Gaschromatographic, Mass- and Infrared-Spectrometric Identification of Cyclic Adenosine-3′:5′-monophosphate (c-AMP) in Maize Seedlings (*Zea mays*)

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Gaschromatographic, mass- and infrared-spectrometric evidence is presented for the identification of adenosine-3′:5′-monophosphate (c-AMP) in maize seedlings (*Zea mays*).

The genuine c-AMP content could be quantitated by GC-detection and refere to authentic c-AMP standards.

Introduction

c-AMP has been identified as a mediator in the action of several hormones and of changes induced by the environment in mammals, bacteria, algae and fungi [1–7]. However, the occurence and function of c-AMP in higher plants is currently a matter of controversy [8–12]. Attempts to detect c-AMP in higher plants using different assay systems have been inconclusive for several reasons [10, 11]. The principal difficulty with plant extracts has been that these materials interfere with the biochemical assays for c-AMP. Consequently, levels of c-AMP ranging from 1 to 10^4 pmol/g fresh weight, have been reported for various higher plants [13–17].

Recent studies refered to the presence of c-AMP in higher plant cells suspended in axenic liquid culture at concentrations up to 12 pmol/g fresh weight [18]. Until now usually biochemical assays have been applied to detect c-AMP in plant tissue although UV controlled HPLC-separation and some IR-data have been published [19].

In this paper the presence of c-AMP in higher plants is shown by a simple extraction method followed by different purification steps, gas chromatography and by mass- and IR-spectral data.

A variety of physiological experiments related to c-AMP in maize seedlings have been reported. One question of interest was the synthesis of c-AMP in coleoptile sections by 3-indole acetic acid [20] and the enhancement of adenylate cyclase activity in coleoptile homogenates [20]. Another question was the distribution of c-AMP in maize seedlings [21] the polarity and rate of c-AMP transport in the coleoptile [22] and the cytochemical localization of adenylate cyclase activity in root tips [23]. Further experiments were concerned with effects of gibberellic acid on the c-AMP content of shoots [24] and a short time effect of c-AMP on coleoptile growth [20]. The significance of these investigations was difficult to estimate as long as the in vivo concentration of c-AMP was not ascertained definitely. Taking into account the quantitative aspect of the problem the extraction was started with 20 kg fresh weight of maize seedlings.

Materials and Methods

Maize seeds (*Zea mays* L., Golden Bantam 8 row and Bear Hybrid WF 9/38 Vaughan's Seed Co.) were kept under running water for 24 h and spread out on steam sterilized moist filter paper in plastic-boxes. The seeds were sprayed with a 0.1% aqueous solution of penicillin G,Na-salt and kept at 20 °C in the dark. Pieces of seedlings were controled periodically for sterility according to Berlin *et al.* [25]. The seedlings were harvested after five days under green safelight and a fresh weight of 20450 g was determined. They were frozen in liquid nitrogen and lyophilized at −20 °C. Thus 1640 g of dry weight representing 8% of the fresh weight was obtained. The dry material was submitted to a series of extraction steps on a Soxhelet-Apparatus, each over 24 h. The procedures for extraction and purification of c-AMP are summarised in Fig. 1.

Extract 3 was evaporated under vacuum and freeze-dried. A brown residue of 830 g (50.6%) was obtained. The residue was dissolved in 1 l redist. water.

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Fig. 1.

Freeze dried tissue → extract 1 (petrolether, 40–60 °C; discarded), extract 2 (ethylacetate, discarded), extract 3 (ethanol-water, 80:20, v/v) containing the nucleotides, → charcoal → anion-exchange-column → 1.TLC → 2.TLC → PC → silylation → IR
↘ GC-MS

To one half of the solution [adenine-U-^{14}C]adenosine-3′:5′-monophosphate (0.5 µCi; specific radioactivity: 238 mCi/mmol; Radiochemical Centre, Amersham Buchler, Braunschweig) was added as a marker in the subsequent purification steps. Both solutions, with and without marker, were treated identically (Table I). The solutions were stirred with 50 g of activated charcoal (Merck) over night and centrifuged at 35000 × *g* for 30 minutes. The supernatants were discarded and the sediments were resuspended and repelleted until the supernatants were colourless. The sediments were extracted by water-ethanol-ammonia 25% (48:50:2,v/v) and dried under vacuum to give light brown residues of 5.420 g ([^{14}C]tracer) and 5.411 g. The residues were dissolved in redist. water and applied to a weak basic anion exchange column (MN-2100, ECTEOLA Cellulose, Machery, Nagel & Co, D-516 Düren; 1.5 cm/100 cm). Fractions of 10 ml in redistilled water were collected and 1 ml aliquots were assayed for radioactivity in 10 ml of a dioxan scintillation cocktail*. The fractions containing radioactivity were pooled and freeze dried. Residues of 510 mg ([^{14}C]tracer) and 505 mg were obtained. In the next step the samples were submitted to a twofold preparative thin-layer chromatography on precoated silica gel plates (Merck, F, 254). For the first chromatography a mixture of *n*-butanol-methanol-ethylacetate-ammonia 25% (7:3:4:4, v/v) was used. The c-AMP zones were detected by UV-light and radioactive scanning (Bertold LB 2722).

The zones were scraped off, eluted with methanol and dried under vacuum to yield residues of 120.3 mg ([^{14}C]tracer) and 119.8 mg. For the second chromatography a mixture of isopropanol-methanol-water-ammonia 25% (10:1:2:5, v/v) was used. After detection as described above the c-AMP zones were eluted with boiling pyridine. The pyridine was removed under vacuum (23 °C; 0.01 Torr) and residues of 9.042 mg ([^{14}C]tracer) and 8.985 mg were obtained. The last purification step used ascending paper chromatography (Schleicher & Schüll, Nr. 2043 b. Mgl. 580 by 600 mm) in isobutyric acid-water-ammonia 25% (66:33:1, v/v; Rf.: 0.56). The chromatograms were dried in a vacuum oven and then scanned for UV-absorption and radioactivity (Metrawatt, LB 280). The paper strips containing c-AMP were eluted with methanol. The eluates were evaporated under vacuum and dried over phosphoruspentoxide at 105 °C under vaccum for 12 h. Residues of 1.160 mg ([^{14}C]tracer) and 1.158 mg were obtained. The residues were stored frozen at – 20 °C.

For GC-MS determination sample aliquots were silylated by the method of Lawson *et al.* [26]. To remove some turbidity the silylated samples were centrifuged and the supernatants were used for the determination. Synthetic c-AMP was also silylated to provide a standard solution. For IR-spectrometry

* The dioxan-cocktail contained 100 g naphtalene and 5 g PPO (2,5-diphenyloxazole) in 1 l dioxane.

Table I.

Purification	Tracer radioactivity		Sample dry weight (tracer added)		Sample dry weight (without tracer)	
	cpm × 10^5	[%]	[mg]	[%]	[mg]	[%]
Extract 3			4.15×10^5	100	4.15×10^5	100
tracer added: adenine-U-^{14}C-c-AMP; 0.5 µCi	6.97	100				
Charcoal	6.67	95.7	5420	1.3	5411	1.3
Anion-exchange-column	5.81	83.5	510	1.2×10^{-1}	505	1.2×10^{-1}
1. TLC	4.86	69.8	120.3	3.0×10^{-2}	118.8	2.8×10^{-2}
2. TLC	3.47	49.8	9.04	2.7×10^{-3}	8.98	2.7×10^{-3}
PC	3.15	45.2	1.16	2.7×10^{-4}	1.15	2.7×10^{4}

the pyridine was removed from the silylated samples and dried under vacuum (23 °C; 0.01 Torr). The residues were methanolysed under nitrogen, evaporated and dried under vacuum over phosphoruspentoxide (105 °C; 0.01 Torr, 12 h). The dried residues were boiled for 20 seconds in 100 µl of absolute pyridine, centrifuged and the supernatants were dried like before. The c-AMP residues were digerated twice with absolute diethylether centrifuged and the ether phases were discarded. The vacuum dried residues were applied to micro KBr discs to obtain the IR-spectra (Beckmann, Mikrolab 600 Computing IR Spectrophotometer).

Results and Discussion

The silylated genuine c-AMP content was quantitated by GC detection and refered to silylated c-AMP standards. The peaks of the silylated genuine c-AMP were examined in a mass spectrometer attached to the gaschromatograph. The c-AMP was identified by MS according to Lawson *et al.* [26] (Fig. 2). The IR-spectrum of the isolated c-AMP was in agreement with the spectrum obtained with

Table II.

	c-AMP-content	
	Minimal amount of c-AMP	Maximal amount of c-AMP
Per g fresh weight of the maize seedlings	382 pmol	710 pmol
Per g dry weight of the maize seedlings	4763 pmol	8853 pmol

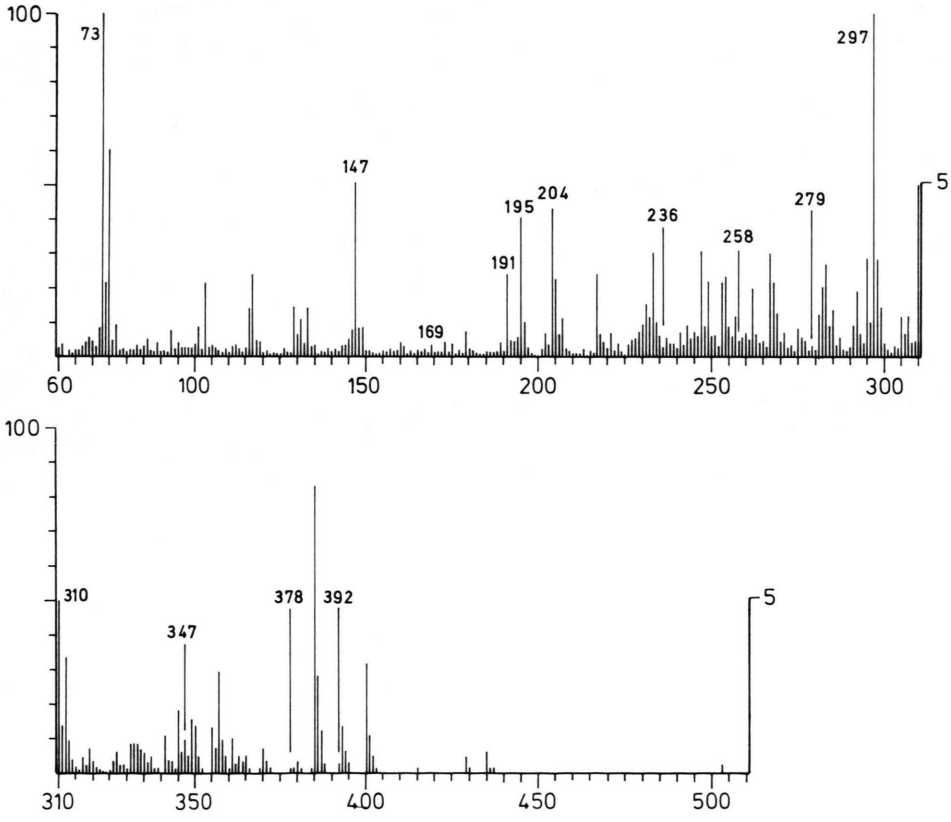

Fig. 2. According to Lawson *et al.* [26] the mass spectra of the trisilylated c-AMP were recorded on a Finnigan GC (9610)/MS Model 4000 instrument with sample introduction through the gas chromatographic inlet; 4 ft × 2 mm i.d. (glass) 3% OV 17 on Chromosorb W/AW-DMCS; M.P. 80/100 mesh, He-30 ml/Min. Inj. 240 °; temperature programmed at 15 °/min. from 100 ° to 250 °; glas-jet-seperator 240 ° and ion source temperatures 220 °; accelerating voltage −2 kV; ionizing energy 70 eV. The molecular ions *m/e* 545 and *m/e*-15 were only observed with sample introduction by direct inlet. The peaks *m/e* 310, 378 and 392 are unique to the 3′:5′-cyclic structure [26, 29].

synthetic c-AMP and with the IR-spectrum published by Lipkin *et al.* [27]. The main data of the IR-spectrum are: (KBr-discs) 3240–3040, 2960, 1670, 1415, 1235, 1055, 1010, 840 and 825 cm^{-1}. The c-AMP levels found are corrected for recovery of tracer [^{14}C]c-AMP which was added to the ethanol/water extract obtained from the freeze dried seedlings. For loss of silylated c-AMP during GC recording a minimum and a maximum amount of c-AMP was estimated (Table II). The concentration of c-AMP found is of the same order of magnitude as found in animal tissue (10^{-7} M) were c-AMP is regulatory [3]. Recently published data on the c-AMP content in different algae [28] (90–934 pmol/g fresh weight) and in Helianthus callus [17] (125 pmol/g fresh weight) are confirmed by the independent chemical method presented in this paper.

Completing this work MS data corresponding to c-AMP out of Phaseolus vulgaris were published by Newton *et al.* [29].

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