CYTIDINE 3',5'-MONOPHOSPHATE (CYCLIC CMP)

I. ISOLATION FROM EXTRACTS OF LEUKEMIA L-1210 CELLS

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SUMMARY: A compound isolated from the acid soluble fraction of intact leukemia L-1210 cells and from hot water extracts of disrupted L-1210 cells, has been identified as 3',5'-cyclic CMP by comparison with the authentic compound with respect to the following characteristics: Rf values in eight solvent systems; electrophoretic mobility in three buffers; uv spectra, mass spectrum; acid hydrolysis and treatment of the products with appropriate enzymes; and effect on initiation of L-1210 cell growth.

INTRODUCTION: Although the presence of 3',5'-cyclic AMP and 3',5'-cyclic GMP in living cells has long been established (1,2), the occurrence of cellular 3',5'-cyclic CMP has not been reported. We have now isolated cyclic CMP from the cold acid soluble fraction of intact leukemia L-1210 cells and from hot water extracts of disrupted L-1210 cells. The search for this cyclic nucleotide was stimulated by the following observation (3): Leukemia L-1210 cells, grown *in vitro* to stationary phase and cooled for 1 hr at 4°, undergo a 2 hr lag before they resume growth upon addition of 9 volumes of fresh (37°) medium; this lag is abolished by cyclic CMP. In contrast, under the same experimental conditions, cyclic AMP prolongs the lag up to 20 hrs, depending upon the concentration of cyclic AMP added.

EXPERIMENTAL:

Materials: Eight week old female DBA/2J mice, weighing 18-20 g were obtained from the RPMI breeding colony. Cytidine 3',5'-cyclic monophosphoric acid ("authentic cyclic CMP") and cytidine 2',3'-cyclic monophosphoric acid were purchased from the Sigma Chemical Co., St. Louis, Mo., and were repurified before use by chromatography in solvents A and B (see below).

3'-Ribonucleotide phosphohydrolase (E.C. No. 3.1.3.6), partially purified from rye grass (14 u/mg; 2'-nucleotidase activity 0.5%; 5'-nucleotidase activity

< 0.2%) and 5'-ribonucleotide phosphohydrolase (E.C. 3.1.3.5) partially purified from Crotalus adamanteus venom (27.6 u/mg) were purchased from Sigma. Crystalline bovine pancreatic ribonuclease (E.C. 2.7.7.16; 40 u/mg) was purchased from Boehringer Corp., New York. [2-^{14}C]CMP (27 mCi/mmole), [2-^{14}C]CDP (24.9 mCi/mmole, and [2-^{14}C]CTP (22 mCi/mmole) were purchased from Schwarz Bioresearch or Schwarz/Mann. 2'-CMP, 3'-CMP, CDP-choline, CDP-ethanolamine and CDP-glycerol were purchased from Sigma. Uridine, cytidine, CMP, CDP and CTP were purchased from P-L Biochemicals, Milwaukee, Wisc.

All Whatman 3 MM papers were prewashed before use with 1 N acetic acid and dried in air. To determine uv spectra with an Aminco DW-2 recording spectrophotometer, the compounds were dissolved in distilled, deionized water, adjusted to pH 7.0 with ammonium hydroxide and to pH 2.0 with HCl. The pH was rechecked following addition of the unknown material, or of the reference compounds.

Paper Chromatography: The following chromatographic systems were used for the separation and identification of the extracted material:

(A) Ethanol-1 M ammonium acetate (7:3 v/v; pH 6.6); (B) Isobutyric acid-2 N ammonium hydroxide (66:34; v/v); (C) n-Butanol-glacial acetic acid-water (50:25:25; v/v); (D) Isoamyl alcohol-5% aq. Na_2HPO_4 (1:1, v/v); (E) Isopropanol-conc. NH_4OH-H_2O) (7:1:2; v/v); (F) Isopropanol-conc. NH_4OH - 0.1 M H_3BO_3 (60:10:30; v/v); (G) Na_2HPO_4 13.8 g into 900 ml H_2O, adjust pH to 6.8 with H_3PO_4, add H_2O to 1 l, add 600 g $(NH_4)_2SO_4$ and 20 ml n-propanol; (H) Dissolve compounds in 0.1 ml of 0.1 M boric acid, apply to chromatogram, air dry and develop in solvent A. All separations were made by ascending chromatography at 20°C for 18 hr on Whatman 3 MM papers.

Paper Electrophoresis: The following buffers were used for the electrophoretic characterization of the extracted material:

1) Na_2HPO_4-NaH_2PO_4, 0.02 M, pH 7.8; 2) KH_2PO_4-NaOH, 0.15 M, pH 4.6; 3) Boric acid-sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) (0.62 g:7.63 g/l, adjust to pH 9.0 with NaOH). Whatman 3 MM papers (15 x 49 cm) were used for all determinations in a water cooled Savant flat plate high voltage electrophoresis unit. A potential of 2500 V was applied for 60 minutes with buffers 1 and 2, 1500 V for 30 minutes with buffer 3.

Isolation of the Material from L-1210 Cells:

a) Isolation from the acid soluble fraction: Fifty DBA/2J mice were inoculated i.p., each with 1 x 10^6 leukemia L-1210 cells. Five days later, the cells were removed from the intraperitoneal cavity immediately upon sacrifice of each mouse, and the harvest from each group of ten mice was placed into 200 ml of ice cold saline and was worked up at once at 4° as follows: The cell suspension was centrifuged at approximately 1500 x g for 10 min and, after decanting the supernatant fluid, the cells were resuspended in 10 ml of saline by gently stirring. The suspensions obtained from the five batches were combined (50 ml) and recentrifuged. The cell pellet was then extracted with 50 ml of ice cold 1 N perchloric acid for 15 min, and the extract was neutralized with 2 N potassium hydroxide (in some experiments 0.6 N acid and 1 N KOH was used). Precipitated $KClO_4$ was removed by centrifugation, and the supernatant solution freeze-dried overnight. The residue was dissolved in 20 ml of

water and 1 ml aliquots were applied, in narrow bands (0.2 x 40 cm), to twenty 46 x 57 cm Whatman 3 MM papers. After ascending chromatography for 18 hours in solvent A, a 2 x 40 cm band was cut from each paper, at the location at which the authentic cyclic CMP marker was located on each paper (Rf = 0.42). The bands were jointly eluted in 500 ml of distilled, deionized water, pH 6.8, and the eluate brought to dryness in vacuo at 30° with a rotary evaporator. The material thus obtained was dissolved in a minimum of water, and was applied in a narrow band to one sheet of Whatman 3 MM paper and rechromatographed in solvent A. Sufficient material was now present at the location corresponding to the Rf of cyclic CMP so as to be visible under UV light. After elution with 50 ml of water, pH 7.0, the material was rechromatographed in solvent B, and following elution with water, its identity was determined as described in the Results.

b) Isolation from hot water extracts of disrupted L-1210 cells. To eliminate the possibility that the isolated material is an artifact of the acid extraction procedure, L-1210 cells harvested from 100 mice were extracted with boiling water. The cells were first washed with saline, frozen quickly at -70°, and the frozen pellet was disrupted by means of a Hughes press. The disrupted cells were then extracted for 15 min by boiling with 1 liter of water, pH 6.8. The pH did not change during the extraction. The debris was removed by centrifugation, and the supernatant solution was brought to dryness in a rotary evaporator at room temperature. The residue was subjected to repeated chromatography, as described for the acid soluble fraction.

Hydrolytic degradation of the isolated material: An aliquot of the isolated material (9.6 $A_{277\ nm}$ units, pH 2.0) (4), was heated in 1 ml of 1N HCl for 2 hr at 100°. The solution was cooled and brought to pH 7.0 with 1N NaOH. To one 0.3 ml aliquot of the neutralized hydrolysate was added 0.1 ml of a solution of 1 mg of 5'-ribonucleotide phosphohydrolase in 1 ml of tris buffer, pH 8.5, and 0.1 ml of 0.1M $MgCl_2$, and the mixture was incubated at 37° for 1 hr with gentle agitation. To another 0.3 ml aliquot of the neutralized hydrolysate was added 0.1 ml of a solution of tris buffer, pH 7.5, containing 0.4 mg of 3'-ribonucleotide phosphohydrolase, and the mixture was incubated for 3 hours at 37° with gentle agitation.

Following incubation, the reaction mixtures were immersed in a boiling water bath for 1 min. and their volume, as well as that of 0.3 ml of an untreated solution, was reduced in vacuo to approximately 0.1 ml. The aliquots were applied to a chromatography sheet, which was developed in solvent A, and the dry chromatogram was then photographed under uv light (Fig. 2).

Effect of RNA-ase on the isolated material: To 3.1 $A_{272\ nm}$ units, pH 7.0, of the material isolated from L-1210 cells, in 0.1 ml of tris buffer, pH 7.1 was added 0.2 ml of a solution of crystalline bovine pancreatic ribonuclease (1 mg/ml of tris buffer, pH 7.1). Incubation with gentle shaking proceeded for 60min at 37°, after which the reaction mixture was immersed into a boiling water bath for 1 min. The entire mixture was then applied to paper, and was chromatographed in solvent A. Equivalent aliquots (based on uv absorbance) of authentic 3',5'-cyclic CMP and 2', 3'-cyclic CMP were treated identically (5).

Mass spectrometry of the isolated compound: The isolated material, as well as authentic cyclic CMP were subjected to mass spectrometry following their conversion to the trimethylsilyl derivatives (6) as follows: To 5.0 $A_{272\ nm}$ units, pH 7.0 of each of the carefully dried compounds was added pyridine, N,O-Bis (trimethylsilyl)

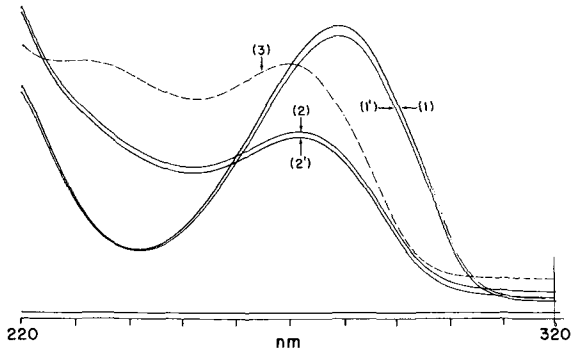

Fig. 1. UV spectra of authentic 3',5'-cyclic CMP at pH 7.0 (1) and pH 2.0 (2), and of the isolated compound at corresponding pH's (1',2'). (3) is the spectrum of 2',3'-cyclic CMP at pH 7.0.

trifluoroacetamide (BSTFA), and trimethylchlorosilane (TMSC) (9:90:1, 0.25 ml), and the mixtures were heated, in sealed vials, at 100° for 3 hr. To obtain medium resolution spectra the solutions were reduced in volume in vacuo to approximately 0.05 ml prior to their introduction into capillary tubes. One end of the tubes was then sealed, and the remaining solvent evaporated in vacuo at room temperature. The medium resolution spectra were obtained on a CEC 21-491 double focusing spectrometer with sample introduction by direct inlet. Probe temperature 110°, source temperature 285°, ionizing energy 70 ev and accelerating voltage 1.2 kv.

RESULTS AND DISCUSSION: From the acid soluble fraction of intact leukemia L-1210 cells and from hot water extracts of disrupted L-1210 cells, harvested from the i.p. cavities of DBA/2J mice on days 5-7 after inoculation, a uv absorbing material was isolated, which was identified as 3',5'-cyclic CMP on the basis of the following criteria:

1) The material had the same Rf values as authentic cyclic CMP upon paper chromatography in the eight solvent systems listed in the experimental section, and exhibited the same relative mobilities as authentic cyclic CMP upon electrophoresis in the three buffer systems listed in that section.

2) The material has uv spectra (Fig. 1) which are essentially indistinguishable from those of authentic cyclic CMP ($\lambda_{max}^{pH\ 7.0}$ = 272 nm, $\lambda_{max}^{pH\ 2.0}$ = 277 nm).

3) Acid hydrolysis of the isolated material produced two uv absorbing products (Fig. 2) which, in the solvents B, C, F and H had the respective Rf values of 5'-

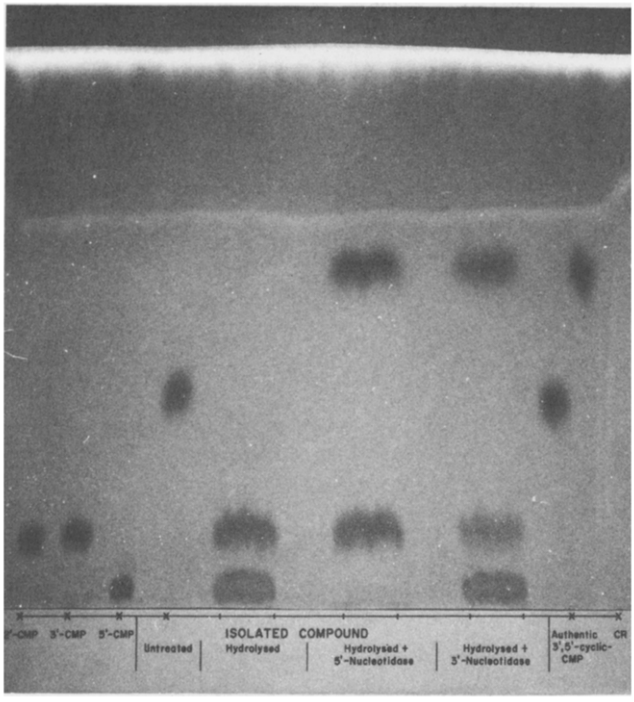

Fig. 2. Paper chromatographic analysis of an acid hydrolysate of the compound isolated from leukemia L-1210 cell extracts. 9.6 $A_{277\ nm}$ units (pH 2.0) of the material were heated in 1 ml of 1 N HCl for 2 hr at 100°. Following neutralization with 1 N NaOH, the hydrolysate was divided into three parts. One was treated with 5'-ribonucleotide phosphohydrolase, another with 3'-ribonucleotide phosphohydrolase and the third served as control. Ascending chromatography of the three reaction mixtures was carried out on Whatman 3 MM paper at 20° for 15 hr in solvent H (see text).

CMP, and 2'(3')-CMP. The same result was obtained upon hydrolysis of authentic cyclic CMP. To confirm the identity of the products, an aliquot of the hydrolysate was treated for 1 hr at 37° with snake venom 5'-nucleotidase. Under these conditions (Fig. 2), the product considered to be 5'-CMP disappeared, and a new, uv absorbing material was seen which, in all four solvents, had Rf values corresponding to cytidine. Following its elution from the paper, the identity of this compound was further confirmed by its deamination, by a cell-free extract of E. coli B, to uridine, the product being identified by chromatography in solvents B and C.

4) Unlike the phosphodiester ring of 2',3'-cyclic CMP, which is opened by

treatment with RNAase (5), that of 3',5'-cyclic CMP was not hydrolysed by treatment with this enzyme. Thus, after incubation of 0.5 μmole of 2',3'-cyclic CMP with eight units of RNAase for 60 min at 37°, this nucleotide could no longer be detected upon paper chromatography. Instead, a new product was encountered which, in the solvents B, F and H had the Rf values of (2'),3'-CMP. Under the same conditions, neither the material isolated from the cells, (3.1 $A_{272\ nm}$ units, pH 7.0) nor authentic cyclic CMP underwent any degradation detectable on the chromatograms.

5) The identity of the compound isolated was also confirmed by mass spectrometry. Like authentic cyclic CMP, the isolated material, following conversion to the trimethylsilyl derivative, gave a molecular ion ($M^{+\cdot}$) of mass 521, corresponding to the presence of three trimethylsilyl functions. The fragmentation pattern of the isolated material was essentially the same as that of authentic tris-trimethylsilyl cyclic CMP.

6) We have observed that resumption of the growth of leukemia L-1210 cells, grown to stationary phase and cooled at 4° for 1 hr, is detectable within 30 minutes following the addition of cyclic CMP (at 10^{-4} - 10^{-8} M) to the culture (3). In the absence of the cyclic nucleotide, growth, as measured by cell count and total protein, lags for approximately 2 hrs. The growth stimulatory effect of the material isolated from the L-1210 cells is indistinguishable from that of authentic cyclic CMP. Such a stimulation of growth is not effected by 2',3'-cyclic CMP, 5'-CMP, 5'-CDP, or 5'-CTP.

The amount of cyclic CMP obtained appears to be proportionate to the number of cells harvested. For instance, 0.94 μmole was obtained by acid (0.6 N) extraction of the L-1210 cells harvested from 30 mice on day 5 after inoculation of 8×10^6 cells; 3.53 μmoles were extracted (1 N acid) from the cells harvested from 100 mice on day 7 after inoculation of 1×10^6 cells, and 5.72 μmoles were obtained from the

hot water extract of the cells (55 cc, 64.3 g wet weight) collected from 100 mice on day 6 after inoculation of 2×10^6 cells. Proportional amounts were obtained in other experiments.

Whereas the identity of the isolated material as 3',5'-cyclic CMP appears to be firmly established by these studies, the question arises whether this cyclic nucleotide, being present in relatively low concentrations in the cell extracts, might be an artifact. Two factors would support the view that the isolated compound is a proper cell constituent. First, the fact that cyclic CMP was isolated from the cells by two different extraction procedures, involving either cold acid or boiling water, pH 6.8, diminishes the possibility that non-enzymatic cyclization of an unidentified precursor occurred during workup. Second, 3'-CMP, 5'-CMP, 5'-CDP, 5'-CTP, CDP-choline CDP ethanol amine and CDP glycerol, which were considered among some of the possible compounds that might give rise, nonenzymatically, to the cyclic nucleotide as a result of the extraction procedures used, remained unchanged when kept in boiling water, pH 6.8, or in cold 1N $HClO_4$ for 15 min. Specifically, 5 μCi of each of [2-^{14}C]CMP, [2-^{14}C]CDP and [2-^{14}C]CTP, or 0.5 mg each of 3'-CMP or of the nucleotide anhydrides were dissolved in a mixture of 32 ml of distilled water and 15 ml of saline, corresponding approximately to the amount of intracellular water and intercellular saline contained in a saline washed pellet of L-1210 cells harvested from 100 mice. The solution was then worked-up exactly as was the cell pellet. Under these conditions no cyclic CMP was detectable on the chromatograms either by uv or, in the case of the labeled compounds, by liquid scintillation spectrometry.

The initiation of cell growth by cyclic CMP, under conditions where cyclic AMP extensively delays the onset of growth, could be construed to suggest that cyclic CMP may play a regulatory role in the cell metabolism. Such a suggestion must not, however, be interpreted as direct proof of the physical existence of cyclic CMP in

the cell. Such proof will have to come from the identification of enzymes which catalyze the formation of the cyclic nucleotide from appropriate precursors, and from the isolation of the compound from cells by procedures other than the ones employed here. These studies are in progress in our laboratory.

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