Acta Bot. Neerl. 23 (5)–(6), October–December 1974, p. 739–746.

SIMILAR EFFECTS OF CYTOKININS AND CARBONYL CYANIDE m-CHLOROPHENYLHYDRAZONE (CCCP) ON AMINO ACID TRANSLOCATION IN LEAVES OF SAGITTARIA GRAMINEA MICHX.

E. SCHENK

Botanisch laboratorium, Utrecht

SUMMARY

Kinetin, zeatin or zeatin riboside applied to parts of excised leaves of *Sagittaria graminea* change the distance profile of ^{14}C-α-amino-iso-butyric acid translocation in these leaves. They probably enhance the rate of accumulation of the basipetally translocated amino acid. The metabolic inhibitor carbonyl cyanide m-chlorophenylhydrazone (CCCP) appeared to act in a very similar way on amino acid translocation; it also caused chlorophyll retention.
Some possible cytokinin-CCCP relations are briefly discussed.

1. INTRODUCTION

Cytokinins are known to cause a great number of growth effects in plants. Together with some other plant hormones they appear to play important parts in the regulation of RNA, DNA and protein synthesis, the regulation of physiological effects in respect with morphogenesis, the retention of chlorophyll in detached leaves and the accumulation of amino acids. Their molecular mode of action, however, is still largely unknown.

From studies on the influence of kinetin in tobacco leaves, MOTHES et al. (1961) have reported that the increased accumulation of the "non-protein amino acid" α-amino-iso-butyric acid (AIB) in the presence of cytokinins was an evidence for a direct effect on accumulation. SKOOG & ARMSTRONG (1970) have suggested another explanation for this observation: cytokinins might have an effect on the synthesis of proteins that are involved in uptake and accumulation. BRÄUTIGAM & MÜLLER (1973) have proposed, from their experiments with *Vallisneria* leaves, that kinetin alters the source-sink relationship rather than that it acts directly on the translocation mechanism. In their studies on the senescence of detached leaves, which may be understood as transport processes in the view of redistribution of organic and inorganic materials, SHIBAOKA & THIMANN (1970) have found evidence that the primary action of kinetin is the inhibition of proteolysis, rather than the promotion of protein synthesis.

In the present paper, the biological activity of 6-furfurylaminopurine (kinetin) and the natural cytokinin, 6-(4-hydroxy-3-methyl-trans-2-butenylamino) purine (zeatin) and its riboside is tested on the uptake and translocation of AIB in leaf strips of *Sagittaria graminea*. These effects will be compared with similar,

but unexpected effects of carbonyl cyanide m-chlorophenylhydrazone (CCCP), generally known as an effective uncoupler of oxidative phosphorylation and also as an inhibitor of photophosphorylation ((HEYTLER 1963; KIMIMURA et al. 1971).

2. MATERIAL AND METHODS

The experiments were carried out with leaf strips of *Sagittaria graminea* Michx., cut to a lenght of about 18 cm. The leaf strips were placed in distilled water for about four hours. In previous experiments (SCHENK 1972) the leaves were "aged" in distilled water for 18 hours, or in 10^{-4} M $CaSO_4$ (according to EPSTEIN 1961, to maintain intact membranes with normal permeability). Since "aged" and freshly cut leaves have remarkably similar translocation profiles, only the last ones were used. The leaves were placed in a flat perspex box on a layer of wet "kleenex" tissue. The use of wet "kleenex" has certain advantages with the application of the chemicals to the leaves. Using agar, the drug has to be dissolved in the molten agar just before it coagulates at about 50 °C, a temperature that may be harmful to the chemical structure of the drug.

The experiments were carried out in series of three leaf strips. The leaf strips were divided into three zones by means of "kleenex" segments separated from each other by a small ring of paraffin, thus preventing them to come into contact with each other. When using drugs, the respective zone was moistened with that drug. A small agar block, containing the radioactive amino acid (AIB) was placed on the tip of each leaf strip. After 48 hours the agar blocks were removed and the leaf strips were freeze dried. Since the experiments were carried out in triplicate, the three leaf strips were scanned together. In a previous paper (SCHENK 1972) more details and advantages of this method have been described.

All experiments took place in light.

Kinetin, zeatin and zeatin riboside were dissolved in dimethylsulphoxide (DMSO) and stored at 4 °C. When used, fresh preparations were made to a final concentration of 1 or 2 mg/l in 0.1 % DMSO. Zeatin and zeatin riboside were obtained from Calbiochem. CCCP was dissolved in a small amount of 95% ethanol. The final concentration was prepared immediately before use because CCCP is hydrolyzed in aqueous solutions fairly rapidly according to LEBLANC Jr. (1971).

CCCP was obtained from Calbiochem and the labelled AIB from the Radiochemical Centre Amersham.

3. RESULTS

3.1. The effect of kinetin on AIB translocation

When AIB is applied to the tip of a *Sagittaria* leaf strip, there is a distinct basipetal transport with an enhanced accumulation at the basal end (SCHENK 1972). In order to study the effects of cytokinins in a more quantitative manner,

the leaf strips of the present experiments were divided into three zones: (I) an apical part, (II) a middle part and (III) a basal part.

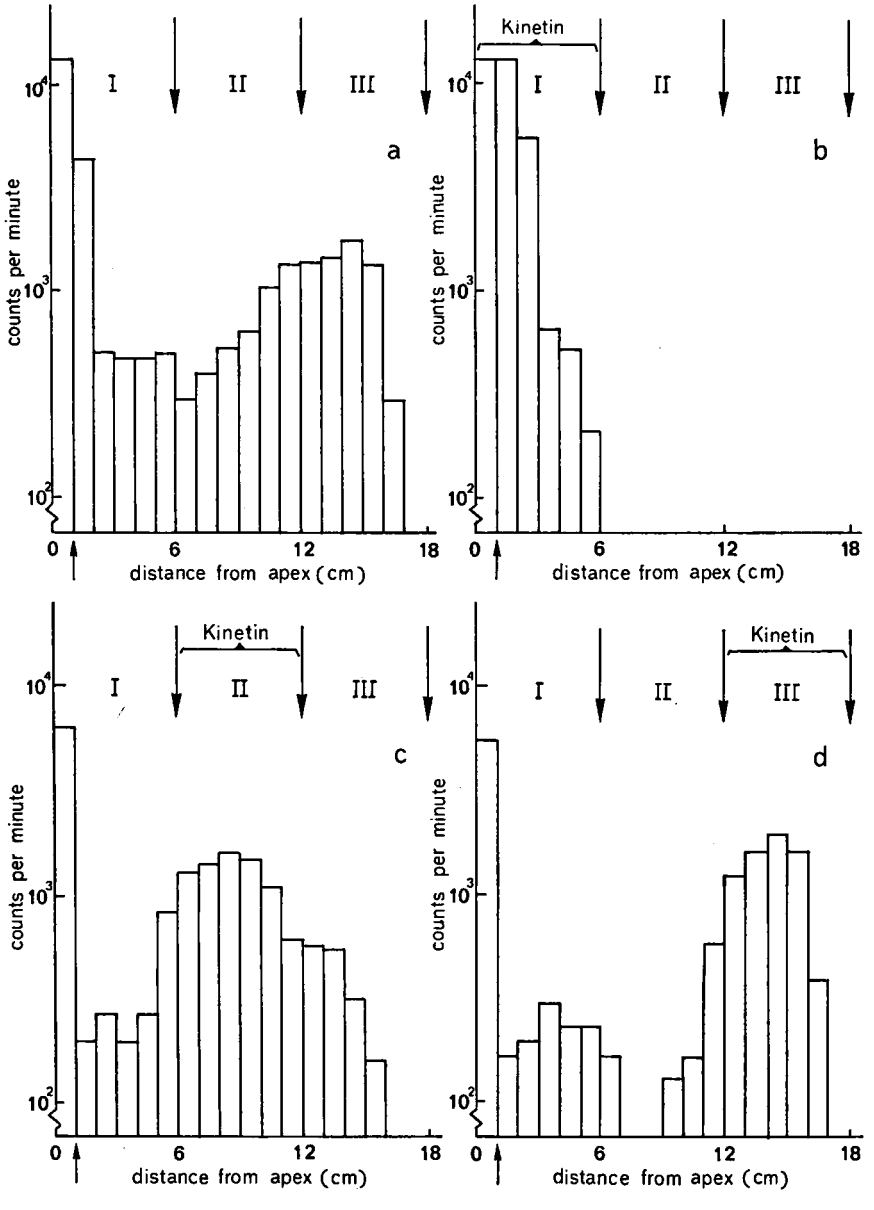

Fig. 1. Distance profiles of apically applied AIB (small arrow) in the leaf strips after 48 hours of translocation. (a) control 0.1 % DMSO (b) kinetin 1 mg/l in zone I (c) kinetin 1 mg/l in zone II (d) kinetin 1 mg/l in zone III.

Table 1. Relative distribution in percentages of the total amount of apically applied AIB in the leaf strips after 48 hours of translocation. The influence of kinetin (2 mg/l), when applied to zone I, II and III, respectively. All percentages are averages of the amounts of AIB in the respective zones in three series of three leaf strips.

	zone I	zone II	zone III
control (0.1% DMSO)	74	5	21
kinetin in zone I	100	–	–
kinetin in zone II	67	33	–
kinetin in zone III	62	6	31

Fig. 1 (a–d) shows distance profiles of AIB translocation, determined 48 hours after its application, either in the presence or in the absence of kinetin. *Table 1* demonstrates the relative distribution of the total AIB content over three zones when one of these zones was treated with kinetin. Note that *fig. 1* (kinetin 1 mg/l) does not correspond with *table 1* (kinetin 2 mg/l).

When kinetin was applied to zone I *(fig. 1b, table 1)*, a distinct increase of the AIB content compared to the same zone in the control *(fig. 1a)* occurred. Out of this zone hardly any translocation took place.

When zone II was treated with kinetin the effect was considerably stronger *(fig. 1c, table 1)*. From *table 1* we see an increase from 5% (control) up to 33%. When zone III was treated an increase was found from 21% up to 31% of the total amount of AIB. With respect to this enlarged accumulation we have to keep in mind that normally (control) the absolute amount of AIB in zone II is remarkable low (generally between 5 and 15%).

Fig. 1d and *table 1* show an increase in zone III above the normally enhanced accumulation owing to the effect of kinetin. Apparently this extra increase gives rise to a decrease of AIB in zone I (see also *table 2* for the same effects of other cytokinins).

3.2. The effect of zeatin and zeatin riboside on AIB translocation

Since kinetin is probably not a natural cytokinin, two in plants occurring cytokinins were tested, viz. zeatin and its riboside. *Table 2* shows the relative distribution of AIB in the three zones found after application of these substances. In spite of a possible tendency to stronger accumulation effects there appeared to be no essential difference with the AIB translocation pattern found in the presence of kinetin.

3.3. The effect of CCCP on AIB translocation

A remarkable observation was a cytokinin-like action of CCCP, at concentrations lower than 10^{-5} M, especially with respect to the translocation of AIB and the retention of chlorophyll. *Fig. 2 (b–d)* shows the influence of CCCP on the course of the AIB distance profiles, when applied to zone I, II and III respectively. The effects strongly resemble the effects found with cytokinins although possibly some minor differences might exist.

The effect of three concentrations of CCCP is shown in *table 3*. With respect to the application in zone I and II, there is a distinct effect on accumulation.

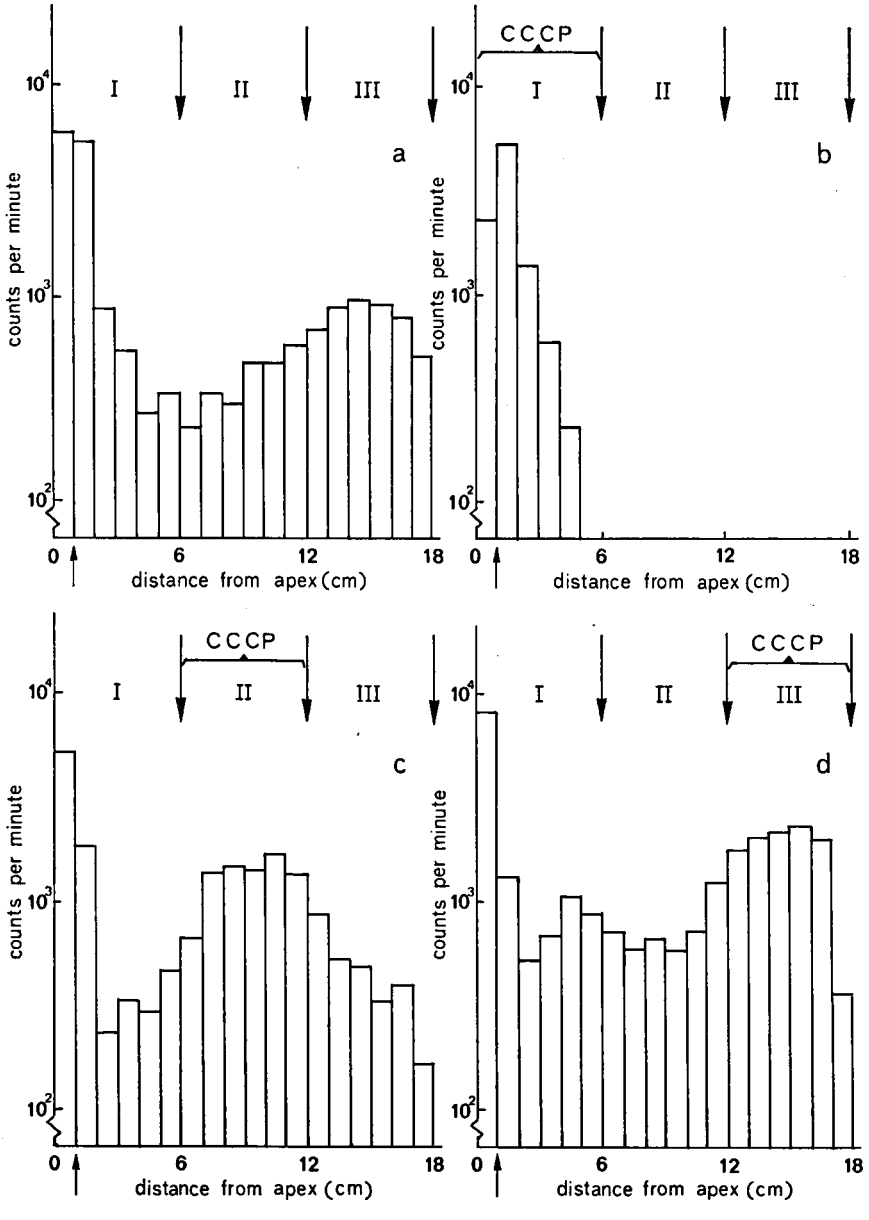

Fig. 2. Distance profiles of apically applied AIB (small arrow) in the leaf strips after 48 hours of translocation. (a) control 0.05% ethanol (b) CCCP $7{,}5.10^{-6}$ M in zone I (c) CCCP $7{,}5.10^{-6}$ M in zone II (d) CCCP $7{,}5.10^{-6}$ M in zone III.

Table 2. Relative distribution in percentages of the total amount of apically applied AIB in the leaf strips after 48 hours of translocation. The influence of zeatin and zeatin riboside (2 mg/l), when applied to zone I, II and III, respectively.
All percentages are averages of the amounts of AIB in the respective zones in three series of three leaf strips.

	zone I	zone II	zone III
control (0.1% DMSO)	67	10	23
zeatin in zone I	100	–	–
zeatin in zone II	66	33	1
zeatin in zone III	29	9	62
zeatin riboside in zone I	100	–	–
zeatin riboside in zone II	57	38	5
zeatin riboside in zone III	50	14	36

In zone III this effect is less clear, probably because an increased accumulation of amino acids is normal in the basal regions of the leaf strips after 48 hours of translocation.

4. DISCUSSION

4.1. The action of cytokinin

When parts of detached leaves of *Sagittaria* are treated with cytokinin, there is a change in the distance profiles of basipetally transported AIB. In those zones there is a distinct accumulation of AIB. These findings are in accordance with those of BRÄUTIGAM (1971), who worked with *Vallisneria* leaves. Although the remarkable observation can be made that hardly any AIB is transported out of a cytokinin-treated part, it appears from *fig. 1 (c* and *d)* that cytokinin

Table 3. Relative distribution in percentages of the total amount of apically applied AIB in the leaf strips after 48 hours of translocation. The influence of different concentrations of CCCP, when applied to zone I, II and III, respectively.
All percentages are averages of the amounts of AIB in the respective zones in three series of three leaf strips.

	zone I	zone II	zone III
control (0.05% ethanol)	63	14	23
CCCP 5.10^{-6} M in zone I	71	14	15
CCCP 5.10^{-6} M in zone II	52	22	26
CCCP 5.10^{-6} M in zone III	56	16	28
CCCP $7{,}5.10^{-6}$ M in zone I	95	4	1
CCCP $7{,}5.10^{-6}$ M in zone II	53	35	12
CCCP $7{,}5.10^{-6}$ M in zone III	44	21	35
CCCP 10^{-5} M in zone I	100	–	–
CCCP 10^{-5} M in zone II	42	47	11
CCCP 10^{-5} M in zone III	49	25	26

favours accumulation of AIB rather than preventing its translocation. The lack of transport out of a cytokinin-treated area may consequently be considered to be a secondary effect, because of the drastically increased accumulation, which hardly leaves any AIB to be transported. These findings are confirmed by the work of BRÄUTIGAM & MÜLLER (1973) who have suggested that the primary action of kinetin is an alteration in source-sink relations, rather than a direct influence on the translocation mechanism.

Although no conclusions can be made as to the possible mode of action of cytokinin, the present results might indicate that the increased accumulation regularly found in the basal region of the control leaf strips has been caused by the presence of a cytokinin, which concentration is higher in the leaf basis than in its apex. This might also explain the observed polarity in amino acid translocation (SCHENK 1972).

4.2. The action of CCCP

None of the CCCP characteristics mentioned in the introduction seem to explain the resemblance between CCCP and cytokinin action. The studies of YAMAMOTO & OHYAMA (1962) and KURAISHI et al. (1968), however, may link their stimulating effect on AIB accumulation. The first authors have found increased levels of NAD in leaves after treatment with cytokinins, the latter, in a study on the interactions between cytokinins and metabolic inhibitors, which might affect the levels of NAD, have found that the morphogenetic effect of cytokinins is stimulated by certain metabolic inhibitors. These inhibitors alone did not have an effect. They have suggested that lower concentrations of inhibitors produce suitable conditions for the action of cytokinins. In the present work, CCCP alone already had distinct effects. These effects are very similar to those of cytokinins although there are some differences.

Comparison of the CCCP and kinetin effects in zone III *(fig. 1d* and *2d)* shows that for kinetin, AIB increase is related to a decrease in zone I, which does not apply to CCCP. Besides, less AIB is transported from a cytokinin-treated zone II to zone III, then from a CCCP-treated one *(tables 1, 2* and *3)*.

From these observations it appears that cytokinins and CCCP, though weaker, have similar effects on AIB translocation and accumulation. The results obtained so far do not exclude a cooperation of CCCP and endogenous cytokinins (KURAISHI et al. 1968). The similarity in action of cytokinins and CCCP is not only restricted to effects on AIB translocation but has also been shown to exist with regard to their action on the turnover of proteins. CCCP-treated leaf strips remained just as green as cytokinin-treated ones after 72 hours and thus exhibited clear chlorophyll-retention activity.

ACKNOWLEDGEMENTS

The author is much indebted to Prof. Dr. J. van Die and Mr. A. J. E. van Bel for valuable suggestions and discussions and to Miss Hannie van der Peut for her technical assistance.

REFERENCES

BRÄUTIGAM, E. (1971): *Einflusz des Kinetins auf den symplasmatischen Transport in Blättern von Vallisneria spiralis L.* Dissertation Universität Halle.

— & E. MÜLLER (1973): Further analysis of the kinetin induced transport processes in Vallisneria leaves. *Proceedings of the research institute of pomology, Poland.* series E (3): 163–170.

EPSTEIN, E. (1961): The essential role of calcium in selective cation transport by plant cells. *Plant Physiol.* **36**: 437–444.

HEYTLER, P. G. (1963): Uncoupling of oxidative phosphorylation by carbonyl cyanide phenylhydrazones. *Biochemistry* **2**: 357–361.

KIMIMURA, M., S. KATOH, I. IKEGAMI & A. TAKAMIYA (1971): Inhibitory side of carbonyl cyanide m-chlorophenylhydrazone in the electron transfer system of chloroplasts. *Biochim. Biophys. Acta* **234**: 92–102.

KURAISHI, S., K. KASAMO, T. TEZUKA, T. USHIJIMA & T. TAZAKI (1968): Inhibitors interacting with cytokinins. *Physiol. Plant.* **21**: 1003–1009.

LEBLANC Jr., O. H. (1971): The effect of uncouplers of oxidative phosphorylation on lipid bilayer membranes: carbonyl cyanide m-chlorophenylhydrazone. *J. Membrane Biol.* **4**: 227–251.

MOTHES, K., L. ENGELBRECHT & H. R. SCHÜTTE (1961): Über die Akkumulation von α-Aminoisobuttersäure im Blattgewebe unter dem Einfluss von Kinetin. *Physiol. Plant.* **14**: 72–75.

SCHENK, E. (1972): Quantitative studies on translocation in Sagittaria graminea Michx. leaves, a new method of measurement. *Acta Bot. Neerl.* **21**: 231–234.

SHIBAOKA, H. & K. V. THIMANN (1970): Antagonisms between kinetin and amino acids. *Plant Physiol.* **46**: 212–220.

SKOOG, F. & D. J. ARMSTRONG (1970): Cytokinins. *Ann. Rev. Plant Physiol.* **21**: 359–384.

YAMAMOTO, Y. & H. OHYAMA (1962): Effects of kinetin on pyridine nucleotide level in leaves. *Bot. Mag. Tokyo* **75**: 368–370.