# ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE – ITS RELEASE IN A HIGHER PLANT BY AN EXOGENEOUS STIMULUS AS DETECTED BY RADIOIMMUNOASSAY

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## 1. Introduction

The occurrence and function of adenosine 3′:5′-cyclic monophosphate (cAMP) in higher plants is a matter of debate [1–3]. Materials separated by biochemical methods or behaving in biochemical systems as expected of cAMP were reported in extracts of higher plants in some cases [4–6], whereas others have found that cAMP was not detected in such extracts [7]. Two major technical problems stood in the way of a reliable determination of cAMP in plants:

(i) Plant extracts contain compounds which interfere with the biochemical methods usually employed for cAMP quantification in animal tissues [8];
(ii) The apparent low levels of this nucleotide in plants [9].

These technical problems were overcome by employing mass and infrared spectroscopy for cAMP identification. Its presence was thus demonstrated in tobacco tissue culture [10] and in maize seedlings [9].

A decisive conclusion, however, requires more than merely the detection of cAMP in higher plants. A physiological reaction of the plant, which requires cAMP, and its response to a stimulus, by producing cAMP, should also be demonstrated. The plant *Nicotiana glutinosa* L. reacts locally when infected with tobacco mosaic virus (TMV). The virus multiplies readily in the leaves for ~24–40 h when, suddenly, its multiplication is halted and in <48 h from inoculation the amount of TMV in the leaves levels off. This phenomenon was associated, at least in part, with the appearance, at this time, of an antiviral factor (AVF) (summary in [11]). It was possible to show that upon TMV infection, a precursor of AVF was being processed to become active AVF, and that in vitro, this reaction required cAMP and guanosine 3′:5′-cyclic monophosphate [12]. Hence, we have chosen the *N. glutinosa*–TMV system to check the in vivo release of cAMP. By employing a sensitive and specific radioimmunoassay we were able to demonstrate not only the presence of cAMP in *N. glutinosa*, but also its pulse-release following TMV infection.

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## 2. Materials and methods

### 2.1. *Plant material and treatments*

*Nicotiana glutinosa* plants were grown in a temperature-controlled green-house at 24 ± 2°C under constant illumination. All leaves were dusted with carborunderm. The plants were then divided randomly into 3 groups:

(i) A control for basal levels of cAMP;
(ii) Mock-inoculated with water, causing injury but not infection;
(iii) Inoculated with TMV (5 μg/ml).

At various time-intervals after injury or inoculation, leaves were removed for cAMP detection.

### 2.2. *cAMP extraction from leaves*

To avoid cAMP degradation during extraction, the leaves were homogenized directly in 7% perchloric acid (PCA; 1 g fresh wt/ml), in a Teflon-coated, motor-driven, tissue-grinder. Extraction of only 3–10 g leaves was sufficient for cAMP determination by the radioimmunoassay reported below. The homogenate was centrifuged for 10 min at 10 000 × *g* and the supernatant fluid was taken for cAMP quantification. The pellets were kept for protein determination.

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### 2.3. *cAMP determination*

The PCA-supernatant fluids were neutralized with 2 N $KHCO_3$, using bromocresol purple as indicator. The samples were acetylated and assayed essentially as in [13]. Adenosine 3′:5′-cyclic phosphoric acid 2′-*O*-succinyl 3-[$^{125}$I]iodotyrosine methyl ester was supplied by The Radiochemical Center (Amersham) and the antiserum against cAMP and the cAMP standards by Collaborative Research (Cambridge MA). The separation between free and antibody-bound ligand was carried out with *Staphylococcus aureus* as in [14].

### 2.4. *Protein determination*

The PCA-pellets were resuspended in a measured volume of 0.1 N NaOH. The pH was checked, and, if necessary, suspensions were titrated to exceeed pH 11. Following centrifugation for 20 min at 30 000 × *g*, the alkaline supernatant-fluids were diluted with water (at least 1:10), and their protein content was determined with Coomassie blue G-250 (Serva) as in [15]. A calibration curve was prepared from PCA-precipitates of bovine serum albumin dissolved in alkali and diluted with water in accordance with the above procedure.

## 3. Results

### 3.1. *cAMP measurements in* N. glutinosa *leaves*

These experiments were done to determine whether radioimmunoassay could overcome the technical difficulties previously encountered with such measurements in higher plants and to determine basal cAMP levels.

Table 1 is a summation of 5 such determinations carried out with different plants at different times. The range of differences in various samples taken randomly from the same batch of plants at the same time is ~20%. However, as demonstrated in table 1, cAMP content in various batches of plants at various times varies between 4–46 mol/mg protein. In a few cases, lower values (0.1–1 mol/mg) were recorded. It should also be noted, that the actual reading of cAMP is within the range of ~7.5–46 fmol, which is below the sensitivity level of conventional biochemical methods.

Table 1
cAMP determination in leaves of various batches of *N. glutinosa* plants

| Batch no. | fmol cAMP/mg protein | fmol . cAMP$^{-1}$ . g fresh wt$^{-1}$ |
|---|---|---|
| 1. | 45.998 | 920.0 |
| 2. | 5.224 | 120.1 |
| 3. | 11.555 | 288.9 |
| 4. | 15.261 | 259.4 |
| 5. | 4.025 | 128.8 |

### 3.2. *cAMP stimulation in* N. glutinosa *leaves following TMV inoculation*

A batch of plants was either mock-inoculated or TMV-inoculated as above. Leaves were harvested randomly at various times post-inoculation and their cAMP content determined as above. In some instances cAMP in non-treated plants of the same batch was also determined.

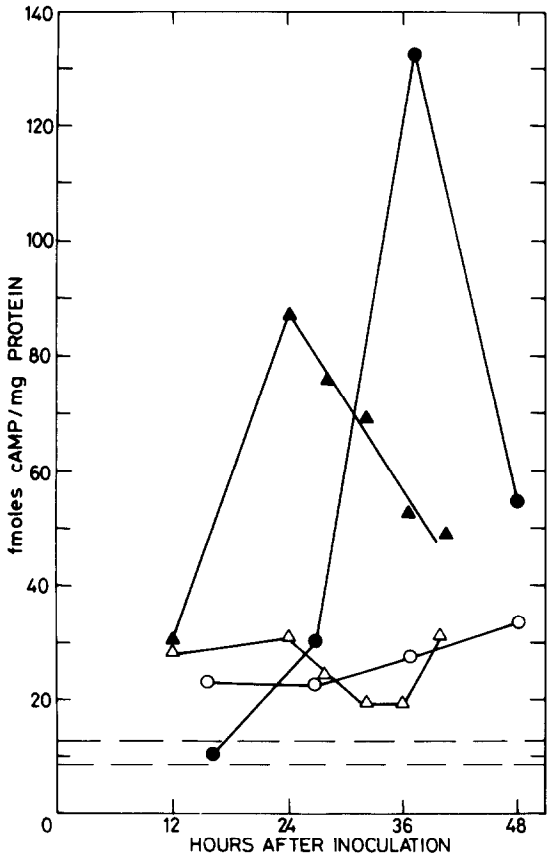


Fig.1. cAMP levels in extracts of *N. glutinosa* leaves at various times after mock-inoculation (○;△) or TMV-inoculation (●;▲). Basal values in untreated leaves fell between the two horizontal lines.

Plants reacted with a pulse-release of cAMP, peaking, in one of the demonstrated experiments, 37 h after inoculation at a 13-fold increase above basal levels and 24 h post-inoculation at a 7-fold increase in the other experiment (fig.1). It is also apparent that the injury itself, caused by mock-inoculation, increased cAMP levels 2–3-fold. In a series of similar experiments, an increase in cAMP levels of ⩾6-fold was always noted, and the peaks centered between 24–40 h after inoculation.

## 4. Discussion

The use of the above-mentioned radioimmunoassay enabled cAMP determination in higher plants. Since it is impractical to assay large volumes, the high sensitivity of the assay is essential. The difficulties and diversities in determining cAMP in plants, as reflected in previous reports, are clarified in table 1, which demonstrates fmol cAMP levels/g fresh tissue. Values readable by earlier methods were obtained only occasionally. However, various parameters such as age, part of plant and environmental conditions, may considerably affect these basal values. cAMP content/mg protein is much lower than the values obtained in animal tissues, e.g., basal levels of cAMP in porcine renal LLC-$PK_1$ cell are 5 pmol/mg protein [16], and in $C_6$-glioma cells 100 pmol/mg protein [17,18].

The present results also indicate that cAMP plays a role in the physiology of higher plants. In [12], in vitro activation of AVF required cAMP. AVF activation was also obtained in vivo by introducing $N^6$,$O^{21}$-dibutyryl cAMP to *N. glutinosa* leaves (in preparation). Here, the demonstration that cAMP is produced by the plant in response to virus infection, indicates not only its presence in the plant, but also its possible role in plants physiological processes.

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