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Extraction, purification, identification and metabolism of 3′,5′-cyclic UMP, 3′,5′-cyclic IMP and 3′,5′-cyclic dTMP from rat tissues

Russell P. NEWTON,* Eric E. KINGSTON,† Nabil A. HAKEEM,* Sa'ad G. SALIH,* John H. BEYNON‡ and Christopher D. MOYSE*

*Department of Biochemistry and ‡Royal Society Research Unit, University College of Swansea, Swansea SA2 8PP, Wales, U.K., and †Research School of Chemistry, The Australian National University, GPO Box 4, Canberra, A.C.T. 2601, Australia

The large-scale extraction and partial purification of endogenous 3′,5′-cyclic UMP, 3′,5′-cyclic IMP and 3′,5′-cyclic dTMP are described. Rat liver, kidney, heart, spleen and lung tissues were subjected to a sequential purification procedure involving freeze-clamping, perchlorate extraction, alumina and Sephadex ion-exchange chromatography and preparative electrophoresis. The samples thus obtained co-chromatographed with authentic cyclic UMP, cyclic IMP and cyclic dTMP on t.l.c. and h.p.l.c. and the u.v. spectra of the extracted samples were identical with those of the standards. Fast atom bombardment of the three cyclic nucleotide standards yielded mass spectra containing a molecular protonated ion in each case; mass-analysed ion kinetic-energy spectrometry ('m.i.k.e.s') of these ions produced a spectrum unique to the parent cyclic nucleotide. The extracted putative cyclic UMP, cyclic IMP and cyclic dTMP each produced a m.i.k.e.s. identical with that obtained with the corresponding cyclic nucleotide standard. Rat liver, heart, kidney, brain, intestine, spleen, testis and lung protein preparations were each found capable of the synthesis of cyclic UMP, cyclic IMP and cyclic dTMP from the corresponding nucleoside triphosphate, of the hydrolysis of these cyclic nucleotides and of their binding, with the exception that cyclic dTMP was not synthesized by the kidney preparation.

INTRODUCTION

Two cyclic nucleotides, namely 3′,5′-cyclic AMP and 3′,5′-cyclic GMP, are key regulatory components of a wide variety of mammalian cellular processes in both endocrine and non-endocrine systems. More recently the natural occurrence of a third cyclic nucleotide, 3′,5′-cyclic CMP, has been established by means of the large-scale extraction of rat tissues and subsequent purification of an analyte followed by its identification as cyclic CMP by h.p.l.c., u.v., i.r. and n.m.r. spectroscopy and by f.a.b. mass spectrometry with m.i.k.e.s. [1–3]. Although it has been suggested that cyclic CMP may constitute a third cyclic nucleotide intracellular mediator [4], this has yet to be established. Nevertheless evidence has been produced of an enzyme, cytidylate cyclase, capable of cyclic CMP synthesis, distinct from adenylate or guanylate cyclase [5–8, R. P. Newton, B. J. Savage & N. A. Hakeem, unpublished work] and of phosphodiesterases capable of cyclic CMP degradation [9], including a cyclic CMP-specific phosphodiesterase [10,11].

The existence of three naturally occurring cyclic nucleotides at least two of which play a central role in the regulation of cellular metabolism, together with the known diversity of purine and pyrimidine nucleotides, led us to investigate whether any other 3′,5′-cyclic nucleotides occur naturally. Apart from the use of specific cyclic-nucleotide-dependent protein kinases, the major means of identification and assay of cyclic AMP and cyclic GMP has been by radioimmunoassay, by some other form of saturation-binding analysis, or by chromatographic means [12]. Although these methods have proved acceptable for this purpose with the latter two cyclic nucleotides, both of these techniques have been criticized as equivocal in the studies of cyclic CMP. For example, there are reports of compounds other than cyclic CMP which display cyclic CMP immunoreactivity [13–15] and of putative cyclic CMP, from a cytidylate cyclase incubation, which co-chromatographed with authentic cyclic CMP in some systems but not in others [16]. Physical methods can provide an alternative and more effective means of identification, but many are impractical for cyclic nucleotides because of the requirement for large quantities of analyte. Although cyclic AMP has been unambiguously identified by means of the electron-ionization m.s. of a volatile trimethylsilyl derivative of this cyclic nucleotide [17,18], our attempts to produce mass spectra of other cyclic nucleotides by both electron ionization of the volatile derivatives and by desorption chemical ionization of the underivatized compounds yielded spectra that were not uniquely characteristic of the cyclic nucleotide under examination, mainly on account of the absence of molecular ions and of large fragment ions. A new method of ionization, f.a.b., is extremely useful for analysis of non-volatile and thermally labile biological compounds [19]. F.a.b. mass spectra contain less fragmentation than spectra produced by other ionization techniques, and although this

Abbreviations used: c.i.d., collision-induced dissociation; f.a.b., fast atom bombardment; m.i.k.e.s., mass-analysed ion kinetic-energy spectrum (spectrometry); PEI, polyethyleneimine; QAE, quaternary aminoethyl; SP, sulphopropyl; Gro, glycerol.

represents a lack of the structural information that is particularly required where isomers are involved, the molecular ion is frequently conserved. M.i.k.e.s. resulting from the c.i.d. of isomeric ion structures have been shown to be capable of easily distinguishing between them [20–22]. We have previously reported the combination of these techniques to identify unambiguously cyclic AMP, cyclic GMP and cyclic CMP as distinct from their 2′,3′-cyclic isomers in tissue extracts [3,23,24], and we have now extended this approach so that the unequivocal identification of microgram quantities of 3′,5′-cyclic UMP, -cyclic IMP, -cyclic XMP and -cyclic dTMP is possible [25]. Here we describe the large-scale extraction and partial purification of endogenous cyclic nucleotides and their subsequent identification. The sequential purification procedure is based upon that previously developed in our laboratory for cyclic CMP [3], with the major change being the replacement of the phenylboronate column chromatography stage with two steps involving QAE-Sephadex and SP-Sephadex; the elution conditions and selection of fractions for retention were determined by pilot experiments using commercially obtained cyclic nucleotide standards. The extracted, partially purified, putative cyclic nucleotides were provisionally identified by u.v. spectroscopy, h.p.l.c. and then confirmed by f.a.b./m.i.k.e.s. spectrometry. The ability of several rat tissues to bind these cyclic nucleotides, to synthesize them from radiolabelled nucleotide triphosphates and to degrade them to mononucleotides was also examined by methods adapted from those used routinely for the estimation of these parameters relative to cyclic AMP and cyclic GMP metabolism.

EXPERIMENTAL

Materials

Radiochemicals were purchased from Amersham International (Amersham, Bucks., U.K.). Cyclic nucleotides were obtained either from the Boehringer Corp. (London W5, U.K.) or from Sigma or BDH Chemicals (both of 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 nucleotides

Adult Lister Hooded rats were maintained at 20–22 °C under a 13 h-light/11 h-dark cycle. Food ('Modified Rat and Mouse Breeding Diet'; Pilsbury, Birmingham, U.K.) and water were available *ad libitum*. Rats were killed, the liver, kidney, heart, spleen and lung tissues removed and homogenized in $HClO_4$, and concentrated neutralized extracts equivalent to 200 g batches of powdered tissue sample produced as described previously [3].

Alumina column chromatography

The extract was applied to a neutral alumina column (55 cm × 3 cm) previously washed with 2 litres of distilled water, and eluted with distilled water at a flow rate of 2.3 ml/min. The first 600 ml of the eluent was retained and freeze-dried.

Ion-exchange chromatography

The dried material eluted from the alumina column was then dissolved in 15 ml of distilled water and applied in three separate 5 ml aliquots to a QAE-25-Sephadex column (Cl^- form, 1.2 cm × 80 cm) and eluted with 0.05 M-HCl at a flow rate of 5.5 ml/min. The first 500 ml were collected, neutralized with 5 ml of ice-cold 5 M-NaOH added in small portions with thorough mixing, then freeze-dried. This freeze-dried sample was resuspended in 10 ml of distilled water and applied to an SP-Sephadex column (formate form, 1.1 cm × 55 cm) and eluted with 0.05 M-formic acid at a flow rate of 6.8 ml/min. The first 350 ml were retained, neutralized and freeze-dried. The samples corresponding to the original 200 g batches were then combined.

Preparative electrophoresis

Preparative electrophoresis was carried out with 300–400 μg samples as previously described for the purification of cyclic CMP [3], with fractions 16–19 and 22–25 being combined into two fractions, retained and designated fractions I and II respectively. The preliminary experiments using standards had shown that fraction I would contain any cyclic dTMP and cyclic IMP present and fraction II any cyclic UMP and cyclic XMP present respectively. These fractions were desalted as previously described [3], then freeze-dried and retained for analysis.

Recovery

The recovery during extraction and purification was examined by adding separately 5×10^5 d.p.m. of each ^{32}P-labelled cyclic nucleotide 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 0.5 ml samples in 5 ml of Aqualuma scintillant in an LKB Rackbeta 1217 instrument.

Control

The entire extraction and purification procedure was repeated with solutions containing 2 mg each of UTP, UDP, 3′-UMP, 5′-UMP, uridine, uracil and 2′,3′-cyclic UMP, and separately with solutions containing 2 mg each of the equivalent derivatives of inosine, xanthosine, deoxythymidine, thymidine, cytosine, adenosine, deoxyadenosine and guanosine. The final fractions were examined by h.p.l.c., u.v. spectrophotometry and f.a.b. m.s.

H.p.l.c. analysis

The concentrated extracts were examined by reverse-phase and paired-ion h.p.l.c., essentially by the methods described previously [26]. The runs were repeated with cyclic nucleotide standards and with mixtures of extracted samples and the appropriate cyclic nucleotide standard.

T.l.c.

The isolated compounds and their products of hydrolysis after incubation with 1 M-NaOH for 10 min were chromatographed with appropriate standards on PEI-cellulose plates, with 50 mM-LiCl as developing solvent.

U.v. absorbance spectroscopy

The u.v.-absorption spectra of the isolated compounds and of the standards were obtained at pH 1, 7 and 11 in a Pye–Unicam SP.1700 spectrophotometer.

M.s.

Positive f.a.b. mass spectra were obtained on a VG ZAB-2F mass spectrometer under conditions as previously specified [3,24–26]. A series of standard solutions of cyclic nucleotides containing from 10 ng·μl^{-1} to 50 μg·μl^{-1} were made up in glycerol/water (1:1, v/v) and then 3–5 μl of the solution were placed on the f.a.b. target; 5 μl samples of the extracts were similarly examined. Sample lifetime for the compounds examined varied from 5 to 20 min. C.i.d. spectra were generated by using N_2 as collision gas in the second field-free region gas cell at a pressure of 800 μPa (6 μTorr). M.i.k.e.s. were obtained in each case by selecting with the magnetic sector the molecular protonated ion and then scanning the electric sector under data-system control. For scans over small regions, at least five sweeps were signal-averaged.

Preparation of tissue homogenates

Rats were killed as described previously [3] and the tissues rapidly removed and homogenized in 50 mM-Tris/HCl, pH 7.4, at 4 °C (1:9, w/v) with a Potter–Elvehjem homogenizer at 4 °C. For the phosphodiesterase and binding-activity assays, the homogenate was centrifuged at 10000 *g* for 20 min, then the supernatant dialysed against 12 vol. of the same buffer with three changes. For the cyclase assays the same preparation procedure was used with the omission of the centrifugation stage. Preliminary experiments indicated that centrifugation at this stage decreased cyclase activities.

Phosphodiesterase

Phosphodiesterase activity with 3′,5′-cyclic UMP, -IMP and -dTMP as substrates was estimated by incubating 100 μl of tissue homogenate with 10 μl of 5 mM unlabelled cyclic nucleotide, 10 μl of ^{32}P-labelled cyclic nucleotide substrate (60000–80000 d.p.m.), 10 μl of *Crotalus adamanteus* venom (10 units/ml) in a total volume of 350 μl of 50 mM-Tris buffer, pH 7.4, containing 5 mM-$MgCl_2$ for 15–20 min at 36 °C. The reaction was stopped by heating at 90 °C for 2 min, reincubating with 1000 units of *Crotalus adamanteus* venom for 15 min, then the substrate and products separated by passage through a QAE-25-Sephadex column (formate form, 0.5 cm × 1.0 cm) and eluted with 30 mM-ammonium formate. The first 2 ml eluted was collected and a 0.5 ml portion was counted for radioactivity in 5 ml of Aqualum scintillant in a LKB 1217 Rackbeta scintillation counter. The appropriate no-enzyme and boiled-enzyme controls were included: the inclusion of a small quantity of *Crotalus adamanteus* venom in the first step of the assay was found to increase reproducibility. (Cyclic XMP phosphodiesterase activity could not be estimated owing to the non-availability of radiolabelled 3′,5′-cyclic XMP.)

Cyclic nucleotide-binding activity

Cyclic nucleotide-binding activity with cyclic UMP, cyclic IMP and cyclic dTMP was estimated by incubating 200 μl of the tissue homogenate with 25 μl of ^{32}P-labelled cyclic nucleotide (40000–100000 d.p.m.), and 10 μl of 10 μM-unlabelled cyclic nucleotide in a total volume of 450 μl of 100 mM-Tris/HCl buffer, pH 6.8, at 35 °C for 3 h. The bound and free cyclic nucleotide were separated by incubating with 500 μl of 20% (w/v) Norit OL charcoal and 2% (w/v) bovine serum albumin in the same buffer for 30 min at 35 °C, then centrifuging at 12000 *g* for 10 min. A 0.5 ml portion of the supernatant was collected and counted for radioactivity as described above. (Cyclic XMP binding capacity could not be estimated due to non-availability of radiolabelled 3′,5′-cyclic XMP.) The protein contents of the samples assayed for cyclic nucleotide binding activities were determined as previously described [24].

Nucleotide cyclase activity

Nucleotide cyclase activity with ITP, UTP, XTP and dTTP was estimated by incubating 20 μl of homogenate with 5 μl of 250 μM-nucleotide triphosphate, 5 μl of ^{32}P-labelled nucleotide triphosphate (200000–250000 d.p.m.) in a total volume of 50 μl of 50 mM-Tris/HCl, pH 7.4, containing 12.5 mM-$MgCl_2$ for 10 min at 37 °C. The reaction was stopped by the addition of 100 μl of 55% (w/v) trichloroacetic acid, the mixture centrifuged at 10000 *g* for 10 min and the supernatant passed through an acidic alumina (0.5 cm × 1.0 cm) and QAE-25-Sephadex (formate form, 2.5 cm × 1.0 cm) column and eluted with 0.03 M-HCl, The first 2.5 ml eluted was retained, concentrated under vacuum to 0.5 ml and counted for radioactivity as described above. The appropriate no-enzyme and boiled-enzyme controls were included. The products were examined and identified by t.l.c. and h.p.l.c. as described above.

RESULTS

The recovery of radioactivity at each stage in the preliminary experiments was between 69 and 91%, and the overall recovery was between 14 and 23%, dependent upon which cyclic nucleotide was being assessed. F.a.b. of the pre-preparative electrophoresis sample, after elution from SP-Sephadex, yielded the mass spectrum partially reproduced in Fig. 1(*a*). Peaks with *m/z* ratio equivalent to those of the molecular protonated ions of each of eight cyclic nucleotides, cyclic-dTMP, -CMP, -UMP, -dAMP, -AMP, -IMP, -GMP and -XMP were evident at *m/z* 305, 306, 307, 314, 330, 331, 346 and 347 respectively, together with a peak for a cyclic GMP–sodium adduct at *m/z* 368. Also apparent were several peaks previously shown to originate from the glycerol matrix used in the f.a.b. process, in particular the protonated glycerol trimer, glycerol trimer plus sodium and glycerol trimer plus two sodium at *m/z* 277, 299 and 321 respectively. The three major peaks at *m/z* 293, 315 and 337 are likely to correspond to a compound of M_r 292 forming a molecular protonated ion, a molecular sodium-adduct ion, and the sodium adduct of its sodium salt. The compound remained unidentified, but these peaks were not present in the spectra produced after further purification of the cyclic nucleotides (Fig. 1*b* and 1*c*). Examination of the spectra produced at the same stage in the control experiment again exhibited peaks emanating from the glycerol matrix and the peaks at *m/z* 293, 315 and 337, but did not contain any significant peaks equivalent to the molecular protonated ions of the eight cyclic nucleotides, indicating that their origin in the extracted sample was not an artefact. In the spectrum of the extracted sample (Fig. 1*a*), the peaks at *m/z* values corresponding to the protonated molecular ions of the cyclic nucleotides were interpreted as evidence that these cyclic nucleotides may have been present in the extract

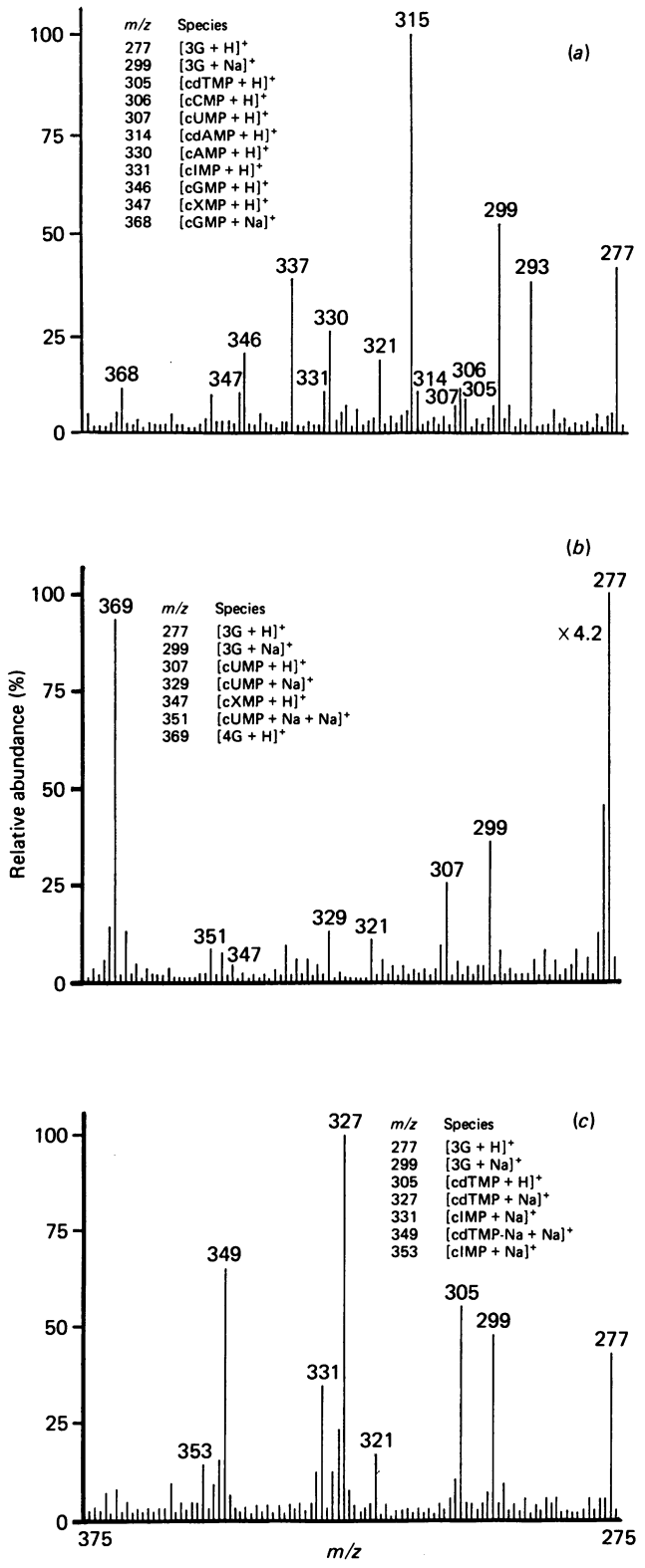


Fig. 1. Mass spectra produced by f.a.b. of partially purified extracted samples

(*a*) F.a.b. mass spectrum of pre-preparative electrophoresis sample, after elution from SP-Sephadex. (*b*) F.a.b. mass spectrum of fraction II obtained from preparative electrophoresis. (*c*) F.a.b. mass spectrum of fraction I obtained from preparative electrophoresis. For further details, see the text. Abbreviations used: cdTMP, cCMP etc., cyclic dTMP, cyclic CMP etc.

but did not constitute conclusive proof without further detailed examination by chromatographic, spectrophotometric and mass-spectrophotometric methods.

After the final stage of the purification, fractions II and I were each found on h.p.l.c. to contain two major peaks of u.v. absorbance. The peaks from fraction II had the same elution times of 11.5 and 13.8 min on paired ion separation and 8.8 and 11.9 on reverse-phase h.p.l.c. as authentic 3′,5′-cyclic UMP and XMP respectively; the two peaks from fraction I had the same elution times of 13.2 and 18.1 min, and 12.4 and 14.2 min, as 3′,5′-cyclic dTMP and -IMP respectively. Only a small peak corresponding to cyclic XMP was evident in each system. The extracted samples also co-chromatographed on t.l.c. with the authentic compounds with R_F values of 0.68, 0.08, and 0.11 and 0.33 for cyclic UMP, -XMP, -dTMP and -IMP respectively. Incubation of these analytes with 1 M-NaOH yielded two u.v.-absorbing spots which co-chromatographed with the corresponding nucleotide 5′-monophosphates at R_F values of 0.20, 0.0, 0.0 and 0.04 and with the corresponding nucleotides at R_F values of 0.91, 0.53, 0.56 and 0.68. The u.v.-absorption spectra of the putative cyclic UMP, -IMP and -dTMP were identical with those of the corresponding standard 3′,5′-cyclic nucleotides at the three pH values tested, but the spectrum of the putative cXMP sample was equivocal in this respect, since only a weak spectrum was obtained, owing to a lack of material available for this analysis.

Fraction II (Fig. 1*b*) produced a f.a.b. mass spectrum containing peaks at *m/z* 277, 299 and 369, corresponding to the matrix, together with peaks at *m/z* 307, 329 and 351, which are coincidental with peaks previously shown to arise from the molecular protonated ions of cyclic UMP and its mono- and di-sodium adducts [25]. The peak at *m/z* 347 corresponds to the molecular protonated ion of cyclic XMP. Any molecular sodium-adduct ions of cyclic XMP would be obscured by the isobaric $[4G+H]^+$ matrix peak at *m/z* 369. The compound of M_r 292 that was present in the pre-electrophoresis sample was no longer evident, but a peak at *m/z* 321, likely to originate from the glycerol matrix, but potentially corresponding to $[\text{cyclic TMP}+H]^+$, was present. Fraction I (Fig. 1*c*) produced a mass spectrum again containing peaks originating from the glycerol matrix, together with major peaks coincidental with the molecular protonated ion of cyclic dTMP and its mono- and di-sodium adducts at *m/z* 305, 327 and 349 respectively and with the molecular protonated ion of cyclic IMP and its monosodium adduct at *m/z* 331 and 353 respectively [25]. Again a peak corresponding to *m/z* 321, which could have arisen from either $[\text{cTMP}+H]^+$ or $[3\text{ Gro–Na}+Na]^+$ was evident. Analysis of the f.a.b. mass spectra and of the h.p.l.c. elution profiles of the electrophoresis fractions I and II for each of the control mixtures showed no peaks analogous to those believed to originate from cUMP, cIMP, cdTMP and cXMP in fractions I and II and from the extracted tissue sample, confirming that their presence in the latter was not an artefact. A peak at *m/z* 321 was evident in the f.a.b. mass spectra of both the fractions I and II from each of the nine control mixtures subjected to the extraction and purification procedure. However, no u.v.-absorbing peak co-chromatographing with cyclic TMP was apparent in any of the h.p.l.c. profiles, and it was therefore concluded that the *m/z* 321 peak originated wholly from the glycerol matrix as $[3\text{ Gro–Na}+Na]^+$. The contribution of the

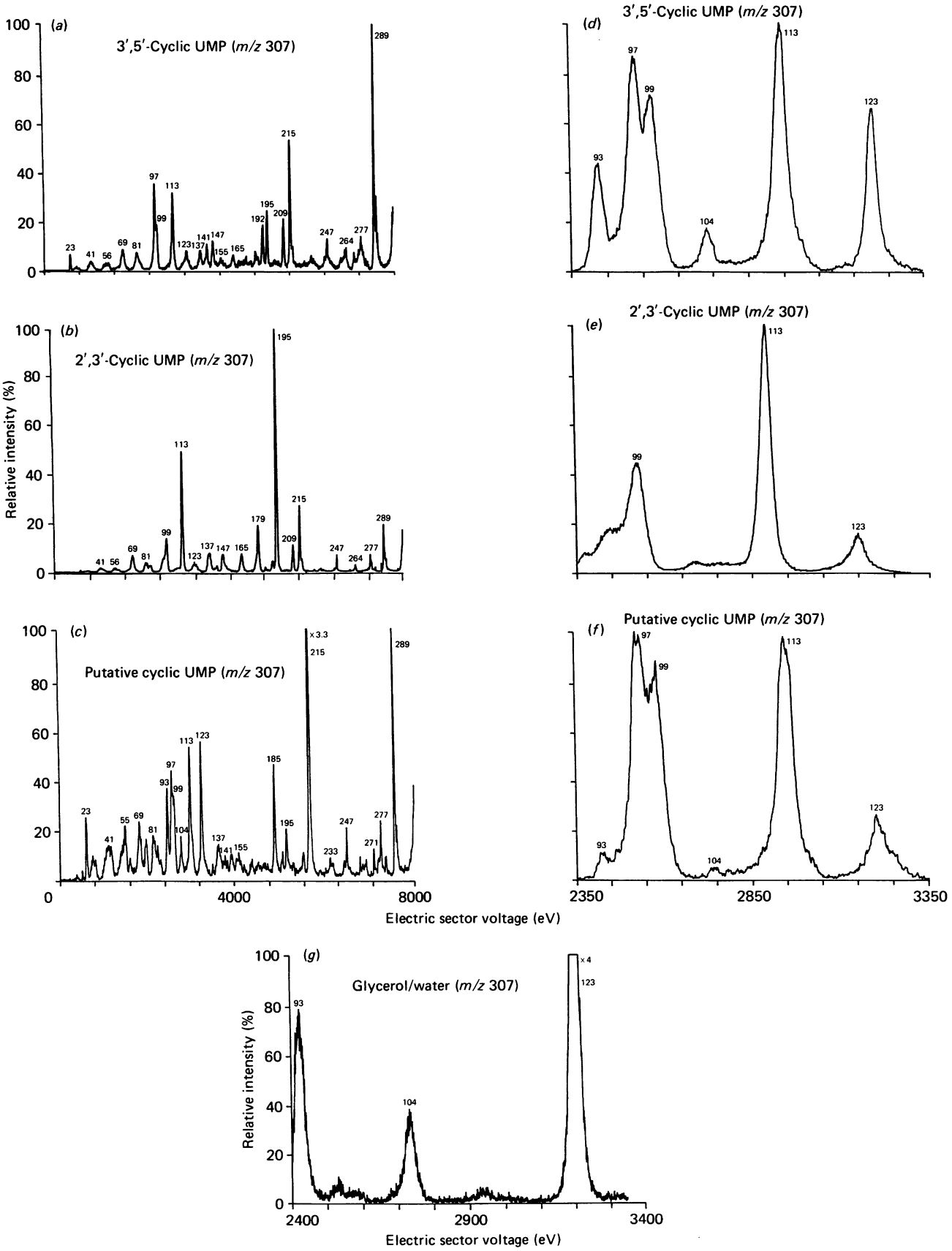


Fig. 2. C.i.d.–m.i.k.e.s. spectra of extracted and standard cyclic UMP

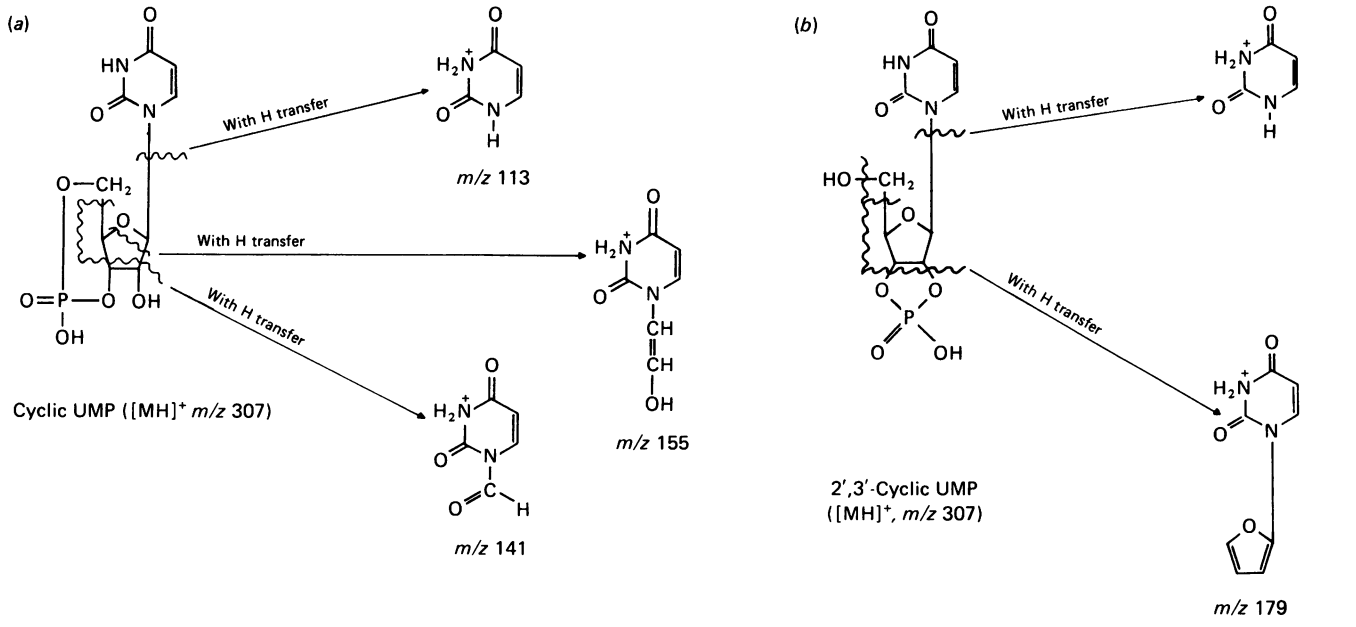
Both full and partial scans are shown, together with a partial scan of the glycerol/water matrix. (*a*) and (*d*), authentic 3′,5′-cyclic UMP; (*b*) and (*e*), authentic 2′,3′-cyclic UMP; (*c*) and (*f*), extracted putative 3′,5′-cyclic UMP; (*g*) glycerol/water matrix. For further details, see the text.

matrix-derived peaks, particularly at *m/z* 277 and 369, was much greater in the spectrum of fraction II (Fig. 1*b*) than in the spectra of fraction I or the pre-preparative electrophoresis sample (Figs. 1*a* and 1*c*), reflecting a lower solubility or stability of fraction II in the glycerol/water matrix. There is also variation between the spectra in the peak heights, relative to one another, of *m/z* 277, 299 and 369, which are the major ions originating from the matrix and which show different degrees of complex- and sodium-adduct formation. These two features emphasise the essentially non-quantitative nature of this technique at present, as previously discussed [23].

The identity of the extracted putative cyclic UMP, cyclic IMP and cyclic dTMP was unequivocally established by the c.i.d.–m.i.k.e.s. scans of the molecular protonated ions produced by f.a.b. of post-electrophoresis retained fractions. In the case of cyclic UMP, comparison of the m.i.k.e.s. spectrum of the *m/z* 307 ion from fraction II with those from 3′,5′- and 2′,3′-cyclic UMP standards clearly shows that the extracted sample contains the 3′,5′-isomer (Fig. 2). Although the spectra of the two standards have several similarities, for example a strong peak at *m/z* 113 corresponding to the protonated base, characteristic differences in the fragmentation of the two isomers are apparent. The 3′,5′ isomer has characteristic ions at *m/z*/ 141 and 155 (Fig. 2*a*) that correspond to the fragments arising from the cleavage pattern shown in Scheme 1(*a*). These ions are much weaker in, or absent from, the spectrum generated from 2′,3′-cyclic UMP (Fig. 2*b*), with the diagnostic peak being at *m/z* 179, corresponding to a different, characteristic, cleavage pattern (Scheme 1*b*). The spectrum from the extracted-sample *m/z* 307 ion is essentially identical with that of the 3′,5′-cyclic UMP standard and contains the diagnostic ions at *m/z* 141 and 155 (Fig. 2*c*). Any apparent differences arise from the lower concentration of the analyte and a consequent higher contribution by the glycerol/water matrix, with peaks at *m/z* 93, 104, 123, 147, 185, 215 and 277 deriving from glycerol. In addition to the presence of the *m/z* 141 and 155 ions and the absence of the *m/z* 179 ion in the m.i.k.e.s. scan extracted-sample *m/z* 307 ion, the identity of the 3′,5′-cyclic UMP in the tissue extract can also be concluded from the expanded m.i.k.e.s. scan in the region between 2350 and 3350 eV. Both the standard 3′,5′-cyclic UMP and the putative cyclic UMP from the extract contain, in addition to the *m/z* 99 (protonated orthophosphoric acid) and *m/z* 113 peaks also present in the 2′,3′-isomer spectrum, a strong peak at *m/z* 97 (Figs. 2*d*–2*f*). This *m/z* 97 peak arises by the consecutive losses of the base (to form the sugar at *m/z* 195) and then the phosphate group (as orthophosphoric acid). For the 2′,3′-isomer the second loss is less favoured, and therefore the *m/z* 297 peak is weak, whereas the *m/z* 195 peak is strong. The peaks at *m/z* 93, 104 and 123 can be seen to have arisen from the glycerol/water matrix (Fig. 2*g*).

Similar comparison of the m.i.k.e.s. scans of the *m/z* 305 and 331 ions, produced by f.a.b. of the fractions I and II from the tissue extract, with those from standard 3′,5′-cycic dTMP and 3′,5′-cyclic IMP respectively established the presence of these two cyclic nucleotides in the extracted sample (Fig. 3). The diagnostic ions present in the spectra from both authentic and putative 3′,5′-cyclic IMP were at *m/z* 137 (protonated base), 165 (base + CHO) and 179 (base + C_2H_3O). For 3′,5′-cyclic dTMP, the characteristic ions at *m/z* 81, 110, 127, 179 and 207 were evident in both m.i.k.e.s. scans. The examination of the extract for cyclic XMP was, however, equivocal (Fig. 4). Although the m.i.k.e.s. scan of the *m/z* 347 ion produced from the standard by f.a.b. contained characteristic ions at *m/z* 136, 153, 165, 181 and 195 (Fig. 4*a*), in the spectrum pertaining to the extracted sample these peaks are obscured significantly by those arising from the glycerol/water matrix, owing to the much lower quantity of *m/z* 347 apparently present in the extract sample (Fig. 4*b*).

In the examination of the metabolism of these cyclic



Scheme 1. Fragmentation pattern of (*a*) 3′,5′-cyclic UMP and (*b*) 2′,3′-cyclic UMP

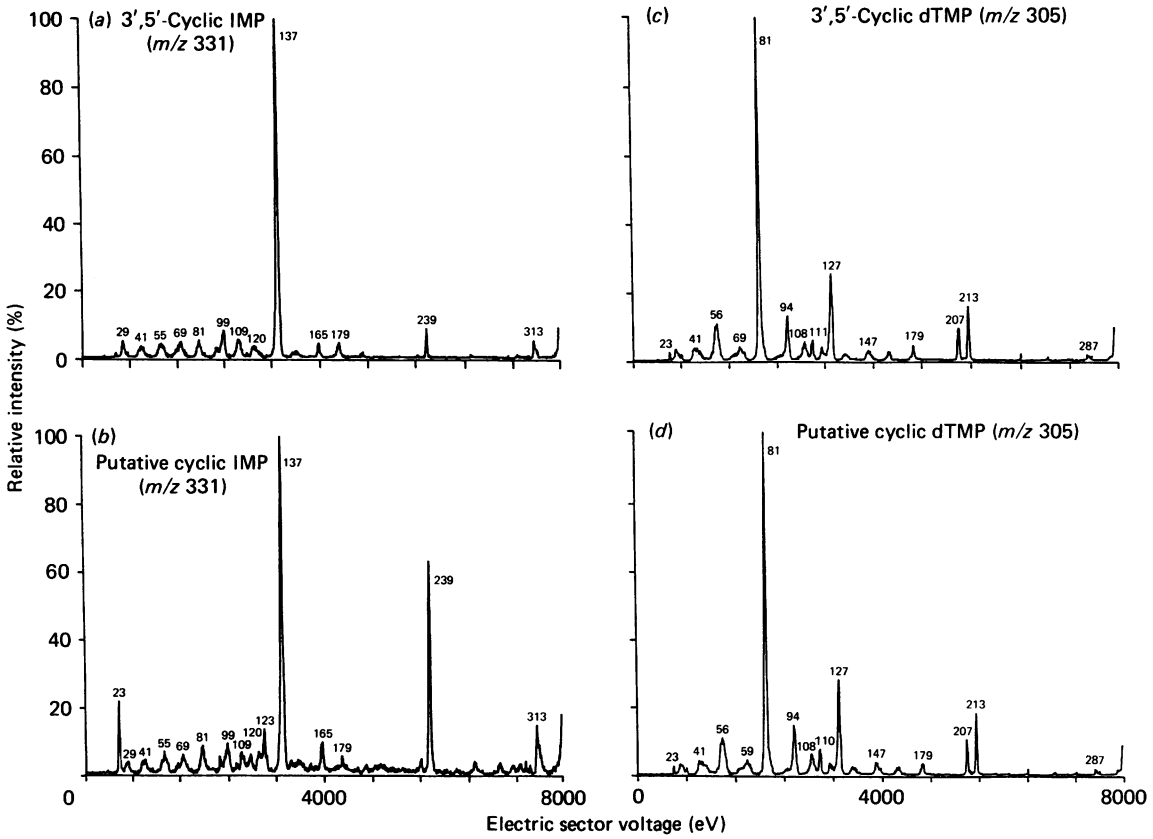


Fig. 3. C.i.d.–m.i.k.e.s. spectra of (*a*) authentic 3',5'-cyclic IMP, (*b*) extracted putative 3',5'-cyclic IMP, (*c*) authentic 3',5'-cyclic dTMP and (*d*) extracted putative 3',5'-cyclic dTMP

For further details see the text.

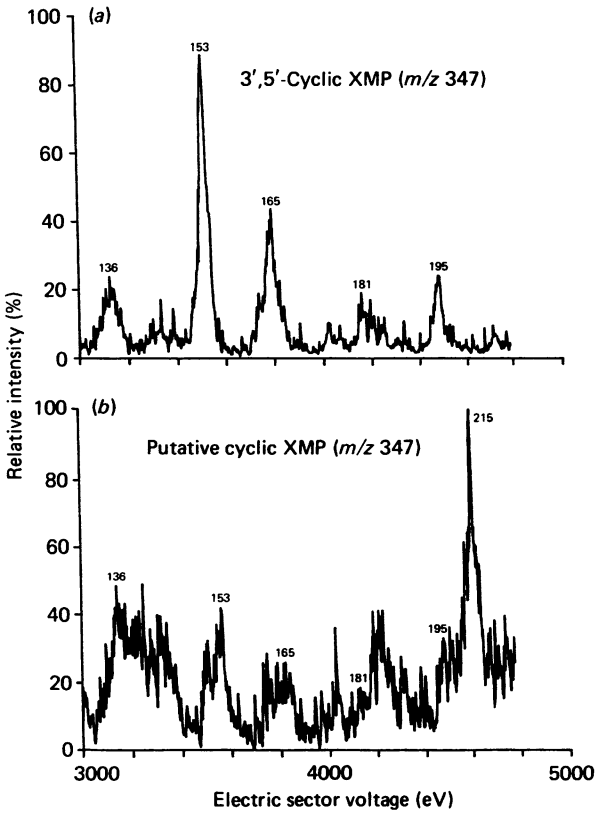


Fig. 4. C.i.d.–m.i.k.e.s. spectra of (*a*) authentic 3',5'-cyclic XMP and (*b*) extracted putative 3',5'-cyclic XMP

nucleotides, it was found that the preparations from rat liver, heart, kidney, brain, intestine, spleen, testis and lung were each able to synthesize radiolabelled cyclic UMP, cyclic IMP and cyclic dTMP from the corresponding ^{32}P-labelled nucleotide triphosphate, with the exception that cyclic dTMP was not synthesized by the kidney preparation. The apparent nucleotide cyclase activities observed vary from 0.4 to 12.6 nmol/min per g original tissue, with the highest rates of synthesis being in the brain preparation for uridylate cyclase, in the lung preparation for inosylate cyclase and in the heart for deoxythymidylate cyclase, at activities of 2.2, 12.6 and 2.8 nmol/min per g of original tissue respectively. The identities of the radiolabelled products estimated were confirmed by chromatography on t.l.c. and h.p.l.c. as described above. These activities represent an incorporation into the cyclic nucleotide products of between 0.4 and 1.8% of the radioactivity added to the incubation as ^{32}P-labelled nucleotide triphosphates, and the radioactivity counted in the incubation mixtures containing the active tissue homogenates was 5–22-fold that counted in the no-enzyme and boiled-enzyme controls. The activities were linear for up to 13 min and for incubations containing 10–30 μl of homogenate. No xanthosylate cyclase activity was obtained. Cyclic UMP, -IMP and -dTMP phosphodiesterase activity was also detected in each of the tissue homogenates tested, the observed activities being within the range 0.9–220 nmol/min per g of original tissue. The highest activities for each of the three substrates were the heart cyclic UMP phosphodiesterase, liver cyclic IMP

phosphodiesterase and testis cyclic dTMP phosphodiesterase, with activities of 221, 18 and 25 nmol/min per g of original tissue respectively. Each of the tissues also possessed the ability to bind cyclic UMP, -IMP and -dTMP to protein preparations, within the range 0.1–12 pmol/mg of protein, the highest binding capacity for each cyclic nucleotide being in the heart for cyclic UMP and in the liver for both cyclic IMP and -dTMP, with activities of 12.1, 8.8 and 6.9 pmol/mg of protein respectively.

DISCUSSION

The combination of a large-scale extraction and purification procedure with a precise analytical technique such as c.i.d.–m.i.k.e.s. has demonstrated unequivocally the natural occurrence of 3′,5′-cyclic UMP, IMP and dTMP. Although several reports in agreement with our observations already existed in the literature, for example of cyclic UMP in rat liver extracts [27] and bacterial culture fluids [28], these conflicted with the reports from other authors of a failure to detect cyclic IMP and cyclic UMP in mammalian tissue extracts [29]. Evidence of the existence of these three cyclic nucleotides, together with the demonstration that protein preparations are able to synthesize, degrade and bind cyclic IMP, UMP and dTMP raises several interesting issues, including their potential involvement in the metabolism and physiological effects of cyclic AMP and cyclic GMP.

The demonstration of the synthesis of cyclic IMP from ITP suggests that the cyclic IMP has at least partially arisen from this mechanism rather than merely by the deamination of cyclic AMP, a pathway previously reported in the toad bladder [30]. The incorporation of radioactivity from ITP into cyclic IMP described here is purely a qualitative experiment, indicating the existence of this pathway, and is not necessarily quantitative, since no triphosphate-regenerating system was included in the assay and the activity recorded therefore should be regarded as a minimum estimate. In addition, the examination of the synthesis, hydrolysis and binding gives no indication of the specificity of these processes. It has, for example, been previously reported that guanylate cyclase from rat lung and sea-urchin sperm [31] is capable of converting ITP into cyclic IMP. Our evidence suggests that this ability to synthesize cyclic nucleotides other than cyclic AMP and cyclic GMP is widespread in rat tissues. The presence of cyclic IMP, and indeed cyclic UMP and cyclic dTMP, may be the result of a metabolic error attributable to a lack of specificity of adenylate or guanylate cyclase and thus not be indicative that these cyclic nucleotides have, in themselves, any function. Nevertheless, their occurrence is of considerable significance, since, for example, cyclic IMP has been shown to stimulate protein kinase activity in crude liver preparations [32] and to block cyclic AMP binding to the regulatory subunit of type I protein kinase [33], and a cyclic IMP derivative is an activator of lobster cyclic GMP-dependent protein kinase [34]. It is thus possible that cyclic IMP, UMP and dTMP may alter the effects of cyclic AMP and cyclic GMP by acting as agonists or antagonists in the interaction of the latter two cyclic nucleotides with protein kinases and phosphodiesterases, thereby altering regulatory systems responsive to them. It is also feasible that cyclic IMP, UMP and dTMP may have a regulatory function distinct from those of cyclic AMP and cyclic GMP; for example, the effects on the central nervous system of the administration of cyclic IMP, UMP and dTMP into the lateral cerebral ventricle of the rat are not explicable merely as interference in the cyclic AMP and cyclic GMP systems [35]. In order to determine whether the three cyclic nucleotides described here have themselves an effect *in vivo*, or if they have a significant effect on the physiological processes responsive to cyclic AMP and cyclic GMP, a systematic survey of the distribution and variation in concentration of these compounds, of the specificity of the proteins capable of their synthesis, hydrolysis and binding, of the effects of the cyclic nucleotides and their derivatives on cellular metabolism, and of the specificity of cyclic AMP- and cyclic GMP-mediated processes, is required.

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