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Mass Fragmentographic Determination of Indole-3-acetic Acid in Callus Tissues of *Panax ginseng* and *Nicotiana tabacum*[1]

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Free indole-3-acetic acid was identified and the amount of which was estimated in extracts of auxin-requiring and nonrequiring calluses of *P. ginseng* and *N. tabacum* by means of gas chromatograph–mass fragmentography (GC–MS–MF). The data well compare with those reported for other plant materials determined by conventional methods. It has been shown that biological samples less than 1 g are sufficient to give a confident result with this method. This will enable to determine rapidly the physiological levels of natural auxins and to discuss the delicate roles of these plant growth regurators.

The root of *Panax ginseng* C.A. Meyer, belonging to Araliaceae family, has been used in the Orient as a tonic since ancient times. It has been claimed, by Shibata,[3] and Takagi and their co-workers,[4] that the pharmacologically active components of this plant are saponins such as ginsenosides Rb_1 and Rg_1. We recently reported that these substances are also found in a significant amount in callus tissues of the plant (hereafter referred to PN-callus) which has been subcultured for 7 years on a Murashige and Skoog's medium containing 0.1 ppm of 2,4-dichlorophenoxy-acetic acid (2,4-D).[5] In an effort to study the effects of auxins on the production of saponins of this callus, one of the present authors (T.I.) has derived a callus which requires no auxin for its growth. This habituated callus (PH-callus) grows readily on an auxin-omitted medium but produced considerably smaller amount of saponins compared to PN-callus.[6]

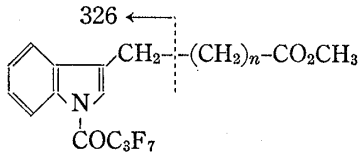
Free indole-3-acetic acid (IAA) has been suggested to be present by the *Avena* curvature test and was estimated to be of about the same order in PN- and PH-callus. In addition to this, it has also been suggested that another auxin-like substance is present in the extract of the latter callus.[6] Here we report an unambiguous result, obtained by gas chromatograph–mass fragmentography (GC–MS–MF), that one of the physiologically active substances is actually IAA. A circumstancial evidence has also been presented for the possible occurrence of the above mentioned auxin-like substance. Successful applications of MS or GC–MS method in the characterizations of IAA or its derivatives from plant sources have recently appeared.[7,8]

1) Part XXVII in the series "Studies in Plant Tissue Cultures": for Part XXVI, see M. Hirotani and T. Furuya, *Phytochemistry*, **14**, 2601 (1975).
2) Location: *a*) *Horikawa, Saiwai-ku, Kawasaki, 210, Japan*; *b*) *Shirokane, Minato-ku, Tokyo, 108, Japan.*
3) Y. Nagai, O. Tanaka, and S. Shibata, *Tetrahedron*, **27**, 881 (1971) and references therein.
4) H. Nabata, H. Saito, and K. Takagi, *Japan. J. Pharmacol.*, **23**, 29 (1973).
5) T. Furuya, H. Kojima, K. Syōno, T. Ishii, K. Uotani, and M. Nishio, *Chem. Pharm. Bull.* (Tokyo), **21**, 98 (1973).
6) T. Ishii, Ph.D. Thesis (1974), Tokyo Educational University.
7) M.S. Greenwood, S. Shaw, J.R. Hillman, A. Ritchie, and M.B. Wilkins, *Planta*, **108**, 170 (1972); M.C. Elliott and M.S. Greenwood, *Phytochemistry*, **13**, 239 (1974); M. Ueda and R.S. Bandurski, *ibid.*, **13**, 243 (1974).
8) R.S. Bandurski and A. Schulze, *Plant Physiol.*, **54**, 257 (1974).

Results and Discussion

The calluses were cultured and harvested as described in the following section. Extractions were performed according to a modified Powell's method.[9,10] Prior to extraction, indole-3-butyric acid (IBA) was added as the internal standard (IS) since a preliminary survey revealed that IBA is absent in these materials. The acidic portions from both extracts were further purified by thin-layer chromatography (TLC), derivatized according to the procedure described for the quantitation of IAA and 5-hydroxy-IAA in human cerebrospinal fluid by Bertilsson *et al.*,[11] and subjected to mass fragmentographic analysis.

Methylated and N-heptafluorobutyrylated authentic samples gave rise to rather simple mass spectra, having major peaks at *m/e* 385 (M^+, 46) and 326 (base peak: 100)[12] for IAA-derivative, *m/e* at 399 (M^+, 50), 339 (37) and 326 (100) for indole-3-propionic acid (IPA) derivative, and at *m/e* 413 (M^+, 48), 339 (100) and 326 (46) for IBA-derivative, respectively. The



		M^+
IAA-deriv.:	$n=0$	385
IPA-deriv.:	$n=1$	399
IBA-deriv.:	$n=2$	413

prominent peak at *m/e* 326 is attributed to the fragment ion resulting from the cleavage of the bond β to the indole nucleus and, therefore, is common to all indolic substances. Retention times (t_R) for authentic samples were 2.5, 3.6 and 5.5 min for these derivatives, respectively, in the GC–MS condition described in the experimental section.

Due to the concomitant presence of many other interfering ingredients (callus tissues contain a lot of impurities compared to those from the intact plants), no detectable peaks corresponding to indolic acids were found when GC analyses of the extracts were performed with flame ionization, electron capture detection or total ion monitoring. In the specific-ion-monitorings, on the other hand, clearly distinguishable peaks in the chromatograms were observed at the respective t_R. Simultaneous monitorings were made with the molecular ion peaks corresponding to IAA-(*m/e* 385), IPA-(399) and IBA-derivative (413), the chromatograms being recorded with a multiple-pen recorder. Monitorings with the fragment ions at *m/e* 326 and 339 were also made in order to obtain some structural informations about the chromatographic peaks. Figures 1—4 illustrate representative mass fragmentograms of the samples presently studied. In all samples presently studied, a clear peak was observed at t_R 2.5 min when monitored with the ions at *m/e* 385 and 326. This is unambiguously ascribed to IAA-derivative and the occurrence of free IAA in callus tissues has thus been firmly established. By comparing the peak areas attributed to IAA with those of IS (IBA: peaks at t_R 5.5 min with *m/e* 413), the endogenous concentrations of free IAA in these materials were estimated;[13] here we assume that the recoveries in the extractions and the yields in the derivatization steps are similar to these compounds. Thus the IAA concentrations were estimated to be around 10 and 45×10^{-9} g/g on a fresh weight basis for PN- and PH-callus, respectively. In a comparative study, measurements were also made with calluses derived from *N. tabacum*. Thus TN-callus (2,4-D-requiring tobacco callus) and TH-callus (habituated tobacco callus) were estimated to contain about 22 and 45×10^{-9} g/g of free IAA, respectively, on a fresh

9) L.E. Powell, *Plant Physiol.*, **39**, 836 (1964).
10) K. Syōno and T. Furuya, *Plant Cell Physiol.*, **13**, 843 (1972).
11) L. Bertilsson, A.J. Atkinson, Jr., J.R. Althaus, Å. Härfast, J.E. Lindgren, and B. Holmstedt, *Anal. Chem.*, **44**, 1434 (1972); L. Bertilsson and L. Palmér, *Science*, **177**, 74 (1972).
12) Relative intensities of the ions are given in parentheses.
13) Equimolecular amount of IAA and IBA gives rise to respective peaks with relative intensities of 1 and 1.33.

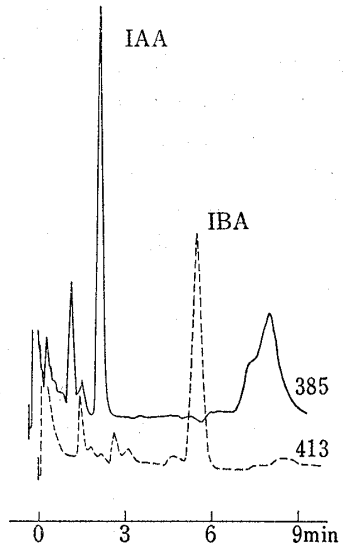


Fig. 1. Mass Fragmentogram of the Extract from PH-Callus monitored with the Ions at *m/e* 385 and 413

This represents 1 μl injection of the diluted sample (in 200 μl of EtOAc, purified from 30 g of fresh callus), containing at most 5 ng of IBA as internal standard. The two pen traces are physically displaced to each other because of the mechanical construction of the recorder. Settings of the instrument were not optimized.

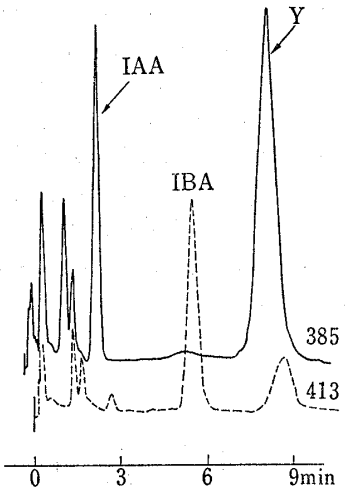


Fig. 2. Mass Fragmentogram of the Extract from TH-Callus monitored with the Ions at *m/e* 385 and 413

See footnotes for Fig. 1.

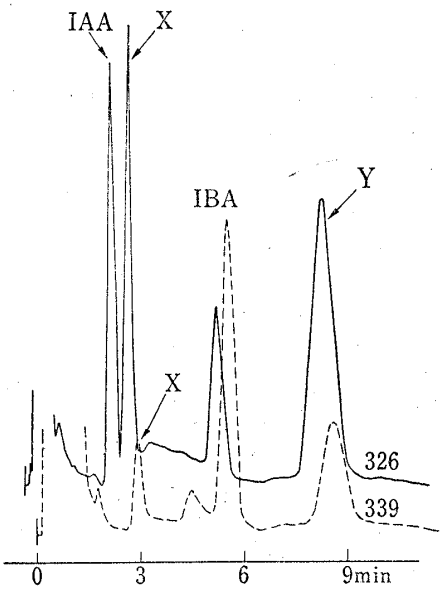


Fig. 3. Mass Fragmentogram of TH-Callus monitored with the Fragment Ions at *m/e* 326 and 339

See footnotes for Fig. 1.

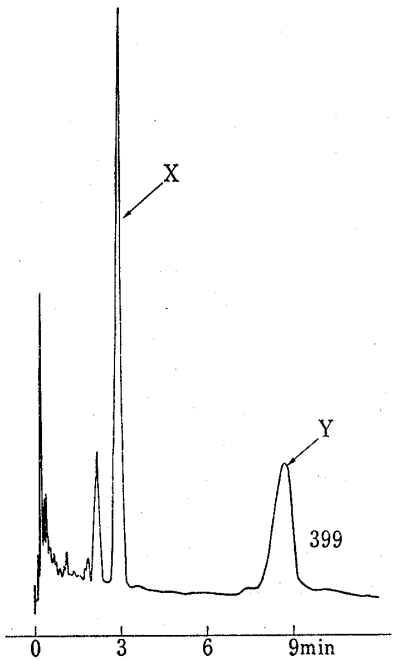


Fig. 4. Mass Fragmentogram of TH-Callus monitored with the Ion at *m/e* 399

The chromatogram was simultaneously run with the pen traces for *m/e* 385 and 413 (Fig. 2) but was recorded on a separate sheet. For other indications, see footnotes for Fig. 1.

weight basis. No IPA was found to be present in these calluses presently studied. The above values are comparable to those reported for other tissues of different origin.[8,14] It may be that the above discrepancy reflects some differences in their physiological state. We have therefore initiated a preliminary survey about the physiological levels of natural auxins in plant materials from wide origins and of differing physiological states.

It would otherwise be noted that an intense peak was recorded at t_R 2.95 min in the all chromatograms determined in the present study when monitored with the ion at *m/e* 399 (see Fig. 4). This coincides to M^+ of IPA-derivative but the above peak cannot be attributed to IPA since its t_R is quite different from that of IPA-derivative (3.6 min). This very peak was also observed when chromatograms were monitored with the ions having *m/e* 326 (intense, see Fig. 3) and 339 (weak). This is indicated as X in these figures. As far as the above observations are concerned, it is possible to speculate that an isomer of indole-propionic acid is present in these materials. Another intense peak appearing at t_R 8.6 min also attracts our interest. This is indicated as Y in these figures. This peak was only recorded for samples from tissues of the habituated calluses (PH- and TH-callus). It might be that this corresponds to the extra auxin-like substance suggested to be present only in the habituated calluses.[6,10] Nothing else is known at present, however, about the nature of the above putative compounds.

The sensitivity of the mass fragmentographic method was found to be surprisingly high: samples as little as 10 picograms could be detected in the present case when the most appropriate ion peak was used for probe. Owing to the high sensitivity and the high specificity of the method, extracts at this purification stage (estimated to be less than 0.1% purity) and with such a minute concentration of IAA can readily be analysed. Biological samples less than 1 g (fresh weight) will therefore be sufficient to give a confident result. It must otherwise be stressed that the purification technique is not critical with this method if an appropriate IS is used; recoveries in the extraction steps and in the derivatization step do not influence the quantitative results. The mass fragmentographic technique will therefore enable to determine, rapidly and accurately, to compare, and then to discuss the delicate roles of auxins as well as of the other plant growth regulators.

Experimental

Tissue Cultures——PN-callus was derived from the petiole of 2 years old *P. ginseng*[5] and has been maintained on the agar medium of Murashige and Skoog (mynus glycine and Edamin) containing 0.1 ppm of 2,4-D, at 25° in the dark and at 4 week intervals. PH-callus was induced from the PN-callus by continuous contacts with media having a low concentration of 2,4-D and finally by culturing on a 2,4-D-omitted medium.[6] The callus tissues at the 20th passage became to grow readily on the basal (auxin-omitted) medium. The induction and the physiological properties of this callus, especially the effects of the exogenously supplied auxins on the growth and the saponin production, will be reported elsewhere.

TN-callus was derived from the stem of *N. tabacum* "Xanthi" and has been subcultured on the above medium containing 1 ppm of 2,4-D. The habituated TH-callus was derived by Syōno and Furuya from an originally IAA-requiring callus by a short treatment with high concentration of a synthetic auxin. This was designated as T222 callus in an earlier paper.[15] The origins and the nature of these calluses are described in our previous papers.[10,15] The above calluses were cultured on the respective media (in the presence or absence of 2,4-D) and were harvested at the end of three-week culture in the dark at 25°.

Extractions——Frozen tissue samples (*ca.* 30 g each) were extracted twice with cold ether at pH 3.5. Prior to extraction, 1 μg of IBA which serves as IS was added to each tissue. The extracts were concentrated under reduced pressure below 40°. Partitioning to obtain the acidic indole fraction from the extracts was performed according to a modified Powell's procedure described in detail in an earlier paper.[10] The residue of an acidic indole fraction was taken up in MeOH and streaked on a TLC plate. Chromatograms were run in MeOAc–isoPrOH–25% NH_4OH (45: 35: 20). The *Rf* section corresponding to the location of synthetic indolic acids (IAA and IBA, detected colorimetrically) was scraped off and was extracted with 0.1 N 80% methanolic KOH. This was filtered and the filtrate was neutralized with 1 N HCl to pH 7.0. This was concentrat-

14) M.H. Bayer, *Plant Physiol.*, **44**, 267 (1969).
15) K. Syōno and T. Furuya, *Plant Cell Physiol.*, **15**, 7 (1974).

ed, adjusted to pH 3.5 and extracted with EtOAc. The solvent was dehydrated with dry Na_2SO_4 and evaporated to dryness.

Derivatization——The above extract was dissolved in MeOH and methylated at −15° with ethereal CH_2N_2. After the elimination of unreacted reagent by blowing N_2 gas into the test tube, an excess of heptafluorobutyryl imidazole was added and the reaction mixture was allowed to stand for 2 hr at 80°. After cooling, this was acidified with 1 N H_2SO_4 and was extracted twice with EtOAc. The solvent was removed and the residue was dissolved in 200 μl of EtOAc. An aliquot (1μl) of this was injected into the column and was subjected to mass fragmentographic analysis.

GC–MS Conditions——A Hitachi RMU-6MG gas chromatograph–mass spectrometer equipped with a glass column (1 m × 3 mm) was packed with 3% OV-17 maintained at 175°. The temperatures of the injector, the glass separator and the ion source were 260°, 280° and 180°, respectively. The pressure of He carrier gas was 2.4 kg/cm². The ionizing potential and the accelerating voltage were 20 eV and around 3.2 kV) respectively. The multiple ion monitor served as an ion-specific detector for the gas chromatograph was adjusted to record exclusively the intensities of two or three ions generated in the mass spectrometer. In the present case were selected the ions corresponding to *m/e* 385, 399 and 413 on one hand and *m/e* 326 and 339 on the other.

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