Preparation of Indole Extracts from Plants for Gas Chromatography and Spectrophotofluorometry [1, 2]

Loyd E. Powell [3]
Department of Biology, Yale University, New Haven, Connecticut

Introduction

Indole compounds have demanded increasing attention from plant physiologists in recent years since it has become evident that most naturally occurring plant auxins and their precursors are indolic (4). A recent review by Stowe (13) of the simple indoles found in plants clearly demonstrates that the investigator must be prepared to work with a relatively large number of these substances which are diverse in their molecular structure. Critical evaluation of naturally occurring plant auxins has been hampered because of the minute concentrations in which they nearly always occur. These difficulties are compounded by the fact that many indole derivatives exhibit some degree of lability with most conventional laboratory techniques, while a few are degraded under even the mildest conditions that can be usefully employed. Bioassays have generally been used to overcome the problem of low concentration, but bioassays at best are tedious and time consuming, and are probably seldom better than semi-quantitative. Furthermore, bioassays respond only to certain indoles, whereas other indoles without biological activity may be at least as important to a particular problem.

Spectrophotofluorometry is a highly sensitive technique that can quantitatively detect nanogram (10^{-9}g) amounts of many fluorescing substances (2). Stowe and Schilke (14) have recently shown that a number of simple indoles can be detected by this method in submicrogram amounts. Since activation and fluorescence spectra generally show broad rather than sharp peaks, other fluorescing substances can interfere with an analysis, even though the activation and fluorescence maxima may not be identical with that of the compound the investigator wishes to measure. Still other compounds may quench fluorescence. For successful spectrophotofluorometry of a substance it is generally necessary for a fairly high level of purification to be effected.

A promising way to obtain much of the necessary purification of indoles prior to spectrophotofluorometry is gas-liquid chromatography (14). A number of acidic and neutral indoles have been assayed with this technique. However, preliminary purification of the extract is generally necessary before gas chromatography can be successfully applied. The major part of this paper will be concerned with methods for preparing plant extracts for gas chromatography and spectrophotofluorometry. It will then be shown that the method has been successfully applied to 2 different plant extracts to show the presence of 2 well known indoles whose existence other investigators have previously established by classical methods.

Experimental Results

In the development of a suitable scheme for the detection of indole derivatives in plants it was assumed that the extracts would have to be partially purified before being gas chromatographed in order to eliminate many substances that might otherwise interfere. After extraction of the sample by any of the commonly used techniques, the sequence of steps finally chosen was as follows: A) fractionation of the extract into major indole groups; B) silica gel partition column chromatography of the major indole groups; C) esterification of acidic indoles; D) gas chromatography; and E) spectrophotofluorometry.

Fractionation. The simple indoles can be conveniently fractionated into 4 groups of substances: (1) acidic, (2) basic, (3) neutral, all of which partition into methylene chloride from aqueous solutions at the proper pH, and (4) water soluble compounds which partition into water from methylene chloride regardless of the pH. A fractionation scheme for separating indoles into the 4 groups is illustrated in figure 1.

This fractionation scheme is believed to be correct for the simple indoles likely to be encountered. Parts of the scheme, for which there was relatively little quantitative evidence in the literature about how certain indoles would partition, were tested with several representative substances. For example, when 5×10^{-7} moles of indole, ethyl indole-3-acetate, and indole-3-acetonitrile were partitioned once between equal volumes of acetonitrile and *n*-hexane, only trace amounts could be detected in the hexane. It seems safe to assume, then, that any of the simple

[1] Received Dec. 16, 1963.
[2] This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research to the author, by United States Public Health Service research grant GM-06921 from the National Institutes of Health to Bruce B. Stowe, and an institutional grant of the American Cancer Society to Yale University.
[3] Present address: Department of Pomology, Cornell University, Ithaca, New York.

neutral indoles can be quantitatively transferred to acetonitrile from hexane with 2 or 3 extractions. This step is convenient for separating many lipids from the neutral indoles (8). The basic indoles tryptamine, gramine, and bufotenine were converted from their salts to the free bases by 1 N NH_4OH, thus enabling them to be partitioned from 1 N NH_4OH into methylene chloride. Those indoles which are classified as water soluble according to this scheme (5-hydroxyindole, 5-hydroxyindole-3-acetic acid, 5-hydroxytryptamine, 5-hydroxytryptophan, tryptophan, abrine, and hypaphorine) were partitioned between methylene chloride and 0.5 M formic acid, methylene chloride and distilled water, and methylene chloride and 1 N NH_4OH. Only with 5-hydroxyindole was there more than a trace amount left in the methylene chloride after one partition with equal volumes of solvent, regardless of which solvent was used. But, even with 5-hydroxyindole the partition was clearly in favor of the aqueous layer at both acid and alkaline pH's, and about equally divided in the case of distilled water, indicating that a quantitative transfer could be achieved with a few extractions. These investigations were not extended to the possibility of any pernicious effects of the acidic or basic pH's on the indoles.

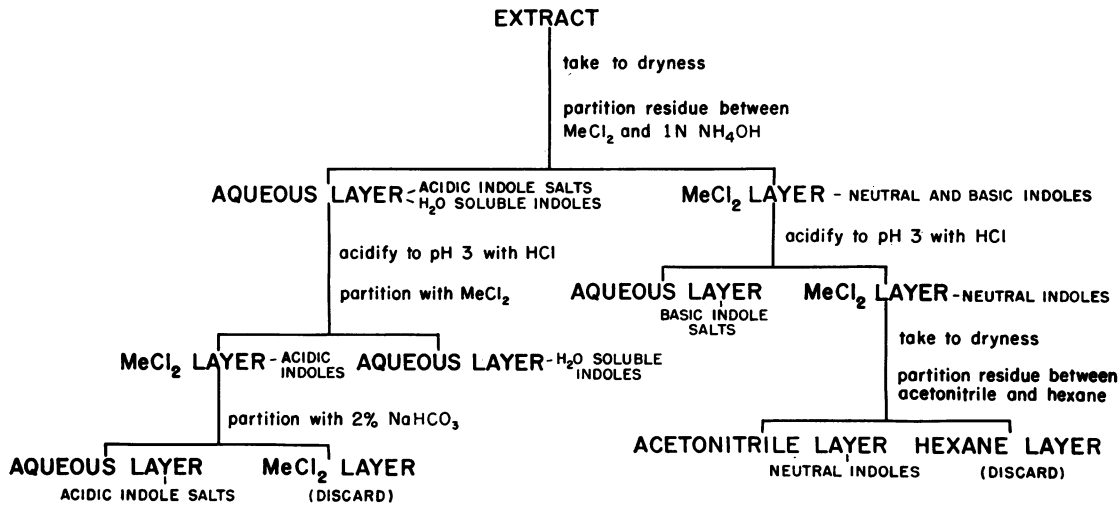
In actual practice a number of the steps in this fractionation scheme can be eliminated, depending on the demands of the particular problem. For example, the step in which acidic indoles dissolved in methylene chloride are further partitioned with 2 % sodium bicarbonate is useful if the investigator wishes to separate the acid indoles from certain phenols. Most phenols do not react with sodium bicarbonate, and thus remain in the methylene chloride, whereas the acidic indoles do react and are partitioned into the aqueous layer. Thus, during such a fractionation as described in figure 1, the indoles are not only logically grouped for later techniques, but a number of nonindolic compounds are eliminated as well.

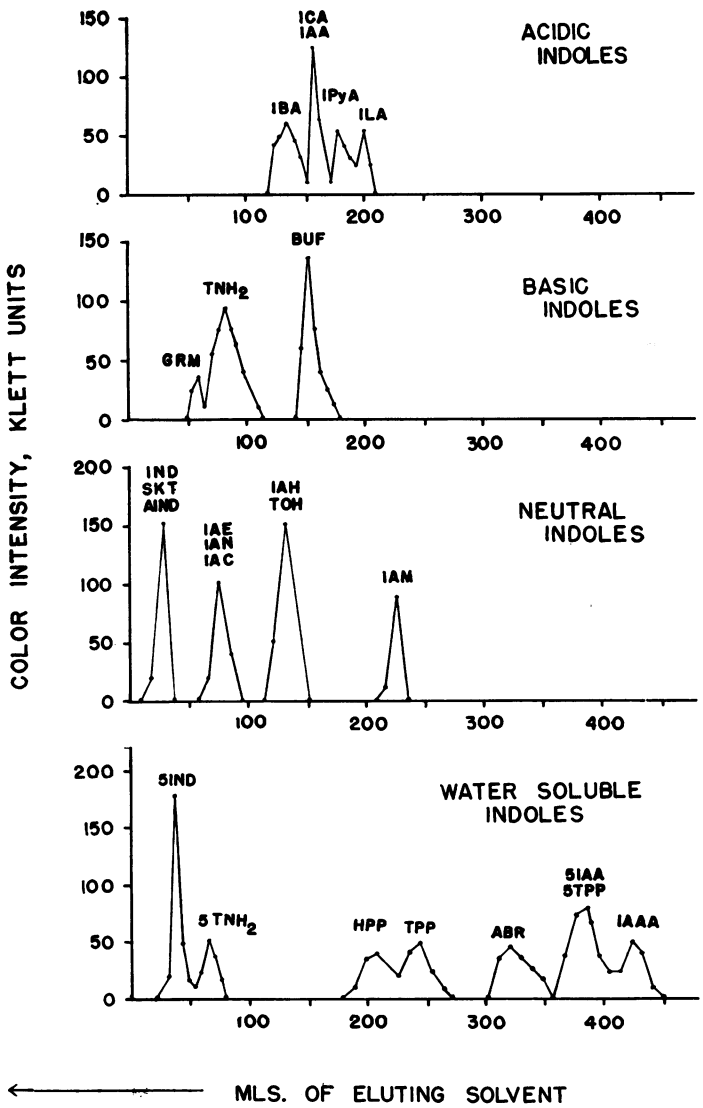
Silica Gel Partition Column Chromatography. This process offers a convenient and mild method for eliminating many nonindolic contaminants from the sample, and can also be used to effect separation of many indoles from each other. Briefly, the operation consisted of a column of silica gel through which the indolic substances were gradient eluted into test tubes in a fraction collector. The indoles were detected with the Salkowski or Ehrlich's reagent after first removing the eluting solvents from the test tubes with the aid of a device especially designed for rapid evaporation of solvents from test tubes at low temperatures (Rotary Evapo-Mix, manufactured by Buchler Instruments, Inc., Fort Lee, New Jersey). In experiments in which the indoles were to be subjected to gas chromatography, the Salkowski and Ehrlich's reagents were, of course, omitted. Instead, test tubes in which the indoles were eluted were bulked, the eluting solvent evaporated, and the residue gas chromatographed. Attempts to replace chemical reagents with spectrophotofluorometry for detecting indoles as they were eluted from the column met with only limited success. Background fluorescence from the column masked all but relatively high concentrations of the indoles. Efforts to eliminate the fluorescence source from the column were not successful.

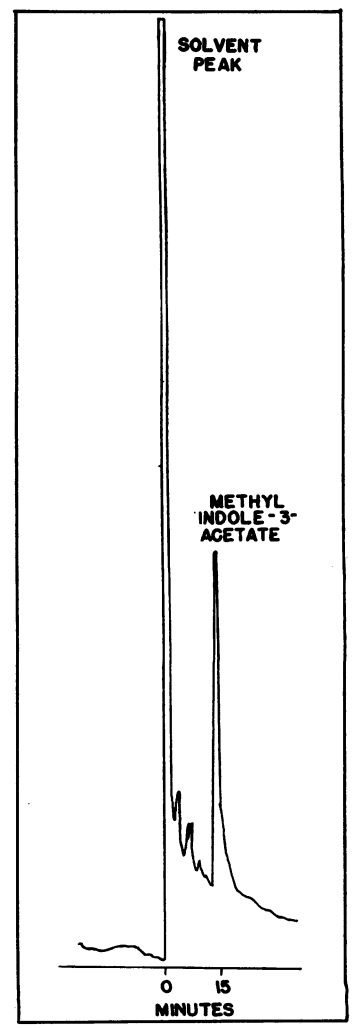
Earlier papers (10, 11) described the silica gel column process in terms of a stepwise elution technique, but since stepwise elution in general may permit the elution of a substance in more than one peak, gradient elution was employed here. Four gradient elution schemes were developed, one for each of the 4 groups of indoles resulting from the fractionation shown in figure 1. The solvent gradient was established with either a 2- or 3-chambered Varigrad (Buchler Instruments, Inc.) that was patterned after the device described by Peterson and Sober (9).

Columns were constructed from silicic acid (Mallinckrodt) which had been washed with 3 N HCl, then water, and finally dried to constant weight at 100°. Eight g of a 100–200 mesh fraction of this silicic acid (silica gel) was hydrated with 5.0 ml of 0.5 M formic acid for chromatography of acidic or neutral indoles, or 5.0 ml of concentrated NH_4OH (sp gr 0.898) in the case of basic or water soluble indoles. The hydrated silica gel was thoroughly mixed until a free flowing powder was obtained, then slurried with *n*-hexane that had been saturated with either 0.5 M formic acid or concentrated NH_4OH, and poured into a 12.5 mm glass column with a stopcock on one end. The column was packed with air pressure from a hand squeeze bulb. Excess hexane was drained from the column. A satisfactory method for introducing acetone soluble indoles to the top of the column consisted of transferring the indole sample to a 15 ml conical glass centrifuge tube, evaporating the carrier solvent, dissolving the indolic residue in a small volume (about 0.25 ml) of acetone, adding one ml of *n*-heptane, and finally evaporating the acetone from the mixture (by use of the Rotary Evapo-Mix) leaving the indoles either dissolved or suspended in the heptane. The heptane-indole mixture was transferred to the column with a pipette. Three such rinsings of the centrifuge tube proved adequate to quantitatively transfer the indoles to the column. The heptane caused no appreciable effect on the gradient. Water soluble indoles not sufficiently soluble in acetone for this method were dissolved in small volumes (about 0.1 ml) of water and transferred to the column with a micro pipette. For most of this work 5×10^{-7} moles of each indole were used, although as little as 5×10^{-9} moles have been successfully handled.

Colorimetric measurement of indoles was achieved with the Gordon-Weber modification of the Salkowski reagent (5) or Ehrlich's reagent (3). The Salkowski reagent was made by dissolving 5.40 g of ferric chloride in 2 liters of 35 % (v/v) perchloric acid. Two ml of this solution were used for each ml of aqueous solution containing the indolic derivative. Ehrlich's reagent consisted of 10 g of *p*-dimethyl-


EXTRACT
take to dryness
partition residue between MeCl2 and 1N NH4OH
AQUEOUS LAYER - ACIDIC INDOLE SALTS, H2O SOLUBLE INDOLES
MeCl2 LAYER - NEUTRAL AND BASIC INDOLES
acidify to pH 3 with HCl
partition with MeCl2
AQUEOUS LAYER BASIC INDOLE SALTS
MeCl2 LAYER - NEUTRAL INDOLES
take to dryness
partition residue between acetonitrile and hexane
MeCl2 LAYER - ACIDIC INDOLES
AQUEOUS LAYER - H2O SOLUBLE INDOLES
partition with 2% NaHCO3
AQUEOUS LAYER ACIDIC INDOLE SALTS
MeCl2 LAYER (DISCARD)
ACETONITRILE LAYER NEUTRAL INDOLES
HEXANE LAYER (DISCARD)


ACIDIC INDOLES
ICA
IAA
IBA
IPyA
ILA
BASIC INDOLES
BUF
TNH2
GRM
NEUTRAL INDOLES
IND
SKT
AIND
IAE
IAN
IAC
IAH
TOH
IAM
WATER SOLUBLE INDOLES
5IND
5TNH2
HPP
TPP
ABR
5IAA
5TPP
IAAA
COLOR INTENSITY, KLETT UNITS
MLS. OF ELUTING SOLVENT


SOLVENT PEAK
METHYL INDOLE-3-ACETATE
0 15
MINUTES

aminobenzaldehyde dissolved in 100 ml of concentrated HCl, sp gr 1.188. Two ml of this reagent were used for each ml of aqueous solution containing the indole derivative, except in the case of indole and 5-hydroxyindole where the reverse ratio was better. It was necessary to employ heat to force color development for some of the indoles.

The elution patterns from the silica gel columns for several indole derivatives are depicted in figure 2. The true position for hypaphorine is in doubt. In some tests it was eluted as shown, but in others its breakthrough volume was only 100 to 150 ml. The explanation for this discrepency is as yet unknown.

Esterification. Of the 4 groups of indoles, only the neutral and acidic ones have been successfully gas chromatographed (14). The neutral ones are sufficiently volatile without further treatment, but acidic indoles must first be esterified. Stowe and Schilke (14) found that the BF_3 catalytic method of esterification was promising with some of the more stable indole acids, including IAA, but that it led to the destruction of such labile indoles as indole-3-carboxylic, indole-3-glycolic, indole-3-pyruvic and indole-3-lactic acids. They suggested that diazomethylation might be a more suitable method, since very mild conditions are employed. Schlenk and Gellerman (12) described a micro method for the generation of diazomethane and its employment for esterification. Their method, modified slightly, was used for the methyl esterification of IAA. The hazards associated with diazomethane are minimized by using a micro method. The apparatus consisted of 3 test tubes (20 × 150 mm) connected in series. A stream of N_2, which was saturated with methylene chloride in the first tube, carried diazomethane generated in the second tube into the third tube where the esterification took place. Rubber stoppers and small bore flexible Teflon tubing were used for connections between tubes. Methylene chloride was substituted for the ether that Schlenk and Gellerman used in their work, in order to overcome the necessity of peroxide elimination. Carbitol, practical grade, [also called diethylene glycol monoethyl ether and 2-(ethoxy-ethoxy) ethanol] was used without further purification, as was N-methyl-N-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Company, Inc.).

To effect esterification the N_2 was first gently bubbled through the methylene chloride of tube 1. Tube 2 contained 1.5 ml of carbitol and 1 ml of 60 % KOH. Tube 3 contained several μg of IAA dissolved in 2 to 3 ml of methylene chloride, and 2 ml of 10 % methyl alcohol. Generation of diazomethane was initiated by adding to tube 2 about 115 mg of N-methyl-N-nitroso-*p*-toluenesulfonamide dissolved in 1.5 ml of methylene chloride. Connection was made immediately with tubes 1 and 3. A yellow tinge became visible in tube 3 within 1 or 2 minutes, indicating that the system was saturated with diazomethane. Esterification is said to be complete at this point (12), but the system was generally allowed to run for about 10 minutes, by which time generation of diazomethane had ceased and the excess gas had been expelled from the solution of tube 3 as judged by visual observation. Tube 3 was then disconnected from the system and further aerated with N_2 for a few minutes to eliminate any remaining traces of diazomethane. The material in tube 3 was taken to dryness in the rotary evaporator in preparation for gas chromatography.

Two recovery experiments were made to determine the per cent conversion of 44 μg of IAA to its methyl ester, using conventional partition methods previously described for separating the 2 substances, and the Salkowski reagent for their detection. In the first experiment 90.2 % of the material was recovered as the methyl ester of IAA, and 3.4 % was not esterified. In the second experiment 95.8 % was

FIG. 1 (*upper*). Scheme for fractionating simple indole compounds into acidic, basic, neutral and water soluble groups. The use of equal volumes of partitioning solvents in each pair is assumed.

FIG. 2 (*lower left*). The elution patterns of acidic, basic, neutral, and water soluble indoles from silica gel columns. Abbreviations used are: IBA, indole-3-butyric acid; IAA, indole-3-acetic acid; ICA, indole-3-carboxylic acid; IPyA, indole-3-pyruvic acid; ILA, indole-3-lactic acid; GRM, gramine; TNH_2, tryptamine; BUF, bufotenine; IND, indole; SKT, skatole; AIND, N-acetyl indole; IAE, ethyl indole-3-acetate; IAN, indole-3-acetonitrile; IAC, indole-3-acetaldehyde; IAH, indole-3-aldehyde; TOH, tryptophol; IAM, indole-3-acetamide; 5IND, 5-hydroxy indole; $5TNH_2$, 5-hydroxy tryptamine; HPP, hypaphorine; TPP, tryptophan; ABR, abrine; 5IAA, 5-hydroxy indole-3-acetic acid; 5TPP, 5-hydroxy tryptophan; IAAA, indole-3-acetylaspartic acid.

The details for the gradient elution chromatography of each group of indoles is as follows: *Acidic indoles.* Column, 8.0 g of silica gel hydrated with 5.0 ml of 0.5 M formic acid; fraction size, 5.4 ml; solvents, Varigrad chamber no. 1—100 ml *n*-hexane saturated with 0.5 M formic acid; Varigrad chamber no. 2—100 ml *n*-hexane saturated with 0.5 M formic acid; Varigrad chamber no. 3—75 ml ethyl acetate saturated with 0.5 M formic acid. *Neutral indoles.* Same as for acid indoles, except fraction size was 9.4 ml. *Basic indoles.* Column, 8.0 g of silica gel hydrated with 5.0 ml of concentrated NH_4OH; fraction size, 5.4 ml; Solvents, Varigrad chamber no. 1—200 ml *n*-hexane saturated with concentrated NH_4OH; Varigrad chamber no. 2—150 ml ethyl acetate saturated with concentrated NH_4OH. *Water soluble indoles.* Column, 8.0 g of silica gel hydrated with 5.0 ml of concentrated NH_4OH; fraction size, 9.4 ml; solvents, Varigrad chamber no. 1—200 ml *n*-hexane saturated with concentrated NH_4OH; Varigrad chamber no. 2, 150 ml *n*-butyl alcohol saturated with concentrated NH_4OH; Varigrad chamber no. 3, 150 ml *n*-butyl alcohol saturated with concentrated NH_4OH. At the end of the gradient elution 50 ml of *n*-butyl alcohol saturated with 1 N NH_4OH were used to elute indoleacetylaspartic acid.

FIG. 3 (*lower right*). Gas chromatogram of presumptive methyl indole-3-acetate from a purified maize extract.

recovered as the methyl ester, while no nonesterified IAA was detected. The usefulness of the method for other indole acids remains to be investigated.

Gas Chromatography. This subject has been discussed in greater detail elsewhere (14). A conventional gas chromatograph equipped with an Argon ionization detector (^{90}Sr) was employed. Six foot, U-shaped glass columns, 6 mm inside diameter were used. The liquid phase substrate for the column was Versamid 900, an ethylene-diamine linoleic acid polyamide of uncertain structure manufactured by General Mills, Inc. This was coated onto Anakrom, Type ABS, 70–80 mesh, a flux-calcinated diatomaceous earth prepared by the Analytical Engineering Laboratories, Inc., to give a 7 % Versamid column. The coating process was carried out by A) dissolving 2 g of Versamid 900 in 150 ml of chloroform: *n*-butyl alcohol, 1: 1 by refluxing, B) adding 27 g of Anakrom ABS to the cooled solution, and C) evaporating the chloroform and butyl alcohol under vacuum at room temperature on a flash evaporator. Powder that adhered to the sides of the evaporator flask was scraped loose and reduced to 60–80 mesh size by gentle grinding. B. B. Stowe (personal communication) has since found that chloroform: isopropyl alcohol works much better than chloroform: *n*-butyl alcohol. Adhesion of the powder to the sides of the flask is minimized, and it is easier to evaporate the carrier solvent from the powder. A column temperature of about 230° was used. Indole samples (1–10 μg), dissolved in 0.5 to 10 μl of ethanol or preferably acetone or acetonitrile, were injected into the vaporizing block kept at a temperature of 70 to 80° above that of the column. Argon flow rates of 85 to 120 ml/min were suitable. Such a system could readily separate a number of neutral indole substances (14). The recovery of the methyl ester of IAA after gas chromatography appears to be satisfactory. Only one such test was made by the author, in which 9.4 μg of the methyl ester was injected into the chromatograph, and 9.8 μg was recovered as determined by spectrophotofluorometry. B. B. Stowe (private communication) reports obtaining similar data from unpublished experiments. Recovery percentages for other indoles are unknown.

Spectrophotofluorometry. A detailed investigation of this technique with indole compounds has been reported (14). As little as 1 nanogram of several of the indoles, including IAA, may be detected and measured with a fair degree of accuracy. Sensitivity such as this compares favorably with the best bioassays commonly used for IAA and other biologically active indole compounds. Fractions from the gas chromatograph that were to be assayed by spectrophotofluorometry were collected by condensing them at room temperature in a loop of small bore Teflon tubing attached to the outlet on the detector oven of the gas chromatograph. The material was then washed from the Teflon tubing with acetone into a test tube .The acetone was evaporated and the residue dissolved in one ml of absolute ethyl alcohol and one ml of a glycine-HCl buffer, pH 2.0 (52 ml of a solution 0.1 M with respect to glycine and NaCl plus 48 ml of 0.1 M HCl) and transferred to a quartz cuvette. Readings were made with an activation wavelength of 280 mμ and a fluorescence wavelength of 360 mμ. These wavelengths are near maxima for most indoles with the exception of the 5-hydroxyindoles which are activated at 295 mμ.

Detection of IAA in Maize. IAA was shown by Berger and Avery (1) to be produced by alkaline hydrolysis of a water soluble precursor from the sweet corn cultivar Country Gentlemen (*Zea mays,* var. rugosa). As a test for the usefulness of the methods described, this material was examined for the presence of IAA. Dry maize seed was ground with water in a homogenizer. The slurry was alkalized to pH 9 to 10 with NH_4OH, then centrifuged to eliminate solids. The supernatant material was fractionated according to the scheme of figure 1, discarding all fractions except the acid one in which IAA would occur. The acid fraction was chromatographed on a silica gel column, using the procedure suggested for acid indoles. Those fractions from the column in which IAA should occur were bulked, taken to dryness, and the residue esterified using diazomethane. The methyl ester of IAA was separated from any nonesterified IAA by partitioning between methylene chloride and 2 % $NaHCO_3$. The methylene chloride, containing the methyl ester, was taken to dryness and the residue transferred with small volumes of acetone to a microtube made by sealing the pointed end of a medicine dropper pipette. The acetone was removed by a stream of N_2 and the residue dissolved in 0.1 ml acetone. Upon gas chromatography of 0.5 μl of extract, representing about 5 g of dry maize seed, a sharp peak in the position of methyl indole-3-acetate was observed (fig 3). Cochromatography with authentic methyl indole-3-acetate yielded only a single peak. This substance was further characterized by collecting it from the gas chromatograph and determining its fluorescence spectrum on the spectrophotofluorometer using an activation wavelength of 280 mμ. Its fluorescence spectrum (fig 4) was typical of that of authentic methyl indole acetate and many other indole compounds. A small aliquot of the extract yielded a strong Salkowski reaction, the color appearing identical to that given by authentic methyl indole-3-acetate, and distinctly different from that given by several other common indoles. Thus, the technique was successful in aiding the isolation and identification of IAA from maize.

Detection of Indole-3-Acetonitrile in Cabbage. As a further test for the usefulness of the method just outlined when applied to plant material, an acetone extract was made of a cabbage head (*Brassica oleracea,* var. capitata). Jones et al. (6) had previously established that indole-3-acetonitrile occurred in cabbage; therefore, the extract was fractionated according to the scheme of figure 1 so as to retain the neutral fraction and thus any indoleacetonitrile.

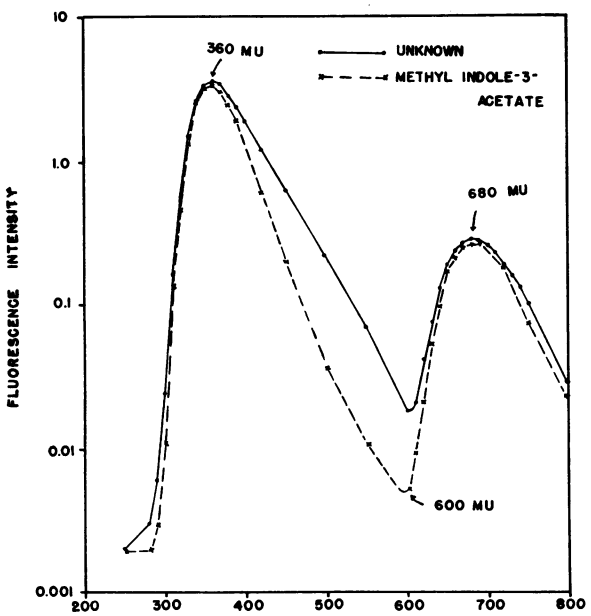


FIG. 4. Fluorescence spectrum of presumptive methyl indole-3-acetate from maize and authentic methyl indole-3-acetate. Activation was at 280 mμ. The peak at 680 mμ may be second order spectrum.

Other fractions were discarded. The neutral fraction was subjected to silica gel column chromatography according to the method previously outlined for neutral indoles. The fractions from the column which could be expected to contain indoleacetonitrile were bulked and taken to dryness. Alcoholic aliquots of 2 to 10 μl of the residue, which was equivalent to 1.2 to 6 g fresh weight of the original cabbage head, were gas chromatographed. A sharp peak in the position of indole-3-acetonitrile was observed. Co-chromatography with authentic indoleacetonitrile yielded a single peak. The fluorescence spectrum of the substance indicated that it was an indole compound. Thus, it is highly likely that it was naturally occurring indole-3-acetonitrile that was detected in cabbage, that conclusion resting on its fractionation properties, silica gel column chromatography, gas chromatography and spectrophotofluorometry.

Discussion

These results suggest that it is now possible to use gas chromatography and spectrophotofluorometry to detect minute quantities of certain indole compounds in plant extracts where, previously, biological means were necessary at similar concentrations. Gas chromatography alone doubtless would prove adequate in many cases, but the 2 techniques together are more definitive since it is possible to show spectral characteristics of the substance being gas chromatographed by employment of the spectrophotofluorometer. This instrument also permits a quantitative check on the gas chromatograph. It proved to be more sensitive to indoles than did the argon ionization detector, in some experiments detecting submicrogram quantities that completely escaped detection by gas chromatography alone. Using spectrophotofluorometry it is possible to detect as little as 10^{-3} μg of several indoles in pure solution (14). The minimum quantities of such compounds necessary for detection in plant extracts has not been studied. Before spectrophotofluorometry can be applied to plant extracts considerable purification must be effected. Fractionation of the crude extracts into major indolic groups, followed by silica gel column chromatography and gas chromatography, were utilized for the necessary purification. There would appear to be no step in the procedure in which losses are inherent, at least for the more stable indoles such as IAA. Good recovery has previously been shown for a similar silica gel column technique (10), while work reported here suggests satisfactory recovery after esterification and gas chromatography.

The method appears to be adapted to all the simple neutral and acidic indole compounds, with the probable exception of those that are very labile. It cannot, of course, differentiate between indoles that are biologically active and those that are not, but since the method is nondestructive, bioassays can be carried out after spectrophotofluorometry.

Although diethyl ether has been the standard organic solvent used for fractionating indoles and similar compounds into acidic and nonacidic groups, Luckwill found that methylene chloride gave cleaner separations (7). Methylene chloride has been used at several points in the technique reported here. It appears to have not only the desirable solvent properties and low boiling point similar to that of ether, but has the further advantages of being nonflamable, much less miscible with water, and without the frequent necessity of peroxide elimination that characterizes ether. However, caution may still need to be exercised since previously it has been shown that a decomposition product, thought to be chlorine, is formed on standing from freshly purified chloroform which causes partial loss of auxin activity (15). A similar situation may exist with methylene chloride. If this were found to be the case peroxide-free ether could be substituted for the methylene chloride.

The technique at its present level of development is not rapid. Several samples could be processed using conventional auxin techniques (single dimensional paper chromatography, etc.) in the time required for one analysis as described here. Assuming that the method is shown to be quantitative, it may be more satisfactory to analyze fewer samples well, than to obtain the ambiguous results that typify so many auxin papers of the past. It should prove extremely useful for characterizing indoles.

The time required for the analysis of a sample may not be as great as indicated if certain steps can be eliminated. Highly contaminated samples will probably have to go through a series of purification steps comparable to, if not identical with, those

described here. It may, however, be possible to omit silica gel column chromatography, and proceed directly from the original fractionation into major indole groups to gas chromatography on samples not containing large amounts of impurities.

Summary

A procedure has been devised for the fractionation and purification of certain indole derivatives occurring in plant extracts so that they may be processed by gas chromatography and spectrophotofluorometry. The sequence of steps encompasses A) fractionation of the indoles in a plant extract into 4 major indole groups—acidic, basic, neutral and water soluble compounds, B) preliminary purification of the indoles in each group by silicia gel column chromatography, C) further purification of the neutral and acidic indoles by gas chromatography, and D) measurement of the fluorescence intensity of individual indoles with a spectrophotofluorometer.

The procedure has been used to confirm the presence of indole-3-acetic acid in maize and indole-3-acetonitrile in cabbage.

Acknowledgments

The author is indebted to Professor Bruce B. Stowe for supplying laboratory facilities for these investigations, for valuable advice, for access to much unpublished data necessary to perform the work described here, and for supplying a number of indole derivatives not commercially available. He also wishes to thank Patrick Malcolm for the privilege of examining unpublished data concerning diazomethylation of acidic indoles.

Literature Cited

1. Berger, J. and George S. Avery. 1944. Isolation of an auxin precursor and an auxin (indoleacetic acid) from maize. Am. J. Botany 31: 199–203.
2. Bowman, R. L., P. A. Caulfield, and S. Udenfriend. 1955. Spectrophotofluorometric assay in the visible and ultraviolet. Science 122: 32–33.
3. Ehrlich, F. and K. A. Jacobsen. 1911. Uber die Umwandlung von Aminosauren in Oxysauren durch Schimmelpilze. Ber. Deut. Chem. Ges. 44: 888–97.
4. Fawcett, C. H. 1961. Indole auxins. Ann. Rev. Plant Physiol. 12: 345–68.
5. Gordon, S. and R. P. Weber. 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. 26: 192–95.
6. Jones, E. R. H., B. Henbest, G. F. Smith, and J. A. Bentley. 1952. 3-Indolylacetonitrile: a naturally occurring plant growth hormone. Nature 169: 485–87.
7. Luckwill, L. C. and C. P. Lloyd-Jones. 1960. Metabolism of plant growth regulators I. 2,4-Dichlorophenoxyacetic acid in leaves of red and of black currant. Ann. Appl. Biol. 48: 613–25.
8. Nitsch, J. P. 1955. Free auxins and free tryptophan in the strawberry. Plant Physiol. 30: 33–39.
9. Peterson, E. A. and H. A. Sober. 1959. Variable gradient device for chromatography. Anal. Chem. 31: 857–62.
10. Powell, L. E. 1960. Separation of plant growth regulating substances on silica gel columns. Plant Physiol. 35: 256–61.
11. Powell, L. E. 1963. Solvent systems for silica gel column chromatography of indole derivatives. Nature 200: 79.
12. Schlenk, H. and J. L. Gellerman. 1960. Esterification of fatty acids with diazomethane on a small scale. Anal. Chem. 32: 1412–14.
13. Stowe, B. B. 1959. Occurrence and metabolism of simple indoles in plants. Progr. Chem. Org. Nat. Prod. 17: 249–97.
14. Stowe, B. B. and J. F. Schilke. 1963. Submicrogram identification and analysis of indole auxins by gas chromatography and spectrophotofluorometry. In: Regulateurs Naturels de la Croissance Végétale, Colloques Internationaux du Centre National de la Reserche Scientifique, 123: 409–19, Paris (1964).
15. Thimann, K. V. and F. Skoog. 1940. The extraction of auxin from plant tissues. Am. J. Botany 27: 951–60.