Isolation, Characterization and Distribution of Adenosine 3':5'-Cyclic Monophosphate from *Pinus radiata*

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Cyclic AMP was extracted in 0.1 M-HCl from tissues of *Pinus radiata* and purified by gel filtration on Sephadex G-10, and chromatography on Dowex AG1 (X2) and polyethyleneimine-cellulose in two separate solvent systems. Presumptive cyclic AMP from 10 kg batches of pine needles was characterized by countercurrent distribution in the presence of cyclic [8-³H]AMP. Statistical analysis of the curves for radioactivity and mass (determined by the Gilman competitive-binding assay) showed that the fit of the curves was highly significant for seven degrees of freedom. The distribution of cyclic AMP within *P. radiata* and various other plant tissues was determined by the Gilman procedure. The results suggest that there is no relationship between variations in cyclic AMP concentrations and the known function of the tissue in which it was measured.

Cyclic AMP plays an essential part in metabolic control processes in bacteria and animals where it has been shown to be involved in such diverse activities as hormone action (for reviews see Posternak, 1974; Jost & Rickenberg, 1971; Hardman *et al.*, 1971; Robison *et al.*, 1968), catabolite repression (Pastan & Perlman, 1970) and cell division (Burger *et al.*, 1972).

Despite the occurrence of this nucleotide in a wide range of biological tissues, reports of the presence of cyclic AMP in higher plants have been subject to debate because of inadequate characterization (Lin, 1974). Investigations of cyclic AMP in higher plants used a radioimmunoassay (Ames, 1976), the Gilman (1970) competitive binding assay (Isherwood & Ring, 1977), and a combination of chromatography and enzymic assays (Ashton & Polya, 1977).

Evidence is now presented for the characterization by countercurrent distribution of cyclic AMP from *Pinus radiata* and its concentration within tissues of that species.

Experimental

P. radiata seed was donated by the Forest Research Institute, Rotorua, New Zealand.

Cyclic [8-³H]AMP (30 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Purity was checked by t.l.c. in the systems described below. PEI (polyethyleneimine)-cellulose, PPO (2,5-diphenyloxazole), dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene], cyclic AMP and 3':5'-cyclic AMP phosphodiesterase (EC 3.1.4.17), from bovine heart, were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. One unit is

defined as the amount of enzyme that will convert 1 μ mol of cyclic AMP per min into AMP. PEI-cellulose plates were bought from Schleicher und Schüll, Dassel, Germany. Sephadex and Blue Dextran 2000 were obtained from Pharmacia, Uppsala, Sweden, and Dowex resin (AG1; X2; 200–400 mesh; Cl⁻ form) was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Cyclic AMP-binding protein was prepared from bovine skeletal muscle by the procedure of Walsh *et al.* (1968), and stored in 5 ml quantities at -15°C.

Methods

Biological material. Pine needles in good condition were collected from 15-year-old trees judged free of disease by foresters at Woodhill Forest, Helensville, Auckland, and batches (10 kg) were extracted. Needles were washed four times with water.

Seedlings were grown from seed treated with HgCl₂ (0.05%, w/v) for 2 min to kill surface contaminants. The seed was then washed thoroughly in sterile water and chilled overnight at 4°C to ensure even germination, before sowing in sterilized potting-mix (peat/pumice/vermiculite, 1:2:1, by vol.). Seedlings were raised in controlled-environment cabinets (25°C, 8 h darkness/16 h light, 3 $\times$ 10⁻⁴ lx), and were given fresh distilled water daily.

Needles described as old in Table 3 were taken from near the trunk of 15-year-old trees. Young needles were taken from the tips of branches of 5-year-old trees. Tissue described as spring shoots comprised the terminal 15–20 cm of branches from 5-year-old trees, and included the apical bud. Seedlings were 3 weeks old when cyclic AMP was extracted.

Apium graveolens (L.) var. dewhurst (celery), *Coleus*

blumei (Benth.) var. *verschaffeltii* and *Cucumis sativus* (L.) var. *anglicus* (cucumber) were raised in potting-mix (peat/pumice, 43:7, v/v) under glasshouse conditions. When harvested, celery plants were 12 weeks old, coleus plants were about 2 years old and cucumber seedlings were 3 weeks old. *Spirodela oligorrhiza* (Kurz.) was raised hydroponically in 5-litre flasks under sterile conditions. Mature plants of *Lupinus arboreus* (Sims.) were collected from Woodhill Forest, Helensville, Auckland, New Zealand. Wheat germ was obtained from the Northern Rolling Mills, Auckland, New Zealand.

All tissue was washed before extraction, as described above.

Extraction of cyclic AMP. Tissue from *P. radiata* was extracted in HCl (0.1M at 100°C for 2 min) to which 50×10^6 d.p.m. of cyclic [^3H]AMP had been added. The extract was adjusted to pH 7 with 6M-NaOH and the preparation was centrifuged at 10000g for 15 min at 4°C in a Sorvall RC2B instrument with GSA or SS34 rotors. The pellet was washed twice with water and the combined supernatant fractions were extracted three times with ethyl acetate equal in volume to the aqueous phase.

Ion-exchange chromatography. Dowex AG1 (X2; 200–400 mesh; Cl^- form) was washed with 1M-HCl and then water; a column (2.5cm $\times$ 25cm) was prepared. The extract (pH 6.5) was applied to the column and batchwise elution was carried out with increasing concentrations of HCl, i.e. 10 mM, 50 mM, 0.1 M, 0.5 M and 1.0 M. The nucleotide was eluted within the range 0.1–0.5 M.

Gel filtration. Sephadex G-10 was soaked overnight in water and fines were removed by decantation ($\times 3$). A column (2.5cm $\times$ 25cm) was packed and the void volume (V_0) determined with Blue Dextran 2000. Extracts (1–2 ml) were applied to the column and eluted with water; fractions (2 ml) were collected at fixed time intervals.

Chromatography of cyclic [^3H]AMP on PEI-cellulose. (a) T.l.c. The purity of cyclic [^3H]AMP was checked as a routine by t.l.c. on PEI-cellulose. Labelled nucleotide (50 nmol) in 0.2 ml was applied in duplicate to a chromatographic plate with authentic non-radioactive cyclic AMP. The chromatogram was first developed in 0.3M-LiCl, dried, rotated through 90° and redeveloped in 0.5M-ammonium acetate/propan-2-ol (1:4, v/v). The carrier was located by u.v. absorption and the radioactive content of the spot was measured by liquid-scintillation spectrometry. Some 96% of cyclic [^3H]AMP migrated with the authentic nucleotide and the labelled compound was used without further purification.

(b) Column chromatography. PEI-cellulose was soaked for 16 h in the solvent to be used for elution. Fines were removed by decantation ($\times 3$), and columns (1cm $\times$ 10cm) were poured. Extracts of tissue from *P. radiata*, which contained cyclic

[^3H]AMP, were applied in volumes of 0.5–1.0 ml. The nucleotide was eluted in a linear gradient of increasing salt concentration (LiCl or ammonium acetate), and 2 ml fractions were collected for analysis.

Determination of cyclic AMP. The procedure used was that described by Gilman (1970). It is based on competition between labelled and unlabelled cyclic AMP for binding sites on the binding protein. Charcoal coated with bovine serum albumin was used to adsorb free cyclic AMP (Brown *et al.*, 1971).

Standard cyclic AMP (50 μl) or plant extract (50 μl) to be assayed was placed in disposable polystyrene tubes (0.7cm $\times$ 3.5cm); 50 μl of cyclic [^3H]AMP (2 pmol; 99000 d.p.m.) and 50 μl of binding protein (22 μg) were added. After tubes were incubated for 2 h at 0–4°C a suspension of albumin-coated charcoal (100 μl) was added to each tube. The mixture was stirred and then centrifuged at 2000g for 10 min in an MSE Mistral 4L centrifuge. A portion (100 μl) of the supernatant fraction (containing protein-bound cyclic AMP) was taken for determination of radioactivity.

To correct for binding inhibitors or promoters present in plant extracts, reference solutions of cyclic AMP were prepared in plant extracts from which endogenous nucleotide had been removed by incubation with cyclic AMP phosphodiesterase from bovine heart. Plant extract was diluted (1:1) with 330 μunits of enzyme in Tris/HCl buffer (20 mM, pH 7.5) containing 4 μmol of MgCl_2/ml . The mixture was incubated at 37°C for 90 min.

The extract to be tested was similarly diluted with the same buffer without phosphodiesterase. After incubation, both extracts were placed in a boiling-water bath for 2 min.

Three dilutions of each sample were assayed, to ensure that at least two lay within the range of reference solutions of cyclic AMP (0–8 pmol). If only one dilution was in this range, the assay was repeated with more appropriate concentrations of the sample. If two dilutions failed to show the expected ratio of cyclic AMP concentration, this suggested that some compound other than cyclic AMP was bound in the assay. Further purification of the plant extract was then undertaken before repeating the measurements.

G.l.c. Trimethylsilyl derivatives of authentic and presumptive cyclic AMP were prepared as described by Lawson *et al.* (1971), with an incubation time of 1 h. Retention times were determined in a Pye-Unicam gas chromatograph series 104 fitted with a flame-ionization detector, and a coiled glass column (1.5m $\times$ 6mm internal diam.) packed with 1% OV 17 on Chromosorb W. The carrier gas was N_2 , attenuation was set at 50×10^2 , and the oven temperature was programmed to rise at 10°C/min from 100 to 260°C.

Determination of radioactivity. Radioactivity was

Fig. 1. Temperature-dependence of K_D of cyclic AMP in 2-methylpropan-1-ol/water

Cyclic AMP (10mg) was dissolved in 10ml of 2-methylpropan-1-ol/water (1:1, v/v) and adjusted to pH 7.0 with 1M-NaOH. The same solvent system (10ml) was added and the system was inverted 50 times in a separating funnel. The phases were left to separate and each was placed in a tared container. The solutions were evaporated overnight at 45°C and the amount of nucleotide in each phase was determined after drying to constant weight. Each point on the graph represents the mean of at least three determinations. Standard error bars are shown.

determined by liquid-scintillation counting in 5ml of toluene/Triton X-100 (2:1, v/v) containing PPO (0.5% w/v) and dimethyl-POPOP (0.01% w/v) in a Packard liquid-scintillation spectrometer model 2650 programmed for automatic discriminator setting. At this setting, channel widths are adjusted for maximum efficiency determined for each vial against an external standard. ^3H was counted at 33% efficiency. The accuracy of radioactivity counting was above 2%.

Determination of partition coefficient. Three samples of cyclic AMP (10mg) were dissolved in 10ml of 2-methylpropan-1-ol-saturated water, neutralized with 1M-NaOH and extracted with an equal volume of water-saturated 2-methylpropan-1-ol in a separating funnel of 50ml capacity. After 50 inversions (Craig & Craig, 1950) the phases were partitioned and each was transferred to a weighed container. Solvent was evaporated overnight at 45°C, and the mass of cyclic AMP in each phase was determined. As the countercurrent apparatus was in a non-thermostatically controlled room, a curve of partition coefficient against temperature was constructed (Fig. 1). Theoretical values of partition coefficients for cyclic AMP were read from this curve.

Countercurrent distribution. Countercurrent distribution was carried out in a Quickfit steady-state

apparatus calibrated for equal volumes of upper and lower phases (25ml). The system was 2-methylpropan-1-ol/water (1:1, v/v), which was left to equilibrate at room temperature with the apparatus.

Conditioning tubes (10–16) were run before addition of the sample. Agitation time was set at 3.5 min (56 inversions) and settling time, 4 min. Only upper phase was transferred. On completion of the distribution, the contents of selected tubes were removed, rinsed with 5 ml of distilled water and tube contents plus rinsings were freeze-dried. Residues were dissolved in 5 ml of water and $3 \times 100 \mu\text{l}$ samples were taken for determination of radioactivity. At the same time, $3 \times 50 \mu\text{l}$ samples were removed for determination of cyclic AMP by protein-binding assay. Random numbers were assigned to samples before analysis, and curves were fitted by the method of Purdy *et al.* (1965).

Freeze-drying. Samples were shell-frozen in 600ml sterile bottles at -40°C for 10 min and then subjected to 93 Pa pressure in a Stokes Freeze Drier for 72 h. Bottles were sealed with air-tight stoppers and stored at room temperature ($18 \pm 3^\circ\text{C}$) until needed.

Results and Discussion

Purification of cyclic AMP from *P. radiata*

In view of criticisms of published procedures for the purification and characterization of cyclic AMP from higher plants (Lin, 1974; Amrhein, 1974; Bressan *et al.*, 1976; Trewavas, 1976), a scheme was devised to ensure maximum yield for unambiguous identification of the nucleotide.

In preliminary experiments with seedlings, acid extraction and extraction with 1M-AMP gave the best recovery of added cyclic $[8\text{-}^3\text{H}]\text{AMP}$, but the former procedure was adopted to obviate difficulties with AMP during purification.

After neutralization, the extract was centrifuged (10000g for 10 min at 4°C), the supernatant fraction was extracted three times with ethyl acetate equal in volume to the aqueous phase, and the aqueous layer was chromatographed successively on four columns: Dowex AG1 (Table 1); Sephadex G-10 (Fig. 2); and two columns of PEI-cellulose (Figs. 3a and 3b).

Characterization of cyclic AMP

Presumptive cyclic AMP isolated from *Pinus* seedlings by the chromatographic procedures described above was treated as described by Lawson *et al.* (1971), but the results were inconsistent and the material was impure on g.l.c. Similar attempts to form the trimethylsilyl ether derivative(s) of the authentic nucleotide were also unsuccessful, so we resorted to countercurrent distribution, with the aim of computing the fit of the distribution curves for authentic

Table 1. Ion-exchange chromatography of cyclic [^3H]-AMP on Dowex AG1 (X2) resin

An extract to which cyclic [^3H]AMP was added was prepared from 60g of *P. radiata* seedlings, and centrifuged as described in the Experimental section. The supernatant fraction was extracted three times with ethyl acetate; the aqueous layer was concentrated under reduced pressure, and applied to a column (2.5cm $\times$ 25cm) of Dowex AG1 resin (X2; 200–400 mesh; Cl^- form) previously washed with 1M-HCl, then water. Batchwise elution was carried out with 50ml volumes of HCl in increasing concentration. Radioactivity was determined by liquid-scintillation spectrometry. The mean of duplicate experiments is shown; variation between results was less than 8%.

HCl concentration (mm)	$10^{-6} \times$ Cyclic [^3H]AMP recovery (d.p.m.)
0	1.9
5	0.2
25	0.0
50	15.5
100	3.9

Fig. 2. Gel filtration of cyclic AMP from *P. radiata*. An extract of needles from *P. radiata* to which was added cyclic [^3H]AMP was chromatographed on Dowex AG1 (X2) and eluted in HCl. The appropriate fractions were pooled, neutralized and concentrated under reduced pressure at 40°C and 1–2ml was applied to a column (2.5cm $\times$ 25cm) of Sephadex G-10. Gel filtration was carried out in water at room temperature. Full details are given in the Experimental section.

cyclic [^3H]AMP and the compound isolated from *Pinus* tissue.

No published values for the partition coefficient of cyclic AMP could be found, so this was determined for the system 2-methylpropan-1-ol/water (1:1, v/v); a theoretical distribution was calculated.

Fig. 3. (a) and (b) Chromatography of extract of *P. radiata* on PEI-cellulose

Pooled fractions obtained by gel filtration of *Pinus* extract on Sephadex G-10 were concentrated and applied in 0.5–1.0ml to a column (1cm $\times$ 10cm) of PEI-cellulose prepared in water. Presumptive cyclic AMP was eluted in a linear gradient of LiCl (0–2.0M) in water at room temperature. Recovery of the nucleotide was monitored by determination of cyclic [^3H]AMP added to the initial extract. Pooled fractions were concentrated and chromatographed on a second column (1cm $\times$ 10cm) of PEI-cellulose prepared in propan-2-ol/water (1:1, v/v). The nucleotide was eluted in a linear gradient of ammonium acetate (0–0.5M) in propan-2-ol/water (1:1, v/v). Details are given in the Experimental section.

Presumptive cyclic AMP, extracted from pine needles, chromatographed on Dowex AG1 (X2) followed by gel filtration on Sephadex G-10, was distributed in 100 transfers, but the results were disappointing in that a normal distribution was not obtained. The peak tube did not coincide with that for a theoretical

Fig. 4. Countercurrent distribution of presumptive cyclic AMP from *P. radiata* and cyclic [8-³H]AMP
The *K_D* of cyclic AMP was determined for the system 2-methylpropan-1-ol/water (1:1, v/v). An extract of *P. radiata*, which had been successively chromatographed on Dowex AG1 (X2) and Sephadex G-10, was subjected to a 100-transfer countercurrent distribution in 2-methylpropan-1-ol/water (1:1, v/v). The contents of tubes 14–20 (inclusive) were pooled, freeze-dried and redistributed in 160 transfers in fresh solvent system. The mass of presumptive cyclic AMP (□) was measured by a modified Gilman procedure, and radioactivity (▲) was determined by liquid-scintillation spectrometry. Details are given in the Experimental section.

Table 2. Countercurrent distribution of presumptive cyclic AMP from *P. radiata* and cyclic [8-³H]AMP: statistical analysis of data

An acid extract of pine needles to which was added cyclic [8-³H]AMP was chromatographed on columns of Dowex AG1 (X2) and Sephadex G-10. Fractions containing presumptive and labelled nucleotide were pooled, freeze-dried and subjected to a 100-transfer countercurrent distribution in the system 2-methylpropan-1-ol/water (1:1, v/v). The contents of tubes 14–20 (inclusive) were combined, freeze-dried and redistributed for 160 transfers as described in the text. The correlation between the deviations from the curve is 0.77, which is not significant for seven degrees of freedom. Abbreviations: *K_D*, partition coefficient, calculated from the distribution; $\bar{p}$, peak tube calculated from *K_D*.

	Unlabelled cyclic AMP	Cyclic [8- ³ H]AMP
<i>K_D</i>	0.145	0.141
Mean tube no.	20.3	19.8
S.D.	4.2	4.2
$\bar{p}$	20.3	19.8

distribution of cyclic AMP in 2-methylpropan-1-ol/water, and this was attributed to the presence in the first few tubes of large quantities of salt, presumed

Table 3. Distribution of cyclic AMP within tissues of *P. radiata* and various other plant species

Tissue extracts were prepared as described in the text and chromatographed on columns (2.5cm×25cm) of Dowex AG1 (X2). The content of cyclic AMP in the eluate fractions was determined by the Gilman (1970) procedure and corrected for recovery of radioactive nucleotide. The maximum error was calculated from that intrinsic to the assay multiplied by the dilution factor. Values are means±s.e.m. for the numbers of determinations given in parentheses.

Plant tissue	Cyclic AMP (pmol/g fresh wt.)	Maximum error of assay (pmol/g fresh wt.)
<i>P. radiata</i>		
Old needles	38.4±0.5 (3)	0.5
Young needles	9.6±0.4 (2)	0.2
Spring shoots	<1.7 (3)	0.1
Male flowers	62.7±1.9 (3)	1.1
Female flowers	<2.4 (2)	0.6
Seed unimbibed	16.8±0.5 (3)	0.6
<i>A. graveolens</i> (celery)		
Leaves	<2.8 (2)	0.9
<i>C. blumei</i>		
Leaves	<1.5 (2)	0.4
Stems	<3.7 (2)	1.1
<i>C. sativus</i> (cucumber)		
Leaves	<6.1 (2)	0.9
Stems	<8.0 (2)	0.7
Roots	53.3±1.7 (2)	0.6
<i>S. oligorrhiza</i>		
Whole plant	11.0±0.7 (2)	0.2
<i>L. arboreus</i> (yellow lupins)		
Roots	<1.2 (2)	1.5
Root nodules	41.2±0.7 (2)	1.4
<i>Triticum sativum</i> (wheat)		
Embryos	11.1±0.6 (2)	0.1

to have been formed by neutralization of the acid extract. (Subsequent experiments showed that the salt could be removed by a second gel-filtration step on Sephadex G-10.) The contents of tubes 14–20 (inclusive) were pooled, freeze-dried and redistributed in fresh solvent system for 160 transfers, and the results are depicted in Fig. 4. Statistical analysis of the data for mass (determined by the Gilman assay) and radioactivity were computed by the method of Purdy *et al.* (1965). Results (Table 2) show that the unknown material behaved as authentic cyclic AMP in this system.

Presence and distribution of cyclic AMP in *P. radiata*

Several tissues of *P. radiata* were extracted with the aim of determining the best source for large-scale purifications of cyclic AMP. It was also hoped that knowledge of the distribution of this nucleotide might provide an indication of its role in plant metabolism.

Table 4. *Distribution of cyclic AMP in various plant tissues as reported by previous investigators*

Plant tissue	Cyclic AMP (pmol/g fresh wt.)	Reference
Oat coleoptiles	7-11	Ownby <i>et al.</i> (1975)
Lettuce seedlings	2-6	Bressan <i>et al.</i> (1976)
Tobacco seedlings	15-25	Ames (1976)
<i>Kalanchoe</i> and <i>Agave</i>	1-6	Ashton & Polya (1977)
Potato tubers	3-30	Isherwood & Ring (1977)

The Gilman assay procedure was used to determine the respective amounts of cyclic AMP in the tissue of *P. radiata*. For the purpose of comparison various other plant tissues were also analysed. The results summarized in Table 3 suggest that there is no relationship between variations in cyclic AMP concentration and the known functions of these tissues. In the main, the quantities shown in Table 3 agree with those reported by other workers (Table 4).

The introduction of exogenous compounds from contaminant sources is a major hazard in extractions of this type where non-sterile tissue was used. However, a bacterial origin can be discounted. The most likely site of infection was the surface of the needles used for extraction, and these were thoroughly washed.

Fungal pathogens present a more plausible alternative. Reports of cyclic AMP determinations in fungi are scarce, but workers with *Mucor* spp. report 25-30 pmol/mg of protein (Larsen & Sypherd, 1974), and 8.4-36.6 pmol/mg of protein (Paveto *et al.*, 1975). These concentrations would necessitate fungal infections of at least 64 g/10 kg of tissue, and it is extremely unlikely that such a level of infection would pass unnoticed.

It seemed possible that the cyclic AMP found might have been formed from ATP during extraction, but no cyclic AMP was detected in two of the tissues investigated (Table 3).

The results reported here indicate the presence of cyclic AMP in *P. radiata* and in several other plants. The role that this nucleotide plays in plant metabolism remains to be determined.

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