STUDIES ON THE EFFECTS OF THE APPLICATIONS OF SYNTHETIC GROWTH SUBSTANCES, ON THE NATURAL HORMONE CONTENT OF PLANTS.

by

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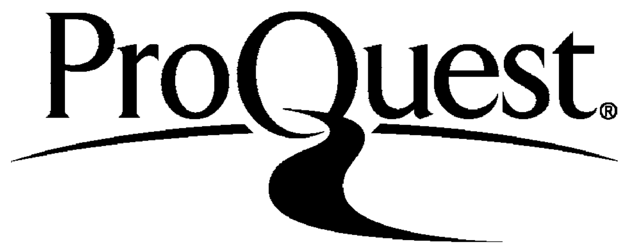

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SUMMARY.

The possible effects of growth regulating substances on the level of β-indolylacetic acid in plants have not been fully studied in the past. This has been mainly due to the lack of sensitive methods for the separation and estimation of natural auxins in extracts of plant tissues. For this reason,seedlings of peas,beans and sunflowers have been treated with 2,4-Dichlorophenoxyacetic acid (2,4-D.); with the supposed anti-auxin 2,3-5-tri-iodobenzoic acid (TIBA); and with maleic hydrazide (MH), a 'growth regulating substance'. These substances have been applied at non-toxic levels,but in sufficient quantities to cause well marked growth responses. Alcoholic extracts of both treated and normal plants have been analysed by a method of paper partition chromatography,and estimations of the quantities of active growth substances determined by a direct,sensitive bioassay of the developed chromatogram.

Indolylacetic acid,and a second inhibitory acidic compound have been recorded in all tissue extracts. At least three other active growth substances have been detected in certain tissues. Only the effects of treatments on the IAA levels have been followed in detail.

2,4-D. treatment of plants has no detectable effect

on the level of IAA in the extracts of either roots or shoots. It is concluded therefore that 2,4-D. exerts its effects on growth directly and not via a disturbance of IAA metabolism.

There are indications that growth of pea seedlings in maleic hydrazide may slightly increase the level of IAA in their roots,but further work is necessary.

Growth of pea seedlings in TIBA solutions,however, causes a marked reduction in the IAA content of the roots. The implications of this result is discussed in relation to the physiological and morphological responses of plants to TIBAtreatment.

Acknowledgements.

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INTRODUCTION.

INTRODUCTION.

Charles Darwin's classical investigations into the tropic movements of plants, formed the starting point of extensive research into the physiology of the hormone control of plant growth. He showed that the bending of a coleoptile, brought about by unilateral light, was due to the perception of the light stimulus by the tip and the transmission from this tip of an 'influence' which stimulated the growing region below. Following on from this work, Paal and Soding demonstrated that the 'influence' was a chemical substance; and in 1926 F.W.Went isolated this substance by allowing it to diffuse into an agar block, which could then function as the coleoptile tip. This was the first separation of a growth hormone from a plant, and utilizing this technique, Went worked out a quantitative test for growth active substances - the *Avena* test.

After these initial discoveries, attempts were made to isolate and identify the chemical nature of this growth stimulant present in plant tissues. Direct and indirect evidence eventually led to the concept that β- indolylacetic acid (IAA) was the chief hormone present in plants, and that

it provided the key to the mechanism of plant growth. This outlook is,however,widening as other naturally occurring active substances are discovered and chemically identified.

Demonstration of the physiological properties of auxin (IAA) followed rapidly; it was found to be essential for growth,and to be able to inhibit or stimulate growth depending on its concentration. It was shown to control apical dominance,stimulate the production of adventitious roots and to be the primary factor controlling fruit set and the development of abscission layers in plants. The level of auxin in plants also seemed to be an important factor in controlling organ differentiation. (Skoog & Tsui 1948.).

Chemists used β - indolylacetic acid as a molecular model and synthesised a number of homologues which had similar physiological properties to those of the natural auxin. Compounds such as naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxy acetic acid (2,4-D.) were shown to stimulate and inhibit growth,stimulate root production and prevent fruit abscission,as did IAA. A large number of such substances were discovered,and although they differed in certain of their specific properties,all were characterised by their ability,in low concentration,to 'stimulate the growth of shoots,freed as far as possible

from their natural auxin content' :- the critical property of a plant "auxin". Auxins show a dual control over all their growth functions, that is, they can either stimulate or inhibit a particular growth phenomenon, depending on their concentration in the tissues. Non-physiological concentrations of these synthetic auxins were found to be toxic to plants, and it became apparent that this toxicity set in at different concentrations in various plant species. This differential sensitivity to high concentrations of auxins has been exploited in the control of weeds in agricultural crops.

A number of the homologues which were tested appeared to antagonise the effect of auxins, for example, indole iso-butyric acid (Burstrom 1951), 1 naphthylmethyl sulphide acetic acid (Aberg. 1951) and 2,3,5-tri-iodobenzoic acid (TIBA). These substances, known as anti auxins were shown to inhibit auxin induced growth and alleviate auxin inhibition (Aberg. 1950). As the investigation of the activity of chemical compounds widened, a number of non-homologous substances were tested. Some of these, although not having the critical stimulatory property of an auxin, did have effects on other plant growth phenomena, e.g. Maleic hydrazide (MH) which was found to break the apical dominance of shoots. (Schoene & Hoffman 1949).

As a result of these investigations, a very large

number of 'plant growth regulating substances' are now known. These have varying physiological properties and are of varying molecular patterns. It is not surprising, therefore, that it has proved impossible to construct a simple theory to explain the actions of all these substances. In recent years, most of the suggestions, for the mode of action of auxins, have been based on the relationship of activity to molecular structure. (Thimann 1951; Veldstra 1953). It is generally considered that the primary step in the regulation of plant growth, by IAA, involves its combination with a 'receptor' to form an active complex. The nature of this 'receptor' is unknown - there is some evidence for the formation of an auxin/protein complex in plants (Siegel & Galston 1953) and some for a coenzyme A/auxin link up. (Leopold 1954). All synthetic auxins have basic molecular similarities to IAA and they are therefore, considered to exert their effects by 'fitting in' to the same receptor, and hence, in a sense, supplementing the natural IAA content. The differences in certain of the effects of these synthetic auxins are attributed to the secondary structural properties of their molecules.

In the same way, the antagonistic action of some 'anti-auxins' can be explained by competition with IAA for this receptor surface. The mechanism of action of the non-homologous growth substances is more obscure and is

probably specific to each substance. Some auxin antagonistic effects, have however, been claimed for maleic hydrazide (Leopold & Klein 1952), but these cannot be explained by a mutual competition with IAA for a receptor surface.

For many years however, arguments have been put forward, that synthetic growth substances act indirectly, by altering the level of the natural auxin in plants. Skoog (1947) suggested that the action of 2,4-D. could be explained by the bringing about of a release of IAA from an inactive protein complex, which then became available to promote growth. The synergistic effect of certain IAA homologues has also been given a similar explanation. (Thimann & Bonner, 1948). Disturbances of the natural auxin metabolism in plants has been suggested as the bases for the effects of auxin antagonists, of both the homologous (TIBA. Aberg. 1953) and non-homologous (MH. Andreae 1954 and Aberg 1953) types.

Most of the support for these suggestions has been of an indirect nature, in that it has been judged on external responses of plants, with no direct attempts to measure their auxin content*. For this reason the present

* Since the commencement of the work, the results of investigations of this kind have been published. (Henderson & Deeoe, 1954; Weintraub, 1953; Pilet, 1953; Fransson & Ingestad, 1955.).

work was started, four years ago.

The problem was two fold, in that (a) a method of extraction of the auxin had to be established; and (b) a method of quantitative estimation of this auxin, developed.

Up to 1951, the study of naturally occurring auxins was limited by the lack of sensitive methods for the separation and chemical identification of the various fractions of plant extracts. The actual extractions were carried out with a wide variety of solvents and the final concentrated extracts tested as a whole, for growth activity, using one of a number of bioassay techniques - mainly the Avena curvature test. (Thimann & Skoog, 1940, 1942; Avery et al, 1940, 1941, 1942). The activity of these extracts, shown by the tests, was attributed to IAA, for various reasons: (1) Molecular weight estimation - determined by diffusion rates. (Kramer & Went, 1949.); (2) Acid labile, alkali stable character. (Berger & Avery, 1944.); (3) Disappearance of the majority of the activity by treatment with IAA oxidase enzyme. It was however reasonable to expect an extract of plant tissues to contain more than one auxin, quite apart from the possibility of inhibitors, which could affect the assay. The values for growth activity obtained by these methods were therefore of little quantitative use.

Methods which were later developed provided means of

separation and specific estimation of the various active growth substances present in a plant extract. These methods included counter current analysis (Holley et al. 1951); paper ionophoresis; column (Linser 1951) and paper chromatography. (Bennet-Clark,1952; Luckwill 1952; Terpstra,1953.).

Chromatography,first developed by Tswett in 1910, has only recently been widely used for the separation of constituents,present in very low concentrations,from complex mixtures. Such a method was ideal for the investigation of the complex extracts obtained from plant tissues. The column,or paper chromatogram could be divided into sections,the contents eluted with various solvents,and a concentration activity curve obtained with any suitable assay technique. The R_F position of any active substances could thus be determined and compared with those of pure synthetic compounds,which would enable their chemical nature to be determined with a fair degree of certainty. If they were in sufficiently high concentrations,substances,once identified,could be detected and estimated colorometrically with suitable sprays. Paper partition chromatography,in particular,provided a method in which very small amounts of active substances could be dealt with,as direct bioassay of the chromatograms was possible. Bennet-Clark et al. (1952) were the first

to describe such a method, using the Avena coleoptile straight growth test to assay chromatograms of the ether extracts of plant tissue. The developed chromatogram was dried, cut into squares, and each was transfered to a dish and moistened with sucrose solution. Sections were grown in these solutions for 24 hours, in the dark, and at 21°C. The coleoptile sections were sensitive to 0·01 parts per million (p.p.m.) of β- indolylacetic acid, and the presence of this and other active compounds on the chromatograms, could be detected by an effect on the growth response of the sections. It was hoped to develop a method of direct bioassay of paper chromatograms which would show a greater sensitivity to IAA and so increase the accuracy of estimations of this substance in plant extracts.

The development of chromatographic methods thus made possible the separation and specific estimation of growth substances present in plant extracts. The remaining problem lay in the selection of a method of extraction of these substances from plants, particularly IAA. The extraction of the latter is complicated by two factors -

(a) The question of the 'form' of the auxin extracted and estimated, and the relation of this to the physiologically active concentration in the plant;

(b) The labile nature of IAA and its possible loss by enzymatic breakdown and photolysis during the extraction processes.

It is generally considered that the extractable auxin of a plant is in two main forms, - (1) Bound auxin, in which the auxin is combined, or 'fixed' in some way to form an inactive complex, and from which it can be released by various processes; and (2) Free auxin, which is thought to be the IAA, physiologically reactive within the plant at the time of extraction. (van Overbeek, 1944; and Gordon, 1953).

The extraction methods which have been developed, can be broadly divided into two groups, according to the 'form' of auxin extracted. There are methods which rely on techniques to bring about the release of IAA from complexes within the plant. These include long term extractions of plant tissues with a variety of solvents, (Avery et al. 1940, 1941 and 1942); hydrolyses of the tissues with strong alkali, (Avery, 1945); and enzymatic digestion techniques. (Thimann et al, 1942, ~~Judkins, 1945~~.). Estimations of the activities of such extracts give values of the 'total' IAA, bound + free. It seems quite likely, however, that these methods of extraction, may bring about the release of IAA from complex molecules, which are not in fact the natural precursors in plant metabolism. The estimated yield of auxin would then bear no relationship, even to that potentially functional in the plant. The extractions of 'free' auxin, in contrast, depend on processes which prevent

the breakdown of the plant complexes :- such as short term extractions at low temperatures,(Kramer & Went,1949); preparatory boiling or freezing of the tissues,(Gustafson, 1941,and van Overbeek,1944); or by the addition of certain enzyme inhibitors. These methods claim to extract that portion of the IAA,reactive in the plants at the time of extraction.

The distinction between 'free' and 'bound' IAA can not,however,be a clear-cut one,as there must be a state of equilibrium between the natural precursors and 'free auxin'. Bound auxin may also include the possible complexes at the actual growth centre. There is no method which can claim to extract and estimate only that IAA which is physiologically reactive,at any one time. The only way to tackle this particular problem seemed to lie in the adoption of a basic method of extraction,to be carried out under carefully controlled conditions,to give comparable results between experiments; the method to be one which limited as far as possible the breakdown of complex compounds,during the process.

It was thought that development of a carefully controlled extraction technique of this type,and chromatographic separation of the extracts,followed by a sensitive bioassay,would show up any effect of the application of growth substances on the natural auxin

content of plants: the 'natural auxin content' to be taken as that level obtained by the particular extraction method under standardised experimental conditions.

METHODS.

1. Growth of material.

2. Extraction techniques.

3. Method of chromatography and plant growth substance assay.

1. Growth of Material.

In the first series of experiments, plants of Vicia faba, Phaseolus vulgaris and Brassica oleracea were grown for periods of up to 5 weeks under normal daylight conditions in a greenhouse.

In the second series of experiments all plants were grown in a constant temperature room with artificial illumination. The temperature was constant at 25°C. and a 14 hour day was supplied by a bank of fluorescent tubes which gave a light intensity of 100-200 foot-candles. The humidity of the room was not controlled, but remained at approximately 35% R.H. Seedlings of peas (Pisum sativum - Meteor) and sunflower (Helianthus annuus) grown under these conditions for periods up to three weeks showed no etiolation and growth appeared normal. Sunflower seedlings were grown in soil in wooden flats; peas were germinated and grown for two days in sand and in darkness and then transferred to tanks of aerated tap water, the seedlings being supported by a perforated perspex sheet which was held at the level of the solution by four glass hooks.

2. Extraction techniques.

A. Methods used for the preliminary extractions :-

In the first series of experiments, the primary solvent of the plant tissue was glass distilled water, followed by extraction of the water/plant mixture with ether. The latter was freshly distilled over 10% Ferrous sulphate, to remove all traces of peroxides which had been shown to affect detection of β-indolylacetic acid. (Seigel & Weintraub 1952).

Details of the basic method. (Method Ia.).

The plant tissue was harvested, the fresh weight recorded and the material ground up in a mortar with a small amount of washed and sterilized sand. The mixture was made up to 100ml. with glass distilled water, 100ml. of ether was added and extraction allowed to continue for 24 hours, in the dark at + 5°C. The ether layer was removed and stored at this temperature; a further 100ml. of ether was added to the brei and left for 8 hours. This layer was separated off, added to the first and the total volume of ether was evaporated down on a water bath to approximately 2ml. A sample of this concentrate was added to a weighed filter paper strip, which was immediately re-weighed to give an estimation of the quantity, but the

rapid evaporation of the ether made this a very inaccurate measurement. The growth activity of the solution on the strip was then assayed by the method to be described.

To increase the accuracy of the measurement of the quantity of extract added to the strip, the final ether extract was evaporated to dryness and the residues taken up in 2ml. of 5% ethyl alcohol. Samples of this solution could be more accurately weighed on the filter paper strips.

A number of experiments were carried out using this method of extraction but the assayed chromatograms gave very varied results. It was thought that part of this variation was due to :-

(a) Differential effects in the initial maceration of the tissues.

(b) The long period of extraction of the water/pulp with ether (32 hours), during which time enzymatic release and/or breakdown of IAA could occur.

(c) The small sample taken from the final concentrate and the inaccuracy of the method of measurement of this quantity, which meant that a fair comparison between experiments could not be made.

Attempts were therefore made to alter the technique to reduce the variation.

Method Ib.

Larger quantities of tissue than were used in the previous extractions, were harvested and broken up in

200ml. of distilled water + 10ml. of ether, for two minutes, in a Wareing Blender. The aqueous mixture was then mechanically shaken with 100ml. of ether for 10 minutes, and left for 14 hours at +5°C. after which time the ether layer was removed. A further 100ml. of ether was added, the flask shaken for ten minutes and the ether layer immediately removed and added to the first. The total ether extract was evaporated down to a small measured quantity (approximately 1ml.) and half of this was placed on a filter paper strip and assayed for growth activity.

A number of extractions were carried out by this method but the results were still variable. The main disadvantage was that the mechanical shaking caused a stable emulsion to form at the water/ether surface so that complete removal of the ether extract was impossible.

Method Ic.

The main technique was as above but the emulusion was partially broken up by filtering off the plant material in an atmosphere of ether just before removal of the ether layer.

Method Id.

A final modification of the technique was tried in an attempt to prevent the formation of an emulsion. The plant material was filtered off immediately after it had been macerated with water in the blender. The filtrate and residue were then separately extracted with ether, the

filtrate was shaken with 100ml. of ether and left for 14 hours and the plant residue was covered with 100ml. of ether for the same period of time. The total ether extract was then evaporated down as before and assayed for growth activity. This treatment did prevent the formation of an emulsion,but it was clumsy to carry out and the results were no less variable.

As a result of the failure of these methods to provide a suitable extract for assay,they were abandoned for a method in which the initial solvent was organic and in which the plant material was removed and discarded before the final extraction with ether.

B. Final extraction method.

Numerous methods of extraction of auxins from plant tissue have been described in recent literature, which in various ways have attempted to prevent concurrent enzyme activity. A method described by Bennet-Clark et al (1953) had been shown to give constant results and was adapted for future extraction experiments.

Details of the technique.

(1) The plant material was harvested, weighed and immediately frozen by placing in a glass vessel in a vacuum flask, packed with dry ice.

(2) The frozen tissue was macerated in a Waring blender for three minutes with 250ml. of approximately absolute alcohol. (In later experiments, the fresh tissue was immediately broken up in alcohol (kept at +5°C.) without prior freezing.)

(3) The macerated tissue was extracted with the alcohol for 24 hours at approximately 0°C. - in the ice box of the refrigerator. The plant residue was then separated off from the alcoholic extract by centrifuging at 2,500 revs. per minute, and was washed with a minimal amount of fresh alcohol, which was immediately separated off in the same way and added to the main extract solution.

(4) The total alcoholic extract was concentrated down by vacuum distillation, at a temperature of 28-30°C. The distillation was carried out in dim light for

approximately $1\frac{1}{2}$ hours until the volume was reduced to about 10ml. The distillation flask was then washed down with distilled water, the solution acidified to pH 3 with Analar phosphoric acid and transferred to a continuous liquid extractor.

(5) The acidified aqueous solution was extracted for $1\frac{1}{4}$ hours with 120ml. of ether, which had been freshly distilled over 10% Ferrous sulphate. A rough calculation estimated that the total ether volume passed five times through the solution in the extractor during this period.

(6) The ether solution obtained was either assayed directly, as in the first extraction experiments, or was further purified by separation into neutral and acidic fractions. The acidic substances were removed by shaking the ether with 3 lots of 20ml. of 5%, ether washed, Sodium bicarbonate solution. The remaining ether solution, containing the neutral substances could be concentrated down and assayed, at least in the non-green tissue extracts, without further purification.

(7) The Sodium bicarbonate solution was acidified to pH 3 with phosphoric acid and the acidic plant substances extracted by shaking the solution with 3 lots of 20 ml. of freshly distilled ether. The latter was washed with distilled water, (acidified with 3 drops of phosphoric acid) and concentrated down in a water bath, kept at 40°C.
The final concentrate (approximately 1ml.) was added to

the filter paper strip,from a sterilized pipette,and the strip was run with the iso propyl/ammonia solvent,dried and assayed.

Conditions of the extraction experiments.

(1) Ground glass apparatus was used for all the processes, so that it could be soaked in alcohol and thoroughly washed between successive experiments.

(2) A full duplicate set of glassware was eventually obtained,for control and treated extractions.

(3) The growth vessels used for the assay of the chromatograms were soaked for at least 24 hours in alcohol, washed in tap,then distilled water and oven dried before each experiment.

(4) To standardise the conditions as much as possible, samples of plant tissue were harvested and macerated with alcohol at approximately the same time of day in each experiment.

3. Method of chromatography and plant growth substance assay.

The method of chromatography was based on that described by Bennet-Clark et al (1952), with elaboration of a new bioassay technique (Audus & Thresh 1953).

Technique of chromatography.

The chromatogram strips were cut $1\frac{1}{2}$ inches broad by 20 inches long from Whatman's No.2 paper. The final ether extract of plant tissue was evaporated to a small volume and dropped from a pipette along a transverse narrow band about 3'' down from the upper end of the strip and allowed to dry. In some experiments in which the final extract was slightly wet, an infra red lamp was used to speed up the drying of the spot on the paper. This excessive heating however, seemed in some cases to impede the running of the substances contained in the extracts.

The strips were suspended in the tanks between glass rods with their upper ends dipping into a glass trough and were left to equilibrate for 1 hour with the vapours of the particular solvent in the base of the tank. The solvent was poured into the trough through a small hole in the glass lid of the tank and allowed to run down the strip for approximately 14 hours in the dark. During this time the solvent travelled about 12-14'' depending on slight

variations of temperature in the laboratory.

The strip was removed, and after the position of the solvent front had been marked it was dried rapidly by means of an electric fan. The chromatogram was cut up into a number of transverse strips, the number varying with the particular needs of the experiment. Each strip was wrapped around a glass square, cut from a microscope slide and inserted into a glass vessel 2·5cm. in diameter and 2cm. high. A known volume of ½% sucrose in glass distilled water was added to flood the filter paper and provide a growth medium for the assay material.

The assay material consisted of 2mm. sections cut 2mm. behind the tip of the 3-day old Meteor pea seedlings. It had been established that sections from this zone showed the greatest extension growth in sucrose solution. (Garrard, 1951). The pea seeds were soaked in running aerated tap water for 24 hours, planted in sterilized sand, moistened with distilled water and grown for two days in a constant temperature room at 25°C. The seedlings were washed in distilled water, the sections cut by means of a special guillotine, and placed in a petri-dish on filter paper moistened with ½% sucrose. Not more than a 100 sections were cut at a time, as during the time taken to weigh a larger number an appreciable amount of growth occurred. Ten sections were taken at random from the

dish, surface dried by rolling gently between filter paper and weighed rapidly to the nearest o.Img. on a microtorsion balance. The weighed sections were transferred immediately to the moistened filter paper strip in a growth vessel, which was fitted with a cork with a central hole to ensure free aeration. The vessels were placed in an incubator in the dark at 27°C. and measurements were taken at intervals during the growth period. The sections were removed, surface dried and re-weighed in the same order as in the initial weighings. The ratio of growth of the ten sections on any one strip, to the mean growth on the control strips was determined and a graph plotted to show the relation between the growth response and the position of the strip on the chromatogram. Two or three control strips were cut from each chromatogram above the original extract spot, to take into account the possible effect of solvent residues on the paper. With the solvent used no significant effect of this nature was observed.

The R_F values of active substances on the chromatogram could be determined from such a graph.

Growth conditions for maximum sensitivity.

Only small amounts of active substances were likely to be present in plant extracts, the assay was therefore required to be as sensitive as possible. Maximum growth, which would give maximum sensitivity could be obtained by

the use of relatively large volumes of sucrose in which the availability of the growth medium would not be a limiting factor. Large volumes of liquid could not be applied to the strips from chromatograms of plant extracts because severe dilution of the growth factors would occur reducing the chances of detection and estimation of these substances. Experiments were therefore carried out to determine the smallest volume of sucrose which would support a satisfactory growth of the sections in the growth vessels.

Quantities of sucrose from 0·1 to 1·0ml. were added to the filter paper strips ($1\frac{1}{2}$'' x ·65'') in the growth vessels and the percentage increase in growth after 24 hours, was determined by weighing. The results are shown in Fig.1.

Very little growth was obtained in 0·1ml. of sucrose; there was a linear increase in growth with increase in the volume of sucrose up to ·6ml. and optimum growth was obtained with 0·7ml. Beyond this point the growth declined rapidly due to water logging of the sections, and only a 40% increase was obtained with 1·0ml. of sucrose. At the optimum volume approximately 140% increase was recorded and this was felt to be sufficient to allow accurate determinations of responses to growth substances.

These experiments were continued over a period of

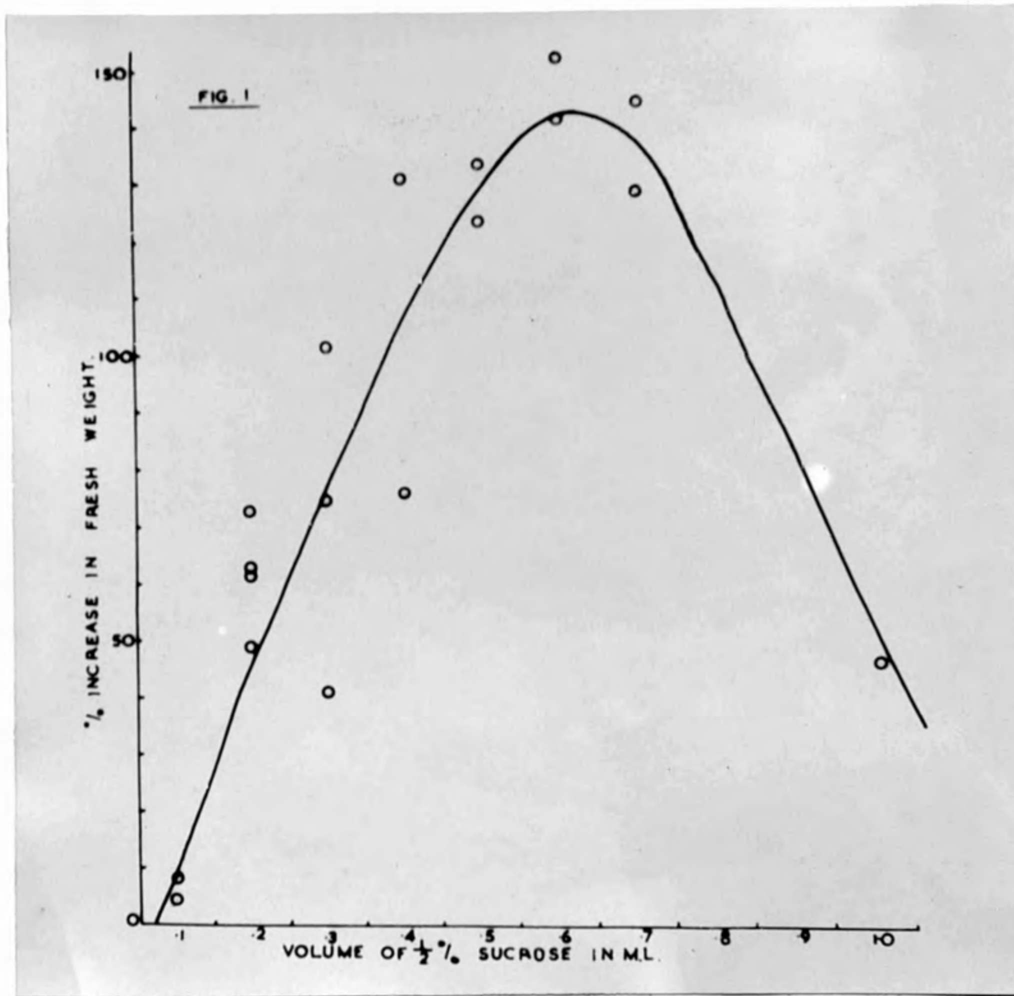


Fig. 1. Growth of pea root sections in varying volumes of sucrose solution.

48 hours and it was noted that even with 0·7ml. sucrose, only 150-160% total increase in growth was obtained. (Experiments carried out at a much later date gave approximately an 180% increase, - due to improved technique.) When larger volumes of sucrose were used and the sections grown in petri dishes a total increase of 220-230% occurred, so obviously the quantity of sucrose in the vessels, in the later stages of growth, was a limiting factor. Because of this fact the growth progress curve over a period of 48 hours was studied to discover the optimal time interval to allow before taking growth measurements. Groups of 10 sections were weighed and placed in each of a series of growth vessels with 0·7ml. of ½% sucrose. The sections were weighed after 3, 6, 12, 23, 28, and 48 hours. The results are shown in Fig.2.

The growth rate of the sections in the vessels remained constant over a period of 24 hours but then quickly fell to zero. When the sections were grown in an unlimiting supply of sucrose, extension continued for 60 hours but the initial rates over the first 24 hours were in no way different in the two growth techniques.

As a result of these experiments, in the final assay technique a standard quantity of 0·7ml. of ½% sucrose was added to each vessel and growth recorded after 24 hours. In some experiments in which very narrow strips had to be

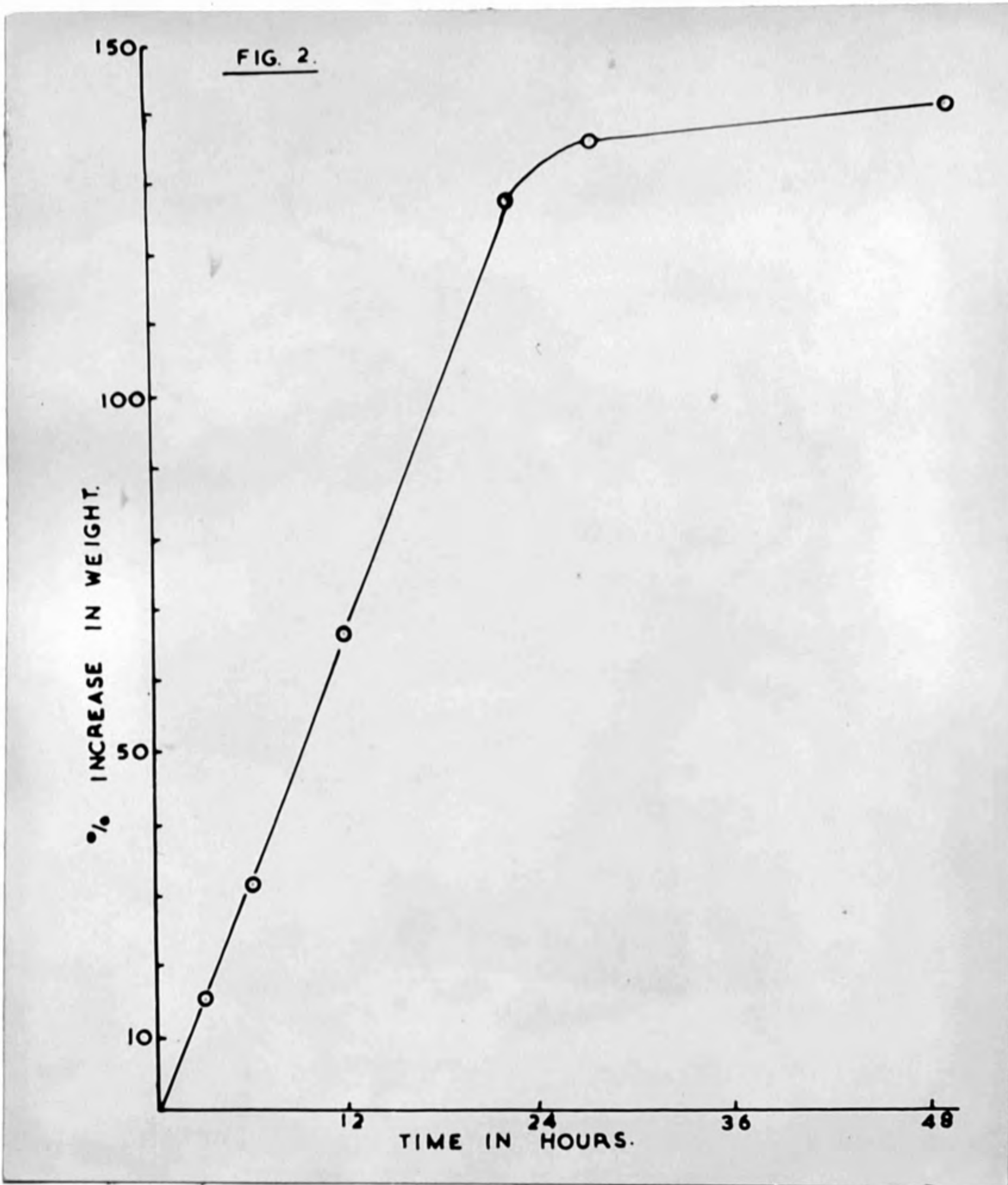


Fig. 2.Growth curve of pea root sections in an optimal volume of sucrose solution.

cut from the chromatogram the volume of sucrose was reduced to 0·6ml.

Choice of the solvent and determination of R_F values.

The choice of the solvent was governed by the following factors :

(a) It had to be completely volatile,so that all traces could be driven off from the chromatogram leaving no toxic residues to impair the root section growth.

(b) It had to provide a suitable separation of the added synthetic chemicals,from the natural plant growth substances.

In the first experiments 2,4-Dichlorophenoxyacetic acid (2,4-D.) was the only synthetic growth substance and β-indolylacetic acid (IAA) the only natural auxin tested chromatographically. A stock solution of 1 part in a 1,000 of the ammonium salt of 2,4-D. was made up :- 0·2g. of the acid was suspended in approximately 100ml. of glass distilled water in a beaker and ammonium hydroxide added until a clear solution was obtained. The excess ammonia was driven off by gently warming the solution on a water bath,when cool it was made up to 200ml. and stored in a refrigerator. 1 part in a 1,000 (10^{-3}) of an alcoholic solution of IAA was also prepared for the tests.

To determine the approximate R_F values of these substances,a spot of a known concentration was placed on

the strip which was run with the particular solvent; the chromatogram was then dried and cut into 10 transverse pieces which were assayed for activity.

The R_F value of 2,4-D. was tested with a wide range of solvents, including ethyl alcohol of varying strengths, at alkaline and acid pH values; propyl alcohol; and 100 parts water saturated butyl alcohol with 10 parts ammonium hydroxide. All gave R_F values lying between ·7 and ·8, with slightly higher values with water and 5% alcohol. The butyl solvent was less volatile and in some experiments the residue on the paper caused inhibition of the section growth throughout the chromatogram.

The final solvent chosen was one suggested by Bennet-Clark, 100 parts isopropyl alcohol, 10 parts water and 10 parts ammonium hydroxide. The approximate R_F values for 2,4-D. and IAA, using this solvent were 0·6 - 0·75 and 0·3 - 0·4 respectively. In these initial experiments the strips were not equilibrated with the vapours of the solvent in the base of the tank before running so rather variable R_F values were obtained. More accurate determinations were carried out, by stricter control of the experimental technique. A spot of a known solution was placed on a strip which was then eqilibrated in the tanks for 1 hour. The propanol/ammonia/water mixture was poured into the trough and the strip allowed to run for

approximately 14 hours overnight. The tanks were kept in the dark and in as uniform a temperature as possible in the laboratory. The strips were removed,dried and marked into 20 transverse pieces which were then assayed. The chromatographic graphs for IAA and 2,4-D. are shown in Fig.3 and Fig.4.

The R_F value of 2,4-D. was 0·65 and of IAA 0·40.

There was a slight shifting of the values from experiment to experiment due to varying conditions of temperature,equilibration time and possibly of the ammonium concentration in the solvent in the base of the tank. The R_F position of the IAA in the actual extraction experiments was always checked by running a parallel strip with a spot of 10^{-3} IAA. The position of this spot could be rapidly detected by spraying the dried chromatogram with a mixture of 5% perchloric acid,50 parts,and ·05M Ferric chloride,1 part; when a pink colour developed in the IAA region. This spray gave a faint pink colour with a spot of 10^{-5} IAA,but was unable to detect 5×10^{-6} solution.

In some of the experiments,the inhibition of the sections caused by the 2,4-D. was recorded over a wide area of the chromatogram. This was due to the large amount originally placed on the strip,and made more obvious by the fact that the response itself was plotted.

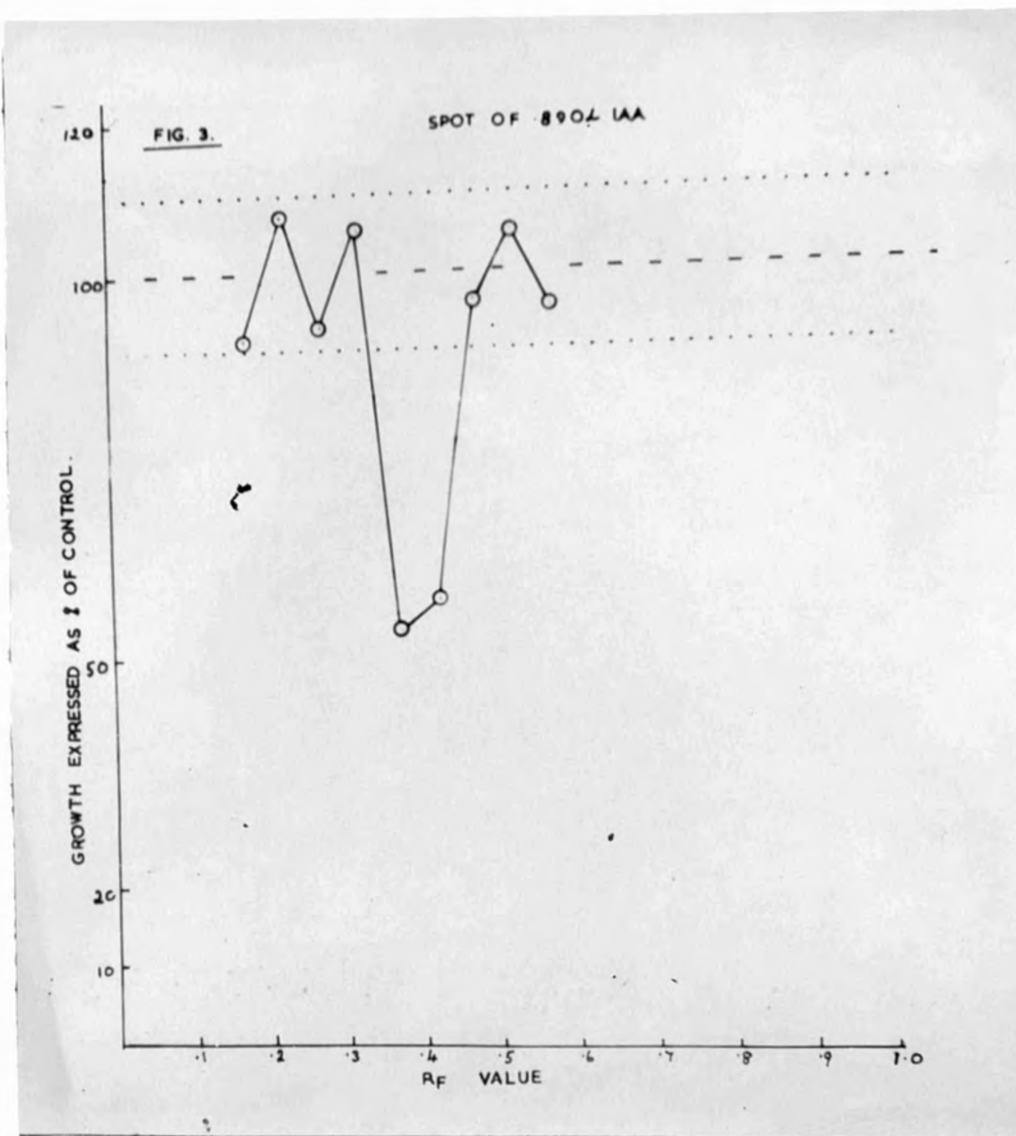


Fig. 3. Chromatogram graph of a solution of IAA.

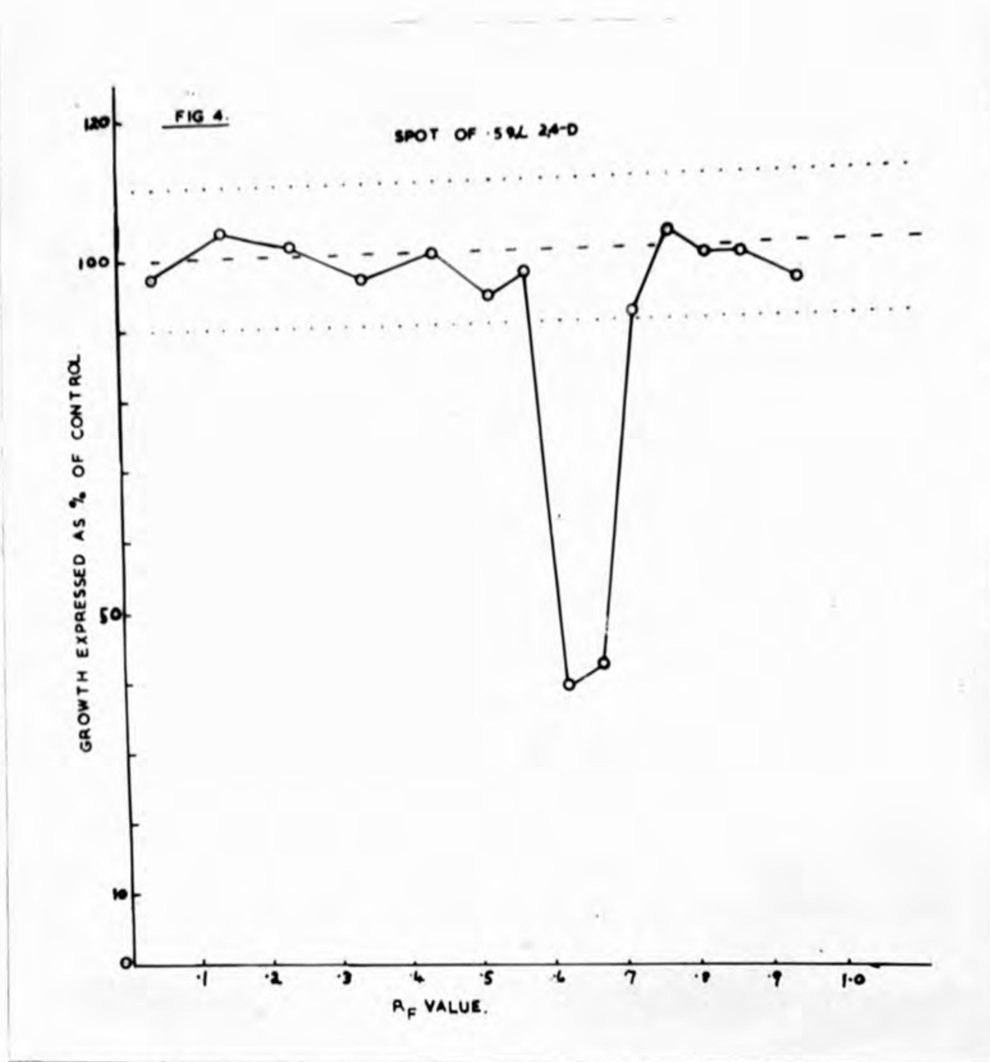


Fig. 4. Chromatogram graph of a solution of the ammonium salt of 2,4-D.

As the growth response was proportional to the logarithm of the concentration this treatment caused a flattening of the curve over the active region on the chromatogram. In subsequent extractions of 2,4-D. treated material, the large amount of the compound in the final concentrated extract caused severe tailing, and inhibition of the root sections almost throughout the chromatogram.

Spots of mixtures of IAA and 2,4-D. were placed on strips and run with the solvent. The results of two of these experiments are shown in Figs.5 and 6. The former was the result of an assay of a chromatogram of a mixture of 10^{-4} IAA and 10^{-4} 2,4-D., and the latter of a mixture of 10^{-8} IAA and 10^{-4} 2,4-D. In both there was a clear separation of the two compounds and even in the second the IAA was detected without difficulty although applied in such a low concentration.

As far as these two compounds were concerned the method provided a means of their separation and detection from unknown solutions.

Two other synthetic compounds; maleic hydrazide (MH) and 2,3,5-tri-iodobenzoic (TIBA) were used in later experiments. Preliminary tests were carried out to determine their R_F position with the alcohol/ammonia solvent.

A 10^{-2} stock solution of the ammonium salt of TIBA

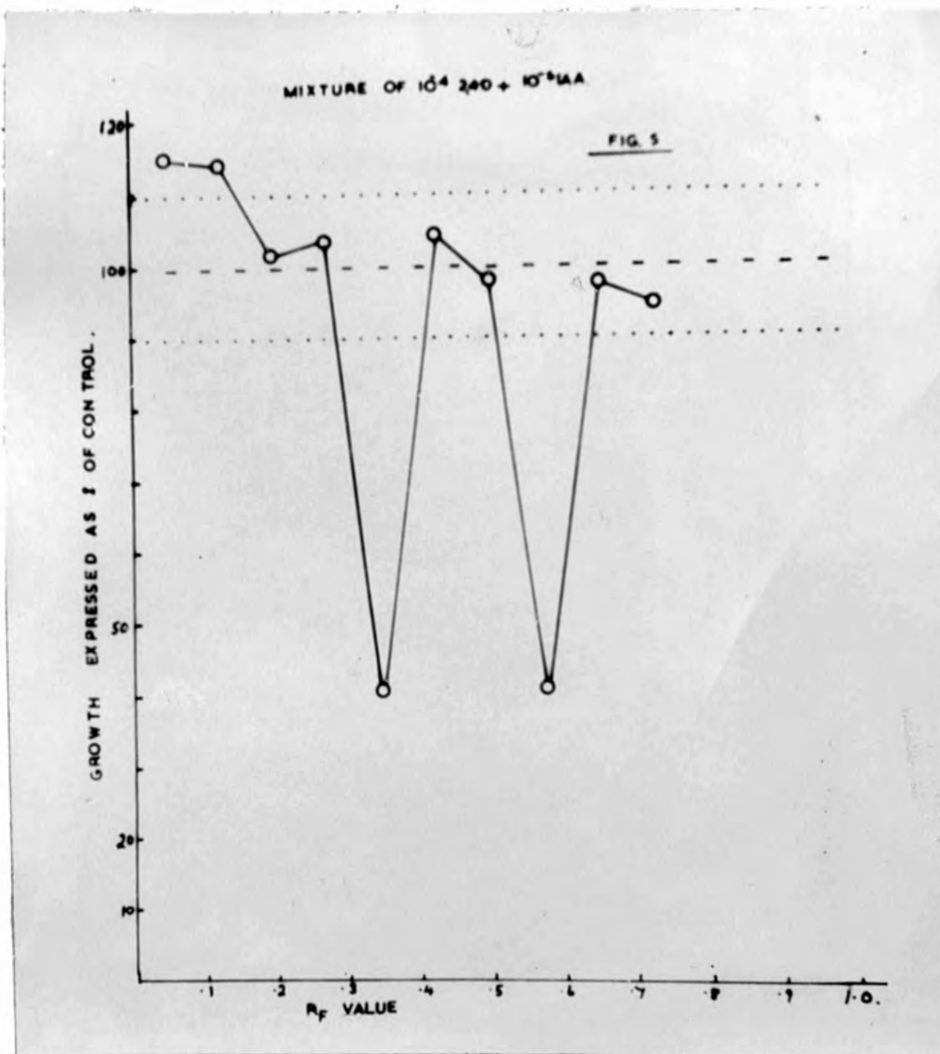


Fig. 5. Chromatogram graph of a mixture of 2,4-D.(10^{-4}) and IAA (10^{-4}).

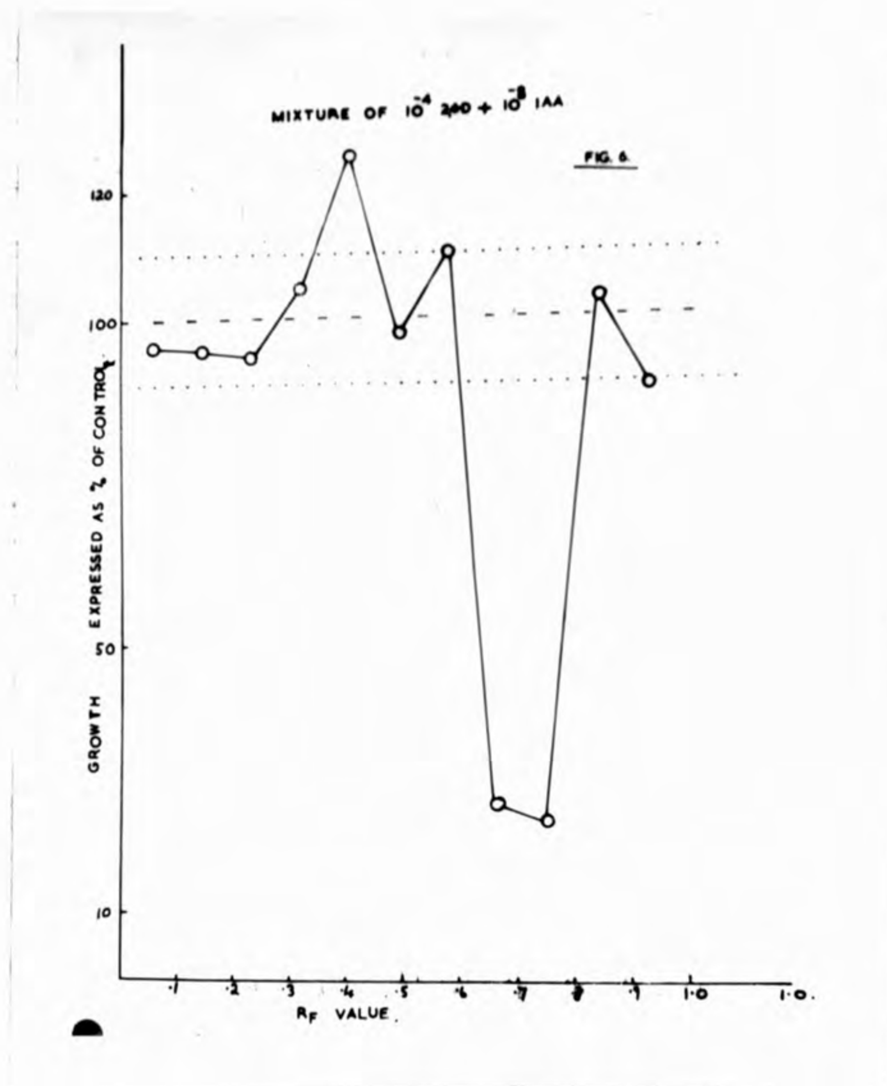


Fig. 6. Chromatogram graph of a mixture of 2,4-D.(10^{-4}) and IAA(10^{-8}).

was prepared as for 2,4-D. and a spot of this solution was placed on a filter paper strip,run with the solvent, dried and assayed as in previous experiments. The chromatogram graph produced is shown in Fig.7. The R_F value of TIBA was between ·60 and ·70.

Maleic hydrazide had been shown to be practically insolule in water and was usually applied to plants as the soluble tri-ethanolamine salt. 2 grams of MH and 2·66 grams of tri-ethanolamine (equivalent amounts) were added to a small volume of glass distilled water. The compound rapidly dissolved and the solution was made up to 200ml. to give a 10^{-2} stock solution of the salt. A sample of this solution was tested in the usual way to determine its R_F value with the solvent. Three chromatograms were run and assayed but no growth activity was detected. A 10^{-2} solution of maleic hydrazide was known to be completely toxic to pea root section growth so that the compound must have been adsorbed on to the paper to become ineffective. The R_F value of MH was determined by studying chromatograms in ultra violet light,when a deep purple fluorescent spot appeared at values between ·18 and ·25. [Dilute solutions of tri-ethanolamine were shown to have no effect on section growth.]

The propanol/ammonia/water solvent would therefore also give adequate separation of naturally occurring

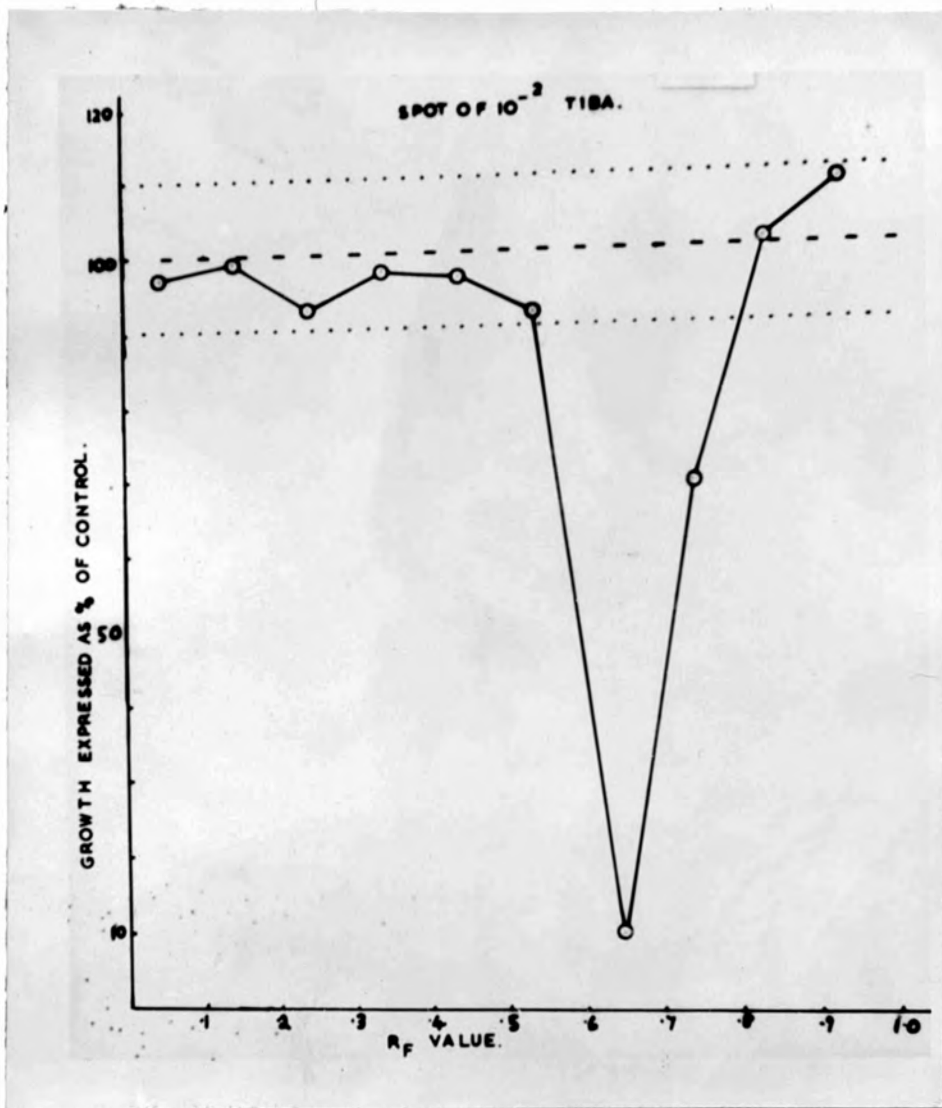


Fig. 7. Chromatogram graph of a solution of the ammonium salt of TIBA.

indolylacetic acid and TIBA (or MH) from extracts of plants which had been treated with either of these compounds. Chromatograms of mixtures of synthetic IAA and TIBA (and MH) were run, to check the possibility of mutual interference resulting in altered R_F values. No such effects were recorded, the R_F values remaining constant.

The relationship of the growth responses of the sections to varying concentrations of IAA; 2,4-D.; TIBA and MH.

A series of experiments were carried out with a range of concentrations for each of these four compounds, to obtain calibration curves for use in the subsequent assay of plant extracts.

It was important to determine if there were any significant differences in the responses of the root sections to these substances, under the conditions of the assay, as compared to those obtained when growth in an unlimiting supply of sucrose. The present assay technique was developed to estimate the yield of natural auxin from plant tissues, so that particular emphasis was laid on the growth reactions of the sections to varying concentrations of IAA.

A 10^{-3} aqueous solution of the sodium salt of IAA was prepared for use in the tests. 0.1mg. of IAA was added to approximately 50ml. of distilled water in a beaker with the calculated equivalent amount of a N/10 solution of sodium hydroxide. The beaker was warmed

gently until a clear solution was obtained. When cool, the quantity was made up to 200 ml. and the solution stored in the refrigerator. A 10^{-3} stock solution of the ammonium salt of 2,4-D. was also made up as before.

A series of dilutions of IAA and 2,4-D., from 1 part in 10^{-12}, to 1 part in 10^{-6} were made up in ½% sucrose. A 0·7ml. sample from each dilution was placed in the growth vessel with 10 weighed root sections. The total increase in weight was measured after 24 hours and expressed as a percentage of the increase obtained when the sections were grown in sucrose alone.

The curves shown in Figs.8 and 9 were drawn from the average values of a large number of experiments. (Appendix, Tables I and II.).

The IAA curve showed that a significant stimulation of growth was obtained in the concentrations of 10^{-11} and 10^{-10}. There was a progressive increase in inhibition beyond 10^{-9}, and the growth fell to 50% of the normal value in 10^{-6}. No stimulation was recorded with 2,4-D. With the latter compound, inhibition began at approximately 10^{-9}, and only 30% of normal growth was obtained in a 10^{-6} solution of 2,4-D. These general results were similar to those obtained when the responses of root sections to varying concentrations of these substances were studied using the petri-dish technique. An attempt was made to

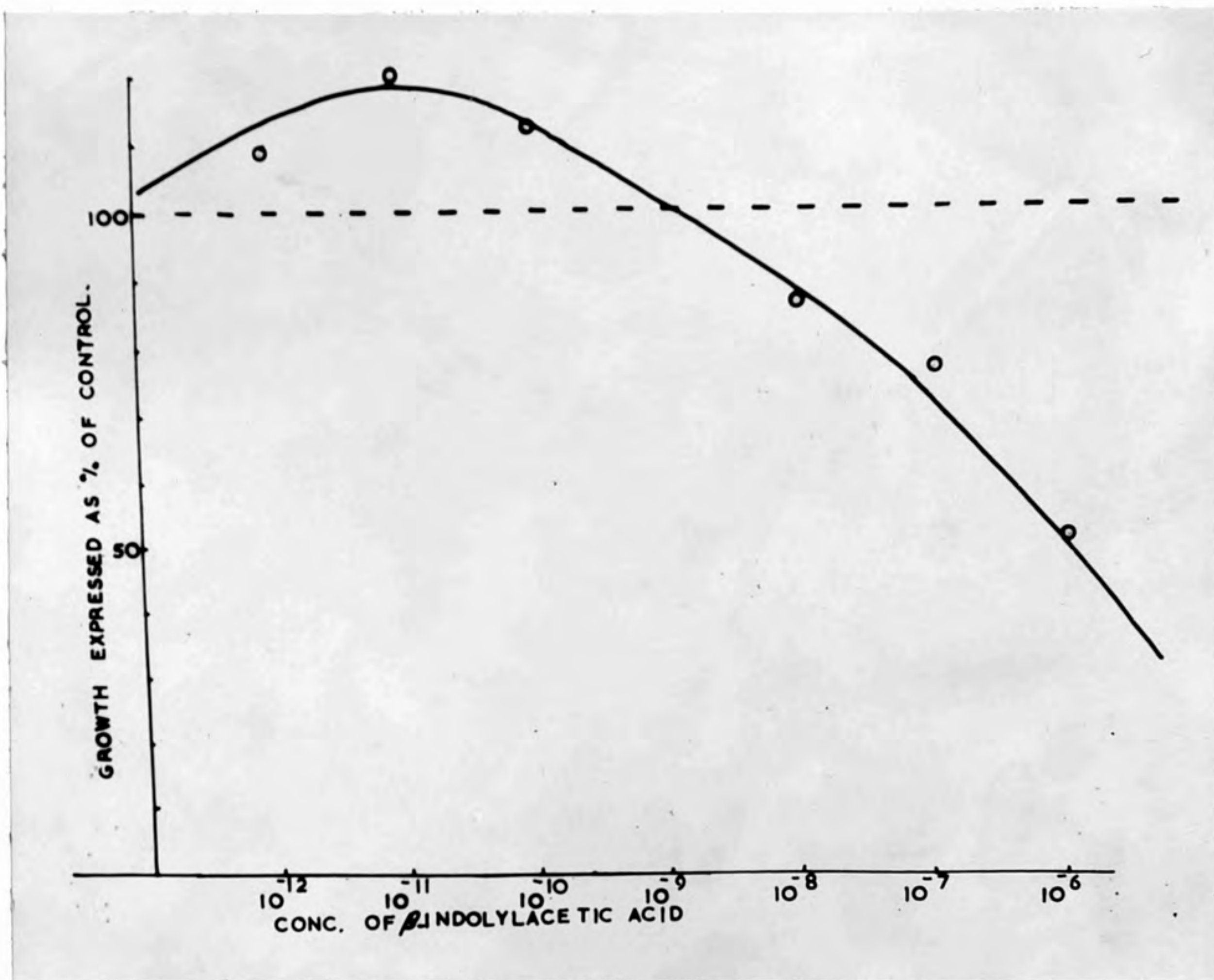


Fig. 8. Calibration curve for IAA.

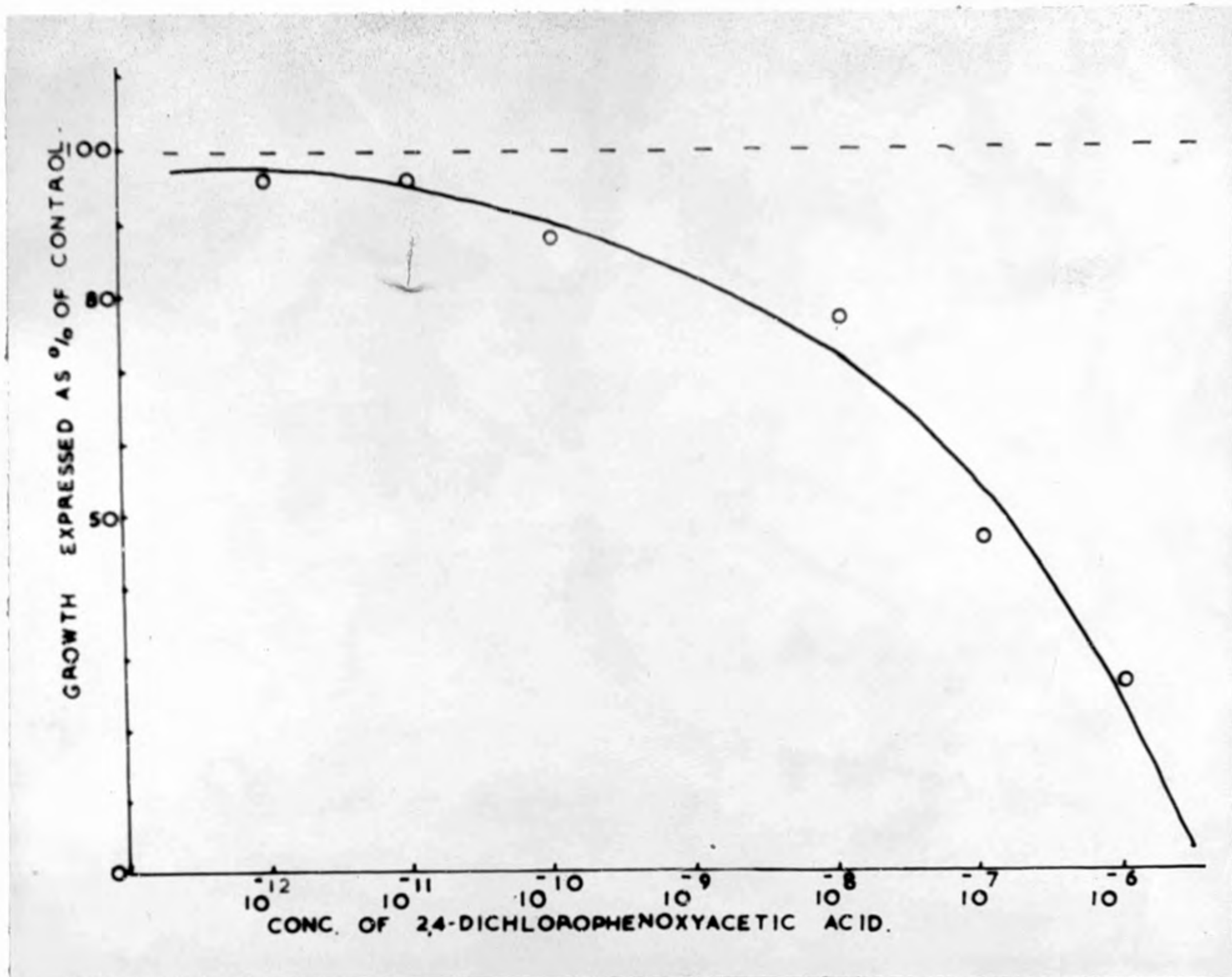


Fig. 9. Calibration curve for 2,4-D.

increase the sensitivity of the method by using a purified sucrose solution for the dilutions of the two compounds. A weighed amount of sucrose was dissolved in a small volume of glass distilled water and shaken in a separating funnel with three successive lots of ether. The sucrose solution was finally separated off, warmed gently to remove all traces of ether, and when cool, diluted to give a ½% solution. The latter was used in a series of experiments covering the concentrations of IAA and 2,4-D. from 10^{-10} to 10^{-12}. The growth responses of the sections to these concentrations made up in the purified medium did not differ significantly from those obtained when using the normal sucrose.

The main concern in the production of these calibration curves was the accuracy with which they could be used to estimate unknown concentrations, (particularly of IAA) in plant extracts, using a single observation of a response. The coefficients of variation were worked out from the data of the IAA curve. Determinations showed that, over the range of concentrations from 10^{-7} to 10^{-12} there were only slight differences in variability at the different concentrations. The mean coefficient of variation over this range was 7·459. From this any single assay departing more than 10% from the mean control level could be taken as an indication of the presence of an

active growth substance. A horizontal line at this level has been drawn in all the chromatographs to give a rapid indication of the significance of the differences of assays from the control mean. At 10^{-6} IAA, the absolute variability decreased, but the coefficient of variation increased, due to the low value of the mean growth.

There was reasonable expectation of being able to detect IAA at a concentration as low as 10^{-11}, as this gave a mean stimulation of 18·44%, (from 10 responses). The amount could not be quantitatively estimated as stimulation could be expected from concentrations varying from 3×10^{-12} to 3×10^{-10}. Concentrations of 10^{-9} IAA would not be detected on the chromatograph as the calibration curve crossed the control line at this point. To overcome this, it would be possible to run a series of chromatograms using a different dilution of the final extract on each. The IAA concentration would then be brought into the stimulatory range, and a rough estimation of the concentration present in the original solution made. In the actual experiments with plant extracts, the amount of plant material used was such as to expect a quantity of IAA which would lie on the inhibitory portion of the curve. The significant inhibition of growth began at approximately a 10^{-8} solution of IAA.

The lowest quantity of 2,4-D. which could be detected was about 3×10^{-10} so the method was not so sensitive for this compound as for IAA.

The chief weakness of the method,in the estimation of IAA from plant extracts,lay in the flatness of the response curve between the concentrations of 10^{-8} and 10^{-7},over which region the majority of the experimental values lay. The mean response at 10^{-8} was 86·5% of the control growth,and at 10^{-7} was 76·9%; a difference of less than 10%. The normal variation of growth at a fixed level of concentration could therefore cause widely varying estimations,based on calculations from the calibration curve. A guard against this error was repitition of experiments.

Rough calibration curves were also obtained for maleic hydrazide and tri-iodobenzoic acid. A series of dilutions of the tri-ethanolamine salt of MH,from 10^{-2} to 10^{-6}; and of the ammonium salt of TIBA,from 10^{-3} to 10^{-9}, were made up in ½% sucrose. 0·75ml. samples of each were assayed by the root section technique,as for IAA and 2,4-D. The calibration curves shown in Figs.10 and 11 were drawn from the average values of a number of assays. (Appendix,Tables III & IV.).

The maleic hydrazide curve (Fig.10) showed that a 3×10^{-4} solution had no effect on section growth but that

a 10^{-2} solution was completely toxic. The root sections were therefore less sensitive to MH than were Avena coleoptile sections, which had been shown (Leopold & Klein, 1952) to be inhibited by a 10^{-7} solution of the compound. The increase in inhibition, with concentration, in the Avena test was much also more gradual than in the pea root assay. No stimulation of growth of the root sections was recorded.

The TIBA curve (Fig.11) was drawn from the averages of only 4 assays at each concentration. No stimulation of growth was observed; inhibition increased very rapidly from a 10^{-5} solution which gave 30% inhibition of growth to 10^{-4}, which caused 75% inhibition. No growth occurred in a 10^{-3} of TIBA. The action curve of this compound in flax roots (Aberg.1953) showed a flattening of the inhibition between 10^{-7} and 10^{-5}M. It was thought that the latter 'stepped' curve was the result of the response of whole roots to increasing concentrations of TIBA, as compared to that of isolated root sections.

[These curves were not used for estimations of concentrations of TIBA and MH in plant extracts, but were produced to obtain information about the relative effectiveness of these substances on section growth and whole plant growth.]

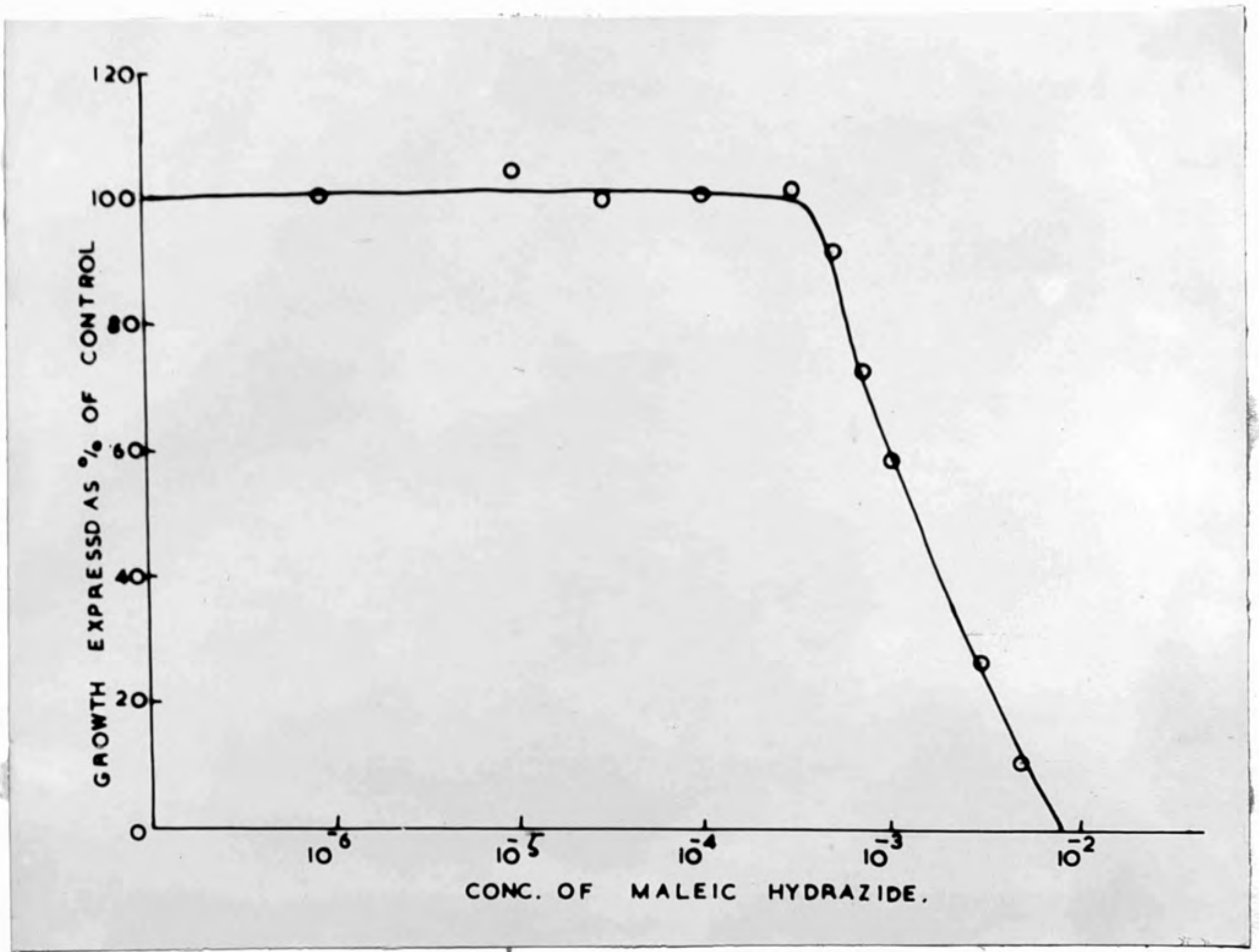


Fig. 10. Calibration curve for maleic hydrazide.

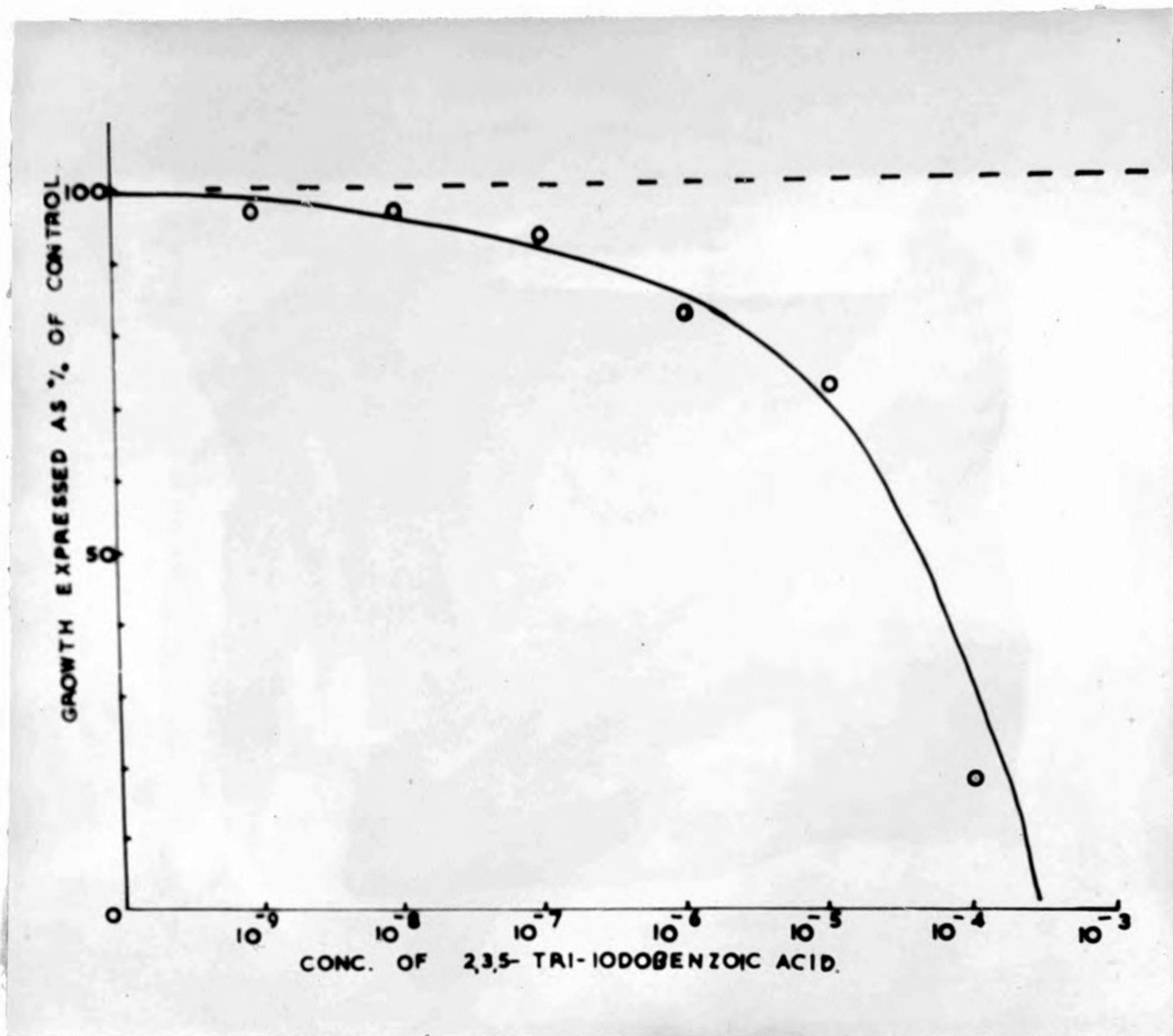


Fig. 11. Calibration curve for TIBA.

<u>Assay of plant extracts</u> (with reference to IAA and 2,4-D. only.).

The errors involved in the application of the calibration curves to the direct assay of unknown concentrations of IAA and 2,4-D. have been discussed. It seemed likely further errors would arise in the quantitative estimation of these substances from chromatograms of plant extracts, due to :-

(a) Decomposition of the active substances during the actual running of the chromatogram.

(b) Lack of complete equilibration between the growth substance on the paper and the sucrose solution in the growth vessel. This would tend to give estimations which were too high, owing to a high local concentration of the substances on the paper in direct contact with the root sections.

(c) Masking of one substance by another which had a similar R_F value. This could be overcome by use of a different developing solvent.

Experiments were carried out to establish a measure of the first two of these errors. The paper strip was weighed to the nearest ·02mg. on a sensitive air-damped balance, a spot of known concentration of IAA (or 2,4-D.) was added and the strip re-weighed immediately, before any extensive evaporation could have taken place. The chromatograms were run with the solvent, dried, and cut up

into 20 transverse pieces. Only that part of the chromatogram over the approximate R_F value of the particular compound being tested, had to be assayed. These strips were placed in the growth vessels with 0·70ml. of ½% sucrose and treated in two ways.

(1) The strips were assayed immediately - 10 sections were added to each vessel, and the percentage increase in growth recorded after 24 hours.

(2) The strips were left with the sucrose solution in the growth vessels for 18 hours, in the dark and at room temperature, to allow time for the equilibration of the auxin between the paper and the sucrose solution. The strips were then assayed normally.

R_F graphs were drawn as in Fig.3 from the results of the assays. The percentage inhibition obtained at each response point, (if more than one) could then be read off and fitted into the calibration curve. The concentration of IAA (or 2,4-D.) which gave this inhibition could be noted, and assuming this to be uniform throughout the 0·7ml. of sucrose, the actual amount of auxin could be calculated. Thus an inhibition of 24% would indicate the presence of 0·7ml. of a 10^{-7} solution of IAA which would be equal to a total of ·07 γ.

A number of chromatograms, to which known amounts of IAA (or 2,4-D.) had been added were (a) assayed

immediately,or (b) assayed after the 18 hour equilibration period with sucrose. Scatter diagrams were constructed of the amount of auxin estimated from the growth responses of the sections,against the amount known to have been added to the strips. The graphs of these results for IAA and 2,4-D. are shown in Figs.12 and 13. (Details - Appendix,Table V. & VI) Fig.12 for 2,4-D. showed that over the range studied there was a linear relationship between the experimentally estimated amount and the actual known amount. A regression coefficient was worked out for the added amount on the estimated amount and gave a value of ·77. This meant that the amount calculated from the chromatogram graphs of unknown solutions was 30% higher than that added to the strip. This factor was not altered by the 18 hour equilibration of the strips with sucrose prior to assay. To obtain an accurate estimation of the amount of 2,4-D. present the calculated values would have to be multiplied by 0·77. The high value obtained from the assays could have been due to the adsorption of the 2,4-D. on to the paper which would give a high local concentration of the substance in direct contact with the root sections. This error was not reduced by standing the strips with the growth medium before assay.

The results of a similar series of experiments with IAA are shown in Fig.13.

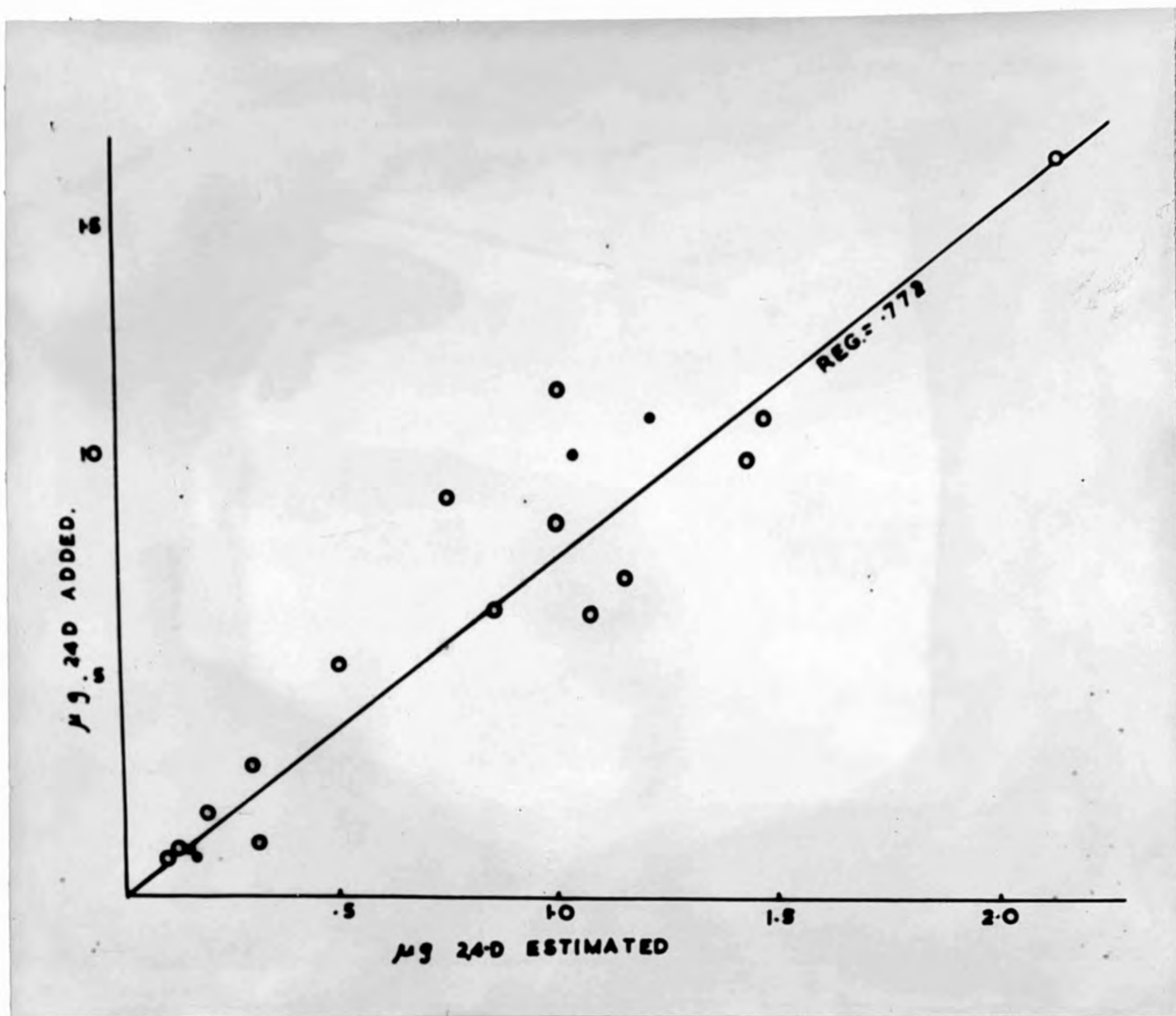


Fig. 12. The relationship between the experimentally estimated amount and the amount of 2,4-D. added to the chromatogram strips.

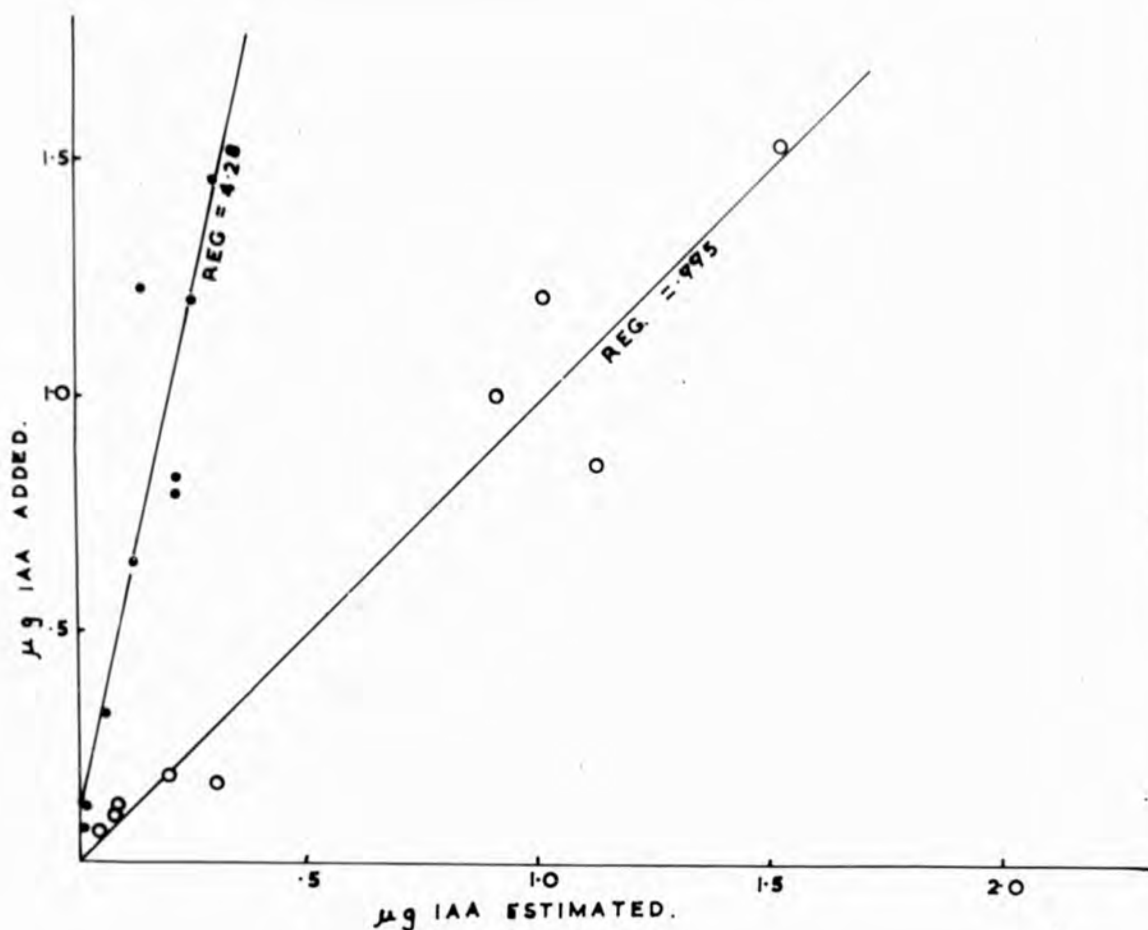


Fig. 13. The relationship of the experimentally estimated amount of IAA to the amount added to the chromatogram strips.

o estimated immediately.

• estimated after 18 hours.

The graph showed that immediate assay of the chromatogram gave an estimated value which had a linear relationship to the known amount added. The regression coefficients were worked out from the two sets of results obtained under the two assay conditions. If assay of the strips was carried out immediately, a regression coefficient of 0·995 was obtained, which meant that the estimated amount of auxin was a direct measure of the quantity in the original spot on the chromatogram. If the strips were left to equilibrate with the sucrose solution before assay, the regression coefficient of the added on the estimated amount worked out to a value of 4·28. This indicated that IAA was lost during the equilibration period. This loss was possibly due to bacterial decomposition in the relatively unsterile conditions of the growth vessels. Such periods of equilibration were therefore ruled out in future assays. The fact that immediate assay of the strips gave such an accurate measure of the amount of IAA in the original spot indicated that none was lost during the running of the chromatogram and that diffusion of the auxin from the paper to the sucrose solution was rapid and complete. It seemed unlikely that decomposition could exactly offset adsorption on the paper, to give such a balanced result.

RESULTS.

1. Preliminary extraction results.

2. Final extraction technique results.

1. Preliminary extraction results.

This series of experiments, in which the initial extraction solvent was water, gave results which proved to be of qualitative value only. The results did however, establish that active growth substances could be detected on chromatograms of plant extracts, by the techniques described.

The details of the material used, with reference to the particular modification of the general extraction technique (described on p. 13 - p 16 .) and the R_F values of the growth activity shown on the assayed chromatograms, are given in Table I. (p. 41)

Graphs of the assayed chromatograms from extractions 2, 3, and 6 are shown in Figs. 14, 15, and 16.

Extractions of tissue, previously treated with 2,4-D. were also assayed for growth activity. In the first experiment, the upper surfaces of the first pair of true leaves, on 15 four week old sunflower plants were painted with approximately 5ml. of a 10^{-3} solution of the ammonium salt of 2,4-D. These and 14 untreated plants were left for $2\frac{1}{2}$ days and the shoots then harvested from above the third pair of leaves. The treated plants showed the typical 2,4-D. response; the leaves, particularly those above the treated zone were folded back on a bent stem and the expansion of new leaf tissue was retarded. The treated sample showed a

TABLE I.

Expt. No.	Material.	Weight.	Method of Extraction.	Activity on chromatograms. (+) = stimulation. (-) = inhibition.				
1.	18 day Meteor pea shoots + cots.	70g.	Ia. (2 c'grams)	·05(-)		·35(-) ·45(-)	·65(-) ·70(-)	1·0(-) 1·0(-)
2.	21 day Meteor pea roots.	35g.	Ib.			·30(-)		1·0(-)
3.	14 day shoots. Scarlet Emperor runner bean.	23·5g.	Ic.		~~1·0~~ 0·1(+)	·35(-)	·60(-)	·70(+)
4.	18 day Ditto.		Ic.		~~1·0~~ 0·1(+)	·40(-)	·65(-)	
5.	14 day Ditto.	33·2g.	Id.			·40(+)	·75(-)	
6.	Tips of 30 day Dwarf bean shoots.	13·5g.	Ib.	·05(-)		·45(-)	·75(-)	

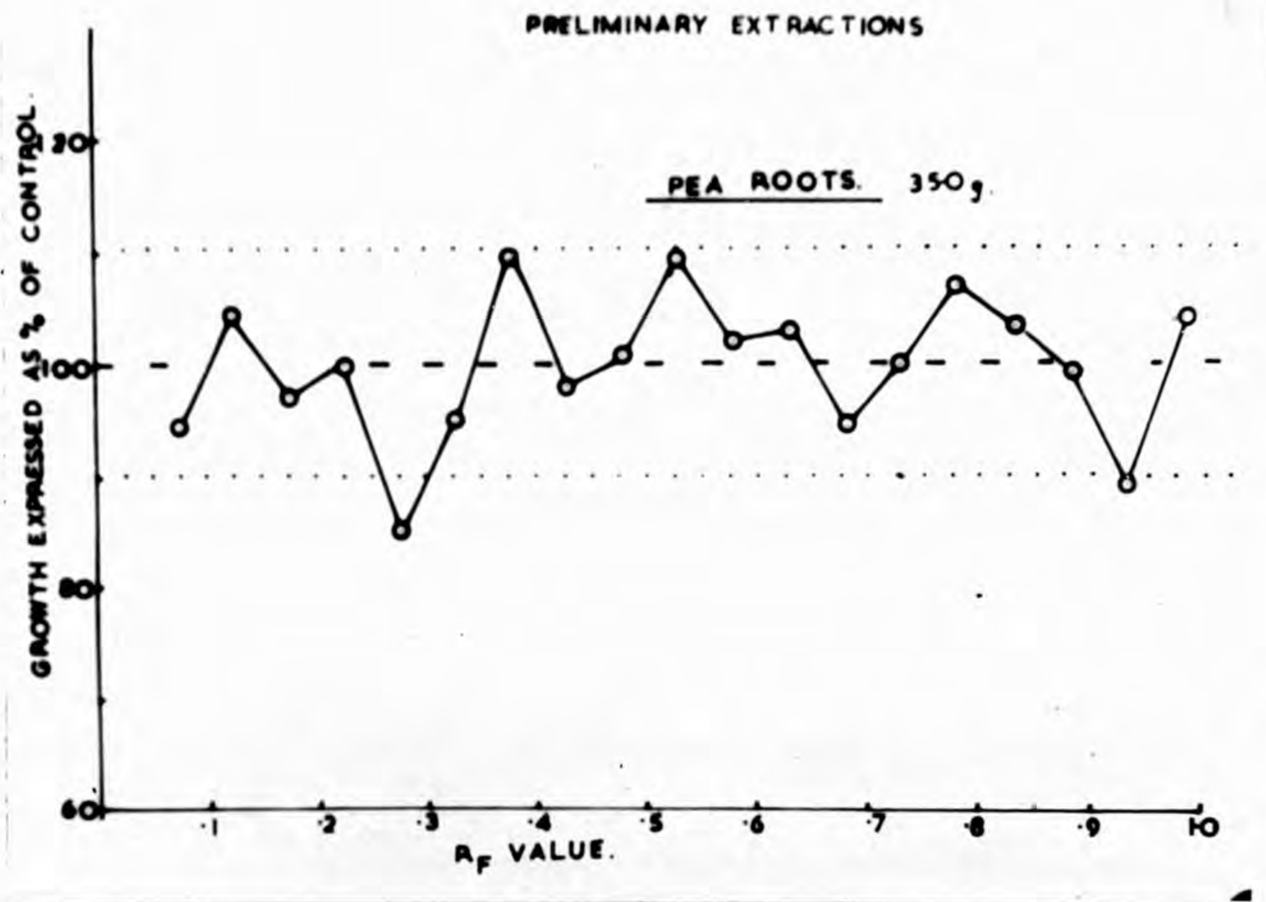


Fig.14. Chromatogram graph of an extraction of pea roots.

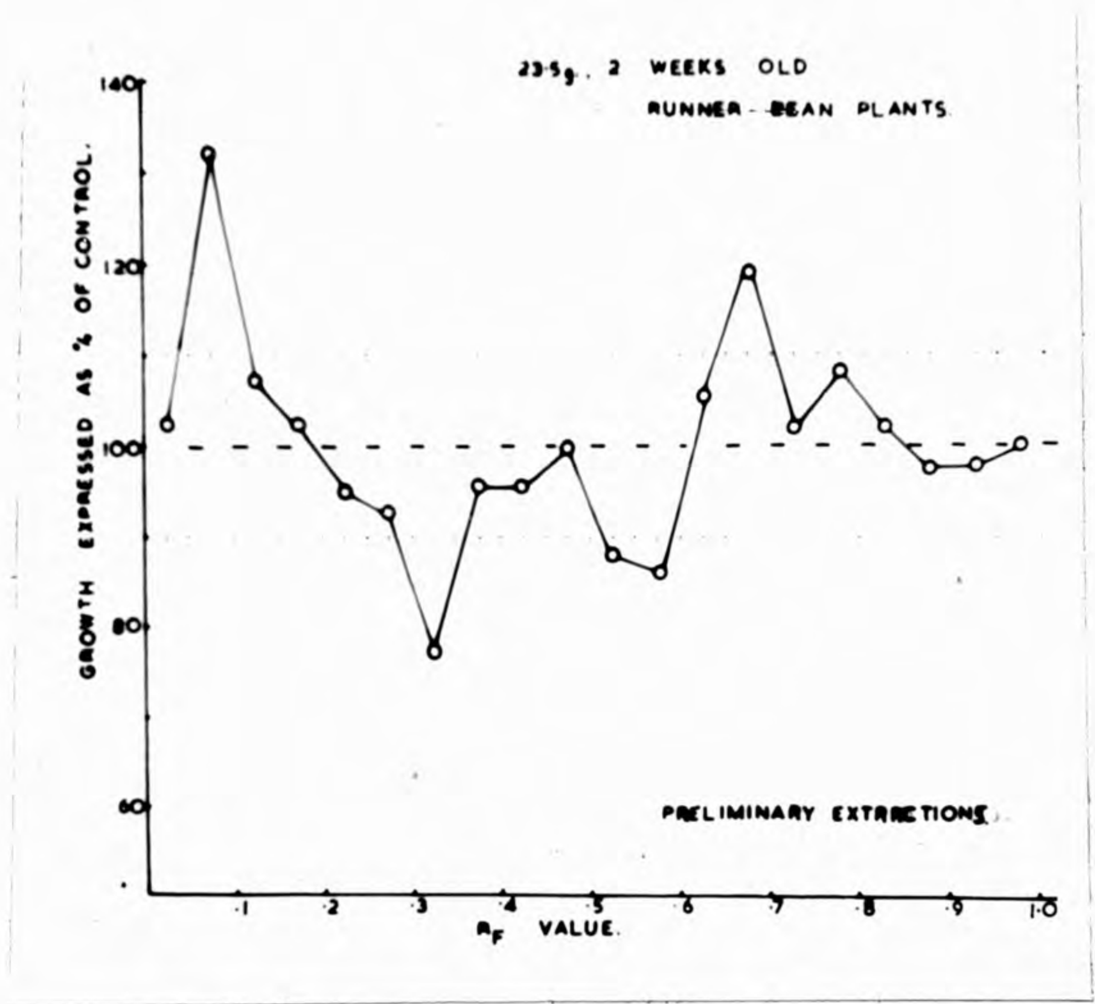


Fig. 15. Chromatogram graph of an extraction of runner bean plants.

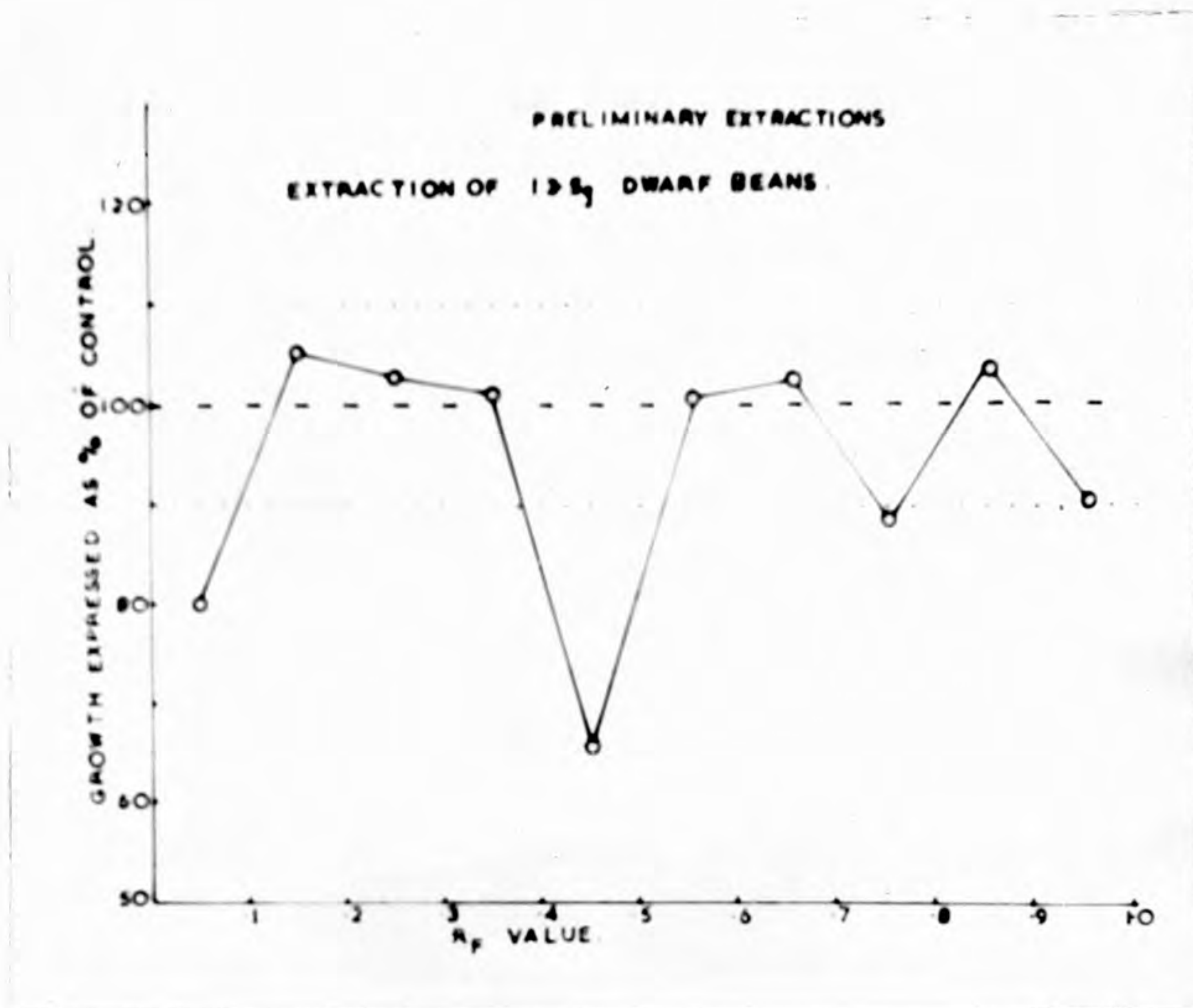


Fig. 16. Chromatogram graph of an extraction of 13.5g. of dwarf beans.(Shoots)

greater fresh weight per plant (·60g.) than the control (·48g.). Extraction of the two samples was carried out by method Ib and each assayed for activity. Both chromatograms showed a stimulation of growth at an R_F value of 0·45; the control extract gave slight inhibition at a value of 0·80 and the treated a large inhibitory area between ·65 and ·80. The latter was obviously due to 2,4-D.,but apart from this there were no striking differences between the activity of the two extracts. The chromatogram graphs are shown in Fig.17.

The experiment was repeated with a smaller number of plants. The assayed chromatograms showed no activity,apart from an inhibitory spot at an R_F of ·75 in the treated extract - due to 2,4-D. This lack of activity may have been due to the small bulk of tissue extracted.

An extraction of Meteor pea seedlings,which had been treated with 2,4-D. was compared with one of similar control plants. Two day old pea seedlings were transferred to tanks of aerated tap water; after 12 days growth,the water in one tank was replaced by a 10^{-5} solution of 2,4-D. and both sets were left for a further two days. There was little obvious external response of the plants to this treatment,apart from a slight thickening of the main root and shoot,and a slower leaf expansion. 53·3g. of control shoots and 38·5g. of treated (including cotyledons)

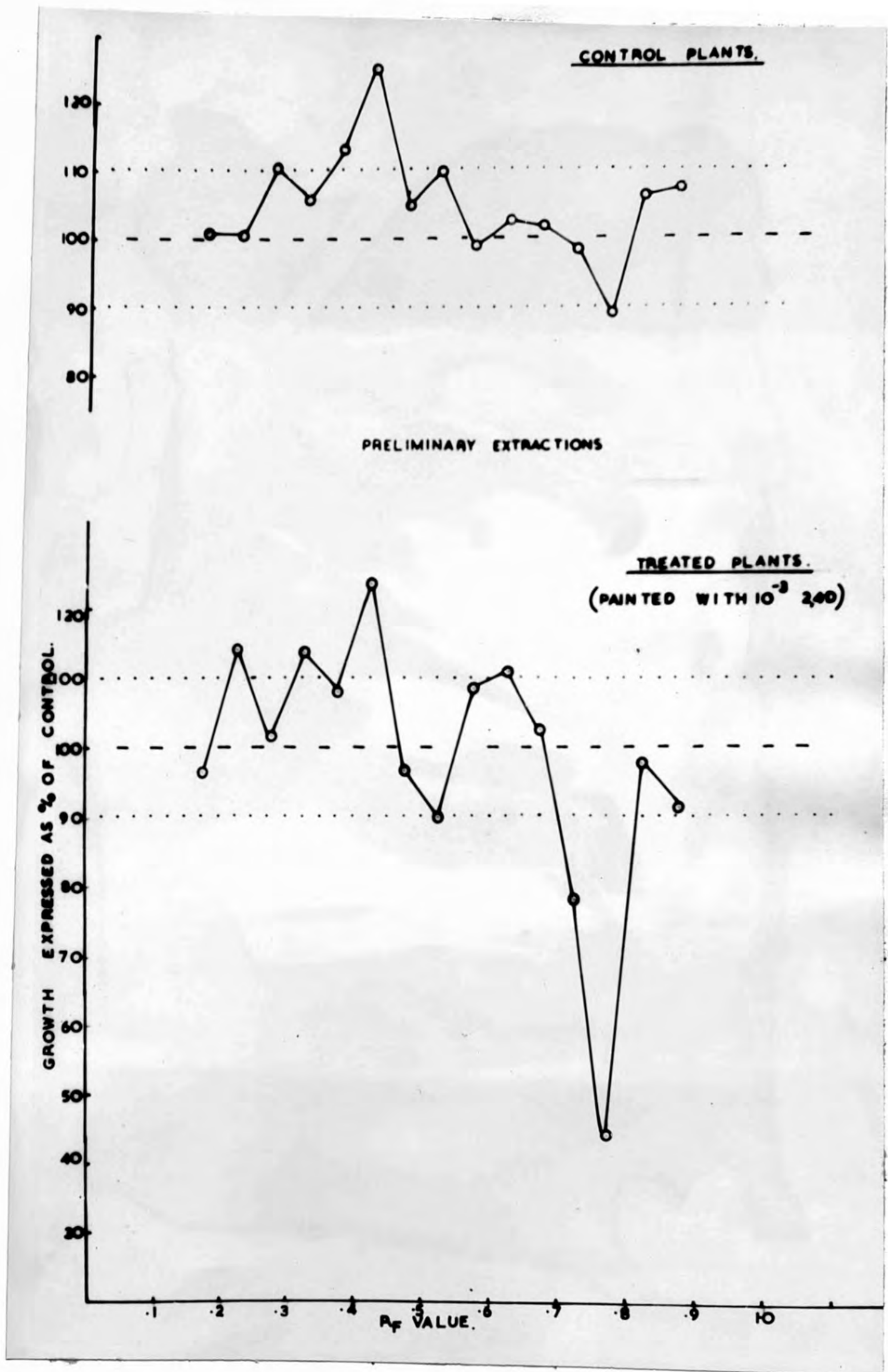


Fig.17 Chromatogram graphs of extracts of normal and 2,4-D. treated shoots of Sunflowers.

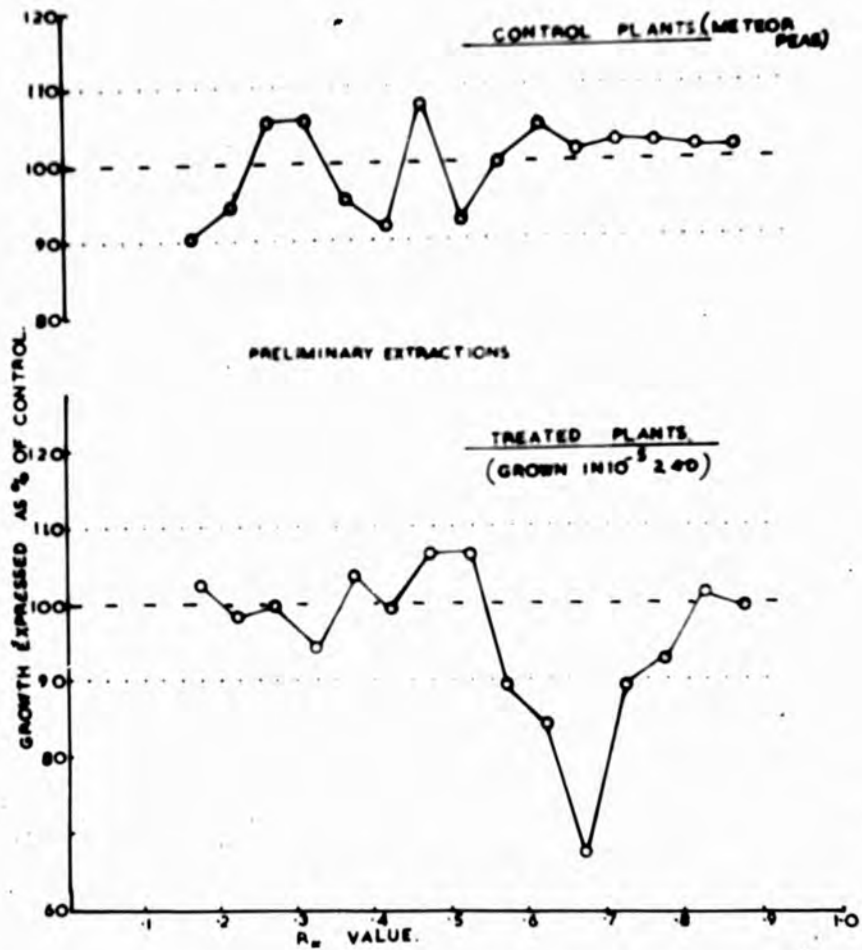


Fig. 18. Chromatogram graphs of extracts of normal and 2,4-D. treated pea shoots.

were extracted by method Ib and assayed for growth activity. The chromatogram of the control extract showed no activity and the treated only showed an inhibitory spot in the 2,4-D. zone. (Fig.18). This result was quite different from the first extraction of pea tissue. (Expt.i.).

In this series of experiments, the results of assayed chromatograms of extracts from similar tissue were very variable. This variability was probably due to the unsterile and relatively un-uniform conditions of the extraction technique. The final ether concentrate assayed on the chromatograms was of an oily and pigmented nature and this seemed to cause uneven running of the solvent down the paper and possibly accounted for the general low level of growth increase of the sections. The technique was abandoned, but certain facts could be drawn from the results.

1. The bioassay technique developed could detect active growth substances on the chromatograms of concentrated extracts of relatively small bulks of plant tissue.

2. An active substance, with an R_F value ranging from ·30 to ·45 was detected from all types of tissues extracted. This was presumed to be IAA because of the similarity in the R_F value with that of the pure compound, and because it occasionally caused stimulation of the root section growth. β- indolylacetic acid was therefore detected from the

green tissue of pea,sunflower,dwarf and runner beans,and from the roots of peas. Accurate estimations of the yields could not be made.

3. An inhibitory substance with an R_F value between ·65 and ·80 was detected on chromatograms of all tissue extracts,with the exception of the pea roots.

4. A stimulatory substance at an R_F value of ·10 was also detected,but only from runner bean shoots.

5. Inhibition occasionally occurred on the first and last strips of the assayed chromatograms. The former inhibition, at the original spot level,seemed to be caused by the oily residues in the final ether concentrate,and these appeared to prevent the absorption of the sucrose solution by the filter paper.

2. Results obtained from the final extraction technique.

A. Untreated plants.

Growth substances from extracts of untreated plants.

The material used was shoots of Sunflower seedlings grown in the constant temperature room. In the first series of experiments, the ether extract was not divided into acidic and neutral fractions, but the whole was used for analysis. The details and results of the extractions are shown in Table II, (p. 46).

The growth-active regions on the chromatograms were generally similar to those obtained in the preliminary extractions. An active substance, with an R_F value between ·32 and ·40 was detected in all the experiments, with the exception of the 24 hour soaked seed. This substance was presumed to be β-indolylacetic acid. (IAA). Two other active spots occurred consistently on the chromatograms, (1) a compound at an R_F of ·10 to ·17 which caused stimulation of section growth and (2) one at an R_F of ·60 to ·80 which brought about growth inhibition. A fourth active zone occasionally appeared - an inhibitory spot at an R_F value of approximately ·90. A typical graph of an assayed chromatogram of a shoot extract is shown in Fig.19.

The yield of IAA, calculated in γ per kilogram fresh

TABLE II.

Expt. No.	Tissue Extracted	IAA. R_F value.	IAA. γ g./kg. F.Wt.	R_F values of other active substances. (+) = stimulation of growth. (-) = inhibition of growth.			
1.	Sunflower seeds. Soaked 24 hours.	-	-		·20(-)		
2.	18 day Sunflower shoots + cotyledons.	·42	1·79		·15(+)	·72(-)	·87(-)
3.	14 day Sunflower shoots + cotyledons.	·32	·83		·12(+)	·72(-)	·92(-)
4.	14 day Sunflower shoots + cotyledons.	·32	2·13	0(-)		·80(-)	
5.	18 day Sunflower shoots + cotyledons.	·40	2·02		·10(+)	·80(-)	
6.	18 day Sunflower shoots above cotyledons.	·40	17·0		·15(+)	·70(-)	
7.	10 day Sunflower shoots above cotyledons.	·40	13·62		·20(+)	·70(-)	
8.	18 day Sunflower shoots above cotyledons.	·40	16·80	0(-)	·17(+)	·67(-)	·92(-)
9.	10 day Sunfl.shoots above cotyledons.(2 chromatograms)	·40 ·40	23·0 18·0	Only IAA zone of chromatograms assayed.			
10.	18 day Sunfl.shoots + cotyledons. Etiolated.	·35	11·5		·10(+)	·62(+)	·72(-)
11.	21 day Sunfl.shoots + cotyledons. Etiolated. 2 chromatograms.	·40 ·40	5·1 3·2		·15(+) ·15(+)	Not assayed below R_F ·5	

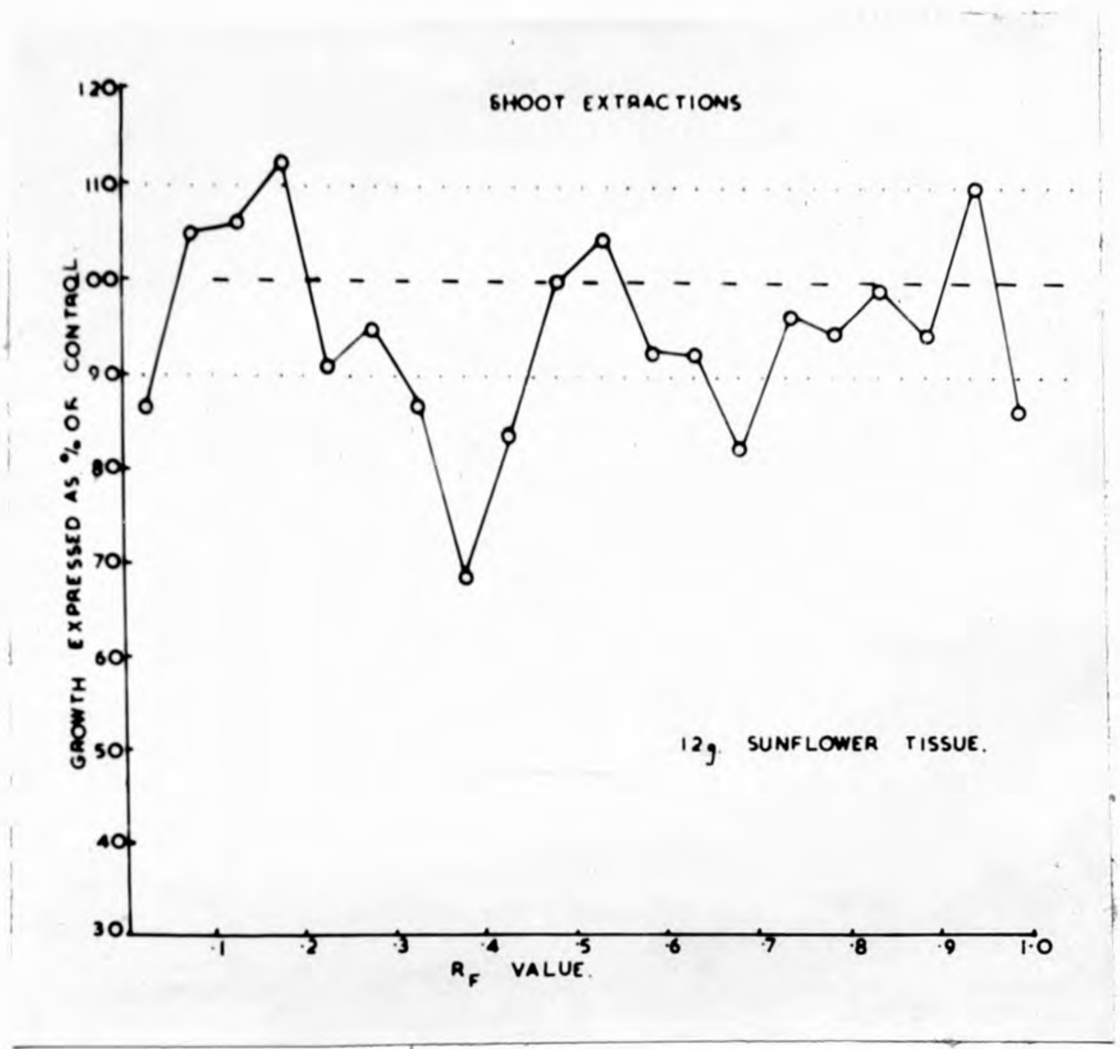


Fig. 19. Chromatogram graph of an extract of Sunflower shoots.

weight was less, if the whole of the plant above the soil was extracted, than if only that part of the plant above the cotyledons was used. The mean yield from the whole shoots (Expts.2 - 5) was 1·69γ/kg. and from the tissue above the cotyledons was 17·68γ/kg. (Expts.6 - 9). Samples of 12·6 and 13·4 grams of cotyledons only, taken from 18 day old Sunflower seedlings, were extracted and assayed. The chromatogram strip from each was divided into 10 transverse pieces; numbers 2 - 5 were assayed and they showed no indication of growth activity. It was concluded from these experiments that the cotyledons of Sunflowers contained little, or no IAA and by accounting for a large part by weight, of a given sample of whole shoots, reduced the yield of auxin worked out on a γ per kilogram fresh weight basis.

Two extractions of etiolated Sunflower shoots were made. (Expts.10 & 11). The yields of IAA were greater than those obtained from green shoots of the same age, but age was possibly not a fair basis for comparison, due to the different stages of seedling growth reached under the different environmental conditions. (The etiolated seedlings consisted mainly of an elongated hypocotyl + cotyledons, with practically no leaf tissue). The stimulation at an R_F value of ·62 was interesting, but needed further investigation.

A sample of cabbage tissue was extracted and the total ether extract assayed for IAA activity. The plants were grown in a greenhouse, until four leaves were well developed, three outer leaves were removed leaving the 'heart' of young tissue. A 21·5 gram sample of this tissue was extracted, and the ether concentrate divided and run on two chromatograms. Only the IAA zones were assayed giving yields of 47·4 and 50·3 γ/kg. fresh weight.

In all the extractions of green tissue, in which the total ether extract solution was used the final concentrate added to the strips contained some oily residues and pigment matter. These, as in the preliminary extraction experiments, occasionally impeded the even running of the solvent and affected the section growth throughout the chromatogram assay. In future experiments the ether soluble acidic and neutral substances were separated by the technique described, and in extractions of green tissue, the acidic portions only were assayed, the pigments and oils remaining in the neutral fraction. In extractions of root tissue, both could be assayed for growth active substances.

Detection of acidic substances:

(a). Green tissue.

Sunflower seedlings were again used for this second series of experiments. The growth activities of the chromatograms were essentially similar to those in the

previous assays - stimulation of growth at an R_F of ·10, inhibition at ·30 to ·45 and a second inhibitory spot at approximately ·70. These growth active substances were all, therefore, of an acidic nature. In order to economise on time and effort most of the analyses in this series were on that part of the chromatogram containing (as assumed) IAA. The chromatograms were marked into 20 transverse pieces, and numbers 4 - 11 inclusively were assayed. Table III gives the details of the tissues extracted and the calculated yields of IAA. (p. 50).

The average yield of 4·81 γ/kg. fresh weight obtained from these 5 extractions was higher than that obtained from the assay of the whole ether fraction of similar tissue. This was probably due to two factors :-

(i) The greater bulk of plant tissue extracted gave a larger quantity of IAA in the final extract. Due to the shape of the calibration curve, this level could be more accurately measured by the assay technique.

(ii) The removal of the oil and pigments in the neutral fraction, gave a 'cleaner' ether concentrate. This resulted in a more even running of the solvent down the paper and better section growth in the assay.

(b). Root tissue.

Extractions of normal pea root tissue were carried out and the final ether solutions assayed to determine the

TABLE III.

Tissue extracted	Yield of IAA. γg./kg. F.Wt.
18 day Sunflower shoots + cotyledons. 56·0g.	9·50
18 day Sunflower shoots + cotyledons. 50·0g.	8·60
18 day Sunflower shoots + cotyledons. 30·0g.	1·65
Ditto. 23·8g.	2·50
Ditto. 30·0g.	1·80

yields, if any, of IAA and to detect the presence of any other growth active substances.

In the first series of extractions, the acidic ether fractions only were assayed. An active substance with an R_F value similar to β-indolylacetic acid was again detected from all extractions. The results of a number of individual experiments are shown in Table IV, giving the calculated yields of IAA and the R_F positions of other active substances detected on the chromatograms. The chromatogram graphs of experiment 3. are shown in Fig.20. Table V gives the average yields of IAA obtained from a number of extractions of pea root tissue of varying ages.

The assayed chromatograms consistently showed inhibition of growth at an R_F value of approximately ·70. This substance, also detected in the previous shoot extracts, was probably identical with the inhibitory compound, of similar R_F value reported by Bennet-Clark (1953) from etiolated pea, broad bean and sunflower seedlings, from the roots of pea, bean and maize and the rhizome of Aegopodium, (called inhibitor β). A second inhibitory compound at an R_F position of ·90 was also detected - from all the young root samples (2 - 4 days) and occasionally from older samples.

The yields of IAA were worked out on a γ g. per kilogram fresh weight and γ g. per 100 root bases. The

TABLE IV.

	Tissue extracted	Yield of IAA		R_F values of other active substances (+)=stimulation (-)=inhibition	
		γg./kg. F.Wt.	γg./100 roots		
1.	2-day Meteor pea roots. (2 chromatograms)		·0043 ·0042	·70(-) ·70(-)	·90(-) ·90(-)
2.	4-day Meteor pea roots.		·0034	·70(-)	·90(-)
3.	4-day Meteor pea roots. (2 chromatograms)		·0074 ·0049	·70(-) ·70(-)	·90(-) ·90(-)
4.	7-day Meteor pea roots. (2 chromatograms)	·63 ·83	·0135 ·0146	·70(-) ·70(-)	
5.	7-day Meteor pea roots.	·74	·027	·70(-)	·90(-)

TABLE V. Pea roots.

Age.	No. of experiments.	Average Yield of IAA γ g./kg. F.Wt.	γ g.per 100 roots.
2 days.	2.	-	·0043
4 days.	4.	-	·00613
7 days.	5.	·59	·015
8 days.	4.	·43	·0156
9 days.	2.	·58	·0315
10 days.	3.	1·30	·043
11 days.	1.	·80	·056
12 days.	3.	1·0	·056

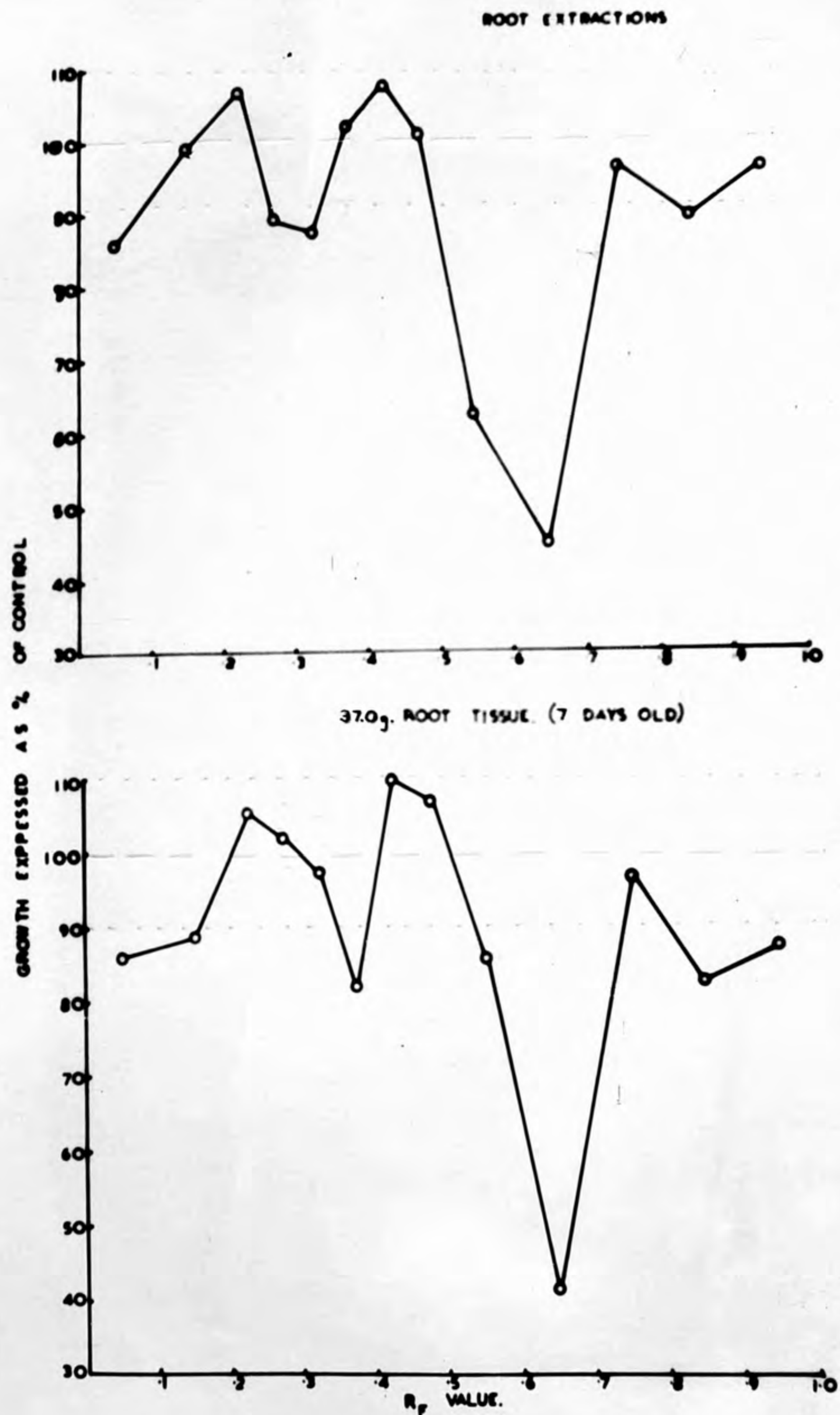


Fig. 20. Duplicate chromatogram graphs from an extract of 37.0 grams of pea roots.

yield calculated on the number of roots showed a marked increase with increase in age of the extracted sample, particularly from 2 - 7 days. Unfortunately the weights of the young root samples (2 - 4) were not recorded, but the yields of IAA worked out on a fresh weight basis also tended to increase with the age of the sample. This does however, serve to illustrate the problems which arise when comparing yields of IAA from tissues of varying ages, etc. Variation in the bases for calculation could possibly reverse the results. In these particular experiments, concerning the age of roots, possibly the yield should have been worked out on a per meristem basis, as lateral roots rapidly developed over the period of time studied (2 - 7 days.).

In the second series of root extractions, the ether fractions containing the neutral substances were assayed. Samples of the roots of 8, 9 and 11 day old pea seedlings were run on chromatograms, but the only activity recorded was inhibition of growth at the starting line, and occasionally at the solvent front.

<u>The effects of acid and alkaline hydrolyses on the growth activity of plant tissue extracts.</u>

The stability of natural growth substances to acid and alkaline treatments, has been used in the past as a method to aid in their identification. Indoleacetic acid was shown to be destroyed by hot acid, but to be resistant to hot alkaline treatment. Pre-treatment of tissues with alkali

has indeed been used to obtain 'total' auxin yields by causing its release from hypothetical inactive precursors. It was important to test the effects of acid and alkaline treatments on the activity of plant extracts, to obtain some information as to the nature of these active substances.

Method of treatment:

The alcoholic extraction of the tissue samples was distilled down to approximately 10ml. The distillation flask was washed down with distilled water and the solution then divided into two equal volumes. The first was immediately extracted with ether, the second was boiled under a reflux condensor with a specific acid (or alkali) for a certain period of time prior to the ether extraction. In the first two experiments the ether extract, as a whole, was assayed, but in the remainder of the extractions the ether was separated into neutral and acidic fractions, and the latter only, assayed for activity.

A number of experiments were carried out, using varying tissues and varying hydrolysing agents. These treatments mainly affected the yields of IAA of the inhibitor at the R_F value of approximately ·70. (Inhibitor β). Details of the hydrolyses are shown in Table VI (p. 56) giving the calculated yields of IAA in γ per kilogram or γ per 100 roots; and the yields of β expressed in γ g. equivalents of IAA per kilogram. (or per 100 roots.).

TABLE VI.

Expt. No.	Tissues	Hydrolysis treatments	IAA γ g. per Kilogram		β. inhibitor in γ g. ≡ IAA per kg.	
			Control	Hydrolysed	Control	Hydrolysed
1.	14 day Sunflower shoots	20ml. N.NaOH for 30 mins.	Too low to calc.	2·53	2·12	337·0
2.	14 day Sunflower shoots	Ditto.	-	-	3·70	135·0
3.	9 day Pea roots.	20ml. N.NaOH for 60 mins.	4·4	25·1	0·64	3·0
4.	9 day Pea roots.	20ml. $\frac{N}{10}$ Hcl. for 90 mins.	5·1	15·6	3·0	60·5
5.	9 day Pea roots.	20ml. $\frac{N}{10}$ Hcl. for 90 mins.	1·05	47·6	11·2	22·4
6.	8 day Pea roots.	20ml. $\frac{N}{100}$ Hcl. for 90 mins.	2·65	Too high to calc.	1·74	3·5
7.	7 day Pea roots.	25ml. H_3PO_4 for 60 mins.	·64	Nil.	·715	7·6
8.	8 day Pea roots.	25ml. H_3PO_4 for 60 mins.	·50	Nil.	·535	3·4
			IAA in γ g. per 100 roots.		β in γ g. ≡ IAA per 100 roots.	
9.	500, 3 day Pea roots.	20ml. N.NaOH for 30 mins.	·0305	·0378	·0302	·0567
10.	400, 4 day	20ml. N.NaOH for 30 mins.	·0021	·132.	·500	·438

Graphs of the assayed chromatograms of experiments 1, 3, 5 and 8 are shown in Figs.21 - 24.

Alkaline hydrolyses brought about an increase in the yield of the compound,presumed to be IAA,from the root tissue and also from the first experiment with green tissue. This result supported the assumption that the compound was β-indolylacetic acid,and also agreed with a large number of reports in the literature of increased growth activity of plant extracts after hot alkaline treatment. The increase of IAA in these experiments must have been from an alcohol soluble 'precursor' (s) extracted from the plant tissue. Determination of the quantity,after such treatment,has been put forward as a means of estimating the 'total' auxin content of a tissue. Little value can be placed on such measurements,as it cannot be known whether this alcohol soluble 'precursor' (s) is the natural source of IAA in the metabolism of the plant.

Alkaline hydrolyses also brought about an increase in the amount of the inhibitor β . (except experiment 10.). The amount of the increase varied,but was greatest in the first two sunflower shoot experiments. The yield of β in these two experiments was increased by approximately 150 and 40 times respectively. The appearance of the inhibited root sections over the β zone of these latter chromatograms was very characteristic,the sections becoming

very yellow in colour and markedly curled.

Acid hydrolyses of root extracts, using 25ml. of syrupy phosphoric acid provided further proof that the active compound at an R_F of ·40 was indolylacetic acid. The assayed chromatograms of these hydrolysed extracts showed no activity over this zone; the untreated halves of the extracts giving the usual activity. (Fig.24). If $\frac{N}{10}$ or $\frac{N}{100}$ Hydrochloric acid was used, however, an increased yield of IAA was recorded. (Fig.23).

Acid hydrolyses, using both dilute hydrochloric and strong phosphoric, brought about increased yields of the inhibitor, from shoot and root tissue.

The three hydrolysing agents were used in blank extractions, i.e. the full extraction process, without any plant tissue. The chromatograms were assayed in the usual way to determine if the chemicals themselves contained any growth active impurities. The extractions with sodium hydroxide and phosphoric acid produced negative chromatograms, apart from a slight inhibition of growth at the original spot level, with the former solution. The hydrochloric acid blank however, gave a chromatogram graph showing 18% inhibition of growth at an R_F of ·70. This may mean that an inhibitor can be present in samples of HCl and due consideration should be given to this before employing the acid as a hydrolysing agent.

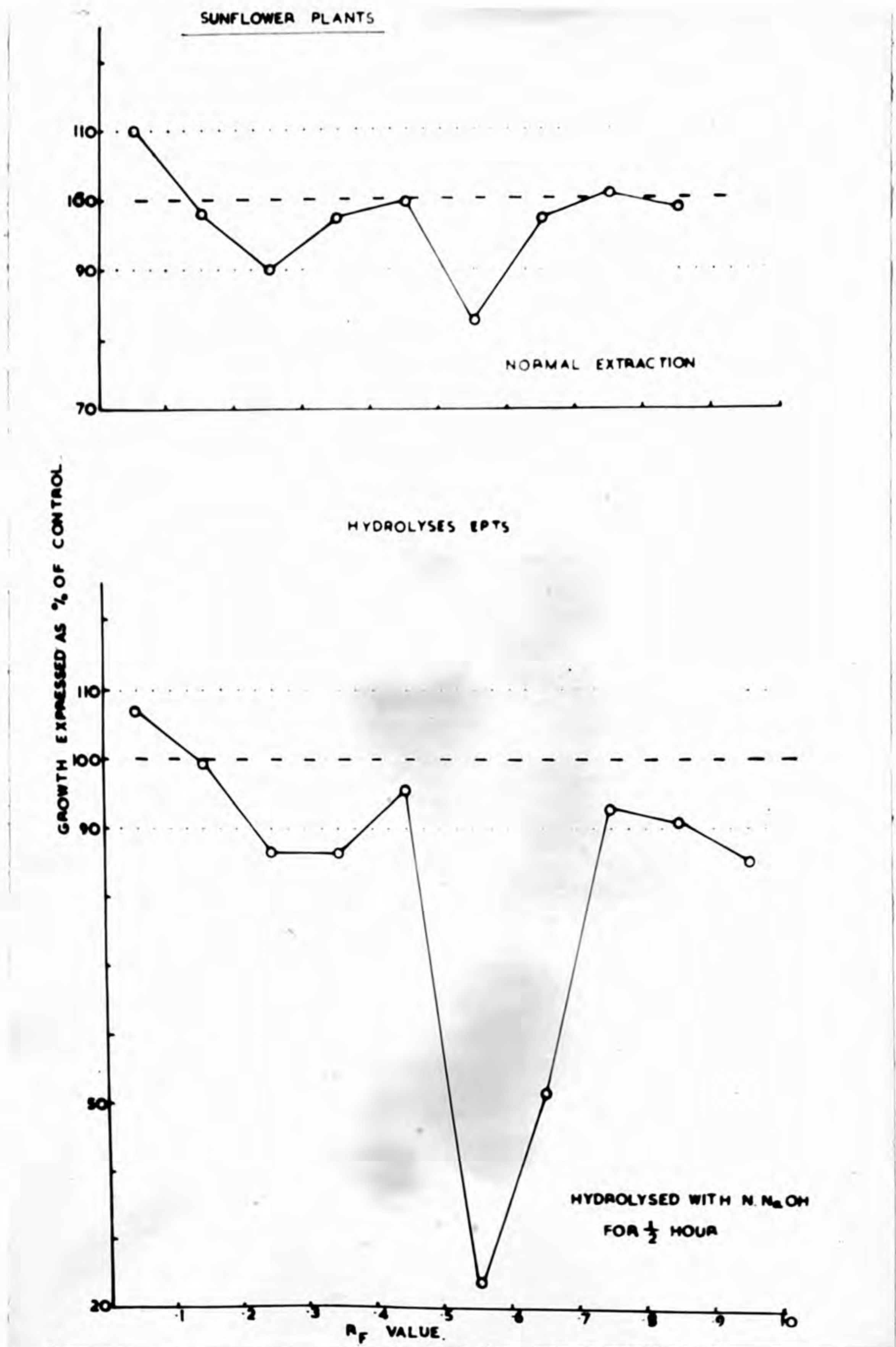


Fig. 21. Chromatogram graphs of normal and hydrolysed portions of an extract of Sunflower shoots.

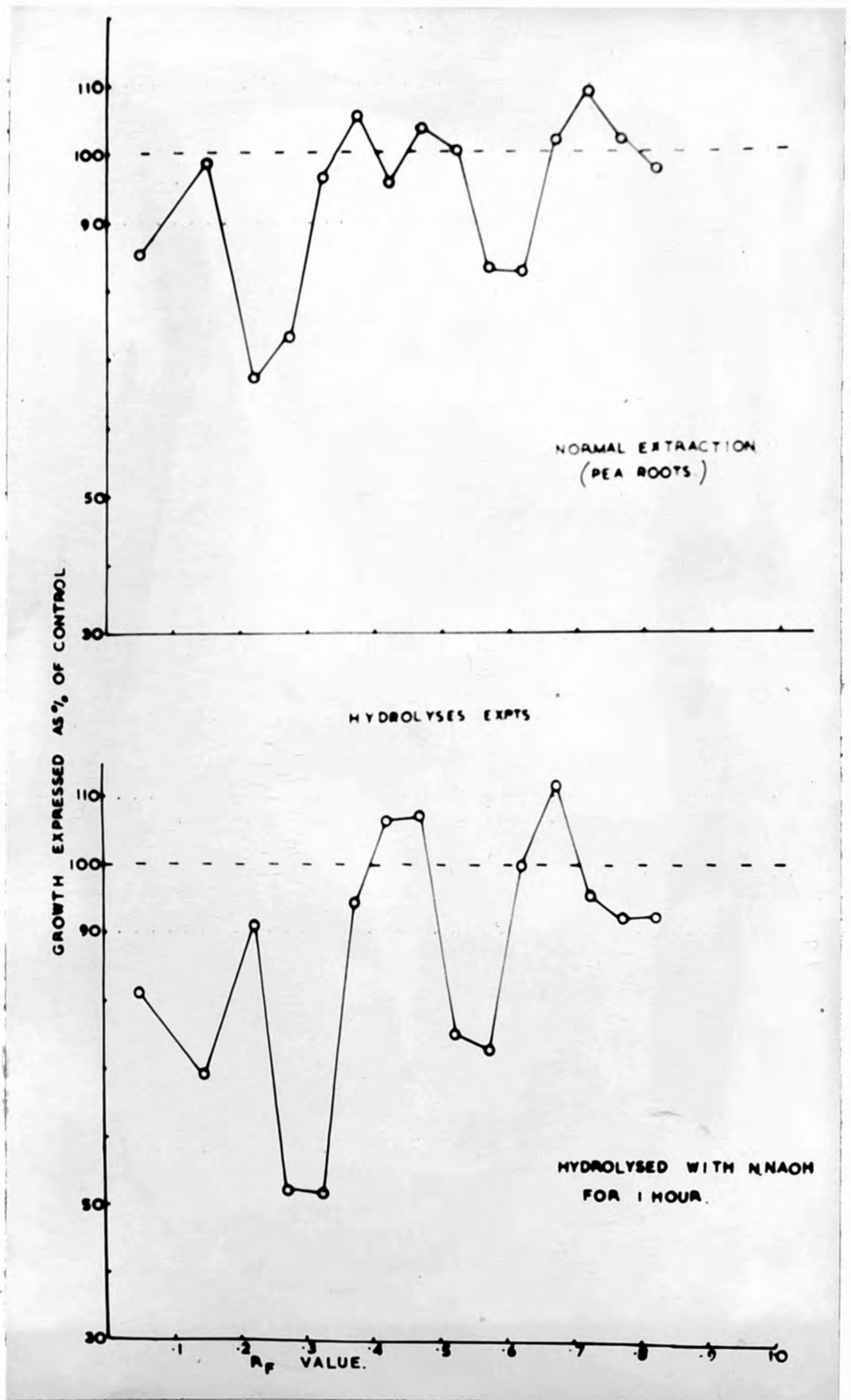


Fig. 22. Chromatogram graphs of normal and hydrolysed portions of an extract of pea roots.

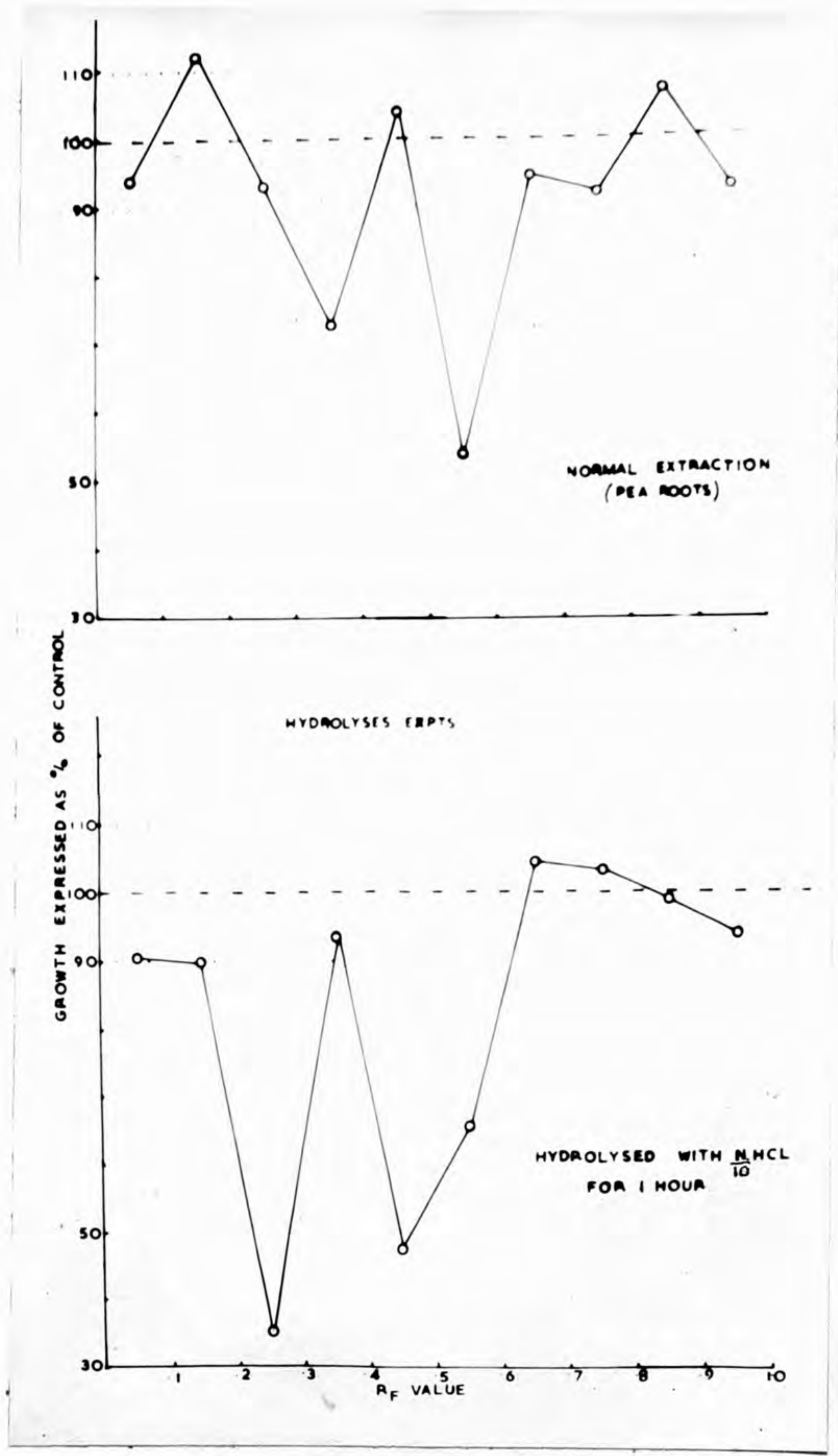


Fig. 23. Chromatogram graphs of normal and hydrolysed (N/10 HCl.) portions of an extract of pea roots.

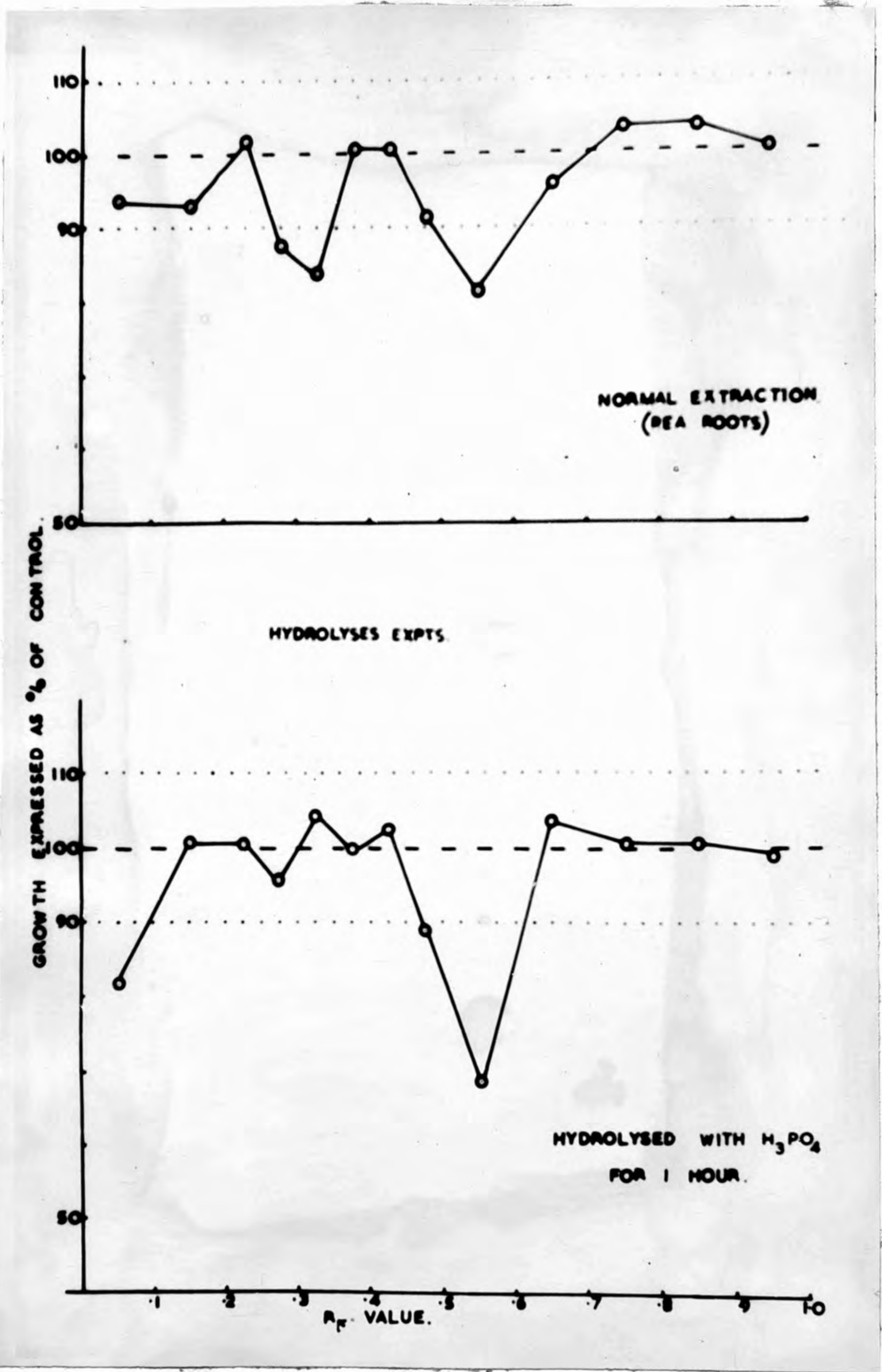


Fig. 24. Chromatogram graphs of normal and hydrolysed (H_3PO_4) portions of an extract of pea roots.

Estimation of the indolylacetic acid content.

The active substance with an R_F of approximately ·35, which was detected on the chromatograms of all tissue extracts was assumed to be IAA, on the following grounds:

(a) It was present in the acid fraction of the ether extractions, and its R_F value with the isopropyl/ammonia/water solvent coincided with that of the pure chemical.

(b) The reaction of this compound to acid and alkaline treatment agreed with the known chemical properties of synthetic IAA.

(c) This inhibitory area on the chromatograms was often preceded by a stimulation of the section growth. This could be explained, if the compound was IAA, by a tailing of the spot; as low concentrations of IAA have been shown to stimulate root section growth.

The purpose of these experiments was to obtain estimates of the yields of IAA from tissue extractions. The results showed a wide variation in these estimations, and it was important to determine how much of this was due to inherent differences in the actual material and how much to errors in extraction and assay.

In a number of experiments, the final acidic ether extracts were divided into two equal portions and each half assayed separately to give the yields of IAA. The results are shown in Table VII, where A and B represent the

replicate assay values. (p. 61 .). By using the figures from these replicated assays it was possible to get a direct measure of the assay error, quite apart from the variations due to plant samples and extractions, (collectively called the 'occasion error'). An analysis of variance was carried out on the logarithmic values of these figures and the results shown in Table VIII. (p. 62).

The analysis showed that the occasion error was very much larger than the assay error and was highly significant. The small assay error could have been expected from the results obtained when chromatographic estimations of IAA from pure solutions were shown to be a direct measure of the quantity added to the strip.

The large occasional error could possibly have been attributed to variable activity of plant enzymes causing destruction of IAA during the extraction. The extraction technique itself, i.e. maceration of fresh tissue with alcohol at + 5°C. should have prevented such activity. An experiment however, was carried out, in which a known quantity of IAA was added to macerated tissue prior to extraction and assay. It was thought that estimation of the quantity of IAA recovered would give some idea of the loss during the extraction process.

Two samples of the shoots of 18 day old Sunflower shoots (from above the cotyledons) were weighed and each

TABLE VII.

IAA content in γg. per k.g. F.Wt.

	Sunflower. 14-18 day old shoots.		Broad bean. 17-27 day old shoots.		Pea. 7-10 day old roots.		Cabbage Central leaves	
	A	B	A	B	A	B	A	B
	9·50	8·60	·95	·87	·83	·64	47·40	50·30
	2·50	1·80	·95	·93	2·20	1·40		
	16·50	10·75	·51	·37	1·70	1·65		
	3·70	2·30						
	3·70	2·40						
	23·00	18·00						
Mean yield IAA	5·96		·72		1·29		48·7	

TABLE VIII.

Analysis of variance of $\log_{10}$ conc. IAA in γ g./kg.

Source of Variance	Sum of Squares	Degrees of freedom	Mean Square Variance
Species	13·9054	3	4·6018
Occasion	1·9185	9	·2022
Assay	·1098	13	·0083

separately macerated with alcohol. 2·5ml. of a 10^{-6} solution of IAA was immediately added to one sample. Both were subsequently extracted by the normal technique and the final acidic ether concentrate from each experiment divided into two equal portions, which were run and assayed in the usual way. The control sample (40 grams, fresh weight) gave yields of ·428 and ·540 γ g. on the two chromatograms, i.e. a total yield of ·968 γ g. The 'sample + IAA' (46 grams, fresh weight) gave yields of 1·213 and 1·740 γ g. i.e. a total of 2·953 γ g. of IAA. If no synthetic IAA had been added to the latter extract, the yield of natural IAA would have been of the order of 1 γ ; this left a 1·953 γ recovery of the 2·5 γ added - approximately an 80% recovery. This result suggested that the loss of IAA due to enzymatic breakdown, or possibly adsorption on to cell proteins, was relatively small.

An experiment was also carried out in which two samples of tissue were extracted on the same day; to give some indication whether this occasion error could be attributed to actual differences in the IAA content of plant samples grown at different times, or simply to variations in the extraction efficiency. It would be assumed that two samples of tissue taken at the same time from a batch of seedlings would contain the same amount of IAA.

64·0 gram and 72·0 gram samples taken from a batch of 18 day old Sunflower seedlings were extracted and assayed, and gave yields of 1·31 γ g. and 8·5 γ g. of IAA per kilogram fresh weight, respectively. This result suggested that the occasion error could be, at least partly attributed to variations in the extraction efficiency.

The reason for the large occasion error was still not fully unexplained, but it was evident from these results that to obtain comparisons of the yields of IAA from normal and untreated tissue samples, both extractions would have to be carried out on the same day.

The mean indolylacetic acid content of the four tissues was calculated and recorded at the foot of Table VII. As would be expected, the IAA contents of the different species, with the exception of pea roots and bean shoots, were significantly different at the 5% level.

B. Growth substances detected from extracts of treated plants:-

1. The effect of 2,4-Dichlorophenoxy acetic acid.

Various tissues, and various methods of application were used to study the effect of 2,4-D. on the IAA content of plants:

(a) Broad beans: A 0·1% solution of the ammonium salt was painted on the upper surfaces of the lowest pair of leaves. Symptoms of epinasty and leaf curl appeared after 24 hours. Samples for extraction were taken from above the treated zone.

(b) Sunflower seedlings: 18 to 30 day old seedlings were treated either by painting the cotyledons, or the lowest pair of foliage leaves with solutions of the ammonium salt of 2,4-D. at concentrations of either 0·1%, 0·01%. These solutions produced the typical responses in the shoots, (leaf curling and epinasty) 24 to 48 hours after application.

(c) Pea seedlings: The seedlings were germinated in sand for two days and then transferred to water culture tanks. One half of the sample was grown in tap water, while the second half was grown in solutions of the ammonium salt of 2,4-D. of concentrations ranging from 0·1 to 0·5 ppm. The seedlings were then grown under these conditions in

the constant temperature room for 6 - 10 days, after which time the roots, or in some experiments, the shoots, were harvested and extracted. The effect of 2,4-D. at these concentrations was to reduce the extension of the main and lateral roots (75% inhibition) and to increase the number of lateral roots by approximately 60%. The net result was a reduction in the fresh weight of root tissue formed, to about 60 - 70% of the controls. There was no visible effect of these treatments on the shoot growth.

The results of assays of the acidic ether fractions of extracts of control and treated tissues are given in Table IX. p.69 Graphs of typical results for Sunflower shoots and Pea roots are shown in Figs.25, 26, and 27.

A number of the experiments with Sunflower tissue failed, due to the large quantity of 2,4-D. in the final ether extract of treated material. This 'tailed' down the chromatograms, causing inhibition of section growth throughout the assay and so prevented detection and estimation of IAA. A typical graph of such an effect is shown in Fig.28. In all these experiments the inhibitory area on the chromatograms, due to 2,4-D. was very wide. To safeguard against this effect either (a) the bulk of treated plant material would have had to be reduced, or (b) the concentration of 2,4-D. lowered. The former would have reduced the accuracy of the estimation of the IAA

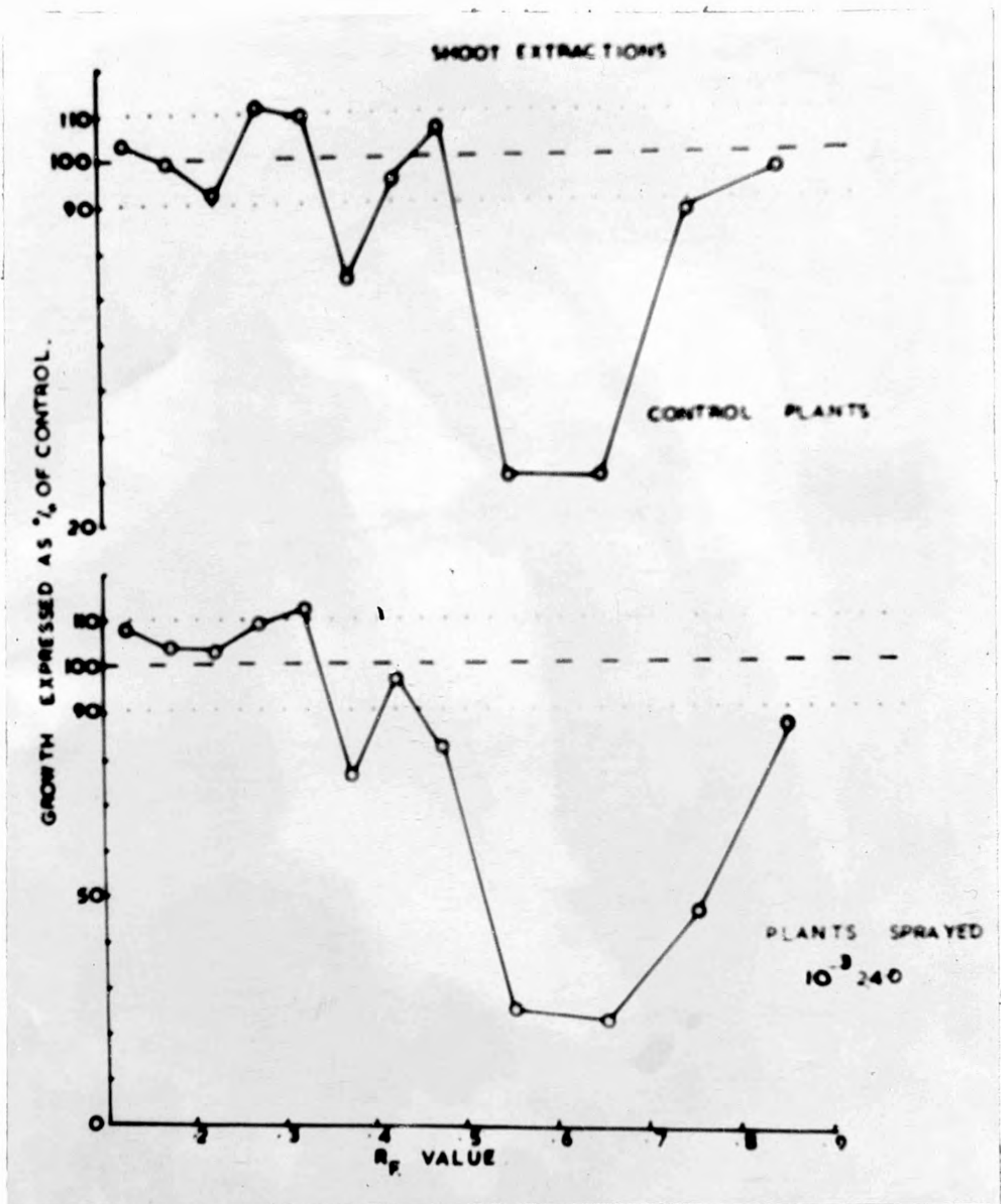
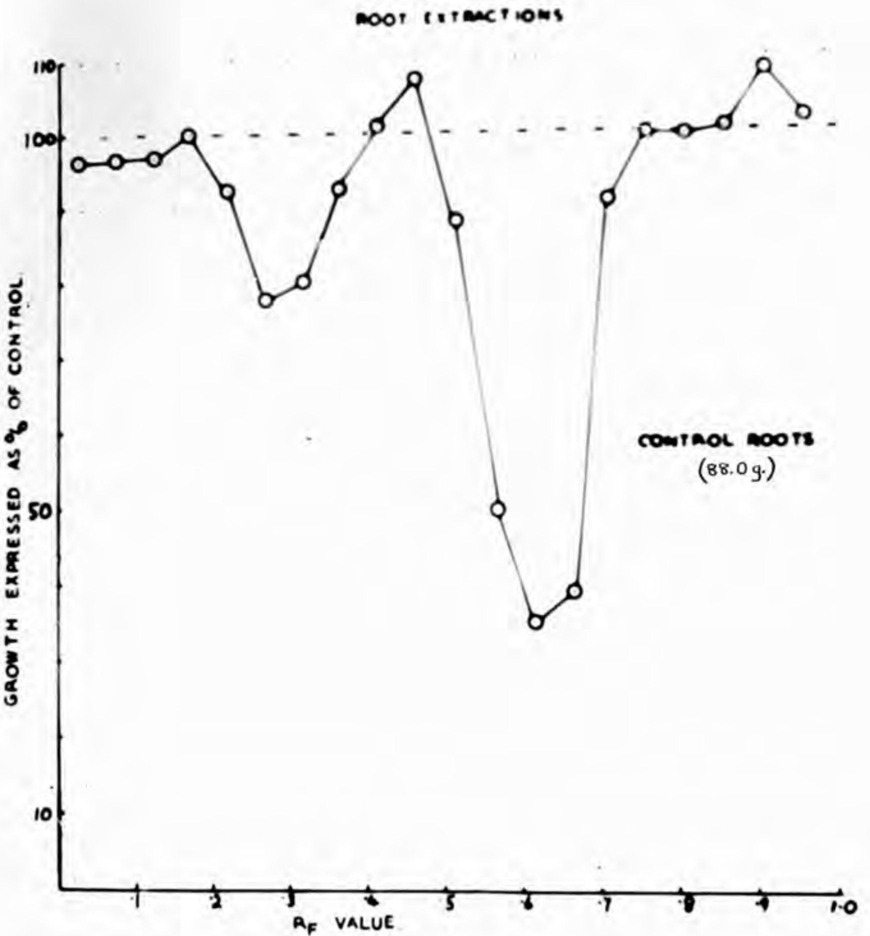


Fig. 25. Chromatogram graphs of extracts of normal and treated (10^{-3} 2,4-D.) Sunflower shoots.



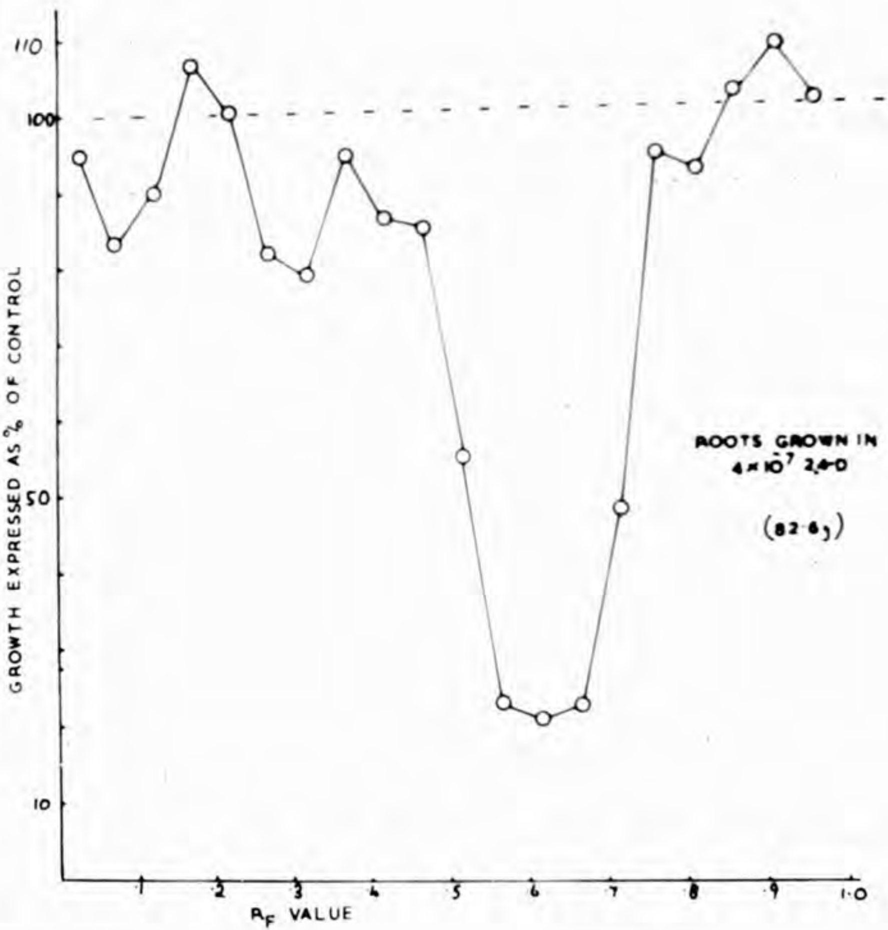
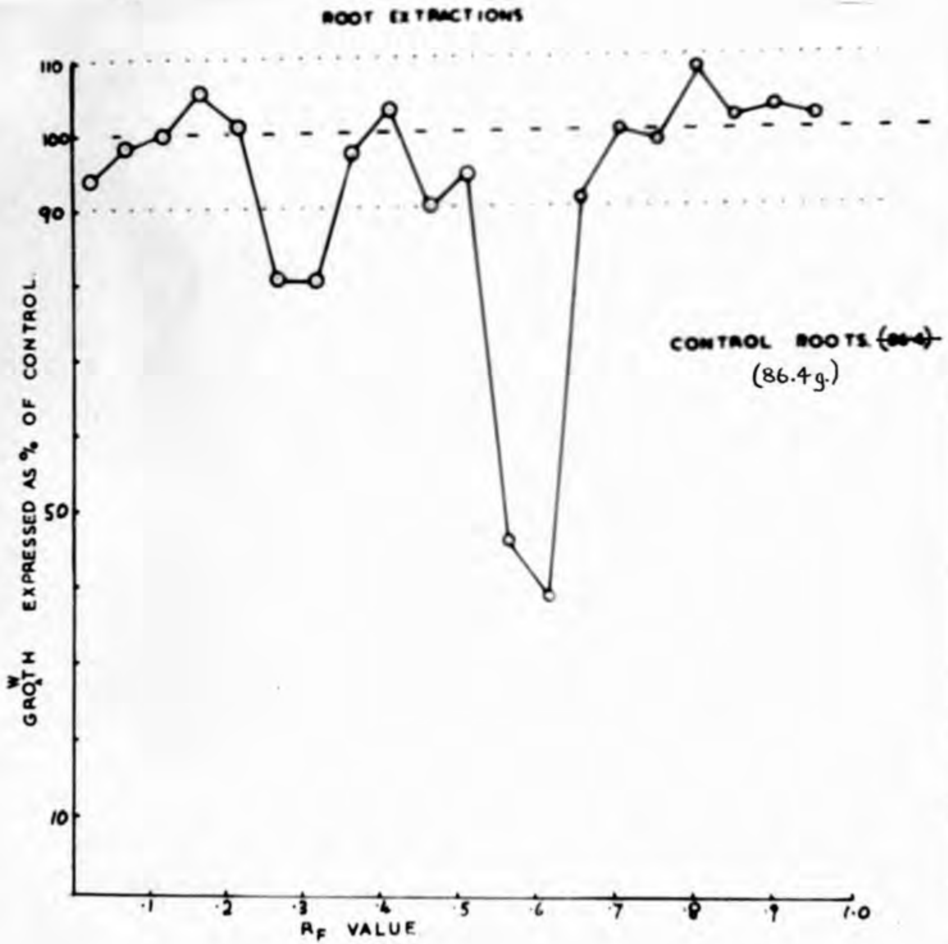


Fig. 26. Chromatogram graphs of extracts (acidic fraction) of normal and 2,4-D (4×10^{-7}) treated pea roots.



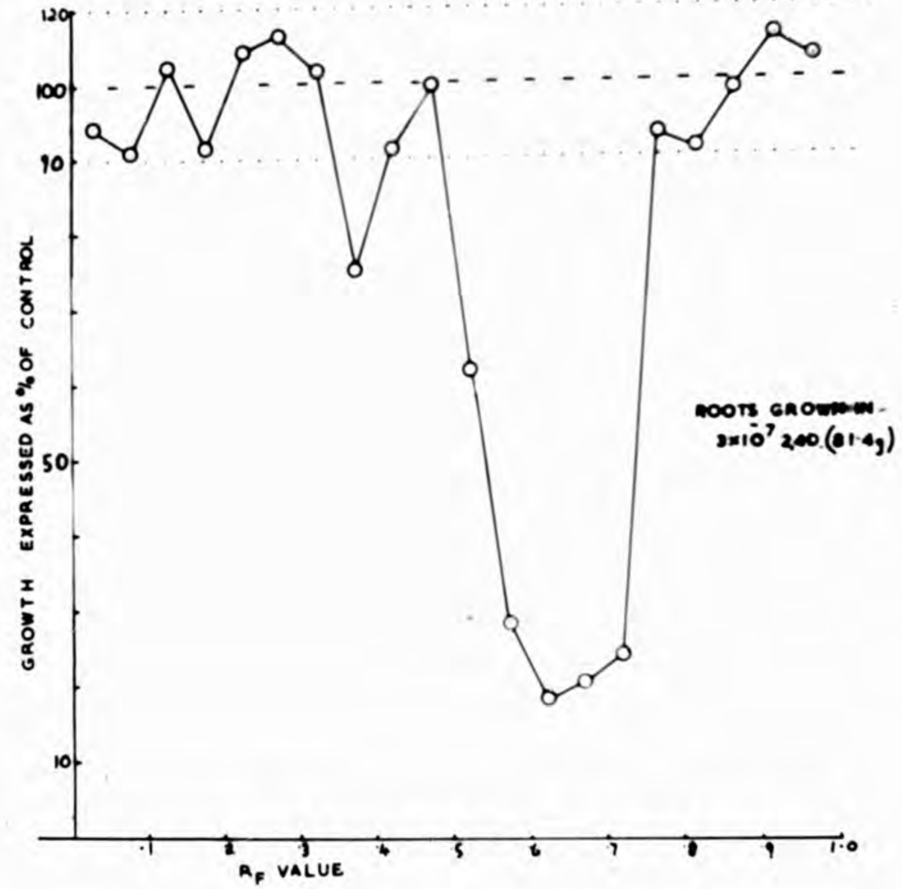


Fig. 27. Chromatogram graphs of extracts (acidic fraction) of normal and 2,4-D. (3×10^{-7}) treated pea roots.

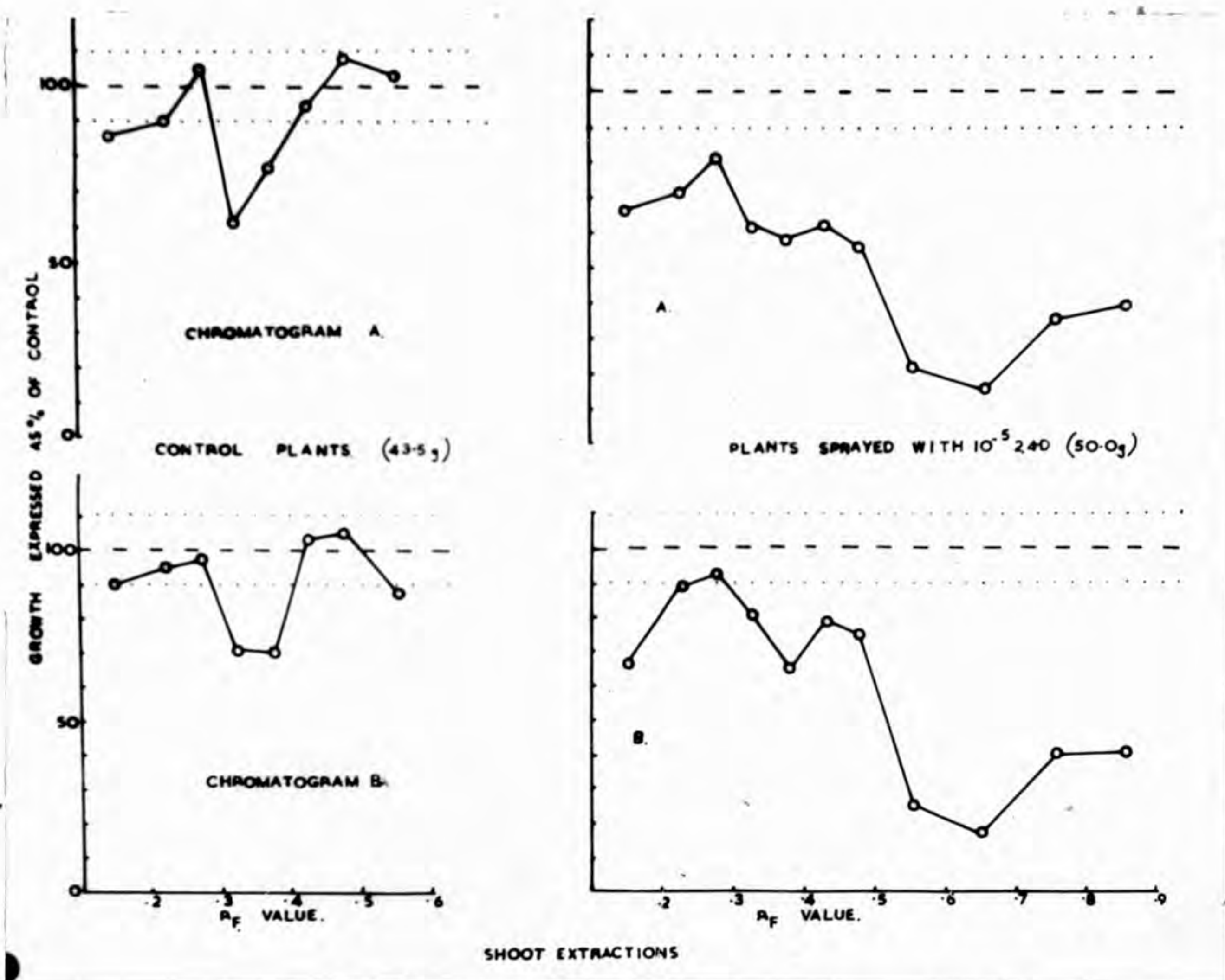


Fig. 28. Chromatogram graphs of extracts of normal and treated Sunflower shoots to show the 'tailing' of the 2,4-D. spot.
Each extract was assayed in two equal portions- A and B.

content, and the latter would have reduced the effect on the plant, and therefore the effect, if any, on the auxin content. Because of this difficulty of striking a balance between an effective concentration of 2,4-D. and one that would not cause streaking down the chromatograms, the majority of the later experiments were carried out on the more sensitive root tissue.

The chromatograms of the control and treated tissue extracts were assayed throughout their lengths. After the first few experiments it was noticed that the inhibition of the sections at an R_F position of approximately ·70 on the control chromatograms had greatly increased. It was thought that the latter must have been due to a carry over of 2,4-D. on the glassware, as a common extraction apparatus was used for both samples. A blank extraction was carried out with the apparatus, cleaned as for experimental use. The assay of this chromatogram did show inhibition in the 2,4-D. zone. A complete duplicate set of apparatus was immediately obtained and used for the control extractions. (All glassware was washed in water and soaked in alcohol for at least 24 hours between experiments.).

The results given in Table IX again showed a wide occasion to occasion variation in the IAA content of all the tissues. There were indications of differences in the auxin content of the different tissues, but no apparent

effect of 2,4-D. on this content. In order to analyse the effect of 2,4-D.,it was thought justifiable to take all the results,of each species,together,ignoring the differences in ages and methods of treatment. If this substance was exerting its effect via IAA levels then this should be apparent using materials of all ages and with all treatments.

An analysis of variance was carried out on the data and the results are given in Table X. p.70 This showed that there was no effect of 2,4-D. treatment on the yields of IAA from any of the materials; as both the treatment and the material/treatment variances were smaller than the residual error. Part of the residual error could be attributed to the assay itself,which as shown in Table VIII was of the order of ·008. This still left an error of ·025 which could be attributed to plant sample differences and variable extraction efficiency.

The occasion variance,i.e. that due to the day to day fluctuations in IAA yields,was significant at the 1% level. This could also be attributed to inherent differences in the plant samples on separate occasions and/or to variations in the extraction efficiency. The fact that this occasion variance was,however,much larger than the residual error did suggest that it might be due mainly to actual differences in the quantity of IAA present

TABLE IX.

Material Analysed	Concentration of 2,4-D. Ammonium salt	Point of application	Duration of treatment	IAA conc. in γ g./kg. Fresh weight	
				Control	Treated
Broad bean shoots (above first pair of leaves)	1000 ppm.	Lowest pr. leaves.	1 day	·87	·90
	"	"	" "	·94	1·30
do.	"	"	10 days	·37	·31
	"	"	10 days	·51	·38
Sunflower shoots. 18 day.	10 ppm.	Cotyledons	2 days	10·75	Streaked
(Above cotyledons)	1000 ppm.	"	14 hours	1·60	1·70
	"	"	24 hours	4·20	3·50
	"	"	" "	7·40	5·40
do.	"	"	" "	2·50	5·80
	"	"	" "	8·10	6·30
	"	"	10 hours	3·00	Streaked
Sunflower shoots. 30 day.	100ppm.	"	24 hours	1·40	1·40
	"	"	" "	2·70	·74
(Above lower pair of leaves)	"	Lower pair leaves.	" "	3·50	5·30
	"	" "	" "	2·90	5·30
	1000 ppm.	" "	9 hours	2·40	3·50
	"	" "	" "	3·70	Streaked
Pea roots 8 days old.	0·5 ppm.	Roots	6 days	·95	·18
12 " "	0·4 ppm.	"	10 "	1·08	1·05
12 " "	0·3 ppm.	"	10 "	1·03	1·48
11 " "	0·3 ppm.	"	9 "	·81	·94
8 " "	0·1 ppm.	"	6 "	·27	·26
Pea shoots. 10 days old.	0·3 ppm.	Roots.	8 days	·35	·44
do.	0·3 ppm.	"	10 "	·87	·93
	0·3 ppm.	"	9 "	·69	·65

TABLE X.

Analysis of variance of $\log_{10}$ conc. IAA in γ g./kg.F.Wt.

Source of Variance	Sum of Squares	Degrees of Freedom	Mean square variance	Variance ratio
Material	5·317	3	1·772	53·69
Occasion	2·34	18	·130	3·94
Treatment	·0035	1	·0035	
Material/ Treatment	·031	3	·00103	
Residual error	·596	18	·033	

in batches of similar tissue,extracted on separated days.

No other consistent effects of 2,4-D. were recorded on the chromatograms of the acidic fractions. The levels of the inhibitor β could not be estimated from the chromatograms of the treated extracts,as 2,4-D. itself had a similar R_F value.

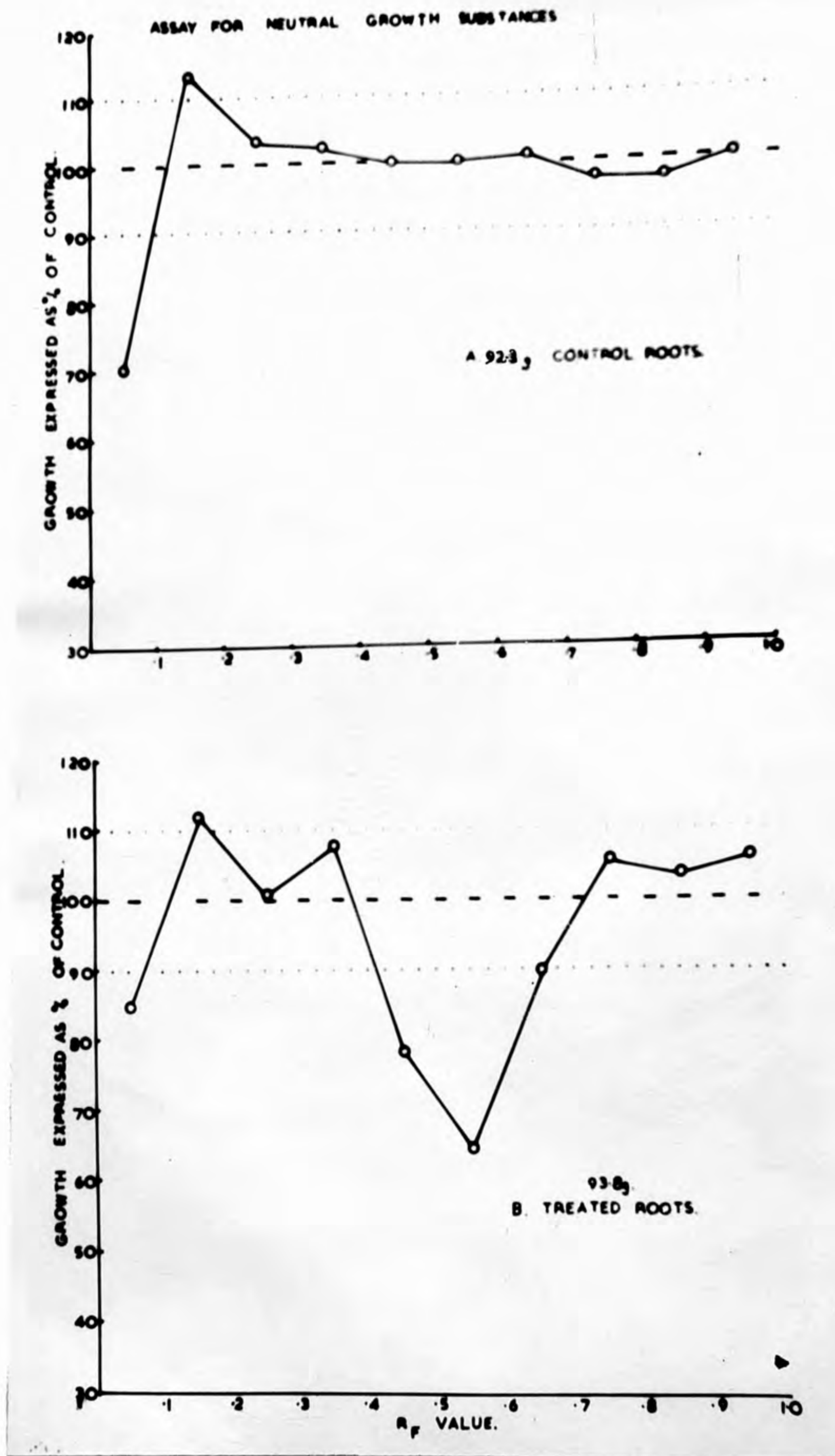
A number of experiments were carried out,in which control and treated roots were extracted and the neutral ether fractions assayed. In all the experiments,the treatment consisted of growing the roots for varying periods of time in a 0·3 ppm. solution of the ammonium salt of 2,4-D. The results of the assays are shown in Table XI,and a typical graph in Fig.29.

Inhibition of the sections occurred at the original spot level on the chromatograms of both control and treated extracts. This activity had been recorded in previous assays of control root extracts. In addition to this, however,slight stimulation was occasionally recorded at an R_F of ·20 and slight inhibition at the solvent front. The chromatograms of the treated root extracts showed a wide inhibitory spot at an R_F ranging from ·4 to ·7,which was not recorded from the control chromatograms. As these R_F values were near to 2,4-D.,it was suspected that these spots were due to residual 2,4-D. left in the neutral fraction as a result of incomplete separation from the

TABLE XI.

Assay of neutral fraction. (Root Extractions).

Age	R_F positions of active substances. (+) = stimulation. (-) = inhibition.						
	Control			Treated			
10 days	·1(-)	·2(+)	·9(-)	·1(-)		·4 - ·5(-)	1·0(-)
10 days	·1(-)			·1(-)		·6(-)	
11 days	·1(-)		1·0(-)	·1(-)		·4(-)	1·0(-)
12 days	·1(-)			·1(-)		·4 - ·5(-)	
10 days	·1(-)			·1(-)		·4 - ·5(-)	1·0(-)
8 days	·1(-)	·2(+)		·1(-)	·2(+)	·5 - ·6(-)	
8 days	·1(-)			·1(-)	·2(+)	·6 - ·7(-)	



Fig, 29. Chromatogram graphs of the neutral fractions of extracts of normal and 2,4-D. treated pea roots.

acid fraction. This was tested by macerating a sample of control tissue and immediately adding to it, 0·1ml. of a 10^{-6} solution of 2,4-D. Extraction was then carried out normally and both the acidic and neutral fractions assayed. No trace of growth activity at an R_F value between ·4 and ·7 was shown on the chromatogram of the neutral fraction. This inhibitory spot must therefore have been due to a neutral substance produced in the living roots by the action of 2,4-D.

2. The effect of maleic hydrazide. (MH).

The assay curve, obtained with increasing concentrations of maleic hydrazide (Fig.10) showed a 60% inhibition of root section growth at a concentration of 1000 p.p.m. When two day old pea seedlings were transfered into culture tanks containing a solution of MH at this concentration, root growth was completely inhibited; indicating that whole seedlings were much more sensitive to this compound than the assay root sections. Pea seedlings were therefore grown in more dilute solutions - 10 to 50 p.p.m. Ten roots were taken at random from normal and treated samples of the seedlings, and the lengths of the primary and lateral roots, together with the numbers of laterals per root, recorded. The mean effects of maleic hydrazide on these characters are given in Table XII (p.75). Photographs of 10 day old roots in control and treatment (20 p.p.m.) tanks are shown in Fig.30.

The length of the primary root was reduced by growth in MH, at all concentrations studied. The percentage inhibition obtained in a 20 p.p.m. solution increased with increase in the duration of the treatment, i.e. 25% inhibition after 5 days treatment, but 61% after 11 days. The length of the lateral roots was also reduced by growth

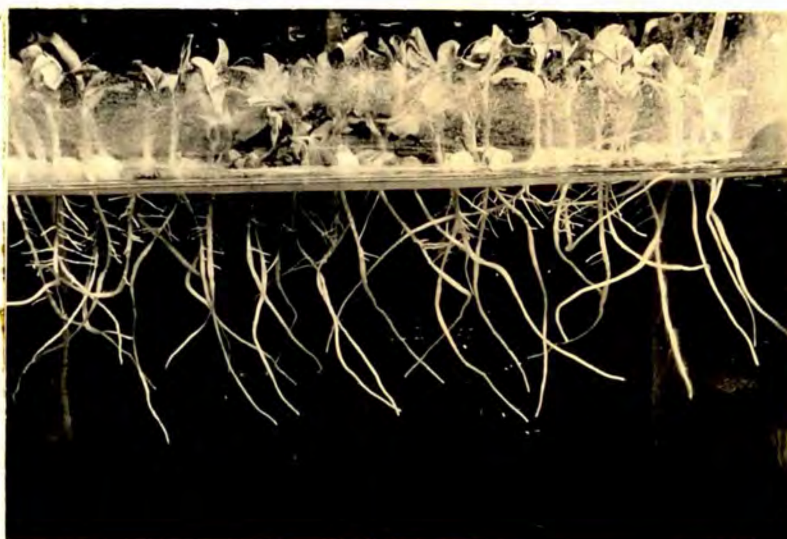
TABLE XII.

Pea roots.

Conc. MH p.p.m.	Duration of treatment.	% Inhibition of Treated Roots			
		Length of tap roots.	No. of Laterals.	Length of Laterals.	Fresh weight.
1000	4 days	-	None	None	100
50	5 "	-	None	None	76
50	7 "	-	None	None	74
30	6 "	-	None	None	59
20	11 "	61	57	65	68
20	10 "	40	55	86	61
20	8 "	22	54	64	51
20	5 "	25	50	84	55
10	12 "	-	-	-	20

- not recorded.

Normal roots.



Roots grown in a 20p.p.m. solution of
Maleic hydrazide.

Fig. 30. Comparison of ten day old pea roots grown
in water and Maleic hydrazide.

in MH,but the percentage inhibition was not correlated with the duration of the treatment,at any one concentration. 50 and 30 p.p.m. solutions prevented lateral root formation; 20 and 10 p.p.m. solutions halved the numbers of laterals produced compared to control seedlings,as well as reducing their length. In one experiment,the pea seedlings were not transferred into a 20 p.p.m. solution of MH until they were three days old. Samples taken after 6 days further growth showed no reduction in the lateral root number compared to a sample of control roots. Lateral roots,in control seedlings,appeared on the third day after germination,so that these results indicated that MH,applied in the second day,prevented (or reduced) the emergence/ initiation of lateral roots.

When the tissues were harvested and macerated in the blender it was noticed that the MH treated roots were much 'harder' and more resistant to the maceration than the controls. This suggested that growth of the seedlings in MH had affected the root histology.

Solutions of 10 and 20 p.p.m. were used for the extraction experiments. Series of control and treated root samples were harvested and extracted on the same day and the acidic ether fractions assayed as in previous experiments. The details and results are given in Table XIII and graphs of assayed chromatograms in Figs.31 and 32.

TABLE XIII.

Pea roots.

Conc. of MH. p.p.m.	Duration of treatment.	IAA γg./kg. F.Wt.		INHIBITOR β. IAA ≡ γg./kg. F.Wt.	
		Control	Treated	Control	Treated
10	12 days	·91	2·0	15·9	10·9
20	10 "	·52	1·65	1·84	2·92
20	10 "	·18	·20	14·8	Too high to estimate
20	5 "	·74	·37	1·6	5·15
20	6 "	·18	·97	3·44	2·05
Mean		·506	1·04	5·69	5·25

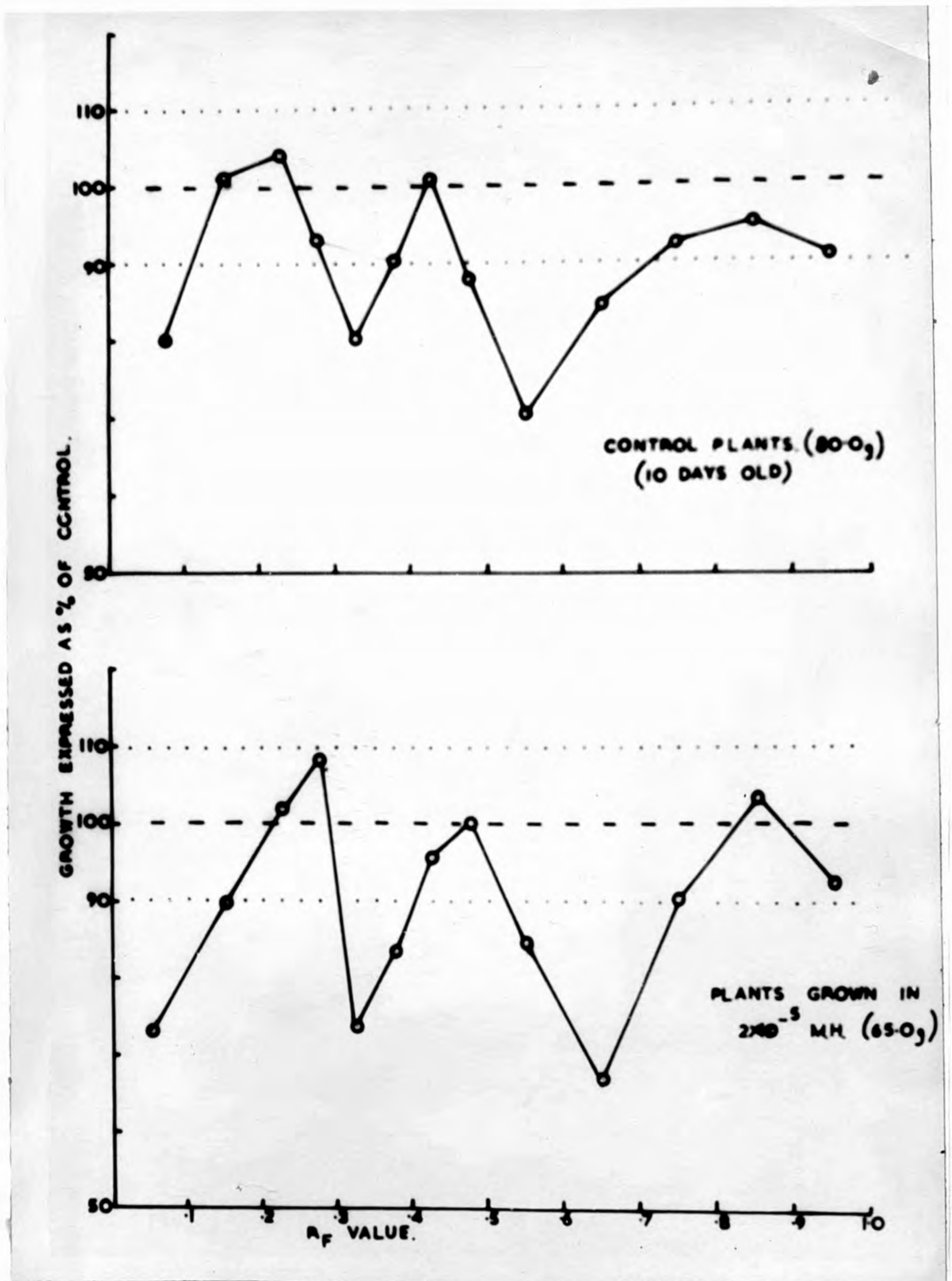


Fig. 31. Chromatogram graphs of extracts (acidic fraction) of normal and treated(20p.p.m.MH.) pea roots.

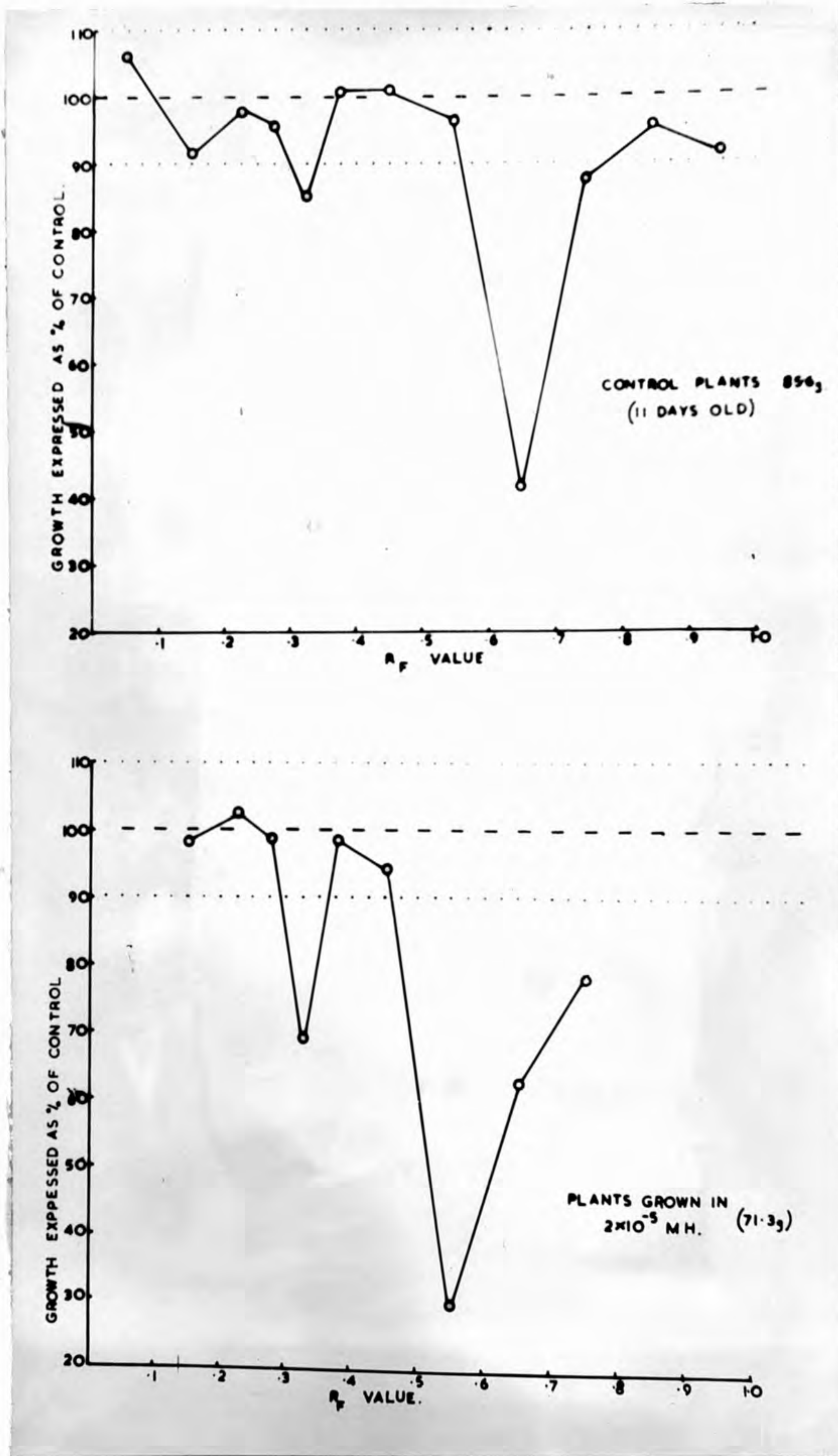


Fig. 32. Chromatogram graphs of extracts (acidic fractions) Of normal and treated (20 p.p.m. MH.) pea roots.

Maleic hydrazide had an R_F value of between ·15 and ·25 with the propanol/ammonia solvent, but at the doses employed in these experiments it could have no effect on the growth of the assay sections. The level of inhibitor β could therefore be determined from the assayed chromatograms of treated and control extracts, in addition to the level of indolylacetic acid. β was expressed in IAA $\equiv$ γ g./kg. fresh weight.

The results again showed a large occasion (or day to day) variation in the IAA content, and to an even greater extent in the level of inhibitor β. Four out of the five experiments showed an increase in the yield of IAA from treated roots, and the mean content for treated roots was twice that of the controls. (Table XIII). A "t" test on the data showed the difference to be almost significant, but obviously further work was necessary. No consistent effect of MH on the quantity of β could be detected - the small differences between the mean quantity in control and treated roots was not significant.

In two of the experiments, the neutral ether fractions of control and treated (20 p.p.m. MH) root extracts were assayed for growth activity. The chromatograms of both control and treated extracts showed slight inhibitory areas at the original spot level, and at the solvent front. In addition to these, the chromatograms of the neutral extracts

of the treated roots showed slight inhibition at an R_F of ·5. The percentage inhibition was much less than that obtained in a similar position on the chromatograms of the neutral ether extracts of 2,4-D. treated roots. Further work was again called for to establish the development of a neutral inhibitor in MH treated pea roots.

3. The effect of 2, 3, 5-tri-iodobenzoic acid (TIBA).

Two day old pea seedlings were transfered to culture tanks and grown for 6 - 9 days in (a) tap water, and (b) TIBA solutions of 10 and 30 p.p.m. Growth of seedlings in solutions of this substance brought about a decrease in the number and length of lateral roots and in the girth of the primary roots. The most striking effect was however on the growth 'form' of the root system, as the lateral roots and to some extent the primary roots grew upwards. (This effect is shown in Fig.33.). The overall effect was a reduction in the fresh weight of the root tissue, e.g. the fresh weight of a sample of roots grown for 7 days in a 10 p.p.m. solution of TIBA was 70% that of an equal number of control roots of the same age. The shoots of the pea seedlings were unaffected at all concentrations.

Samples of root tissue from control and treatment tanks were harvested, extracted and the acidic ether fractions assayed as in the 2,4-D. experiments. Details and results of these experiments are shown in Table ~~XIII~~, XIV, and a typical pair of graphs in Fig.34.

In all the experiments it appeared that treatment of roots with TIBA caused a marked reduction in their IAA content. (This effect was even more striking if the IAA yields of control and treated samples were compared on

TABLE XIV.

Pea roots.

Duration of treatment	Concentration of TIBA p.p.m.	IAA content γ g./kg. F.W.	
		Control	Treated
6 days	10	1·3	·0002*
7 days	10	1·07	·0002*
7 days	10	1·89	·50
8 days	10	1·40	·35
9 days	10	·77	·0001*
8 days	30	·51	0 ø or ·01

* Stimulation on chromatogram.

ø No response on chromatogram.

Fig. 33. The roots of pea seedlings grown for 7 days in a 10 p.p.m. solution of the ammonium salt of TIBA.

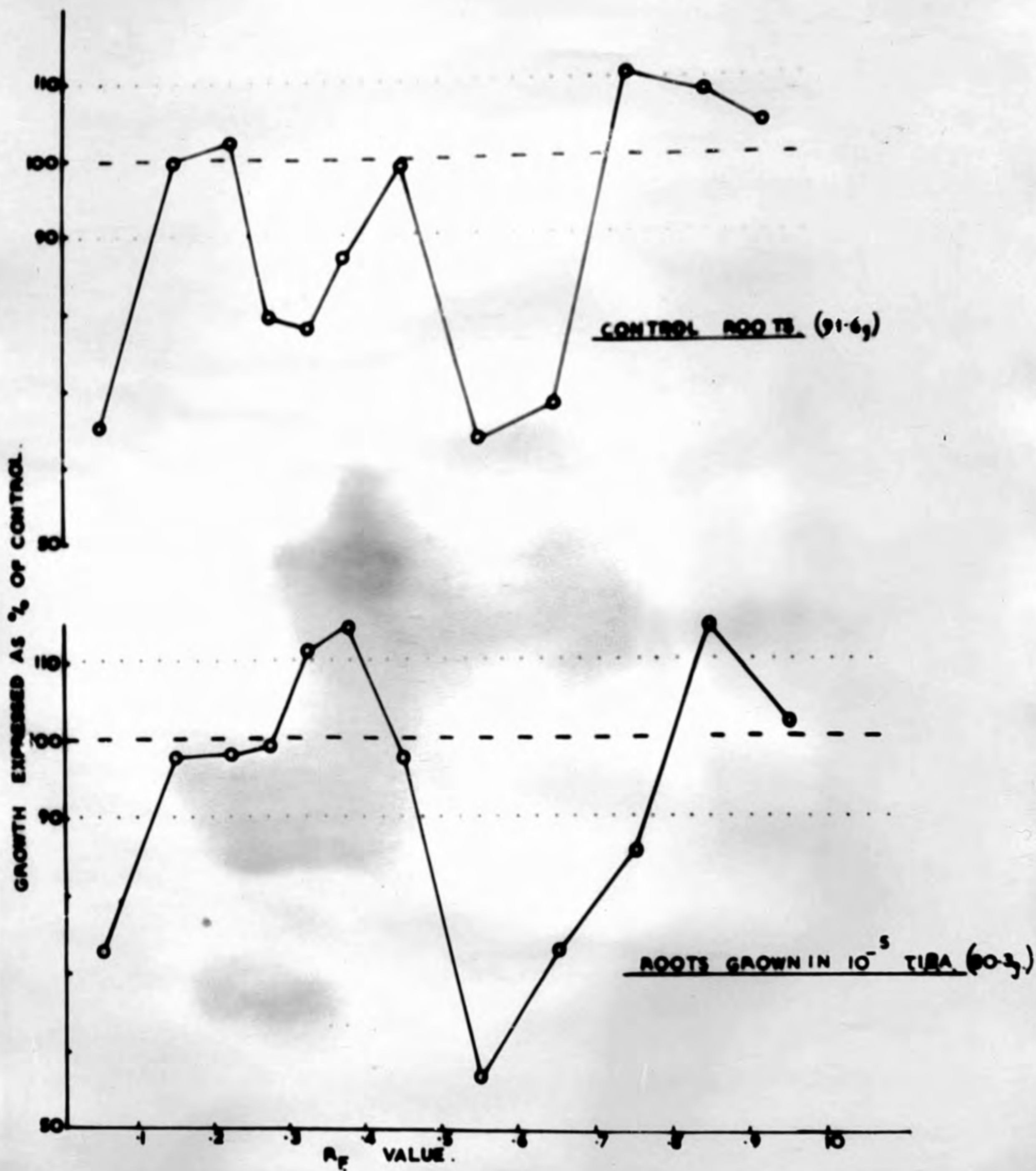


Fig. 34. Chromatogram graphs of extracts (acidic fraction) of normal and treated (10 p.p.m. TIBA) pea roots.

a γ g. per 100 root basis.). In three out of the six experiments the IAA was reduced from an inhibitory to a stimulatory level, and consequently estimation could only be approximate. In one experiment, no IAA was detected in the treated sample, but this could possibly have been due to the presence of an approximately 10^{-9} solution, where the assay curve for this substance cuts the control level. In spite of these difficulties in the estimations, there could be no doubt that TIBA had dramatically reduced the IAA level in the roots of pea seedlings.

The neutral ether fractions of three of these extractions were also assayed for growth activities. Apart from a slight inhibition at the original spot level, no activity was recorded from the chromatograms of either the control or treated root extracts.

DISCUSSION.

DISCUSSION.

In the assessment of the qualitative and quantitative aspects of the experimental results, it is important to realize the limitations of the methods employed, and the criticisms which can be levelled against them.

Considering the qualitative aspects first; the techniques used in the determination of the growth substances present in plants, included (1) extraction of the plant substances; (2) chromatographic separation of the extracted substances, and finally (3) detection on the developed chromatograms by the root assay method.

The extraction technique sets a limit on the substances detected, in that they must all be alcohol soluble. The maceration and extraction processes may bring about the breakdown of the active compounds which are actually functional in the plant, and these breakdown products may, or may not be growth active. Active substances which are highly photo or thermo-labile would be partially lost during the extraction processes, some of which are carried out in the laboratory, at temperatures up to 30°C.

The chromatographic separation of the growth substances present in the final ether concentrate is dependent on the choice of the solvent. The ammonia/iso propyl/water solvent was used in all the experiments and was tested for

suitability to separate indolylacetic acid and synthetic 2,4-Dichlorophenoxy-acetic acid,(or TIBA). Its potentialities for the complex plant extracts are unknown and it may well be that some active compounds are not separated with this solvent. Since the commencement of the work, it has been shown that the presence of ammonia in the developing solvent can bring about interconversion of certain compounds. (Nitsch, 1955).

Finally, the assay technique itself sets a limitation on the detection of growth active substances. The different tests employed vary in their sensitivity to growth active substances, for example pea roots are very much less sensitive to indole acetonitrile than _Avena_ coleoptile sections. (Bentley & Bickle, 1952). There is also the possibility that active compounds may be firmly adsorbed on the chromatographic strip, and they would not then be detected by the assay method. An example of this adsorption has been demonstrated with synthetic maleic hydrazide, which could not be detected on chromatograms, although present in toxic concentrations.

Accepting the above limitations and criticisms of the methods, at least 5 active substances were detected on the chromatograms of plant tissue extracts:

The active acidic substance, at an R_F value of ·35, which was detected in all the tissue extracts (with the

exception of the sunflower seeds) was assumed to be β- indolyl acetic acid. IAA was ~~therefore~~ recorded from the green shoots of peas, sunflowers, cabbages, dwarf and runner beans, and from the etiolated shoots of sunflowers and roots of peas. The ubiquitous presence of this compound agrees with the literature.

A second acidic compound, always inhibitory, was also detected in all the plant extracts. This substance had an average R_F value of ·65 with the propanol/ammonia solvent. It was recorded in the extracts obtained by the preliminary and final extraction techniques, and was probably identical to an inhibitory substance, with a similar R_F value, reported by Bennet-Clark (1953) from etiolated pea, broad bean and sunflower seedlings, from the roots of pea, bean and maize and the rhizomes of Aegopodium. (Inhibitor β).

An attempt was made to determine the growth relationships of root sections, with varying concentrations of this inhibitor β . 225 grams of the roots of 9 day old pea seedlings were extracted in 2 separate samples. The acidic ether fractions were combined and placed on a 2" wide filter paper strip. The latter was run in the usual manner, dried and cut into two (R_F 0 to ·45 and ·45 to ·90). The lower portion was reset in the chromatography tank and run again with the solvent. The latter was allowed to run off the strip, into a test tube, and the eluate

collected after 24 hours. This solution was evaporated to dryness, taken up in 2ml. of ½% sucrose, and a series of dilutions made and assayed in the growth vessels by the root section technique. The growth curve obtained was similar to the calibration curve with decreasing concentrations of 2,4-D., showing no stimulation of section growth. This was a similar relationship to that shown by Kefford (1955) for inhibitor β, using the coleoptile assay technique. This compound is therefore not an auxin. It is resistant to acid and alkali treatment, and it gives no colour with the indole spray. This negative response is not conclusive, as the concentration on the chromatogram may have been too low to react with the spray. Despite its regular occurrence in plants, nothing is known of this compound, beyond the fact that it is an acidic, stable inhibitor.

A third substance, which was stimulatory, was occasionally recorded at an R_F value of approximately ·15. This substance was detected from runner bean tissue only, in the preliminary extraction experiments. It was occasionally detected on the chromatograms of the acidic ether fractions of extracts (rapid technique) from sunflower shoots. In contrast to the 32% stimulation recorded from the runner bean tissue, at this R_F value; in the sunflower extracts it was rarely more than 10% and

often not significant. No activity was shown in this position on the assayed chromatograms of the <u>acidic</u> ether fractions of root extracts. An acidic stimulatory compound (alpha) was demonstrated by Bennet-Clark (1952), at this R_F position on the chromatograms of extracts of etiolated shoots and roots, and was shown to have considerable root growth promoting activity. It seems likely that α is identical with the compound detected in these experiments. Indole pyruvic acid (IPyA) has been reported from the extracts of sweet corn (Stowe & Thimann, 1954) and this has an R_F value of ·15 with the propanol/ammonia solvent. Kefford (1955) suggested that, due to the chromatographic evidence, the α stimulatory compound could be IPyA. The fact that in this series of experiments, the compound is spasmodically recorded, suggests that the activity at an R_F of ·15 might be due to IPyA, as the latter compound is known to be chemically unstable and to undergo spontaneous breakdown. The identification of IPyA in a variety of tissues would be interesting, as it has been postulated as one of the intermediates in the production of IAA from tryptophane. (Gordon, 1954).

A fourth zone of activity, inhibition of growth at an R_F value of ·90 was recorded from all the chromatograms of the acidic ether fractions of extracts of young

roots (3 - 4½ days old). Similar activity was recorded on the chromatograms of the total (i.e. acidic + neutral fractions) ether extracts of sunflower seedlings. In the later extracts of green tissues, only the IAA zone of the chromatograms of the acidic ether fractions were assayed, so that it could not be certain whether the substance detected was acidic or neutral. Indole aceto nitrile (IAN), a neutral compound, was reported by Jones et al (1952) to be present in large quantities in members of the Cruciferae, and using the propanol/ammonia solvent it was shown to have an R_F value close to the solvent front. They also demonstrated that quantities of this compound, which could be detected by the Avena coleoptile straight growth test, were left in the acidic fraction of the ether extracts. It seemed possible then, that the activity at R_F ·90 on the chromatograms of the acidic ether fractions of young roots, was due to residual, neutral IAN. Unfortunately the neutral ether fractions of these extractions had been discarded. Contrary to this suggestion, (a) the neutral fraction of older roots, although occasionally showing activity at the actual solvent front, do not show any activity at an R_F of ·90; and (b) pea root sections are relatively insensitive to IAN. A rough test showed that a solution of 1 p.p.m. of IAN in ½% of sucrose had no effect on the growth of

root sections, and a 10 p.p.m. solution caused a 22% inhibition of growth. It seems unlikely then, that a detectable quantity of IAN can be left in the ether, from which the neutral substances have been supposedly removed. It is likely therefore, that this is an acidic, inhibitory substance, consistently present in young pea roots, and detected occasionally in other tissues.

Inhibition of growth was always recorded at the starting line of chromatograms of the neutral ether fraction of all extractions. It was also occasionally detected on the chromatograms of the acidic fraction. It is suggested that this inhibition is correlated with the presence of oily substances in the concentrated extracts (particularly obvious with neutral ether concentrates). These substances do not move on the paper, and may have direct toxic effects on growth, and also seem to affect the adsorption of the nutrient sucrose solution by the paper.

The effects of acid and alkaline hydrolyses on the alcoholic extracts of plant tissues, were studied with particular emphasis on IAA and inhibitor β .

Alkaline hydrolyses (with Sodium hydroxide) brought about increased yields of indolylacetic acid and inhibitor β , from pea roots and sunflower shoots. These increases must have been due to release from alcohol

soluble plant compounds. An increased yield of auxin, following alkaline hydrolysis has been widely reported in the literature (Avery et al.1941,1942) and is evidence for the presence of 'bound' auxin in the plant. The increase in β is interesting, as it indicates the necessity for caution when considering the increased yields of 'auxin' from whole extracts of hydrolysed plant tissues, i.e. extracts which have not been analysed by chromatographic methods.

Hydrolyses of the alcoholic extracts of roots with syrupy phosphoric acid, caused the loss of indolylacetic acid. Pure IAA is known to be destroyed by hot acid treatment, so that this result is chemical evidence for the identification of this active compound, as IAA. When $\frac{N}{10}$ and $\frac{N}{100}$ hydrochloric acid was used as the hydrolysing agent, however, an increased yield of this IAA was detected on the chromatograms. There have been occasional reports in the literature of increased growth activity of plant tissues, following acid hydrolyses, (Skoog, 1947; Larsen, 1951) but this has been suggested as being due to release of an acid stable auxin, not IAA. The contradictory results presented here, are possibly due to the concentration differences of the two acid treatments, and their differential effects on the balance

of two chemical reactions:

(1) Release of IAA from a alcohol soluble plant precursor(s).

(2) Destruction of IAA, by acid treatment.

With the application of relatively dilute hydrolysis ($\frac{N}{10}$ and $\frac{N}{100}$ HCl), the first reaction dominated over the second, resulting in an increased yield of IAA. If more drastic treatment was applied (syrupy phosphoric acid) the destruction of IAA completely dominated over its release, and none was therefore detected in the hydrolysed extract. Experiments should have been carried out, using a wide range of concentrations of the acids, to prove that the differential effect, was due to acid strength and not acid type.

Hydrolyses with either HCl or phosphoric acid resulted in an increased yield of β from roots and shoots. This compound is therefore released from alcohol soluble plant products by acid and alkali hydrolyses, and is stable under all treatments.

Consideration of the quantitative aspects of the results is more complex. It is immediately apparent when estimations of auxin quantities are made, that there is a large day to day variation in the yields of IAA from similar tissues. The variation due to the assay technique is small, so that the day to day, or occasion variance must

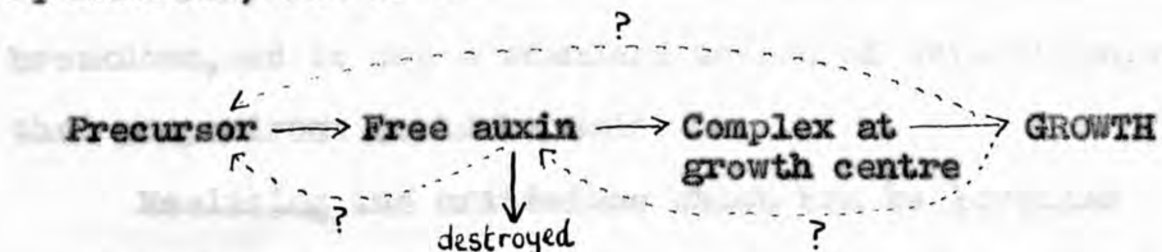
be due to inherent differences in the IAA content of similar tissues,grown at different times,and / or to variations in the extraction efficiency. In relation to this problem,it is important to consider the 'nature' of the auxin that is extracted and estimated. It has been generally accepted that auxins extracted from plants could come from three sources :

(1) From a precursor,(or precursors) which is in itself inactive. This precursor may represent the natural source of auxin in the plant,but could include other substances,which under certain experimental conditions might break up to release IAA.

(2) Free auxin.

(3) Hypothetical auxin complex at the growth centre.

The extraction technique used claims to prevent any release of IAA from the first category,and to remove only the 'free' auxin from plant tissues. The nature of the third possible source is unknown,so that the effect of this extraction method is problematical. The various auxin forms must be in a state of dynamic equilibrium,such as :-



and it is difficult to see how the measurement of one

form, strictly separated from the other, is possible. The extraction method employed might affect the balance of this 'equation'. It must however be accepted that the technique, as far as can be ascertained, does extract only the 'free auxin'. The relation of this auxin extracted and estimated, to that which is physiologically functional in the plant, is unknown.

Another problem which emerges, in the comparison of IAA yields of tissues, is the choice of the basis, on which to place the estimations - i.e. per gram fresh weight; per plant; or possibly per meristem. This seemed particularly important, when comparing yields from treated and control plants, in which the fresh weight per plant was altered by the treatment.

A third problem in the estimations of IAA yields is the possible effect of enzymes, causing the breakdown of this compound in the material prior to extraction and during the actual extraction processes.

The one way to tackle these problems was to adopt a carefully controlled extraction technique, which limited as far as possible release of bound auxin and enzymatic breakdown, and to use a standard method of estimation, so that comparisons could be made.

Realizing the criticisms which can be levelled against the methods employed, certain facts, concerning the yields of IAA (worked out on a fresh weight basis)

are drawn from the results:

(1) The cotyledons of 2 - 3 week old sunflower seedlings contain less (possibly none) IAA, than the shoots.

(2) Young roots (3 - 4½ days) contain less auxin than older ones (8 - 12 days). This agrees with the results of Pilet (1953) working with the roots of Lens culinaris. The increase is possibly correlated with the development of lateral roots, as apices seem to be the primary loci of auxin formation.

(3) There was no significant difference in the yields of IAA from bean shoots, pea shoots or pea roots, but these differed significantly from sunflower and cabbage seedlings. The latter also differed significantly from the sunflower and gave the highest yields of IAA.

The effects of the applied growth substances are as follows:-

1. The effect of the application of 2,4-Dichlorophenoxy acetic acid:

The treatment of plants with the synthetic auxin, 2,4-D., at concentrations which bring about marked responses in the root and shoot, show no consistent effect on the IAA levels as estimated and calculated, on a fresh weight basis. These facts support the view that 2,4-D. is acting directly in the growth system itself, and not indirectly, via a disturbance of IAA metabolism. An alteration in the level of IAA, necessary to evoke such

responses would have been clearly detectable. This direct action of 2,4-D. would explain the close parallel between the responses brought about by 2,4-D. and IAA.

The results also indicate that there is a regulatory mechanism in the plant controlling the level of free auxin:- if 2,4-D. fits into the same receptors at the growth centre, then when applied in high concentrations it would be expected that the level of IAA would build up ('equation'). No increase is detected, which suggests that when a synthetic auxin is applied to a plant, in a high concentration, either release of IAA from the precursor is reduced, or its breakdown is stimulated.

The well known differences in the responses of plants to high levels of applied IAA and 2,4-D. may be explained by the secondary differences in molecular structure as the 2,4-D. would not be lowered in the plant, either by 'binding' to proteinaceous plant products, or by enzymatic destruction. The fact that treatment of plants with IAA + 2,4-D. produces a lowered response than application of 2,4-D. alone (Hitchcock & Zimmerman, 1952), could be accounted for by competition of IAA with 2,4-D. for the growth centres. Assuming the IAA molecules would be more successful, the 2,4-D. would be partly excluded from the growth centre, and the inhibition, due to the inability of the synthetic auxin to function

normally in the whole growth 'cycle',would be reduced.

The experiments indicated however,that the applied 2,4-D. itself undergoes some change in the plant cell, and that a fraction is converted into a neutral substance. This substance may be an inhibitor,or an inactive neutral substance,either converted back into 2,4-D. during the chromatographic analysis,or by the root growth assay. (The R_F value is closely similar to that of 2,4-D.). The neutral substance is formed by enzymatic activity of the living cell and is not found in mixtures of 2,4-D. with tissue homogenates. Leaper and Bishop (1951) have suggested that the phenoxyacetic acids are converted to quinones in the plant,and quinones are in general,highly toxic. Unfortunately it is not known how such compounds would react to extraction and chromatographic analysis.

The results obtained are directly opposed to those reported by Weintraub (1953) of a lower auxin content in the buds of 2,4-D. treated plants; and to the results of Henderson and Deese (1954) which show a lower auxin content in 2,4-D. treated bean,pea and sunflower seedlings, and an increase in the yield of auxin from 2,4-D. treated oat seedlings. Both sets of results were obtained using the Went _Avena_ test,which meant that the _whole_ plant extract was assayed,without separation of its possible constituents. Although 2,4-D. has been shown to be

virtually inactive in this test,other plant substances may have affected the assay,so that the validity of these results is doubtful.

The reports by Goldacre (1949) of stimulation of the IAA oxidase enzyme (prepared from etiolated pea epicotyls) by 2,4-D. were later shown by him (Goldacre et al.1953) to be due,not to the 2,4-D. itself,but to a ·2% impurity of 2,4-dichlorophenol (D.C.P.). It seems possible that the report by Henderson and Deese (1954) that 2,4-D. increases the destruction of IAA added in vitro to sections of pea epicotyl and sunflower hypocotyl, might be due to an accelerating effect of a DCP impurity on the IAA oxidase system of the sections.

2. <u>The effect of application of maleic hydrazide</u>:

Maleic hydrazide was first reported as a growth inhibitor by Schoene and Hoffman (1949). Since that date the reports of the various effects of maleic hydrazide on the growth of plants,present a confused picture. It is primarily an inhibitor of cell division,at concentrations which have little effect on extension growth - shown by the fact that whole pea seedlings are much more sensitive to the compound than the root sections,and that the main effect on the former is suppression of lateral roots. It has also been shown that maleic hydrazide, (1) prolongs dormancy (Wittwer and Sharma 1950); (2) suppresses

flowering (Nickell,1953, Strukmeyer,1953); (3) removes apical dominance, (Naylor and Davies,1950); (4) induces formation of abscission layers,(Weintraub et al,1952); and (5) shows a competitive antagonism of IAA action in the standard pea and Avena tests (Leopold and Klein,1952) and of the inhibitory action of IAA in pea roots (Leopold and Klein,1952) and in flax roots,(Aberg,1953).

The last three effects of MH could be,presumably, brought about by a lowering of the IAA level,and have caused maleic hydrazide to be termed an 'anti auxin'. It seems unlikely the anti auxin action could be brought about by a mutual molecular competitive action with IAA for growth receptors,because of the non-homologous structure of MH. The claim that maleic hydrazide stimulates the rate of IAA destruction (in vitro) by the IAA oxidase enzyme (Andreae,1954) indicates the possibility that the antagonistic effect is an indirect one,mediated via IAA metabolic enzymes. (Aberg,1953).

On the other hand,the first two effects,on flowering and dormancy,resemble more the action of an auxin,than that of an anti auxin. It should however,be realized that there is evidence,at least for the potato tuber,that dormancy is not controlled by auxin levels. (Hemberg,1952, 1954.). The importance of the role of auxin in flower initiation is also in doubt,and it can only be said that

there appears to be a correlation of flowering with a low auxin content and vice versa.

Apart from these growth responses,maleic hydrazide causes metabolic and histological disturbances. Increase in sucrose has been reported (Naylor and Davies,1950), increase in polysaccharides (Currier et al.1951) and collapse of phloem tubes. (Greulach,1951).

In spite of the 'anti auxin' action of maleic hydrazide,previous investigations have shown it to have no effect on the IAA level in the plant. Pilet (1954) showed it to have no effect on the IAA level of the roots of Lens culinaris,and Braun (1954) reported that MH inhibited plant tumour growth,but did not alter the IAA level. In the present investigations on pea roots,with concentrations sufficient to inhibit both meristematic and extension growth,distinct indications of a raised IAA level were obtained.

It is suggested that the antagonistic effects of maleic hydrazide are due to a blocking,may be enzymatic, of the free auxin $\longrightarrow$ growth receptor process,not a molecular competitive action for the receptors. If this effect is coupled with a disturbance of the metabolic production of IAA,it explains the increase in IAA in the treated pea roots.

There was some evidence for the production of a

neutral inhibitor, in the maleic hydrazide treated roots. Further work is necessary to establish this, but it may be important in relation to the growth inhibitory action of maleic hydrazide, and to its effects on dormancy.

There seems little doubt that maleic hydrazide, like other growth regulatory substances exerts a number of different actions, depending on its concentration and the tissue in which it is present.

3. The effect of the application of 2,3-5 tri-iodobenzoic acid:

The results showed a marked reduction in the yield of IAA from the treated pea roots. This effect of TIBA, at these non-toxic levels would explain a number of the physiological activities of this compound on plants: (1) its general inhibition of extension growth in internodes (Galston, 1947; Snyder, 1949.); (2) the fact that it induces abscission (Galston, 1947; Weinstraub et al. 1952; (3) the suppression of apical dominance that has been reported, (Galston, 1947); and (4) stimulation of flowering, if the idea that flowering is associated with low auxin levels, is accepted. TIBA antagonises the action of IAA in the *Avena* test (Galston, 1947) and in the inhibition of root growth (Aberg, 1953; ~~Aberg and Johnson, 1955)~~. This could be explained either by molecular competitive action, or by a reduction in IAA level, if this

is caused by enhanced destruction by tissue enzymes. The antagonism by IAA,of the effect of TIBA on abscission can be similarly explained. The rather unusual blocking effects observed on IAA transport in sweet potato petioles (Kuse,1953) can also be explained if the lowering of auxin levels by TIBA is due to a *destroying* action, which could be localised in the TIBA treated zone of the petiole.

However,there are a number of well authenticated actions of TIBA which cannot be so explained. One is its synergistic action on IAA activity at low concentrations in the *Avena* cylinder test and pea test,(Thimann and Bonner,1948) an action which is supposed to underlie the small auxin activity claimed for this compound. (Thimann and Bonner,1948; Muir and Hansch,1951). It will not explain the small synergisms of IAA in the inhibition of root growth. (Aberg,1953; ~~Aberg and Jönsson,1955~~). The concentration of TIBA used in the present experiments (10 p.p.m. $\equiv$ 2 x 10^{-5}M) was of the same order as those which gave growth stimulations and IAA synergisms in coleoptiles and pea epicotyls (Thimann and Bonner,1948; Muir and Hansch,1951) and those which gave antagonisms of root growth inhibition by IAA. (Aberg,1953). In roots however,the synergistic action on endogenous auxin that has been claimed by Aberg,takes place at a lower

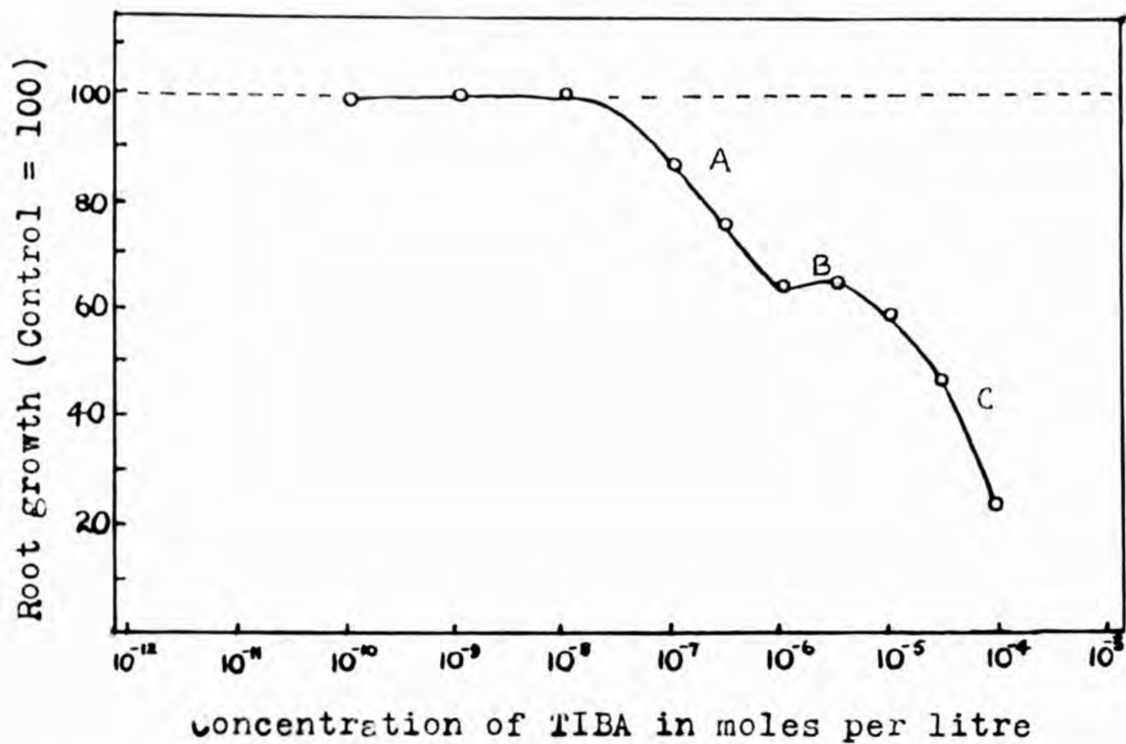
concentration. It is possible therefore that in these responses to TIBA we have yet another example of the much higher sensitivity of roots to a growth substance, both synergisms and antagonisms setting in at a much lower concentration level in those organs than in coleoptiles and epicotyls. In this investigation, only roots subjected to antagonistic concentrations were studied. Further experiments on both shoots and roots with a widely extended range of TIBA concentrations are needed to elucidate this possible double action.

Aberg, in his studies of the action curve of TIBA in flax roots (Aberg, 1953) and pea roots (~~Aberg and Jönsson, 1955~~) distinguished three distinct TIBA actions. (See Fig. 35). Inhibition in low concentrations (10^{-8} to 10^{-6}M) is postulated as due to synergism of natural auxin (presumed present in supra-optimal concentrations). If this is true, then at this level the depressing effect of TIBA on the IAA content of roots is not being exerted; otherwise a stimulation rather than an inhibition of root growth would be expected. A flattening of the inhibition curve in slightly higher concentrations was supposed to be due to a balance between the synergistic effect and an antagonistic effect which sets in at these higher concentrations. This corresponds

to the concentrations used in the current experiments, and could be explained by the partial disappearance of natural IAA, induced by this TIBA concentration. If this was the only effect of TIBA, one would expect the growth inhibition to be reversed, and the curve to rise to and even exceed the control level. Aberg explains the rapidly increasing inhibition, following the plateau portion of the curve, in terms of a rapid onset of "toxicity" at the higher concentrations.

This presents a picture of a triple action of TIBA based largely on indirect evidence. Thus its supposed synergistic interaction in low concentrations with IAA, is based on the very small synergistic effects obtained with externally applied IAA. Such small effects could not explain the substantial TIBA inhibitions observed in these low concentrations. The explanation for the curve, also assumes that the effective concentrations of endogenous IAA in roots are supra-optimal, which does not appear always to be the case. Assuming this to be so, a ~~similar~~ simpler explanation of the stepped curve of Aberg (Fig. 35) can be put forward, on a basis of the TIBA effect reported here:

In the absence of TIBA, the root grows at a certain rate, which is determined by the level of natural IAA, which may be (in the case of flax roots) either at an



Effect of TIBA on root growth of flax seedlings (after Aberg, 1953)

Fig. 35. The 'stepped' calibration curve for TIBA using whole flax roots.

optimal or sub-optimal level. When TIBA is applied in increasing concentrations this endogenous IAA is lowered progressively, producing the first part of the inhibition curve (A). The root growth falls to the 'plateau' region of the curve (B), which must correspond to a point where no IAA is left to enter the growth complex. (It is important to realize that the flax and pea root tests, which have shown this TIBA activity curve are carried out over a short period of time; as assuming this plateau region of the curve to correspond to what might be called a zero concentration of IAA, one questions the statement "no auxin, no growth".) With further increase in TIBA concentration, growth stays at this level until the direct toxic action of the compound sets in, giving the third portion of the activity curve (C).

Only two actions of TIBA are necessary therefore to give this stepped curve:

1. A direct inhibiting action in high concentrations.
2. An action, which greatly reduces the level of 'free' auxin setting in at a much lower concentration. This could be due either to a blocking of IAA synthesis, or a stimulation of its breakdown. The latter seems unlikely, as there is a report that TIBA inhibits the activity of IAA oxidase enzyme. (Goldacre, Galston and Weintraub, 1953).

Obviously the above explanation of the stepped activity curve of TIBA, involves many suppositions, and a

careful study of the relationship between IAA levels and various TIBA treatment concentrations is needed.

The synergistic action of TIBA with auxins applied externally to the coleoptile, epicotyl and root are probably quite unrelated to the two effects postulated (1 and 2) and its cause still remains a mystery.

The growth of pea seedlings in solutions of TIBA causes a modification of the geotropic response of the root, in that the primary and the lateral roots tend to grow upwards. The fact that such an altered response is coupled with a lowering of the level of IAA, indicates that this substance plays a fundamental role in tropic movements. It is reported by Leopold (1955) that Geiger - Hüber and Hüber found that repeated decapitation of roots, which lowers the auxin content, also causes them to grow upwards.

These results are further evidence that tropic movements are brought about by differential auxin levels in the particular organ.

SUMMARY.

1. Detection and estimation of active growth substances in plant material involved the development and standardisation of the following techniques :-

(i) Extraction. Preliminary investigations showed that water was unsatisfactory as a primary solvent and it was replaced by an established technique using alcohol extraction followed by ether. The ether extract could be divided into acidic and neutral fractions.

(ii) Chromatographic separation. An ammonia/ isopropyl alcohol/water solvent was found to give satisfactory chromatographic separation of indole acetic acid from (a) 2,4-Dichlorophenoxy acetic acid, (b) Maleic hydrazide, and (c) 2,3,5,Tri-iodobenzoic acid.

(iii) Detection and Estimation. A direct bioassay of the paper chromatogram was developed using the pea root section technique.

The relationship of the growth response of the root assay to varying concentrations of IAA, 2,4-D., TIBA and Maleic hydrazide was determined. It was shown that the assay detected IAA at a concentration as low as 10^{-11} (10^{-5} p.p.m.).

Estimations were made of the reliability of the combined chromatographic and root assay methods for estimations of IAA in unknown solutions. It was found that immediate assay of a chromatogram of an IAA solution gave an estimation which was a direct measure of the quantity originally added to the paper.

2. Evaluation of the growth substances present in normal plant tissues gave the following results :-

(i) At least five active substances were recorded. The extracts consistently contained IAA at an R_F value between 0·30 and 0·45, and an inhibitor at an R_F value between 0·60 and 0·75 (β).

(ii) There were significant differences between the IAA yields from different species and from different tissues of the same species. In addition there was a wide day to day variation in the yield of IAA from similar tissues which was not due to errors in the assay technique.

(iii) Alkaline hydrolyses of the alcoholic plant extracts brought about an increase in the yield of IAA and the inhibitor β . Acid hydrolyses caused an increase in the yield of β and either increased the yield of IAA, or destroyed the IAA present in the extract. It was suggested that the latter differential effect was

due to differences in the strengths of the acids used.

3. Estimations were made of the growth substances in plants which had been treated with 2,4-D., Maleic hydrazide and TIBA :-

(i) Treatment with 2,4-Dichlorophenoxy acetic acid had no effect on the IAA yield of shoots or roots. This was taken as evidence that 2,4-D. has a direct action on growth, not one mediated via an altered level of IAA within the plant.

The 2,4-D. treatment caused the production of a neutral inhibitor in pea roots.

(ii) Treatment with Maleic hydrazide appeared to increase the yield of IAA from pea roots but further work was necessary to establish this effect. No consistent effect on the level of the inhibitor β was detected.

There were again indications of the production of a neutral inhibitor with a closely similar R_F value to that detected in the 2,4-D. treated roots.

(iii) Treatment with 2,3,5 Tri-iodobenzoic acid caused a reduction in the yield of IAA from pea roots. This result could explain a number of the effects of TIBA treatment on whole plants. It is suggested that TIBA affects the metabolism of IAA.

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A P P E N D I X.

TABLE I.

Calibration Results: Growth expressed as a percentage of control after 24 hours.

Indolylacetic acid.

1 p.p.m.	·1 p.p.m.	·01 p.p.m.	·0001 p.p.m.	·00001 p.p.m.	·000001 p.p.m.
24·80	84·80	86·50	123·8	126·2	106·2
35·75	80·00	75·60	127·6	105·2	114·4
28·00	85·80	86·00	125·2	110·8	108·2
49·25	78·40	90·00	111·5	119·0	105·0
57·80	66·40	86·60	103·8	130·2	101·0
48·60	82·40	83·50	94·0	135·0	104·5
49·40	84·50		113·0	107·2	108·4
52·10	82·50		102·0	109·5	103·2
	78·00			115·5	114·8
	79·00			125·8	92·8
	71·20				
	72·80				
	64·30				
	73·00				
	68·00				
	79·50				

TABLE II.

Calibration Results: Growth expressed as a percentage of control after 24 hours.

2,4-Dichlorophenoxyacetic acid.

1 p.p.m.	·1 p.p.m.	·01 p.p.m.	·0001 p.p.m.	·00001 p.p.m.	·000001 p.p.m
25·60	57·80	92·35	106·00	108·30	110·00
24·80	44·30	86·50	108·20	101·20	105·00
23·70	46·85	71·35	87·20	103·80	98·00
26·80	52·75	71·85	94·90	90·10	103·00
20·15	25·60	78·00	80·00	91·20	88·60
15·80	37·80	71·75	79·50	95·00	89·60
24·10	39·20	72·80	80·75	96·50	112·10
	37·00	94·75	77·00	91·50	92·00
	25·60		113·50		86·00
	44·10		69·00		80·10
			85·00		93·50
			74·50		80·00
			86·00		
			84·00		
			84·00		
			86·00		
			112·00		
			110·00		
			83·10		
			80·80		
			92·50		

TABLE III.

Calibration Results: Growth expressed as a percentage of control after 24 hours.

Maleic hydrazide.

5000 p.p.m.	3000 p.p.m.	1000 p.p.m.	700 p.p.m.	500 p.p.m.	300 p.p.m.	100 p.p.m.
11·30	29·40	56·60	71·60	88·60	100·20	100·20
11·20	29·60	56·60	73·00	89·50	103·50	103·50
10·80	27·70	64·20	69·80	78·10	96·50	96·50
		56·60				
		61·50				

TABLE IV.

Calibration Results: Growth expressed as a percentage of control after 24 hours

2,3,5 tri-iodobenzoic acid.

100·0 p.p.m.	10·0 p.p.m.	1·0 p.p.m.	·10 p.p.m.	·01 p.p.m.	·001 p.p.m.
15·60	69·00	83·00	97·80	99·50	103·00
19·10	72·00	86·20	91·40	90·00	97·00
21·40	74·60	76·80	90·60	102·50	101·50
18·70	72·00	82·20	91·50	101·20	98·60

TABLE V.

Actual and experimentally calculated quantities of IAA on the chromatogram strips.

Quantity of IAA in γg.

Assay technique	Actual	Experimental
Strips equilibrated for 18 hours.	·80	·21
	1·46	·30
	·83	·23
	1·85	·90
	·65	·12
	1·24	·14
	·34	·06
	·074	·007
	·130	·024
Strips used immediately.	1·21	·997
	1·00	·90
	1·52	1·50
	·64	·71
	1·12	1·70
	·89	·97
	·117	·077
	·062	·045
	·101	·075
	·181	·195

TABLE VI.

Actual and experimentally calculated quantities of 2,4-D. on the chromatogram strips.

Quantity of 2,4-D. in γgm.

Assay technique	Actual	Experimental
Strips equilibrated for 18 hours.	·99	1·05
	1·07	1·20
	1·08	1·46
	·65	·89
	·087	·165
Strips used immediately.	1·64	2·10
	·72	1·15
	·85	·99
	·98	1·43
	·64	1·068
	·91	·75
	·83	·60
	·105	·128
	·11	·31
	·15	·14
	·017	·015