*Phytochemistry*, 1977, Vol. 16, pp. 305–307, Pergamon Press, Printed in England.

# SUSTAINED RELEASE OF CYTOKININS FROM NATURAL POLYMERS

SHMUEL BITTNER*, ISRAEL PERRY* and YEHUDA KNOBLER†

*Department of Chemistry, Ben Gurion University of the Negev, Beer Sheva, Israel; †Department of Organic Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

(*Received 10 April 1976*)

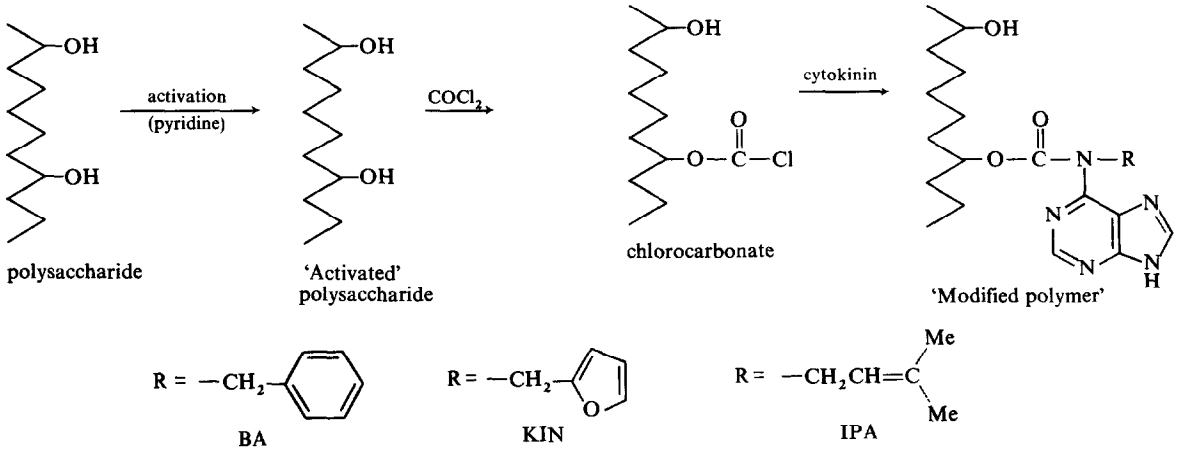
**Key Word Index**—Controlled release; cytokinins; 6-benzyl-amino purine; 6-furfurylaminopurine; 6-(4-methyl-3-pentyl-aminopurine); plant hormones; polysaccharides; synthesis; starch; cellulose; kinetic of release.

**Abstract**—The cytokinins 6-benzylaminopurine, 6-furfurylaminopurine and 6-(3-methyl-2-butenylamino) purine, were attached to starch and to cellulose by means of a carbamatic bond. The modified polysaccharides contained about one cytokinin molecule per 50 glucose units. The rate of release of the cytokinins was followed spectrophotometrically in aqueous solutions at different pHs. A good agreement with a first order model was found and the kinetic constants for the various systems were determined. The clear dependence on the pH of the medium supports a mechanism in which hydrolysis of the carbamatic bond is the rate determining step. The hormonal activity of the modified polymers was determined in the soybean callus bioassay and compared with the activities of non-bonded cytokinins. While high concentrations of free hormones cause inhibition of growth, such concentrations of bound hormone did not show inhibition.

## INTRODUCTION

The last few years have seen tremendous progress in the field of controlled release systems. The concept is not new, but the present studies of sustained or prolonged release of drugs [1, 2], fertilizers [3] and plant protection agents [4] are in a state of rapid development. The effective release methods are based on elementary principles and include (i) adequate formulations (ii) various types of microencapsulations. Insecticides, fertilizers, insect sexual stimulants, pheromones and therapeutic agents have been encapsulated by permeable or semipermeable polymers [5] (iii) chemical attachment to either synthetic or natural polymers [6, 7]. The active component is attached to the polymer through a covalent bond or by complexation (e.g. ion exchange resin). The covalent bond may be breakable (esteric, amidic, glycosidic) or unbreakable (carbon–carbon) under normal physiological conditions. Prolongation of activity of various antibiotics bound to synthetic copolymers was observed [8]. Toxic and other secondary effects which follow use of synthetic polymers focused attention on the use of biopolymers like starch, albumines or dextrins as carriers [9]. (iv) Entrapment in polymeric matrices [10, 11]. Simple physical inclusion of the active material within the chains of a synthetic polymer can be achieved either during the polymerization itself or by dissolution in a preformed polymer. Such entrapment also protects sensitive compounds from external influences [12].

This laboratory is carrying out systematic research on the prolongation and control of the release of plant hormones. In a previous work [7] we described some




Scheme 1. Bridging of cytokinins to polysaccharides via a carbamatic bond.

preliminary experiments with hormones bound to synthetic polymers. It was indicated that the rate of release depended not only on the type of polymer or copolymer but also on the amount of bound hormone and pH of the medium. The present paper reports the results of experiments in which cytokinins were attached to natural polymers.

## RESULTS AND DISCUSSION

### *Preparation of bound cytokinins*

Three representatives of the cytokinin hormones were selected for this study: 6-benzylaminopurine (BA), 6-furfurylaminopurine (KIN) and 6-(3-methyl-2-butenylamino) purine (IPA). The first two are synthetic cytokinins while the third is a naturally occurring hormone. The cytokinins were attached to starch (S) and to cellulose (C), by use of a modification of a known method [9] (Scheme 1). First the polysaccharides were activated by treatment with an aqueous solution of pyridine (5%) followed by azeotropic distillation and drying. This process induces the swelling of the polymers, breaks part of their intramolecular hydrogen bonds and facilitates the diffusion of the reagents. The activated polysaccharides were next reacted with excess of phosgene, which combined with free hydroxy groups, yielding chlorocarbonates. These active groups reacted readily with the cytokinins to form carbamatic bonds, bridging between the polysaccharide and the $N^6$-substituted adenines.

Table 1. Rate of release of IPA from the polymer C-IPA at pH 1 [102 mg of modified polymer containing $4.2 \times 10^{-6}$ mol IPA were suspended in 200 ml aq. soln $10^{-1}$ M HCl at 30°]

| Time (hr) | $A_{260\,nm}$ | Hormone released (mol × $10^6$) | $k$ ($10^{-7}$ $sec^{-1}$) |
|---|---|---|---|
| 0 | 0.218 | 0 | — |
| 1 | 0.240 | 0.23 | [0.68]* |
| 4 | 0.255 | 0 44 | [0.34]* |
| 24 | 0.285 | 0.74 | 9.8 |
| 47 | 0.300 | 0.90 | 8.2 |
| 74 | 0.356 | 1.44 | 6 9 |
| 146 | 0.405 | 1.92 | 5 0 |
| 195 | 0.450 | 2.33 | 5 0 |
| 243 | 0 495 | 2 73 | 5.3 |
| 315 | 0.510 | 2.85 | 4 3 |
| 360 | 0.550 | 3.16 | 4.7 |
| 530 | 0.625 | 3.67 | 4.8 |
| 650 | 0.702 | 4 19 | † |

* At this range, the experimental error is very high. † Total release; no more bound cytokinin.

The IR absorption at 1800 $cm^{-1}$, showed that the modified polymer still contains chlorocarbonate groups; boiling for a short while in neutral conditions causes total hydrolysis. The modified polymers were obtained, as stable amorphous powders. Both elemental analysis and spectrophotometric measurements (after total hydrolysis), showed that the polymer contains one molecule of cytokinin per 40–50 glucose units. Synthesis at higher temperature (80°) results in a higher density of cytokinin.

### *Kinetics of cytokinin release*

The rate of release of KIN and IPA from their polymeric carriers in aqueous suspension, was followed at pH 1, 5 and 12, by measuring the A at 260 nm (purine absorption; see Experimental). Molar absorbances of the free cytokinins (at pH 1) were: $\varepsilon_{KIN} = 17760$, $\varepsilon_{BA} = 17\,733$, and $\varepsilon_{IPA} = 17\,420\ cm^{-1}\ M^{-1}$. All eleven systems studied showed a rapid initial increase of cytokinin concentrations in solution. This initial burst was followed by a leveling tendency behaviour typical of first order kinetic processes. Consequently, the data can be treated by using the familiar relationship $\ln C_0/(C_0 - C) = kt$, where $C_0$ = initial concentration of cytokinin attached to the polymer, $C$ = concentration of cytokinin at time $t$. $k$ = first order rate constant ($sec^{-1}$). Table 1 shows the rate of release of IPA from the polymer *C*-IPA at pH 1, obtained by using the above equation. The process was followed for over 600 hr until total release was obtained. Similar calculations were performed with the other modified polymers at the various pH's. Table 2 shows the kinetic constants for the systems studied.

Table 2. Kinetic constants ($k$) for the release of cytokinins attached to polysaccharides

| System | $10^{-6} \times k$ ($sec^{-1}$) | | |
|---|---|---|---|
| | pH = 1 | pH = 5 | pH = 12 |
| S + KIN | 0.59 | — | 19.5 |
| S + IPA | 0.72 | 1.25 | 3.30 |
| C + KIN | 0.62 | 1 33 | 1.88 |
| C + IPA | 0 56 | 1.08 | 2.50 |

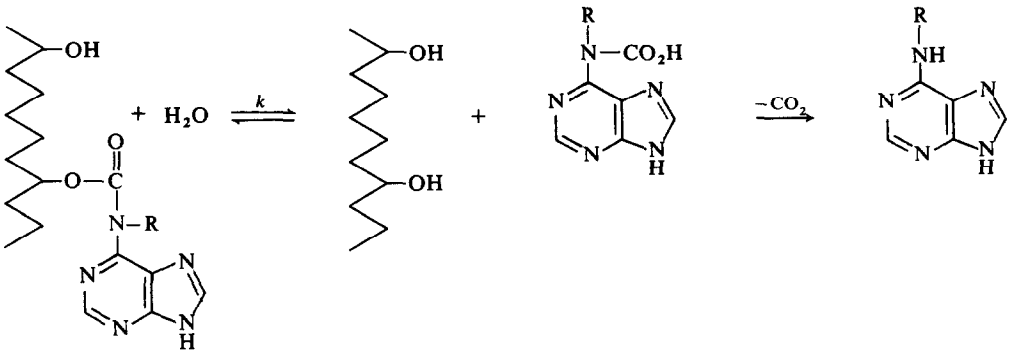
The results of Table 2 show a clear dependence of the kinetic constants on pH, the fastest process taking place in basic conditions. This kind of pH dependence supports a hydrolytic mechanism of release in which the hydrolysis of the carbamylic bond is the slowest and hence the limiting step of the process. The release mechanism might take place as shown in Scheme 2.

Hydrolytic fission of the carbamoyl linkage yields carbamic acid, which eliminates $CO_2$ to give the free cytokinin. The last stage, is known to be spontaneous in acidic solutions, while the release rate of the hormone is faster in basic media. This fact *inter alia* shows that the first stage (the hydrolysis), is the rate-determining step. Another fact which points towards the same conclusion is that no significant differences have been found between the rates of release from starch or cellulose. As in both polymers the type of bonding to the cytokinin is the same, it can be concluded that the hydrolytic step (and not diffusion, steric conformation, etc.) is the rate-determining step.

### *Cytokinin activity*

The cytokinin activity found in the modified polymers was measured in a typical test of serial concentrations in the soybean assay, in comparison to that of free cytokinins. It was found that with free cytokinins the range over which growth increase is nearly linear is 0.005–0.5 ppm. Response with bound cytokinin could be observed in the 0.1 ppm range, with linear growth increase of up to 100 ppm. Obviously only a small fraction of the bound cytokinin was released and became available to the callus. Over-optimum amounts of free cytokinin inhibited growth and thus higher concentration

(>0·5 ppm) of BA profoundly inhibited growth. With bound cytokinin it was possible to introduce into the medium 20-fold the optimal quantity of hormone, without causing any inhibitory effects. This study indicates that cytokinins covalently bound to natural polymers are released over an extended period. The slow release of these biologically active cytokinins eliminates the inhibitory effects which result from excess of free cytokinin.



Scheme 2. Hydrolytic release of cytokinins from polysaccharides.

## EXPERIMENTAL

UV spectra were measured in $H_2O$, TLC was performed on Si gel G and IR spectra were measured in KBr micropellets.

*Activation of starch.* A suspension of 575 mg (3.19 mmol) of soluble starch, $C_5H_5N$ (1 ml) and $H_2O$ (1 ml) was warmed under stirring until a clear soln was obtained. $C_5H_5N$ (20 ml) was added and distillation was carried out until a constant bp was reached (115°). The mixture at this stage contained 10 ml in which part of the starch is colloidal and another part precipitated (altogether about 0.5 g of activated starch).

*Chlorocarbonation of activated starch.* The mixture containing the activated starch was cooled to −18° and 8 ml phosgene in $C_6H_6$ (12%; 0.01 mol) and 8 ml of toluene were added slowly and with stirring. The stirring was continued for 12 hr at room temp. and the mixture was used without further treatment in the next stage.

*Bridging 6-benzylaminopurine to starch.* The mixture from the former stage was cooled to 5° and 6-benzylaminopurine (1 g) dissolved in tetrahydrofuran, was added dropwise for 60 min. The stirring was continued for an additional 30 min at this temp. and for 24 hr at room temp. Addition of EtOH caused precipitation of the polymeric material. It was stirred for 30 min in EtOH, filtered and washed several times with hot EtOH to remove any adsorbed $C_5H_5N$, HCl (tested with $AgNO_3$) or unbonded cytokinin (tested by TLC). The *modified starch* (0.650 g) was washed with $Et_2O$ and dried *in vac* over $P_2O_5$. (Found: C, 40.86; H, 6.20; N, 0.86%). From the N content, it was calculated to contain one molecule of 6-benzylaminopurine per 44 glucose units.

[IR: 1800 (CO—Cl), 1720 (OCONH); 1620; 1150; 720 $cm^{-1}$].

Repeating the same procedure with stirring for 24 hr at 80° gave a product for which: C, 39.64; H, 6.45; N, 0.98%. This product contained one molecule of cytokinin per 38 glucose units.

*Bridging of 6-furfurylaminopurine to starch.* According to the same procedure. At room temp. 0.55 g of modified polymer were obtained (Found: C, 41.15; H, 6.32; N, 0.86; Cl, 0.73%), which accords with one molecule of cytokinin per 50 glucose units (IR 1800; 1720 $cm^{-1}$).

*Bridging of 6-(3-methyl-2-butenylamino) purine to starch.* According to the same procedure, 0.55 g of modified polymer were obtained. (Found: C, 40.86; H, 6.02; N, 0.46; Cl, 0.80%) which accorded with one hormone molecule per 95 glucose units (IR 1820; 1730 $cm^{-1}$).

*Determination of released cytokinin.* For the release expts 100 mg of modified polymer was suspended in 200 ml aq. soln at the desired pH with stirring. Aliquots (5 ml) were taken at different times and the cytokinin content was determined spectrophotometrically, using the *A* at 260 nm.

*Bioassay procedures.* Determination of cytokinin activity was based on the soybean callus assay, as described in ref. [13]. Four conical flasks were used for each bound cytokinin (BA, KIN and IPA). Each flask contained 3 explants grown on 25 ml nutrient medium. All the explants from 1 flask were weighed together. The medium contained the following mineral salts: $MgSO_4$, $KH_2PO_4$, $NH_4NO_3$, $KNO_3$, KCl, $ZnSO_4$, $H_3BO_3$, $MnSO_4$, KI, $Ca(NO_3)_2$ sequestrene, and the following organic constituents: 30 g/l. sucrose, 10 g/l. Difco agar, 560 μM *myo*-inositol, 11.4 μM IAA and 1·2 μM thiamine HCl. pH was adjusted to 5.7 and the temp. maintained at 25°.

*Acknowledgements*—The authors wish to thank Drs O. Levy and Y. Markowitz for their critical reading of the manuscript and comments. Thanks are also due to Miss E. Ostrovski for technical assistance.

## REFERENCES

1. Nairn, G. (1969) *Can. Pharm. J.* **102**, 336.
2. Willains, A. (1969) in *Sustained Release Pharmaceuticals*, Noyes Development Corporation, Park Ridge, New York.
3. Lunt, O. R. (1971) *J. Agr. Food Chem.* **19**, 797.
4. Jvy, E. E. (1973) 166th ACS-Meeting, Chicago, **33**, No. 2, 554.
5. Sliwka, W. (1975) *Angew. Chem. Internat. Edit.* **14**, 539.
6. Tahan, M., Lander, N. and Zilkha, A. (1973) *Isr. J. Chem.* **11**, 835; Tahan, M., Calderon, J. and Zilkha, A. (1974) *Isr. J. Chem.* **12**, 785.
7. Bittner, S. (1972) Progress Report, The Negev Institute of Arid Zone Research, Beer Sheva, Israel; Perry, I. (1973) Ph.D. Thesis, Hebrew University, Jerusalem.
8. Jatskevitz, R. (1954) *Z. Physiol. Chem.* **297**, 149 and (1955) *Naturforsch.* 106.
9. Kratzl, K., Kaufman, E., Kraupp, O. and Storman, H. (1961) *Monatsh.* **92**, 371, 379, 384.
10. Bittner, S. and Perry, I. (1975) *Proc. 43rd Ann. Meet. Isr. Chem. Soc.* 128.
11. Stigmann, A., Narkis, M. and Tirosh, N. (1975) *Proc. Symp. Israel–France on Behaviour of Pesticides in Soil*, 11.
12. Norris, R. D. and Powelkiewicz, J. (1975) *Phytochemistry* **14**, 1701.
12. Miller, C. O. (1963) in *Modern Methods of Plant Analysis*, vol. 6, p. 194. Springer-Verlag, Berlin.