ISOLATION OF CYCLIC 3',5'-PYRIMIDINE MONONUCLEOTIDES FROM BACTERIAL CULTURE FLUIDS

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Summary: Cyclic 3',5'-cytidine and 3',5'-uridine monophosphates were isolated from culture fluid of Corynebacterium murisepticum or Microbacterium species both of which excrete a large amount of cyclic 3',5'-adenosine monophosphate.

The presence in nature and the physiological roles of cyclic 3',5'-pyrimidine mononucleotides have, so far, been unknown yet. This paper reports the isolation of cyclic 3',5'-cytidine monophosphate and 3',5'-uridine monophosphate found in trace amounts in the culture fluid of Corynebacterium murisepticum No. 7 or Microbacterium species No. 205 both of which excrete a large amount of cyclic 3',5'-adenosine monophosphate (cAMP) (1).

Experimental.

Microbacterium species No. 205 isolated by the author was grown with agitation at 30°C under aeration in a 3000 liters stainless steel fermentor containing 1000 liters of the cAMP production medium. The culture medium consisted of glucose 50 g, urea 5 g, $(NH_4)_2SO_4$ 5 g, K_2HPO_4 10 g, KH_2PO_4 10 g, $MgSO_4 \cdot 7H_2O$ 10 g, $ZnSO_4 \cdot 7H_2O$ 100 mg, peptone 10 g, yeast extract 5 g, inosine 5 g per liter of distilled water; adjusted to pH 8.0 with KOH. The methods of two dimensional paper chromatography and ion exchange chromatography were described in a previous paper (1).

Sample preparation for cyclic 3',5'-pyrimidine mononucleotides: Culture fluid (3000 liters) was clarified by a Sharples continuous flow centrifuge. The supernatant was acidified to pH 2.0 with concentrated HCl and passed through a charcoal column. The charcoal column was thoroughly washed

with water and the adsorbed nucleotides were eluted with 50% ethanol containing 1% NH_4OH. The eluate was concentrated *in vacuo* until the concentration of cAMP attained to about 20 mg per ml and adjusted to pH 8.0 with concentrated NH_4OH. Eight volumes of ethanol were added to this solution and the precipitate was removed by decantation after cooling at 4^oC for 16 hours. The supernatant was acidified to pH 2.0 with concentrated HCl and stood overnight at 4^oC. The cAMP crystals formed were separated by filtration. The filtrate was further concentrated *in vacuo* until the above cAMP concentration was obtained and kept at 4^oC overnight. The cAMP crystals were removed again by filtration. To remove cAMP exhaustively the filtrate was treated twice more by the same procedure as described above except for some modifications as follows: Six volumes of ethanol, 48 hours cooling and centrifugal separation of the precipitate at 0^oC (10,000 x g, 30 min.) were applied to the respective process instead of 8 volumes of ethanol, overnight cooling and decantation at room temperature.

Ion exchange column chromatography: The final supernatant was again passed through a charcoal column and cyclic 3',5'-pyrimidine mononucleotides were eluted with 50% ethanol containing 1% NH_4OH. Excess NH_4OH in the eluate was removed under the reduced pressure and the eluate was exactly adjusted to pH 8.0 with concentrated NH_4OH and applied to the column (4 x 70 cm) of Dowex 1 (formate form), anion-exchange resin. After the column was washed with distilled water (20 liters), adsorbed nucleotides were stepwisely eluted by Bergkvist's method (2) with formic acid and sodium formate. Formic acid and sodium formate were removed from the eluate by the same charcoal treatment method described by Kobata *et al* (3).

Identification of the nucleotides: The nucleotides in each peak fraction of the anion exchange chromatography were separated by two dimensional paper chromatography with Toyo filter paper No. 51A. After development, U. V. absorbing areas on the triplicate sheets of paper under the Pen Lay 11 SC-type lamp (Ultra-Violet Products, Inc., U. S. A.) were marked, cut out, and extracted overnight at 5°C with distilled water, 0.1N HCl, and 0.1N NaOH, separately. The

base and sugar moieties and the whole structure of each nucleotide were identified as described by Kobata et al. (3), Drummond et al. (4) and Smith et al (5).

Results.

Fig. 1 shows a typical chromatogram of culture fluid of Microbacterium sp. No. 205 with a Dowex 1 column (4 x 70 cm). Many peak fractions were found to contain U. V. absorbing compounds. The presence of cCMP in the peak 9 fraction, cyclic 3',5'-deoxyadenosine monophosphate (deoxy cAMP) in the peak 12 fraction, cAMP in the peak 13 fraction, cUMP in the peak 14 fraction, and cyclic 3',5'-inosine monophosphate (cIMP) in the peak 17 fraction was revealed by two dimensional paper chromatography on reference to the chromatogram of authentic cyclic 3',5'-mononucleotides (Fig. 2). Similar results were also obtained from culture fluid of Corynebacterium murisepticum No. 7 isolated by the author. This paper deals with only the peak fractions found to contain cyclic 3',5'-pyrimidine mononucleotides.

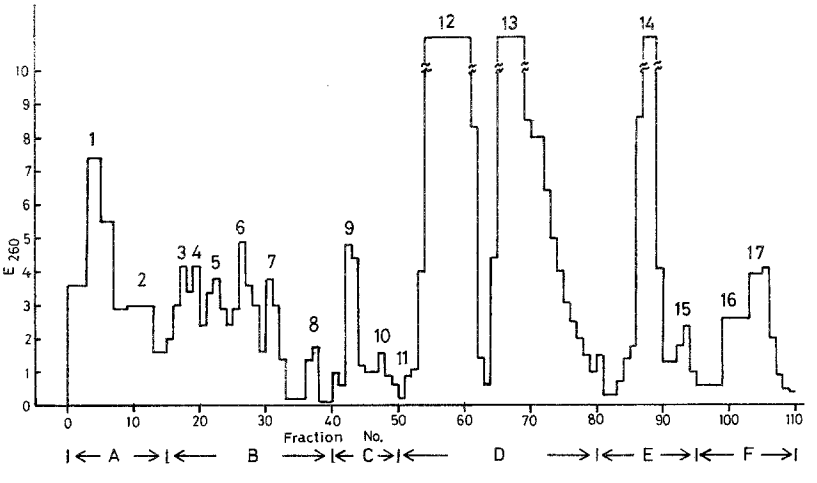

Fig. 1. Ion exchange chromatogram of sample for large scale isolation of cyclic 3',5'-pyrimidine mononucleotides from culture fluid of Microbacterium species No. 205. Exchanger: Dowex 1, x-4, 100 to 200 mesh, formate form, 4 x 70 cm. Eluting agents: A, 0.001 N formic acid; B, 0.005 N formic acid; C, 0.01 N formic acid; D, 0.05 N formic acid; E, 0.1 N formic acid + 0.1 M sodium formate; F, 0.15 N formic acid + 0.3 M sodium formate. Each fraction: 1000 ml.

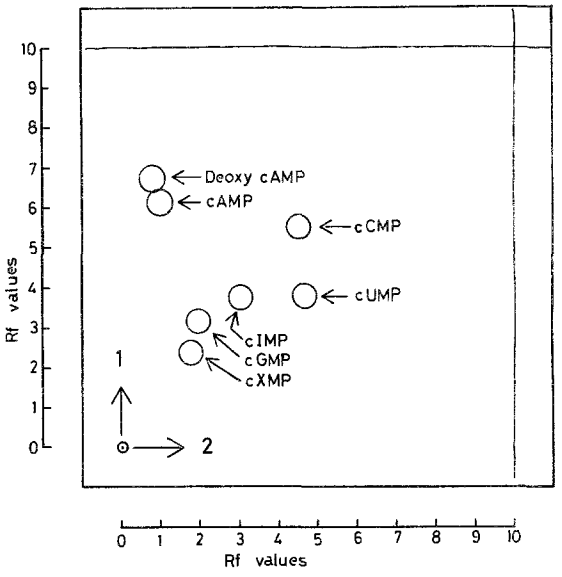

Fig. 2. Two dimensional paper chromatogram of authentic cyclic 3',5'-mononucleotides. Solvent system: 1, isobutyric acid : 1 N acetic acid : 1 N NH_4OH (10 : 1 : 5); 2, isopropanol : saturated ammonium sulfate solution : 1 M sodium acetate (2 : 80 : 20).

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cCMP in peak 9: Rechromatography of the peak 9 fraction through a Dowex 1 (formate) column (3.5 x 10 cm) gave 5 peaks (Fig. 3). The two dimensional paper chromatogram of the aliquot from the fraction of the 9-5 peak revealed two U.V. absorbing spots. Fig. 4 illustrates the rechromatography of the peak 9-5 fraction in Fig. 3 on a Dowex 1 (formate) column (3.5 x 10 cm). The peak 95-2 in Fig. 5 was recognized as the fraction containing cCMP by two dimensional paper chromatography. After charcoal treatment, the peak 95-2 fraction was applied to an Amberlite IRC-50 (sodium form) and the passed solution was concentrated *in vacuo* at 30°C. To this concentrated solution isopropanol was added until crystallization started. After cooling at 3°C for 40 hours, 58 mg of sodium cCMP was obtained as fine white needles from 3000 liters of culture fluid. The infrared absorption spectra of isolated sodium cCMP and authentic sodium cCMP were perfectly identical (Fig. 5).

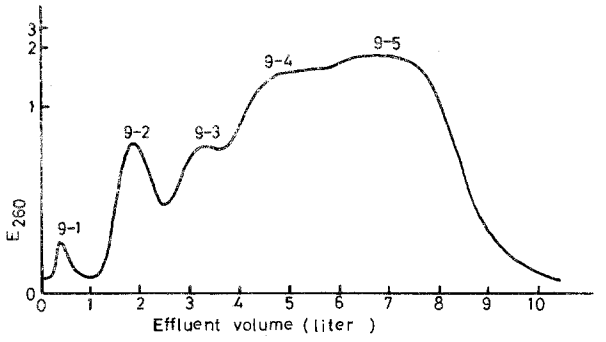

Fig. 3. Ion exchange chromatogram of the peak 9 fraction in Fig. 1. Dowex 1, x-4, 100 to 200 mesh, formate form, 3.5 x 10 cm. Eluting agent: 0.01 N formic acid.

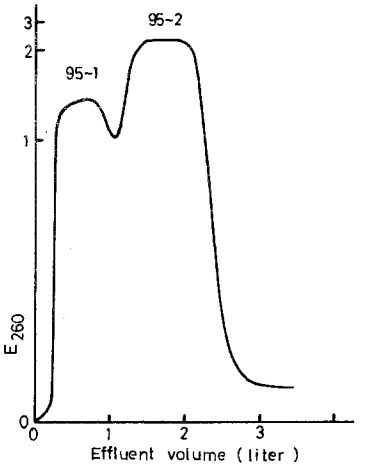

Fig. 4. Ion exchange chromatogram of the peak 9-5 fraction in Fig. 3. Exchanger: Dowex 1, x-4, 100 to 200 mesh, formate form, 3.5 x 10 cm. Eluting agent: 0.01 N formic acid.

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cUMP in peak 14: The two-dimensional paper chromatogram of the peak 14 fraction in Fig. 1 gave four U. V. absorbing spots. The Rf values of one of their spots coincided with those of authentic cUMP. The nucleotide in this spot was identified as cUMP by various chemical tests for the extract. After charcoal treatment, the peak 14 fraction was passed through a basic alumina

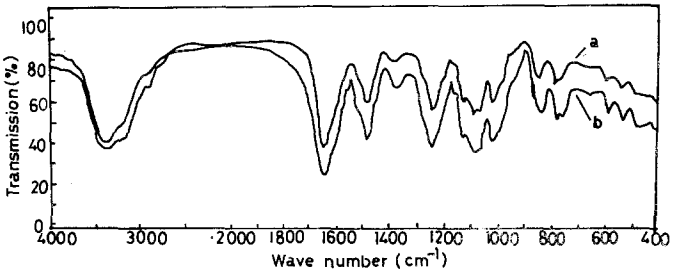

Fig. 5. Infrared absorption spectra of (a) the isolated compound from the peak 95-2 fraction in Fig. 4 and (b) the authentic cCMP.

column, and then the column was washed with water. The passed solution and washings were combined and concentrated to a small volume. The concentrated solution was spotted in a line on many microcrystalline cellulose (Avicel F) precoated sheets and thin layer chromatography was done with the solvent system of saturated $(NH_4)_2SO_4$ solution - 1 M sodium acetate - isopropanol (80 : 20 : 2). The band corresponding to the spot of cUMP was scraped off from the sheets and extracted with water. The extract was treated with a charcoal column to remove $(NH_4)_2SO_4$ and sodium acetate. Since the eluate from the charcoal column contained ethanol and NH_4OH, it was concentrated under the reduced pressure to remove ethanol and excess NH_4OH. The concentrated solution was passed through a SP-Sephadex (sodium form) column and washed with water. The passed solution and washings were combined, concentrated to a small volume and isopropanol was added until crystallization commenced. After cooling at 3°C for 24 hours, 25 mg of crystals of sodium cUMP was obtained by filtration from 3000 liters of culture fluid. The infrared absorption spectrum of isolated sodium cUMP agreed completely with that of authentic sample (Fig. 6).

Discussion.

It is apparent from the above data that cCMP and cUMP are present in nature in addition to cAMP, deoxy cAMP and cGMP. Presence of cyclic 3',5'-

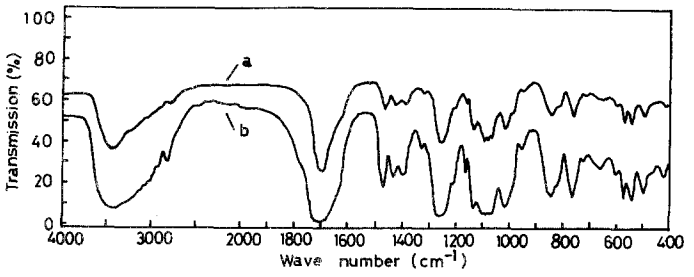

Fig. 6. Infrared absorption spectra of (a) the compound isolated from the spot 3 in Fig. 7 and (b) the authentic cUMP.

pyrimidine mononucleotides in bacterial culture fluid is explained as follows: (a) non-stringent substrate specifity of adenylate cyclase, (b) the presence of cytidylate cyclase and uridylate cyclase, or (c) the presence of adenylate cyclase or cytidylate cyclase and cCMP deaminase in the bacteria employed.

The possibility that the above cyclic 3',5'-pyrimidine mononuceotides might be contained in peptone or yeast extract as the constituent of the cAMP production medium is not plausible, since the author found that the above microbes excreted a large amount of cCMP (0.5 to 2 mM) in the culture medium supplemented with cytosine, uracil or their derivatives (6).

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