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STUDIES ON THE BIOSYNTHESIS AND DEGRADATION OF
3-INDOLEACETIC ACID AND GRAMIN IN BARLEY SHOOTS

by

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A thesis submitted to the Faculty
of Graduate Studies in partial fulfillment
of the requirements for the degree
of
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ABSTRACT

The purpose of the study was to elucidate the inter-relationships of soluble, simple indoles in barley shoots, with particular reference to the biosynthesis and breakdown of indoleacetic acid and gramine.

Feeding experiments were carried out with DL-tryptophan-3-C¹⁴, D-tryptophan, L-tryptophan, gramine-1-C¹⁴, tryptamine-2-C¹⁴, indolelactic acid-3-C¹⁴, indoleacetaldehyde, tryptophol, indoleacetoneitrile, indoleacetamide, indoleacetic acid-2-C¹⁴, indoleglycolic acid, indoleglyoxylic acid and indolealdehyde. In most experiments, the indole compounds were fed to excised shoots of 14-day old seedlings for a period of 24 hrs in a growth chamber in the light. Then gramine was fed to shoots, the experiment was continued for 72 hrs. The shoots were then macerated in methanol and washed with acetone. The combined extract was evaporated to dryness, and the residue dissolved in water. After removal of chlorophyll, the extract was partitioned into the ACID ether, NEUTRAL ether and AQUEOUS fractions. The indole compounds present in each fraction were separated by paper and thin-layer chromatography. Identification of indoles rested on co-chromatography with authentic compounds, autoradiography, color reactions and UV absorption spectra.

The native indoles present in young barley shoots were shown to be indoleacetic acid, indolealdehyde, tryptophan, malonyltryptophan, aminomethylindole, methylaminomethylindole and gramine. When DL-tryptophan-3-C¹⁴ was fed to excised

shoots, radioactivity was found in malonyltryptophan, indolelactic acid, aminomethylindole, methylaminomethylindole, gramine, indoleacetic acid, and indolealdehyde. Both malonyltryptophan and indolelactic acid were probably of the D configuration. When labelled gramine was fed, no unequivocal identification of the radioactive metabolites found in the ether and aqueous fractions could be made. C¹⁴-labelled indolelactic acid principally gave rise to tryptophan and two-water-soluble compounds, but labelled gramine, methylaminomethylindole, aminomethylindole and indoleacetic acid were also found in small amounts. Although no evidence was found for the involvement of tryptamine, tryptophol, indoleacetaldehyde or indoleacetonitrile as intermediates in the metabolism of tryptophan, feeding experiments with these compounds showed that enzymes are present in barley shoots which are able to convert all these compounds to indoleacetic acid.

Indoleacetic acid-2-C¹⁴ was mainly converted to indoleacetyl-aspartic acid and several water-soluble compounds. Indoleacetic acid was also converted to indolealdehyde, but the postulated intermediates in this reaction, indoleglycolic acid and indoleglyoxylic acid, were not found after feeding radioactive indoleacetic acid. These compounds could themselves give rise to indolealdehyde when fed to barley, and indolealdehyde itself was further oxidised to indolecarboxylic acid.

It is concluded that, although indoleacetic acid could be formed from any of the precursors tested, the most likely

pathway for its biosynthesis was from tryptophan via indolepyruvic acid and indoleacetaldehyde, possibly by a reaction analagous to that catalysed by pyruvic oxidase in respiratory metabolism. Exogenous indoleacetic acid was mainly converted to indoleacetyl-aspartic acid, several unknown water-soluble compounds and indolealdehyde. Gramine and its precursors were formed, as expected, from tryptophan, but the nature of the intermediate between tryptophan and aminomethylindole remains unknown, and no breakdown products of gramine were identified.

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LIST OF ABBREVIATIONS

IAA	:	Indoleacetic acid
IAW	:	Isopropanol : ammonia : water solvent system
BuAW	:	Butanol : acetic acid : water solvent system
IBeW	:	Isopropanol : benzene : water solvent system
Isobuff	:	Isopropanol : buffer solvent system
BeAW	:	Benzene : acetic acid : water solvent system
8% NaCl	:	Sodium chloride : water solvent system
DmAC	:	Dimethylaminocinnemaldehyde reagent
DNPH	:	Dinitrophenylhydrazine reagent

INTRODUCTION

Although the Avena coleoptile test has been a cornerstone of investigations of plant growth regulators for thirty-five years, no detailed study has been made of the metabolism of simple indoles in monocotyledonous plants. To amend this gap, work was undertaken on the barley seedling. Barley is readily available, easily grown to produce a uniform population of seedlings, and metabolises applied indoles well. Seedlings of barley have been used previously to study the biosynthesis of tryptophan (Wightman, Chisholm and Neish 1961) and contain gramine, an interesting simple alkaloid which has been the subject of considerable research.

Thus, the aim of the present study was to identify the naturally occurring indoles of barley, and to relate these to the biochemical pathways involved in the biosynthesis and degradation of 3-indoleacetic acid and gramine in excised shoots of this plant. To this end, C¹⁴-labelled tryptophan, indoleacetic acid and gramine were fed to shoots, and after a 24 hr or 72 hr treatment period, the radioactive metabolites present in methanol extracts of the tissue were identified by means of chromatography, autoradiography and UV spectroscopy.

II. REVIEW OF LITERATURE

A. METABOLISM OF TRYPTOPHAN

1. Biosynthesis of Indoleacetic acid

a. Early history

Modern work on plant growth hormones, with its wide theoretical and practical implications, grew out of the interest of 19th century physiologists in such familiar phenomena as insect galls (Beyerinck, 1888), and the response of grass coleoptiles to light (Darwin, 1880).

Darwin's work on phototropism was extended by Boysen-Jensen (1910-1913) who showed that the bending of unilaterally illuminated coleoptiles was due to greater growth of the shaded side. A mica barrier, placed in a horizontal cut on the shaded side, abolished the curvature in regions below the cut, suggesting that transport of the phototropic stimulus was basipetal. Boysen-Jensen also showed that, while decapitated coleoptiles gave no phototropic response, the response could be restored by replacing the coleoptile tip, providing the experiment was carried out in a moist atmosphere.

The classical work of Frits Went appeared in 1928. Went placed coleoptile tips on small agar blocks, and showed that the blocks could later replace coleoptile tips in restoring the phototropic response to decapitated coleoptiles. He concluded that the plant growth hormone was a diffusible substance, and calculated its molecular weight from its diffusion constant. The concentration of this 'auxin' in higher plants was very low, and further progress on the

biochemistry of this compound depended for a time principally on the study of fungus cultures.

In 1932, Boysen-Jensen showed that a substance produced by liquid cultures of Aspergillus niger promoted the growth of Avena coleoptiles. Nielson (1928, 1930) found a similar growth promoting substance, which he called 'rhizopine', in cultures of Rhizopus suinus. Rhizopine was soluble in water and ether, sensitive to oxidation, and heat stable. In 1932, Dolk and Thimann showed that rhizopine was an acid of approximately the same strength as acetic acid, sensitive to warm acid, but stable to warm alkali. Then in 1934, K $\ddot{o}$ gl, Haagen-Smit and Erxleben isolated indole-3-acetic acid (IAA) from human urine, and showed that it had a striking promotive effect on coleoptile growth. Went (1965) described the events which led up to this discovery:

"When K $\ddot{o}$ gl started to work on the extraction of growth hormones in plants, he looked for starting materials which were high in the substance. Therefore, he started to look for natural products which might contain the hormone.

There was a slight possibility that the female sex hormone would have plant growth activity. When he received from Germany an impure preparation of female sex hormones, this turned out to be highly active, whereas the pure product was inactive. Therefore, the material from which the female sex hormone was extracted apparently contained the plant hormone. This was urine of pregnant women. Afterwards, it was found that urine from all persons contained auxin, and since urine turned out to be very rich in it, they continued to extract auxin from it."

K $\ddot{o}$ gl and Kostermans (1934) concluded that the growth substance produced by fungi such as yeast and Aspergillus, was also IAA.

b. Tryptophan as a precursor of Indoleacetic acid

Because of its close chemical similarity to IAA, and wide biological distribution, tryptophan was a logical choice as the precursor of IAA. Thimann (1935) was the first to implicate tryptophan directly in IAA biosynthesis. Using Rhizopus suinus, he isolated one gram of crystalline IAA from 1500 litres of medium, and confirmed its identity by positive Ehrlich and Salkowski color reactions, melting point, vacuum distillation temperature and sensitivity to acid. He also noticed that the production of IAA by this fungus was greatest in Witte peptone medium, which had a high tryptophan content.

In 1938, Berthelot and Amoreux showed that crown gall bacteria readily converted tryptophan to auxin, and later it was reported that the tumor tissue provoked by the bacterium could also perform this conversion (Link and Eggers 1943).

About this time, interest returned to higher plants. Haagen-Smit, Dandliker, Wittwer and Murneek (1946) isolated crystalline IAA from alkaline extracts of corn kernels, proving the existence of the hormone in a higher plant. Then in 1947, Wildman, Ferri and Bonner infiltrated spinach leaves with a tryptophan solution, at a concentration of 0.25 mg per ml, and showed a ten-fold increase in auxin content by bioassay of a crude leaf extract. Gordon and Nieva, (1949) obtained similar results with pineapple leaves and leaf breis. Feeding of tryptophan to bean and cress roots caused inhibition of growth, hypertrophy of the root tissue and induction of lateral roots, effects similar to those produced by IAA (Terroine 1948, Pohl 1952).

PATHWAYS OF TRYPTOPHAN METABOLISM IN PLANTS AND ANIMALS

Since about 1950, the use of paper chromatography has allowed more critical separation and identification of auxin from a variety of plant materials. For example, Yamaki and Nakamura (1952), by using chromatography to separate their reaction products, showed that corn embryo juice could convert tryptophan to IAA. Similarly, Wolf (1952) showed the formation of IAA from tryptophan by Ustilago and Gymnosporangium.

Another major technical advance was the advent of C¹⁴-labelled tryptophan, with the advantages of sensitivity of detection without tedious bioassay, and greater assurance that the IAA formed did in fact originate from the tryptophan administered.

Dannenberg and Liverman (1957) studied the metabolism of DL-tryptophan-2-C¹⁴ in watermelon slices and demonstrated a labelled, Ehrlich positive spot having the R_f and IAA in two solvent systems.

Pilet (1961) reported that when root and epicotyl fragments of Lens were incubated with DL-tryptophan-C¹⁴ labelled IAA could be identified chromatographically. Srivastava and Shaw, (1962) incubated mycelium and uredospores of Melampsora lini with DL-tryptophan-2-C¹⁴ and identified radioactive IAA as an Ehrlich positive spot having the relation to the front (R_f) of authentic IAA in two solvent systems, and active in the Avena test. A very low yield of IAA was obtained (0.016% in 8 hrs), but this experience is common in such experiments. Wightman (1962, 1964) showed production of labelled IAA from DL-tryptophan-3-C¹⁴ by cabbage and tomato shoots. Identification of IAA rested on its R_f values in three solvent systems, its color

reactions, and activity in the wheat coleoptile bioassay.

Several lines of indirect evidence also support the role of tryptophan in IAA biosynthesis. First, tryptophan shows a delayed response in the Avena test. Secondly there is a parallelism between the concentration of tryptophan and auxin in different parts of the plant, and also between auxin concentration and the activity of the enzyme converting tryptophan to auxin (e.g. Yamaki and Nakamura 1952, Lund 1956, Wildman and Bonner 1948). Thirdly, zinc deficiency, which affects tryptophan biosynthesis by reducing the activity of the zinc metallo-enzyme tryptophan synthetase, also reduces the concentration of auxin in the plant (Tsui 1948). Lastly, low doses of ionising radiation cause parallel reductions in auxin concentration and the activity of the enzymes converting tryptophan to auxin (Gordon 1961).

c. Indolepyruvic acid as a Precursor of Indoleacetic acid

i) Occurrence of Indolepyruvic acid

Indolepyruvic acid was early suggested as an intermediate between tryptophan and IAA. Thimann (1935) noticed that formation of IAA from tryptophan by Rhizopus suinus was an aerobic process, and advanced the hypothesis that tryptophan was oxidatively deaminated to indolepyruvic acid via the unstable intermediate, indoleiminopropionic acid.

Indolepyruvic acid is itself unstable, especially under alkaline conditions, and this has greatly hampered evaluation of its role in IAA biogenesis. Bentley, Farrar, Housley, Smith and Taylor showed the production of seven spots after two-way chromatography of synthetic indolepyruvic acid in isopropanol : ammonia : water followed by isopropanol : acetic acid : water. The compounds formed included IAA and possibly indoleglycolic acid; no indolepyruvic acid remained after chromatography. Jepson (1958) identified indolecarboxylic acid and indolecarboxamide as breakdown products of indolepyruvic acid after ammoniacal chromatography. However, Dannenberg and Liverman (1957) concluded that decomposition of indolepyruvic acid during chromatography was not complete, so that it could be identified after chromatography in ammoniacal solvents.

Despite the lability of indolepyruvic acid, several authors claim to have identified this compound in various plant tissues. Stowe and Thimann (1954) extracted a compound from kernels of Country Gentleman sweet corn which had an R_f value of 12 in isopropanol : ammonia : water, gave a positive Salkowski reaction, and was active in the pea and Avena tests. They concluded that this was indolepyruvic acid. Vlitos and Meudt (1954) identified as indolepyruvic acid a compound found in soybean and tobacco leaves. The compound was Ehrlich positive, and had an R_f identical with synthetic indolepyruvic acid in isopropanol : ammonia : water. However, the compound present in the plant extracts was stable for several weeks at 5°C, while synthetic indolepyruvic acid was highly unstable. Bloemmaert (1954) chromatographed extracts of potato peelings in

butanol : ammonia : water and bioassayed the chromatograms using the Avena test. He found a growth promoting compound with a lower Rf value than IAA and suggested that this might be indolepyruvic acid. In more recent work, Srivastava (1964) examined extracts of Country Gentleman sweet corn kernels and tomato seedlings for the presence of indolepyruvic acid. He used a water : acetic acid solvent in which synthetic indolepyruvic acid was stable, but was unable to detect any indolepyruvic acid colorimetrically.

In summary, there is as yet no convincing evidence for the presence of native indolepyruvic acid in a higher plant. It is possible that some of the earlier workers may have identified, as indolepyruvic acid, Ehrlich positive spots which were in fact due to indoleacetylaspatic acid or malonyltryptophan.

ii) Formation of Indolepyruvic acid from Tryptophan

Several authors have studied the conversion of tryptophan to indolepyruvic acid. Dannenberg and Liverman (1957), after feeding DL-tryptophan-2-C¹⁴ to watermelon slices, showed the formation of a radioactive spot which they identified as indolepyruvic acid. This spot agreed with the Rf of synthetic indolepyruvic acid in isopropanol : ammonia : water and in isopropanol : acetic acid : water, and showed yellow UV fluorescence after spraying with 1,2-diamino-4-nitrobenzene, a relatively specific test for keto groups. The unknown and synthetic indolepyruvic acid also gave a yellow-green color

with Ehrlich reagent. It would be particularly desirable to repeat this work to decide whether this compound was not in fact malonyltryptophan, (discovered by Good and Andreae in 1957).

The best evidence for the formation of indolepyruvic acid from tryptophan is that of Kaper and Veldstra (1958). These workers first showed that the decomposition of indolepyruvic acid during chromatography produced a complex of seven spots, each giving distinctive colors with the Ehrlich and Salkowski reagents. They then fed C^{14} -labelled tryptophan to cultures of Agrobacterium tumefaciens, and showed the same characteristic pattern of breakdown products on chromatograms of an extract of the culture medium. They also showed, after feeding tryptophan-9- C^{14} , and tryptophan-11- C^{14} , that all the indolepyruvic acid decomposition spots were radioactive, a further proof of their origin from tryptophan. Recently, Srivastava (1964) has isolated the indolepyruvic acid formed from tryptophan by Agrobacterium tumefaciens, and identified it by its IR and UV spectra and color reactions.

Libbert and Brunn (1961) showed the formation of indolepyruvic acid during conversion of tryptophan to IAA by a pea enzyme system. Indolepyruvic acid was identified by its Rf in acetic acid : water, and its reaction with silver nitrate, and the Salkowski and Ehrlich reagents. Srivastava and Shaw (1962) tentatively identified indolepyruvic acid as a product of the metabolism of DL-tryptophan-2- C^{14} by Melampsora lini. However, Srivastava (1964) was unable to show formation of indolepyruvic acid during conversion of tryptophan to IAA by corn kernels, corn coleoptiles or tomato seedlings.

Indolepyruvic acid could be formed from tryptophan either by oxidative deamination (Equation 1) or by transamination:

Gordon (1961) has summarised evidence to show that transamination is the more likely pathway in plants. Stowe (1955) showed that cell-free extracts of Esherichia coli, Pseudomonas fluorescens and Agrobacterium tumefaciens carried out a vigorous transamination of tryptophan, which was completely dependent on the presence of keto acids, and strongly stimulated by pyridoxal phosphate. Transamination of tryptophan by higher plants has been shown by Wilson, King and Burris (1954) in lupin, by Murakami and Hayashi (1957) in rice grains, and by Hasse and Homann (1962) in Lupinus, Ricinus and Nicotiniana. Libbert (1962) showed formation of IAA from tryptophan by pea enzymes in presence of α -ketoglutarate, but the reaction was inhibited by pyridoxal phosphate.

Gordon (1961) states that amino acid oxidases, on the other hand, are generally lacking in higher plants. Furthermore, animal amino acid oxidases are highly stereospecific, while the tryptophan to IAA system in plants is not stereospecific.

iii. Conversion of Indolepyruvic acid to Indoleacetic acid

Since indolepyruvic acid is spontaneously converted to

IAA, enzymatic conversion of indolepyruvic acid to IAA is difficult to prove. However, Wildman, Ferri and Bonner (1947) infiltrated indolepyruvic acid into spinach leaf discs, and showed an increase in the auxin activity of the leaf extract. Gordon and Nieva (1949) showed that pineapple leaves could also carry out this conversion.

Two alternate pathways between indolepyruvic acid and IAA have been suggested: decarboxylation to indoleacetaldehyde, and conversion to indoleacetonitrile via an oxime intermediate. These pathways are discussed below.

d. The Indoleacetaldehyde Pathway

i. Occurrence of Indoleacetaldehyde

Unequivocal demonstration of the presence of indoleacetaldehyde in plant tissues has not been easy. The concentration of indoleacetaldehyde, when it is present, is very low, and pure synthetic indoleacetaldehyde was not available for purposes of comparison until 1952 (Brown, Henbest and Jones).

Larsen (1944) working with etiolated pea seedlings, showed the presence of a neutral indole auxin, and concluded that this substance was indoleacetaldehyde. Gordon and Nieva (1949) identified indoleacetaldehyde in pineapple leaves by inactivation with dimedon, and by formation of a bisulfite addition product, from which the indoleacetaldehyde could be regenerated. Linser, Kiermayer and Youssef (1958) chromatographed extracts of stems of Brassica oleracea var. sabauda

in propanol : ammonia : water, and reported the presence of a growth promoting substance at the Rf of indoleacetaldehyde. This substance gave a yellow color with ferric chloride and perchloric acid, similar to that given by synthetic indoleacetaldehyde. Larsen and Aasheim (1959) contributed the best evidence yet obtained for the presence of indoleacetaldehyde in plants. The neutral ether fraction of extracts of etiolated pea epicotyls was chromatographed in a hexane : water solvent, which clearly separated indoleacetaldehyde from indoleacetonitrile. A positive reaction to 0.1 per cent dimethylaminocinnemaldehyde solution (DMAC) was obtained at the Rf of indoleacetaldehyde. The region containing the suspected indoleacetaldehyde was eluted from the chromatogram with ether and bioassayed with the Avena test before and after treatment with soil. Activity after treatment with soil was several hundred times higher than the original activity of the extract, a form of behaviour which was also characteristic of synthetic indoleacetaldehyde.

In summary, pea seedlings have been shown to contain a neutral, DMAC positive compound, having the Rf of indoleacetaldehyde in one solvent system, and convertible to IAA by soil microorganisms. It seems reasonable to conclude that this compound was, in fact, indoleacetaldehyde. Larsen and Aasheim (1959) could not, however, detect any indoleacetaldehyde in Brassica.

ii) Formation of Indoleacetaldehyde from Tryptophan

Addition of tryptophan to preparations of pineapple leaf (Gordon and Nieva 1949), mung bean seedling (Weber and Gordon 1953) or pea epicotyl (Larsen 1951) caused an increase in the neutral auxin, presumed to be indoleacetaldehyde. This neutral compound was convertible to acid auxin by treatment with soil, or with Schardinger's enzyme, which is behaviour characteristic of indoleacetaldehyde. None of these studies exclude the possibility that the neutral auxin was indoleacetoneitrile, or the ethylester of IAA.

iii) Formation of Indoleacetaldehyde from Indolepyruvic acid

Conversion of indolepyruvic acid to indoleacetaldehyde has never been directly demonstrated, principally because of the low concentration, lability and rapid metabolism of both compounds.

Gordon (1956) noted that during conversion of indolepyruvic acid to IAA by pineapple and mung bean preparations, there was no increase in free indoleacetaldehyde, and suggested that the conversion of indolepyruvic acid to indoleacetaldehyde may be analogous to the pyruvic carboxylase system. Indolepyruvic acid would then be decarboxylated to the indoleacetaldehyde diphosphothiamine intermediate, and IAA formed via the indolylacetyl-lipoate and CoA esters. In support of this hypothesis, Gordon (1961) found that the conversion of tryptophan to IAA by cell-free extracts of Coleus buds was accelerated by lipoic acid.

iv) Conversion of Indoleacetaldehyde to Indoleacetic acid

In 1947, Wildman, Ferri and Bonner showed that indoleacetaldehyde was not converted to auxin by spinach leaves. However, Gordon and Nieva (1949) point out that the synthetic preparation used by Wildman et al. probably contained little or no indoleacetaldehyde.

Larson (1949) showed that an impure preparation of indoleacetaldehyde, prepared from tryptophan and isatin, was rapidly converted to acid auxin by excised Avena coleoptiles and by coleoptile juice. Gordon and Nieva (1949) used an indoleacetaldehyde preparation purified from pineapple leaf extracts via the bisulfite addition product. This purified preparation was converted to acid auxin by pineapple leaf breis. Larson (1951) showed that an enzyme system present in Avena coleoptile juice could convert crystalline naphthalene acetaldehyde to naphthalene acetic acid, a reaction which was also catalysed by Artemesia root preparations (Ashby 1951).

Bentley and Housley (1952) reported that pure, crystalline indoleacetaldehyde was active in the Avena test, and that an acid auxin was produced in the reaction. Cell free homogenates of mung bean (Phaseolus aureus) seedlings can also convert indoleacetaldehyde to auxin (Gordon 1956).

v) Relationships between Indolepyruvic acid, Indoleacetaldehyde, Tryptophol and Indolelactic acid

Larsen (1951) showed that when naphthalene acetaldehyde

was metabolised by Avena coleoptiles, 2 moles of the aldehyde were consumed for every mole of acid produced. This led him to suggest that a dismutation reaction was occurring, in which naphthalene acetaldehyde was simultaneously oxidised to naphthalene acetic acid and reduced to the corresponding alcohol.

Using Acetobacter xylinum, Larsen, Harbo, Klungsoyr and Aasheim (1962) showed, by paper chromatography of an extract of the culture medium, that both IAA and tryptophol were produced when washed cells were incubated in a medium containing tryptophan. When sodium bisulfite was added to the medium, there was a reduction in the amount of IAA and tryptophol produced, and a small amount of indoleacetaldehyde could be demonstrated. This result supported the hypothesis that indoleacetaldehyde was the precursor of both IAA and tryptophol.

This reaction, however, is probably not a true dismutation, Racker (1949) showed that purified 'aldehyde mutase' preparations from beef liver actually contained two separate enzymes, an alcohol dehydrogenase and an aldehyde dehydrogenase.

Kaper and Veldstra (1958) reported the presence of both tryptophol and indolelactic acid in the culture medium of Agrobacterium tumefaciens grown in the presence of tryptophan. Under anaerobic conditions these were the main products of tryptophan metabolism. Both indolelactic acid and tryptophol were produced when the bacteria were incubated with indolepyruvic

acid under aerobic conditions, but under anaerobic conditions only indolelactic acid was found. Incubation with indolelactic acid did not yield tryptophol. Kaper and Veldstra therefore suggested the following relationships:

Libbert and Brunn (1961) identified tryptophol as a product of tryptophan metabolism by a pea enzyme system, after removal of an ethanol-soluble native inhibitor of aldehyde oxidase. Bailey and Gentile (1962) tentatively identified tryptophol as a metabolite of tryptophan in the culture medium of Diplodia natalensis. Indolepyruvic acid, indoleacetaldehyde and tryptamine were absent, and indolelactic acid was present in greater amount than was IAA.

Little further evidence is available concerning the role of indolelactic acid in plants. Dye, Clark and Wain (1962), studying the metabolism of normal and crown-gall infected tomato stems, identified a 'compound X' whose Rf values were similar to those of indolelactic acid. Wightman (1964) has shown that native indolelactic acid is present in young tomato plants, and that radioactive indolelactic acid can be demonstrated when DL-tryptophan-3-C¹⁴ is fed to these plants. Wightman has suggested the following sequence in tomato for the biosynthesis of IAA:

Tryptophan $\rightarrow$ Indolelactic acid $\rightarrow$ Tryptophol

4) $\rightarrow$ Indoleacetaldehyde $\rightarrow$ IAA

Wightman, Chisholm and Neish, (1961) showed that barley shoots could readily convert indolelactic acid to tryptophan and gramine. Gamborg, Wetter and Neish (1962) showed that the reverse of one of these reactions, the oxidation of indolelactic acid to indolepyruvic acid, was catalysed by glycolate oxygen oxidoreductase, an enzyme found in salvia, wheat angelica and buckwheat leaves. Further work is clearly needed to clarify the relationships of these interesting compounds.

e. The Indoleacetonitrile Pathway

i) Occurrence of Indoleacetonitrile

The first isolation of an auxin from the vegetative parts of a higher plant was the crystallisation of indoleacetonitrile from immature cabbage heads by Henbest, Jones and Smith in 1953. The same workers also showed the presence of indoleacetonitrile in other crucifers, such as brussel sprout, radish, cauliflower, turnip and swede. Subsequent reports of the presence of indoleacetonitrile in Brassicae have been made by Denffer, Behrens and Fischer (1952), Linser, Mayr and Maschek (1954) and Fischer (1954). Indoleacetonitrile has been found in non-cruciferous plants by several workers, e.g. in potato and Aegopodium podagraria (Bennet-Clark and Kefford, 1953)

and in tobacco ovaries (Lund, 1956). Unfortunately, many of these identifications rest on an R_f value in a single solvent, and often use only bioassay to locate the compound on the chromatogram. Such identifications can only be regarded as tentative.

ii. Formation of Indoleacetonitrile from Tryptophan.

Jones, Henbest, Smith and Bentley (1952) made the plausible suggestion that indoleacetonitrile, like other simple indoles, might originate from tryptophan. Dannenberg and Liverman (1957) incubated young watermelon slices with DL-tryptophan-2- C^{14} , and demonstrated the formation of a neutral, radioactive compound having R_f values similar to those of indoleacetonitrile in three solvents. No Ehrlich color was detected, however. Kutacek, Prochazka and Grunberger (1960) fed DL-tryptophan-3- C^{14} to young cabbage plants, and identified indoleacetonitrile by chromatography in three solvents and by its color reaction.

Gmelin, Saarivirta and Virtanen (1961) showed the presence in young cabbage heads of the S-glucoside, glucobrassicin, a precursor of indoleacetonitrile and ascorbigen.

Glucobrassicin

Prochazka, Grunberger, Kutacek and Stakjova (1962) found that ascorbigen was radioactive when C¹⁴-labelled tryptophan was fed, but could not detect glucobrassicin.

Most recently, Wightman (1952) showed the production of radioactive indoleacetonitrile from DL-tryptophan-3-C¹⁴ by young cabbage plants, the acetonitrile being identified by R_f in three solvents, bioassay, and color reactions with DMAC and the nitrite-nitric reagent. Ascorbigen was also tentatively identified.

iii. Formation of indoleacetonitrile from Indolepyruvic acid

Three possible pathways for the biosynthesis of indoleacetonitrile have been suggested. The earliest suggestion was made by Jones, Henbest, Smith and Bentley (1952) as follows:

Ahmad and Spenser (1960) criticised this hypothesis on several grounds. Firstly, amino acid oxidases, which would be required to carry out the first step are rare in plants. Secondly, the second step would be unusual in a biological system, where α-amino acids generally undergo rapid spontaneous hydrolysis to α-keto acids. Lastly, the third step is without chemical or biological precedent.

Dannenberg and Liverman (1957) suggested that indoleacetonitrile might arise by condensation of hydroxylamine with

indoleacetaldehyde, forming an oxime, which would then be dehydrated to indoleacetonitrile.

Participation of the aldehyde was inferred from the fact that dimedon, thought to be a specific trapping agent for aldehydes, inhibited the formation of indoleacetonitrile from tryptophan. Ahmad and Spenser (1960) pointed out that the last step in the reaction, although it can be accomplished chemically, was without biological precedent. However, Mahadevan (1963) showed that indoleacetaldoxime could be converted to indoleacetonitrile by banana leaf Aspergillus niger, Penicillium chrysogenum, Fusarium oxysporum and Gibberella fujikuroi, but not by cabbage or barley.

The third reaction sequence was proposed by Stowe (1959) and Ahmad and Spenser (1960), and involves transoximation of indolepyruvic acid to form indolepyruvic acid oxime.

Housley and Bentley (1956) found in cabbage leaves an acid, water soluble, ether insoluble compound, which was converted to indoleacetonitrile by heating to 98-100°C at pH 5.6 for 25 minutes. Ahmad and Spenser (1960) showed that the Rf values, UV fluorescence and color reactions of

this indoleacetonitrile precursor were identical with those of synthetic indolepyruvic acid oxime. Furthermore, indolepyruvic acid oxime was converted to indoleacetonitrile in 95% yield by heating in 0.05M H_2SO_4 for 3 hrs. Conversion of synthetic indolepyruvic acid oxime to indoleacetonitrile was blocked by dimedon, showing that dimedon inhibition did not necessarily mean the participation of aldehyde in the reaction. Synthetic indolepyruvic acid oxime showed 20-25% of the activity of IAA in the Avena test, while Housley and Bentley's precursor was active in the cress root test. α -Keto acid oximes have been shown to occur in plants (Virtanen and Laine 1939), and transoximases, catalysing transfer of $=NOH$ groups between keto acids, have been demonstrated by Yamafujii, Aoka and Omura (1956). Thus, there is strong evidence that indoleacetonitrile may be formed in plants by transoximation of indolepyruvic acid.

iv) Conversion of Indoleacetonitrile to Indoleacetic acid

Bentley and Housley (1952) found that indoleacetonitrile was more active than IAA in the Avena test, but could detect little IAA as a product of its metabolism. They suggested indoleacetamide as a possible intermediate between indoleacetonitrile and IAA, but the same group (Jones et al. 1952) noted the very slight activity of this compound in the Avena test.

Thimann (1953) showed that Avena coleoptiles could convert indoleacetonitrile to IAA with about a 50% yield. Stowe and Thimann (1954) presented chromatographic evidence for the

formation of IAA in the reaction, but could detect no indoleacetamide. Thimann (1953) also noted the complete inactivity of indoleacetonitrile in the pea curvature test, and its low activity in similar tests with corn coleoptiles and Lupinus embryos.

Seeley, Fawcett, Wain and Wightman (1956) showed chromatographically that pea and tomato tissue were incapable of converting indoleacetonitrile to IAA, but could form IAA from indoleacetamide. On the other hand wheat tissue, which had only slight ability to convert indoleacetamide to IAA, readily produced IAA from indoleacetonitrile. These results gave further evidence that indoleacetamide is not an intermediate between indoleacetamide and IAA.

Ballin (1962) showed that some species e.g. Brassica oleracea and Triticum aestivum, which showed a growth response to indoleacetonitrile, were also capable of converting indoleacetonitrile to IAA. Other plants, e.g. Helianthus annuus and Solanum lycopersicum also showed a growth response, but no IAA production could be demonstrated. Ballin considered that indoleacetonitrile could act as an auxin either per se, or after conversion to IAA, depending on the enzymatic constitution of the plant.

Lastly, Mahadevan (1963) showed that barley and cabbage, both of which convert indoleacetonitrile to IAA (Thimann and Mahadevan, 1958), could also metabolise indolacetaldoxime to IAA but not to indoleacetonitrile. It is possible that

indoleacetaldoxime is an intermediate between indole-acetonitrile and IAA, although, as mentioned above, several tissues convert indoleacetaldehyde oxime to both indole-acetonitrile and IAA, making the sequence of events uncertain.

f. The Role of Tryptamine and 5-Hydroxyindole Pathway

i) Occurrence of Tryptamine

Native tryptamine has been reliably identified in a plant on only one occasion. White (1944) crystallised tryptamine from flowers of "Acacia" which contained 0.5 per cent tryptamine per gram fresh weight, and identified the compound by its melting point, and preparation of the hydrochloride and picrate derivatives.

Hember (1948) found an Ehrlich and ninhydrin positive compound in maize kernels which had the same R_f values as tryptamine. However, his published R_f values are very ill-defined (e.g. R_f in 70% EtOH 35-75). West (1959) reported the presence of tryptamine in tomato fruits, on the basis of bioassay and chromatography in three solvents. Srivastava (1964) could find no evidence of tryptamine in corn kernels.

ii) Occurrence of 5-Hydroxyindole Derivatives

The importance of 5-hydroxytryptamine (serotonin) as a neurohumoral and vasoconstrictor agent has stimulated considerable research on 5-hydroxyindole compounds in animals. Their occurrence in plants, however, appears to be sporadic.

Waalkes, Sjoerdsma, Greveling, Weissbach and Udenfriend (1958) identified 5-hydroxytryptamine in relatively large amounts (up to $65 \mu\text{g}$ per gram fresh weight) in banana pulp and peel. Their identification was based on Rf in one solvent, Ehrlich color, and fluorescence activation wavelength. West (1959) found 5-hydroxytryptamine as well as tryptamine in tomato fruits, and showed a rise in both compounds during ripening.

Fairly large doses of 5-hydroxytryptamine can apparently be tolerated by animals if taken orally, but injection of even minute amounts into skin causes intense pain and irritation. For example, Collier and Cheshier (1956) showed by pharmacological and chromatographic tests that the sting of the nettle, Urtica dioica, contains 5-hydroxytryptamine. The irritant trichomes ('cowhage') of Mucuna pruriens also contained 5-hydroxytryptamine as shown by Rf, color reactions and UV spectrum (Borden, Brown and Batty, 1954).

Bufotenine, 5-hydroxy-NN'-dimethyltryptamine, found in toad venom, was isolated from the poisonous fungus Amanita by Wieland, Motzel and Merz (1953); 15 g of the monopicrate were obtained from 100 kg fungus. Bufotenine is also present in the toxic pods of the legume Piptadenia peregrina (Fish, Johnston and Horning, 1955) where it is accompanied by bufotenine oxide, NN'-dimethyltryptamine and NN'-dimethyltryptamine oxide. 5-Hydroxyindoleacetic acid is also present in Piptadenia pods (Horning, fide Waalkes et al. 1955), but was not found in bananas.

5-Hydroxytryptophan has been reported to be present in peach leaves (Weaver and Jackson, 1963) and after feeding of tryptophan to watermelon slices (Dannenberg and Liverman, 1957). In the latter paper it is possible that the suspected 5-hydroxytryptophan was in fact malonyltryptophan, which had not been discovered at that time.

Thus, tryptamine is by no means widespread in the plant kingdom. It seems to be associated with flowers and ripening fruits, while its 5-hydroxy derivatives are concerned more with defence mechanisms than with growth processes.

iii) Formation of Tryptamine from Tryptophan

The most likely origin of tryptamine in the plant would seem to be by oxidative decarboxylation of tryptophan.

Tryptophan decarboxylase has never been demonstrated in plants. Indeed, even in animals, the principal pathway seems to be via 5-hydroxytryptophan decarboxylase (Udenfriend, Clark and Titus, 1953).

Dannenberg and Liverman (1957) found a labelled spot having the R_f of tryptamine in two solvents after feeding DL-tryptophan-2- C^{14} to watermelon slices. This compound appeared in the basic ether fraction, but did not give any color with Ehrlich reagent.

Grady and Wolf (1959) showed that cultures of Taphrina deformans and Dibotryon morbosum, which produced IAA in

tryptophan media, accumulated a compound having the same Rf as tryptamine in two solvents. Cultures of Ustilago zeae, which also produced IAA from tryptophan, did not form tryptamine (Wolf, 1952).

iv) Formation of Indoleacetic acid from Tryptamine

Some tissues are apparently able to convert tryptamine to IAA. These include Avena (Skoog, 1937), pea (Went and Thimann, 1937), pineapple (Gordon and Nieva, 1949), and corn embryo (Yamaki and Nakamura, 1952). On the other hand, Ustilago (Wolf, 1952), mung bean (Weber and Gordon, 1953) and spinach (Wildman, Ferri and Bonner, 1947) cannot carry out this conversion. Also, Gordon (1956) showed that in pea tissue the presence of inhibitors of amine oxidase abolished the conversion of tryptamine to IAA, while conversion of tryptophan to IAA was unimpaired, suggesting that tryptamine is not an obligate intermediate between tryptophan and IAA.

Plant amine oxidases also seem to be limited in distribution. Werle and Zabel (1958) made an extensive survey of histaminase activity in the plant kingdom. Activity was present in the Papilionaceae, Umbelliferae, Oleaceae, Labiateae and Compositae, but absent in many other dicot families, the monocots, and the gymnosperms. Clark and Lann (1957) described the extensive purification of a tryptamine oxidase from peas and showed that the product of the reaction was indoleacetaldehyde, by isolation of the DNPH and dimedon derivatives of the indoleacetaldehyde. Yields of

indoleacetaldehyde up to 90 per cent of the added tryptamine were reported at low tryptamine concentrations, but the enzyme was rapidly inactivated by high concentrations of tryptamine. Tryptamine could also be oxidised by peroxidase, and since Kenten (1953) had shown that the oxidation of phenylacetaldehyde by plant peroxidase resulted in the formation of benzaldehyde, Clark and Mann suggested that tryptamine might be oxidised to indoleacetaldehyde by a similar reaction.

In summary, the sporadic occurrence of both tryptamine and tryptamine enzymes in plants suggest that tryptamine is not a major intermediate in the conversion of tryptophan to IAA.

2. Biosynthesis of Gramine

a) Occurrence of Gramine

The tryptophan molecule is the precursor of several complex and interesting alkaloids such as the harmans, and the ergot and Rauwolfia alkaloids. At the present time, however, I shall discuss only the simple alkaloid 3-dimethylaminomethylindole, or gramine.

10)

Euler and Hellstrom, 1933, while studying the UV absorption characteristics of chlorophyll mutants of barley, crystallised a compound which they concluded was a methylated indole base. Euler and Erdtmann (1935) prepared derivatives of this compound, and identified it as gramine. Gramine was

found in five out of eighteen Swedish barley strains which were examined, and the gramine-containing strains were more resistant to nematode infection (Brandt, Euler, Hellström and Löfgren 1935). These workers also found that the concentration of gramine increased from the base to the tip of the leaf. During the first ten days after germination the amount of gramine present remained constant, but thereafter the percentage decreased and gramine had disappeared one month after germination. Brandt et al. concluded that the gramine of barley was identical with the 'donaxine' isolated from the Asiatic reed, Arundo donax, by Orehov, Norkima and Maximova (1935). This plant was apparently investigated because of its distastefulness to grazing camels.

Wiewiorski and Podkowinska (1962) identified gramine in Lupinus luteus, but this claim was later withdrawn (Wiewiorski, 1964).

b. Precursors of Gramine

Marion and coworkers have carried out a series of experiments on the precursors of gramine in barley (var. Charlottown). In all cases, precursors were fed to six-day old barley through the roots, and the plants were harvested on the eleventh day. These long feeding times have caused some conflicts with the results of other investigators, and this may be due to the effect of bacterial contamination on the feeding solutions used by Marion and coworkers.

Bowden and Marion (1951a) fed DL-tryptophan-3-C¹⁴ to barley seedlings and isolated labelled gramine. Systematic degradation of the gramine showed that radioactivity was present only in the carbon of the side chain. Bowden and Marion (1951b) also showed that the major site of formation of gramine was at the leaf tip, and that this was not an artefact due to transport of gramine upwards and accumulation at the tip.

Leete and Marion (1953) fed a mixture of DL-tryptophan-2-C¹⁴ and DL-tryptophan-3-C¹⁴ to barley seedlings and showed that the gramine formed was labelled only in the 2 position, and in the methylene carbon of the side chain. The ratio of the activity in the two positions was the same in the gramine as in the administered tryptophan, indicating that tryptophan is converted to gramine without cleavage of the methylene link.

Breccia and Marion (1959) showed that C¹⁴-labelled IAA, indoleglyoxylic acid, indolealdehyde and indoleacetamide were not converted to gramine. However, when indolepyruvic acid-3-C¹⁴ and indoleacrylic acid-3-C¹⁴ were fed, radioactivity was found in gramine, though greater labelling was produced by indoleacrylic acid than by indolepyruvic acid.

The work of Wightman, Chisholm and Neish (1961) conflicts with these results to some extent, possibly because the latter workers administered the labelled compounds to the shoot, and allowed metabolism to proceed for only 72 hrs.

Wightman et al. found that indoleacrylic acid was a very poor precursor of gramine; however, they agreed with Breccia and Marion in finding that IAA and indolealdehyde were also poor precursors. Indolelactic acid, tryptophan and tryptophan precursors, such as shikimic acid, anthranilic acid and serine, were good precursors of gramine. Unlike the other compounds tested, serine-3-C¹⁴ labelled the N-methyl groups of gramine, due to its ability to enter the 1-carbon pool.

O'Donovan and Leete (1963) fed a mixture of DL-tryptophan-3-C¹⁴ and DL-tryptophan-3-H³ to barley seedlings. The radioactive gramine formed was labelled solely in the methylene group of the side chain, and the ratio of C¹⁴ and H³ was the same in the gramine as in the administered tryptophan. This suggests that the methylene group of the side chain maintains its integrity during conversion to gramine, and that indolecrylic acid therefore cannot be an intermediate.

The origin of the methyl groups of gramine has been studied by Mudd (1960, a,b). Mudd first showed that enzymes from barley seedlings were capable of synthesising S-adenosyl-methionine-Me-C¹⁴. He then showed that seedlings incubated with S-adenosylmethionine-Me-C¹⁴ showed labelling in gramine, and that L-methionine, L-methionine sulfoxide and S-methyl-L-methionine did not act as methyl donors. Mudd concluded that pathways of methylation in plants were probably similar to those already established in animals.

In 1961, Mudd showed the presence of two other gramine precursors in 4-day old barley seedlings. Native 3-amino-

methylindole was demonstrated by chromatography in an isopropanol : buffer solvent, and by color reactions. When aminomethylindole was incubated with S-adenosylmethionine- ^{14}C and a barley enzyme, the presence of labelled 3-methylaminomethylindole could be demonstrated.

Thus, at the present time, known precursors of gramine include tryptophan and its precursors, indolelactic acid, possibly indolepyruvic acid, aminomethylindole and methylaminomethylindole. S-adenosyl-L-methionine is the methyl donor, and possible biosynthetic schemes must take into account the integrity of the side-chain methylene group during conversion of tryptophan to gramine.

3. Formation of Malonyl-D-tryptophan

Good and Andreae (1957) reported that indoleacetyl-aspartic acid was formed when tryptophan was fed to excised pea epicotyls. They later discovered, however, that the compound formed had slightly different R_f values from indoleacetylaspargic acid and showed that it was, in fact, a conjugate of tryptophan and malonic acid. Malonyl-tryptophan has since been shown to be present in untreated tomato, spinach, pea and oat tissues, and once formed, does not appear to be metabolised.

Zenk and Scherf (1963) isolated malonyltryptophan from Garagena and apple fruits, hydrolysed the purified compound and isolated pure tryptophan by paper chromatography. This tryptophan was oxidised to indolepyruvate by D-amino acid oxidase, but not by L-amino acid oxidase, showing that it had the D configuration. In a further study, 148 angiosperms were fed with D-tryptophan; 134 produced large amounts of malonyltryptophan while none was produced by incubation with L-tryptophan. It therefore seemed unlikely that D-tryptophan was being produced by racemisation. Naturally occurring malonyltryptophan was found in 14 genera of ferns and angiosperms.

From this work, Zenk and Scherf concluded that the conjugate was α -N-malonyl-D-tryptophan. This was the first discovery of a natural D-amino acid from a higher plant, and the first report of D-tryptophan in nature.

4. Degradation of Tryptophan by Cleavage of the Pyrrole Ring

a. Pathways in Animals and Microorganisms

Very extensive research has been carried out on tryptophan degradation in microorganisms and animals, and is only summarised briefly here. A recent review is that of Henderson, Gholson and Dalgleish (1962).

Mammals, Neurospora, and Pseudomonas cleave the pyrrole ring of tryptophan by the enzyme tryptophan pyrrolase.

The resulting formylkynurenine is then hydrolysed to kynurenine

by kynurenine formamidase.

Kynurenine may be metabolised along three main routes. Firstly, transamination yields an unstable α -keto acid, which cyclises spontaneously to 4-hydroxyquinoline-2-carboxylic acid, or kynurenic acid. This is excreted in the urine of animals, but can be metabolised by *Pseudomonas* to glutamic acid, alanine, acetic acid and CO_2 (Taniuchi and Hayaishi, 1962).

Secondly, kynurenine may be hydrolysed to anthranilic acid and alanine by the enzyme kynureninase.

Anthranilic acid is a precursor of tryptophan, and Matchett and DeWitt (1963) have demonstrated a tryptophan-anthranilic acid cycle in *Neurospora*. Anthranilic acid can also be degraded by *Pseudomonas* to Krebs cycle acids, via pyrocatechol and β -ketoadipic acid (Behrman, 1962).

Thirdly, kynurenine may be hydroxylated to 3-hydroxykynurenine. 3-Hydroxykynurenine can then be converted to 4,8-dihydroxyquinoline-2-carboxylic acid, or xanthurenic acid, by a reaction analagous to the kynurenine-kynurenic acid reaction. Xanthurenic acid is excreted in the urine of mammals.

3-Hydroxykynurenine can also be hydrolysed to 3-hydroxy-anthranilic acid, an important intermediate in the formation of NAD in Neurospora and rat liver:

b. Occurrence of Kynurenine Pathway Intermediates in Plants

In contrast to animals and microorganisms, information on the kynurenine pathway in plants is very meager. During breakdown of tryptophan by a plant peroxidase, Wiltshire (1953) demonstrated the appearance of a compound with UV absorption maxima at 270 $m\mu$ and 300 $m\mu$, and suggested that this might be 3-hydroxykynurenine. Weaver and Jackson (1963) reported the presence of kynurenic acid and xanthurenic acid in peach leaves. These compounds were identified by their R_f values in four solvents, and color reactions with the Ehrlich, sulfanilic acid and nitrite-nitric reagents. R_f values for the authentic compounds were reported for only two solvents, however, and no

color reactions were given for authentic kymurenic acid, which does not react with Ehrlich reagent.

Teas and Anderson (1951) showed the accumulation of anthranilic acid in young leaves and anthers of a corn mutant. Anthranilic acid may be either a product or a precursor of tryptophan (Matchett and DeMoss 1963, Wightman et al., 1962), and therefore the presence of anthranilic acid does not prove the existence of the kymurenine pathway.

c. Feeding Experiments with Unlabelled Precursors

Early work on the biosynthesis of nicotinic acid in plants seemed to support the hypothesis that plant and animal pathways for the formation of this compound are the same. Gustafson (1949) showed increased synthesis of nicotinic acid by leaves of broccoli, cabbage and tomato when DL-tryptophan was administered through the petiole. Galston (1949) showed that tryptophan, kynurenine and kymurenic acid were converted by etiolated pea epicotyls to a substance showing nicotinic acid activity in the Lactobacillus bioassay. In 1950, Nason found that excised corn embryos showed a significant increase in nicotinic acid when fed tryptophan. 3-Hydroxyanthranilic acid caused an even greater increase in nicotinic acid, but IAA had no effect.

d. Feeding Experiments with Labelled Precursors

More recent experiments with labelled precursors have not supported the hypothesis that tryptophan is the source

of the pyridine ring in plants. For example, pea plants did not convert tryptophan-3-C¹⁴ to trigonelline, the methyl betaine of nicotinic acid (Leete, Marion and Spenser, 1955). Similarly, Aranoff (1956) showed that 3-hydroxyanthranilic-acid-7-C¹⁴ was not a precursor of trigonelline in soybean leaves.

Dawson, Christman, D'Adamo, Solt and Wolf showed that C¹⁴-labelled nicotinic acid was converted to nicotine by excised Nicotiniana roots. However, Leete (1957) showed that Nicotiniana was unable to convert DL-tryptophan-7a-C¹⁴ to nicotine, and Grimshaw and Marion (1958) found that anthranilic acid-1-C¹⁴ was not a precursor of nicotine.

Thus, although plants may be able to utilise the preformed pyridine ring, it is likely that the normal path of biosynthesis of pyridine nucleotides is different from that of animals and microorganisms, and does not originate in tryptophan.

B. METABOLISM OF INDOLEACETIC ACID

1. Oxidative degradation

Physiological and biochemical aspects of the enzyme, indoleacetic acid oxidase, have recently been reviewed by Hare (1964). The present review will therefore be restricted to a discussion of the postulated products of the oxidase reaction. The nature of these products is still not known with certainty, but three main possibilities have been investigated, namely, the aldehyde pathway, the oxindole pathway, and the acetophenone pathway.

a. The Aldehyde Pathway

Tang and Bonner (1947) found that dialysed pea homogenates rapidly inactivated IAA. They showed that for each mole of IAA destroyed, 1 mole of oxygen was consumed and 1 mole of CO₂ produced, suggesting that IAA was oxidatively decarboxylated to indolealdehyde, possibly by the following reaction sequence.

Subsequent work has established that indolealdehyde is indeed produced during the metabolism of IAA, though in some tissues it appears only in small quantities, and is certainly not the principal reaction product. Racusen (1955) studied the oxidation of IAA by a purified IAA oxidase preparation from etiolated pea epicotyls. Indolealdehyde was

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identified as a product by its chromatographic properties, color reactions, and the absorption spectrum of its 2,4-dinitrophenylhydrazone. However, the yield of indolealdehyde was only 5 per cent, and several other reaction products were detected. Notable among these was a compound having the chromatographic and electrophoretic mobility of indoleglycolic acid.

Stutz (1957), using a highly purified IAA oxidase preparation from green lupin seedlings, found no indolealdehyde among the reaction products when IAA was provided as a substrate. Three neutral and four acidic products were found, and one acid and one neutral compound gave indole color reactions. However, when the purified lupin IAA oxidase was coupled to a cytochrome c-cytochrome oxidase system of animal origin, indolealdehyde became the main product, and could be isolated and identified by its IR absorption spectrum (Stutz 1958). Indoleglycolic acid was readily converted to indolealdehyde in presence or absence of the cytochrome system, but indoleglyoxylic acid was not metabolised, showing that it was not an intermediate in the oxidation pathway.

Pilet (1961) incubated a Lens root brei with IAA-2-C¹⁴, and found three ether-soluble radioactive products. One of these products was identified as indolealdehyde by its R_f in one solvent system, and from its positive reaction with four color reagents. In a more recent study, Wightman (1964)

fed IAA-2-C¹⁴ to excised tomato shoots, and clearly demonstrated the presence of radioactive indolealdehyde in a neutral ether extract of the tissue.

Thus there is ample evidence that IAA may be oxidised to indolealdehyde by excised shoot tissue, plant breis, and purified enzyme systems, though in no case was the reaction found to be stoichiometric.

The final end product of the indolealdehyde pathway may be indolecarboxylic acid.

Seeley, Fawcett, Wain and Wightman (1956) isolated this compound from wheat tissue which had been incubated with indoleacetonitrile, and confirmed its identity from its R_f value, color reactions, melting point and I_k spectrum. Wightman (1962) identified radioactive indolecarboxylic acid in cabbage tissue, as a product of the metabolism of DL-tryptophan-3-C¹⁴. In both these investigations, the aldehyde precursor of indolecarboxylic acid probably arose by α -oxidation of the acetonitrile, and was therefore present in relatively high concentration. Until recently, indolecarboxylic acid has not been observed in plants fed IAA, perhaps due to the small amount of indolealdehyde formed in such cases. However, Wightman (1964) has shown that radioactive indolecarboxylic acid is formed in excised tomato shoots supplied with IAA-2-C¹⁴.

b. The Oxindole Pathway

Ray and Thimann (1955, 1956) and Ray (1956) carried out an extensive spectrophotometric study of the IAA oxidase reaction in the fungus Omphalia, and concluded that degradation of IAA took place in two steps. In the first step, IAA was enzymatically converted to an unstable intermediate. This intermediate was then converted to the final product in a non-enzymatic, acid catalysed reaction. The intermediate was neutral, formed a compound with bisulfite, and was not indolealdehyde, an acetophenone or hydroxylated IAA. Although the final product was not crystallised, and was contaminated with various impurities due to side reactions of the unstable intermediate, its UV and IR spectra suggested that it was an oxindole. The following reaction pathway was suggested for the formation of this compound.

A similar scheme was suggested by MacLachlan and Waygood (1956), from experiments on the degradation of IAA by an oxidase preparation from wheat leaves.

c. The Acetophenone Pathway

In studies on the destruction of IAA by purified pea IAA oxidase, Manning and Galston (1955) found two products which gave positive color reactions with Ehrlich reagent. They considered the possibility that IAA might be degraded by cleavage of the pyrrole ring, in a reaction analagous to the formation of kynurenine from tryptophan as found in Pseudomonas.

The R_f values and UV spectra of the reaction products showed, however, that they were not indolealdehyde, o-formamidoacetophenone (I), o-aminoacetophenone (II) or 4-hydroxyquinoline (III). Manning and Galston therefore suggested that the unknown compounds might be hydroxy derivatives of acetophenone.

This idea was severely criticised by Abramovitch and Ahmed (1961) for three reasons. First, it was unlikely that the R_f values of phenolic derivatives of the acetophenones would be so similar to those of the acetophenones themselves. Secondly, if such phenols were formed, oxygen uptake should be at least 50 per cent greater than that observed, and, lastly, indolealdehyde could have been present but masked by the other compounds, which have similar R_f values.

Abramovitch and Ahmed oxidised IAA with manganiversene, and isolated the reaction products by vapor phase chromatography. The products were crystallised and identified by their melting points, mixed melting points and IR spectra. The compounds found were a small amount of o-aminoacetophenone, o-formamidoacetophenone (9-13% yield), indolealdehyde (12.3% yield) and 3-methyloxindole (25% yield). Thus, oxidation by manganiversene followed all the methods of attack which had previously been suggested. Oxidation of IAA by a wheat leaf enzyme preparation supplied by Waygood led to the formation of indolealdehyde and 3-methyloxindole, but no acetophenones were found.

Cleavage of the indole ring of IAA was also suggested by the work of Strydom and Hartmann (1960) who found that IAA-2-C¹⁴ fed to plum stem cuttings gave rise fairly rapidly to C¹⁴C₂, the peak of radioactive CO₂ evolution being 2-5 days after feeding. These authors suggested that cleavage of the indole ring was followed by degradation of the sidechain either by β -oxidation or by two successive α -oxidations.

Geronimo, Catlin and Maxie (1964) using the same system, also found a peak of C¹⁴O₂ evolution 2-5 days after feeding. After 6 hrs, over 99% of the carbon-14 was in the aromatic fraction, and only two indoles were present, IAA and an unknown compound which produced IAA on alkaline hydrolysis.

In summary, it appears that plants can oxidise IAA to indolealdehyde and 3-methyloxindole. Side reactions from unstable intermediates may cause many other minor products,

and the carbon skeleton of the acetic acid side-chain may be catabolised to CO_2 . However, the complete series of reactions has not yet been established in any plant.

2. Formation of Peptides and Esters

Andreae and Good (1955) were the first to describe the enzymatic formation of a peptide from IAA. They found that pea stem sections incubated with IAA formed large amounts of an acidic compound which gave a positive reaction with Salkowski reagent. On hydrolysis the compound yielded IAA and aspartic acid. It showed the same color reactions, R_f values, UV spectrum and biological activity as synthetic indoleacetylaspargic acid. As a result of this finding, Good, Andreae and Van Ysselstein (1956) studied the metabolism of IAA in twelve crop plants, including barley, and showed that indoleacetylaspargic acid was formed in all cases. Andreae, Robinson and Van Ysselstein (1961) showed that radioactive indoleacetylaspargic acid accumulated in excised pea root tips after incubation with IAA-1- C^{14} , IAA-2- C^{14} or IAA-7- C^{14} . All the radioactivity extractable from the tissue by ethanol was in the form of indoleacetylaspargic acid.

With regard to the natural occurrence of indoleacetylaspargic acid in higher plants. Klamt (1961 a,b) identified this peptide as a component of the tissues of several flowering plants and it has been reported to occur naturally in young tomato plants (Row, Sandford and Hitchcock, 1961).

Deverall and Daly (1964) studied the metabolism of IAA-1-C¹⁴ by normal and rust-infected first leaves of wheat. After 6 hrs, decarboxylation accounted for only 20 per cent of the IAA entering the plant while 60 per cent of the IAA was converted to four water-soluble compounds. Three of these compounds had R_f values indistinguishable from that of indoleacetylaspatic acid in isopropanol : ammonia : water, but gave lower R_f values than the authentic peptide in butanol : acetic acid : water. The fourth compound was indistinguishable from indoleacetamide in isopropanol : ammonia : water, but had widely different R_f values to the acetamide in three other solvent systems.

Zenk (1961) isolated a new compound from leaves of Colchicum neapolitanum fed with IAA. This compound yielded IAA and glucose on acid or alkaline hydrolysis, and enzymatic hydrolysis established its structure as 1-(indole-3-acetyl)- β -D-glucose. Indoleacetylglucose was found to hydrolyse during ammoniacal chromatography, yielding glucose and indoleacetamide, which suggests that the indoleacetamide identified by Good et al. (1956) as a product of IAA metabolism in several plants may have been a chromatographic artefact. By analogy with the formation of glucuronic acid conjugates in animals, Zenk suggested that indoleacetylglucose was probably formed in plants from IAA and uridine diphosphate glucose.

Farrar (1962) reported the presence of a series of

compounds ("zeanins") in immature corn kernels which gave positive DMAC and Salkowski reactions. These compounds were active in the Avena test, had low Rf values when chromatographed in butanol : water, and broke down under conditions of ammoniacal chromatography to yield IAA. Farrar suggested that these compounds may be related to indolepyruvic acid, ascorbigen or indoleacetylglucose.

In summary, previous work indicates that the major fraction of exogenously supplied IAA is converted in most plants to water-soluble compounds including indoleacetyl-aspartic acid and indoleacetylglucose. The significance of such compounds in the normal metabolism of the plant is not yet known.

III. MATERIAL AND METHODS

A. GENERAL PROCEDURES

1. Plant Material and Cultural Practices

Barley seed (Hordeum vulgare var. Atlas 57) was obtained from the University of California at Davis. The seed was soaked in water overnight, then sown in 5 inch pots in vermiculite, at a rate of 4 g (dry seed) per pot. The pots were covered with glass until the shoots emerged on the 4th day. The plants were grown in a greenhouse with natural light, plus supplementary light to give a daylength of 16 hrs, and were fertilised twice a week after emergence with a commercial 20:20:20 fertiliser, at a concentration of 3 g per liter

2. Harvesting and Feeding

The shoots were harvested 14 days after sowing, by immersing the pots in water, and excising the shoots underwater with scissors.

Immediately after cutting, the shoots were placed in 100 ml beakers containing water. The water was then replaced by the feeding solution, full details of which are given later during the description of each experiment. The beakers were placed in a growth chamber under continuous light for 24 hrs. The light intensity was about 1300 f.c., the temperature 74°F, and the relative humidity 55-65%.

When the volume of the feeding solution was small, as was the case in radioactive experiments, distilled

water was added at intervals as the solution was taken up by the plants. The total amount of solution absorbed was 80-100 ml per 100 g shoots per 24 hrs.

3. Maceration and Separation into Fractions

The shoots were macerated in cold methanol in a Waring blender. The macerate was kept in the refrigerator for 24 hrs, then filtered on a Buchner funnel and the residue washed thoroughly with acetone. The plant residue was then discarded.

The filtrate was evaporated to a small volume in a rotary evaporator at 50°C and 10-15 mm Hg pressure, then transferred to a beaker and evaporated to dryness in a stream of air. The sample was taken up in about 200 ml of distilled water and filtered through a celite pad to remove precipitated chlorophyll. The residue was washed well with distilled water and then discarded.

The filtrate and washings were adjusted to pH 7.0 with 2N NaOH, and extracted three times with 250 ml of freshly distilled ether. The extracts were combined to give the NEUTRAL ether fraction.

The residual aqueous phase was then adjusted to pH 3.0 with $2\text{NH}_2\text{SO}_4$ and re-extracted three times with 250 ml of freshly distilled ether, to give the ACID ether fraction.

The majority of the water was removed from both ether fractions by evaporating the ether rapidly in a stream of air, when the water froze out, and the ether

could be removed by decantation. The ether was then evaporated to dryness on a steam bath, and the solid residue dissolved in methanol at a concentration equivalent to 50 g tissue per ml.

The aqueous phase remaining after ether extraction was evaporated to dryness in a rotary evaporator, and the residue dissolved in 50% methanol at a concentration equivalent to 5 g tissue per ml. This was called the AQUEOUS fraction.

4. Chromatography

a. Paper chromatography

Whatman No. 1 paper and all-glass Shandon tanks were used throughout. Tanks measuring 13.5" x 7" x 14.5" were used for ascending chromatography while the tanks used for descending chromatography measured 20" x 8" x 20".

i) Solvent systems

The following solvent systems were used:

Ascending

IAW

Isopropanol : conc. ammonia : water, mixed 8:1:1, v/v/v

Time required for 10 inch run : approximately 13 hrs.

BuAW

Butanol : glacial acetic acid : water, mixed 60:15:25, v/v/v

Time required for 10 inch run : approximately 12 hrs.

IBeW

Isopropanol : benzene : water, mixed 55:30:11, v/v/v

Time required for 10 inch run : approximately 8 hrs.

8% NaCl

Sodium chloride : water, mixed 8:100, wt/v

Time required for 10 inch run : approximately 2 hrs.

Isobuff

Isopropanol : buffer, mixed 71:29, v/v

Time required for 10 inch run : approximately 11 hrs.

The buffer solution was a mixture of 97 parts 0.2M dipotassium hydrogen phosphate and 3 parts 0.1M citric acid. The papers were dipped in this buffer and dried before use.

Descending

BeAF

Benzene : glacial acetic acid : water, mixed 2:2:1 v/v/v

Time required for 10 inch run : approximately 3 hrs.

The upper, organic phase of this two-phase solvent was used to develop the chromatogram. The lower, aqueous phase was placed in the bottom of the tank to saturate the atmosphere with the stationary phase.

CTA

A mixture of carbon tetrachloride and glacial acetic acid, mixed 50:1 v/v was used as the mobile phase.

An immiscible mixture of carbon tetrachloride : glacial acetic acid : water, mixed 1:1:1 v/v/v was placed in the bottom of the tank to provide the aqueous stationary phase.

ii) Detection agents

The following detection reagents were used:

DMAC

0.05 g of p-dimethylaminocinnamaldehyde were dissolved in 10 ml 12N HCl. 90 ml of acetone were added just before use. The chromatograms were developed by dipping in the reagent and then heating at 50°C in an oven for 3 minutes. Heating was not necessary for colour development, but drove off the HCl, which otherwise quickly corroded the densitometer.

DNPH

A saturated solution of 2:4-dinitrophenylhydrazine in 2N HCl was mixed 1:1 with ethanol just before use, and applied to the chromatogram as a spray.

Ninhydrin

1 g ninhydrin and 0.1 g 8-hydroxy-quinoline were dissolved in 100 ml methanol. The chromatograms were dipped in this solution and then heated at 60°C for 30 minutes in an atmosphere saturated with methanol vapor.

Ninhydrin-acetic

9 volumes of 0.2% ninhydrin in acetone were mixed with 1 volume of glacial acetic acid. The chromatograms were dipped in this solution, heated at 110°C for 2 minutes, then examined under UV light.

b. Thin-layer chromatography

Thin layer chromatography was only used to separate indoleacetylaspartic acid and malonyltryptophan, which could not be separated by paper chromatography. A 1 mm layer of Silica Gel G (Merck) was prepared, and activated by heating to 110°C for 20 minutes. The developing solvent was chloroform : ethyl acetate : formic acid, mixed 35 : 55 : 10 v/v/v (Zenk 1964). Detection of indoles was accomplished by autoradiography or by spraying the plates with DMAC.

5. Densitometry

Chromatograms which had been developed in DMAC were frequently examined in a Photovolt Model 52C densitometer equipped with a Varicord variable response recorder. A 575 mμ filter was used. The use of the densitometer allowed precise location of the center of colored bands, and therefore more accurate calculation of R_f values.

6. Location and Measurement of Radioactivity

a. Strip counting

For rapid location of radioactive compounds on one dimensional paper chromatograms, a Nuclear Chicago C100B Actigraph, with 1620B Analytical count ratemeter, D47 gas flow counter and R1000 Rectilinear recording milliammeter was used. The operating voltage was 1350 v, and the gas pressure 5 lbs per sq inch. A 1½" strip of the chromatogram was run through the instrument at a rate of 3/4" per minute,

using a $\frac{1}{2}$ " slit width and a time constant of 10. The K value varied from 500 to 10,000 cpm depending on the amount of radioactivity present on the chromatogram.

b. Autoradiography

Autoradiography of chromatograms was used when more critical resolution was required, particularly with two-dimensional chromatograms and with thin-layer plates. 12 inch x 10 inch Kodak No-Screen Medical x-ray film was used, with an exposure time varying from one to four weeks.

c. Quantitative Measurement of Radioactivity

Only one experiment involving quantitative measurement of radioactivity was attempted. The radioactive solutions were pipetted on to ridged copper planchettes, dried, and counted using a Nuclear Chicago 183B scaling unit with end window counter.

7. Ultra-Violet Absorption Spectra

UV absorption spectra were obtained using a Bausch and Lomb 505 recording spectrophotometer. Matched silica cuvettes of 1 cm path length were used, and the solvent in all cases was water.

8. Bioassay of Chromatograms

Bioassays were carried out only to confirm the presence of indoleacetic acid in the ACID ether fraction from unfed plants. The procedure employed was that described by Fawcett,

Wain and Wightman (1960). Each chromatogram was cut transversely into 20 segments of equal size and the growth promoting activity of substances present in each segment was then determined by means of the wheat coleoptile straight growth test.

9. Sources of Authentic Compounds

a. Radioactive compounds

All radioactive compounds were labelled in the side chain, and arabic numerals have been used to indicate the position of the isotopic carbon.

The following compounds were purchased from commercial sources:

From The Radiochemical Centre, Amersham:

DL-Tryptophan-3-C¹⁴, specific activity 32.5 mc per mM.

Indoleacetic acid-2-C¹⁴, specific activity 9.2 mc per mM

From New England Nuclear:

Tryptamine-2-C¹⁴-bisuccinate, specific activity 1.3 mc per mM

The following compounds were synthesised by T. Vollrath:

DL-Indolelactic acid-3-C¹⁴, specific activity 0.06 mc per mM, prepared by the method of Gortakowski and Armstrong (1957).

3-dimethylamino-methyl-C¹⁴-indole (gramine-1-C¹⁴), specific activity 0.13 mc per mM, prepared by the method of Kuhn and Stein (1937).

b. Unlabelled Compounds

The following compounds were purchased from commercial sources:

From Calbiochem:

DL-tryptophan, D-tryptophan, L-tryptophan, kynurenine, kynurenic acid, tryptamine, tryptophol, 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, N-methyltryptamine.

From Nutritional Biochemicals Co.:

NN'-dimethyltryptamine, DL-indolelactic acid, indolealdehyde, gramine.

From Regis Chemical Co.:

Indoleacetaldehyde-bisulfite, indoleacetoneitrile.

From Fisher Scientific Co.:

Indoleacetic acid.

From Mann Research Laboratories:

Indoleacetamide.

The following compounds were synthesised;

Indoleglyoxylic acid was synthesised by T. Vollrath, using the method of Shaw, McMillan, Gudmundson and Armstrong (1957).

Indolepyruvic acid was synthesised by T. Vollrath, using the method of Bentley, Farrar, Housley, Smith and Taylor (1956).

Indoleacetylaspatic acid was synthesised by I. McGregor using the method of Good (1956).

Malonyl-DL-tryptophan was synthesised by

I. McGregor, using the method of Zenk (1962).

The following compounds were received as gifts:

From Dr. R. Heacock:

Indolecarboxylic acid, o-aminoacetophenone,

o-formamidoacetophenone, 3-methyloxindole

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Aminomethylindole.

B. NON-ENZYMATIC DESTRUCTION OF INDOLES

1. Introduction

Several indole compounds are known to be unstable, particularly to light and under acid conditions. For example, IAA is destroyed by light in the presence of dyes (Gordon 1954), and by acid (Boysen-Jensen 1936). Indolepyruvic acid is notoriously labile, particularly under alkaline conditions (Bentley et al., 1956), and free indoleacetaldehyde is unstable, and must be stored as the bisulfite complex (Larsen and Klungsøyr, 1964).

The following experiment is a brief study of the stability of several indole compounds to the conditions used in subsequent feeding experiments. No attempt was made to distinguish between impurities of the original compound and breakdown products due to exposure to light, ether fractionation and chromatography.

The compounds included in the study were tryptophan, indolelactic acid, gramine and indoleacetic acid, which were available with carbon-14 labelling, and indoleacetaldehyde and indolepyruvic acid which were known to be unstable. Tryptamine was not included because insufficient labelled material was available.

2. Materials and Methods

50 mg of each of the following compounds were used:

DL-tryptophan-3-C¹⁴ (total activity 25 μ c), DL-indolelactic acid-3-C¹⁴ (total activity 15 μ c), gramine-1-C¹⁴ (total activity 90 μ c)

indoleacetic acid-2-C¹⁴ (total activity 25 μ c) and unlabelled indolepyruvic acid. 100 mg indoleacetaldehyde bisulfite were used, and the indoleacetaldehyde was regenerated by the method of Larsen and Klungsøyr (1964).

Each indole compound was dissolved in 200 ml distilled water; the acid indoles were first dissolved in 0.5 ml. 2N NaOH and the gramine in 5 ml ethanol before dilution. The pH of each of the final solutions was adjusted to 7.0.

The solutions were then exposed to light in a growth chamber in covered beakers for 24 hrs except in the case of gramine which remained in the chamber for 72 hrs. After exposure to light, all the solutions were clear and colorless with the exception of indolepyruvic acid, which was dark brown.

Each solution was then evaporated to dryness, redissolved in 100 ml of water and extracted with ether at pH 7.0, and again at pH 3.0. The NEUTRAL and ACID ether fractions were freed of water and evaporated to dryness in the standard way, and then dissolved in 10 ml of methanol. The AQUEOUS fraction was evaporated to dryness and dissolved in 10 ml of 50% methanol.

3. Chromatography

100 μ l aliquots of each fraction were applied to the origin of a chromatogram as a 2" streak and the chromatogram was developed in IAW. In the case of indolepyruvic acid, similar chromatograms were run in BeAW and BuAW, as indolepyruvic acid is unstable in IAW. In addition, 50 per cent of the NEUTRAL ether fraction from gramine was chromatographed in BeAW as a 6" band.

Autoradiographs were prepared from the tryptophan, indolelactic acid, gramine and IAA chromatograms, and these are illustrated in Figs 1-5. Longitudinal strips were cut from the chromatogram of each fraction and developed with DMAC.

The Rf values and DMAC reactions of the compounds found are summarised in Table 1.

As seen from Figure 1, most of the tryptophan was unchanged (Bands 1,4 and 8), but small amounts of six other compounds were present. Indolelactic acid was almost completely stable (Fig. 2). Band 1 represented unchanged indolelactic acid and Band 2, which was present in small amounts in the AQUEOUS fraction, was the only contaminant. Most of the radioactivity present on the IAA chromatogram (Fig. 3) was due to unchanged IAA (Band 1). However, smaller amounts of at least three other compounds (Bands 2,3 and 4) were present in the ACID and NEUTRAL fractions. Band 2, which was wide and diffuse, may represent more than one compound. The gramine solution contained relatively large amounts of at least three other compounds (Bands 2,3 and 4) after exposure to light (Fig. 5). Indoleacetaldehyde, on the other hand, appeared to be relatively stable. A small DMAC positive band appeared at Rf 26 in the AQUEOUS fraction, but no indication of the presence of IAA was found in the ACID fraction. Indolepyruvic acid, as expected, was almost completely destroyed. Chromatography of the indolepyruvic acid extracts in BeAW revealed a small blue-green band at Rf 34, which was probably

Fig. 1. Autoradiograph of ACID (AF), NEUTRAL (NF) and AQUEOUS (AQ) fractions from a solution of DL-tryptophan-3-C¹⁴ after exposure to light. Chromatogram developed in isopropanol : ammonia : water.

Fig. 2. Autoradiograph of ACID (AF), NEUTRAL (NF) and AQUEOUS (AQ) fractions from a solution of DL-indolelactic acid-3-C¹⁴ after exposure to light. Chromatogram developed in isopropanol : ammonia : water.

Fig. 3. Autoradiograph of ACID (AF), NEUTRAL (NF) and AQUEOUS (AQ) fractions from a solution of indoleacetic acid- 2-C^{14} after exposure to light. Chromatogram developed in isopropanol : ammonia : water.

Fig. 4. Autoradiograph of ACID (AF), NEUTRAL (NF) and AQUEOUS (AQ) fractions from a solution of gramine-1-C¹⁴ after exposure to light. Chromatogram developed in isopropanol : ammonia : water.

Fig. 5. Autoradiograph of NEUTRAL fraction of gramine-1-C¹⁴ solution after exposure to light. Chromatogram developed in benzene : acetic acid : water.

Table 1. Rf values and color reaction of compounds formed after exposure of tryptophan, indolelactic acid, gramine, indoleacetic acid, indoleacetaldehyde and indolepyruvic acid to the standard feeding and extraction procedures.

Compound	Solvent	ACID		NEUTRAL		AQUEOUS	
		Rf	DMAC	Rf	DMAC	Rf	DMAC
Tryptophan	IAW	22	NR	22	NR	0	NR
		33	NR	70	NR	29	blue
		47	NR	80	NR	68	NR
Indole-lactic acid	IAW	34	blue	34	blue	36	blue
						80	NR
Indole-acetic acid	IAW	31	blue	30	NR		
		47	NR	46	NR		
		77	blue	80	NR		
		83	NR	85	NR		
Gramine	IAW			25	NR	25	NR
				60	NR	58	NR
				75	NR	74	NR
		86	violet	86	violet	86	violet
	BeAW			8	violet		
				33	NR		
				45	NR		
Indole-acetaldehyde	IAW	75	blue	77	blue	26	blue
Indole-pyruvic acid	BuAW	90	blue	90	blue	90	blue
		65	pink			60	pink
	BeAW	0	pink			0	pink
		35	green-blue	35	green-blue	34	green-blue
		62	blue	61	blue	60	blue
						87	blue

NR : No reaction

the remaining indolepyruvic acid. IAA was present as a strong blue band at Rf 62 in this solvent, and at least two other compounds were also found.

4. Conclusions

Tryptophan, indolelactic acid, indoleacetaldehyde and IAA were relatively stable to the procedures employed. Indolepyruvic acid was completely unstable, and it was concluded that as IAA was one of the principal breakdown products, it would not be profitable to test indolepyruvic acid as a precursor of IAA in a feeding experiment. Gramine was either unstable or impure. This question will have to be resolved before further metabolism experiments with gramine are undertaken.

Due to exigencies of time, no attempt was made to identify the unknown breakdown products or impurities which were encountered in this experiment. It would be valuable, however, to investigate the identity or non-identity of these compounds with some of the unknown substances found when the parent indoles were fed to barley.

IV. EXPERIMENTAL

A. NATIVE INDOLES

1. Introduction

Previous workers had shown the presence of the following native indoles in barley: IAA (Srivastava, 1962), gramine (Euler and Hellström, 1933), 3-aminomethylindole and 3-methylaminomethylindole (Mudd, 1961) and tryptophan (Wightman *et al.*, 1961).

Preliminary experiments with unfed shoots of Atlas barley showed that the following compounds could not be demonstrated: indolepyruvic acid, indolelactic acid, indoleglyoxylic acid, indoleglycolic acid, tryptophol, 5-hydroxytryptophan and 5-hydroxyindoleacetic acid. At the same time, tentative identifications of the following compounds were made: indoleacetic acid, tryptophan, malonyltryptophan, aminomethylindole, methylaminomethylindole, gramine, tryptamine, indoleacetaldehyde, indolealdehyde and indoleacetonitrile. The following experiments were designed to confirm the presence of the latter group of compounds.

2. Methods and Materials

1.247 kg of 14-day old Atlas barley shoots were harvested, macerated and fractionated into ACID ether, NEUTRAL ether and AQUEOUS fractions, as described previously.

3. Chromatography

a. ACID ETHER FRACTION

Chromatography of Crude Acid Fraction

An aliquot of the ACID fraction, equivalent to 1 kg of barley shoots, was streaked on a chromatogram at the rate of 100 g tissue per inch, and then developed in IAW. A $1\frac{1}{2}$ " strip of this chromatogram was treated with DMAC and the absorbance of the colored bands measured with a recording densitometer. The resulting densitometer trace is shown in Figure 6. Six DMAC positive compounds were present in the extract. Only Bands 1 and 3 will be discussed here as the remaining compounds were found more abundantly in the AQUEOUS fraction.

Band 1: Suspected malonyltryptophan

Figure 6 shows a compound running at Rf 22, which gave a light blue color with DMAC. It was suspected that this compound was malonyltryptophan. Although authentic malonyltryptophan has an Rf of 11 in IAW this value is frequently increased in crude extracts. Accordingly, this region of the rest of the chromatogram was eluted with water, and the eluate rechromatographed in BuAW. A strip from this chromatogram was treated with DMAC, and a blue band appeared at Rf 76 (authentic malonyltryptophan, Rf 78). The remainder of the band was eluted with water for determination of its UV absorption spectrum, which is shown in Figure 7.

Fig. 6. Densitometer scan of ACID fraction from unfed barley shoots. Chromatogram developed in isopropanol : ammonia : water, then treated with DMAC.
B = Blue band

Fig. 7. UV absorption spectra of suspected malonyl-tryptophan from unfed barley shoots (continuous line), and of authentic malonyl-tryptophan (closed circles).

The spectra of Band 1 and of authentic malonyltryptophan are clearly not identical. Further work with much larger amounts of material is required to decide whether the discrepancy is real, or whether it is due to other contaminating substances. With this reservation, Band 1 was tentatively identified as malonyltryptophan.

Band 3: Suspected Indoleacetic Acid

An aliquot of the ACID ether fraction, equivalent to 1 kg of shoot tissue, was chromatographed in BeA^W, which separates IAA from most other acid indoles, such as indolelactic acid and indoleglycolic acid. The region containing the suspected IAA was eluted and rechromatographed in IAW, together with a marker of authentic IAA. A 1½" strip of this chromatogram was treated with DMAC, and the densitometer trace subsequently obtained is shown in Figure 8. A faint blue band of suspected IAA was visible at Rf 43, while the distinct blue band of authentic IAA had an Rf of 45.

The remainder of the chromatogram was bioassayed, using the wheat coleoptile straight growth test. Figure 9 shows a region of strong growth promoting activity between Rf 40 and 45, coinciding with the position of both authentic and suspected IAA as indicated by the DMAC reaction. It was therefore concluded that IAA was present in the unfed barley tissue.

Fig. 8. Densitometer scan of chromatogram of suspected indoleacetic acid from unfed barley shoots, and authentic indoleacetic acid. Chromatogram developed in isopropanol : ammonia : water, then treated with DMAC. B = Blue band

Fig. 9. Histogram showing bioassay results from chromatogram of suspected indoleacetic acid from unfed barley shoots. Chromatogram developed in isopropanol : ammonia : water.

b. NEUTRAL ETHER FRACTION

Indolealdehyde

An aliquot of the NEUTRAL ether fraction was applied to Whatman No. 1 paper at the rate of 25 g tissue per inch, and the chromatogram was then developed in BeAW. On spraying a strip of this chromatogram with DNPH, an orange-brown band appeared at Rf 54. A marker spot of authentic indolealdehyde also gave an orange-brown spot at Rf 54.

The region of the rest of the chromatogram containing the suspected indolealdehyde was eluted, and the eluate rechromatographed in BuAW, together with a marker spot of authentic indolealdehyde. A strip of this chromatogram was sprayed with DNPH, and both suspected and authentic indolealdehyde gave an orange-brown spot at Rf 91.

The remainder of the suspected indolealdehyde was eluted with water, and its UV absorption spectrum compared with that of synthetic indolealdehyde (Fig. 10). The two spectra were very similar, and since indolealdehyde has a characteristic spectrum, due to the presence of a conjugated double bond in the side chain, this was particularly good evidence that the compound extracted from barley was indeed indolealdehyde.

Fig. 10.

Fig. 11.

Fig. 10. UV absorption spectra of suspected indolealdehyde from unfed barley shoots (continuous line), and of authentic indolealdehyde (closed circles).

Fig. 11. UV absorption spectra of suspected indoleacet-aldehyde from unfed barley shoots.
a = compound eluted from original BeAW chromatogram
b = compound having R_f value of 82 in IAW
c = compound having R_f value of 42 in IAW

Indoleacetaldehyde

A further aliquot of the NEUTRAL ether fraction was applied to paper at the rate of 25 g tissue per inch, and chromatographed in BeAW. When a strip of the chromatogram was sprayed with DNPH, a yellow band was seen at Rf 75. Authentic indoleacetaldehyde gave a yellow spot at Rf 76.

The suspected indoleacetaldehyde was eluted and rechromatographed in BuAW. Both suspected indoleacetaldehyde and authentic indoleacetaldehyde gave a yellow band at Rf 91 when developed with DNPH.

The UV spectrum of a sample of suspected indoleacetaldehyde eluted from the original BeAW chromatogram is shown in Figure 11(a). Although a peak is present at $280\text{ m}\mu$, the shoulder at $308\text{ m}\mu$ is not typical of indoleacetaldehyde.

Accordingly, the suspected indoleacetaldehyde was chromatographed in IAW. Two DNPH positive bands were found at Rf 42 and 82 : authentic indoleacetaldehyde gave a single band at Rf 82.

The UV spectra of the two compounds found after chromatography in IAW are shown in Figure 11, curves (b) and (c). The $280\text{ m}\mu$ peak in curve (a) of Figure 11 was clearly due to the compound running at Rf 42 (curve c), which cannot be indoleacetaldehyde. The compound at Rf 82 showed no clearly defined spectrum (curve b), and could not therefore be identified as indoleacetaldehyde.

Thus no indoleacetaldehyde was demonstrated in the NEUTRAL fraction.

Fig. 12. UV absorption spectra of suspected indoleacetonitrile from unfed barley shoots (continuous line), and of authentic indoleacetonitrile (closed circles).

Fig. 13. Densitometer scan of AQUEOUS fraction from unfed barley shoots. Chromatogram developed in iso-propanol : ammonia : water, then treated with DLAC.
P = Purple band
B = Blue band
V = Violet band

Indoleacetonitrile

Generally speaking when the NEUTRAL ether fraction was chromatographed in BeAW, no DMAC reaction was found at Rf 80, which is the Rf of indoleacetonitrile in this solvent.

In one experiment, however, when the NEUTRAL fraction from 1 kg of shoots was chromatographed at a rate equivalent to 100 g tissue per inch, a band was found in this region which gave a strong purple reaction with DMAC, and had a UV spectrum very similar to that of authentic indoleacetonitrile (Figure 12).

However, this compound had negligible growth promoting activity in the wheat coleoptile bioassay. Furthermore, it had an Rf of 0 in CTA, while the Rf of indoleacetonitrile in this solvent was 48. The identity of this compound is unknown, but it is clearly not indoleacetonitrile.

Compounds of the gramine group also appeared in the NEUTRAL fraction, but as they were more abundantly found in the AQUEOUS fraction, they will be discussed below along with other results from that fraction.

c. AQUEOUS FRACTION

Chromatography of Crude Aqueous Fraction

An aliquot of the AQUEOUS fraction was applied to paper at the rate of 0.5 gm tissue per inch and chromatographed in IAW. The chromatogram was treated with DMAC, and the resulting densitometer trace is shown in Figure 13. Six DMAC-positive bands, numbered 1-6, were present.

In order to identify these bands, a partially purified sample of each band was obtained as follows. An aliquot of the AQUEOUS fraction, equivalent to 5 g tissue, was streaked on each of 15 chromatograms. The chromatograms were run in IAW, and a narrow strip of each chromatogram was treated with DMAC to locate the bands. The individual bands were then eluted and purified by rechromatography in IAW.

The purified sample of each band was then divided into six parts. These were chromatographed in the following six solvents; IAW, BuAW, Isobuff, NaCl, BeAW and CTA. Strips from each chromatogram were treated with DMAC and examined in the densitometer, Rf values being obtained from the maxima of the densitometer traces. Appropriate marker spots of authentic indoles were run at the same time. The results for all six bands are presented in Table 2 and are discussed individually below.

Band 1: Suspected Tryptophan

Band 1 had Rf values identical to those of authentic tryptophan in all solvents tested, and gave the same colors as authentic tryptophan with DMAC (strong blue-purple) and ninhydrin (grey-purple).

A sample of Band 1 was purified further by chromatography first in isopropanol : ammonia : water (6:1:1), then in 8% NaCl. Its UV absorption spectrum was then determined (Fig. 14), and found to be similar to that of authentic tryptophan.

Table 2. Rf values of indole compounds from AQUEOUS fraction of unfed barley shoots, and of authentic tryptophan, aminomethylindole, tryptamine and gramine.

Compound	Rf					
	IAW	BuAW	Iso- buff	8% NaCl	BeAW	CTA
Band 1	38	47	45	58	0	-
Tryptophan	39	50	49	62	0	-
Band 2	58	46	43	34	0	0
Band 3	65	64	64	49	4	0
Aminomethyl indole	66	64	64	50	2	0
Band 4	70	62	72	49	0	0
Tryptamine	71	65	70	53	0	0
Band 5	75	64	76	59	9	0
Band 6	81	70	86	64	7	0
Gramine	80	71	86	69	5	0

Fig. 14.

Fig. 15.

Fig. 14. UV absorption spectra of suspected tryptophan from unfed barley shoots (continuous line), and of authentic tryptophan (closed circles).

Fig. 15. UV absorption spectra of suspected aminomethylindole from unfed barley shoots (continuous line), and of authentic aminomethylindole (closed circles).

Band 2

Band 2 gave a green-blue color with DMAC and was ninhydrin-negative. No satisfactory UV spectrum was obtained, and the identity of this compound is unknown.

Band 3: Suspected aminomethylindole

Band 3 showed R_f values closely similar to those of authentic aminomethylindole, and agreed with authentic aminomethylindole in giving a violet color with DMAC and a yellow color with ninhydrin.

A sample of Band 3 was purified by chromatography in isopropanol : ammonia : water (6:1:1), followed by 8% NaCl. The UV absorption spectrum of the purified compound was essentially identical with synthetic aminomethylindole, as shown in Figure 15.

Band 4

Band 4 had R_f values very close to those of Band 5 in all solvents used. However, it gave a clear blue color with DMAC, while Band 5 gave a violet reaction. The R_f values of Band 4 were identical with those of authentic tryptamine in the solvents tested.

Bands 4 and 5 could be separated by descending chromatography in 8% NaCl. The UV spectrum of Band 4, obtained in this way, is shown in Figure 17, where it can be seen that both Band 4 and tryptamine have typical indole spectra.

Fig. 16.

Fig. 17.

Fig. 18.

Fig. 16. UV absorption spectra of suspected methylamino-methylindole from unfed barley shoots (continuous line), and of authentic gramine (closed circles).

Fig. 17. UV absorption spectra of unknown tryptamine-like compound from unfed barley shoots (continuous line), and of authentic tryptamine (closed circles).

Fig. 18. UV absorption spectra of suspected gramine from unfed barley shoots (continuous line), and of authentic gramine (closed circles).

However, when Band 4 was tested with the ninhydrin-acetic reagent, which is specific for tryptamine and a narrow range of substituted tryptamines, no reaction was obtained. It must be concluded, therefore, that Band 4 is not tryptamine.

Band 4 is present in quite large amounts, and is a basic compound, being extracted into ether at pH 12, like the members of the gramine group. It is tentatively suggested that this compound may be the 'missing link' between tryptophan and aminomethylindole.

Band 5: Suspected Methylaminomethylindole

Unfortunately, no synthetic methylaminomethylindole was available for comparison with this compound. It gave a violet color with DMAC, similar to that given by aminomethylindole and gramine, and had Rf values slightly below gramine. Its Rf value in Isobuff agrees with that given by Mudd (1961) for methylaminomethylindole.

A sample of Band 5 was purified by chromatography in isopropanol : ammonia : water (6:1:1), which removed traces of Band 6, and descending chromatography in 8% NaCl, which removed Band 4. The UV absorption spectrum of the purified compound was identical with that of gramine, as shown in Figure 16.

Band 6: Suspected Gramine

Band 6 had Rf values identical with those of authentic gramine, and also gave the same color reactions with DMAC

(violet) and ninhydrin (weak yellow).

The UV absorption spectrum of a sample of Band 6 (purified by chromatography in IAW) is shown in Figure 18. It is identical with that of authentic gramine.

4. Conclusions

The evidence presented above shows that indoleacetic acid, indolealdehyde, tryptophan, aminomethylindole, methylaminomethylindole, gramine, and probably malonyl-tryptophan were normal constituents of 14-day-old barley shoots. Indoleacetonitrile, indoleacetaldehyde and tryptamine were not present, though relatively large amounts of a compound similar to tryptamine were found. This latter compound may be an intermediate in the biosynthesis of gramine.

B. METABOLISM OF TRYPTOPHAN

1. Introduction

The large number of compounds which can arise from tryptophan may be divided into five groups;

1. Compounds of the IAA pathway.
2. Hydroxyindoles.
3. Alkaloids, such as gramine.
4. Peptides.
5. Compounds of the kynurenine pathway.

The role of tryptophan as a protein precursor was not considered in the present study.

The following experiment was designed to discover whether tryptophan was a precursor, in barley, of the following representative members of these five groups;

1. Indolepyruvic acid, indolelactic acid, tryptamine, indoleacetonitrile, indoleacetaldehyde, tryptophol, indoleacetic acid, indoleacetylaspatic acid, indoleacetamide, indoleglycolic acid, indolealdehyde.
2. 5-Hydroxytryptophan
3. Aminomethylindole, methylaminomethylindole, gramine.
4. Malonyl-D-tryptophan.
5. Kynurenic acid, xanthurenic acid.

2. Materials and Methods

42.5 mg DL-tryptophan and 0.63 mg DL-tryptophan-3-C¹⁴ total activity, (200 μ c) were dissolved in 125 ml of water, and the pH of the solution was adjusted to 7.0.

425 g of 14-day old barley shoots were harvested, and divided between twenty-five 100 ml beakers. 5 ml of the radioactive tryptophan feeding solution were added to each beaker, and the plants were placed in a growth chamber under continuous light. Distilled water was added as the solution was taken up by the plants.

After 24 hrs, the plants were macerated and fractionated as described previously.

3. Chromatography

a. ACID ETHER FRACTION

Two aliquots of the ACID fraction were applied to paper at a rate equivalent to 25 g tissue per inch. The chromatograms were run in IAW and BeAW respectively, and autoradiographs of these chromatograms are shown in Figures 19 and 21. Figure 20 shows a densitometer trace of the chromatogram illustrated in Figure 19, after treatment with DMAC.

Compounds which might be expected to be present on these chromatograms are indolepyruvic acid, indolelactic acid, indoleacetic acid, indoleglycolic acid, malonyltryptophan, indoleacetylaspatic acid, kymurenic acid and xanthurenic acid. The evidence for the presence of these compounds is discussed below.

Fig. 19. Autoradiograph of ACID fraction from barley shoots fed DL-tryptophan-3- C^{14} . Chromatogram developed in isopropanol : ammonia : water.

Fig. 20. Densitometer scan of ACID fraction from barley shoots fed DL-tryptophan-3- C^{14} . Chromatogram developed in isopropanol : ammonia : water, then treated with DMAC.

P = Purple band
Pk = Pink band
B = Blue band

Fig. 21. Autoradiograph of ACID fraction from barley shoots fed DL-tryptophan-3- C^{14} . Chromatogram developed in benzene : acetic acid : water.

Fig. 22. Autoradiograph of two-dimensional chromatogram of ACID fraction from barley shoots fed DL-tryptophan- 3-C^{14} , together with authentic indoleacetic acid and indolepyruvic acid. Chromatogram developed first in butanol : acetic acid : water, then in benzene : acetic acid : water.

Fig. 23. Composite drawing of autoradiograph shown in Figure 22, and of the parent chromatogram after treatment with DMAC.

Dotted line - Radioactivity
Continuous line - Colored zones

Indolepyruvic Acid

Synthetic indolepyruvic acid was completely degraded by chromatography in IAW and therefore no indolepyruvic acid would be expected on the chromatogram shown in Figure 19, which shows the ACID fraction chromatographed in IAW.

However, indolepyruvic acid is reasonably stable in BeAW, having an R_f of 34. A faint band was present at R_f 36 when the ACID fraction was chromatographed in BeAW (Figure 21, Band 2).

An attempt was then made to identify indolepyruvic acid by two-dimensional chromatography in two acid solvents. An aliquot of the ACID fraction was applied to the origin of a two-dimensional chromatogram, together with authentic indolepyruvic acid and IAA. The chromatogram was developed first in BuAW, and then in BeAW. An autoradiograph of this chromatogram is shown in Figure 22, and Figure 23 shows a composite drawing of this autoradiograph and of the parent chromatogram after treatment with DMAC. Spot 7 probably represents the compound present as Band 2 on the chromatogram shown in Figure 21. However, the authentic indolepyruvic acid applied to the two-dimensional chromatogram could not be detected chromogenically, and therefore spot 7 could not be conclusively identified as indolepyruvic acid.

Indolelactic Acid

A fairly strong radioactive band, showing a faint blue DMAC reaction, appeared at R_f 49 after chromatography of the

ACID fraction in IAW (Figure 19, Band 7).

It was suspected that the compound forming band 7 was indolelactic acid. Accordingly, this band was eluted and rechromatographed in BeAW and BuAW. Table 3 shows that the Rf values of suspected and authentic indolelactic acid were similar in these two solvents.

Table 3. Rf values of suspected indolelactic acid from barley shoots fed tryptophan-3-C¹⁴, and of authentic indolelactic acid

Compound	Rf	
	BeAW	BuAW
Suspected indolelactic acid	2	82
Authentic indolelactic acid	10	86

The amount of indolelactic acid produced when DL-tryptophan was fed to barley shoots was small. However, a much larger amount of indolelactic acid was found when D-tryptophan was fed. As this experiment was principally designed to show the formation of malonyl-D-tryptophan, details of the feeding procedure are recorded under that heading.

Figure 24 shows densitometer scans of equivalent amounts of the ACID fractions obtained from barley shoots fed either D-tryptophan or L-tryptophan. The chromatograms were run in IAW and developed with DMAC. A prominent blue band of indolelactic acid was present at Rf 52 on the

chromatogram from the D-tryptophan fed material, while no band was visible on the chromatogram from the L-tryptophan treatment. The suspected indolelactic acid from the D-tryptophan treatment was eluted and rechromatographed in BeAW, when the suspected indolelactic acid ran at Rf 15 and authentic indolelactic acid at Rf 10.

It was concluded that D-tryptophan is fairly readily converted to indolelactic acid by barley shoots. L-tryptophan is not a precursor of indolelactic acid, and may even be inhibitory, as the amount of indolelactic acid produced by feeding DL-tryptophan was small.

Indoleacetic acid

A well-defined band of radioactivity appeared at Rf 40-45 when the ACID fraction was chromatographed in IAW (Figure 19, Band 6). This band was rechromatographed in BeAW and BuAW, with the results shown in Table 4. These Rf values clearly indicate that band 6 was not IAA.

Table 4. Rf values of suspected indoleacetic acid from barley shoots fed DL-tryptophan-3-C¹⁴, and of authentic indoleacetic acid

Compound	Rf	
	BeAW	BuAW
Suspected indoleacetic acid	0	70
Authentic indoleacetic acid	70	89

When the ACID fraction was chromatographed in BeAW, a faint band of radioactivity was observed at Rf 60 (Figure 21, Band 3). This band was eluted and rechromatographed in IAW, together with authentic IAA. A radioactive band appeared at Rf 30, coincident with the synthetic IAA.

Spot 8 on Figure 23 represents the compound present as Band 3 in Figure 21. It can be seen that this spot coincides exactly with authentic IAA which was run on the same chromatogram.

It was concluded that Band 3 in Figure 21, and Spot 8 in Figure 23 represent small amounts of radioactive IAA.

Indoleglycolic acid

A strong, narrow band of radioactivity appeared at Rf 26-29 on chromatography of the ACID fraction in IAW (Figure 19, Band 4). This band gave a rapid pink-purple color with DMAC, similar to that given by authentic indoleglycolic acid.

The suspected indoleglycolic acid was eluted and rechromatographed in BuAW, in which it had an Rf of 80, coincident with that of authentic indoleglycolic acid. No DMAC color was obtained on this chromatogram, possibly due to the small amount of the compound present.

These results are consistent with the hypothesis that Band 4 is indoleglycolic acid, but insufficient to prove this identification conclusively.

Fig. 24. Densitometer scan of ACID fractions from barley shoots fed D- and L-tryptophan. Chromatogram developed in isopropanol : ammonia : water, then treated with DMAC.
P = Purple band
Pk = Pink band
B = Blue band

Fig. 25. UV absorption spectra of suspected malonyl-tryptophan from barley shoots fed DL-tryptophan-3-C¹⁴ (continuous line), and of authentic malonyl-tryptophan (closed circles).

Malonyl-D-Tryptophan and Indoleacetylaspatic acid

When the ACID fraction was chromatographed in IAW, a highly radioactive compound giving a blue-purple DMAC reaction was present at Rf 18. (Figure 19, Band 4). This compound was also present in the AQUEOUS fraction (Figure 29, Band 1), and on the basis of its Rf values and color reaction with DMAC, it was tentatively identified as malonyl-D-tryptophan.

Suspected malonyltryptophan was eluted from the ACID fraction, and rechromatographed in BuAW. Suspected malonyltryptophan from the AQUEOUS fraction was also eluted and purified by chromatography in BuAW and IAW. The two samples of suspected malonyltryptophan were then chromatographed in BuAW, NaCl and BeAW, together with marker spots of the authentic compound. The results are presented in Table 5.

Table 5. Rf values of suspected malonyltryptophan from barley shoots fed DL-tryptophan-3-C¹⁴, and of authentic malonyltryptophan and indoleacetylaspatic acid

Compound	Rf		
	BuAW	NaCl	BeAW
Suspected malonyltryptophan from ACID fraction	73	-	-
Authentic malonyltryptophan	78	-	-
Authentic indoleacetylaspatic acid	78	-	-
Suspected malonyltryptophan from AQUEOUS fraction	85	77	0
Authentic malonyltryptophan	83	67	0

- Value not determined

Fig. 26. Autoradiograph of thin-layer chromatogram of suspected malonyltryptophan from barley shoots fed DL-tryptophan-3-C¹⁴.

1. ACID fraction
2. ACID fraction + authentic indoleacetylaspartic acid
3. ACID fraction + authentic malonyltryptophan
4. Purified suspected malonyltryptophan from ACID fraction
5. AQUEOUS fraction
6. Purified suspected malonyltryptophan from AQUEOUS fraction

A further sample of suspected malonyltryptophan from the AQUEOUS fraction was purified by chromatography in IAM and BuAW. The UV spectrum of this purified sample was very similar to that of authentic malonyltryptophan, as shown in Figure 25.

However, malonyltryptophan and indoleacetylaspartic acid cannot be separated by paper chromatography, so that the possibility remained that the suspected malonyltryptophan was in fact indoleacetylaspartic acid. Accordingly, thin-layer chromatography was employed to differentiate the two compounds. The following samples were chromatographed:

1. ACID fraction.
2. ACID fraction plus authentic indoleacetylaspartic acid.
3. ACID fraction plus authentic malonyltryptophan.
4. Purified suspected malonyltryptophan from ACID fraction.
5. AQUEOUS fraction.
6. Purified suspected malonyltryptophan from AQUEOUS fraction.

An autoradiograph of the thin-layer chromatogram obtained is shown in Figure 26. A strong radioactive spot was visible at Rf 50 in all cases (Spot A). This coincided with the Rf of authentic malonyltryptophan run on the same plate, and showed the same blue color with DMAC. In sample 3, where an aliquot of the ACID fraction and malonyltryptophan were run together, the size of Spot A was increased; this did not happen when the ACID fraction and indoleacetylaspartic acid were run together (Spot 2). It was therefore concluded that Spot A was malonyltryptophan.

Authentic indoleacetylaspartic acid ran at Rf 66 in this solvent. This was close to, but not identical with the Rf of Spot B, present in the ACID fraction. Spot B gave a pink color with DMAC, while indoleacetylaspartic acid gave a pink-purple reaction. In sample 2, where the ACID fraction and authentic indoleacetylaspartic acid were run together, the size of Spot B was not increased. It was concluded that no indoleacetylaspartic acid was present in the ACID fraction from shoots fed DL-tryptophan.

Evidence on the configuration of the malonyltryptophan was obtained from a feeding experiment with unlabelled D- and L-tryptophan. 450 g of 14-day old barley shoots were harvested, and divided among thirteen 100 ml beakers. 40 ml of D-tryptophan solution, containing 0.5 mg per ml, were added to each beaker and the plants were placed in a growth chamber under continuous light. After 24 hours, 360 ml of solution from a total of 520 ml had been absorbed by the plants. A comparable sample of shoots (450 g) was fed with a solution of L-tryptophan (0.5 mg per ml) in the same way; these plants absorbed 304 ml of solution in 24 hours. The plants were macerated and the extract fractionated as described previously.

The chromatographic results obtained from the ACID fractions from these treatments have already been presented in Figure 24. No malonyltryptophan was present in the ACID fraction from the L-tryptophan treatment. In the D-tryptophan ACID fraction, a strong, blue-purple band was present at Rf 23. The high concentration of malonyltryptophan present on the chromatogram

caused the center of the band to fade, which resulted in the apparent double peak appearing on the densitometer scan (Figure 24).

The chromatograms of the AQUEOUS fractions from the D-tryptophan and L-tryptophan feedings are shown in Figure 27. These chromatograms were also run in IAW and developed with DMAC. A strong peak of malonyltryptophan was present only in the D-tryptophan-fed material.

Thus, the evidence from paper and thin layer chromatography, DMAC color reactions and UV spectroscopy lead to the conclusion that DL-tryptophan was metabolised in barley shoots to malonyltryptophan, but not to indoleacetylaspatic acid. As malonyltryptophan was formed from D-tryptophan, but not from L-tryptophan, the configuration of the compound must have been malonyl-D-tryptophan.

Kynurenic acid

Since kynurenic acid is not an indole and does not react with DMAC, the only evidence for its presence would be that of radioactivity. In IAW, authentic kynurenic acid runs at an Rf slightly less than that of indolelactic acid. Since Band 7 on the chromatogram shown in Figure 19 was identified as indolelactic acid, it was possible that Band 6 was kynurenic acid. However the Rf of Band 6 in BuAW was 70 while that of authentic kynurenic acid was 58. It was concluded that there was no evidence for the presence of kynurenic acid in barley shoots fed DL-tryptophan.

Xanthurenic acid

Like kymurenic acid, xanthurenic acid does not react with DMAC, and therefore could be detected only by radioactivity. A faint band of radioactivity appeared at Rf 8 when the ACID fraction was run in IAW (Figure 19, Band 1). The Rf of xanthurenic acid in this solvent is 10. When Band 1 was eluted and rechromatographed in BuAW, it had an Rf of 55; the Rf of authentic xanthurenic acid in this solvent was 51.

These observations are consistent with the hypothesis that Band 1 was xanthurenic acid, but they are not sufficient to prove the nature of this compound decisively.

Unknown Compounds in the Acid Fraction

Bands 2, 4, 6, 9 and 10 in Figure 19 are unknown compounds. With the possible exception of Band 6, they are all DMAC negative. Their Rf values in IAW and BuAW are summarised below, in Table 6.

Table 6. Rf values of unknown compounds in the ACID fraction of barley shoots fed DL-tryptophan-3-C¹⁴

Compound	Rf	
	IAW	BuAW
Band 2	13	24
Band 6	43	70
Band 8	56	65
Band 9	77	85
Band 10	83	90

Fig. 27. Densitometer scan of AQUEOUS fractions from barley shoots fed D- or L-tryptophan. Chromatograms developed in isopropanol : ammonia : water, then treated with D₂O.

B = Blue band
P = Purple band
V = Violet band

Fig. 28. Autoradiograph of NEUTRAL fraction from barley shoots fed DL-tryptophan-3-Cl₄. Chromatogram developed in benzene : acetic acid : water.

b. NEUTRAL ETHER FRACTION

The NEUTRAL fraction was chromatographed in BeAW, at an application rate equivalent to 25 g tissue per inch. An autoradiograph of this chromatogram is presented in Figure 28.

Compounds which might be present in the NEUTRAL fraction include indolealdehyde, indoleacetaldehyde, tryptophol, indoleacetonitrile, indoleacetamide and the gramine group. Evidence for these compounds is discussed below, except that for the gramine group which is considered later with the results from the AQUEOUS fraction.

Indolealdehyde and Indoleacetamide

Indolealdehyde has an Rf of 40 in BeAW, which differentiates it sharply from other neutral indoles, with the exception of indoleacetamide which has an Rf of 45. In Figure 28, a diffuse band of radioactivity can be seen between Rf 35 and 45. This band was eluted and rechromatographed in BuAW, and gave a DNPH positive spot at Rf 86, identical with that of a marker spot of indolealdehyde. Indoleacetamide is DNPH negative, and has an Rf in BuAW of 83. It was concluded that radioactive indolealdehyde, but not indoleacetamide, was present in barley shoots after feeding labelled DL-tryptophan.

Indoleacetonitrile, Indoleacetaldehyde and Tryptophol

As seen in Figure 28, a weak band of radioactivity is present at Rf 80 (Band 3). This band gave no DMAC reaction. Band 3 was eluted and rechromatographed in CTA, and although

Fig. 29. Autoradiograph of AQUEOUS fraction from barley shoots fed DL-tryptophan-3-C¹⁴. Chromatogram developed in isopropanol : ammonia : water.

Fig. 30. Densitometer scan (solid line) and Actigraph strip count (dotted line) of AQUEOUS fraction from barley shoots fed DL-tryptophan-3-C¹⁴. Chromatogram developed in butanol : acetic acid : water, then treated with DMAC.
 B = Blue band
 V = Violet band

the amount of radioactivity present was small, a weak band appeared at Rf 0. This eliminated indoleacetonitrile, indoleacetaldehyde and tryptophol, which have Rf values of 48, 36 and 22 respectively in this solvent. It is concluded that the metabolism of DL-tryptophan by barley shoots does not give rise to a perceptible amount of indoleacetonitrile, indoleacetaldehyde or tryptophol.

c. AQUEOUS FRACTION

Possible components of the AQUEOUS fraction include malonyltryptophan, 5-hydroxytryptophan, tryptophan, tryptamine, gramine and gramine precursors. These compounds are discussed below, with the exception of malonyltryptophan, which has already been considered under the ACID fraction.

An aliquot of the AQUEOUS fraction was chromatographed in IAW, at an application rate equivalent to 0.5 g of tissue per inch. An autoradiograph of this chromatogram, which showed eight bands of radioactivity, is shown in Figure 29.

An aliquot of the AQUEOUS fraction equivalent to 5 g of tissue was applied to each of 15 chromatograms, and run in IAW. Narrow strips of each chromatogram were developed with DMAC to locate the bands. Bands 3,5,6,7 and 8 were cut out, eluted, and further purified by rechromatography in IAW. Each band was then chromatographed in IAW, BuAW, NaCl and BeAW, together with appropriate marker spots. The resulting Rf values are shown in Table 7.

Table 7. Rf values of radioactive indole compounds from the AQUEOUS fraction of barley shoots fed DL-tryptophan-3-C¹⁴, and of authentic tryptophan, aminomethylindole, tryptamine and gramine

Compound	Rf			
	IAW	BuAW	NaCl	BeAW
Band 3	38	51	53	0
Tryptophan	39	52	58	0
Band 5	69	64	47	5
Aminomethylindole	69	67	51	3
Band 6	72	70	49	1
Tryptamine	71	68	55	3
Band 7	74	70	58	8
Band 8	81	69	66	10
Gramine	81	66	68	8

All these compounds were present in the AQUEOUS fraction from unfed barley shoots. They were identified as follows:

Band 3: Tryptophan

Band 5: Aminomethylindole

Band 6: Unknown (Band 4 of the AQUEOUS fraction from unfed tissue)

Band 7: Methylaminomethylindole

Band 8: Gramine

The two remaining bands, 2 and 4, were not investigated further.

5-Hydroxytryptophan

5-Hydroxytryptophan has an R_f of 25 in BuA^W, which is widely different from most other indoles. To determine whether this compound was formed in the present feeding experiment, an aliquot of the AQUECUS fraction was chromatographed in BuA^W, together with a sample of authentic 5-hydroxytryptophan. As shown in Figure 30, there was no radioactive peak at R_f 25; a slight peak appeared at R_f 20, but this radioactive band showed no DMAC reaction, while authentic 5-hydroxytryptophan gave a bright blue color with DMAC. Therefore, it was concluded that 5-hydroxytryptophan was absent.

4. Discussion

Evidence for the participation of tryptophan in the biosynthesis of IAA was quite scanty, and three similar feeding experiments gave the same results. Tryptamine, indoleacetonitrile, indoleacetaldehyde, indoleacetamide and tryptophol were absent, and evidence of labelling in indolepyruvic acid and IAA was tenuous. Thus, if barley synthesises IAA from tryptophan, the only recognized pathway which seems possible is the indolepyruvic acid- indoleacetaldehyde scheme. Both these compounds are labile, and would not be expected to accumulate. There was weak evidence for the presence of labelled indoleglycolic acid, and radioactive indolealdehyde was definitely found. Indolealdehyde may arise from IAA via indoleglycolic acid, but may also be the result

of more direct oxidations, (e.g. α -oxidation of IAN). No indoleacetylaspatic acid was found. Therefore not only was little IAA formed, but there was no evidence for rapid flow of label through IAA to IAA metabolites. The yield of IAA obtained by feeding tryptophan tends to be small; for example, Srivastava and Shaw (1962) showed that the yield of labelled IAA obtained by feeding DL-tryptophan-2-C¹⁴ to mycelium of Melampsora lini was only 0.016% in 8 hrs. Galston and Hillman (1961) point out that control of plant growth hormones "obviously requires that they be constantly in short supply, i.e. that they be limiting to overall growth rate".

On the basis of the present results, one of the factors ensuring a short supply of growth hormone appears to be the rate of conversion of tryptophan to IAA.

Small amounts of indolelactic acid were formed from DL-tryptophan, and considerably larger amounts from D-tryptophan. This suggests that indolelactic acid itself has the D-configuration. If this is the case, indolelactic acid probably is not formed via indolepyruvic acid, since racemisation would take place during this reaction.

The failure to find 5-hydroxytryptophan is at variance with the results of Dannenberg and Liverman (1957), but, as already suggested, it is possible that the compound identified by these authors as 5-hydroxytryptophan was actually malonyltryptophan. On the other hand, Dannenberg and Liverman worked with watermelon fruits, and since most

identifications of 5-hydroxyindoles in plants have been made from reproductive structures, their identification may have been correct.

Tryptophan clearly acted as a good precursor of aminomethylindole, methylaminomethylindole and gramine, and also of the unknown compound (Band 6) of the AQUEOUS fraction. The heaviest labelling appeared to be in methylaminomethylindole, but, as no specific activity measurements were made, it was not possible to draw firm conclusions on the sequential relationships of these compounds.

Quantitatively, the most important metabolite of tryptophan was malonyl-D-tryptophan. The role of this compound in the normal metabolism of the plant remains obscure.

Tryptophan does not seem to be metabolised via the kynurenine pathway in barley. Although xanthurenic acid may have been present, kynurenic acid was absent, and there was no evidence for the presence of kynurenine, which reacts positively with DMAC and therefore would have been easily detected. Since the tryptophan administered was labelled in the 3-carbon of the side chain, anthranilic acid and later members of the pathway, even if present, would not have been detected.

C. METABOLISM OF INDOLELACTIC ACID

1. Introduction

The role of indolelactic acid in plants has received little attention. Wightman, Chisholm and Neish (1961) showed that indolelactic acid was a good precursor of tryptophan and gramine in barley shoots. Kaper and Veldstra (1958) showed that indolelactic acid was not converted to tryptophol by Agrobacterium tumefaciens, but Hope (1963) has suggested that this reaction may occur in tomato plants and this has been recently confirmed by Wightman (1964).

In the present experiment, answers were sought to the following questions:

1. Does indolelactic acid give rise to tryptophol, IAA and indolealdehyde?
2. Is indolelactic acid metabolised to tryptophan, gramine, and the gramine precursors (aminomethyl-indole and methylaminomethylindole)?
3. Does indolelactic acid form peptides similar to those formed from tryptophan and IAA?

2. Materials and Methods

40 mg DL-indolelactic-acid-C¹⁴ (total activity 12.4 μ c) were dissolved in 90 ml of distilled water, and the pH of the solution adjusted to 7.0. 360 g of barley shoots were harvested, and divided among eighteen 100 ml beakers. 5 ml of indolelactic acid feeding solution were added to each

Fig. 31. Autoradiograph of ACID fraction from barley shoots fed indolelactic acid-3-C¹⁴. Chromatogram developed in isopropanol : ammonia : water.

Fig. 32. Densitometer scan of ACID fraction of barley shoots fed indolelactic acid-3-C¹⁴. Chromatogram developed in isopropanol : ammonia : water, then treated with D₂O.

P = Purple band
B = Blue band
Pk = Pink band

Fig. 33. UV absorption spectra of suspected indolelactic acid from barley shoots fed indolelactic acid-3- Cl^4 (continuous line), and of authentic indolelactic acid (closed circles).

Fig. 34. Densitometer scan (solid line) and Actigraph strip count (dotted line) of chromatogram of suspected indoleacetic acid from barley shoots fed indolelactic acid-3- Cl^4 . Chromatogram developed in isopropanol : ammonia : water, then treated with $Dim\dot{A}C$.
B = Blue band

beaker, and the shoots were placed in a growth chamber under continuous light. Distilled water was added from time to time as the feeding solution was absorbed by the plants.

After 24 hrs, the plants were macerated and the methanol extract fractionated in the usual way.

3. Chromatography

a. ACID ETHER FRACTION

The ACID fraction was chromatographed in IAW, at an application rate equivalent to 50 g tissue per inch. An autoradiograph of this chromatogram is presented in Figure 31 and shows two major and seven minor bands of radioactivity. A strip of this chromatogram was treated with DMAC, and a densitometer scan of the strip (Figure 32) showed that three of the radioactive bands gave purple or blue-purple color reactions with DMAC. The possibility that these bands were indolelactic acid, IAA and a peptide of indolelactic acid was examined.

Indolelactic acid

The strong DMAC positive band of radioactivity between R_fs 50 and 60 (Figure 31, Band 7) seemed likely to be due to indolelactic acid. This band was eluted and rechromatographed in BeAW and BuAW. The R_f values of suspected and authentic ILA in these solvents were similar, as shown in Table 8.

Table 8. Rf values of suspected indolelactic acid from barley shoots fed indolelactic acid-3-C¹⁴, and of authentic indolelactic acid

Compound	Rf	
	BeAW	BuAW
Suspected indolelactic acid	7	77
Authentic indolelactic acid	8	86

Band 7 was purified by chromatography in BuAW and IAW. The UV spectrum of the purified compound was close to that of authentic indolelactic acid (Figure 33).

It was concluded that Band 7 was, in fact, unmetabolised indolelactic acid.

Indoleacetic acid

The Rf of Band 6 in IAW (Figure 31) and its color reaction with DMAC were similar to those of authentic IAA. Accordingly, this band was eluted and rechromatographed in BeAW and BuAW, with the results shown in Table 9. Clearly, Band 6 was not IAA.

Table 9. Rf values of suspected indoleacetic acid from barley shoots fed indolelactic acid-3-C¹⁴, and of authentic indoleacetic acid

Compound	Rf	
	BeAW	BuAW
Suspected indoleacetic acid	2	80
Authentic indoleacetic acid	70	89

Lack of material prevented further investigation of Band 6.

It was not identical with Band 2 of the AQUEOUS fraction; it may have been an acidic peptide of indolelactic acid.

In an effort to obtain some evidence for the presence of radioactive IAA, a second feeding experiment was performed in which 100 mg of DL-indolelactic acid-3-C¹⁴ (total activity 31 μ c) were fed to 330 g barley shoots. The ACID fraction obtained was first chromatographed in BeAW, and the region of the chromatogram from Rf 50-70 was then eluted and rechromatographed in IAW. A faint blue DMAC reaction was observed at Rf 25, coinciding with an extremely small radioactive peak (Figure 34). Thus, the evidence for the production of IAA from indolelactic acid was very slight.

Unknown compounds in the ACID fraction

Table 10 summarises the Rf values of the unknown radioactive compounds found in the ACID fraction.

Table 10. Rf values of unknown compounds found in the ACID fraction of barley shoots fed indolelactic acid-3-C¹⁴

Band	Rf		
	IAW	BuAW	BeAW
1	8	-	-
2	11	-	-
3	15	-	-
4	20	90	-
5	31	80	2
6	37	80	-
8	68	85	-
9	84	90	-

—: Value not determined

Fig. 35. Autoradiograph of NEUTRAL fraction from barley shoots fed indolelactic acid-3-C¹⁴. Chromatogram developed in benzene : acetic acid : water.

b. NEUTRAL ETHER FRACTION

The NEUTRAL fraction was chromatographed in BeAW, at a rate of application equivalent to 40 g tissue per inch. An autoradiograph of this chromatogram revealed no bands of radioactivity (Figure 35). Faint radioactivity near the origin was due to the presence of labelled gramine, and this will be discussed under the AQUEOUS fraction. Thus, there was no evidence for the formation of tryptophol, indoleacetaldehyde or indolealdehyde from indolelactic acid.

c. AQUEOUS FRACTION

The AQUEOUS fraction was chromatographed in IAW, at a rate of application equivalent to 0.2 g tissue per inch. An autoradiograph of this chromatogram is presented in Figure 36, and the corresponding densitometer trace is shown in Figure 37. Seven bands of radioactivity were present, and all of these were DMAC positive with the exception of Band 1.

Tryptophan

A strong band of radioactivity appeared at Rf 35 (Figure 36, Band 3). This compound gave a blue purple reaction with DMAC, and was tentatively identified as tryptophan.

To confirm this identification, a sample of Band 3 was purified by rechromatography in IAW, then chromatographed

Fig. 36. autoradiograph of AQUEOUS fraction from barley shoots fed indolelactic acid-3-C¹⁴. Chromatogram developed in isopropanol : ammonia : water.

Fig. 37. Densitometer scan of AQUEOUS fraction from barley shoots fed indolelactic acid-3-C¹⁴. Chromatogram developed in isopropanol : ammonia : water, then treated with DMAC.

Pk = Pink band
 B = Blue band
 V = Violet band

in IAW, BuAW, NaCl and BeAW, together with marker spots of authentic tryptophan. As seen in Table 11, the Rf values of Band 3 and tryptophan were similar.

Table 11. Rf values of suspected tryptophan from barley shoots fed indolelactic acid-3-C14 and of authentic tryptophan

Compound	Rf			
	IAW	BuAW	NaCl	BeAW
Suspected tryptophan	39	47	59	0
Authentic tryptophan	39	53	55	0

The UV spectrum of Band 3 was also very similar to that of authentic tryptophan (Figure 36). It was concluded that Band 3 was radioactive tryptophan.

Aminomethylindole, Methylaminomethylindole, and Gramine

Bands 5, 6 and 7 of Figure 36 coincided in Rf and color reaction with the compounds previously identified in extracts from unfed and tryptophan-fed shoots as aminomethylindole, methylaminomethylindole and gramine.

Partially purified samples of these three bands were obtained by re-chromatography in IAW, and the Rf value of each compound was then determined in IAW, BuAW, NaCl and BeAW (Table 12).

Table 12. Rf values of suspected aminomethylindole, methylaminomethylindole and gramine from barley shoots fed indolelactic acid-3-C¹⁴, and of authentic aminomethylindole and gramine

Compound	Rf			
	IAW	BuAW	NaCl	BeAW
Band 5	75	78	47	0
Aminomethylindole	78	72	50	0
Band 6	87	78	59	0
Band 7	90	70	66	4
Gramine	89	71	64	9

The amount of radioactivity in Band 5 was very low, and the Rf values recorded above are essentially based on the color reaction of this compound with DMAC. However, as aminomethylindole was present in unfed barley shoots, the above Rf values are not especially significant. Nonetheless since carbon 14 was definitely present in this compound on the autoradiograph (Figure 36), which is a more sensitive measurement of radioactivity than that obtained with the Actigraph scanner, it was concluded that aminomethylindole was in fact labelled by the feeding of indolelactic acid-3-C¹⁴. Thus, indolelactic acid can act as a precursor of aminomethylindole, methylaminomethylindole and gramine in barley.

Peptides

Strong radioactive bands are seen in Figure 36 at Rfs 6-13 and 22-29 (Bands 1 and 2). Band 2 gave a strong blue reaction with DMAC. A pink DMAC reaction appeared to be

Fig. 38. UV absorption spectra of suspected tryptophan from barley shoots fed indolelactic acid-3-C¹⁴ (continuous line), and of authentic tryptophan (closed circles).

Fig. 39. UV absorption spectra of Band 1 (closed circles) and Band 2 (continuous line) from AQUEOUS fraction of barley shoots fed indolelactic acid-3-C¹⁴, and of authentic kynurenine (open circles).

associated with Band 1, but as many non-indole compounds have low Rf's in the IAW solvent, it is possible that the pink color was due to some other compound.

Samples of Bands 1 and 2 were purified by rechromatography in IAW (Band 1) and IAW and BuAW (Band 2), and were then further chromatographed in IAW, BuAW, NaCl, BeAW and CTA. The results are shown in Table 13.

Table 13. Rf values of Band 1 and Band 2 from the AQUEOUS fraction of barley shoots fed indolelactic acid-3-C¹⁴

Compound	Rf				
	IAW	BuAW	NaCl	BeAW	CTA
Band 1	18	30	56	0	0
Band 2	24	47	79	0	0

Figure 39 shows the UV absorption spectra of Bands 1 and 2. Band 2 gave a typical indole spectrum, whereas Band 1 showed strong peaks at 270 $m\mu$ and 340 $m\mu$, and had a spectrum somewhat similar to that of kynurenine.

It seems probable that Band 2 is either a hydroxylated derivative or a peptide compound of indolelactic acid. Its water soluble nature and comparatively low Rf in BuAW suggest that the former possibility is more likely. Band 1 may be a compound in which cleavage of the pyrrole ring of ILA has occurred, in a reaction analagous to the formation of kynurine from tryptophan.

Indolelactic acid

A faint radioactive band can be seen at Rf 50 in Figure 36 (Band 4). This band gave a faint blue reaction with DMAC, and was identified as unmetabolised indolelactic acid which had not been completely extracted into the ACID fraction. The Rf values of Band 4 and of authentic indolelactic acid in four solvent systems are recorded in Table 14.

Table 14. Rf values of suspected indolelactic acid from the AQUEOUS fraction of barley shoots fed indolelactic acid-3-C¹⁴, and of authentic indolelactic acid

Compound	Rf			
	IAW	BuAW	NaCl	Isobuff
Suspected indoleacetic acid	47	82	62	74
Authentic indoleacetic acid	46	82	69	71

4. Discussion

The principal metabolites of indolelactic acid-3-C¹⁴ in barley shoots were labelled tryptophan and two unidentified water soluble compounds. There was only doubtful evidence of the presence of radioactive IAA, and no labelled tryptophol or indolealdehyde could be demonstrated. Such label as was present in IAA may well have arisen via tryptophan. Gramine, aminomethylindole and methylaminomethylindole were definitely labelled, probably also via tryptophan.

Although the results of earlier feeding experiments have shown that barley shoots are capable of forming indolelactic

acid from tryptophan, the results of the present experiment, coupled with the fact that no indolelactic acid was found in unfed barley, suggest that this acid does not play an important role in the normal indole metabolism of this plant.

D. METABOLISM OF TRYPTAMINE

1. Introduction

Tryptamine is a potential precursor of IAA. It may also be metabolised to indolealdehyde, either via IAA, or more directly by the action of a peroxidase (Clarke and Mann, 1957). 5-Hydroxytryptamine and 1-substituted tryptamines have also been found in plants (Waalkes *et al.*, 1958, Fish *et al.*, 1955).

The purpose of the present experiment was to discover whether tryptamine can act as a precursor of IAA, indolealdehyde, 5-hydroxytryptamine, NH^1 -dimethyltryptamine or gramine in barley shoots.

2. Materials and Methods

a. Experiment A

20 mg tryptamine-2- Cl^{14} bisuccinate (total activity 94 μc) and 40 mg unlabelled tryptamine hydrochloride were dissolved in 90 ml distilled water, and the pH of the solution adjusted to 7.0. 360 g of barley shoots were harvested and divided among eighteen 100 ml beakers, and 5 ml of tryptamine feeding solution were added to each beaker. The shoots were placed in a growth chamber under continuous light and distilled water was added as required. After 24 hrs, the shoots were macerated, and the extracts prepared for chromatography as described previously.

Fig. 40. Autoradiograph of ACID fraction from barley shoots fed tryptamine-2-C¹⁴. Chromatogram developed in isopropanol : ammonia : water.

Fig. 41. Densitometer scan of ACID fraction from barley shoots fed tryptamine-2-C¹⁴. Chromatogram developed in isopropanol : ammonia : water, then treated with DMAC.

B = Blue band
Pk = Pink band

b. Experiment B

In the second feeding experiment, 110 g of shoots were fed with 20 mg tryptamine-2-C¹⁴ bisuccinate (total activity 94 μ c), dissolved in 30 ml of water, and 220 g of shoots were fed with 46 mg unlabelled tryptamine hydrochloride dissolved in 55 ml water. The extracts made from these two treatments were combined before chromatography.

3. Chromatography

a. ACID ETHER FRACTION

Indoleacetic acid

The ACID fraction from Experiment A was chromatographed in IAW, at an application rate equivalent to 40 g of tissue per inch. Figure 40 shows that only one strong radioactive band appeared on the chromatogram (Band 3, Rf 50), and this band gave no color reaction with DLAC (Figure 41). Very faint radioactive bands were present at Rf 20, 38 and 58 (Bands 3, 4 and 6). Clearly, if IAA was present, it was present only in very small amounts.

The ACID fraction from Experiment B was chromatographed in BeAW. An Actigraph strip count and a densitometer scan of this chromatogram are shown in Figure 42. A small peak of radioactivity was present at Rf 65; the compound forming this peak was eluted and rechromatographed in IAW and CTA, giving the Rf values shown in Table 15.

Fig. 42. Densitometer scan (solid line) and Actigraph strip count (dotted line) of ACID fraction from barley shoots fed tryptamine-2- C^{14} . Chromatogram developed in benzene : acetic acid : water, then treated with DMAC.

P = Purple band
B = Blue band
Pk = Fink band

Fig. 43. Densitometer scan (solid line) and Actigraph strip count (dotted line) of NEUTRAL fraction from barley shoots fed tryptamine-2- C^{14} . Chromatogram developed in benzene : acetic acid : water, then treated with DMAC.

B = Blue band

Table 15. Rf values of suspected indoleacetic acid from barley shoots fed tryptamine-2-C¹⁴, and of authentic indoleacetic acid

Compound	Rf	
	IAW	CTA
Suspected indoleacetic acid	30	25
Authentic indoleacetic acid	34	25

Both the suspected IAA and authentic IAA gave a blue color reaction with D₂AC. The amount of suspected IAA present was too small to obtain a UV spectrum, but on the evidence of its Rf in 3 solvents and D₂AC color, it was concluded that this compound was labelled IAA.

B. NEUTRAL ETHER FRACTION

Indolealdehyde

The NEUTRAL fraction from Experiment B was chromatographed in BeAW, and an Actigraph strip count and densitometer scan of this chromatogram are shown in Figure 43. A small radioactive peak, giving a weak blue color reaction with D₂AC was present at Rf 55. This band was eluted and rechromatographed in IAW and CTA, with the results shown in Table 16.

Fig. 44. Autoradiograph of NEUTRAL fraction of barley shoots fed tryptamine-2- C^{14} . Chromatogram developed in benzene : acetic acid : water.

Fig. 45. Autoradiograph of suspected N-substituted tryptamine from NEUTRAL fraction of barley shoots fed tryptamine-2-C¹⁴. Chromatogram developed in isopropanol : ammonia : water.

Fig. 46. Densitometer scan of suspected N-substituted tryptamine from NEUTRAL fraction of barley shoots fed tryptamine-2-C¹⁴. Chromatogram developed in isopropanol : ammonia : water, then treated with DMAC.
V = Violet band

Table 16. Rf values of suspected indolealdehyde from barley shoots fed tryptamine-2-C¹⁴, and of authentic indolealdehyde.

Compound	Rf	
	IAW	CTA
Suspected indolealdehyde	85	12
Authentic indolealdehyde	86	7

The NEUTRAL fraction of Experiment A was chromatographed in the same way. The autoradiograph of this chromatogram (Figure 44) showed considerable streaking. However, a band of denser radioactivity was present at Rf 40 (Band 3). This region was eluted and re-chromatographed in BuAW, when a radioactive peak giving an orange-brown reaction with DNPH appeared at Rf 86. Authentic indolealdehyde gave a similar color reaction with DNPH at the same Rf. It was therefore concluded that labelled indolealdehyde was present in this fraction.

N-methyl- and NN'-dimethyltryptamine.

In the autoradiograph shown in Figure 44, a wide band of radioactivity is present near the origin (Band 1). The Rf values of N-methyl- and NN'-dimethyltryptamine in BeAW are 5 and 7 respectively, and so these compounds could be present in this band.

Band 1 was eluted and rechromatographed in IAW.

Figure 45 shows that most of the radioactivity appeared as a diffuse band at Rf 82-93. Authentic N-methyl- and N,N'-dimethyltryptamine have Rf values of 89 and 92 in this solvent.

The densitometer trace of this chromatogram (Figure 46) showed a violet band at Rf 86, which was undoubtedly mainly due to gramine. Reference to Figures 47 and 48 will show, however, that gramine found in the AQUEOUS fraction was not labelled; therefore the radioactivity found in Band 1 of this fraction is not due to gramine. N-methyl tryptamines give a blue rather than a violet color with DMAC, but this color could be masked by the large amount of gramine which was present. It is therefore possible that Band 3 was an N-substituted tryptamine.

c. AQUEOUS FRACTION

An aliquot of the AQUEOUS fraction from Experiment A was chromatographed in IAW at an application rate equivalent to 0.1 g tissue per inch. Two major and three minor bands of radioactivity were observed on the autoradiograph of this chromatogram (Figure 47). Figure 48 shows an Actigraph strip count and densitometer scan of a similar chromatogram from the AQUEOUS fraction of Experiment B.

Fig. 47. Autoradiograph of AQUEOUS fraction from barley shoots fed tryptamine-2-C¹⁴. Chromatogram developed in isopropanol : ammonia : water.

Fig. 48. Densitometer scan (solid line) and Actigraph strip count (dotted line) of AQUEOUS fraction from barley shoots fed tryptamine-2-C¹⁴. Chromatogram developed in isopropanol : ammonia : water, then treated with DMAC.

P = Purple band
 B = Blue band
 V = Violet band

Band 5. Tryptamine

The major band of radioactivity in the AQUEOUS fraction was Band 5, which gave a blue DMAC reaction and had Rf values identical with those of authentic tryptamine, as shown in Table 17.

Table 17. Rf values of suspected tryptamine from barley shoots fed tryptamine-2-C¹⁴, and of authentic tryptamine

Compound	Rf			
	IAW	BuAW	NaCl	BeAW
Suspected tryptamine	83	71	57	6
Suspected tryptamine	85	73	56	5

When band 5 was chromatographed in 8% NaCl, three minor radioactive bands were found at Rfs 30, 70 and 80. These corresponded with the Rf values of the breakdown products found when authentic tryptamine was chromatographed in this solvent. Band 5 was therefore identified as ^{un-}metabolised tryptamine.

Band 4. Suspected 5-hydroxytryptamine

The blue DMAC reaction given by this compound, together with its Rf value in IAW, suggested that it might be 5-hydroxytryptamine. However, its Rf values in two other solvent systems did not support this hypothesis, as shown in Table 18.

Table 18. Rf values of suspected 5-hydroxytryptamine from barley shoots fed tryptamine-2-C¹⁴, and of authentic 5-hydroxytryptamine

Compound	Rf		
	IAW	NaCl	BuAW
Suspected 5-hydroxytryptamine	72	72	46
Authentic 5-hydroxytryptamine	68	39	32

The nature of Band 4 remains unknown.

Gramine

Native gramine was present in the AQUEOUS fraction, as shown by the violet band at Rf 86 in Figure 48. However, Figure 47 shows no radioactivity at Rf values higher than that of tryptamine (Band 5), and therefore the gramine was not labelled. Thus, tryptamine was not a precursor of gramine.

4. Conclusions

Most of the tryptamine fed to barley shoots remained unmetabolised after 24 hrs. A small amount of IAA and a greater amount of indolealdehyde was formed, and there was some evidence for the presence of N-methylated tryptamine. Since the conversion of aminomethylindole to gramine by barley is so facile, it seems reasonable to suppose that the analagous methylation of tryptamine might also be possible. However, due to the high concentration of native gramine, it was impossible to study this reaction in the young plant. Gramine disappears from the plant after about six weeks, and older plants might provide

better material. However, since the enzyme methylating aminomethylindole probably also disappears in older plants, tryptamine methyltransferase might not be demonstrable even in older tissues. As some strains of barley do not produce gramine it would be interesting to know whether such strains can carry out N-methylation of tryptamine.

E. METABOLISM OF INDOLEACETALDEHYDE

1. Introduction

In most schemes for the biosynthesis of IAA, indoleacetaldehyde is a key intermediate, providing a junction between the indole-pyruvic acid and tryptamine pathways, and possibly even with the indoleacetonitrile pathways.

Indoleacetaldehyde is active in the Avena test, and can be converted to IAA by coleoptile juice, mung bean seedlings, Acetobacter and Agrobacterium. Larsen et al. (1962) showed that indoleacetaldehyde gives rise to tryptophol in approximately the same amounts as IAA and suggested that this might be a mutase reaction.

The purpose of the following experiments was to answer the following two questions:

1. Is indoleacetaldehyde metabolised to IAA by barley shoots?
2. Is indoleacetaldehyde metabolised to tryptophol by barley shoots?

2. Materials and Methods

Indoleacetaldehyde was regenerated from 0.657 g indoleacetaldehyde bisulfite by the method of Larsen and Klungsoyr (1964). The indoleacetaldehyde was dissolved in 500 ml of water, and the pH of the solution was adjusted to 7.0.

437 g 14-day old Atlas barley shoots were harvested, and divided among twenty-five 100 ml beakers. 20 ml indoleacetaldehyde solution were added to each beaker and the barley was then placed in a growth chamber in continuous light for 24 hrs.

Fig. 49. Densitometer scan of ACID fractions from barley shoots fed indoleacetaldehyde (IAAld), and from unfed barley shoots. Chromatogram developed in isopropanol : ammonia : water, then treated with D₁AC.
 P = Furple band
 B = Blue band
 BG = Blue-green band

Fig. 50. UV absorption spectra of suspected indoleacetic acid from barley shoots fed indoleacetaldehyde (continuous line), and of authentic indoleacetic acid (closed circles).

During this period, 385 ml of the feeding solution were taken up by the plants. A control group of 446 g barley shoots was fed only water.

Maceration of the shoots and preparation of the extracts for chromatography were carried out as described previously.

3. Chromatography

a. ACID ETHER FRACTION

Identification of Indoleacetic acid

The ACID fraction was applied to paper at the rate of 25 g per inch, and chromatographed in IAF. A densitometer scan of the resulting chromatogram, after treatment with DMAC, is shown in Figure 49.

An intense blue-purple band was present at R_f 50 on the chromatogram from indoleacetaldehyde-fed tissue, but not on that from the unfed tissue. This band was tentatively identified as IAA.

The band of suspected IAA was eluted and rechromatographed in BeAW, SuAW and IAF, together with markers of authentic IAA. The results obtained are presented in Table 19.

Table 19. Rf values of suspected indoleacetic acid from barley shoots fed indoleacetaldehyde, and of authentic indoleacetic acid

Compound	Rf		
	BeAW	BuAW	IAW
Suspected indoleacetic acid	75	89	36
Authentic indoleacetic acid	75	89	38

Clearly, the Rf values of the suspected and authentic IAA were identical in all three solvents.

A sample of suspected IAA was purified by chromatography in IAW and BeAW, and its UV absorption spectrum was then compared with that of authentic IAA. As seen in Figure 50, the two spectra are closely similar.

From the evidence of Rf values in three solvents, color reaction with DMAC, and UV spectrum, it is concluded that IAA was produced when indoleacetaldehyde was fed to barley shoots.

Further examination of Figure 49 reveals a strong purple band at Rf 27, which is very probably indoleacetylaspatic acid. Malonyltryptophan has the same Rf (and is present in the unfed tissue), but it is hardly likely to be formed from indoleacetaldehyde. The presence of appreciable amounts of indoleacetylaspatic acid provides further evidence that IAA has been formed.

Fig. 51. Densitometer scan of NEUTRAL fractions from barley shoots fed indoleacetaldehyde (IAAld), and from unfed barley shoots. Chromatogram developed in benzene : acetic acid : water, then treated with DEAC.

Fig. 52. UV absorption spectra of suspected tryptophol (continuous line) from barley shoots fed indoleacetaldehyde, and of authentic tryptophol (closed circles).

b. NEUTRAL ETHER FRACTION

Identification of Tryptophol

An aliquot of the NEUTRAL fraction was chromatographed in BeAW at an application rate of 2 g tissue per inch (i.e. 1/10 of the rate used in chromatography of the ACID fraction). A strip of the chromatogram was treated with DMAC, and the resulting densitometer scan is shown in Figure 51.

A very strong, blue-green band with a grey center appeared at Rf 65 on the chromatogram of the NEUTRAL fraction from indoleacetaldehyde fed tissue, but not on the chromatogram from control tissue. This color reaction with DMAC is unusual, and typical of tryptophol.

A purified sample of the suspected tryptophol was obtained by chromatographing an aliquot of the NEUTRAL fraction in distilled water. This solvent separated the suspected tryptophol from an unknown compound which gave an intense, slow pink reaction with DMAC. The purified sample was then chromatographed in BeAW, BuAW and IAW, together with authentic tryptophol markers. As seen in Table 20, suspected and authentic tryptophol had the same Rf values in the three solvent systems.

Table 20. Rf values of suspected tryptophol from barley shoots fed indoleacetaldehyde, and of authentic tryptophol

Compound	Rf		
	BeAW	BuAW	IAW
Suspected tryptophol	73	92	82
Authentic tryptophol	76	91	81

A second sample of purified suspected tryptophol was obtained by chromatographing an aliquot of the NEUTRAL fraction in 8% NaCl, which also separated the suspected tryptophol from the compound giving a pink DMAC reaction. The UV absorption spectra of this sample of suspected tryptophol and of authentic tryptophol were identical, as shown in Figure 52.

4. Conclusions

This experiment was designed to decide whether indoleacetaldehyde was metabolised to IAA and tryptophol in barley shoots. Rapid metabolism of indoleacetaldehyde took place; no indoleacetaldehyde could be detected in the plants after 24 hrs and large amounts of IAA and tryptophol were produced.

If we assume that the sensitivity of the DMAC reaction for IAA and tryptophol is approximately the same it appears at first glance that considerably more tryptophol than IAA was produced, (remembering that the acid fraction was chromatographed at a rate of application 10x that of the neutral fraction).

However, IAA is rapidly metabolised by barley shoots to indoleacetylaspartic acid and numerous DMAC negative compounds. The question of the metabolism of tryptophol will be considered in the following experiment.

F. METABOLISM OF TRYPTOPHOL

1. Introduction

As far as the author is aware, there are no reports in the literature on the metabolism of tryptophol in plants. However, Libbert and Brunn (1961) have shown that this alcohol appeared as an intermediate product during the enzymatic degradation of tryptophan by extracts of pea tissue, and Wightman (1964) has reported that radioactive tryptophol was formed in tomato when excised shoots were supplied with tryptophan-3-C¹⁴. It is also known from experiments with Acetobacter, that indoleacetaldehyde can be converted to both tryptophol and IAA (Larsen et al. 1962). Indoleacetaldehyde has been postulated in the present work to be an important intermediate in the conversion of tryptophan to IAA in barley, and since tryptophol does not accumulate in this plant, it seems that metabolism of the tryptophol must occur.

The following experiment was therefore carried out to determine whether tryptophol could act as a precursor of IAA in barley shoots.

2. Materials and Methods

64 mg of tryptophol were dissolved in 5 ml of ethanol, then diluted to 640 ml with water. The pH of the solution was adjusted to 7.0. 500 g of 14-day old barley shoots were harvested and distributed among sixteen 100 ml beakers. 40 ml of tryptophol solution were added to each beaker, and the plants were placed in a growth chamber under continuous light

Fig. 53. Densitometer scan of ACID fraction from barley shoots fed tryptophol. Chromatogram developed in isopropanol : ammonia : water, then treated with D₂O.

P = Purple band

B = Blue band

BG = Blue-green band

Fig. 54. UV absorption spectrum of unknown compound at Rf 63 on chromatogram of ACID fraction from barley shoots fed tryptophol.

for 24 hrs. 475 ml of solution were taken up by the plants during the feeding period.

Maceration and fractionation of the extract were carried out in the standard manner.

3. Chromatography

a. ACID ETHER FRACTION

Identification of Indoleacetic acid

An aliquot of the ACID fraction was chromatographed in IAW at an application rate equivalent to 25 g tissue per inch. A densitometer scan of this chromatogram, after treatment with DMAC, is shown in Figure 53. It is at once apparent that the pattern of metabolites on this chromatogram is almost identical with that obtained from the ACID fraction of shoots fed with indoleacetaldehyde (see Figure 49). In both instances, a prominent band of suspected IAA appeared.

This band (Rf 39) was eluted from the present chromatogram, and rechromatographed in BeAW, BuAW and IAW. The Rf values obtained are shown in Table 21.

Table 21. Rf values of suspected indoleacetic acid from barley shoots fed tryptophol, and of authentic indoleacetic acid

Compound	Rf		
	BeAW	BuAW	IAW
Suspected indoleacetic acid	66	86	33
Authentic indoleacetic acid	70	87	34

A further sample of the suspected IAA was purified by chromatography in IAW and BeAW, and a comparison was made of the UV spectrum of this compound with that of authentic IAA. The spectra obtained are shown in Figure 58; clearly, the two spectra are closely similar. Thus, feeding of tryptophol to barley resulted in the formation of IAA.

Figure 53 shows a strong purple band at Rf 15, which is probably indoleacetylaspartic acid. This peptide was also found in appreciable quantities after feeding indoleacetaldehyde (see Figure 49).

Investigation of Unknown Compound of Rf 63

A compound giving a strong blue-purple reaction was prominent at about Rf 60 on the chromatograms of the ACID fraction from both the indoleacetaldehyde and tryptophol feeding experiments (figures 49 and 53). It seemed possible that this compound was tryptamine, and, to test this possibility, the band was eluted and its Rf values and UV spectrum compared with those of authentic tryptamine. The results are presented in Table 22 and Figure 54.

Table 22. Rf values of unknown compound at Rf 63 from ACID fraction of tryptophol-fed barley, and of authentic tryptamine

Compound	Rf			
	BeAW	BuAW	IAW	NaCl
Unknown compound at Rf 63	0	67	70	52
Authentic tryptamine	5	75	60	62

Fig. 55. Densitometer scan of NEUTRAL fraction of barley shoots fed tryptophol. Chromatogram developed with benzene : acetic acid : water, then treated with DMAC.
B = Blue band
V = Violet band
BG = Blue-green band

Although both tryptamine and the unknown compound had similar color reactions with DMAC and typical indole spectra, their R_f values did not coincide. Furthermore, tryptamine gave a purple reaction with ninhydrin, whereas the unknown compound was ninhydrin negative. The characteristics of the unknown compound were similar to those of the unknown native compound (Band 4 of the AQUEOUS fraction). However, the latter was water-, rather than ether-soluble.

b. NEUTRAL ETHER FRACTION

Figure 55 shows the densitometer scan of the chromatogram of the NEUTRAL fraction run in BeAW and treated with DMAC. The fraction was applied to the chromatogram at a rate equivalent to 12 g tissue per inch. A strong blue-green band was present at R_f 65, indicating that a considerable amount of the tryptophol remained unmetabolised.

When a strip of the same chromatogram was sprayed with DNPH, no yellow reaction was obtained at R_f 67, indicating that no indoleacetaldehyde was present.

4. Conclusions

Evidence has been presented to show that tryptophol was converted by barley shoots to IAA and IAA metabolites, in a pattern very similar to that obtained when indoleacetaldehyde was fed to shoots. A considerable amount of tryptophol, however, remained unmetabolised after 24 hrs. This situation differed from that in the preceding experiment, when a greater amount of

indoleacetaldehyde was fed to a lesser amount of tissue, yet no indoleacetaldehyde was detected after 24 hrs. It seems, therefore, that, although both indoleacetaldehyde and tryptophol can be metabolised to IAA, the rate of conversion of indoleacetaldehyde to IAA is much greater than that of tryptophol to IAA. This fact, together with the very similar pattern of metabolites produced by the two compounds, suggests that the sequence of events may be as follows:

G. METABOLISM OF INDOLEACETONITRILE AND INDOLEACETAMIDE

1. Introduction

Thimann and Mahadevan (1958) showed that barley was active in transforming indoleacetonitrile to IAA. Wightman (1962) found that wheat coleoptile tissue also readily converted indoleacetonitrile to IAA, while indoleacetamide, which had been postulated as an intermediate in the reaction, was a poor precursor of IAA. Fawcett *et al.* (1958) showed that indoleacetonitrile could be converted to indolecarboxylic acid by α -oxidation, and that indolealdehyde was a stable intermediate.

The purpose of the present experiment was to answer the following questions:

1. Is indoleacetonitrile metabolised to IAA by barley shoots?
2. Is indoleacetamide metabolised to IAA by barley shoots?
3. If so, is the amount of IAA produced from indoleacetonitrile greater or less than that produced from indoleacetamide?
4. Is indoleacetonitrile converted to indolealdehyde by barley shoots?

2. Materials and Methods

64 mg of indoleacetonitrile were dissolved in 5 ml of ethanol. The solution was diluted to 640 ml with distilled water, and adjusted to pH 7.0. A solution of indoleacetamide containing 64 mg in 640 ml water was prepared in the same manner.

Fig. 56. Densitometer scan of ACID fraction from barley shoots fed indoleacetonitrile. Chromatogram developed in isopropanol : ammonia : water, then treated with DMAC.

B = Blue band
P = Purple band
Pk = Pink band

Fig. 57. Densitometer scan of ACID fraction from barley shoots fed indoleacetamide. Chromatogram developed in isopropanol : ammonia : water, then treated with DMAC.

B = Blue band
P = Purple band

Fig. 58. UV absorption spectra of authentic indoleacetic acid (curve a) and of suspected indoleacetic acid from barley shoots fed either tryptophol (curve b) indoleacetonitrile (curve c) or indoleacetamide (curve d).

Two batches of 500 g of 14-day old barley shoots were harvested as described previously, and each lot was distributed among sixteen 100 ml beakers. The indoleacetonitrile feeding solution was added to one group of beakers. The indoleacetonitrile feeding solution was added to one group of shoots, at 40 ml per beaker, and the indoleacetamide was added to the second group in the same way. The plants were placed in a growth chamber under continuous light for 24 hrs, during which time the indoleacetonitrile treatment absorbed 410 ml of solution, and the indoleacetamide treatment absorbed 450 ml.

Maceration of the plants and fractionation of the extracts were carried out as described previously.

3. Chromatography

a. ACID ETHER FRACTION

Identification of Indoleacetic acid

An aliquot of the ACID fraction from each treatment was applied to paper at a rate equivalent to 25 g tissue per inch, and chromatographed in IAW. Densitometer scans of these chromatograms, after treatment with D₂O, are shown in Figures 56 and 57.

A blue band of suspected IAA appeared at R_f 38 in each case. The compound forming this band was eluted from each chromatogram, and rechromatographed in BeAW, BuAW and IAW. Table 23 shows that the R_f values of the two samples of suspected IAA were identical with those of authentic IAA.

Table 23. Rf values of suspected indoleacetic acid from barley shoots fed either indoleacetonitrile or indoleacetamide, and of authentic indoleacetic acid

Compound	Rf		
	BeAW	BuAW	IAW
Suspected indoleacetic acid from barley fed indoleacetonitrile	67	88	34
Suspected indoleacetic acid from barley fed indoleacetamide	70	90	35
Authentic indoleacetic acid	70	87	34

Further samples of the suspected IAA were purified by chromatography in IAW and BeAW and their UV spectra compared with that of authentic IAA. These spectra are shown in Figure 58. The suspected IAA from the indoleacetonitrile treatment showed a spectrum similar to that of authentic IAA, but the amount of suspected IAA produced by the indoleacetamide treatment was too small for a satisfactory spectrum to be obtained.

b. NEUTRAL ETHER FRACTION

Estimation of Indolealdehyde

In this experiment the amount of indolealdehyde produced when indoleacetonitrile was fed was compared with that produced by feeding tryptophol. The feeding of indoleacetonitrile and tryptophol was carried out at the same time and on the same batch of barley, so that the two experiments were comparable. The amount of indolealdehyde produced by feeding indoleacetamide

could not be determined, since the large amount of unmetabolised indoleacetamide present in the NEUTRAL fraction interfered with the estimation. A sample of barley shoots which had been fed only water for 24 hours acted as a second control.

Indolealdehyde was estimated as follows. An aliquot of the NEUTRAL fraction equivalent to 50 g of barley shoots was applied to a 4" strip of a chromatogram. A marker spot of NEUTRAL fraction, together with 4 spots of known amounts of authentic indolealdehyde (5,10,15 and 20 μ g), were run on the same chromatogram, the solvent employed being BeAW.

The indolealdehyde was located by spraying the marker spot of NEUTRAL fraction with DNPH. The indolealdehyde region was then cut out of the main chromatogram of the NEUTRAL fraction and out of the standard spots of authentic indolealdehyde. These pieces of paper, together with a blank, were weighed and then eluted in 10 ml water for 25 minutes. The extinction of the eluates was then measured at 300 $m\mu$. A standard curve was constructed, and the amount of indolealdehyde in each sample of plant material calculated by comparison with the standard curve. The results are presented in Table 24.

Table 24. Estimation of the amount of indolealdehyde in barley shoots fed either indoleacetonitrile or tryptophol, and in unfed barley shoots

Sample	E ₃₀₀ /mg paper x 1000	Indolealdehyde/ 100 g tissue
Blank	0.4037	-
Indolealdehyde 5 μ g	0.8187	-
10 μ g	1.0890	-
15 μ g	1.4250	-
20 μ g	2.0467	-
Indoleacetonitrile- fed shoots	1.8160 (diluted x2)	72
Tryptophol-fed shoots	0.7783	11
Unfed shoots	0.8600	13

4. Conclusions

From the above evidence, it was concluded that both indoleacetonitrile and indoleacetamide were metabolised to IAA by barley shoots. Inspection of Figures 56 and 57 shows clearly that the amount of IAA produced from indoleacetonitrile was much greater than that produced from indoleacetamide. The area of the IAA peak (Rf 38), which gives a semiquantitative measure of the amount of IAA present, was 179 units in the indoleacetonitrile treatment and only 18 units in the indoleacetamide treatment, a 10-fold difference. The area of the indoleacetylaspartic acid peak (Rf 15) was also greater in the indoleacetonitrile treatment.

Furthermore, the indoleacetamide treatment showed a large band of unmetabolised indoleacetamide at Rf 72, while no indoleacetamide band appeared in the indoleacetonitrile treatment. These results confirm the work of Stowe and Thimann (1954) and Seeley *et al.* (1956), who showed that indoleacetamide is not an intermediate in the conversion of indoleacetonitrile to IAA.

Two possible pathways for the biosynthesis of indolealdehyde are known, the first involving oxidation of IAA, and the second direct α -oxidation of indoleacetonitrile. Now tryptophol was found to be an even better precursor of IAA than indoleacetonitrile (by comparing the results in Figures 53 and 56), and therefore presumably both compounds had equal opportunity to give rise to indolealdehyde by the IAA route. Yet, feeding tryptophol failed to raise the level of indolealdehyde above that present in unfed material, while indoleacetonitrile caused a seven-fold increase in this compound. Therefore we can conclude that the α -oxidation pathway probably does occur in barley. Indolecarboxylic acid is the final end product of this pathway, as shown by Fawcett *et al.* (1958), but no indolecarboxylic acid was found in the present experiment. Barley tissue is capable of metabolising indolealdehyde to indolecarboxylic acid are only produced when a high concentration of indolealdehyde is present.

H. THE TIME COURSE OF BIOSYNTHESIS OF TRYPTOPHAN AND GRAMINE IN BARLEY SEEDLINGS

1. Introduction

It has been known since the time of the discovery of gramine (Brandt et al., 1935) that this compound is found only in the seedling. The purpose of the following experiment was to trace the time course of synthesis and destruction of gramine in some detail, and to try to correlate its behaviour with that of its precursor, tryptophan.

2. Materials and Methods

a. Culture and harvest of plants

26 lots of 100 barley seeds (var. Atlas), were soaked in distilled water for 18 hours. Each lot of 100 seeds was then planted in vermiculite in a 6" pot, and grown in an airconditioned greenhouse with a diurnal temperature range of 65-75°F, and a daylength of 16 hours. The plants were fertilized twice a week with a commercial 20:20:20 fertilizer.

Samples, consisting of two pots, were taken on days 4,6,7,8,9,10,11,12,13,14,15,18, and 20, day 1 being the day of planting. Samples, consisting of 200 unsoaked seeds, and 200 seeds soaked for 18 hours were also taken. The plants from the two pots of each sample were carefully washed free of vermiculite, counted, blotted dry, and divided into roots, seed, 1st, 2nd and 3rd leaves. The seed was defined as those structures lying within the husk, and consequently, later samples of this fraction consisted mainly of the base of the

stem, Each leaf was cut off at its point of emergence from the previous leaf, and the coleoptile was included with the first leaf. One pot of plants from each sample was used for fresh weight and dry weight determinations. The second pot was used for measurement of tryptophan and gramine, and the fresh weight only was determined.

b. Preparation of extracts

The samples of tissue intended for tryptophan and gramine analysis were macerated in cold methanol for 1 min. in a Waring Blendor. The insoluble residue was filtered off on a Buchner funnel, and washed with methanol. The combined methanol extract and washings were evaporated to dryness in a stream of air, and the residue taken up in warm water and filtered through celite. The aqueous filtrate was evaporated to dryness in a rotary evaporator at 40°C, and the residue dissolved in 10 ml of 50% methanol.

c. Determination of Tryptophan

In determining the tryptophan content of each methanol extract, advantage was taken of the fact that tryptophan has an R_f of 55 in 8% NaCl, while the other free amino acids found in barley run between R_f 75 and 100.

An aliquot of the extract to be analysed, representing between 0.1 and 1.0 g fresh weight of tissue, was streaked on a 4" line on the origin of a sheet of Whatman #1 paper. The chromatogram was developed for 2 hrs in 8% NaCl. When dry,

the chromatogram was dipped in a 1% solution of ninhydrin in methanol, containing 0.1% 8-hydroxyquinoline, and heated for 30 minutes at 60°C in a methanol saturated atmosphere. The purple band corresponding to tryptophan was cut out and weighed, as was a blank from the same region of the chromatogram. Sample and blank were then placed in test-tubes, and 5 ml of a solution consisting of 1 part 95% EtOH and 1 part of 0.025M phosphate buffer at pH 7.0 was added to each. The test-tubes were capped and allowed to elute in the refrigerator for 24 hrs, after which time the absorbance of the solution was read at 570 mμ. The amount of tryptophan in the sample was then calculated by comparison with a standard curve. Beer's Law was obeyed up to a tryptophan concentration of 4 μg per ml of eluant, and the standard error of the method was 5%.

Two practical details should be noted. Firstly the amount of tryptophan was small in comparison with that of the other amino acids present, which ran at higher Rf. Consequently, when the sheets were dipped in ninhydrin, they were then hung in such a manner that the front of the chromatogram was at the bottom of the sheet. This prevented the color from the other amino acids from running onto and obscuring the tryptophan band. Secondly, after the samples had been allowed to elute in the cold, small crystals of salt often formed in the eluting medium. These were allowed to settle for a few moments before the absorbance reading was taken, so that erroneously high readings were avoided.

d. Determination of Gramine

Gramine was found only in the leaves. It was determined spectrophotometrically by the following procedure, each determination being made in duplicate.

An aliquot of each extract, representing 5-50 mg of tissue, was streaked on a 1½" line on Whatman No. 1 paper, together with a second aliquot as a marker. The chromatogram was developed for 16 hrs with an ascending solvent consisting of 70 parts isopropanol : 30 parts 0.1M K_2HPO_4 . The R_f of gramine in this solvent was 75.

With the aid of the marker strip, the area of the chromatogram containing the gramine was located, cut out, weighed, and placed in a test tube. A blank segment from each chromatogram served as a control. The samples and blanks were eluted for 2 hrs with 5 ml 0.01M phosphate buffer at pH 8.0, and the absorbance of each solution was then determined at 278 $m\mu$. The gramine content was then calculated by comparison with a standard curve, which plotted the absorbance of known amounts of gramine which had been chromatographed and eluted in the same way as the unknowns, to allow for possible losses during these procedures. Beer's Law was obeyed at concentrations below 10 μg gramine per ml of eluant, and the standard error of the determinations was 3%.

Fig. 59. Fresh weight of the entire seedling, and of the seed, root, and first, second and third leaves of barley during the first 20 days of growth.

Fig. 60. Dry weight of the entire seedling, and of the seed, root, and first, second and third leaves of barley during the first 20 days of growth.

Fig. 61. Concentration of free tryptophan in the entire seedling and in the seed, root, and first, second and third leaves of barley during the first 20 days of growth.

Fig. 62. Concentration of gramine in the entire seedling, and in the first, second and third leaves of barley during the first 20 days of growth.

3. Results

a. Fresh Weight and Dry Weight Determinations

The weight measurements (Figures 59 and 60) illustrate the classical behaviour of germinating seedlings.

The dry weight of the seed fell precipitously from the start of germination to about the 9th day, indicating rapid loss of the food reserves of the endosperm. It then declined slowly and continuously to the 20th day.

The first leaf, appearing about the 3rd day, showed a rapid linear increase in dry weight with time until the 10th day; a much slower though still linear increase occurred until the 20th day.

The second leaf showed a linear increase in both fresh and dry weight from its emergence on the 9th day to the last measurement on the 20th day. Its rate of growth was 1.0 mg dry weight per plant per day, which was much slower than that of the first leaf (1.8 mg dry weight per plant per day). However, the second leaf maintained its original growth rate for at least 11 days, while the original rapid growth of the first leaf stopped after 4 days. Consequently, on the 20th day, the dry weight of the first and second leaves was almost identical. The morphological appearance of the two leaves was quite different, the second leaf being much longer, narrower, more pointed and lighter green than the first.

Only two measurements were taken on the third leaf. It was morphologically similar to the second leaf, and appeared to have a similar initial growth rate.

The root showed a linear increase in fresh weight and dry weight from the 6th to the 20th day. The rate of fresh weight increase during this period was, however, considerably lower than that achieved between the 4th and 6th days (8 mg fresh weight per plant per day versus 40 mg fresh weight per plant per day).

The total dry weight of the plant fell rapidly until the 8th day. A slow increase occurred between the 8th and 15th days, when a linear rate of increase of 4.1 mg dry weight per plant per day was established. After the 5th day, the total fresh weight of the plant increased linearly throughout the growth period at an average rate of 25 mg fresh weight per plant per day.

b. Tryptophan Content

The results obtained for the tryptophan content of the various parts of the barley seedling during the first 20 days of growth are shown in Figure 61.

In the first leaf, free tryptophan increased rapidly until the 9th day, then fell until the 12th day. A second increase occurred with a maximum on the 13th day, followed by a decrease to the 20th day.

In the second leaf, a single maximum occurred on the 12th day, while in the root, two maxima appeared again, with peaks on the 8th and 12th days. The two measurements which were taken of the third leaf indicate a rising concentration on a per plant basis.

The seed showed a strong maximum on the 4th day, falling almost to zero on the 6th. Two subsequent peaks on the 10th and 13th days reflect the previously mentioned fact that the 'seed' by this time consists mainly of the base of the stem, enclosed within the husk.

If the tryptophan content was expressed in terms of μg per g fresh weight, similar relationships were again evident, except that the youngest sample of each tissue showed a very high concentration of tryptophan, relative to later samples.

c. Gramine Content

The changes in gramine content of barley seedlings during the early stages of growth are shown in Figure 62. Gramine was found only in shoot tissues, and was present in much higher concentrations than free tryptophan. The maximum concentration was 340 μg per plant, as compared with a maximum tryptophan concentration of 14 μg per plant. In the first leaf, there was a rapid increase in gramine content which reached a first maximum on the 9th day; this was followed by a fall and a second sharp rise to a maximum on the 18th day. The second leaf showed a small peak on the 12th day, with a second and much larger peak on the 18th day. The third leaf contained a negligible amount of gramine. As with free tryptophan, the absolute amount of gramine in the first leaf was much higher than in the second, and the youngest tissues contained the highest concentration of gramine.

4. Discussion

The results of this experiment may be interpreted as follows. From the 1st to the 4th day there was a considerable build-up of free tryptophan in the seed, presumably due to hydrolysis of endosperm proteins. From the 4th to the 6th days, the increase in tryptophan content of the first leaf was paralleled by a decrease in seed tryptophan, so that the free tryptophan of the plant as a whole remained almost stationary.

From the 6th to the 9th days, the first leaf and the roots grew rapidly at the expense of the remaining seed reserves, which were exhausted by about the 9th day. The free tryptophan and gramine content increased rapidly during this period, and some synthesis of tryptophan probably occurred in the first leaf. With the exhaustion of the seed reserves at about the 10th day, a temporary fall in tryptophan occurred in the root and first leaf. The gramine content of the first leaf also fell.

From the 10th to about the 16th day, an extremely rapid synthesis of tryptophan occurred, mainly in the first leaf. Most of this tryptophan was further metabolised to gramine, and even assuming no gramine degradation, a total of 200 μg of gramine per plant was produced during this period. On about the 18th day, gramine synthesis stopped, and this event was presumably preceded by a considerable decline in tryptophan synthesis. From this time onward, a rapid fall in gramine concentration occurred.

Mann and Mudd (1963), and Mann, Steinhart and Mudd (1963) have shown that changes in the amount of hordenine (N^N'-dimethyl-tyramine) in the barley root follow a pattern very similar to that observed for gramine in the barley shoot in the present experiment, and that the rise and fall in hordenine concentration are paralleled by changes in the enzyme tyramine methylperase, which transfers methyl groups from S-adenosylmethionine to tyrosine.

I. METABOLISM OF GRAMINE

1. Introduction

Since the catabolism of gramine had not previously been studied, the aim of the following experiment was to discover whether breakdown of gramine could be demonstrated in 14-day old shoots. It was expected that if any breakdown occurred, it would do so at a slow rate, and therefore a longer feeding period than usual was used.

2. Materials and Methods

400 g of 14-day old barley shoots were harvested and divided among twenty 100 ml beakers. 40 mg of gramine-1-C¹⁴ (total activity 73 μ c) were dissolved in 5 ml of ethanol, and the solution was diluted to 100 ml with distilled water. The pH of the solution was adjusted to 7.0, and 5 ml of the feeding solution added to each beaker. The plants were placed in a growth chamber under continuous light for 72 hrs, and distilled water was added to the beakers from time to time as required. The plants were then macerated, and the extract fractionated in the usual manner.

3. Results

Figures 63, 64 and 66 show respectively an autoradiograph of the ACID fraction chromatographed in IAW, an autoradiograph of the NEUTRAL fraction chromatographed in BeAW, and an autoradiograph of the AQUEOUS fraction chromatographed in IAW. Figure 65 shows an autoradiograph of Band 1 of the NEUTRAL

Fig. 63. Autoradiograph of the ACID fraction from barley shoots fed gramine-1-C¹⁴. Chromatogram developed in isopropanol : ammonia : water.

Fig. 64. Autoradiograph of the NEUTRAL fraction from barley shoots fed gramine-1-C¹⁴. Chromatogram developed in benzene : acetic acid : water.

Fig. 65. Autoradiograph of Band 1 shown in Figure 64, after elution and rechromatography in isopropanol : ammonia : water.

Fig. 66. Autoradiograph of the AQUEOUS fraction from barley shoots fed gramine-1-C¹⁴. Chromatogram developed in isopropanol : ammonia : water.

fraction, after elution and rechromatography in IAW.

Clearly, very little breakdown of gramine occurred. The ACID and AQUEOUS fractions contained only gramine, and by far the largest part of the radioactivity in the NEUTRAL fraction was also due to gramine (Figure 65, Band 2). Two additional compounds were also present in the NEUTRAL fraction, a faint band seen at Rf 43 in Figure 64 (Band 2), and a much stronger band at Rf 72 in Figure 65 (Band 1).

However, similar bands were found when gramine was not fed to plants, but merely exposed to light in the growth chamber for 72 hours. (see Figures 4 and 5). No firm conclusions can therefore be drawn until the following points are explored:

- a) Are the compounds found in the control experiment without plant material breakdown products or impurities?
- b) If they are breakdown products, is breakdown increased by exposing the gramine to the enzyme systems of the plant?
- c) Since the metabolism of gramine is so dependent on the developmental stage of the plant, it would be valuable to make several feedings at different time intervals after germination. There appears to be an abrupt fall in the concentration of gramine in the plant at about the third week after germination, and this would seem to be the most profitable time to explore. However, an experiment performed with three-week old plants gave results very similar to those reported above.

4. Conclusions

Two radioactive compounds, other than gramine, were found after feeding gramine-1-C¹⁴ to barley shoots, but as similar compounds were found in absence of plant material, it is not known whether the two compounds resulted from impurity of the gramine, chemical breakdown during the treatment period, or enzyme action. However, the results clearly indicate that the majority of the gramine was not metabolised.

J. METABOLISM OF INDOLEACETIC ACID

1. Quantitative Distribution of Indoleacetic acid Metabolites

a. Introduction

Earlier work on the metabolism of IAA concentrated mainly on the identification of ether soluble compounds. However, the recent work of Andreae et al., Zenk and Klämbt has shown that water-soluble compounds are quantitatively predominant among the metabolites of exogenously supplied IAA. In addition, various workers have shown that auxin can bind to cellular proteins (Wildman and Gordon 1942, Siegel and Galston 1953).

To examine the situation in barley shoots, the following experiment was carried out to assess the distribution of IAA metabolites among the ACID and NEUTRAL ether, AQUEOUS and bound fractions in quantitative terms. The experiment also served as a check on the general losses incurred during the standard fractionation procedure.

b. Materials and Methods

100 g of 14-day old barley shoots were fed with a solution containing 22.5 mg IAA-2-C¹⁴ (total activity 25 μ c), dissolved in 15 ml of water, at pH 7.0. The shoots remained in the growth chamber under continuous light for 24 hrs, during which time an additional 90 ml of distilled water were added to the plants. After 24 hrs, 38 ml of feeding solution remained, and this was retained for determination of residual radioactivity. The shoots were macerated and fractionated according to the flow sheet shown in Figure 66A.

Fig. 66A. Flow sheet of operations carried out during fractionation of barley shoots for quantitative measurement of the metabolites of indoleacetic acid-2-C¹⁴.

Table 25. Radioactivity present in various fractions after feeding indoleacetic acid-2-C¹⁴ to barley shoots.

Fraction	Total cpm	cpm	
		% Original Feeding Soln.	% Filtrate 2
Original Feeding Solution	487,500	100.0	
Residual Feeding Solution	114,500	23.4	
Hydrolysate Filtrate	15,000	3.0	
Filtrate 1.	<u>340,000</u>	<u>69.8</u>	
	469,500	96.2	
Loss during maceration and hydrolysis	18,000	3.8	
Celite Filtrate	14,500	2.9	
Filtrate 2	340,000	69.8	100.0
NEUTRAL Ether Fraction	10,000	2.1	2.9
ACID Ether Fraction	61,000	12.5	17.9
AQUEOUS Fraction	<u>237,000</u>	<u>48.6</u>	<u>69.7</u>
	308,000	63.3	90.5
Loss during ether fractionation	32,000		9.5

The radioactivity present in the following fractions was determined:

1. Original feeding solution.
2. Residual feeding solution (not taken up after 24 hrs).
3. Hydrolysate filtrate (a measure of bound radioactivity).
4. Filtrate 1 (total methanol-acetone soluble fraction).
5. Celite filtrate.
6. Filtrate 2 (Methanol-acetone soluble fraction after celite Filtration)
7. NEUTRAL ether fraction
8. ACID ether fraction
9. AQUEOUS fraction

Radioactivity was determined by plating aliquots of the fractions on to ridged copper planchettes, evaporating to dryness, and counting in a Nuclear Chicago 183B scaling unit with end window counter. Self-absorption was corrected by counting three samples of increasing volume for each fraction, plotting cpm per ml against volume plated, and extrapolating to zero volume. Self absorption was only a serious problem in the aqueous fraction because of the high concentration of both radioactivity and sugars in this fraction. The results of the experiment are presented in Table 25.

c. Discussion

As seen from the data in Table 25, about 75 per cent of the administered IAA was absorbed by the barley shoots in 24 hrs. Of the IAA which entered the plant, almost 70 per cent was in

the AQUEOUS fraction, 18 per cent in the ACID ether fraction, and only 3 per cent in the NEUTRAL ether fraction. The ether fractions may have been under-represented to some extent and the AQUEOUS fraction correspondingly over-represented, since the water which was frozen out of the ether fractions after separation was added to the AQUEOUS fraction, to prevent unknown losses. It is unlikely, however, that any serious error was incurred by this procedure.

Relatively little activity was associated with the solid residue. An amount of activity equivalent to 4 per cent of the radioactivity which entered the shoots could be released from the solid residue by alkaline hydrolysis. If we assume that the entire loss during maceration and hydrolysis was accounted for by activity bound to the solid residue and not released by hydrolysis, then a maximum of 8.9 per cent of the total radioactivity entering the plant was associated with the alcohol-acetone insoluble fraction. Although the activity in this fraction is quantitatively small, it may be qualitatively important, as it could include the hypothetical auxin-acceptor complexes which control the growth process.

Recovery of IAA metabolites was relatively quantitative, 91.7 per cent of the activity supplied to the plants being recovered. The largest loss occurred during ether fractionation, and it is possible that some volatile components were lost during evaporation of the ether fractions on the steam bath, particularly from the NEUTRAL ether fraction. The CO₂

respired by the plants was not collected, and may have contained some radioactivity, but losses from this source cannot account for more than 3.8 per cent of the total radioactivity supplied. In this connection, previous work has shown that the radioactive carbon of IAA-2-C¹⁴ fed to plum stem cuttings did not appear as C¹⁴O₂ in significant amounts until 48 hrs after treatment (Strydom and Hartman, 1960; Geronimo, et al., 1964).

In summary, the results of this experiment confirm the importance of water-soluble compounds in the metabolism of IAA. Indolealdehyde and other neutral compounds appear to be of minor importance, and not more than 3.8 per cent of the radioactivity supplied was lost as C¹⁴O₂.

2. Qualitative Nature of Indoleacetic acid Metabolites

a. Introduction

As indicated in the review of literature, the principal compounds which have been implicated as metabolites of IAA are the following:

- a) Indoleglycolic acid, indoleglyoxylic acid, indolealdehyde, indolecarboxylic acid.
- b) Indoleacetamide.
- c) o-Formamidoacetophenone, o-aminoacetophenone, 3-methyloxindole.
- d) 2-Hydroxyindoleacetic acid, 5-hydroxyindoleacetic acid.
- e) Indoleacetylaspatic acid, indoleacetylglucose, 2-hydroxyindoleacetylglucose.

The purpose of the following experiment was to discover whether any of these compounds are formed during the metabolism of

IAA by barley shoots.

b. Materials and Methods

Three metabolism experiments were performed, but as the results of all three were qualitatively identical, feeding details for only one of the experiments are given below.

65 mg unlabelled IAA and 1.52 mg IAA-2-C¹⁴ (total activity 100 μ c) were dissolved in a small volume of 2M NaOH. The solution was diluted to 85 ml with distilled water and the pH adjusted to 7.0. 690 g of 14-day old barley shoots were harvested and distributed among seventeen 100 ml beakers. 5 ml of the radioactive IAA solution were added to each beaker, and the plants were placed in a growth chamber in continuous light. After 24 hrs, the plants were macerated and the indole metabolites fractionated as described previously.

c. Chromatography

1. ACID ETHER FRACTION

The compounds which might be present in the ACID fraction were IAA, indoleglycolic acid, indoleglyoxylic acid, indolecarboxylic acid, o-formamidoacetophenone, o-aminoacetophenone and indoleacetylaspatic acid.

Two-dimensional chromatography and co-chromatography with authentic compounds were used to identify the components of the ACID fraction. Aliquots of this fraction, equivalent to 1.6 g. of plant material, were applied to the origin of two-dimensional chromatograms, which were then run in one of the two following

Fig. 67. Autoradiograph of ACID fraction from barley shoots fed indoleacetic acid-2- C^{14} . Chromatogram developed first in isopropanol : ammonia : water, then in butanol : acetic acid : water.

Fig. 68. Composite drawing of the autoradiograph described in Figure 67, and of the parent chromatogram after treatment with DLAC.
Dotted line = Radioactivity
Continuous line = Colored zones

Fig. 69. Autoradiograph of ACID fraction from barley shoots fed indoleacetic acid-2-C¹⁴, together with authentic indoleacetic acid (IAA), indoleacetylaspatic acid (IAA_{sp}), indoleglycolic acid (IGOL), indoleacetamide (IAAm) and o-aminoacetophenone (AAF). Chromatogram developed first in isopropanol : ammonia : water, then in butanol : acetic acid : water.

Fig. 70. Composite drawing of the autoradiograph described in Figure 69, and of the parent chromatogram after treatment with DMAC.
Dotted line = Radioactivity
Continuous line = Colored zones

Fig. 71. Autoradiograph of the ACID fraction from barley shoots fed indoleacetic acid-2-C¹⁴, together with authentic indoleglyoxylic acid (IGOX). Chromatogram developed first in isopropanol : ammonia : water, then in butanol : acetic acid : water.

Fig. 72. Composite drawing of the autoradiograph described in Fig. 71, and of the parent chromatogram after treatment with DNP. Dotted line = Radioactivity Continuous line = DNP color

Fig. 73. Autoradiograph of ACID fraction from barley shoots fed indoleacetic acid-2- C^{14} , together with authentic indoleacetylaspatic acid (IAA_{sp}), indoleacetic acid (IAA), indoleglycolic acid (IGOL), indolecarboxylic acid (ICA) and indoleacetamide (IAAm). Chromatogram developed first in isopropanol : ammonia : water, then in benzene : acetic acid : water.

Fig. 74. Composite drawing of the autoradiograph described in Figure 73, and of the parent chromatogram after treatment with DEAC.
Dotted line = Radioactivity
Continuous line = Colored zones

solvent combinations:

a) IAW/BuAW

First direction : IAW ascending

Second direction : BuAW ascending

b) IAW/BeAW

First direction : IAW ascending

Second direction : BeAW descending

The chromatograms were also run with appropriate authentic compounds, applied together with the aliquot of the ACID fraction.

Autoradiographs were made of the chromatograms, and the chromatograms were then treated with either the DMAC or DMPH reagent. The positions of the authentic indoles, as revealed by their color reactions, were then compared with those of the radioactive spots found on the autoradiographs.

Figure 67 shows an autoradiograph of the two-dimensional chromatogram obtained when an aliquot of the acid fraction along was developed in IAW/BuAW. Five major spots (numbered 5,6,7,8 and 9) were observed and seven faint spots (numbered 1,2,3,4,10,11 and 12) could also be distinguished. Figure 68 is a composite drawing of the autoradiograph and of the same chromatogram after treatment with DMAC. Radioactive spots are indicated by dotted lines and colored spots by continuous lines. Spots 5,7,8, and 9 were DMAC positive.

Figures 69 and 70 show the autoradiograph and colored chromatogram obtained by chromatographing the ACID fraction, together with authentic IAA, indoleacetylaspartic acid, indole-

Table 26. Rf values and color reactions of radioactive compounds found in the ACID fraction of barley shoots fed indoleacetic acid-2-C¹⁴, and of corresponding authentic indoles.

Spot number	DnAC color	Rf			
		IAW	BuAW	IBeW	BeAW
1	NR	22	27	-	-
2	NR	11	37	-	-
3	NR	12	58	-	-
4	NR	26	63	-	-
5	Blue-purple	8	74	0	0
Indoleacetyl- aspartic acid	Blue-purple	10	77	10	2
6	NR	42	75	45	0
Indoleglyoxylic acid	Slow weak blue	48	87	73	14
7	Pink-purple	15	87	20	0
Indoleglycolic acid	Pink-purple	33	93	8	3
8A	Blue	36	84	87	59
Indoleacetic acid	Blue	40	84	90	62
8B	Slow yellow	40	80	87	40
Indolecarboxylic acid	Slow blue green	30	90	96	57
9	Blue	50	83	22	12
Indoleacetamide	Blue	75	83	83	45
10	NR	62	92	-	15
11	NR	62	90	61	33
12	NR	83	87	89	-
o-Aminoaceto- phenone	Brown-pink	98	90	96	92

NR; No reaction

-; Value not determined

glycolic acid, indoleacetamide and o-aminoacetophenone, in IAW/BuAW. Figure 71 shows an autoradiograph of the ACID fraction, together with authentic indoleglyoxylic acid, developed in IAW/BuAW. The colored spots obtained after this chromatogram was sprayed with DMF reagent are shown in Figure 72.

Figure 73 shows an autoradiograph of the ACID fraction, developed with authentic IAA, indoleacetylaspatic acid, indoleglycolic acid, indoleacetamide and o-aminoacetophenone, in the second combination of solvents, IAW/BuAW. Most of the radioactive spots found in Figure 69 were again observed, and an additional major spot, 6B appeared. Figure 74 is a composite drawing of this autoradiograph, and of the same chromatogram after treatment with DMAC.

Table 26 gives the Rf values and color reactions of the thirteen radioactive spots found in the ACID fraction. In the case of spots 5,7,8A,9 and 11, these Rf values were obtained by preparing partially purified samples of the compounds by one dimensional chromatography, then running each compound in each of the four solvent systems separately. The Rf values of the remaining spots, and the Rf value for spot 9 in BeAW, were obtained from the autoradiographs of the two-dimensional chromatograms.

Further work to identify the compounds referred to as spots 5,6,7,8A,9,11 and 12 is discussed below. Spots 1, 2,3,4, and 10 are unknown compounds present in trace amounts, and will not be discussed further.

Spot 5. Suspected Indoleacetylaspartic acid

This compound showed a strong blue-purple DMAC reaction when the ACID fraction was chromatographed alone (Fig. 68). When this fraction was co-chromatographed with authentic indoleacetylaspartic acid, the size of spot 5 increased, and the boundaries of the radioactive spot coincided with those of authentic indoleacetylaspartic acid in both pairs of solvent systems (Figs. 70 and 74). The R_f values of spot 5 also agreed with those of authentic indoleacetylaspartic acid in four one-dimensional solvent systems, as shown in Table 26.

Spot 5 also appeared in the AQUEOUS fraction (Fig. 81), and its UV spectrum and hydrolysis products are discussed under that heading.

It was concluded that spot 5 was indoleacetylaspartic acid.

Spot 7. Suspected Indoleglycolic acid

This spot gave a rapid pink-purple DMAC reaction, very similar to that of authentic indoleglycolic acid. However, it can be seen from Figs. 70 and 74 that neither spot 7 nor any other radioactive spot co-chromatographed with indoleglycolic acid. It was concluded that spot 7 was not indoleglycolic acid, and that indoleglycolic acid was not, in fact, among the metabolites of IAA in barley shoots.

Fig. 75. UV absorption spectra of suspected indoleacetic acid from barley shoots fed indoleacetic acid-2-C¹⁴ (continuous line), and of authentic indoleacetic acid (closed circles).

Spot 8A. Suspected Indoleacetic acid

Spot 8A gave a blue reaction with DMAC, and co-chromatographed with authentic IAA in IAW/BuAW and IAW/BeAW (Figs. 70 and 74). Table 26 shows that the R_f values of this compound in four one-dimensional solvent systems were identical to those of authentic IAA, and as seen in Fig. 75, the UV spectra of suspected and authentic IAA were also identical. Spot 8A, therefore, represented unmetabolised IAA.

Spot 8B. Suspected 2-Hydroxyindoleacetic acid or Indolecarboxylic acid

Spot 8B was not distinguishable from IAA in IAW/BuAW. It separated slightly from IAA in IAW/BeAW, and showed an unusual slow, yellow-green DMAC reaction, which turned to green when a high concentration of the compound was present and did not co-chromatograph with spot 8B (Figure 74). No authentic 2-hydroxyindoleacetic acid was available for comparison, but the R_f values of spot 8B are fairly similar to those quoted by Klämbt (1959) for this compound.

Spot 8B was therefore not indolecarboxylic acid, but may have been 2-hydroxyindoleacetic acid. No indolecarboxylic acid was detected in the ACID fraction.

Spot 6. Suspected Indoleglyoxylic acid

This radioactive spot gave no color reaction when treated with DMAC. Authentic indoleglyoxylic acid did not co-chromatograph with spot 6, nor with any other radioactive spot present on the

chromatogram developed in IAW/BuAW. The glyoxylic acid did co-chromatograph with spot 7 in IAW/BeAW, illustrating the importance of using more than one pair of solvents for co-chromatographic analysis.

Thus, spot 6 was not indoleglyoxylic acid, and no indoleglyoxylic acid was detected.

Spots 9 and 11. Suspected Indoleacetamide

Spot 9 gave a blue reaction with DNAC, but did not co-chromatograph with indoleacetamide in either solvent pair. Spot 11 gave no color reaction with DNAC, but appeared within the boundaries of the authentic indoleacetamide spot in both pairs of solvent systems. However, co-chromatography with indoleacetamide did not increase the size of spot 11, as would be expected if spot 11 were indoleacetamide. Therefore, it was concluded that neither spot 9 nor spot 11 were indoleacetamide.

Spot 12.

Spot 12 was present in very small amounts, gave no color reaction with DNAC, and co-chromatographed with o-aminoacetophenone in IAW/BuAW. However, this spot was not observed on the chromatogram developed in IAW/BeAW, so that its identity remains uncertain.

Conclusions

Of the thirteen radioactive compounds found in the ACID fraction, two of the major spots were identified as indoleacetylaspartic acid and unmetabolised IAA. A third major

spot may have been 2-hydroxyindoleacetic acid, and four major and seven minor spots remain unidentified.

No evidence was found for the presence of indoleglycolic acid, indoleglyoxylic acid, indolecarboxylic acid or indoleacetamide, and there was only weak evidence for the presence of trace amounts of o-aminoacetophenone.

Table 25 shows that the acid fraction contained about 18 per cent of the carbon-14 which entered the plant. Inspection of Figure 67 suggests that about half the radioactivity present in this fraction (i.e. 9 per cent of the radioactivity in the plant) is found as indoleacetylaspatic acid. Spot 6 would account for about 4 per cent and Spot 8 (unmetabolised IAA unknown 8B) for about 3 per cent of the radioactivity entering the plant. Thus it is clear that the amount of free IAA in the treated shoots after 24 hrs was only a small fraction of that which was absorbed during the feeding period.

2. NEUTRAL ETHER FRACTION

The principal neutral metabolites of IAA are indolealdehyde and 3-methoxyindole. Indolecarboxylic acid also appears preferentially in the neutral fraction, although it has a lower pK value than IAA (3.7 vs 4.7, Pilet, 1961).

An aliquot of the NEUTRAL fraction equivalent to 0.4 g of tissue was chromatographed in the two-dimensional pair of solvents IAW/SeAW, together with authentic IAA, indolecarboxylic acid and indoleacetamide. Figure 76 shows that six major spots (numbered 8,16,16A,20,23 and 24) and six lesser spots

Fig. 76. Autoradiograph of NEUTRAL fraction from barley shoots fed indoleacetic acid-2-C¹⁴, together with authentic indoleacetic acid, indolecarboxylic acid and indoleacetamide. Chromatogram developed first in isopropanol : ammonia : water, then in benzene : acetic acid : water.

Fig. 77. Composite drawing of the autoradiograph described in Figure 76, and of the parent chromatogram after treatment with DMAC.
Dotted line = Radioactivity
Continuous line = Colored zones.

Table 27. Rf values and color reactions of radioactive compounds found in the NEUTRAL fraction of barley shoots fed indoleacetic acid-2-C¹⁴, and of corresponding authentic indoles.

Spot number	Color reaction			Rf	
	DMAC	DMPH	BeAT	IAW	SuAW
8 Indoleacetic acid	Blue	NR	57	39	88
	Blue	NR	62	40	84
16A	Brown-pink	NR	0	70	-
16 Indolecarboxylic acid	NR	NR	50	37	-
	Slow blue-green	NR	50	37	90
17	NR	NR	20	80	-
18	NR	NR	25	70	-
19	NR	NR	47	60	-
20 Indolealdehyde	Slow weak blue	Orange	42	78	92
	Slow weak blue	Orange	44	82	92
21	NR	NR	57	74	-
22	NR	NR	90	20	-
23 3-methyloxindole	Slow pink	NR	73	80	85
	yellow	--	90	88	89
24	NR	NR	95	82	-

NR: No reaction
- : Value not determined

(numbered 15,17,18,19,21 and 22) were present on the autoradiograph. Figure 77 shows a composite drawing of this autoradiograph, and of the same chromatogram after treatment with DMAC. The NEUTRAL fraction contained, as expected, a large amount of gramine and methylaminomethylindole. Large spots of these compounds can be seen in the lower right-hand corner of Figure 77, between spots 16A and 17, but no radioactivity was associated with these compounds.

The R_f values and color reactions of the neutral metabolites of IAA are presented in Table 27. The R_f values were obtained from the two-dimensional chromatogram discussed above (see Fig. 76), except in the case of spots 8,20 and 23, which were obtained from separate one-dimensional chromatograms of partially purified samples.

Identification of the radioactive spots 8,16,20, and 23 is discussed in more detail below.

Spot 8. Suspected Indoleacetic acid

This spot co-chromatographed with IAA in BuAW/BeAW, and gave a blue color with DMAC when chromatographed in the absence of authentic IAA. Spot 8 also had R_f values identical with those of authentic IAA in three one-dimensional solvent systems, and was therefore identified as unmetabolised IAA.

Spot 16. Suspected Indolecarboxylic acid

Spot 16 co-chromatographed with authentic indolecarboxylic acid in 1AW/BeAW, but gave no reaction to DMAC when the NEUTRAL

Fig. 78. UV absorption spectra of suspected indolealdehyde from barley shoots fed indoleacetic acid-2-C¹⁴ (continuous line), and of authentic indolealdehyde (closed circles).

fraction was chromatographed alone. However, the color reaction of indolecarboxylic acid with DMAC is weak and slow, and the amount of radioactive material present in spot 16 was not large. The compound forming this spot was tentatively identified as indolecarboxylic acid.

Spot 20. Suspected Indolealdehyde

This large spot gave an orange color with DMFH, and its R_f values agreed with those of authentic indolealdehyde in three one-dimensional solvent systems. A partially purified sample of the compound forming this spot was prepared by one-dimensional chromatography of an aliquot of the NEUTRAL fraction. Figure 78 shows that the UV spectrum of this sample was identical to that of authentic indolealdehyde.

It was therefore concluded that spot 20, the most prominent component of the neutral fraction, was indolealdehyde.

Spot 23.

Spots 23 and 24 were very close together and the blurred appearance of this area suggests that several compounds may be present. Spot 23 gave little color with DMAC, although a faint pink color appeared after a time. The R_f values of spot 23 are rather similar to those of authentic 3-methyloxindole in three one-dimensional solvent systems (Table 27) although they tend to be somewhat lower.

Fig. 79. UV absorption spectra of spot 23 from NEUTRAL fraction of barley shoots fed indoleacetic acid-2-C¹⁴ (continuous line), and of authentic 3-methyl-oxindole (open circles) and o-aminoacetophenone (closed circles).

spectrum compared with those of 3-methyloxindole and o-amino-acetophenone. As seen in Fig. 79, the spectrum of spot 23 did not coincide with the spectra of either of the above authentic compounds, but as the purity of the sample was in doubt, no firm conclusions could be drawn.

Conclusions

The NEUTRAL fraction contained at least twelve radioactive compounds, of which six were present in small amounts. Two of the major spots were identified as unmetabolised IAA and indolealdehyde, and a third was tentatively identified as indolecarboxylic acid. No conclusive evidence for the presence of 3-methyloxindole was obtained.

Clearly, IAA can be metabolised to indolealdehyde, but it appears that the quantitative importance of this pathway is slight since the entire neutral fraction contained only 3 per cent of the radioactive metabolites of IAA. It should be borne in mind, however, that indolealdehyde is found in unfed barley shoots while compounds such as indoleacetylaspartic acid are not. The indolealdehyde pathway may be of real importance in the normal metabolism of the plant, while formation of indoleacetylaspartic acid is merely a response to a wholly artificial situation.

Since the total activity of the unmetabolised IAA found in the ACID and NEUTRAL ether fractions cannot exceed 4 per cent of the radioactivity which entered the shoots, it is evident that metabolism of exogenously supplied IAA by young barley shoots is rapid and complete.

Fig. 80. Autoradiograph of AQUEOUS fraction from barley shoots fed indoleacetic acid-2-C¹⁴. Chromatogram developed first in butanol : acetic acid : water, then in isopropanol : benzene : water.

Fig. 81. Drawing of autoradiograph described in Figure 80 to illustrate system of numbering spots.

Fig. 82. Autoradiograph of AQUEOUS fraction from barley shoots fed indoleacetic acid-2-C¹⁴. Chromatogram developed first in butanol : acetic acid : water, then in isopropanol : ammonia : water.

Fig. 83. Drawing of autoradiograph described in Figure 82 to illustrate system of numbering spots.

Table 28. Rf values and color reactions of radioactive compounds found in the AQUEOUS fraction of barley shoots fed indoleacetic acid-2-C¹⁴, and of corresponding authentic indoles.

Spot number	DMAC color	Rf		
		BuAW	IBeV	IAW
1	NR	25	0	-
2	NR	57	0	-
3	NR	70	2	7
4	NR	52	7	D
5 Indoleacetyl- aspartic acid	Purple	80	2	2
	Purple	77	10	10
6	NR	70	20	-
7	Faint blue-green	43	35	0
8	NR	70	36	-
9	NR	79	25	-
10	NR	80	37	-
11	Faint blue-green	52	56	65 (D)
12	NR	70	60	40 67
13	NR	67	74	-
14	NR	74	85	-
15	NR	87	97	-

NR: No reaction

-: Value not determined

D: Decomposed during chromatography

3. AQUEOUS FRACTION

The AQUEOUS fraction might be expected to contain conjugation compounds of IAA, such as indoleacetylaspartic acid, indoleacetylglucose and 2-hydroxyindoleacetylglucose, and also ring-hydroxylated derivatives of IAA such as 5-hydroxyindoleacetic acid.

Figure 80 shows an autoradiograph of the chromatogram obtained when an aliquot of the AQUEOUS fraction, equivalent to 0.25 g tissue, was developed in BuAW/IBeW. A total of fifteen radioactive spots could be distinguished (Fig. 81) but only six of these (spots 3,4,5,6,11 and 12) were present in appreciable amounts. Figure 82 shows an autoradiograph of the AQUEOUS fraction developed in BuAW/IAW. Many of the spots observed in Fig. 80 could again be identified, but spot 11 and possibly spot 4 showed breakdown and streaking (Fig. 83).

Table 28 lists the R_f values and color reactions of the radioactive spots observed on chromatograms of the AQUEOUS fraction. The R_f values for spots 3,4,7,11 and 12 were obtained from one-dimensional chromatograms, while values for the remaining spots were calculated from the two-dimensional autoradiographs (Figs. 80 and 82). Identification of the compounds present in spots 3,4,5,6, and 11 is discussed below in greater detail.

Spots 3 and 4. Suspected 2-hydroxyindoleacetylglucose

In a report on the metabolism of IAA-2-C¹⁴, Klumbt (1964) presented an autoradiograph of an extract from wheat coleoptiles,

Fig. 84

Fig. 85

Fig. 84. UV absorption spectrum of spot 3 from AQUEOUS fraction of barley shoots fed indoleacetic acid-2-C¹⁴.

Fig. 85. UV absorption spectra of suspected indoleacetylaspargic acid from barley shoots fed indoleacetic acid-2-C¹⁴ (continuous line), and of authentic indoleacetylaspargic acid (closed circles).

chromatographed in the two-dimensional system, butanol : formic acid : water / isopropanol : ammonia : water. The principal radioactive metabolite on this chromatogram formed an elongated spot, which was very close to the region occupied by spots 3 and 4 in Figure 83. Klammt identified this major metabolite as the β -D-glucoside of 2-hydroxyindoleacetic acid. As no authentic sample of this compound was available during the present study, the possible identification of spots 3 and 4 as the β -D-glucoside could not be further investigated. This was unfortunate, as these two compounds together accounted for at least half of the radioactivity present in the AQUEOUS fraction, and therefore for about 35 per cent of the radioactivity present in the shoots.

The UV spectrum of spot 3 is shown in Figure 84. It was rather similar to the typical indole spectrum but was shifted towards the longer wavelengths by about 55 m μ .

Spot 5. Suspected Indoleacetylaspatic acid

Spot 5 gave a blue-purple color when treated with DMAC, and had R_f values identical with those of authentic indoleacetylaspatic acid in three one-dimensional solvent systems (Table 28). The UV spectrum of this compound was identical to that of authentic indoleacetylaspatic acid (Figure 86).

A sample of spot 5 was purified by chromatography in IAW and BuAW, then hydrolysed in 2N HCl for 90 minutes at 100°C in vacuo. When the hydrolysate was chromatographed in IAW, a radioactive compound was found at R_f 42. This gave a blue reaction with DMAC, and was identified as IAA. When the

hydrolysate was chromatographed in BuAW, a ninhydrin positive compound appeared at Rf 20; both the Rf value and the ninhydrin color of this compound were identical with those of authentic aspartic acid. It was therefore concluded that spot 5 was indoleacetylaspatic acid.

Spot 11. Suspected Indoleacetylglucose

This spot had an Rf of 52 in BuAW. Zenk (1961) reported the Rf of authentic indoleacetylglucose to be 56 in a BuAW solvent of slightly different composition (5:1:2.2). The Rf of spot 11 in IbeW was 56, while Zenk reported that of indoleacetylglucose to be 39 in this solvent system. Spot 11 decomposed in IAW to give two principal spots at Rf 40 and 65. Authentic indoleacetylglucose similarly decomposed in IAW to give IAA (Rf 39) and indoleacetamide (Rf 73). Thus, the chromatographic properties of spot 11 and those of indoleacetylglucose, while not identical, are very similar.

It was concluded that spot 11 was probably either indoleacetylglucose, or a closely similar compound.

Conclusions

The AQUEOUS fraction contained six major and nine minor radioactive metabolites. Three compounds were predominant; one of these was identified as indoleacetylaspatic acid, and a second may have been 2-hydroxyindoleacetylglucose. A smaller amount of a compound similar to indoleacetylglucose was also found. 5-Hydroxyindoleacetic acid, if present, would have

appeared as a DMAC positive spot slightly above spot 5 in SuAW/IAW. No such spot was found, and it was therefore concluded that 5-hydroxyindoleacetic acid was not present in the AQUEOUS fraction.

At least one third of the radioactivity present in this fraction was due to indoleacetylaspatic acid. When this activity was added to that of the indoleacetylaspatic acid found in the ACID fraction, a total of about 35 per cent of the radioactivity in the plant could be accounted for in the form of this compound.

3. Indoleglycolic acid and Indoleglyoxylic acid as Precursors of Indolealdehyde

a. Introduction

Tang and Bonner (1947) were the first to suggest that IAA may be oxidatively decarboxylated to indolealdehyde via indoleglycollic acid and indoleglyoxylic acid.

This possibility was examined by Stutz (1958) who found that purified lupin IAA oxidase readily converted indoleglycolic acid to indolealdehyde, but had no action on indoleglyoxylic acid. Neither indoleglycolic acid nor indoleglyoxylic acid has ever been reliably identified from plant material, even after feeding IAA.

In the experiment described on pages 183-211 of the present study, it was shown that when IAA-2-C¹⁴ was fed to barley shoots, a small amount of radioactive indolealdehyde

was formed, but indoleglycolic acid and indoleglyoxylic acid could not be detected. The purpose of the following experiment was to discover whether indoleglycolic acid and indoleglyoxylic acid, when fed alone to barley shoots, can be converted to indolealdehyde.

b. Materials and Methods

Three samples of 14-day old barley shoots, weighing 187 g, 200 g and 189 g respectively, were harvested and the plants in each sample divided between eight 100 ml beakers.

40 ml of a 0.5 mg/ml solution of sodium-3-indoleglycollate were added to each beaker containing the first group of shoots and 40 ml of a 0.5 mg/ml solution of sodium-3-indoleglyoxalate were added to the beakers containing the second group of shoots. The pH of both these solutions was adjusted to 7.0. Distilled water was added to the beakers containing the third group of shoots.

The plants were placed in a growth chamber under continuous light for 24 hrs, during which time 165 ml of solution were taken up by each of the treatments. The shoots were then macerated and fractionated in the standard way.

The NEUTRAL ether fraction of each treatment was chromatographed in BeAF. Part of the chromatogram was sprayed with DNPH to locate indolealdehyde, and the corresponding portion of the rest of the chromatogram (representing 60 per cent of the NEUTRAL fraction) was cut out and eluted in 5 ml of water. The UV spectra obtained from the three eluates are shown in Fig. 86,

Fig. 86.

Fig. 87.

Fig. 86. UV absorption spectra of authentic indolealdehyde (curve a) and of suspected indolealdehyde from unfed barley shoots (curve d) and from shoots fed either indoleglycolic acid (curve b) or indoleglyoxylic acid (curve c).

Fig. 87. UV absorption spectra of suspected indoleglyoxylic acid peptide from barley shoots fed indoleglyoxylic acid (continuous line), and of authentic indoleglyoxylic acid (closed circles).

together with the spectrum of authentic indolealdehyde.

As seen from the curves in this Figure, the absorption spectrum of the indolealdehyde formed after feeding either indoleglycolic acid or indoleglyoxylic acid was identical with that of authentic indolealdehyde. Some increase in indolealdehyde was observed after feeding indoleglyoxylic acid, but a much larger increase was obtained by feeding indoleglycolic acid. It was later found that the extraction of indolealdehyde into the NEUTRAL fraction was incomplete, since some of this compound was present in the ACID ether fraction. For this reason, the exact amount of indolealdehyde produced by the different feeding treatments could not be determined. However, at least three times as much indolealdehyde was present in the shoots after feeding of indoleglycolic acid as after feeding of indoleglyoxylic acid. Thus, it is clear that indoleglyoxylic acid cannot be an intermediate between indoleglycolic acid and indolealdehyde, as was suggested by Tang and Bonner.

The metabolism of indoleglycolic acid and indoleglyoxylic acid to compounds other than indolealdehyde was not investigated in detail. However, it is pertinent to note that indoleglycolic acid was rapidly metabolised to a number of acidic compounds which gave positive DNAC reactions. A large amount of indoleglyoxylic acid remained unmetabolised after 24 hrs. The principal metabolite of this compound showed an R_f value of 15 in IAT, and gave a positive reaction with DNPH, but no reaction with DNAC. The spectrum of this metabolite is compared with that of indolegly-

oxylic acid in Figure 87; it seems likely that it is a peptide conjugate of indoleglyoxylic acid. Clearly, the metabolic stability of indoleglyoxylic acid contrasts sharply with the lability of its higher homologue, indolepyruvic acid.

c. Conclusions

The results of the present experiment show that feeding of indoleglycolic acid and indoleglyoxylic acid to barley shoots increased the level of indolealdehyde in the tissues. Indoleglycolic acid was a much more effective precursor of indolealdehyde than indoleglyoxylic acid, indicating that indoleglyoxylic acid was not an intermediate between indoleglycolic acid and indolealdehyde. Indoleglycolic acid was rapidly metabolised, which may account for the fact that this compound could not be identified as a metabolite of IAA. On the other hand, indoleglyoxylic acid was relatively stable in the plant, and this would suggest that, if it were an intermediate between IAA and indolealdehyde, it should be detectable after feeding IAA. As no indoleglyoxylic acid could be detected under these conditions, it was concluded that it did not participate in the indolealdehyde pathway.

4. Indolealdehyde as a Precursor of Indolecarboxylic acid

a. Introduction

Indolecarboxylic acid has been identified in plant tissues in which the concentration of indolealdehyde was probably high (Seeley et al. 1956; Wightman 1962, 1964). In the present study,

indolecarboxylic acid was tentatively identified as a product of the metabolism of IAA. No indolecarboxylic acid was found when indoleglycolic acid was fed, but since the carboxylic acid gives a weak color reaction with DMAC, its presence on the chromatogram could have been masked by the many other compounds present which reacted strongly with DMAC.

The following experiment was therefore carried out to determine whether barley shoots are, in fact, capable of converting indolealdehyde to indolecarboxylic acid.

b. Materials and Methods

690 g of 13-day old barley shoots were harvested and distributed between seventeen 100 ml beakers. 65 mg of indolealdehyde were dissolved in 85 ml of distilled water, and the pH of the solution was adjusted to 7.0. 5 ml of this feeding solution were added to each beaker, and the plants were then placed in a growth chamber under continuous light. In most instances, the indolealdehyde solution was completely absorbed before the end of the 24-hour feeding period, and where this was the case, a further 10 ml of distilled water was added to each beaker.

After 24 hours, maceration and fractionation of the methanol extract were carried out in the standard way.

c. Chromatography

When the ACID and NEUTRAL ether fractions were chromatographed in BeA^W, a compound giving a slow, blue-green color with DMAC appeared on both chromatograms near R_f 60. The

Fig. 88. Densitometer scan of NEUTRAL fraction from barley shoots fed indolealdehyde. Chromatogram developed in benzene : acetic acid : water, then treated with DMAC.
BG = Blue-green band

Fig. 89. UV absorption spectra of suspected indolecarboxylic acid from barley shoots fed indolealdehyde (continuous line), and of authentic indolecarboxylic acid (closed circles).

densitometer scan obtained from the chromatogram of the NEUTRAL fraction is illustrated in Figure 89.

The compound running at Rf 60 was eluted and re-chromatographed in BeAW, BuAW, 2% acetic acid, and isopropanol : ammonia : water (10/1/1). The Rf values obtained are summarised in Table 29. Clearly, the Rf values of suspected and authentic indolecarboxylic acid were closely similar.

Table 29. Rf values of suspected indolecarboxylic acid from barley shoots fed indolealdehyde, and of authentic indolecarboxylic acid

Compound	BeAW	BuAW	2% acetic acid	IAW 10/1/1
Suspected indolecarboxylic acid from neutral fraction	62	87	36	20
Suspected indolecarboxylic acid from acid fraction	55	86	35	20
Authentic indolecarboxylic acid	57	87	33	26

The UV spectrum of the suspected indolecarboxylic acid was obtained from a sample purified by chromatography in BeAW and 2% acetic acid. It was found to be very similar to that of authentic indolecarboxylic acid, as shown in Figure 90.

d. Conclusions

Feeding indolealdehyde to barley shoots resulted in the formation of indolecarboxylic acid, but the amount of indolecarboxylic acid formed was small when compared to the amount of indolealdehyde administered.

V. SUMMARY

The nature of the precursors of IAA in barley plants was the most important question implicit in this study. The answer to this question, however, still remains obscure. IAA was shown to be present in unfed plants, so that the question of its origin is not merely an academic one. It is also fairly certain that the feeding of labelled tryptophan resulted in the formation of radioactive IAA, but the yield of IAA was very small. Thus, an excess of the substrate, tryptophan, resulted in little increase in the yield of the product, indoleacetic acid. This finding clearly suggests that the concentration of the enzyme, or enzymes, converting tryptophan to IAA is limiting; the nature of the control of this enzyme system therefore becomes extremely interesting.

Any compound which is an intermediate between tryptophan and IAA must, of course, give rise to IAA when administered to the plant. One might also expect to detect the intermediate after feeding tryptophan, unless it was turned over at a very rapid rate. Compounds which have been suggested as intermediates between tryptophan and IAA are indolepyruvic acid, indolelactic acid, tryptamine, indoleacetaldehyde, tryptophol, indoleacetonitrile and indoleacetamide. None of these postulated intermediates was found in unfed barley shoots. However, when each of these compounds was fed to barley in high concentrations (about 100 μg per gram Fresh Wt.), indoleacetaldehyde gave large amounts of IAA, indoleacetylaspartic acid and tryptophol,

and was itself not detectable after 24 hrs; tryptophol, indoleacetonitrile and indoleacetamide gave relatively large amounts of IAA but were not completely metabolised, and indolepyruvic acid, which was not fed to plants, was found to decompose spontaneously in aqueous solution to IAA and other compounds. Indolelactic acid and tryptamine gave rise to very small amounts of IAA. Tryptamine was largely unmetabolised, while indolelactic was transformed to tryptophan and other water-soluble compounds. Since indolelactic acid was readily converted to tryptophan, the IAA arising from indolelactic acid may have been formed by this indirect route. Thus, of the suspected intermediates in IAA formation, only indolelactic acid was definitely identified as a product of the metabolism of tryptophan. However, indolelactic acid was mainly formed from D-tryptophan, and the tryptophan-IAA system is not stereospecific (Gordon 1961).

It seems most likely that although barley can form IAA from any of the possible intermediates considered above, the normal pathway for the conversion of tryptophan to IAA involves the intermediary formation of indolepyruvic acid. Indolepyruvic acid would then give rise to IAA either via indoleacetaldehyde, or by direct spontaneous decomposition. The hypothesis of Gordon (1961), that the conversion of indolepyruvic acid to IAA is analagous to the pyruvic oxidase system of respiratory metabolism, is an extremely attractive one; the lack of free indoleacetaldehyde is explained, and the reactants would be

restrained on the surface of a complex enzyme and not subject to non-specific, purely chemical degradation.

The role of tryptophan as the precursor of gramine was confirmed. Gramine, methylaminomethylindole and aminomethylindole were present in the untreated plant, together with relatively large amounts of a DMAC positive, ninhydrin negative basic compound with Rf values similar to those of tryptamine. There was a correlation between the concentration of free tryptophan and the concentration of gramine in barley seedlings during the first three weeks of growth. Gramine was formed only in the shoots, and the major fraction of this alkaloid was found in the first leaf. Feeding radioactive tryptophan resulted in the labelling of aminomethylindole, methylaminomethylindole, gramine, and the unknown, tryptamine-like compound. Indolelactic acid was also a precursor of aminomethylindole, methylaminomethylindole and gramine, but this conversion probably took place via tryptophan, to which indolelactic acid was readily metabolised. Tryptamine and indoleacetic acid were not precursors of gramine. The nature of the intermediates between tryptophan and aminomethylindole is still unknown; a possible candidate is the unknown, tryptamine-like compound mentioned above. When radioactive gramine was fed to 14-day old barley shoots, no unequivocal evidence for the further metabolism of the gramine was obtained.

Indolealdehyde was present in untreated shoots. Precursors of indolealdehyde include tryptophan, tryptamine, indoleacetonitrile,

IAA, indoleglycolic acid and indoleglyoxylic acid. The role of the last three compounds as precursors of indolealdehyde constitutes qualitative evidence for the presence of the indolealdehyde pathway of IAA degradation in barley tissue. Indoleglycolic acid may have been an intermediate between IAA and indolealdehyde, although no indoleglycolic acid was detected after feeding of IAA to shoots. Indoleglyoxylic acid was not an intermediate between indoleglycolic acid and indolealdehyde, and the aldehyde itself could be further metabolised to indolecarboxylic acid. The quantitative importance of this pathway was small when large amounts of IAA were fed, but it may represent the main pathway of normal IAA metabolism. Indoleacetonitrile gave rise to an unusually large amount of indolealdehyde, which suggests that enzymes are present in barley shoots which will catalyse α -oxidation of the side chain.

Calculations based on results recorded in the present study indicate that 1 g fresh weight of 14-day old barley leaf tissue contains about 1250 μ g of gramine, 50 μ g of free tryptophan, 0.1 μ g of indolealdehyde and 0.01 μ g of IAA. In the metabolism experiments described in this thesis, the feeding rate for each compound was of the order of 100 μ g per gram fresh weight of tissue, an amount which was high with respect to the amount of most of the native indoles present in the plant. Because of this high feeding rate, the formation of conjugation compounds became prominent to a degree which does not reflect their importance in the normal metabolism of the plant. Thus for example, feeding D-tryptophan resulted in the formation of

appreciable quantities of malonyl-D-tryptophan, a compound which was found only in small amounts in the normal, unfed tissue. Similarly, at least 35 per cent of the IAA fed to barley shoots was converted to indoleacetylaspatic acid, and three other prominent water-soluble products, which possibly included indoleacetylglucose and 2-hydroxy-indoleacetylglucose. Indolelactic acid was metabolized to two watersoluble compounds, one of which was probably a conjugation compound with an amino acid. Indoleglyoxylic acid was also mainly metabolised to an acidic, peptide-like compound.

There was little evidence for the formation of 5-hydroxy compounds in barley shoots. No 5-hydroxytryptophan or 5-hydroxy-indoleacetic acid were found in any of the metabolism experiments and no 5-hydroxytryptamine was found when tryptamine was fed to shoots. The kynurenine pathway for tryptophan metabolism was absent, and there was little evidence for the analagous o-aminoacetophenone pathway of IAA degradation. Indolelactic acid may possibly have undergone a ring cleavage, analagous to the formation of kynurenine from tryptophan. Many unknown compounds were detected in all feeding experiments, though most of these compounds gave no color reaction with DMAC and were therefore probably not indoles.

In conclusion, it is clear from the present study that the routes by which simple indoles are metabolised in barley are of considerable complexity; they also possess a great degree of flexibility, as witnessed by the fact that IAA can be formed

from eight different precursors. This flexibility in the biochemical mechanisms of barley must surely have adaptive value, ensuring that if the main pathway of IAA biosynthesis is destroyed, some other pathway is able to take over the formation of this important growth hormone.

The biochemical relationships established in this study are summarised in diagrammatic form in Figure 90.

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