INDOLEACETIC ACID IN CAMBIAL TISSUE OF RADIATA PINE

By K. R. Shepherd* and K. S. Rowan†

[*Manuscript received November* 11, 1966]

Summary

The major growth substance detected by mesocotyl bioassay in extracts from cambial tissue of mature radiata pine (*Pinus radiata* D. Don) was identified as indoleacetic acid by movement on chromatograms and electrophoretograms, by Salkowski test, and by absorption and fluorescence spectroscopy. Indoleacetic acid concentration increased in the top stem in late winter and in the mid and lower stem in mid-spring, consistent with auxin moving down the stem at this time. Since material chromatographically similar to indoleacetic acid was not formed when [^{14}C]tryptophan was added to the extracting medium, the indoleacetic acid was not formed by oxidation of tryptophan during extraction.

I. Introduction

The hormone theory for the control of cambial activity in trees states that auxin moving from newly developing buds and subsequently from the expanding leaves initiates formation and maintains activity of the cambium (Wareing 1958; Larson 1962). Thus the concentration of auxin moving basipetally in the stem controls cell size across the annual ring at any height (Priestley 1930). Zimmerman (1936), Avery, Burkholder, and Creighton (1937), Mirov (1941), Hatcher (1959), and Brown and Wetmore (1959) provide evidence consistent with this theory. Although interaction between several hormones is likely to control cambial activity (Kefford and Goldacre 1961; Thimann 1963, 1965; Wareing, Hanney, and Digby 1964), a number of workers have activated the cambium with applications of a single substance (Snow 1935; Brown and Cormak 1937; Larson 1962; Wareing, Hanney, and Digby 1964). The work reported in this paper identifies a major growth-promoting substance, indole-3-acetic acid (IAA), in the cambial tissue of radiata pine (*Pinus radiata* D. Don) and provides evidence that IAA concentration regulates growth of the cambium.

When Whitmore and Zahner (1964) extracted IAA from stem tissue of *Pinus* sp. by blending in buffer, the yield at pH 9 was double that at pH 2·8. They attributed this to synthesis of IAA at the higher pH through oxidation of endogenous tryptophan by quinones, a reaction described by Gordon and Paleg (1961). These workers found that the reaction

$$\text{tryptophan} \xrightarrow{\text{quinone}} \text{indolepyruvic acid} \rightarrow \text{indoleacetic acid}$$

occurred spontaneously *in vitro* at pH 8 and above, and at pH 6 and above when phenolase was present. When Whitmore and Zahner (1964) prepared extracts from tissue with diethyl ether–ethanol mixtures or with diethyl ether alone, they found

* Department of Forestry, University of Melbourne; present address: Forestry Office, Forestry Commission of New South Wales, Tumut, N.S.W.

† Department of Botany, University of Melbourne.

radioactive material with R_F approximately equal to IAA when they added [^{14}C]tryptophan to the extracting medium. Because this material was extracted from boiled tissue, they suggested that IAA could be formed by non-enzymic synthesis during extraction. The experiments reported here show that tryptophan is not oxidized during methanolic extraction from tissues of *P. radiata*.

II. Materials and Methods

Methanol (for analysis) was supplied by E. Merck & Co., Daamstadt, Germany. Ethyl acetate (Analar) was redistilled after treating with $CaCl_2$ to remove water-soluble impurities. IAA and indoleacetonitrile (IAN) were supplied by Sigma Chemical Co., St. Louis, Missouri. Other chemicals were of Analar grade.

[*methylene*-^{14}C]Tryptophan supplied 99% pure by Amersham Radiochemical Centre (32·5 mc/m-mole) was dissolved in methanol to give 10 μc/ml and stored at −15°C. Whatman No. 3MM paper washed in 2N acetic acid was used for both descending and ascending chromatograms. The precautions recommended by Nitsch and Nitsch (1960) for preventing distillation of indoles from marker spots to chromatograms intended for bioassay were observed. When necessary, chromatograms were stored in darkness at −15°C before bioassay. Radioactive material run on paper strips 24 mm wide was detected by passing under a thin end-window Geiger tube (20th Century Electronics Ltd., type EW2C) and recorded in counts/minute using a ratemeter (Ecko Electronics Ltd., type N522C with probe unit type N558) and recorder (Elliott Bros. Ltd., type 203). The following chromatography solvents were used:

(1) Isopropanol–acetic acid–water (4 : 1 : 1 v/v);

(2) Isopropanol–ammonia–water (8 : 1 : 1 v/v);

(3) Water, following equilibration overnight;

(4) n-Butanol–acetic acid–water (4 : 1 : 1 v/v).

Indoles were detected on paper chromatograms by fluorescence in ultraviolet light.

For electrophoresis, solutions were applied to strips of acid-washed Whatman No. 3MM paper moistened with ammonium acetate (50 mM) and 25 V/cm applied across the paper using the apparatus described by Markham and Smith (1952). If intended for bioassay, strips containing unknown and control solutions were run simultaneously in separate baths of carbon tetrachloride.

For bioassay of auxin, the straight-growth test using mesocotyl of *Avena* (cv. Forkedeer or Laurel) was used (Nitsch and Nitsch 1956). Sections were cut using a Thimann coleoptile microtome (Faculty Workshops, Melbourne University). The response of mesocotyl sections to standard solutions of IAA was measured for each batch of sections, since the response varied between days; the concentration of auxin at positions on chromatograms and electrophoretograms corresponding to IAA was calculated from the response to these standard solutions.

Cambial tissue from mature trees of radiata pine was obtained by outlining the area required (64 cm^2 was adopted as a standard) and paring away the outer corky bark. The soft tissue including phloem, cambium, and immature xylem was collected by first peeling off the fibrous layer and then scraping away the softer tissues with a

Perspex scraper. No attempt was made to separate the various fractions of this tissue as was done by Bonga and Clark (1963). The bared surface was washed with ice-cold methanol and the total collection (approx. 400 ml) sealed and chilled with solid CO_2 for transport to the laboratory.

On return to the laboratory the sample tissue was homogenized in an M.S.E. Ato Mix bottom-drive blender and the brei stored overnight at $-15°C$. The next day the brei was filtered in the cold and evaporated to the aqueous phase using a Rinco rotary film evaporator. The pH was adjusted to 7 using $NaHCO_3$ and partitioned twice with three volumes of light petroleum to remove possible inhibitors and some pigments. After removing traces of light petroleum by evaporation, the aqueous phase was adjusted to pH 3 with HCl and partitioned with ethyl acetate. The solution was evaporated to dryness and the residue stored at $-15°C$ until required for chromatography, when it was dissolved in methanol. Small samples (6·25 cm^2) prepared from logs cut from the trunk and taken to the laboratory before extracting were treated in the same way, except that the tissue was ground in a glass pestle and mortar with a little acid-washed sand and the extract recovered by centrifugation. [^{14}C]Tryptophan was included in the extracting medium when required.

Sections of stem 1–3 cm long were cut 2 cm below the apex of a main or lateral branch of seedlings approximately 1·5 m high. Leaves were cut from the stem section which was weighed and ground in an ice-cold pestle and mortar in 2–4 ml cold methanol containing 1 μc [^{14}C]tryptophan if required. The extract was recovered by centrifugation and evaporated to dryness.

III. Results

(*a*) *Contaminants in* [^{14}*C*]*Tryptophan*

[^{14}C]Tryptophan (1 μc) examined by paper chromatography in solvent 1 was resolved into a major peak (tryptophan) and a small peak equivalent in area to 1% of the total ^{14}C on the chromatogram at an R_F value of 0·85. Several indoles, including IAA and IAN run in this position (Stowe and Thimann 1954). A part of the contaminating material could be extracted from an aqueous solution of the [^{14}C]tryptophan (pH 3) with diethyl ether or ethyl acetate and shown to contain material similar to IAA and IAN when examined by chromatography in solvents 2 and 3 and by paper electrophoresis at pH 7·5.

(*b*) *Auxin Extraction from Tissue with* [^{14}*C*]*Tryptophan Included in the Extracting Medium*

(i) *Cambial Tissue.*—Extracts with [^{14}C]tryptophan added to the methanol were prepared from small samples of cambial tissue. The fraction soluble in light petroleum and the fraction extracted with ethyl acetate at pH 3 were analysed by chromatography in solvent 1 with a fraction extracted with ethyl acetate from [^{14}C]tryptophan (1 μc) at pH 3 as control (Fig. 1). Since radioactive material in the extract with R_F 0·85 did not increase above the control level, [^{14}C]tryptophan was not oxidized when extracting IAA by this method.

(ii) *Stem Tissue of Seedlings.*—A 1-cm section of the stem (0·90 g) was ground with methanol (3 ml) containing 1 μc [^{14}C]tryptophan. The extract recovered by

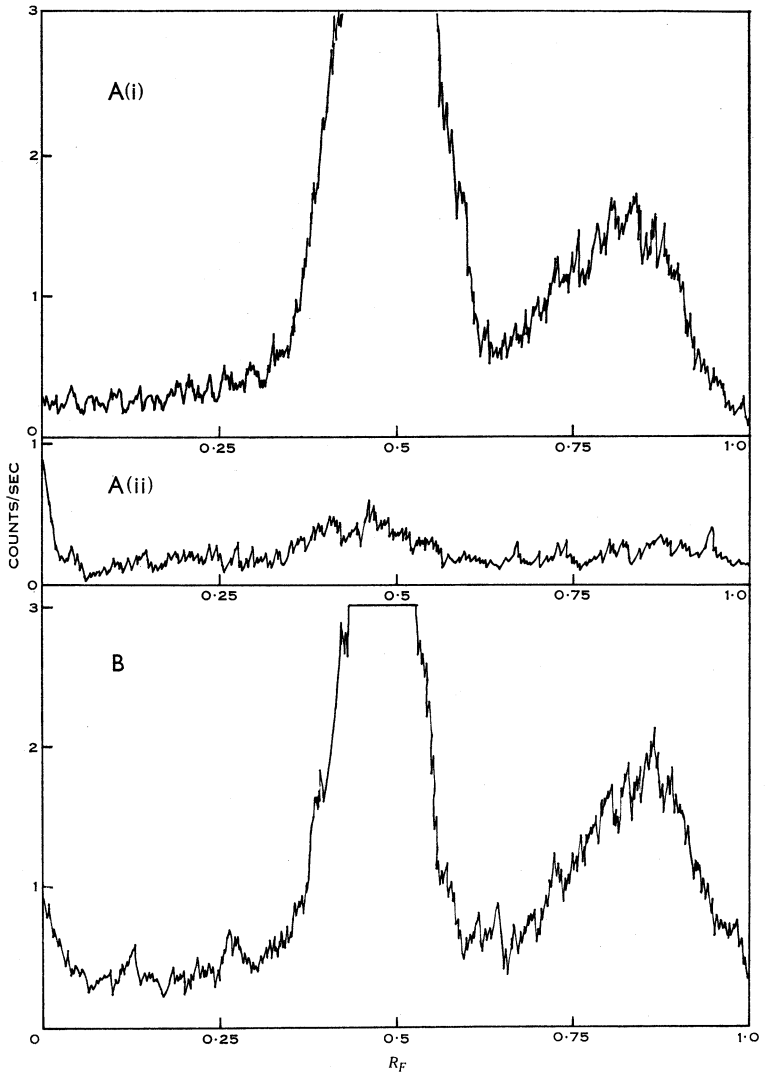

Fig. 1.—Scanning diagram from chromatograms run in solvent 1. *A*, Fractions prepared from extracts from cambial tissue of *P. radiata* (1 g fresh wt.) with 1 μc [^{14}C]tryptophan added to the extracting medium. The methanolic extract was evaporated and redissolved in 1 ml aqueous solution. (i) Fraction extracted at pH 7 with light petroleum. (ii) Fraction extracted at pH 3 with ethyl acetate. *B*, Fraction extracted at pH 3 with ethyl acetate from 1 ml aqueous solution containing 1 μc [^{14}C]tryptophan.

centrifugation was evaporated and applied to a chromatogram in 0·2 ml methanol. [^{14}C]Tryptophan (1 μc) was applied to a control chromatogram and both developed

in solvent 1. Radioactivity found at R_F 0·85 in the tissue extract did not increase above the control (Fig. 2).

A stem section (0·38 g) was ground in methanol (5 ml) containing 2 μc [^{14}C]tryptophan. Two samples of the supernatant solution (1·8 ml) were evaporated to dryness; one was dissolved in 1 ml $NaHCO_3$ (5% w/v) while the other was dissolved in 1 ml $NaHCO_3$ previously adjusted to pH 3. Both samples were fractionated with

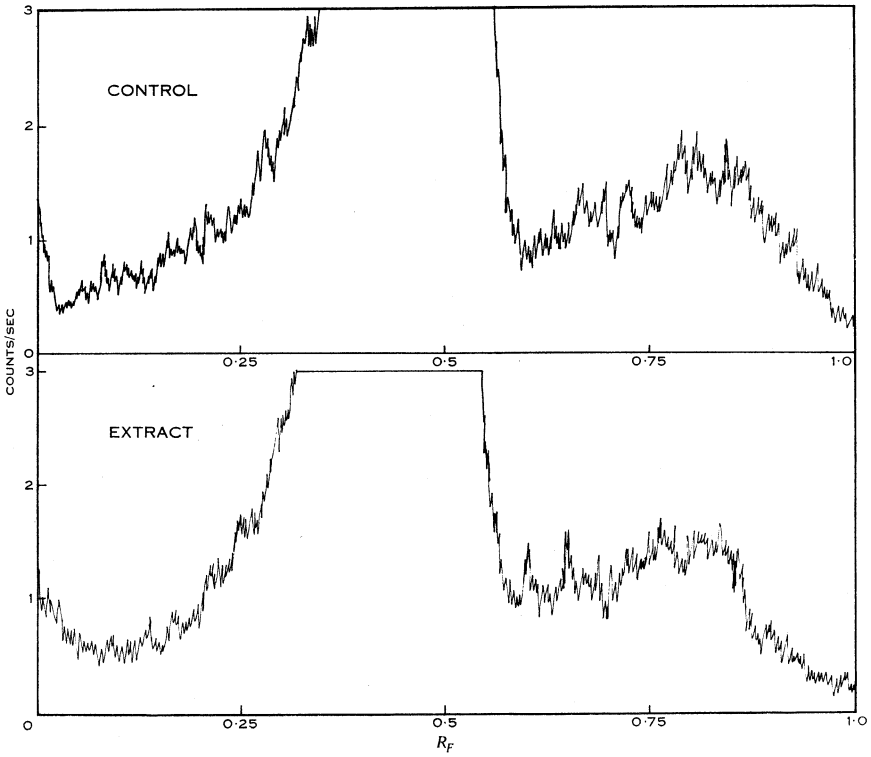

Fig. 2.—Scanning diagram from chromatograms run in solvent 1. Upper: 1 μc [^{14}C]tryptophan. Lower: extract from stem (0·09 g) of *P. radiata* prepared with 1 μc [^{14}C]tryptophan in the extracting medium.

ethyl acetate. The pH of the alkaline sample was then adjusted to pH 3 and a second fraction prepared in ethyl acetate which would contain any acidic oxidation products of tryptophan formed while the solution was alkaline. Dissolving the extract from stem tissue in alkaline solution did not oxidize tryptophan to IAA since the radioactive material at R_F 0·85 in solvent 1 did not increase above that in the extract dissolved at pH 3.

(*c*) *Estimation of Tryptophan in Stem Tissue of* P. radiata

Sections (6 cm) were cut 2 cm below the apex of lateral branches of 10 seedlings. Leaves were removed, the sections (5·4 g) blended for 3 min in 50 ml cold methanol, and the extract recovered by filtration. The residue was blended again in 50 ml

methanol. Filtrates were combined, evaporated to dryness, and resuspended in 5 ml distilled water. Pigments were extracted in 2 ml chloroform and the aqueous phase evaporated to dryness and resuspended in 3 ml methanol. An insoluble residue was removed by centrifugation and the supernatant solution evaporated and redissolved in 0·5 ml methanol. Of this, 0·10 ml was run as a line on a chromatogram in solvent 4. A zone with R_F equal to authentic tryptophan was cut out with an appropriate blank. Both were soaked overnight in methanol (5 ml) and α-amino nitrogen estimated in 3 ml samples (Moore and Stein 1948; Anderson and Rowan 1965). The stem tissue contained 0·36 μmole tryptophan per gram fresh weight.

TABLE 1

DETAILS OF TREES FELLED FOR SAMPLING AND POSITION OF SAMPLES

Tree No.	Date	Diameter (cm)	Height (m)	Height to Lowest Green Branch (m)	Sample Position (m)		
					Base	Mid	Top
1	19.iii.63	59·6	36·0	19·5	1·3	22·3	32·8
2	3.iv.63	33·3	29·0	10·2	1·6	15·4	24·6
3	23.viii.63	34·0	30·5	10·8	2·0	18·7	26·2
4	8.x.63	36·3	31·5	15·0	1·6	15·4	26·2

(*d*) *Estimation of IAA in Cambial Tissue*

Cambial samples were taken from mature trees in a 30-year-old plantation of radiata pine near Creswick, Victoria. These were all dominant trees growing close to a study plot with a mean diameter of 27·9 cm and dominant height of 30·1 m. Three samples were taken from each tree (Table 1). One sample was taken at the top of the tree about 4 m below the apex (top crown: T), the second at the base of the green crown but above the lower, moribund live branches (mid stem: M), and the third near the base of the stem but above the butt swell region (base stem: B).

Extracts prepared from these samples were resolved by chromatography (solvent 2) on paper strips 2 cm wide. Bioassay of duplicate chromatograms showed poor replication of active zones with some extracts from trees 1 and 2, though the mean R_F of the active zone was at the value of authentic IAA. In later experiments the zone in the position of IAA (R_F 0·3–0·6) was eluted from the chromatograms with methanol; the eluate was evaporated to dryness, redissolved in water, and resolved by electrophoresis. Each extract from cambial tissue was run in quadruplicate using four mesocotyl sections for bioassay of each piece of paper. A set of standard solutions of IAA was bioassayed for each chromatogram or electrophoretogram run. The estimate of IAA concentration in extracts by bioassay was corrected for IAA lost during manipulation as determined in preliminary experiments. The results of

bioassay of the extract from the mid stem of tree 2 resolved by chromatography only and by chromatography followed by electrophoresis are shown in Figure 3.

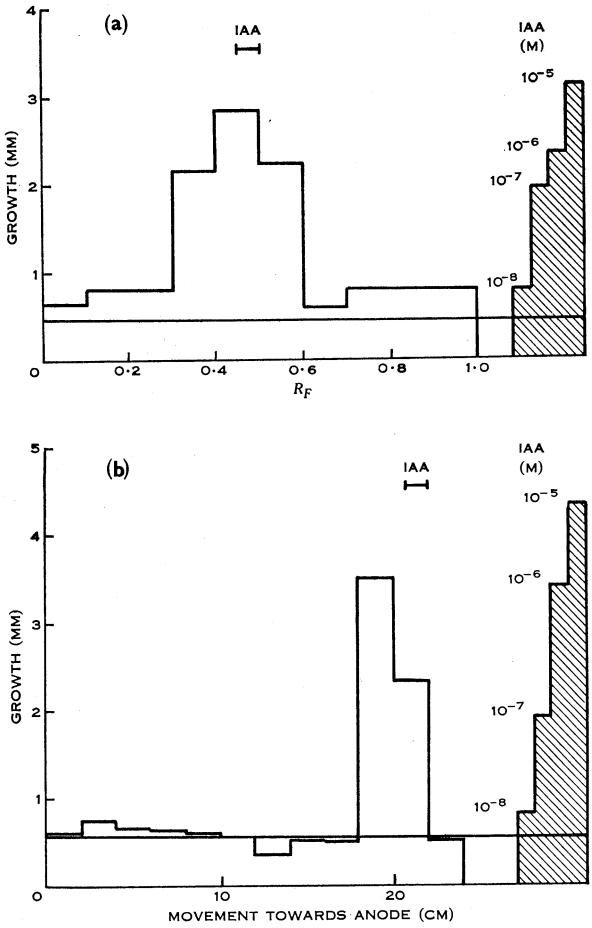

Fig. 3.—Bioassays (*Avena* mesocotyl) of the extract from 6·4 cm² of cambium at the mid stem of tree 2 resolved by: (*a*) chromatography in solvent 2, and (*b*) chromatography in solvent 2 followed by electrophoresis at pH 7·5. Growth responses are means of duplicate determinations in (*a*), and of quadruplicate determinations in (*b*). The position of authentic IAA and the responses of mesocotyl sections to standard solutions of IAA (shaded areas) are shown.

Bioassay of the three extracts from one tree are shown in Figure 4, while results for all trees are shown in Table 2.

(*e*) *Identification of IAA in Tissue Extracts*

In addition to similarity of R_F value and electrophoretic mobility, the following tests on material eluted with ethanol from active zones of a number of chromatograms and electrophoretograms confirm that the major auxin in the extracts was IAA.

(1) The Salkowski test (Gordon and Weber 1951) was positive and concentrations were of the same order as those shown in Table 2. (2) The maximum absorption measured in a Unicam SP 500 spectrophotometer was at 279–281 mμ, thus corresponding with authentic IAA. (3) Using an Aminco-Bowman spectrophotofluorometer, the excitation and emission maxima were equal to authentic IAA. An analysis of one electrophoretogram strip is shown in Figure 5. The strip was cut into zones 2 cm long, each piece eluted in ethanol (final volume: 2 ml), and fluorescence intensity measured. Concentrations compared with standard solutions of authentic

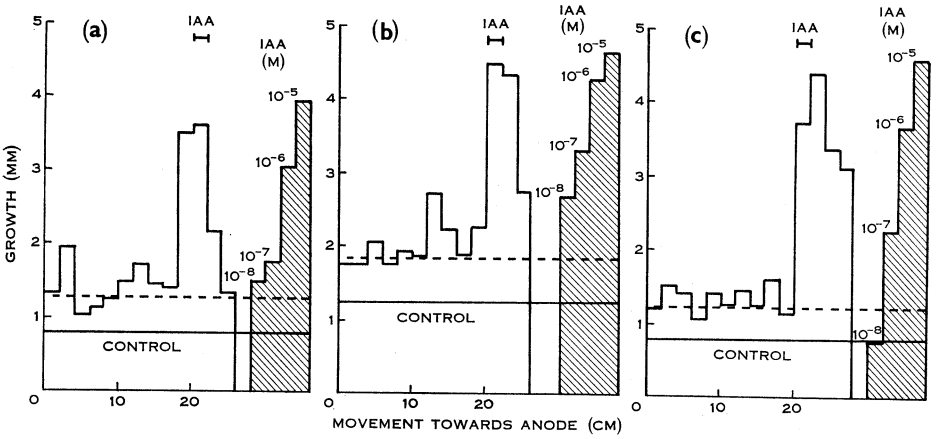

Fig. 4.—Bioassays (*Avena* mesocotyl) of extracts from 6·4 cm^2 of cambium at the top crown (*a*), mid stem (*b*), and base stem (*c*) of tree 4. Each extract was resolved by chromatography in solvent 2 followed by electrophoresis at pH 7·5. Growth responses are means of quadruplicate determinations. The position of authentic IAA and the responses of mesocotyl sections to standard solutions of IAA (shaded areas) are shown. The broken horizontal line shows the growth response significantly greater than control growth at $P = 1\%$.

IAA in these preliminary experiments appear lower than those in Table 2. Since internal standards were not used (Udenfriend 1962) this is due probably to fluorescence quenching. Burnett and Audus (1964) have described similar methods for estimating indoles in tissue extracts.

IV. Discussion

Whitmore and Zahner (1964) reported that [^{14}C]tryptophan added to the extracting medium was oxidized to IAA by quinones during extraction from tissues of *Pinus* sp., thus implying that IAA extracted was an artefact. Oxidizing 1% of the tryptophan reported here in stem tissue of *P. radiata* would give 3·6 mμmoles IAA per gram fresh weight, an amount of the same order as that found in cambial tissue. However, since radioactive material at R_F 0·85 in solvent 1 (Figs. 1 and 2) would double, 1% oxidation of [^{14}C]tryptophan would have been detected, and we conclude that IAA extracted in the experiments reported here is not an artefact. Oxidation of [^{14}C]tryptophan in the experiments of Whitmore and Zahner (1964) remains doubtful since they have not shown control chromatograms of the [^{14}C]tryptophan used.

Although IAA in cambial tissue never fell below 1 mμmole per gram fresh weight (Table 2), at the end of winter (tree 3) the concentration increased at the top

TABLE 2

IAA EXTRACTED FROM 6·4 CM² OF CAMBIAL TISSUE AT THE TOP CROWN, MID STEM, AND BASE STEM OF MATURE TREES OF *P. RADIATA*

Estimations were made by mesocotyl bioassay following chromatography (C) or by chromatography followed by electrophoresis (E). Values are means from two chromatograms or four electrophoretograms

Tree No.	IAA (mμmoles) Extracted from Tissue from:					
	Base Stem		Mid Stem		Top Crown	
	C	E	C	E	C	E
1					6·4*	
2	9·3		6·3	3·4	1·9	
3		1·0		3·4		13·7
4		25·1		16·3		22·0

* Single chromatogram only.

of the tree, an observation consistent with patterns of shoot growth (Fielding 1955), finally increasing by an order of magnitude at all positions by the late spring (tree 4).

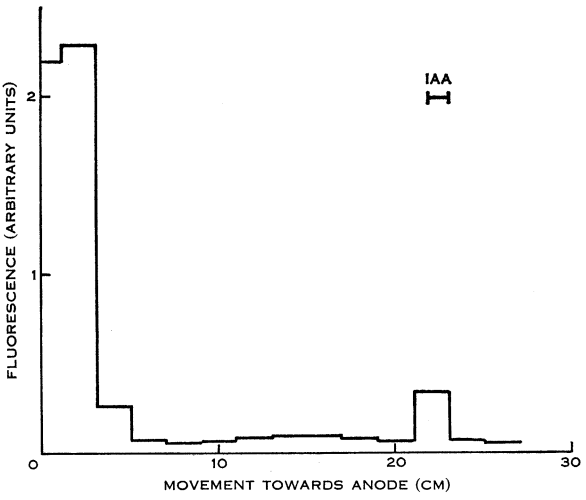

Fig. 5.—Analysis of an electrophoretogram of an extract using spectrophotofluorometry. The electrophoretogram was cut into pieces 2 cm long, each eluted with 2 ml ethanol, and fluorescence measured in the Aminco-Bowman spectrophotofluorometer. The position of authentic IAA is shown.

Thus the distribution of IAA in time and space is consistent with regulation of cambial activity by the hormone produced in the upper crown moving down the stem in spring, and is consistent with lack of *diffusible* auxin detected by previous

workers (Avery, Burkholder, and Creighton 1937; Gunckel and Thimann 1949; Brown 1958) in trees during winter.

Since Bottomley *et al.* (1963) have reported kinin-like activity in extracts from cambial tissue of pine, IAA is not the only growth-promoting compound in the tissue. Gibberellins stimulate growth of mesocotyls but probably do not occur in high concentration in cambial tissue, though the minor zone of activity in some electrophoretograms (Fig. 4) is in approximately the position of gibberellic acid (Spring and Rowan, unpublished data).

V. Acknowledgments

We would like to thank Professor F. W. Gibson for allowing us to use the Aminco-Bowman spectrophotofluorometer. One of us (K.R.S.) was supported by the Forestry Commission of New South Wales and Australian Paper Manufacturers Ltd.

VI. References

Anderson, J. W., and Rowan, K. S. (1965).—*Biochem. J.* **97**, 741–6.
Avery, G. S., Burkholder, P. R., and Creighton, H. B. (1937).—*Am. J. Bot.* **24**, 51–8.
Bonga, J. M., and Clark, J. (1963).—*Can. J. Bot.* **41**, 1133–4.
Bottomley, W., Kefford, N. P., Zwar, J. A., and Goldacre, P. L. (1963).—*Aust. J. biol. Sci.* **16**, 395–406.
Brown, A. B., and Cormack, R. G. H. (1937).—*Can. J. Res.* C **15**, 433–41.
Brown, C. L. (1958).—In "The Physiology of Forest Trees". (Ed. K. V. Thimann.) p. 511. (Ronald Press Co.: New York.)
Brown, C. L., and Wetmore, R. H. (1959).—*Am. J. Bot.* **46**, 586–90.
Burnett, D., and Audus, L. J. (1964).—*Phytochemistry* **3**, 395–415.
Fielding, J. M. (1955).—Leafl. Commonw. For. Timb. Bur., Canberra. No. 75.
Gordon, S. A., and Paleg, L. G. (1961).—*Pl. Physiol., Lancaster* **36**, 838–45.
Gordon, S. A., and Weber, R. P. (1951).—*Pl. Physiol., Lancaster* **26**, 192–5.
Gunckel, J. E., and Thimann, K. V. (1949).—*Am. J. Bot.* **36**, 145–51.
Hatcher, E. S. J. (1959).—*Ann. Bot., Lond.* (N.S.) **23**, 409–23.
Kefford, N. P., and Goldacre, P. L. (1961).—*Am. J. Bot.* **48**, 643–50.
Larson, P. R. (1962).—In "The Physiology of Forest Trees". (Ed. K. V. Thimann.) p. 97. (Ronald Press Co.: New York.)
Markham, R., and Smith, J. D. (1952).—*Biochem. J.* **52**, 552–7.
Mirov, N. T. (1941).—*J. For.* **39**, 457–64.
Moore, S., and Stein, H. W. (1948).—*J. biol. Chem.* **176**, 367–88.
Nitsch, C., and Nitsch, J. P. (1960).—*Pl. Physiol., Lancaster* **35**, 450–4.
Nitsch, J. P., and Nitsch, C. (1956).—*Pl. Physiol., Lancaster* **31**, 94–111.
Priestley, J. H. (1930).—*New Phytol.* **29**, 316–54.
Snow, R. (1935).—*New Phytol.* **34**, 347–60.
Stowe, B. B., and Thimann, K. V. (1954).—*Archs Biochem. Biophys.* **31**, 501–16.
Thimann, K. V. (1963).—*A. Rev. Pl. Physiol.* **14**, 1–18.
Thimann, K. V. (1965).—*Recent Prog. Horm. Res.* **21**, 579–96.
Udenfriend, S. (1962).—"Fluorescence Assay in Biology and Medicine." (Academic Press, Inc.: London.)
Wareing, P. F. (1958).—*J. Inst. Wood Sci.* **1**, 34–42.
Wareing, P. F., Hanney, C. E. A., and Digby, J. (1964).—In "The Formation of Wood in Forest Trees". (Ed. M. H. Zimmerman.) p. 323. (Academic Press, Inc.: London.)
Whitmore, F. W., and Zahner, R. (1964).—*Science, N.Y.* **145**, 166–7.
Zimmermann, W. A. (1936).—*Z. Bot.* **30**, 209–52.