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CYTIDINE 3',5'-MONOPHOSPHATE (CYCLIC CMP) FORMATION BY HOMOGENATES OF MOUSE LIVER[1]

Stella Y. Cech and Louis J. Ignarro[2]

Department of Pharmacology, Tulane University

School of Medicine, New Orleans, Louisiana 70112

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Summary: Cyclic CMP[3] has been identified as a product of the reaction between mouse liver homogenate, CTP and Mn^{2+} at neutral pH and 37°. This reaction appears to be enzymatic in character in that product formation is pH-, temperature-, time-, and substrate-dependent, and is inhibited by boiling the homogenate. Cyclic CMP formation is enhanced with 0.3 mM Mn^{2+} or Fe^{2+} and inhibited with 3 mM Mn^{2+} or detergents. Cyclic CMP was identified as one of the reaction products by comparison with authentic compound in several systems including: chromatography on neutral alumina columns, Dowex 1-formate columns, polyethyleneimine cellulose columns and thin layer plates; crystallization to constant specific activity; radioimmunoassay.

The occurrence of cyclic CMP in cells (leukemia L-1210 cells) was first reported by Bloch (1), who also reported that cyclic CMP abolishes the temperature-dependent lag phase and stimulates resumption of growth of leukemia L-1210 cells (2). Preliminary experiments in this laboratory revealed the presence of a cyclic CMP generating system in murine myeloid leukemic tumors and in normal mouse liver and spleen (3). In view of the knowledge that adenylate and guanylate cyclase catalyze the conversion of ATP and GTP to cyclic AMP and cyclic GMP, respectively, we elected to study the conversion of CTP to cyclic CMP. We report here that mouse liver homogenates possess the capacity to form cyclic CMP from CTP under defined experimental conditions.

[1] A brief report of these findings was presented at the Third International Conference on Cyclic Nucleotides, New Orleans, Louisiana, July 17-22, 1977.

[2] Recipient of Research Career Development Award No. 1-K04 AM 00076 from the USPHS.

[3] Abbreviations: cyclic CMP, cytidine 3',5'-monophosphate; cyclic AMP, adenosine 3',5'-monophosphate; cyclic GMP, guanosine 3',5'-monophosphate.

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The formation of cyclic CMP is due to the presence of cytidylate cyclase activity in mouse liver.

MATERIALS AND METHODS

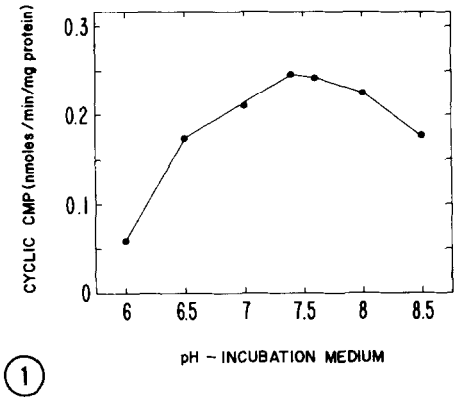
Materials: The following materials were purchased from Sigma Chemical Co., St. Louis, Mo.: cytidine 3',5'-cyclic monophosphoric acid, cytidine 5'-triphosphate sodium salt, cytidine 5'-diphosphate sodium salt, cytidine 5'-monophosphate sodium salt, Dowex-1 chloride form (1 x 8-200), polyethyleneimine (PEI) cellulose. Nitex No. 110 nylon filament bolting cloth (50 μm pore size) was purchased from Tobler, Ernst & Traber, Inc., Elmsford, N.Y. Aluminum Oxide Woelm-neutral for column chromatography (activity grade 1) was obtained from ICN Pharmaceuticals, Inc., Cleveland, Ohio. α-[32-P] CTP tetra (triethylammonium) salt, 13-25 Ci/mmole, was purchased from New England Nuclear, Boston, Mass., and [3-H] cyclic CMP ammonium salt, 21 Ci/mmole, from Amersham/Searle Corp., Arlington Heights, Ill. PEI cellulose TLC ready plastic sheets with luminescer was obtained from Schleicher & Schull, Dassel, W. Germany.

Preparation of Whole Homogenates from Mouse Liver: BAGG-Swiss male mice (15-20 g) were sacrificed by cervical dislocation and the livers were rapidly excised, minced and homogenized (20% w/v) in ice-cold 10 mM Tris HCl (pH 7.4) containing 10 mM NaCl, 10 mM KCl and 0.005 mM disodium EDTA. Homogenization was performed with a Potter-Elvehjem tissue grinder by executing 8 complete strokes of a motor driven (1,200 r.p.m.) Teflon pestle (0.008 inch clearance). Homogenates were filtered through Nitex No. 110 nylon filament bolting cloth to remove unbroken cells and connective tissue, and were used 1 hr after preparation. In the subcellular distribution experiments, homogenates were subjected to differential centrifugation in 0.32 M sucrose containing the components of the homogenization medium described above. The individual subcellular fractions analyzed were the whole homogenate and the sediments and supernatants obtained from centrifugations of 10,000g x 10 min and 100,000g x 60 min.

Formation of Cyclic CMP from CTP: Assays were conducted in 13 x 100 mm glass tubes for 10 min at 37° in a final volume of 1.0 ml. Incubation media consisted of 40 mM Tris HCl (pH 7.4), 1.0 mM CTP, 3 x 10^5 cpm of α-[32-P] CTP (13-25 Ci/mmole), 0.3 mM $Mn(C_2H_3O_2)_2$, and 0.1 ml of homogenate equivalent to 2.8-3.4 mg of protein (4). Incubations were initiated by rapid, successive additions of $Mn(C_2H_3O_2)_2$ and CTP, and were terminated by addition of 0.1 ml of ice-cold 60 mM disodium EDTA and cooling the samples to 4°. Termination of product formation was rapid and complete. Acidification and boiling were not necessary and nonenzymatic product formation was negligible. Tris buffer (0.5 ml), containing 3 x 10^4 cpm of [3-H] cyclic CMP (21 Ci/mmole), was added to the samples which were then chromatographed on neutral alumina columns (5). Alumina chromatography resulted in the retention of over 99.99% of the added CTP. Incubated blanks (tissue omitted) and nonincubated blanks (tissue included) contained less than 0.01% (10-30 cpm) of total α-[32-P] CTP added. Chromatography of authentic CTP, CDP, CMP and cyclic CMP revealed that only cyclic CMP was recovered in the first 3 ml of eluate after elution with neutral Tris buffer. These data are in agreement with those of White and Zenser (6). Recoveries of cyclic CMP were 60-80%. Eluates were added to scintillation cocktail and the radioactivity was measured as described previously (5). Neither radioisotope required further purification prior to use.

RESULTS AND DISCUSSION

One of the earliest experiments conducted was designed to identify the



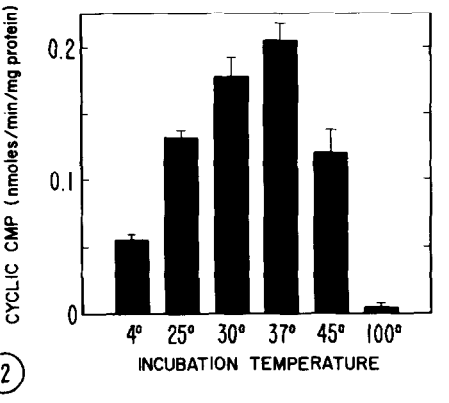
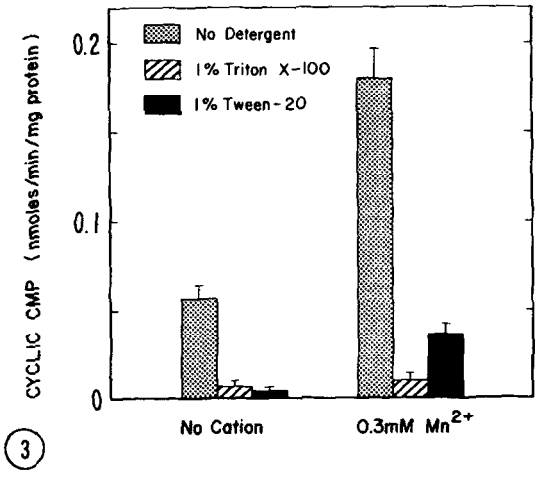


Figure 1: Effect of pH of incubation medium on cyclic CMP formation by mouse liver homogenate. Tris maleate buffer was used for pH 6 and 6.5; Tris HCl buffer was used for pH 7, 7.4, 7.6, 8 and 8.5. Incubations were conducted with 1.0 mM CTP and 0.3 mM Mn^{2+} for 5 min at 37° as outlined in the text. Data are expressed as the mean values of 6-9 determinations from 2-3 separate experiments.

Figure 2: Effect of incubation temperature on cyclic CMP formation by mouse liver homogenate. Incubations were conducted at the indicated temperatures except for that noted as 100°; where the homogenate was boiled for 5 min and subsequently incubated at 37°. Incubations were conducted with 1.0 mM CTP and 0.3 mM Mn^{2+} for 5 min at pH 7.4 as outlined in the text. Data are expressed as the mean ± S.E.M. of 6 determinations from 2 separate experiments.

most suitable tissue fraction with which to study the formation of cyclic CMP. Maximal formation of cyclic CMP occurred with the whole homogenate fraction. The sedimentable fractions resulting from centrifugations of 10,000g x 10 min and 100,000g x 60 min had little, but significant, cyclic CMP forming activity. Approximately 15% of the total activity was associated with the soluble fraction (100,000g x 60 min supernatant). Studies are in progress to determine whether cyclic CMP generating activity is localized to the nuclear fraction.

After identifying the most active fraction and conducting pilot experiments to determine the optimal pH (7.4) (Figure 1) and incubation temperature (30°-37°) (Figure 2), a more detailed analysis of the ionic requirements, including the effects of detergents, was conducted (Figure 3). Although cyclic CMP formation occurred in the absence of added divalent cation, 0.3 mM Mn^{2+}



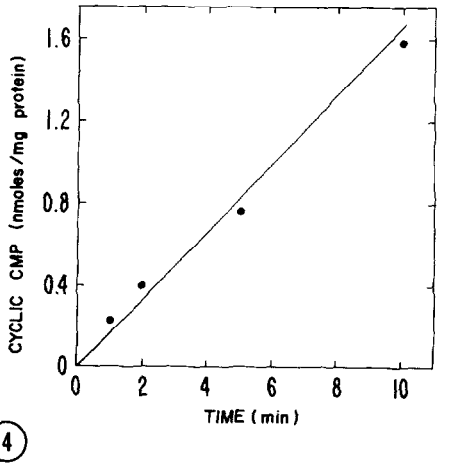
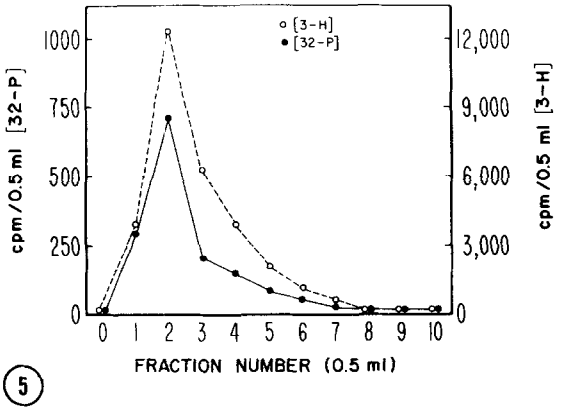


Figure 3: Effects of manganese and detergents on cyclic CMP formation by mouse liver homogenate. Incubations were conducted with 1.0 mM CTP and 0.3 mM Mn^{2+} for 5 min at 37° and pH 7.4 as outlined in the text in the presence or absence of detergent in the incubation medium. Data are expressed as the mean ± S.E.M. of 6-9 determinations from 2-3 separate experiments.

Figure 4: Effect of incubation time on cyclic CMP formation by mouse liver homogenate. Incubations were conducted with 1.0 mM CTP and 0.3 mM Mn^{2+} at 37° and pH 7.4 as outlined in the text. Data are expressed as the mean values of 15 determinations from 5 separate experiments.

stimulated the formation of this pyrimidine cyclic nucleotide. In each case, the inclusion of nonionic detergents decreased the extent of cyclic CMP formation. The optimal concentration of Mn^{2+} was 0.3 mM. Higher Mn^{2+} concentrations (1-3 mM) were markedly inhibitory. Preliminary experiments indicated that 0.3 mM Fe^{2+} is just as active as, and 1 mM Fe^{2+} is 2-3 times more active than, 0.3 mM Mn^{2+} in stimulating cyclic CMP formation. Addition of 6 mM disodium EDTA inhibited completely the formation of cyclic CMP in the presence or absence of added cation, thus indicating the presence in the diluted homogenate of one or more naturally occurring cations capable of stimulating or facilitating cyclic CMP formation. Cyclic CMP formation was reduced markedly at 4° and was negligible if homogenates were boiled for 5 min just prior to use (Figure 2).

Subsequent to securing some of the optimal conditions for demonstrating cyclic CMP generation by liver homogenates, the time course of cyclic CMP



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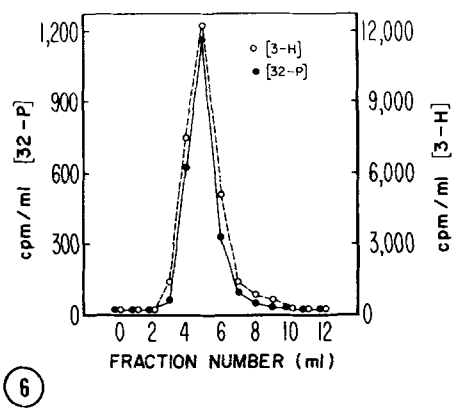

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Figure 5: Column chromatography of incubation mixtures on neutral alumina. One ml of incubation mixture, containing [32-P] labelled reaction product and [3-H] authentic cyclic CMP, was applied to columns of neutral alumina and the columns were eluted with 50 mM Tris HCl, pH 7.4. Fraction 0 represents 1 ml (resulting from addition of 1 ml sample) and all other fractions are 0.5 ml. Data represent mean values from 3-4 separate experiments.

Figure 6: Column chromatography of neutral alumina eluates on Dowex 1-formate. One ml of the 3 ml eluate resulting from column chromatography of incubation mixtures on neutral alumina was applied to columns of Dowex 1-formate, and the columns were eluted with 0.5 N formic acid. Data represent mean values from 4 separate experiments.

formation was studied (Figure 4). Cyclic CMP formation was linear for at least 10 min at 37°. Although the data are not illustrated, cyclic CMP formation was linear throughout a protein concentration range of 0.25 to 3.6 mg per ml of reaction volume incubated at 37° for 10 min. A double-reciprocal plot of velocity vs. substrate concentration yielded a straight line ($r=0.99$); the K_m and V_{max} were 1.6×10^{-4}M and 9.27×10^{-10} moles min^{-1}, respectively. Neither ATP nor GTP served as effective substrates for cyclic CMP formation. The inclusion of 1 mM cyclic CMP, 3 mM 1-methyl-3-isobutylxanthine, 15 mM phosphocreatine and 150 Units of phosphocreatine kinase in the enzyme reaction mixture failed to alter the rate of cyclic CMP formation, thus, suggesting that neither the substrate nor product is undergoing significant degradation under the experimental conditions employed. Cyclic CMP formation was not affected by 0.1 mM concentrations of ATP, GTP, epinephrine or acetylcholine.

Cyclic CMP was verified as the principal product of the reaction involving mouse liver homogenate, CTP and Mn^{2+} conducted at pH 7.4 and at 37°, by several different procedures. The [32-P] product of the reaction cochromatographed with [3-H] cyclic CMP and authentic unlabelled cyclic CMP by neutral alumina column chromatography (Figure 5) and Dowex 1-formate column chromatography (Figure 6), thus, suggesting that the [32-P] labelled product was cyclic CMP. Chromatography of alumina eluates (resulting from column chromatography of incubation mixtures) on columns of polyethyleneimine cellulose resulted in identical elution profiles for the [32-P] labelled product and [3-H] cyclic CMP. Both product and authentic cyclic CMP eluted with 0.05 M LiCl in the first 3 ml; elution with 1 M LiCl was required to remove CTP from the column. Thin layer chromatography of neutral alumina eluates on polyethyleneimine cellulose plates (developed in 0.1 M LiCl for 60 min) revealed one spot, containing both [32-P] and [3-H] activity, possessing an R_f of 0.34. Authentic cyclic CMP and CTP yielded R_f values of 0.34 and zero. The [32-P] labelled product, after isolation from incubation mixtures by neutral alumina column chromatography, and [3-H] cyclic CMP crystallized together on three successive occasions with excess unlabelled authentic cyclic CMP to constant specific activity. Crystallization was conducted either by cooling samples to 4°C or by addition of ice-cold isopropanol. The ranges of [32-P] and [3-H] cpm during the three successive crystallizations were 75-83 and 560-625, respectively. The [32-P] product, isolated from incubation mixtures by chromatography on neutral alumina columns, was identified as cyclic CMP by specific radioimmunoassay. These experiments were conducted by H. L. Cailla and M. A. Delaage (Centre de Biochimie et Biologie Moleculaire-CNRS, Marseille, France). The radioimmunoassay indicated that both 0.3 mM Mn^{2+} and 1 mM Fe^{2+} stimulated cyclic CMP formation. In agreement with measurements of product formation by the radiolabelling procedure described in this report, radioimmunoassay of the reaction product indicated that 1 mM Fe^{2+} was 2-3 times more active than

0.3 mM Mn^{2+}. Details of this new radioimmunoassay for cyclic CMP were presented at the Third International Conference on Cyclic Nucleotides, New Orleans, Louisiana, July 17-22, 1977.

The data presented in this report illustrate that normal mouse liver possesses the capacity to form cyclic CMP from CTP and that the generation of this pyrimidine cyclic nucleotide is facilitated by Mn^{2+} (and Fe^{2+}). Cyclic CMP formation under defined experimental conditions is undoubtedly attributed to a cyclase, namely, cytidylate cyclase. The properties of cytidylate cyclase differ from those of adenylate and guanylate cyclase in several respects including substrate specificity, optimal stimulation with low (0.3 mM) rather than high (3-5 mM) concentrations of Mn^{2+}, stimulation with Fe^{2+}, and lack of effect of ATP, GTP or epinephrine. The demonstration of cytidylate cyclase activity in mammalian tissue supports the earlier findings by Bloch (1) on the identification of cyclic CMP as a natural constituent of murine L-1210 cells in culture.

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REFERENCES

1. Bloch, A. (1974) Biochem. Biophys. Res. Commun., 58, 652-659.
2. Bloch, A., Dutschman, G., and Maue, R. (1974) Biochem. Biophys. Res. Commun., 59, 955-959.
3. Cech, S. Y., Gross, R. A., and Ignarro, L. J. (1976) Pharmacologist, 18, 223.
4. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem., 193, 265-275.
5. Ignarro, L. J., Gross, R. A., and Gross, D. M. (1976) J. Cyclic Nucl. Res., 2, 337-346.
6. White, A. A., and Zenser, T. V. (1971) Anal. Biochem., 41, 372-396.