BRIEF PAPERS

FURTHER STUDIES OF SOME PLANT INDOLES AND AUXINS BY PAPER CHROMATOGRAPHY[1,2]

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The characterization of plant growth substances by paper chromatography has expanded rapidly in the past few years, and since the review of Gordon in 1954 (6) many new papers have appeared (1, 2, 4, 7, 11, 12, 17, 19, 20, 21, 22, 24, 26). This increasing use of the procedure makes it desirable to report both its successes and limitations when applied to new plant materials and to the solution of existing problems.

Since all the auxins so far conclusively identified in plants are indole derivatives, it was felt that an examination of plant material known to be rich in indole compounds might bring out some new auxin relationships. Chromatograms of two indole-rich materials have accordingly been prepared and studied. The indole derivatives of a representative tissue culture and of the medium on which it was grown have also been examined, in order to determine whether the indoleacetic acid in the culture could have been taken up from the medium.

Indole itself has been reported to be present in the perfume of, and extracts from, many flowers, notably by Hesse (8, 9) who succeeded in isolating the substance in from 0.1 to 2.5 % yield from large quantities of Citrus and other flower oils. Since no modern investigation of this material has been recorded, a study was made of orange blossoms obtained through the courtesy of Dr. W. C. Cooper, U. S. Department of Agriculture, Weslaco, Texas. The blossoms were picked and shipped by air mail the same day. The material was wilted and somewhat browned on arrival, but was at once placed in the deep freeze at – 10° C. Later, the 139 gm of entire flowers were extracted with 800 ml of peroxide-free ether for 2 hours at 5° C. The strongly yellow ether extract was fractionated into neutral and acid substances by extraction of the acid materials with 1 % $NaHCO_3$, separation of the aqueous layer, acidification to pH 3 with 15 % tartaric acid, and re-extraction with ether. Both fractions were strongly fragrant; on evaporation the acid fraction yielded about 0.4 ml of a yellow oil, while the neutral fraction produced a copious waxy orange-colored residue. This latter was triturated with several small volumes of ethanol, which were combined and evaporated in vacuo, resulting in an orange oil. Samples of this alcohol triturate oil, the acid fraction oil and a small piece of the neutral fraction wax were applied to individual Whatman No. 1 paper strips and chromatographed in 8–1–1, isopropanol–28 % NH_4OH–H_2O. The appearance of the strips under ultraviolet light and after spraying with modified Salkowski reagent is indicated in table I.

Methyl anthranilate, also reported by Hesse as a constituent of orange blossom oil, was run on the control strips where it appeared as a bright blue fluorescing spot, but no similarly appearing compound was noted on chromatograms of the extracts. The presence of indole is confirmed by the orange Salkow-

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TABLE I

CHROMATOGRAPHIC BEHAVIOR OF SALKOWSKI-POSITIVE SUBSTANCES IN FRACTIONS OF ETHER EXTRACT OF CITRUS FLOWERS

COMPOUND	CONTROLS		ACID FRACTION		NEUTRAL WAXY FRACTION		NEUTRAL ALCOHOL TRITURATE	
	SALKOWSKI COLOR	R_f	SALKOWSKI COLOR	R_f	SALKOWSKI COLOR	R_f	SALKOWSKI COLOR	R_f
Indole, 40 μgm	Diffuse orange	0.8	Weak orange	0.8–0.9	Diffuse orange	0.7–0.9	Orange	0.7–0.9
Methyl anthranilate, 20 μgm	Blue (in UV)	0.77	Faint brown	0.53			Yellowing	0.4–0.7
IAA, 20 μgm	Crimson	0.25	Crimson	0.20–0.25	Bright yellow (in UV)	0.2–0.4	Bright yellow (in UV)	0.2–0.35
			Yellow (in UV)	0.12	Bright yellow (in UV)	0.0–0.1	Bright yellow (in UV)	0.04–0.1
					Green	0.0		

Equilibrated 18 hrs, run 10 hrs at 26° C, solvent: isopropanol–water–ammonia, 8–1–1.

ski reaction at high R_f's on all three strips; apparently so much was present that it was carried over into the acid fraction as well. As has been noted elsewhere (24) indole does not form compact spots under these chromatographic conditions. Two brilliant yellow fluorescing compounds are present in the neutral extracts. Indole acetic acid (IAA), probably extracted from the ovaries of the flowers, was indicated by a compact crimson area at R_f 0.20 to 0.25.

The acid fraction was further studied by biological assay. Portions of the chromatogram were placed in 2 ml of 2.5 % sucrose solution together with twelve 3-mm subapical Avena coleoptile sections and the growth over 20 hours was measured. The detection of an area of strong growth promotion at an average R_f of 0.25, corresponding with a control spot of synthetic IAA, supported the above colorimetric identification of IAA in the orange blossom extract. IAA has previously been reported to occur in flower and fruit parts (18, 20). In addition to the area of growth promotion considered due to IAA, an area of growth inhibition was detected on the above chromatogram. The latter area corresponded in R_f with the unknown substance present in a number of tissues and termed inhibitor β (12). Kefford (13) has recently found evidence that inhibitor β may be an indole or a phenol derivative.

Another indole derivative, abrine (n-methyltryptophan) constitutes about 1 % of the dry weight of Jequirity seeds, *Abrus precatorius* L. (5). Therefore, 400 gm of these seeds obtained from the Cheney Drug Company, Boston, Massachusetts were subjected to the isolation procedures for abrine described by Cahill and Jackson (3). No difficulty was experienced in obtaining it as a pure crystalline compound. Nevertheless, several chromatograms of the same methanol extract from which abrine was later isolated pure failed to show any convincing reaction with either the Salkowski or Ehrlich reagents. The isolated abrine, when chromatographed alone, was readily detected on the paper by both these reagents at an R_f of 0.29 (cf 24). Evidently, therefore, the large quantity of pigments removed from the seed coat, as well as the lipids present in the extract, interfered with the chromatography and color-developing reagents. The experience is cited to demonstrate how easily compounds present in considerable quantities in an extract may be completely masked by other substances.

The isolation of a strain of corn endosperm which grows vigorously in vitro has been reported by La Rue (16), and its cultural requirements have been described by Straus and La Rue (25). The tissue is designated as CE Clone 1-C and has been maintained for several years on a mineral salts–sucrose–yeast extract medium.

We obtained from Dr. Jacob Straus of these laboratories (cf 25) a group of 16 cultures of this endosperm material of the same age. The combined fresh weight of the tissue was 19 gm, and being very friable, it was not ground but extracted directly with 200 ml of peroxide-free ether in the cold room overnight.

TABLE II

R_f AND SALKOWSKI REACTION OF ETHER EXTRACT (ACID FRACTION) FROM CORN ENDOSPERM CULTURED ON YEAST EXTRACT MEDIUM

MATERIAL	R_f	SALKOWSKI REACTION
IAN, 40 μgm	0.84	Blue
IAA, 40 μgm	0.30	Crimson
Acid fraction of ether soluble extract	0.30	Crimson
	0.20	Two close crimson spots
	0.17	

Equilibrated 6 hrs, run 15 hrs at 26° C, solvent: isopropanol–ammonia–water, 8–1–1.

Fractionation and chromatography of this extract was carried out as outlined above for the orange blossom material; in this case both the neutral and acid fractions of ether-soluble material yielded only very small quantities of residue. Table II presents the results for the acid fraction and controls only; the chromatograms of the neutral fraction showed no spots other than faint fluorescent areas.

From the observed color density and spot area, at least 20 μgm of IAA were present in this 19 gm of tissue, or approximately 1 mg/kg fresh weight. This concentration is of the same order as those found by Hinsvark et al (10) in developing corn kernels, but it is remarkably high when compared with other growing tissues (e.g., etiolated seedlings) in which IAA has been assayed. The two close but distinctly separated spots of lower R_f value than IAA had the same R_f and gave the same crimson color with the Salkowski reagent as two bands found in alcoholic extracts of sweet corn seed and potato tuber skin (13). These spots are in the region to which indole pyruvic acid migrates, and this compound gives a similar color reaction (24). However, since direct comparison with a new sample of synthetic material was not possible at this time, precise identification of these spots must be deferred.

The large amount of IAA (1 mg/kg) present in the maize endosperm must either have been synthesized by this tissue, or have been concentrated from the medium. The IAA content of the medium was therefore examined. The medium used to grow the 16 cultures totalled about 160 ml and included some 800 mg of Difco yeast extract. Accordingly, 2.5 gm of Difco yeast extract was dissolved in 100 ml of water. One half of this solution was then autoclaved 20 minutes at 15 lbs to see if this procedure would release IAA from labile precursors. (The yeast extract used for the tissue culture medium was Seitz-filtered and had not been autoclaved.) When cool both the autoclaved and untreated extracts were brought to pH 3 with 15 % tartaric acid and extracted twice with 100 ml of peroxide-free ether. A very small amount of tan-colored residue resulted from the evaporation of the ether. Chromatograms were run as described above. Although both chromatograms showed several small compact spots of fluorescent ma-

terials, no trace of any color was developed by the Salkowski reagent.

Since the total amount of substances which might interfere was very small it is unlikely that IAA actually present could have been masked as was the abrine in the Jequirity seeds extract. The modified Salkowski reagent could readily detect 1 μgm of IAA on clean strips such as these. It can be concluded, therefore, that less than 1 μgm of IAA is present in a gram of yeast extract, and that autoclaving at its natural pH does not release any detectable amount of IAA or other Salkowski-reacting compounds from yeast extract in solution.

However, yeast extract must contain some IAA or other ether-soluble auxin, since IAA has been isolated from yeast (14), and more recently Skinner and Street (23) found it necessary to treat yeast extract with ether to remove auxin-like substances interfering with their root cultures. To check this point, portions of a chromatogram of the ether-soluble acids from 2.5 gm of Difco yeast extract were assayed with Avena coleoptile sections. An area of growth activity corresponding in R_f value with IAA, and equivalent to about 0.4 μgm of IAA, was detected. This corresponds to about 0.15 μgm IAA/gm (Skinner and Street estimated 0.4 μgm/gm). Hence, if the endosperm cultures had taken up all the IAA from the 800 mg of yeast extract present in the medium, they would only have gained 0.13 μgm. But the IAA content of the tissues, as shown above, was certainly a hundred-fold more than this, namely of the order of 20 μgm. Thus, it is clear that the endosperm culture synthesizes its IAA de novo, and does not merely absorb it from the medium. This provides independent support of the conclusion of Kulescha and Gautheret (15) based on bioassay alone, that some tissue cultures are capable of producing an auxin de novo.

In this and related studies it has been observed that the Salkowski reaction is by no means specific for indoles. Many phenolic compounds give strong reactions. Some colors and R_f's are: catechol, brown-pink, 0.4; *p*-hydroxybenzaldehyde, faint yellow, 0.4; resorcinol, crimson, 0.7; α-naphthol, blue, 0.75; orcinol, yellow-green, 0.8. This fact should be noted in studying plant extracts.

Summary

Paper chromatography, using a modified Salkowski reagent and biological assay, was applied to a number of plant extracts believed to contain indole derivatives. Orange blossoms were shown to contain quantities of indole and indole-3-acetic acid (IAA) together with an unknown growth inhibitor.

Although crystalline abrine was readily obtained from a methanolic extract of Abrus seeds, no abrine could be detected on a chromatogram of this extract, because of interference by lipides and pigments.

Yeast extract (Difco) contains IAA at a concentration of about 0.15 μgm/gm.

Corn endosperm grown in vitro, using the yeast extract as medium, contains 1 mg IAA per kg fresh weight, as well as two other Salkowski reacting substances. These compounds are thus synthesized by the tissue and not merely taken up from the medium.

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AN AUXIN INACTIVATION SYSTEM INVOLVING TYROSINASE[1,2]

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Since the discovery by Thimann (12) that leaf extracts of *Vicia Faba* and *Helianthus annuus* would inactivate native auxins in solution, much work has been done to elucidate the nature of the auxin-inactivating systems in extracts of plants. Because auxin destruction tended to be pronounced in tissue extracts in which browning occurred, it was suggested that phenolases might participate in auxin destruction (12, 15). Wagenknecht and Burris (13) found inhibitions of IAA oxidation by copper-chelating agents, which further implicated a copper enzyme. These results were not widely confirmed, however, and more recently the emphasis has shifted to the iron enzyme, peroxidase. There is conclusive evidence that peroxidase can inactivate IAA, although the mechanism by which this oxidation comes about remains uncertain (4, 5, 6, 7, 9, 11).

In recent work (2) on extracts of the fern *Osmunda cinnamomea* L., peroxidase appeared to be responsible for at least part of the auxin-destroying activity found in tissue extracts. However, occasional inhibitions by copper-chelating agents were observed. Since these extracts also contained an active tyrosinase, it appeared worthwhile to investigate the possibility that this enzyme might act on IAA.

Tyrosinase, prepared from the mushroom *Psalliota campestris*, was obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio. Tyrosinase activity was determined by the method of Adams and Nelson (1). The tyrosinase-containing extract of Osmunda was prepared by extracting dormant leaves as described previously (2), and dialyzing the centrifuged extract against 0.067 M, pH 6.1 phosphate buffer for 5 days (with five changes of buffer), at 4° C. It contained 0.74 catecholase units per ml, or 2.1 units per gm fresh weight of tissue.

IAA inactivation was measured by incubating a 7×10^{-7} M solution of IAA, containing appropriate addenda and made up to 10 ml, at 30° C, in the dark, with occasional shaking. Aliquots were removed at the times specified, boiled for 5 minutes to inactivate the enzyme, and made into agar blocks for auxin bioassay by the standard Avena curvature test (14). Within the range of variability of the test, Avena curvature was roughly proportional to the concentration of auxin in the aliquot. Therefore, all results are expressed as degrees Avena curvature, and percentage inactivation is calculated from these values.

The results shown in table I indicate that, while mushroom tyrosinase alone or in the presence of tyrosine or 3,4-dihydroxyphenylalanine (DOPA) did not inactivate IAA, appreciable auxin destruction occurred when the enzyme acted on pyrogallol or catechol. These phenols alone had no effect on IAA, and all four of them were oxidized by the enzyme, as was

TABLE I

INACTIVATION OF IAA BY MUSHROOM TYROSINASE

ADDENDA *	TIME	AVENA CURVATURE	INACTIVATION
	hrs	*degrees*	*%*
None	6	33	..
Enzyme	0	32	
	6	35	0
Pyrogallol	6	34	0
Enzyme + pyrogallol	0	32	
	6	23	27
Catechol	6	31	6
Enzyme + catechol	0	32	
	6	14	58
DOPA	6	33	0
Enzyme + DOPA	0	33	
	6	32	4
Tyrosine	6	32	4
Enzyme + tyrosine	0	32	
	6	33	0

* Basal reaction mixture was 7×10^{-7} M IAA, pH 6.1. Tyrosinase was added to give 0.12 catecholase units per ml. Phenols were added to make 10^{-3} M. Final volume 10 ml.

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