GROWTH HORMONE AS A POSSIBLE CAUSE OF TWISTED-STEM DISEASE IN ONIONS

A THESIS SUBMITTED TO THE

AHMADU BELLO UNIVERSITY

Z A R I A

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF MASTER OF
SCIENCE IN ANALYTICAL CHEMISTRY

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APRIL, 1978

ACKNOWLEDGEMENT

I wish to express my profound gratitude and deep appreciation to my Supervisor, Dr. H.T. Bozimo, who was patient enough to render fraternal guidance and technical assistance through all the various stages of this work. I am also grateful to the Head of Department of Chemistry, Professor U.D. Gomwalk and other members of the teaching staff who were involved in the handling of the Analytical Chemistry course for giving me the opportunity to do this work.

I wish also to thank Dr. A.C. Ebenebe of the Institute of Agricultural Research (IAR), Ahmadu Bello University, Zaria, for letting us into the details of some other aspects of this work and for providing us with some of the onion plants.

Finally, I thank all the technical staff of this department for their assistance.

ABSTRACT

As a result of the outbreak of an unidentified disease among onion plants, these plants were examined quantitatively for their auxin contents. Both the bulb and the stem were found to contain detectable levels of Indole-3-acetic acid (IAA), The stem appeared to have generally a lower level of IAA than the bulb. The concentration of IAA in twisted (diseased) onion plant was also determined. Though only one specimen of the twisted onion was available for the present work, it is very likely the twisted onion contains a higher level of IAA than do the healthy ones. This will have to be further confirmed during the rainy season when the twisted plants are usually available.

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CHAPTER I

INTRODUCTION

Growth Hormones or auxins are characterised by their ability to influence the rate of cell enlargement and hence basic growth. They also affect many aspects of development. Indole-3-acetic acid (IAA) was the first authentic auxin to be isolated from plant tissues and is widespread in plants. It is at present regarded as the most important growth regulator in plants. Abnormal variation in the concentration of this auxin may lead to abnormal characteristics in plant growth.

The first stage in the growth of a plant is the germination of the seed. This seed contains, inside the thin seed coat, an embryonic plant, complete with leaves, root and shoot, and possesses, either in the embryonic plant itself or in a tissue surrounding it, a large reserve of food for the early growth stages. During the early stage of germination auxins are produced. These auxins presumably come from stored inactive precursors in the dry seed. The supply of this auxin does not limit the rate of growth of the young plant at this stage. It rather largely stimulates it.

After germination the growth rate becomes largely determined by the net rate of carbohydrate production in the presence of light (= photosynthesis). Auxin supply also goes on to enhance growth except under certain adverse nutrient conditions. On the other hand deficiency of zinc may limit growth rate of the plant. This is because zinc deficient plants are very low in auxins and this is correlated with a low tryptophan content. Zinc is therefore necessary for the synthesis of tryptophan from which the auxin is derived. This deficiency leads to excessive inactivation of the natural auxin supply so that the resulting low auxin concentration limits growth. This imbalance can be restored by treatment of the plant with the synthetic auxin.

Relatively few quantitative determinations of the IAA content of the vegetative parts of plants have been made. There is no standard method for extracting auxins from a plant; the methods are largely empirical. Consequently differences in extraction and assay methods often make comparison of results difficult. In particular no determination of the IAA content of onions seems so far to have been made.

OBJECTIVES OF THE PRESENT WORK

For some time now onion farmers in this part of the country have had to sustain heavy losses from their farms as a result of recurring outbreak of a kind of disease which attacks these plants. The disease, hitherto un-identified, manifests itself in a number of ways. The young plant has twisted stem carrying pale yellow leaves. The bulb is narrow and elongated so that it is no longer possible to distinguish between the bulb and the stem. If the bulb is cut open there is no coloration of the tissue but occasionally very light brown coloration is observed especially near the bulb. Those plants which escapt early attack normally develop normal bulbs and so do not show this coloration. Despite all attempts to keep them dry during storage, affected plants (bulbs) rot quite easily.

Attempts have been made by some plant pathologists to find a possible cause of this disease and possibly find a cure for it. Some of the work so far carried out includes[1]

(i) Innoculation experiments - here efforts were made to isolate the pathogens. After innoculating some healthy plants some element of twisting was observed but the symptoms were quite different from those obtained in the normal twisted (affected) plants.

(ii) Ecological studies - influence of different environments were studied. Investigations were carried out to determine the influence of pH of the soil as well as the soil type. Results obtained showed that neither the pH nor the type of soil around this area was responsible for the disease.

(iii) Mode of spread in the field. In this investigation experiments were carried out to find out whether the disease is transmitted from one plant to another through the soil or by the plants themselves. Several pots were planted with the healthy plants and in the middle of each pot was planted one twisted plant. After several days it was observed that only those plants very close to the twisted plant got affected. It was concluded from these results that the disease was not transmitted through the soil but by the plant itself.

(iv) Effect of zinc and fungicides. Investigations were carried to find out whether there was deficiency of zinc ions in the soil or in the plant, or whether there was attack by fungus. Analyses of both the healthy and twisted plants showed that there was no zinc deficiency in either of the species. Instead it was even seen that the twisted plants had more zinc than the healthy ones. It was therefore obvious that zinc deficiency was not responsible for the disease. Similarly it was observed that the disease was not brought about by fungal attack.

The results from the above studies did not throw any light on the possible cause of the disease. It was thought that since auxins play a vital role in plant growth and development a quantitative determination of concentrations of these plant growth substances in both the healthy and diseased (twisted) plants was necessary. Indole-3-acetic acid (IAA) being the most important of these plants growth substances was to be screened and its concentration determined. Abnormal level of concentration of IAA, if found in the twisted plant, could be a pointer to the possible cause of the disease.

CHAPTER II

REVIEW OF PLANT GROWTH HORMONES

Plant growth substances may be defined as organic compounds which, at low concentrations, promote, inhibit, or qualitatively modify growth. Their effect does not depend on their caloric value or their content of essential elements. By "low concentrations" is generally meant concentration lower than 10^{-4} molar when the compound is applied externally in aqueens solution to suitable test objects[2]

These substances which affect the growth of plants are classified according to the qualitative character of their biological effect. Some of these individual groups are listed below:

(i) Auxins - these are growth substances capable of inducing elongation in shoot cells when applied in suitable concentrations. Auxins may, and generally do, affect other processes besides elongation, but the effect on elongation is considered critical. They are generally acids with an unsaturated, cyclic nucleus. Indole-3-acetic acid is commonly used as a standard in determining auxin activity.

(ii) Antiauxins are growth substances which competitively inhibit the action of auxin. Their natural occurrence has not been definitely established.

(iii) Growth inhibitors are substances which retard growth in both shoot and root cells and have no stimulatory range of concentrations.

(iv) Fructigenic substances are growth substances which induce development of the tissues supporting the ovules, i.e. development of fruits in the broadest sense.

(v) Wound Hormones are growth substances which induce cell division and are liberated upon injury of tissue.

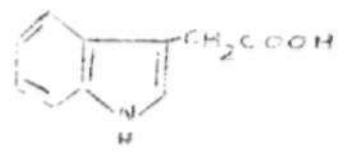
This review will cover those substances which affect the extension of the cell wall i.e. auxins.

The first naturally-occurring compounds having auxin activity were detected in various commercial enzyme preparations (Seubert 1925)[3], in various fungal cultures (Nielsen 1928,)[4] and in human urine (Kögl and Haagen-Smit 1931)[5]. Kögl and co-workers finally isolated an acid, termed auxin a, from urine and both auxin a and a new acid auxin b, from corn germ oil and malt.[6] These acids were characterised and found to be di-sec-butyl cyclo-pentene derivatives. However, it has not been possible to repeat the original isolations, in spite of numerous attempts by many workers including Kogl himself, which makes the general occurrence of these substances doubtful.

Naturally-occurring Auxins

In contrast to work on auxins a and b, work on extracts of plant material has revealed the existence of a number of indole compounds with auxin activity. Bentley[7] has shown that indole compounds occupy a central place in the hormone physiology of plants.

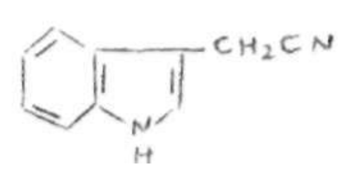
Indole-3-acetic acid (IAA)



IAA was first isolated from human urine and yeast plasmolyzate by Kőgl and co-workers in 1934[8]. At first it was thought IAA was a product only of microorganisms and not a hormone of higher plants, and it was accordingly named heteroauxin. With the realization that IAA is one of the main auxins of higher plants, the term was discontinued. IAA was subsequently isolated from alkali-hydrolysed corn meal and the endosperm of immature corn grains[9], and dormant mature maize after alkaline hydrolysis[10]. Slight traces of IAA are produced from tryptophane in a number of proteins on treatment with alkali[11]. Recently the application of paper partition chromatopaphic techniques has enabled IAA to be detected in a wide range of plant tissues.

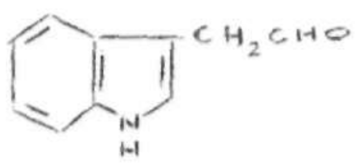
Although many reports have confirmed the existence of IAA in plant tissues, one can not also ignore the fairly large number of reports in which workers have been unable to detect IAA in their extracts. IAA could be lost during the process of extraction and purification, for example by the prolonged action of low pH or ultra-violet light, both of which destroy IAA to a certain extent. Also more IAA is needed to give a colour test than can be detected by bio-assay, so that failure of a colour reaction does not necessarily mean the absence of IAA. Free IAA may only be operative in tissues at certain stages of development. For example, it could be found in the colourless, inner leaves of brussel sprouts, but not in the outer, green leaves. There is also a growing body of evidence that it may only be operative as a complex, in a bound form. Vlitos and Meudt[12] could detect IAA after alkaline hydrolysis of tobacco leaf and stem tissues but not otherwise.

3 - Indoleacetonitrile (IAN)



IAN was originally isolated in the crystalline form from immature cabbage heads, where it was present in aabout 0.0002%[13]. This was the first isolation of a growth hormone from the leaves and stems of actively growing higher plants. High activity in neutral extracts of brussel sprouts, radish, cauliflower, swede and turnip was investigated and was also probably due to IAN. In addition, IAN is now reported to occur in a variety of other tissues[14].

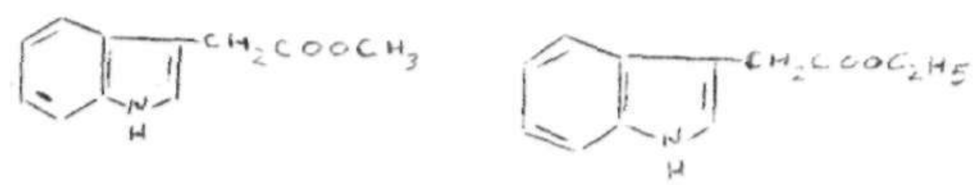
3-Indoleacetaldehyde



Evidence for the occurrence in plants of 3-Indoleacetaldehyde is largely circumstantial. It was discovered that a neutral, growth promoting compound in plant extracts could be converted to an acid, probably by soil, some enzyme, coleoptile juice and leaf extracts.

The most conclusive evidence for the existence of aldehyde in plant extracts is the formation of an addition product with sodium bisulphite and inactivation with dimendon, both reactions typical of aldehydes[15]. 3-Indoleacetaldehyde is difficult to detect by chromatographic methods because of its extreme instability. It easily polymerises and changes to IAA. Earlier work on the physiological effects of 3-Indoleacetaldehyde was hampered by the fact that pure, synthetic samples were not available, and the crude preparations used probably contained extremely small amounts, if any, of the desired substance. Only recently has the synthesis of the crystalline product been achieved by Brown and co-workers[16], and it should therefore be possible to attempt an examination of its behaviour on chromatography.

Methyl and ethyl 3-indolylacetate

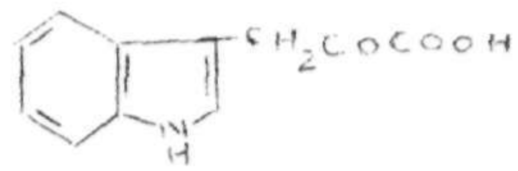


The methyl ester of IAA is biologically active, but it has not so far been demonstrated in plant extracts. It was first isolated from human urine after esterification of IAA by refluxing a semi-purified extract with MeOH plus

1.5% Hcl[17].

IAEt has been isolated in pure form from immature maize kernels. It has also been reported in apple endorsperm.

3-Indolylpyruvic acid[18]



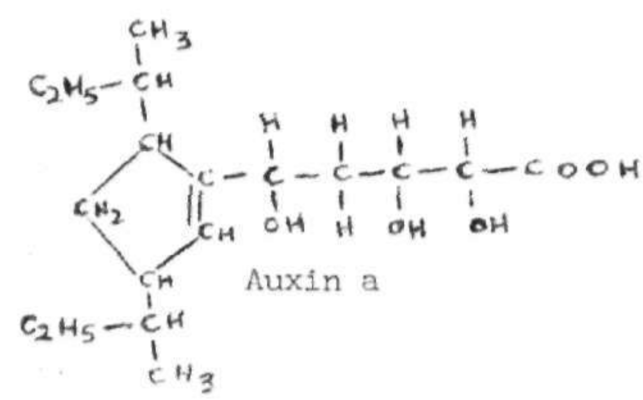
This hormone has been reported in maize endosperm, and in leaves of soybean and tobacco. It is also present in potato peelings.

Apart from indole compounds there are quite a number of other non-indole compounds which have auxin activity. These compounds particularly the chlorinated derivatives of phenoxyacetic acids, have in the last few years become perhaps the most important of the auxins, in view of their application to agriculture and horticulture. The compounds that are in most frequent use are 2,4-dichlorophenoxyacetic acid, 2-methyl-4-chlorophenoxyacetic acid and 2,4,5-trichlorophenoxy-acetic acid. Unfortunately no satisfactory specific chemical tests have yet been devised for the detection and estimation of these compounds in low concentrations. However the presence of 2,4-dichlorophenoxyacetic acid can be demonstrated in the form of its uranyl salt[19] but the limits of concentration which are admissible for this test are 200 - 400 µg per ml.

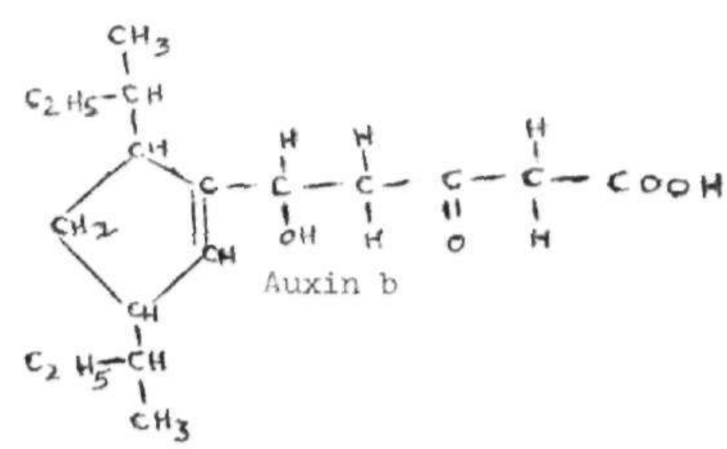
CHAPTER III

CHEMISTRY OF AUXINS

First attempts at isolating the auxins in sufficient quantity and purity to allow investigation of their chemical structure resulted in the preparation of crystals of three distinct chemical substances - auxin a from urine, auxin b from malt and maize germ oil and heteroauxin (Indole-3-acetic acid) from urine. Auxin a and auxin b proved to be very similar in chemical structure. They were characterised and found to be di-sec-butylcyclopentene derivatives and the final formulae proposed for them were as follows:[20]

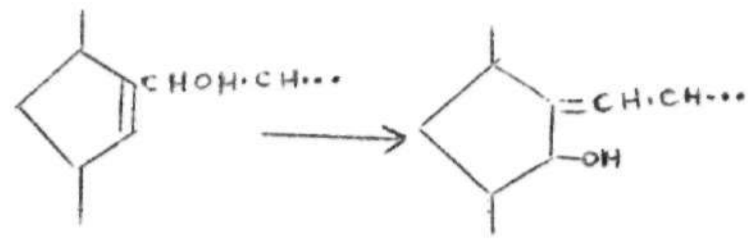


Auxin a

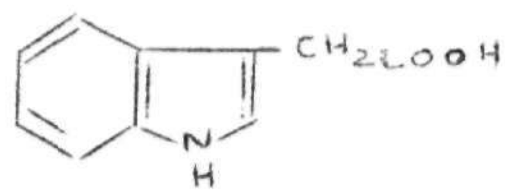


Auxin b

These two substances were previously unknown to chemical science and were found to be very unstable in vitro. In vacuo, isomerisation takes place, resulting in a shift of the double bond to the side-chain, and giving rise to an inactive pseudo-auxin with the following structure.



This probably does not happen in the plant. Heteroauxin or indole-3-acetic acid which has now been isolated from a number of plant sources including corn meal, malt, spinach leaves etc has quite a different structure. Its formula is



It has been shown to be produced from the amino acid, tryptophan, by the activity of bacteria (Escherichia communis)[2]

The first isolation of auxin a and heteroauxin were made from urine. Attention was also given in those early days to the identity of the naturally occurring hormones of plants. Because of the extremely small quantities obtainable from the plant materials studied direct chemical tests were not feasible as the latter were far too insensitive. Attention was therefore directed to indirect identification methods. These methods involved

(a) the acid-alkali stability relationships

and (b) determination of the diffusion constants in agar.

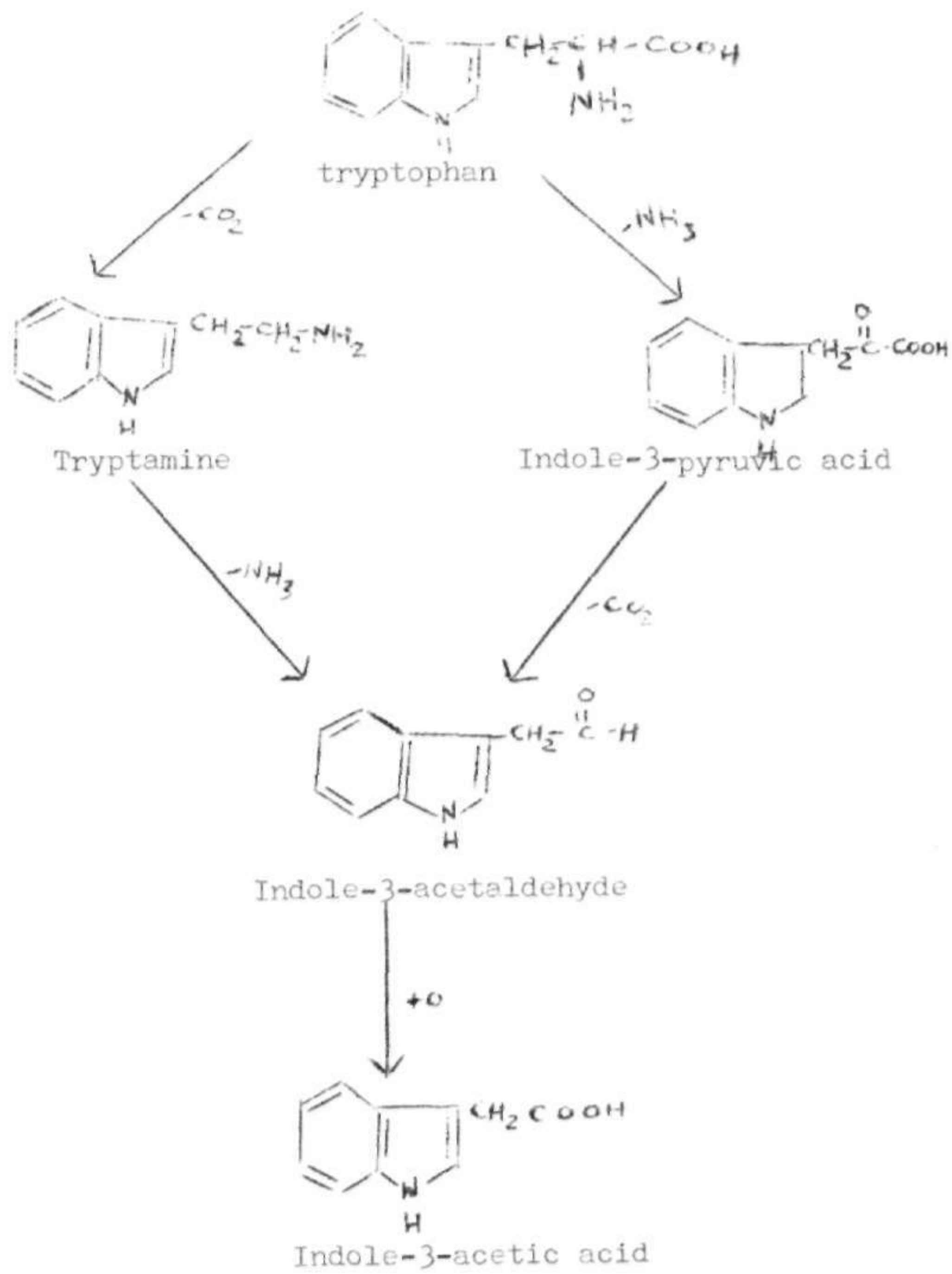
It was shown that auxins a and b were destroyed by hot alkali whereas indole-3-acetic acid was stable under the same conditions. On the other hand, hot acid decomposed indole-3-acetic acid and auxin b, but not auxin a. Thus a method of distinguishing the natural plant hormones in extracts was established, assuming that they are limited to these three compounds. On this basis it was suggested that auxin a was the hormone of the oat coleoptile and other seedling roots.[22] Later on some workers found that the auxin in the oat coleoptile tip was acid-labile and therefore could not be auxin a or auxin b. Although it bore close resemblance to IAA, it was resistant to hot dilute ammonia, a treatment that destroyed the latter hormone. Paper partition chromatography showed that IAA was the auxin that was likely to be present. Most of it was acid-labile, but a small residue left after many hours refluxing with HCL, was alkali-stable and so could not have been auxin a or b.[23] This method of differentiating and identifying natural auxins is therefore very unreliable.

Diffusion constant measurements have been obtained by allowing diffusion to take place through a series of identical rectangular agar blocks arranged linearly and in close contact. Determination of the distribution of the auxin concentration through the series is done by assaying the individual blocks. From the distribution curves obtained, the value of the diffusion constant can be calculated, thereby giving the molecular weight. Early determinations on the auxin from the oat coleoptile and on pure auxin a gave a molecular weitht of 376 which agreed reasonably well with that for the proposed formula of auxin a (molecular weight = 328) and not with IAA (molecular weight = 175). More recent applications of the same technique to potato, tomato, spinach, pineapple and a number of other higher plants gave results characteristic of IAA rather than of auxin a or b. Again a series of analysis of coleoptile auxin using paper chromatography have shown activity only at R_f values corresponding with IAA.[24] It is therefore only when actual crystalline hormones have been isolated that a final decision on these questions can be made. Auxins a and b have not yet been isolated from plant materials by other workers other than Kogl and his colleagues but IAA has been. The existence of auxins a and b is therefore doubtful.

The existence of IAA as the main natural auxin in plants comes from chemical studies of auxin precursors in the plant. It was shown that the mould Rhizopus suinus, one of the earliest natural sources of IAA, developed a high auxin activity by the oxidation of amino acids from peptone[25]. Later it was also shown that this mould could convert the amino acid tryptophan to IAA[26].

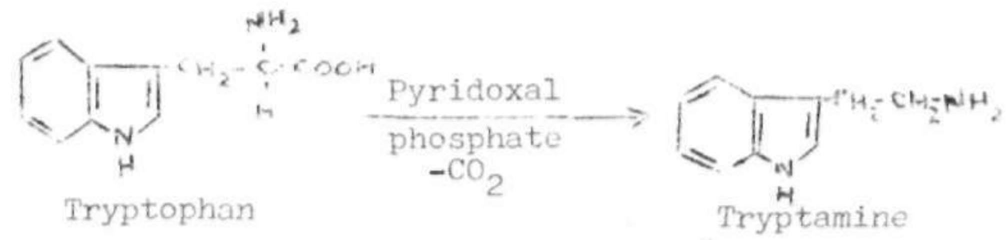
The pathway of this metabolic process is still a subject of much investigation and debate. Very low molecular weight indole compounds have been detected in the plant tissue, implying that these must be intermediates in one pathway or another. It is possible, therefore, that different biosynthetic pathways are used preferentially by different species.

Wightman[27] proposed two metabolic pathways to indole-3-acetaldehyde, which was considered to be the labile intermediate precusor of IAA. One pathway involved the decarboxylation of tryptophan to tryptamine followed by an oxidative deamination to indole-3-acetaldehyde. The other pathway involved, first, an oxidative deamination to indole-3-pyruvic acid followed by decarboxylation to the aldehyde:

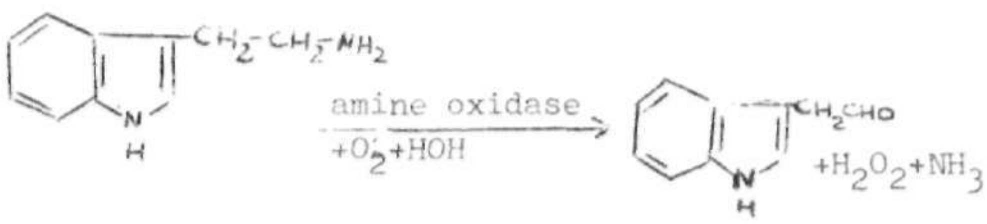


The production of auxin (IAA) from tryptophan is carried out by some enzyme systems[28].

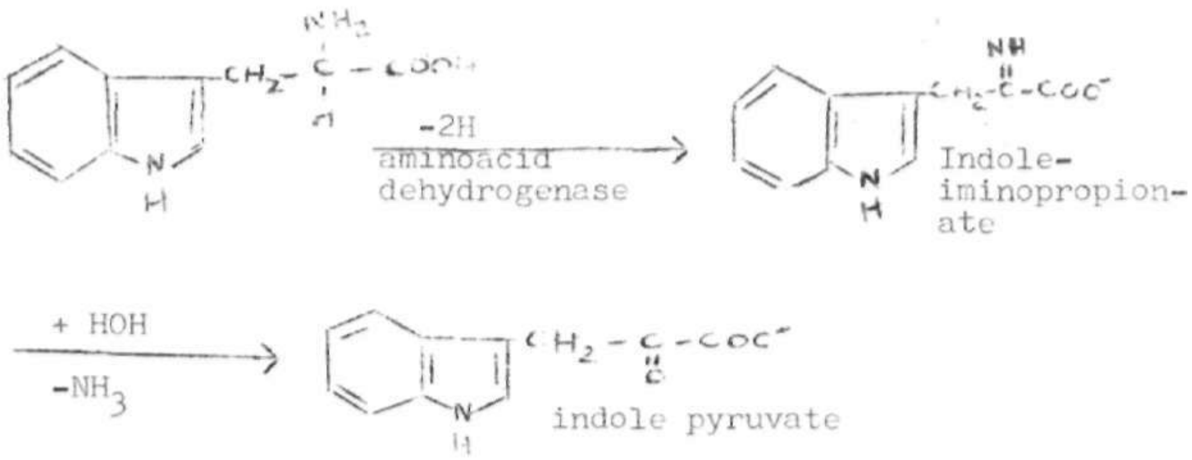
The decarboxylation of tryptophan follows the following sequence[29].



then oxidative deamination



The second pathway involving oxidative deamination of tryptophan is postulated to follow the following sequence of reactions



Indo-pyruvate is unstable, sponteneously decomposing to yield, in part IAA.

The credibility of any of these pathways will depend on whether these supposed intermediates - indole-3-pyruvic acid, tryptamine and indoleacetaldehyde actually exist in plants. It is now widely accepted that this is so for the indole-3-acetaldehyde. It has been shown by Larsen[30] that this substance can reach a concentration of 4 mg per kg in cabbage leaves and that it can be easily oxidised to IAA by an enzyme system (aldehydrase) present in soil.

The establishment of the Keto-acid as a possible intermediate in auxin formation is obscured by the instability of the compound which decomposes sponteneously into acid and neutral compound. Its occurrence in any appreciable quantity in plants is still in some doubt. Its presence in acetone extracts of maize embryos, wheat roots etc has been claimed on the basis of R_f value on a paper chromatogram[31]. Under some conditions 3-indolepyruvic acid can be converted to IAA by crude enzyme preparations from pineapples[32]. Also its role as an essential intermediate in the interconversion is strongly indicated by the accumulatio under anaerobic conditions, of Indole-3-lactic acid, which could be produced from it by a reduction catalysed by an appropriate dehydrogenase. However, no isolation of this compound from vegetative tissue has yet been achieved.

The evidence for tryptamine as a possible intermediate in the interconversion of tryptophan to IAA is rather unconvincing. So far, it has been identified only once, in the flowers and shoots of Acacia floribunda and Acacia pruinosa, where it probably has nothing to do with auxin metabolism[33]. Enzyme preparations from some plants will convert it to IAA but other preparations, e.g. from spinach leaves (which can produce auxin from tryptophan), will not.

The question whether the path of conversion of tryptophan to IAA goes through tryptamine or through indole-3-pyruvic acid, or through another intermediate still remains unsettled. It is therefore possible that different plants solve this problem in different ways.

CHEMICAL STRUCTURE & GROWTH ACTIVITY OF AUXINS

In studies of physiologically active compounds, investigations are usually made to relate changes in the chemical structure of an active compound to its activity. Such studies of structure-activity relationships helps to formulate structural rules which a substance must fulfil in order to be active, and thus provides suggestions for the synthesis of additional active compounds and the exploration of the mechanisms by which the observed effect is produced. Many studies of this type have been carried out for the plant growth substances because of the relatively simple chemical structure of the native auxins isolated so far; which has made the synthesis of analogous substances fairly easy.

From these studies have emerged the following rules[34] which give the minimum requirements for high auxin activity in any compound:

(1) A ring-system as a nucleus

(2) At least one double bond in this ring

(3) A side-chain possessing a - COOH group (or a group easily convertible into a - COOH group).

(4) At least one carbon atom between the ring and the - COOH group in the chain.

(5) A particular spatial relationship between the ring-system and the - COOH group.

All the auxins so far mentioned conform to these requirements. Maximum activity has been shown by compounds having one bridging carbon atom between the nucleus and the -COOH group. Increase in the number of these bridging atoms results in a fall in activity. Much greater activity is shown by these compounds having an odd number of bridging carbon atoms in the side chains than by these with an even number.

The requirement for the ring and its double bonds has not been fully accepted since some active auxins which lack such a ring system are known [35].

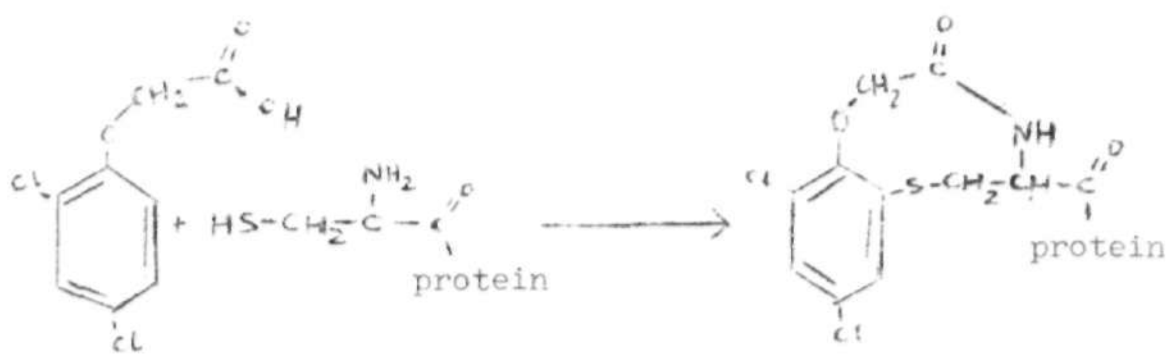
The position of the side-chain may have a great effect in the ring-systems. This is illustrated in the α-and β-naphthylacetic acids: the former has an activity in various auxin assays tests as high as that of IAA, whereas the activity of the β - compound is very slight. It is possible that this surprising difference may be due to secondary properties of the molecule.

The possession of at least one hydrogen atom attached to the α- carbon atom of the side-chain has also been claimed as a necessary condition for auxin activity. This activity disappears when a hydroxyl group is substituted for the hydrogen in this side-chain.

In most cases, active compounds have a terminal - COOH or a group easily convertible into one. The activity of the esters of IAA decreases with increasing size of the e esterifying alcohol and probably runs parallel with the ease of enzymatic hydrotysis of the ester in the plant. Sodium salts of the auxins have been found to possess greater specific activity than the corresponding acids. This may be due to the ease of penetration of the site of action or from a specific stimulation effect of alkaline ions on auxin activity.

Aldehydes are easily converted to the corresponding acids by enzymes present in the plants. Indole-3-acetonitrile isolated from cabbage leaves is easily converted to IAA[36] and this might account for its high auxin activity.

Substitution in the pyrrolene ring of IAA causes a reduction in activity. This reduction is least for a - CH_3 group and greater for C_2H_5 - . Activity is completely destroyed by an - OCH_3 group in any position. Substitution of the hydrogen atom attached to the nitrogen on the indole brings about the greatest reduction in activity. On the other hand, monosubstitution of halogens or a methyl group in the phenylene moiety of the indole gives increased activity, the greatest effect being obtained in the 4 or 6 position[37]. Single substitution gives less active compounds than double substitutions. There are strong indications that substitutions in both ortho-positionsin phenoxyacetic acids completely destroys activity[38]. Hence only one ortho position is substituted in 2,4-dichlorophenoxyactic acid. This suggests that in the plant essential chemical reactions take place in which auxins are involved in the combination at the free ortho carbon atom. This observation has led to the two-point attachment theory[39] of auxin action. In this theory, the auxin is supposed to combine at its carboxyl group and at a free ortho position with a cysteinyl unit of a protein in the following way:



Di-ortho substitution blocks the attachment to the ring and thus prevents the compound acting as an auxin.

PHYSICO-CHEMICAL ATTRIBUTES OF AUXINS

The search for greater knowledge of the mechanism of auxin action in the plant led to attention being directed to those chemical and physical properties of the active molecules which are closely correlated with hormone activity. It was then observed that extension-growth was much greater in acidic than in neutral or alkaline media. The optimum pH lies between 4 and 5. A possible explanation on an auxin basis was the fact that all known auxins were weak acids.

The role of the double bond in the nucleus has also been investigated. Based on the analogy of the mode of action of certain known enzyme systems, it was suggested that the auxins might be playing the part of organic catalysts in some metabolic process essential for extension-growth and that the catalytic action might depend on a reversible oxidation - reduction of this double bond. Experiments[40] designed to check this led to a property of the auxin molecule which shows a considerable degree of correlation with the growth-promoting activity.

This was the tendency of active compounds to be adsorbed at a water-mercury interface. Thus compounds of high auxin activity were strongly adsorbed at the interface while those of low activity were not. This discovery led to a modification of the structural requirements postulated by Koepfli, Thimann and Went[41]. This modification states that the primary requirement of the ring is that it shall have a high surface activity and the - COOH dipole must have a component perpendicular to the plane of the ring. The action of the auxins therefore depends on their adsorption on to the lipoid constituents of the colloidal membranes which form the outer boundary of the cell protoplast. A powerful turgescent action is then exerted on the membrane, thus increasing its permeability to dissolved substances. In this way food supply to the growing cell, and the latter's growth rate are regulated.

CHAPTER IV

METHODS OF DETERMINING AUXINS

For more than three decades after the discovery of plant auxins biological assays remained the mainstray of all work on these naturally-occurring auxins. Over the whole of this period they have remained the most sensitive of tests and, for the first twenty years, the solution of the enigma of the respective roles of auxin a auxin b and 'heteroauxin' (i.e. indole-3-acetic acid) was sought in various empirical tests involving the use of such assays. With the advent of paper chromatography, the biological assay of auxins was adapted to this new analytical technique, but only when large quantities of extracted plant material were used could colorimetric tests be applied for the more precise characterization of the active substances.

The pea root segment test[42] is about the most sensitive of the biological assays and can detect as little as 10^{-5} µg of indole-3-acetic acid (IAA) but cannot distinguish between an auxin sensu stricto and a growth inhibitor. One of the most sensitive of the true auxin assays for use with chromatography involves segments of Avena internodes[43] and can detect IAA in concentrations of approximately 0.01 μg/ml. Compared with these the most sensitive colorimetric tests for indoles are very poor. Thus tests involving ferric chloride (eg. Salkowski reaction) cannot detect concentrations much less than 2 μg/ml[44]. In the Ehrlich reaction the sensitivity is a little greater (colours can be obtained with as little as 1.0 $\mu g/cm^2$ of chromatographic paper). But biological assays are completely non-specific, and using them alone, the only indication of the nature of an active substance on a chromatogram is its R_f value. Further-more, biological assay, unless many times repplicated, is very imprecise and with the usual variation of response of biological material, cannot measure auxin concentration with an error much less than ± 40%. The unsatisfactory nature of this situation has been succintly stated by Steward and Shantz[45]:

"It is no longer particularly instructive to multiply examples of these alleged growth-promoting 'substances' which are designated solely by areas on the chromatogram ... unless the responsible compounds can be isolated and identified."

Attention has therefore been devoted to chemical and physico-chemical methods which would give more reliable results than the biological assay methods. Almost all the methods used for the analysis of auxins rely on some type of preliminary separation, of which solvent extraction and chromatography are the most commonly used. The separated auxins are then usually determined by both chemical and physical means.

INDOLE AUXINS

By far the most important plant indole auxin is indole-3-acetic acid (IAA), the naturally-occurring growth regulator of universal occurrence in the plant Kingdom. Its detection, estimation and identification is fundamental to the study of plant physiology.

There are three major problems in the study of auxins. The first is the very low concentration normally present in plants. For example, the amount of IAA in vegetative tissue normally ranges between 0.5 and 15 µg per Kg of fresh plant material.

A second problem is the fact that IAA is present in equilibrium with bound forms and any estimation must include measurement of the concentrations of such compound if proper precautions are not taken. Bound forms include the simple glucose or inositol esters, the corresponding nitrile, aldehyde or lactic acid and more complex forms such as gluco-brassicin.

A third problem is the fact that IAA exhibits some degree of lability with most conventional laboratory techniques. It can be degraded under even the mildest conditions that can be usefully employed. For instance it is slowly destroyed in basic conditions. It therefore requires necessary precautions to avoid losses when handling this auxin..

IAA is synthesized in plants from the aromatic amino acid, tryptophan. Any method of estimation depends for its success on separating it from the precursor tryptophan, from intermediates in the pathway and also from any other indoles that may be present in a particular plant. These include tryptophan, serotonin and indican. After extraction from the plant material it is determined by any of the following analytical techniques or by a combination of some of these.

(i) FLUORIMETRY[46]

A quantitative determination of IAA in aqueons solution by a fluorimetric method has been worked out by Ebert[47]. It is a highly sensitive technique that can quantitatively detect nanogram amounts of many fluorescing substances.

IAA concentration can be determined and the sensitivity is such that differences in concentration of 0.05-0.1 µg/ml can be distinguished. The method depends on the intensity of fluorescence when $CuSO_4$ and H_2SO_4 are added to the solution. The intensity of the fluorescence obtained by irradiation with U.V. light is measured photoelectrically in a suitable fluorimeter, in which the fluorescence is filtered through secondary filters. The fluorescence at low concentration is green and, yellow at high concentration.

One major problem with this technique is the fact that other fluorescing substances can interfere with an analysis since activation and fluorescence spectra generally show broad rather than sharp peaks, even though the activation and fluorescence maxima may not be identical with that of the compound under investigation. Secondly other compounds may quench fluorescence. It is therefore necessary for successful fluorimetric measurement of a substance that a fairly high level of purification be carried out. A promising way of doing this is gas-liquid chromatography.

(ii) PAPER/THIN LAYER CHROMATOGRAPHY

Kefford[48] has shown that there is a linear relationship between spot area and logarithm of quantity of IAA over the range of 0.25 to 8.00 μg when chromatogram is sprayed with ferric-perchloric reagent. For paper chromatography this reagent is a mixture of 0.001M $FeCL_3$ and 5% perchloric acid.

The Ehrlich reagent is also used. It gives rather stable pink, purple or blue colours with indole derivatives, except some hydroxy-indolylacetic acid. For spraying use is made of 2% p-dimethyl-aminobenzaldehyde in 2N HCl in 80% ethanol. For dipping, one volume of 10% (wt/vol.) p-dimethylaminobenzaldehyde is used in concentrated HCl to 4 volumes of acetone[49]. This dipping technique is capable of detecting 0.05 μg of IAA on a chromatogram.

For TLC best resolutions are obtained on neutral unbuffered silicagel G, the compound being located with either of the following chromogenic reagents:

a. p-dimethylaminobenzaldehyde (1g) dissolved in 25% HCl (50 ml) and 96% ethanol (50ml). Each chromatoplate is sprayed with about 10 ml of the solution. Ammonia from the developing solvent is first removed by warming the plate to 50°c for 5 minutes. After the sprayed plate has been warmed to 50°c for 20 minutes, it is exposed to the vapours from aqua regia. Indole derivatives appear as blue, violet or red spots.

b. 35% formaldehyde solution (10 ml) diluted with 25% HCl (10 ml) and 96% ethanol (20 ml). The chromatoplate is heavily sprayed (with 10 ml of the reagent) and heated to 100°c after about 5 minutes. Indole derivatives form yellowish brown spots. In U.V. light these spots have an orange fluorescence. Exposure of the sprayed films to the vapours of aqua regia causes an intensification of the fluorescence.

IAA is decomposed by light, so that it must be chromatographed in the dark.

(iii) ABSORPTION SPECTRA - U.V.

The absorption spectra of IAA can be measured in very dilute solution against a solvent blank using an automatic recording spectrophotometer.

If the location of the compound being examined is known on a chromatogram, an attempt can be made to obtain its absorption spectrum in U.V. light. Nitsch[50] demonstrated that this can be done by spraying the paper with paraffin oil to render it translucent and readings taken twenty minutes later. The general shape of the curve obtained, and the region of maximum absorption, fit reasonably well with that given by IAA in solution.

Alternatively, the compound can be located by one of the chromogenic reagents and the absorption spectrum in visible light determined. The absorption maxima can be used to determine quantitatively and directly on the paper, amounts of IAA as low as 0.5 μg. For this determination to be precise the spots should be kept small and no interferring substances should be present.

Also the substance can be eluted from the paper and the absorbancy measured in organic solvents. The eluting solvent is usually methanol (three rinses with 2 ml portions, allowing 5 minutes per rinse).

iv. GAS-LIQUID CHROMATOGRAPHY

Powell[51] employed this technique for the estimation of IAA in barley. After extraction of the sample the sequence of steps chosen was as follows:

(i) Fractionation of the extract into major indole groups

(ii) Silica Gel partition chromatography of the major indole groups

(iii) Esterification of the acidic indoles

(iv) gas-liquid chromatography.

The simple indoles were conveniently fractionated into three groups of substances namely acidic, basic and nuetral all of which partition into methylene chloride from aqueons solutions at the proper pH, and water soluble compounds which partition into water from methylene chloride regardless of the pH.

After fractionation silica Gel partition column chromatograph was used to eliminate many non-indolic contaminants from each sample. This process also helped to effect separation of many indole derivatives from each other. Briefly, the separation consisted of a column of Silica gel through which the indolic substances were gradient eluted into test tubes in a fraction collector.

Before the sample was gas chromatographed, esterification of the acidic fraction containing mainly IAA was effected using diazomethane. The diazomethane used was generated from N-methyl-N-nitroso-p-toluenesulphonamide using a micro method. Three test tubes were connected in series. To effect esterification N_2 was first gently bubbled through methylene chloride contained in the first test tube. The second test tube contained 1.5 ml of carbitol and 1 ml of 60% KOH. The third test tube contained several µg of IAA dissolved in 2 to 3 ml of methylene chloride and 2 ml of 10% methanol. Generation of diazomethane was initiated by adding to the second tube about 115 mg of N-methyl-N-nitroso-p-toluenesulphonamide dissolved in 1-5 ml of methylene chloride. Connection was immediately made with test tubes 1 and 3. A yellow tinge became visible in the third tube within about 2 minutes, indicating that the system was saturated with diazomethane. Esterification was then said to be complete at this point. This third test tube was then disconnected from the system and further aerated with N_2 for a few minutes to eliminate traces of diazomethane. The material obtained in tube 3 was taken to dryness in a rotary evaporator in preparation for gas chromatography. The samples (1-10 µg) were dissolved in 0.5 to 10 µl of ethanol and then chromatographed. The recovery of the methyl ester of IAA after gas chromatography was found to be satisfactory.

(v) COLORIMETRIC METHOD.

Among the naturally-occurring auxins, only IAA and its derivatives can at present be determined by colorimetric methods. Of the colour reactions given by indole compounds only three which have been studied more extensively and used in colorimetric determinations will be described.

(a) The Salkowski Reaction:

Small amounts of IAA form an intense red colour with $FeCl_3$ in the presence of high concentration of a mineral acid. This reaction, the Salkowski reaction, has been used for qualitative and quantitative estimation of IAA by various workers among whom are Gordon and Weber[52]. The reagents used were 1.0 ml of 0.5 $FeCl_3$ and 50 ml 35% $HClO_4$. 2.0 ml of this reagent were added to 1.0 ml of aqueous solution of the auxin (containing a total of 0.2 - 45 µg of IAA). Colour intensity obtained after about 20 - 25 minutes was determined in a colorimeter at 530 m u. Readings obtained were converted to concentrations of IAA by means of a standard curve.

The limit of detection with this method is 0.2-45 µg/ml of IAA.

(b) The Adamkiewicz Reaction (Hopkins - Cole Reaction.)

The addition of glyoxylic acid and strong sulphuric acid to a solution of an indole derivative brings about a reddish-yellow coloration. Sutter[53] added copper sulphate to the reagent mixture as a modification of this test and used it for the quantitative determination of IAA.

A minimum of 1.5-3 µg of IAA in 3 ml of test solution yields the colour, but an accurate determination requires at least 12 µg.

(c) The Nitroso-Indole Reaction

Indole compounds yield a red colour when treated with sodium nitrite and nitric acid. This reaction, the nitroso-indole reaction, was used as the basis for a quantitative method for the determination of IAA by Mitchell and Brunstetter[54]

To 50 ml of the acid solution (i.e. solution of IAA) were added 0.5 ml of 5% gum arabic solution, 2.0 ml of 5% potassium nitrite solution, and 0.4 ml of concentrated nitric acid - in that order. The solution was shaken and left to stand for 2 hours at room temperature when a red colour developed. The intensity of this red colour was estimated in a colorimeter against a standard IAA solution. The smallest amount of IAA which can be measured by this method is of the order of 10 - 25 µg per ml.

CHAPTER V

E X P E R I M E N T A L

MATERIALS AND METHODS

Plant material:

The onion plants used in this work were obtained from the Institute of Agricultural Research Plant Pathology Department. Other plant materials were also obtained from an onion farm at Sabon Gari near Zaria town.

Sample Preparation:

The bulb was separated from the stem and each section extracted separately. The plant material was macerated in prechilled methanol (3 ml/g fresh weight) in a Pifco blender and kept at 1-2^{o}C for 24 hours to ensure complete extraction. The solid residue was filtered off, and the filtrate evaporated at 45-50^{o}C under reduced pressure using Rotary Vacuum Evaporator until a small volume of water remained. The extract was filtered through Hyflosupercel (filter aid) to remove precipitated chlorophyll. The aqueous filtrate from Hyflosupercel was adjusted to pH 7.0 and then extracted three times with peroxide-free ether. The ether phase which contained the neutral fraction was discarded. The aqueous phase was adjusted to pH 3.0

and then extracted three times with peroxide-free ether. The ether phase contained the acidic fraction (indole-3-acetic acid). This acidic fraction was then concentrated into a small volume (about 2-5 ml) for further use in gas chromatography.

Esterification:

Diazomethane was used as the esterifying agent. It was prepared according to a procedure described by De Boer and Backer[55] using p-toluenesulphonyl-methylnitrosamide (Aldrich chemical Co. Inc.). A distilling flask (250 ml) was fitted with a condenser and a long-stem dropping funnel. The condenser was connected to a 250 ml Erlenmeyer flask by means of an adapter. An outlet tube was fitted through a cecond hole in the adapter of the Erlenmeyer flask. This outlet tube was bent so as to pass into and nearly to the bottom of a second Erlenmeyer flask. Both receivers were cooled in an ice-salt mixture. In the first receiver was placed 10 ml of ether and in the second 35 ml of ether. The inlet tube passed below the surface of the ether in the second receiver.

The distilling flask contained a solution of 6g of potassium bydroxide dissolved in 10 ml of water, 35 ml of ethanol, 10 ml of ether and a teflon coated magnetic stirring bar. The dropping funnel was adjusted so that the stem is just above the surface of the solution in the distilling flask. The distilling flask was heated in a water bath at 70-75°C and a solution of 21.5 g (0.1 mole) of p-toluenesulphonylmethylnitrosamide in 125 ml of ether was added through the dropping funnel at a regular rate for about 20 minutes. When the dropping funnel was empty about 50-100 ml of additional ether was added slowly, and the distillation continued until the distillate was colourless. The combined distillate from the two receiving flasks contained the diazomethane.

The actual esterification was carried out by adding the ethereal solution of diazomethane obtained above in small portions to the ice-cooled ethereal solution of the sample until the solution acquired a pale yellow colour. The esterified sample was left in the fume cupboard for a few minutes to allow excess of the diazomethane to evaporate and the resulting methyl ester was used for gas chromatographic analysis. Similarly a 10 ml solution (2.8 μg) of the authentic material (IAA) was esterified for use as a standard.

Gas Chromatographic Determination of IAA

A Gow-Mac Gas Chromatograph (Gow-Mac Instrument Co.) Model 69-102 equipped with a thermal conductivity detector, and fitted with a 4' x ¼" Carbowax M column, was used to obtain the chromatograms on a Pye-Unicam AR 55 linear recorder. The carrier gas used was nitrogen.

Operating conditions:

Column temperature:	202 ± 1°C
Column Condition:	Isothermal
Carrier gas flow rate:	7 ml/min
Filament Current:	100 mA
Chart Speed :	60 secs/cm
Attenuation :	8
Recording Sensitivity:	1 mV
Sample Size :	5 µl

The samples were injected with a mocrolitre syringe (Hamilton Co., Inc., Whitter, Calif.). Retention times were measured from the initial emergence of the solvent to the peak of the ester in question. Mixtures of the samples with authentic IAA yielded only single peaks.

CHAPTER VI

RESULTS AND DISCUSSION

TABLE I

Amounts of IAA present (µg/Kg fresh weight) in the bulbs of onion plants.

Sample Reference Number	Height x Width at half height	Cutting & weighing	Triangulation	Mean
1	7.877	7.710	8.108	7.898
2	14.768	17.421	17.885	16.691
3	18.216	25.276	23.006	22.166
4	15.357	14.617	14.161	14.712
5	3.662	4.211	4.075	3.983
6	9.875	10.474	9.653	10.000
7	4.743	5.405	5.999	5.382
8	8.998	10.165	10.010	9.724
Mean	10.437	11.910	11.695	11.320
Standard Deviation	5.220	6.976	6.244	6.130

TABLE II

Amounts of IAA present (μg/Kg fresh weight) in the stems of onion plants.

Sample Reference Number	Height x Width at half height	Cutting & weighing	Triangu-lation	Mean
9	4.760	5.165	5.154	5.026
10	5.668	7.632	6.637	6.646
11	6.350	7.096	7.361	6.935
12	6.348	6.919	6.446	6.571
Mean	5.782	6.703	6.400	6.295
Standard Deviation	0.753	1.069	0.919	0.860

The peak areas of the samples 1-12 (see figures 1 & 2) were calculated using all of the following three methods:

i. Height & width at half height

ii. Cutting and weighing

iii. Triangulation

The peak areas obtained were compared with that obtained

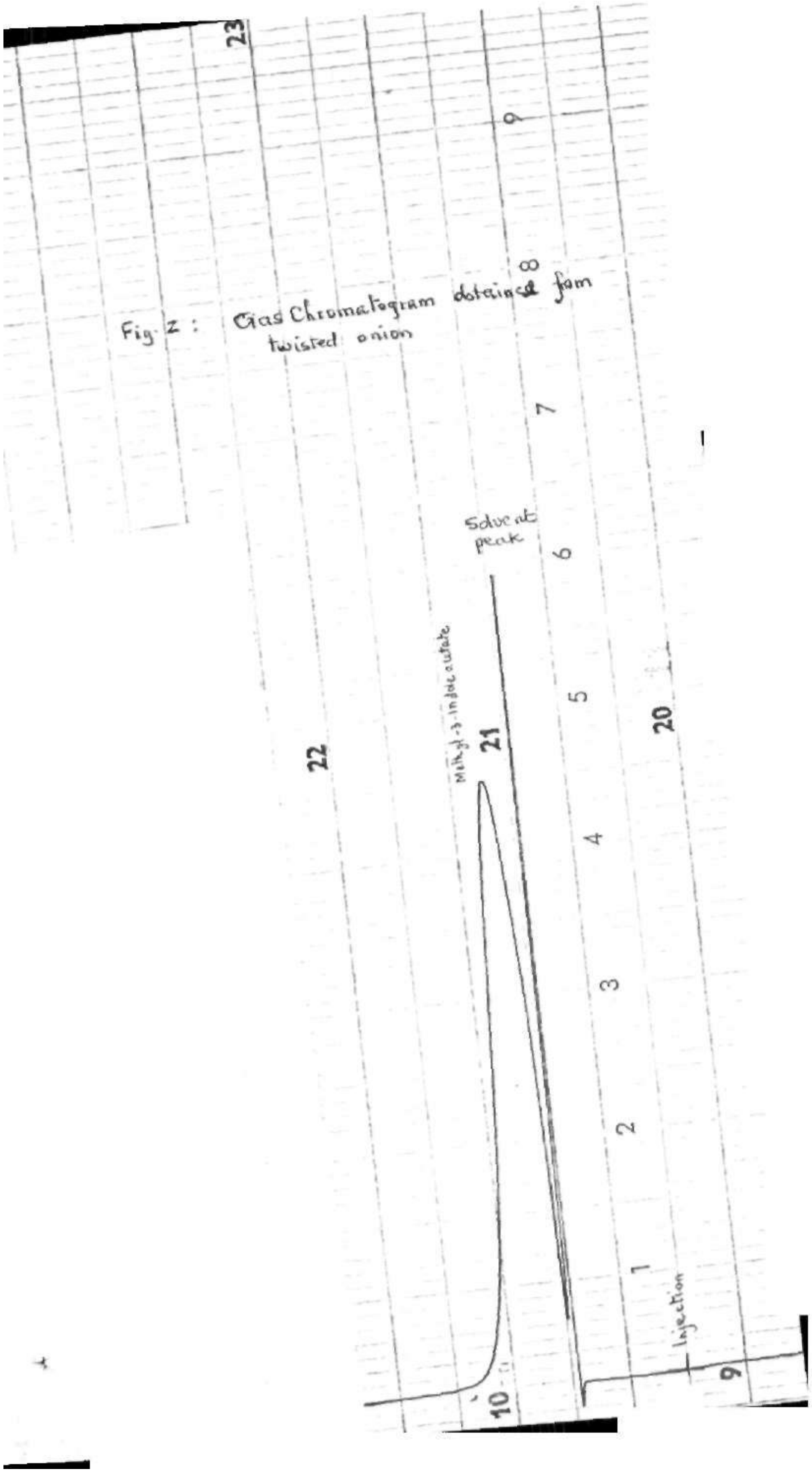


Fig. 2 : Gas Chromatogram obtained fom twisted onion

using the standard sample. Since peak areas are proportional to the weights of samples injected, the actual weight/concentration of the samples were calculated per kilogram weight of the fresh plant material used. The results obtained are summarised in tables I and II for the bulb and for the stem.

Most of the values of IAA content obtained fall in the range 0.5-15 µg/kg fresh weight. The high values obtained in some of the bulbs (samples 2-4) may have arisen partly due to artefacts liberated from IAA-complexes during chemical manipulation. This could occur during the extraction process because of the constant power failures lasting several hours resulting in the temperature rising well above 4°C during the 24 hour extraction period.

Another reason which could have accounted for the wide variation in the values obtained in the bulbs is the age of the plants. The plants used varied widely in their ages and since IAA occurs in higher concentrations in young and rapidly growing plants than in mature plants it is not therefore very surprising that the values obtained varied. There is also the possibility of the existence of endogenous rhythms in auxin level. Thus comparison of the auxin contents can be valid only if the plants were harvested at the same time of the day and the IAA extracted. Unfortunately, such control was not imposed on the collection schedule for the plant specimens.

On the whole the mean value obtained for the bulbs is 11.320 µg/Kg fresh weight with a standard deviation of 6.130. Had an electronic integrator been used a higher precision may have been obtained.

The values obtained for the stems were more consistent, with a mean of 6.295 µg/Kg fresh weight and a standard deviation of 0.860. It is therefore obvious that there is less IAA in the stem than in the bulb (see tables I & II).

Only one twisted (diseased) plant was obtained for analysis. The IAA content was found to be 30.584 µg/Kg fresh weight. More plants could not be obtained because this investigation was carried out during the dry season at which time the twisted plants do not survive. They are obtained only during the rainy season which is also the planting season. Even the few twisted plants which were obtained earlier on got rotted in the refrigerator because of the constant power failures lasting several hours.

To see if this value for the twisted plant represents an actual departure from the normal (healthy) ones, or if given the large variance in the latter, the figure 30.584 µg/Kg could fall within the normal variance of the values for the healthy plants, a calculation of a students' t-statistic (test) was carried out. For 95% confidence limits, the value of IAA expected for the healthy plants lies between 6.422 and 16.329 µg/Kg using the data from table I. The value of 30.584 µg/Kg obtained for the twisted plant lies outside this limit. This therefore indicates that the twisted plant contains an abnormally high level of concentration of IAA. Again this has to be further confirmed when more twisted plants are available for analysis.

Some other values (nos. 2,3,5 & 7 - table I) that lie outside this range can be accounted for in terms of the complications that gave rise to the wide variation mentioned earlier on. However, if these other values (nos 2,3,5, & 7 - table I) were to be discarded, then the new average value for the remaining ones would be 10.584 µg/Kg which is not much different from the average value of 11.320 µg/Kg obtained for all the data in the table. Also 95% confidence limits using Students' t-statistic (test) gives a range between 6.361 and 14.807 µg/Kg which is not very much removed from the previous range already calculated when all the values in table I were taken together.

Similarly calculations of the Students' t-statistic (test) was applied to the values obtained for the stem. (table II). The range of values obtained was 5.101 and

7.487 µg/Kg. In this case no.9 was slightly outside this range. The results obtained in this case showed more consistency.

CONCLUSION

The results obtained in this work showed that the bulbs of onions contain higher concentrations of IAA than the stems. The values obtained showed also that they lie in the range 0.5-15 μg/Kg fresh weight which is the usual range of concentration of IAA in plants. The value obtained for the bulbs is 11.320 μg/Kg fresh weight while the stems had an average value of 6.295 μg/Kg fresh weight. It was not possible to obtain the exact concentration of the auxin because of a number of constraints earlier mentioned. Some of these constraints include frequent power failures lasting several hours, and variation in the ages of the plants analysed. Temperature fluctuations above $4^{o}C$ during the 24 hour extraction period could lead to liberation of artefacts from the IAA -complexes thus giving a biased idea of the actual contents of free auxin present.

Again absence of an electronic integrator made the values of peak areas obtained not very precise.

Because of the time constraint imposed on this work it was not possible to wait for the next rainy season to obtain more twisted plants for analysis and for effective comparison with the healthy plants. Any future work on this should be planned to coincide with the period when lots of the twisted plants would be available.

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