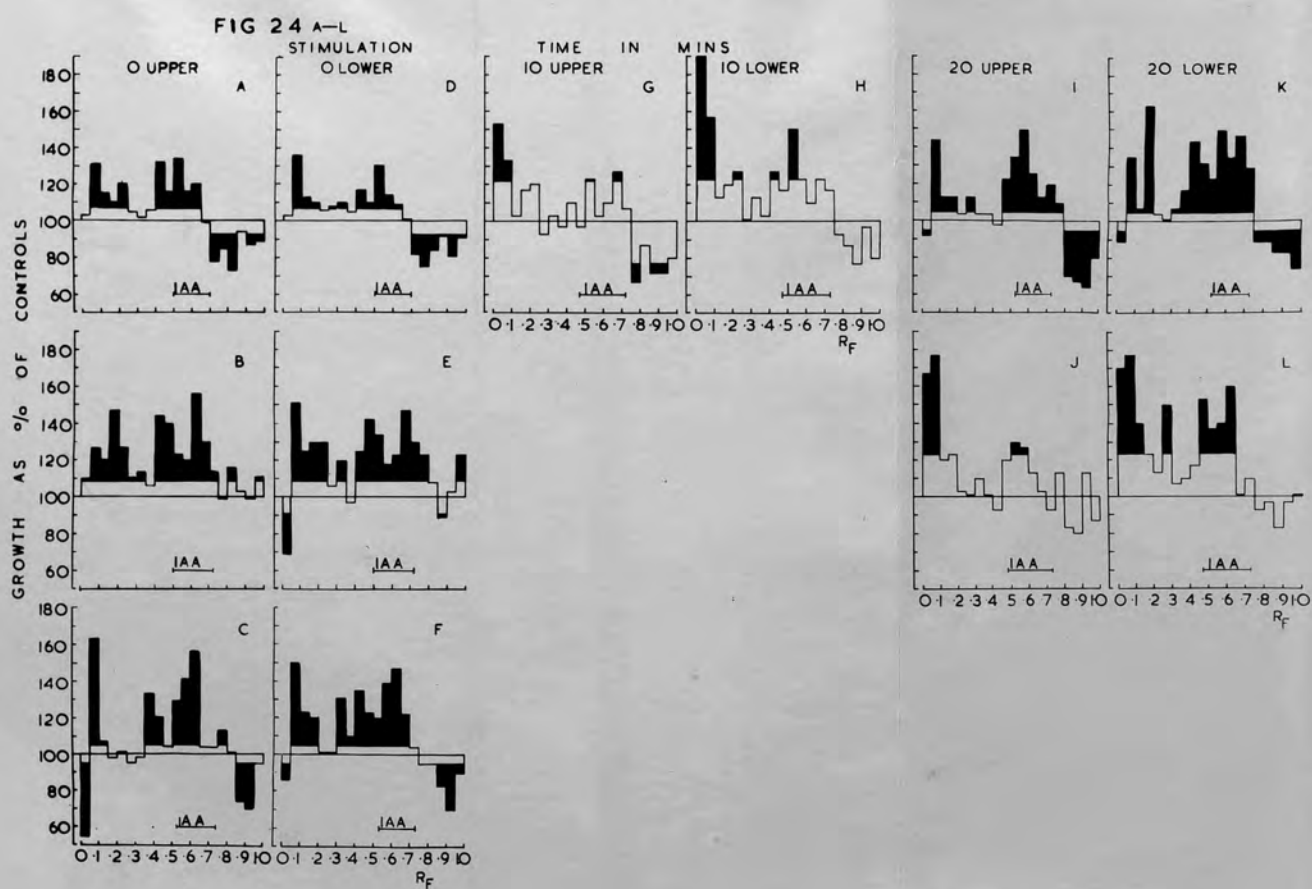


FIG. 1. Growth of the root tip of the assay was made. The upper and lower root tips were treated with the indicated concentrations of the test substance. The growth was measured as a percentage of the control. The histograms show the distribution of the growth percentages. The upper and lower root tips were treated with the indicated concentrations of the test substance. The growth was measured as a percentage of the control. The histograms show the distribution of the growth percentages.

Fig. 24 (a - 1)

Oat coleoptile assay of the ether insoluble water soluble auxins in vertical and geotropically stimulated longitudinally split root tips. Growth response as % of controls plotted against Rf values.

The words upper and lower indicate from which half of the root tip the assay was made.



GROWTH AS % OF CONTROLS

Fig. 24 (cont.) (t - m)

(a) soluble assay of the ether insoluble water
 soluble enzyme in verticillium and geotrichum
 stimulated longitudinally split root tips.
 Growth as % of controls plotted against R₂
 values.

Fig. 24 (m - t) (cont.)

Oat coleoptile assay of the ether insoluble water soluble auxins in vertical and geotropically stimulated longitudinally split root tips. Growth as % of controls plotted against Rf values.

FIG 24 M-T

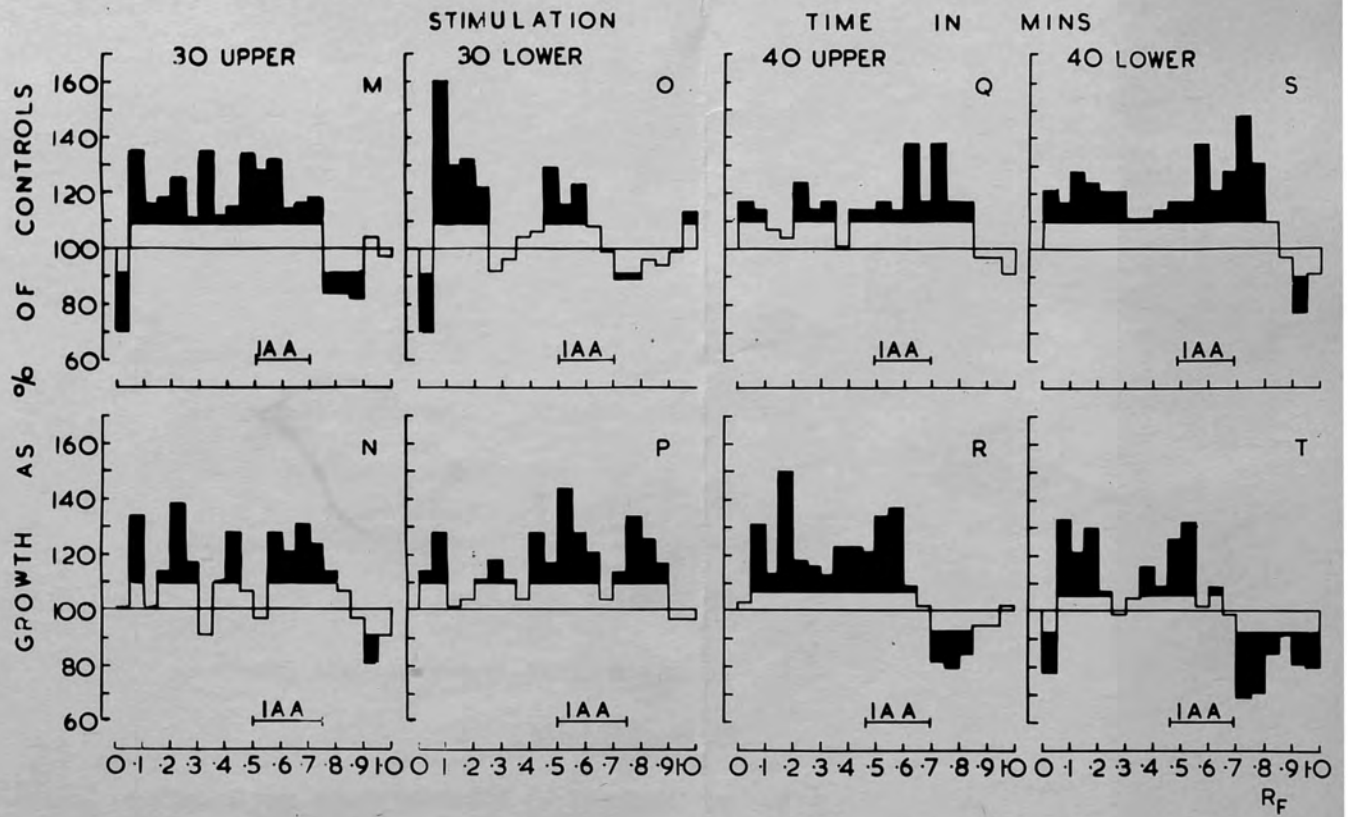


FIG 25

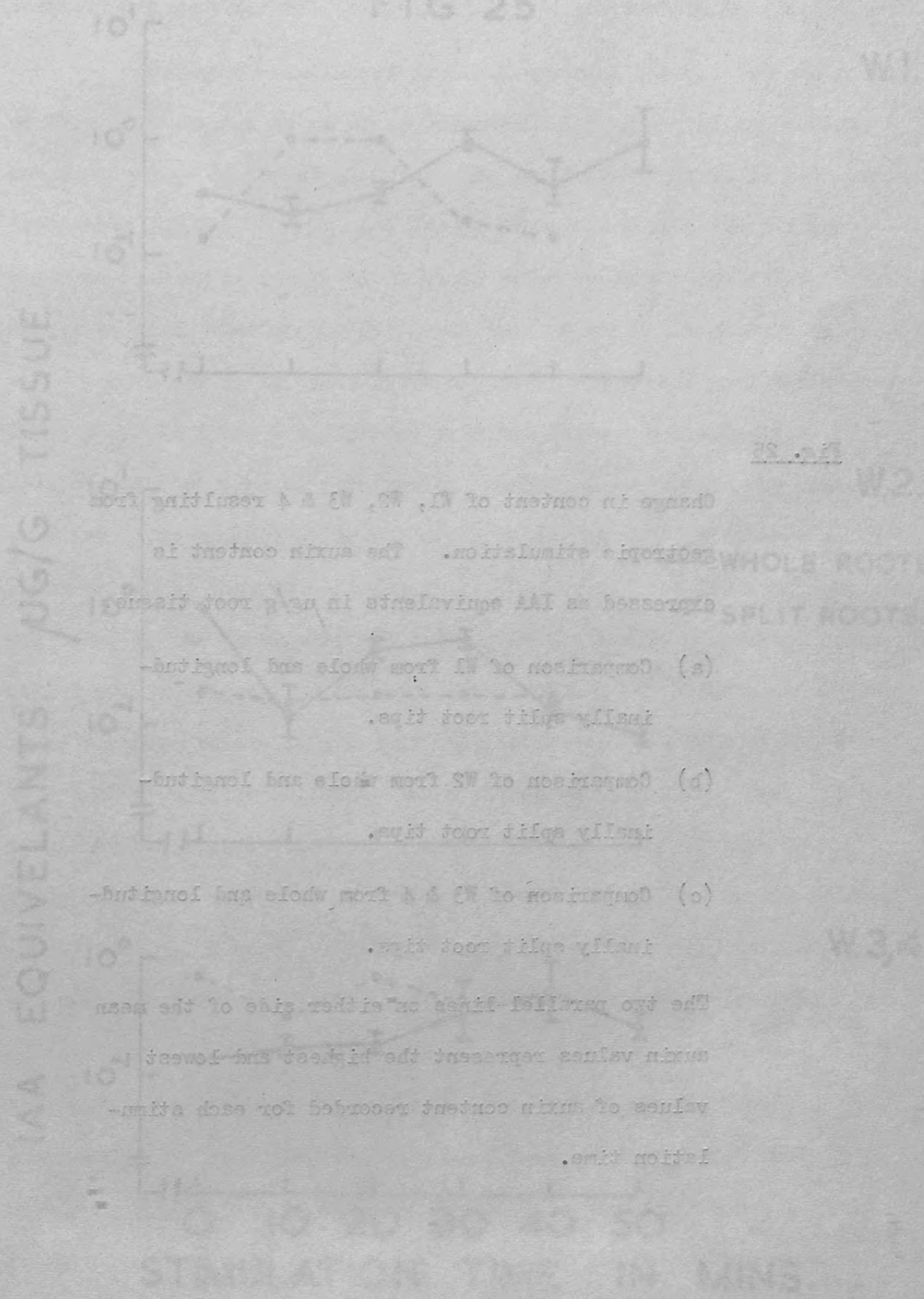


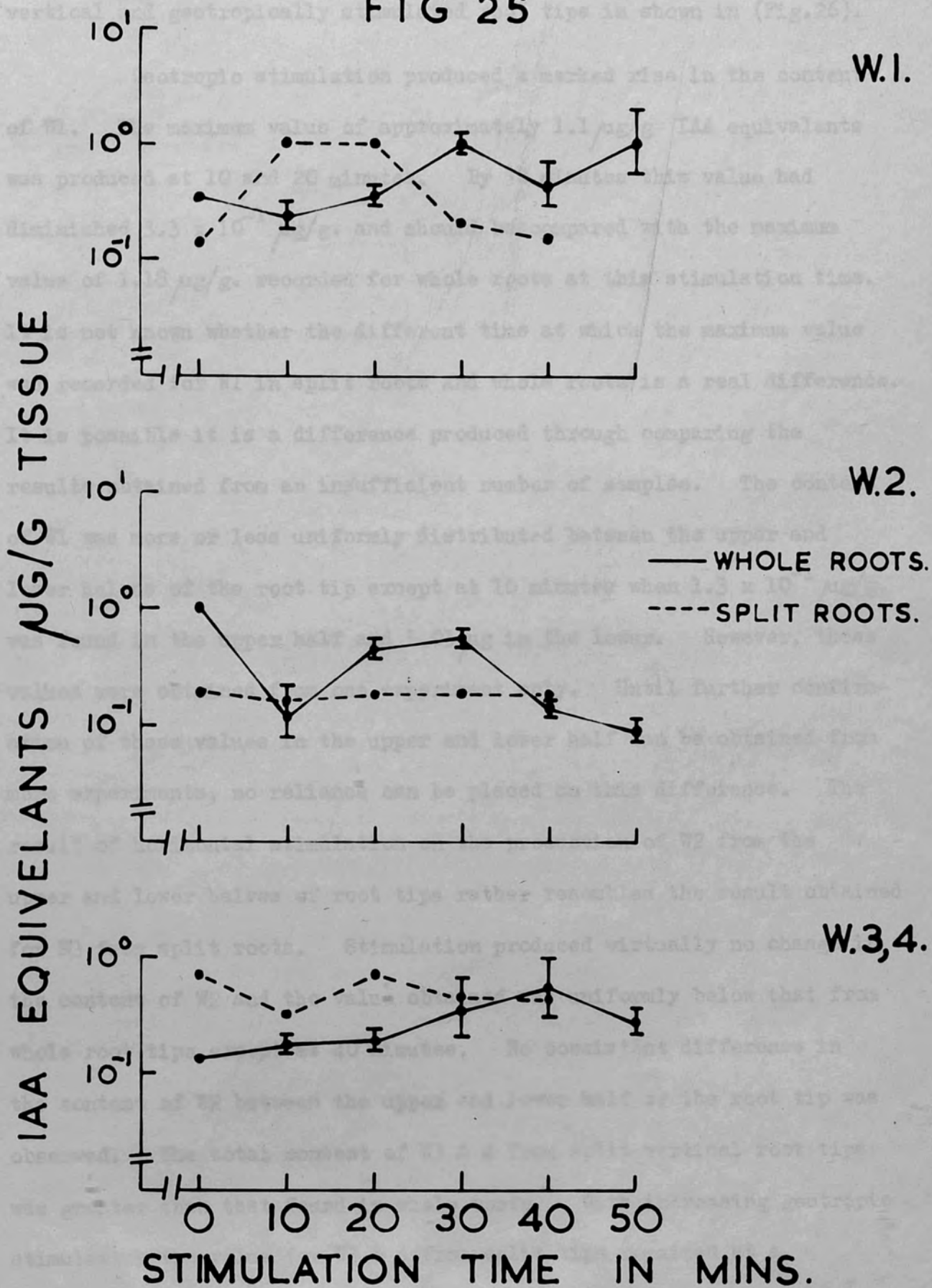
Fig. 25

Change in content of W1, W2, W3 & 4 resulting from geotropic stimulation. The auxin content is expressed as IAA equivalents in $\mu\text{g/g}$ root tissue.

- (a) Comparison of W1 from whole and longitudinally split root tips.
- (b) Comparison of W2 from whole and longitudinally split root tips.
- (c) Comparison of W3 & 4 from whole and longitudinally split root tips.

The two parallel lines on either side of the mean auxin values represent the highest and lowest values of auxin content recorded for each stimulation time.

FIG 25



of W1, W2, W3 & 4 in the upper half relative to the lower half of vertical and geotropically stimulated root tips is shown in (Fig.26).

Geotropic stimulation produced a marked rise in the content of W1. The maximum value of approximately $1.1 \mu\text{g/g}$ IAA equivalents was produced at 10 and 20 minutes. By 30 minutes this value had diminished $3.3 \times 10^{-1} \mu\text{g/g}$. and should be compared with the maximum value of $1.18 \mu\text{g/g}$. recorded for whole roots at this stimulation time. It is not known whether the different time at which the maximum value was recorded for W1 in split roots and whole roots is a real difference. It is possible it is a difference produced through comparing the results obtained from an insufficient number of samples. The content of W1 was more or less uniformly distributed between the upper and lower halves of the root tip except at 10 minutes when $1.3 \times 10^{-1} \mu\text{g/g}$. was found in the upper half and $1.03 \mu\text{g}$ in the lower. However, these values were obtained from one experiment only. Until further confirmation of these values in the upper and lower half can be obtained from more experiments, no reliance can be placed on this difference. The result of horizontal stimulation on the production of W2 from the upper and lower halves of root tips rather resembles the result obtained for E3 from split roots. Stimulation produced virtually no change in the content of W2 and the value obtained was uniformly below that from whole root tips except at 40 minutes. No consistent difference in the content of W2 between the upper and lower half of the root tip was observed. The total content of W3 & 4 from split vertical root tips was greater than that found in whole roots. With increasing geotropic stimulation the value for W3 & 4 from split tips remained at a

FIG 26



IA EQUIVALENTS IN GIG TISSUE

change in content of W1, W2, W3 & A resulting from two
 from stimulation. The main content is expressed in
 IA equivalents in mg. root tissue.

(a) Comparison of W1 from the upper half in relation
 to W1 from the lower half of the root tip.

(b) Comparison of W2 from the upper half in relation
 to W2 from the lower half of the root tip.

(c) Comparison of W3 & A from the upper half in relation
 to W3 & A from the lower half of the root tip.

The two parallel lines on either side of the main curve
 values represent the highest and lowest values of main content
 recorded for each stimulation time. Where a 0 has been placed
 below the minimum value at least one of these experiments did not
 produce a statistically significant growth response.

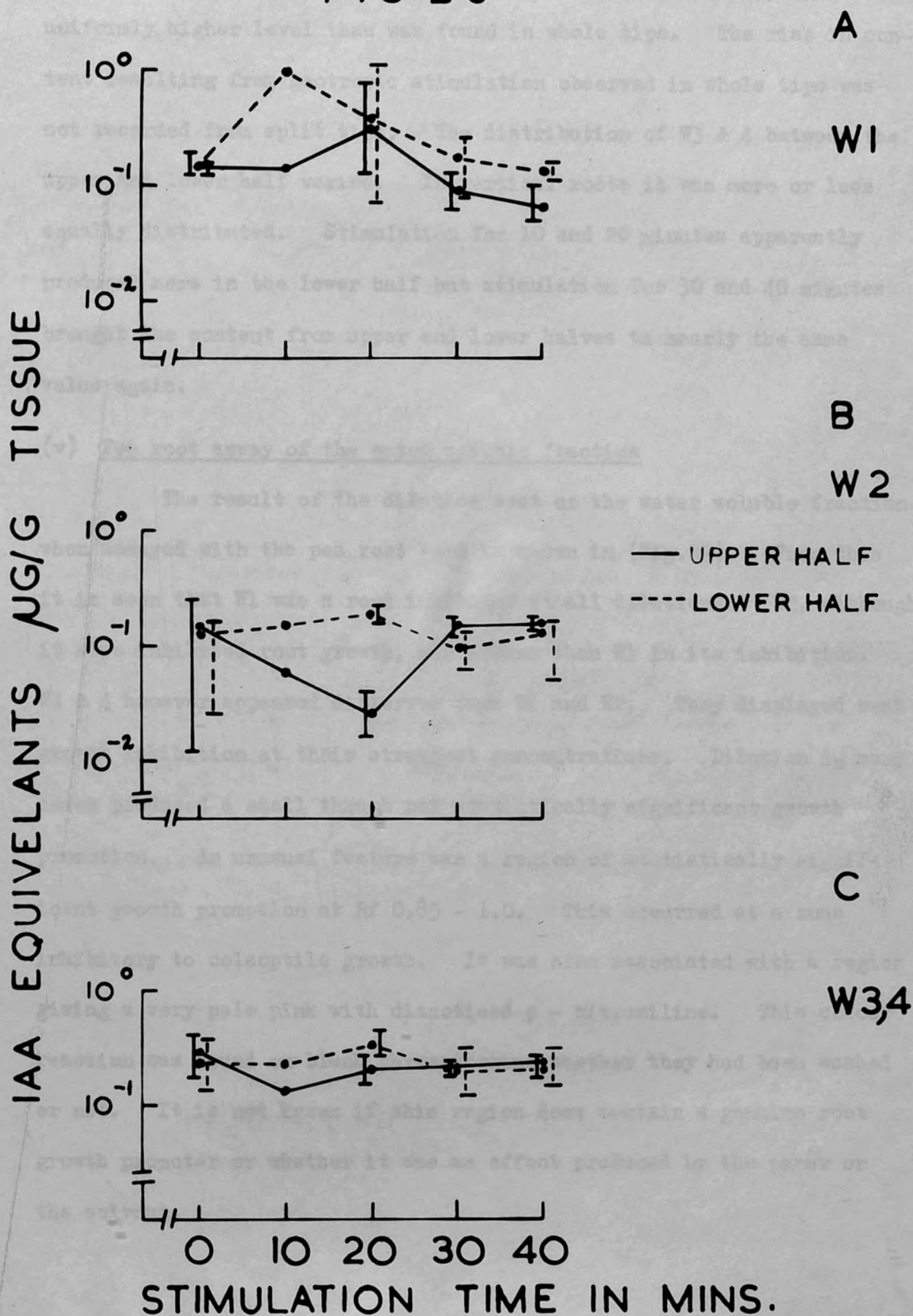
Fig. 26

Change in content of W1, W2, W3 & 4 resulting from geotropic stimulation. The auxin content is expressed as IAA equivalents in $\mu\text{g/g}$. root tissue.

- (a) Comparison of W1 from the upper half in relation to W1 from the lower half of the root tip.
- (b) Comparison of W2 from the upper half in relation to W2 from the lower half of the root tip.
- (c) Comparison of W3 & 4 from the upper half in relation to W3 & 4 from the lower half of the root tip.

The two parallel lines on either side of the mean auxin values represent the highest and lowest values of auxin content recorded for each stimulation time. Where a 0 has been placed below the minimum value at least one of these experiments did not produce a statistically significant growth response.

FIG 26



uniformly higher level than was found in whole tips. The rise in content resulting from geotropic stimulation observed in whole tips was not recorded from split tips. The distribution of W3 & 4 between the upper and lower half varied. In vertical roots it was more or less equally distributed. Stimulation for 10 and 20 minutes apparently produced more in the lower half but stimulation for 30 and 40 minutes brought the content from upper and lower halves to nearly the same value again.

(v) Pea root assay of the water soluble fraction

The result of the dilution test on the water soluble fraction when assayed with the pea root test is shown in (Fig.27). From this it is seen that W1 was a root inhibitor at all dilutions. W2, although it also inhibited root growth, was weaker than W1 in its inhibition. W3 & 4 however appeared different from W1 and W2. They displayed weak growth inhibition at their strongest concentrations. Dilution in many cases produced a small though not statistically significant growth promotion. An unusual feature was a region of statistically significant growth promotion at Rf 0.85 - 1.0. This occurred at a zone inhibitory to coleoptile growth. It was also associated with a region giving a very pale pink with diazotised p - nitraniline. This colour reaction was found on blank chromatograms whether they had been washed or not. It is not known if this region does contain a genuine root growth promoter or whether it was an effect produced by the paper or the solvent.

History of the growth response of pea root sections to dilutions of the water soluble fraction. The external concentration of the dilution from each twentieth part of the chromatogram was diluted one in ten and then one in a hundred. These dilutions have been expressed as

- 0 = original dilution
- 1 = one in ten dilution
- 2 = one in a hundred dilution

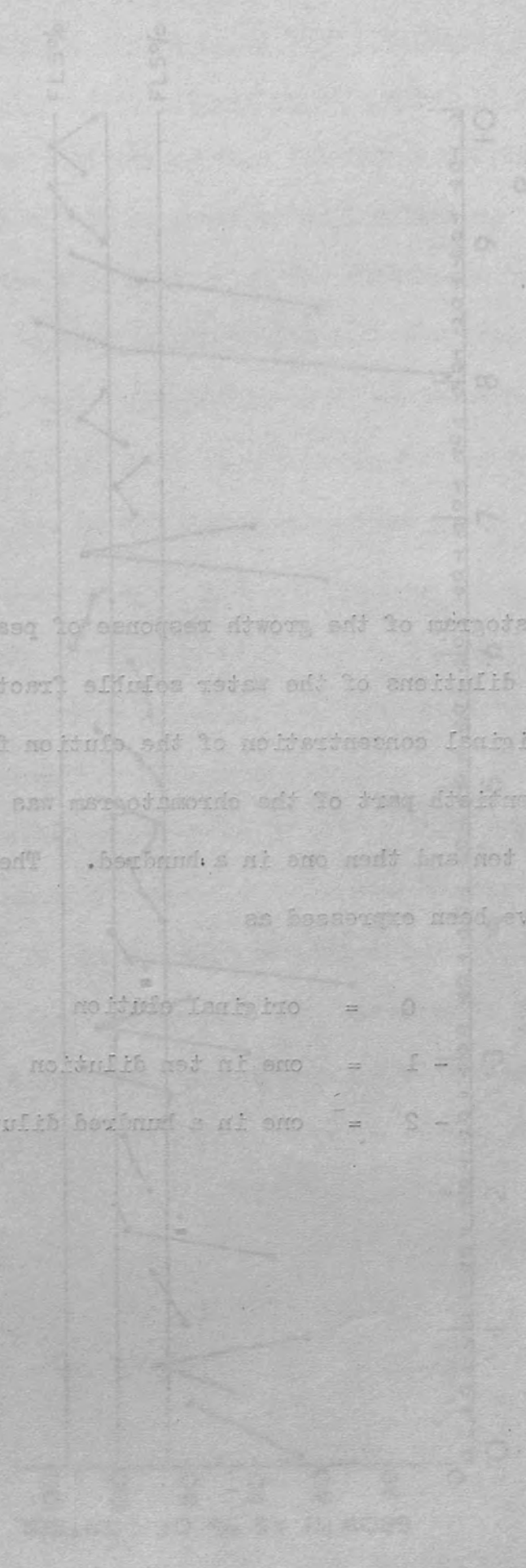


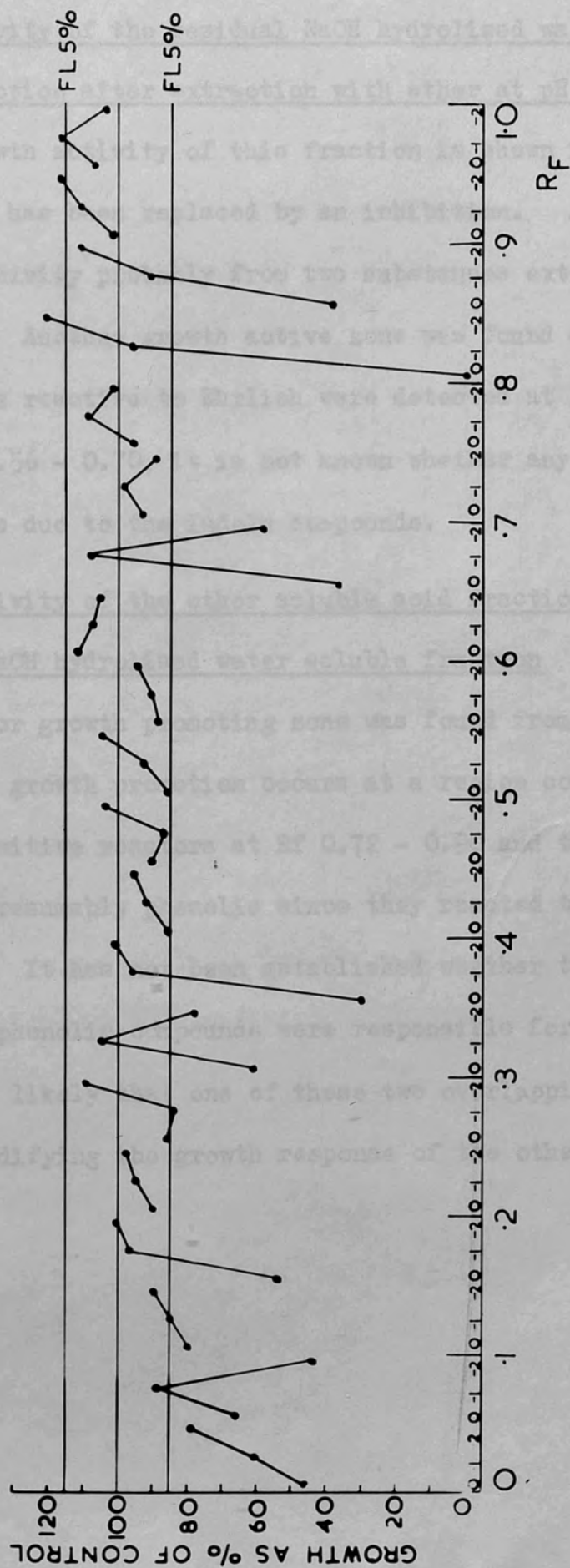
Fig. 21

181

Fig. 27

Histogram of the growth response of pea root sections to dilutions of the water soluble fraction. The original concentration of the elution from each twentieth part of the chromatogram was diluted one in ten and then one in a hundred. These dilutions have been expressed as

- 0 = original elution
- 1 = one in ten dilution
- 2 = one in a hundred dilution



(vi) Growth activity of the residual NaOH hydrolysed water soluble fraction after extraction with ether at pH 3.0

The growth activity of this fraction is shown in (Fig.28a). The promoter, W1, has been replaced by an inhibition. A large zone of high growth activity probably from two substances extends from Rf 0.15 to 0.50. Another growth active zone was found at Rf 0.65 - 0.75. Although compounds reactive to Ehrlich were detected at Rf 0.12 - 0.20, 0.27 - 0.42 and 0.56 - 0.70, it is not known whether any of the new growth activity is due to the indole compounds.

(vii) Growth activity of the ether soluble acid fraction from the NaOH hydrolysed water soluble fraction

One major growth promoting zone was found from Rf 0.65 - 0.85 (Fig.28b). This growth promotion occurs at a region containing both purple Ehrlich positive reactors at Rf 0.72 - 0.90 and three substances Rf 0.75 - 0.90, presumably phenolic since they reacted to diazotised p - nitraniline. It has not been established whether the indolic compounds or the phenolic compounds were responsible for the growth activity. It is likely that one of these two overlapping groups of substances was modifying the growth response of the other.

FIG 28

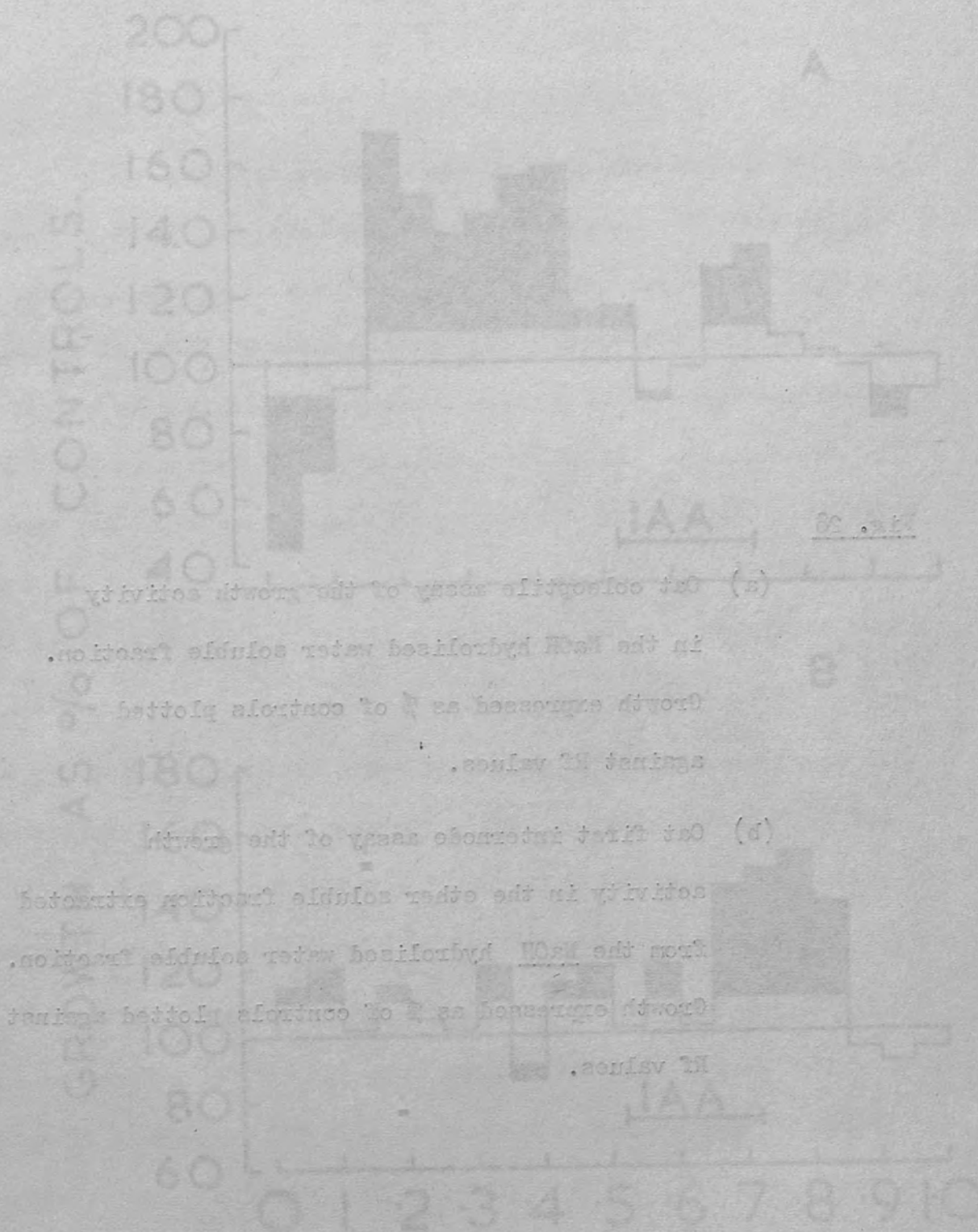
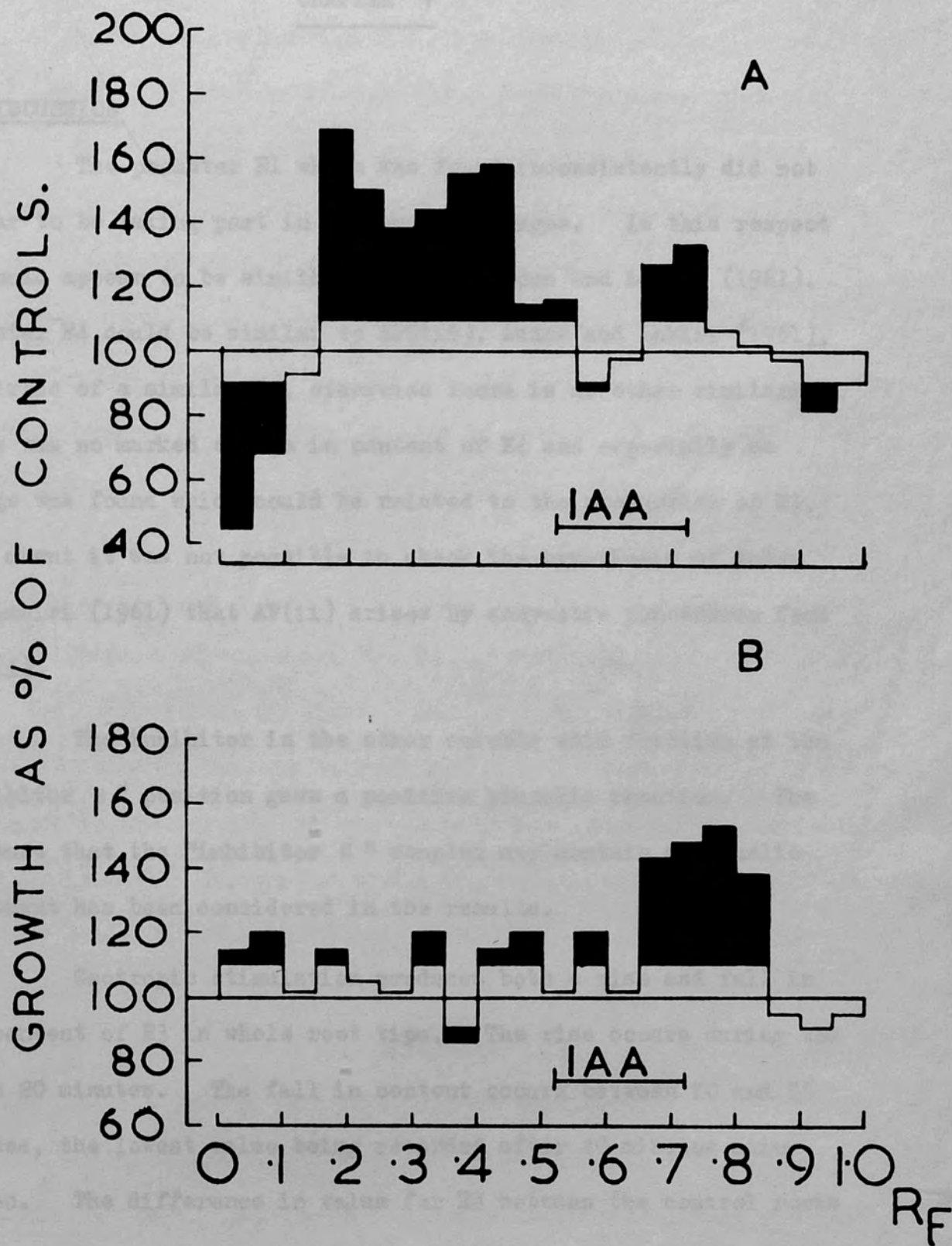


Fig. 28

- (a) Oat coleoptile assay of the growth activity in the NaOH hydrolysed water soluble fraction. Growth expressed as % of controls plotted against Rf values.
- (b) Oat first internode assay of the growth activity in the ether soluble fraction extracted from the NaOH hydrolysed water soluble fraction. Growth expressed as % of controls plotted against Rf values.

FIG 28



CHAPTER V

DISCUSSION

The promoter E1 which was found inconsistently did not appear to be taking part in any growth changes. In this respect E1 would appear to be similar to AP(i), Audus and Lahiri (1961). Promoter E4 could be similar to AP(iii), Audus and Lahiri (1961), by virtue of a similar Rf, otherwise there is no other similarity. There was no marked change in content of E4 and especially no change was found which could be related to the production of E3. This meant it was not possible to check the hypothesis of Audus and Lahiri (1961) that AP(ii) arises by enzymatic conversion from AP(iii).

The inhibitor in the ether soluble acid fraction at the "inhibitor β " position gave a positive phenolic reaction. The evidence that the "inhibitor β " complex may contain a phenolic component has been considered in the results.

Geotropic stimulation produces both a rise and fall in the content of E3 in whole root tips. The rise occurs during the first 20 minutes. The fall in content occurs between 20 and 25 minutes, the lowest value being recorded after 40 minutes stimulation. The difference in value for E3 between the control roots and the maximum value when expressed as IAA equivalents represented a 12-fold increase. The difference between the maximum and minimum values, i.e. 20 minutes and 40 minutes, in IAA equivalents,

represented a 60-fold drop in content.

These results confirm the rapid burst of production of AP(ii) produced by geotropic stimulation found by Audus and Lahiri (1961). However, some differences were found between the two sets of results. Audus and Lahiri recorded an increase in concentration with stimulation of approximately 1000 times the concentration found in vertical roots. The increase in content recorded in these experiments was 12 times that found in unstimulated roots. The second difference between the two sets of results lies in the period of stimulation required to produce the maximum auxin content. Audus and Lahiri found that 40 minutes stimulation was required, whereas in these experiments the maximum auxin content was formed at 20 minutes. A regression line of curvature for Vicia roots cuts the time axis at 36 minutes. Therefore it would appear that the auxin content and the change in auxin content between 20 and 50 minutes must be of great importance in determining the geotropic growth response of horizontally stimulated roots. During the phase of positive curvature Audus and Brownbridge (1957a) found that the overall growth rates were considerably reduced, a point confirmed by Bennet-Clark et al (1959). To explain this reduced growth rate Audus and Brownbridge required the accumulation of a root growth inhibitor on the lower side of the root. Audus and Lahiri suggested that the rapid accumulation of AP(ii) which reached its maximum at 40 minutes could be this inhibitor. Audus and Brownbridge (1957a) indicated that the root growth inhibitor could not be IAA. In their experiments with extracts from Vicia

roots using a starch column Bennet-Clark et al (1959) could find little or no IAA but did find another active growth substance running very close to IAA. Audus and Lahiri (1961) gave reasons to indicate that AP(ii) probably arose by enzymatic conversion from AP(iii) which shows changes inversely correlated with those of AP(ii). They suggested that AP(ii) was produced by a "de novo" synthesis from an inactive precursor. Bennet-Clark et al (1959) could not find the release of auxin found by Audus and Lahiri. Since they made their extractions on vertical roots and roots which had been stimulated for 5 hours, it is possible they missed these specific changes which appear to take place within one hour. Bennet-Clark et al also suggest that release of auxin from the vacuole to the cytoplasm was more likely. The results obtained from these experiments could not distinguish whether E₃ was released from a bound form or generated by a de novo synthesis from an inactive precursor. Though it is tentatively suggested that E₃ may be formed from an inactive neutral ether soluble precursor.

It is suggested that the fall in content of E₃ after 20 minutes stimulation could be attributed to one of three causes. Firstly the increase in free auxin content could be enzymatically destroyed. The destruction could be brought about by the formation of an adaptive IAA like oxidase enzyme system Galston and Dahlberg (1954). The increased concentration of E₃ would induce the formation of the oxidase after a lag period of 10-20 minutes. The increase in rate of destruction of E₃ over the rate of formation

would account for the decrease in content from 20 minutes to 40 minutes. If E3 is being destroyed by an induced oxidase enzyme system then it is likely that some other auxin and not E3 is responsible for the decreased growth in roots following geotropic stimulation. The regression line of curvature for Vicia roots cuts the time axis at 36 minutes. The decreased growth rate in geotropically stimulated Pisum roots recorded by Audus and Brownbridge (1957) required the accumulation of an auxin in the root tip. If E3 is being destroyed between 20 and 40 minutes stimulation then it is difficult to see how E3 could be regulating the growth of the cell. Secondly the excess auxin could be detoxicated by being bound to an amino acid or peptide; for example the binding of IAA to form indolyl acetyl peptides when excess IAA is fed to tissues. Thirdly it is suggested the increased free auxin content could be bound to proteins, especially those proteins contiguous to the cytoplasmic membrane and cell wall. Once bound to these proteins the auxin would be too firmly attached to be eluted off either by alcohol or ether. This would explain the "disappearance" of this auxin. Wildman and Gordon (1942) have found auxin so strongly attached to proteins that it could not be eluted off with alcohol or ether. It is suggested that attachment of the auxin to the protein would make it physiologically active in controlling the growth of the root cells. There is a little evidence that auxin is active in the cell when in a "bound" form. Vlitos and Meudt (1953) could only find IAA in tobacco after alkaline hydrolysis. Bonner

and Foster (1956) have applied kinetics to auxin induced growth and have suggested that IAA can form growth active complexes and that the growth rate (of coleoptiles) was proportional to the amount of complex formed. Gordon (1954) however, thinks that bound auxins are probably not physiologically active. Bentley (1961) suggests that IAA is not the auxin physiologically active in normal growth. She thinks that endogenous and exogenous IAA may not be active at the cellular level until it has been transformed into "some other active agent or complex."

The Cholodny-Went theory of a lateral redistribution of auxin under gravity has been regarded as the primary theory of geotropism. This theory depended on there being no increase in the total free auxin content but merely a deflection from the upper surface of the reacting organ to the lower. However, the results of Audus and Brownbridge (1957), Audus and Lahiri (1961) and the present work indicate that the theory of geotropic response based solely on a lateral redistribution of auxin cannot be correct. The results shown in (Fig. 17) indicate a production of auxin, to a level greater than that found in vertical roots, was formed by geotropic stimulation.

It was hoped to demonstrate a change in content of E₃ in the upper and lower halves of root tips. It was presumed that the auxin content from each half, of a vertical split root, when added should be nearly the same as the auxin content found in whole vertical roots. This assumption was not found to be correct. The E₃ content of split roots was found to be approximately three times less than the content of whole unstimulated roots. When the roots were stimulated the

level of E3 remained at a uniform value of approximately 3.3×10^{-2} $\mu\text{g/g}$. IAA equivalents, from 10 minutes to 40 minutes. No increase in content as was found in whole stimulated root tips was detected. The level of E3 in either half with respect to each other was nearly the same and certainly no large increase in the content of E3 in the lower half of the root was observed. This result was unexpected. Re-examination of the results of Lahiri (1959 pp.232 and 242) seemed to indicate a similar marked decrease in the content of AP(ii) from split roots as compared to the content of AP(ii) from whole roots. Both sets of roots had been stimulated for 60 minutes.

It was thought that cutting the root along the longitudinal media plane was responsible for the difference in auxin content between whole and split roots. The whole technique of slicing roots longitudinally as compared to cutting whole roots was critically considered. In the slicing technique a stainless steel blade was used to minimise activation of enzyme action by metal ions. There were only two differences between whole roots and split roots. The first difference lay in the time the cut surface was exposed to air. In whole roots this was approximately 15 seconds before the root froze. In split roots two cuts had to be made, the first at right angles to the longitudinal axis of the root and the second along the longitudinal axis. The time taken for these two cuts to be made varied between 20 and 30 seconds. Then there was the time taken for the tissue to freeze again approximately 15 seconds. The second difference lay in the surface area of the root which was cut and exposed to air.

In sliced roots this is obviously very much larger.

In the results reference has been made to the inactivation or destruction of auxin, especially by an oxidase similar to IAA oxidase. However, no reference can be found as to the rapidity of auxin destruction and especially the destruction which could occur within 30-45 seconds.

Consideration of the problem has given rise to two trains of thought. Firstly that an inactivation or destruction of auxin, probably enzymatic, was occurring. This had to take place within 45 seconds before the tissue froze. The second suggestion is the production of a substance which could be extracted and interfere with the bioassay response. Each of these two possibilities will be considered more fully. The first postulate requires an inactivation or destruction of the auxin, in this case E3. The process would take place primarily at the cut surface or just below and could be physical or enzymatic or both. Whichever it was it would stop or occur very very slowly once the root was frozen to -70°C . This gives a maximum time of 45 seconds in which the inactivation or destruction could occur. While it is known that certain enzymatic processes in cells can take place well within 30 seconds, is it possible that virtually the total auxin content of stimulated root tips could be inactivated or destroyed.

The second postulate requires the formation of an ether soluble acid. This substance could be produced enzymatically or more probably by direct chemical change at the cut surface. The

formation of this acid would probably involve the oxidative or hydrolytic decomposition of a conjugate or complex of which this substance was part. On chromatography of the ether soluble acid fraction this substance would have an R_f similar to or slightly greater than E3. It is suggested that this substance would interfere with and reduce the first internode response to E3. There is evidence of a substance from whole roots which, it is thought, may interfere with the bioassay response to E3, and this is possibly a phenolic compound. From the colorimetric analysis of the water soluble fraction the presence of phenolic conjugates or complex phenolic compounds is suspected. Many of these phenolic complexes are unstable, Hathway (1960). It should be noted that phenolic acids in neutral chromatography solvents do not produce compact spots but form long "tails" which could overlap and affect the response of growth promoters. It is thought that the low auxin content from split roots could result from a combination of a partial destruction or inactivation coupled with the production of an ether soluble acid which could lower the bioassay response.

The auxins of the water soluble fraction were examined. The high growth activity of the water soluble auxins is in agreement with previous work, Britton et al (1956), Audus and Gunning (1958), Lahiri and Audus (1960), Thurman and Street (1960). A figure of 2.96 µg/g. root tissue IAA equivalents was recorded for the total auxin content of the water soluble fraction in unstimulated roots. Audus and Gunning (1958) state that most of the auxin potential in roots is not IAA itself but a system of water soluble auxin precursors

present in the relatively high concentration of greater than $10 \mu\text{g}$ IAA equivalents/ 100 g. root tissue. They point out that the activity of the water soluble auxins decreases rapidly with time. Eleven days from harvesting of the roots to the assay gave a value of $9.7 \mu\text{g}$ IAA equivalents; after fourteen days this had dropped to $5.8 \mu\text{g}$ equivalents. It should be pointed out that the figure of $2.96 \mu\text{g}$ IAA equivalents per g. root tissue may not be as absurdly high as first thought. The figure of $2.96 \mu\text{g}$ IAA equivalents could come down to nearly $2.0 \mu\text{g}$ IAA equivalents/ g. root tissue when more experiments were performed. This figure is only ten times greater than the value of Audus and Gunning and there is a lapse of no more than four days between harvesting the roots and bioassay.

A change in content resulting from geotropic stimulation was observed for all the promoters of the water soluble fraction. Because the function of these promoters in regulating root growth and geotropic response is unknown, the importance of the change in content cannot be assessed. The results of the split root experiments were varied. It was hoped that content of the water soluble promoters from split roots would equal the content from whole roots. With W1 this was found to happen, the one anomalous feature being that the peak of production of W1 in split stimulated roots occurred sooner than in stimulated whole roots. Until more experiments have been done no significance can be attached to these differences which could be the result of sampling variance. With W2 on the other hand no apparent change of content resulting from geotropic stimulation was found in split roots. The reason for this lack of change is

not known, except that it might have been destroyed or inactivated by the longitudinal cutting. W3 & 4 displayed the reverse trend in that the content from split roots was higher than that found in whole roots. It could be that the content in 100 half roots was now less than supra-optimal for growth and gave a higher response than the whole root sample. This is not thought to be likely and may be a consequence of a sampling variation. Gunning (1961) was able to find water soluble promoters which on elution and rechromatography gave a higher response than originally. He was not able to give an adequate explanation of this phenomenon.

The relationship of growth activity in the pea root dilution test to activity in the coleoptile test of the water soluble fraction has been made. These results indicated a difference in character and function between W1, W2, W3 & 4. W1 was an inhibitor, W2 was less of an inhibitor and W3 & 4 displayed a very small growth inhibition. It was thought that W1 might have a function in regulating root growth since it appeared to be a strong root growth inhibitor. By its formation or disappearance from the upper and lower half of the root tip it could regulate growth and geotropic response.

No bioassay experiments were performed on the phenomenon of interconvertibility but several experiments were made to find interconvertibility using colour tests. Although no interconvertibility was found in nearly all experiments, in one, a positive result was found. The region at Rf 0.4 when eluted and re-run gave a positive phenolic response at Rf 0.0 and 0.40.

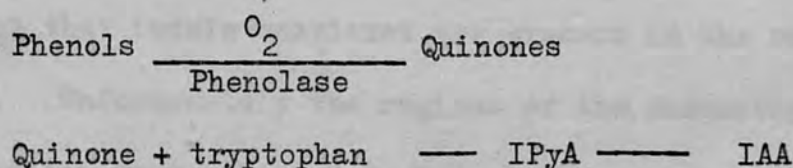
The identity of the components of the water soluble fraction was investigated using colour reactions and fluorimetric analysis. Two compounds were identified with a fair degree of certainty and the presence of one more is tentatively suggested. Tryptophan was identified on the basis of its reaction to Ehrlich, its Rf, and especially the close agreement in the fluorimetric analysis between the presumed sample of tryptophan and a pure sample of D.L. tryptophan. DOPA or a substance very similar to DOPA was identified by its reaction to diazotised sulphanilic acid, its Rf and a fairly close agreement in the fluorimetric analysis between the presumed sample of DOPA and a pure sample of DOPA. It is very tentatively suggested that phenylalanine might be present in the water soluble fraction. This conclusion was come to after comparing the Rf, and colour reactions to Ehrlich and ninhydrin of β -phenylalanine with chromatograms of the water soluble fraction. From fluorescence and colour reactions it is thought that p -coumaric acid may be present in the ether soluble acid fraction following alkaline hydrolysis of the water soluble fraction.

The reaction of the water soluble growth promoters to colour reagents was studied. Surprisingly only one positive purple indole reaction was found, this was tryptophan. This result led to the suspicion that a non indole system of growth promoters was present. However, after alkaline hydrolysis the presence of no less than six indole positive areas was revealed, four in the water soluble fraction and at least one each in the ether soluble acid and neutral fractions.

From preliminary experiments on the hydrolysis of tryptophan it was evident these could not have come from tryptophan. This result meant that indole complexes were present in the water soluble fraction which did not give a positive response to Ehrlich. Because they did not give a positive response to Ehrlich it is likely they were not indolylacetyl peptides since these usually give a purple colour to Ehrlich. From the variety of indole compounds produced on hydrolysis it seems likely that several different indole complexes might be present in the water soluble fraction. It was recognised that some of the indole compounds formed might have arisen from secondary breakdown during hydrolysis of indoles formed initially in the hydrolysis. The water soluble fraction after alkaline hydrolysis was assayed for growth activity. It was noticed that promoter W1 had been replaced by an inhibitor. There was growth activity at some of the indole areas but until these substances have been identified these results have little significance.

The widespread occurrence in the water soluble fraction of substances giving phenolic reactions was observed. It was thought from Rf data that those phenolic substances near the starting line might be conjugated phenols since conjugation lowers the Rf of phenols in a butanolic solvent, Hathway (1960). One of the phenolic substances was identified as DOPA. The coincidence of phenolic areas to regions of high growth activity, cf. W1 and W2, was noticed. Apparently the water soluble phenolic compounds either do not inhibit growth activity or they actively promote or aid growth promotion themselves. The growth activity of tryptophan in the water soluble

extracts was low, however on either side of the area giving a purple reaction to Ehrlich were two areas of very high growth activity and these were associated with phenolic reactions. This provoked the suspicion that there was an interaction between tryptophan and a phenolic compound resulting in high activity possibly by the conversion of tryptophan to IAA. Henderson and Nitsch (1962) have shown that the growth of first internode sections to tryptophan is considerably increased by the presence of ortho dihydric phenols in solution, e.g. caffeic and chlorogenic acids. They thought that the dihydric phenols would doubly augment the presence of IAA by enhancing its biosynthesis from tryptophan and by inhibiting its destruction by an IAA oxidase enzyme system. Gordon and Paleg (1961) have given evidence that IAA can be formed in plants by the following pathway.



However Gordon and Paleg do not think that this pathway is operative in normal intact cells. They suggest the phenols and phenoloxidasases in intact cells are very probably spatially separated.

If it can be proved that one of the phenolic compounds located on either side of the tryptophan zone is an ortho - dihydric phenol then the following suggestion is made. The growth promotion at the W2 promoter position Rf 0.2 - 0.3 may not be caused by a genuine endogenous growth promoter but may be the result of a fort-

uitous juxtaposition of tryptophan and a suitable phenol on the chromatogram.

It is not known if the water soluble promoters were growth active indole complexes or not. There is evidence both for and against the concept of auxin complexes being growth active. Gordon (1954) and Aberg (1957) both suggest that auxin complexes are not growth active. Ascorbigen is a water soluble complex which is growth inactive, Prochazka et al (1957). Ascorbigen will yield IAA on alkaline hydrolysis. Bonner and Foster (1956) suggest that growth ^{active} auxin complexes can be formed. Vlitos and Meudt (1953) could only find IAA after alkaline hydrolysis and it was thought that the IAA might be operative only in a "bound" form. Andreae and Good (1955) discovered that IAA could form an indolyl-acetylpeptide and this complex was growth active in the Avena coleoptile test. There does seem no doubt that indole complexes are present in the water soluble fraction. Unfortunately the regions of the chromatogram from which the indoles were released on hydrolysis were not defined and related to specific growth areas. Consequently no decision can be made whether or not the growth promoters of the water soluble fraction are growth active indole complexes.

It has not been possible to confirm that IAA is present in roots. Nor was it possible to ascertain if the auxin, E3, present in the ether soluble acid fraction was released from a bound form or produced by a de novo synthesis on geotropic stimulation. The increased auxin production in geotropically stimulated roots was confirmed. The lower content of E3 from split roots could result

from a combined inactivation or destruction together with a lower growth response caused by an interfering substance on the chromatogram. The high growth activity of the water soluble fraction was confirmed. The relation of phenolic substances to at least two strong growth promoting areas was noticed. A number of indole complexes unreactive to Ehrlich was observed. These indole complexes will release Ehrlich positive indoles on alkaline hydrolysis.

To answer some of the questions which have been raised in this work the following aspects should be investigated.

The promoter E3 at the IAA region should be freed from the impurity and identified. The reasons for the low content of E3 from split roots should be investigated. One preliminary experiment would be to see what effect an ether soluble acid extract from split roots had on the growth response of E3 from whole roots and on IAA.

Many more experiments should be made on the water soluble fraction from teotropically stimulated whole and split roots. These results should indicate trends in the content of W1, W2, W3 & 4. It might be possible to relate the production and disappearance of these substances both to the formation of ether soluble auxins and to geotropic curvature.

Bulk extracts of the water soluble ether insoluble fraction should be chromatogrammed. These chromatograms should be divided into small areas and after elution hydrolysed with NaOH. Those areas from which indole compounds were released could be investigated more carefully. The indoles should be identified and from this some

indication of the original complex could be gained.

The phenolic complexes should be separated from the indolic ones. Identification of the complexes should be undertaken. The effect of the phenolic complexes on growth should be noted. The possibility of substituted ~~cinnamic~~^{cinnamic} acid complexes occurring in the water soluble fraction should not be overlooked.

An accurate analysis of the change in tryptophan content during geotropic stimulation should be made. This could be done most easily on the Aminco Bowman photofluorimeter.

SUMMARY

- 1 An investigation was made on the effect of geotropic stimulation on the auxin content of Vicia faba root tips. The distribution of auxin between the upper and lower halves of root tips was studied to check the applicability of the Cholodny-Went theory to the geotropic response of roots.
- 2 Modifications were made to the chromatography apparatus and root stimulation apparatus. Two new root cutters, one for whole roots and one for splitting roots longitudinally were designed and made.
- 3 More rapid freezing of the root tips of Vicia faba was brought about by using a solid CO₂ - alcohol freezing bath instead of ice water. Freeze drying of tissues was introduced.
- 4 IAA marker chromatograms were run in the same tank with chromatograms of extracts. The possibility of the Nitsch "transfer" artefact occurring was investigated. Transfer of IAA from the marker to adjacent chromatograms was not observed.
- 5 Inhibitors were found in the industrial methylated spirits used as extractant. A change was made to methanol in which fewer inhibitors were found. Growth inhibitors were detected in the chromatography paper. These could be removed by washing the paper with isobutanol, methanol,

SUMMARY continued

water for two days. An inhibitor was also found in the chromatography room air. The chromatography was subsequently done elsewhere.

- 6 Tryptophan was subjected to the alkaline hydrolysis methods used in the hydrolysis of extracts. No ether soluble Ehrlich positive acids were detected nor were any other Ehrlich positive zones found in the water soluble fraction except for tryptophan itself.
- 7 Four ether soluble acid promoters and one inhibitor were found in methanolic extracts of root tips. Promoters E1, 0.0 - 0.25 (AP(i)) ? E2, 0.35 - 0.55; E3, 0.55 - 0.75 (IAA)? and E4, 0.85 - 1.00 (AP(iii)) ? 0.75 - 0.85 (Inhibitor β).
- 8 The ether soluble fraction gave no colour reactions except for 2 phenolic reactions at Rf 0.30 - 0.45 and 0.7 - 0.80 (probably inhibitor β)
- 9 Four water soluble ether insoluble promoters and one inhibitor were found in methanolic extracts of root tips. The Rf of the promoters were W1, 0.0 - 0.15; W2, 0.2 - 0.3; W3, 0.40 - 0.55; W4, 0.60 - 80. Inhibitor, Rf 0.8 - 1.0
- 10 Colour tests on the water soluble fraction gave a variety of results. Only one purple Ehrlich positive zone was found and this due to tryptophan. An abundance of phenolic reactions at regions of high growth activity

SUMMARY continued

- 11 Alkaline hydrolysis released 6 indole positive reactors. Four in the water soluble fraction and one each in the ether soluble acid and neutral fractions. These could not have come from tryptophan.
- 12 The growth activity of the ether soluble acid auxins in root tips was studied using the oat first internode assay.
- 13 Geotropic stimulation produces an increased auxin production in the ether soluble acid fraction of E3 (The IAA region)
- 14 This increased auxin content was not found in split root tips. The apparent decrease in auxin content was thought to be brought about by splitting the roots. The factor responsible for the apparent decrease was not known.
- 15 Geotropic stimulation produced a change in the content of all the water soluble auxins. The auxin content of longitudinally split root tips also showed changes but these did not conform to the pattern of the auxin change in whole roots.

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TABLE I

Growth of cat first intermede sections to end-gone 13A. Section growth expressed as % of controls.

Section number of 1st intermede	1st		2nd		3rd		4th		5th		6th		7th		8th		Mean	
	Set	Set	Set	Set	Set	Set	Set	Set	Set	Set	Set	Set	Set	Set	Set	Set		
10 ⁰	355.5	333.3	338.8	328.8	320.0	334.9	325.3	337.3	338.2									
10 ¹	294.4	261.1	272.7	279.1	271.1	294.2	291.8	282.2	260.7									
10 ⁻¹	227.2	227.7	227.7	223.3	223.3	200.9	210.5	215.3	220.6									
10 ⁻²	138.8	177.7	175.0	168.8	151.1	169.8	160.2	145.9	160.9									
10 ⁻³	94.4	88.8	92.6	95.5	100.0	112.4	112.4	-	93.3									
10 ⁻⁴	92.6	94.4	92.6	97.7	84.4	90.6	97.8	-	92.3									
Mean control length	13.80	13.80	13.80	14.252	14.252	14.055	14.055	14.055	14.055									
Control length	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00									
S.D. of controls	0.1826	0.1826	0.1826	0.1394	0.1394	0.0988	0.0988	0.0988	0.0988									

(APPENDIX

TABLE I

Growth of oat first internode sections to ^{ex}ogenous IAA. Section growth expressed as % of controls.

Concentration of IAA mg./l.	1st Set	2nd Set	3rd Set	4th Set	5th Set	6th Set	7th Set	8th Set	Mean
10 ¹	355.5	333.3	338.8	328.8	320.0	334.9	325.3	337.3	338.2
10 ⁰	294.4	261.1	272.7	279.1	271.1	294.2	291.8	282.2	280.7
10 ⁻¹	222.2	227.7	227.7	225.3	235.5	200.9	210.5	215.3	220.6
10 ⁻²	138.8	177.7	175.0	168.8	151.1	169.8	160.2	145.9	160.9
10 ⁻³	94.4	88.8	91.6	95.5	100.0	112.4	112.4	-	93.3
10 ⁻⁴	91.6	94.4	91.6	97.7	84.4	90.6	95.6	-	92.3
Mean control length	13.80	13.80	13.80	14.252	14.252	14.085	14.085	14.085	14.085
Original length	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00
S.D. of controls	0.1826	0.1826	0.1826	0.1394	0.1394	0.09888	0.09888	0.09880	0.09888

TABLE 2

Growth of oat coleoptile sections to ~~endogenous~~^{exogenous} IAA. Section growth expressed as % of controls.

Concentration of IAA mg./L.	1st Set	2nd Set	3rd Set	4th Set	5th Set	Mean
10^1	286.1	281.5	307.9	251.4	307.9	287.0
10^0	201.5	207.7	201.9	210.4	211.8	206.7
10^{-1}	141.5	133.8	142.6	146.8	145.4	142.0
10^{-2}	107.6	107.6	103.1	100.2	103.1	104.3
10^{-3}	98.4	96.9	90.3	96.0	90.3	94.4
10^{-4}	95.3	89.2	94.6	103.1	91.8	94.8
Mean control length	15.250	15.25	15.535	15.535	15.535	
Original length	12.00	12.00	12.00	12.00	12.00	
S.D. of controls	0.1291	0.1291	0.10	0.10	0.10	

TABLE 3

Change in fluorescent intensity with increasing pH
Fluorescence values in arbitrary units.

<u>pH</u> <u>Values</u>	<u>Fluorescence Figures</u>	
	<u>DOPA</u>	<u>Unknown sample</u>
0.1	17	25.7
1.0	27	35.0
2.2	62.5	64.0
3.0	97	90.0
4.0	100	100.0
5.0	100	100.0
6.0	90.5	90.0
7.0	80.5	72.5
8.0	55.5	48.0
8.93	46.5	27.0
10.14	3.35	-

TABLE 4

Change in fluorescent intensity with increasing pH
 Fluorescent values in arbitrary units.

<u>pH</u> <u>Values</u>	<u>Fluorescence figures</u>	
	<u>D.L. Tryptophan</u>	<u>Unknown sample</u>
0.1	4.4	5.5
1.0	11.0	17.4
2.2	24.4	23.7
3.0	31.75	36.0
4.0	39.0	41.5
5.0	42.0	45.5
6.0	45.0	51.0
7.0	47.5	51.0
8.0	51.0	53.0
8.93	58.5	58.0
10.14	89.5	88.5
11.07	100.0	100.0
12.10	92.5	91.0
12.97	22.3	23.7
13.20	16.6	22.0
14.00	2.4	6.80

TABLE 7

Ether soluble acid auxins in *Vicia faba* whole root tips. Effect of geotropic stimulation on auxin production. Weight of tissue in all experiments 1.0 g. Oat first internode assay. Growth expressed as total extension.

Figure No. in text	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u
Vial No.																					
1	1.08	1.3	1.90	1.75	0.85	1.85	1.85	1.02	0.88	0.93	2.25	1.40	2.25	1.00	0.70	0.80	2.10	1.05	0.65	2.20	0.90
2	0.97	1.42	2.25	1.75	1.10	1.95	1.90	1.20	0.98	1.05	1.95	1.10	2.20	1.15	0.65	1.08	2.25	0.98	1.03	2.40	1.00
3	0.88	1.25	2.15	2.00	0.85	2.05	2.35	1.50	0.90	0.98	1.85	1.27	1.90	0.85	0.97	0.75	2.30	0.93	0.95	2.20	0.95
4	0.92	1.35	2.00	2.20	0.93	2.05	2.17	1.78	0.95	1.05	1.85	1.35	2.10	0.88	0.80	0.92	2.00	1.10	1.05	2.85	1.05
5	1.05	1.63	2.00	2.10	0.95	2.20	2.10	2.00	0.88	1.05	2.05	1.35	2.00	0.78	0.88	0.82	2.15	1.05	1.13	2.65	1.20
6	1.00	1.30	2.30	2.40	0.87	2.00	1.85	1.70	0.95	0.90	2.50	1.40	2.00	1.00	0.88	0.90	2.15	0.95	0.93	2.25	0.95
7	1.03	1.47	2.10	1.75	0.95	1.90	1.75	1.30	0.92	1.00	2.75	1.60	2.30	1.08	0.98	0.92	2.30	1.10	0.97	2.15	1.05
8	0.95	1.35	2.00	1.85	1.15	2.05	2.05	1.32	1.10	0.95	2.05	1.60	2.00	1.05	1.10	1.08	2.40	0.90	1.05	2.05	0.95
9	0.88	1.32	2.45	2.05	1.05	2.40	2.27	1.50	0.98	1.22	2.20	1.75	2.10	1.15	1.10	0.98	2.30	0.90	1.05	2.50	0.95
10	1.00	1.42	2.35	1.65	1.15	2.65	3.10	1.68	1.07	1.20	1.35	1.53	2.50	1.73	1.00	1.67	2.60	1.03	1.03	2.45	1.10
11	2.15	1.20	2.25	2.35	1.02	3.05	3.67	1.58	0.95	1.15	3.50	1.32	2.45	2.00	0.95	1.25	3.05	0.95	1.65	2.45	1.20
12	1.30	1.20	4.30	2.15	2.35	2.70	2.85	1.60	1.00	1.50	3.25	1.55	2.55	1.13	1.88	0.95	2.45	0.90	1.65	2.35	1.05
13	0.90	1.90	3.75	1.90	2.60	2.95	3.80	1.60	1.65	2.95	4.25	1.75	2.95	1.17	1.53	0.95	3.30	1.05	1.15	3.70	1.05
14	0.78	2.50	2.05	1.60	1.45	5.65	2.75	1.90	2.60	1.73	2.00	1.60	2.85	0.75	1.05	0.70	2.30	1.22	1.20	2.75	0.80
15	0.85	1.85	1.50	1.50	1.03	3.25	1.40	2.05	2.00	1.22	1.35	1.73	4.80	0.98	0.65	0.92	1.85	1.20	0.75	2.00	0.85
16	0.95	1.40	1.95	1.55	1.05	2.00	1.70	1.35	1.00	0.70	2.00	1.40	2.55	0.85	0.78	0.75	2.10	0.85	0.87	2.10	0.95
17	0.85	1.15	2.15	1.73	0.95	2.25	2.25	1.12	0.85	0.93	2.35	1.15	1.75	0.92	0.90	0.92	2.10	0.98	0.85	2.20	0.95
18	0.75	1.20	1.80	1.70	0.85	2.15	1.90	1.40	0.88	0.85	2.25	1.30	1.75	0.85	0.95	0.60	2.05	1.03	0.93	2.25	0.85
19	0.80	1.40	1.95	2.00	0.80	1.80	1.80	1.35	0.95	1.00	2.45	1.45	1.85	0.92	0.80	0.85	2.45	0.90	0.97	2.35	1.00
20	0.90	1.32	2.00	1.95	0.78	1.80	2.25	1.50	0.85	1.20	1.85	1.50	2.05	0.95	1.00	0.95	2.35	0.93	1.10	2.50	0.95
Mean total extension of controls	0.88	1.21	2.20	1.83	0.87	2.08	1.83	1.21	0.88	0.87	2.08	1.21	2.04	0.88	0.88	0.88	2.20	0.87	0.89	2.20	0.89
Standard deviation of controls	.0753	.099	.133	.171	.047	.149	.171	.099	.0753	.047	.149	.099	.102	.0753	.0753	.0753	.133	.047	.088	.133	.088

TABLE 8

Ether soluble auxins in upper and lower halves of root tips. Effect of geotropic stimulation on auxin production. Weight of tissue in each half, 0.5 g. Cat first internode assay. Growth expressed as total extension.

Figure No. in text	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t
Vial No.1	1.37	1.40	1.20	1.67	1.37	1.55	1.9	1.80	2.10	1.45	1.60	0.75	1.45	0.65	1.15	1.55	1.08	1.55	1.00	0.95
2	1.39	1.58	1.45	1.55	1.60	1.58	2.4	1.50	2.40	1.47	1.30	0.85	1.00	0.85	1.05	1.53	1.45	1.77	1.35	1.15
3	1.23	1.35	1.55	1.15	1.25	1.35	1.95	1.83	1.75	1.39	1.45	0.90	1.55	0.80	1.25	1.25	1.15	1.63	1.45	1.15
4	1.74	1.45	1.60	1.23	1.32	1.68	2.10	1.20	1.75	1.39	1.20	0.75	1.28	0.80	1.00	1.25	1.25	1.40	1.35	1.30
5	1.67	1.88	2.18	1.50	1.58	1.70	2.00	1.60	2.05	1.20	1.45	0.80	1.35	0.95	1.00	1.45	1.10	1.88	1.25	1.25
6	1.83	1.65	1.85	2.00	1.85	1.77	2.25	1.30	2.40	1.80	1.40	0.95	1.60	1.10	1.13	1.57	1.40	1.67	1.30	1.18
7	1.83	1.75	1.65	1.60	1.65	2.15	2.35	1.35	2.50	1.60	1.15	0.75	1.60	0.80	1.25	1.50	1.50	1.45	1.25	1.10
8	1.67	1.45	1.65	1.75	1.33	1.80	2.40	1.39	2.25	1.45	1.22	1.10	1.68	1.05	1.28	1.67	1.20	1.57	1.15	1.28
9	1.43	1.38	1.80	1.30	1.42	1.60	1.95	1.74	2.05	1.80	1.40	0.85	1.70	0.90	1.15	1.35	1.40	1.40	1.15	1.20
10	1.95	1.60	1.75	1.83	1.60	1.90	2.30	1.30	2.22	1.50	1.65	0.95	1.25	1.00	1.10	1.65	1.33	1.95	1.25	1.45
11	1.67	1.38	1.60	1.50	1.40	1.50	2.35	1.74	2.45	1.85	1.00	0.95	2.05	0.75	1.23	1.63	1.45	1.67	1.15	1.25
12	1.47	1.65	2.68	2.00	1.52	1.70	2.55	1.87	2.80	1.85	1.05	0.85	1.60	0.75	1.00	1.50	1.25	1.93	1.15	1.20
13	1.87	1.47	1.80	1.90	1.45	3.40	2.15	1.65	2.40	1.95	1.30	1.10	1.47	0.70	1.30	1.40	1.25	1.82	1.23	1.15
14	2.15	2.48	1.92	2.30	2.15	2.50	2.80	1.95	2.55	1.60	1.22	0.70	1.05	0.85	1.65	1.50	1.55	2.40	1.30	1.40
15	1.53	1.65	1.40	2.15	2.12	1.40	2.15	1.05	2.05	1.00	0.90	0.95	0.95	0.75	1.45	1.65	1.40	1.15	1.15	1.20
16	1.43	1.25	1.45	1.20	1.48	1.40	1.95	1.45	1.40	1.35	1.10	0.80	1.10	0.60	1.65	1.40	1.90	1.05	1.55	1.55
17	1.23	1.45	1.95	1.45	1.25	1.68	1.55	1.74	1.70	1.30	1.10	0.60	1.35	0.80	1.15	1.20	1.30	1.25	1.15	1.05
18	1.37	1.32	1.65	1.20	1.25	1.85	1.40	1.67	1.75	1.39	1.15	0.55	0.95	0.85	0.95	1.25	0.95	1.35	1.00	0.90
19	1.67	1.38	1.68	1.74	1.47	1.50	1.70	1.90	2.00	1.67	1.48	0.65	1.10	0.95	1.15	1.63	0.65	1.40	0.85	1.03
20	1.77	1.35	1.55	1.95	1.70	1.58	2.00	2.15	2.25	2.00	1.28	0.95	1.40	0.85	1.15	1.73	0.95	1.45	1.00	1.15
Mean total extension of controls	1.47	1.41	1.78	1.47	1.41	1.78	2.05	1.47	2.05	1.47	1.30	0.89	1.30	0.89	1.16	1.41	1.16	1.41	1.16	1.16
Standard deviation of controls	0.0894	0.0888	0.09	0.0894	0.0888	0.09	0.145	0.0894	0.145	0.0894	0.141	0.0876	0.141	0.0876	0.047	0.0888	0.047	0.0888	0.047	0.047

TABLE 9

Ether insoluble (water soluble) auxins in whole root tips. Effect of geotropic stimulation on auxin production. Weight of tissue in all experiments 1.0 g. Oat coleoptile assay. Growth expressed as total extension.

Figure No. in text	a	b	c	d	e	f	g	h	i	j	k
Vial No. 1	2.45	2.75	2.00	2.80	2.65	2.85	2.70	2.60	2.55	2.45	2.55
2	2.60	2.45	2.45	2.30	2.30	2.70	2.90	2.45	2.35	2.45	2.85
3	1.95	1.95	1.80	1.80	2.05	1.70	2.15	1.65	1.90	2.00	2.25
4	2.05	1.80	1.60	2.25	1.80	2.75	2.00	2.20	1.95	1.80	1.65
5	3.00	2.55	1.75	2.80	2.80	2.05	3.00	1.95	2.15	1.90	2.00
6	2.85	2.25	1.95	2.20	1.80	1.55	2.25	1.70	1.90	1.55	1.40
7	1.80	1.85	1.75	1.90	1.85	1.60	1.90	2.10	1.90	1.65	1.80
8	1.80	1.95	1.80	1.85	1.70	1.65	1.90	2.00	1.50	1.90	1.70
9	1.55	2.00	1.85	1.80	1.85	1.90	1.80	1.75	2.40	2.00	1.75
10	1.55	2.00	1.90	1.45	1.85	2.00	1.70	2.15	2.00	1.85	2.00
11	2.00	2.00	1.80	1.80	1.85	1.90	1.95	2.00	2.05	1.95	1.60
12	1.70	1.80	1.45	2.20	2.15	2.10	2.20	2.55	2.00	2.30	2.10
13	1.90	1.90	1.50	2.05	2.25	2.50	2.00	1.95	2.15	1.65	2.05
14	2.05	1.65	1.80	1.85	1.95	1.95	2.10	1.80	1.75	1.75	1.55
15	1.80	1.60	1.90	1.90	1.75	1.90	1.70	2.05	1.60	2.00	1.20
16	1.35	1.50	1.85	2.00	1.50	1.45	1.60	1.25	1.95	1.60	1.40
17	1.50	1.50	1.25	1.55	1.25	1.60	1.65	1.30	1.45	1.45	1.20
18	1.50	1.20	1.30	1.50	1.35	1.30	1.55	1.40	1.40	1.60	1.20
19	1.25	1.30	1.40	1.25	1.35	1.70	1.40	1.60	1.60	1.40	1.25
20	1.30	1.20	1.30	1.40	1.45	1.70	1.50	1.50	1.50	1.85	1.20
Mean total extension of controls	1.63	1.63	1.58	1.63	1.58	1.50	1.63	1.50	1.58	1.50	1.50
Standard deviation of controls	.0966	.0966	.115	.0966	.115	.173	.0966	.173	.115	.173	.173

TABLE 10

Ether insoluble (Water soluble) auxins in upper and lower halves of root tips. Effect of geotropic stimulation on auxin production. Weight of tissue in each half, 0.5 g. Oat coleoptile assay. Growth expressed as total extension.

Figure No. in text	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	
Vial No.1	1.85	1.60	0.90	1.85	1.00	1.40	2.30	2.90	1.50	2.50	1.45	2.55	1.02	1.50	1.02	1.70	1.75	1.85	1.80	1.40	
2	2.35	1.85	2.65	2.43	2.20	2.45	2.00	2.35	2.35	2.65	2.20	2.65	1.97	2.00	2.34	1.90	1.70	2.35	1.75	2.38	
3	2.05	1.75	1.75	2.02	1.82	2.00	1.55	1.70	1.85	1.80	1.75	2.10	1.70	1.50	1.90	1.50	1.60	2.03	1.90	2.17	
4	1.97	2.15	1.60	1.97	1.90	1.95	1.75	1.80	1.85	1.85	2.65	1.85	1.72	1.70	1.92	1.55	1.55	2.68	1.85	2.32	
5	2.15	1.85	1.65	1.90	1.90	1.65	1.80	1.90	1.70	1.55	1.70	1.70	1.82	2.05	1.78	1.65	1.85	2.12	1.80	1.92	
6	1.87	1.60	1.55	1.93	1.55	1.65	1.40	1.50	1.85	1.50	1.65	2.25	1.75	1.75	1.35	1.75	1.70	2.08	1.80	1.77	
7	1.83	1.65	1.60	1.97	1.75	2.15	1.55	1.70	1.70	1.65	1.75	1.60	1.97	1.35	1.40	1.65	1.75	2.02	1.65	1.88	
8	1.90	1.55	2.16	1.87	1.41	1.80	1.45	1.55	1.70	1.50	1.90	1.65	1.62	1.65	1.52	1.55	1.50	2.20	1.65	2.08	
9	2.37	2.10	1.95	2.10	1.82	2.20	1.65	1.90	1.60	1.40	2.35	1.75	1.68	1.90	1.55	1.90	1.70	2.20	1.70	1.95	
10	2.07	2.05	1.70	1.97	2.07	2.00	1.45	1.75	2.00	1.80	2.15	2.30	1.95	1.60	1.88	1.75	1.70	2.17	1.75	2.25	
11	2.40	1.80	2.10	2.33	1.95	1.95	1.85	2.25	2.20	1.95	2.00	2.05	1.87	1.45	1.40	2.15	1.75	2.40	1.75	2.37	
12	2.07	1.75	2.30	2.05	1.72	2.25	1.55	1.85	2.45	1.90	2.45	2.10	1.92	1.90	1.80	1.90	1.70	2.45	2.05	1.82	
13	2.15	1.27	2.55	1.95	1.80	2.40	1.65	1.65	2.05	1.70	2.20	2.40	1.67	1.80	1.57	1.80	2.05	1.95	1.80	1.95	
14	1.78	1.90	1.70	1.80	2.15	2.00	1.90	1.85	1.85	1.55	2.40	1.50	1.70	1.95	1.45	1.55	1.75	1.83	1.90	1.77	
15	1.40	1.67	1.70	1.47	1.90	1.70	1.60	1.75	1.95	1.40	2.10	1.70	1.72	1.85	1.30	1.70	2.05	1.47	2.20	1.23	
16	1.53	1.45	1.85	1.35	1.80	1.55	1.45	1.40	1.80	1.70	1.45	1.40	1.22	1.70	1.30	2.00	1.75	1.43	1.95	1.27	
17	1.30	1.20	1.65	1.42	1.57	1.55	1.30	1.30	1.15	1.25	1.45	1.45	1.22	1.60	1.40	1.88	1.75	1.52	1.65	1.52	
18	1.68	1.50	1.20	1.65	1.30	1.35	1.10	1.15	1.10	1.20	1.35	1.25	1.20	1.40	1.37	1.75	1.40	1.70	1.40	1.65	
19	1.55	1.45	1.15	1.45	1.50	1.15	1.15	1.45	1.05	1.70	1.35	1.45	1.52	1.20	1.45	1.45	1.45	1.70	1.15	1.45	
20	1.60	1.62	1.55	1.63	1.80	1.55	1.20	1.20	1.30	1.30	1.20	1.50	1.42	1.35	1.65	1.45	1.35	1.83	1.35	1.43	
Mean total extension of controls	1.79	1.46	1.63	1.79	1.46	1.63	1.50	1.50	1.63	1.50	1.63	1.50	1.46	1.49	1.46	1.49	1.49	1.49	1.79	1.49	1.79
Standard devi- ation of controls	.083	.064	.0395	.083	.064	.0395	.173	.173	.0395	.173	.0395	.173	.064	.0745	.064	.0745	.0745	.083	.0745	.083	

TABLE 11

Water soluble auxins from 2.0 g. root tips. 2 tenfold dilutions made on each original concentration. Assay - Pea root extension test. Growth expressed as total extension.

Vial No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Original concentration	4.4	6.25	7.55	5.00	8.45	8.10	5.75	2.45	8.15	8.60	8.1	8.35	10.57	3.5	8.75	9.05	0.90	3.68	9.60	10.1
Dilution 10^{-1}	5.7	8.4	8.05	9.10	9.00	8.05	9.94	9.10	8.70	8.20	8.8	8.55	10.17	10.25	9.35	10.30	9.1	8.6	10.50	11.03
10^{-2}	7.45	4.15	8.50	9.40	9.25	10.3	7.40	9.45	9.00	9.80	9.9	9.00	10.00	5.6	8.45	9.65	11.4	10.5	11.0	9.85
Mean total extension of controls	9.43																			
Standard deviation of controls	0.7225																			