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# Cyclic nucleotide content of tobacco BY-2 cells

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**Abstract**

The cyclic nucleotide content of cultured tobacco bright yellow-2 (BY-2) cells was determined, after freeze-killing, perchlorate extraction and sequential chromatography, by radioimmunoassay. The identities of the putative cyclic nucleotides, adenosine 3′,5′-cyclic monophosphate (cyclic AMP), guanosine 3′,5′-cyclic monophosphate (cyclic GMP) and cytidine 3′,5′-cyclic monophosphate (cyclic CMP) were unambiguously confirmed by tandem mass spectrometry. The potential of BY-2 cell cultures as a model system for future investigations of cyclic nucleotide function in higher plants is discussed.


*Keywords:* Tobacco; BY2; Cyclic AMP; Cyclic GMP; Cyclic CMP; Mass spectrometry; Electrospray

## 1. Introduction

While the natural occurrence and physiological function of cyclic nucleotides in higher plants has been a topic of contention for a long period, it is now clear that these compounds perform important roles in higher plants (for example, Bolwell, 1995; Newton et al., 1999). The initial scepticism over the identity of the putative cyclic nucleotides (Keates, 1973; Amrhein, 1974; Lin, 1974), was overcome by the use of mass spectrometric analysis (Kingston et al., 1984, 1985) which has provided unequivocal evidence of the natural occurrence of 3′,5′-cyclic AMP, 3′,5′-cyclic GMP, and other cyclic nucleotides in higher plants. Fast-atom bombardment MS readily provided molecular mass information of non-volatile polar compounds including nucleotides, but as a soft ionization technique the major drawback of the obtained spectra is the absence of diagnostic fragments. To overcome this problem collision induced dissociation (CID) of the protonated molecule selected from the FAB mass spectrum provides a mass-analyzed kinetic energy (MIKE) spectrum that can be used to generate structural information including the differentiation of cyclic nucleotide isomers (Kingston et al., 1984, 1985; Newton et al., 1984,1986). This tandem MS enabled for example the unambiguous identification of cAMP, cGMP, cIMP, cUMP, cCMP and cdTMP in partially purified extracts of meristematic and non-meristematic tissue from *Pisum sativum* (Newton et al., 1991). It has also been used to demonstrate the activities of the enzymes, adenylyl- and guanylyl cyclases, responsible for the synthesis of two of the cyclic nucleotides (Pacini et al., 1993; Roef et al., 1996; Witters et al., 1996; Newton, 1996).

Electrospray ionization (ESI) mass spectrometry has replaced many of the FAB–MS applications by virtue of its greater sensitivity. When coupled to separation techniques such as capillary HPLC electrospray mass spectrometry becomes a very powerful analytical technique with detection limits as low as 25 femtomoles (Witters et al., 1996, 1997, 1998) and has enabled a cell cycle-regulated cAMP accumulation to be demonstrated in a *Nicotiana tabacum* BY2 cell culture (Eshan et al., 1998).

Various functions have subsequently been proposed for cyclic nucleotides in higher plants as discussed at length in a recent Tansley review (Newton et al., 1999),

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including a role for cyclic AMP in plant cell defence mechanisms (Bindschelder et al., 2001; Cooke et al., 1989, 1994), in pollen tube growth and orientation (Moutinho et al., 2001), and in the cell cycle (Ehsan et al., 1998); for cyclic GMP in the mediation of light-induced effects (Brown et al., 1989; Bowler et al., 1994a,b), and as a component of the signal pathways mediating auxin-induced stomatal opening (Coussson, 2001); and for both cyclic AMP and cyclic GMP in the regulation of ion channels (Anderson et al., 1992; Sentenac et al., 1992; Hoshi, 1995; Gaymard et al., 1996; Kurosaki, 1997). Despite an impressive accumulation of recent evidence, the precise physiological functions of these compounds in plant cells, known as key regulatory molecules in mammals and other organisms, still remain to be elucidated. In higher plants the knowledge state of cyclic nucleotide systems plants is intriguingly different: the biochemistry of cyclic AMP, adenylyl cyclase, phosphodiesterase has slowly unfolded over the last quarter of a century, but comparatively little has been determined relating to the molecular biology of these components. In contrast a significant research effort has been applied to molecular biology studies of cyclic GMP and guanylyl cyclase, but comparatively little advance made into the biochemical parameters relating to the cyclic GMP system in higher plants. Other than their natural occurrence in higher plants little is known of other cyclic nucleotides such as cyclic CMP (Newton et al., 1999). A major cause in our view of this disparity and of imprecise knowledge of cyclic nucleotide function in plants is that detailed studies of cyclic nucleotide biochemistry and molecular biology have been carried out on a diverse variety of plant systems. It is thus opportune to concentrate such investigations onto a model system, and tobacco BY-2 cell cultures offer some advantages as such a system. Generally, BY-2 cultures show high homogeneity and high growth rates, can be highly synchronized (Nagata et al., 1992) and are readily transformed (Geelen and Inze, 2001). While the use of *Arabidopsis* as a model system for studying cell biology has been important in the development of novel tools and methods (Geelen and Inze, 2001), for some studies the small size of *Arabidopsis* cells in culture and the difficulty in synchronizing cultures mean they are not ideal, especially when studies of the cell cycle are involved. BY-2 cells have been used successfully in studies of the cytoskeleton (cited in Geelen and Inze, 2001), regulation of the cell cycle and cell growth (cited in Geelen and Inze, 2001), the role of cyclic AMP as a second messenger (Laukens et al., 2001), cellulose synthesis (Nakagawa and Sakurai, 1998), isoprenoid synthesis (Andrea et al., 2000), gene expression (Yang et al., 2001) and defence response (Houot et al., 2001). Here we describe the extraction, partial purification, identification and estimation of the cyclic nucleotides present in tobacco BY-2 cells as a prelude to their use as a model system for the investigation of cyclic nucleotide biochemistry, molecular biology and physiological function in plants.

## 2. Results and discussion

Direct loop injection of an aliquot of the cyclic nucleotide fraction of the BY-2 extract into positive mode ESIMS (Fig. 1) yielded a spectrum consistent with the presence of peaks corresponding to $[MH]^+$, $[MNa]^+$ and $[MNa_2]^+$ for one or more cyclic AMP isomers, at $m/z$ 330, 352 and 374, for one or more cyclic GMP isomers, at $m/z$ 346, 368 and 390 and for one or more cyclic CMP isomers, at $m/z$ 306, 328 and 350. The UV chromatograms of six cyclic nucleotide standards run independently demonstrated retention times of 1.3, 2.3, 2.4, 3.5, 5.1 and 7.1 min for 2′,3′-cCMP, 3′,5′-cCMP, 2′,3′-cGMP, 3′,5′-cGMP, 2′,3′-cAMP and 3′,5′-cAMP, respectively. The UV chromatogram of the cyclic nucleotide fraction of the BY-2 extract (Fig. 2) shows six major peaks eluting at retention times consistent with their identification as the above six cyclic nucleotides. The ESI mass spectra of these six each contained peaks isobaric with $m/z$ for the protonated molecules of the above six cyclic nucleotides at $m/z$ 306, 306, 346, 346, 330 and 330, respectively. No substantive differences were apparent between the mass spectra obtained from pairs of isomers, either from the extract or from standards, which would enable differentiation of 2′,3′- and 3′,5′-cyclic nucleotides.

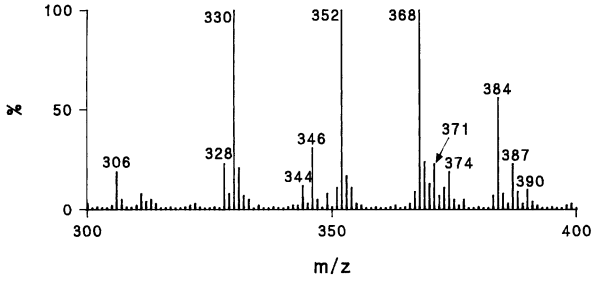


Fig. 1. Positive mode electrospray mass spectrum from direct loop injection of an aliquot of the cyclic nucleotide fraction of the BY-2 extract.

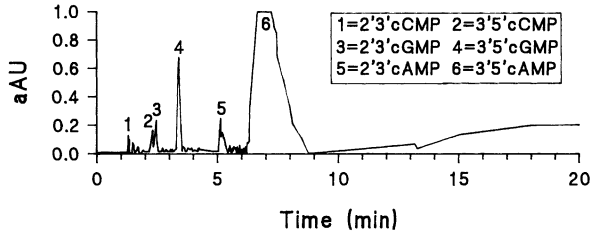


Fig. 2. UV chromatogram of the cyclic nucleotide fraction of the BY-2 extract.

The product ion spectra, however, can be used to identify isomeric nucleotides as they exhibit different fragmentation patterns in ESI-MS/MS due to the position of the phosphate group on the ribose unit. Thus to identify the cyclic nucleotides unequivocally, the ESI tandem mass spectra of these putative protonated molecules were obtained. The product ion spectra of peaks 1 and 2 (Figs. 3 and 4, respectively) are essentially identical to those of the 2′,3′- and 3′,5′-cCMP standards. The main feature of these spectra is that the most abundant fragment corresponds to the protonated cytidine base $[BH_2]^+$ formed by the cleavage of the glycoside bond at $m/z$ 112 which subsequently loses $NH_3$ to form $m/z$ 95. Other product ions common to both spectra in Figs. 3 and 4 include $m/z$ 289, 226, 208 and 190 formed by the loss of $NH_3$, $HPO_3$, $H_3PO_4$ and $H_3PO_4 + H_2O$, respectively. The intensity of all four of these product ions is higher in the 3′,5′- isomer of cCMP. These differences along with the formation of $m/z$ 178 by the loss of $CH_2O$ from $m/z$ 208, observed only in 2′,3′-cCMP allow the isomers to be distinguished.

The product ion spectra of peaks 3 and 4 (Figs. 5 and 6, respectively) are essentially identical to those of the 2′,3′ and 3′,5′-cGMP standards. The protonated guanine base at $m/z$ 152 is the most abundant product ion in both spectra but only in the spectrum of 3′,5′-cGMP does the base show the loss of $NH_3$ resulting in $m/z$ 135. Further ions in this spectrum at $m/z$ 328, 230, 202 and 110 (formed by the loss of $H_2O$, $H_3PO_4$ and $C_2H_4$, respectively) are not observed in that of 2′,3′-cGMP.

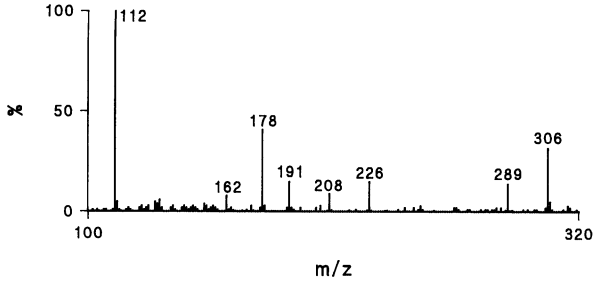


Fig. 3. Product ion spectrum from protonated molecule at $m/z$ 306 in ESI mass spectrum from peak 1 of the cyclic nucleotide fraction of the BY-2 extract.

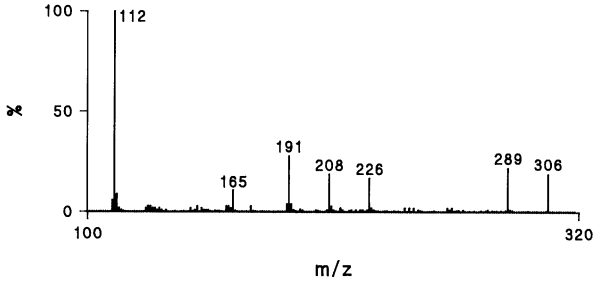


Fig. 4. Product ion spectrum from protonated molecule at $m/z$ 306 in ESI mass spectrum from peak 2 of the cyclic nucleotide fraction of the BY-2 extract.

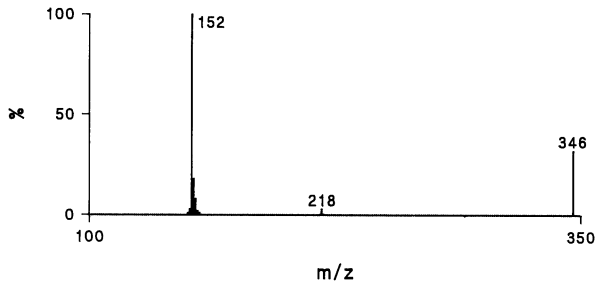


Fig. 5. Product ion spectrum from protonated molecule at $m/z$ 346 in ESI mass spectrum from peak 3 of the cyclic nucleotide fraction of the BY-2 extract.

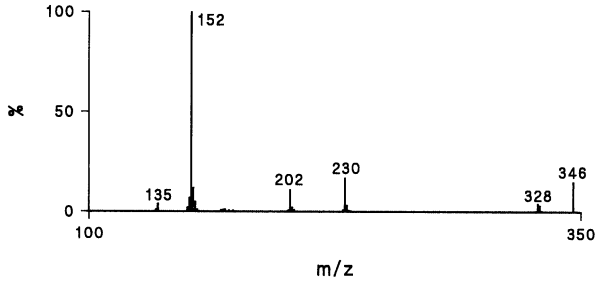


Fig. 6. Product ion spectrum from protonated molecule at $m/z$ 346 in ESI mass spectrum from peak 4 of the cyclic nucleotide fraction of the BY-2 extract.

The product ion spectra of peaks 5 and 6 (Figs. 7 and 8, respectively) are essentially identical to those of the 2′,3′- and 3′,5′-cAMP standards. As with the isomers of cCMP, these spectra allow the isomers of cAMP to be

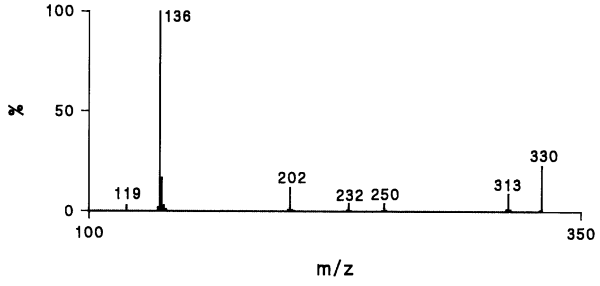


Fig. 7. Product ion spectrum from protonated molecule at $m/z$ 330 in ESI mass spectrum from peak 5 of the cyclic nucleotide fraction of the BY-2 extract.

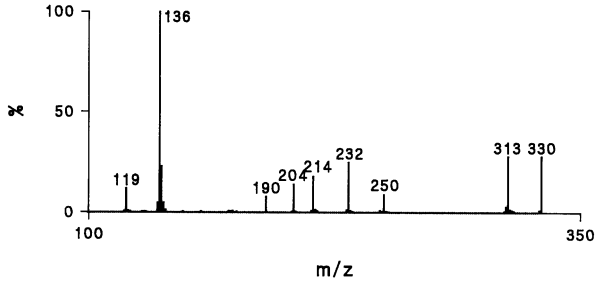


Fig. 8. Product ion spectrum from protonated molecule at $m/z$ 330 in ESI mass spectrum from peak 6 of the cyclic nucleotide fraction of the BY-2 extract.

easily distinguished. The protonated adenine base appears in both spectra at *m/z* 136, though it is the base peak only for 2′,3′-cAMP. Product ions having *m/z* 313, 250 and 232 formed by the loss of $NH_3$, $HPO_3$ and $H_3PO_4$, respectively, are present in both spectra, though they are higher in abundance when formed from 3′,5′-cAMP. The most distinguishing feature in the spectrum of the 3′,5′- isomer is that *m/z* 313 is the most abundant ion rather than the protonated base. The remaining features which also distinguish the spectra include the presence of *m/z* 202 in Fig. 7 only and *m/z* 214, 204 and 190 in Fig. 8 only. The loss of $H_2O$, $C_2H_4$ and $CH_2O$ from *m/z* 232 forms *m/z* 214, 204 and 202, respectively.

Having established the presence of three 3′,5′-cyclic nucleotides in the tobacco BY-2 extract, their levels were determined by radioimmunoassay as adenosine 3′,5′-cyclic monophosphate, $22.79 \pm 0.295$ pmol $g^{-1}$ wet wt, guanosine 3′,5′-cyclic monophosphate, $5.267 \pm 0.623$ pmol $g^{-1}$ wet wt, cytidine 3′,5′-cyclic monophosphate, $0.312 \pm 0.082$ pmol $g^{-1}$ wet wt. The value for cyclic AMP is in good agreement with data previously published for this cyclic nucleotide in BY-2 cells (Ehsan et al., 1998). This is the first report of cyclic GMP and cyclic CMP in BY-2 cells and only the second report of cyclic CMP in any plant tissues. In other plants, the presence of cGMP, together with activities of the associated enzymes of synthesis and degradation, is now well established (Newton et al., 1999). Furthermore, cyclic GMP appears to mediate between several signals and the responses they elicit. For example, cyclic GMP promotes organogenesis in callus (Mangat and Janjua, 1987), and has been implicated as a component of the signal pathways mediating- auxin-induced stomatal opening (Cousson, 2001), phytochrome-controlled chloroplast development (Bowler, et al., 1994a,b), gibberellin-induced transcription of the alpha-amylase gene in aleurone (Penson et al., 1996) and expression of defence responses (Bolwell 1999; Durner et al., 1998). In the latter case there is evidence suggesting that cyclic GMP is part of a signal chain involving NO.

The only other reported occurrence of cyclic CMP in higher plant tissues was in *Pisum* roots (Newton et al., 1989), indicating a significantly higher concentration of cyclic CMP in the meristems. The greater quantity of cyclic CMP in the meristem may reflect a role in the rapidly dividing cells, analogous to that posed for cyclic CMP in mammalian cells (Newton, 1995), while other analogies suggest different roles, for example, the possibility of a role in intercellular communication in higher plants, similar to the functional extrusion of cyclic CMP from bacterial cells (Newton et al., 1998), as similar extrusion processes have already been reported for cyclic AMP in lower plants (Francko and Wetzel, 1980a,b).

## 3. Experimental

### 3.1. Growth and maintenance of tobacco BY-2 suspension cultures

The original suspension culture of TBY-2 was a gift from Dr S. Conlan (School of Biological Sciences, University of Wales Swansea). Suspension cultures were maintained on the modified medium of Lindsmaier and Skoog (1965) in which $KH_2PO_4$ and thiamine HCl are increased to 370 and 1 mg $l^{-1}$ respectively, and sucrose and 2,4-dichlorophenoxyacetic acid are supplemented to 3% (w/v) and 0.2 mg $l^{-1}$, respectively (Nagata et al., 1981). Sub-culturing was carried out at 1 wk intervals, by aseptic transfer of 1.0 ml culture into 50.0 ml of fresh sterile medium in a 250 ml conical flask. Cultures were maintained on an orbital shaker (130 rpm) at $27.0 \pm 1.0$ °C, in the dark and were used for experimental purposes 5 days after sub-culturing.

### 3.2. Extraction and partial purification of cyclic nucleotides

Cells of 5-day-old cultures of TBY-2 were recovered by filtration through four layers of 'Miracloth' (Calbiochem, USA) and homogenized (4 min, 4 °C) using a Silverson homogenizer (Silverson machines Ltd.) with a screen with 23 holes (1 mm diameter) $cm^{-2}$ in 0.6 M ice-cold perchloric acid (0.5 ml per $g^{-1}$ fr. wt cells). The homogenate was allowed to stand for 30 min at 4 °C, filtered through 4 layers of 'Miracloth' and centrifuged (48,000 *g*, 15 min). The pH of the supernatant was adjusted to 7.0 with 1.0 M KOH, following which the supernatant was centrifuged (48,000 *g*, 15 min) and freeze-dried. The freeze-dried extract was resuspended in 15.0 ml of 5.0 mM HCl, centrifuged (48,000 *g*, 25 min, 4 °C) and the supernatant loaded onto a column of acid alumina (25.0 g, Sigma, UK) that had been washed with 50.0 ml of $H_2O$ prior to sample application. Following complete loading of the sample, the column was washed with 50.0 ml of 5.0 mM HCl, followed by 70.0 ml of 0.1 M ammonium acetate (pH 6.5). Cyclic nucleotides were then eluted with 200.0 ml 0.1 M ammonium acetate (pH 6.5), which was collected and freeze-dried.

The freeze-dried fraction was resuspended in 2.0 ml $H_2O$ and loaded onto a column (105×11 mm) of sulphopropyl (SP) Sephadex cation exchange medium (40–125 μm bead diameter, Sigma-Aldrich Company Ltd., UK) that had been pre-swollen in 0.1 M formic acid and then washed extensively with $H_2O$. The cyclic nucleotides were eluted from the column with 30.0 ml $H_2O$, which was collected and loaded onto a column (105×11 mm) of diethyl-[2-hydroxypropyl]-aminoethyl (QAE) Sephadex anion exchange medium (40–125 μm bead diameter, Sigma-Aldrich company Ltd., UK). The column

was washed with 30.0 ml $H_2O$ before the cyclic nucleotides were eluted with 30.0 ml of 0.5 M formic acid. This eluant was collected and freeze-dried prior to resuspension in 3.0 ml of 250.0 mM ammonium acetate, pH 8.8. This sample was loaded onto an 8×1.5 cm Affi-Gel 601 (Boronate gel, Bio-Rad Laboratories Ltd., UK) affinity column that had been pre-swollen in 250.0 mM ammonium acetate, pH 8.8. The cyclic nucleotides were eluted using 30.0 ml of 250.0 mM ammonium acetate, pH 8.8. Preliminary experiments with $2\times10^6$ dpm [$^{14}$C]-cAMP indicated a recovery of >87%.

### 3.3. LC-MS

#### 3.3.1. Chemicals for LC-MS

HPLC grade water and methanol (Fisher Scientific, Loughborough, UK), AnalaR grade ammonium acetate (BDH Chemical Ltd., Poole, UK) and 99+% acetic acid (Aldrich Chemical Company Inc., Dorset, UK) were used. All solvents were filtered through a 0.45 um membrane filter and degassed prior to analysis. Standard cyclic nucleotides (Sigma, Poole, UK) were prepared in water as 10 mg (±0.2 mg)/10 ml stock solutions in volumetric flasks (±0.2 ml), then diluted as necessary for analysis.

#### 3.3.2. LC/ESI-MS analysis

Conventional HPLC was performed using an Agilent 1100 LC system with a Luna phenylhexyl column (100×4.6 mm, 3 μm particle size; Phenomenex, Macclesfield, UK). Separations were carried out at ambient temperature with starting conditions of 60% ammonium acetate (10 mM, pH 5), solvent A, and 40% methanol and water (2:1, v/v), solvent B. and a programmed gradient of 0 min 40% B, 4 min 40% B, 6 min 80% B, 9 min 80% B, 10 min 40% B and 20 min 40% B. The pump was set to 1 ml/min and the UV detector to 254 nm. LC/ESI-MS was conducted by splitting the LC eluent in a ratio of 1:4 (MS: waste) using a T-piece so that a flow of 200 pl/min entered the mass spectrometer. A Finnigan MAT LCQ ion trap mass spectrometer was used with ESI, which required a heated capillary temperature of 250 °C, sheath and auxiliary gas flows of 60 and 30 (arbitrary units), respectively, and a 4.5 kV spray voltage. Collision energies were applied reducing the molecular ion to approximately 10–20% in the product ion spectrum. Data were initially acquired in both positive and negative scanning modes, but positive mode was utilized as the tandem mass spectra were more complex and thus more effective for diagnostic purposes.

## 4. Radioimmunoassay

Prior to radioimmunoassay the cyclic nucleotide-containing extract was fractionated to separate cyclic nucleotides. The extract was applied to plastic columns (11×1 cm) containing layers of 0.5 cm of Dowex 1X8, 0.5 cm of neutral alumina and 2.0 cm of QAE-Sephadex and eluted with HCl. As previously reported (Newton et al., 1994) cyclic CMP was eluted with 0.03 M HCl after 3–8 ml, with cyclic AMP eluting between 9 and 16 ml and cyclic GMP eluted in the next 10 ml after increasing HCl concentration to 0.09 M HCl. The fractions were then concentrated by freeze-drying before radioimmunoassay.

The standard protocol adopted for each cyclic nucleotide was based upon the method of Newton et al. (1994). In brief, between 1.5 and $3\times10^4$ c.p.m. of $^{125}$I-labelled 2′-*O*-succinyl cyclic nucleotide tyrosinyl methyl ester were incubated together with unlabelled cyclic nucleotide standards in quantities ranging from 10 fmol to 10 nmol with antiserum, raised in rabbits against succinyl cyclic nucleotide-thyroglobulin conjugate, diluted up to 50,000-fold, with 10 ul of a mixture of 1 vol. acetic anhydride and 2.5 vol. triethylamine in a total volume of 500 ul of 0.1 M citrate buffer pH 6.2 for 20–24 h at 4 °C. The bound radiolabelled antigen was then separated and counted by adding 200 ul of a 2% charcoal suspension in the same buffer containing in addition 0.25% bovine serum albumin, incubating for 90 min, followed by centrifugation at 14,500 *g* for 10 min and counting the aspirated supernatant in LKB-Wallac Wizard II gamma-counter. Each sample was counted for 5 min and counted three times. Four replicates of each incubation were carried out. Controls to determine background counts, total counts, non-specific radiolabelled-antigen binding, and total radiolabelled-antigen binding in the absence of unlabelled antigen (zero binding) were routinely included. Samples of unknown cyclic nucleotide concentration were included in place of the cyclic nucleotide standards in the above protocol.

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