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THE FORMATION OF POLYPHENOLS IN TREES I. ADMINISTRATION OF ^{14}C GLUCOSE AND SUBSEQUENT DISTRIBUTION OF RADIOACTIVITY

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Abstract—After administration of uniformly labelled ^{14}C-glucose to a kino vein in *Eucalyptus sieberiana*, labelled polyphenols were found in the kino. The distribution of radioactivity and the presence of labelled sugars and polyphenols in the sapwood, indicated *in situ* formation of polyphenols from sugars.

INTRODUCTION

WHEN the cambium layers of several genera of woody plants are damaged under certain conditions, they form viscid liquids known as resins or carbohydrate-gums or kinos, etc. These liquids frequently exude to the outside of the tree and harden, or, depending on the species and severity of the wound, they are confined to "veins" (transversed by radial parenchyma bridges) or to "pockets" or "blisters" (in which the bridges are absent) by the xylem which subsequently forms over the damaged areas.

The amount of liquid formed by a single injury varies considerably depending on the nature of injury, the type and health of the tree or shrub, and the genus to which it belongs. In at least one genus, there is also a wide variation in the amount formed by different species; *Eucalyptus calophylla* Lindl. (Myrtaceae) and *E. sieberiana* F. Muell. frequently form kino after injury, and in the former the amount can be large (2 l. or more), whereas *E. microcorys* F. Meull. rarely forms more than a very small amount, if any, after injury.

The type of compound formed by the injured cambium is characteristic of the genus; for example, carbohydrate-gums are formed by *Acacia* species and kinos (composed almost entirely of polyphenols) by *Eucalyptus* species. Several types of eucalypt kinos are known, some, such as *E. sieberiana* kino, are largely composed of labile polymerized flavans, while others contain in addition significant amounts of monomeric polyphenols, some of which have not yet been found in large amounts elsewhere.

With many eucalypt species, kino begins to exude within six weeks of cambial injury and continues to do so for several days and sometimes weeks. Consequently, kino appears to be another material which could be used in the study of the biosynthesis of certain compounds, and the mode of linkage of polymerized flavans. Furthermore, in those species so far studied, the composition of the polyphenols in undamaged cambium differs markedly from that in kino, so that a study of the formation of the latter may reveal some of the biochemical factors controlling the formation of polyphenols in different parts of the tree.

This paper describes attempts to determine whether labelled compounds can be satisfactorily incorporated in kino. It also describes the extent of distribution of radioactivity in neighbouring tissues after one of these experiments. Uniformly labelled ^{14}C-glucose was used for the injection experiments as this sugar is the basic metabolite for cell processes, and is translocated throughout the plant in the form of sucrose and other sugars.[1] The

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[1] M. H. ZIMMERMANN, *Ann. Rev. Plant Physiol.* **11**, 167 (1960).

subsequent distribution of radioactivity in the specimen would indicate the loss to be expected when other precursors of polyphenols were injected into the tree. Furthermore, an examination of the radioactive extractives of different tissues might indicate the nature of the translocated precursor of polyphenols. Finally, both the A and B rings of the flavan units in the kino would be labelled by glucose so that the polymer would be suitable for the initial degradative studies.

TABLE 1. KINO COLLECTED AFTER INJECTION OF ^{14}C GLUCOSE

Collection time, days after injection	No. of sample	Kino from chisel cuts mg	cpm/mg	No. of sample	Kino from saw cut mg	cpm/mg
5 (a.m.)	a	316	532	k	76	1·9
8 (a.m.)	b	567	84	l	126	0·6
9 (p.m.)	c	253	44	m	97	6·4
12 (p.m.)	d	298	12	n	36	3·3
13 (a.m.)	e	273	17			
13 (p.m.)	f	315	17			
14 (a.m.)	g	86	20			
15 (p.m.)	h	1290	18			
16 (p.m.)	i	171	12			
17 (a.m.)	j	115	10			

RESULTS AND DISCUSSION

Several methods of administering a liquid to an active kino vein were tried. The best results were obtained by adding the solution of the precursor to chisel incisions into such a vein in a vigorously growing young tree. The kino was then able to exude through the incisions to the outside of the tree soon after it was formed.

The exceptionally high ambient temperatures were probably responsible for the variations in the daily yield of kino (Table 1), the radioactivity of which progressively decreased. Autoradiograms of chromatograms of the kino samples showed that activity was present in both the polyphenolic and carbohydrate components. The high radioactivity of the dialyzates from two samples of kino (Table 2) was also found to be due to both the carbohydrate and polymerized polyphenolic components. The amounts of radioactive glucose

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN KINO SAMPLES

Kino sample	Dialyzed material: Yield %	Dialyzed material: Activity cpm/mg	Dialyzed material: Activity % of total	Undialyzed material. Activity cpm/mg
b	14·4	533	95	4
h	12·8	86	64	7

and traces of sucrose and fructose were all too small to be detected by chromogenic sprays. About 6 per cent of the radioactivity originally injected was recovered in the samples of undialyzed exuded kino.

The autoradiograms of the cross-sections of the tree (Fig. 2, see also Fig. 1) show that, with the type of injection used, most of the radioactivity in the xylem is confined to discs 2–6, that is within 5 cm of the injection level (in disc 4). Samples of thin tangential sections

of phloem adjacent to the cambium showed a similar distribution of activity. Sample H (Fig. 1) taken from the level of disc 5 had an activity of 58·1 cpm/mg, whereas samples I and G at the level of discs 1 and 7 had activities of 2·0 and 1·8 cpm/mg respectively. In addition, the activities of the kino (sample E, disc 6, Fig. 1, 21·5 cpm/mg and sample F, disc 2, 5·2 cpm/mg) exposed by the removal of the bark at the conclusion of the experiment were comparable to those of phloem samples H and I. The extent of translocation in the cambium may have been greater than that in the phloem or xylem as the activity at the base of the dead epicormic shoot in disc 2 is relatively strong (Fig. 4a, b). The above observations indicate that the degree of movement of ^{14}C-glucose from the injection area is much less than that of dyes or other non-metabolic materials.

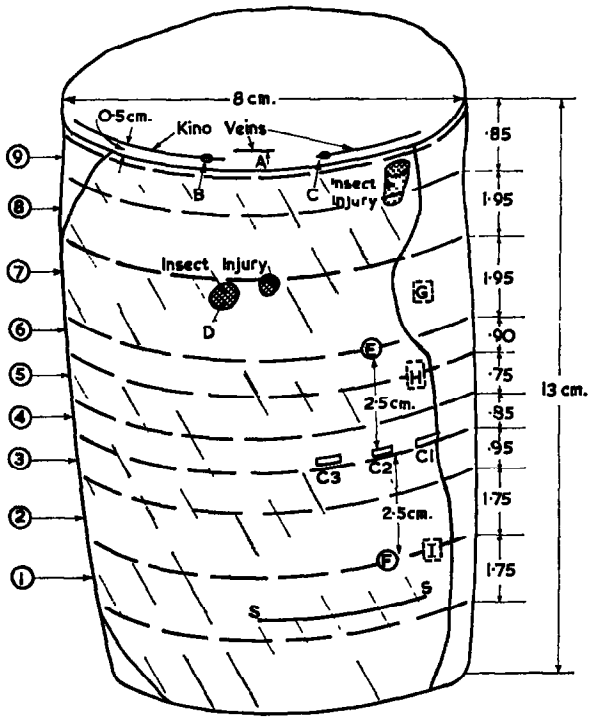

FIG. 1. EXPERIMENTAL SECTION OF THE *Eucalyptus sieberiana* TREE SHOWING POSITION OF THE DISCS NUMBERED 1–9, CHISEL INCISIONS (C1, C2, C3) AND SAW CUT (S——S)

The sloping broken lines represent the direction of the ridges on the sapwood in the kino vein area. The activity of the kino from positions A-F was 0·0, 0·0, 0·4, 1·4, 21·5 and 5·2 cpm/mg respectively. The activity of the ground inner phloem from positions G–I was 2·0, 58·1 and 1·8 cpm/mg respectively.

Centripetal movement of radioactive material was greatest at the chisel incisions C1 and C3 (disc 4, Fig. 2 and 3). The lower degree of movement at incision C2 is probably due to the crushing of the parenchyma bridges in a xylem kino vein when the incision was made; a similar interruption is shown in disc 6 (Fig. 4c) where the bridges were broken during growth of the tree. The horizontal distribution of radioactivity ceased at a sharply defined boundary about three growth rings from the cambium (e.g. disc 6, Fig. 2) which in all cases coincided with the periphery of the heartwood (Fig. 3).

Both the polyphenolic and carbohydrate components of the methanol extracts of the phloem and sapwood samples from the level of disc 5 were radioactive, and the activity of

the sucrose was slightly greater than that of glucose and fructose. After the phloem and sapwood samples had been extracted with methanol and alkali they possessed activities of 20 and 10 cpm/mg respectively, indicating that some incorporation of activity into the parenchyma cell walls had taken place.

The above evidence shows that in early summer and with the method of injection used, labelled glucose (or sucrose) moves from the phloem to the sapwood, and within 19 days reaches the sapwood–heartwood boundary. The evidence does not prove that this is the normal translocation path of sugars, but it is consistent with other evidence[2–6] that polyphenols are formed *in situ* from sugars translocated in this manner. The appearance of labelled polyphenols in the kino sample collected within 5 days of injection of labelled glucose suggests that kino is formed directly from sugars and not from disintegrated cell wall. The amount of labelled carbon incorporated in kino is low, but sufficiently great to permit a study of the linkages in polymerized leucoanthocyanins (Hillis and Hasegawa, unpublished data). Attempts are being made to increase the degree of incorporation.

EXPERIMENTAL

Administration of ^{14}C-glucose

The tree chosen for this particular study was a 7-yr-old, polled, *Eucalyptus sieberiana* which had begun to exude kino through the bark 7 days previously. Subsequently, it was found that insect attack was the probable cause of this kino production. The loose material was removed from the bark and, at the position where kino had previously exuded, three incisions (at about 45° to the vertical) were made (at 9.30 a.m. on 21st December) into the tree with a sharp 1·2 cm chisel. The bark was forced slightly downwards when the chisel was removed. A horizontal saw cut was then made 4 cm below these incisions (Fig. 1). Within 2–3 min, a solution of D-glucose-^{14}C-(U) (Radiochemical Centre, Amersham, England) (0·1 mc, in 1 ml distilled water) was placed, a few drops at a time, at the bottom of the chisel incisions with a hypodermic syringe. After 0·5 hr, the solution had been absorbed, and it was followed by the rinse water (2 ml) used for washing of the receptacles, and the whole area covered with polythene film.

The kino, which subsequently exuded, was collected at early morning (7 a.m.) or late evening (7 p.m.) from both chisel and saw cuts. The day air temperature during the period of exudation was between 16° and 40°C. Nineteen days after the commencement of the experiment the tree was cut down, the bark removed from the experimental section, and samples of kino collected from portions of the cambial kino vein. A longitudinal saw cut was made in the section for reference purposes and a hole bored through the centre. The section was then cut into a number of discs (Fig. 1) and planed to a smooth surface; in this process 10–20 per cent of the volume of each disc was lost.

Detection of radioactivity

The radioactivity of a thin film or layer of evaporated extract, kino sample or finely ground (-200 mesh) phloem or wood was measured by an end window G.M. counter (with a Philips Predetermined Counter Scaler PW4035).

Autoradiograms of the top and bottom of each disc were made by placing the discs on a metal rod followed by a suitable holed X-ray film and then aluminium foil. When all

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[3] W. E. HILLIS and ANN CARLE, *Biochem. J.* **74**, 607 (1960).
[4] W. E. HILLIS and ANN CARLE, *Biochem. J.* **82**, 435 (1962).
[5] W. E. HILLIS, F. R. HUMPHREYS, R. K. BAMBER and ANN CARLE, *Holforschung* **16**, 114 (1962).
[6] A. B. WARDROP and J. CRONSHAW, *Nature* **193**, 90 (1962).

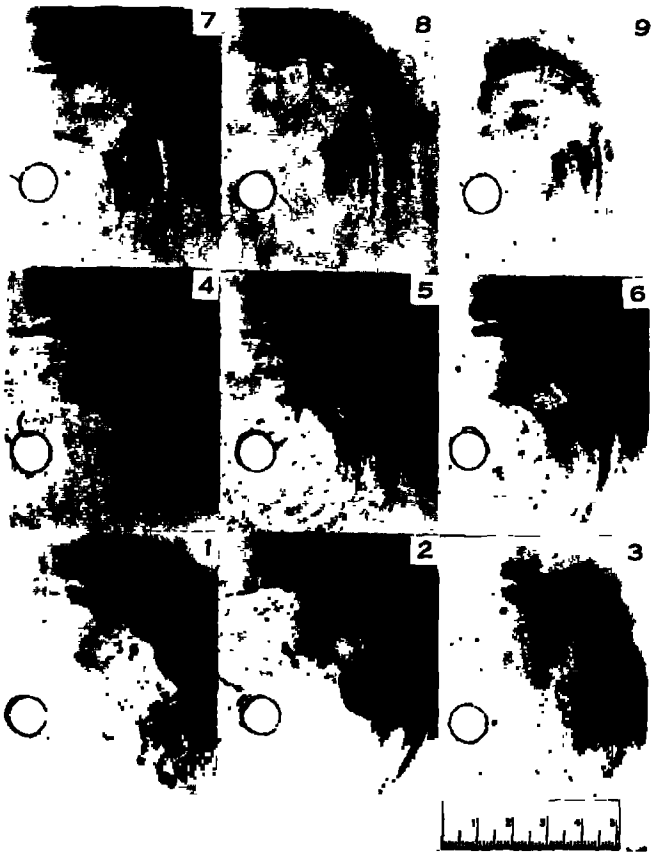

FIG. 2. AUTORADIOGRAMS OF THE RADIOACTIVE PORTION OF THE SURFACES ILLUSTRATED IN FIG. 3, SHOWING HIGH ACTIVITY IN KINO VEINS AND ACTIVITY IN REGIONS OF THE SAPWOOD UP TO THE HEARTWOOD PERIPHERY. TIME OF EXPOSURE—2 WEEKS.

The randomly-spaced spots on the left of the active areas are artefacts arising during preparation of the samples. Scale in centimetres.

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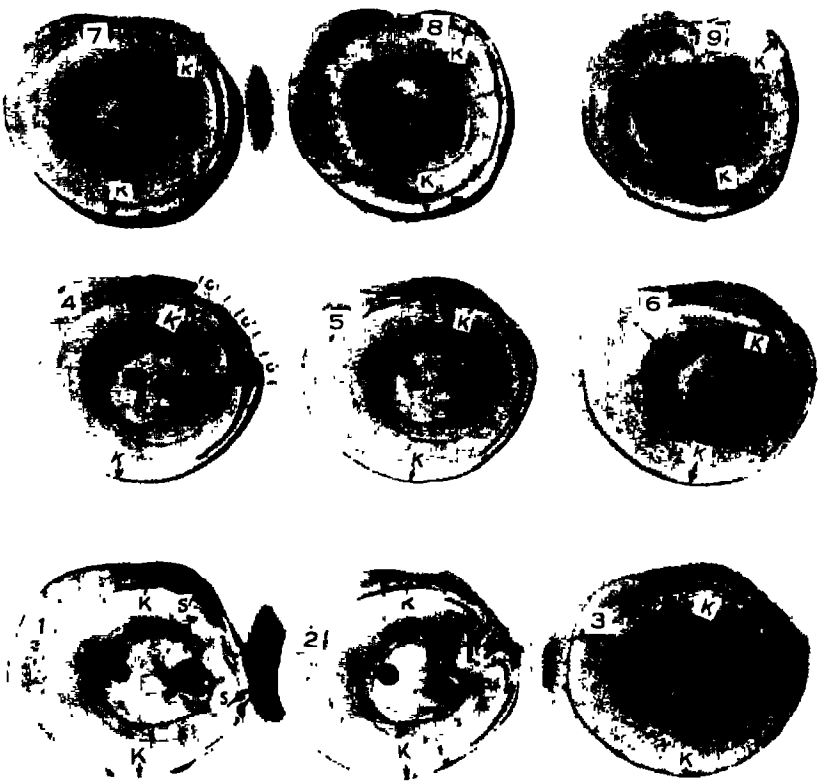

FIG. 3. DISCS CUT FROM *Eucalyptus sieberiana* (FIG. 1).

C1, C2, C3 show the positions of the chisel incisions in disc 4, S——S (disc 1) and K——K show the limits of the saw cut and the cambial kino vein respectively. The sapwood–heartwood boundary has been outlined. Scale in centimetres.

FIG. 4. (a) DISC 2 SHOWING STUB OF DEAD EPICORMIC SHOOT. (b) AN AUTORADIOGRAM (AFTER EXPOSURE FOR 3 DAYS) OF DISC 2 SHOWING HIGH ACTIVITY IN THE CAMBIAL REGION AT THE BASE OF THE STUB, AND ACTIVITY IN THE SAPWOOD UP TO THE HEARTWOOD BOUNDARY. (c) AN AUTORADIOGRAM (AFTER EXPOSURE FOR 3 DAYS) OF DISC 5 SHOWING HIGH ACTIVITY IN THE SAPWOOD ON THE PITH SIDE OF THE INTACT PARENCHYMA BRIDGES IN THE KINO VEIN.

the discs were in position the whole was tightly clamped and set aside for exposure. Autoradiograms of the unsprayed chromatograms were prepared by clamping the chromatogram and X-ray film between thick sheets of glass for 1–5 months. The film used was "Kodirex" Medical X-ray Film "No Screen" (Kodak Pty. Ltd., Melbourne, Australia).

Sapwood and phloem samples

The sapwood sample (1 × 1 cm) was taken from a position adjacent to the heartwood boundary in disc 5 and above the chisel incision C3 (Fig. 3). The large phloem sample originally covered the cambial kino vein in disc 5. The samples were sliced or shredded with a sharp knife, extracted with methanol in a soxhlet apparatus for 48 hr, and then boiled with 0·5 N NaOH (50 ml) filtered and washed with boiling water (500 ml).

The thin tangential sections of phloem (G, H, I, Fig. 1) were obtained by removing the first 10 mm of the inner phloem after thoroughly scraping away the cambium.

Dialysis

The kino solution (250 mg (sample b) or 532 mg (sample h) in 25 ml water) was dialysed through Visking Seamless Tubing (average pore size, 24 Å; Union Carbide) into stirred distilled water (400 ml) at 20–30° C for 36 hr, when the water was changed and the dialysis repeated. The liquors were concentrated under vacuum at less than 50°. The activities of both the undialyzed and dialyzed fractions were determined and the distribution of activity calculated from these figures.

Chromatographic examination

Two dimensional chromatograms of the kino samples, the dialyzed and undialyzed fractions and the methanol extracts of the phloem and sapwood were developed by the ascending method using (1) 6% acetic acid followed by (2) butan-1-ol-acetic acid–water (6 : 1 : 2). The polyphenols were revealed by examining the chromatograms under u.v. light before and after exposure to ammonia vapour, by spraying with diazotized *p*-nitroaniline, or ferric chloride–potassium ferricyanide or vanillin–conc. HCl. The chromatograms of the phloem and sapwood extracts, which were identical with those previously reported for this species,[2,3] showed the presence of ellagitannins, ellagic acid, gallic acid, catechin and polymerized flavans. Almost the whole of the kino formed a streak on the acetic acid axis and gave a brilliant red colour with vanillin-HCl indicating the presence of polymerized flavans. The delphinidin and traces of cyanidin formed when the kino was heated with butanol–hydrochloric acid were identified chromatographically as previously reported.[3] The streak, on the acetic acid axis of the two dimensional chromatograms of the different samples of extracts and kino, reacted strongly with the above reagents for polyphenols and was shown by the radioautograms to be strongly radioactive.

The sugars were resolved by developing one-dimensional chromatograms of the kino or extracts (together with sucrose, glucose, fructose and galactose as markers) with butan-1-ol-acetic acid–water (2 : 1 : 1), ethyl acetate-pyridine-water (12 : 5 : 4) and butyl acetate-pyridine-ethanol-water (8 : 2 : 2 : 1). The markers were revealed with silver nitrate–ammonia or aniline–phosphoric acid and the *Rs* values (distance travelled by sugar/that by sucrose) of glucose and fructose respectively in the three solvent systems were 1·43 and 1·69, 1·17 and 1·08, 3·18 and 5·55. The radioactive components revealed by the autoradiograms of these chromatograms were identical in chromatographic properties with those of the sucrose, fructose and glucose markers.