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Gas Chromatographic Analysis of Acidic Indole Auxins in Nicotiana[1]

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Abstract. **Acidic indole auxins have been extracted from *N. glauca, N. langsdorffii* and their 2 tumor-prone 4n- and 2n-hybrids. After purification of the extracts and thin-layer chromatography, acidic indoles were subjected to esterification and gas chromatography. The esters of 4 indole acids were detected and determined: indole-3-acetic acid, indole-3-carboxylic acid, indole-3-propionic acid and indole-3-butyric acid. The indolic nature of fractionated samples was confirmed by spectrophotofluorometry and the physiological significance of the indole esters proven in a biotest. A substantial increase in extractable indole-3-butyric acid in the tumor-prone hybrids suggests an additional pathway of auxin synthesis in these tissues.**

An indole auxin, presumably indole-acetic acid (IAA), was described as the main growth hormone in several *Nicotiana* species, their hybrids and hybrid derivatives (2, 3, 4). Identification of IAA was based on ether extraction and separation by paper- and thin-layer chromatography, coupled with an *Avena* biotest. A quantitative comparison of the extracted acidic indole compound revealed a higher content in the tumor-prone genotypes of *Nicotiana*, as compared to their non-tumor forming parent species. However, obtaining more detailed information about other naturally occurring indole auxins in the plant extracts was made difficult by the large number of these substances and the minute concentrations in which they usually occur.

Because of similarities in molecular structure these indoles overlap on chromatograms or possess similar R_F values in the same partition solvent systems (24, 30). Therefore, a highly sensitive method is necessary for a more detailed identification of indole-auxins at physiological concentrations. Gas-liquid chromatography and spectrophotofluorometry have proven to be valuable for investigations of synthetic indoles (6, 9, 15, 21, 27). The present communication describes the application of these analytical procedures to biological material. Specifically, extracts of *Nicotiana glauca, N. langsdorffii* and their tumor-prone 4n- and 2n-hybrids have been examined for the occurrence of different acidic indole auxins. The most striking aspect observed was an unusually high level of indole-butyric acid in tumor-prone hybrids.

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Materials and Methods

Plant Material. Plants of *N. glauca, N. langsdorffii* and their 2 tumor-prone hybrids, the amphidiploid hybrid GGLL (4n) and the F_1-hybrid GL (2n) were grown in the greenhouse for 4 to 5 months. Before the onset of flowering and tumor-formation, the top 10 to 15 cm stems of the plants were excised, the leaves removed and the pieces cut and frozen immediately. For each extraction 30 g of tissue (fr wt) from 4 to 5 plants was used.

Preparation of Samples for Gas Chromatography. In order to investigate indole containing samples in the physiological range of concentrations by gas chromatography, a thorough and selective purification of tissue extracts is essential to eliminate many interfering substances (27). The sequence of steps was as follows: A) fractionation of plant extracts into major indole groups; B) thin-layer chromatography of the acidic indole group on silica gel; C) methylation of acidic indoles; D) gas chromatography; E) spectrophotofluorometry; F) biotest.

Frozen tissue samples were extracted for 20 hr at 3° with cold, peroxide-free ether at pH 3.5. The ether was evaporated and the extract taken to dryness under reduced pressure. The fractionation scheme described in detail by Powell (21) was used to obtain the acidic indole fractions of the various *Nicotiana* extracts. Any remaining non-indolic compounds like phenols and phenol derivatives were removed by partitioning the acidic indole-fraction with 2% $NaHCO_3$. The residue of the aqueous indole-fraction was taken up in a known volume of purified methanol and streaked on thin-layer chromatographic sheets (Eastman Chromagram Sheet, type K301R, Silicagel). Chromatograms were run in *n*-butanol-water-ammonia (10:10:1, upper phase). Four to 5 μg of synthetic indole-3-acetic acid (IAA)

were run as controls and the R_F of IAA determined by the method of Gordon and Weber (14) with ferric chloride-perchloric acid. The dried chromatograms, 8 cm in length, were divided into 10 equal transverse strips and the R_F sections corresponding to the location of synthetic IAA in controls and known to contain natural acidic indole derivatives, were scraped off and transferred to a millipore filter syringe. The auxin containing silica gel was eluted twice each with 1 ml of methylene chloride and 1 ml of 10% methyl alcohol. The combined eluates were then subjected to esterification, using diazomethylation (20, 22) and a micromethod described by Powell (21) for the methylation of small indole samples.

As references for naturally occurring indole acids, the following synthetic indole acids which are commercially available, were esterified by the same methods: indole-acetic acid (IAA), indole-carboxylic acid (ICA), indole-propionic acid (IPA), indole-butyric acid (IBA), indole-lactic acid (ILA), indole-acrylic acid (IAcA) and 5-hydroxyindole-acetic acid (5-OH-IAA). Although the physiological significance of indole-pyruvic acid as an intermediate product of IAA synthesis from tryptophan is recognized, this indole compound could not be included in the present investigation because of its rapid decomposition in the alkaline solvent system (*n*-butanol-ammonia-water) used for thin-layer chromatography (1, 23, 28). Ethyl indole-3-acetate (IAA-EE), obtained from commercial sources, was selected as a standard since it has an intermediate retention volume and is useful over a wide range of column temperatures (27).

Gas Chromatography. The gas chromatograph used was a Glowall Model 310 connected to a Photovolt Chart Recorder. It was equipped with an interchangeable hydrogen flame detector and run at a 300 volt DC current. The coiled columns were 6 feet long and had an inner diameter of 6 mm. They were packed either with 3% SE-30 (methyl vinyl silicone rubber, General Electric Company) or 3% silicone QF-1 (a Dow-Corning Corporation Fluorinated Silicone). The column temperature was kept at 190°, the vaporizing block temperature at 235° and the detector oven at 255°. Argon was used as the carrier gas at a flow rate of 30 ml/min at 20 to 22 lbs/in^2 pressure. Two to 5 μg samples of esterified indoles dissolved in acetone were injected into the vaporizing block.

In an attempt to analyze fractions of the gas chromatographed samples by spectrophotofluorometry and bioassay, the hydrogen flame detector was replaced by an argon ionizing detector (run on 1000 DC volts; radium source: 22.5 μcuries) which allows collection of fractionated samples after passing through the detector chamber. Small bore teflon tubing was connected to the detector outlet and led through an ice filled Dewer container. Condensation of the indole esters occurred in the cooled teflon tubing which was consequently rinsed with acetone. For the spectrophotofluorometric measurements the acetone was evaporated and the residue dissolved in 1 ml of ethyl alcohol and transferred to a quartz cuvette. Readings were made at an activation wavelength of 280 mμ and a fluorescence wavelength of 360 mμ. These wavelengths are near maxima for most indoles (except for 5-OH indoles which are activated at 295 mμ).

For bioassay the condensed residues from the teflon tubing were rinsed with ethanol water, transferred to agar blocks (2.5 × 2.5 × 1.3 mm) and subjected to an *Avena* curvature test (2, 4, 26).

Results

A) *Gas Chromatography.* Diazomethylation proved to be a successful method for esterification of naturally occurring indole acids. In recovery experiments with samples of synthetic indoles esterification was nearly complete, *i.e.* 90 to 96% was recovered as methyl ester. These data are in agreement with those of Stowe and Schilke (27) and Grunwald *et al.* (15). Since paper- and thin-layer chromatograms of some *Nicotiana* extracts had suggested that other acidic growth hormones might be concealed at the location of IAA (3, 4) a gas chromatographic analysis was performed of those acidic indoles which are of physiological interest and which show the same or very similar R_F values on paper- and thin-layer chromatograms (table I). Gas chromatographic data obtained with 2 to 5 μg samples of methylated synthetic indole acids are summarized in table II and calculated according to the recommended practice for gas chromatography by Ettre (11). Relative retention values r are given with respect to ethylindole-3-acetate and the resolution is expressed as effective plate number N. All indoles gave responses with the SE-30 and QF-1 columns. Retention times varied between 5 min (IAA-ME) and 20 min (IAcA-ME). The crowding at the beginning of the chromatogram could not be eliminated by lowering flow rates or by lowering the temperature. In the latter situation the substances are insufficiently volatile (27).

Gas chromatography of naturally occurring indole esters in *Nicotiana* revealed several peaks which coincided in their location on chromatograms with those of the synthetic indole esters. Since these substances were not always found in the extracts of the investigated *Nicotiana* plants, each experiment was repeated at least 12 times and simultaneously

Table I. *R_F-Values of Indole Acids on Thin-Layer-Chromatograms, Run in* n*-butanol-H_2O-ammonia (10:10:1), Upper Phase*

Substance	R_F
Indole-3-acetic acid	0.35
Indole-3-carboxylic acid	0.22
Indole-3-propionic acid	0.44
Indole-3-butyric acid	0.55
Indole-3-lactic acid	0.48
Indole-3-acrylic acid	0.45

Table II. *Comparison of Retention Times (t'_R), Relative Retention Times (r), and Effective Plate Values (N) of 2 to 5 μg Samples of Various Indole Methyl-Esters on 3 % SE-30 and Silicone QF-1 Columns*

Column temperature: 190°; carrier gas flow rate: 30 ml/min.

Indole esters	3 % SE-30			3 % Silicone QF-1		
	t'_R	r	N	t'_R	r	N
Ethyl-indole-3-acetate	6.2	1.00	784	5.6	1.00	608
Methyl-indole-3-acetate	5.1	0.82	816	4.6	0.82	336
Methyl-indole-3-carboxylate	5.6	0.90	784	5.6	1.00	480
Methyl-indole-3-propionate	8.0	1.30	688	6.6	1.18	352
Methyl-indole-3-butyrate	9.7	1.58	994	8.3	1.48	576
Methyl-indole-3-lactate	10.0	1.64	970	9.6	1.72	848
Methyl-indole-5-hydroxyacetate	14.6	2.37	784	16.5	2.94	800
Methyl-indole-3-acrylate	17.1	2.82	702	20.8	3.71	752

compared with controls. In compensation experiments *Nicotiana* indole esters and the methylated synthetic controls were injected into the columns simultaneously. Overlapping of peak areas was indicative for the specificity of the extractable indole acids.

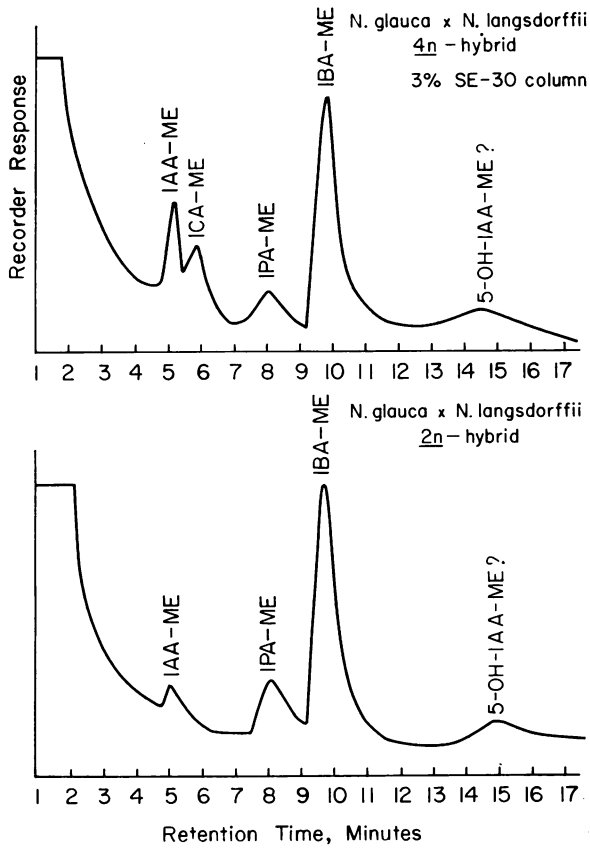

FIG. 1. Gas chromatogram of methylated indole acids derived from thin-layer chromatograms (run in *n*-butanol-ammonia-water) at R_F's 0.25 to 0.55 of *Nicotiana glauca* and *N. langsdorffii* extracts. Column: 3 % SE-30; temperature 190°. Argon flow rate: 30 ml/min. IAA-ME: indole-acetic acid methyl ester; ICA-ME: indole-carboxylic acid methyl ester; IPA-ME: indole-propionic acid methyl ester; IBA-ME: indole-butyric acid methyl ester; 5-OH-IAA-ME: 5-hydroxyindole-acetic acid methyl ester.

In *N. glauca* and *N. langsdorffii* 4 different methylated indole acids could be identified, *i.e.* ICA-ME, IAA-ME, IPA-ME, IBA-ME (fig 1). The corresponding acids are known to be of plant physiological significance since they act either as growth promoters in many plant tissues or are intermediates of IAA synthesis *in vivo* (5, 8, 10, 25,30).

The IAA content in *N. glauca* and *N. langsdorffii* is rather low (the recorder response indicates a concentration of about 0.7 μg IAA in 30 g fr tissue) but the indole-auxins ICA, IPA, and IBA are present at concentrations of about 1.0 μg each in 30 g tissue. Tissues of the tumor-prone 4n- and 2n-hybrids, however, show a considerable increase in the auxin IBA, the concentration of which lies around 1.8 to 2.2 μg per 30 g fresh tissue (fig 2).

On thin-layer chromatograms with *n*-butanol-H_2O-NH_3, the R_F of IBA lies considerably higher than that of the other investigated indole acids, *i.e.* at about 0.55 (table I). Therefore, purified fractions of 4n-hybrid extracts were divided into 2 groups, 1 containing the R_F sections 0.25 to 0.45 containing the indole acids ICA, IPA, and IAA (fraction I); the other R_F fraction 0.45 to 0.65, containing IBA and possibly other, unidentified indole compounds (fraction II). Figure 3 shows the gas chromatographic separation of the methyl esters of these indole acids. IBA-ME is present in the 0.45 to 0.65 thin-layer chromatogram fraction, whereas the other indole acids occur in the 0.25 to 0.45 fraction. Compounds with higher retention times (t'_R) than 9.7 (for IBA-ME) could be found in all investigated tissue extracts. Purification procedures of the plant extracts, thin-layer chromatography and esterification of the fractionated samples identifies them as indole esters. It may be suspected that broad peaks between retention times (t'_R) of 14 to 15 min (fig 2) represent the methyl ester of naturally occurring 5-OH-IAA. The low concentration of this substance in the plant material used, however, does not permit a clear identification of this auxin.

B) *Spectrophotofluorometry.* If a suspected acidic indole is separated and purified, spectrophotofluorometry can be employed to confirm the indolic nature of the compound (7, 16, 27). Therefore, col-

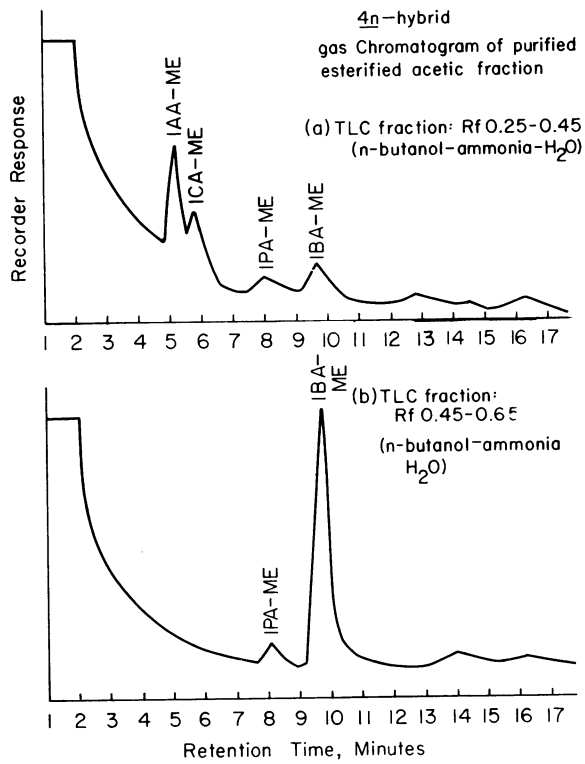

FIG. 2. Gas chromatograms of methylated indole acids, derived from thin-layer chromatograms at R_F's 0.25 to 0.55 of *N. glauca* × *N. langsdorffii* 4n and 2n hybrids. Specifications as in figure 1.

lected fractions from the gas chromatograms were transferred to quartz cuvettes and the measurements carried out on an Aminco-Bowman instrument. All investigated fractions had activation maxima in the 275 to 285 mμ range, with their major fluorescence peaks between 360 and 375 mμ. As anticipated, the spectrophotofluorometric spectra of the methyl esters

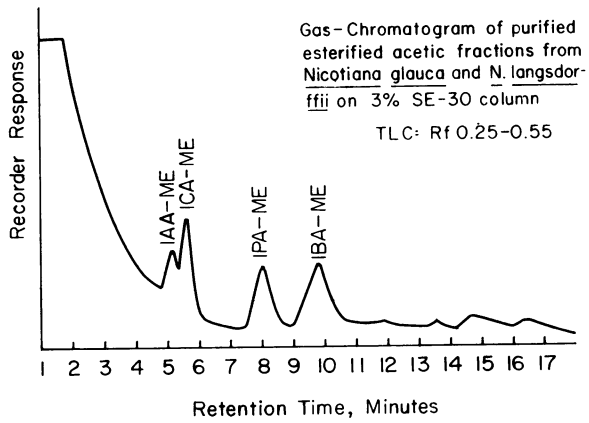

FIG. 3. Gas chromatogram of methylated indole acids, derived from thin-layer chromatograms of 4n hybrid extracts at a) R_F 0.25 to 0.45, and b) R_F 0.45 to 0.65. Specifications as in figure 1.

studied here were found to be almost identical to the indole acid spectra themselves. Spectrophotofluorometric measurements, therefore, confirmed the indolic nature of the isolated fractions. Actual identification of the compounds, however, rests most securely on the chromatographic properties cited above (15).

C) *Bioassay.* In several experiments, collected fractions of 4n hybrid extracts were directly transferred to agar blocks and measured by an *Avena* curvature biotest. Fraction I, containing the methyl esters of ICA, IAA, and IPA, gave an average coleoptile curvature of −8.5°, whereas the methyl esters of fraction II (containing IBA-ME) gave an average curvature of −5°. The relatively weak response of the *Avena* coleoptiles to these otherwise potent growth hormones is explained by the structural changes during methylation of these indole acids, since it has long been known that esterification of indole acids reduces their growth-promoting activities (29). Nevertheless, the growth response of *Avena* coleoptiles shows the auxinic nature of the investigated compounds.

Discussion

The gas chromatographic analysis of esterified indole acids in *Nicotiana* plants revealed several indole acids, IAA, ICA, IPA, and IBA. All 4 indoles could be isolated from *N. glauca* and *N. langsdorffii* extracts at low concentrations, whereas in the tumor-prone 4n- and 2n-hybrids the content of IBA was significantly increased. Although all identified indoles were present in the parental and the 4n hybrid tissues, 2n hybrids showed no detectable ICA. The significance of this possible deficiency remains to be studied in the future.

The synthesis of IAA from tryptophan is considered possibly to follow 2 different routes (10, 12) which include such intermediates as ICA and IPA together with several neutral indole compounds. The pathway of IAA synthesis in *Nicotiana* seems to follow the same proposed reaction chains. A separate pathway for the synthesis of ICA from tryptophan seems to be possible in some plant tissues, and ICA has been found by Klambt (17) in wheat coleoptiles and by Clarke *et al.* (8) in tomato crown gall tissue extracts. IPA with auxin activity in higher plants has been described by Fischer (13), Linser *et al.* (18) and Melchior (19).

Since the proposed pathway for IAA synthesis from tryptophan does not include IBA as an intermediate it may be that this auxin is the product of a separate auxin-producing pathway in *Nicotiana*, in which case it may be a storage form of IAA. IBA has been found to be a naturally occurring auxin in another one of the *Solanaceae*, that is, in *Solanum tuberosum* (5). The evidence for the occurrence of IBA in potato tissues was based on paper-chromatographic separations. The present identification of IBA in *Nicotiana* suggests a wider distribution of this auxin in higher plants.

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