Phytochemistry, 1965, Vol. 4, pp. 891 to 904. Pergamon Press Ltd. Printed in England

GROWTH SUBSTANCES IN THE ROOTS OF *VICIA FABA*—II*

D. BURNETT†, L. J. AUDUS and H. D. ZINSMEISTER‡

Bedford College, University of London

(*Received* 10 *December* 1964)

Abstract—An extension of previous studies[1] on the auxins of *Vicia faba* roots has used cellulose and cellulose derivative column chromatography and spectrofluorimetry, for the separation and identification of the components of methanol extracts. Purification of the IAA-like ether-soluble acid auxin has been carried out on DEAE cellulose. Owing to the presence of a fluorescent compound (activation max. 330 mμ, emission max. 445 mμ) in the growth active region of chromatograms, IAA could not be positively identified by its u.v. absorption or fluorescent behaviour but cannot be present in quantities greater than 2–5 μg/kg fresh weight of root material. The accompanying fluorescent material may be an auxin very closely related to the "citrus auxin" of Khalifah *et al.*[2] In the water-soluble ether-insoluble fraction, tryptophan and 3,4-dihydroxyphenylalanine (DOPA) have been unambiguously demonstrated and accurately estimated to be present in concentrations of 12 mg and 20 mg/kg fresh weight of root respectively. Tryptophan may contribute most, if not all, the auxin activity to the zone on the chromatogram corresponding to WP(*i*) in pea roots and P_1 previously reported for *Vicia* roots. Other substances, some probably indoles, are present in the zone but have not been identified. Three other auxin zones (corresponding to WP(*ii*), WP(*iii*) and WP(*iv*) in pea roots) have been shown to contain neutral and basic substances, probably indoles, but not yet identifiable by their fluorescent characteristics. The fact that mild oxidation of DOPA with silver oxide produces substances with auxin activity and properties resembling those of the water-soluble components suggests that the latter may be partly artefacts of DOPA oxidation during extraction and chromatography.

INTRODUCTION

SEVERAL studies have already been made of the auxins of *Vicia faba* roots (Audus and Lahiri, 1961;[1] Lahiri and Audus, 1960[3]). Several substances with marked activity in the *Avena* first internode and coleoptile segment tests have been demonstrated in methanol and ethanol extracts but none have been identified with certainty. In view of doubts concerning the role of endogenous indole-3-acetic acid (IAA) in growth control in roots[3–6] it seemed important to make further studies of two major problems revealed by this early work. They were (*a*) the nature of the ether-soluble acidic auxin which closely resembled IAA, but which was not apparently identical with it and which seemed to be concerned with geotropic response, and (*b*) the nature of the water-soluble, ether-insoluble auxins whose combined activities in the extracts far exceeded that of the IAA-like substance.

* The greater part of the experimental work described in this paper was submitted as part of a thesis for the Ph.D. degree of the University of London by D. Burnett.

† Present address: Botany Department, Rothamsted Experimental Station, Harpenden, Herts.

‡ Dr. H. D. Zinsmeister, as a guest in the department, collaborated closely in the work on the four water-soluble unknowns An_1–An_4. Present address: Botanisches Institut, Der Universitat, München.

[1] L. J. AUDUS and L. N. LAHIRI, *J. Exp. Botany* **12**, 75 (1961).
[2] R. A. KHALIFA, L. N. LEWIS and C. W. COGGINS, JR., *Science* **142**, 399 (1963).
[3] A. N. LAHIRI and L. J. AUDUS, *J. Exp. Botany* **11**, 341 (1960).
[4] L. J. AUDUS and M. E. BROWNBRIDGE, *J. Exp. Botany* **8**, 105 (1957).
[5] L. J. AUDUS and M. E. BROWNBRIDGE, *J. Exp. Botany* **8**, 235 (1957).
[6] L. J. AUDUS and N. DAS, *J. Exp. Botany* **6**, 328 (1955).

THE ACID, ETHER-SOLUBLE AUXINS

Separation of Fractions

The ether-soluble fraction (see Methods) of 12-day-old *Vicia faba* roots was evaporated to dryness, redissolved in distilled water and applied to the top of a charged DEAE/cellulose (anionic) column. Elution with distilled water removed neutral indole compounds leaving only acidic compounds on the column. Unfortunately indole-3-acetic acid (IAA) seems to be partially decomposed when 0·1 N H_2SO_4 is used for elution but is satisfactorily removed with 0·05 M Na_2SO_4. This eluate can be re-extracted with ether after acidifying to pH 3 giving with microgram quantities, recoveries of 85–97 per cent. This is superior to methods

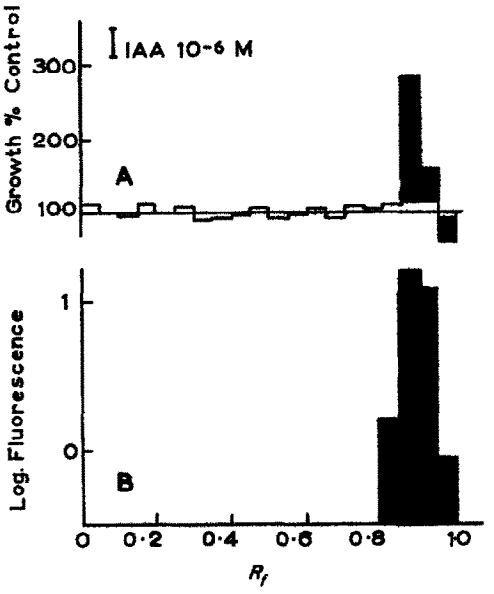

FIG. 1.

(A) *Avena* internode bioassay of paper chromatogram of ether-soluble fraction after elution from DEAE cellulose column with 0·05 M Na_2SO_4. Equivalent content of 40 g fresh weight of roots. The shaded areas mark responses beyond the 5 per cent fiducial limits; (B) Chromatogram of IAA marker. Quantitative estimation by fluorescence.

involving extraction of acids with 5% sodium bicarbonate, which, in a series of preliminary tests on similar small quantities of IAA and other indole acids (indole-3-carboxylic, -butyric, -propionic and -lactic), followed with the spectrophotofluorimeter, gave recoveries of the order of 50 per cent only.

Paper Chromatography

The ether extracts of the column eluate were taken down to a small volume (1–2 ml) and spotted onto standard chromatograph paper strip (see Methods). After running in the standard solvent, strips were divided longitudinally into three equal parts; one was assayed by the *Avena* first internode assay and the other two were used for chemical and fluorescence tests respectively. A parallel chromatogram was always run, with IAA as a marker.

The results of a typical analysis are shown in Fig. 1A together with chromatograms of the

IAA marker which were similarly cut into twenty strips, each strip being eluted and IAA estimated by the spectrophotofluorimeter (Fig. 1B). It will be seen that very considerable auxin activity occurs at the same R_f as IAA. This area is Ehrlich-positive. However at the solvent front there is significant growth inhibition which coincides with substances giving positive reactions with *p*-nitraniline and ferric chloride, indicating the presence of phenolic acids.

Spectrophotofluorimetry

Further attempts at identification of the auxin involved elution of the strips containing active material, followed by fluorimetry in phosphate citrate buffer at pH 5. Preliminary tests, run with IAA in amounts giving activity comparable with that of the extract, gave

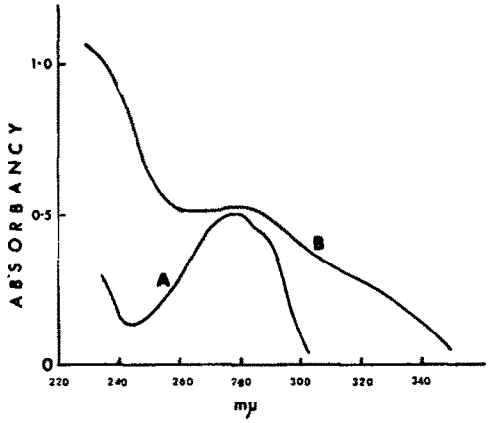

FIG. 2. ULTRA-VIOLET ABSORPTION SPECTRA.

(A) 10^{-4} M IAA and (B) acid ether-soluble eluate from DEAE cellulose column at a concentration equivalent to contents of 50 g fresh weight of root per ml of phosphate-citrate buffer, pH 7·0.

recoveries of 77·8 ± 3·3 per cent. From the chromatograms strips were cut between R_f 0·77 and 0·97 (faint Ehrlich reaction). The eluate of these strips showed the presence of one compound with single activation and emission maxima at 330 mμ and 445 mμ respectively. The corresponding values for IAA are 285 mμ and 365 mμ. This corresponds to no known indole compound that has been tested so far (cf. [7]).

However the possible presence in this region of the chromatogram of phenolic and other compounds having u.v. absorption bands at or near the maxima recorded above might well distort the spectra of this unknown compound. To check this a series of dilutions were made on an eluate and their spectral characteristics re-examined. No change in the wavelength of the spectral maxima took place on dilution and the relationship between fluorescence intensity and concentration was linear up to values equivalent to the contents of 100 g fresh weight of root per ml of solution. This showed the absence of any gross interference. Ultra violet absorptions of similar solutions (≡ 50 g fresh weight of root/ml) were compared with that of a 10^{-4} M IAA solution (Fig. 2). There was a main absorption peak at 285 mμ corresponding very closely with that of IAA but there were obviously other u.v. absorbing compounds present. Thus the existence of another absorbing peak at 320–330 mμ, possibly corresponding to the unknown fluorescent compound, can just be detected. If we assume that the absorption at

[7] D. BURNETT and L. J. AUDUS, *Phytochem.* 3, 395 (1964).

285 mμ is due entirely to IAA this represents a total of 17·5 μg in 50 g of roots. This concentration of IAA would have been self-quenching, but even so should have given a fluorescence intensity of well over 100 units at the corresponding IAA wavelengths; in fact an intensity of only 2·75 units was found.

This very strongly suggests that IAA could not constitute more than about 3 per cent of the total material absorbing u.v. at that wavelength. However biological assays of several eluates show that an IAA content from 2–10 μg/kg fresh weight of roots could have accounted for the recorded growth activities and this would have been equivalent to 1·3–7·2 units of fluorescence at the dilutions used, these values overlapping the 2·75 units actually observed. One might have expected such an amount to have given a distinct shoulder on the emission spectrum of the main fluorescent compound but none was ever detected.

THE WATER SOLUBLE FRACTION

The aqueous residue left after successive extractions with ether at pH 3 (the water-soluble fraction) was taken to pH 7 with saturated barium hydroxide and the resulting precipitate spun down at about 500 g. The golden-coloured supernatant was then used in the following analyses after reduction to suitable volume under vacuum at 26°.

Paper Chromatography

The paper chromatograms were loaded on a transverse line at a rate equivalent to the contents of 10–15 g of root material. After running in the standard solvent the 6-cm strips were usually divided longitudinally into three equal 2-cm strips for bioassay, colour tests and elution for further studies respectively.

Bioassays of typical chromatograms are seen in Fig. 3, both corresponding to the content of 3·3 g fresh weight of roots. Auxin activity between R_fs 0·05 and 0·3 has been consistently found in all chromatograms so far run. In addition occasional significant responses appear at other R_f values, e.g. 0·65–0·7 and 0·9–0·95, but these are not so consistent.

This zone of auxin activity between R_fs 0·05 and 0·3 corresponds very closely with two chemically-reacting spots. The first (R_f 0·05–0·1) gives immediate deep purple and green colours with *p*-nitraniline and ferric chloride respectively and has been called *Unknown A*. From its reactions it was thought to be a dihydroxyphenolic compound. The second (R_f 0·15–0·28) gives a purple colour with Ehrlich's reagent and has been called *Unknown B*. Because of the great auxin activity in this region these two spots have been examined in some detail.

There are however very considerable quantities of amino acids in this region of the chromatogram. These have been separated and partially identified by subsequent two-way paper chromatography but only one, tryptophan (see later), is likely to have been responsible for any of the auxin responses. The benzidine reaction shows that sugars are also present but these could not have caused a response since the biological assays were always carried out in optimal sucrose buffer. Paper chromatography in other solvents shows that these sugars are almost entirely sucrose, glucose and fructose.

Two Ehrlich yellow reactors (*Y*1 and *Y*2) also occur at R_fs 0–0·03 and 0·22–0·32 respectively. They have no auxin activity. They have not yet been identified but the following possibilities have been ruled out on a basis of R_f in our standard solvent: urea, allantoin, *o*-, *m*- and *p*-aminobenzoic acids, kynurenine, citrulline, indole-3-acrylic acid and 3- and 5-hydroxyanthranilic acids.

The identity of Unknown A. The indication that *A* was a dihydroxyphenolic compound pointed to L-β(3,4-dihydroxyphenyl)alanine (DOPA). This is of wide occurrence in plants and its R_f values in the standard solvent were found to be identical with those of *Unknown A*.

It was subsequently identified unequivocally as DOPA by the following comparisons: (i) The effects of pH on fluorescent behaviour were identical. In citrate–phosphate buffer at pH 4 and 5 the activation maximum was 285 mμ and the emission maximum 330 mμ. In glycine–NaCl–NaOH buffer at pH 12 the activation maximum was at 340 mμ and the emission maximum at 475 mμ and in borate–NaOH buffer at pH 11 the activation maximum was 330 mμ and the fluorescent maximum 395 mμ. In alkaline pHs the solutions became pale yellow indicating a chemical change as the basis of the shift in fluorescent maxima.

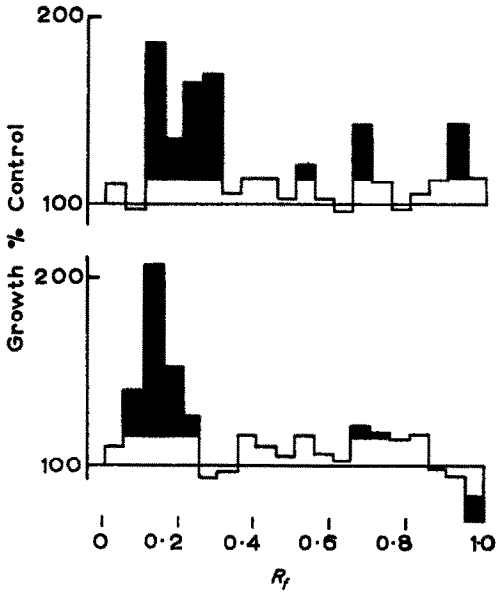

FIG. 3. *Avena* INTERNODE BIOASSAYS OF CHROMATOGRAMS OF WHOLE WATER-SOLUBLE FRACTION FOR DIFFERENT ROOT SAMPLES ON SEPARATE OCCASIONS.

The shaded areas mark responses beyond the 5 per cent fiducial limits. Material equivalent to content of 3·3 g fresh weight of roots for each sample.

In glycine–NaCl–NaOH buffer the fluorescent product was four times more fluorescent than the unchanged DOPA and in the borate–NaOH buffer it was some twenty times more fluorescent, a phenomenon which may prove to be of considerable value in the quantitative estimation of DOPA in very small quantities. In addition the pH–fluorescence curves for unchanged DOPA and the *Unknown A* over the pH range 0·1–8·0 were identical (see Fig. 4A).

(ii) Their behaviours on ion exchange columns were identical; both passed through cellulose powder columns but were retained by cellulose phosphate and DEAE cellulose, as would be expected of an amphoteric compound.

(iii) Chromatographic behaviour on paper is also very similar (Table 1). In phenol–ammonia both DOPA and *Unknown A* show a similar pattern of breakdown products as detected by visible inspection, u.v., Ehrlich and $FeCl_3$ reactions. Fluorescence–dilution

curves of the two substances allowed an accurate determination of DOPA concentration in *Vicia* roots. This proved to be 20·1 μg/g fresh weight of root system.

The identity of Unknown B. The colour reaction of this compound (purple with Ehrlich

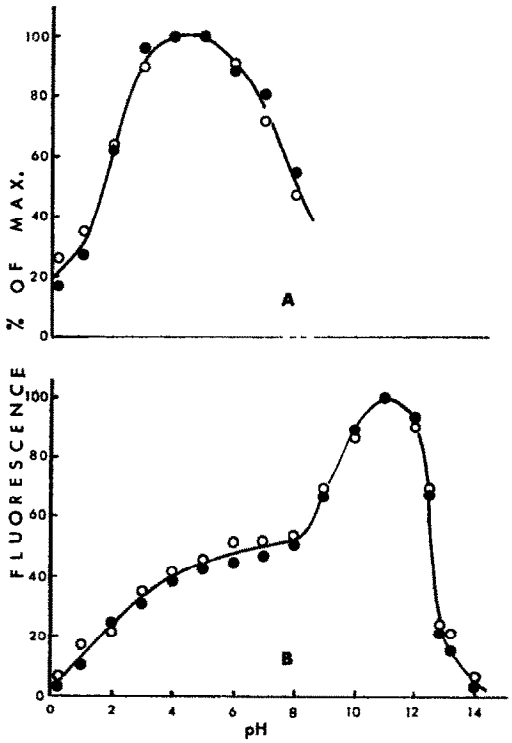

FIG. 4. pH–FLUORESCENCE CURVES.
(A) pH–fluorescence curves for *Unknown A* (○) and 3,4-dihydroxyphenylalanine (●);
(B) pH–fluorescence curves for *Unknown B* (○) and tryptophan (●).

TABLE 1. CHROMATOGRAPHIC COMPARISON OF DOPA AND *Unknown A*

Solvent system	R_f Values	
	DOPA	*Unknown A*
Standard solvent	0·07	0·09–0·13
n-Propanol:water (75:25)	0·27	0·25
iso-Propanol:water (80:20)	0·18	0·17
n-Butanol:glacial acetic acid:water (120:30:50)	0·24	0·26–0·28
Phenol:ammonia (200:1)	0·34	0·37–0·38

and with ninhydrin) immediately suggested that it might be tryptophan, which in preliminary experiments on ether–water partition separated completely into the water-soluble fraction. This has subsequently been established in the following tests.

(i) They both show the same fluorescent properties with activation maxima at 280 mμ and emission maxima at 360 mμ at pH 7·0. Above pH 10 both show an increase of 5 mμ in

the wavelength of the emission maxima. Fluorescence of *Unknown B* shows the same unusual stepped pH-curve (Fig. 4B).

(ii) Both pass cellulose powder columns but are retained by cellulose phosphate and DEAE cellulose.

(iii) Two dimensional paper chromatography in butanol–acetic acid and then in KCl give similar R_f values for both (Table 2).

TABLE 2. CHROMATOGRAPHIC COMPARISON OF TRYPTOPHAN AND *Unknown B*

	Butanol:acetic acid:water (120:30:50)	20% KCl
Unknown B	0·32–0·48	0·51–0·55
Tryptophan	0·49	0·54

(iv) Paper electrophoresis at pH 1·9 showed that *Unknown B* moved towards the cathode to the same extent as tryptophan and *N*-methyltryptophan. However the immediate purple reaction with Ehrlich changes overnight to blue in the case of *N*-methyltryptophan, but to green in the case of tryptophan and *Unknown B*.

(v) Fluorescence–dilution curves have identical slopes for both. As for DOPA the use of these curves allowed the accurate estimation of tryptophan concentration in *Vicia* roots. This amounted to 12·2 μg/g fresh weight.

Component Separation on Ion-Exchange Columns

Cellulose, cellulose-phosphate and DEAE cellulose columns were also used in an attempt to separate the various active components of the water-soluble fraction. Columns 20 × 2 cm were sufficient to retain all the adsorbable material in extracts from 50 g fresh weight of roots. The water eluates, 200 or 400 ml, from such loaded columns were collected, reduced under vacuum to a small volume and then chromatographed on paper. They were then bioassayed.

It was found that the chromatograms of the cellulose column eluates were remarkably similar to those of the crude water-soluble fraction, showing that very little physiologically active material had been retained (Fig. 5A). The colour reactions were also similar but *Y*2 was retained. As was to be expected *Unknown A* (DOPA) and *Unknown B* (tryptophan) were retained by both the cellulose phosphate (cation) and the DEAE cellulose (anion) columns. Both these columns passed *Y*1, which is obviously a neutral compound. The cation column passed some phenolic materials running at R_f 0·12 and 0·5 in the standard solvent but these were retained by the anion columns. Most but not all of the amino acids were retained by both columns. The cellulose phosphate column passed very little active auxin showing that most of the water-soluble auxins are basic in nature (Fig. 5B).

Since the anion column retains tryptophan and DOPA but lets through most of the remaining auxin activity (Fig. 5C) and since the paper chromatograms closely resemble those obtained previously from water-soluble fractions of pea roots[8] and *Vicia* roots[3] subsequent attention was concentrated on an analysis of the eluates from the DEAE cellulose anion columns.

[8] L. J. AUDUS and B. E. GUNNING, *Physiol. Plantarum* 11, 685 (1958).

The Components of the Eluate from DEAE Cellulose Columns

Identification of components in zones An_1–An_4. The eluate has been analysed by paper chromatography etc. as for the ether-soluble eluate. One feature of these eluates was that the subsequent chromatographic pattern was greatly affected by the loading of the column. Columns, 10 × 2 cm, loaded with material equivalent to 100 g of root material gave paper chromatograms showing biological activity over most of their length with maxima corres-

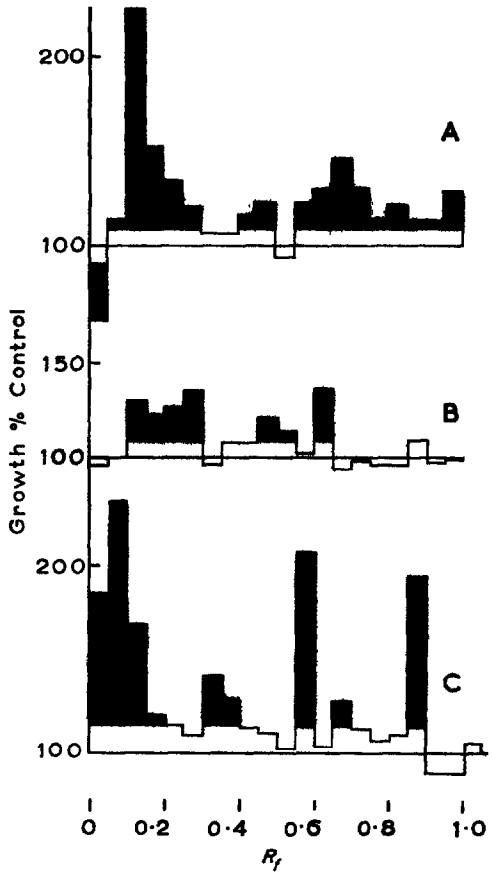

FIG. 5. *Avena* INTERNODE ASSAY OF PAPER CHROMATOGRAM OF WATER-SOLUBLE FRACTION.
(A) Water eluate from cellulose powder column; (B) Water eluate from cellulose phosphate column (cationic); and (C) Water eluate from DEAE cellulose column (anionic). A and B, material equivalent to content of 3·3 g fresh weight of roots. C, material equivalent to content of 5·0 g roots. The shaded areas represent responses beyond the 5 per cent fiducial limits.

ponding with the R_f of tryptophan, whose occurrence was confirmed by Ehrlich and ninhydrin sprays (Fig. 6A). Clearly adsorption sites had been saturated, leading to a leakage of tryptophan and possibly other acidic auxins. With lower rates of loading (i.e. material from 50 g roots on a 20 × 2 cm column) tryptophan largely, if not completely, disappeared as shown by the absence of bioassay response and colour reactions at its R_f of 0·1–0·2 (Fig. 6B). At the same time there appeared four additional zones reacting pink to Ehrlich and corresponding more or less with the zones of growth activity. The nature of these four basic compounds

(confirmed by the fact that they are all retained by cellulose phosphate columns) has been further studied. They have been given the symbols An_1–An_4. Their properties are set out in Table 3 and their electrophoretic behaviour in Fig. 7.

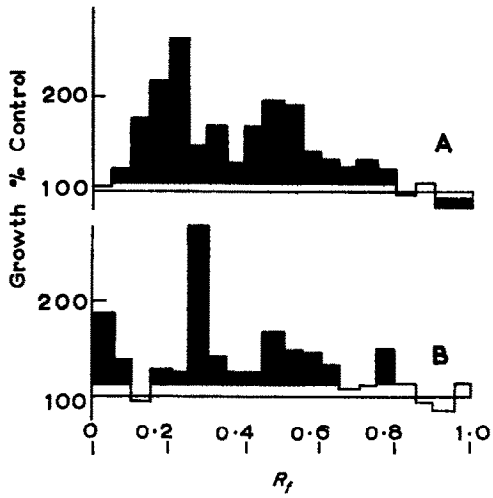

FIG. 6. PAPER CHROMATOGRAMS.

(A) *Avena* internode assay of paper chromatogram of water-soluble eluate of a 10 cm DEAE cellulose column; (B) *Avena* coleoptile segment assay of paper chromatogram of water-soluble eluate of 20 cm DEAE cellulose columns. Material equivalent to 5 g fresh weight of roots. The shaded areas represent responses beyond the 5 per cent fiducial limits.

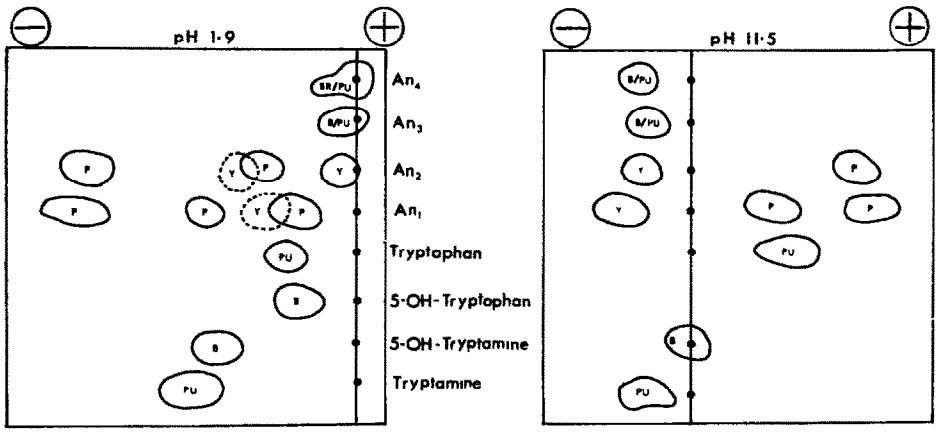

FIG. 7. ELECTROPHORETOGRAMS OF ELUATES FROM ZONES An_1–An_4 AT pH 1·9 AND 11·5 COMPARED WITH PURE SAMPLES OF TRYPTOPHAN, 5-HYDROXYTRYPTAMINE, 5-HYDROXYTRYPTOPHAN AND TRYPTAMINE.

Electrophoresis for 6 hr at 15 V/cm.

It is clear that An_1 is a mixture of at least two indole compounds of which tryptophan, from the electrophoretic patterns, is probably one. It had obviously "leaked" from the anion column. The other, which also appears to be amphoteric, does not correspond in its behaviour on electrophoresis with the other markers 5-hydroxytryptophan, tryptamine and

TABLE 3. PROPERTIES OF COMPOUNDS An_1–An_4

Zone	R_f in standard solvent	Ehrlich colour	Ultra-violet absorption max (mμ)	Fluorescence properties: Activation max (mμ)	Fluorescence properties: Fluorescence max (mμ)	Fluorescence properties: pH max emission	Fluorescence properties: pH ½-max emission	Electrophoresis, No. components and charge (Fig. 7): pH 1·9	Electrophoresis, No. components and charge (Fig. 7): pH 11·5	Ninhydrin–acetic (Jepson 1960[9])	Xanthydrol
An_1	0·10–0·15 0·19–0·25	Pink Pink-purple	265	350	430	4	<0 13·2	3(+)	2(−)	Purple no fluorescence	Pink
An_2	0·32–0·39	Pink	280	315	340	4–5	0·5 13·5	2(+)	1(−)	Neg.	Blue
An_3	0·48–0·6	Pink then purple	—	280	330	14	<0 >14	1(+)	1(−)	Neg.	Blue
An_4	0·68–0·72	Pink	—	280	330	1	<0 >14	1(+)	1(−)	Neg.	Neg.

[9] J. B. JEPSON, *Chromatographic and Electrophoretic Techniques* (Edited by I. SMITH) Heinemann, London (1960).

5-hydroxytryptamine. Although Jepson's ninhydrin–acetic acid reagent[9] gave an intense purple colour in this zone on the original chromatogram, the fact that it did not fluoresce under u.v. also rules out the presence of tryptamines. A third pink reactor appeared in acid solution and could have been produced at that pH from one of the other components.

An Ehrlich yellow reactor, remaining positively charged in both acid and alkali is also present. A very flat pH–fluorescence curve with little change from pH 1–11 tells us little, in view of the fact that An_1 is a mixture.

The electrophoretic behaviour of An_2 suggests that one component reacting pink to Ehrlich is the same as one unknown of An_1 again indicating incomplete separations on the chromatograms. The second pink reactor may also be an artefact produced only at pH 1·9. It does not seem to be the same as that produced from An_1.

An_3 seems to be a single basic substance. Since it gives a strong Xanthydrol reaction it is most probably an indole compound. An_4 is also a single basic compound. However although it gives the same pink and yellow colours with Ehrlich and modified Ehrlich respectively, An_4 gives no reaction with Xanthydrol, although this could be due to concentrations being subthreshold for this particular test.

The interconvertibility of compounds in zones An_1–An_4. Previous investigations on the water-soluble ether-insoluble auxins of *Pisum sativum*[8] and *Vicia faba*[3] had produced strong evidence that four compounds, very similar in chromatographic behaviour to An_1–An_4, are interconvertible in that each spot, on elution and further chromatography, yielded up to four spots corresponding to the four original compounds. Two-way chromatography also showed the same situation.

The possible interconvertibility of substances in An_1–An_4 was similarly tested by running chromatograms in two directions with the same standard solvent. These experiments showed that in zones An_2, An_3 and An_4 there was no detectable trace of interconversion since all three formed single spots strictly along the diagonal of the paper. On the other hand An_1, which ran as a single spot on the first run (R_f 0·22–0·23), resolved itself into two spots in the second run (R_f 0·05 and 0·20). This suggests that in An_1 there is production of at least one other Ehrlich-reacting compound between the two runs, possibly during the drying of the paper (cf. electrophoresis at pH 1·9). There is thus no confirmation from these experiments that the four compounds are interconvertible.

Possible production of water-soluble auxins from DOPA-oxidation. The fact that relatively large quantities of tryptophan and DOPA are present in the water-soluble fraction and that they are both fairly labile compounds has raised the possibility that one or more of the compounds in An_1–An_4 might be artefacts produced from tryptophan or DOPA on the DEAE column. This was checked by applying about 600 μg of tryptophan and about 1800 μg of DOPA (approximate equivalent contents of root extracts) each to two freshly made DEAE columns and eluting in the usual way with distilled water. No chromogenic or growth active compounds could be detected in either eluate.

However the possibility still remains that in the extract itself, i.e. in the presence perhaps of unknown catalytic components, DOPA oxidation might still account for some growth active products. This is shown by the following preliminary experiment. A 0·03% solution of DOPA was partially oxidized by finely divided silver oxide for 3 min at room temperature. The red solution was taken down to small volume and aliquots run on paper chromatograms in the standard solvent. Four distinct fluorescent bands separated out at R_f 0·01–0·02, 0·07–0·1, 0·12–0·16 and 0·19–0·22 respectively. Bioassay with the *Avena* first internode test showed marked growth activity associated with the area occupied by the spots (R_f 0–0·25)

and corresponding to that occupied by substance(s) An_1 although we have no further evidence that they have anything else in common (Fig. 8). However distinct and significant growth activity was present at higher R_f values where no fluorescent or chromogenic material could be detected, although of course concentrations may have been below the threshold of the tests applied.

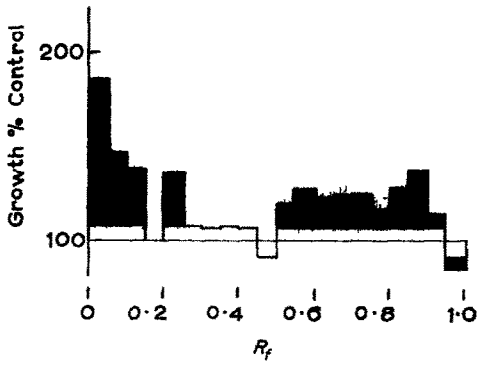

FIG. 8. *Avena* INTERNODE ASSAY OF PAPER CHROMATOGRAMS OF THE PRODUCTS OF OXIDATION OF 5·9 MG OF DOPA BY SILVER OXIDE.

The shaded areas represent responses beyond the 5 per cent fiducial limits.

DISCUSSION

The problem of whether IAA is present in *Vicia* roots still remains unsolved. The results reported in this paper suggest that it can be present only in very small amounts (of the order of 2–3 μg/kg) while the main auxin activity may be due to an unidentified compound, running at the same R_f, but with quite different fluorescent properties (activation maximum, 330 mμ; fluorescent maximum, 445 mμ). This compound bears a very close resemblance indeed to the "citrus auxin"[2] which ran in two solvents at R_f values extremely close to those of IAA and showed an activation maximum at 350 mμ and an emission maxima at 460 mμ. It was suggested that the "citrus auxin" could be related to one of the naphthols.

However, we have been able to demonstrate unequivocally and to measure accurately the relatively large amounts of free tryptophan that occur in these roots. Concentrations are in the region of 10 μg/g fresh weight of roots, which means that it is present in amounts at least 10,000 times greater than the elusive IAA could be. It would be surprising if this were not metabolized to some extent in root cells forming small quantities of IAA and possibly other indole compounds with auxin activity. There is little doubt that tryptophan is the major compound giving auxin activity in the water-soluble fraction in the R_f region 0·1–0·3 but it is clearly not the only one. Growth peaks appear consistently at higher R_f values in the standard solvent, the whole picture corresponding within the wide limits of R_f variability now familiar to auxin workers, to the four water-soluble auxins WP(*i*) to WP(*iv*) of pea roots[8] and the similar P_1–P_4 of *Vicia* roots.[3] In contrast to the previous results with *Vicia*, all four compounds give a pink colour reaction with Ehrlich reagent, suggesting that they might be indolic in nature. These discrepancies may be due to the larger quantities of root material extracted in the current series of experiments, bringing the concentrations on the chromatograms above the threshold for the colour development.

Zone An_1 (R_f 0·1–0·25) corresponds closely to WP(*i*) from pea roots (R_f 0·05–0·25) and P_1 previously found in *Vicia* root (R_f 0·05–0·2). It seems however to be a mixture of at least three compounds, one of which is undoubtedly tryptophan, which may account for the whole auxin activity of the zone.

Zone An_2 (R_f 0·25–0·45) corresponds to the substance WP(*iv*) occasionally seen on one and two-way chromatograms of water-soluble components of pea roots (R_f 0·13–0·45) and possibly P_2 (R_f 0·3–0·5) previously recorded for *Vicia faba* roots. Apart from some contaminants from zone An_1, one of which may be tryptophan, the main component is a basic indole compound, although electrophoretic migration seems to rule out tryptamine and 5-hydroxytryptamine, and the fluorescence characteristics are unlike any indole so far studied.

Zone An_3 (R_f 0·45–0·65) corresponds exactly with WP(*ii*) of pea roots. It contains one indole-like substance which is neutral in acid but becomes positively charged in an alkaline medium suggesting that its chemical nature is changed at high pH values.

An_4 (R_f 0·65–0·80) does not run quite as fast as the component WP(*iii*) of peas (R_f 0·70–0·95) or the previously reported P_4 from *Vicia* (R_f 0·80–1·0) but could easily be the same substance.

The unusual fluorescence behaviour of both An_3 and An_4 showing virtually no quenching even at pH 14 is instructive. Normally quenching of the indole fluorescence at high pH is thought to be due to the abstraction of a proton from the imino-N by the OH^- of the solvent while lack of quenching, as seen here with An_3, has been reported in indole compounds methylated on the imino-nitrogen. It has also been shown[10] that a similar maximum at high pH is seen in indole-2-carboxylic acid. It is suggested that both these compounds might be -N- or -2- substituted indoles.

In these experiments the zone An_1–An_4, in contrast to the four water-soluble auxins of pea and those previously reported for *Vicia*, do not seem to be interconvertible. This lends substance to the criticisms of Thurman and Street,[11] that the apparent interconversions were artefacts from a too heavily loaded paper, the spot elution and subsequent re-chromatographing brings about a further separation of mixtures of substances. But there is a difference in the two series of experiments that may partly account for these discrepancies; it is that tryptophan and DOPA had been first removed from the water-soluble fraction before chromatography. It is not inconceivable that these two components might interact on the paper or in the eluate (they run very close together on the chromatogram) to give indole derivatives with the properties of An_1–An_4. For example Gordon and Paleg[12] have shown that tryptophan and catechol can interact to form small quantities of IAA and thought that dihydroxyphenols might augment IAA biosynthesis from tryptophan in this way.

Although we have not yet checked directly to see whether such a reaction between tryptophan and DOPA will proceed, yet we have evidence that the mild oxidation of DOPA will produce auxins which run at positions on chromatograms similar to those of An_1–An_4. Although these need to be followed up carefully yet they do suggest in an unambiguous manner the dangerous ease with which artefacts may be produced from mixtures of substances subjected to even the mildest of conditions of chromatography and its associated procedures.

[10] D. Burnett, Ph.D. Thesis, London University (1963).
[11] D. A. Thurman and H. E. Street, *J. Exp. Botany* **11**, 118 (1960).
[12] S. A. Gordon and L. G. Paleg, *Plant Physiol.* **36**, 838 (1961).

METHODS

Extraction and Fractionation

The whole root systems of 12-day-old *Vicia faba* plants were harvested, weighed and quickly frozen in cardice. They were then macerated in redistilled precooled methanol in a Wareing blender while in the frozen state, and allowed to extract for 18 hr at −14°. The cell debris was then removed by filtration through sintered glass and the filtrate reduced to dryness under vacuum at 26° in a rotary evaporator. The dry residue was taken up in a small quantity of water, the solution adjusted to pH 3 with 0·1 N phosphoric acid and then extracted with five successive aliquots of redistilled ethyl ether. The pooled ether extracts constituted the ether-soluble fraction; the aqueous residue constituted the water-soluble fraction.

Cellulose Column Chromatography

The cellulose, cellulose phosphate and DEAE cellulose were used sometimes in powder but usually in floc form. Columns were 10–20 cm long by 2 cm in diameter and were thoroughly washed before use to remove traces of fluorescent impurities. Cellulose was washed with distilled water; cellulose phosphate was washed successively with distilled water, 0·1 N NaOH, water, 0·1 N H_2SO_4 and finally water; DEAE cellulose was similarly treated but the H_2SO_4 preceded the NaOH wash.

Paper Chromatography

Whatman 3 mm paper strips (6 × 36 cm) were prewashed in running solvent (isobutanol: methanol: water, 75:10:15) and dried. This solvent is termed the standard solvent. The solution for analysis was applied on a transverse line 4 cm from one end of the paper. Ascending chromatography was used after 6 hr of equilibration and the solvent allowed to rise a distance of 22–25 cm in the dark at 20–22°. After running, chromatograms were dried in the dark in the draught from a fan.

Biological Assay

Avena sativa var. Victory I (Svalof) or *A. sativa* var. Blenda grains were soaked in tap water for 2 hr and germinated in sterile sand in darkness at 25° for 72 hr. For the first internode test 3 mm segments were cut 2 mm below the coleoptilar node from internodes which were 20–23 mm long. Segments were left to stand on wet muslin for 1 hr before random distribution into the test solutions. For the coleoptile test seedlings were illuminated for 6 hr in dim red light 18 hr before harvesting. Segments, 3 mm long, were then cut at a distance of 3 mm from the tip of coleoptiles 20–25 mm long. All these operations were carried out in yellow-green light (Wratten 0A filter over a 60 W lamp).

Assay was performed in small glass tubes of 2·5 × 2·5 cm dimensions in 1 ml of phosphate–citrate buffer containing 2% sucrose at pH 5·0. Chromatogram segments were finely shredded into the vials before the addition of the *Avena* segments. During the 20 hr of segment growth the tubes were stoppered with corks with a central cotton wool plug and gently agitated on a mechanical shaker at 25° in the dark. Enlarged records of final segment length were made by aligning each set of samples on a glass plate and projecting an image onto bromide paper with a photographic enlarger. Ten segments were placed in each tube and for each chromatogram ten control tubes were used, each containing a shredded strip of washed chromatogram paper. The fiducial limits of the chromatogram assays were determined from the variance of the means of the ten control samples.