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OCCURRENCE OF ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE IN PLANT TISSUES

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Abstract—A procedure is described which unequivocally demonstrates the presence of adenosine 3′:5′-cyclic monophosphate in *Phaseolus vulgaris*. Its concentration was determined spectrophotometrically at 2·6–9·2 nmol g^{-1} of tissue (dry wt) for 6-day-old seedlings and about one-tenth of this in 13-day-old plants.

ALTHOUGH much information is available relating to the occurrence of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in animal tissues, there is still relatively little concerning plant tissues. Much of the evidence for the presence of cyclic AMP in tissues of higher plants is of a presumptive nature, derived from observed physiological effects of exogenously supplied cyclic AMP (see e.g. Ref. 1).

Attempts have been made to demonstrate directly the presence of cyclic AMP in plant tissues. For example, incorporation by plant tissue of radioactivity from [U-^{14}C]-adenine into a chromatographic fraction which elutes at the same point as cyclic AMP has been cited as evidence.[2] Saloman and Mascarenhas[3] extended this approach by using $Ba(OH)_2$ to produce from the suspected cyclic AMP, substances having chromatographic behaviour similar to that of 3′AMP and 5′AMP in two chromatographic solvent systems. Using the same two systems, the product obtained by treating the original material with HNO_2 was shown to have a similar R_f to 3′:5′-cyclic IMP. Narayanan *et al.*[4] used a method, based on that of Johnson,[5] involving use of a cyclic AMP phosphodiesterase preparation to produce AMP from suspected cyclic AMP; AMP was estimated enzymically.

Most reports concerning the occurrence of cyclic AMP in plant tissues can be criticized in that the chromatographic or enzymic data upon which they are based are equivocal. Using TLC or PC, it is often difficult to separate isomeric nucleotides such as 2′:3′-cyclic AMP and 3′:5′-cyclic AMP, or, 2′, 3′, and 5′-AMP and simply to record a compound having similar chromatographic properties to cyclic AMP in one or two solvent systems is not enough. Identification by enzymic methods can only be relied upon when the enzyme has been demonstrated to have an absolute specificity for its substrate. In the present work, the aim has been to develop methods for the extraction, unambiguous identification, and estimation of cyclic AMP in plant tissues.

Seeds of *Phaseolus vulgaris* cv. The Prince (Bees Ltd., Sealand, Cheshire) were soaked in

[1] SALOMAN, D. and MASCARENHAS, J. (1971) *Z. Pflanzenphysiol.* **65**, (1971) 385.
[2] POLLARD, C. J. (1970) *Biochim. Biophys. Acta* **201**, 511.
[3] SALOMAN, D. and MASCARENHAS, J. (1971) *Life Sci.* **10**, 879.
[4] NARAYANAN, A., VERMEERSCH, J. and PRADET, A. (1970) *Compt. Rend.* **t271D**, 2406.
[5] JOHNSON, R. A., HARDMAN, J. G., BROADUS, A. E. and SUTHERLAND, E. W. (1970) *Anal. Biochem.* **35**, 91.

running water for 24 hr and germinated in moist compost at 25°. Seedlings (6-days-old) were immersed in liq. N_2 and freeze-dried: samples (200 g) of the freeze-dried powder were homogenized in an ice-cold monophasic mixture of MeOH–$CHCl_3$–M-formic acid (12:5:3).[6] Care was taken to obviate a rise in temp. during homogenization.

The homogenate was filtered through a sintered glass Büchner funnel (por. 2) and the residue re-extracted three times by resuspension in the MeOH–$CHCl_3$–formic acid mixture (MCF). Chloroform and water was added to the combined filtrate and washings until the ratio of MeOH–$CHCl_3$–formic acid became 12:11:10, by vol, at which composition the system became biphasic. After separating the phases by centrifuging at 2000 *g* for 5 min, the aqueous phase was evaporated to dryness under reduced pressure and the residue redissolved in *ca.* 100 ml H_2O. The pH of the extract was adjusted to 4·0 with HOAc, 750 mg of prepared Norit OL charcoal[7] were added and the suspension stirred continuously for 30 min at 4°. By using a sintered glass Büchner funnel (por. 2) containing a layer (5–10 mm depth) of Hyflo Super-cel, the charcoal was filtered from the suspension and washed 3 × by resuspension in H_2O (3 × 20 ml). Elution was effected in a similar way using 3 × 30 ml portions of ethanolic ammonia.[7]

The combined eluates from the charcoal adsorption procedure were evaporated to dryness under reduced pressure and the residue redissolved in *ca.* 500 ml H_2O. After adjusting the pH to 7·0 with 0·1 M KOH, and using the method of Bradham and Woolley,[8] the extract was fractionated by ion-exchange chromatography, first on a column of Dowex-2 (X8; Cl^-; 200–400 mesh) then on Dowex-50 (X4; H^+; 200–400 mesh). It was found necessary to modify the published procedure, using M formate in place of 0·3 M formate for the final elution of the Dowex-50 column. The eluate was evaporated to dryness under reduced pressure, redissolved in EtOH (50%) and used directly for TLC together with authentic samples of cyclic AMP. Layers of either cellulose or silica gel G were used in conjunction with solvent *d* (Table 1).

Developed plates were sprayed with an ethanolic solution of 2′,7′-dichlorofluorescein (0·01% w/v) containing a few drops of conc. NH_3[9] and spots were visualized in UV light. Areas of interest were scraped from the TLC plate, eluted with aq. EtOH (50%) and the

TABLE 1. ELECTROPHORETIC BUFFERS AND CHROMATOGRAPHIC SOLVENTS USED IN IDENTIFYING 3′:5′-CYCLIC AMP

Electrophoretic buffers (Paper–Whatman 3MM)	TLC solvents (Adsorbent–Silica Gel G)	PC solvents (Paper–Whatman No. 1)
a $Na_2B_4O_7$ (50 mM; pH 9·2)	*d* *iso*PrOH–NH_4OH–H_2O (14:3:3)	*g* *n*-PrOH–NH_4OH–H_2O (6:3:1)
b HOAc–HCO_2H (pH 2·0)[10]	*e* *iso*PrOH–MeOH–NH_4OH–H_2O (11:1:6:2)	*h* *n*-PrOAc–HCO_2H (90%) (11:5:3)
c Ammonium acetate (250 mM; pH 3·6)	*f* EtOH–250 mM $Na_2B_4O_7$ (71:29; pH 8 0)	*i* EtOH–250 mM $Na_2B_4O_7$ (71:29, pH 8 0)
		j EtOH–100 mM $Na_2B_4O_7$ (63:32, pH 9 0)
		k EtOH–100 mM $Na_2B_4O_7$ (67:33, pH 10 0)

[6] BIELESKI, R. L. (1964) *Anal. Biochem.* **9**, 431.
[7] BROWN, E. G. (1962) *Biochem. J.* **85**, 633.
[8] BRADHAM, L. S. and WOOLLEY, D. W. (1964) *Biochim. Biophys. Acta* **93**, 47.
[9] DUNPHY, P. J., WHITTLE, K. J. and PENNOCK, J. F. (1965) *Chem. Ind. (London)* 1217.

eluate applied to a paper strip for examination by high voltage electrophoresis in buffer *a* (Table 1) using a voltage gradient of 20 V/cm. After 1·5–2·0 hr the band migrating towards the anode at the same rate as a marker of authentic cyclic AMP, was removed and eluted with aq. EtOH (50%). The eluate was concentrated by evaporation and rechromatographed on a TLC plate as before. This time, however, dichlorofluorescein was not used to facilitate visualization of the spots. Finally the spot believed to be cyclic AMP was eluted in EtOH (50%) and the eluate examined by further TLC, PC and high voltage electrophoresis using the systems listed in Table 1. The compound obtained was also examined spectrophotometrically at several pH values and its identification as an adenosine derivative confirmed. In each of the 8 solvent systems detailed in Table 1, a single spot was obtained which cochromatographed with an authentic sample of cyclic AMP. Similarly, electrophoresis in buffers, *a*, *b* and *c* produced bands migrating with the reference band of cyclic AMP. Solvent *d* was shown to distinguish clearly between the 3′:5′- and the 2′:3′-cyclic AMP isomers.

The % recovery of [U-^{14}C]-cyclic AMP which had been added to the initial extraction medium was assessed at each stage of the procedure. This assessment of the method as a whole was repeated several times and showed an overall recovery of 50–60%. The major part of the loss was attributable to, (a) the initial extraction procedure and subsequent recovery of cyclic AMP from the $CHCl_3$–MeOH–formic acid mixture, and (b) the charcoal adsorption and elution step.

Using the procedure outlined and determining the product spectrophotometrically,[8] tissues of several plants were examined for their contents of cyclic AMP. Mature leaves of *Rumex sanguineus*, *Tussilago farfara* and *Ilex aquifolium* proved difficult to extract and cyclic AMP could not be detected in 10-day-old seedlings of *Vicia faba*. Allowing for recovery losses, values obtained for *Phaseolus vulgaris* indicate tissue levels of between 2·6 and 9·2 nmol g^{-1} of tissue (dry wt) in 6-day-old plants and about one-tenth of this value in 13-day-old plants.

These findings unequivocally demonstrate the presence of cyclic AMP in a higher plant and suggest that the concentration of this nucleotide is at its highest, on a dry wt basis, during the early stages of growth and development. Present work is evaluating the applicability to plant extracts of the 'binding-protein' method,[11,12] used for determining cyclic AMP concentrations in animal tissues.

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[10] EFRON, M. L. (1959) *Biochem. J.* **72**, 691.
[11] GILMAN, A. G. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 305.
[12] BROWN, B. L., ALBANO, J. D. M., ELKINS, R. P. and SGHERZI, A. M. (1971) *Biochem. J.* **121**, 561.