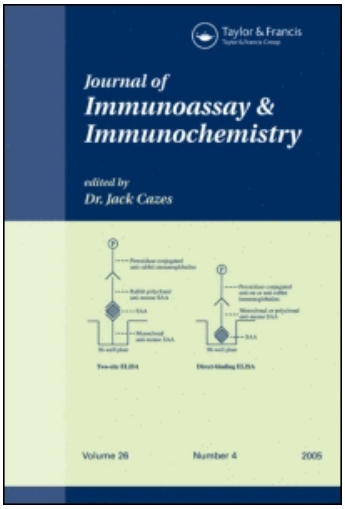

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Radioimmunoassay of Cytidine 3',5'-Cyclic Monophosphate: Unambiguous Assay by Means of an Optimized Protocol Incorporating a Trilayer Column Separation to Obviate Cross-Reactivity Problems

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RADIOIMMUNOASSAY OF CYTIDINE 3',5'-CYCLIC MONOPHOSPHATE: UNAMBIGUOUS ASSAY BY MEANS OF AN OPTIMIZED PROTOCOL INCORPORATING A TRILAYER COLUMN SEPARATION TO OBVIATE CROSS-REACTIVITY PROBLEMS.

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ABSTRACT

Previous assays for cytidine 3',5'-cyclic monophosphate (cyclic CMP) have been criticized as being ambiguous. Here a modified RIA protocol, in which the production of assay components has been optimized and a novel trilayer chromatography column separation introduced which successfully separates cyclic CMP from compounds, endogenous to living tissues, which cross-react with anti-cyclic CMP sera, is described. The assay is capable of assaying cyclic CMP between 0.1 and 5 pmol, can be increased in sensitivity by means of an additional acetylation step, and enables the separation of cyclic CMP, cyclic AMP and cyclic GMP so that all three can be estimated in a single sample.

INTRODUCTION

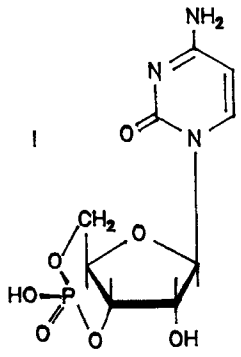
The role of cyclic AMP as a biochemical second messenger, acting as the intracellular mediator of the actions of a range of mammalian hormones and neurotransmitters is well established; a second cyclic nucleotide, cyclic GMP, performs a similar regulatory role but of a more restricted and specific nature, being involved in

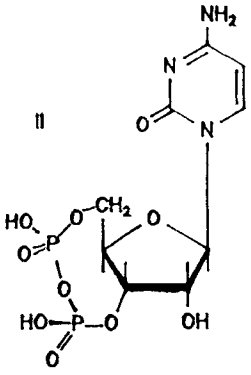
the transduction of the visual signal in the mammalian eye and in the regulation of the water/electrolyte balance in the blood stream and the intestine. Evidence of the natural occurrence of a third cyclic nucleotide, cytidine 3',5'-cyclic monophosphate (cyclic CMP) was first provided by Bloch and his coworkers (1), who identified the compound in extracts from mammalian tissues and leukaemic cell cultures by cochromatography with standards, and by acid hydolysis, mass spectrometry and u.v. absorption spectroscopy. A range of effects of cyclic CMP upon cellular metabolism were reported (2), and the later development of radioimmunoassays (3-6) and an enzymeimmunoassay (7) for cyclic CMP appeared to provide a more rapid means of determining endogenous cyclic CMP levels. The demonstration of enzyme activities capable of the synthesis (8) and degradation of cyclic CMP (9), plus identification of a range of factors which appeared to alter endogenous cyclic CMP levels, led to suggestions that cyclic CMP had a role as a biochemical second messenger analogous to those of cyclic AMP and cyclic GMP.

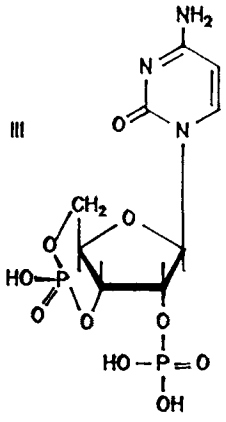
Other evidence contradicted such a hypothesis. The identity of the extracted putative cyclic CMP was contested by reports that the analyte could be separated chromatographically from authentic radiolabelled cyclic CMP (10) and that several different cyclic CMP-immunoreactive materials were naturally occurring (11). As RIA and chromatography had constituted the major means of identification and assay these reports created serious difficulties, which were compounded by observations that the major radiolabelled product synthesized by the putative cytidylyl cyclase was

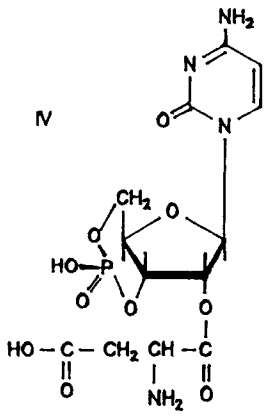
chromatographically separable from cyclic CMP (12), and that more than one cyclic CMP-immunoreactive compound was found among the products of the reaction (11). Taken together with a report that the phosphodiesterase hydrolysing cyclic CMP was not specific (13), the evidence collectively casts serious doubt upon the existence of any metabolic regulatory function of cyclic CMP and, indeed, even upon its natural occurrence.

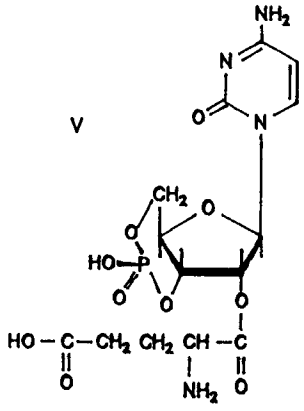
Cyclic CMP has now been unequivocally established as endogenous to both mammalian and plant tissues by means of tandem mass spectrometric (MS) analysis of sequentially purified tissue extracts. Tandem MS analysis provides unambiguous qualitative identification of submicrogram quantities of cyclic nucleotides (14) and its application to purified analytes extracted from mammalian (15) and plant tissues (16), in combination with numerous controls designed to obviate problems of artefactual production, has demonstrated that cyclic CMP is endogenous to these tissues. The plurality of cyclic CMP-immunoreactive compounds may be explained by the identification of four novel cytidine cyclic nucleotide derivatives (17) which are produced in addition to cyclic CMP (I) as side-products of the cytidylyl cyclase reaction. These four compounds, cytidine 3',5'-cyclic pyrophosphate (cCDP, II), cytidine-2'-monophosphate-3',5'-cyclic monophosphate (P-cCMP, III), cytidine-2'-O-aspartyl-3',5'-cyclic monophosphate (asp-cCMP, IV) and cytidine-2'-O-glutamyl-3',5'-cyclic monophosphate (glu-cCMP, V), are cross-reactive with some anti-cyclic CMP sera and offer a potential explanation of the discrepancies in the radioimmunoassays for cyclic CMP previously reported.











Here we report upon the modification of previous cyclic CMP RIA methodology, and the development of separatory steps which remove the four compounds II - V from the sample under test and which also separate cyclic CMP, cyclic AMP and cyclic GMP from one another, thereby permitting estimation of the concentrations of three cyclic nucleotides in a single preparation.

MATERIALS AND METHODS

Materials

Cyclic nucleotides and ion-exchange materials were obtained from the Sigma Chemical Co., Poole, Dorset, UK; all other biochemicals and chemicals were purchased either from BDH Chemicals Ltd., Poole, Dorset, UK, or from the Aldrich Chemical Co., Gillingham, Kent, UK. All radiolabelled chemicals were purchased from Amersham International plc, Aylesbury, Bucks, UK. Dutch white rabbits were used to raise antisera; the rabbits were purchased from Foxfield Rabbits, Broadway Farm, Foxfield, UK, housed in a Home Office approved and licensed animal facility at 20-22°C with a 12 hour light/dark cycle and fed on SGI rabbit and guinea pig diet with added coccidostat (clopidol), purchased from Pilsbury's Ltd, Birmingham, UK.

Synthesis of 2'-O-succinylcytidine 3',5'-cyclic monophosphate

2'-O-succinylcytidine 3',5'-cyclic monophosphate (ScCMP), an acyl derivative of cyclic CMP necessary for production of a cyclic CMP-protein conjugate and for synthesis of a high, specific activity radioiodinated cyclic CMP derivative, was synthesized by a

modification of those originally reported by Cailla (3) and Murphy and Stone (4) as previously described (18). The ScCMP was separated from unchanged reagents and sideproducts by passage through a Dowex 1-X8 column (formate form) (18) and the purified freezedried product identified as ScCMP by tandem mass spectrometry (18).

Synthesis of ScCMP-protein conjugates

As ScCMP is not itself immunogenic, it is necessary to conjugate it to a protein prior to injection. ScCMP was conjugated to proteins by methods previously described for cyclic AMP, to keyhole limpet haemocyanin (19), bovine serum albumin (19), human serum albumin (3) and thyroglobulin (20). The molar ratios were calculated as 7:1, 18:1, 14:1 and 90:1 respectively by examination of the UV absorption spectra.

Production of antisera

Antisera were raised by injecting rabbits with 0.25mg of the cyclic CMP conjugate mixed with 1ml of Freund's adjuvant and 0.9% (w/v) saline in a total volume of 1.5ml. Each animal received 40 or more intradermal injections of 50µl according to the procedure of Vaitukaitis et al (21). Booster injections of the same size were administered at approximately 4-6 week intervals and immunized animals were bled from the ear veins 7-12 days after each injection. After clotting, sera were decanted, freezedried and stored at -70° until use.

Synthesis of high specific activity radiolabelled antigen

A radioactively labelled antigen was prepared by esterifying ScCMP with tyrosine methyl ester then labelling with ^{125}I. The 2'-O-

succinyltyrosinylmethyl ester of cytidine 3',5'-cyclic monophosphate (ScCMPTME) was synthesized by adding 14µl of trioctylamine and 1.5µl of ethylchloroformate to 5mg of freshly prepared ScCMP in 200µl of dimethylformamide, followed by 5mg tyrosine methyl ester in 100µl dimethylformamide then 9.5µl trioctylamine, and allowing reaction to take place for 3 hours at room temperature. ScCMPTME was then eluted from a Dowex 1-X8 formate column as previously described (18); the identify of the putative ScCMPTME was confirmed by tandem MS analysis (18).

For the synthesis of radiolabelled ^{125}I-ScCMPTME, 5µg of ScCMPTME in 5µl of 0.1M phosphate buffer, pH 7.5, was incubated with 2mCi of $Na^{125}I$; 20µl of chloramine T (1 $mgml^{-1}$) was added in 3µl aliquots over a period of 30 secs., and reaction allowed to proceed for 90 secs in one incubation, and for 180 secs in a second, otherwise replicate experiment, carried out to determine optimum reaction time. The reaction was stopped by addition of 50µl of sodium metabisulphite solution (0.6 $mgml^{-1}$), then the reaction vial washed out with 4 x 500µl aliquots of 0.1M citrate buffer, pH 6.2, containing 0.5 gl^{-1} of NaN_3 and pumped on to a Sephadex G-10 column (60 x 0.6cm i.d.) surrounded by a water jacket at 2-4°, then eluted with the same citrate buffer at a flow rate of 1 $mlmin^{-1}$. Fractions of 3ml were collected and 50µl aliquots were counted in an LKB Rackgamma counter. Fractions 45-58, containing the iodinated antigen, were combined, diluted with 0.1M citrate buffer, pH 6.2, containing 0.5g/l NaN_3 and 2g/l bovine serum albumin, and divided into aliquots containing approximately 4 x 10^6 d.p.m. ml^{-1}, frozen and stored at -20° until use.

Radioimmunoassay protocol

A range of protocols were examined, with variations in volume, incubation composition, time-course and free antigen/bound antigen separation method being compared to optimize the process. To separate bound and free antigen, precipitation with ammonium sulphate and with polyethylene glycol, gel filtration, electrophoresis, equilibrium dialysis, immunological precipitation with anti-rabbit-γ-globulin, both soluble and covalently linked to magnetic particles, and charcoal adsorption were each examined. The standard protocol adopted after comparison of the preliminary studies was as follows. Between 1.5 and 3 x 10^4 c.p.m. of labelled antigen were incubated together with unlabelled cyclic CMP standards in quantities ranging from 10fmol to 10nmol with antiserum, diluted up to 50,000-fold, in a total volume of 500µl of 0.1M citrate buffer pH 6.2 for 20-24 hours at 4 . The bound radiolabelled antigen was then separated and counted by adding 200µl of a 2% charcoal suspension in the same buffer containing in addition 0.25% bovine serum albumin, incubating for 90 min, followed by centrifugation at 14,500g for 10 min and counting the aspirated supernatant in the γ-counter. Four replicates of each incubation were carried out. Controls to determine background counts, total counts, non-specific radiolabelled-antigen binding, and total radiolabelled-antigen binding in the absence of unlabelled antigen (zero binding) were routinely included. Samples of unknown cyclic CMP concentration, and of other cyclic nucleotides and analogues in cross-reactivity tests, were included in place of the cyclic CMP standards in the above protocol. Where increased sensitivity was necessary, the

above assay protocol was modified to include addition of 10µl of an acetylation mixture of 1 vol. of acetic anhydride and 2.5 vol. of triethylamine in the incubation mixture.

Extraction of cyclic CMP

Cyclic CMP was extracted from tissue samples as previously described (22); the tissue samples were freeze-killed using aluminium clamps prechilled in liquid N_2, freeze-dried, then homogenized in 0.6 M perchloric acid (1:1.5 w/v), and the precipitated protein removed by centrifugation. The extracts were neutralized with 1 M KOH and the resultant precipitate of $KClO_4$ removed by centrifugation. All operations were carried out at 4^0; the final extract was stored at -20^0 prior to chromatographic separation.

Separation of cyclic CMP from cross-reactants

A range of column sizes and a variety of eluents were used to assess the ability of QAE-Sephadex, Dowex, alumina and AffiGel 601 (boronate) to separate cyclic CMP from its four analogues (II-V) and from cyclic AMP and cyclic GMP. The efficiencies of the processes were determined by collecting a series of 1ml fractions and determining the radiolabel present after applying 10nmol of compound containing 200-300,000 d.p.m.

Biological samples

Swiss white mice and Lister hooded rats were all bred on campus in a licenced Animal House, according to Home Office regulations and guidelines, and fed on a rat and mouse breeding diet

cube purchased from Havanolac Ltd., Blackthorn, Oxford, U.K. Rabbits were obtained and kept as described above. Prior to extraction of tissue samples the animals were sacrificed by a sharp blow to the base of the skull.

Patient urine and blood samples were provided by a local hospital, Fucus spiralis was collected from the local seashore and *Phaseolus vulgaris* var. The Prince was germinated in moist vermiculite and seedlings grown at 23^0 in a light cycle of 16 hr light/8 hr dark.

RESULTS

The radioiodination of ScCMPTME was found more difficult than the corresponding syntheses of the derivatives of cyclic AMP and cyclic GMP, with precise reaction conditions being critical. Key steps included carrying out the reaction in the smallest practicable volume with the minimum quantity of chloramine T, keeping pH below 8.0 to obviate additional substitutions, use of Dowex in purification of ScCMPTME to avoid use of a NaCl gradient with QAE Sephadex, use of carrier-free iodine, and absence of any reducing substances; a major feature was the length of the reaction time. The time course of non-radioactive iodination of ScCMPTME had earlier been followed by monitoring the appearance and disappearance of the characteristic peaks of ScCMPTME, and of its mono- and di-iodo derivatives, in the fast atom bombardment mass spectrum (18), the data suggesting the optimum time for iodination was 90 secs. Comparison of the incorporation of radiolabelled iodine after 90 secs and 180 secs (Figure 1) confirmed this conclusion, with only 15% of ^{125}I being in the I-ScCMPTME fractions in the longer incubation

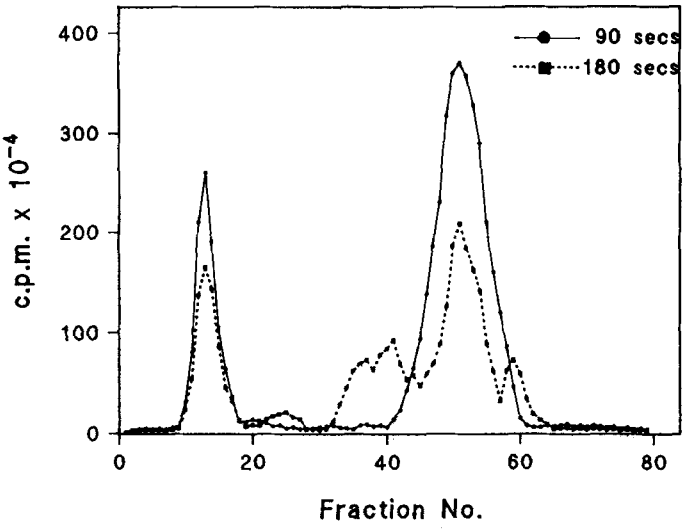


FIGURE 1. Incorporation of radiolabelled iodine into the 2'-O-succinyltyrosmymethyl ester of cytidine 3',5'-cyclic monophosphate. Plot of radioactivity against fractions eluted from Sephadex G-10 column after 90 and 180 second radioiodination reactions; the required mono-iodo derivative is eluted between fractions 45 and 58.

compared to 30% in the shorter, and other labelled side products (fractions 30-44) being formed in the longer reaction. (The peak at fractions 10-13 is unreacted iodide).

Comparison of the antisera raised against different cyclic CMP-protein conjugates showed titres which appear to reflect the molar ratio of cyclic CMP to protein. To bind 50% of the radiolabel present in the standard assay protocol, antisera raised against the keyhole limpet haemocyanin-conjugate could be diluted 2,000 - 5,000-fold, human serum albumin conjugate 5,000 - 20,000-fold, bovine serum albumin conjugate 10,000 - 40,000-fold, and the thyroglobulin-conjugate 25,000 - 50,000-fold. Use of the antisera raised against

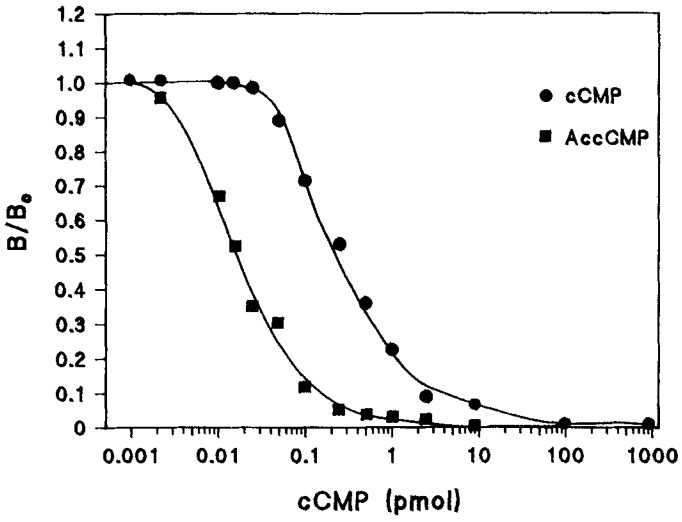


FIGURE 2. Standard curves for radioimmunoassay of cyclic CMP with and without acetylation.

the thyroglobulin-conjugate produced a standard curve which enabled determination of the cyclic CMP content of unknowns between 100fmol and 5pmol (Figure 2); acetylation increased the sensitivity of the standard curve by 8- to 40-fold.

Cross-reactivity with the cyclic CMP analogues and with cyclic AMP and cyclic GMP varied between antisera, with cyclic CMP-thyroglobulin conjugates producing antisera of better specificity than the others. As indicated in a representative plot from a single antiserum (Figure 3), greatest cross-reactivity was observed with the 2'-O-aminoacyl derivatives of cyclic CMP and with cyclic AMP (Table 1).

The use of single columns of QAE-Sephadex, neutral alumina, Dowex-1 or AffiGel 601 did not produce adequate separations of

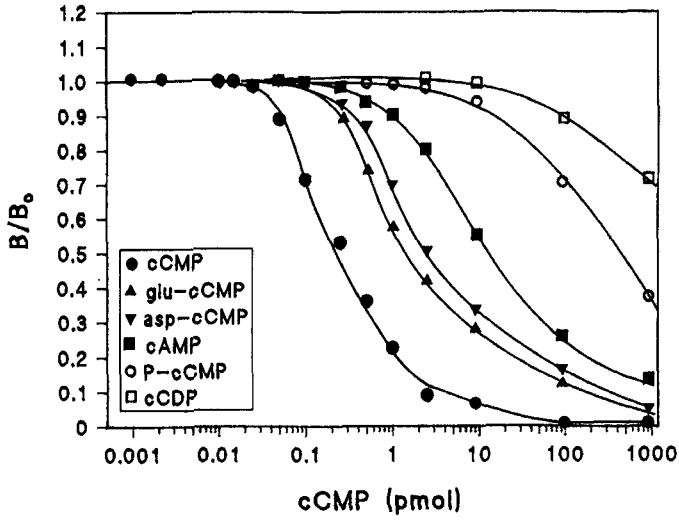


FIGURE 3. Standard radioimmunoassay curves for cyclic CMP and potential cross-reactants.

TABLE 1

Cross reactivity of anti-cyclic CMP sera.

Compound	Range of cross reactivity* of antisera raised in different animals
2',3'-cyclic CMP	>2000
CTP	>2000
CDP	800 - >2000
CMP	600 - >2000
Cytosine	>2000
Cytidine	>2000
Cyclic AMP	40 - 700
Cyclic GMP	400 - >2000
Cyclic UMP	800 - >2000
Cyclic IMP	>2000
2'-P-cCMP	600 - 1200
cCDP	800 - >2000
Glu-cCMP	8 - 300
asp-cCMP	12 - 200

* Cross reactivity is expressed as the ratio of concentration of the ligand giving 50% displacement of the radiolabelled antigen to the concentration of cyclic CMP giving the same displacement.

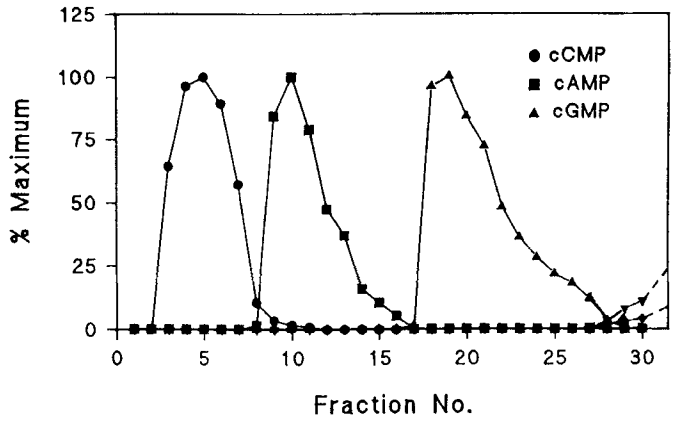


FIGURE 4. Separation of cyclic CMP, cyclic AMP, cyclic GMP and cyclic CMP derivatives. Compounds are eluted from a trilayer column with 16ml 0.03M HCl, followed by 16ml 0.09M HCl. Elution of cyclic CMP, -AMP and -GMP was complete; P-cCMP and cCDP commenced elution in fraction 28.

cyclic CMP from its immunoassay cross-reactants with column lengths and elution conditions which gave acceptable recoveries of the radiolabelled cyclic CMP. Sequential combination of columns was successful, for example chromatography on a neutral alumina column (4 x 0.5cm) eluted with 10ml of 0.2M phosphate buffer, pH 7.4, followed by AffiGel 601 (4 x 0.5cm) eluted with 10ml of HEPES-NaCl, pH 8.4, satisfactorily resolved cyclic CMP from the other compounds with a reproducible recovery of ca. 75%. However the separation proved laborious and time-consuming; an alternative approach in which three chromatographic matrices, which could be eluted by a common agent, were layered within a single column, proved more efficient. Plastic columns (11 x 1cm) containing 0.5cm of Dowex 1-X8, 0.5cm of neutral alumina and 2.0cm of QAE-Sephadex were eluted

with HCl. Cyclic CMP was eluted with 0.03M HCl after 3-8ml, with cyclic AMP eluting between 9-16ml (Figure 4); further elution was achieved by increasing HCl concentration to 0.09M HCl, with cyclic GMP being eluted in the next 10ml, with the cyclic CMP analogues commencing to elute towards the end of this cyclic GMP-containing fraction (Figure 4). Recovery of radiolabelled cyclic CMP was 87-92% when applied alone in solution to the column, and 82-89% when added together with tissue extract.

Application of the partial purification followed by radioimmunoassay enabled the determination of cyclic CMP concentrations in a number of tissue extracts (Table 2). Cyclic CMP levels in tissue extracts from mice, rats and rabbits were generally of the same order with the highest concentrations found in the brain and testis of rabbit and mouse tissues respectively. Mouse testis contained approximately seven-fold more cyclic CMP than the ovary while concentration in the mouse testis was more than sixty-fold that found in rabbit ovary. In the rat comparison of the adult heart and liver with that of the foetus showed a slightly higher level in the foetal heart with the level in the foetal liver being approximately ten-fold higher than the level in adult liver. Cyclic CMP levels in urine obtained from leukaemic patients were very much elevated in comparison to normal human samples, while in plant samples, the highest concentration was found in young seedlings of *Phaseolus vulgaris* of four days growth, the level decreasing to around 14% of this value after a further 13 days. The cyclic CMP concentration of the culture medium of *Corynebacterium murisepticum* was found to be 5.2nM; inclusion of 0.5mM cytidine in the medium 10

TABLE 2

Concentration of cyclic CMP determined by RIA

Tissue		Conc[a]. [a] pmol/g dry wt. [b] pmol/mmol creatinine [c] pmol/10^9 white blood cells [d] nM [e] pmol/mg protein	(n)
Mouse			
	Liver	[a]4.9 ± 1.9	(8)
	Heart	[a]1.1 ± 0.3	(8)
	Lung	[a]0.9 ± 0.2	(8)
	Kidney	[a]0.7 ± 0.2	(8)
	Testis	[a]8.9 ± 2.1	(6)
	Ovary	[a]1.3 ± 0.7	(6)
	Uterus	[a]3.8 ± 1.1	(6)
Rat			
	Liver	[a]3.7 ± 1.8	(6)
	Liver (foetal)	[a]34.7	(3)
	Heart	[a]0.8 ± 0.2	(6)
	Heart (foetal)	[a]1.1	(3)
Rabbit			
	Liver	[a]3.8 ± 1.1	(4)
	Heart	[a]0.6 ± 0.2	(4)
	Testis	[a]11.8 ± 4.1	(4)
	Ovary	[a]0.7 ± 0.2	(4)
	Brain	[a]14.7 ± 4.8	(4)
Human			
	Urine (normal)	[b]0.8 ± 0.2	(20)
	Urine (leukaemic)	[b]37.3 ± 0.9	(4)
	Blood (normal)	[c]11.8 ± 4.9	(20)
	Blood (leukaemic)	[c]3.3 x 10^3	(2)

TABLE 2: Continued

Phaseolus vulgaris			
	Seed	[b]0.7 ± 0.4	(4)
	Leaf (4 days after germination	[b]5.7 ± 1.1	(4)
	Leaf (9 days after germination	[b] 1.3 ± 0.2	(4)
	Leaf (17 days after germination)	[b]0.8 ± 0.2	(4)
Corynebacterium murisepticum			
	Culture medium	[d]5.2	(1)
	Culture medium 10 hours after addition of 5 mM cytidine	[d]183	(1)
Fucus spiralis		[t]43.2	(1)
Euglena gracilis		[t]92.1	(1)

Concentrations of individual samples were determined as means of four replicates; all replicates were within ±8% of mean. Concentrations expressed here are means of concentrations of n samples, ± S.E.M., unless n = 3 or less.

hours prior to extraction resulted in a medium cyclic CMP concentration of 183nM, suggesting that rapid biosynthesis after precursor uptake was taking place.

DISCUSSION

The study of the cross-reactivity of the anti-cyclic CMP serum with the four naturally occurring cyclic CMP-analogues indicates that the latter compounds are a likely cause of the discrepancies in the literature relating to cyclic CMP identification and estimation. The relative cross-reactivity observed with the cyclic CMP analogues is as would be anticipated on the basis of molecular structure, since the 2'-O-aminoacyl derivatives show analogy to the 2'-O-succinylaminoacyl residues present in the conjugate against which

the antibodies were raised. The method developed of separating cyclic CMP from these cross-reactants has good reproducibility and running separatory matrices as a single column of three layers has significant logistic advantages over the use of sequential columns, and has the additional benefit of resolving cyclic CMP, cyclic AMP and cyclic GMP, thereby facilitating their estimation in a single sample. The separation of cyclic CMP from cyclic AMP prior to RIA for the former is also desirable, since although cyclic AMP shows only limited cross-reactivity in this system, endogenous concentrations of cyclic AMP are one to two orders of magnitude greater than those of cyclic CMP and can constitute a cross-reactivity problem.

The optimization of the synthesis of the radiodinated tracer, the use of thyroglobulin as carrier protein in the immunization schedule and the separation of bound and free antigen in the immunoassay incubation by adsorption of the former onto charcoal have, in our laboratory, greatly improved the reproducibility of both the synthesis of the individual components and the performance of the RIA itself. The revised assay should be capable of providing unambiguous determination of endogenous cyclic CMP levels; the limited application of the assay shown here supports the concept that cyclic CMP may be involved in the regulation of cell differentiation and proliferation (2), with the faster growing foetal tissues containing elevated cyclic CMP concentrations compared to normal tissues, and the urine of leukaemic patients containing significantly higher levels than the control urines. The presence of cyclic CMP in each of the samples examined, and the

rapid increase in synthesis of cyclic CMP by a bacterial cell in response to provision of cytidine in the medium, are also compatible with such a general function of this cyclic nucleotide. Recent suggestions, for example that cyclic CMP may be useful as a marker of ovarian cancer (23) and that it may be involved in the inhibition of arachidonic acid turnover and eicosanoid release (24), together with evidence of the regulation of cyclic CMP metabolizing enzymes and observations of the intracellular effects of dibutyl cyclic CMP (25), support the concept that cyclic CMP may perform a significant role in metabolic regulation; it is hoped that application of this unambiguous assay for cyclic CMP will resolve the contention aroused by previous studies and play an important role in studies designed to elucidate the natural function of this third cyclic nucleotide.

ACKNOWLEDGEMENTS

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