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THE FORMATION OF POLYPHENOLS IN TREES—III.

THE EFFECT OF ENZYME INHIBITORS*

W. E. HILLIS and T. INOUE†

Division of Forest Products, C.S.I.R.O., South Melbourne, Australia

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Abstract—Gallotannin is almost the only polyphenol in the sapwood of *Rhus succedanea* L., whereas the main polyphenols in the heartwood include also fisetin, fustin, garbanzol, 3,7,4′-trihydroxyflavone, gallic and ellagic acids. Fisetin 7-glucoside is also present in small and variable amounts in the heartwood. A change in metabolism, which is one possible factor responsible for the formation of heartwood extractives, has been initiated in the sapwood of this *Rhus* sp. by administering enzyme inhibitors. The response to inhibitors indicates that a blockage in the utilization of acetate units in the TCA cycle may be a factor in the formation of flavonoids in the heartwood.

INTRODUCTION

HEARTWOOD has been defined as "the inner layers of the wood which, in the growing tree, have ceased to contain living cells, and in which the reserve materials (e.g. starch) have been removed or converted into heartwood "substance".[1] Although heartwood is an important feature of many trees its presence is difficult to explain biochemically.

Heartwood is often, but not always, distinguished from sapwood by a number of other features[2] in addition to the above, prominent among which are a pronounced increase in extractives content and lower moisture and higher gas ratios . Of the different classes of extractives which occur, only the polyphenolic type will be considered in this paper.

There is evidence supporting the view that polyphenols are not translocated[3–5] and that those found in the heartwood extractives are formed *in situ* or at the sapwood–heartwood boundary from stored or translocated carbohydrate[6–9]. This conversion requires at the heartwood boundary (*a*) a zone with increased activity of certain enzymes catalysing polyphenol biosynthesis (this could be due to the disorganization of some enzyme systems resulting in the polyphenol reaction becoming the dominant activity of the cell), (*b*) increased channelling of precursors to polyphenol formation, and (*c*) a ready availability of substrate.

As a result of her observations of anatomical features, Chattaway[10] postulated that in the

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† On leave from Hoshi College of Pharmacy, Tokyo, Japan.

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[2] H. E. DADSWELL and W. E. HILLIS, *Wood Extractives* (Edited by W. E. HILLIS), p. 3. Academic Press, New York (1962).

[3] W. E. HILLIS, *Wood Extractives* (Edited by W. E. HILLIS), p. 60. Academic Press, New York (1962).

[4] E. C. BATE-SMITH, *Wood Extractives* (Edited by W. E. HILLIS), p. 133. Academic Press, New York (1962).

[5] A. H. WILLIAMS, In *Phenolics in Plants in Health and Disease* (Edited by J. B. PRIDHAM), p. 41. Pergamon Press, Oxford (1960).

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[7] W. E. HILLIS, F. R. HUMPHREYS, R. K. BAMBER and A. CARLE, *Holzforschung* **16**, 114 (1962).

[8] W. E. HILLIS and A. CARLE, *Biochem. J.* **82**, 435 (1962).

[9] A. B. WARDROP and J. CRONSHAW, *Nature* **193**, 90 (1962).

[10] M. M. CHATTAWAY, *Australian Forestry* **16**, 25 (1952).

formation of heartwood, the death of the ray cell is preceded by a period of great physiological activity and then the protoplasmic membrane disintegrates to liberate the extractives. In recent years, several workers have examined this problem at the cytological level, but with conflicting results. Plasmolytic and electrical properties and the response to certain dyes,[11] indicate a steady decrease in vitality of the ray parenchyma cells in *Fagus sylvatica* from the cambium to the mature wood zone. Frey-Wyssling and Bosshard[12] concluded, from a cytological analysis of the ray cells of several other European trees, that with the passing of time and at an appreciable distance from the heartwood boundary, the parenchyma cells of all tree species undergo irreversible changes which result in the degradation of the protoplasm and the disorganization (without activation) of the cell's oxidizing system. Similar properties have been observed in Japanese trees.[13] On the other hand, after an extensive examination of these aspects in several European trees, Lairand[14] was unable to confirm some of the assumptions concerning the vitality of the nucleus that were suggested by the above workers. Also, he found the nuclei disappeared close to the heartwood boundary in *Pinus sylvestris* and *Larix europea*, a fact which has been observed also in *Tamarix aphylla*[15] and *Pinus radiata*.[16]

Although there is no direct evidence of an increase in the size or activity of the nuclei, which would be expected for the formation of heartwood extractives, there is information of increased cellular activity in the intermediate zone between sapwood and heartwood. Zelaswki[17] confirmed the findings of Djaparidze by showing that there is a zone of intensified physiological activity in the inner sapwood rings of *Quercus pedunculata*. During storage after felling, respiration of the living sapwood cells increases, particularly in the zone adjacent to the heartwood. Also, Lairand[14] found an increased peroxidase activity in the rings adjacent to the heartwood of pine and larch, as Wardrop (personal communication) has found in several Australian species. However this enzyme may not have a direct association with heartwood formation as activation[18] or synthesis[19] of peroxidase and polyphenol oxidase[18] has been noticed in several mechanically[20] or disease[21] injured tissues. Activation of these enzymes appears to be an unspecific mechanism and although it is associated with an increased polyphenol content, its role is more likely to be concerned with polymerization (browning) of the polyphenol monomers after their formation. Kondo[22] has pointed out that all the enzyme systems, and particularly those in the cell wall, are not necessarily de-activated when the nuclei disintegrate and the normal physiological functions stop. He found the activity of a sucrose hydrolysing enzyme in different tissues to be in the following order: intermediate zone $\gg$ sapwood > heartwood > control (sterilized wood). The activity of an enzyme oxidizing catechol followed the same pattern (T. Kondo, personal communication).

The protein content at the sapwood–heartwood boundary of pines does not increase,[23] and if this is representative of other species, the formation of large amounts of heartwood extractives must be the result of an activation of some enzymes or a disturbance of the equilib-

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[12] A. FREY-WYSSLING and H. H. BOSSHARD, *Holzforschung* **13**, 129 (1959).
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[19] M. A. STAHMANN, I. URITANI and K. TOMIYAMA, *Phytopathology* **50**, 655 (1960).
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[23] G. BECKER, *Holz Roh- Werkstoff* **70**, 368 (1962).

rium of different enzyme systems and not to the formation of greater amounts of enzymes. Several of the above aspects of the formation of heartwood are under investigation in these laboratories, and the present communication reports an attempt to initiate the formation of heartwood polyphenols in the sapwood by a disturbance of the enzyme systems. We have found *Rhus succedanea* L. provides a most suitable experimental material as the marked difference in composition of the polyphenols in sapwood and heartwood facilities the examination of changes following the disturbance in certain enzyme systems. For example the heartwood polyphenols have an intense yellow fluorescence under u.v. light and their formation is readily detected.

RESULTS AND DISCUSSION

Polyphenols in Rhus *Wood*

Fisetin and dihydrofisetin (fustin) (which have previously been found in this and other *Rhus* species)[24–27] have been found to be the major components in the heartwood extractives (Table 1). 3,7,4′-Trihydroxyflavone and the corresponding dihydro-compound (garbanzol)[28] have also been identified. Purification of the small quantities of dihydroflavonols isolated was difficult (particularly with garbanzol) owing to their instability.

TABLE 1. PROPERTIES OF POLYPHENOLS ISOLATED FROM *Rhus succedanea*

Compound	Chromatographic properties* PC R_f (× 100) in Solvent Systems BAW	6HA	30HA	TLC I	II	III	u.v. absorption maxima (mμ) EtOH	EtOH + NaOEt
(1) Ellagic acid†‡	29	1	10	0	0	0	—	—
(2) Gallic Acid†§	54	39	55	8	35	37	276	—
(3) Methyl Gallate†	70	42	67	17	44	45	—	—
(4) Gallotannin	40	7	55	0	0	0	284	—
(5) Fisetin†	62	1	12	4	24	26	251, 320, 366	—
(6) Fustin†	78	40	68	6	29	36	281, 311 (sh.)	254, 340
(7) Garbanzol†	87	41	75	15	44	46	279, 313	256, 340
(8) 3,7,4′-Trihydroxy-flavone†	81	1	17	16	34	38	319, 360	—
(9) Unknown	42	77	—	—	—	—	—	—
(10) Unknown	43	67	—	—	—	—	—	—
(11) Unknown	81	0	12	12	39	44	275, 396	—
(12) Fisetin glycoside	13	4	25	0	1	1	256, 320, 371	decrease in intensity
							EtOH + NaOAc no effect	EtOH + $AlCl_3$ 272, 322, 432

* See Experimental for key to solvents; PC = paper chromatography; TLC = thin-layer chromatography.
† The identity of these components was confirmed by direct comparison with authentic compounds.
‡ R_f in Forestal = 0·32.
§ R_f in *m*-C = 0·07.

[24] A. G. PERKIN, *J. Chem. Soc.* **71**, 1194 (1897).
[25] T. OYAMADA, *Ann. Chem.* **538**, 44 (1939).
[26] M. HASEGAWA and T. SHIRATO, *Nippon Kagaku Zasshi* **72**, 223 (1951).
[27] K. WEINGES, *Ann. Chem.* **627**, 229 (1959).
[28] E. WONG, P. I. MORTIMER and T. A. GEISSMAN, *Phytochem.* **4**, 89 (1965).

When Compound 12 (Table 1) was hydrolysed approximately equi-molecular amounts of fisetin and glucose were obtained. The R_f values yield supporting evidence that the compound is a glycoside and the spectral data (lack of response to sodium acetate)[29] shows that the 7-hydroxyl is substituted. The properties of compound 12 are consistent with those of fisetin 7-glucoside and it is apparently the first fisetin glycoside isolated. The relative amount of this compound in heartwood varied and it appeared to be greater in heartwood of recent formation. Traces of other flavonoids were also detected but not identified; gallic and ellagic acids and gallotannin (Compound 4; Table 1) were also present in appreciable amounts.

Apparently, the sapwoods of *Rhus* species have not been examined previously and in our samples (from three different trees) a gallotannin (Compound 4, Table 1) was almost entirely the only polyphenol present. It was accompanied by very small amounts of gallic acid, two components with the chromatographic and colour properties of β-glucogallin and *m*-digallic acid, methyl gallate (probably an artefact arising during extraction)[30] and traces of ellagic acid and compounds that are formed on acid degradation of Compound 4. Insufficient amounts of the gallotannin (Compound 4) were obtained to permit identification. Its R_f values were similar to those of tannic acid and 1,3,6-trigalloyl glucose, but the appearance of the latter under short-wave u.v. light was distinctly different. Hydrolysis of Compound 4 for increasing periods of time showed that gallic acid appeared in 10 min and after 7 hr was the only polyphenol present; on complete hydrolysis, glucose was the only sugar detected. Compound 4 had disappeared in 2 hr hydrolysis but ten other components were resolved and those with chromatographic properties close to the original compound disappeared on further heating. β-Glucogallin appeared after 1 hr hydrolysis, then became a prominent compound and was the last (apart from gallic acid) to disappear. Purified tannic acid behaved in a very similar way under the same conditions except that *m*-digallic acid was also formed. Compound 4 has chromatographic properties very similar if not identical to those of penta-*O*-galloyl-glucose[31] and present evidence indicates that the two compounds are the same.

The composition of the sapwood and heartwood of *Rhus succedanea* is consequently similar to that of similar tissues in some other members of the Anacardiaceae e.g. *Schinopsis* species.[32]

Effect of Enzyme Inhibitors

The gallic acid moiety of the gallotannin in sapwood is probably formed directly from shikimic acid[33–35] whereas the flavonoids of the heartwood require later intermediates from this pathway (via C_9 compounds) and in addition those from the acetate pathway.[36] It is not known how much of the energy requirements of sapwood involve one or more of the Embden–Meyerhof–Parnas and pentose phosphate sequences or of the TCA cycle. If the energy were largely supplied by the latter then it can be postulated that the energy requirements of the sapwood cells do not release acetate units to enable the A ring of flavonoids to be formed. In this context, it is noteworthy that the major phenolic material (lignin) formed during the

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[36] T. SWAIN, *Wood Extractives* (Edited by W. E. HILLIS), p. 277. Academic Press, New York (1962)

active growth of the wood cell lacks acetate engendered fragments, presumably because of energy requirements of the TCA cycle. Because arsenite and iodoacetic acid are known[37,38] to block the utilization of acetate in this cycle, these two inhibitors were tested for their ability to promote the formation of flavonoids in sapwood. When solutions of these two compounds were added to holes drilled through branches of *Rhus succedanea*, a marked increase in the formation of flavonoids was observed in the surrounding sapwood above that of controls. Thus the postulate is supported. There was no detectable difference in the composition of the polyphenols from the normal heartwood and from the sapwood treated with the two inhibitors. Although the conclusions drawn from experiments involving injury to tissues cannot be decisive, it should be pointed out that the results obtained in experiments carried out in each of three years were mutually confirmatory. Moreover addition of iodoacetic acid and mercuric chloride to sweet potato slices has also been shown to induce the formation of chlorogenic acid and other components in the adjacent sound tissue.[39]

In contrast to an earlier trial of this technique[40] on *Eucalyptus sieberi* (syn. *E. sieberiana*) and *E. regnans*, this *Rhus* sample shows there is no relationship between the hydroxyl pattern of the B ring of the flavonoids and the aromatic moiety of the gallotannins. It would appear there are separate pathways accounting for the hydroxylation patterns in these two groups of compounds.

As gallic and ellagic acids and their derivatives were detected in the sapwood without any trace of cinnamic acid or its derivatives, the results could be explained by postulating a blockage in the biosynthetic pathway between shikimic and related acids and the cinnamic acid derivatives and related C_9 precursors of the B-ring of the flavonoids. This blockage may have a greater influence on the absence of flavonoid components in the sapwood than a shortage of acetate units, but, as the results show, its effect (if it exists) is removed by treatment with both arsenite and iodoacetic acid (see also[40]). Gallotannin was found in the heartwood extractives, but because the amount appeared to be significantly lower than that in the sapwood, its synthesis at the heartwood periphery seems unlikely.

Origin of Heartwood Polyphenols

The amount of extractives in the *Rhus* heartwood (15·5 per cent) is larger than in the sapwood (10·3 per cent). Some of the heartwood extractives may have arisen from the phenolic extractives in the sapwood, but the additional amount would have probably arisen from translocated carbohydrate, as the starch content in the sapwood adjacent to the heartwood appears to be much lower than in the outer sapwood layers (compare[7,15]). Appreciable amounts of phenylpyruvic acid would be involved in the formation of the B ring of the heartwood extractives so that presumably an efficient transamination system exists during its formation from phenylalanine in order to avoid the necessity of translocation of the latter (cf.[41]). The decrease in starch content towards the heartwood boundary suggests the source of energy for extractives formation is spread over many cells. Verification of this aspect requires the determination of ATP content. Much of the current evidence indicates that the nuclei disintegrate before heartwood formation[12,13] and consequently before the extractives are formed in large amounts. Inactivation of enzymes (particularly those of the mitochondrial system) and insolubilization of proteins would take place when the tonoplast ruptures and liberates the

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polyphenols already formed. This inactivation would proceed rapidly when the polyphenols are oxidized to quinones by the peroxidase which in certain cases is known to be present in considerable amounts. Obviously, further cytoplasmic studies of this region are needed before the mechanism of the formation of heartwood extractives can be postulated.

Initiation of Heartwood Formation

The loss of water from (or entry of gas into) woody tissues has frequently been suggested[3,42,43] as the first change in sapwood that leads to heartwood formation. After a detailed examination of heartwood formation in *Fagus sylvatica*, Zycha[44] came to the same conclusion and claimed supporting experimental evidence. We have repeated and extended[45] his work using *Rhus succedanea* because very small quantities (6×10^{-9} g) of the fisetin found in heartwood can be readily detected under u.v. light. However unequivocal support for this theory could not be obtained.

The increase in polyphenol content at the heartwood boundary of many species and in the naturally damaged regions of this *Rhus* species, resembles the expression of metabolic dysfunction induced in host cells by fungal infection.[46] However, no evidence of hyphae were evident on microscopic examination, although this does not preclude the possibility that hyphae elsewhere in the tree secrete toxins which are translocated along the vessels to induce polyphenol formation. Clearly, the natural causes of the disturbance in the enzyme systems which lead to heartwood formation require further study.

EXPERIMENTAL

Chromatographic Examination

Two dimensional chromatograms were prepared using first, *n*-butanol–acetic acid–water (6:1:2, BAW), then 6% acetic acid (6HA). One dimensional chromatograms were prepared using these solvents and also *m*-cresol–acetic acid–water (50:2:48, m-C), 30% acetic acid (30 HA) and hydrochloric acid–acetic acid–water (3:30:10, Forestal).

The chromatograms were examined under u.v. light (254 and 366 mμ) before and after exposure to ammonia vapour. The chromogenic sprays used were; a solution (0·1%) of a stabilized diazotized *p*-nitroaniline in 20% sodium acetate, and ferric chloride–potassium ferricyanide[47] for polyphenols and aniline phosphate[48] for sugars. Chromatoplates of Silica Gel G.F. 254 (E. Merck, A. G. Darmstadt) were run in a constant temperature room at 20°. The following solvents were used; I, chloroform–acetic acid (8:2); II, toluene–ethyl formate–formic acid (5:4:1); III, chloroform–ethyl acetate–formic acid (5:4:1).

Methanol-soluble Material

Portion of a branch containing five growth rings was separated into sapwood devoid of yellow fluorescent material, and heartwood containing very largely yellow fluorescent tissues from which brown non-fluorescent material was removed. These two portions were ground

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[43] B. HUBER, *Handbuch der Pflanzenphysiologie* (Edited by W. RUHLAND), Vol. III, p. 541. Springer-Verlag, Berlin (1956).
[44] H. ZYCHA, *Fortwiss. Centr.* **67**, 80 (1948).
[45] W. E. HILLIS, Unpublished work.
[46] I. A. M. CRUICKSHANK, *Ann. Rev. Phytopathol.* **1**, 351 (1963).
[47] G. M. BARTON, R. S. EVANS and J. A. F. GARDNER, *Nature* **170**, 249 (1952).
[48] A. S. F. ASH and T. M. REYNOLDS, *Australian J. Biol. Sci.* **7**, 435 (1954).

and extracted exhaustively with methanol when 10·3 and 15·5% soluble material, respectively was obtained.

Isolation of Polyphenols

A seven-year-old branch containing wound heartwood as well as sapwood was removed from a garden-grown *Rhus succedanea* and ground (109 g), then extracted with hot methanol and the extract concentrated. *Compound* 1 (Table 1; crude material, wt. 107 mg) was removed, the viscous filtrate extracted with light petrol (b.p. 40–60°) and the residue evaporated *in vacuo* to yield a brown powder (6·8 g). The latter (4·2 g) was dissolved in methanol, streaked onto No. 3 Whatman papers, resolved with BAW., the sheets cut into five bands and separately extracted with methanol in a soxhlet-type apparatus.

Extract I (from band with lowest R_f) contained polymeric material and was not examined further. Extract II on concentration precipitated *Compound* 1 (crude material, wt. 94 mg), of which the tetra-carbethoxy derivative was made (m.p. 247° and mixed m.p. 245–248°), thus confirming the identification of *Compound* 1 as ellagic acid. The filtrate from *Compound* 1 was placed on a polyamide column (1·5 × 8 in.) and eluted with water and then aqueous methanol of increasing methanol concentration. The major compound(s) removed by water was *Compound* 1, by 20 and 30% methanol were *Compounds* 1, 2 and 3, by 40, 50, 60 and 70% methanol were *Compounds* 2 and 3, and by 80 and 100% methanol were *Compounds* 4 and 5. All fractions contained traces of compounds that were formed on acid degradation of *Compound* 4 amongst which β-glucogallin was identified chromatographically; *m*-digallic acid was similarly identified but this compound is not a degradation product of *Compound* 4. The 50–100% eluates contained trace amounts of *Compound* 12 and two other unidentified yellow fluorescent components.

Compounds 2 and 3 were identified as gallic acid and methyl gallate by chromatographic examination and colour reactions in comparison with authentic materials.

The 80 and 100% methanol eluates were evaporated to dryness, the ethyl acetate soluble portion removed and to this solution chloroform was added dropwise until precipitation occurred. The precipitate was collected, dissolved in ethyl acetate and reprecipitated twice more to yield a colourless specimen of *Compound* 4 (10·5 mg). This Compound was hydrolysed with 5% sulphuric acid for 8 hr, neutralized with barium carbonate, and the sugar in the filtrate identified as glucose by direct chromatographic comparison with authentic material using aniline hydrogen phthalate as chromogenic spray. Samples (50–100 μg) of *Compound* 4 were sealed in capillaries with 5% sulphuric acid and heated in boiling water for periods of 10, 20, 30, 40, 50, 60 min, 2, 3, 4, 5, 6 and 7 hr and then chromatographed immediately in 6% acetic acid and then BAW.

Extract III contained compounds present in other extracts.

Extract IV was resolved on a polyamide column as above. Water removed *Compound* 2 (gallic acid) which crystallized in fine needles (19 mg). The 60% ethanol extract was further separated on No. 3 Whatman paper using BAW. to yield in addition to gallic acid, *Compounds* 5 and 6 which were identified as fisetin (20 mg, m.p. and mixed m.p. 235°) and fustin (about 18 mg) respectively (Table 1).

Extract V was further separated on No. 3 Whatman papers, using BAW., into fisetin and a mixture with higher R_f values. The latter was separated into *Components Va* and *Vb* by preparative layer chromatography using Silica Gel G.F. 254 and solvent II. The low R_f component (*Va*) was further separated on No. 3 Whatman paper using 6% acetic acid into fustin and traces of *Compounds* 9 and 10. The high R_f component (*Vb*) was further separated

twice more using thin-layer chromatography with solvent I to yield *Compounds* 7 and 8 and a trace of *Compound* 11; the last two compounds were separated on thick paper with 30% acetic acid. *Compounds* 7 and 8 were identified as garbanzol and its related flavonol (3,7,4'-trihydroxyflavone) respectively. The small amounts and instability of garbanzol prevented its complete purification.

A three-year-old branch was found to contain fluorescent material in only the middle growth ring and appeared to arise from injury to that ring. The parenchyma surrounding the vessels were full of extractives which also exuded into the vessels. The fluorescent area was cut out, and its methanol soluble material (700 mg) found to contain an unusually large amount of *Compound* 12, which was separated by streaking the extract on No. 3 Whatman papers, resolving with BAW. and later resolving the extract of the appropriate band with 30% acetic acid. The chromatographically homogeneous compound (2 mg) obtained from the latter, was pale yellow in colour, with an intense yellow fluorescence but could not be crystallized and had a m.p. 202–205° (decomp.). Hydrolysis yielded fisetin and glucose identified chromatographically. A portion (1 mg) was hydrolysed with 5% sulphuric acid at 100° for 45 min, and the fisetin estimated spectrophotometrically and the glucose by the method of Pridham.[49] The molecular ratio was found to be fisetin–glucose, 1·00:1·09.

Administration of Enzyme Inhibitors to Rhus *branches*

Holes ($\frac{1}{16}$ in. dia.) were drilled almost through several branches at mid-day on warm breezy days and, except for the controls, solutions (about 0·5 ml) of inhibitors were added immediately after drilling. The experiments were done on two trees and in the early-summer periods of 1962–64 and examined 6–9 months later. The enzyme inhibitor solutions used were; (i) 0·1 M sodium arsenite; (ii) 0·1 M iodoacetic acid; (iii) 1 mM 2,4-dinitrophenol; (iv) 0·1 M sodium fluoride; (v) 0·1 M sodium fluoroacetate; (vi) 0·1 M potassium cyanide; (vii) 0·1 M hydroxylamine. The last five inhibitors formed a small layer ($\frac{1}{16}$–$\frac{1}{8}$ in.) of yellow fluorescent wood around the injection hole and formed wings of less than 0·5 in. above and below the holes. These inhibitors also killed the tissues to slight but differing extent. The amount of yellow fluorescent zone was no greater than in the controls. In addition the wounds formed a black resinous non-fluorescent material.

Sodium arsenite produced larger fluorescent areas than the controls, but the most marked effect was with iodoacetic acid. In one experiment the leaves on the branch became increasingly bright red after 8 days, on the 11th day appreciable quantities of brown non-fluorescent resin began to exude from the injection hole, and on the 18th day the bright red leaves, containing a few green patches, began to absciss. Six months later, most of the 13 in. of wood on the apex side of the injection point was dead and brown, but an intensely yellow fluorescent portion extended from this point in diminishing amounts for a length of 6 in. On the other side of the injection hole, the dead tissues were present for 1·75 in. but the yellow fluorescent areas extended for at least 3 in.

In an *in vitro* experiment the end portion of a branch (4·5 in. long × 0·6 in. dia.) was placed in sodium arsenite solution for 18 hr then transferred to a tube and stoppered. The gas pressure in the tube showed metabolism continued for 9 days. Examination of the stem after 11 days failed to reveal any fluorescence.

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