SOME PROPERTIES OF A PLANT GROWTH INHIBITOR PRESENT IN XYLEM SAP OF WOODY SPECIES

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Summary

An ether-soluble, non-toxic inhibitor of plant growth was shown to be present in varying amounts in xylem sap from shoots of various woody species. Some chromatographic properties of the inhibitor are described. The inhibitor reduced growth of wheat coleoptile sections and lettuce hypocotyls, and inhibited germination of lettuce and cress seeds. The inhibitor did not interact competitively with either auxin in the wheat coleoptile elongation test or with gibberellic acid in the lettuce hypocotyl test. Gibberellic acid did not overcome the inhibitory effect on seed germination. The inhibition of coleoptile section growth was reversed on transfer from inhibitor solution to sucrose solution.

In willow sap (*Salix fragilis* L.), inhibitor levels were higher in the dormant winter period than in midsummer. In the evergreen species, Cape honeysuckle (*Tecomaria capensis* Spach.), there was little change in sap inhibitor concentration throughout the year.

I. Introduction

A number of growth inhibitors have been extracted from various plant tissues and isolated by chromatography. Many of these have been shown to occur in a way which suggests some connection with growth phases in the plant (Bentley 1958; Hemberg 1961).

Recently an inhibitor was found in xylem sap from various woody species (Davison 1963). The method used to collect the xylem sap samples involved a minimum of tissue destruction so that in this instance the extracted sap can be regarded as essentially the transport fluid largely free of contaminating cell material. This appeared to be the first record of a strong inhibitor moving within the sap stream and its presence raised the question of its significance in growth processes. Some further properties of the inhibitor have now been investigated and these are described below.

II. Materials and Methods

(a) Plant Material

For these experiments xylem sap from four different species was tested. These included the deciduous species apple [*Malus sylvestris* (L.) cv. Sturmer], peach [*Prunus persica* (L.) Batsch cv. Golden Queen], and willow (*Salix fragilis* L.), and the evergreen semi-climber Cape honeysuckle (*Tecomaria capensis* Spach.). Regular samples were collected from only the latter two species. The Cape honeysuckle was growing as a hedge in a well-drained, light volcanic soil in almost frost-free conditions. This species is a winter flowering plant showing some leaf growth during the winter

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months. The willow trees were growing in a low lying swampy area with saturated soil conditions throughout the year. In 1963, leaf fall of these willows commenced early April and was mainly completed by the end of April. Bud break occurred about mid-September. Trees of all species sampled were mature and shoots selected for use were of wood more than one year old.

(b) Collection of Xylem Sap

Xylem sap was collected from shoots 1–2 cm in diameter by a method described by Bollard (1953). Suction was applied to the base of a shoot while small pieces (1 cm) of the top were removed at regular intervals. Xylem sap so extracted is a clear, watery fluid. After collection the sap was filtered through Whatman No. 1 filter paper and stored at −40°C until used.

Inhibitor concentrations are expressed as the corresponding volumes of xylem sap from which the active extract was made.

The separate experiments reported in this paper in which willow sap was used as the source of the inhibitor were carried out with different bulk collections of sap made at different times of the year. Since the inhibitor concentration in sap is not constant throughout the year [see Section III(b)] the amounts of inhibitor used in different experiments, expressed as they are on a volume equivalent basis, will not be the same in the different experiments.

(c) Extraction of Inhibitor from Xylem Sap

The inhibitor was usually removed from the sap by adjusting the pH to 2 and shaking three times with peroxide-free ether. In some experiments the inhibitor was used without further purification, by redissolving the evaporated ether residue in aqueous solution (designated unpurified inhibitor). At other times the inhibitor was further purified by one-way chromatography and elution of the active region by ethanol (designated partially purified inhibitor).

(d) Detection of Inhibitor

The presence of the inhibitor in extracts or on regions of chromatograms was detected by the growth response of 6-mm subapical sections of wheat coleoptiles, cv. Aotea. Test plants were grown in sand. These tests were carried out after the method of Nitsch and Nitsch (1956) in small test tubes mounted on a revolving wheel, 10 sections being used per tube. Test solutions were buffered to pH 5 with a potassium phosphate–citric acid buffer and contained 2% sucrose unless otherwise stated. Section lengths were recorded after 16–20 hr at 26°C. No sign of section injury was noted at the inhibitor concentrations tested.

(e) Paper Chromatography

Chromatography was carried out on Whatman No. 1 paper. An ascending system, with a running length of approximately 25 cm, was used. After drying, segments of the chromatograms (usually 10) were placed directly in the assay tubes and a small volume (0·5 ml) of buffered sucrose added.

III. RESULTS

(a) Extraction and Separation

The relative amounts of inhibitor, as shown by reduced coleoptile section growth, extracted by ether at different pH values from 10-ml aliquots of willow sap are shown in the following tabulation. The inhibitor solutions obtained from the evaporated ether residues were tested without further purification:

pH	Final Section Length (mm)*	Percentage Inhibition of Extension
Control	13·3	0
7·0	12·4	11
5·5	10·4	39
4·0	9·6	50
2·0	9·8	53

* Initial section length 6·0 mm.

The amount of inhibitor extracted increased as the pH was lowered, showing the acidic nature of the substance. The inhibitor may be similarly extracted from sap by ethyl acetate and chloroform, but these solvents are less effective than ether.

The presence of the inhibitor in willow xylem sap can be shown also by directly evaporating an aliquot of sap to dryness and testing in the bioassay. When similar evaporated residues of Cape honeysuckle sap were transferred by ethanol to paper and chromatographed in isopropanol–ammonia–water (10 : 1 : 1 v/v), an inhibitor zone was located at an R_F value similar to that of the "unpurified inhibitor" obtained by ether extraction at pH 2. These results indicate that the inhibitor is present in the sap *in vivo* and is not formed by the ether-extraction procedure.

Ether extracts of willow sap chromatographed in several solvents showed inhibitor activity at the following R_F values:

	R_F
Isopropanol–ammonia (sp. gr. 0·880)–water, 10 : 1 : 1 (v/v)	0·60–0·65
Benzene–acetic acid–water, 125 : 72 : 3 (v/v)	0·95–1·00
Butanol–ammonia (sp. gr. 0·880)–water, 200 : 6 : 36 (v/v)	0·50–0·60

In these solvents the active zone runs slightly ahead of salicylic acid. Unlike the latter, the region of inhibition does not fluoresce under ultraviolet light. The inhibitor is stable to long periods of storage as a frozen solution and appears unaffected by autoclaving.

(b) Inhibitor Content of Various Saps

Figure 1 shows the response of wheat coleoptile sections to serial dilutions of willow sap extracts (unpurified inhibitor) without the addition of sucrose. There is no evidence of any stimulatory effect of the inhibitor at low concentrations as, for example, has been demonstrated for some carboxylic and tannic acids by Varga (1958).

Assays of the relative amounts of unpurified inhibitor in saps extracted from three species, apple, willow, and Cape honeysuckle, on the same day (August 1962)

are shown in Figure 2. Results indicate that there may be a considerable difference in the inhibitor content, high amounts being found in willow in particular.

Ether extracts, prepared from xylem sap collected from these three species and peach, were chromatographed in isopropanol–ammonia–water (10 : 1 : 1 v/v). Results of bioassays of these chromatograms are shown in Figure 3, *A–D*. With each extract a zone of marked inhibition is present at an R_F value between 0·5–0·8, suggesting that similar inhibitors might be present in each sap.
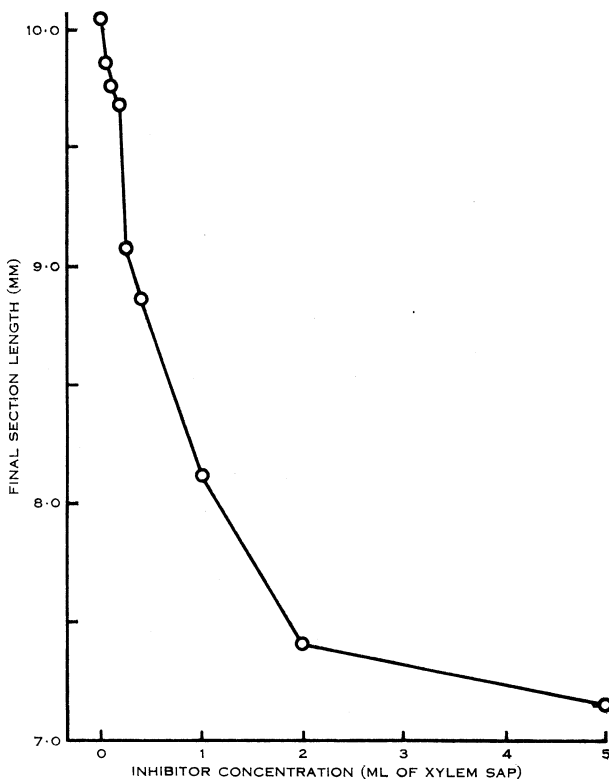

Fig. 1.—Effect of inhibitor concentration on growth of wheat
coleoptile sections.

In a further experiment, an extract of 150 dormant willow buds (brown scales removed) was made by placing the tissue in ether overnight at 4°C. This extract was shaken with sodium bicarbonate to remove the acidic fraction which was then recovered by adjusting the aqueous solution to pH 2 and reshaking with ether. Results of a bioassay of a chromatogram run with this extract are given in Figure 3,*E*. The inhibitory zone is shown at approximately the same R_F as the inhibitory zone from the willow sap extract. This would suggest that the sap and bud extracts contain similar inhibitory substances. The position of the zone of inhibition seems generally similar to that of β-inhibitor (Bennet-Clark and Kefford 1953), which has been shown to occur widely in root, stem, and leaf tissue (Hemberg 1961).

(c) Seasonal Changes

If the inhibitor was related to growth phases of a tree, there might be some change in level in the sap during the year. Seasonal changes in the inhibitor content of xylem sap from willow and Cape honeysuckle have been followed.

The willow extracts were compared in the same assay using the equivalent of 3 ml of sap for each sample. The Cape honeysuckle extracts were assayed similarly in a separate experiment using 2 ml sap. The extracts used from both species were unpurified. Results of these series are shown in Figure 4. In willow, a marked build up in inhibitor concentrations from May to June was observed, commencing soon
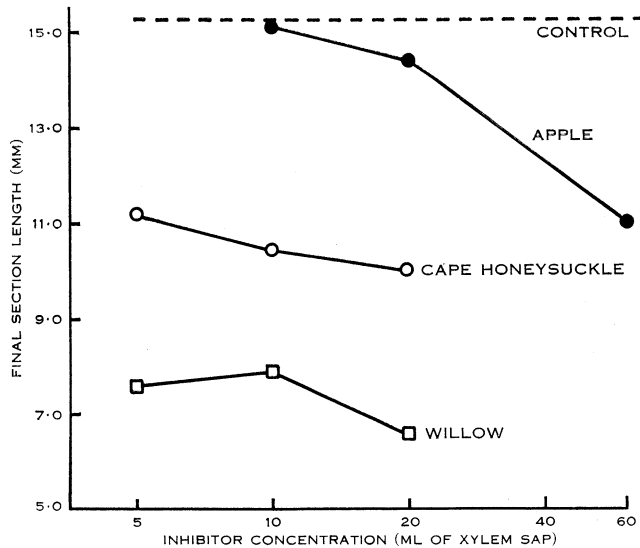

Fig. 2.—Assay of inhibitor levels in xylem sap of various species (August 1962).

after leaf fall. From other evidence the inhibitor levels attained during the winter were estimated as being at least five times those found at midsummer. High levels persisted until November–December, after which concentrations again decreased to a level approximating those of the previous January–April period. In contrast, the inhibitor level in xylem sap of Cape honeysuckle showed only relatively small irregular fluctuations throughout the year.

(d) Reversibility of Effect on Extension Growth

Wheat coleoptile sections were grown in partially purified inhibitor solutions containing sucrose (equivalent of 6 ml of willow sap) for 6 and 11 hr and then transferred to sucrose solutions. The growth curves obtained are shown in Figure 5. Each progress point on the curve was obtained by withdrawing from the experiment two tubes of the particular treatment at the time of measurement.

The sections placed in inhibitor for 6 hr showed a low growth rate for a further 5 hr after transfer to sucrose, but recovered to attain a final length at 47 hr close to that of the controls. The sections placed in inhibitor for 11 hr showed decreased growth at 24 hr but again these sections showed a considerable recovery later to reach a final length only slightly less than that of the 6-hr treatment. The action of the inhibitor is thus clearly reversible.
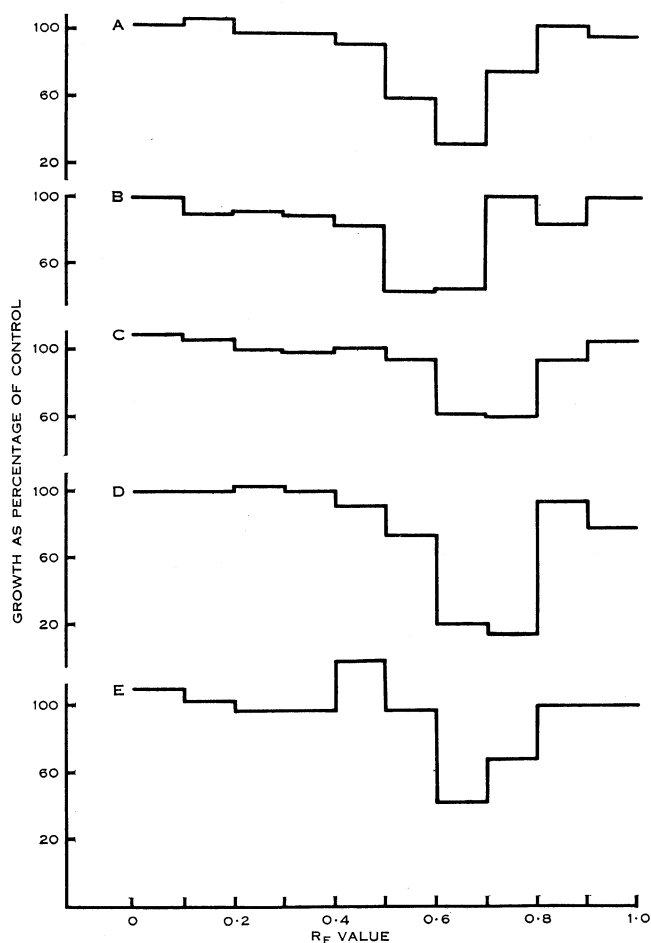

Fig. 3.—Bioassay of chromatographed ether extracts of xylem sap and buds: *A*, 30 ml Cape honeysuckle sap; *B*, 70 ml apple sap; *C*, 30 ml peach sap; *D*, 15 ml willow sap; *E*, 150 dormant willow buds.

(e) *Interaction of Inhibitor with Auxin*

The possibility of a direct interaction of the inhibitor with auxin in extension growth was tested by placing coleoptile sections in a series of concentrations of 3-indolylacetic acid (IAA) with or without partially purified inhibitor (equivalent of 3 ml willow sap). Results are shown in Figure 6. The nature of the curves indicates

that increase in concentration of IAA to 1 p.p.m., which gives close to the maximum response with these sections, does not overcome the inhibition effect. If auxin and
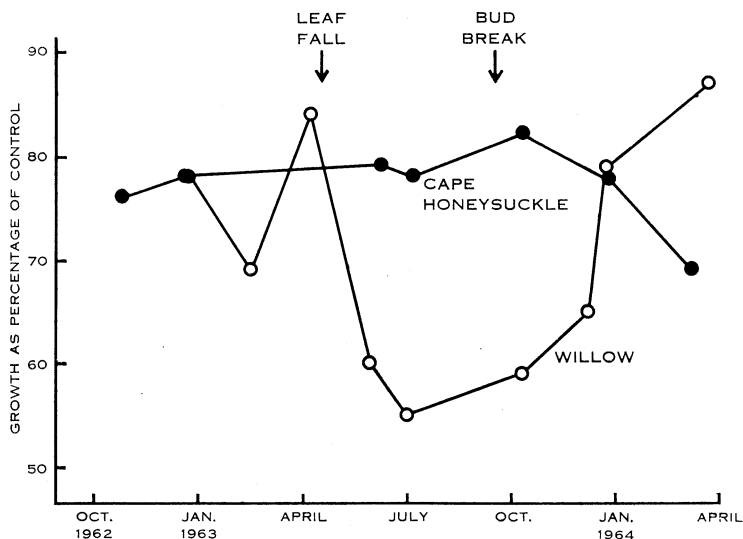

Fig. 4.—Seasonal changes in the inhibitor content of xylem sap of willow and Cape honeysuckle. The equivalent of 3 ml and 2 ml of sap, respectively, was tested at each sampling time.

inhibitor were acting competitively it would be expected that the high concentration of auxin supplied within this "physiological range" would tend to eliminate the
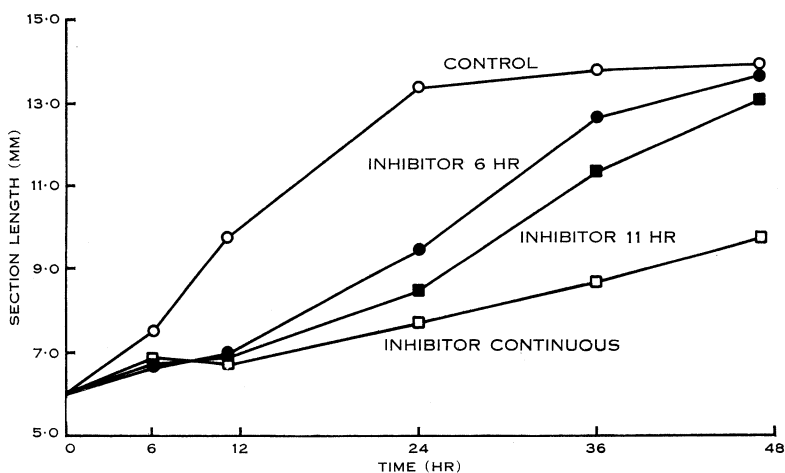

Fig. 5.—Effect on subsequent growth of transferring coleoptile sections from inhibitor solutions to sucrose after treatment for 6 and 11 hr.

inhibition. As this does not occur no competitive relationship between the inhibitor and auxin is suggested.

(f) Interaction of Inhibitor with Gibberellic Acid

The possibility of interaction with gibberellic acid was also tested with lettuce seedlings, cv. Great Lakes, the hypocotyl of which shows a small response to gibberellic acid (Frankland and Wareing 1960). For this experiment 15 selected germinated seeds of lettuce 24 hr old were transferred to 4-cm filter paper circles moistened with 1 ml of solution and contained in 5-cm petri dishes. Two dishes were used per treatment. Hypocotyl length was measured after growth for 48 hr in darkness at 26°C. Results of an experiment in which a series of gibberellic acid concentrations
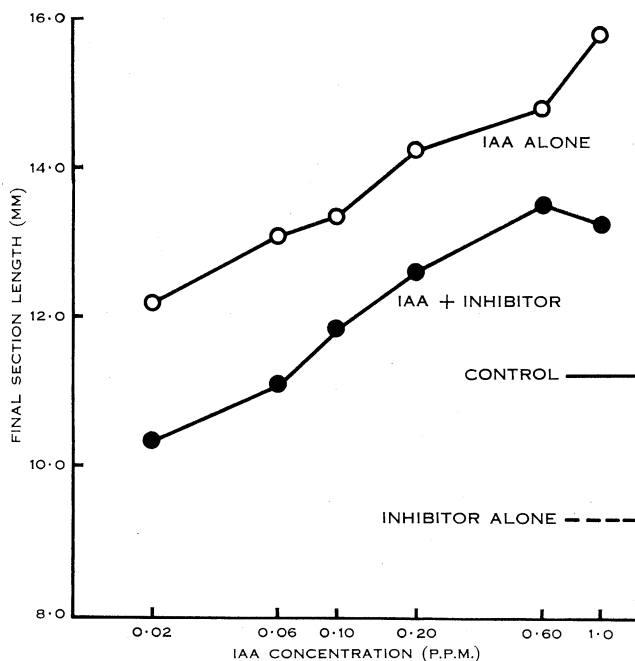

Fig. 6.—Effect of IAA alone or in combination with sap inhibitor on growth of wheat coleoptile sections. Points on the two curves are significantly different ($P < 0.05$) at all IAA concentrations.

was tested alone or with partially purified inhibitor (equivalent of 25 ml willow sap) are shown in Figure 7. Again it would be expected that if these two substances were acting competitively an increase in the concentration of gibberellic acid would tend to overcome the effect of the inhibitor. Results show that the inhibitor alone reduced markedly the final length of hypocotyls. The response to gibberellic acid is also decreased by the inhibitor but there is no evidence that the increased concentrations of gibberellic acid reduced the inhibitor effect. Again the parallel form of the two response curves does not suggest any competitive interaction between the two substances.

(g) Effect of Inhibitor on Seed Germination

The effect of the inhibitor alone or in combination with gibberellic acid on germination of lettuce seed (cv. Great Lakes) was also tested. For this experiment

40 seeds were germinated on 4-cm circles of double-thickness Whatman No. 1 filter paper moistened with 1 ml of inhibitor solution and contained in 5-cm petri dishes. Two dishes were used per treatment. The tests were carried out at 20°C in darkness, the number of germinated seeds being recorded after 24 hr. Details and results of this experiment are recorded in Table 1. Extracts of willow sap (unpurified inhibitor), the equivalent of 10 and 20 ml sap, were found to inhibit strongly the germination of seed of lettuce. Addition of gibberellic acid at concentrations of 10, 50, and 100 p.p.m. did not overcome this inhibition. Gibberellic acid alone had no effect on germination. Extracts similarly inhibited the germination of cress seeds. Further tests have shown a close correspondence between the regions of chromatograms causing inhibition of section extension and those inhibiting seed germination.
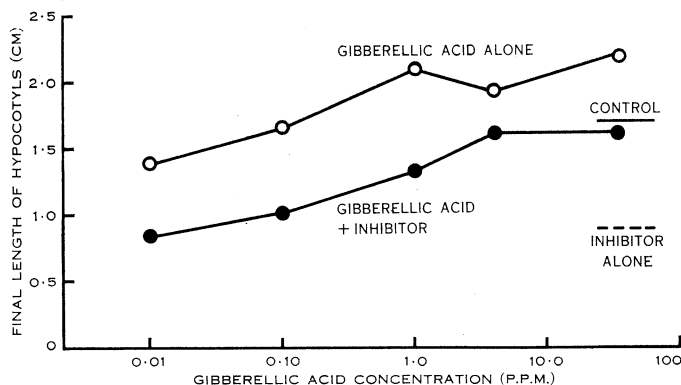

Fig. 7.—Effect of gibberellic acid alone or in combination with sap inhibitor on growth of lettuce seedling hypocotyls. Points on the two curves are significantly different $(P < 0.05)$ at all gibberellic acid concentrations.

IV. DISCUSSION

It is of considerable interest that a strongly inhibiting non-toxic substance is found in a freely transportable form in the xylem stream of woody species. Chromatographic evidence suggests that it is a single substance which occurs in the sap of the various species tested. Such a substance will undoubtedly move into the growing points and other aerial parts with the transpiration stream and might be expected to have some effect on growth processes there. The apparent similarity in R_F values of the inhibitory regions of chromatograms from bud extracts and sap extracts could be taken as evidence that the sap inhibitor does indeed find its way into these organs.

Investigations of the possible action of the inhibitor have shown it is strongly active in a variety of growth tests including germination. Reversibility of inhibition might be considered a prerequisite for any regulatory function of an inhibitor in growth processes in a plant. This certainly has been demonstrated for the sap extract in the section-elongation experiments. Similar reversibility has been described for other inhibitors (Barlow, Hancock, and Lacey 1955; Van Steveninck 1959). The experiments to test the interaction between the sap inhibitor and either IAA or

gibberellic acid in specific elongation tests have not demonstrated any competitive relationship with these regulators. With both the coleoptile and hypocotyl tests the inhibitor lowers the response of the organ at all concentrations of IAA or gibberellic acid, but there is no indication that increase in concentration of either of the latter substances overcomes the inhibition. Thus the inhibitor and these promoters are in all probability acting at different points in the growth chain. Gibberellic acid will not apparently overcome the inhibition of seed germination caused by sap extracts. Phillips (1962) found that a natural inhibitor, naringenin, while responding similarly to the sap inhibitor in such elongation tests with auxin and gibberellic acid showed an apparent competitive effect on seed germination.

TABLE 1

EFFECT OF WILLOW SAP INHIBITOR ALONE AND IN COMBINATION WITH
GIBBERELLIC ACID ON GERMINATION OF LETTUCE SEED (CV. GREAT
LAKES)

Extract equivalent to 10 ml sap used as source of inhibitor in all
cases except one, when extract equivalent to 20 ml used

Additions	Germination after 24 hr (%)
Inhibitor alone ($\equiv$ 10 ml sap)	53
Inhibitor alone ($\equiv$ 20 ml sap)	3
Inhibitor plus:	
Gibberellic acid, 10 p.p.m.	58
Gibberellic acid, 50 p.p.m.	53
Gibberellic acid, 100 p.p.m.	55
Gibberellic acid alone:	
10 p.p.m.	83
50 p.p.m.	93
100 p.p.m.	90
Control	93

The contrast in pattern of seasonal change of inhibitor level in xylem sap between the two species tested, willow and Cape honeysuckle, is notable. It is possible that the lack of any pronounced seasonal variation in inhibitor levels in sap of the Cape honeysuckle may be related to the fact that this plant does not appear to have any deep rest period and therefore never becomes truly dormant in the present situation.

In willow, however, the inhibitor levels rose sharply with the onset of the deepest phase of dormancy (after leaf fall) and were highest throughout the dormant winter period. This trend suggests that the sap inhibitor could play some role in dormancy control. High levels are still present, however, during bud break and through until November–December, by which time trees will have made a considerable amount of shoot growth. If the sap inhibitor was to play a part in dormancy control then these high levels, which it is assumed move upward to the buds, must be directly dispersed there or have their effects offset in some manner at bud break. It is noteworthy that Hendershott and Bailey (1955), Blommaert (1955), and Hemberg

(1949) have all shown a build up of bud-inhibitor levels during the rest period, but their data also showed a decrease just before bud break.

Recent results (Phillips 1962; Eagles and Wareing 1963) seem to implicate gibberellic acid in the process of dormancy breaking of tree buds. This had led the latter authors to suggest that dormancy may be regulated by a gibberellin–inhibitor balance. There is the possibility that high bud-inhibitor levels arising from translocated inhibitor could be overcome by increased gibberellin levels which have been shown to occur as a response to chilling (Smith and Rappaport 1961). The present experiments, however, have not shown any direct interaction between gibberellic acid and the translocated inhibitor either in the hypocotyl test or in a seed germination test.

The chemical nature of the inhibitor is unknown. Its general chromatographic behaviour in several solvents suggests a similarity with the β-inhibitor complex extracted from a variety of plants. Some workers (Varga 1957; Bentley 1958) have identified a number of separate substances in this region of chromatograms, whereas others have found that, at least in some species, only a single inhibitor substance may be involved (Robinson, Wareing, and Thomas 1963; Lane and Bailey 1964). Chromatographic evidence available from other experiments with the sap inhibitor suggests that it is probably a single substance giving the inhibition. It is important to re-emphasize in this respect that the extract as removed from the shoot by suction is a homogeneous clean one, compared with that obtained from macerated or chopped tissues. Therefore it would not contain additional substances arising as a result of tissue maceration coupled with the extended leaching period often used for such experiments.

V. Acknowledgment

VI. References

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