THE BIOPRODUCTION OF INDOLE-3-ACETIC ACID AND RELATED COMPOUNDS IN ROOT NODULES AND ROOTS OF LUPINUS LUTEUS L. AND BY ITS RHIZOBIAL SYMBIONT

J. DULLAART

Department of Experimental Botany,
Botanisch Laboratorium, Leiden.

SUMMARY

The bioproduction of indole-3-acetic acid (IAA) and indole-3-carboxylic acid (ICA) from L-tryptophan in root nodules and roots of *Lupinus luteus* and by the symbiotic *Rhizobium lupini* was studied *in vitro*, and IAA degradation and the conversion of a number of related indole derivatives were also investigated. The results permit the conclusion that at least a substantial part of the large amounts of IAA present in the root nodules is produced via plant enzymes as a result of a metabolic alteration induced by the rhizobial infection. Young roots appeared to possess a high activity of IAA production from L-tryptophan, but most of this production is masked by enzymatic degradation of IAA.

According to the results of the IAA destruction experiments, cell-free enzyme extracts of mature roots show the lowest activity, and the non-dialyzed young root enzyme extract exhibits significantly greater activity than the dialyzed preparations. For the root nodule enzyme preparations this situation was found to be reversed, suggesting that the balance between promoting and inhibiting substances influencing enzymatic IAA degradation in nodule tissue is changed in favour of inhibition as a result of the rhizobial infection. Some interesting data concerning differences in IAA destruction behaviour between different strains of *Rh. lupini* were also obtained.

The results of the comparative investigations concerning the conversion of a number of related indole derivatives indicated that there are some significant differences with respect to the conversion capacities of the different incubation systems.

1. INTRODUCTION

Evidence presented in a previous paper led to the conclusion that root nodules of yellow lupine contain substantially more indoleacetic acid (IAA) than the roots (DULLAART 1967). This result can be considered as a confirmation of older work on the auxin content of leguminous root nodules and roots (THIMANN 1936, 1939; LINK & EGGERS 1940; PATE 1958); and the combined use of paper chromatography and spectrofluorometric analysis showed that IAA is indeed the main auxin present in the nodule tissue.

However, the very important question of the origin of the high amounts of IAA in root nodules is still unanswered. Since it is well known that the bacterial symbiont, *Rhizobium* spec., is able to produce IAA from tryptophan in culture media (LINK 1937; CHEN 1938; THIMANN 1939; GEORGI & BEGUIN 1939; KEFFORD *et al.* 1960; BULARD *et al.* 1963; HARTMANN & GLOMBITZA

1967), it has been suggested that the endophytic organism is directly responsible for the high IAA content of the nodules. But the possibility that the relatively large quantities of IAA might also be the result of an alteration of the indole metabolism of the tissue itself as a reaction to the rhizobial infection cannot be excluded. In this respect the nodule formation might be comparable to the crown-gall disease and other phytopathogenic phenomena (SEQUEIRA 1963). In fact, there is some evidence for such an alteration of the indole metabolism: quantitative estimations of the indolecarboxylic acid (ICA) contents of root nodules and roots of lupine, made concurrently with the IAA determinations, revealed that the nodular tissue contained much less ICA than the roots (DULLAART 1967). It has also been shown that ICA is one of the products formed by *Rhizobium* from tryptophan (RIGAUD *et al.* 1965), but this does not make it easier to understand why the ICA content of nodules is substantially lower than that of the roots, since the nodule tissue contains large amounts of the rhizobial endophyte. This situation can be interpreted as a change of the indole metabolism induced by the rhizobial infection.

The need for detailed investigations into the indole metabolism in root nodules and roots as compared to the indole metabolism of the rhizobial symbiont is evident. Attention must of course be directed first to the metabolic aspects of IAA production. Since L-tryptophan is generally considered to be the main precursor in the biogenesis of IAA in higher and lower plants, a comparative study was made of the IAA production from L-tryptophan in sterile, cell-free, crude enzyme preparations from root nodules, roots, and the microbial symbiont, *Rhizobium lupini*; and the production of IAA from tryptophan by *Rhizobium* cells was also investigated. The ultimate aim of this study was to determine whether the high IAA content of the nodules is due to synthesis by the endophyte or to an increased production by the plant cells themselves. This made it necessary to examine the metabolic pathways from tryptophan to IAA in the bacteria and the plant tissue, including the formation of related indole compounds and their possible fate with special attention to ICA in connection with the already mentioned high ICA content of roots as compared to that of the nodules.

Because it forms the subject of a separate research project of our laboratory, the metabolism of the degradation of IAA was not considered in this study except with respect to its possible influence on the yield of the IAA production in the reaction mixtures.

2. MATERIALS AND METHODS

2.1. Plant materials

Lupinus: Root nodules and roots (minus the nodule zones and having diameters of less than 2 mm) of yellow lupine (*Lupinus luteus* L. "bittere gele lupine"), grown for 60 days in the field (*Rijkskwekerij Drakenburg* at Baarn) were collected, thoroughly washed with tap and distilled water, and lyophilized.

Young, non-nodulated roots were harvested from seedlings of lupine from seeds, surface-sterilized with 96% H_2SO_4 for 20 minutes, and grown in sterilized vermiculite in the laboratory under constant conditions: day-length 16 hours light, (light source Philips TL 40w/33, distance from the light tubes 150 cm, energy on plant level 20,000 erg/sec/cm^2), 8 hours dark; temperature 20 °C; 70–80% relative humidity. These roots were also thoroughly washed and lyophilized.

The lyophilized materials were stored at – 20 °C.

Rhizobium: In all experiments on tryptophan conversion by bacterial cells and cell-free enzyme preparations of bacterial cells, the effectively nodulating *Rhizobium lupini* strain A98 was used. This relatively fast-growing strain was obtained from Dr. van Schreven of the *Rijksdienst voor de IJsselmeerpolders* at Kampen. The organism was grown under continuous aeration in the following culture medium: 15 g mannitol, 100 ml yeast extract, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 0.2 g NaCl, and 2 g $CaCO_3$ per litre distilled water. If necessary, the pH was adjusted to 7.0. The medium was filtered through Whatman No. I filter paper to remove superfluous $CaCO_3$ before sterilization for 40 minutes at 110 °C. Before inoculation, a drop of sterilized anti-foam preparation (Rhodorsil Antimouse 426) was added. Rhizobial cultures were grown for 60 hours in the dark at a temperature of 25 °C, after which the cultures were centrifuged at 2,000 g for 15 minutes. The precipitated cells were washed twice with 0.05 M phosphate buffer (pH 6.5) by resuspension and centrifugation. This procedure was performed under avoidance of contamination by other microbes. The resulting cell paste was stored at – 20 °C.

2.2. Enzyme extracts

Lupinus: Lyophilized nodules or roots in portions equivalent to 100 g fresh weight were powdered in a mortar with some sand. A volume of 0.05 M phosphate buffer (pH 6.5) equal to the volume of water lost during the lyophilization was added and the brei was homogenized for 30 minutes at 0 °C and filtered through cheese cloth. The filtrate was centrifuged at 80,000 to 100,000 g for 30 minutes at 0 °C, after which the clear supernatant was sterilized by filtration through Sartorius membrane filters with a maximum pore diameter of 0.35 μ, and then stored in sterile tubes in a deepfreezer (– 20 °C).

In many cases the supernatant fluid was dialyzed for 24 hours at 2.5 °C against 1 litre phosphate buffer (pH 6.5) with two changes of this volume of external buffer. This dialyzed enzyme preparation was also membrane-filter-sterilized and stored at – 20 °C.

Rhizobium: Five-gram samples of the stored rhizobial cells were thawed and suspended in 20 ml phosphate buffer (pH 6.5). The bacterial cell walls were fractured in a French pressure cell. The resulting suspension was centrifuged at 80,000 to 100,000 g for 30 minutes. The supernatant was diluted by addition of an equal volume of phosphate buffer. The whole procedure was performed at a temperature of 0 °C.

Part of the resulting enzyme preparation was immediately membrane-filter-sterilized, the other part was dialyzed against the 0.05 M phosphate buffer as described above and also filter-sterilized. The sterile enzyme preparations were stored in sterile tubes at – 20 °C.

The measurement of protein was done according to LOWRY *et al.* (1951). Since plant tissues, and especially the root nodule tissue, contain large amounts of phenolic substances, and since the Folin & Ciocalteu's reagent used in this method is a specific phenol reagent, the free phenolic compounds had to be removed from the protein preparations before quantitative protein estimations were made. For this purpose, 2 ml of the protein solution was mixed with 2 ml 10% trichloroacetic acid (TCA) and centrifuged for 15 minutes at about 2,000 g.

After the supernatant had been discarded, the sediment was suspended in 5 ml 5% TCA and again centrifuged before being resuspended in 3 ml 0.5 N NaOH. The resulting suspension was held in a boiling water bath for 10 minutes and stirred from time to time. Turbid solutions were centrifuged again. Aliquots of 1.0 ml were used for the determination according to Lowry *et al.* The entire procedure was performed in duplicate for each enzyme extract.

As a measure of the effectiviness of the TCA precipitation method, it can be said that the results of the application of the Lowry method for non-dialyzed nodule enzyme extracts were about twice as high when no TCA precipitation was applied.

2.3. Incubation methods

For the incubation experiments performed for quantitative estimation of the production of IAA and ICA from L-tryptophan, the reaction mixtures contained 0.5–5.0 ml sterile enzyme extract, depending on the protein content, the end concentration of protein being in the order of magnitude of 0.5 mg per ml, boiled enzyme extract or buffer. Usually, the total volume of the mixtures was 10 ml. They contained 1.0 mg tryptophan per ml and some facultative additives: α-ketoglutaric acid (720 μg per ml) and pyridoxal phosphate (100 μg per ml). Detailed data about other additives are presented together with the description of the individual experiments. The basal incubation solution was 0.05 M phosphate buffer (pH 6.5). All solutions were sterilized by membrane filtration before incubation in sterile 50 ml erlenmeyer flasks for 20 hours at 25 ± 0.1 °C in the dark in a Gallenkamp shaking incubator.

In the experiments on enzymatic conversion of a number of indole derivatives (including tryptamine, indolelactic acid, indoleacetic acid, indolepyruvic acid, indolecarboxylic acid, tryptophol, indoleacetamide, indoleacetaldehyde, and indolealdehyde) the 5-ml incubation mixtures contained per ml 0.4 to 1.0 mg protein and 100 μg of the indolic substance. The incubations were performed as described above. Only non-dialyzed enzyme extracts were used in these experiments.

Reactions were routinely stopped by placing the incubation flasks in an ice bath and lowering the pH to 2.0 with 0.5 N HCl.

Bacterial incubation experiments were performed in essentially the same way as the enzyme incubations, except that the mixtures contained about 2.6×10^{10} rhizobial cells instead of enzyme extract. In addition to IAA and ICA production, the bacterial conversions of the various indole derivatives were investigated in the same way as conversions by the enzyme preparations.

Sterility check: To ascertain whether the conditions of enzyme incubations had been strictly sterile, 0.05-ml samples of the incubation mixtures were taken at the end of the incubation period and applied to nutrient agar plates (25 g yeast extract (Difco), 10 g glucose, 5 g proteose peptone, and 20 g agar per litre distilled water) in petri dishes. The agar plates were incubated at 25 °C for more than 5 days. When these plates showed microbial contamination, the corresponding experimental results were discarded.

2.4. Purification and estimation of the indole compounds

The acidified, cold mixtures were extracted three times with 10 ml peroxide-free diethylether. The combined extracts of each mixture were dried with anhydrous $MgSO_4$ and evaporated to dryness in a rotary-film evaporator at 30 °C under reduced pressure; the residues were dissolved in 0.5 ml redistilled 96% ethanol. This fraction contained the acidic and neutral ether-soluble indole compounds.

In some cases the alkaline ether-soluble fraction was isolated to investigate the presence of alkaline indoles such as tryptamine. The pH of the incubation mixtures from which acidic and neutral indoles had been extracted was adjusted to 8.0 with 0.5 N $NaHCO_3$, after which three extractions with 10 ml peroxide-free ether were performed. The pooled extracts were then dried with anhydrous $MgSO_4$ and evaporated to dryness. The residues were dissolved in 0.5 ml 96 % ethanol. All ethanol fractions were kept in glass-stoppered vessels at – 20 °C.

The thin-layer and two-fold paper chromatography techniques have been described in detail elsewhere (DULLAART 1967).

To obtain qualitative and quantitative information about the presence of IAA and ICA, the various ethanol eluates of the specific paper-chromatographic zones were examined spectrofluorometrically with a Baird-Atomic spectrofluorometer (type Fluorispec).

The purity of the eluates was consistently checked by determination of the fluorescence spectrum at the excitation maximum and the excitation spectrum at the fluorescence maximum. These spectrofluorograms were compared with those of pure IAA in ethanol (activation max. 290 mμ, fluorescence max. 348 mμ) or ICA (activation max. 288 mμ, fluorescence max. 337 mμ).

When the purity of the eluates was sufficient, the maximum fluorescence at the excitation maximum was estimated with the fluorometer, which was calibrated for sensitivity according to the manufacturer's instructions. The concentration of IAA or ICA in the eluates was determined by interpolation in a calibration curve. Most of the determinations were performed in duplicate or triplicate; the values in the tables represent the averages and the deviations of the measurements.

It was to be expected that the losses would be rather high after extraction and twofold paper chromatography (see *e.g.* KUTAČEK & PROCHAZKA 1964). To make it possible to evaluate the losses of IAA and ICA during the procedure and calculate the amounts of these compounds produced in the incubation mixtures, it was necessary to know the recovery. Therefore, a number of IAA and ICA solutions of different concentrations were extracted, after which the

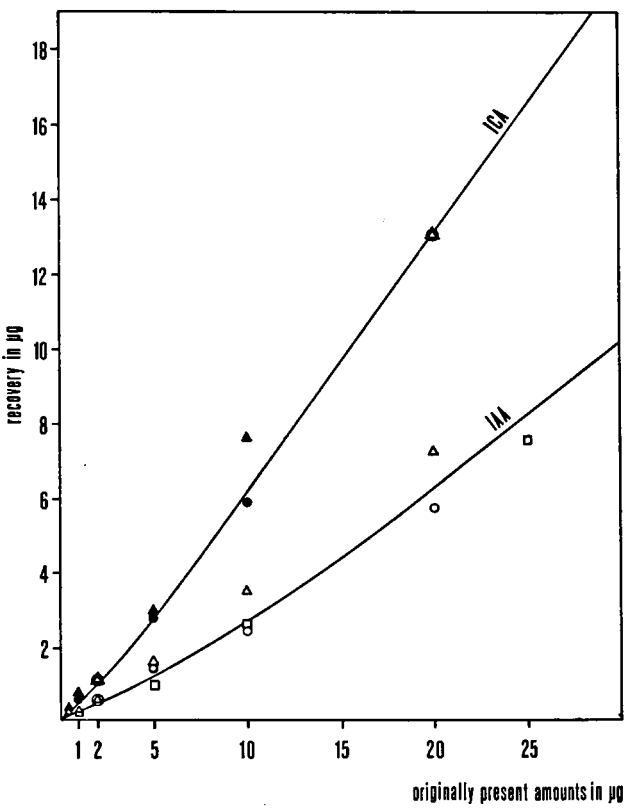

Fig. 1. Recovery curves of IAA and ICA, determined after extraction and twofold chromatography.

IAA and ICA were isolated by the twofold chromatography method and estimated spectrofluorometrically.

Fig. 1 shows the curves obtained for the recovered IAA and ICA as functions of the original amounts. The lines are not strictly linear, but are curved in the lower region.

2.5. Sources of chemicals, with abbreviations used

The chemicals used in the experiments and the sources from which they were obtained are: L-tryptophan (L-TTP), indole-3-acetic acid (IAA), indole-3-pyruvic acid (IPyA), 5-hydroxy-indole-3-acetic acid (5-OH-IAA), indole-3-acetamide (IAAm), indole-3-propionic acid, tryptophol (T-OH), tryptamine (T-NH_2), indole-3-acetonitrile (IAN), indole-3-glyoxylic acid (IGA), indole-3-glyoxylamide, indole-3-aldehyde (IAld), DL-kynurenin, anthranilic acid, indole, skatole, α-ketoglutaric acid, pyridoxal-5'-phosphate, dimedone, all obtained from Fluka AG Chemische Fabrik, Buchs SG, Switzerland; indole-3-carboxylic acid (ICA) from Kepec Chemische Fabrik GMBH, Siegburg, Germany; indole-3-acetaldehyde bisulfite addition (IAAld bis.) and indole-3-glycollic acid (IGolA) from K & K Laboratories Inc., Plainview, N.Y. U.S.A.; indole-3-lactic acid (ILA) from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

All organic solvents used in the extraction and chromatographic procedures were redistilled.

3. RESULTS

3.1. Incubation experiments with sterile cell-free enzyme preparations of root nodule tissue

With respect to these and similar experiments it must be kept in mind that nodules are very complicated systems. Rhizobial infection of the already differentiated cortical root tissue causes various changes in this tissue, as shown, for instance, by the meristematic activity. Moreover, the cells of the nodule tissue are filled with the rhizobial bacteria, most of which have been altered to the so-called bacteroids. Consequently, comparison of this tissue with normal root tissue offers many difficulties, particularly because a good reference measure is lacking.

Problems were encountered in the preparation of the enzyme extract. When the clear, crude enzyme preparation obtained after ultracentrifugation was held at 4 °C, a grey precipitate formed in the brown solution after a few hours. This process was more rapid at room temperature and was independent of the presence or absence of oxygen in the atmosphere, probably due to a chemical bonding of phenoles to the proteins (LOOMIS & BATAILE 1966). Addition of granulated, hydrated nylon (see Loomis & Bataile) before the addition of buffer to the tissue powder failed to give any visible improvement, but dialysis eliminated the precipitate formation. When undialyzed enzyme extracts were to be used, these extracts had to be filter-sterilized immediately after ultracentrifugation and then frozen, although some precipitate formed at thawing. However, the protein contents of the sterile undialyzed and the dialyzed enzyme extracts did not differ essentially, and the presence of some precipitate in the undialyzed enzyme preparation was therefore accepted.

Table 1. Production of IAA and ICA in incubation mixtures with nodule enzyme preparations.

Enzyme		Substrate + additives			Products			
					IAA		ICA	
dialyzed	boiled	L-TTP	α-ketoglutaric acid	pyridoxal phosphate	Total IAA (in μg)	IAA in μg per mg protein[1]	Total ICA (in μg)	ICA in μg per mg protein[1]
−	−	+	−	−	1.9 ± 0.3	0.22 ± 0.03	0.4	0.05
−	−	+	−	−	2.1 ± 0.0	0.25 ± 0.00	0.3	0.04
−	−	+	+	+	10.1 ± 0.8	1.24 ± 0.10	3.1[2]	0.38[2]
−	−	+	+	+	8.3 ± 0.3	1.01 ± 0.03	3.9[2]	0.48[2]
+	−	+	−	−	2.0 ± 0.4	0.26 ± 0.06	−	−
+	−	+	+	+	13.3 ± 0.5	1.82 ± 0.07	2.3	0.31
+	−	+	+	+	13.2 ± 1.7	1.80 ± 0.23	2.1	0.29
+	−	+	+	−	8.7 ± 0.2	1.16 ± 0.02	1.5	0.20
+	−	+	−	+	1.3 ± 0.3	0.11 ± 0.05	0.2	0.01
+	−	−	+	+	0.3 ± 0.2	−	0.1	−
−	−	−	−	−	0.1 ± 0.0	−	−	−
−	+	+	+	+	0.4 ± 0.1	−	0.1	−
+	+	+	+	+	0.4 ± 0.1	−	0.2	−
no enzyme		+	−	−	0.1 ± 0.1	−	0.0	−
no enzyme		+	+	+	0.2 ± 0.0	−	0.0	−

[1] corrected for spontaneous production of IAA and ICA in the incubation mixtures and for the amounts of these substances already present in the enzyme extracts.
[2] spectrofluorometrically impure.

3.1.1. Enzymatic production of IAA and ICA from tryptophan

Table 1 shows the quantitative data on the enzymatic production of IAA and ICA from L-TTP. With respect to the data concerning the enzymatic IAA production, some conclusions can be drawn:

1. In the absence of additives there is no difference between the IAA production in the presence of non-dialyzed and dialyzed enzyme extracts.

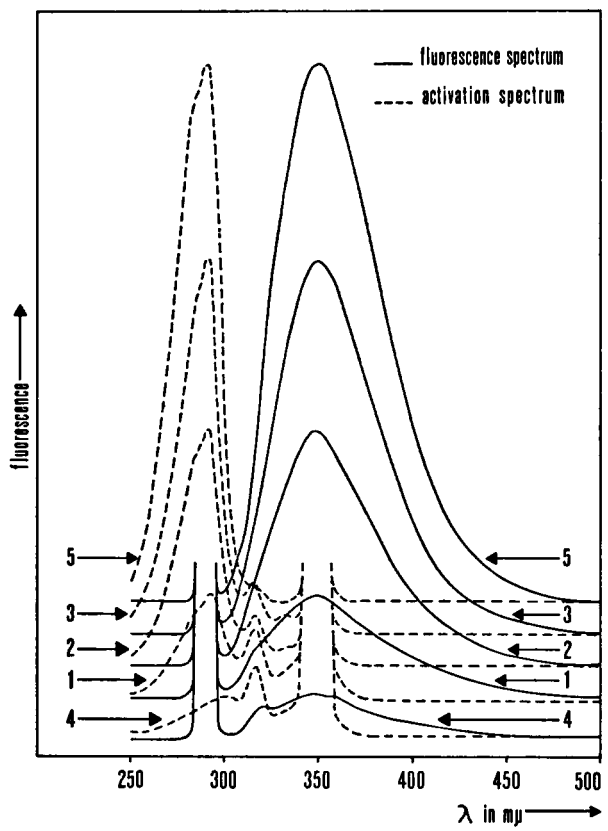

Fig. 2. Spectrofluorograms of eluates of the IAA zones of chromatograms of extracts prepared after incubation of the following mixtures and of pure IAA:
1. nodule enzyme + L-TTP
2. nodule enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
3. dialyzed nodule enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
4. boiled dialyzed nodule enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
5. pure IAA

2. In the presence of additives the dialyzed enzyme extract shows slightly higher IAA yield than the non-dialyzed enzyme extract.
3. The presence of α-ketoglutaric acid is much more important than that of pyridoxal phosphate, and in general the addition of both these compounds to the incubation mixture is necessary for good IAA production.

Fig. 2 shows some spectrofluorograms indicating that the compounds isolated from the chromatogram spots with the Rf of IAA are identical with pure IAA.

With respect to the data on ICA production from tryptophan it must be said that they are presented with reservations. The spectrofluorograms of the compounds isolated from the spots with the Rf value of ICA were sometimes, but not always, not quite identical with those of pure ICA (*cf. fig. 3*). This

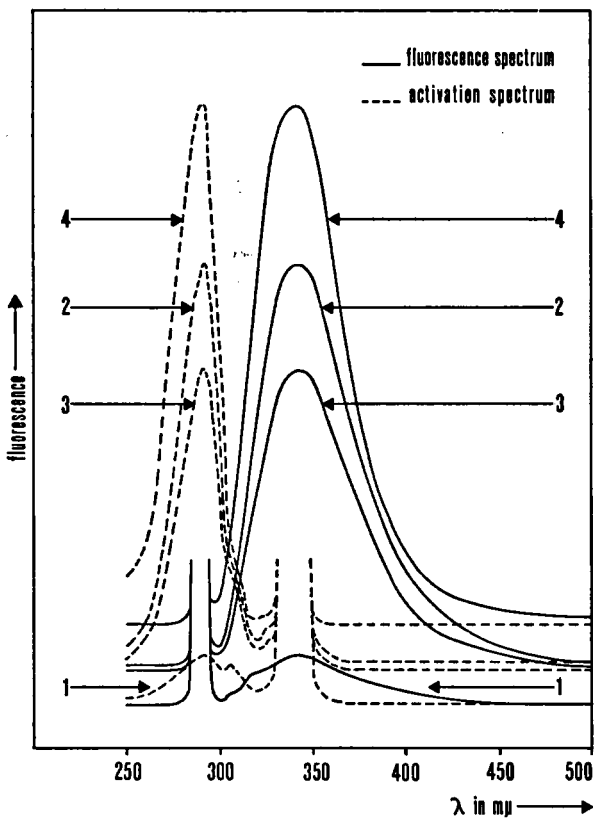

Fig. 3. Spectrofluorograms of eluates of the ICA zones of chromatograms of extracts prepared after incubation of the following mixtures and of pure ICA:
1. nodule enzyme + L-TTP
2. nodule enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
3. dialyzed nodule enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
4. pure ICA

could be due to the presence of an impurity. Consequently, these data can only be considered to give a rough impression of ICA production.

Figs. 4 and *5* show drawings of two two-dimensional thin-layer chromatograms (TLC) of the acid + neutral fraction of the extracts of two incubation mixtures. The first represents the Salkowski-positive compounds extracted from L-TTP incubated with only non-dialyzed enzyme preparation, the second the Salkowski-positive components extracted from an incubation mixture containing dialyzed enzyme extract, L-TTP, and the cofactors α-ketoglutaric acid and pyridoxal phosphate. It is evident that they contain essentially the same substances, chromatographically identical with: IAA, ICA, IAld, IGA; moreover, both TLCs show two unidentified spots, called here A and B. However, the pattern of the second TLC is much more distinct than that of the first, which must be ascribed to the higher concentrations of all the components. Consequently, the addition of the cofactors only promotes the already detectable enzymatic TTP conversion; in other words: the presence of the cofactors does not induce any other pathway of TTP conversion.

The fact that the IAA production from L-TTP is promoted by the addition of pyridoxal phosphate and α-ketoglutaric acid offers evidence that a transamination reaction is involved in this conversion. Replacement of α-ketoglutaric acid by sodium pyruvate gave the same result (*cf. table 4*). This indicates that indole-pyruvic acid (IPyA) is formed as the first intermediate in this pathway.

To investigate a possible alternative pathway of IAA bioproduction from L-TTP via tryptamine (T-NH_2), basic ether-extracts of the incubation mixtures

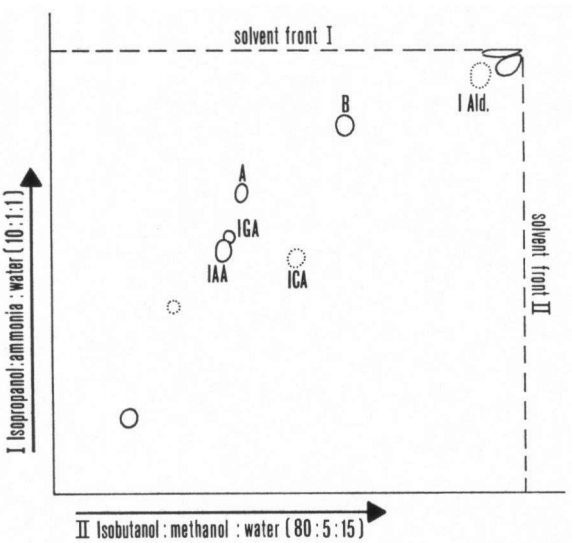

Fig. 4. Drawing of a two-dimensional TLC of the acid + neutral fraction of an extract of an incubation mixture containing nodule enzyme and L-TTP; sprayed with Salkowski's reagent.

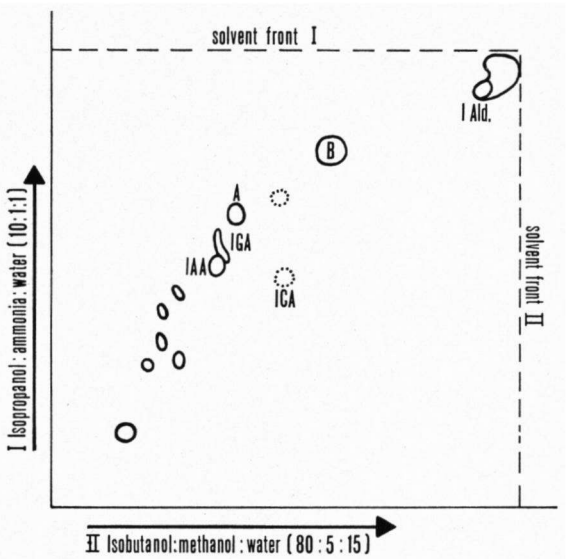

Fig. 5. Drawing of a two-dimensional TLC of the acid + neutral fraction of an extract of an incubation mixture containing dialyzed nodule enzyme, L-TTP, and pyridoxal phosphate + α-ketoglutaric acid; sprayed with Salkowski's reagent.

were studied by TLC. However, T-NH_2 was never detected, not even in minuscule amounts.

3.1.2. Enzymatic degradation of IAA and ICA

Since IAA and ICA production in the incubation mixtures would be negatively influenced by any degradation processes, some observations were made concerning the possible destruction of IAA and ICA. The results are presented in *table 2.*

It is clear from these results that breakdown of IAA occurs, whether or not enzyme extract is present. Consequently, for the interpretation of the IAA pro-

Table 2. Degradation of IAA and ICA in the presence of nodule enzyme extracts.

Incubation mixture (5 ml)	Amounts degraded (μg)
IAA[1] + dialyzed enzyme[2]	29.1 ± 1.6
IAA[1] + dialyzed enzyme[2]	23.3 ± 4.2
IAA[1] + non-dialyzed enzyme[3]	21.0 ± 0.5
IAA[1] + non-dialyzed enzyme[3]	15.5 ± 3.5
IAA[1] without enzyme	8.0 ± 3.0
ICA + non-dialyzed enzyme[3]	0
ICA without enzyme	0

[1] 50 μg per 5 ml
[2] 3.5 mg protein per 5 ml
[3] 3.9 mg protein per 5 ml

duction results the destruction during incubation must be taken into account. ICA appeared not to be degraded.

3.1.3. Conversion of some related indole derivatives

Experiments were performed to collect more information about the indole metabolism in the different enzyme and rhizobial systems for several reasons:

1. the possibility exists that there are alternative pathways of IAA and ICA production;
2. to explain the origin of some substances present in the TLCs of extracts of enzyme-incubation mixtures with L-TTP (see 3.1.1. and *figs. 4* and *5*);
3. to study the origin of some indole substances detected in extracts of root and nodule tissues (DULLAART 1967);
4. to compare the different potential enzymatic conversion reactions present in the different systems.

Table 3 shows the reaction products (identified by TLC) extracted from incubation mixtures of non-dialyzed enzyme extracts and a number of indole compounds. The TLCs of these extracts were consistently compared with those of incubations without enzyme extracts to distinguish spontaneously formed products from those produced enzymatically. In this table IPyA and IGolA are omitted, because of the extreme instability of these substances during the procedure, as a result of which comparison of the TLCs of the extracts of incubation mixtures with and without enzyme preparation did not yield significant differences.

Apart from the conversion of IAAm, the reaction products presented in *table 3* were produced in very small amounts. Since this also holds for IAA formation from T-NH_2, it supplies another indication for the improbability of the occurrence of a significant IAA production pathway from tryptophan via T-NH_2.

The production of IGA together with ICA from IAld is very striking and means that an enzymatic carboxylation must have taken place. This specific reaction has not been described earlier.

The slight conversion of IAAld bisulfite addition product probably implies

Table 3. Enzymatic conversion of some indole substances in incubation mixtures with non-dialyzed nodule enzyme extract.

Incubated indole compound	Reaction products
tryptamine	IAA, ICA, IGA.
tryptophol	IAA.
indolelactic acid	no conversion.
indoleacetic acid	no conversion.
indolecarboxylic acid	no conversion.
indoleacetamide	IAA (large amounts).
indoleacetaldehyde-bis	IAA, ICA, IAld.
indolealdehyde	ICA, IGA.

that IAAld can be converted to a minor extent by this enzyme extract.

The production of large amounts of IAA from IAAm seems very interesting. However, it does not appear justified to suggest that IAAm might be an important intermediate product in the IAA production from L-TTP, because of the promotion of this IAA production by α-ketoglutaric acid and pyridoxal phosphate. Moreover, not even a trace of IAAm was ever detected in the extracts of the enzyme-incubation mixtures with L-TTP.

There seem to be good grounds to pay special attention to the two unidentified spots, A and B, present in the TLCs of extracts of incubation mixtures of L-TTP and enzyme extracts. These two "mysterious" spots always show identical colour reactions with the various spray reagents: with Salkowski's reagent both are violet, with Ehrlich's reagent violet, and with DMCA bluish-violet. These compounds were only produced from L-TTP and not from any of the other indole derivatives applied. It is evident that the production of these substances was stimulated by the addition of α-ketoglutaric acid and pyridoxal phosphate, as was the case for the production of IAA. After fractionation of the extracts these substances are present in the acid fraction. They do not show fluorescence on TLC inUV light. Since these compounds are not produced from IPyA, it seems likely that they are intermediate products of the IPyA formation from L-TTP. So far, however, not the slightest evidence about their identity has been obtained.

3.1.4. The metabolic pathway of IAA production from tryptophan

The great likelihood that IPyA is an intermediate in the IAA production pathway from L-TTP gives rise to some important questions, the main one being whether the measured amounts of IAA were already present in the extracts of the incubation mixtures or were formed as a product of spontaneous degradation of IPyA during the chromatographic separation. Since it has been demonstrated (KAPER 1957; KAPER & VELDSTRA 1958, KAPER *et al.* 1963 a,b) that IPyA undergoes very rapid spontaneous destruction during chromatography with ammoniacal solvents and gives rise to the formation of a stable pattern of spots among which IAA is an important one, and the first part of the twofold chromatography procedure includes an ammoniacal solvent, it seems plausible that the IAA was formed during chromatography from IPyA. Therefore the degradation behaviour of IPyA was studied in relation to the chromatographic data of the extracts of the incubation mixtures. *Fig. 6* presents a drawing of the stable pattern of spots resulting from a two-dimensional TLC of authentic IPyA, performed in the same way as for the extracts and including an ammoniacal isopropanol solvent in the first dimension. As can be seen, among some unidentified spots IAA, ICA, IAld, IAAm, IGolA, and IGA belong to the stable spot pattern. Comparison of *fig. 6* with *figs. 4* and *5* shows that the latter two pictures lack spots of IGolA and IAAm as well as at least one of the unidentified spots. Actually, no IAAm was detected on the TLCs of the extracts, and although the presence of IGolA was suspected in a few cases, it could not be demonstrated. Thus, it is very unlikely that IPyA was ever

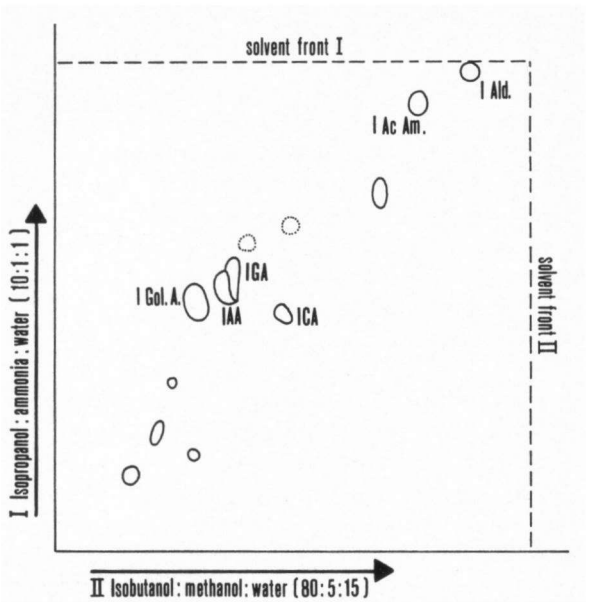

Fig. 6. Drawing of a two-dimensional TLC of authentic IPyA; sprayed with Salkowski's reagent.

present in significant amounts in the extracts of the incubation mixtures, which gives support to the belief that at least most of the measured amounts of IAA had already been present in the extracts.

This finding gives rise to the important question how IAA is formed from IPyA in the incubation mixtures. It is generally believed that in plants IPyA is decarboxylated to indoleacetaldehyde (IAAld), which would in turn be converted to IAA by oxidation. This implies that IAAld would appear as a free intermediate in this reaction pathway. To check this, a few incubation experiments were carried out in which $NaHSO_3$ (which is known to give an addition product with aldehydes) in a concentration equimolar to the L-TTP concentration, or dime-

Table 4. Production of IAA and ICA in different incubation mixtures with dialyzed nodule enzyme preparation.

Standard incubation mixture	Additives	Products	
		IAA in μg	ICA in μg
dialyzed enzyme preparation + L-TTP.	α-ketoglutaric acid	12.3 ± 1.3	3.3
	α-ketoglutaric acid + pyridoxal phosphate	22.5 ± 2.5	1.4 ± 0.4
	α-ketoglutaric acid + pyridoxal phosphate + $NaHSO_3$	0.1	–
	α-ketoglutaric acid + pyridoxal phosphate + dimedone	18.8 ± 1.3	2.8
	Na-pyruvate + pyridoxal phosphate	24.8 ± 2.3	4.3

done (which is known to react specifically with aldehydes, resulting in insoluble products) in the same molar concentration as L-TTP, was added to the incubation mixtures. The results of these experiments are presented in *table 4*, together with some supplementary experimental results.

These results show that $NaHSO_3$ completely inhibits IAA production. This might be due to the fact that $NaHSO_3$ reacts not only with aldehydes but also with keto-acids, so that the whole reaction is blocked, and furthermore, the probable presence of some SO_2 under the incubation conditions might inhibit the enzymatic functions. Thus, $NaHSO_3$ cannot be the appropriate substance, because it is not sufficiently specific.

With regard to the effect of dimedone, it is shown that IAA production is slightly but far from completely inhibited. This can only be understood by assuming that IAAld does not play a very significant role as a free intermediate under these conditions. A breakdown of IPyA without IAAld as an intermediate might be conceivable as an enzymatic process, e.g., an oxidative decarboxylation. On the other hand, the possibility of a non-enzymatic sequence must also be taken into consideration, since under the present incubation conditions there is a spontaneous degradation of IPyA with IAA as one of the important products. This can be seen from *fig. 7*, which shows a two-dimensional TLC of the neutral + acid fraction of an extract of IPyA incubated under sterile conditions without enzyme preparation. It is interesting to compare this picture with *fig. 6*. It is striking that in *fig. 7* the spot of IAAm is lacking, and the spot of IGolA is very weak in comparison with the corresponding spot in *fig. 6*.

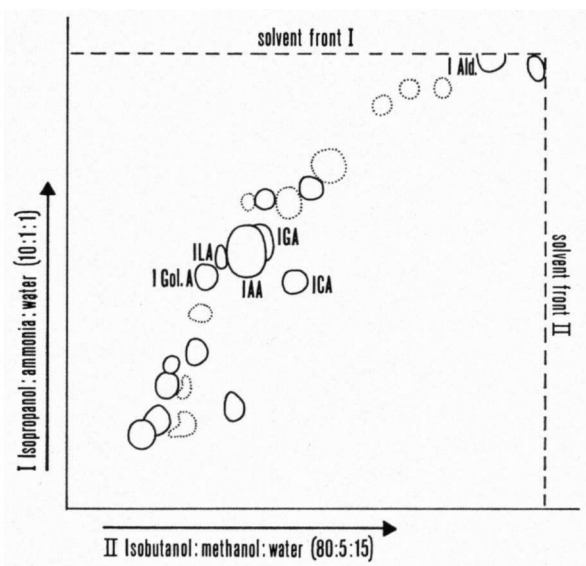

Fig. 7. Drawing of a two-dimensional TLC of the acid + neutral fraction of an extract of IPyA incubated under sterile conditions without enzyme preparation. Chromatogram sprayed with Salkowski's reagent.

This might mean that this extract contained no IPyA or only a very small amount, which would indicate that most of the incubated IPyA was broken down during incubation. Two-dimensional TLC of an extract of a comparable IPyA incubation mixture to which dimedone was added showed that IAA is formed in spite of the presence of dimedone.

Consequently, the formation of IAA from IPyA by non-enzymatic breakdown must be considered as a real possibility,although it seems rather illogical that the bioproduction of so important a substance could be dependent upon such a spontaneous process. It must be kept in mind, however, that these experiments were carried out with crude, cell-free enzyme preparations. It remains possible that the enzymes necessary for IAA production are actually present in a multi-enzyme system, so that there would be little or no free IAAld in the cell. In other words, the possibility exists that the formation of IAA from IPyA *in vivo* actually runs via IAAld or via a direct oxidative decarboxylation.

3.1.5. Pathways of ICA formation from tryptophan

ICA may be considered as a by-product of IAA formation from L-TTP via IPyA. It can be formed in different ways and is probably one of the products directly resulting from the spontaneous degradation of IPyA. But, as shown in *table 3*, it is also formed enzymatically from IAld, which is also one of the degradation products of IPyA. Moreover, it was demonstrated that during incubation ICA is spontaneously formed from IGA, which is in turn a non-enzymatic degradation product of IPyA. It must be stressed, however, that an enzymatic or non-enzymatic production of ICA from IAA was never observed in the present series of incubation experiments.

Unfortunately, the quantitative estimations of the amounts of ICA produced from L-TTP during the incubation experiments were not completely reliable. But these data nonetheless undoubtedly reflect the right order of magnitude. This is important with respect to the possibility of evaluating the quantitative relation between the amounts of IAA and ICA produced.

3.2. Incubation experiments with sterile, cell-free enzyme preparations of roots

For purposes of comparison, the metabolism of IAA production from L-TTP in mature nodule-bearing roots required investigation with exclusion of the nodule zones.

A study of the IAA production from L-TTP by enzyme extracts of roots with higher meristematic activity was also necessary. For this investigation 14-day-old seedling roots of lupine, grown in the laboratory under semi-sterile conditions – thus non-nodulated – were used (to be called young roots).

Homogenization of the mature root material proved to be rather laborious, but this was not the case for the young roots.

As was to be expected, the protein content of these enzyme preparations was rather low: about ten times lower than that of the nodule-enzyme preparations. For comparability of the results of these incubation experiments, the

volume of enzyme preparation was taken ten times higher than that used in the nodule-enzyme incubation experiments.

3.2.1. Enzymatic production of IAA and ICA from tryptophan

Tables 5 and *6* show the quantitative data concerning the enzymatic production of IAA and ICA, *table 5* for the mature roots and *table 6* for the young roots.

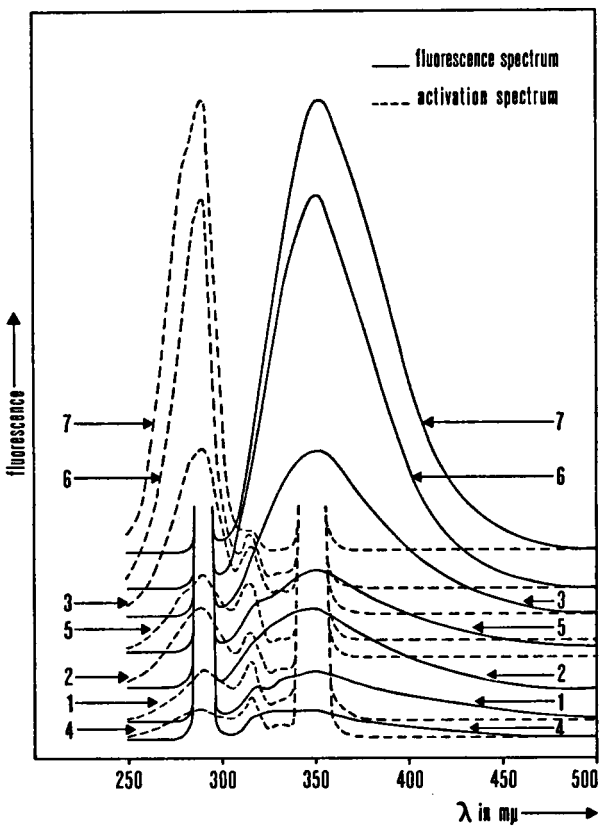

Fig. 8. Spectrofluorograms of eluates of the IAA zones of chromatograms of extracts prepared after incubation of the following mixtures and of pure IAA:
1. mature root enzyme + L-TTP
2. mature root enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
3. dialyzed mature root enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
4. young root enzyme + L-TTP,
5. young root enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
6. dialyzed young root enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
7. pure IAA

Table 5. Production of IAA and ICA in incubation mixtures with mature root enzyme preparations.

Enzyme		Substrate + additives			Products			
					IAA		ICA	
dialyzed	boiled	L-TTP	α-ketoglutaric acid	pyridoxal phosphate	Total IAA (in μg)	IAA in μg per mg protein[1]	Total ICA (in μg)	ICA in μg per mg protein[1]
−	−	+	−	−	0.8	0.08	0.4	0.05
−	−	+	+	+	2.4 ± 0.1	0.31 ± 0.02	0.3	0.03
−	−	+	+	+	2.1 ± 0.5	0.26 ± 0.07	0.3	0.03
+	−	+	+	+	4.7 ± 0.0	0.62 ± 0.00	0.2	0.04
+	−	+	+	+	4.2 ± 0.5	0.56 ± 0.07	0.3	−
+	−	−	−	−	0.1	−	0.0	−
−	−	−	−	−	0.2	−	0.1	−
+	+	+	+	+	0.2	−	0.1	−
no enzyme		+	+	+	0.2 ± 0.0	−	0.0	−
no enzyme		+	—	—	0.1 ± 0.1	−	0.0	−

[1] corrected for spontaneous production of IAA and ICA in the incubation mixtures and for the amounts of these substances already present in the enzyme extracts.

Table 6. Production of IAA and ICA in incubation mixtures with young root enzyme preparations

Enzyme		Substrate + additives			Products			
					IAA		ICA	
dialyzed	boiled	L-TTP	α-ketoglutaric acid	pyridoxal phosphate	Total IAA (in μg)	IAA in μg per mg protein[1]	Total ICA (in μg)	ICA in μg per mg protein[1]
−	−	+	−	−	0.4 ± 0.0	0	−	−
−	−	+	+	+	2.4 ± 0.1	0.44 ± 0.01	1.6	0.31
−	−	+	+	+	2.2 ± 0.1	0.40 ± 0.02	1.6	0.30
+	−	+	−	−	1.5 ± 0.5	0.21 ± 0.09	0.9	0.14
+	−	+	+	+	11.8 ± 0.3	2.09 ± 0.05	1.7	0.29
+	−	+	+	+	11.8 ± 0.8	2.09 ± 0.14	2.1	0.37
+	+	+	+	+	0.0 ± 0.0	−	0.3 ± 0.0	−
−	+	+	+	+	0.2	−	0.3 ± 0.0	−
−	−	−	−	−	0.3	−	0.3	−
+	−	−	−	−	0.3	−	0.1	−
no enzyme		+	+	+	0.2 ± 0.0	−	0.0	−
no enzyme		+	−	−	0.1 ± 0.1	−	0.0	−

[1] corrected for spontaneous production of IAA and ICA in the incubation mixtures and for the amounts of these substances already present in the enzyme extracts.

From the data on the enzymatic IAA production from L-TTP by enzyme preparations deriving from both tissue sources it may be concluded that addition of α-ketoglutaric acid and pyridoxal phosphate gives a strong promotion of the IAA formation and that dialysis of the enzyme preparations results in a larger output as compared to the IAA production obtained with non-dialyzed enzyme preparations, both in the presence of the cofactors. The marked enhancement of the IAA production caused by dialysis of the young root enzyme

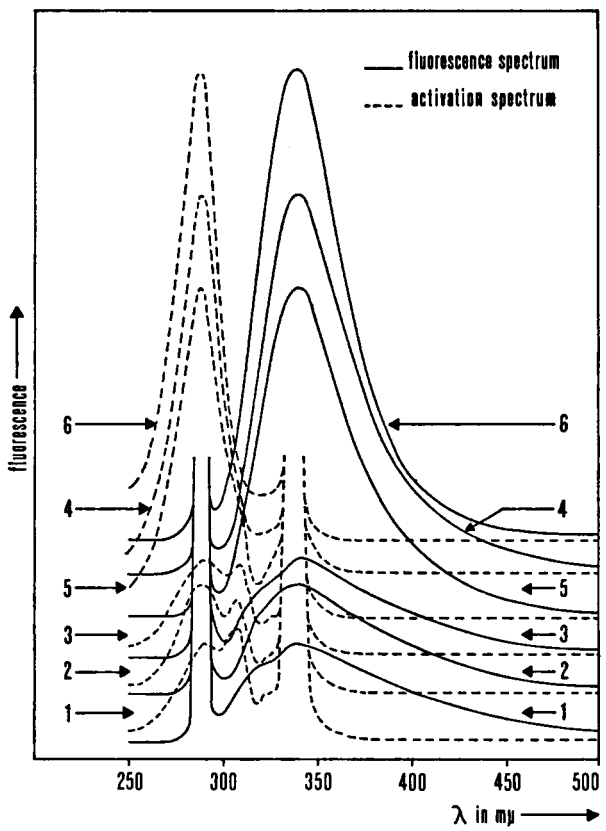

Fig. 9. Spectrofluorograms of eluates of the ICA zones of chromatograms of extracts prepared after incubation of the following mixtures and of pure ICA:
1. mature root enzyme + L-TTP
2. mature root enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
3. dialyzed mature root enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
4. young root enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
5. dialyzed young root enzyme + L-TTP + pyridoxal phosphate + α-ketoglutaric acid
6. pure ICA

preparation is very striking, the more so since there was little or no effect on the ICA production.

Spectrofluorometric checks demonstrated that the substances isolated from the incubation mixtures were indeed identical with authentic IAA (*fig. 8*) or ICA (*fig. 9*). The results of the ICA estimations in the extracts of incubation mixtures with mature root enzyme extracts must be regarded with some reservation, since the measured amounts were very small and distinct spectrofluorograms could not be obtained. However, as was shown by TLC, ICA was produced from L-TTP enzymatically. The data may be considered to give a correct impression of the order of magnitude of the ICA production in the different incubation mixtures with the mature root enzyme preparations.

Figs. 10 and *11* represent two-dimensional TLCs of the Salkowski-positive substances present in the acid + neutral fraction of L-TTP incubation mixtures with young root enzyme preparations. The same volumes of the extracts were spotted in all cases. *Fig. 10* shows a drawing of a TLC of the extract of the incubation of L-TTP plus non-dialyzed enzyme extract, *fig. 11* one of a TLC of the substances extracted from the incubation mixture containing dialyzed enzyme preparation, L-TTP, α-ketoglutaric acid, and pyridoxal phosphate. The spot patterns of the TLCs of the extracts of the identical incubation mixtures with mature root enzyme preparations were very similar and are therefore not illustrated.

The spot patterns in *figs. 10* and *11* are essentially the same, except that the latter is much more distinct than the former. Both show IAA and ICA, the largest amounts being present in the latter, while this TLC also contained IAld

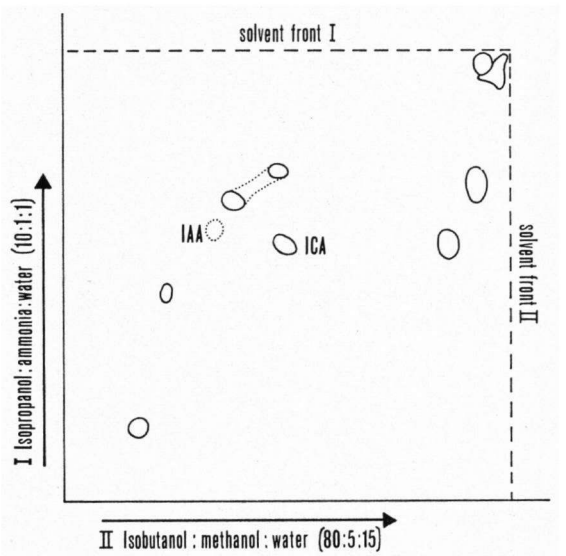

Fig. 10. Drawing of a two-dimensional TLC of the acid + neutral fraction of an extract of an incubation mixture containing young root-enzyme preparation and L-TTP; sprayed with Salkowski's reagent.

and some IGA. The presence of the two spots A and B, already known from the extracts of the nodule enzyme incubation mixtures with L-TTP (*cf.* 3.1.1., *figs. 4* and *5*), is very striking. The presence of some unidentifiable yellowish spots near the second solvent front was not specific for incubation with L-TTP, since they were also found for extracts of incubation mixtures with other indole derivatives.

These results demonstrate that the production of IAA from L-TTP with both root enzyme preparations is promoted by addition of α-ketoglutaric acid and pyridoxal phosphate, which suggests that a transamination reaction is involved in this conversion. This indicates that IPyA is formed as the first intermediate in this pathway.

The failure to detect T-NH_2 in alkaline ether extracts of these incubation mixtures indicates that this substance is not produced from L-TTP.

3.2.2. Enzymatic degradation of IAA and ICA

Since IAA and ICA production during incubation would be negatively influenced by destruction, the possibility of IAA and ICA breakdown was studied. The results presented in *table 7* show that breakdown of IAA occurs during incubation in both the presence and absence of enzyme preparations. Consequently, the occurrence of destruction must be taken into account in the interpretation of the IAA production results.

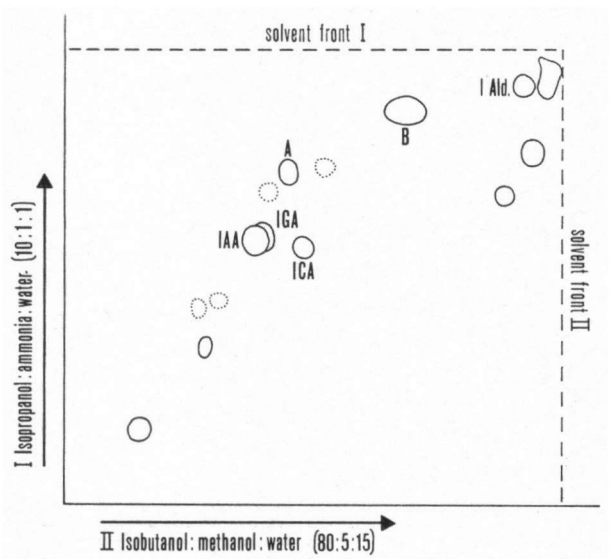

Fig. 11. Drawing of a two-dimensional TLC of the acid + neutral fraction of an extract of an incubation mixture containing dialyzed young root-enzyme preparation, L-TTP, pyridoxal phosphate, and α-ketoglutaric acid. Chromatogram sprayed with Salkowski's reagent.

It is evident from these results that the level of IAA destruction in the presence of mature root enzyme extracts is lower than that in the presence of young root enzyme preparations. Furthermore, the non-dialyzed young root enzyme extract shows a significantly higher IAA destruction than the dialyzed young root enzyme extract, which might be explained by the presence of some cofactor substance(s) of enzymatic IAA destruction processes in the former. The two mature root enzyme extracts do not show any significant difference in this respect.

No ICA degradation was found in either the presence or absence of these enzyme preparations.

The greater IAA destruction in the presence of non-dialyzed young root enzyme extract suggests that this difference is responsible for the strong alteration of the IAA/ICA production ratio after dialysis of the enzyme preparation (*cf.* 3.2.1.). This might mean that in young, meristematic root tissue there is actually a substantial production of IAA from L-TTP via IPyA, as suggested by the fact that the IAA production per mg protein in the presence of this dialyzed enzyme is quite comparable with the specific IAA production in the presence of dialyzed nodule enzyme preparation (*cf.* 3.1.1.). However, in this root tissue most of the IAA is readily destroyed. ICA, also produced but not destroyed, will remain, and consequently the amount of this substance will increase. This seems to offer an acceptable explanation of the observed high ICA content in relation to the IAA content of roots of lupine (DULLAART 1967).

3.2.3. Conversion of some related indole derivatives

Table 8 shows the TLC-identified reaction products extracted from incubation mixtures of non-dialyzed mature root and young root enzyme extracts and a

Table 7. Degradation of IAA and ICA in the presence of enzyme preparations of mature and young roots.

	Incubation mixture (5 ml)	Amount degraded (μg)
mature roots	IAA[1] + dialyzed enzyme[2]	21.0 ± 1.5
	IAA[1] + non-dialyzed enzyme[3]	21.6 ± 2.4
	IAA[1] without enzyme	8.0 ± 3.0
	ICA[1] + non-dialyzed enzyme[3]	0
	ICA[1] without enzyme	0
young roots	IAA[1] + dialyzed enzyme[4]	25.2 ± 0.2
	IAA[1] + non-dialyzed enzyme[5]	30.4 ± 1.6
	IAA[1] without enzyme	8.0 ± 3.0
	ICA[1] + non-dialyzed enzyme[5]	0
	ICA[1] without enzyme	0

[1] 50 μg per 5 ml
[2] 2.6 mg protein per 5 ml
[3] 2.2 mg protein per 5 ml
[4] 3.7 mg protein per 5 ml
[5] 4.4 mg protein per 5 ml

number of indole compounds. The TLCs of these extracts were compared in all cases with the TLCs of incubations without enzyme preparations as a check for the presence of spontaneously formed products.

Table 8. Conversion of some indole substances in incubation mixtures with non-dialyzed enzyme extracts of mature and young roots, as demonstrated by TLC.

Incubated indole compound	Reaction products	
	mature root enzyme extract	young root enzyme extract
tryptamine	IAA, ICA, IGA	IAA, ICA, IGA
tryptophol	IAA(?), ICA	IAA, ICA
indolelactic acid	no conversion	no conversion
indoleacetic acid	no conversion	no conversion
indolecarboxylic acid	no conversion	no conversion
indoleacetamide	IAA	IAA
indoleacetaldehyde-bis	ICA, IGA, IAld	ICA, IGA, IAld
indolealdehyde	ICA	ICA

The reaction products mentioned in *table 8* were found in very small amounts. The similarity of the results for the two enzyme preparations indicates that they possess the same enzymatic conversion systems.

The formation of IAA from T-NH_2 by both enzyme extracts is noteworthy. However, the very low effeciency of this reaction indicates that it is improbable that tryptamine is an important intermediate substance in the enzymatic IAA production pathway from L-TTP in the incubation mixtures witn these root enzyme preparations.

The presence of spots A and B on the TLCs of the extracts of L-TTP incubation mixtures with both root enzyme extracts is very striking. Since these compounds had already been observed on the TLCs of the nodule enzyme incubation extracts (*cf.* 3.1.3.), their nature becomes even more intriguing.

3.2.4. The metabolic pathway of IAA production from tryptophan

As already mentioned, IPyA is presumably an intermediate substance in this metabolic pathway. Since spots of IAAm and IGolA were not detected on any of the TLCs of the extracts, it may be assumed that, according to the argumentation presented in 3.1.4., most of the IPyA had been degraded before extraction, possibly spontaneously.

The stimulation of IAA production by dialysis of the enzyme extracts is very conspicuous. Since in the case of the mature root enzyme extracts there was no significant difference of IAA destruction in the presence of both non-dialyzed and dialyzed enzyme preparations (*table 7*), while for the dialyzed young root enzyme extract this stimulation seems too high in relation to the IAA output with non-dialyzed enzyme extract to be entirely explained by the difference in IAA destruction, it seems possible that dialysis led to the loss of some inhibitor of the enzymatic IAA formation process.

3.2.5. Production of ICA from tryptophan

The possible pathways of ICA formation from L-TTP via IPyA have already been discussed (3.1.5.). As stated above (3.2.1.), the quantitative ICA production data for mature root enzyme preparations are not entirely reliable because of the very low outputs, but they undoubtedly give the right impression of the order of magnitude. The data on ICA production for young root enzyme preparations are reliable, however. As will be discussed below, the quantitative relation of these ICA production data to the IAA production data may be very important with respect to the main problem investigated in this study.

3.3. Incubation experiments with cell suspensions of the bacterial symbiont, *Rhizobium lupini* (Schroeter) Eckhardt *et al.*

Several earlier studies on IAA production from L-TTP by *Rhizobium* in various culture media are available (LINK 1937; CHEN 1938; THIMANN 1939; GEORGI & BEGUIN 1939), and more recently detailed data on rhizobial TTP conversion have been presented by KEFFORD *et al.* (1960), working with different strains of *Rhizobium trifolii*, *Rh. meliloti*, and *Rh. japonicum*, and by BULARD *et al.* (1963), RIGAUD & BULARD (1965), RIGAUD (1966, 1967) for *Rh. meliloti*, and HARTMANN & GLOMBITZA (1967) for *Rh. leguminosarum*. These studies have shown that together with IAA several indole compounds, such as IAAld, ILA, T-OH, IAld, and ICA, are produced from L-TTP.

There are, however, no publications concerning TTP conversion experiments with *Rh. lupini*. Since it is well-known that *Rh. lupini* is the specific symbiotic micro-organism in lupine root nodules and because there may be metabolic differences between the various *Rhizobium* species, the L-TTP conversion by *Rh. lupini* in general and the production of IAA and ICA in particular were investigated within the framework of this study.

The rhizobial strain: Although *Rh. lupini* is known to be a generally slow-growing species, some of the strains of *Rh. lupini* obtained in 1965 from Dr. Van Schreven of the *Rijksdienst voor de IJsselmeerpolders*, Kampen, The Netherlands, showed rather rapid growth in culture media. Since the slimy substances produced during growth interfered with the harvesting of the cells by centrifugation, the strain showing the least slime production, A 98, was chosen for the incubation experiments.

According to a letter from Dr. Van Schreven accompanying the shipment, the strains had just been checked with respect to their nodulating capacity and had proved to be very active. Recently (1969) a nodulation check experiment performed with strain A 98 and lupine seedlings yielded one plant possessing one effective nodule. An A 98 culture was also sent to the *Rijksdienst voor de IJsselmeerpolders* for a nodulation check. Their results showed no lupine nodulation at all but a rather good nodulation of lucerne, suggesting that this may actually be a *Rh. meliloti* strain. Exchange of cultures can be excluded, since our section has done no work with or possessed any cultures of *Rh. meliloti*. Our collection contained only *Rh. lupini* strains. Consequently, there must be an other explanation for these phenomena. According to Dr.

Van Schreven (personal communication), it is known that after culturing in the laboratory for some time, especially *Rh. lupini* cultures lose their nodulation capacity. It is also known that *Rhizobium* cultures easily become modified. Moreover, since the A 98 strain was chosen because of its rapid growth and low slime production, this strain is in all likelihood an atypical *Rh. lupini* strain with some special characteristics. The lucerne nodulation of this strain is very peculiar, but the cross-inoculation groups of *Rhizobium* (ALLEN & ALLEN 1958) are not yet completely defined and need much more study.

In 1968 a new culture sample of strain A 98 was received together with other *Rh. lupini* strains from the *Rijksdienst voor de IJsselmeerpolders*. The results of some experiments performed to check this fresh culture are shown in *table 10* and discussed below.

3.3.1. Bacterial production of IAA and ICA from tryptophan

Table 9 gives the quantitative data on the rhizobial production of IAA and

Table 9. Production of IAA and ICA in incubation mixtures with rhizobial cells.

Bacterial cells	Substrate + additives		Products	
	L-tryptophan	α-ketoglutaric acid + pyridoxal phosphate	IAA (μg)	ICA (μg)
+	+	−	30.2 ± 0.6	16.4 ± 1.4
+	+	−	23.6 ± 2.4	14.2± 1.2
+	+	+	34.8 ± 0.2	23.8 ± 0.4
+	+	+	34.4 ± 2.0	28.0 ± 0.4
+	−	−	0.8	0.2 ± 0.1
+	−	−	1.3 ± 0.0	0.3 ± 0.2
−	+	+	0.2 ± 0.0	0.0
−	+	−	0.1 ± 0.1	0.0

ICA from L-TTP, obtained by spectrofluorometry.

These results clearly demonstrate an enhancement of the production of IAA and ICA due to the addition of α-ketoglutaric acid and pyridoxal phosphate. The somewhat stronger increase of the ICA production as compared to the IAA production in the presence of these cofactors is difficult to explain.

The reliability of these results was demonstrated by spectrofluorometry of the isolated IAA and ICA solutions.

Fig. 12 presents a drawing of a two-dimensional TLC of the Salkowski-positive substances present in the acid + neutral fraction of an ether extract of an incubation mixture containing rhizobial cells and L-TTP.

The pattern of spots was not changed by the presence of α-ketoglutaric acid and pyridoxal phosphate in the incubation mixture. As can be seen from this figure, IAA, ICA, ILA, IGA, IGolA, IAld, and IAAm were present. All these substances belong to the spot pattern of chromatographically degraded authentic IPyA, indicating that a significant amount of IPyA was extracted from the incubation mixture. This fact, together with the enhanced IAA and ICA production due to the addition of α-ketoglutaric acid and pyridoxal phosphate to the

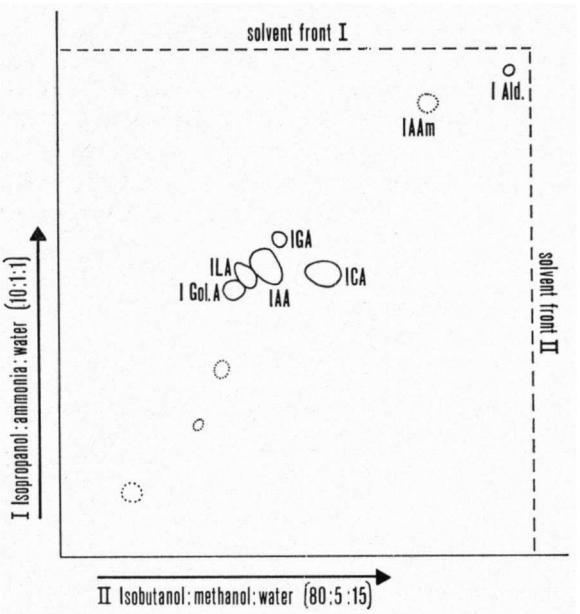

Fig. 12. Drawing of a two-dimensional TLC of the Salkowski-positive substances present in the acid + neutral fraction of an extract of an incubation mixture containing rhizobial cells and L-TTP.

incubation mixture, indicates the involvement of IPyA as an intermediate substance in this pathway.

Attempts to detect T-NH_2 in basic ether extracts of these incubation mixtures by TLC gave negative results, indicating that this compound was not produced from L-TTP.

Table 10 gives some quantitative data on IAA and ICA production from L-TTP by strain A 98–1968. All the experimental conditions were identical with those applied in the experiments with the older strain (A 98–1965) presented above.

These results are rather surprising in relation to the comparable results obtained with strain A 98–1965 (*table 9*). The final amount of IAA is very low as compared to the amount of ICA. There is only a slight enhancement of the

Table 10. Production of IAA and ICA in incubation mixtures with strain A 98–1968[1].

Incubation mixture	IAA (μg)	ICA (μg)
A 98–1968 + L-TTP	2.9 ± 0.2	13.2 ± 1.5
A 98–1968 + L-TTP	2.4 ± 0.4	13.2 ± 1.5
A 98–1968 + L-TTP + cofactors[2]	3.6 ± 0.1	13.3 ± 0.9
A 98–1968 + L-TTP + cofactors[2]	3.6 ± 0.1	13.3 ± 1.0

[1] The number of cells present in these mixtures was in the order of 10^{10}.
[2] α-ketoglutaric acid + pyridoxal phosphate.

IAA production due to addition of the cofactors, and the ICA yield remained constant.

The spectrofluorometric checks demonstrated that these results are reliable. TLCs of the extracts showed that they contained some IPyA.

Since the low IAA output as compared to that of ICA could have been due to the destruction of IAA by the A 98 strain, the possibility of IAA degradation by both the A 98–1968 and the A 98–1965 strains was investigated.

3.3.2. Rhizobial IAA degradation

Table 11 shows some experimental results concerning IAA and ICA destruction by the two A 98 strains.

Table 11. Degradation of IAA and ICA by two strains of *Rh. lupini* A 98.

Incubation mixture (5 ml)	Amount degraded (μg)
IAA[1] + A 98–1965[2]	11.5 ± 0.3
IAA[1] + A 98–1968[3]	49.5 ± 0.2
IAA[1] + without bacteria	8.0 ± 3.0
ICA[1] + A 98–1965[2]	0
ICA[1] + A 98–1968[3]	0
ICA[1] without bacteria	0

[1] 50 μg per 5 ml.
[2] 1.3×10^{10} cells per 5 ml.
[3] $\pm 10^{10}$ cells per 5 ml.

These results demonstrate that no destruction of ICA takes place. The IAA destruction by strain A 98–1965 is very slight. Strain A 98–1968 destroyed all the IAA during incubation, which confirms the suggestion that it possesses the capacity to degrade IAA (3.3.1.). The IAA yields presented in *table 10* could possibly be explained by the presumption that this IAA was produced during chromatography from residual IPyA present in the extract, as suggested by the TLC results.

It may therefore be concluded that the pattern of IAA destruction can differ considerably among *Rhizobium* strains. In this connexion a curious observation must be mentioned. Some years ago we isolated from a culture of strain A 98–1965 a modification that showed divergent colony growth and rapid IAA destruction, in contrast with the normal form, for which IAA destruction could not be detected (Dullaart & De Deckere, unpublished results). This may imply that IAA destruction by *Rhizobium* is an easily modifiable property. The divergent results obtained with *Rh. leguminosarum* by RIGAUD (1969) and HARTMANN & GLOMBITZA (1967) might be explained on this basis.

3.3.3. Conversion of some related indole derivatives

Table 12 gives the reaction products isolated by ether extraction from incubation

Table 12. Conversion of some indole compounds by rhizobia in incubation mixtures.

Incubated indole compound	Reaction products
tryptamine	IAA, T-OH
tryptophol	IAA
indolelactic acid	IPyA[1]
indoleacetic acid	no conversion
indolecarboxylic acid	IAld (?)
indoleacetamide	no conversion
indoleacetaldehyde-bis	IAA (large amounts), T-OH
indolealdehyde	ICA (large amounts)

[1] identified by its chromatographic degradation pattern.

mixtures of rhizobial cells (A 98–1965) with some indole compounds and identified by TLC. Spontaneously formed products are omitted. The production of IPyA from ILA (which is formed from L-TTP, possibly via IPyA, see 3.3.1) and the production of large amounts of ICA from IAld are very interesting. The importance of the ICA production will be discussed below (3.3.5.). The formation of ILA from L-TTP also seems important with respect to a distinction of the L-TTP conversion metabolisms of the rhizobial symbiont and the plant, since ILA has been detected in nodule extracts in very small amounts (DULLAART 1967) and the rhizobial cells produce this compound in rather large amounts from L-TTP.

With respect to the formation of IAA and T-OH from T-NH_2, the low efficiency of this conversion indicates that it is improbable that T-NH_2 is an important intermediate substance in the rhizobial IAA production pathway from L-TTP in the incubation mixtures.

The strong conversion of IAAld bisulfite into IAA and T-OH probably means that conditions permitting further conversion reactions of IAAld are present in the rhizobial cells.

3.3.4. The metabolic pathway of IAA production from tryptophan

Since *fig. 12* shows the complete pattern of substances ascribable to chromatographic degradation of IPyA (*fig. 6*) including IAAm and IGolA, it can only be concluded that significant amounts of IPyA must have been present in the extracts of the incubation mixtures with L-TTP. This means that IPyA was produced from L-TTP and that the quantitative data on IAA and ICA production are not completely reliable, since part of the IAA and a smaller part of the ICA (see *table 13*) are the result of chromatographic degradation of this IPyA.

The stimulation of the IAA and ICA production from L-TTP by addition of α-ketoglutaric acid and pyridoxal phosphate suggests that the normal production of these substances by this strain is not optimal.

3.3.5. Production of ICA from tryptophan

The quantitative data on ICA production are very striking, especially as

compared to the data on ICA production from L-TTP with the enzyme preparations of nodules and mature and young roots. These high ICA yields must certainly be ascribed to the high and specific rhizobial conversion of IAld (which is a product of the spontaneous degradation of IPyA) to ICA (*table 12*).

This high ICA production as compared to the IAA production seems especially important in relation to the central problem of the origin of the substantially higher IAA content of nodules in relation to that of roots. To make certain that the different quantitative relations of IAA and ICA yields of the L-TTP incubations in the presence of the enzyme extracts of nodules, roots, and rhizobial cells were not the result of differences in the spontaneous degradation of IPyA during incubation and chromatography, it was necessary to perform a comparative quantitative study of these breakdown phenomena. The results of these experiments are presented in *table 13*. From these results it is clear that as compared to IAA, ICA is a minor component among the spontaneous degredation products of IPyA. This indicates that the high ICA production from L-TTP by the rhizobial cells must be due to the above-mentioned specific rhizobial conversion reaction of IAld to ICA.

Table 13. Spontaneous formation of IAA and ICA from IPyA during incubation, extraction, and twofold chromatography.

Experimental conditions	IAA (μg)	ICA (μg)
IPyA[1], only chromatographed	18.0	2.5
IPyA[1] in buffer[2], extracted, and chromatographed	6.9	1.8
IPyA[1], incubated[2], extracted, and chromatographed	6.5	2.0

[1] 200 μg.
[2] 5 ml incubation mixture.

Furthermore, it may be concluded that most of the IPyA was degraded during incubation, since the IAAm and IGolA spots were significantly less distinct for the extract of incubated IPyA than for non-incubated IPyA extract and authentic IPyA.

The bacteroids. It is well known that in nodule tissue most of the rhizobial cells occur in the bacteroidal form. According to FRED *et al.* (1932) these bacteroids are unable to multiply and their metabolic activity differs from the normal rhizobial metabolic pattern. For instance, the nitrogen-fixing ability is due exclusively to the bacteroids (BERGERSEN & TURNER 1967, 1968).

Unfortunately it is not yet possible to prepare from nodule tissue bacteroid fractions free of normal bacterial cells. For this reason, incubation experiments with bacteroid fractions were not performed. When absolutely pure bacteroid fractions can be obtained, it will become much easier to approach the main problem with which this study is concerned.

3.4. Incubation experiments with sterile, cell-free enzyme preparations of *Rhizobium lupini*

The fact that most of the cells of root nodule tissue are completely filled with

the symbiotic rhizobial cells raises the question whether part of the protein present in the cell-free enzyme extracts prepared from root nodule tissue originates form the bacterial cells. Although with the procedure used here for enzyme preparation (2.2.) it seems rather improbable that many rhizobial cell walls will be disrupted, it was thought desirable to obtain some experimental information about the enzymatic production of IAA from L-TTP in the presence of cell-free enzyme preparations of rhizobial cells.

The ultimate value of this information is of course rather limited, since a large proportion of the rhizobial cells present in the nodule cells are changed into bacteroids. Because of the differences in the metabolic activity of bacteroids and normal bacterial cells, however, it is conceivable that the bacteroidal metabolism of IAA production also undergoes changes as compared to the normal rhizobial IAA production from L-TTP.

All the incubation experiments were carried out with enzyme extracts prepared from *Rh. lupini* strain A 98-1965 (3.3.).

Several methods for preparation of the enzyme extract were tried: grinding with aluminium oxide powder (Alcoa), ultrasonic cell disruption, and cell fracture by means of a French pressure cell. The last of these methods gave the highest yield of proteins from the cell-free crude enzyme preparations, and was therefore adopted; with this method, 2.2×10^{11} rhizobial cells yielded 1.0 mg protein.

3.4.1. Enzymatic production of IAA and ICA from tryptophan

Table 14 shows quantitative data on the enzymatic production of IAA and ICA from L-TTP. From these results it may be concluded that the production of both IAA and ICA from L-TTP is strongly enhanced by the presence of α-ketoglutaric acid and pyridoxal phosphate in these incubation mixtures. This indicates that a transamination reaction is involved in these conversions and thus that IPyA is formed as first intermediate substance. The somewhat lower yields obtained with dialyzed enzyme preparation suggest that some cofactor of this enzymatic pathway was lost during dialysis.

Spectrofluorometric checks showed that the substances isolated from the incubation mixtures were identical with authentic IAA or ICA.

Figs. 13 and *14* show drawings of 2-dimensional TLCs of the Salkowski-positive substances present in the acid + neutral fraction of L-TTP-containing incubation mixtures with rhizobial enzyme preparations. Equal volumes of the extracts were spotted. *Fig. 13* represents a TLC of an extract of the incubation of L-TTP plus non-dialyzed enzyme extract, *fig. 14* a TLC of the substances extracted from an incubation mixture containing non-dialyzed enzyme preparation, L-TTP, α-ketoglutaric acid, and pyridoxal phosphate. *Fig. 13* shows only a weak spot of IAA and a trace of IAld, whereas *fig. 14* shows the presence of IAA, ICA, IAld, and the two mysterious spots A and B. The presence of some IGA on the latter TLC must be considered dubious.

Attempts to detect $T\text{-}NH_2$ in the alkaline ether-soluble fractions of these

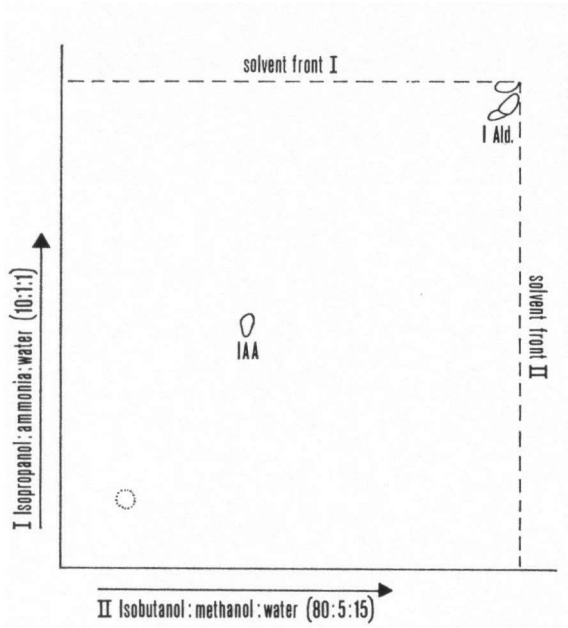

Fig. 13. Drawing of a two-dimensional TLC of the acid + neutral fraction of an extract of an incubation mixture containing rhizobial enzyme extract and L-TTP; chromatogram sprayed with Salkowski's reagent.

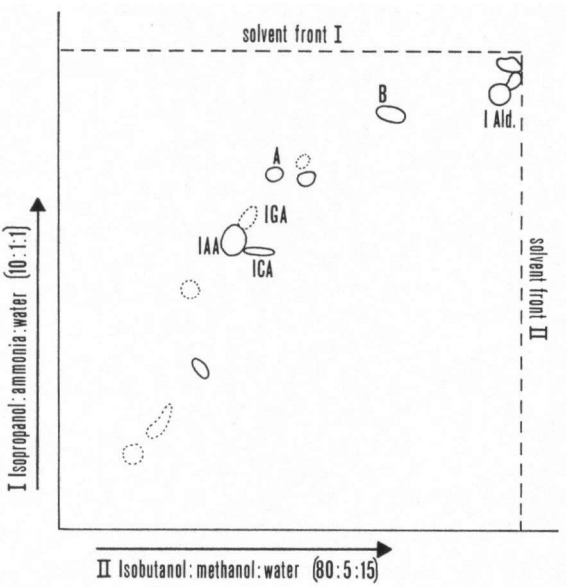

Fig. 14. Drawing of a two-dimensional TLC of the Salkowski-positive substances present in the acid + neutral fraction of an extract of an incubation mixture containing rhizobial enzyme preparation, L-TTP, pyridoxal phosphate, and α-ketoglutaric acid.

Table 14. Production of IAA and ICA in incubation mixtures with rhizobial enzyme preparations.

Enzyme		Substrate + additives			Products			
					IAA		ICA	
dialyzed	boiled	L-TTP	α-ketoglutaric acid	pyridoxal phosphate	Total IAA (in μg)	IAA in μg per mg protein[1]	Total ICA (in μg)	ICA in μg per mg protein[1]
−	−	+	−	−	2.2 ± 0.2	0.70 ± 0.07	0.4 ± 0.1	0.15 ± 0.03
−	−	+	+	+	23.7 ± 4.9	7.87 ± 1.60	2.5 ± 0.0	0.83 ± 0.00
−	−	+	+	+	18.8 ± 2.3	6.20 ± 0.80	2.3 ± 0.5	0.76 ± 0.18
+	−	+	−	−	2.2 ± 0.2	0.68 ± 0.10	0.6 ± 0.2	0.19 ± 0.05
+	−	+	+	+	11.1 ± 0.3	3.62 ± 0.10	1.1 ± 0.1	0.35 ± 0.02
+	−	+	+	+	17.3 ± 0.7	5.70 ± 0.20	1.6 ± 0.6	0.54 ± 0.19
−	+	+	−	−	0.5	−	0.0	−
−	+	+	+	+	0.3	−	0.3	−
no enzyme		+	−	−	0.1 ± 0.1	−	0.0	−
no enzyme		+	+	+	0.2 ± 0.0	−	0.0	−

[1] corrected for spontaneous production of IAA and ICA in the incubation mixtures.

incubation mixtures did not yield any trace of this compound, indicating that this substance is not produced from L-TTP.

3.4.2. Enzymatic degradation of IAA and ICA

Some information was collected about destruction of IAA and ICA in the incubation mixtures, as shown in *table 15*.

Table 15. Degradation of IAA and ICA in incubation mixtures containing rhizobial enzyme extracts.

Incubation mixture (5 ml)	Amounts degraded (μg)
IAA[1] + dialyzed enzyme[2]	16.0 ± 2.3
IAA[1] + non-dialyzed enzyme[3]	18.5 ± 0.0
IAA[1] without enzyme	8.0 ± 3.0
ICA[1] + non-dialyzed enzyme[3]	0
ICA[1] without enzyme	0

[1] 50 μg per 5 ml
[2] 3.0 mg protein per 5 ml
[3] 3.3 mg protein per 5 ml

These results show that there is some loss of IAA due to the presence of the enzyme preparations. In the interpretation of the IAA production results, therefore, the destruction during incubation must be taken into account.

ICA does not appear to be destroyed at all.

3.4.3. Conversion of some related indole derivatives

In *table 16* the reaction products extracted from incubation mixtures of non-

Table 16. Conversion of some indole substances in incubation mixtures containing non-dialyzed rhizobial enzyme extracts.

Incubated indole compound	Reaction products
tryptamine	IAA
tryptophol	IAA
indolelactic acid	no conversion
indoleacetic acid	no conversion
indolecarboxylic acid	no conversion
indoleacetamide	no conversion
indoleacetaldehyde-bis	IAA (large amounts), IAld
indolealdehyde	ICA

dialyzed rhizobial enzyme preparation and a number of indole compounds, all identified by TLC, are presented. The TLCs of these extracts were always compared with the TLCs of incubations without enzyme extract to check for spontaneously formed products.

Except for the conversion of IAAld-bisulfite, the amounts of the reaction products were very small. This was also the case for IAA formation from

T-NH_2, supporting the improbability of the occurrence of an important IAA production pathway from L-TTP via T-NH_2.

The production of large amounts of IAA from IAAld-$NaHSO_3$ addition product indicates that IAAld can be converted by this enzyme preparation despite its presence as bisulfite addition product.

In this connexion some special attention must again be given to the two spots A and B present in the TLCs of extracts of incubation mixtures of L-TTP, α-ketoglutaric acid, pyridoxal phosphate, and enzyme preparations. The presence of these two unidentified spots is very interesting, since they were also found in TLCs of extracts from incubation mixtures of L-TTP with nodule and root enzyme extracts (see 3.1.3. and 3.2.3.), and is the more surprising since these substances were not produced in the incubation mixtures with L-TTP and rhizobial cell suspensions. Consequently, the possibility must be considered that these substances are artifacts arising from *in vitro* incubation of these crude enzyme preparations with L-TTP. In any case, the question of the identity of these compounds remains intriguing.

3.4.4. The metabolic pathway of IAA production from tryptophan

As mentioned above, IPyA is very probably involved as the main intermediate substance in this metabolic pathway. Since no spots of IAAm and IGolA occurred on the TLCs of the incubation extracts, it may be assumed that, according to the argumentation presented in 3.1.4., most of the formed IPyA was degraded before extraction, possibly spontaneously, yielding IAA as one of the degradation products.

3.4.5. Production of ICA from tryptophan

Some possible pathways of ICA production from L-TTP have already been described (3.1.5.). The low ICA production as compared to that of IAA is conspicuous in relation to the ICA/IAA yield ratios of the rhizobial L-TTP conversions (3.3.1.). This difference must be caused by the slight conversion of IAld to ICA in the presence of the enzyme preparation (3.4.3.) as compared to the rhizobial production of ICA from IAld (3.3.3.), indicating that certain enzymatic conversion properties were changed during the preparation of the enzyme extract.

3.4.6. Comparison of the efficiency of the IAA production in the presence of rhizobial cells and enzyme preparations

Since no data on the efficiency of isolation of soluble proteins from the rhizobial cells are available, it is rather difficult to compare the data on IAA production from L-TTP in the presence of rhizobial cell suspensions with those obtained in the presence of enzyme preparations. The L-TTP incubation mixtures containing enzyme extracts had 3.0 mg protein, corresponding to 6.6×10^{11} cells, whereas the bacterial incubation mixtures contained 2.6×10^{10} cells, the difference amounting to a factor of about 25. It does not seem likely that the efficiency of soluble protein isolation is only 4 per cent. If it assumed that the

soluble protein fraction is responsible for the IAA production from L-TTP, this would mean that the bacterial IAA production possesses a considerably higher efficiency than the same conversion in the presence of the crude enzyme preparations (*cf.* 3.3.1. and 3.4.1.).

4. DISCUSSION

4.1. Origin of IAA

The present experiments have demonstrated that all the investigated systems possess the capacity to produce IAA from L-TTP and that in all cases the same bioproduction pathway via IPyA is followed. Consequently it appears to be impossible, on the basis of this biochemical evidence, to distinguish whether the IAA in root nodule tissue is of plant origin or of rhizobial origin.

It was found that on the basis of protein content the rhizobial cells showed the highest IAA production rate from L-TTP, followed by the bacterial cell-free enzyme preparation, the nodule and the young root enzyme preparations giving nearly equal results, and the result with mature root enzyme preparation being the lowest (*cf.* the results of IAA production from L-TTP with dialyzed enzyme preparations in presence of α-ketoglutaric acid and pyridoxal phosphate). As to the difficulty of disrupting rhizobial cells by specific grinding methods used for the preparation of cell-free enzyme (3.4.), it does not seem very probable that a significant part of the proteins in the nodule enzyme preparations is of bacterial origin, since the homogenization of the lyophilized nodule tissue in a mortar provided with sand seems too coarse a method to break many bacterial or bacteroidal cells. Moreover, in microscopic preparations the bacteroids appear to be undamaged. If this assumption is correct, it may be concluded that most of the IAA production capacity of crude nodule enzyme preparations is of plant origin. In this connexion the comparable specific activity of IAA production from L-TTP in the presence of dialyzed bacteria-free young root enzyme preparations seems very interesting, since it indicates that young root tissue with high meristematic activity and root nodule tissue, i.e., dedifferentiated root tissue also having a high meristematic activity, are comparable with respect to the ability to produce IAA from L-TTP in the presence of crude, cell-free enzyme extracts of these materials. Consequently, it does not seem necessary to suppose that all the IAA present in nodule tissue was produced by the rhizobial endophytes, or, in other words, it seems highly probable that at least a part of this IAA was actually produced by the enzymatic system of the plant nodule cells. This is the more evident because the total protein content of the nodule enzyme extracts was about 5 to 10 times higher than the protein content of the young root enzyme extracts. Since the enzyme preparation methods were identical for these tissues, this finding may indicate that, on the basis of fresh weight, considerably more IAA is produced in nodule tissue than in root tissue. It therefore seems reasonable to suggest that the infection of already differentiated root cortex tissue by the rhizobial symbiont causes some kind of rejuvenation of the cells, possibly involving a new synthesis of the plant enzyme system responsible for the IAA bioproduction.

4.2. Significance of the ICA production data

Other evidence is available indicating that a substantial part of the IAA found in the nodule tissue is probably of plant origin, the strongest being the data on ICA production from L-TTP in relation to the IAA production in the various incubation systems investigated. These ratios can be presented as quotients: [IAA]/[ICA].

It is known that the quotient of the amounts of IAA and ICA present in the extracts of root nodules varies between 2.6 and 5.0, which is markedly higher than the values found in roots (DULLAART 1967). It is very interesting to compare this result with the quotients of production data found in the present study. The quotient for the produced amounts of IAA and ICA in the presence of nodule enzyme preparation (dialyzed and in the presence of α-ketoglutaric acid and pyridoxal phosphate[1]) is about 6.0, whereas for the rhizobial cells (strain A 98–1965, with and without the additives) this value ranges between 1.8 and 1.2. For strain A 98–1968 this quotient is still much lower, demonstrating the same trend to a greater degree. It can be seen that the quotient for the nodule extracts lies between the limits represented by the production quotients of nodule enzyme preparation and the rhizobial cells. Moreover, it must be taken into consideration that the degradation of IAA during the growth of the nodules (there being no ICA destruction) tends to reduce the quotient of the nodule extract results.

Consequently, if it is assumed that the endophytic rhizobial cells – normal cells and bacteroids – have the same L-TTP metabolism as that underlying the IAA and ICA production from L-TTP by rhizobial cells in the incubation experiments, the role of the endophytic cells with regard to the IAA production in the complete nodule would seem to be of minor importance. However, it is obvious that data on the L-TTP conversion metabolism in the presence of bacteroids are required to check this hypothesis.

Moreover, great caution must be exercised in the interpretation of the *in vivo* situation in terms of results of *in vitro* experiments (BETZ *et al.* 1968). This is illustrated by the great difference between the ratios of the IAA and ICA results obtained in the incubation experiments with the rhizobial cells and the cell-free enzyme preparations of these cells.

However, in spite of these objections the present results seem to suggest that the relatively high IAA content of the nodule tissue is at least partially due to stimulation of the IAA bioproduction – possibly by *de novo* synthesis of the required enzymes as a reaction to the rhizobial infection. This leads to the question whether the IAA production in nodule tissue is greater than in young roots, the latter offering the best basis for comparison with the nodules as regards meristematic activity. Conclusions concerning this point can only be drawn if the degradation of IAA is taken into account.

[1] Results with non-dialyzed enzyme extract are left out of consideration because of the unreliability of the ICA data.

4.3. The role of IAA destruction

Any differences between the rates of IAA destruction in nodule tissue and roots would of course have great influence on the IAA contents of these tissues. Since this problem forms the subject of another research program of our department, it was not extensively investigated during this study. Nevertheless, the rather simple experiments performed yielded some valuable results.

As already mentioned, it was found that the dialyzed nodule enzyme extract showed a significantly higher IAA destruction rate than the non-dialyzed nodule enzyme preparation. It seems likely that some inhibitor substance was lost during dialysis. On the other hand, the dialyzed young root enzyme preparation appeared to destroy less IAA than the non-dialyzed young root enzyme extract, possibly due to the loss of some substance promoting enzymatic IAA breakdown during dialysis. These results may indicate that as a result of rhizobial infection the balance between substances promoting and inhibiting enzymatic IAA destruction in nodule tissue undergoes a change favouring inhibition. It is well-known, for instance, that phenolic substances play an important role in enzymatic IAA destruction, and therefore a parallel might be drawn between these observations and a number of phytopathogenic phenomena (SEQUEIRA 1966).

The rather low rate of IAA destruction observed for mature root enzyme preparations, and the absence of a significant difference for dialyzed and non-dialyzed enzyme extracts are not so very surprising, since the metabolic activity of these extracts would be expected to be rather low. This is also suggested by the low specific IAA production activity in the presence of these preparations in comparison with that with the other enzyme extracts.

From the previous results already referred to (DULLAART 1967) it is known that mature roots (parental roots) contain relatively large amounts of ICA and considerably smaller amounts of IAA, the quotient [IAA]/[ICA] being 0.3 to 0.4.

As mentioned in 3.2.2., this might be explained by the plausible assumption that during growth of the roots in the meristematic region considerable amounts of IAA are produced from L-TTP via IPyA, ICA being a by-product, whereas most of the IAA was destroyed enzymatically and the ICA remained untouched by destruction reactions, resulting in a relative accumulation of ICA in relation to IAA. Since, however, the ICA content of the parental roots on a fresh weight basis is substantially higher than the ICA content of nodule tissue (DULLAART 1967), this may imply that considerably more IAA is produced in roots than in nodules. If it is assumed that the ratio between the amounts of IAA and ICA produced from L-TTP is the same for nodule tissue and roots, this would mean that the newly induced IAA production, as a rejuvenation reaction of the already differentiated root cortex tissue to the rhizobial infection, does not reach the level of the IAA production in young root tissue.

4.4. Some conclusions drawn from the results of the comparative investigations on the enzymatic conversion of a number of related indole derivatives[1]

Comparison of the tables summarizing the results of the conversion of some indole substances in the presence of nodule enzyme extract and root enzyme extracts shows that there is only one essential difference, i.e., in the production of IAA from IAAm in the presence of a nodule enzyme preparation. Since this conversion was not found for either rhizobial cells or rhizobial enzyme extracts, it must be concluded that this enzymatic ability is induced by the rhizobial infection as a reaction in the plant cells.

The production of large amounts of IAA from IAAld-$NaHSO_3$ by rhizobial cells and rhizobial enzyme extract, whereas nodule enzyme extract showed no significant conversion of this substance, provides additional evidence that the nodule enzyme preparation does not contain significant amounts of rhizobial proteins attributable to the preparation procedure. Moreover, since the root enzyme preparations also do not show significant IAAld-$NaHSO_3$ conversion, it is clear that rhizobia have a much greater capacity to convert this compound than do the plant cells.

The conspicuous production of large amounts of ICA from IAld by the rhizobial cells, apparently a specific property of the bacterial cells, has already been discussed (3.3.5.).

With regard to the production of significant amounts of ILA from L-TTP via IPyA by the rhizobial bacteria, the presence of very small amounts of ILA in extracts of nodule tissue has been reported (DULLAART 1967). Since the formation of ILA from L-TTP was not observed in the present incubation experiments with nodule and root enzyme extracts, it must be concluded that the presence of small amounts of ILA in nodule extracts is due to rhizobial activity, indicating the occurrence of some L-TTP conversion by the endophytic organism. In this connexion it may be remarked that the formation of IPyA from ILA seems to be another specific property of the bacterial cells.

4.5. Evidence concerning the metabolic pathway of IAA production *in vivo*

The *in vitro* experiments have demonstrated that IAA is produced from L-TTP via IPyA, in which pathway spontaneous degradation of IPyA may play an important role. In fact, the simultaneous formation of ICA, IAld, and IGA can only be properly understood by assumption of such a spontaneous breakdown of IPyA, in which the keto-enol equilibrium must be concerned (KAPER 1957; KAPER & VELDSTRA 1968; KAPER *et al.* 1963 a, b).

In all likelihood, the results of estimations of IAA and ICA in extracts of root nodules and roots of lupine (DULLAART 1967) give a good picture of the *in vivo* situation. Consequently, the fact that in these tissues both ICA and IAA

[1] A diagram summarizing the conversions observed in the different incubation systems is given in *fig. 15.*

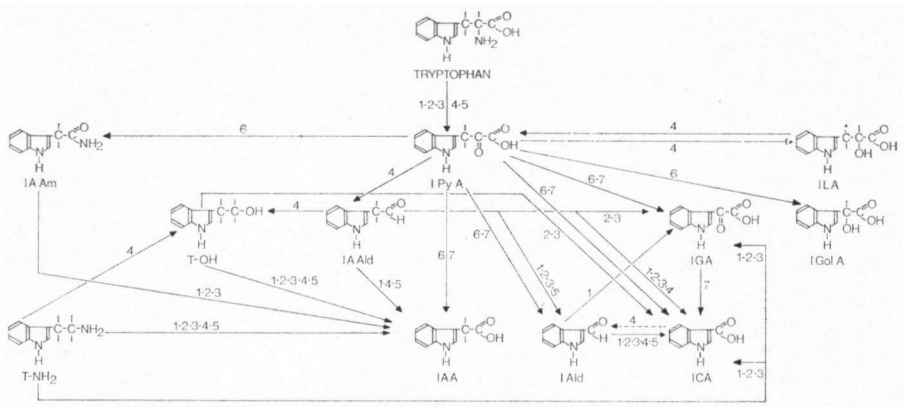

Fig. 15. Diagram summarizing the conversions observed in the different incubation systems.
1. nodule enzyme system
2. mature root enzyme system
3. young root enzyme system
4. *Rhizobium*
5. rhizobial enzyme system
6. chromatography in ammoniacal solution
7. spontaneous formation during incubation

have been demonstrated to be present and also that the relations of the amounts of these substances are rather satisfactorily explained by the results of the *in vitro* investigations, provide reliable evidence that spontaneous IPyA degradation occurs *in vivo*. Moreover, it seems probable that L-TTP is the main precursor of this metabolic pathway. It might be asked whether enough L-TTP is available for this reaction sequence *in vivo*, since the efficiency of the IAA production *in vitro* was found to be very low. Presumably, the efficiency of this reaction sequence *in vivo* is higher. Furthermore, root nodule tissue and young root tissue possess high meristematic activity, which would involve a high turnover of proteins (SHELDRAKE & NORTHCOTE 1968 a, b,c). It seems therefore very likely that the breakdown of proteins into amino acids can liberate the L-TTP required for this specific conversion sequence.

4.6. Some general considerations

It is interesting to view these data in relation to the role of phytohormones in root nodulation and other abnormal growth phenomena caused by infections.

NUTMAN (1956) and BRIAN (1957) suggested on the basis of their results that IAA causes leguminous root hair deformation leading to the rhizobial infection, and KEFFORD *et al.* (1960) demonstrated rhizobial IAA formation from tryptophan excreted by leguminous roots. The work of SAHLMAN & FÅHREUS (1962) and unpublished results from investigations in progress in our laboratory raise serious doubts about this interpretation, since they suggest the production of some other root-hair-deforming factor by rhizobia in the leguminous rhizosphere. Nevertheless, IAA seems to play some role in the

leguminous root nodule development, *e.g.*, the initiation of cell divisions leading to this development (LIBBENGA 1970).

Seen in this light, the relatively high IAA content of leguminous root nodules becomes even more interesting. In this connexion it is of interest to mention that the root nodules of the non-legume *Alnus glutinosa* (L.) Vill., which are caused by a microbial infection (probably an actinomycete), also contain much larger amounts of IAA than the roots (DULLAART, 1970). On the basis of the known facts it seems justifiable to suggest that root nodulation is the result of a complex of factors, of which IAA is one of the important ones. But other growth substances (*e.g.* gibberellins, cytokinins, dormins) might also play a role. In fact, the results of recent investigations have demonstrated that root nodules of lupine contain significantly higher amounts of gibberellin-like substances than the roots (Dullaart & Duba, in preparation), which offers confirmation of RADLEY's results (1956). Moreover, it was demonstrated that in incubation mixtures containing a dialyzed root nodule enzyme preparation, L-TTP, α-ketoglutaric acid, and pyridoxal phosphate, the last of these can be replaced by GA_3, yielding amounts of IAA of the same order of magnitude. (Dullaart & Duba, in preparation). This result, suggesting some correlation between these different growth substances, is in agreement with some of the data in the literature (VALDOVINOS & SASTRY 1968; VALDOVINOS *et al.* 1967; LANTICAN & MUIR 1967).

In the phytopathological literature much attention has been paid to the connection between IAA and abnormal growth patterns (SEQUEIRA 1963; MANI 1964). Most of the investigations have been devoted to the crown-gall disease caused by infection with *Agrobacterium tumefaciens*, which like *Rhizobium* spec. belongs to the bacterial family of the *Rhizobiaceae* (BREED *et al.* 1957). Crown-gall tumours develop after an irreversible alteration of the physiological pattern of (in principle) one cell, induced by the wounding and infection of certain plant species by *Agrobacterium tumefaciens*. One expression of this changed physiological behaviour is an autonomous production of sufficient amounts of IAA, indicating that these tumours are not dependent on the IAA production from tryptophan by the parasitic microorganism. Therefore, on the grounds of the results of this study it seems very likely that leguminous root nodulation and crown-gall tumour development are analogous phenomena with regard to the stimulated host production of IAA induced by the microbial infection.

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