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CYTIDYLATE CYCLASE: THE PRODUCT ISOLATED BY THE METHOD OF CECH AND IGNARRO IS NOT CYTIDINE 3',5'-MONOPHOSPHATE

R. M. Gaion and G. Krishna
Section on Drug-Tissue Interactions, Laboratory of Chemical Pharmacology,
National Heart, Lung, and Blood Institute,
National Institutes of Health, Bethesda, Maryland 20014

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Summary

When mouse liver homogenates were incubated with [α-^{32}P]-CTP according to the method of Cech and Ignarro (4,5), a [^{32}P] product (100-120 pmoles/mg protein/minute) was isolated by chromatography on alumina column, in the same fraction as [^{3}H]-cyclic CMP.[1] However this product did not behave as cyclic CMP in various chromatographic systems. Moreover the amount of this [^{32}P] product isolated was markedly reduced by removal of protein prior to chromatography on alumina. Chromatography of the enzymatic reaction product either before or after alumina purification on Dowex 1 - formate column indicated that 5'-CMP and CDP are the major products, with no formation of cyclic CMP. These results indicate that the [^{32}P] labeled material isolated by Cech and Ignarro is mainly if not entirely 5'-CMP and CDP.

INTRODUCTION

Recently Bloch reported the presence of cyclic CMP in mouse leukemia cells (leukemia L1210 cells) and in regenerating liver (1). Bloch also reported that cyclic CMP, when added to growth media, was able to abolish the low temperature induced lag phase in the growth of leukemia L1210 cells (2).

During the last few years we have made various attempts with very little success to measure cyclic CMP formation from [α-^{32}P]-CTP by the homogenate of leukemia L1210 cells and by homogenates of various tissues including rat fetal and regenerating liver. For this purpose we had devised a simple method for the assay of cytidylate cyclase using a procedure similar to one developed for the assay of guanylate cyclase (3). Even though this method is capable of detecting the formation of as little as 1-2 pmoles of cyclic CMP, so far we have been unable to detect cytidylate cyclase activity in homogenates of the following tissues under various conditions: mouse liver, rat normal, fetal and regenerating liver, kidney, lung, heart, bovine rod outer segments, and sea urchin sperms.

[1]Abbreviations: Cyclic CMP, cytidine 3',5'-monophosphate.

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Recently Cech and Ignarro reported a method for the assay of cytidylate cyclase in which alumina columns were used for the separation of cyclic CMP from other nucleotides (4,5). These authors reported the formation of a product by mouse liver homogenate which co-chromatographed with authentic cyclic CMP in various chromatographic systems. Since there was a major discrepancy between our findings and those of Cech and Ignarro we repeated the assay with mouse liver homogenate using the same conditions reported by the authors (4,5). Even though we were able to obtain a product which co-chromatographed with authentic cyclic CMP in the first alumina column in amounts comparable to those reported by Cech and Ignarro (4,5), this product was demonstrated not to be cyclic CMP by various chromatographic systems. In our experiments mouse liver homogenates did not convert [α-^{32}P]-CTP to cyclic CMP but formed mainly 5' CMP and CDP.

MATERIALS AND METHODS

Materials : The following materials were purchased from Boehringer Mannheim Corp, New York: cytidine 3':5'-monophosphate monosodium salt, cytidine 5'-triphosphate trisodium salt, cytidine 5'-diphosphate, cytidine 5'-monophosphate disodium salt, cytidine 5'-diphosphocholine monosodium salt. Bio Rad AG-1 X 8, formate form (100-200 mesh), and Bio Rad AG-50 H^+ x 4 (200-400 mesh) were from Bio Rad Laboratories, New York, N.Y. Woelm neutral aluminum oxide (activity grade super I type W200) was obtained from ICN Life Sciences Group, Cleveland, Ohio. [α-^{32}P]-Cytidine-5'-triphosphate tetraethylammonium salt (10-30 Ci/mmole), [methyl-^{14}C]-cytidine diphosphocholine (40-60 mCi/mmole) and Aquasol were purchased from New England Nuclear Corp. Boston, Mass. [5-^{3}H]-Cytidine-5'-diphosphate ammonium salt (10 Ci/mmole), [5-^{3}H]-cytidine 5'-monophosphate, ammonium salt (13 Ci/mmole) and [5-^{3}H]-cytidine 3',5'-cyclic phosphate ammonium salt (18 Ci/mmole) were from Amersham Corp., Arlington Heights, Ill. Other chemicals were from standard source.

Preparation of whole homogenates from mouse liver : BAGG-Swiss mice (20-25 g) were killed by cervical dislocation, and the liver lobes were removed and homogenized in four volumes of ice cold 10 mM Tris HCl (pH 7.4), containing 10 mM KCl, 10 mM NaCl and 0.005 mM EDTA and filtered through nylon cloth as described by Cech and Ignarro (4,5). Protein was estimated by the method of Lowry using bovine albumin as standard (6).

Formation of cyclic CMP from CTP : Mouse liver homogenates (3-3.2 mg protein) were incubated at 37°C for 10 min in a medium (1 ml) containing 40 mM Tris-HCl (pH 7.4) 0.1 mM [α-^{32}P]-CTP (specific activity 3 cpm/pmole) 0.3 mM Mg $(C_2H_3O_2)_2$. Incubations were terminated by addition of 0.1 ml of ice-cold disodium EDTA (60 mM) and cooling the samples to 4°C. [^{3}H]-Cyclic CMP (30,000 cpm) in 0.1 ml of 100 mM Tris-HCl (pH 7.4) was added to each tube in order to monitor the recovery of cyclic CMP during chromatography of the samples. The samples were poured onto dry alumina columns (0.5 x 4 cm) and eluted with 2 ml of 100 mM Tris-HCl (pH 7.4). All the eluates were collected in counting vials and 17 ml of Aquasol were added. Both [^{3}H] and [^{32}P] were determined by liquid scintillation spectrometry. The recovery of [^{3}H]-cyclic CMP was routinely 70-80%.

Chromatograph on Dowex 1-formate columns : Dowex 1-formate columns (Bio Rad AG-1 x 8-formate form, 100-200 mesh, 0.5 x 4 cm) were prepared as described earlier (7,8). The samples were applied to the columns and eluted in the following sequence: (1) water (5 ml); (2) 0.01 N HCOOH (6 ml); (3) 0.04 N HCOOH (8 ml); (4) 0.5N HCOOH (8 ml); (5) 2 N HCOOH (30 ml). Fractions (1 ml) were collected and radioactivity was determined by scintillation spectrometry.

Chromatography on Dowex 50 and Alumina : Columns (0.5 x 4 cm) were packed with Dowex 50-H^+ (BioRad AG 50 x 4, 100-200 mesh) as described previously (3). After applying the samples, the columns were eluted with water. The first 2 ml eluted from the columns was discarded and the second 2 ml eluted were allowed to pass directly through dry alumina columns (0.5 x 4 cm). The alumina columns were eluted with 2 ml of 100 mM Tris-HCl (pH 7.4). The eluates were collected and the [^{3}H] and [^{32}P] radioactivities were determined by scintillation spectrometry.

RESULTS AND DISCUSSION

When mouse liver homogenates were incubated with [α-^{32}P]-CTP and Mn^{++}, a [^{32}P] product was isolated along with [^{3}H]-cyclic CMP by chromatography of the samples on alumina columns. The formation of this product was linear for two minutes of incubation, after which the product accumulated at a slower rate. The rate of product formation (100-120 pmoles/mg protein/min) was similar to that reported by Cech and Ignarro. Since a large amount of protein was employed in the assay, some protein appeared in the eluate from the alumina columns. The removal of protein from the parallel incubated samples by precipitation with trichloroacetic acid followed by extraction of the trichloroacetic acid with water-saturated diethyl ether resulted in a decrease in the amount of [^{32}P] recovered (Fig. 1). The eluates from alumina chromatography were rechromatographed on a second set of alumina columns and these were eluted with 2 ml of Tris-HCl (pH 7.4). This procedure completely eliminated from the samples all [^{32}P] radioactivity without significant loss of [^{3}H]-cyclic CMP. Thus most of the product isolated by chromatography on alumina columns was not cyclic CMP. Instead the large amount of protein present in this incubation medium was responsible for carrying over most of the [^{32}P] product found in cyclic CMP fraction and this could be eliminated either by the removal of protein prior to separation or by chromatography with a second alumina column. Moreover, by utilizing combined Dowex 50-alumina chromatography we were able to show that no [^{32}P]-cyclic CMP was formed (Table 1).

In order to examine the nature of the [^{32}P] product isolated along with [^{3}H] cyclic CMP in the presence of large amounts of protein, we developed a method for

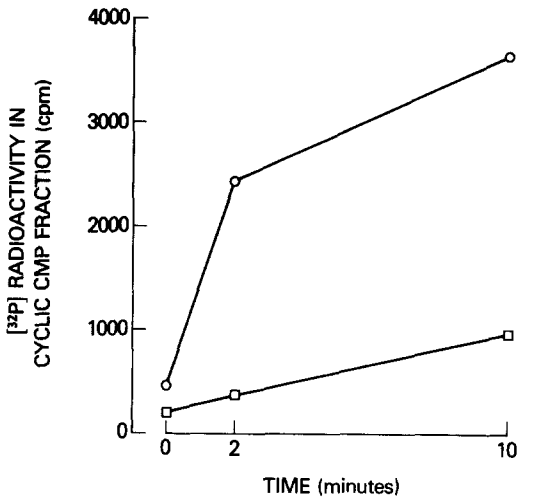

Figure 1. Effect of removal of protein prior to alumina chromatography on the isolation of "cyclic CMP". Mouse liver homogenate were incubated as described in Methods and were chromatographed on dry alumina columns either before (circles) or after (squares) removal of protein by precipitation with trichloroacetic acid (TCA) followed by removal of TCA from the supernatant fluid by repeated extraction with water-saturated ether. The results are expressed as [^{32}P] radioactivity (cpm) recovered in the cyclic CMP fraction and corrected for the recovery of authentic [^{3}H]-cyclic CMP.

Table 1. Effect of combined use of Dowex-50 and alumina chromatography on the recovery of [^{32}P] in cyclic CMP fraction

Chromatography	[^{32}P] radioactivity in cyclic CMP fraction (cpm)	
	0 min	5 Min
Alumina	280	1520
Dowex 50-H^+ + Alumina	17	12

Incubation was performed as described in methods. The samples were pooled either on to dry alumina columns without removal of protein, or on to Dowex 50-H^+ (0.5 x 4 cm, BioRad AG-50 - H^+ x 4, 200-400 mesh) and eluted with water. The first 2 ml eluates were discarded and the next 2 ml eluates from the Dowex-50 columns were allowed to pass directly through a second column of dry alumina. Samples added to the alumina columns (0.5 x 4 cm) were eluted with 2 ml of 100 mM Tris-HCl (pH 7.4). Both [^{32}P] and [^{3}H] were determined and [^{32}P] has been corrected for the recovery of [^{3}H] cyclic CMP.

separation of all possible cytidine nucleotides by chromatography on Dowex 1-formate columns. Utilizing a stepwise elution with increasing concentrations of formic acid we were able to separate cyclic CMP from 5'-CMP, CTP, CDP, and CDP-choline. Since the presence of protein in the incubation mixture could alter this separation, the

separation of all cytidine nucleotides was performed after their addition to the incubation medium. The chromatography of the samples after incubation at 0, 2 and 10 minutes indicated the formation of two main products: one co-chromatographed with 5'-CMP, and the other with CDP. A third, unidentified product was seen in samples incubated for 10 min (Fig. 2a). There was no [^{32}P] radioactivity associated with cyclic CMP. More than 60% of the [α-^{32}P]-CTP was converted to [^{32}P]-5'-CMP in 2 min. CTP could be eluted with a 5 ml of a solvent consisting of 8 N formic acid and 1.0 N ammonium formate. In order to determine whether a small amount of [^{32}P]-cyclic CMP was formed during the incubation of mouse liver homogenates, the samples were first chromatographed on alumina as described by Cech and Ignarro (4,5). The eluates from alumina columns were then rechromatographed on Dowex 1-formate. As shown in Fig. 2b [^{32}P] radioactivity was present only in the fractions containing 5'-CMP, CDP and the third, unidentified compound. Even though alumina was able to remove more than 99% of the radioactivity associated with 5'-CMP and CDP, about 1% of the radioactivity was recovered after alumina chromatography. This was mainly due to the presence of protein which was able to carry over this amount of radioactive 5'-CMP and CDP. Even though Cech and Ignarro (5) employed Dowex 1 - formate chromatography to identify the product obtained, these authors eluted the columns with 0.5 N HCOOH which would elute 5'-CMP as well as cyclic CMP. We have employed various thin layer chromatographic systems, including those employed by Cech and Ignarro (4,5), and we have not been able to detect the formation of [^{32}P]-cyclic CMP by mouse liver homogenates (7).

The data presented in this report clearly indicate that the product formed by incubation of mouse liver homogenates with [α-^{32}P]-CTP is not cyclic CMP, but represents 5'-CMP, CDP and an unidentified product. We have examined other tissues as well as mouse leukemia L1210 cells for cytidylate cyclase activity with very little success (7).

Recently Kuo *et al.* (9) and Cheng and Bloch (10) have reported the presence of a specific phosphodiesterase for cyclic CMP in various tissues. The presence of cyclic CMP in tissues has also been reported (11,12). The existence of a specific phosphodiesterase and the presence of cyclic CMP in tissues does not necessarily

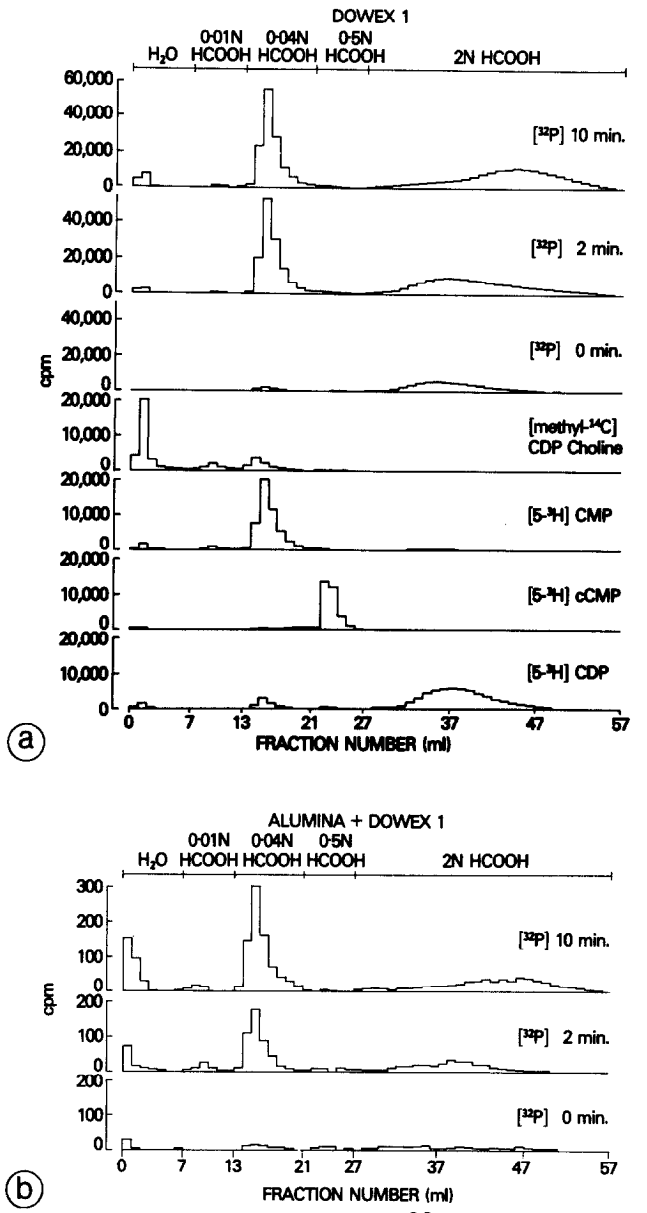

Figure 2. Dowex-1 chromatography of [^{32}P] product obtained in the assay of Cecl and Ignarro before or after alumina chromatography. Incubations were performed with mouse liver homogenates as described in Methods. (a) The enzymatic reactions were terminated by addition of 0.1 ml of 60 mM Na_2EDTA and cooling the sample to 4°C. [^{3}H]-Cyclic CMP (30,000 cpm), [^{3}H]-5'-CMP (62000 cpm), [^{3}H]-CDP (100,000 cpm) were added to 0 min, 2 min and 10 min samples, respectively. [Methyl-^{14}C]-CDP choline (43,000 cpm) was added to another set of zero time samples. These samples were chromatographed on Dowex 1-formate columns (0.5 x 4 cm, BioRad AG-1 x 8, 100-200 mesh formate form). The columns were eluted sequentially as described in the text. One ml fractions were collected, and [^{32}P], [^{3}H] and [^{14}C] were determined. (b) Another set of incubations were performed as described above and were chromatographed on alumina columns. These were then eluted with 2 ml of Tris-HCl (pH 7.4) and the eluates were rechromatographed on Dowex-1 as described above. A recovery of 70% of added [^{3}H] cyclic CMP was obtained in fractions 21-26.

imply the presence of cytidylate cyclase. The possibility that cyclic CMP is formed by some other pathway may have to be clarified before we fully understand cyclic CMP metabolism and its possible role in cell proliferation.

REFERENCES

1. Bloch, A. (1974) Biochem. Biophys. Res. Comm. 58, 652-659.
2. Bloch, A. (1975) Adv. in Cyclic Nucleotide Res. Vol 5 (G. I. Drummond, P. Greengard, G.A. Robison, Eds.) Raven Press. N.Y. pp 331-338.
3. Krishna, G. and Krishnan, N. (1975) J. Cyclic Nucleotide Res. 1, 293-302.
4. Cech, S.Y. and Ignarro, L.J. (1977) Science 198, 1063-1065.
5. Cech, S.Y. and Ignarro, L.J. (1978) Biochem. Biophys. Res. Comm., 80, 119-125.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
7. Gaion, R.M., and Krishna, G. (1978) Pharmacologist 20, 148.
8. Krishnan, N. and Krishna, G. (1976) Anal. Biochim. 70, 18-31.
9. Kuo, J.F., Brokett, N.L., Shoki, M., and Tse, J. (1978) J. Biol. Chem. 253, 2518-2521.
10. Cheng, Y.C., and Bloch, A. (1978) J. Biol. Chem. 253, 2522-2524.
11. Cailla, H.G., Goridis, C., Roux, D.R., Reutter, W., and Delaage, M.A. (1978) In Advances in Cyclic Nucleotide Research, Vol. 9 (George, W.J., and Ignarro, L.J. Eds.), Raven Press, New York, pp 768.
12. Murphy, B.E., and Stone, J.E. (1978) Pharmacologist 20, 232.