

QUANTITATIVE ESTIMATION OF INDOLEACETIC ACID AND INDOLECARBOXYLIC ACID IN ROOT NODULES AND ROOTS OF LUPINUS LUTEUS L.

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SUMMARY

By means of a double chromatographic separation technique applied to the acid fraction of methanol extracts of root nodules, parental roots, and 14 day old roots of *Lupinus luteus* L., indoleacetic acid and indolecarboxylic acid were demonstrated spectrofluorometrically and the amounts determined. The difference between the IAA content of nodules and parental roots on a fresh-weight basis was not as great as is generally believed: we found about 3 times more IAA in nodules than in roots. The 14 day old roots contained the lowest amount of IAA: about half the amount found in roots on which nodules had been present. The ICA content of roots that had been in contact with nodules was about 3 times higher than the ICA content of the nodules. The ICA content of 14 day old roots was the lowest. These results are discussed in the light of a possible alteration of the indole metabolism of the root caused by infection with the bacterial symbiont *Rhizobium lupini*, as an indication for a fundamental alteration of growth-regulation leading to the genesis of nodules.

1. INTRODUCTION

Several studies on the auxins in root nodules of leguminous plants have been published. Shortly after the beginning of the auxin era, the presence of relatively large quantities of auxin in leguminous root nodules was reported.

THIMANN (1936, 1939) used the *Avena* coleoptile curvature test and demonstrated that root nodules contain a much larger quantity of diffusible auxins than neighbouring root tissues. LINK & EGGERS (1940) found nodule tissue to be very rich in extractable auxins. It was also demonstrated that the bacterial symbiont, *Rhizobium*, produces indoleacetic acid (IAA) from tryptophan in various culture media (LINK 1937, CHEN 1938, THIMANN 1939, GEORGI & BEGUIN 1939).

More recently, PATE (1958) used an ethanol extraction procedure and, with an *Avena* straight growth test after paper-chromatographic separation, demonstrated three growth-promoters and two inhibitors in extracts of nodule tissue. One of the growth-promoters he identified as IAA from its R_f-value on the chromatogram, its colour-reactions with Ehrlich and Salkowski reagents, and its fluorescence on the chromatogram in UV light. Pate worked with material from field-grown pea plants, and compared extracts from nodules with extracts from root tissue. He estimated that IAA-extractable activity in nodule tissue is some 40 to 60 times higher than in root tissue.

Before investigating the origin of this difference in IAA content – the existence of which is generally accepted – it was thought necessary to re-examine the higher IAA content of root nodules, especially quantitatively.

The main motivation for the present study was found in the investigation of BURNETT c.s. (1965) into the growth substances in the roots of *Vicia faba*. These authors used column and paper-chromatography for the separation, and spectrofluorimetry and an *Avena* test for the identification of the growth-promoting components in methanol extracts of 12 day old roots. They were unable to identify IAA positively by its UV-absorption or fluorescence, and stated that it could not be present in quantities greater than 2–5 μ g/kg fresh weight of root material. However, they found another fluorescent compound in the chromatogram region showing growth activity; this compound had a fluorescence characteristic that was quite different from that of IAA. They consider it to be an auxin very closely related to the "citrus auxin" of KHALIFAH c.s. (1963). This report raised several questions in our mind concerning the auxin situation in root nodules, the most important question being: Is the main extractable auxin in root nodules IAA? An attempt was then made to examine quantitatively the IAA content of the parental roots as compared to that of root nodules. During this investigation it was found that indolecarboxylic acid (ICA) was also present in relatively large quantities in the extracts, and an attempt was then made to determine the amounts of this compound.

The work of Burnett c.s. gives an excellent demonstration of the fact that a biotest alone cannot give adequate evidence for the conclusion that all auxin activity present in the chromatogram region with the Rf-value of IAA is actually caused by IAA. A bio-assay cannot give exact information about the nature of auxins. Therefore, spectrofluorimetry was used for the identification and quantitative estimation of small amounts of IAA.

2. MATERIALS AND METHODS

2.1. Plant material

Lyophilized nodules and roots – minus the nodule zones – of lupine (*Lupinus luteus* L., "bittere gele lupine") were used. Unless otherwise mentioned, the plants were grown in the field and were harvested when 45 days old. For some of the experiments, roots were taken from plants grown in the laboratory on vermiculite for 14 days.

2.2. Extraction

A standard low-temperature methanol extraction procedure was used. The lyophilized tissue (usually in 10 g portions) was powdered in a mortar with sand and mixed with 20 times its weight of precooled absolute methanol. Extraction was allowed to proceed for 20 hours at -15°C . The extract was filtered, the tissue powder rinsed three times with small volumes of methanol, and after addition of some distilled water the combined methanol extract was evaporated under reduced pressure at $\pm 50^{\circ}\text{C}$ until only a water solution remained. To this solution the same volume of a 1 N NaHCO_3 solution was added, the end concentration of the NaHCO_3 solution being 0.5 N. This solution was washed three times by shaking it with the same volume of peroxide-free diethylether in

a separatory funnel to remove lipoidal impurities, large amounts of which are always present. The NaHCO_3 solution was then acidified to pH 3.0 and extracted three times with the same volume of peroxide-free ether. The pooled ether extract was dried with anhydrous MgSO_4 and evaporated under reduced pressure until dry. The residue was taken up in a small volume of purified ethanol. This solution, which was an ethanol solution of the acid ether-soluble fraction of the methanol extract, was applied in portions for chromatography.

2.3. Chromatography

2.3.1 Thin-layer chromatography

For chromatographic identification, a two-dimensional TLC technique was used. The plates were 0.25 mm thick (Silicagel G "Merck"), and were activated at 100°C for 30 minutes before being used.

Solvents: isopropanol : ammonia (sp.gr. 0.91) : water = 10:1:1
 isobutanol : methanol : water = 80:5:15

Spray reagents: DMAC. (2 g. p-dimethylaminocinnamaldehyde dissolved in a mixture of 100 ml 96% ethanol + 100 ml 6 N hydrochloric acid)

Ehrlich (2% p-dimethylaminobenzaldehyde + 2 N hydrochloric acid in 80% ethanol)

Salkowski (50 ml 35% perchloric acid (HClO_4) + 1 ml 0.5 M FeCl_3 + 50 ml 96% ethanol)

2.3.2 Paper chromatography

For separation and purification, an ascending technique was used. It proved necessary to chromatograph twice. It was also necessary to wash the paper (Whatman no. I) with purified ethanol to remove fluorescent compounds: eluates of small pieces of unwashed paper showed a fluorescence behaviour very much like that of indole derivatives.

A known quantity of the ethanol solution to be analyzed was applied on a transverse line about 3 cm long.

Solvent: isopropanol : ammonia (sp. gr. 0.91) : water = 10:1:1.

One hour of equilibration was followed by a run of 16 hours in the dark at 20–22°C. Marker spots of IAA and ICA were run at the same time. The chromatograms were then dried with hot air and inspected under UV light for fluorescence. The zones corresponding to the marker spots were cut out and eluted in purified ethanol. The ethanol was evaporated under reduced pressure; the residue was then taken up in a small volume of purified ethanol and applied as a small spot on a new strip of prewashed Whatman no. I paper. The solvent was now: benzene : acetic acid (glacial) : water = 2:2:1. Equilibration for 1 hour was followed by a 4-hour run in the dark at 20–22°C. After drying with hot air and inspection under UV light, the zones corresponding to the marker spots were again cut out and eluted in 3 ml purified (double-distilled) ethanol. A piece of paper taken under the starting line was also eluted as a blank in all

cases. These eluates were then examined spectrofluorometrically with a "Baird-Atomic" spectrofluorometer.

As was to be expected after double-chromatography, the losses were rather high. Thus, for IAA determinations the following parallel extractions were also made:

1. of tissue alone,
2. of tissue plus a known quantity of IAA,
3. of a known quantity of IAA.

This was done to obtain a measure of the loss of IAA during the procedure and to be able to calculate the original amount of IAA extracted from the tissue.

3. RESULTS

3.1 Chromatography

For orientation, two-dimensional thin layer chromatography was used. *Fig. 1* shows the result of the acid fraction of a nodule extract; *fig. 2* shows the result of the acid fraction of a root extract.

In nodule extracts, the IAA, ICA, and indolelactic acid could be identified by their R_f values and their colour reactions with the spray reagents (DMAC was the most sensitive). Indoleglycolic acid may have been present, but this could not be established with certainty; the spot had the right R_f values in both solvents but in some cases the colour with DMAC was not quite identical to that of authentic indoleglycolic acid.

In the chromatograms of root extracts the spots were always very vague, as indicated by the dashed lines in *fig. 2*. Only IAA and ICA could be identified by their R_f values and their colour reactions.

3.2 Spectrofluorometric IAA determination

Fig. 3 shows a spectrofluorogram of pure IAA dissolved in double-distilled ethanol: activation max. 290 $m\mu$, fluorescence max. 348 $m\mu$. *Fig. 4* is representative of spectrofluorograms of eluates from the zones corresponding to the IAA marker spot, after being chromatographed twice. Eluate 1 represents the IAA in a nodule extract, eluate 2 that of the nodule extract + added IAA, eluate 3 that of correspondingly treated IAA, and eluate 4 a spectrofluorogram of an eluate of blank chromatography paper taken under the starting line and excited at 290 $m\mu$.

The same peaks are found as for IAA: activation max. 290 $m\mu$, fluorescence max. 348 $m\mu$. The broad peaks (activation max. 315 $m\mu$, fluorescence max. 410 $m\mu$) indicate the presence of an impurity in the eluates. This is probably ascribable to the solvent used during the second chromatography; it was also found in the blank eluate. In the determination it was of no importance.

Calculation of the original IAA content in root nodules and roots yielded the following results:

- Root nodules: 250–400 μg IAA/kg fresh tissue.
- Parental roots: 70–160 μg IAA/kg fresh tissue.

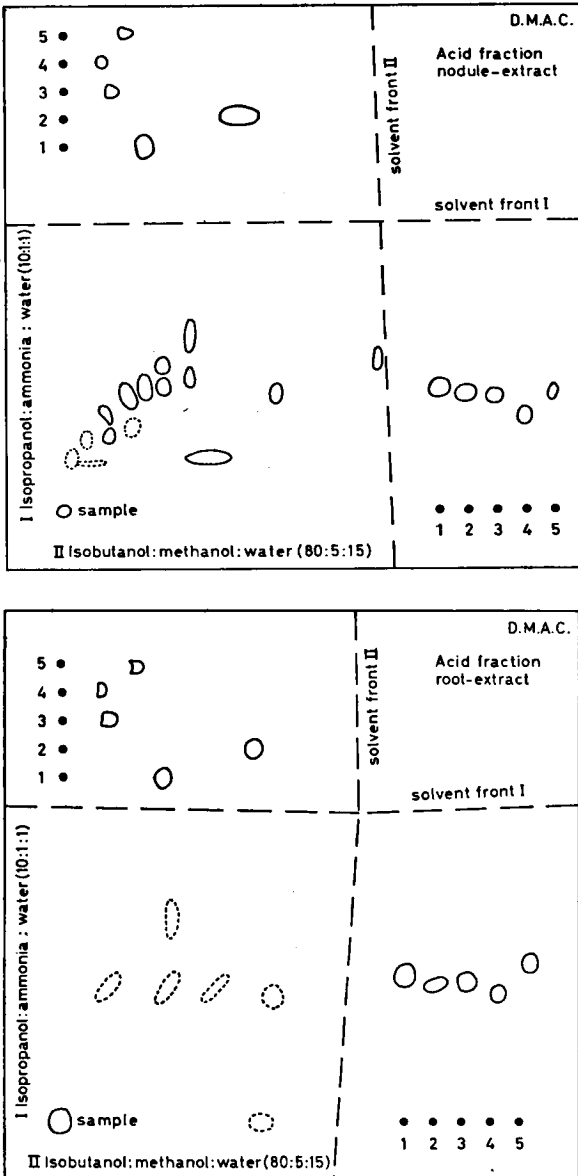


Fig. 1-2. Drawings of two thin-layer chromatograms of the acid fraction of a nodule extract (Fig. 1) and of a root extract (Fig. 2). Reference spots: 1: indoleacetic acid; 2: indolecarboxylic acid; 3: indolelactic acid; 4: indoleglycolic acid; 5: indoleglyoxylic acid.

The identity of the extracted substance with IAA was confirmed by the following evidence: it gave the right concentration response in our *Avena* straight growth assay; it had the correct Rf values of IAA in chromatography with different solvents, and it gave the right colour reaction with different spray reagents. Consequently, the high IAA content of root-nodules had been de-

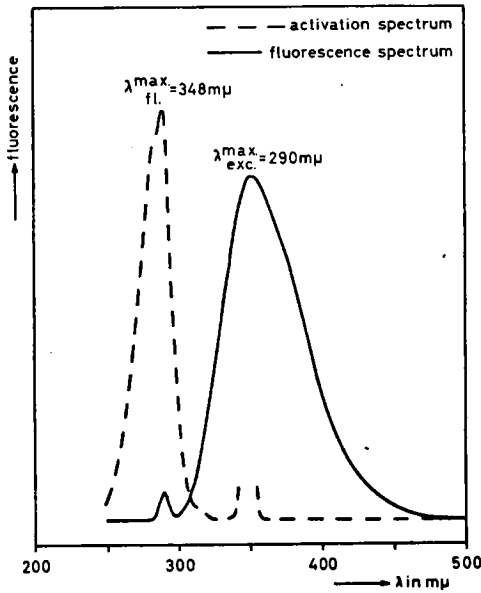


Fig. 3. Spectrofluorogram of IAA. (10^{-6} g/ml in ethanol)

monstrated quantitatively. But the IAA content of the parental-roots is also rather high. This could be explained by the fact that these roots had been in contact with IAA-producing root nodules. It therefore became highly interesting to know the amount of IAA in roots lacking nodules. We therefore prepared extracts of 14 days old root material grown in vermiculite cultures without the

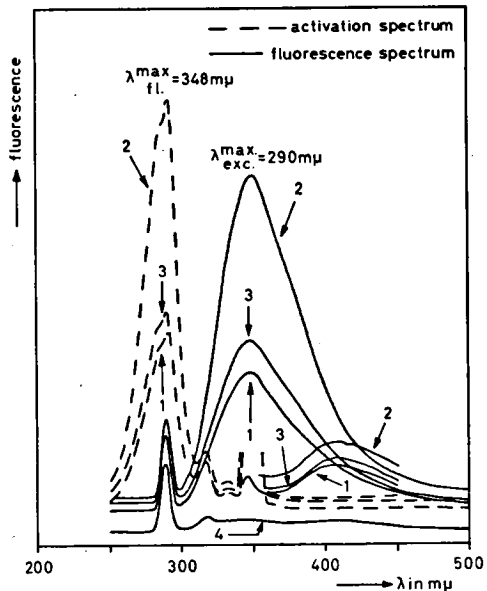


Fig. 4. Spectrofluorograms of eluates from the zones corresponding to the IAA marker spot, after twofold chromatography.
 1 = eluate of a nodule extract.
 2 = eluate of a nodule extract to which some IAA was added.
 3 = eluate of correspondingly treated IAA.
 4 = blank eluate.

presence of *Rhizobium* in the root medium. The result of the spectrofluorometric IAA determination was:

Young roots: 30–60 μg IAA/kg fresh tissue.

The thin-layer chromatographic orientation showed that some other acid indole derivatives were present in the extracts, the most abundant being ICA. We therefore attempted to determine the amounts of ICA in the extracts in the same way as for IAA.

Ethanol solutions of ICA have nearly the same fluorescence characteristics as ethanol solutions of IAA, but the peaks lie at slightly different places: activation max. 288 $\text{m}\mu$, fluorescence max. 337 $\text{m}\mu$. The chromatographic separation was sufficient to yield ICA in a nearly pure state. The results of the spectrofluorimetric determination were:

Root nodules: 50–150 μg ICA/kg fresh tissue.

Parental roots: 200–400 μg ICA/kg fresh tissue.

Young roots: 25–35 μg ICA/kg fresh tissue.

Indolelactic acid (ILA) was detected in nodule extracts only in very small amounts, but the evidence is nevertheless rather strong: after being separated from the extracts during the two paper-chromatography procedures, the eluate gave the typical fluorospectrum – although very weakly – of indole-derivatives. With thin-layer chromatography this eluate gave a weak spot with the same R_f as given by authentic ILA and also gave the same colour with the very sensitive DMAC reagent.

With the separation technique it was not possible to determine spectrofluorometrically whether indoleglycolic acid was present in the extracts. This indole compound undergoes a very rapid destruction in acid solvents.

4. DISCUSSION

It has been demonstrated that the root-nodules have a relatively high IAA content. But the difference as compared to the parental roots is not as high as Pate estimated, i.e. 40 to 60 times as much IAA extractable activity in nodules as in the roots. It must be stressed, however, that he worked with pea plants and that his investigations were semi-quantitative, as he himself stated. He applied chromatography only once, and used an *Avena* bioassay for detection and determination. It is well known that the separation in a single chromatography run is often insufficient, so there may have been synergistic effects in his biotest.

Concerning the results of the IAA determination in extracts of the 14 day old roots, it may be said that the IAA content is lower than that of the roots on which nodules had been present, but is still rather high with respect to the results of Burnett *c.s.*, who worked with *Vicia faba*.

What is the origin of the relatively large quantities of IAA in root nodules? Is this IAA produced only by the symbiotic *Rhizobia* (KEFFORD *c.s.* 1960, BULARD *c.s.* 1963, HARTMANN & GLOMBITZA 1967, and our own unpublished

data), or is it also caused by an alteration of the indole metabolism of the tissue itself, as is the case with crown-gall and other phytopathogenic phenomena (SEQUEIRA 1966)? In connection with this point the difference between the ICA content of nodules and the parental roots may be very interesting.

It has been found that ICA is one of the products formed by *Rhizobia* from tryptophan (RIGAUD *c.s.* 1965; our unpublished data). However, this fact does not make it easier to understand why the ICA content of nodules is substantially lower than that of the parental roots. It has been demonstrated that in tomato stem tissue ICA occurs as a degradation product of IAA (WIGHTMAN 1964). But at this moment there is no direct evidence, either from enzyme preparations of nodule tissue or from enzyme preparations of root tissue, of an ICA production from IAA. It seems possible, however, that this difference in ICA content is the result of a difference in IAA degradation in root tissue and nodule tissue.

An indication pointing in the direction of an alteration of the indole metabolism of the tissue was found in the difference between the relations of IAA to ICA in extracts of root nodules and of roots; these relations, as quotients

$\left(\frac{[IAA]}{[ICA]}\right)$ of the earlier results are:

Root nodules	2.6-5.0
Parental roots	0.3-0.4
14 day old roots	1.2-1.7

The differences are very striking. It is tempting to suggest with regard to the quotient for the 14 day old roots, that nodule tissue shows some aspects of the rejuvenation of root tissue. This seems likely because of the rather high meristematic activity in nodule tissue. In relation to the differences in the IAA and ICA content of nodules and roots, one observation from the crown-gall literature seems very interesting: ether extracts of tumour tissue from tomato plants contained IAA and ICA, but extracts of normal tissue contained only ICA and no IAA (CLARKE *c.s.* 1959). This phenomenon might be very closely related to that of nodule tissue, as presented here.

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