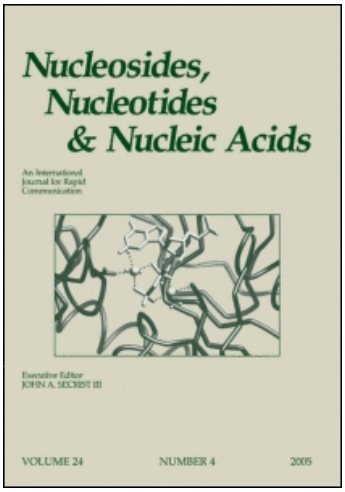

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Application of Fast Atom Bombardment Mass Spectrometry and Mass-Analysed Ion Kinetic Energy Spectrum Scanning to Studies of Cyclic Nucleotide Biochemistry

Russell P. Newton[a]; A. Gareth Brenton[b]; Terence J. Walton[a]; Frank M. Harris[b]; Dipankar Ghosh[b]; Andrea M. Jenkins[a]; Eric E. Kingston[b]
[a] Biochemistry Research Group, School of Biological Sciences, [b] Mass Spectrometry Research Unit, Department of Chemistry, University College of Swansea, Swansea, United Kingdom

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APPLICATION OF FAST ATOM BOMBARDMENT MASS SPECTROMETRY AND MASS-ANALYSED ION KINETIC ENERGY SPECTRUM SCANNING TO STUDIES OF CYCLIC NUCLEOTIDE BIOCHEMISTRY

*†Russell P. Newton, °A. Gareth Brenton, †Terence J. Walton, °Frank M. Harris, °Dipankar Ghosh, †Andrea M. Jenkins and °Eric E. Kingston
†Biochemistry Research Group, School of Biological Sciences and °Mass Spectrometry Research Unit, Department of Chemistry, University College of Swansea, Swansea SA2 8PP, United Kingdom

Abstract: FAB mass spectrometry with MIKES scanning provides an effective means of identifying cyclic nucleotides. Its application readily distinguishes 2',3'- and 3',5'-isomers, and has enabled the demonstration of the natural occurrence of cCMP, cUMP, cIMP and cdTMP in mammals and higher plants, the identification of novel side-products generated by cytidylyl cyclase activity and of synthetic cyclic nucleotide derivatives, and the quantitation of phospho-diesterase activity.

Because of their low tissue concentrations and lability in solution, analysis of cyclic nucleotides has inherent difficulties. While long-established techniques of mass spectrometry have been of limited success in cyclic nucleotide analysis due to difficulties in the synthesis and stability of volatile derivatives, fast atom bombardment mass spectrometry and mass-analysed ion kinetic energy spectrum scanning has proved very effective in such analyses. The application of FAB/MIKES to cyclic nucleotides provides a very effective means of unambiguous identification, easily distinguishing the 3',5'-cyclic nucleotides, the putative second messengers, from their 2',3'-isomers. In particular, MIKE spectra from the molecular ions of 3',5'-cyclic nucleotides contain, in addition to protonated base ($[BH_2]^+$) and protonated base plus 28 mass units ($[BH_2 + 28]^+$), a prominent peak corresponding to protonated base plus 42 mass units ($[BH_2 + 42]^+ \equiv [BH\text{-}CH = CHOH]^+$), while spectra obtained from 2',3'-isomers do not contain this prominent peak (1).

Application of the FAB/MIKES analysis technique to large scale tissue extracts subjected to a sequential purification procedure involving freeze-clamping, perchlorate extraction, alumina, QAE sephadex and SP sephadex ion exchange chromatography has demonstrated the natural occurrence of cytidine-, uridine-, inosine- and deoxythymidine-3',5'-cyclic monophosphates (cCMP, cUMP, cIMP and cdTMP) in mammalian tissues (2,3).

Application of a modified procedure to plant tissue extracts has shown that cyclic AMP, -GMP, -CMP were present in the rapidly proliferating, meristematic areas of roots, while cyclic AMP, -GMP, -CMP, -IMP and -dTMP were present in the more slowly proliferating, non-meristematic regions of the root (4). The relative concentration of cyclic CMP was found to be significantly higher in the meristematic tissue than in the non-meristematic regions, whilst cyclic UMP was present at higher relative concentrations in the non-meristematic tissue than in the meristem. The differences in cyclic nucleotide concentration in these tissues is of potential significance in view of current concepts involving cyclic nucleotides in the regulation of cell division.

FAB/MIKES analysis has also proved invaluable in the analysis of synthetic derivatives of cyclic nucleotides (5). Since cyclic nucleotides do not readily permeate membranes, lipophilic derivatives are synthesized to provide a molecule capable of rapidly passing through cell membranes. Upon entering the cell, the derivative has its lipophilic side chains removed by hydrolysis, thus releasing the parent cyclic nucleotide within the cell and its effects can then be monitored. However the synthesis of a derivative of this type is *via* a sequential synthesis and other products are possible, in which the cyclic phospho-diester moiety has been hydrolyzed, in which only one butyryl group has been added, or in which butyryl moieties have been added to the incorrect positions. Contamination by, or misidentification of, such potential side-products would pose serious problems in interpretation of data obtained after administration of these preparations; simple chromatographic analysis often lacks the resolution to distinguish these compounds. However the absence of an ion at $[MH + 18]^+$ in the FAB mass spectrum confirms that no dibutyryl mononucleotide has been produced by hydrolysis of the cyclic phosphodiester group, and the number of butyryl substituents can also be readily determined from the mass spectrum.

MIKE spectra produced from $[MH]^+$ for dibutyryl cyclic nucleotides contain characteristic peaks corresponding to protonated base, protonated butyrylated base and also the protonated butyrylated ribose cyclic phosphate, for example at m/z 112, 182 and 265 respectively in the case of dibutyryl cCMP, thus substitution of ribose or base moiety can be easily indicated.

Similar problems of identification have existed over derivatives of cyclic nucleotides required for immunoassay. Cyclic nucleotides are not themselves immunogenic, thus to raise antisera it is necessary to conjugate them to a carrier protein prior to immunization. To facilitate conjugation it is necessary to first synthesize a 2'-O-succinyl derivative, which in turn is coupled to a carrier protein prior to immunization. To facilitate conjugation it is necessary to first synthesize a 2'-O-succinyl derivative, which in turn is coupled to a carrier protein. In addition, in order to produce a radiolabelled antigen, it is necessary to produce a tyrosinyl methyl ester derivative which in turn can be readily radioiodinated. FAB/MIKES analysis of the succinyl and succinyl tyrosinyl methyl ester derivatives of cyclic nucleotides can be used to confirm successful synthesis by indicating position of substitution and retention of the cyclic phosphodiester group.

Application of the FAB/MIKES technique has also been utilized in biochemical studies of the cyclic nucleotide-related enzymes. Conclusive evidence of the existence of cytidylate cyclase, an enzyme capable of the conversion of CTP to cyclic CMP, has been obtained by a combination of techniques including dual labelling, selective hydrolysis, chromatography and FAB/MIKES analysis of the enzyme products (6). In addition to demonstrating cyclic CMP as a product, the existence of four novel cytidine compounds, cytidine-3',5'-cyclic pyrophosphate, cytidine-2'-monophosphate-3',5'-cyclic monophosphate, cytidine-2'-O-aspartyl-3',5'-cyclic monophosphate, and cytidine-2'-O-glutamyl-3',5'-cyclic monophosphate has also been shown.

Application of the FAB/MIKES analysis to incubates containing cyclic nucleotide phosphodiesterase has now enabled the development of quantitation of cyclic nucleotides (7). Characteristic peaks of the substrate, cyclic AMP, and product, AMP, were identified in the mass spectra and MIKES scans of the protonated molecules. By spiking enzyme

incubates with known quantities of cyclic AMP and AMP and measuring peak heights in the MIKE spectra of both spiked and unspiked samples, the concentrations of cyclic AMP and AMP in solution at the end of the enzyme incubation have been estimated. From the data obtained the K_m and V_{max} of the enzyme were calculated as 181 μM and 28.6 nmol/min respectively, showing excellent agreement with the values obtained for the same enzyme preparation by the conventional radioactive assay of K_m = 205 μM and V_{max} = 33.2 nmol/min.

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