Some Constituents of Xylem Sap and their Possible Relationship to Xylem Differentiation

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ABSTRACT

Bleeding sap of *Actinidia chinensis* and *Betula populifolia* and guttation fluid of *Avena sativa* were analysed for sugars, amino-acids, auxin, and certain enzymes. A wide range of amino-acids was found in all three. Auxin was not detected in the bleeding sap, but was present in *Avena* guttation fluid (5·1 μg IAA equivalent/l). 'IAA oxidase', acid phosphatase, ribonuclease, deoxyribonuclease, and protease were detected in the bleeding sap and guttation fluid. The possibility that some of the substances found in sap and guttation fluid are products of autolysing, differentiating xylem cells in the roots is discussed.

INTRODUCTION

ALTHOUGH it has been known for a long time that xylem cells die as they differentiate there have been very few attempts to investigate the biochemistry of the autolytic process involved, or to analyse the substances released. One possible line of attack was suggested by the ingenious theory of root pressure put forward by Priestley (1922). He pointed out that in the root-hair region where most absorption is taking place, xylem cells are differentiating, and that during this process the contents disappear, presumably as a result of hydrolysis. This general hydrolysis would result in a large increase in the number of molecules present, and thus in a great rise in osmotic pressure. Water would move in and the sap would rise. Although the mechanism Priestley suggested is no longer thought to play a very significant part in root pressure, it does focus attention on the possibility that some of the substances released as the xylem cells die will be carried up in the xylem sap. It might therefore be possible to detect these substances by analysing xylem sap, which can be collected as bleeding sap or as guttation fluid.

Sap has been a popular object of analysis for many years. The studies have mostly been concerned with sugars, salts, or organic nitrogen. Some enzymes have been detected, for example diastase and peroxidase (Wormall, 1924) and amylase (Meeuse, 1952). Recently, both kinins (Kende, 1964) and giberellins (Sitton, Richmond, and Vaadia, 1967) have also been found in bleeding sap. But none of these investigations was directly concerned with finding substances which might have been released by differentiating xylem cells.

We have analysed sap for auxin and for hydrolytic enzymes which might be associated with autolysis: acid phosphatase, ribonuclease, deoxyribonuclease, and protease. Sugars and amino-acids were also investigated. These

determinations were carried out on guttation fluid from seedlings of *Avena sativa* and bleeding sap from *Betula populifolia* and *Actinidia chinensis*. The latter two species were selected because it was possible to collect quite large volumes of bleeding sap from them.

MATERIALS AND METHODS

Seeds of *Avena sativa* L. var. Padarn were obtained from Messrs. Ison of Cambridge. They were soaked in water for 3 h before being sown in sand in plastic boxes with transparent lids in which holes had been bored. They were grown in a room whose temperature was maintained at 25 °C.

Bleeding sap was collected from trees of *Betula populifolia* Marsh and *Actinidia chinensis* Planch growing in the Cambridge University Botanic Garden.

Indol-3yl-acetic acid (IAA) was obtained from Roche Biochemicals Ltd.

Collection of sap and guttation fluid

The bleeding sap was collected in March from trees before bud-break. Twigs, about 2 m from the ground, were cut with a scalpel; the bark was peeled off for about 5 cm below the cut and thoroughly washed toy balloons, containing about 1 ml of toluene, were placed over the cut end of the twigs and held on by means of sticking plaster. The sap that dripped out of the cut end of the twigs and accumulated in the balloons was collected daily and stored in the deep freeze at −15 °C.

The *Avena* seedlings, grown in sand in the plastic boxes with their lids on, exuded guttation fluid because of the high humidity of the atmosphere surrounding them. The guttation fluid from several thousand seedlings was collected at 3–4 hourly intervals with Pasteur pipettes and was stored in the deep freeze.

Analysis of sap and guttation fluid

In order to separate charged and uncharged compounds, the sap was passed through an ion-exchange column which retained the amino-acids. Zeocarb 225 SRC-14 resin was used (Permutit Ltd., London) in a column 1 cm in diameter and about 10 cm long. The fraction that passed through, and the washings, were collected, concentrated, and used for sugar estimations. The compounds retained were eluted with 0·5 N ammonia; the eluate was concentrated and used for amino-acid determinations on a Technicon automatic amino-acid analyser. The sugars were separated by descending chromatography on Whatman No. 1 paper using ethyl acetate/pyridine/water (8 : 2 : 1 v/v) (Jermyn and Isherwood, 1949).

For the detection and estimation of neutral sugars, the aniline hydrogen phthalate method of Wilson (1959) was used. The reagent was prepared by adding aniline to a hydrogen phthalate solution immediately before use. The papers were dipped, dried, and then heated at 100 °C until the spots developed. Uronic acids could be detected but not estimated by this method. Keto sugars were detected by spraying with a urea/HCl reagent and then heating at 100 °C (Bell, 1955).

For estimation, elutions were carried out for 3 h in the dark with ethanolic/HCl. (420 ml ethanol + 28 ml HCl + water to 500 ml) (Wilson, 1959). Optical densities were measured at 390 nm for hexoses and 360 nm for pentoses. Standard curves were prepared from known amounts of sugars which had been chromatographed, detected, and eluted as above.

Total reducing sugars were estimated by the Nelson method as described by Bell (1955). Sucrose was determined by the difference observed between the total amount of reducing sugar before and after hydrolysis with 1·0 N—HCl at 100 °C for 10 min.

Enzyme assays

Blanks and test samples were estimated in duplicate in all assays and each was repeated at least three times.

Acid phosphatase. The assay is based on the splitting of *p*-nitrophenyl phosphate (Lowry, 1957). The reagent (1 ml), containing sodium *p*-nitrophenyl phosphate (8 mM), acetate buffer (0·05 M, pH 5) and $MgCl_2$ (0·02 M), was mixed with 0·2 ml of sap and incubated for 2 h at 37 °C. The mixture was then cooled in ice and 3 ml of 0·1 N-NaOH were added. The optical density was measured at 410 nm.

Ribonuclease and deoxyribonuclease. These assays were performed by the method of de Duve Pressman, Gianetto, Wattiaux, and Appelmans (1955). RNA or DNA (1 mg) in 2 ml of acetate buffer (0·1 M, ph 5) was incubated with 0·2 ml of sap for 2 h at 37 °C. The nucleic acid was precipitated by perchloric acid in the cold (and also with uranyl acetate in the case of RNA), separated by centrifugation and the optical density of the supernatant was measured at 260 nm.

Acid protease. The assay depended on the increase in α-amino groups produced by splitting a protein substrate. The reaction mixture, which allowed long incubation times, was composed of 0·2 ml of 1 per cent bovine serum albumen (fraction V) in acetate buffer (0·1 M, pH 5), 0·2 ml sap, and 0·025 ml 0·2 per cent thiomersal (an antiseptic). One set of experimental samples and blanks (in duplicate) was put in the deep freeze; another set was incubated at 37 °C for 24 h. The α-amino groups in both were then estimated by the method of Cocking and Yemm (1954). The increase in α-amino groups, and hence proteolytic activity was calculated.

'*IAA oxidase*'. A reagent (1 ml) containing phosphate buffer (0·05 M, pH 6·6) and 10 μg of IAA was incubated for 2 h at 37 °C with 0·2 ml sap. The amounts of IAA present were then estimated by the method of Gordon and Weber (1951). A standard curve enabled the amount of IAA destroyed to be calculated.

Polysaccharidases. Determinations of cellulase, xylanase, and mannanase activity were carried out by the methods of Myers and Northcote (1958).

Extraction and estimation of auxin

The sap was acidified to pH 3, using methyl orange as an internal indicator, and shaken three times in the cold with peroxide-free ether. For estimation by the coleoptile straight-growth method, about 100 ml of the tree saps and 50 ml of *Avena* guttation fluid were used for each determination. In experiments employing the mesocotyl extension bioassay, only about 10 ml of guttation fluid were used. The samples in ether were combined and evaporated to dryness under reduced pressure. They were taken up in a small volume of ethanol and applied to chromatograms.

For the determination of auxin in tree saps and in some estimations of auxin in guttation fluid descending paper chromatograms were used; they were developed, eluted, and the auxin was estimated by an *Avena* coleoptile straight-growth bioassay by methods which we have described elsewhere (Sheldrake and Northcote, 1968*a*).

Thin-layer chromatography was also used for some *Avena* guttation fluid extracts, 20 × 20 cm glass plates were coated with cellulose and developed with methyl acetate/isopropanol/ammonia (Randerath, 1963). In this system IAA was found to have an Rf of 0·65. The position of IAA marker spots was detected by a perchloric acid/$FeCl_3$ spray (Larsen, 1955). The cellulose was scraped off in appropriate zones and placed in small tubes in which an *Avena* mesocotyl extension bioassay was performed, by the method of Nitsch and Nitsch (1956). In each assay tube 1 ml of solution was used. Standard curves were prepared using IAA (1–500 ng/ml) and the amount of auxin was thus determined.

RESULTS

pH values

The pH of the saps was found by means of a pH meter to be: *Betula*, pH 5·89; *Actinidia*, pH 5·45; *Avena*, pH 5·65.

Sugars

The total amount of reducing sugars (g glucose equivalents/l) present in the saps were: *Betula*, 3·540; *Actinidia*, 0·960; *Avena*, 0·043.

The *Betula* and *Actinidia* saps contained glucose and fructose. No other sugars were detected in the *Actinidia* sap, but traces of sucrose were present in *Betula* sap. In the *Avena* guttation fluid a much wider range of sugars was found; uronic acids, glucose, galactose, fructose, xylose, arabinose, and traces of ribose. These were analysed quantitatively with the following results (mg/l): glucose 2·3; galactose, 13·0; fructose, 10·3; arabinose, 1·6; xylose, 3·7; ribose, trace; sucrose, 7·3.

Amino-acids

The positions of the peaks and their quantitative values were known from previous runs on the Technicon automatic amino-acid analyser using standard amino-acid solutions. On this system the glutamine peak coincides with that of serine: the amounts of these two amino-acids were calculated after a second analysis had been carried out on a sample which had been hydrolysed by heating with 6 N-HCl at 100 °C in a sealed tube for 6 h. The results are shown in Table 1.

TABLE 1

Amino-acids in sap

μmoles/l	*Avena*	*Betula*	*Actinidia*
Aspartic	2·22	1·31	33·7
Threonine	1·48	1·16	3·15
Serine	3·80	2·00	1·35
Glutamic	2·14	2·71	570·0
Citrulline	..	5·25	..
Proline	0·66	..	3·27
Glycine	3·26	1·30	5·87
Alanine	3·22	1·12	10·1
Valine	1·15	1·58	73·5
Methionine	..	..	9·45
Isoleucine	0·47	1·15	33·3
Leucine	0·93	0·97	7·5
Tyrosine	0·78	0·37	1·20
Phenylalanine	0·54	2·40	14·7
Lysine	3·73	8·72	32·4
Tryptophan	Trace	..	..
Ornithine	1·45	2·62	6·67
Histidine	1·92	4·85	14·1
Arginine	4·20	4·80	1·26
Glutamine	..	..	69·70
Cysteine	..	0·66	..

Auxin

No auxin was detected in the *Actinidia* and *Betula* sap. However, it was always found in the *Avena* guttation fluid. Auxin activity was detected only in the zone of the chromatogram corresponding to IAA (Fig. 1). Analyses were carried out on four separate batches of guttation fluid. The average amount of auxin present was 5·1 μg IAA equivalents/l.

Enzyme assays

Although the tree saps were collected over toluene and the *Avena* guttation fluid was collected at regular intervals and immediately stored in the deep freeze, the possibility remained that significant numbers of bacteria might influence the enzyme assays. In order to remove any bacteria present the saps were centrifuged at 20,000 *g* for half an hour and the supernatant was then passed through an oxoid filter. The assays were carried out on this bacteria-free sap.

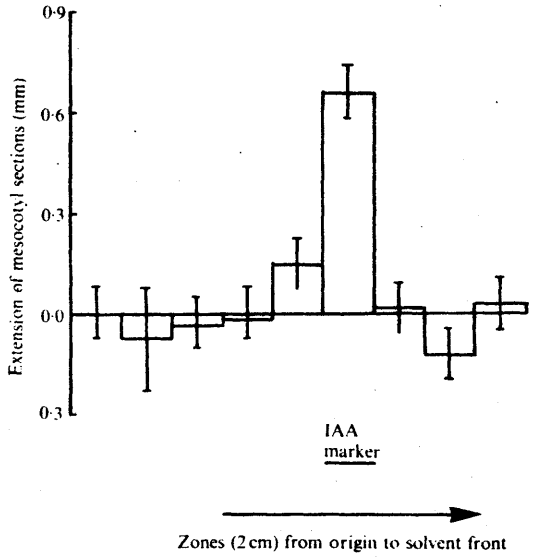

FIG. 1. Bioassay of a thin-layer chromatogram (cellulose powder) of an acidic, ether extract of the guttation fluid of *Avena*. The auxin activity occurs in the region of the IAA marker.

The *Betula* and *Actinidia* saps were collected in March 1966, stored in the deep freeze, and preliminary assays done in the next few months. In March 1967 further samples of *Betula* sap were collected from the same tree, but it was impossible to obtain any more *Actinidia* sap because the tree had been cut down. The second series of assays were carried out on *Betula* sap and *Avena* guttation fluid which had been stored in the deep freeze for no more than a few weeks; but the *Actinidia* sap had been there for over a year. In some of these assays no activity was detected in the case of *Actinidia*, but this may mean that the relevant enzyme had been denatured or destroyed during storage. The units in which the results are expressed are arbitary, representing optical density increase/ml sap/h, unless otherwise stated. The units enable activities of different saps to be compared, and also show qualitatively whether enzyme activity is present or not. The results of the assays are shown in Table 2.

TABLE 2

Enzyme activities of bleeding sap and guttation fluid

Arbitary units, see text

Enzyme	Sap or guttation fluid		
	Avena	*Betula*	*Actinidia*
Acid phosphatase	0·624	0·176	1·248
Acid ribonuclease	5·00	0·12	*
Acid deoxyribonuclease	0·310	0·063	0·060
Acid protease	0·010	0·023	0·018
'IAA oxidase' (μg IAA destroyed/ml sap/hour)	1·83	5·40	*

* No activity detected after storage until 1967 but activity present when tested in 1966.

Definite activity was detected for acid phosphatase, ribonuclease, deoxyribonuclease, protease, and 'IAA oxidase'. No cellulase, mannanase, nor xylanase activity was detected.

DISCUSSION

The contents of xylem sap represent the resultant of a number of processes, and to draw conclusions about any one of these from an analysis of the sap is difficult. However, in view of the sparseness of information about the process of xylem differentiation at a biochemical level, it seems worth examining the results of an analysis of sap from this point of view. In addition to xylem differentiation in the roots, the factors responsible for the contents of the sap are likely to be leakage and secretion of substances into the xylem from surrounding tissues (possibly including the endosperm in the case of seedlings) and absorption by cells adjacent to the xylem. The roles of absorption and secretion are not clear, but from the studies of Pate, Walker, and Wallace (1965) it is known that internodes of the field pea can absorb at least some of the nitrogenous substances passing upwards in the xylem sap. Presumably sugars can also be absorbed. Any interpretations of what is being released by the differentiation of xylem cells in the root based on the analysis of sap must therefore be guarded.

The bleeding sap from the two trees had passed through at least 3 m of xylem before it was collected. The interpretation of the data for the sugars in the sap would therefore be difficult, and in fact no conclusions can be drawn about the role of xylem differentiation in their production. However, the *Avena* guttation fluid had passed through only about 5 cm of xylem and thus much less time would have been available for absorption. The range of sugars found was similar to that reported for guttation fluid of seedlings of rye, wheat, and barley: arabinose, xylose, fructose, glucose, galactose, ribose, and sucrose (Goatley and Lewis, 1966). The amounts detected in *Avena* guttation fluid were of the same order as those reported for rye, wheat, and

barley. It would be difficult to explain this range of sugars on a secretion theory. It seems more likely that they would have been released by differentiating xylem cells. Sucrose might have been released as the vacuole broke down, and some of the glucose and fructose could have been derived from this by hydrolysis. The hydrolysis of the end walls of the developing xylem cells, which break down and disappear, could account for the uronic acids, galactose, and arabinose (from pectin) and arabinose and xylose (from hemicellulose). The ribose, present in smaller amounts, could not come from the hydrolysis of wall polysaccharides since they contain none; it could, however, have been released by the hydrolysis of RNA and nucleotides.

The wide range of amino-acids found in all the saps analysed could similarly be due to a general hydrolysis of proteins in differentiating xylem cells. Some of them, present in larger amounts, could also have been secreted. In the *Actinidia* sap there were large amounts of aspartic and glutamic acids and glutamine, and these are the types of amino-acids which are known to play a large role as nitrogenous export compounds from roots to shoots (Pate *et al.*, 1965). But the presence of so many amino-acids in relatively small amounts suggests a less specific origin for most of them. It seems likely that they could have been produced by the breakdown of the protoplasm of the xylem cells.

The hydrolytic enzymes detected—acid phosphatase, ribonuclease, deoxyribonuclease, protease—are all of the type known to be present in lysosomes in animals, and are precisely the sorts of enzymes which would be expected to be responsible for the autolysis of differentiating xylem cells. Very little is known about this autolytic process: vesicles containing acid phosphatase have been studied by histo-chemical techniques, in differentiating xylem cells and they behave in the way one might expect lysosomes to behave in the process of differentiation (Gahan and Maple, 1966); but the evidence is based only on acid phosphatase localization and there is no strong argument, other than by analogy with animals, that these vesicles are lysosomes, or indeed that lysosomes exist in plants. But some lysosome-like system must be involved in the autolysis of the xylem cell, and the presence of the hydrolytic enzymes in sap is indirect evidence for this. Presumably pectinases, hemicellulases, and cellulase are also involved in the breakdown of the xylem end walls. We had hoped to detect these in sap; but perhaps the enzymes are more labile than the lysosomal type enzymes, or perhaps the assays used were too insensitive. It might be possible with improved techniques to find these enzymes in sap.

We have obtained evidence which shows that auxin can be produced as a consequence of cellular autolysis (Sheldrake and Northcote, 1968*a*), and we have suggested that differentiating xylem cells may be an important source of auxin in the plant (Sheldrake and Northcote, 1968*b*). The presence of auxin in *Avena* guttation fluid is circumstantial evidence for this. The amounts were not large and none was detected in the tree sap. However, auxin in the sap might be absorbed as it travels up the xylem and the presence of an

IAA-destroying enzyme would reduce or eliminate any IAA initially present. Thus the absence of auxin from tree sap is not evidence against its production by differentiating xylem. In fact the existence of an enzymic system which destroys IAA would be necessary for the hypothesis of controlled auxin production by differentiating xylem. For if all comparable xylem cells produce similar amounts of auxin as they differentiate, the same amounts would be formed by differentiating xylem in roots and shoots. But roots form much less auxin than shoots, and are inhibited by auxin at concentrations which are stimulatory for shoots. Therefore in roots the net auxin production by autolysing xylem cells would have to be damped down greatly and this could be effected by the presence of an auxin-destroying system in the differentiating xylem cells of the root. The 'IAA oxidase' detected in the bleeding sap and guttation fluid, which originate in the roots, is therefore consistent with the hypothesis of auxin formation by differentiating xylem.

We have suggested (Sheldrake and Northcote, 1968*b*) that kinins are also formed by autolysing cells as a consequence of the breakdown of nucleic acids. The presence of kinins in bleeding sap (Kende, 1964) could be explained by their release into the sap from differentiating xylem cells.

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