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STUDIES ON PHLOEM EXUDATION FROM YUCCA FLACCIDA HAW. XIV. METABOLISM OF 8-^{14}C-ZEATIN IN AN EXCISED INFLORESCENSE STALK, IN PHLOEM EXUDATE AND IN FLOWER SAP

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SUMMARY

The metabolism of zeatin-8-^{14}C in detached inflorescence stalks of *Yucca flaccida* has been studied. The compound has been fed either through the xylem or has been added directly to phloem exudate.

The results strongly indicate that after uptake by the stalk zeatin is converted into the corresponding mono-, di- and tri-phosphate ribonucleotides, whereas incubation of phloem sap with zeatin only resulted in the formation of the mono-ribonucleotide.

1. INTRODUCTION

There is considerable evidence for the synthesis of cytokinins in the roots and their translocation to above parts of plants (KENDE 1965). Presumably they are translocated through the xylem. In recent years the occurrence of cytokinin active compounds in phloem exudate from a variety of plants has also been established (HALL & BAKER 1972, PHILIPS & CLELAND 1972, HOAD 1973, VONK 1974).

One of the gaps in our knowledge on the fate of cytokinins in whole plant systems is the possible participation of the shoot tissue in the conversion of cytokinins during transport. SONDHEIMER & TZOU (1971) reported the possible formation of mono-, di- and tri-nucleotides in bean axes with 8-^{14}C-zeatin.

PARKER & LETHAM (1973), studying ^{3}H-zeatin metabolism in excised radish seedlings found zeatin-5′-monophosphate as a second dominant metabolite in hypocotyls and petioles. These authors suggested cytokinin nucleotide formation by lateral movement of zeatin from the transpiration stream into the cotyledons and transport in basipetal direction in the phloem of hypocotyls.

Recently, formation of N^6-(Δ^2-isopentenyl) adenosine and N^6-benzyl adenine nucleoside-5′-triphosphates was described for isolated tobacco and *Acer* cells (LALOUE, TERRINE & GAWER 1974).

In a previous paper the detached inflorescences of *Yucca* were used to study the metabolic properties leading to the formation of products found in the phloem exudate e.g. sugars (TAMMES, VONK & VAN DIE 1967). The main cytokinin activity which was found in the phloem exudate of this monocotyledon appeared to be due to a nucleotide (VONK 1974).

The present paper reports the formation of ^{14}C-labelled nucleotides after feeding 8-^{14}C-zeatin to a detached inflorescence. Evidence will also be given for de conversion of ^{14}C-zeatin into its corresponding mono-ribonucleotide when applied directly to phloem exudate collected from an untreated attached inflorescence. Finally the breakdown of zeatin ribonucleotide into zeatin riboside by press-sap from flower parts will be described.

2. MATERIALS AND METHODS

2.1 Application of radio activity

Phloem exudate of young detached inflorescences has been collected as previously described in detail (VAN DIE & TAMMES 1966; TAMMES, VONK & VAN DIE 1967). Stalks, 20 cm long freshly harvested were placed with their basal end in a small beaker containing 2.0 ml 1% sucrose solution and 4 μCi 8-^{14}C-zeatin.

The exudate was collected at different time intervals and 10 μl samples used for radioactivity measurement. The volume between each interval was determined by weight. The remaining exudate and stalk at the end of the experiment were stored at −20°C until analysis.

2.2 Paper- and thin layer chromatography

Total exudate (2.1) was chromatographed on Whatman no 3 paper using butanol: conc. ammonia: water (6:1:2 v/v/v), upper phase as a solvent (solvent A). The chromatograms were divided equally in 1 cm strips and radioactivity was measured (2.8).

Nucleotides form the exudate were separated from each other in two different ways: A) By means of two dimensional T.L.C. on cellulose MN 300 20 × 20 cm plates without binder (Magery & Nagel). Plates and solvents were prepared as described by FEIGE et al. (1969). B) On DEAE cellulose exchanger 5 × 20 cm layers 0.25 mm thick. An aqueous solution of NaCl (0.15 M) was used as a solvent. From the resulting chromatograms autoradiograms were made on "Kodak-no-screen' X-ray films. Exposure time was at least one week.

Zeatin and zeatin riboside were separated from each other on Polychram Sil-N-HR/UV 254 plates. Non-radioactive zeatin, zeatin riboside and inosine were spotted on the plates, after which the radioactive samples were spotted on the same place. After running the chromatograms in ethanol:water (8:2 v/v), spots detected by U.V. absorption were scraped from the plates and prepared for counting of the radioactivity.

2.3 Paper electrophoresis

Paper electrophoresis was carried out in a 0.05 M Tris-citrate buffer pH 3.5 on Whatman 3 MM paper, 25V cm^{-1} at 4°C for 3 hours.

2.4 Column chromatography

To separate zeatin nucleotides from free base zeatin and zeatin nucleoside, phloem exudate was acidified to pH 3.5 with 0.01 N HCl and run over a cellulose

phosphate cation exchanger column (Sigma 0.99 meq. gram^{-1}) 23 cm length and 1.5 cm in inner diameter (NH_4^+ form). The column was equilibrated to pH 3.5 with 0.02 N HCl before use (WILSON et al. 1974). After introduction of the active sample, the column was washed with acidified water pH 3.5 and the effluent collected in a bottle surrounded with melting ice. This fraction, containing nucleotides which were not retained by the column, was freeze dried, whereas zeatin and zeatin nucleoside were retained by the column. The whole procedure was carried out in a cold room at 4°C.

2.5 Incubation of sap from flower parts with a nucleotide cytokinin fraction

Five grams fresh weight of *Yucca* flowers were stripped of petals and frozen at −20°C for five minutes. The frozen material was inserted in a pre-cooled press-tube of a French pressure cell press and pressed at 840 Bar. The sap was centrifuged at 0°C at 1000 × g for 10 minutes and 1.0 ml added to a purified nucleotide fraction from a cellulose phosphate column (2.4). The mixture was incubated for half an hour at 35°C. As a control, 1.0 ml of the nucleotide fraction, diluted with 1.0 ml water, was treated in the same way.

Chromatography of the incubated mixture was carried out on Whatman 3 paper as described (see section 2.2).

2.6 Incubation of phloem exudate with ^{14}C-zeatin

The high molecular weight fraction of 10 ml *Yucca* exudate collected as described by VAN DIE & TAMMES (1975–1976), stored for one month at −20°C, was obtained after filtration through a Sephadex G-25 column (41 × 2,5 cm) in 0.01 M Tris-buffer pH 8.2 at 4°C. The combined 280 nm absorbing material was freeze dried, taken up in another 6.0 ml exudate and divided in three portions. One ml was kept in a boiling water bath; 2.5 ml was stored at 0°C (melting ice) and the remaining 2.5 ml was kept at room temperature. After 4 hours ^{14}C-zeatin (0.063 mCi) was added to these samples. Actinomycin, a bacteriostatic agent, was given in a final concentration of 10 mg litre^{-1} to an aliquot of one ml of the sample stored at room temperature. The remaining non-treated 1.5 ml was used as a control. The different vials were incubated at 35°C, at intervals of 1, 4, 9 and 20 hours. Portions of 0.1 ml were withdrawn and chromatographed on paper (2.2). The reaction of the remaining solutions was stopped by adding 1.0 ml methanol and the vials stored immediately at −20°C until electrophoresis.

2.7 Enzymes

The hydrolysis of nucleotides was essentially carried out as described bij VONK (1974) but with a 12 hours incubation with alkaline phosphatase at 37°C.

Apyrase treatment (Sigma grade I) of the nucleotides was carried out according to LALOUE, TERRINE & GAWER (1974). Radio-active *Yucca* exudate (0.5 ml) was adjusted to pH 6.4 with 0.2 M Tris-maleate buffer pH 5.8. Total activity

of the solution at zero time was 26000 dpm ml^{-1}. Samples of 50 µl were taken at different time intervals (*table 2*) and electrophoresized (2.3).

2.8 Counting

Paper-chromatograms and thin layer plates were cut in segments of 1 cm and 0.5 cm respectively and the radio-activity was counted in a Mark II scintillation spectrometer (scintillation fluid on Dioxane Butyl P.B.D. basis).

2.9 Radioactive zeatin

Radioactive 8-^{14}C-zeatin has been purchased from and synthesized by Dr. R. Horgan, Dept. of Botany and Microbiology, University College of Wales, Penglais, Aberystwyth, U.K. Radio purity was established by chromatographing a sample on paper (n-butanol: NH_4OH 4:1 v/v) and silicagel thin layer plates (chloroform: methanol 9:1 v/v or ethylacetate: n-propanol: water 4:1:2 v/v/v).

The sample contained 95% zeatin. Specific activity was 11.7 mCi $mMol^{-1}$.

3. RESULTS

3.1 Uptake of ^{14}C-zeatin by a detached inflorescence

In a previous paper it has been shown that detached inflorescence stalks of *Yucca* can be fed at the basal end with labelled substances. The label can be recovered in the phloem exudate of the stalks (TAMMES, VONK & VAN DIE 1967). *Table 1* shows the occurrence of radioactive compounds in the phloem exudate of a detached inflorescence stalk at different time intervals after application of ^{14}C-zeatin. Three hours after the start of the uptake experiment, the first radioactive sample could be collected. The concentration of radioactivity increased during a total period of nine hours. Accumulation in the phloem exudate was not observed, as the initial concentration of 4.44×10^3 dpm μl^{-1} was never reached. As will be shown in the next paragraph the main active compounds appeared to be nucleotides of zeatin.

3.2 Identification of the radioactive products

To determine the nature of the radioactive phloem exudate compounds collected, the exudate was subjected to paper chromatography. The conversion of ^{14}C-zeatin, given to the basal end of the inflorescence stalk, into compounds with a low Rf value is demonstrated in *fig. 1 A* and *B*, respectively. Since nucleotides do not move appreciably during paper chromatography these results indicated the presence of one or more nucleotides.

All samples collected at the different time intervals (*table 1*) were analysed in the same way by paper chromatography. Radioactivity found at the Rf value of nucleotides accounted for ninety-six to ninety-eight percent of the total activity in the exudate.

To separate the nucleotide fraction from nucleoside or free base cytokinins, total exudate was purified by means of a cellulose cation exchanger column

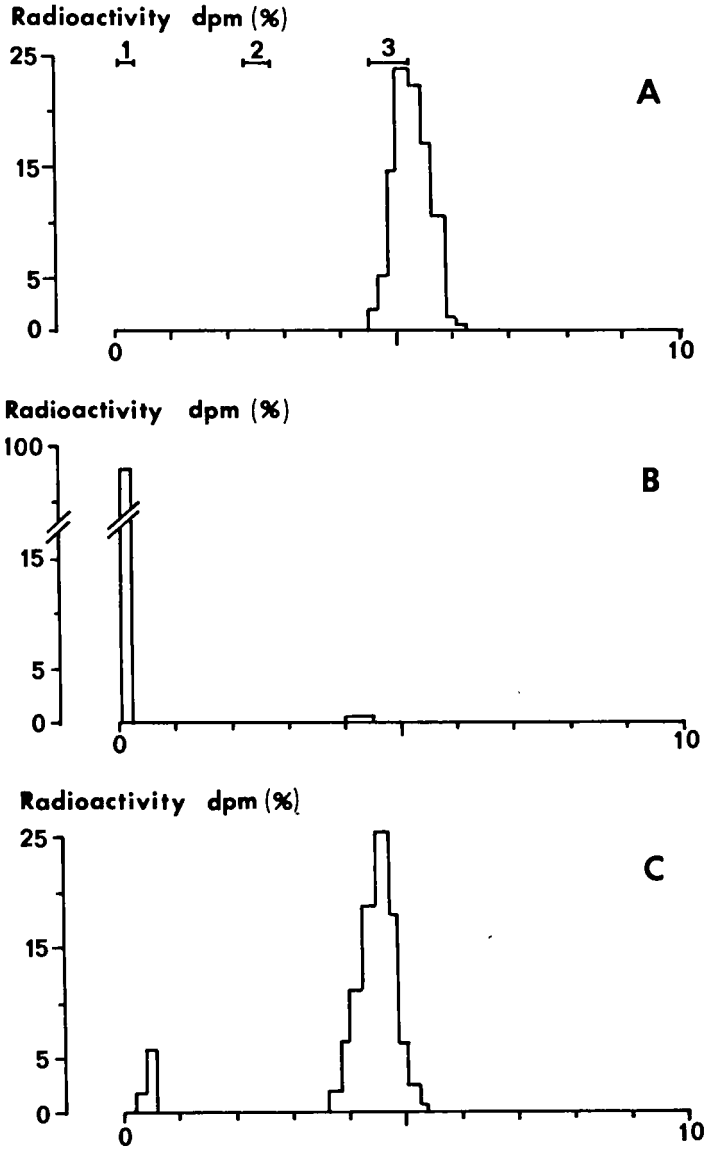

Fig. 1. A–C. Histogram of radioactive compounds after running phloem exudate of a detached *Yucca* inflorescence stalk together with non-radioactively marker compounds on Whatman 3 paper (solvent A). A. ^{14}C-zeatin + non-radioactive exudate. B. Exudate after uptake of ^{14}C-zeatin by a detached stalk. C. As B, but the exudate was purified first on a cellulose phosphate exchanger column, treated with alkaline phosphatase, and subsequently chromatographed.
The marker compounds were: AMP (1), adenine and adenosine (2), zeatin and zeatin riboside (3).

Table 1. Time course experiment of phloem exudate collected from a detached inflorescence stalk of *Yucca* after feeding 4 μCi (72 μg) 8-^{14}C zeatin to the basal end.

time (min.) after application of 8-^{14}C-zeatin	volume (mg) collected	d.p.m. per 10 μlitre
0–180	660	330
180–330	474	1740
330–420	244	4380
420–540	106	5702

(2.4). The negatively charged eluant from the column was treated with alkaline phosphatase and an aliquot chromatographed on paper. Ninety percent of radioactivity was found at a spot with a Rf equal to that of free base zeatin or zeatin riboside (*fig. 1* C).

It is, however, known that nucleotides such as ATP, ADP and AMP, when subjected to an alkaline solution at pH 8.2 at 35°C, are converted to inosine (Vonk, unpublished results). This observation made it appropriate to investigate in more detail the alkaline phosphatase treated fraction from the column.

To investigate the possible occurrence of inosine, an aliquot of the alkaline phosphatase treated nucleotide fraction was co-chromatographed on Polychram plates in the solvent ethyl alcohol: water (8:2 v/v). The Rf values of zeatin, zeatin nucleoside and inosine were 0.71; 0.80 and 0.88 respectively (*fig. 2*).

Moreover the formation of inosine could be ruled out because this compound appeared to be inactive in the *Amaranthus* bio-assay when tested in a concentration range of 10^{-8} till 5×10^{-6} M, whereas alkaline phoasphatase treated phloem exudate reacted strongly positive (VONK 1974).

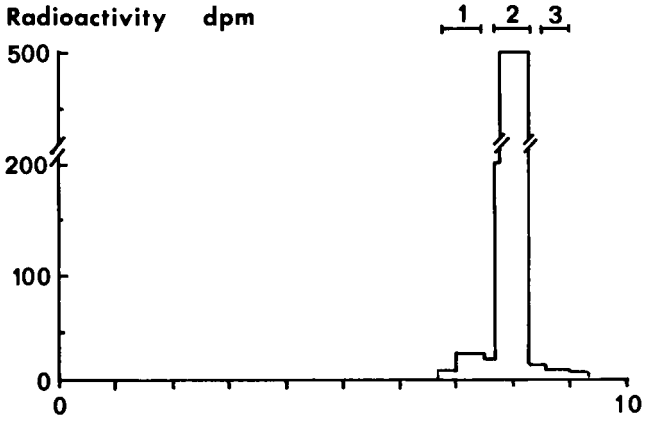

Fig. 2. Histogram of a radioactive nucleotide containing fraction treated with alkaline phosphatase, after T.L.C. on Polygram plates. The marker compounds were: zeatin (1), zeatin riboside (2) and inosine (3).

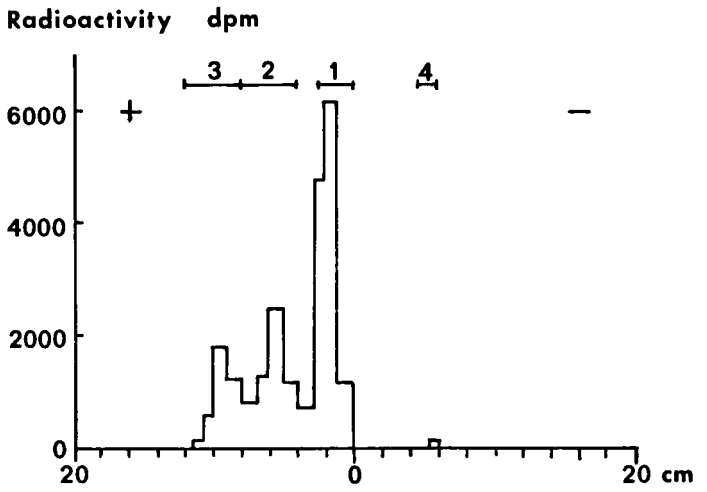

Fig. 3. Electrophoretic separation of radioactive nucleotides of exudate obtained from a detached *Yucca* inflorescence stalk, after application of 8-^{14}C zeatin. The marker compounds were: AMP (1), ADP (2), ATP (3) and zeatin riboside (4).

These results indicate the formation of zeatin riboside, after treatment of the radioactive nucleotide fraction with alkaline phosphatase. A notable observation is that a zeatin nucleotide fraction may contain a number of nucleotides, such as the 5′-mono, di- and triphosphates. In all cases zeatin-riboside will be found after alkaline phosphatase treatment of the non-homogenous nucleotide fraction.

Running samples of the active phloem exudate on cellulose thin layer plates and DEAE cellulose layers in different solvents (2.2) in both cases gave three different spots visible after exposure to "Kodak no screen" X-ray film. The electrophoretic mobilities (2.3) were found to be equal to nucleotide 5′-mono-, di- and triphosphate, respectively (*fig. 3*). As nucleotides of zeatin are not commercially available, AMP, ADP and ATP were used as markers. From the above mentioned separation procedures the composition of the nucleotide fraction in the phloem exudate could be determined. The following composition was found; nucleotide mono-phosphate 57%, nucleotide diphosphate 26% and nucleotide tri-phosphate 17%. The ratio 97% nucleotide (anode) and 3% nucleoside (cathode) of the activity recovered from the electrophoretic strips, was in good agreement with the ratio nucleotide, nucleoside found after paper chromatography of the different fractions collected.

Additional evidence on the occurrence of tri- and di-phosphate nucleotides was obtained by the action of apyrase on the exudate. This unspecific enzyme degrades nucleotide triphosphates into monophosphates with diphosphates as an intermediate. A rapid decrease within ten minutes of the tri-phosphate nucleotide, was observed after the incubation of the sample began (*table 2*).

Table 2. Apyrase action on the nucleotides from *Yucca* exudate obtained after application of 8-^{14}C zeatin for different periods (T) to a detached inflorescence stalk. Figures represent per cent radioactivity after electrophoretic separation.

Incubation time (min)	T_0	T_{300} (without enzyme)	T_{10}	T_{40}	T_{80} (with enzyme)	T_{160}	T_{300}
Nucleotide mono-phosphate	79.3	78.0	79.9	79.0	86.0	88.4	90.4
Nucleotide di-phosphate	13.5	16.6	18.0	21.0	14.0	11.6	9.6
Nucleotide tri-phosphate	7.2	5.4	2.3	0.0	0.0	0.0	0.0

The decrease of nucleotide diphosphate also occurred, by the action of ADP-ase activity in this emzyme mixture. A lower initial percentage di- and tri-phosphate was found as compared with the results depicted in *fig. 3*. This is probably due to a decrease in the pH (see 2.7) of the exudate.

3.3 Decomposition of nucleotides by pressed flower-parts

One of the important questions arising from the presence of nucleotide cytokinins in phloem sap is whether the flower parts (ovary, stamen and stigma) contain enzymes able to convert the nucleotides into nucleosides or free base cytokinins. To answer this question the press sap of the flower parts was added to the nucleotide fraction (2.5).

Three different radioactive peaks were obtained after incubation of the press-sap for half an hour (*fig. 4*). The results of the blanc were similar to those in *fig. 1* B. After elution with water, peak no 1 (25%) could be converted into

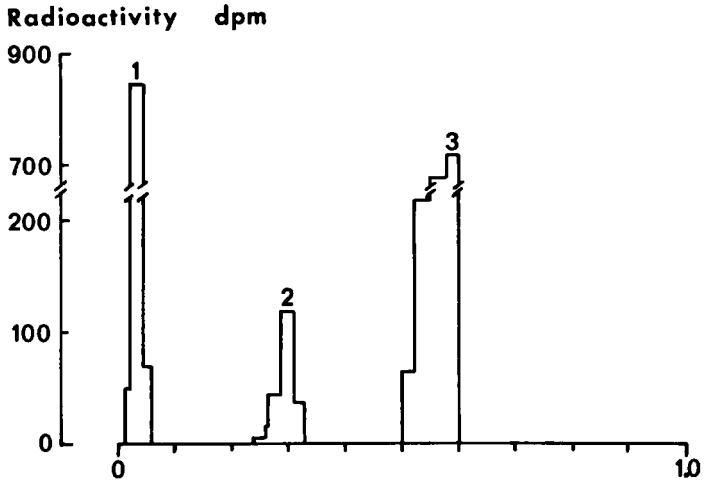

Fig. 4. Histogram of the radioactive nucleotide fraction treated with sap pressed from flower parts after paper chromatography (solvent A). The marker compounds were: AMP (1), adenine and adenosine (2), zeatin and zeatin riboside (3).

peak no 3 after treatment with alkaline phosphatase, indicating that most probably an incubation time of half an hour was too short to get a complete degradation of the nucleotide fraction.

The nature of peak no 2 (8% of the total radioactivity) was not investigated further, while the Rf value of peak no 3 co-chromatographed with zeatin riboside on Polychram plates (2.2). Peak no 3 accounted for 67% of the total radioactivity.

From the results it was concluded that the ovary, stamens and stigma of *Yucca* contain enzymes able to convert cytokinin nucleotides in cytokinin nucleosides.

3.4 Conversion of ^{14}C-zeatin into zeatin nucleotides by phloem exudate

According to BECKER, KLUGE & ZIEGLER (1971), ATP is a major metabolite in phloem exudate of *Yucca* and *Tilia platyphyllos* after incubation of the sieve tube with $^{32}PO_4^{3-}$. For this reason it was investigated whether *Yucca* sap also contains enzymes to convert ^{14}C-zeatin into zeatin nucleotides. Since the total protein fraction of phloem exudate is low, (TAMMES & VAN DIE 1964) the

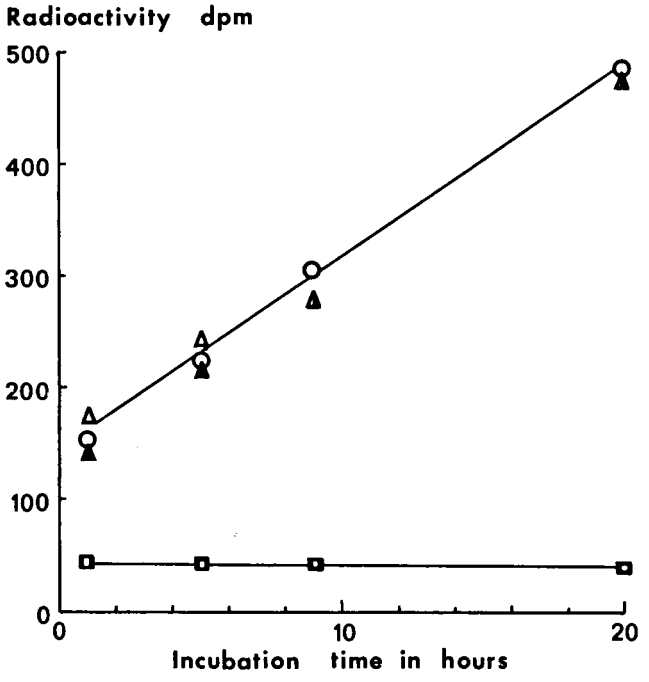

Fig. 5. Nucleotide formation in *Yucca* exudate after incubation of 8-^{14}C zeatin. Treatment: boiled (□—□), four hours at 0 °C before incubation (○—○), four hours room temperature before incubation (△—△) and four hours room temperature before incubation + actinomycin (▲—▲).

protein fraction was first concentrated on a Sephadex G-25 column (2.6). Another difficulty is that although the exudate obtained was relatively pure, bacterial contamination could not be excluded. Therefore the concentrated sap was treated in three different ways (0 °C, room temperature, and room temperature + actinomycin) before ^{14}C-zeatin was added (see 2.6). If any contamination had taken place a faster conversion in the sample stored at room temperature could be expected, as compared with that stored at 0 °C and that into which actinomycin was added before incubation. Activity found on nucleotide Rf after paper chromatography at different time intervals is demonstrated (*fig. 5*). The results obtained showed that the nucleotide activity found in the samples was not due to bacterial contamination but to enzyme activity. This view is supported by the fact that no nucleotide activity was found in the boiled sample. However, data derived from electrophoretic mobilities showed that only the mono-phosphate nucleotide was formed by the exudate (data not given), moreover only 6% ^{14}C-zeatin was converted into this nucleotide. The remaining non-nucleotide activity, appeared to be zeatin free base as detected on Polychram plates (2.2).

4. DISCUSSION

In a previous paper it has been shown that radioactive compounds which enter the open xylem vessels of a detached inflorescence stalk of *Yucca* can be translocated to the adjacent phloem (VAN DIE & TAMMES 1966; TAMMES, VONK & VAN DIE 1967).

After feeding of ^{14}C-zeatin to the basal end of the stalk it has been shown that the phloem exudate collected from a detached inflorescence contains nucleotides of zeatin. Overall, the results indicate nearly a complete conversion of ^{14}C-zeatin into mono-, di- and triphosphate nucleotides.

These data are in good agreement with earlier results that cytokinin activity in phloem exudate of whole *Yucca* plants was mainly nucleotide bound (VONK 1974).

The presence of these compounds was suggested to occur in a bean axis by SONDHEIMER & TZOU (1971). The occurrence of the mono-, di- and tri-nucleotides in tobacco and *Acer* cells has been reported by LALOUE, TERRINE & GAWER (1974).

According to KENDE (1965), SITTON et al. (1967), YOSHIDA et al. (1971), the root tips are the main site of free base- and riboside cytokinin production.

The present results strongly indicate a lateral movement of these compounds from the xylem vessels into the sieve tubes, as suggested by PARKER & LETHAM (1973). The results also show that the nucleotide cytokinins can be formed in the inflorescence axis of *Yucca flaccida* and that enzymes are present in phloem exudate which are capable of converting ^{14}C-zeatin into the nucleotide monophosphate. Apparently the presence of leaves is not required for these processes as leaves were absent in these experiments.

It seems surprising that only the monophosphate nucleotide was found after

incubation of phloem exudate with ^{14}C-zeatin, whereas application of ^{14}C-zeatin to a detached inflorescence resulted in phloem exudate containing the mono-, di- and tri-nucleotides. These differences may be attributed to the fact that the exudate used for incubation was not freshly prepared but stored for one month at -20 °C before use; hence enzyme inactivation could have taken place. Alternatively, there is the possibility of the occurrence of different enzymes within the xylem/phloem parenchyma cells and within the sieve tubes themselves (the exudate).

Free base and/or riboside cytokinins are mainly found in young growing parts (VAN STADEN & WAREING 1972). These authors also suggested that nucleotide is the main form in which cytokinins are translocated and/or stored within the plant.

In this respect it is worth mentioning that young fruits of *Cocos nucifera* and *Phoenix dactylifera* may be regarded as being fed through the mobile aqueous phase of the sieve tube system (VAN DIE 1974). The occurrence of cytokinin-like substances in the endosperm of *Cocos* fruits and coconut water is well known (NITSCH 1970, VAN STADEN & DREWES 1975). Nucleotide cytokinin has not been isolated from *Cocos* endosperm so far.

The experiments presented show that flower parts of *Yucca* possessed enzymes which degrade the cytokinin nucleotides to zeatin riboside.

The physiological implications of nucleotide formation in the inflorescence, and the breakdown into zeatin riboside by the flower parts, are not yet known. It may be speculated, however, that these compounds play a role in processes leading to flowering and/or fruiting. The effect of cytokinins on the accelaration of flowering has been demonstrated (TEPFER, KARPOF & GREYSON 1966; MAHESWARI & VENKATARAMAN 1966). Recently TSE et al. (1974) showed that removal of young leaves and application of the cytokinin N-benzyl-α-(tetrahydro-2H-pyran-2-yl) adenine promoted inflorescence development in *Bougainvillea*. Cytokinins also play a role in flowering of *Iris*.

Forcing of *Iris* cv Wedgwood under low light intensity and short days can easily result in flower bud abortion (bud blast). Bud blast could be overcome by injecting cytokinins into the flower bud of dark treated *Iris* plants (MAE & VONK 1974). As light is beneficial to phloem transport in these plants, it is possible that the translocation of cytokinins through the phloem stream is also affected by light treatment.

These studies emphasize the need for further investigations on the metabolism of cytokinins and their possible role in the flowering process.

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