Phytochemistry, Vol. 28, No. 9, pp. 2243–2254, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00
Maxwell Pergamon Macmillan plc.

IDENTIFICATION OF CYCLIC NUCLEOTIDE CONSTITUENTS OF MERISTEMATIC AND NON-MERISTEMATIC TISSUE OF *PISUM SATIVUM* ROOTS

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(*Received* 22 *September* 1988)

Key Word Index—*Pisum sativum*: Leguminoseae; pea; meristem; cyclic nucleotides; fast atom bombardment; mass-analysed ion kinetic energy spectrometry; 3′,5′-cyclic-AMP, 3′,5′-cyclic-GMP, 3′,5′-cyclic-CMP, 3′,5′-cyclic-UMP, 3′,5′-cyclic-IMP and 3′,5′-cyclic-dTMP.

Abstract—The extraction of endogenous 3′,5′-cyclic-AMP, 3′,5′-cyclic-GMP, 3′,5′-cyclic-CMP, 3′,5′-cyclic-UMP, 3′,5′-cyclic-IMP and 3′,5′-cyclic-deoxyTMP from meristematic and non-meristematic regions of *Pisum* roots is described. Extracts were subjected to a sequential purification procedure involving adsorption chromatography on alumina, ion-exchange chromatography, and preparative electrophoresis. Purified samples were compared with cyclic nucleotide standards by HPLC and UV absorbance spectrophotometry; further analysis was carried out by fast atom bombardment mass spectrometry and mass-analysed ion kinetic energy spectrometry. 3′,5′-Cyclic-AMP, -GMP, -CMP and -UMP were found to be present in *Pisum* meristematic tissue and 3′,5′-cyclic-AMP, -GMP, -CMP, -IMP and -dTMP are present in the non-meristematic regions. Data indicate that there is a significantly higher concentration of cyclic CMP in meristematic tissue relative to that in non-meristematic regions.

INTRODUCTION

The presence of adenosine 3′,5′-cyclic monophosphate (cyclic-AMP) and guanosine 3′,5′-cyclic monophosphate (cyclic-GMP) in mammalian tissues is well established, and their roles as mediators of hormone action and as metabolic regulators are substantially understood. In higher plants, although the presence of both compounds and of enzymes capable of their biosynthesis and degradation has also been established, their respective functions are not yet understood (for reviews see [1, 2]). In addition to cyclic-AMP and cyclic-GMP, it has more recently been established that cytidine 3′,5′-cyclic monophosphate (cyclic-CMP), uridine 3′,5′-cyclic monophosphate (cyclic-UMP), inosine 3′,5′-cyclic monophosphate (cyclic-IMP) and 2′-deoxythymidine 3′,5′-cyclic monophosphate (cyclic-dTMP) are endogenous nucleotides of mammalian tissues [3–7]. The development of specific procedures for the unambiguous identification of cyclic nucleotides in tissue extracts has been a necessary component of such studies, since identification by individual chromatographic and enzymic methods, and by radioimmunoassay, can be ambiguous. This is because the 2′,3′-cyclic nucleotide isomers and some other nucleotides can interfere in these processes [1, 2]. Although classical methods of mass spectrometry, such as electron-impact mass spectrometry (EIMS), have been of value in studies of cyclic-AMP, these procedures are inadequate for studies of the other cyclic nucleotides because of the low volatility of the requisite trimethylsilyl derivative, its thermal instability, and the relatively large quantity of sample required. The advent of a new method of soft ionization, fast atom bombardment (FAB) [8], has enabled the ready generation of a quasi-molecular ion in the mass spectra of underivatized cyclic nucleotides. This technique, together with the production of mass-analysed ion kinetic energy (MIKE) spectra resulting from the collision-induced dissociation (CID) of ions generated by FABMS, has facilitated the unequivocal identification of cyclic nucleotides, readily distinguishing between 3′,5′-cyclic nucleotides and their 2′3′-isomers [9, 10], and has been applied successfully to the identification of cyclic nucleotides in purified mammalian tissue extracts [3–7]. The basis of this identification is the recognition of characteristic fragments equivalent to the protonated purine or pyrimidine base, the protonated base + 28 mass units, and the protonated base + 42 mass units in the MIKE spectrum arising from the protonated molecular ion [9, 10].

It has recently been established that in *Lactuca* cotyledons a cyclic nucleotide phosphodiesterase is present which is capable of hydrolysing not only cyclic-AMP and cyclic-GMP, but also cyclic-CMP and cyclic-UMP [11–13]. The properties of this enzyme are similar in many respects to a multifunctional phosphodiesterase found in mammalian tissues [14] which is believed to have a role in the hydrolysis of both purine and pyrimidine cyclic nucleotides. In view of the existence of this enzyme in *Lactuca*, the known occurrence of cyclic-AMP and cyclic-GMP in plants, and current interest in pyrimidine cyclic nucleotides it was decided to apply FAB /MIKES analysis to nucleotide preparations from plant tissues. This paper describes the identification of cyclic nucleotides, extracted and partially purified by chro-

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matography and electrophoresis, from meristematic and non-meristematic areas of the roots of *Pisum* seedlings, by HPLC, UV spectrophotometry and FAB/MIKES analysis. Pea seedlings were selected as the tissue source in preference to lettuce on the basis of the easier separation of the requisite quantities of meristematic and non-meristematic tissue. It was hoped that any differences in the cyclic nucleotide composition of the meristematic and non-meristematic regions may provide some clue as to the function of the cyclic nucleotides.

RESULTS

Fast atom bombardment of the extracted samples, after elution from SP-Sephadex but before electrophor-

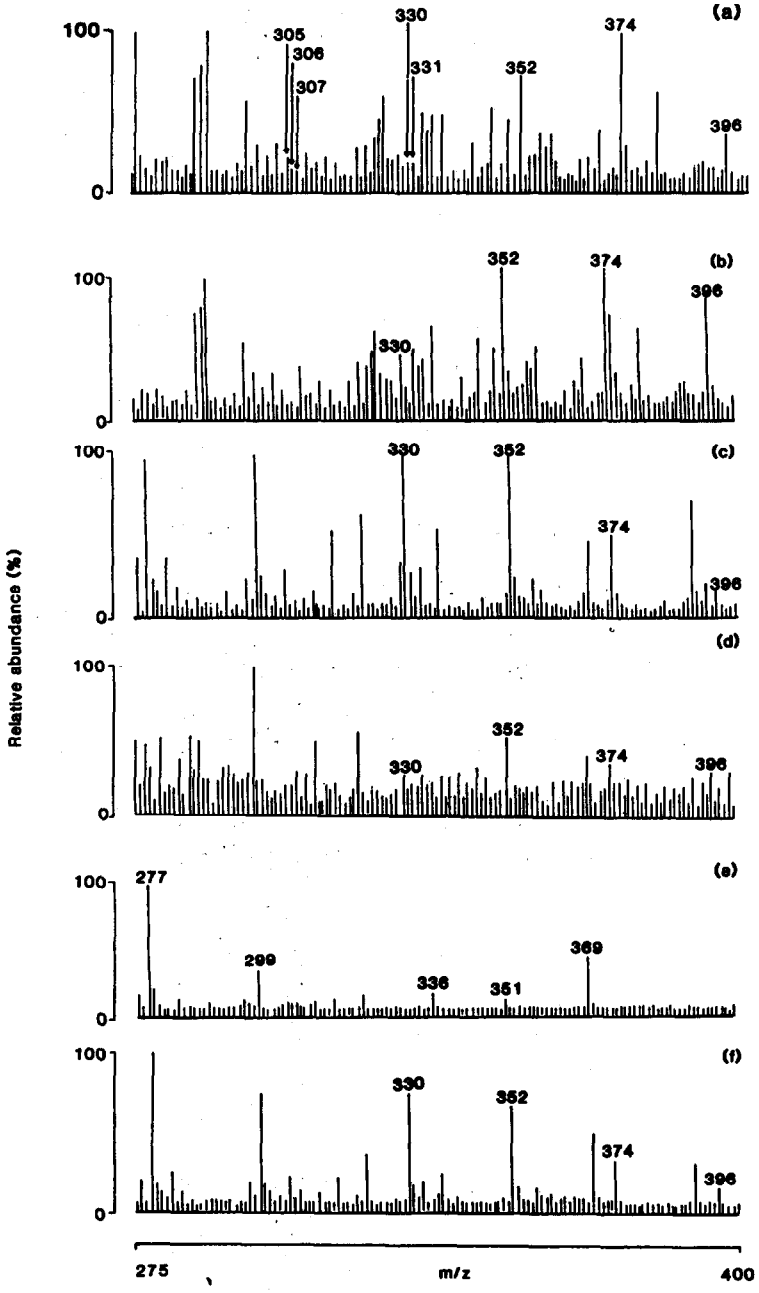

Fig. 1. Positive-ion fast atom bombardment mass spectra of the total nucleotide extracts A and B from meristematic tissue (a and c) and an extract from non-meristematic tissue (d) of *Pisum sativum*. The FAB spectrum (b) was obtained following addition of 5 $\mu g/\mu l$ authentic adenosine 3',5'-cyclic monophosphate to meristematic extract A. The FAB spectrum obtained from meristematic extract A previously incubated with phosphodiesterase is shown in (e). Also presented is the FAB spectrum of fraction I obtained by further purification of the total nucleotide extract from meristematic tissue (f). Details of the cyclic nucleotide isolation procedures and mass spectrometry are given in the Experimental.

esis, yielded the mass spectra partially reproduced in Fig. 1. The partial spectra from ions of *m/z* 275 to 400, a range which would contain the protonated molecular ions of any cyclic nucleotides and of their sodium adducts, for duplicate meristematic extracts designated A and B, and for the non-meristematic extract C, are shown in Fig. 1a, c and d, respectively. They exhibit a considerable number of peaks of greater relative intensity than those simply originating from the glycerol–water matrix, indicating the multi-component complexity of the extracts even after three stages of chromatographic purification. Peaks with a *m/z* ratio equivalent to those of the molecular protonated ions of cyclic-dTMP, -CMP, -UMP, -AMP, -IMP and -GMP were evident at *m/z* 305, 306, 307, 330, 331 and 336 respectively, but in most cases were not significantly larger than adjacent peaks. Similarly, the peak heights of the mono- and di-sodium adducts of these molecular ions were not very different in height from those of the surrounding background peaks. The exceptions were the presence of large peaks at *m/z* 352, 374, and 396 in the spectrum of sample A (Fig. 1a), isobaric with the mono-, di- and tri-sodium adducts of cyclic-AMP. In the second meristematic extract B, the mono- and di-sodium adduct peaks at *m/z* 352 and 374 were again significant (Fig. 1c), but in this case the protonated molecular ion of cyclic-AMP at *m/z* 330 was very strong whereas that of the tri-sodium adduct at *m/z* 396 was weak. This different relative intensity of the peaks in the two meristematic samples reflects the different amount of sodium ions present in the plant extracts. In the non-meristematic sample C (Fig. 1d), significant peaks were present at *m/z* 330, 352 and 374, but the relative intensity of the latter compared to other peaks in the *m/z* 275–400 range was much less than that observed for *m/z* 374 in the meristematic extracts, suggesting that cyclic AMP is present as a lesser component in the non-meristematic regions. Also in this spectrum from C, the ion at *m/z* 346, isobaric with the protonated molecular ion of cyclic-GMP, was much more prominent than in the spectra from A and B, suggesting that this second nucleotide is present in greater relative proportions than is the case in the meristematic extracts.

Whilst in no way conclusive, the presence of these peaks is consistent with the presence in the three extracts of cyclic-AMP and, in smaller quantities, the other cyclic nucleotides. Further support is added by the increased relative intensity of the peaks at *m/z* 352, 374 and 396 (Fig. 1b) following addition of standard cyclic-AMP to extract A (essentially identical spectra were also obtained after spiking samples B and C with standard cyclic-AMP), and by the absence of these diagnostic peaks from the spectra obtained from the controls. The controls, which consisted of the extracts treated with phosphodiesterase to hydrolyse cyclic nucleotides and of nucleotide mixtures subjected to the purification procedure to see if cyclic nucleotides could arise as artefacts, gave essentially identical spectra as represented by control A obtained following phosphodiesterase treatment (Fig. 1e).

Presence of cyclic-AMP in the meristematic extracts and of cyclic-AMP and cyclic-GMP in the non-meristematic extracts was also indicated by the paired-ion HPLC traces of these samples, in which each possessed a large UV-absorbing peak at 20.7 min co-chromatographing with a reference sample of cyclic-AMP; the non-meristematic sample trace contained also a large peak at 20.7 min together with a much smaller peak (*ca* 8% of the major peak) at 14.8 min co-chromatographing with authentic cyclic-GMP.

In order to identify the above compounds unambiguously and to determine if other cyclic nucleotides are also present as minor components of the total cyclic nucleotide extracts, FAB mass spectra and MIKES scans of the ions isobaric with the protonated molecular ions of the appropriate cyclic nucleotides were obtained from the fractions which would contain individual cyclic nucleotides if present. These fractions were, first, further purified by electrophoresis and additional chromatography. The mass spectra of fraction I (cyclic AMP, see Experimental) from each of the three extracts were essentially identical and that obtained from meristematic sample A is reproduced in Fig. 1f. In addition to the matrix-derived background peaks of $[3Gro+H]^+$, $[3Gro+Na]^+$ and $[3Gro+2Na]^+$ at *m/z* 277, 299 and 321 respectively, each spectrum contained large peaks at *m/z* 330, 352 and 374, corresponding to the protonated molecular ion of cyclic-AMP and its mono- and di-sodium adducts, respectively.

Identification as cyclic-AMP was supported by HPLC, with a large peak at 20.8 min on paired-ion chromatography and by UV absorption spectrophotometry, in which the sample was found to have λ_{max} at 257, 259 and 260 min at pH 1, 7 and 11 respectively; essentially similar profiles were obtained with standard cyclic-AMP. The sample was unambiguously identified as cyclic AMP by the MIKES scan of the protonated molecular ion at *m/z* 330 (Fig. 2). In each spectrum the major peak was at *m/z* 136 corresponding to the protonated base adenine. Presence of the characteristic fragments of the 3′,5′-cyclic isomer at *m/z* 164 and 178 [9, 10] and absence of the strong peak at *m/z* 202, characteristic of 2′,3′-cyclic-AMP [9, 10], establish the compound as 3′,5′-cyclic-AMP. MIKES scanning of the *m/z* 330 ion present in control samples produced spectra which did not contain diagnostic peaks at *m/z* 136, 164 and 178. Collectively these data provide unequivocal evidence that 3′,5′-cyclic-AMP is an endogenous component of both meristematic and non-meristematic regions of *Pisum* roots.

The same approach was used to analyse fractions II–VI from the three extracts and their equivalent controls. The FAB mass spectra of fraction II (cyclic-GMP, see Experimental) from the three extracts contained large peaks at *m/z* 346, 368 and 390, corresponding to the protonated molecular ion of cyclic-GMP and its mono- and di-sodium adducts; no large peaks were observed in the spectra from the controls. Paired-ion HPLC analysis demonstrated a UV-absorbing peak at 14.6 min in each of the three extracts, and this had λ_{max} at 255, 252 and 259 nm at pH 1, 7 and 11 respectively, and it exhibited spectral characteristics of a compound containing guanine. MIKES scanning of the *m/z* 346 precursor ion yielded spectra (Fig. 3) which contained large peaks at *m/z* 152, corresponding to the protonated base guanosine, and at *m/z* 180 and 194, characteristic of 3′,5′-cyclic GMP. Peaks at *m/z* 195, 214 and 217 which are characteristic peaks of 2′,3′-cyclic GMP, were absent. No diagnostic peaks were observed in the controls and it could thus be concluded that cyclic GMP also is an endogenous component of both meristematic and non-meristematic regions of *Pisum*.

FABMS of the fractions III (cyclic-CMP, see Experimental) from each extract indicated a difference between the meristematic and non-meristematic extracts. The meristem extracts A and B contained strong ions at *m/z*

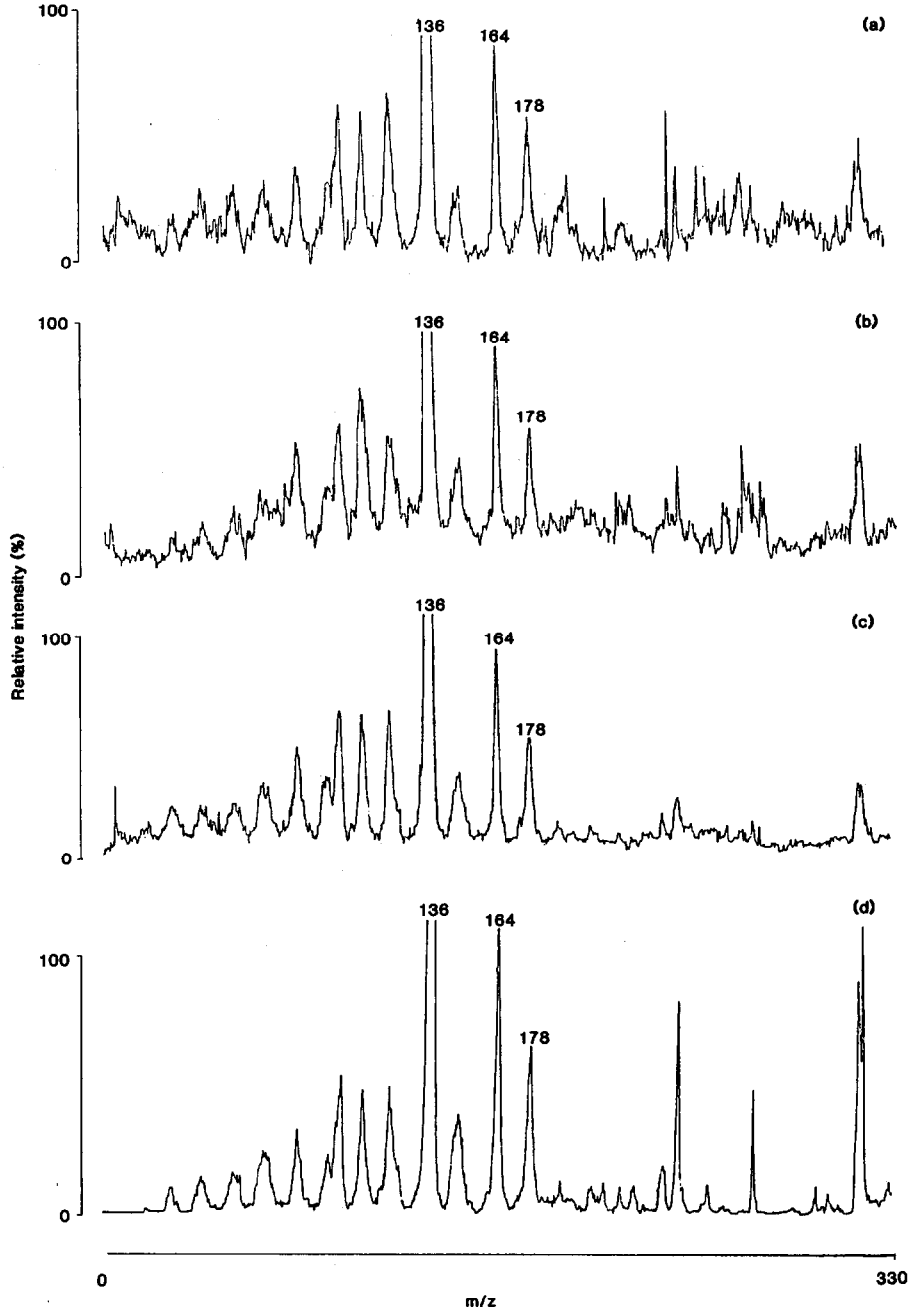

Fig. 2. Mass-analysed ion kinetic energy spectrum scans of *m/z* 330, generated by fast atom bombardment of fraction I (see Experimental) from meristematic tissue (a and b) and non-meristematic tissue (c), together with the MIKES scan of authentic adenosine 3′,5′-cyclic monophosphate (d). The *m/z* values of diagnostic peaks are indicated.

306, 328 and 350 corresponding to the protonated molecular ion and mono- and di-sodium adducts of cyclic-CMP (Fig. 4a and b). The spectrum from C, the non-meristematic sample (Fig. 4c), did not exhibit (such) large peaks at these positions, and this difference was further highlighted upon HPLC, in which the meristematic extracts produced a much larger UV absorbing peak at 8.9 min, cochromatographing with cyclic-CMP. This had λ_{max} at 279, 271 and 272 at pH 1, 7 and 11 respectively, and the spectral characteristics of a cytidine-containing compound. The MIKES scans of the three sample extracts (Fig. 5) confirmed the presence of cyclic-CMP in each sample, with the characteristic peaks at *m/z* 112, 140 and 154. The substantially lower quantity of cyclic-CMP present in the non-meristematic sample C can be seen from the much greater contribution made to the spectrum by two matrix-derived background peaks at *m/z* 115 and 214 (Fig. 5c) when compared to the MIKE spectra

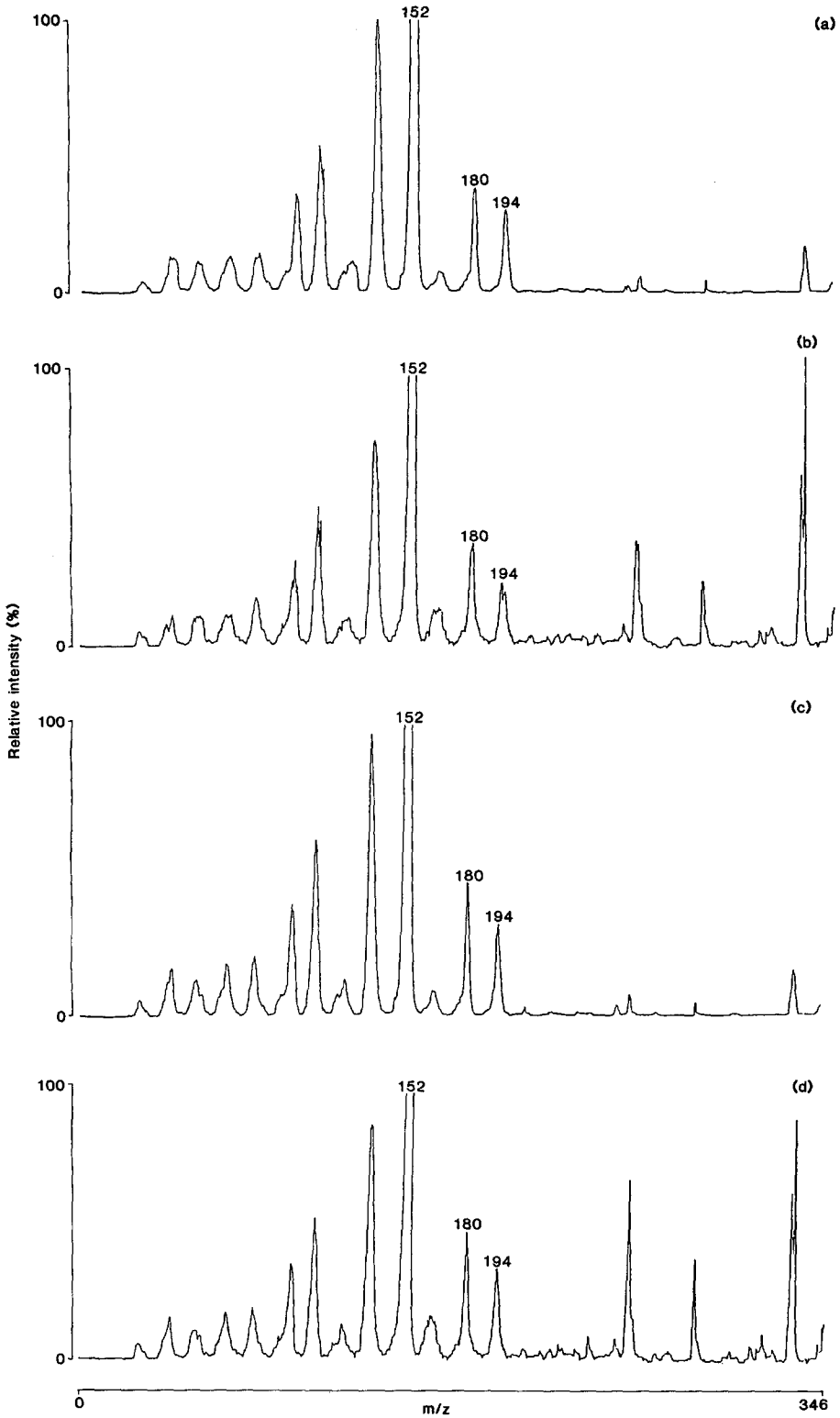

Fig. 3. Mass analysed ion kinetic energy spectrum scans of *m/z* 346 generated by fast atom bombardment of fraction II (see Experimental) from meristematic tissue (a and b) and non-meristematic tissue (c), together with the MIKES scan of authentic guanosine 3',5'-cyclic monophosphate (d). The *m/z* values of diagnostic peaks are indicated.

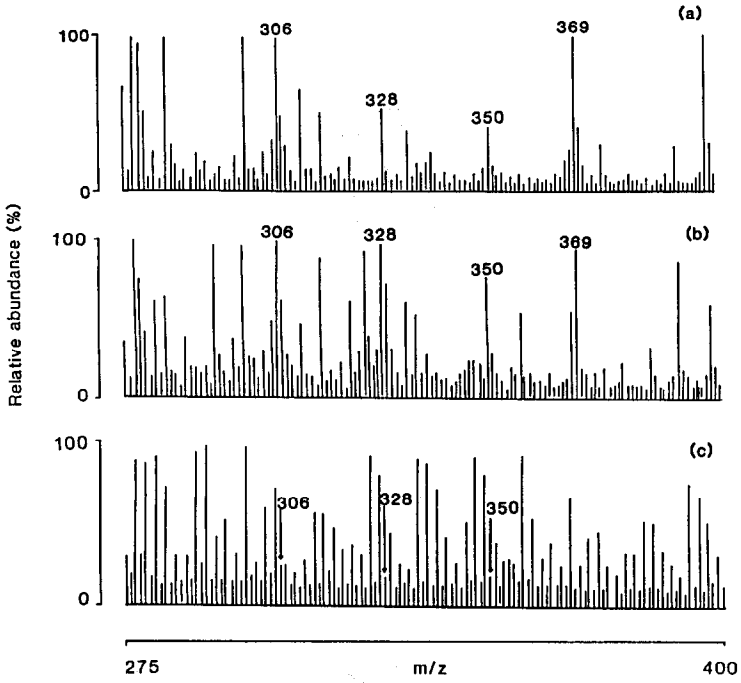

Fig. 4. Positive-ion fast atom bombardment mass spectra of fraction III (see Experimental) from meristematic tissue (a and b) and non-meristematic tissue (c).

obtained from the meristematic samples (Fig. 5a and b). No relevant sample-related peaks were observed in the controls; it was thus concluded that cyclic-CMP is present in both meristematic and non-meristematic tissue, but at significantly lower concentrations in the latter.

Examination of fraction IV, which would contain any cyclic-UMP present, and of fraction V, which would contain any cyclic-IMP present (see Experimental), indicated that the relative distribution of these two cyclic nucleotides between meristematic and non-meristematic tissue is the opposite of that with cyclic-CMP. The FAB mass spectra obtained for fraction IV from the two meristematic samples contained only weak peaks at *m/z* 307, 329 and 351, corresponding to the protonated molecular ion and mono- and di-sodium adducts of cyclic-UMP. This contrasted with the much stronger peaks observed in the spectrum from fraction IV from the non-meristematic sample. Similarly the mass spectra for fraction V from the meristematic samples had no significant peaks at *m/z* 331, 353 or 375, characteristic of cyclic-IMP, while the non-meristematic sample contained peaks of strong intensity at these *m/z* values. This was consistent with the absence of any UV-absorbing peaks on HPLC of the fractions IV and V from the meristematic samples, the presence of a UV-absorbing peak at 11.3 min in fraction IV and at 17.9 min in fraction V from the non-meristematic sample. The peaks had λ_{max} of 262, 263 and 261 nm at pH 1, 7 and 11, and of 248, 249 and 251 nm at pH 1, 7 and 11, respectively; their retention times were identical to those of authentic reference samples of cyclic-UMP and IMP and their spectral characteristics were those of uracil- and inosine-containing compounds. Provisional identification of these compounds as cyclic-UMP and cyclic-IMP was confirmed by the MIKES scans. The MIKE spectrum produced from the *m/z* 307 ion from the non-meristematic sample contained the characteristic peaks of cyclic-UMP at *m/z* 113, 141 and 155 (Fig. 6e), but these peaks were absent in the meristematic sample A and very weak in sample B (Fig. 6a and b); the MIKE spectra obtained from the *m/z* 331 ions generated from the non-meristematic sample contained ions at *m/z* 137, 165 and 179, characteristic of cyclic-IMP, which were absent from the meristematic samples (Fig. 7). No relevant peaks were observed in the controls and it could thus be concluded that both cyclic-IMP and -UMP are present in non-meristematic tissue, but that little or no detectable cyclic UMP and no detectable cyclic IMP is present in the meristematic tissue.

With each of the extracts A–C, examination of FAB mass spectra obtained from fraction VI (cyclic-dTMP, see Experimental) revealed a significant peak at *m/z* 305, isobaric with the protonated molecular ion of cyclic dTMP. With the meristematic samples there were only minor peaks at *m/z* 327 and 349, corresponding to the mono- and di-sodium adducts. These peaks were much larger in the spectrum obtained from the non-meristematic sample. HPLC analysis of the three samples did not yield any well-defined UV-absorbing peak. MIKES scanning of the *m/z* 331 ion from each of the three samples produced a spectrum from the non-meristematic sample containing the peaks characteristic of 3',5'-cyclic-dTMP at *m/z* 81, 110, 127, 179 and 207 (the characteristic CID fragmentation pattern of cyclic dTMP does not follow the same pattern as that of the other cyclic nucleotides because of the absence of oxygen at the 2'-position [7]), whereas the corresponding MIKE spectra from the two meristematic samples did not contain these diagnostic peaks (Fig. 8). As these peaks were absent from the

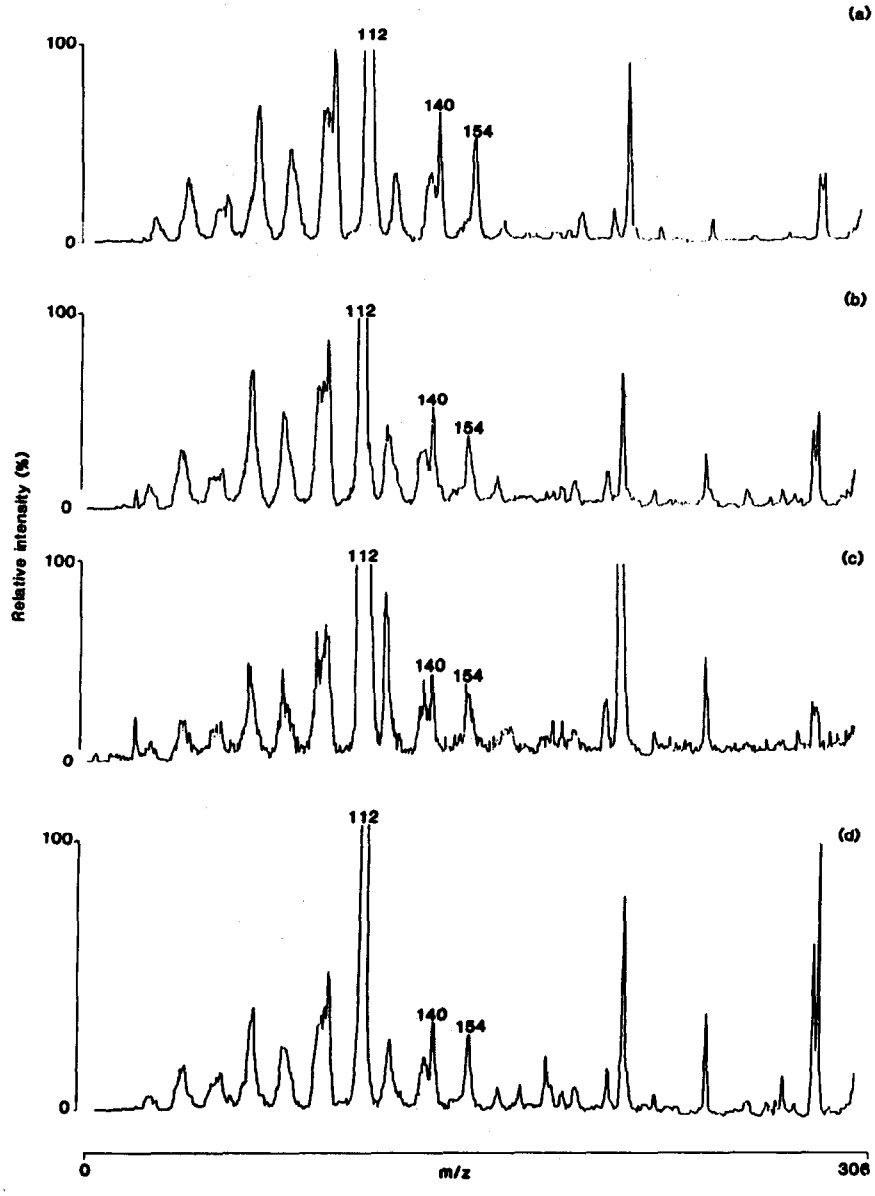

Fig. 5. Mass-analysed ion kinetic energy spectrum scans of *m/z* 306 generated by fast atom bombardment of fraction III (see Experimental for details) from meristematic tissue (a and b) and non-meristematic tissue (c), together with the MIKES scan of authentic cytidine 3',5'-cyclic monophosphate (d). The *m/z* values of diagnostic peaks are indicated.

spectra obtained from the controls, it was concluded that cyclic-dTMP is present in the non-meristematic tissues in low concentration relative to the levels of the other cyclic nucleotides, and that it is not present in detectable quantities in the meristematic tissues.

DISCUSSION

The data described above indicate that cyclic-AMP, -GMP, -CMP and -UMP are present in *Pisum* root meristematic tissue, and that cyclic-AMP, -GMP, -CMP, -IMP and -dTMP are present in the non-meristematic regions. The relative concentration of cyclic-CMP is shown to be significantly higher in the meristematic tissue than in the non-meristematic regions. Conversely cyclic UMP is present at higher relative concentrations in the non-meristematic tissue than in the meristem. The combination of immersion in liquid nitrogen, extraction with perchloric acid, chromatographic purification and FAB/CID-MIKE analysis have provided unequivocal evidence of the identity of these endogenous cyclic nucleotides and this technique offers great potential for identification of cyclic nucleotides and structurally similar compounds in plant extracts. At present, the data described here can only be considered as semi-quantitative, but there are current developments in technique which enable quantification of cyclic nucleotide phosphodiesterase activity through the estimation of cyclic-

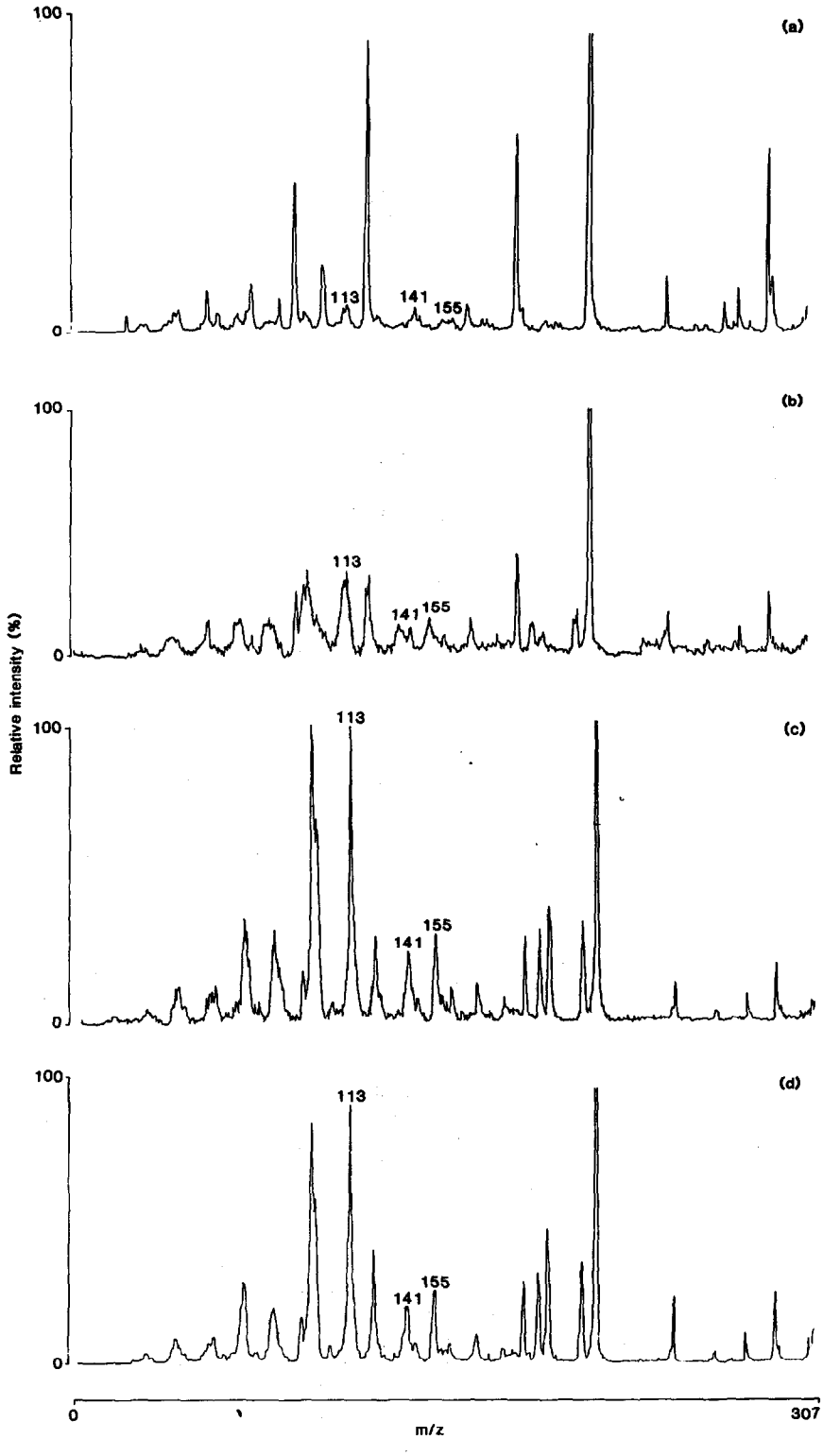

Fig. 6. Mass-analysed ion kinetic energy spectrum scan of *m/z* 307 generated by fast atom bombardment of fraction IV (see Experimental for details) from meristematic tissue (a and b) and non-meristematic tissue (c), together with the MIKES scan of authentic uridine 3',5'-cyclic monophosphate (d). The *m/z* values of diagnostic peaks are indicated.

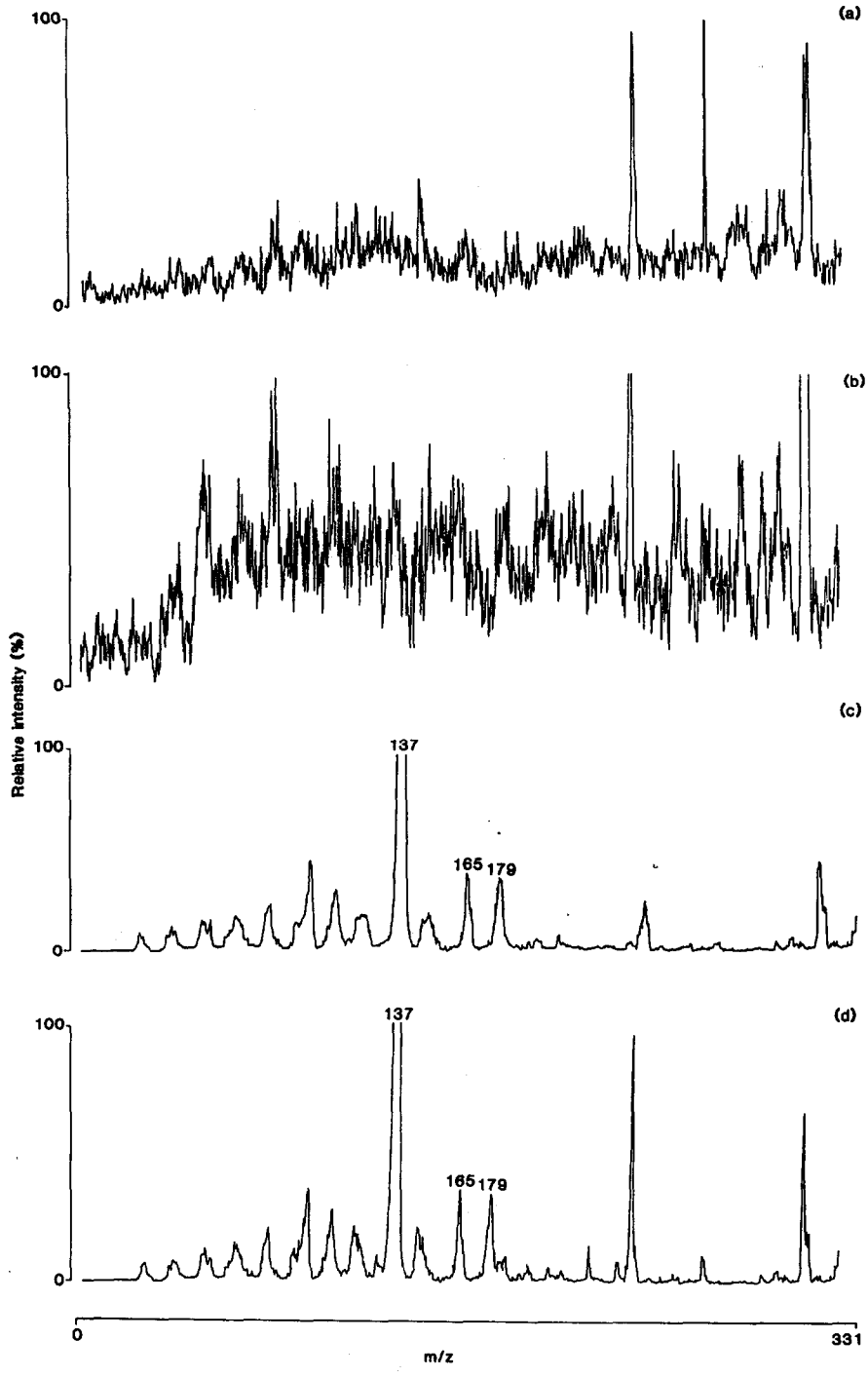

Fig. 7. Mass-analysed ion kinetic energy spectrum scan of *m/z* 331 generated by fast atom bombardment of fraction V (see Experimental for details) from meristematic tissue (a and b) and non-meristematic tissue (c), together with the MIKES scan of authentic inosine 3′,5′-cyclic monophosphate (d). The *m/z* values of diagnostic peaks are indicated.

AMP/AMP ratios by means of FAB/MIKES [17]. The application of such a procedure to identify and quantify cyclic nucleotide concentrations in tissue extracts should obviate ambiguities of identification and difficulties of cross-reactivity which have until now hampered progress in research into cyclic nucleotide biochemistry in higher plants [1, 2].

The presence of cyclic AMP and cyclic GMP in higher plant tissues has previously been reported (see reviews [1, 2]); in addition to confirming, by a technique novel in plant biochemistry, the presence of these two cyclic nucleotides in *Pisum*, this constitutes the first report of the natural occurrence of cyclic-UMP, -IMP, -CMP and -dTMP in plant tissues. The presence of these four novel

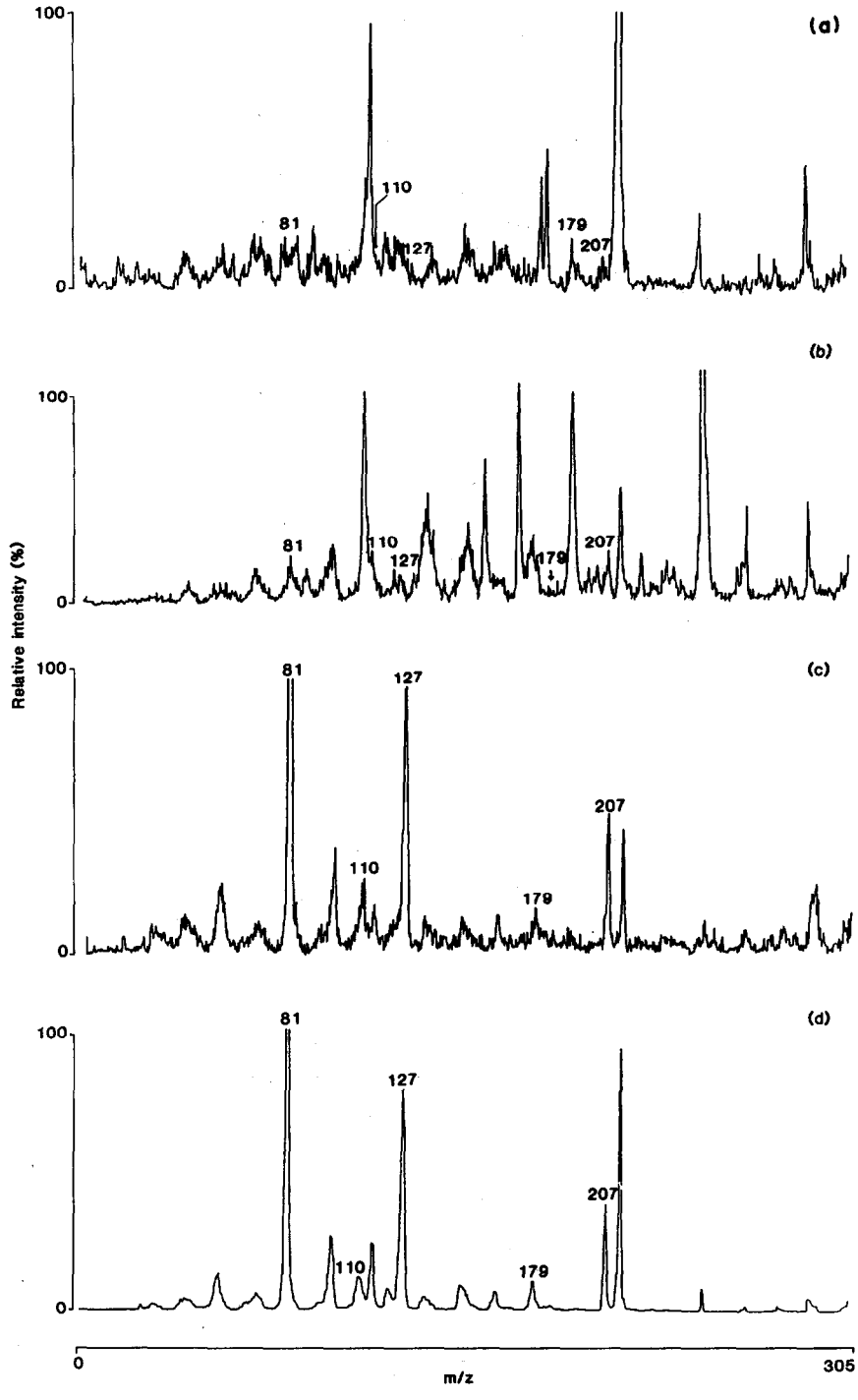

Fig. 8. Mass-analysed ion kinetic energy spectrum scans of *m/z* 305 generated by fast atom bombardment of fraction IV (see Experimental for details) from meristematic tissue (a and b) and non-meristematic tissue (c), together with the MIKES scan of authentic 2′-deoxythymidine 3′,5′-cyclic monophosphate (d). The *m/z* values of diagnostic peaks are indicated.

cyclic nucleotides poses the question of whether they possess specific biochemical functions. Without detailed study of the relevant enzymes however, it is not possible to say whether each of these compounds is synthesized by a specific nucleotide cyclase or if it arises merely from a lack of specificity by adenylate or guanylate cyclase, enzymes shown previously to occur in higher plant species [18, 19]. It has previously been suggested that there is a difference in meristematic and non-meristematic tissues in their cyclic AMP concentration [20]. The work

described here suggests that there is a significant difference in total cyclic nucleotide composition of meristematic and non-meristematic plant tissue.

The greater quantity of cyclic-CMP in the rapidly dividing cells within the meristematic regions in comparison to the cells of non-meristematic tissues is particularly interesting in view of current theories concerning the role of cyclic-CMP in mammalian tissues. In rapidly proliferating mammalian tissues the cyclic-CMP concentration is significantly elevated and is suggested to have a function in the regulation of cell division and growth [21–25]. The presence of higher relative concentrations of cyclic UMP in non-meristematic cells than in meristematic cells, taken together with the presence of cyclic-IMP and -dTMP in the non-meristematic regions and their absence in detectable quantity from the meristematic regions, may also reflect the difference in proliferation rate of the two types of tissue. Other analogies suggest different roles for these cyclic nucleotides. For example, the presence of cyclic-CMP and cyclic-IMP offers the possibility of a role in intercellular communication in higher plants similar to the functional extrusion of cyclic CMP and cyclic IMP from bacterial cells [26]; similar extrusion processes have, in fact, already been reported for cyclic-AMP in lower plants [27, 28].

Even if cyclic-CMP, -UMP, -IMP and -dTMP do not have specific functions *per se*, their presence is nevertheless of considerable potential significance since it will affect the potency of cyclic-AMP and cyclic-GMP in the same micro-environment. For example, we have previously shown that the phosphodiesterase activity of *Lactuca* is capable of hydrolysing not only cyclic-AMP and cyclic-GMP, but also cyclic-UMP and cyclic-CMP, with each affecting the hydrolysis of each of the others [11–13]. Similarly, of two plant protein kinases sensitive to cyclic-AMP and cyclic-GMP in *Lemna* [29], one is activated by cyclic-IMP and one inhibited by it.

It is thus feasible that cyclic-CMP, -UMP, -IMP and -dTMP may modify the effects of cyclic-AMP and cyclic-GMP by acting either as agonists or antagonists in the interaction of the latter two cyclic nucleotides with protein kinases and phosphodiesterases, thereby affecting regulatory systems responsive to them. It is also possible that cyclic-CMP, -IMP, -UMP and -dTMP have regulatory functions distinct from those of cyclic-AMP and cyclic-GMP. This appears to be the case in mammals in, e.g. the central nervous system in which the administration of cyclic-CMP, -IMP, -UMP and -dTMP produces effects that are not explicable merely as interference in the cyclic-AMP and cyclic-GMP systems [30]. In order to determine whether these cyclic nucleotides have themselves an effect *in vivo*, a systematic survey of the distribution and variation in concentration of these compounds, of the specificity of the proteins capable of their synthesis, binding and hydrolysis, and of the effects of the cyclic nucleotides and their derivatives on cellular metabolism is required.

EXPERIMENTAL

Materials. Seeds of *Pisum sativum* cv. Lincoln (C. Sharpe and Co. Ltd., Sleaford, Lincs.) were surface sterilized and germinated as previously described [11–13]. Cyclic nucleotide standards were obtained from Sigma or BDH Chemicals (both of Poole, Dorset, U.K.), CC materials were obtained from Sigma, and all other chemicals either from BDH Chemicals or Aldrich (Gillingham, Dorset, U.K.) unless otherwise specified. All items were of the highest purity commercially available.

Separation of meristematic and non-meristematic root tissue. After imbibition, the seeds were germinated and seedlings grown in the dark at 25° on moist Agriperlite for 3 days. The root meristematic tips (0–2 mm) of 3-day-old seedlings were detached by scalpel blade and frozen immediately in liquid N_2 at −80°. To collect non-meristematic material the roots of 3-day-old seedlings were cut at 1 cm from the tip and the lower material discarded. 2 cm of upper axes were collected and immediately stored at −80°. Two meristematic samples, designated A and B, and one non-meristematic sample designated C were so obtained.

Extraction and purification of cyclic nucleotides. The frozen tissue samples (100 g) were ground to a powder in a mortar and pestle precooled with liquid N_2. Perchloric acid extracts of these tissues were prepared and neutralized as described previously [7]. They were then subjected to CC through alumina, QAE Sephadex and SP Sephadex following the procedure in ref. [7]. The 3 post-SP Sephadex preparations were designated 'total cyclic nucleotide extracts'.

Separation of cyclic nucleotides. Cyclic nucleotide-containing fractions obtained by prep. electrophoresis (200 μg samples) were further separated by TLC and desalted, all as previously described [5, 7], then freeze-dried and retained for analysis. These fractions were designated fractions I–VI respectively, and would contain cyclic-AMP, -GMP, -CMP, -UMP, -IMP and -dTMP respectively as indicated by previous experimentation [5, 7].

Controls. Two sets of controls were used: (i) the complete extraction and purification procedure was repeated with aqueous solution containing 2 mg each of ATP, ADP, 5'-AMP, 3'-AMP, deoxy-5'-AMP, adenine, adenosine and 2',3'-cyclic AMP, and separately with solutions containing 2 mg each of the equivalent derivatives of inosine, xanthosine, uracil, thymidine, cytosine and guanosine. The final fractions were examined by HPLC, UV spectrophotometry and FABMS. (ii) Aliquots of the 3 total cyclic nucleotide extracts were incubated with the mammalian multifunctional cyclic nucleotide phosphodiesterase [14] and the resultant non-cyclic nucleotides chromatographically removed, all as previously described [15]. The resultant fractions, designated controls A, B and C, were examined directly by HPLC and by FABMS. After separation by electrophoresis, TLC and desalting, they were also examined by FABMS and FAB/MIKES.

HPLC analysis. Samples of the 3 total cyclic nucleotide extracts and the controls, of the separated cyclic nucleotides, and of cyclic nucleotide standards, were examined by paired ion HPLC, essentially by the method described previously [16].

UV absorption spectrophotometry. The UV spectra of the isolated compounds and of standards were recorded at pH 1, 7 and 11.

Mass spectrometry. Positive-ion FAB mass spectra were obtained with a VG ZAB-2F mass spectrometer (VG Analytical, Wythenshaw, U.K.) under previously specified conditions [3–7, 9, 10, 16]. A series of standard solutions of cyclic nucleotides containing from 0.01 to 10 μg/5 μl were made up in glycerol–H_2O (1:1) and 3 μl placed on the FAB target; 3 μl samples of the extracts were similarly examined. Sample lifetime for the compounds examined varied from 2 to 10 min on the FAB probe under bombardment from 8 keV Xe atoms (1–2 mA). The FAB source accelerating potential was set to 8 kV. All CID spectra were generated by using N_2 as collision gas in the second field-free region gas cell at an indicated ion gauge pressure of 10^{-6} torr, equivalent to 40–50% attentuation of the parent-ion signal. MIKE spectra were obtained in each case by selecting, with the magnetic sector, the appropriate protonated molecular

ion and then scanning the electric sector under data-system control. For scans over small regions, at least 4 sweeps were signal averaged.

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