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RADIOIMMUNOASSAY OF CYTIDINE 3',5' MONOPHOSPHATE (cCMP)[1]
I. DEVELOPMENT OF THE ASSAY

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SUMMARY: A method is presented for the production of reagents for a radioimmunoassay for cCMP. cCMP was succinylated at the 2'O position with [1,4 ^{14}C] succinic anhydride, and the monosuccinyl cCMP coupled to Keyhole limpet hemocyanin and injected into rabbits. Antibodies to cCMP were produced that showed minimal cross-reactivity with other cyclic nucleotides. Monosuccinyl cCMP was coupled to tyrosine methyl ester, then labeled with ^{125}I, and used as the radiolabeled ligand in the immunoassay of cCMP. By use of this assay, the concentration of cCMP in various tissues of rat and guinea pig have been determined.

The isolation of pyrimidine cyclic nucleotides from tissue was first reported by Bloch (1-3). Bloch demonstrated that cCMP could be isolated from L-1210 mouse leukemia cells. Determination of the function of this cyclic nucleotide has been hampered by the lack of a sensitive assay for its measurement (4-6).

In 1975, we began synthesis of the reagents for a radioimmunoassay of cCMP, using a modification of the method of Steiner et al. (7). Because cCMP is relatively insoluble in pyridine (8), succinylation was carried out in water. [^{14}C] succinic anhydride was used to aid in the characterization of the compound and facilitate isolation. We report here a method for the preparation of all reagents for this immunoassay, which can detect as little as 50 femtomoles of cCMP without succinylation prior to assay.

MATERIALS AND METHODS:

MATERIALS: The following were purchased from the Sigma Chemical Corp.: cytidine 3',5'-monophosphate monosodium salt, cytidine 5'-monophosphate, uridine 5'-monophosphate, uridine 3',5'-monophosphate, Dowex 1-X8 formate resin (200-400 mesh), and Dowex 50 cation resin (200-400 mesh). [1,4 ^{14}C]-succinic anhydride (7.3 mCi/mmol) and [^{3}H]-cAMP (37.7 Ci/mmol) were purchased from New England Nuclear Corp. Chloramine-T and triethylamine were purchased from Mallinckrodt Chemical Corp. [^{3}H]-cCMP (21.0 Ci/mmol) was purchased from Amersham Chemical

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Corp. Keyhole limpet hemocyanin and 1-ethyl-3(3-dimethylaminopropyl) carbodiimide HCl (EDC) were purchased from Calbiochem, and ^{125}I was from Union Carbide. Dibutryl cCMP was the gift of Dr. Wendell Wierenga, Upjohn Company, Kalamazoo, Michigan (9).

PREPARATION OF MONOSUCCINYL CYCLIC CMP (ScCMP): cCMP (45 mg) was dissolved in 5.0 ml of water with 500 µl of triethylamine and 0.20 µCi of [^{14}C] succinic anhydride. After vortexing, 200 mg of freshly sublimed succinic anhydride was added The mixture was shaken for 10 minutes. Aliquots of 10 µl were taken at 60 second intervals and applied to cellulose thin-layer (TLC) plates. Ascending chromatography in butanol:acetic acid:water (12:3:5) showed rapid conversion of cCMP (Rf 0.10) to a new compound (Rf 0.20). This new compound was radiolabeled, indicating the presence of [^{14}C] succinic anhydride. Purification of the reaction mixture on a Dowex 1-X8 formate column (1.5 X 20 cm), using increasing concentrations of formic acid (0.1 M to 0.4 M), yielded a single peak of [^{14}C]-labeled material that had the same UV absorption maximum as cCMP. After mild alkaline hydrolysis for 10 minutes in 0.05 M NaOH, this compound reverted to cCMP with concomitant loss of the [^{14}C] label, thus confirming succinylation at the 2'O position (10,11).

PREPARATION OF cCMP-HEMOCYANIN CONJUGATE: Immunogenic cCMP was prepared by the method of Steiner et al. (7). The hemocyanin-ScCMP conjugate was [^{14}C] labeled and showed maximum UV absorbance at 272 mu, pH 7.0.

PREPARATION OF ANTI-cCMP SERA: Fifteen female rabbits were immunized with 0.4 mg of hemocyanin-ScCMP (0.1 mg/foot pad) in complete Freund's adjuvant. Using the immunization schedule of Steiner et al. (7), suitable antibody was produced by two animals after 6 months (Fig. 2).

PREPARATION OF MONOSUCCINYL cCMP-TYROSINE METHYL ESTER (ScCMPTME): 5.0 mg of [^{14}C]ScCMP (0.1 µCi) was dissolved in 200 µl of dimethylformamide (DMF) which had been vacuum distilled over CaH_2. Trioctylamine (14.0 µl) in DMF was added and the mixture was kept at 0°C. Ethyl chloroformate (1.5 µl), in DMF was added and the mixture was stirred for 15 minutes at 0°C. Tyrosine methyl ester HCl (5 mg) and 9.5 µl of trioctylamine were added. After stirring at room temperature for 3 hours, the reaction was stopped by dilution with water and the mixture separated on Dowex 1-X8 (1.5 X 20 cm) with increasing concentrations of formic acid (0.1 M to 1.0 M). The separated products were placed on cellulose TLC plates (butanol:acetic acid:water, 12:3:5) and the following compounds were identified: (a) unreacted starting material, [^{14}C]-ScCMP, at Rf 0.20; (b) cCMP, amounting to 20% of starting material, Rf 0.10; (c) 2'-O-monosuccinyl cCMP-tyrosine methyl ester, Rf 0.25. This new compound was characterized by the following: nitrosonapthol-positive for tyrosine, [^{14}C] label present on the succinyl moiety, UV spectrum the sum of ScCMP and TME at 272 mu, pH 7.0, and alkaline hydrolysis with 0.05 M NaOH for 20 minutes produced cCMP and [^{14}C] succinyl tyrosine. The reversion of the new compound to cCMP and [^{14}C] succinyl tyrosine (nitrosonapthol-positive) indicates tyrosination of the free carboxyl group of ScCMP.

PREPARATION OF [^{125}I]-MONOSUCCINYL cCMP-TYROSINE METHYL ESTER [^{125}I]-(ScCMPTME): ScCMPTME was iodinated by a method used for the iodination of cyclic AMP (7). The iodination mixture was chromatographed on Sephadex G-10 and eluted with 0.2 M sodium acetate buffer, pH 5.8. Three peaks of radioactivity were found, one of which specifically binds the cCMP antibodies.

PREPARATION OF BIOLOGICAL MATERIAL: Male Sprague-Dawley rats (95-105 gm) were killed by decapitation. Organs were excised, weighed quickly, and immediately frozen in dry ice and acetone. Four volumes of ice cold water were added and the tissue homogenized for 10-15 seconds. Aliquots of the homogenate were flash-frozen and stored at -30°C for later use. To the remaining homogenate was added an equal volume of 2.5 N $HClO_4$ and the sample was vortexed for 30 seconds. After centri-

fugation at 10,000 g for 10 minutes, the supernatant was neutralized with 5.0 N KOH. The precipitated $KClO_4$ was pelleted by centrifugation and the supernatant placed on a Dowex 1-X8 formate column (0.75 X 4 cm). The column was washed with 12 ml of water followed by 20 ml of 0.10 N formic acid. cCMP was eluted in the 20 ml 0.10 N formic acid fraction (Fraction II) and cAMP was retained on the column (Fig. 1). Fraction II was lyophilized and reconstituted in a small volume of water. To determine the recovery of cCMP, [^{3}H]-cCMP (15,000 dpm) was added to the tissue prior to homogenization. The recovered radioactivity from extracted samples was 69.4 ± 8.2% (n=30). cCMP concentrations were corrected for [^{3}H]-cCMP added and for percent recovery. The other cyclic nucleotides, cAMP, cGMP, and cUMP were quantitatively retained on the column using the chromatographic system described.

Urine samples were kept frozen until just prior to chromatography on Dowex 1-X8. Fraction II was collected and assayed.

The osteosarcoma samples assayed was a surgical specimen. It was frozen quickly and later extracted and chromatographed as described above.

THIN LAYER CHROMATOGRAPHY OF LIVER EXTRACTS: Fraction II obtained from liver was lyophilized and adjusted to a volume of 0.5 ml with water. 10 µl were spotted on a cellulose TLC plate with authentic markers (10 µg) of cAMP, cGMP, 5'CMP, and cUMP and subjected to ascending chromatography in ethanol:ammonia acetate (5:2). The plate was air dried and 1 cm^2 sections of cellulose were scraped off and extracted with water. After centrifugation at 1,000 g for 10 minutes, the supernatant was assayed for immunoreactive material by radioimmunoassay. The only immunoreactive material was found at the same Rf as authentic cCMP, Rf 0.53. The amount of immunoreactive material present in Fraction II eluted from the Dowex 1 column was equal to the amount located at the Rf of cCMP on TLC.

ASSAY PROCEDURE: Assay tubes contained 25-200 µl of cCMP standard or of biological extract (Fraction II) in 0.10 M sodium acetate buffer, pH 6.8, 100 µl of

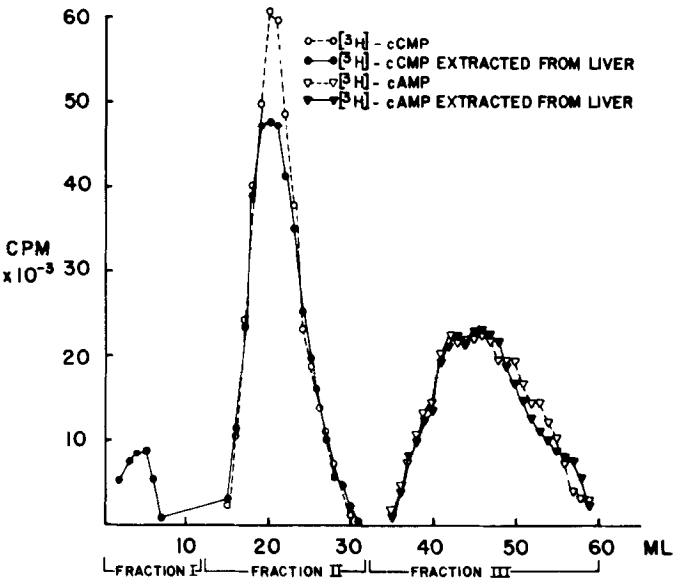

FIGURE 1 - Elution pattern of cCMP and cAMP from Dowex 1-X8 formate. [^{3}H]-cCMP 415,000 cpm, and [^{3}H]-cAMP 435,000 cpm were added to liver and extracted as described in the methods. The extract was placed on Dowex 1-formate and after washing with 12 ml of water (Fraction I), cCMP was eluted with 20 ml of 0.1 N formic acid (Fraction II) and cAMP with 25 ml of 0.2 N formic acid (Fraction III).

[^{125}I]-ScCMPTME (0.01 picomoles, or 12,500 cpm), and 100 µl of antiserum in acetate buffer (final dilution 1:200,000). The mixture was incubated for 18 hours at 4°C and the antibody-bound [^{125}I]-ScCMPTME was separated from free [^{125}]-ScCMPTME by filtering through a Millipore filter (presoaked in acetate buffer, pore size 0.45 µm). Each assay was run in duplicate or triplicate with a variation between tubes of 4.1 ± 0.92%. The inter-assay coefficient of variation was 12.2 ± 7.0%. Tissue extracts were run in duplicate or triplicate and assayed at 3 dilutions.

RESULTS AND DISCUSSION:

Figure 2 demonstrates the range of sensitivity of the newly developed radioimmunoassay for cCMP. As shown in Figure 2, the assay is highly specific for cCMP; cAMP and cUMP being 100-200 times less reactive with the antibody. The possible interference of these latter nucleotides with the assay of cCMP was

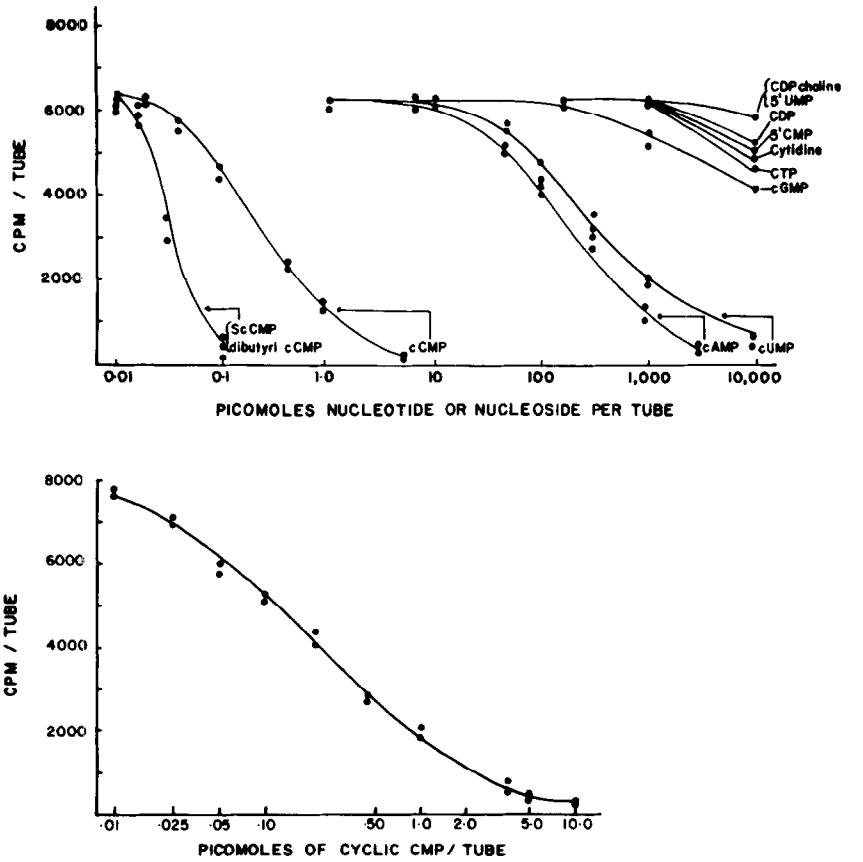

FIGURE 2 - Cross-reactivity and Standard Immunoassay curve with Anti-cCMP antibody. The upper figure shows cross-reactivity of various nucleotides and nucleosides. Each tube contained antibody at a final dilution of 1:200,000 and less than 0.01 picomoles of [^{125}I]-ScCMPTME (12,600 cpm) in a total volume of 400 µl. The lower figure is a typical standard curve run at the same assay conditions described, except that [^{125}I]-ScCMPTME was 13,800 cpm.

TABLE I

CYCLIC CMP CONTENT (FRACTION II) IN VARIOUS TISSUES

TISSUE	cCMP pmoles/gm WET WEIGHT (N)	RANGE*
Rat Liver	7.1 (43)	1.6-22.6
Brain	9.6 (5)	1.1-17.2
Lung	7.7 (5)	0.2-26.2
Heart	2.4 (5)	0.4-4.6
Guinea pig - fetal liver	47.0 (3)	20.4,25.1,95
- adult liver	122.6 (4)	111.8-133.4
- fetal kidney	373.3 (1)	------
- adult kidney	44.5 (4)	23.7-60.8

*For N=3 or less all values are given.

excluded by chromatography of the tissue extracts on Dowex 1 prior to assay. Cytidine diphosphocholine, 5'CMP, CDP, and CTP did not bind significantly to the antibodies at concentrations below 5×10^{-4}M.

The levels of cCMP measured by this assay in various tissues are listed in Table 1. In the rat tissues examined, the concentrations of cCMP detected ranged from 2.4 - 9.6 pm/gm. In guinea pig tissues, levels extended from 44.5-373 pm/gm. The cCMP content in the urine collected over a 3 day period from one male donor varied from 11.5 to 108 pm cCMP/ml, the peak value occurring between 7 and 11 a.m. Bloch has reported isolation of cCMP in the urine of acute leukemia patients (12). The one surgical specimen of human osteosarcoma had a content of 28.5 pm cCMP/gm.

Recently, Cailla et al., have reported an RIA procedure for cCMP using succinylation of samples prior to assay (5). The cCMP concentration determined by these workers in various rat tissues are about 80-90% less than our own values. Two liver samples which were prepared by the method of Cailla et al. (5) and chromatographed on Dowex 1, without a water wash preceding the formic acid elution, contained 0.7 and 0.4 pm cCMP/gm wet weight as measured by our RIA. Samples from the identical tissues were prepared by our chromatographic procedure, with

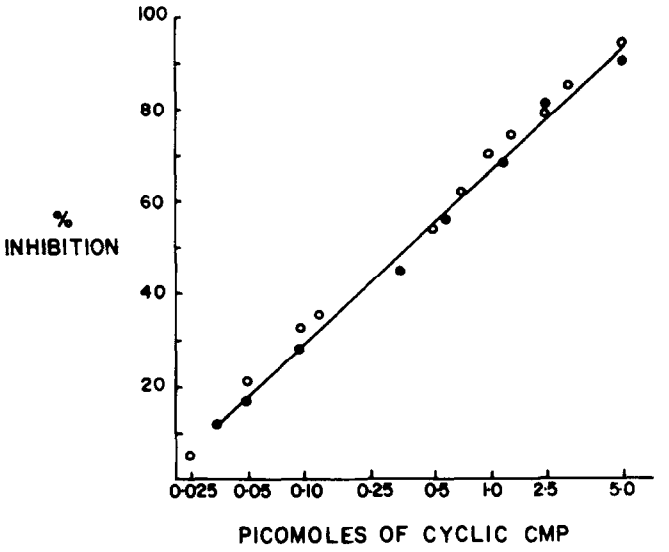

FIGURE 3 - Verification of the cyclic CMP radioimmunoassay. Cyclic CMP was added to small aliquots of liver homogenates and immediately extracted as described in methods. After correcting for recovery (69.4 ± 8.2%, n=30), and subtracting the endogenous cCMP concentration, the data were plotted (open circles). A standard cCMP curve in acetate buffer (without previous extraction) is also shown (closed circles). Linear regression analysis showed that the lines for both sets of data had identical slopes and correlation coefficients of > .98.

the formic acid wash preceded by the water wash contained 8.5 and 6.7 pm/gm wet weight. It is possible that the differences in the results obtained by the two different procedures is attributable to an inhibitor(s), which is eluted with the initial water wash used in our method.

That the immunoreactive material measured in our RIA is cCMP is supported by: (a) its separation from cAMP on Dowex 1-X8 as shown in Fig. 1; (b) by the same Rf on TLC as authentic cCMP; (c) by inhibition curves using known concentrations of authentic cCMP in acetate buffer which were compared with inhibition curves obtained by extracting and chromatographing known concentrations of cCMP added to liver, as shown in Figure 3; and (d) by the lack of inhibition of anti-cCMP-[^{125}I]-ScCMPTME binding to CMP, CTP, CDP, or CDP choline. Another possible validation of the identity of the immunoreactive compound being cCMP is hydrolysis by cCMP-phosphodiesterase (C-PDE) isolated independently by Kuo et al. (13), and Cheng et al. (14). However, this cannot be carried out at present because of the low activity of the enzyme preparations.

Increased levels of cCMP have been reported in regenerating liver and decreased levels of C-PDE have been reported in regenerating liver and fetal tissues (12,15). In agreement with these reports, we found that fetal guinea pig kidney had several fold more cCMP than the adult tissues, however, fetal liver appeared to have less cCMP than adult liver. This report is the first description of the procedures used by us in making a radioimmunoassay for cCMP. This report further describes a method for the measurement of cCMP and its concentration in various tissues of rat and guinea pig. Investigations of cCMP's cellular functions are now being conducted.

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