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Extraction, purification and identification of cytidine 3′,5′-cyclic monophosphate from rat tissues

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The large-scale extraction and purification to homogeneity of cyclic CMP and its unequivocal identification are described. Rat liver, kidney, heart, spleen and lung tissues were subjected to a sequential purification procedure involving freeze-clamping, perchlorate extraction, alumina and boronate column chromatography, polyacrylamide-gel column electrophoresis and high-voltage paper electrophoresis. The purified sample co-chromatographed with authentic cyclic CMP on t.l.c. and high-pressure liquid chromatography and was positive in a cyclic CMP radioimmunoassay. The u.v., i.r. and p.m.r. spectra were each essentially identical with those of authentic cyclic CMP. Fast-atom bombardment of authentic cyclic CMP yielded a mass spectrum containing a molecular protonated ion: mass-ion-kinetic-energy scanning of this ion produced a spectrum unique to 3′,5′-cyclic CMP. The extracted nucleotide produced an identical mass-ion-kinetic-energy spectrum.

From the initial observations that cyclic AMP mediated the action of glucagon and adrenaline there developed the 'secondary messenger' concept (Sutherland & Rall, 1960), and it is now established that cyclic AMP is, with the exception of the anucleate erythrocyte, ubiquitous in mammalian cells (Jost & Rickenberg, 1971) and has a central function in endocrine regulation (Herman & Taunton, 1980).

In addition, cyclic AMP has a role in many non-endocrine processes such as blood clotting (Steer & Saltzman, 1980) and visual responses (Bitensky *et al.*, 1971) in mammals, and has been implicated in non-mammalian regulatory systems, including those of bacteria (Botsford, 1981), slime moulds (Gerisch & Malchow, 1976) and higher plants (Brown & Newton, 1981).

A second cyclic nucleotide, cyclic GMP, was later demonstrated to occur in most tissues and is cited as an intracellular regulator, both as a mediator of hormone action and in non-endocrine systems (Herman & Taunton, 1980). Many observations of apparently antagonistic effects of cyclic AMP and cyclic GMP can be explained in terms of the 'Yin-Yang' hypothesis (Goldberg *et al.*, 1974, 1975) in which the two cyclic nucleotides are described as exerting opposing regulatory actions. The absolute and relative concentrations of cyclic AMP and cyclic GMP are thus crucial factors in metabolic regulation. The cellular effects of these two cyclic nucleotides, together with the fact that pyrimidine nucleotides often display similar properties and functions to those of purine nucleotides, led to investigations of the possible occurrence and involvement in cellular regulation of other cyclic nucleotides.

Evidence for the natural occurrence of 3′,5′-cyclic CMP was first described by Bloch (1974), who isolated from leukaemia L-1210 cell extracts a compound that co-chromatographed with authentic cyclic CMP and that also possessed the same u.v.-absorption spectrum and mass spectrum. A chromatographically identical compound was isolated from normal and regenerating liver cells with a greater than 100-fold increase in concentration in the latter, leading to the suggestion that it may perform a role in the regulation of hepatic growth (Bloch, 1975). Supporting evidence of the identity of the isolated compound as 3′,5′-cyclic CMP was later produced by radioimmunoassay (Cailla *et al.*, 1978; Murphy & Stone, 1979; Hachiya *et al.*, 1980; Sato *et al.*, 1982) and enzyme immunoassay (Yamamoto *et al.*, 1982), and various factors have been cited as altering endogenous concentrations of 3′,5′-cyclic CMP, including luteinizing-hormone-

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; f.a.b., fast-atom bombardment.

releasing hormone (luliberin) (Hierowski *et al.*, 1981; Arisawa *et al.*, 1982), long-acting thyroid stimulator (Ochi *et al.*, 1982) and increased cell-proliferation rates (Bloch, 1975; Cailla *et al.*, 1978; Murphy & Stone, 1979; Scavennec *et al.*, 1981). Indirect evidence for the natural occurrence of 3',5'-cyclic CMP is provided by reports of an enzyme, cytidylate cyclase, capable of its synthesis (Cech & Ignarro, 1977, 1978) and of phosphodiesterases capable of cyclic CMP hydrolysis (Cheng & Bloch, 1978; Kuo *et al.*, 1978; Helfman *et al.*, 1981).

However, the identification of the extracted compound is not unequivocal (Stone & Murphy, 1981), and it has subsequently been claimed that 3',5'-cyclic CMP is not an endogenous nucleotide (Wikberg *et al.*, 1981; Wikberg & Wingren, 1981). The latter authors reported the existence of cyclic CMP-immunoreactive material that could be separated from 3',5'-cyclic CMP by chromatographic methods. As radioimmunoassay has constituted the major means to date of identification and assay of the putative 3',5'-cyclic CMP, these observations, together with the wide variations in reported endogenous concentrations of 3',5'-cyclic CMP, ranging from 1.5nmol/g (Bloch, 1975) to 0.2pmol/g of tissue (Yamamoto *et al.*, 1982), cast serious doubt on the validity of the cyclic CMP radioimmunoassay technique (Anderson, 1982). In addition, Gaion & Krishna (1979) questioned the existence of cytidylate cyclase, since their investigations suggested that the products of the reaction could be chromatographically separated from 3',5'-cyclic CMP, and Stone & Murphy (1981) found more than one cyclic CMP-immunoreactive compound among the products of the enzymic reaction. Furthermore, the cyclic CMP phosphodiesterase activity is multifunctional, being able to hydrolyse both 3',5'- and 2',3'-cyclic nucleotides and having activity with both purines and pyrimidines (Conrad & Bloch, 1980; Helfman & Kuo, 1982).

Several authors (e.g. Wikberg & Wingren, 1981; Anderson, 1982; Stone & Murphy, 1981) have consequently expressed the requirement for more positive and substantial evidence of the identity of 3',5'-cyclic CMP from tissue extracts before it can be considered as a natural constituent and its possible functions seriously investigated. The work described in the present paper represents the large-scale extraction and sequential purification to homogeneity of 3',5'-cyclic CMP from rat tissues followed by a combination of techniques, including chromatography, radioimmunoassay, enzymic hydrolysis, u.v., n.m.r. and i.r. spectroscopy, and mass spectrometry, which, taken collectively, are capable of providing unambiguous identification of the analyte.

Experimental

Materials

Radiochemicals were purchased from Amersham International (Amersham, Bucks., U.K.). Cyclic nucleotides were obtained either from the Boehringer Corp. (London W.5, U.K.) or from Sigma Chemical Co. (Poole, Dorset, U.K.). Affigel 601 was obtained from BioRad Laboratories (Watford, Herts., U.K.). All other biochemicals were from either Sigma Chemical Co. or BDH Chemicals (Poole, Dorset, U.K.), and all other chemicals from either BDH Chemicals or the Aldrich Chemical Co. (Gillingham, Dorset, U.K.), unless otherwise specified. All items were of the highest purity commercially available.

Extraction of cyclic CMP

Adult Lister Hooded rats were maintained at 20–22°C under a 13h/11h light–dark cycle. Food (Modified Rat and Mouse Breeding Diet; Pilsbury, Birmingham, U.K.) and water were available *ad libitum*. Rats were killed by a blow to the base of the neck and immediately decapitated. Liver, kidney, heart, spleen and lung tissues were rapidly exposed and freeze-clamped between two silver blocks fitted to a pair of tongs and precooled in liquid N_2. The frozen tissue samples were then ground to a powder in a mortar and pestle precooled with liquid N_2 and weighed. A 15g sample of the powdered tissue was homogenized in 50ml of ice-cold 0.6M-$HClO_4$ (1:3, w/v) in a Potter–Elvehjem homogenizer for 3min at 0–4°C, and the resultant homogenate was centrifuged at 14000*g* for 20min at 4°C. After being decanted off, the supernatant was neutralized with ice-cold 1M-KOH. The resultant precipitate was removed by centrifugation at 14000*g* for 20min at 4°C and discarded. The supernatants corresponding to 200g batches of powdered tissue sample were combined and concentrated to minimum volume under reduced pressure.

Alumina column chromatography

The extract was applied to a neutral alumina column (60cm × 2.5cm) previously washed with 1.5 litres of distilled water, and eluted with distilled water at a flow rate of 2.7ml/min. The first 500ml of the eluent was retained and concentrated under vacuum at 28°C to dryness.

Boronate column chromatography

The material eluted from the alumina column was then dissolved in distilled water, applied to an Affigel 601 phenylboronate column (8cm × 1.5cm) and eluted with 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaCl buffer, pH8.4, at a flow rate of 1ml/min. The first 20ml of

eluent was collected and retained. Samples corresponding to the original 200g batches were freeze-dried, weighed and combined.

Preparative electrophoresis

Preparative electrophoresis was carried out with a 7900 Uniphor Column Electrophoresis system (LKB Instruments, Croydon, Surrey, U.K.). A column (34cm × 2.5cm) was set up containing 0.2% polyacrylamide in 50mM-Trizma base, pH7.4, with a cathode buffer containing 15g of glycine/l and 3g of Trizma base/l at pH8.4 and an anode buffer containing 30mM-H_2SO_4 and 8g of Trizma base/l at pH7.1. Samples (300μg) in 5ml of distilled water were applied to the column at 10°C and electrophoresed at a current of 60mA at 1.2–1.4kV and 11kW for 7h. The column was then eluted with anode buffer at a flow rate of 4ml/for 13h, at the same current, power and voltage, and 2ml fractions were collected. Fractions 10, 11 and 12 were combined and freeze-dried.

Paper electrophoresis

The freeze-dried material from the preparative gel electrophoresis was redissolved in the minimum quantity of water and then subjected to high-voltage paper electrophoresis in a Shandon Southern model 124 apparatus. Samples were applied to a paper strip (61cm × 23cm; Whatman 3MM) together with a marker solution of 25μg of 3',5'-cyclic CMP in 20μl of water at each end, and electrophoresed in a 50mM-sodium borate buffer, pH9.2, for 110min at 30mA and 1.4kV. The two edges of the electrophoretogram were sprayed with an ethanolic solution of 2',7'-dichlorofluorescein (0.01%, w/v) containing a few drops of conc. NH_3 and spots were detected under u.v. light. The spot from each sample applied migrating towards the anode at an equivalent rate as the marker of authentic cyclic CMP was removed and eluted with aq. 50% (v/v) ethanol. The eluates were concentrated under vacuum, combined and desalted by gel filtration through Sephadex G-10 (Pharmacia, Uppsala, Sweden) (12cm × 1cm), then freeze-dried.

Recovery

The recovery during extraction and purification was examined by adding 10^5 d.p.m. of 3',5'-cyclic [5-^{3}H]CMP to extracts at each stage of the procedure in preliminary experiments. The radioactivity recovered in the retained fraction at the end of each stage was determined by counting the radioactivities of 0.5ml samples in 5ml of Aqualuma scintillant in a LKB Rackbeta 1217 instrument.

Control

The entire extraction and purification procedure was repeated with a solution containing 2mg of each of CTP, CDP, 3'-CMP, 5'-CMP, 2'-CMP, cytosine, cytidine, 2',3'-cyclic CMP and CDP-choline replacing tissue and the final fraction was examined by h.p.l.c., t.l.c. and u.v. spectrophotometry.

H.p.l.c. analysis

The concentrated extract was examined by reverse-phase and paired-ion h.p.l.c. essentially by the method described by Brown *et al.* (1982). The run was repeated with standard 3',5'-cyclic CMP and with a mixture of both extracted sample and marker.

U.v. and i.r. spectroscopy

The u.v.-absorption spectrum of the isolated compound was obtained at pH1, 7 and 11 in a Pye–Unicam SP.1700 u.v. spectrophotometer. The i.r.-absorption spectra of both standard 3',5'-cyclic CMP and the extracted sample were produced from KBr discs in a Pye–Unicam SP.1050 instrument.

Radioimmunoassay

Radioimmunoassay was carried out by use of a kit obtained commercially from Collaborative Research (Waltham, MA, U.S.A.) in essentially the manner recommended by the supplier, based on the methods of Cailla *et al.* (1978) and Murphy & Stone (1979).

Enzymic hydrolysis

Authentic 3',5'-cyclic CMP and the extracted sample were incubated with cyclic CMP phosphodiesterase by the procedure of Thompson *et al.* (1979) as modified by Newton & Salih (1983), but in the absence of nucleotidase. The products of the reaction were chromatographed on PEI-(polyethyleneimine-)cellulose plates, with 25mM-LiCl as developing solvent.

N.m.r. spectroscopy

N.m.r. spectra were obtained on a Varian XLFT-100 instrument with Fast Fourier Transform and at a reference frequency of 100.08MHz. Microgram quantities of the sample were dissolved in the minimum volume of ^2H_2O and, with sodium trimethylsilyl[$^2H_2$]propionate as an internal standard, the field was scanned at ambient temperature. In the case of standard cyclic CMP, 80 transients were adequate. With the limiting quantity of material, 1250 scans were required for the extracted sample.

Mass spectrometry

Mass spectra of the trimethylsilyl derivatives of authentic cytidine nucleotides were produced as described for cyclic AMP (Newton *et al.*, 1980). Positive f.a.b. mass spectra were obtained on a VG ZAB-2F mass spectrometer fitted with a VG FAB source and ion gun. Argon atoms were used to bombard the samples, the ion-gun conditions being typically 8kV accelerating potential and 1–2mA discharge current as measured by the constant-current power supply. The source accelerating voltage was 8kV. Samples of standards and analyte were prepared by dissolving in 50μl of glycerol/water (1:1, v/v) and placing 3–5μl of the solution containing up to 3μg of sample on the f.a.b. target. Under these conditions the sample lifetime was 10–15min.

To generate collision-induced-dissociation spectra, nitrogen was used as a collision gas in the second-field-free-region gas cell at an indicated pressure of 6×10^{-6}Torr (8mPa). Mass-ion-kinetic-energy spectra were obtained by scanning the electric-sector voltage under control of a data system. For scans over small regions, at least five sweeps were averaged by the computer. To maintain a steady ion current, the f.a.b. sample probe and power-supply discharge current were rigorously controlled throughout the acquisition of mass-ion-kinetic-energy spectra.

Results

The recovery of radioactivity at each stage in the preliminary experiments was between 72 and 87%, and the overall recovery was estimated at 27%. The final sample produced a single peak, on two h.p.l.c. systems, with the same elution times of 6.1 and 9.2min on reverse-phase and paired-ion separation respectively as authentic 3',5'-cyclic CMP, in a system previously shown to be capable of resolving all the known naturally occurring 2',3'- and 3',5'-cyclic nucleotides (Brown *et al.*, 1982). The u.v.-absorption spectrum of the analyte had $\lambda_{max.}$ at pH1, 7 and 11 of 279nm, 271nm and 271nm, again identical with authentic 3',5'-cyclic CMP, and exhibiting the characteristics of a cytidine-containing compound.

The putative and authentic 3',5'-cyclic CMP had the same R_F on t.l.c. of 0.58, and, after hydrolysis by cyclic CMP phosphodiesterase of both extract and standard, two u.v.-absorbing spots were detected after t.l.c., a minor one at the original R_F and the major one at R_F 0.14 that co-chromatographed with 5'-CMP, thus suggesting the presence of a phosphodiester linkage. Radioimmunoassay, with anti-(cyclic CMP) serum, produced a positive reaction. These observations are consistent with the isolated sample being 3',5'-cyclic CMP, but are not unequivocal evidence of its identity. The possibility that the end product was

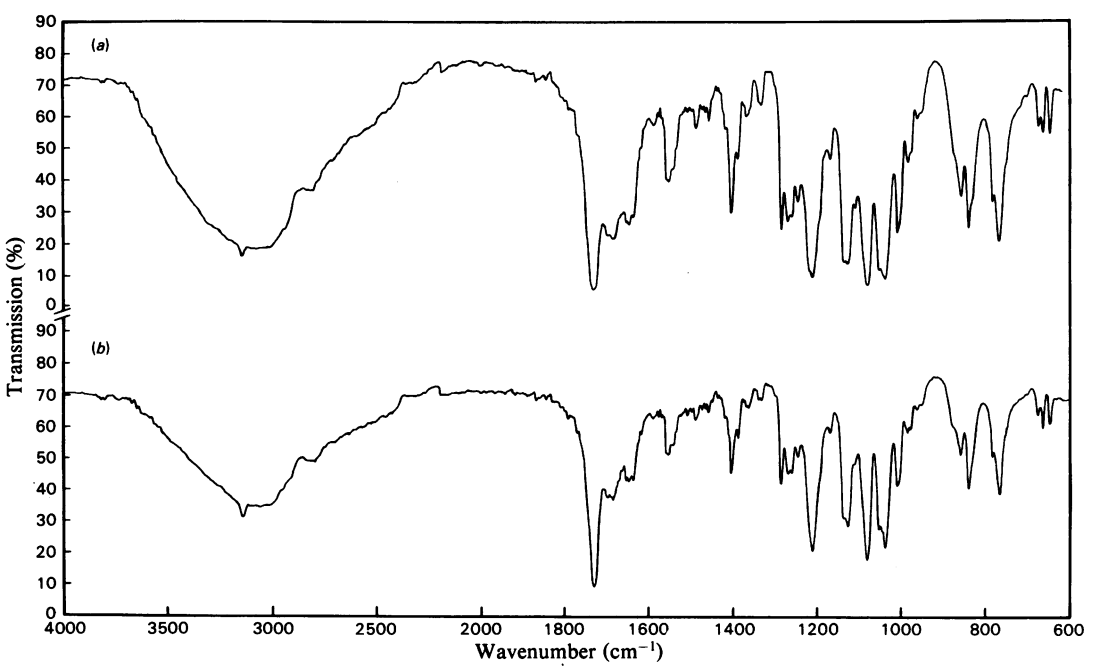

Fig. 1. *I.r.-absorption spectra of* (*a*) *authentic 3',5'-cyclic CMP and* (*b*) *extracted sample*
For details see the text.

an artifact in origin was discounted by the absence of any u.v.-absorbing material in the equivalent final fraction when the mixture containing cytidine nucleotides other than 3',5'-cyclic CMP was put through the same procedure.

The identification of the analyte as 3',5'-cyclic CMP was confirmed by i.r. and n.m.r. spectroscopy and by mass spectrometry. In view of the limited quantity of extracted material, it was considered more practical in each case to examine the spectra on a comparative basis rather than carry out exhaustive individual analyses of individual spectra. The i.r. spectra of the putative and authentic 3',5'-cyclic CMP (Fig. 1) are essentially identical. Both contain the broad absorption between 2400 and 3600 cm^{-1}, making assignment of individual OH, CH and NH absorbancies nonproductive, but the characteristic intense C=O bond has been shifted to 1740 cm^{-1} by the presence of the pyrimidine ring in both cases, and the C=C, C=N, C−N and C−O bonds at 1630, 1695, 1230 and 1100 cm^{-1} exactly coincide. This replication in the i.r. 'fingerprint' of the putative and authentic 3',5'-cyclic CMP is strong evidence that the compounds are identical, as can also be deduced from the n.m.r. spectra.

Reliable analysis of the couplings of the proton-n.m.r. spectra of 3',5'-cyclic CMP is virtually impossible because of the tight coupling of the spin systems (Blackburn *et al.*, 1973), and the problems are normally overcome by either using ^{13}C-n.m.r. (Lapper & Smith, 1973), or inducing a lanthanide shift (Kainosho & Ajisaka, 1975). The small amount of material available for examination by n.m.r. precluded the latter two approaches, and so the proton-n.m.r. spectra are considered purely from a comparative viewpoint. The proton-n.m.r. spectra of the putative and authentic 3',5'-cyclic CMP are shown in Fig. 2. Minor differences between the spectra arise from the necessary variations in acquisition times arising because of sample concentration, resulting in the less-effective solvent suppression in the spectrum of the putative sample (*b*), together with greater background 'noise'. Nevertheless the major peaks emanating from the two samples can be seen to be

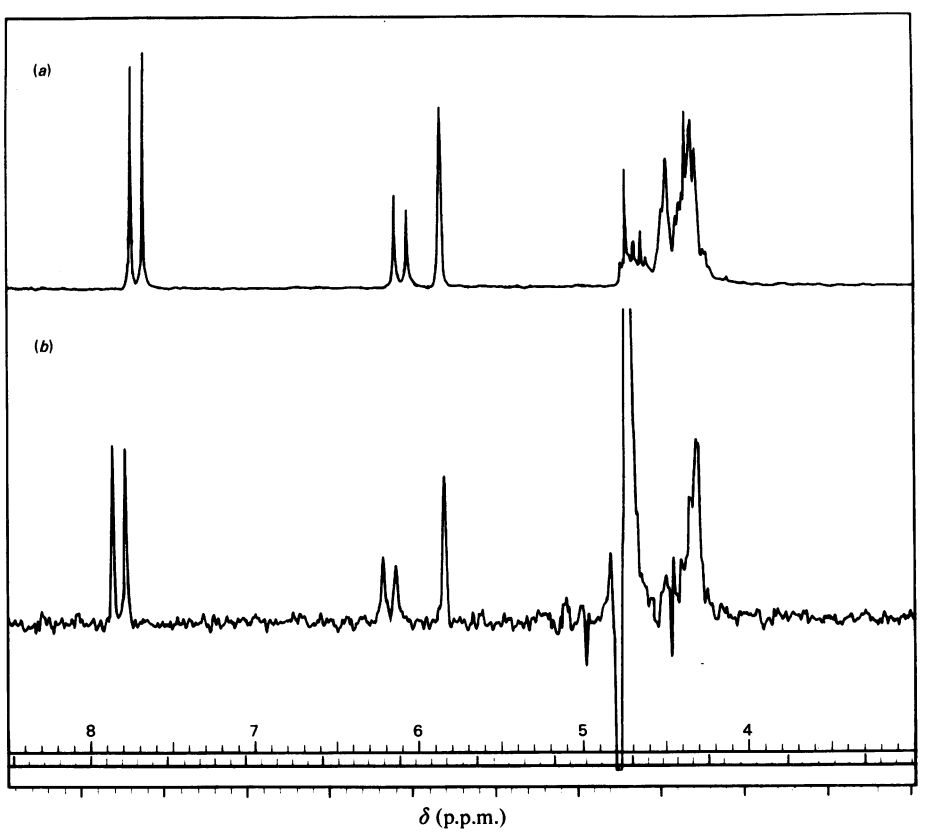

Fig. 2. *Proton-n.m.r. spectra of* (*a*) *authentic 3',5'-cyclic CMP and* (*b*) *extracted sample*
For details see the text.

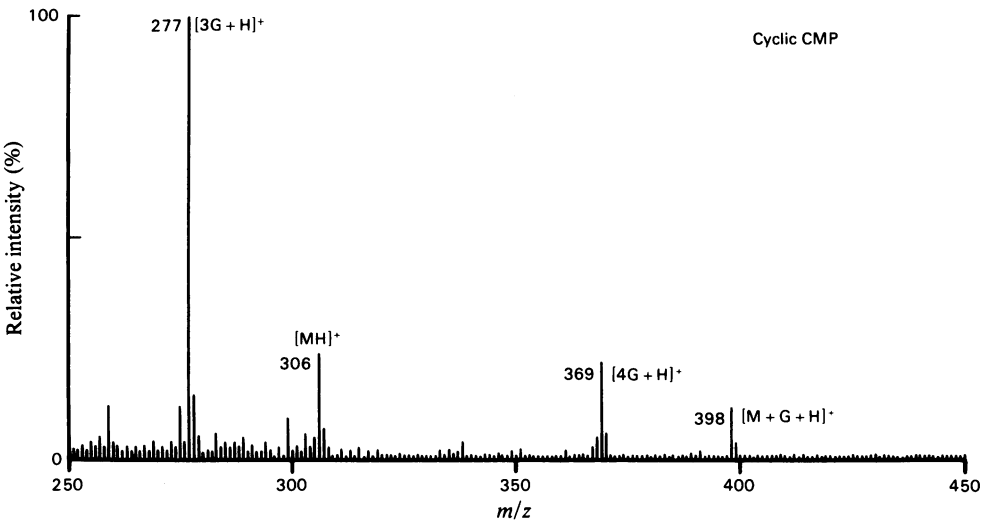

Fig. 3. *Positive-ion f.a.b. mass spectrum of 3',5'-cyclic CMP*
For details see the text.

essentially the same, with the $C_{(6)}$ and $C_{(5)}$ doublets at 7.76 and 6.14 and 1' singlets at 5.81 p.p.m. and at 7.71, 6.10 and 5.82 p.p.m. respectively for the extracted sample and authentic 3',5'-cyclic CMP, closely corresponding to one another in shift and to the reported spectrum of a cyclic CMP derivative (Wierenga & Woltersom, 1977).

In the initial effort to obtain mass spectra for comparison, attempts to obtain a molecular ion both from unmodified 3',5'-cyclic CMP and from its trimethylsilyl derivative as described for cyclic AMP (Newton *et al.*, 1980) were unsuccessful. Although mass spectra could be obtained in this way, they were not clearly distinguishable from those obtained from other cytidine nucleotides, which displayed similar fragmentation patterns, and therefore an alternative approach had to be utilized. The positive-ion f.a.b. mass spectrum of authentic 3',5'-cyclic CMP (Fig. 3) contained a molecular protonated ion at *m/z* 306, and major peaks arising from the glycerol matrix (Kingston *et al.*, 1982) at *m/z* 277, 369 and 398 corresponding to protonated glycerol trimer, protonated glycerol tetramer and protonated glycerol plus 3',5'-cyclic CMP. Attempts to enhance the molecular protonated ion by variation of conditions were unsuccessful. The spectrum obtained under the same conditions from the extracted sample contained the *m/z* 306 species and was indistinguishable from that of authentic 3',5'-cyclic CMP. The f.a.b. mass spectra obtained from CTP, CDP, 3'-CMP, 5'-CMP and cytidine did not contain the *m/z* 306 species, although molecular parent ions for each compound were evident. 2',3'-Cyclic CMP did contain an *m/z* 306 species. Thus, although the mass spectrum alone was insufficient to distinguish between the two cyclic CMP isomers, it indicated that the analyte was one, or a mixture, of these two isomers.

The $[M+H]^+$ peak *m/z* 306 was investigated further by using the collision-induced-dissociation/mass-ion-kinetic-energy scanning technique, and spectra obtained for (*a*) 3',5'-cyclic CMP, (*b*) 2',3'-cyclic CMP and (*c*) the purified extract are shown in Fig. 4. The major fragmentation in all three produces an *m/z* 112 species. Although the spectra of the two standard isomers are similar, there are a number of differences that allow unequivocal differentiation between them. One part of the spectrum was chosen for further investigation, and the result of averaging a number of sweeps is shown. 3',5'-Cyclic CMP yielded fragments at *m/z* 140 and *m/z* 154, whereas 2',3'-cyclic CMP showed a different fragmentation pattern: *m/z* 140 is weak, *m/z* 154 is absent and a strong *m/z* 178 is observed. The spectra obtained from the extracted sample (*c*) show clearly that it is 3',5'-cyclic CMP and not the 2',3'-isomer.

Discussion

The purification method utilized was designed to provide a homogeneous sample for identification, and was not intended for routine assay purposes. The evidence obtained provides unequivocal evidence that 3',5'-cyclic CMP is an endogenous nucleotide. The previous doubts over its identification in tissue extracts have arisen

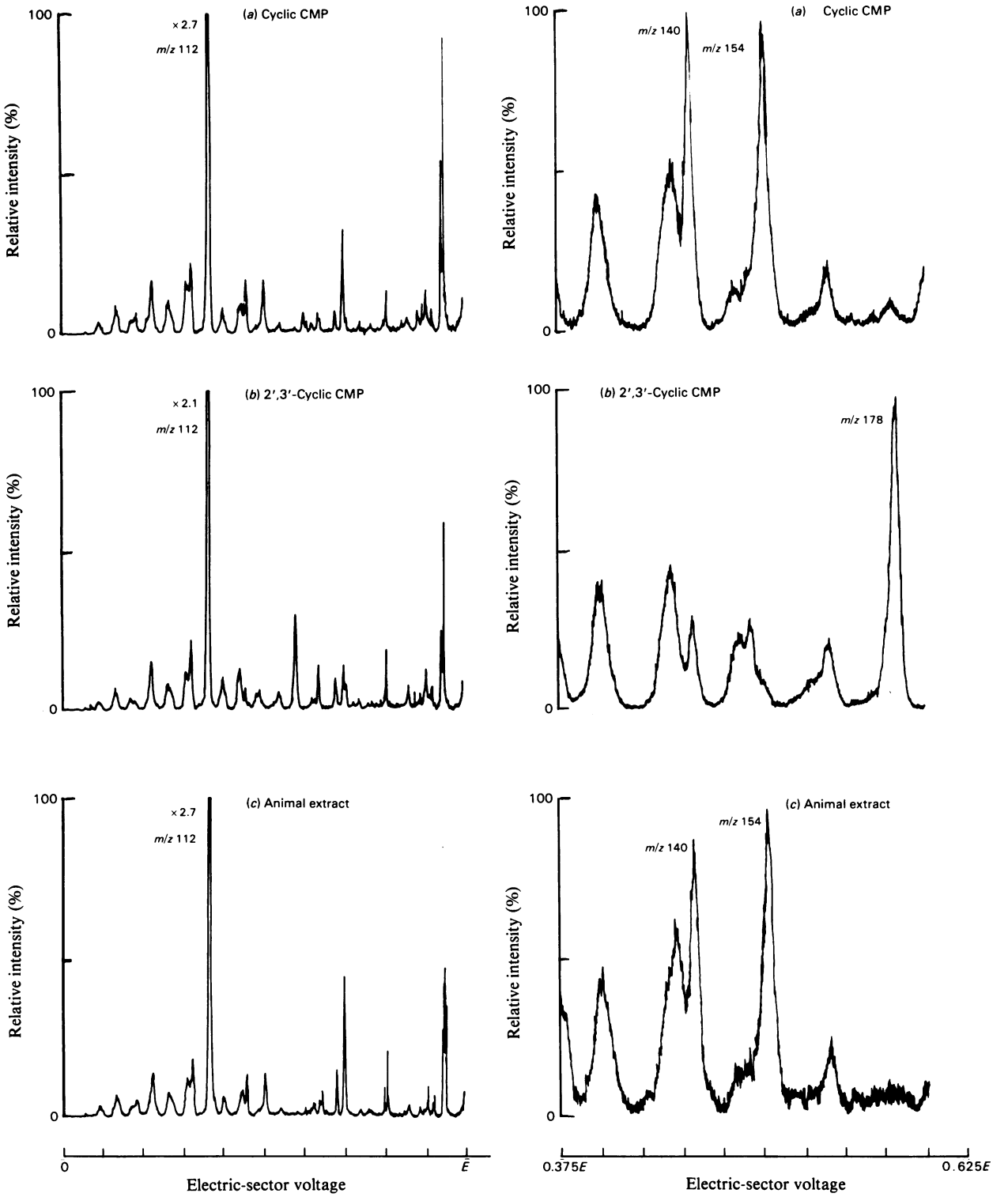

Fig. 4. *Collision-induced-dissociation/mass-ion-kinetic-energy scans of (a) authentic 3′,5′-cyclic CMP, (b) authentic 2′,3′-cyclic CMP and (c) extracted sample*
Both full and partial scans are recorded. For details see the text.

because of discrepancies relating to 3',5'-cyclic CMP immunoreactivity (Wikberg & Wingren, 1981; Wikberg *et al.*, 1981) and the properties of enzymes responsible for its synthesis (Gaion & Krishna, 1979) and degradation. In our hands, also, radioimmunoassay of 3',5-cyclic CMP in crude and partially purified extracts has yielded results with a lack of reproducibility. The observation that 3',5'-cyclic CMP synthesized by cell homogenates is bound to at least one protein or peptide immediately after formation (Ignarro, 1979; Ignarro & Cech, 1979) provides a feasible explanation of the difficulties. The binding of the product of the cytidylate cyclase reaction to protein would explain its anomalous chromatographic behaviour reported by Gaion & Krishna (1979): the radioimmunoassay of a mixture containing 3',5'-cyclic CMP and 3',5'-cyclic CMP linked to one or more amino acids resulting from breakdown of the 3',5'-cyclic CMP-protein complex during extraction would produce different results with antisera of differing specificity, with the relative proportion of 3',5'-cyclic CMP and 3',5'-cyclic CMP-amino acid a potential variable in replicate experiments.

The demonstration that 3',5'-cyclic CMP is a natural cellular constituent lends extra significance to observed changes, for example in proliferation rate (Anderson *et al.*, 1981) and behaviour (Benton & Newton, 1983), elicited by exogenous application of 3',5'-cyclic CMP or its derivatives. This evidence, the existence of a hormone and calmodulin-sensitive phosphodiesterase displaying absolute specificity for cyclic CMP as substrate (Newton & Salih, 1983), of a cyclic nucleotide-dependent protein kinase capable of activation by cyclic CMP (Vardanis, 1980), and of a protein capable of binding cyclic nucleotides but with greatest affinity for 3',5'-cyclic CMP (R. P. Newton & S. G. Salih, unpublished work), in combination with earlier data and in view of the effects of cyclic AMP and cyclic GMP, suggests that 3',5'-cyclic CMP should be seriously examined as a potential intracellular modulator.

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