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OCCURRENCE OF GUANOSINE 3′,5′-CYCLIC MONOPHOSPHATE (CYCLIC GMP) AND ASSOCIATED ENZYME SYSTEMS IN *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*, Leguminosae, guanosine 3′,5′-cyclic monophosphate, cyclic GMP, guanylate cyclase, phosphodiesterase

Abstract—Cyclic GMP, isolated from *Phaseolus vulgaris*, has been unequivocally identified by NMR and FAB-mass spectrometry with MIKES-scanning Radioimmunoassay, previously used by others to estimate concentrations of putative cyclic GMP in plant tissues, was shown to be subject to interference by other plant constituents Partial purification of the cyclic GMP extract, before assay, eliminated the interference Using this modified assay procedure, the cyclic GMP content of *Phaseolus vulgaris* tissue was determined and a value of 33 pmol/10 g fresh wt recorded Intact chloroplasts were shown to contain both cyclic GMP phosphodiesterase and a guanylate cyclase It is therefore concluded that the potential exists in this higher plant tissue for cyclic GMP-mediated metabolic control mechanisms

INTRODUCTION

Adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) are known to play important regulatory roles in mammalian [1, 2] and bacterial metabolism [3, 4] The involvement of these two nucleotides in the control of lower plant metabolism has also been described [5] The presence of cyclic AMP in the tissues of higher plants has been unequivocally demonstrated [6] and there are a number of indications, reviewed recently by Brown and Newton [7, 8], that it is involved in the control of higher plant metabolism Furthermore, the enzymes adenylate cyclase and cyclic nucleotide phosphodiesterase, which respectively catalyse the synthesis and hydrolytic degradation of cyclic AMP, have also been shown to be present in higher plant tissues together with a specific cyclic AMP binding protein [9–12] These are, in effect, the essential components of a functional cyclic nucleotide regulatory system

The occurrence of cyclic GMP in plants was first indicated by the work of Lundeen *et al* [13] In those studies, a radioimmunoassay for cyclic GMP was used to investigate changes in concentration during cell enlargement and cell division in excised pith tissues of *Nicotiana tabacum* Radioimmunoassay was also employed by Ames *et al* [14] in their investigation of cyclic GMP concentrations in a tumour-prone *Nicotiana* amphiploid Cyclic GMP has been reported to occur in bean root tissue [15], pine pollen [16] and in relatively high concentrations in the medicinal plants *Evodia rutaecarpa*, *E officinalis* and *Zizyphus jujuba* [17–19] However, just as the earlier evidence for the occurrence of cyclic AMP had been challenged by some authors, similar criticism could be levied against these identifications of the compound as cyclic GMP

In order to establish the possibility of cyclic GMP playing a regulatory role in higher plants, as it does in animal tissues and bacteria, it is necessary to demonstrate (a) unequivocal evidence for the identification of the putative cyclic GMP, and (b) the existence in plants of a functional guanylate cyclase together with a functional cyclic GMP phosphodiesterase This, then, was the objective of the present investigation We now report on the unequivocal identification of cyclic GMP, from a plant source, and on the validity of applying radioimmunoassay to determine its concentration in plant extracts The presence of guanylate cyclase and cyclic GMP phosphodiesterase in the same tissue is also shown

RESULTS AND DISCUSSION

Identification of cyclic GMP

The extracted nucleotide exhibited UV absorption spectra characteristic of a guanosine nucleotide and identical to those of an authentic sample of cyclic GMP with λ_{max} at pH 1, 7 and 11, of 255 nm, 252 nm and 259 nm respectively Because of the tight coupling of the spin systems, interpretation of ^{1}H NMR spectra of 3′,5′-cyclic GMP is difficult [20], and insufficient of the extracted sample was available for ^{13}C NMR examination The ^{1}H NMR data obtained, both for the authentic and putative cyclic GMP samples, are shown in Table 1 Proton peaks were assigned by analogy with previously reported NMR spectra for cyclic nucleotides [20–22] The spectra of the two present samples were closely similar and whilst not unequivocally identifying the extracted sample, strongly supported its identification as 3′,5′-cyclic GMP In particular, the large difference in shift between the protons at position 8 and 1 is characteristic of cyclic nucleotides [21, 22], and the peak at 7 88 ppm is similar to that reported for 2′,3′-cyclic GMP [22]

In a mass spectrometric approach to the identification problem, the molecular weight regions of the FAB mass spectrum of an authentic sample of cyclic GMP were recorded (Fig 1) Cyclic GMP yielded a protonated

Table 1 NMR spectral data for an authentic sample of 3',5'-cyclic GMP and a putative sample from *P vulgaris*

Assignment	$^1H\delta$* Cyclic GMP (authentic)	$^1H\delta$* Cyclic GMP (putative)
8	7 885	7 883
1'	5 981	5 975
2'	4 761	4 765
3'	4 771	4 791
4'	4 366	4 341
5'	4 096	4 131
5''	4 056	4 091

*100 08 MHz, TMSP-D_2 as internal standard = 0

molecular ion $[M+H]^+$ at *m/z* 346 of comparable intensity to that of the protonated glycerol trimer $[3G+H]^+$ derived from the matrix [23] The only other ions emanating from the cyclic nucleotide were at *m/z* 368 $[M+Na]^+$, 390 $[(Na\ salt)+Na]^+$ and 438 $[M+G+H]^+$

The FAB spectrum derived from a commercial sample of 2',3'-cyclic GMP and that from the cyclic nucleotide isolated from *Phaseolus* were indistinguishable from the spectrum of 3',5'-cyclic GMP shown in Fig 1, with the characteristic large peaks at *m/z* 346 and 368 Although the FAB mass spectra alone were thus not sufficient to distinguish between the cyclic GMP isomers, comparison with the FAB mass spectra obtained from guanosine, GMP, GDP and GTP, all of which possessed $[M+H]^+$ and $[M+Na]^+$ peaks but did not produce an *m/z* 346 ion, confirmed that the guanosine nucleotide isolated from *Phaseolus* was one of the two cyclic GMP isomers The large peak at *m/z* 346 was studied further using the CID-MIKE technique

The CID-MIKE spectra of the *m/z* 346 ions of 3',5'-cyclic GMP, 2',3'-cyclic GMP and the isolated nucleotide are shown in Fig 2 The predominant fragmentation process for both isomers is glycosidic cleavage [24] of the protonated base, yielding an *m/z* 152 ion There are distinct differences between the spectra of the two isomers in the region chosen for closer examination, shown in Fig 2 In this, 3',5'-cyclic GMP fragments to give two major peaks *m/z* 180 and *m/z* 194, whereas 2',3'-cyclic GMP exhibits a much weaker *m/z* 180 and the *m/z* 194 ion is virtually absent In addition, three new peaks are observed in this region, at *m/z* 195, 214 and 217 The two isomers of cyclic GMP can thus be readily differentiated and the compound from *Phaseolus* is clearly seen to be the 3',5'-cyclic GMP monophosphate

Validity of the radioimmunoassay of cyclic GMP in plant extracts

Although the cyclic GMP content of plant tissues has previously been determined by radioimmunoassay (RIA), the procedure used [13, 15–19] was essentially that devised for use with animal tissue extracts Since it is possible that plant constituents such as phenolics interfere with this RIA, the results could be unreliable In earlier work here [8] it was shown that this was certainly the case with the 'binding-protein' assay for cyclic AMP which had also originally been designed for analysis of animal tissue extracts Consequently, as a preliminary to the present studies, the RIA was examined for possible interference by constituents of the plant extracts to be assayed The procedure adopted was similar to that used in assessing interference by plant products with the 'binding-protein' assay for cyclic AMP [9] In this procedure, the plant extract was subjected to a series of purification steps which remove non-nucleotide compounds and eventually 'non-cyclic' nucleotides The sample obtained at each purification stage was examined for effect by plotting a standard cyclic GMP assay curve in its presence and absence Whereas the endogenous cyclic GMP content of the extract would be expected to displace the curve, its slope should remain unchanged Alterations to the slope must indicate interference and hence erroneous determination of cyclic GMP concentrations The results of this experiment (Table 2) show that the crude plant extract does contain interfering compounds but as indicated by the correlation coefficient and the standard deviation, the position improves as the extract is purified Interference is still present after ion-exchange chromatography on Dowex-1 x 8 (Fraction 1) Fraction 2, which was prepared from Fraction 1 by further column chromatography on

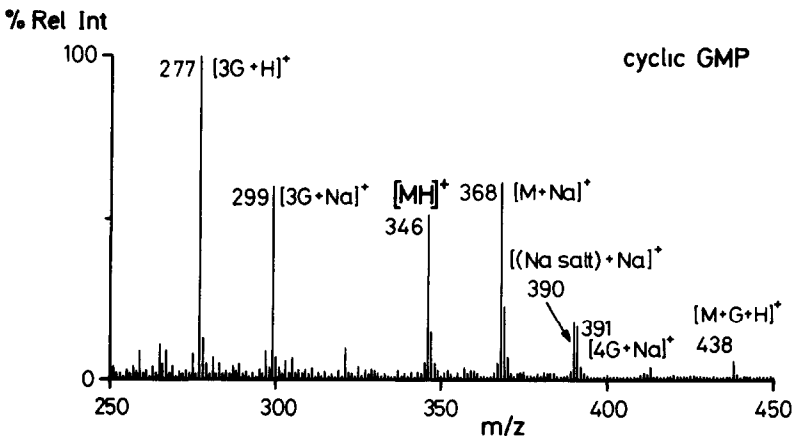

Fig 1 FAB-mass spectrum of 3',5'-cyclic GMP

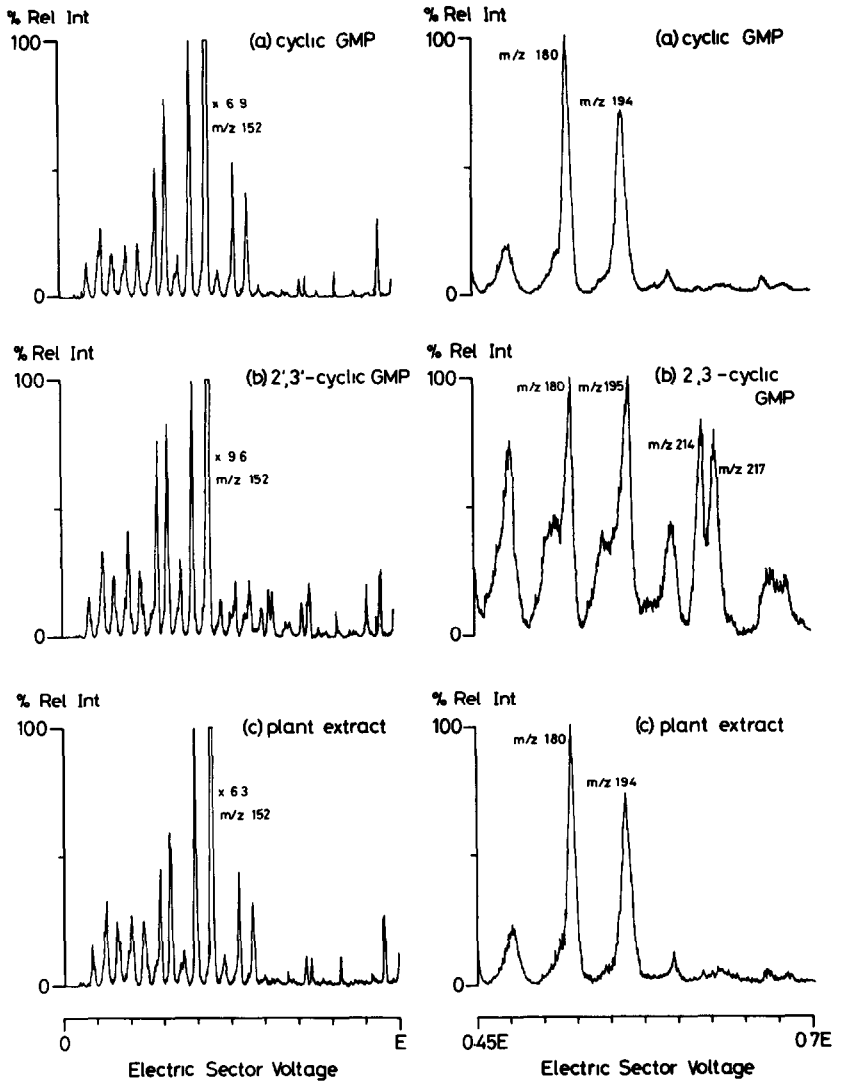

Fig 2 CID-MIKE spectra of the m/z 346 ions of (a) 3',5'-cyclic GMP, (b) 2',3'-cyclic GMP, and (c) the guanosine nucleotide extracted from *Phaseolus vulgaris*

Table 2 Effect of partial purification of the plant extract on results obtained with the standard RIA assay procedure for cyclic GMP

RIA conditions	Correlation coefficient	Standard deviation	Gradient ($pmol^{-1}$)	Intercept (pmol of cyclic GMP)
Standard plot (5)	1·00	±0·1	0·49	2·02
S + crude extract*	0·91	±0·27	0·43	3·74
S + fraction 1*	0·96	±0·17	0·44	3·47
S + fraction 2*	0·98	±0·11	0·47	3·35
S + TLC preparation*	0·99	±0·09	0·49	3·15

*Stages in the purification of a cyclic GMP extract (for details, see text)

Dowex-50 × 8, also contained interfering substances However, after the TLC stage, interference had been eliminated and the data then obtained closely paralleled the standard curve for cyclic GMP It is concluded, therefore, that it is valid to use the standard RIA procedure for cyclic GMP determination in plant extracts but only if the cyclic GMP fraction is first purified to remove interfering plant constituents

The intercept of the TLC fraction plot on the concentration axis (Table 1) gives a value of 1·1 pmol/100 μl

sample which corresponds to an original concentration of 11 pmol/10 g tissue fresh wt Estimation of the recovery of cyclic GMP during the pre-RIA purification procedure gave a figure of 33%, i e a corrected value for the overall cyclic GMP content of *Phaseolus vulgaris* seedlings is 33 pmol/10 g fresh wt This compares with the values reported by Haddox *et al* [15] of 4–200 pmol/10 g fresh wt where the upper value is for meristematic root tissue These latter authors observed that the values for the root meristematic tissue were up to 10-fold greater than those for the surrounding area which would give 20 pmol/10 g fresh wt compared to our overall estimate of 33 pmol/10 g fresh wt

Cyclic GMP phosphodiesterase

Our earlier studies with *Phaseolus vulgaris* and *Spinacea oleracea* had shown presence of 3',5'-cyclic nucleotide phosphodiesterase activity [9–11] More detailed examination of *Spinacea* showed the plurality of this enzyme with a form, exhibiting relatively high specific activity, in the chloroplasts [10, 11] In the present work, the chloroplasts of *Phaseolus vulgaris* were examined for cyclic GMP phosphodiesterase activity By means of the assay procedure described in the Experimental, cyclic GMP phosphodiesterase was demonstrated in preparations obtained from intact chloroplasts by freezing and thawing Using ammonium sulphate, fractional precipitation of these preparations from *Phaseolus vulgaris* resulted in the bulk of the activity being found in the 0–20% saturation fraction (sp act 1 5 nmol cyclic GMP/min/mg protein) Determination of K_m by a Lineweaver–Burk plot, with cyclic GMP as substrate, gave a value of 77 μM The ratio of the initial rate of reaction with cyclic GMP to that with cyclic AMP was 3 45 1

Guanylate cyclase

Intact chloroplasts from *Phaseolus vulgaris* seedlings were broken by freezing and thawing (see Experimental) and the resulting preparation tested for guanylate cyclase activity The procedure was essentially that of Garbers and Murad [25] in which [8-^{3}H]GTP was used as substrate The product, [8-^{3}H]cyclic GMP, was separated from excess GTP and from the degradation products GDP and GMP by co-precipitation with $ZnCO_3$ followed by anion-exchange chromatography on a column of polyethyleneimine cellulose [25] Measured over 60 min incubation periods, the chloroplast preparation synthesized 1 18 pmol cyclic GMP/min/mg of protein

Overall significance of the experimental data

The results described above unequivocally demonstrate the presence of cyclic GMP in the tissues of a higher plant Taken with our previous unambiguous mass spectrometric demonstration of the occurrence of cyclic AMP in plant tissues [6], together with the presence of cyclic AMP phosphodiesterases [9–11], adenylate cyclase [12], cyclic AMP binding protein [12] and calmodulin [26] in plants, the present findings further emphasize the existence of all the necessary components for cyclic nucleotide regulated control systems in plant metabolism

There is now ample evidence that, in animals, cyclic GMP is a unique component in a complex network of biological regulation [27] This involves lipid metabolism, the visual process, lymphocyte proliferation, antibody production, leukocyte chemotaxis, cell-mediated toxicity and there is also evidence for cyclic GMP involvement in the regulation of the synthesis or expression of specific mRNA molecules in both prokaryotic and eukaryotic systems, including microbial and animal systems Although cyclic AMP and cyclic GMP play a number of roles independently of one another, there are also biological systems in which they play opposing roles (Yin-Yang hypothesis) [28, 29] This should be borne in mind in appraising the older physiological experiments, reviewed in ref [7], which were designed to investigate the effects of cyclic AMP on various plant processes In order to make meaningful interpretation of analytical results, it is advisable to determine the concentrations of both nucleotides Future investigations of the role of cyclic AMP in plants should therefore be accompanied by parallel studies of cyclic GMP

EXPERIMENTAL

Plant material Seeds of *Phaseolus vulgaris* cv The Prince (Bees Seeds Ltd, Sealand, U K) were germinated in moist vermiculite and the seedlings grown at 23° in a light cycle of 16 hr light (6 klx) and 8 hr dark

Chloroplast preparation Chloroplasts were isolated from leaves of 8-day seedlings of *Phaseolus vulgaris* The procedure was essentially that of Lominski and Rienits [30] with minor modifications After removing the midribs and using a Waring blender at half-speed for 30 sec, leaves (100 g) were homogenized at 4° in a medium (180 ml) containing 0 4 M sucrose, 10 mM NaCl, 50 mM Tris–HCl (pH 8 0) and 82 mM cysteine The homogenate was filtered through eight layers of cheese-cloth with a thin lining of cotton wool Cell debris was removed by centrifuging the filtrate at 200 g for 3 min The chloroplast pellet was resuspended in 10 mM NaCl (100 ml) and centrifuged at 7000 *g* for 10 min Chloroplasts were finally resuspended at 4° in the 0 4 M buffered sucrose, described above The suspension was then subjected to rapid freezing and thawing This process was repeated six times

Extraction and purification of cyclic GMP Leaves (1 5 kg) from 8-day *Phaseolus* seedlings were extracted by a minor modification of the procedure previously developed for the isolation of cyclic AMP [31] This involved rapid freezing in liquid N_2, followed by freeze-drying and homogenization of the freeze-dried powder in an ice-cold monophasic mixture of MeOH–$CHCl_3$–HCO_2H After adding water and $CHCl_3$, the aqueous phase was taken, adjusted to pH 4 and subjected to a charcoal-adsorption step Elution of the charcoal with ethanolic NH_3 yielded a soln of crude cyclic GMP This was subjected to ion-exchange chromatography, first on a column (15 cm × 1 5 cm) of Dowex 1 × 8 (200–400 mesh) and then on a similar size column of Dowex 50 × 8 (200–400 mesh) A flow rate of 0 5 ml/min was used The fraction obtained after chromatography on Dowex-1 was designated Fraction 1, and that after chromatography on Dowex-50 was designated Fraction 2 The elution profile of cyclic GMP on these two columns was determined using [^{3}H]-cyclic GMP (10^4 dpm) Recovery of cyclic GMP was optimized by substituting 0 1 M HCl for the HCO_2H eluent in the original cyclic AMP procedure Cyclic GMP eluted from the Dowex-1 column between 12–26 ml and from the Dowex-50 column between 10–35 ml The cyclic GMP eluate was evaporated to dryness at 30° under red pres, redissolved in 50% aq EtOH and chromatographed by TLC on silica gel G60 A 0 3 mm thickness of adsorbent was used and the solvent consisted of *iso*-PrOH–NH_3

(sp gr 0 88)–H_2O (14 3 3) After spraying the plates with 2',7'-dichlorofluorescein (0 01 % w/v in EtOH containing a few drops of NH_3) bands were visualized in UV-light The area corresponding to that of an authentic sample of cyclic GMP, run simultaneously, was eluted with 50 % aq EtOH This was designated 'TLC fraction' As previously described for the isolation of cyclic AMP [31] the eluate was subjected to high voltage paper electrophoresis (23 V/cm/2 hr)

The sample eluted from the electrophoretogram was re-run in the previous TLC system but treatment with dichlorofluorescein was omitted The band, visualized in UV-light and which corresponded in electrophoretic behaviour to an authentic sample of cyclic GMP, was eluted The whole procedure was repeated and the extracts combined until an adequate quantity of material was available for analysis

NMR and MS NMR spectra were obtained with a Varian XLFT-100 instrument with a reference frequency of 100 08 MHz Microgram quantities of the sample were dissolved in the minimum volume of D_2O and, using trimethylsilylpropionate-D_2 (Na salt) as an internal standard, the field was scanned at ambient temperature spin rate 28 rps RF field 63 dB pulse width 28 μsec acquisition time 2 sec with 600 transients and 8000 points

Positive ion FAB-mass spectra were obtained with a VG Analytical ZAB-2F mass spectrometer using a VG FAB source and ion gun The sample was bombarded with argon atoms under ion gun conditions of 8 kV accelerating potential, 1–2 mA discharge current, and a source accelerating voltage of 8 kV The sample was prepared in a glycerol–water (1 1) matrix and 3 μl (containing up to 200 μg of cyclic nucleotide in suspension) were placed on the FAB target Under these conditions, the sample lifetime was 10–15 min

Collision-induced dissociation (CID) spectra were generated using N_2 as a collision gas, in the second field-free region gas cell, at a pressure of 6×10^{-6} torr Mass-analysed ion kinetic energy (MIKE) spectra were obtained by scanning the electric sector voltage under the control of a data acquisition system For partial scans, at least five sweeps were averaged by the computer To maintain a steady ion current, the FAB sample probe and power supply discharge current were rigorously controlled throughout the acquisition of MIKE spectra

Radioimmunoassay of cyclic GMP RIA kits for determining concentrations of cyclic GMP in tissue extracts were obtained from Amersham International plc, Amersham, U K The protocol was modified, as necessary, to facilitate examination of the effects of plant constituents other than cyclic GMP, present at the various stages of the cyclic GMP purification procedure

Measurement of protein concentrations The method of Lowry *et al* [32] as modified by Rider [33] was used throughout the present investigation

Determination of guanylate cyclase activity Guanylate cyclase activity was measured by the method of Garbers and Murad [25] with minor modifications Samples (50 μl) of the broken chloroplast suspension were incubated at 30° in a medium made by mixing 50 μl of 10 mM GTP with 50 μl of 100 mM $MnCl_2$ and 100 μl of 175 mM Tris assay buffer (pH 7 9) containing 8 $\times 10^4$ dpm [8-^{3}H]cyclic GMP The reaction was stopped by adding to each incubate 0 25 ml of a soln of Zn diacetate–cyclic GMP (0 2 M Zn diacetate soln containing 2 2 mM cyclic GMP) and then placing them in an ice-bath After adding 0 25 ml of Na_2CO_3 soln (200 mM) the incubate was rapidly frozen and thawed and the precipitate removed by centrifuging The supernatant was transferred to a PEI-cellulose column (110 mm $\times$ 15 mm) and the column washed with 15 ml of 50 mM HOAc followed by 15 ml of H_2O Cyclic GMP was eluted from the column in 20 ml of 20 mM LiCl soln The radioactivity of aliquots was measured in an LKB Rackbeta 1217 scintillation spectrometer, programmed for automatic quench correction by the internal standards method Aqualuma scintillant (LKB Ltd, Croyden, U K) was used (5 ml/200 μl sample)

Cyclic GMP phosphodiesterase assay The method used in measuring cyclic GMP phosphodiesterase activity was based on that of Thompson *et al* [34, 35] Incubation mixtures consisted of 100 μl [8-^{3}H]cyclic GMP ($6–8 \times 10^4$ dpm), 100 μl 1 mM cyclic GMP and 200 μl enzyme preparation After incubation at 30° for 50 min, 100 μl of nucleotidase preparation (1 mg/ml) was added and incubation continued at the same temperature for a further 10 min The reaction was stopped by placing incubation tubes in a boiling water bath for 1 min After rapid cooling in ice, the tubes were centrifuged to remove the protein precipitate The supernatant was passed through a column (110 mm $\times$ 15 mm) of Dowex-1 $\times$ 8 (200–400 mesh) Guanosine was eluted in 5 ml water and its radioactivity determined

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NOTE ADDED IN PROOF

Identification of cyclic GMP from a plant source has also recently been described by B Janistyn (1983) who has used a GC/MS technique (*Planta* **159**, 382)