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Failure to Detect Cyclic 3′,5′-Adenosine Monophosphate in Healthy and Crown Gall Tumorous Tissues of *Vicia faba*[1]

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ABSTRACT

Attempts were made to provide proof for the occurrence of cyclic 3′,5′-adenosine monophosphate in healthy and crown gall tissues of *Vicia faba*. Although our purified extracts gave positive readings in the Gilman binding assay for cyclic AMP, they were not digested by a specific cyclic 3′,5′-adenosine monophosphate phosphodiesterase from beef heart. The extracts were digested, however, by a partially purified cyclic nucleotide phosphodiesterase from carrot tissue, which attacks both cyclic 2′,3′- and 3′,5′-nucleotides. The data indicate that the substances detected in the *V. faba* extracts are perhaps cyclic 2′,3′-nucleotides, a possible RNA degradation product.

Various laboratories have claimed they have detected cyclic 3′,5′-adenosine monophosphate in higher plant tissues (8, 11). Other reports have presented evidence for a change in cyclic AMP levels after treatment of plant tissues with gibberellic acid (7), auxin (9), or irradiation with far red (3).

A cyclic nucleotide phosphodiesterase has been isolated and partially purified from a number of plant tissues (4, 6, 10). The enzyme is quite different in many of its properties from the cyclic AMP specific phosphodiesterase of mammalian cells. Despite the work of Wood *et al.* (12), there is no clear proof for the presence of an adenyl cyclase in plant tissues.

This report is concerned with our attempts to provide unambiguous evidence for the occurrence of cyclic AMP as a normal constituent of higher plant tissue.

Broad bean (*Vicia faba* L.) plants were grown and maintained in a greenhouse at a temperature of 28 C ± 3. Two weeks after planting, one group of plants was inoculated by needle puncture at 12 separate sites in the first two internodes with a 48-hr culture of *Agrobacterium tumefaciens* (strain 806 obtained from Dr. T. T. Stonier, Manhattan College), a bacterium which incites autonomous plant tumors. The second group was untreated, but the first two internodes were marked for later reference. Four weeks after inoculation, both treated and untreated internodes were harvested and immediately frozen on Dry Ice. The tissue was then minced, suspended in an equal volume of 0.1 N HCl, and 10,000 dpm of cyclic H^3-AMP [4.2 mc/μmole] were added for an estimation of cyclic AMP recovery after purification. After homogenization for 1 min at high speed in a Waring Blendor, the extract was clarified by squeezing through four layers of cheesecloth. The filtrate was then boiled for 17 min, neutralized, and reboiled for 17 min. This step precipitated almost all the protein and polysaccharides. The pellet obtained by centrifugation (10,000*g* for 15 min) was saved for protein determination, while the supernatant extracts were acidified and purified by passage through 15 × 0.7 cm columns of Dowex AG-50-X8 and the cyclic AMP fraction eluted with 0.05 N HCl. This eluate was neutralized in 0.01 M tris-HCl, pH 7.4, and was applied to a second column (5 × 0.7 cm of Dowex-2 equilibrated with 0.01 M tris-HCl, pH 7.4), and the cyclic AMP fraction again eluted with 0.05 N HCl. Dowex-2 eluates were lyophilized; the residues were dissolved in 0.01 M tris-HCl, pH 7.4, and the pH was adjusted to 7.4 with 0.1 N NaOH. Recoveries were routinely 50 to 60%.

Duplicate samples from each treatment and from column blanks were assayed for cyclic AMP by the method of Gilman (2). The experimental results are expressed as picomoles of cyclic AMP/mg protein ± the standard error of the mean determined from three to seven separate experiments.

Both crown gall and healthy plant tissues yielded measurable values of cyclic AMP by the Gilman assay. Crown gall tumor tissue had significantly lower amounts of cyclic AMP than normal tissue (Table I). However, the Gilman assay is not entirely specific for cyclic 3′,5′-AMP since cyclic 2′,3′-AMP will also bind to the protein kinase.

To test the authenticity of the cyclic AMP from plant tissue, the samples were reacted with highly purified beef heart cyclic AMP phosphodiesterase for 5 hr at 30 C in 40 mM tris-HCl buffer, pH 7.4. This phosphodiesterase is specific for cyclic 3′,5′-AMP and will not degrade cyclic 2′,3′-AMP (1). After termination of the reaction by boiling, the treated samples were assayed by the Gilman procedure. Although the authentic sample of 1μM cyclic 3′,5′-AMP was 100% degraded, the plant samples were not digested since they gave approximately the same reading in the Gilman assay as before treatment (Table II).

Possibly some compound in the purified plant extracts was interfering with the action of the cyclic AMP phosphodiesterase. To test this hypothesis an aliquot of the plant extracts was mixed with an equal volume of authentic 1μM cyclic AMP and then incubated with autoclaved or active phosphodiesterase. When the digested combined samples were assayed by the Gilman procedure, the authentic cyclic AMP of the mixture was completely degraded, while the plant extract portion remained intact (Table III). The plant extract thus appears to have no inhibitor effect upon the action of beef heart cyclic AMP phosphodiesterase.

In previous studies we established the presence of a cyclic nucleotide phosphodiesterase in carrot tissue (6). Experiments

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on the specificity of this enzyme revealed that it was active on both cyclic 3′,5′-AMP and cyclic 2′,3′-AMP. We then repeated our digestion experiments, this time using carrot cyclic nucleotide phosphodiesterase with the same reaction conditions previously described except substituting 40 mM sodium acetate, pH 5.0, for tris-HCl buffer. The results of the Gilman assay in the reaction products show that the active component of the plant extract had been degraded (Table IV).

Thus the evidence suggests that the cyclic AMP in our purified plant extracts is cyclic 2′,3′-AMP. This conclusion becomes strengthened by the report of Lin and Varner (4) that

Table I. *Amount of Cyclic AMP Detected by the Gilman Assay in Purified Extracts from Normal and Tumor-bearing Internodes of Vicia faba*

The amount of cyclic AMP found in column blanks was subtracted from experimental data of each experiment. Values shown are the means ± SEM from seven separate experiments.

Tissue	Cyclic AMP
	pmoles/mg protein
Normal	60.6 ± 8.5
Tumor	26.0 ± 5.0

Table II. *Effect of Beef Heart Cyclic 3′,5′-AMP Phosphodiesterase on Cyclic AMP from Purified Extracts of Vicia faba*

Values shown are not corrected for recovery or protein content and are the means ± SEM from five separate experiments.

Extract	Cyclic AMP	
	Active phosphodiesterase	Boiled phosphodiesterase
	pmoles	
Normal tissue	4.4 ± 1.3	3.9 ± 0.5
Tumor tissue	2.6 ± 0.6	2.7 ± 0.4
1 μM cyclic AMP	0.0 ± 0.0	34.5 ± 2.6

Table III. *Effect of Purified Plant Extracts from Vicia faba on Beef Heart Cyclic 3′,5′-AMP Phosphodiesterase Digestion of Authentic Cyclic AMP*

Values shown are not corrected for recovery or protein content and are the means ± SEM from five separate experiments.

Sample	Cyclic AMP	
	Active phosphodiesterase	Boiled phosphodiesterase
	pmoles	
Normal tissue + equal volume of tris pH 7.4 buffer	2.4 ± 0.5	2.7 ± 0.6
Tumor tissue + equal volume of tris pH 7.4 buffer	1.3 ± 0.8	1.8 ± 0.4
1 μM cyclic AMP + equal volume of tris pH 7.4 buffer	0.0 ± 0.0	19.0 ± 1.7
Normal tissue + 1 μM cyclic AMP	2.1 ± 0.9	22.8 ± 1.1
Tumor tissue + 1 μM cyclic AMP	1.5 ± 0.6	21.5 ± 0.9

Table IV. *Treatment of Purified Plant Extracts of Vicia faba with Carrot Cyclic Nucleotide Phosphodiesterase*

Purified plant extracts were treated exactly as described in the text for the beef heart cyclic AMP phosphodiesterase experiments. Values shown are not corrected for recovery or protein content and are the means ± SEM for three separate experiments.

Sample	Cyclic AMP	
	Active phosphodiesterase	Boiled phosphodiesterase
	pmoles	
Normal tissue	0.0 ± 0.0	4.0 ± 0.5
Tumor tissue	0.0 ± 0.0	2.5 ± 0.7
1 μM cyclic 2′,3′-AMP	0.0 ± 0.0	35.7 ± 4.8

plant RNases yielded a cyclic 2′,3′-nucleotide product. The difference we observed between the cyclic AMP content of tumorous and normal tissue probably reflects a lower cyclic nucleotide pool in tumor tissue due to its increased RNA synthesis (5).

The results presented in this communication, together with our failure to detect plant adenyl cyclase under a variety of different assay conditions (pH, metal ions, ATP regenerating system, ATP analogs, reaction with plant hormones such as IAA, GA_3, and cytokinen), suggest that in *Vicia faba* at least cyclic 3′,5′-AMP is not a metabolic constituent.

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LITERATURE CITED

1. Butcher, R. W. and E. W. Sutherland. 1962. Adenosine 3′,5′-phosphate in biological materials: Purification and properties of cyclic 3′,5′-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3′,5′-phosphate in human urine. J. Biol. Chem. 237: 1244–1251.
2. Gilman, A. G. 1970. A protein binding assay for adenosine 3′,5′-cyclic monophosphate. Proc. Nat. Acad. Sci. U.S.A. 67: 305–312.
3. Janistyn, B. 1972. 3-Indole acetic acid induced efflux of nucleotides concomitant with an increased synthesis of adenosine-3′,5′-monophosphate (C-AMP) in maize coleoptile sections. Z. Naturforsch. 27: 273–276.
4. Lin, P. and J. E. Varner. 1972. Cyclic nucleotide phosphodiesterase in pea seedlings. Biochim. Biophys. Acta 276: 454–474.
5. Niles, R. M. and M. S. Mount. 1973. Chromatin-directed ribonucleic acid synthesis: A comparison of chromatins isolated from healthy, avirulent *Agrobacterium tumefaciens* inoculated, and crown-gall tumor tissues of *Vicia faba*. Plant Physiol. 52: 368–372.
6. Niles, R. M. and M. S. Mount. 1974. Partial purification and properties of cyclic nucleotide phosphodiesterase from carrot. Phytochemistry. In press.
7. Pollard, C. J. 1970. Influence of gibberellic acid on the incorporation of 8-^{14}C adenosine into adenosine 3′,5′-cyclic phosphate in barley aleurone layers. Biochim. Biophys. Acta 201: 511–514.
8. Raymond, P., A. Narayanan, and A. Pradet. 1973. Evidence for the presence of 3′,5′-cyclic AMP in plant tissues. Biochim. Biophys. Res. Comm. 53: 1115–1121.
9. Solomon, D. and J. P. Mascarenhas. 1971. Auxin-induced synthesis of cyclic 3′,5′-adenosine monophosphate in *Avena* coleoptiles. Life Sci. 10: 879–883.
10. Vandepente, J., R. C. Huffaker, and R. Alvarez. 1973. Cyclic nucleotide phosphodiesterase activity in barley seeds. Plant Physiol. 52: 278–282.
11. Wood, H. N. and A. C. Braun. 1973. 8-bromoadenosine 3′,5′-cyclic monophosphate as a promoter of cell division in excised tobacco pith parenchyma tissue. Proc. Nat. Acad. Sci. U.S.A. 70: 447–450.
12. Wood, H. N., M. C. Lin, and A. C. Braun. 1972. The inhibition of plant and animal adenosine 3′,5′-cyclic monophosphate phosphodiesterases by a cell-division-promoting substance from tissues of higher plant species. Proc. Nat. Acad. Sci. U.S.A. 69: 403–406.